DETECTION, CHARACTERIZATION, AND CONTROL OF *BOVINE VIRAL DIARRHEA VIRUS* IN DAIRY HERDS

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ABSTRACT

Effective *Bovine Viral Diarrhea Virus* (BVDV) control on dairy farms is multifaceted and includes methods to accurately detect virus, remove BVDV persistently infected (PI) cattle, prevent virus introduction using comprehensive biosecurity plans, and optimize herd immunity through continuous vaccination against BVDV. The work in this thesis takes into consideration the above means to achieve effective BVDV control more specifically by attempting to: 1) determine the herd infection status by screening newborn calves for precolostral BVDV serum antibodies; 2) eradicate BVDV from a large commercial dairy herd through a combination of test and removal procedures and biosecurity measures; 3) characterize BVDV in PI calves from the Upper Midwestern United States by nucleic acid sequencing in order to more fully understand the changes that are occurring in the BVDV genome that may affect detection and elimination protocols, and; 4) implement a quantitative real-time RT-PCR (qRT-PCR) for quantification of BVDV RNA in a variety of clinical samples obtained from PI cattle.

Although many tests have been developed to detect BVDV PI cattle, there are few strategies to detect endemic BVDV infections at the herd level, especially in those herds that routinely administer BVDV vaccines. Many BVDV infections result from direct exposure to BVDV PI cattle. The detection and removal of BVDV PI cattle are essential steps towards reducing virus exposure within the herd and are critical components of national BVDV eradication efforts, such as those in Scandinavia. Veterinary diagnosticians and researchers have developed a variety of accurate tests to detect BVDV PI cattle in dairy herds. For example, screening bulk milk from dairy herds for BVDV by

RT-PCR is popular due to the ease of sample collection and the large number of animals that can be screened with one sample. A disadvantage of screening bulk milk is that it will not detect PI cattle in the non-lactating herd nor in youngstock. Alternatively, non-vaccinated sentinel calves can be used to detect BVDV PI exposure in youngstock; however, the sensitivity of sentinel calves in large herds with multiple groups of cattle is not known.

Chapter 2 of this thesis investigated a novel screening approach to detect BVDV by screening newborn calves for BVDV serum antibodies prior to colostrum feeding. Newborn calves that are seropositive for BVDV antibody prior to colostrum feeding indicate fetal infection during the last two trimesters of gestation. The number of newborn calves seropositive for BVDV serum antibodies at birth is estimated to be greater than the number of PI calves. Because the number of BVDV seropositive calves is greater than the number of BVDV PI calves fewer calves need to be tested by precolostral serum antibody screening to detect BVDV fetal infections and the probability of one or more PI cattle in the pregnant herd is likely. In addition to requiring fewer test animals, precolostral screening detects infections in lactating, non-lactating, and pregnant youngstock populations and is not confounded by vaccination.

Rapid consolidation of the United States dairy industry has resulted in fewer and larger dairy herds. Chapter 3 of this thesis describes the elimination of BVDV PI animals in a large commercial dairy herd with a RT-PCR test. Previous testing in the study herd indicated that approximately 5% of the calves were born with BVDV precolostral serum antibodies. The birth of BVDV seropositive calves also roughly coincided with an

increase in post-partum diseases that failed to respond to proven therapies. The herd owners elected to test all animals for BVDV PI with a serum BVDV RT-PCR test. Accurate detection of BVDV PI cattle is important in all herds, but less than perfect sensitivity and the potential of a false negative result are amplified in large herds with PI cattle. False negative test results would lead to the retention of one or more PI cattle and ultimately the continued persistence of BVDV within the herd. Serum samples from all cattle on the premises, and heifer calves born during the following 9 months, were tested for BVDV by RT-PCR and those determined to be BVDV PI on confirmatory tests were removed from the herd. Whether or not BVDV persisted in, or was eliminated from, this herd was determined by monitoring newborn calf precolostral serum antibodies for BVDV one year after the test and removal of all PI cattle. The chapter describes the detection of BVDV PI cattle, genetic characterization of BVDV isolated from the PI cattle, the detection of BVDV acute infections, and the precolostral monitoring results before and after the removal of PI cattle.

Bovine viral diarrhea virus is a single-stranded RNA virus that lacks a proof-reading mechanism resulting in mutations and recombination of the viral genome. Point mutations and recombination of viral RNA can result in novel, unique viruses. Few animal disease laboratories perform nucleic acid sequencing for BVDV, thus changes in the BVDV genome are not well described. The objective of chapter 4 was to successfully sequence a portion of the viral RNA and compare the viral genome sequences of forty PI cattle detected on dairy farms in the Upper Midwestern United States. The 5' untranslated region (5'UTR) region of the BVDV genome contains conserved regions

and is commonly used for PCR detection tests. This project described the use of primers targeting 5'UTR that produce a PCR product for nucleic acid sequence comparisons between vaccine and field strains and allow for differentiation between subgenotypes BVDV 1a, BVDV 2a, and BVDV 1b.

Testing many animals for BVDV PI requires appreciable amount of supplies and labor. The ear notch (skin) sample is a convenient tissue for testing and detecting BVDV PI animals because it is an easy sample to collect and requires minimal amounts of supplies and equipment. Ear notch skin samples offer some flexibility because they can be tested for BVDV by immunohistochemistry (IHC), antigen-capture ELISA (ACE), or RT-PCR. Pooling ear notch phosphate buffered saline (PBS) supernatant for RT-PCR is a popular method to screen large numbers of animals at a reduced cost. This method involves soaking the ear notch in a small amount (~2 ml) of PBS and then pooling the supernatant. The pooled supernatant is then tested for BVDV by RT-PCR. If the pooled supernatant is positive, the originally submitted samples can be tested individually to determine the PI animal. While ear notches have become the sample of choice for PI testing, there is little information available on the quantity of viral RNA in ear notches and the PBS supernatant that contains the soaking ear notch. The objective outlined in Chapter 6 was to implement a quantitative real-time RT-PCR (qRT-PCR) for quantification of BVDV RNA in a variety of clinical samples obtained from PI cattle. Serum, whole blood, nasal swabs and skin samples were collected from PI cattle and analyzed by qRT-PCR. The data derived from qRT -PCR allowed for an estimation of RNA copies in the variety of samples obtained from PI calves.

This thesis will give bovine veterinarians, diagnosticians, and researchers additional information on the dynamics and manifestations of BVDV in dairy herds. The precolostral screening method of newborn calves appears feasible and has potential application in commercial dairy herds. The performance and utility of a highly sensitive and specific RT-PCR test was assessed and appeared successful in a large commercial dairy herd. Additionally, nucleic acid sequence analysis of BVDV obtained from PI dairy cattle were compared to PI cattle from other farms and well-described viruses strains listed in GenBank. Quantification of BVDV RNA in clinical samples provided essential information needed to estimate the size of pools and potential variations in detectable RNA from diagnostics samples.

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CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

In March 1946, Olafson, MacCallum and Fox described an acute, infectious and contagious disease of cattle. The disease was characterized by leukopenia, high temperatures, salivation, nasal discharge, diarrhea, depression, anorexia, dehydration and occasional abortions pregnant cattle. Infected cattle had ulcers of mucous membranes lining the lips, cheeks, tongue, pharynx and esophagus. In the initial cases, the cattle had decreased milk production and mortalities ranged from four to eight percent. The cattle described by Olfason, MacCallum and Fox are thought to be the first described cases of *Bovine Viral Diarrhea Virus* (BVDV) infection. Since then, thousands of peer-reviewed manuscripts and at least three books have been written on BVDV. This review of literature will cover topics relevant to this dissertation.

1.1.1 Taxonomy, morphology and virus features

Recent taxonomy and classification of BVDV is described in a text entitled; Virus Taxonomy: Classification and Nomenclature of Viruses: Eighth report of the International Committee on the Taxonomy of Viruses (van Regenmortel MHV et al.: 2005). The following paragraphs summarize the properties and features of BVDV as reported in the text.

Bovine viral diarrhea virus **(**BVDV) is an enveloped, positive sense ssRNA virus in the *Flaviviridae* family and *Pestivirus* genus. The virus is approximately 12.3 kb in size and most of the viral genome is spanned by a single ORF. Virions are composed of a nucleocapsid protein (C) and three enveloped glycoproteins, E^{rns}, E1, and E2.

Members of the pestivirus genus infect pigs and ruminants, including cattle, sheep, goats, and wild ruminants. Transmission occurs by direct and indirect contact. Virion infectivity is stable over a relatively broad pH range, but unstable at temperatures above 40°C and the virion is inactivated by solvents and detergents. Transplacental and congenital transmission occurs in all host species. Infections can be subclinical or produce a range of clinical conditions including diarrhea, acute hemorrhagic syndrome, acute fatal, and a wasting disease.

Viral replication is initiated by receptor-mediated endocytosis involving one or more cell surface molecules and viral glycoproteins E^{rns} and E2. After endocytosis and uncoating, the genome RNA serves as the mRNA. Cap-independent internal initiation mechanisms initiate translation and involves an internal ribosome entry site (IRES) within the 5' untranslated region (UTR) of the RNA.

Pestiviruses are antigenically related and epitopes cross react within all pestiviruses. Separate antigenic determinants defined by monoclonal antibodies (Mabs) have also been identified and Mab binding patterns are generally consistent with the genetic relatedness of viruses. Antigen variation is particularly pronounced among BVDV isolates.

Infected animals mount potent antibody responses to structural glycoproteins E^{rns}, E2 and to the NS3 proteins, while antibody responses to all other virus-encoded polypeptides are weak or non-existent. Maternal antibodies reactive with the E^{rns} and E2 proteins can neutralize virus infectivity.

Pestivirus species differentiation considers several parameters and their relationship to the type viruses of the currently recognized species. Nucleotide sequence relatedness is and important criterion for pestivirus species differentiation. Parameters for differentiation often include: 1) at least 25% difference at the nucleotide level across the entire genome; 2) at least 10-fold difference in neutralization titre in cross-neutralization tests with polyclonal immune sera; and 3) differences in host or origin, range, and disease can assist in species identification.

1.2 Transplacental and intrauterine BVDV infections

Fetal infection is a key feature of BVDV transmission. Virus that crosses the placenta and causes fetal infection can produce a variety of outcomes including, but not limited to, abortion, congenital abnormalities, fetal mummification, and the development of persistently infected (PI) calves. Fetal exposure to BVDV with virus clearance and seroconversion is also a common outcome during the second and third trimesters of gestation when the fetus is immunocompetent. Both cytopathic and non-cytopathic BVDV biotypes are capable of crossing the placenta and infecting the fetus. Cytopathic types of BVDV cause significantly more damage to the conceptus as compared to non-cytopathic strains. Fetal infections with cytopathic isolates commonly result in fetal loss and cytopathic strains did not support persistent viremia and the birth of PI calves in heifers infected during early gestation experimentally (Brownlie et al., 1989).

In utero exposure to non-cytopathic BVDV between 42 and 125 days gestation has resulted in the birth of BVDV PI calves (McClurkin et al., 1984). These calves are

immunotolerant to BVDV and recognized the virus as "self." Failure of PI calves to mount an immune response results in consistent and prolonged viral shedding. Exposure to cytopathic BVDV between 125 and 140 days gestation has led to the birth of calves with a variety of congenital defects. The birth of stunted and deformed calves was a feature of BVDV fetal infections in a beef cow-calf herd thought to have been exposed to a cytopathic BVDV strain during a window of approximately 120-140 days gestation (Taylor et al., 1997).

The developing bovine fetus becomes fully immunocompetent and has completed organogenesis by approximately 125 days gestation. Fetal organ maturation and growth continues from 125 days gestation until parturition at approximately 280 days gestation in the bovine. BVDV fetal infection after 125 days gestation, and upon the development of a competent immune system, leads to the birth of clinically normal calves with pre-colostral BVDV antibodies. Fetuses exposed to BVDV during the last two-thirds of gestation usually seroconvert to BVDV within 30 days of fetal infection (Casaro et al., 1971; Kendrick 1971). Late term abortions, stillbirths and weak calves have been reported in late-gestation BVDV infection (Ward et al., 1969).

Fetal infection with BVDV appears to be a relatively common event in the United States. During a 10-year period from 1980 to 1990, BVDV was detected in 4.54% of bovine abortions and stillbirth submissions to the South Dakota Animal Disease Research and Diagnostic Laboratory (Kirkbride 1992). Fetal calf serum obtained from abattoirs is commonly contaminated with BVDV and BVDV antibodies. BVDV was isolated from 332 of 1,608 (20.6%) lots of raw fetal calf serum obtained specifically for examination

and 93 of 190 (49%) lots of fetal calf serum used for commercial sale. Virus neutralization detected antibodies against BVDV in 224 of 1,608 (13.9%) lots of raw serum. The detection of BVDV and antibody in this study does not give an accurate representation of the percentage of viremic and seropositive fetuses because the lots were pooled in groups of two or three. The number of fetuses represented in the commercially available lots of serum was not known, but is likely much greater than 2-3 animals per pool (Bolin et al., 1991).

The fetus can be protected by vaccination of the dam, yet immunity to prevent fetal infection is not complete (Cortese et al., 1998; Dean et al., 2003; Zimmer et al., 2002). Prophylactic maternal pre-exposure to homologous strains affords good fetal protection on subsequent exposure. Jersey heifers exposed to BVDV prior to pregnancy had lower incidence of fetal death and intrauterine growth retardation than heifers naive prior to virus challenge during the first trimester (Duffell et al., 1984). Fetal infection secondary to modified-live vaccination is unlikely, but must be considered when modified-live vaccines not used according to label directions (USDA-APHIS 2004 Veterinary Services Memorandum No. 800.110).

Reproductive and ovary dysfunctions have also been associated with BVDV infections. Acute BVDV infections resulted in differences in ovarian follicular growth for two estrous cycles following an acute infection (Grooms et al., 1998). BVDV was isolated from the ovaries of heifers six and eight days post-infection and stromal cells in the ovaries contained viral antigen in heifers acutely infected with BVDV. Changes in ovarian function secondary to BVDV infection would have likely have resulted in

reduced fertility (Grooms et al., 1998). Cytopathic BVDV was also isolated and detected in the ovaries from cows and heifers vaccinated with a modified-live vaccine (Grooms et al., 1998). More recent research indicates that bovine follicular cells and oocytes can be infected with BVDV at all stages of follicular development and acute infection is associated with a transient fall in estradiol secretion. Acute BVDV infection is suspected to reduce fertility by impairment of oocyte quality and disruption of gonadal steroidogenesis (Fray et al., 2000).

1.3 Mucosal disease

The noncytopathic BVDV biotypes are the only strains that cause infection that result in the development of PI cattle. Mucosal disease is the result of a super-infection involving antigenically-related noncytopathic and cytopathic strains. Post mortem examination of PI calves that succumbed to mucosal diseases revealed high levels of cytopathic virus in enteric tissues (Brownlie et al., 1984). Healthy PI calves inoculated with cytopathic isolates resulted in lesions, clinical signs, and an immune response consistent with mucosal disease and BVDV infection (Bolin et al., 1985). In calves with fatal mucosal disease, RNA recombination events were detected by sequence analysis. The recombination events between ncp and cp strains are referred to as a virus pair. Molecular examination of the viral pair revealed that the respective cp strains arise by RNA recombination from ncp viruses. Recombination of the ncp primarily affects the nonstructural NS2/3 polypeptide and results in the production of NS3 from cytopathic strains (Tautz et al., 1998).

1.4 BVDV and the immune system

The relationship between BVDV and immunosuppression in immunocompetent non-PI cattle has been demonstrated in both natural and experimental studies. Both cytopathic and noncytopathic strains of both genotypes have been described as having a negative impact on the innate and cell mediated immune system. Macrophages, neutrophils, interferon production, and circulating lymphocytes are all negatively affected in acute infections (Potgieter 1995). Early studies examining the innate immune system indicated that cattle experimentally infected with BVDV have impaired degranulation of PMNs (Roth et al., 1981). Calves intranasally infected with type 2 BVDV showed a significant drop in the number of circulating neutrophils, lymphocytes and monocytes by 3 and 5 post-exposure (Archambault et al., 2000). Monocytes infected with noncytopathic strains of BVDV had decreased ability to stimulate allogeneic and memory CD4(+) T cell responses (Glew et al., 2003). Cytopathic biotypes induced apoptosis in peripheral blood mononuclear cells. Apoptosis was also detected in bovine CD4(+) and CD8(+) T cells (Lambot et al., 1998). Interferon is an important component in the activation of the innate immune response. Noncytopathic biotypes of BVDV failed to induce interferon type I in cultured bovine macrophages, whereas cytopathic biotypes were capable of inducing the interferon response (Peterhans et al., 2003/6). The failure of noncytopathic BVDV to induce interferon is speculated to be an important feature in the development of PI calves.

T lymphocytes populations in the thymus and B lymphocytes populations in Peyer's patches were both depressed in calves experimentally infected with BVDV

(Brodersen and Kelling, 1999). There are numerous studies describing the effect of BVDV on the immune system; however, additional details will not be included in this review of literature.

1.5 BVDV and concurrent infections

BVDV's role in causing disease is likely attributed to the negative effect the virus has on the immune system and not a direct infection of the cells and tissues of the pulmonary and alimentary system. Calves concurrently infected with BVD and BRSV developed more severe clinical signs and lesions as demonstrated by histopathological and IHC exam. BVDV was also shed in greater concentrations and for a longer duration in calves concurrently infected with both BVD and BRSV (Brodersen and Kelling, 1998). In calves infected with both BRSV and BVD, BRSV was detected in lung lavage fluid for a longer duration than those calves infected with BVDV only. Calves dually infected also seroconverted later and with lower BRSV titers (Elvander et al., 1998). Clinical signs were most severe in calves experimentally co-infected with BVDV and *Mannheimia haemolytica.* The duration of elevated concentrations of acute phase proteins was significantly longer in the co-infected group as compared to the BVDV infected group (Ganheim et al., 2003).

In gnotobiotic calves, BVDV infection resulted in villus atrophy in the duodenum and submucosal intestinal inflammation. In those calves, bovine rotavirus (BRV) infections were more severe in calves that were concurrently infected with BVDV versus BRV and BVDV alone (Kelling et al., 2002). In beef calves, BVDV was isolated more

frequently from sick stocker calves than healthy calves. BVDV type 1 strains were involved in acute respiratory disease of calves with pneumonic *Mannheimia haemolytica* and *Pasteurella multocida* (Fulton et al., 2002). Exposure to PI cattle was associated with an increased respiratory tract disease of non-PI in-contact feedlot cattle (Loneragan et al., 2005).

1.5 Epidemiology and herd screening methods

Cattle persistently infected with BVDV are an important reservoir for infection, but is must be noted that a few epidemiological studies have indicated that PI cattle are rare within the herd and relatively few herds contain PI cattle. In two Michigan counties, 3 of 20 (15%) dairy herds contained PI cattle (Houe et al., 1995). The overall prevalence of BVDV positive bulk milk was 12.4% in over 90 northeastern United States dairy herds. The prevalence of BVDV PI in these dairy herds may be an underestimation because screening bulk milk only detects lactating cattle and not PI cattle in youngstock populations and non-lactating, dry cows (Renshaw et al., 2000). Although few herds contain PI cattle, rapid herd consolidation throughout the dairy industry will result in fewer, larger herds and the potential introduction of PI cattle.

A veterinarian's clinical impression is neither sensitive nor specific toward a diagnosis of endemic BVDV infections and the presence of PI cattle. Endemic BVDV infections are often subtle and difficult to detect by clinical observation. Variability in virus virulence, in addition to host factors, such as age, reproductive and immune status, can all confound the clinical impression. A reduction in clinical signs attributed to

vaccination and other endemic pathogens such as Salmonellosis, Johne's disease and Bovine Respiratory Disease Complex (BRDC) will also confound the clinical impression. In 52 beef herds suspected of having BVDV PI cattle based on history and clinical signs, only 10 (19%) actually had BVDV PI cattle (Wittum et al., 2001). In unvaccinated European herds, PI cattle were detected in 10 of 19 Danish dairy herds with an unknown status (Houe and Meyling, 1991). Endemic BVDV infections are also masked by slow transmission rates, as described in a study examining the transmission of BVDV in two dry-lot dairy youngstock populations (Rush et al., 2001). In those two California dry-lot dairies with similar management practices, transmission rates ranged from 0.5%/day to >1.3%/day, and the proportion of calves infected with BVDV by age 9 months was 67% and 36%, respectively. Although few studies have formally assessed the accuracy of a veterinarian's clinical impression, it is likely the accuracy of a practitioner's clinical impression is low.

Monitoring dairy herds for PI cattle can be attempted using the following options; 1) monitoring reproduction efficiency, 2) monitoring neonatal and postnatal morbidity and mortality, 3) serological evaluation of sentinel animals, 4) laboratory examination aborted fetus and neonatal deaths, 5) attempts to demonstrate seroconversion with acute and convalescent serology, and 6) screening milk for BVDV by RT-PCR or virus isolation. If BVDV infections are detected, a whole-herd testing for PI cattle should be initiated. In addition to testing all ruminants within the herd, all the calves from pregnant females should be tested for at least 9 months following the entire herd test to detect *in utero* PI calves.

Monitoring dairy herds for BVDV infections by investigating low reproductive efficiency is problematic. Many producers and herd managers are constantly striving to improve reproductive efficiency. The causes of poor reproductive efficiency are numerous and usually multi-factorial. In herds that artificially inseminate, variations in fertility can be significantly influenced by human factors. Heat detection efficiency, artificial insemination technique, routine changes in feedstuffs, and environmental factors can all have a significant impact on conception rates and reproductive efficiency. Post-partum metabolic disease and infectious diseases that directly impact the reproductive tract can result in subsequent losses of reproductive efficiency. Although there are numerous man-made and environmental factors affecting reproductive efficiency on dairy herds, endemic BVDV infections will contribute to decreased reproductive performance; however, endemic BVDV infections are not expected to have a profound, widespread impact. In one study examining 128 US beef herds, the fall pregnancy rate was 5% lower in herds with PI calves than those herds without PI calves (Wittum et al., 2001). Studies examining and comparing the reproductive efficiency in dairy herds with and without endemic BVDV infections are lacking.

BVDV infections have a negative impact on neonatal and postnatal calf health, yet detecting the infections can be challenging. Screening calves for BVDV infection can be accomplished by attempting to demonstrate virus in sick calves by virus isolation, RT-PCR, or immunohistochemistry. In many cases, testing young calves (< 4 months old) can only be done by demonstrating virus (VI, RT-PCR, IHC) in acute infections and PI cattle. Widespread use of vaccines and high levels of serum antibody obtained from

colostrum has limited the usefulness of serology in young calves. BVDV antibodies in colostrum fed to calves from vaccinated dams will circulate for up to 4 months. Calves are predicted to have a mean antibody titer of 1:32 for type I BVDV and 1:16 for type II BVDV by three months and congenitally infected calves, i.e. those born with BVDV antibody and had seroconverted *in utero,* had higher antibody titers than non-infected cohorts (Munoz-Zanzi et al., 2002).

Serologic evaluation of unvaccinated sentinel calves has been used to detect the presence of BVDV PI cattle in dairy and beef herds. The presence of non-vaccinated, seropositive calves serves as indirect evidence of virus exposure and the likelihood of at least one PI calf within the herd. In 14 Michigan dairy herds, BVDV PI cattle were detected when 3 of 5 unvaccinated 6- to 12-month-old heifers had antibody titers > or = 128. In the this herd screening strategy, evaluating 5 sentinel calves for the presence of BVDV PI calves had a sensitivity of 66% and a specificity of 100%. When virus isolation was included with SN test to detect PI animals, the sensitivity increased to 83%. In this study, one of the sentinel calves was PI and because PI calves rarely seroconvert, the PI sentinel calves was classified as non-exposed (Pillars and Grooms, 2002). In a more recent study, PI cattle were predicted with high accuracy in Japanese dairy herds by the use of serum neutralization and virus isolation in three unvaccinated calves 6- to 12-months-old. When both SN and virus isolation were used, 5 of 20 herds were corrected identified as containing PI cattle (sensitivity 100%). In herds without PI cattle, the testing strategy classified 13 or 15 herds as negative (specificity 86%). In the Japanese study, the SN cut-off titer was 64 and the herd was considered infected if two of three

non-PI calves had titers greater than 64. The majority of the herds in this study were dairy (19/20) and herd size ranged from 23 to 183 animals (Seki et al., 2006).

In beef herds, the use of sentinel samples was also attempt to predict PI cattle. Three of 10 randomly selected calves had a titer greater than 1:1000 in 53% of the herds with PI cattle. Unfortunately, at least 3 of 10 calves also had titers greater than 1:1000 in 20% of the herds that did not have a PI calf. Serological evaluation of a subset of beef calves in Canadian beef cattle herds had a sensitivity of 53% and a specificity of 80%. Serologic evaluation of calves or cows did not accurately predict the presence of PI cattle in these beef cattle herds. Determining a herd as infected may be a result of fenceline contact with other herds or the sharing of pastures with neighboring cattle. The beef cow herds ranged in size from 54 to 280 cattle (Waldner and Campbell, 2005).

The limited sensitivity of sentinel calves may result from variations in herd size and low stocking density that limits exposure to PI cattle. Testing large herds with multiple groups of cattle will result in decreased sensitivity of sentinel calf groups. Sentinel calves would need prolonged, direct contact with almost all cattle in the herd to be effective sentinels. Without direct contact with a PI animal, seroconversion in the sentinel calves will not happen. The probability of direct contact with a PI animal decreases when there are multiple groups of cattle spread over a larger geographical area. The sensitivity of sentinel calf groups can be improved by having multiple groups or repeating groupings over time.

The specificity of sentinel calves appears high. In the two dairy herd examples mentioned previously, the specificity of sentinel calves was 100 and 86%. In the beef herd study, the arbitrary SN cut-off value resulted in herds being classified as false positive, which decreased the specificity of the screening approach. Many of the beef cattle in that study were vaccinated and non-vaccinated sentinel calves were not used.

Acute and convalescent serology is a popular and reliable way to detect exposure to numerous pathogens in many animal species. Vaccination, especially modified-live virus vaccines, will produce high titers that can confound interpretation of serum neutralization SN titers. There is no accurate method of differentiating BVDV antibody produced by vaccination, field infection, or antibody transferred from dam to offspring through colostrum. In addition, many SN tests offered at diagnostic laboratories use two live viruses (genotypes 1a and 2a) to determine cytopathic effects in cell culture. Most of the BVDV that has been characterized today is BVDV subgenotype 1b, and examination of how well type 1a and type 2a viruses cross react with 1b antibody is not known. In one report, comparison of neutralizing antibodies to type 1a, 1b and 2a from experimentally infected and vaccinated cattle cautioned against using SN titers alone to differentiate natural infections from vaccination with MLV vaccines (Jones et al., 2001). Cytopathic BVDV 1b viruses are available, but not currently offered in an SN tests in diagnostic laboratories.

An antibody response to BVDV infection can be detected 2-3 weeks postinfection with a plateau 8-10 weeks post infection (Lambot et al., 1997). Timing the collection of acute and convalescent samples in vaccinated animals is confounded seroconversion

rates. Acute and convalescent serology has the additional drawback in that virus transmission in dairy herds can be slow (Rush et al., 2001). Selecting acute and convalescent sampling dates are also difficult because many BVDV infections are subclinical.

1.7 Detecting BVDV PI cattle

Efficient and reliable tests are essential for BVDV control programs. Accurate detection and elimination of PI cattle is essential for controlling the transmission of virus. Historically, detection of PI cattle involved cell culture isolation followed by virus detection through immunofluorescence or immunoperoxidase monolayer assay (IPMA) methods. Later, immunohistochemistry (IHC) on formalin-fixed skin samples (ear notches) was added as a routine test for PI detection. More recently, the detection of BVDV by reverse transcription-polymerase chain reaction (RT-PCR) and antigen capture enzyme-linked immunosorbent assay (ACE) has show to be more sensitive and rapid than by cell culture isolation. Currently, a variety of tests are currently used by diagnostic laboratories to detect PI cattle. These tests often include IHC on skin biopsies (ear notches), ACE on fresh skin samples, virus isolation (VI), and RT-PCR on a variety of samples, including blood, serum and ear-notch supernatant (phosphate buffered saline)

1.7.1 Reverse transcription-polymerase chain reaction (RT-PCR):

Numerous clinical samples including serum, blood (buffy coats), tissues, milk, nasal swab and soaked skin supernatant (PBS) can all be tested by RT-PCR methods.

In dairy herds, screening bulk tank milk by RT-PCR is a popular method to detect BVDV in lactating dairy cattle (Radwan et al., 1995). Bulk milk contains somatic cells (leukocytes) that can be screened for BVDV by RT-PCR procedures. In one study, a persistently infected (PI) cow was detected in a bulk milk sample that contained 162 lactating cows. In the same study, 19 other herds were screened with bulk milk and individual RT-PCR blood testing. A positive bulk milk test was detected in all ten herds with one lactating PI animals (Drew et al., 1999). In 2000, Renshaw *et al.,* reported that VI and RT-PCR are both suitable for detection of BVDV in bulk milk samples when used independently, but to increase the probability of successful detection and to provide cross-checks against assay contamination, it is desirable to utilize both methods in parallel (Renshaw et al., 2000). The primary limitation of bulk milk RT-PCR is that the screening method will not capture infections in nonlactating cows, calves and heifers.

Pooling ear notch phosphate buffered saline (PBS) for RT-PCR is a popular method to screen many animals at a reduced cost. The pooling method usually involves soaking the ear notch in a small amount (approximately 2 ml) of PBS and pooling the supernatant. The pooled supernatant is then tested for BVDV by RT-PCR. If the pooled supernatant is positive by RT-PCR, the originally submitted samples are tested individually.

Ear notches are the preferred samples for PI testing because they are easy samples to collect and require minimal tools. Kennedy *et al.,* examined pooling supernatant from ear notches and reported that RT-PCR detected a single positive ACE positive ear notch in pools up to 100 animals (100% , n = 36) (Kennedy et al., 2006). The pooling could

provide an initial, rapid, cost-effective method of screening cattle herds for BVDV PI animals. Although the pooled procedure appeared sensitive, there is no data on the quantity of viral RNA copies in skin and the PBS fluid.

The major limitation of RT-PCR is the lack of methods standardization between laboratories that could compromise the consistent identification animals of animals infected with BVDV.

1.7.2 Immunohistochemistry (IHC)

Immunohistochemical (IHC) staining of skin biopsy samples from cattle has been used as a method for the early detection of persistent BVDV infection. Initial reports described pronounced staining in the keratinocytes and in hair follicle epithelium, hair matrix cells of the hair bulb, and the dermal papilla of PI cattle. Staining in calves acutely infected, and not BVDV PI, had different and distinct staining confined to small foci in the nonfollicular epidermis and follicular ostia (Njaa et al., 2000; Baszler et al., 1995). Another study reported similar data and concluded the staining of skin biopsy samples is a reliable method for screening neonatal calves for BVDV PI (Grooms and Keilen, 2002). Skin biopsy represents an effective method for identifying PI cattle.

1.7.3 Comparison of tests

In one study involving 59 PI Angus calves, skin biopsy samples (ear notches) were collected and IHC and ACE were compared. Both IHC and ACE detected 100% of PI calves. In that study, RT-PCR and virus isolation were used as the gold standards. However, IHC and ACE also detected six and eight acutely infected calves, respectively,

at initial screening. Both IHC and ACE are accurate at detecting BVDV-infected calves, but veterinarians and producers should be advised that both tests detect some calves acutely infected with BVDV. Repeat testing using VI or RT-PCR on buffy coat samples should be performed at 30 days after initial screening to conclusively discriminate between acute and PI (Cornish et al. 2005). In 2007, Edmondson et al. evaluated the diagnostic proficiency of current methods for detecting BVDV in infected cattle using intra- and inter-laboratory comparisons. Samples were collected from 4 animals more than 7 months of age (2 BVDV negative animals, a PI animal, and a PI animal that previously lacked detectable virus in serum as determined by VI). Samples were submitted to 23 participating diagnostic laboratories using the respective laboratory's standard submission protocol. Samples collected for submission included: 1) serum for ACE, RT-PCR, and VI; 2) whole blood for RT-PCR and VI; and 3) skin biopsies for ACE and IHC. The ACE performed on skin provided the greatest consistency in detecting positive samples and a perfect level of agreement among laboratories. Reverse transcription-polymerase chain reaction and IHC performed well by correctly identifying > or = 85% of samples positive for BVDV. Virus isolation performed on serum yielded the lowest consistency in detecting positive samples and the lowest level of agreement. The level of agreement between laboratories for detecting BVDV in persistently infected cattle ranged from perfect to less than expected by chance. The variation between laboratories suggests a need for training opportunities in standardized laboratory protocols and proficiency testing (Edmondson et al., 2007).

1.8 Literature review summary

Over sixty years after the initial description, BVDV continues to be an important disease of cattle worldwide. Decades of research has revealed that the detection and removal of PI cattle is a critical step in stopping infections. After reviewing the literature, there appears to be a need for simple, affordable screening methods for large dairy herds that routinely vaccinate for BVDV. In addition, few case reports have described the manifestations of BVDV infections in beef and dairy herds, but none have extensively characterized the infections and documented the control efforts in large (>500 cow) dairy herds. Documenting and reporting the details of a herd control programs will give veterinarians and producers the confidence to pursue investigations in their clients herds.

Accurate diagnostic tests are critical for BVDV programs. Screening large groups of cattle by ear-notching is popular with producers, yet, details on the quantity and potential variation in pooled skin samples are limited. Additional data are needed to allow for an estimation and potential variability of detectable BVDV RNA copes in the variety of samples collected from PI calves.

SECTION B: Detection of *Bovine Viral Diarrhea Virus* in dairy herds

CHAPTER 2

SEROLOGICAL EVALUATION OF PRECOLOSTRAL SERUM SAMPLES TO DETECT *BOVINE VIRAL DIARRHEA VIRUS* INFECTIONS IN LARGE COMMERCIAL DAIRY HERDS

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2.1 Introduction

Bovine viral diarrhea virus (BVDV; family *Flaviviridae,* genes *Pestivirus*) continues to be an important pathogen affecting ruminants worldwide. Despite the widespread use of modified-live and killed vaccines, BVDV persists as a pathogen causing a wide variety of sub-clinical and clinical infections manifested by respiratory disease, immunosuppression, and decreased reproductive performance (Brock 2004).

Serological screening strategies to detect herds with endemic BVDV infections are limited, especially in herds that routinely vaccinate against BVDV. One approach for detecting endemic BVDV infection involves demonstrating seroconversion in a subset of animals by testing paired sera (acute and convalescent). The interpretation of this approach (acute and convalescent serology) is confounded by the widespread use of BVDV vaccines. Also, seroconversion in a subset of animals is difficult to demonstrate due to subclinical infections and to the slow intraherd spread of BVDV. Slow intraherd transmission rates in two California dry-lot dairies with similar management practices ranged from 0.5%/day to >1.3%/day, and the proportion of calves infected with BVDV by age 9 months was 67% and 36%, respectively (Rush et al., 2001). The presence of subclinical infections and slow intraherd transmission creates a diagnostic challenge when attempting to select the acute and convalescent sampling dates.

Serological evaluation of nonvaccinated sentinel calves has been attempted in both dairy and beef herds and has been marginally successful (Pillars and Groom, 2002; Waldner and Campbell, 2005). The presence of nonvaccinated seropositive calves serves

as indirect evidence of virus exposure and the likelihood of a BVDV persistently infected (PI) calf within the herd. In Michigan dairy herds containing less than 200 lactating dairy cattle, screening nonvaccinated heifers (6–12 months old) had a herd sensitivity of 66% and a herd specificity of 100% for detecting herds with endemic BVDV infections and BVDV PI cattle. The limited sensitivity of sentinel calf screening in dairy and beef herds may be a result of low stocking density and the limited exposure to BVD PI cattle. In addition, large cattle farms have numerous, variable sized cattle groups that could reduce exposure of BVD PI cattle to sentinels. Commingling or fence-line contact of cattle to positive adjacent herds can result in false-positive results and further confound the use of sentinel calves. Nevertheless, sentinel calves continue to be useful to screen for other pathogens including infectious bovine rhinotracheitis virus (IBRV) and Leptospira. Simple, easily interpretable strategies to detect endemic BVDV infections in vaccinated herds with a wide variety of management practices are necessary to provide herd-level information essential for a BVDV control program.

Antigen and nucleic acid detection tests for BVDV, such as immunohistochemistry (IHC) on skin, antigen-capture enzyme-linked immunosorbent assay (ACE) on skin, and reverse transcription-polymerase chain reaction (RT-PCR) on various tissues and serum are routinely used for the detection of BVDV PI cattle among vaccinated and nonvaccinated cattle (Goyal 2005). All three tests have high sensitivity and specificity for detecting BVDV PI cattle (Cornish et al., 2005; Fulton et al., 2006; Grooms and Keilen, 2002; Njaa et al., 2000). However, a large number of animals must be tested to obtain a high level of confidence that the herd is free of BVDV PI cattle

because such animals often represent less than 1% of animals within the herd. Pooling strategies to detect BVDV PI cattle using bulk tank milk (Radwan et al., 1995) or pooled ear notch supernatants (Kennedy et al., 2006) are popular alternatives because of reduced testing fees. When sampled correctly, the bulk tank milk–screening test is reliable at detecting BVDV infections, yet screening bulk milk for BVDV does not capture infections in nonlactating cows and heifers. Pooling saline from soaked skin samples (ear notches) is another method to screen many animals and to reduce testing fees; however, the sensitivity of RT-PCR on pooled ear notch fluids has not been extensively validated and pooling can result in decreased sensitivity.

Newborn calves can serve as a useful sentinel animal for BVDV infection in a herd of pregnant cattle. After 125 days gestation, the developing bovine fetus is immunocompetent and has completed organogenesis. Fetal infection with BVDV after 125 days of gestation (after the development of a competent immune system) usually leads to the birth of normal calves with precolostral BVDV antibodies (Casaro et al., 1971; Kendrick 1971). Therefore, detecting fetal infection gives a clear indication that BVDV is circulating within the herd, crossing the placenta, and causing fetal infections.

2.2 Materials and Methods

The current study was conducted on four large commercial dairy farms of at least 1000 lactating cows each; two herds were in California (herds A and B) and two were in Minnesota (herds C and D). The data collected from the California herds has been previously published in an article examining the health impact of natural congenital

BVDV infection (Munoz-Zanzi et al., 2003). Serum samples were collected from 961 newborn calves before colostrum ingestion (Figure 2.1). After blood collection (approximately 6 ml per calf), the calf was fed colostrum and managed according to farm protocols.

A single-tube *Taq*Man RT-PCR was performed on all precolostral serum samples from the Minnesota herds to detect *in utero* BVDV infection (Mahlum et al., 2002). Briefly, RNA was extracted and purified from 200 ul of serum using a commercially available kit (QIAamp 9604 Kit, Qiagen Inc., Valencia, CA). The viral RNA was subjected to reverse transcription and amplification using a *Taq*Man dual-labeled fluorescent probe. End-point analysis of the amplified products was performed on an ABI Prism 7000 Sequence Detection System (ABI Prism 7000 Sequence Detection System, Applied Biosystems, Foster City, CA). Calves that tested positive for BVDV by RT-PCR prior to colostrum feeding were retested approximately 2 weeks following the initial result to confirm BVDV PI status. Retesting included RT-PCR, IHC on formalin-fixed ear notches, and virus isolation. There was complete agreement between RT-PCR, IHC and virus isolation on all re-tested samples.

A serum neutralization (SN) test was used to detect antibody for BVDV-1 and -2 in the California herds, and a commercially available ELISA kit (IDEXX® Herd Check BVDV Antibody Test Kit, IDEXX Laboratories Inc., Westbrook, ME) was used to determine BVDV serum antibody in the Minnesota herds. The SN test was performed by the use of serial 2-fold dilutions of heat-inactivated serum, a 100- to 500-tissue culture infective dose ($TCID_{50}$) of BVDV-NADL (National Animal Disease Laboratory; type 1)

or BVDV-125c (type 2), bovine fetal testicle cells, and 96-hr incubation in 5% CO_2 at 37°C. The antibody titer was reported as the highest serum dilution that caused complete inhibition of BVDV-induced cytopathic effects. Titers of ≥1:4 were considered evidence of specific antibody against BVDV, and end-point dilutions were reported as ≥1:4,096. In the Minnesota herds, antibodies against BVDV-1 and -2 were evaluated with a commercially available indirect enzyme-linked immunoassay. Briefly, 25 µl of serum was added to 100 µl of sample diluent, mixed gently by tapping the plate, and incubated for 90 min. The wells were washed 5 times, the liquid contents aspirated, and 100 µl of horseradish peroxidase conjugate was added. After a 30-min incubation and wash cycle, 100 µl of tetramethylbenzidine (TMB) substrate was added, then 100 µl of stop solution was added to the well and the absorbance was measured at 450 nm. Sample-to-positive ratios (S/P ratios) were calculated against negative controls.

Serum samples that tested positive for BVDV antibody were further analyzed for total immunoglobulin to detect unintentional colostrum feeding or suckling prior to blood collection. If the serum sample had less than 400 mg/dl of antibody as detected by a qualitative assay (zinc sulfate turbidity), the calf was considered to be congenitally infected with BVDV with *in utero* antibody seroconversion.

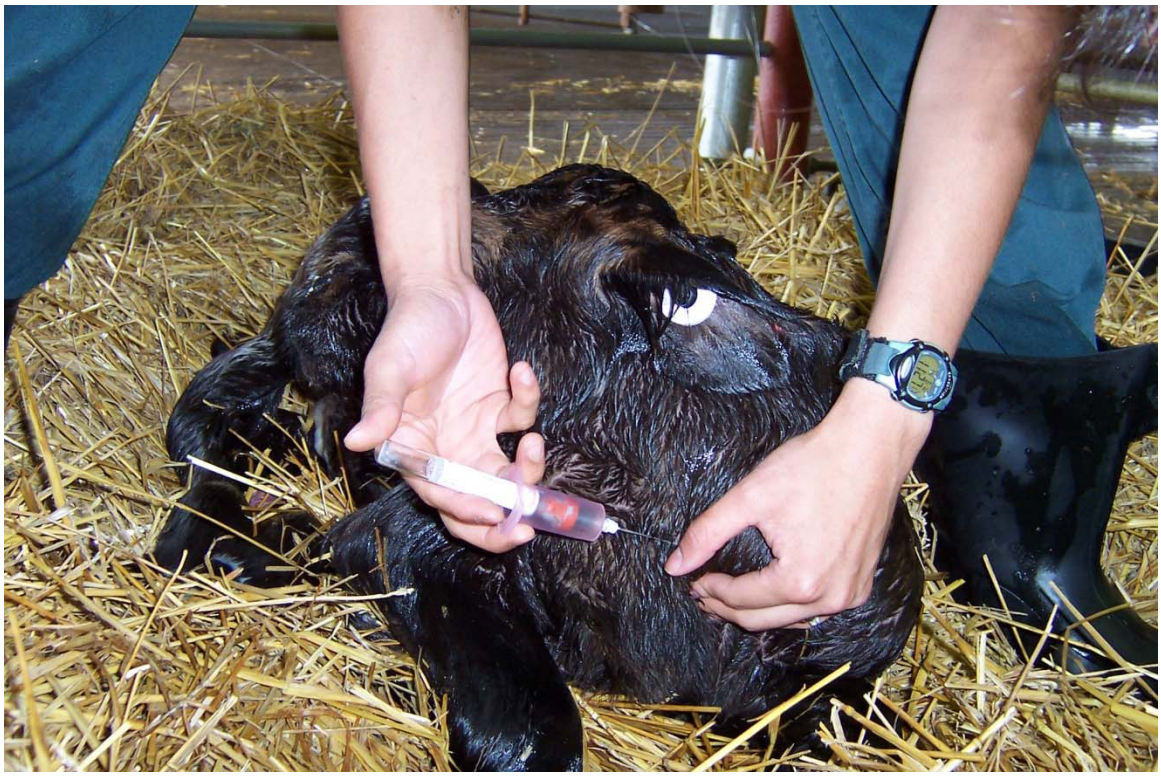

Figure 2.1: Collection of precolostral blood from a newborn dairy calf.

2.3 Results

In the four herds examined, the percentage of BVDV RT-PCR–positive calves at birth was 2.6% (25/961) as compared to 0.8% (8/961) of calves later determined to be PI. The data from all herds re-emphasize that calves testing positive by RT-PCR should be isolated and retested to rule out transient or acute BVDV infections. In one Minnesota herd (herd D), the unknown introduction of a PI heifer to a group of late gestation cows likely resulted in more calves being RT-PCR positive at birth. Bulk milk screening in the Minnesota herds C and D was not performed. Minnesota herd D documented BVDV PI lactating cattle prior to the initiation of precolostral screening and this herd was enrolled

to document fetal infections in a herd with known endemic BVDV infections. Minnesota herd D managers did not attempt to detect the lactating PI cattle because they concluded that additional testing was cost prohibitive. All cattle in Minnesota herd C were tested for PI by RT-PCR and two heifers were detected in the youngstock population. There were no lactating PI cattle detected in Minnesota herd C at the time of testing; therefore, bulk milk testing in Minnesota herd C would likely have been negative at the time of test and removal. Bulk milk screening in was not attempted in either of the California herds. No PI calves were born during the sampling period in California herd B.

The detection of BVDV antibody in precolostral serum samples correlated with the birth of PI calves in 3 of 4 herds examined (Table 2.1). BVDV serum antibody was detected in 6.8% (range 5.3–8.1%) of live newborn calves. The ratio of BVDV antibody positive to PI in newborn calves across all four herds indicated that for every PI calf born, there are approximately eight calves congenitally infected and seropositive for BVDV antibody prior to colostrum feeding.

Table 2.1: Prevalence of BVDV antibody and BVDV RNA in precolostral serum samples collected from four large commercial dairy herds.

Herd	State	No. of Calves Sampled	No. (%) positive for BVDV Antibody	No. (%) positive by RT-PCR*	No. (%) of BVDV PI	BVDV Antibody: BVDV PI ratio
A**	CA	236	19 (8.1%)	4 (1.8%)	2 (0.9%)	9.5:1
B**	CA	210	14 (6.7%)	3 (1.4%)	0 (0%)	NA
C	MN	226	12 (5.3%)	2 (0.9%)	1 (0.4%)	12:1
D	MN	289	20 (6.9%)	16 (5.5%)	5 (1.7%)	4:1
Total		961	64 (6.8%)	25 (2.6%)	8 (0.8%)	8:1

* BVDV RT-PCR positive newborn calves can be viremic and later clear the virus. Calves that tested negative for BVDV on follow-up tests were determined acutely infected and not BVDV PI. ** Data previously published (Munoz-Zanzi et al., 2003)

2.4 Discussion and Limitations

There is a lack of simple and cost-effective strategies to detect endemic BVDV infections in large commercial dairy herds that routinely vaccinate for BVDV. The current "all-antigen" and nucleic acid–detection strategies used to detect BVDV in vaccinated herds has limitations, and eliminating vaccination to determine seroconversion is not appealing to producers and veterinarians. The clinical signs associated with BVDV infection are often subclinical and non-descript. Because of this, livestock producers and veterinarians are often reluctant to invest the time and resources needed to rule-in or rule-out an endemic BVDV infection based solely on nonspecific clinical impression. Hundreds of cattle in a herd of thousands would need to be tested for PI status to achieve statistical significance that the herd is free of BVDV infections.

In dairy herds with year-round breeding schedules, endemic BVDV infections, and slow intraherd transmission rates, the risk of fetal BVDV infection would be expected to be equal across the entire gestational period. The odds of a fetus being infected during the last half of gestation, a period of approximately 160 days, would be 3 to 4 times greater than the period when fetal infection results in a PI animal (approximately 50 days). Therefore, screening precolostral serum samples for BVDV antibodies would theoretically yield 3 to 4 times more BVDV antibody–positive calves than PI calves. The higher percentage of BVDV seropositive calves than that of BVDV PI calves reduces the number of animals tested to achieve a high confidence of detecting

endemic fetal infection. In the 4 herds examined, there were approximately 8 BVDV seropositive calves for every calf determined PI.

The relatively high number of calves classified as acutely infected at birth in Minnesota herd D was unusual and unexpected. The number of acutely infected calves at birth was twice that of persistently infected calves. Yet, this finding may be explained by how pregnant cows and heifers are co-mingled on dairy farms during late gestation. On most dairies, pregnant dry cows (50-60 days prior to calving) and pregnant heifers (50-60 days prior to calving) are first commingled during late gestation. The comingling of these two groups of cattle shortly before calving is unique to dairy cattle and not beef cow-calf herds. When these two groups are comingled, a PI dairy heifer or cow has the potential to infect naïve, late gestating cows or heifers in either group. If these two groups of cattle were commingled at the beginning of gestation, fewer viremic calves at birth would be expected, but commingling during late gestation would likely result in more viremic calves.

Precolostral screening of newborn calves appears to have many advantages. When newborn calves from first-calf heifers and second lactation and older cows are screened for precolostral BVDV antibodies, both bred heifers and dry cows are screened simultaneously. This approach is advantageous over the young stock sentinel program because it detects virus in pregnant dry cows and first-calf heifers. Herds can continue to vaccinate nonpregnant cattle with BVDV vaccines because BVDV modified-live virus

vaccine has been shown not to shed and infect nonvaccinated animals (Kleiboeker et al., 2003).

Although precolostral screening does not appear to be confounded by vaccination, it must be noted that many vaccine companies have received USDA exemptions to use modified live IBRV and BVDV vaccine in pregnant cows. With the USDA exemption, modified live virus vaccines can be used in pregnant cows and heifers provided they were vaccinated, according to label directions, with an approved vaccine prior to breeding. In regards to BVDV, the USDA exemption study parameters included pre-suckling serum sampling from at least 400 randomly chosen calves from cattle vaccinated with modified live vaccinate during the second and third trimester of pregnancy. All pre-suckling serum samples were tested and found negative for antibodies to types 1 and 2 BVDV, thus demonstrating lack of fetal exposure to BVDV MLV *in utero*. Therefore, there is no evidence to suggest that fetal seroconversion will occur in cattle vaccinated according to label directions. Improperly administering a BVDV modified-live vaccine to pregnant cattle during the last two trimesters of pregnancy could result in BVDV fetal infection and fetal seroconversion that could compromise a precolostral surveillance program. Therefore, following label directions when vaccinating against BVDV is critical to maintaining a successful precolostral surveillance program.

Fetal exposure to BVDV and subsequent fetal seroconversion are expected when late gestation cattle are exposed to BVDV. If BVDV infections are occurring in nonpregnant cattle, congenital infections will not occur and screening for precolostral

BVDV antibody in newborn calves will fail to detect infections in nonpregnant cattle and cattle in early gestation. Although precolostral screening needs to be validated across many herds and compared to other screening tests such as bulk tank milk PCR and calf sentinel programs, preliminary data suggest that screening newborns for BVDV antibody can reliably detect endemic infections in large dairy herds and be used to monitor the progress of BVDV control programs over time.

SECTION C: Detection, characterization and control of *Bovine Viral Diarrhea Virus* in a dairy herd

CHAPTER 3

CASE REPORT: DETECTION, CHARACTERIZATION, AND CONTROL OF *BOVINE VIRAL DIARRHEA VIRUS* IN A LARGE COMMERCIAL DAIRY HERD

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3.1 Case description

A large Holstein dairy herd in central Minnesota had been experiencing an increased rate of post-partum metritis and pneumonia for more than two years. During the more severe episodes, the post-partum metritis cases approached 40% and the pneumonia cases approached 30% in post-partum cows and heifers. The pneumonia and metritis disease episodes did not correlate with known management changes and were described by the owners and employees as unpredictable events. In addition to metritis and pneumonia, many post-partum cows and heifers developed diarrhea and *Salmonella montevideo* was routinely cultured from the feces of diarrhetic cows. Many diarrhetic cows were also seropositive for *Mycobacterium paratuberculosis* antibody by ELISA.

The dairy herd consists of approximately 1,200 lactating cows and 1,800 replacement heifers (Figures 3.1 and 3.2). The herd is similar to many expanding dairy herds in Minnesota and was originally assembled from small dairy herds, sale-barn heifers, and heifers from other suppliers. This dairy retains and raises all heifer calves and does not commingle cattle with other herds. The vaccination protocol includes vaccinating heifers twice prior to breeding and vaccinating lactating cows once approximately 30 days post-partum with a commercially available modified-live virus (MLV) vaccine containing bovine viral diarrhea virus (BVDV), bovine herpes virus (BHV-1), parainfluenza 3 virus (PI-3), and bovine respiratory syncytial virus (BRSV). Once pregnant, the cows and heifers are not vaccinated.

Figure 3.1: Heifers at a large commercial dairy farm. The farm in this case report retained all heifers. This pen of heifers contained a PI heifer that had fence-line contact with pregnant dry cows.

Figure 3.2: Dairy free-stall barn full of lactating cows. This herd was originally assembled from small dairy herds, auction-derived heifers, and heifers from suppliers.

Beginning in February 2004 and continuing through July 2005, tissues, feces, and serum from diseased post-partum cattle were submitted to the Minnesota Veterinary Diagnostic Laboratory for necropsy and diagnostic examination. Bacteriology, histopathology, parasitology, immunohistochemistry, and molecular diagnostics tests were performed on these samples, when applicable. Results of the diagnostic procedures are summarized (Table 3.1).

Post-partum pneumonia and metritis cases approached 40% of all cows and heifers during July and August 2005. All post-partum cows and heifers were examined 3-4 days a week during August 2005. Upon observation, we concluded that the owner's and employee's diagnosis of the disease events was accurate. In addition to the high rates of post-partum diseases, the affected cattle responded poorly to antibiotic and supportive therapy and many developed chronic disease.

Endemic BVDV infections had been suspected by the herd veterinarians; yet, serum samples collected from 19 diseased cattle at least 30 days after a recorded post-partum illness failed to demonstrate high serum neutralizing (SN) antibody titers to BVDV type 1a and BVDV type 2a. Serum neutralizing BVDV type 1a titers ranged from 1:32 to 1:512 and BVD type 2a titers ranged from 1:8 to 1:512, with one BVDV type 2a titer at 1:1024. Additional herd diagnostic strategies to detect endemic BVDV infections were not attempted.

The goal of this case investigation was to detect endemic BVDV infections by precolostral screening of newborn calves, pursue BVDV control by testing and removing PI cattle, and genetically characterize BVDV isolates by RNA sequencing techniques.

Table 3.1: Summary of laboratory submissions from diseased lactating dairy cows and heifers.

Date(s)	Clinical signs	Specimen	Diagnostic Summary
May 2004	Pneumonia	Lung	*Mannheimia hemolytica* pleuropneumonia
Mar 2004	Pneumonia	Lung	*Mannheimia hemolytica* pleuropneumonia
Feb 2004	Pneumonia	Lung	Pleuropneumonia – *Pasteurella multocida* and *Arcanobacterium pyogenes* isolated
Feb 2004	Metritis	Uterine swab	*Streptococcus sp., Haemophilus somnus, Eubacterium sp., Peptostreptococcus sp. Bacteroides sp.*, and *Fusobacterium sp.* isolated.
Feb 2004	Metritis	Uterine mucus	*Mannheimia hemolytica,* non-hemolytic *Escherichia coli,* and *Arcanobacterium pyogenes* isolated.
July 2005	Metritis	Live cow	Severe endometritis – *Arcanobacterium pyogenes* isolated
Nov 2004 – July 2005	Diarrhea	Fecal samples (8)	*Salmonella montevideo* isolated.
July 2004 – April 2005	Diarrhea	Serum samples (11)	*M. paratuberculosis* seropositive (ELISA)

3.2 Initial precolostral serum sampling

Employees of the dairy were trained to collect a blood sample from newborn calves prior to colostrum feeding. Trained farm staff collected approximately 6 ml of blood in Vacutainer® SST tubes (Vacutainer SST, Becton, Dickinson and Company, NJ). The blood tubes were centrifuged and stored in a refrigerator until bi-weekly delivery to the Minnesota Veterinary Diagnostic Laboratory. The restraint and collection of blood from newborn calves was approved by the University of Minnesota Institutional Animal Use and Care Committee (IACUC).

Serum samples were collected from calves when it was convenient for the employees and not all calves were sampled. Approximately 75% of live newborn calves were sampled. During a four month sampling period (September 2005 through December 2005), BVDV antibody was detected in 5.3% (12/226) of newborn calf serum samples using a commercially available ELISA kit (IDEXX Herdchek BVDV Antibody Test Kit, Westbrook, ME). Serum samples from calves that tested positive for BVDV antibody were then tested for total immunoglobulin by zinc sulfate turbidity to rule-out inadvertent colostrum ingestion. Calves with less that 400 mg/dl IgG were classified as BVDV congenitally infected with *in utero* seroconversion. All precolostral serum samples were tested for BVDV by RT-PCR and 0.8% (2/226) of newborn calves tested positive for BVDV. One of the calves that tested positive for BVDV died within a day of birth and the other calf tested negative for BVDV on follow-up tests. None of the 226 calves sampled during the four month period were confirmed PI.

3.3 Herd testing for BVDV PI

Precolostral screening for BVDV antibody in newborn calves gave clear indication that BVDV was circulating in the pregnant cow and heifer populations. Serum samples were collected from all animals on the farm (3065 cattle) and tested for BVDV by RT-PCR. The BVDV RT-PCR procedure, including the primers and protocol, has been described in a previous publication (Mahlum et al., 2002).

Serum samples from eight of the 3065 cattle tested positive for BVDV by RT-PCR on the initial test. All cattle that tested positive on the initial test were non-lactating heifers. Whole blood, serum, and a formalin fixed ear-notch were collected from each of the eight heifers approximately two weeks following the initial positive RT-PCR test result. Two of the eight heifers tested positive for BVDV on the follow-up tests. In those two heifers, follow-up testing detected BVDV antigen in a formalin fixed ear-notch by immunohistochemistry (IHC), BVDV was again detected in a serum sample by RT-PCR, and BVDV was isolated from whole blood on bovine turbinate cells. The two heifers that tested positive on follow-up tests were classified as PI and removed from the herd. The six remaining heifers that tested negative on follow-up tests were determined as acutely infected, non-BVDV PI heifers, and were retained in the herd.

The two PI heifers were in the youngstock population and all acute infections were in heifers with direct contact with the PI heifers. PI cattle and acute BVDV infections were not detected in lactating cattle. The oldest BVDV PI heifer (#7771) was 16-months-old and was located in a pen with approximately 125 heifers being bred by

artificial insemination. The breeding pen containing PI heifer 7771 had direct fence-line contact with a large pen of approximately 125 dry cows (220-250 days gestation) and a pen of approximately 80 heifers exposed to a bull. Heifer 7771 had been in the pen for approximately five months and had direct fenceline contact with more than 300 pregnant dry cows during the 5 month period. The younger heifer (#8527) was 6-months-old and was in a treatment pen with 8 other calves. Heifer calf 8527 had developed chronic pneumonia and died during follow-up testing.

In the nine months following the completion of herd testing, three newborn PI calves were detected. Two of the three PI calves were bull calves that had persistent central nervous systems signs including head tremors with caudal neck extension to an opisthotonic posture, ataxia, and an inability to stand. The affected bull calves had a bright and alert mentation, but they failed to thrive and were euthanized by intravenous injection of sodium pentabarbitol. There were no significant lesions noted on necropsy or histopathological examination of the affected bull calves. Although histopathological examination of the brain was unremarkable, the neurons in the medulla, pons and midbrain stained intensely positive for BVDV antigen by IHC, a feature of noncytopathic PI. Formalin fixed ear notches and tissues from the affected bull calves were also positive for BVDV by IHC and RT-PCR, respectively.

3.4 Follow-up precolostral serum sampling

Precolostral serum sampling of newborn calves continued for 17 months after the removal of PI heifers. As expected, BVDV seropositive calves continued to be born

during the 5 months following the removal of BVDV PI animals, indicating historical fetal exposure with *in utero* seroconversion. In the 12 months following the five month period of seropositive calves, two of 450 (0.4%) calves tested positive for BVDV antibody by ELISA. The two calves that tested positive for BVDV by ELISA had less than 400 mg/dl IgG and were also positive for BVDV antibody by serum neutralization. All of the 450 precolostral serum samples were negative for BVDV by RT-PCR.

3.5 Molecular characterization of field viruses

To further characterize the virus detected in the PI heifers, the nucleotide sequence of the 5' untranslated region (5' UTR) was analyzed. The 5' UTR represents a conserved region of the BVDV genome (Ridpath et al., 1993; Boye et al., 1991). Comparison of the BVDV 5' UTR classifies the virus into three genotypes; namely, BVDV type 1a, BVDV type 1b and BVDV type 2a (Ridpath and Bolin, 1998).

Two sequencing primers were selected from previously published data (Ridpath, 2005). Viral RNA was extracted from the serum using a commercially available RNA extraction kit (QIAamp Viral RNA Mini Extraction Kit, Qiagen Inc, Valencia, CA), and RT-PCR was conducted utilizing commercially available kits (Qiagen OneStep RT-PCR enzyme mix, buffer and dNTPs, Qiagen Inc, Valencia, CA). The reagents, PCR primers and extracted viral RNA were subjected to thermocycling and a product of approximately 240 base pairs was detected on agarose gel. The RT-PCR products were subjected to Sanger dye-terminator sequencing and were analyzed using an automated sequence machine (ABI 3730 DNA Analyzer, Applied Biosystems, Foster City, CA) at the

BioMedical Genomics Center, University of Minnesota. Viral nucleotide sequences were compared to published BVDV sequences in Genbank by using computer software (Dnastar-Megalign sequence analysis program, Dnastar Inc, Madison,WI).

Phylogenetic tree analysis of the BVDV detected from the PI cattle and BVDV sequences obtained from Genbank revealed that there were two distinct viruses detected on this farm. The strain detected in PI heifer 7771 was similar to the subgenotype 1b Draper reference strain and the strain detected in calf 8527 was similar to the Oregon C24V type 1a reference strain (Figure 3.3).

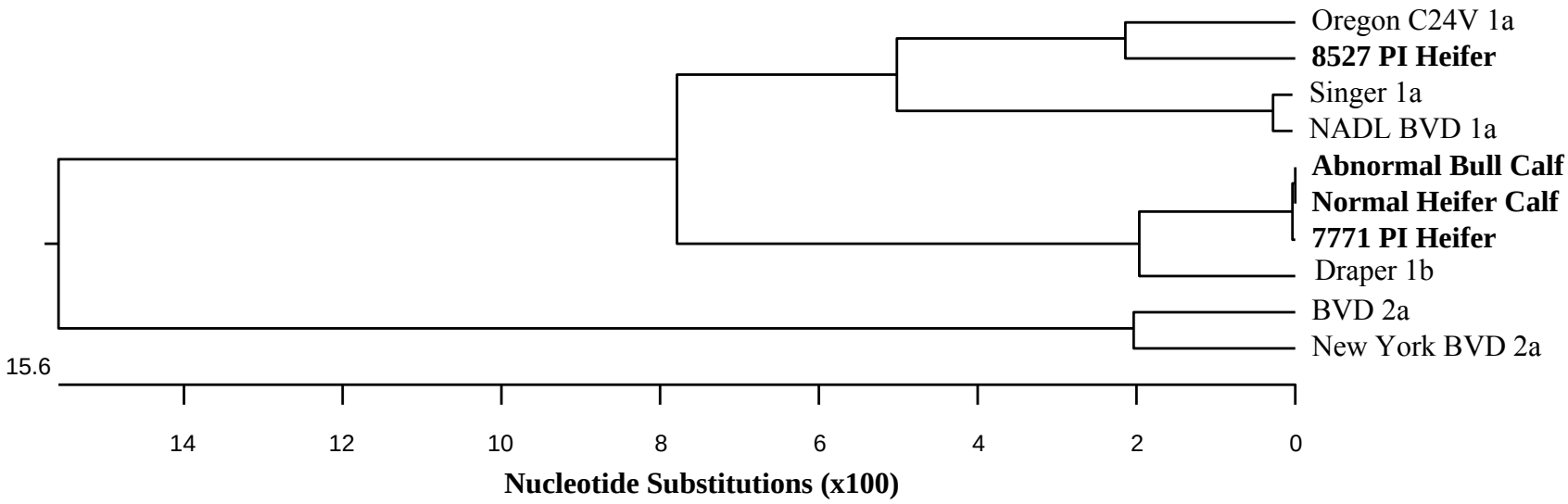

Figure 3.3: Phylogenic tree analysis of BVDV detected in the PI cattle (bold) and sequences obtained from Genbank. The two newborn calves and the oldest BVDV PI heifer 7771 are identical to each other and most similar to the Draper subgenotype 1b reference strain over a 240 base pair segment of the 5' UTR. The 8527 PI calf (6-month-old heifer) is most similar to the Oregon C24V type 1a strain over a 240 base pair segment of the 5' UTR.

Percent Identity

	1	2	3	4	5	6	7	8	9	10		
1		**100.0**	**93.1**	83.5	86.8	82.0	81.5	59.8	63.7	**100.0**	**1**	**Abnormal PI Bull Calf**
2	0.0		**93.1**	83.5	86.8	82.0	81.5	59.8	63.7	**100.0**	**2**	**Normal PI Heifer Calf**
3	3.9	3.9		81.4	84.1	83.1	82.5	61.9	62.4	**93.1**	**3**	Draper 1b
4	17.9	17.9	16.3		**96.8**	84.6	84.0	60.1	60.1	83.5	**4**	Oregon C24V 1a
5	13.7	13.7	12.2	3.3		87.8	87.3	60.8	63.7	86.8	**5**	**8527 PI calf**
6	16.3	16.3	14.8	11.2	7.4		99.5	61.9	63.5	82.0	**6**	Singer 1a
7	17.0	17.0	15.5	11.8	8.0	0.		63.5	65.1	81.5	**7**	NADL BVD 1a
8	35.1	35.1	31.3	35.6	31.6	30.5	29.6		96.8	59.8	**8**	BVD 2a
9	29.6	29.6	28.6	32.6	26.7	27.8	26.9	3.3		63.7	**9**	New York BVD 2a
10	0.0	0.0	3.9	17.9	13.7	16.3	17.0	35.1	29.6		**10**	**7771 PI Heifer**
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**		

Figure 3.4: Percent identity chart of BVDV detected in the PI heifers and sequences obtained from Genbank. The upper right half of the percent identity chart compares the nucleotide sequences directly. The BVDV PI cattle in this herd are in bold print. The two calves (#1 and 2) and the oldest PI heifer 7771 (#10) have identical nucleotide sequences to each other and are 93.1% similar to the Draper BVDV type 1b strain (#3) over a 240 base pair segment of the 5' UTR. The 8537 PI calf (#5) is 96.8% similar to the Oregon C24V 1a BVDV strain (#4).

The birth and detection of a PI calf is the result of a historical infection. When a cow or heifer gives birth to known BVDV PI calf, the infection indicates that the dam was infected with BVDV during the first trimester of pregnancy. Occasionally, the dam of the PI calf is herself PI, but this is a less frequent observation (Wittum et al., 2001). Finding the dam of the PI calf and researching the dam's location during the first trimester of pregnancy will indicate the probable location of BVDV exposure. In this herd, both dams of the PI cattle (7771 and 8527) were purchased as pregnant heifers. The dams of both of the PI cattle tested negative for PI. The location of the dams of BVDV PI heifers 7771 and calf 8527 during the first trimester of gestation is unknown. Therefore, BVDV infections that resulted in the birth of heifers 7771 and calf 8527 did not occur at this dairy. In addition, the BVDV detected in heifers 7771 and calf 8527 had different viral sequences. The viral sequence in heifer 7771 was similar to genotype 1b and the viral sequence in calf 8527 was similar to genotype 2a (Figure 3.4).

The three BVDV PI calves born on the dairy after the test and removal of BVDV PI heifers were born from non-lactating, first-calf heifers. The viral sequence of BVDV obtained from the three newborn calves indicated that the virus had an identical genetic sequence to PI heifer 7771. On-farm records indicate that PI heifer 7771 had exposed the dams of all three newborn PI calves to BVDV during the first trimester of pregnancy in the heifer breeding pen. PI calf 8527 was approximately 6-months-old and did not have direct contact with pregnant cows or heifers.

3.6 Discussion

Diagnosing subclinical, endemic BVDV infections in large dairy herds is challenging for bovine veterinarians. Demonstrating BVDV seroconversion in a subset of dairy cattle with acute and convalescent serology is confounded by the widespread use of modified-live vaccines, slow intra-herd transmission rates (Rush et al., 2001), and a wide variety of concurrent infections that can mimic BVDV infections. Screening bulk tank milk for BVDV by RT-PCR is a popular test to detect BVDV in lactating dairy cattle (Radwan et al., 1995), but bulk milk RT-PCR will obviously not capture infections in non-lactating cows and heifers. Non-vaccinated sentinel calves have been used to detect endemic BVDV infections and PI cattle in small dairy herds (Pillars and Grooms, 2002; Seki et al., 2006), but the sensitivity of this approach in large herds with multiple animal groups is unknown. Also, many months would pass before sentinel calves would seroconvert which would delay the diagnosis. Simply testing many animals for PI with ACE, IHC, or RT-PCR is not appealing to veterinarians, owners, and herdsmen due to labor and testing fees. In addition to the labor and fees, several cattle in a large herd would need to be tested for BVDV PI to achieve statistical certainty that the herd is free of PI cattle. In this dairy herd with 0.07% (2/3065) prevalence of PI, over 800 cattle were tested before the first BVDV PI heifer was detected.

Precolostral serum sampling of newborn calves was selected as the herd screening method for the following reasons; 1) detecting fetal infection gave clear evidence that BVDV was circulating within the herd, crossing the placenta, and infecting fetuses, 2)

screening fetuses captured fetal infections in the non-lactating pregnant cow and heifer populations, 3) precolostral serum sampling is not confounded by the potential shedding of modified-live vaccine virus from vaccinated animals to non-vaccinated pregnant cattle, 4) there was a higher incidence of precolostral BVDV antibodies compared to those that are PI, so fewer calves need to be screened to detect an endemic infection, and 5) if BVDV infections in this herd had a negative impact on post-partum heath, fetal infections should be occurring simultaneously.

Establishing a cause-effect relationship between the increase in post-partum diseases and endemic BVDV infections in this herd was problematic. We documented that PI heifer 7771 had direct fence-line contact with late gestation cows and heifers for 5 months prior to her detection and removal. Precolostral screening of newborn calves from cows and heifers exposed to PI heifer 7771 indicated fetal exposure, seroconversion, and in two newborn calves, acute BVDV infection. Additional diagnostic tests to determine which pregnant cows and heifers may have been exposed in late gestation were not attempted. In hindsight, serum samples from all post partum cattle could have been collected and archived for serum neutralization assays using a BVDV type 1b virus, but the detection and characterization of BVDV type 1b on this farm was not completed for more than a year after PI cattle were detected and removed. In addition, there are few reports available on how to interpret BVDV type 1b antibody titers in cattle vaccinated with MLV vaccines. In one report, comparison of neutralizing antibodies to type 1a, 1b and 2a from experimentally infected and vaccinated cattle cautioned against using SN titers alone to differentiate natural infections from

vaccination with MLV vaccines (Jones et al., 2001). In the absence of determining which cows and heifers were infected during late gestation, direct quantification of BVDV-associated losses could not be measured or estimated. Nevertheless, the owners and staff of this dairy reported an improvement in post-partum cow and calf health after the removal of the PI heifers and concluded that the investment in detecting and removing the PI cattle was justified. The cost of RT-PCR BVDV testing, labor, and supplies for sampling 3,065 cattle were paid by the dairy. The investigators paid for the initial and follow-up precolostral screening testing and the genetic sequencing.

The relationship between BVDV and its ability to cause immunosuppression in immunocompetent non-PI cattle has been demonstrated in both natural and experimental studies. Both cytopathic and noncytopathic strains of BVDV have been described as having a negative impact on the innate and cell mediated immune system. Macrophages, neutrophils, interferon production, and circulating lymphocytes are all negatively affected in acute BVDV infections (Potgieter 1995). Early studies examining the innate immune system indicated that cattle experimentally infected with BVDV have impaired degranulation of polymorphonuclear leukocytes (Roth et al., 1981) and calves intranasally infected with BVDV type 2 showed a significant drop in the number of circulating neutrophils, lymphocytes and monocytes by 3 and 5 days post-exposure (Archambault et al., 2000). Clinical pneumonia was also the most severe in calves experimentally co-infected with BVDV and *Mannheimia hemolytica* and the duration of elevated concentrations of acute phase proteins was significantly longer in the co-infected group as compared to the BVDV infected group (Ganheim et al., 2003). In this herd,

BVDV's role in causing post partum disease was likely attributed to the negative effect the virus had on the immune system rather than to a direct infection and pathology of the pulmonary, reproductive, and gastrointestinal systems.

Cattle that are BVDV PI are an important reservoir of virus (Werdin et al., 1989) and shed large amounts of virus throughout their lives (Duffell and Harkness, 1985). The variety of tests used to detect BVDV PI, namely ACE, IHC and RT-PCR, have all reported high sensitivity and specificity for detecting BVDV PI cattle when tested individually (Cornish et al., 2005; Grooms and Keilen, 2002; Fulton et al., 2006; Njaa et al., 2000), but ACE on fresh ear notch appears to be the most robust test for the detection of BVDV PI across many Veterinary Diagnostic Laboratories (Edmondson et al., 2007). In this herd, RT-PCR was selected because the author wanted to document acute BVDV infections. Acute infections were only detected in cattle with direct contact with the PI heifers. If acute infections were detected in groups without PI cattle, the investigation would have continued attempting to determine why virus would have persisted without direct contact with PI cattle.

Accurate detection of PI cattle is important in all herds, but less than perfect sensitivity and the potential of false negative test result is amplified in large herds with PI cattle. If RT-PCR would have produced a false negative test result and a PI animal would have been retained in the herd, fetal infections, as detected by the follow-up precolostral screening methods, would have been expected to continue. Precolostral screening of newborn calves continued for 12 months after the birth of the last known PI

calf (17 months after test and removal of BVDV PI cattle). Of the 450 calves sampled, two precolostral serum samples tested positive for BVDV antibody. Both serum samples had less than 400 mg/dl IgG by the qualitative zinc sulfate turbidity test and both serum samples were positive for BVDV antibody by serum neutralization. By previous definition, these two calves were congenitally infected, non-BVDV PI calves with *in utero* seroconversion. Although the two newborn calves were seropositive for BVDV and fit the previous definition of *in utero* exposure to BVDV, there was a significant reduction (Fisher's exact test, $p < 0.01$) from 5.3% (12/226) seropositive calves before detection and removal of PI heifers to 0.44% (2/450) seropositive calves after the removal of PI heifers.

The detection of serum antibodies in the two seropositive calves may not have been a result of fetal exposure and seroconversion to circulating BVDV. If the two calves unknowingly suckled a small amount of colostrum, but failed to achieve high levels of immunoglobulin transfer (>400 mg/dl IgG), the precolostral serum sample would have tested positive for BVDV antibodies. The detection of BVDV serum antibody in newborn calves prior to colostrum feeding might also occur if the epitheliochorial bovine placenta did not prevent complete *in utero* transfer of antibodies from the dam to the fetus. In rare occurrences, an incomplete placental barrier may result in the transfer of antibodies from the dam to the fetus. If BVDV antibody from the dam crosses the placenta to the fetus, the fetal serum will test positive for BVDV antibody prior to colostrum feeding. Additional evidence to support that fetal infection stopped

after the detection and removal of PI heifers includes that BVDV was not detected by RT-PCR in any of the 450 precolostral samples after the removal of the PI heifers.

Bovine viral diarrhea virus is a single-stranded RNA virus that lacks a proof-reading mechanism resulting in mutations and recombination of the viral genome. Point mutations and recombination of viral RNA can result in novel, unique viruses. Few animal disease laboratories perform nucleic acid sequencing for BVDV; thus, changes in the BVDV genome are not well described. Tracking viral changes is important for control and eradication strategies. In this herd, the virus detected in three of four BVDV PI cattle was genetically sequenced and determined to be closely related to BVDV the subgenotype 1b Draper reference strain, a virus that is similar to BVDV 1a, but not included in commercially available vaccines. Many commercial vaccines contain genotypes 1a and 2a. Field strains of BVDV will likely evolve away from vaccine strains.

In summary, endemic BVDV fetal infections were documented by a precolostral screening method, PI heifers were detected and removed, and there was a significant reduction in fetal infections after the removal of the PI heifers. Although specific health data were not recorded by the dairy, the owners and herdspersons reported a noticeable improvement in animal health after the removal of the PI heifers. This herd appears to have achieved a negative PI status and has implemented a protocol to PI test all new introductions and the offspring of purchased pregnant cattle. In addition, the herd managers are aware of the potential risk of fence-line transmission from neighboring

herds. Currently, the cattle on this farm do not have direct contact with neighboring cattle.

CHAPTER 4

POSTMORTEM EXAMINATION OF ABORTED FETUSES, STILLBORN CALVES AND NON-VIABLE WEAKBORN CALVES IN A LARGE DAIRY HERD ENDEMICALLY INFECTED WITH *BOVINE VIRAL DIARRHEA VIRUS*

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4.1 Introduction

The percentage of live-born dairy calves in the United States has dropped from 93.4% in 1996 to 88.8% in 2002. In addition, the all-herd abortion rate on dairy farms increased from 3.5% in 1996 to 4.0% in 2002. Large dairy herds (> 500 cows) had the highest percentage of abortions at 4.9% (USDA-APHIS NAHMS Dairy 1996 and 2002). The reported stillbirth rate (calves born dead) in American dairies is 8% (Silvia del Rio et al., 2007). In another study, the loss of the calf was compounded by a significantly increased risk of culling/death throughout the lactation and increased days open in the cows that had stillbirths (Bicalho et al., 2007). This chapter summarizes the post-mortem examination findings of aborted fetuses, stillborn calves, and non-viable weakborn calves (death within the first day of life) from a large dairy herd endemically infected with BVDV. The percentage of BVDV infections in aborted fetuses, stillborn, and non-viable weakborn calves were compared to live-born BVDV antibody and RT-PCR positive calves. This study examined if BVDV infections were more common in aborted fetuses, stillborn, and non-viable calves than live calves in a single large dairy herd. The gross necropsy finding in all stillborn calves and aborted fetuses were recorded and are reported.

4.2 Materials and Methods

Sixty stillborn calves and twelve aborted fetuses were collected from a commercial dairy of approximately 1,200 lactating cows. Aborted fetuses, stillborn, and

non-viable weakborn calves (perinatal mortalities) were saved, picked-up, and taken to the Minnesota Veterinary Diagnostic Laboratory for necropsy exam and BVDV testing. Abnormal necropsy findings of interest included, but were not limited to, meconium staining, aspiration of amniotic fluid, aerated lung, congenital anomalies and post-mortem decomposition. Fresh lung, thymus, spleen, and brain were collected, homogenized and tested for BVDV by RT-PCR (Mahlum et al., 2003). Thoracic fluid was tested for BVDV antibodies with a commercially available antibody ELISA kit (IDEXX® Herd Check BVDV Antibody Test Kit, IDEXX Laboratories Inc., Westbrook, ME). Precolostral serum samples from clinically normal, live-born calves were simultaneously collected for testing and with the same tests used on the necropsied calves and fetuses.

4.3 Results

There were 708 calvings during the 5 month collection period starting in August and ending in December 2005. The stillborn rate over the five month time period was 8.5% (60/708). Twelve aborted fetuses were identified and collected over the same time period giving a yearly calculated abortion rate of 2.4%.

Gross exam and necropsy revealed meconium staining in 17% (10/60) of stillborn calves. Meconium is the fecal material found in the colon of newborn calves. If a calf experienced hypoxia secondary to distress or a difficult delivery, it will release meconium staining the surrounding amniotic fluid and hair (Dufty and Sloss, 1977). Meconium staining is not always fatal because some live-born calves will have staining,

yet staining is common in calves that experience hypoxia. The cause of fetal hypoxia is not always known and can have multiple causes.

If a fetus is deprived of oxygen for an extended period, it will eventually take a breath. If the nose is submerged in amniotic fluid when it takes a breath, it will aspirate amniotic fluid into the trachea. In the sixty stillborn calves examined, 9 (15%) had aspirated amniotic fluid.

Approximately 25% (15/60) of the stillborns were from cattle that recorded an assisted calving (dystocia). In four of the dystocia stillborns, necropsy revealed traumatic injuries. Two of the calves had hemothorax and hemoabdomen, one calf had fractured ribs, and one calf had a torn diaphragm. Many of the dystocia calves that did not have hemorrhage or fractures had congested and swollen heads and tongues consistent with prolonged delivery.

The lungs of 17% (10/60) of the stillborn/weakborn calves floated in formalin. This finding is consistent with respiration and indicates the calf was born live and took a breath. By definition, these calves are not stillborns, but would be classified as postnatal mortalities.

Four of the sixty stillborn calves were in various stages of decomposition. Calves with one or more of the following gross features were classified as decomposed; 1) cloudy cornea, 2) hair sloughs, 3) generalized edema (anasarca), 4) dehydration, and 5) malodorous.

Bovine viral diarrhea virus infections were not over-represented in the aborted fetuses, stillborn calves, and non-viable weakborn calves. Only two (2.7%, 2/72) of the necropsied stillborns had thoracic fluid that was positive for BVDV antibody and only one of the stillborn calves had tissues that were positive for BVDV by RT-PCR. Simultaneous screening of live calves indicated that approximately 5.3% (12/226) of the live-born calves had BVDV antibodies prior to colostrum feeding and approximately 0.9% (2/226) of live-born calves were RT-PCR positive for virus at birth as previously described in chapter 2. Although BVDV infections had other negative impacts in this herd, BVDV infections were not statistically higher in the aborted fetuses, stillborn calves, and non-viable weakborn calves when compared to the live-born calves (Fisher's Exact Test, p-value = 0.5).

One stillborn calf had a cleft palate and another had an enlarged heart with a ventricular septal defect. Both are uncommon, but not unique congenital anomalies of cattle. There were no significant lesions detected in the remaining stillborn calves (19/60, 32%).

Twelve aborted fetuses were collected and examined. One fetus had hydrocephalus, one was macerated, and another had midline medial facial cleft. Two of the aborted fetuses were decomposed and there were no significant lesions detected in the remaining seven fetuses. None of the aborted fetal tissue tested positive for BVDV by RT-PCR and none of the available thoracic fluid tested positive for BVDV antibody by

ELISA. The necropsy and test results of all stillborn calves and aborted fetuses are summarized in table 4.1.

4.4 Discussion

Fetal loss and abortion caused by BVDV infections have been reported in case reports (Woodard 1994; Kirkbride 1992; Bezek and Mechor, 1992). Endemic BVDV infections in this herd did not appear to result in increased abortions or stillborn calves. Virus virulence and cytopathogenicity (cytopathic vs. non-cytopathic) are important factors when considering BVDV as a potential cause for reproductive losses. Both cytopathic and non-cytopathic BVDV biotypes are capable of crossing the placenta and infecting the fetus. Fetal infections with cytopathic isolates commonly result in fetal loss and cytopathic strains were unable to support persistent viremia and the birth of PI calves in heifers infected during early gestation (Brownlie et al., 1989). As described in Chapter 3, the virus detecting and isolated from a PI heifer in this herd was similar to the 1b subgenotype and was non-cytopathic in cell culture. The impact of BVDV infections on fertility, such as early embryonic death and irregular returns to estrus, was not examined.

A majority (90%, 54/60) of the stillborn calves did not have features of decomposition and appeared to be alive at the initiation of parturition. The cause of stillborn can not be definitely determined, but it must be noted that bovine fetus appears relatively susceptible to anoxia. Experimental studies indicate that most fetuses will die following 6-8 minutes of anoxia. Calves that survive hypoxia and anoxia are often born weak and unable to maintain and upright position and many of these calves die within the

first week of life (Dufty and Sloss, 1977). Many of the stillborns calves (32%, 19/60) did not have lesions, yet calves that die of acute anoxia or metabolic acidosis are not expected to have distinctive lesions.

Fetal malpresented calves (backwards, head back, etc.) have a two times higher risk of dystocia and a five times higher risk of stillbirth (Mee 1991). In a more recent study, the duration of second stage labor and presentation/position/posture of the fetus were significant factors for stillbirth (Gundelach et al., 2008). Posterior malpresentation (backwards calves) often have their nose submerged in amniotic fluid and are at greater risk of aspirating amniotic fluid when hypoxic and this pnenomenon was documented in 9 of 60 stillborn calves in this herd.

Fifteen calves were born from cows that recorded a dystocia. Excessive obstetrical assistance and trauma to the calf was documented in four of these stillborns, yet trauma to the calf secondary to obstetrical assistance did not appear to be a significant cause of stillbirth in the remaining 11 calves that recorded dystocia.

Four of the sixty stillborn calves were in various stages of decomposition. Decomposed, malodorous stillborns are usually the result of putrefactive bacteria gaining access to the fetus. The bacteria detecting in stillborns and fetuses are usually a mixed culture of bacteria that are common in the bovine environment (Non-hemolytic *Escherichia coli, Arcanobacterium pyogenes,* etc.). If many stillborns appear decomposed and there are an abnormally high number of abortions, additional diagnostics should be initiated to rule-out infectious pathogens such as IBRV,

Brucellosis, *Campylobacter sp.,* Listeriosis, Salmonellosis, Neospora caninum, Q fever, or Leptospirosis.

Congenital defects were rare (0.5%, 4/708) and were observed in both the stillborn calves and aborted fetuses. The anomalies (heart defect, cleft palate, etc.) are uncommon, but not unique to cattle. Defects of the heart wall are amongst the most common heart defects in stillborn calves (Mee 1991). Multiple calves with congenital defects and a common genetic ancestry should initiate a thorough genetic investigation.

In summary, BVDV infections were not over-represented in the aborted fetuses, stillborn, or weak born calves in a herd endemically infected with BVDV. The herd's stillborn rate of 8.5% was similar to the 8% reported in American dairies (Silvia del Rio N et al., 2007), and the abortion rate of 2.5% was less than the 4.9% reported by USDA-APHIS (USDA-APHIS NAHMS Dairy 1996 and 2002). Most of the stillborns appeared to have been alive at the onset of parturition and necropsy revealed many lesions consistent with fetal hypoxia or anoxia.

Table 4.1: Summary of necropsy and BVDV test results

Probable cause of death	**Necropsy lesions**	**No. stillborn (%) n=60***	**No. abortions (%) n=12**
Hypoxia/Anoxia	Meconium staining	17 (28)	0
	Tracheal amniotic fluid	9 (15)	0
Reported Dystocia	Total (15)	15 (25)	0
	Internal bleeding (2)	2 (3.3)	
	Diaphragmatic hernia (1)	1 (1.7)	0
	Multiple rib fractures (1)	1 (1.7)	0
Postnatal mortality	Aerated lungs	10 (17)	0
Death *in utero*	Decomposed carcass	4 (6.7)	3 (25)
BVDV infection	BVD virus/antibody detected	2 (3.3)	0
Congenital anomalies	Heart wall defect, cleft palate, hydrocephalus	2 (3.3)	2 (16)
Unknown	No lesions	19 (32)	7 (58)

* Some stillborns had more than one lesion. For example, a stillborn can have both aspirated amniotic fluid and meconium staining

Figure 4.1: Image of an aborted bovine fetus and three stillborn calves collected from a large commercial dairy farm with endemic BVDV infections.

SECTION D: Molecular Epidemiology: Characterization of BVDV in PI calves by nucleic acid sequencing

CHAPTER 5

BOVINE VIRAL DIARRHEA VIRUS: GENETIC ANALYSIS AND SIGNALMENT OF PERSISTENTLY INFECTED DAIRY CALVES IN THE UPPER MIDWESTERN UNITED STATES

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5.1 Introduction

Bovine viral diarrhea virus (BVDV; family *Flaviviridae,* genus *Pestivirus*) is an economically important virus affecting ruminants worldwide. The reproductive, respiratory, gastrointestinal, circulatory, immunologic, lymphatic, musculoskeletal, integumentary and central nervous systems have all been negatively affected by BVDV (Brock, 2004). Cattle persistently infected (PI) with BVDV are an import reservoir for the virus and BVDV control and eradication strategies focus on the detection and removal of PI cattle.

PI cattle occur when a fetus is infected with noncytopathic BVDV during the first trimester of pregnancy (Brownlie et al., 1989; Liess et al., 1987; McClurkin et al., 1984). Fetuses that survive a noncytopathic BVDV infection during the first trimester are immunotolerent to, and persistently infected with, BVDV. If a PI calf is detected and confirmed, the dam of the calf should be tested for persistent infection. If the dam of a PI calf is not herself PI, researching the signalment of the dam can provide additional information about BVDV infections within the herd. Determining the location of the dam of the PI calf during the first trimester of pregnancy will reveal the time and location of fetal infection. When a PI calf is detected, the time and location of BVDV infection of the dam can be retrospectively calculated to a time approximately 5 to 9 months prior to the birth date of the PI calf. The "point-in-time" infection that results in the birth of a PI animal provides information that cannot always be obtained when documenting acute infections by seroconversion in non-PI cattle.

The manifestations of BVDV exposure and infection are expected to be different on dairy farms than on beef farms. In addition to year-round breeding schedules, the dairy industry is experiencing rapid consolidation and specialization. It is not uncommon for a dairy calf to be born, raised, and bred on one or more farms prior to lactation. Off-site calf ranches are becoming more common as dairy farms become larger and these specialized farms often commingle and raise cattle from more than one farm. According to 2007 NAHMS data, at least 22.5% of heifers from large herds (500 or more) were bred off-site and nearly two-thirds (63.8 percent) of farms sent heifers to a raiser where the cattle had contact with cattle from other farms (USDA-APHIS 2007). Failure to separate cattle according to farm origin can result in an increased risk of disease transmission among the commingled animals. The development of large heifer raising facilities will likely lead to an increased risk of dairies purchasing PI replacement heifers or pregnant non-PI heifers gestating PI fetuses (Larson 2005).

Dairy cattle persistently infected with BVDV are rare. Data in chapter 2 revealed that, 8 of 961 (0.8%) dairy calves born on four large dairy farms in Minnesota and California were PI. Obtaining as much information from PI cattle can provide important information in BVDV control strategies. In addition to determining the location of the PI calf's dam during the first trimester, the virus shed by the PI animal can be sequenced and compared to those obtained from other PI cattle and those detected in acute infections. Yet, few animal disease laboratories routinely perform nucleic acid sequencing for BVDV and sequences are rarely documented and compared to PI cattle and acute infections within and between farms.

The purpose of this study was to detect and confirm PI dairy cattle, research the dam's location during the first trimester of gestation, and genetically sequence a portion of the 5' untranslated (UTR) region of viral RNA to determine the prevalence of different genotypes of PI dairy cattle in the upper Midwest.

5.2 Materials and Methods

Only dairy cattle that were confirmed BVDV PI were included in this study. Persistently infected cattle reported in this study had at least two positive test results, at least 3 weeks apart, and were tested with either antigen-capture enzyme-linked immunosorbent assay (ACE) on skin or reverse transcription-polymerase chain reaction (RT-PCR) on skin or serum. The ACE was performed on phosphate-buffered saline (PBS) containing a soaking skin (ear notch) sample. The ACE test was performed according to the instructions outlined in the test-kit package insert provided by the manufacturer (Bovine Virus Diarrhea Antigen Test Kit®, IDEXX Laboratories, Westbrook, ME). The single-tube *Taq*Man RT-PCR test has been described previously (Mahlum et al., 2002). Briefly, RNA was extracted and purified from 200 ul of serum or PBS soak using a commercially available kit (QIAamp 9604 Kit, Qiagen Inc., Valencia, CA). The viral RNA was subjected to reverse transcription and amplification using a *Taq*Man dual-labeled fluorescent probe. Endpoint analysis of the amplified products was performed on an ABI Prism 7000 Sequence Detection System (ABI Prism 7000 Sequence Detection System, Applied Biosystems, Foster City, CA).

Once an animal was determined PI, the farm records were examined to determine the dam of the PI. When available, the dam was tested for PI. The dam's lactation number (1st calf heifer, 2nd lactation cow, etc.) when she bore the PI calf was obtained from farm records.

To further characterize the virus detected in BVDV PI cattle, a 270 base pair segment of the 5' untranslated region (5' UTR) was analyzed. The two sequencing primers were selected from previously published data (Ridpath 2005). The 5' forward primer (5' – CATGCCCTTAGTAGGAC – 3') extends from position 109-125 and the 3' primer (5' – CTCCATGTGCCATGRACAG – 3') extends from position 381-399 of the 5' UTR BVDV1-SD-1 genome. The 5' UTR represents a conserved region of the BVDV genome (Boye et al., 1991; Ridpath et al., 1993). Comparison of the 5' UTR classified the virus into three genotypes; namely, BVDV type 1a, BVDV type 1b and BVDV type 2a (Ridpath 1998). Phylogenetic analysis of BVDV isolates using the 5' UTR region of the genome generates results similar to those using the E2, NS2/3 and N^{pro} genes (Becher et al., 1999; Evermann and Ridpath 2002; Fulton et al., 2000; Pellerin et al., 1994; Ridpath et al., 1994; Stokstad et al., 2004; van Rijn et al., 1997).

Viral RNA for sequencing was extracted from the PBS containing the soaking ear notch or the serum using a commercially available RNA extraction kit (QIAamp Viral RNA Mini Extraction Kit, Qiagen Inc, Valencia, CA), and RT-PCR was conducted utilizing commercially available kits (Qiagen OneStep RT-PCR enzyme mix, buffer and dNTPs, Qiagen Inc, Valencia, CA). Sequencing was performed directly from the ear

notch PBS, serum, and one paraffin-embedded formalin-fixed ear notch (PI calf 31). None of the sequences were performed on viral isolates. The reagents, PCR primers and extracted viral RNA were subjected to thermocycling and a product of approximately 240 base pairs was detected on agarose gel. The RT-PCR products were subjected to Sanger dye-terminator sequencing and were analyzed using an automated sequence machine (ABI 3730 DNA Analyzer, Applied Biosystems, Foster City, CA) at the BioMedical Genomics Center, University of Minnesota. Nucleotide sequences were compared among each other and with published NADL, C24V, Singer and Draper BVDV reference sequences obtained from Genbank by using computer software (Dnastar-Megalign sequence analysis program, Dnastar Inc, Madison, WI).

5.3 Results

Almost all of the PI calves (36/40, 90%) included in this study were shedding a virus similar to the BVDV 1b subgenotype and all 36 1b sequences were greater than 95% similar to each other (Figure 4.1). Three PI calves (4, 15 and 20) were shedding strains similar to genotype 1a, and calf 39 from dairy F was shedding a strain similar to genotype 2a.

Across all dairies, a majority (23/37, 62%) of the BVDV PI dairy calves were offspring of first calf heifers exposed to other growing heifers, and presumably one or more PI heifers, within the non-lactating youngstock population. A minority of PI calves (14/37 38%) were calves from lactating dairy cows in a second or greater lactation where the fetal infection would have occurred in the herd of lactating cows (Table 4.1).

At the herd level, 5 of the 6 dairies with available dam data had PI calves that were the offspring of 1st lactation heifers, 4 of the 6 dairies had PI calves that were offspring of lactating cows, and dairies D, E, and F had PI calves born from both heifers and cows.

Three PI calves were confirmed in dairy A. Viruses from calf 7 and heifer 5 in dairy A had identical sequences over a 240 base pair segment of the 5' UTR and were most similar to the reference Draper 1b subgenotype. On farm records indicated that the dam of PI calf 7 was a heifer of the same age of PI heifer 5 and both the PI heifer and the dam of PI calf 7 were in the same pen during the first trimester of gestation. Therefore, the development of PI calf 7 was the result of direct exposure to PI heifer 5. Calf 4 from dairy A was a 6-month-old calf that was shedding a virus similar to the 1a genotype reference strains.

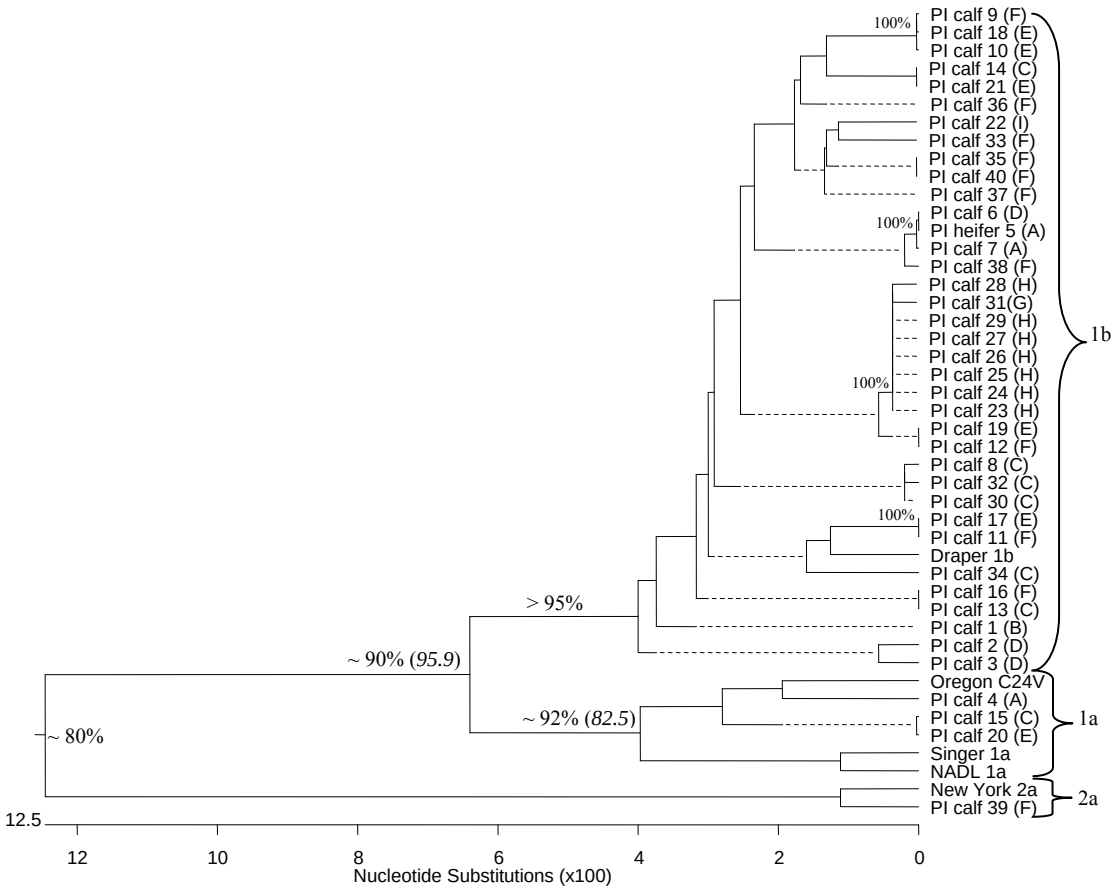


Figure 5.1: Phylogenetic tree constructed from 240 nucleotides of *Bovine viral diarrhea virus* 5' UTR. The dairy from which the PI calf was detected is in parenthesis. The values (%) in normal text at the nodes are taken from the percent identity table and the bootstrap values follow the percent identity values and are in italics and parenthesis.

Table 5.1: Location and dam signalment of 40 PI dairy cattle detected on 9 dairies in the upper Midwestern United States.

Dairy	Location	Total PI calves	Dam = Heifer	Dam = Cow	Calf ID
A	MN	3	3	0	4,5,7
B	MN	1	unknown	unknown	1
C	WI	7	7	0	8,13-15,30,32, 34
D	MN	3	2	1	2,3,6
E	MN	5	1	4	17-21
F	MN	12	10	2	9-12,16,33,35-40
G	IL	1	unknown	unknown	31
H	SD	7	0	7	23-29
I	MN	1	unknown	unknown	22
Total		40	23	14	

Seven PI calves were detected on dairy C. Three of the PI calves (8, 30, 32) were shedding stains similar to the 1b subgenotype that were more than 99.6% similar to each other. Three of the PI calves in dairy C had sequences that were identical to those detected in PI calves from dairies E and F. Calf 14 was shedding a 1b strain that was identical to PI calf 21 in herd E, PI calf 15 was shedding a 1a strain that was identical to calf 20 in herd E, and calf 13 was shedding a strain that was identical to a PI calf 16 in herd F. Dairy C purchased pregnant heifers from multiple sources over multiple years and the dams of the PI calves in dairy C were all first-calf heifers. All newborn calves in dairy C were tested for PI with ACE over three years and none of the lactating cows in dairy C produced a PI calf. Therefore, there was no evidence of BVDV infections within the lactating population of dairy C.

The three PI calves in dairy D were all shedding stains similar to the 1b subgenotype and PI calves 2 and 3 were similar (98.9%) to each other. The virus sequence in PI calf 6 in dairy D was identical to viruses from heifer 7 and calf 5 in herd A. Dairies D and A were both in central Minnesota and had purchased pregnant heifers from suppliers, but neither dairy used a common heifer raiser.

Dairy E had five confirmed PI calves. Four of the PI calves were shedding strains similar to the 1b genotype and one PI calf was shedding a strain similar to the 1a genotype. Three of the four 1b strains were unique, but three viral sequences from PI calves in dairy E were identical to sequences from PI calves detected in dairy F. Viral sequences from calves 10 and 18 were identical to sequences from calf 9 in dairy F. Calf

19 had an identical sequence to calf 12 in dairy F, and calf 17 had a sequence identical to calf 11 in dairy F. Both dairies E and F had many of their heifers raised at multiple custom heifer farms throughout Minnesota.

Dairy F was the largest dairy and had 12 confirmed PI calves. This dairy had multiple heifer raisers and also purchased cattle from multiple sources in multiple states. Eleven of the 12 PI calves were shedding stains similar to the 1b subgenotype and one PI calf was shedding a strain similar to the 2a genotype. Only calves 35 and 40 in dairy F had identical genetic sequences and the 10 remaining 1b strains had sequence identities that ranged in similarity from 96.0 to 99.0% (percent identity table not provided).

All seven PI calves in dairy H had identical sequences over the 270 base pair segment of the 5" UTR and all seven calves were born within three weeks of each other at a South Dakota dairy. The dams of all the PI calves were cows that were purchased as dry cows when the dairy was constructed and multiple cattle were purchased and assembled.

Dairy B had one PI calf that was unique to the other PI cattle and was more the 2.5% different than the remaining strains. The PI calf in dairy B (Figure 4.3) was purchased from a central Minnesota sale-barn and the only information available was that the calf was from a small dairy.

One PI calf was detected in herd I and it had a viral sequence identical to a PI calf 37 in herd F. Herd I was a small dairy in north central Minnesota and would occasionally buy pregnant heifers.

Calf 31 from dairy G was detected in Illinios and was shedding a strain 99.3% similar to the seven calves at dairy H in South Dakota. Additional information from PI calf 31 was not available.

Figure 5.2: Image of persistently infected Holstein bull calf (PI calf #1, dairy B) purchased at a Minnesota auction facility

5.4 Discussion

Genetic sequencing data showed that most PI cattle (36/40, 90%) from these dairies are infected with BVDV strains similar to the 1b subgenotype. This is consistent with previous reports on PI beef cattle in central plains feedlots (Fulton et al., 2006; Fulton et al., 2002). In another study, analysis of 53 BVDV isolates from dairy herds between 1998 and 2001 showed that 40% of the field isolates were genotype 2 and 60% were genotype 1. Of the genotype 1 isolates, 81% were subgenotype 1b (Tajima and Dubovi, 2005). Strains most similar to BVDV genotype 1b appear to persist despite the widespread use of vaccines and most commercially available vaccines do not contain virus similar to the 1b subgenotype.

Like most RNA viruses, BVDV lacks a proof-reading mechanism, resulting in mutations and recombination of the viral genome. Point mutations and recombination of viral RNA can result in novel, unique viruses. Tracking and documenting changes in the virus can provide important information for BVDV control and eradication programs. Genetic sequencing of the highly conserved 5" UTR can also be a useful tool at the farm level when attempting to eliminate BVDV PI cattle from a herd. If the herd tests and removes all PI cattle but infections are discovered at a later date, the viral sequences of the 5' conserved region of the genome can be analyzed to determine if the original strain persisted, or if a new virus was introduced in the herd. The 5" UTR region appears highly conserved. In dairy A, heifer 7 had infected the dam (cohort heifer) of PI calf 5

and the genetic sequence remained identical throughout the entire gestation and birth of PI calf 5.

Genetic sequencing can also be used to determine if a farm has more than one genotype of the virus. Genetic analysis of the 40 PI cattle on these dairies revealed that if a farm had more than 1 PI calf, they likely had calves that were shedding unique viruses. Dairy H was the only dairy where all seven calves had identical genetic sequences over a 240 base pair segment of the 5' UTR. Not all cattle were tested in dairy H leaving open the possibility that there may have been other virus subtypes.

The dams of most of the PI calves (23/37, 62%) were heifers and dairies C, D, E and F had used one or more custom heifer raisers to raise and breed heifers. In addition to using heifer raisers, all four dairies had reported buying pregnant heifers from suppliers. Dairies C, D, E and F reported that the heifer raisers had commingled cattle from other farms. Commingling animals from multiple sources is not an unusual practice for heifer raisers. One publication reported that off-site heifer facilities rarely separated heifers according to farm of origin during the rearing period (Wolf 2003). This information suggests that the risk of exposure to BVDV, presumably from another PI animal, is greater in a youngstock heifer population than in a lactating cow population.

When heifer raising farms commingle cattle from two or more farms and they do not have a PI testing protocol, a lactating dairy farm can potentially send a non-PI calf to a heifer raiser for rearing and breeding where she can then become pregnant, infected, and gestate a PI fetus. If an original lactating dairy was free of PI cattle, the calves from

heifers raised at an off-site farm can be a source of PI calves. If the dairy does not test for PI and retains ownership of a PI calf, a PI calf has the potential to return to a heifer rearing facility, become pregnant, and enter the lactating herd. Even if the PI calf fails to become pregnant and lactate, it has the potential to sustain infections within the youngstock population by exposing early gestation heifers to BVDV and making more PI calves within the pregnant heifer population. In this manner, PI calves would need to only live to breeding age to sustain infection within the youngstock population resulting in the birth of more PI calves.

In summary, most PI calves detected on these large Midwest dairies shed a BVDV similar to the 1b subgenotype and the highest risk for infection and the subsequent birth of PI calves was in the youngstock heifer population. Genetic sequencing documented that many PI calves had highly similar or, in 8 separate sets of calves on 5 different dairies, identical sequences over the conserved region of the 5' UTR of the BVDV genome.

SECTION E: Quantification of BVDV RNA copies in clinical samples obtained from PI calves using quantitative RT-PCR

CHAPTER 6

QUANTITY OF *BOVINE VIRAL DIARRHEA VIRUS* RNA IN CLINICAL SAMPLES OBTAINED FROM A COHORT OF NATURALLY EXPOSED PERSISTENLY INFECTED CALVES USING QUANTITATIVE RT-PCR

Introduction:

An ear notch skin sample is a convenient tissue for detection of *Bovine viral diarrhea virus* (BVDV) persistently infected (PI) cattle because it is easy to collect and requires minimal supplies and equipment. Ear notch skin also offers the flexibility of testing by immunohistochemistry (IHC), reverse-transcriptase polymerase chain reaction (RT-PCR), and antigen-capture ELISA (ACE). All three tests have high sensitivity and specificity for detecting PI cattle (Cornish et al., 2005; Fulton et al., 2006; Grooms and Keilen 2002; Njaa et al., 2000), and none appear to be negatively affected by passively acquired antibodies. ACE on fresh ear notch appears to be the most robust test for PI detection across many Veterinary Diagnostic Laboratories (Edmondson et al., 2007).

Pooling ear notch phosphate-buffered saline (PBS) supernatant for RT-PCR is a popular method to screen many cattle at a reduced cost. The pooling method usually involves soaking the ear notch skin samples in a small amount (approximately 1-2 ml) of PBS and pooling a portion of the supernatant for RT-PCR testing (Kennedy et al., 2006). If the pooled supernatant tests positive by RT-PCR, the samples are then tested individually. While ear notches have become the sample of choice for PI testing, there is minimal information on the quantity of viral RNA copies in the PBS supernatant containing the soaking skin and how the skin sample compares to blood, serum, or a nasal swab from the same animal. Constructing pools without data on the quantity of RNA copies in clinical samples is a significant limitation when attempting to determine pool size.

Many conventional RT-PCR tests are only qualitative. Quantitative RT-PCR assays can be used to determine the initial template concentration by monitoring the progress of the reaction in real-time. The TaqMan real-time system detects PCR products using the 5' nuclease activity of Taq DNA polymerase on fluorogenic DNA probes during each extension cycle. The TaqMan probe contains a fluorescent reporter dye at the 5' end and a quencher dye at the 3' end. The quencher dye in the intact probe prevents the emission of the reporter dye. If the target sequence is present, the probe anneals to the target and is cleaved by the 5' nuclease activity of Taq DNA polymerase during primer extension. Cleavage of the probe separates the reporter from the quencher dye and reporter dye fluorescence increases as a function of cycle number. The cycle number at which the reporter dye fluorescence passes a fixed threshold above baseline is the threshold cycle (Ct). The greater the initial concentration of the target RNA (or DNA), the sooner a significant increase in fluorescence is observed (McBride et al., 1998; Reischl and Kochanowshi 1999; Williams et al., 1998). Using the standard curve generated with a target RNA sequence of known copy number, the starting amount of target RNA in an unknown sample can be calculated. With this in mind, quantitative RT-PCR has two prerequisites: (1) a standard curve demonstrates the range over which PCR product yield provides a reliable measure of mRNA input, and (2) testing of numerous samples allows for statistical analysis of differences in PCR product yield (Halford 1999).

The purpose of this study is to develop a quantitative real-time RT-PCR (qRT-PCR) for quantification of BVDV RNA in four diagnostic samples obtained from a

cohort of naturally exposed PI cattle. Data obtained from these calves will give an estimation of the quantity of detectable RNA in clinical samples. Serum, whole blood, nasal swabs and ear notch skin samples were collected from a cohort of live PI cattle over 8 months, extracted within 2 days of collection, and analyzed by qRT-PCR at the completion of sample collection. The data collected from this study provides an estimation of RNA copies in clinical specimens obtained from PI calves and attempts to document changes in virus quantity over an 8 month sampling period.

Materials and Methods:

A DNA product of 199 base pairs was generated from a BVDV isolate obtained from National Veterinary Services Laboratories (NADL BVDV type 1a) by a single-step RT-PCR reaction. The PCR product was confirmed and visualized on an ethidium bromide-stained agarose gel and purified with a commercially available kit (Qiagen QIAquick PCR purification Kit Cat. No. 2806). The purified RT-PCR product was cloned in to a plasmid vector PCRII-TOPO according to manufacture's specifications (Invitrogen TOPO-TA cloning kit for sequencing; Invitrogen). The recombinant plasmid was incubated in and propagated from competent *Escherichia coli* TOP10F' cells.

The recombinant plasmid DNA was purified from the TOP10 F' *Escherichia cells* using a commercial kit (Qiagen QIAprep Spin Miniprep Kit Cat. No. 27106). The copy number per volume for the purified plasmid was calculated from the concentration of extracted DNA quantified by spectrophotometry (NanoDrop 1000). A standard curve was calculated from 10-fold serial dilutions of recombinant purified standard plasmid

DNA in an estimated range of 10^{-1} to 10^{6} copies/ul. The minimum detection limit using the plasmid template was defined as the lowest plasmid copy number producing a threshold cycle significantly different from reactions with no template DNA. The minimum detection limit was determined to be approximately 50 – 100 copies/ml of PBS supernatant, buffy coat extract and serum.

Seven PI dairy calves obtained and isolated from a dairy were sampled 10 times from February 9 through October 18, 2009. The seven PI heifer calves were born within 2 weeks of each other. The samples were collected approximately every 3 to 4 weeks and the calves were 6 months old on the first sample date and 14 months old at the completion of the study. The virus shed by all calves had identical nucleotide sequences over a portion of the 5' UTR. The virus sequence was compared to BVDV reference strains obtained from GenBank and was >95% similar to the Draper BVDV 1b subgenotype reference strain.

Samples collected from the PI calves included a whole blood sample collected in an EDTA blood tube, a blood sample that was allowed to clot and centrifuged for serum, a triangular ear notch approximately 5 mm from the base of the haired ear margin to the cut tip obtained from the lower ear margin, and a cotton nasal swab collected from one nostril. The ear notch skin and cotton nasal swab were placed in 1 ml of PBS and all samples were packaged in a cooler with frozen ice packs and taken to the Minnesota Veterinary Diagnostic Laboratory (MVDL) for RNA extraction and storage at -80 C. Buffy coats were removed from the whole blood samples by Ficoll separation and 200 ul

of each sample was extracted using a commercially available kit (QIAamp 9604 Kit, Qiagen Inc., Valencia, CA). All samples were stored at -80 °C until tested by qRT-PCR. A single-tube *Taq*Man RT-PCR (Mahlum et al., 2002) was performed on all samples during one week. Briefly, the viral RNA was subjected to reverse transcription and amplification using a *Taq*Man dual-labeled fluorescent probe. Analysis of the amplified products was performed on an ABI Prism 7000 Sequence Detection System (ABI Prism 7000 Sequence Detection System, Applied Biosystems, Foster City, CA). Absolute quantitative values were converted to the $\log_{10}$ scale and analyzed with statistical software (SAS MIXED PROC).

Additional validation on pooling was accomplished by collecting supernatant from negative ear notch skin samples routinely submitted to the MVDL. Twenty five ul of ear notch supernatant from the PI calves was added to 1.25, 2.50, and 5.0 ml of supernatant from test negative calves to generate pools of 1:50, 1:100, and 1:200, respectively.

Results:

All 70 buffy coats, ear notch and nasal swab supernatant samples tested positive by qRT-PCR (100%, 95% confidence interval .95 – 1.0). Sixty nine of the seventy serum samples tested positive by qRT-PCR (99%, 95% confidence interval .93 – .99). The mean $\log_{10}$ RNA copies in one ml of ear notch, nasal swab supernatant, and buffy coat extract were 4.67, 5.20 and 4.93, respectively. The mean $\log_{10}$ RNA quantity in serum was 4.48. There were no cattle that served as negative controls. The negative control for

RNA extraction was fetal horse serum and the negative control for qRT-PCR was purified water. All negative controls used in the extraction methods or qRT-PCR tested negative.

Calves 1 and 2 had significantly lower ($p<0.01$) $\log_{10}$ RNA copies in the ear notch supernatant and buffy coat samples than calves 3 – 7 (Table 6.1). The mean $\log_{10}$ RNA copies in the ear notch supernatant were 3.92 and 3.78 in calves 1 and 2, respectively, and the mean $\log_{10}$ RNA copies in the ear notch supernatant from the other five calves ranged from 4.76 to 5.35. The mean $\log_{10}$ RNA copies in buffy coat extract from calves 1 and 2 were also significantly lower ($p<0.01$) at 3.88 and 4.25, respectively. Buffy coat extract mean $\log_{10}$ RNA copies in the five other calves ranged from 5.15 to 5.39. The mean standard deviations across all calves and all samples were highest in the buffy coat extracts (±0.84) followed by ear notch supernatant (±0.78), nasal swab supernatant (±0.75), and serum (±0.63) (Table 6.1).

During the 8 month sampling period, there was a slight, statistically significant decrease in viral RNA in the ear notch supernatant and buffy coat extract across all calves. Ear notch supernatant RNA decreased 0.05 $\log_{10}$ RNA ($p = 0.01$) and buffy coat extract decreased 0.10 $\log_{10}$ RNA ($p < 0.0001$). There was no significant increase or decrease of viral RNA in the serum or nasal swab samples over the 8 months. Serum RNA decreased 0.014 $\log_{10}$ ($p = 0.38$) and nasal swab decreased 0.01 $\log_{10}$ RNA ($p = 0.62$) (Figure 6.1).

Ear notch, nasal swab supernatant, and buffy coat samples produced higher mean $\log_{10}$ RNA copies, wider ranges and higher standard deviations than serum. The range for ear notch supernatant was 2.23 to 5.89 $\log_{10}$ RNA copies / ml, the range for nasal swab supernatant was 2.95 to 6.37 $\log_{10}$ RNA copies / ml, and the buffy coat samples ranged from 3.42 to 6.60 $\log_{10}$ RNA copies / ml. The range in 69 of the 70 positive serum samples was 3.93 to 5.30 $\log_{10}$ RNA copies per ml. A serum sample from calf 4 collected on the test collection date tested negative.

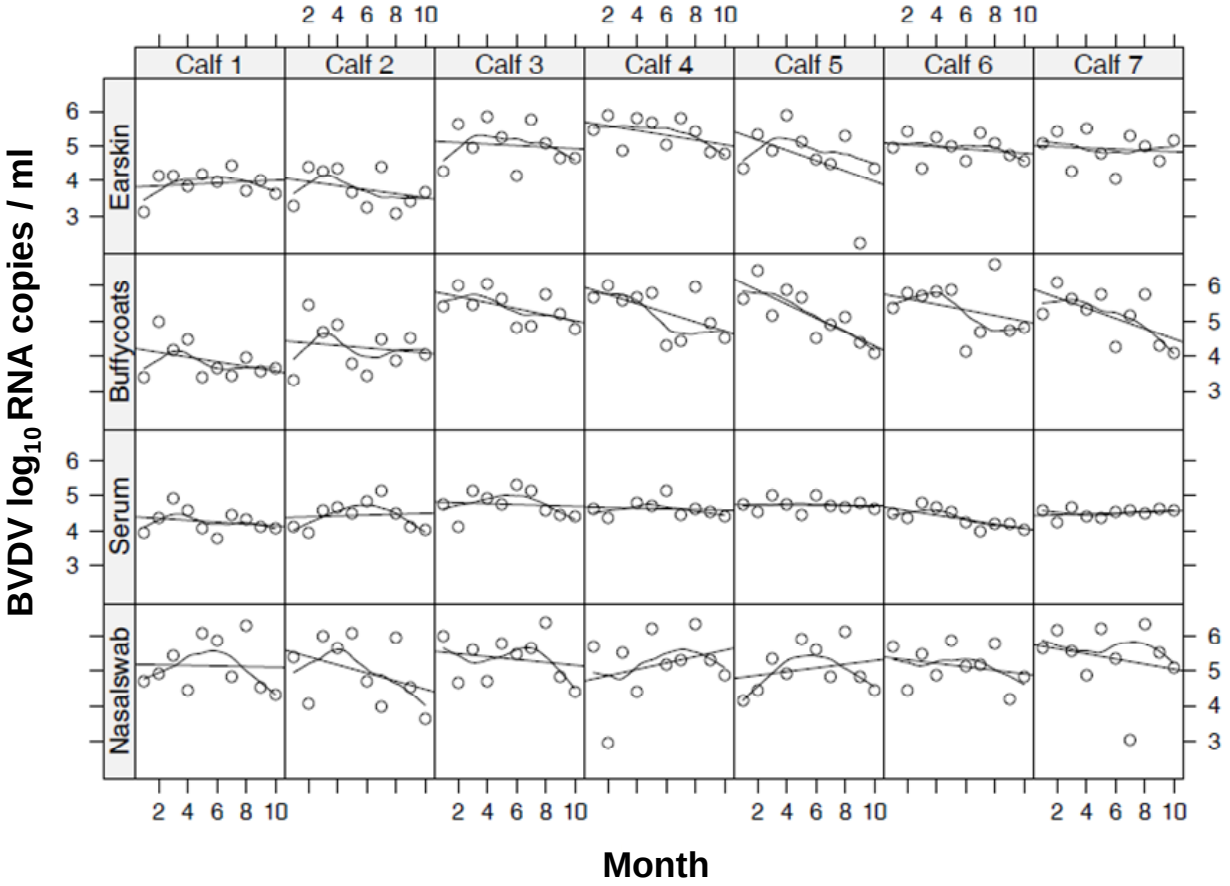
Pools generated with negative ear notch supernatant and spiked samples from the PI calves produced positive results in 209 of 210 of pools. All seventy pools of 1:100 and 1:200 tested positive. Sixty nine of 70 pools at 1:50 tested positive by RT-PCR. One pool of 1:50 tested negative.

Table 6.1. Mean, standard deviation (SD), and range of log_{10} RNA in one ml of supernatant (ear notch skin & nasal swab), Ficoll buffy coat extract, and serum obtained from 7 PI calves every 3-4 weeks over 8 months.

	BVDV log_{10} RNA / ml of fluid			
	Ear notch supernatant	Buffy coat extract	Nasal swab supernatant	Serum
All calves, all samples:				
Mean ± SD	4.67 ± 0.78	4.93 ± 0.84	5.20 ± 0.75	4.48 ± 0.63
Range: Min.-Max.	2.23 - 5.89	3.42 - 6.60	2.95 - 6.37	0[a] - 5.31
Individual calves:				
Calf 1: Mean ± SD	3.92[b] ± 0.35	3.88 [b] ± 0.52	5.14 ± 0.72	4.37 ± 0.33
Calf 2: Mean ± SD	3.78 [b] ± 0.50	4.25 [b] ± 0.68	5.00 ± 0.92	4.45 ± 0.39
Calf 3: Mean ± SD	5.03 ± 0.60	5.39 ± 0.47	5.34 ± 0.65	4.76 ± 0.37
Calf 4: Mean ± SD	5.35 ± 0.44	5.30 ± 0.67	5.17 ± 0.97	4.17 ± 1.48
Calf 5: Mean ± SD	4.76 ± 1.02	5.18 ± 0.74	5.15 ± 0.58	4.74 ± 0.18
Calf 6: Mean ± SD	4.93 ± 0.37	5.35 ± 0.74	5.24 ± 0.46	4.36 ± 0.27
Calf 7: Mean ± SD	4.92 ± 0.48	5.15 ± 0.71	5.37 ± 0.96	4.51 ± 0.13

[a] One serum sample from calf 4 tested negative on the third test day (3/22/2009)
[b] Significantly lower than calves 3 – 7 ($p < .01$)

Figure 6.1. Comparison of the $\log_{10}$ BVDV RNA in ear notch PBS, buffy coat extract, serum and nasal swab PBS on 10 collection dates in 7 PI calves over 8 months. RNA in ear notch supernatant decreased 0.05 $\log_{10}$ ($p = 0.01$) and buffy coat extract decreased 0.10 $\log_{10}$ ($p<0.0001$) over the 8 month sampling period. Serum and nasal swab supernatant did not significantly decrease.



Discussion:

Viral quantification can provide valuable information both in viral diagnostics and pathogenesis research. In this study, the goal was to estimate the quantity of BVDV RNA copies in four diagnostic samples collected from seven PI calves over 8 months. In addition to diagnostic and pathogenesis research, estimating the quantity of RNA copies in diagnostic samples can help determine the limitations of pooling diagnostic samples. In these BVDV PI calves, the quantity of $\log_{10}$ RNA across all samples ranged from 2.23 to 6.60 and was higher, but more variable, in the cell-rich heterogeneous ear notch, nasal swab supernatant and buffy coat extract when compared to serum.

Only a few studies have documented the quantity and variability of virus in clinical samples obtained from PI cattle. Variation of virus quantity in serum from 7 PI cattle ranged from 5×10^3 to 5×10^5 and decreased by 1 to 10-fold over at least a 2-year period (Brock et al., 1998). The decrease in serum virus in that study was associated with the development of virus neutralizing antibody. The development of virus neutralizing or passively acquired antibodies in BVDV PI cattle and its effect on viral BVDV RNA copies in skin samples is likely negligible on the assay because RT-PCR is not affected by the presence of antibody. Yet, the quantity and attendant variability of virus in skin, whole blood and nasal swabs and the effect antibody might have on the overall virus burden was not examined.

The diagnostic samples collected from the seven PI calves in this study consistently produced high quantities of detectable RNA. Variability in virus quantity in the ear notch, buffy coat and nasal swab samples was expected due to variability when pipetting small aliquots (200 ul) of a heterogeneous sample for extraction. Variation in the proportions of pipetted blood, epithelial cells, mucus and bacteria in the diagnostic sample will result in variability of detectable RNA. Endogenous and exogenous substances in ear notch supernatant, such as hair, enzymes, ribonucleases (RNases), feces, and bacteria can also affect RT-PCR efficiency and generate variability (Akane et al., 1994; Izraeli et al., 1991). Also, the use of a plasmid vector clone and the dilution series used to generate a standard curve gives a good estimate of the efficiency of the PCR assay, but, more importantly, the generation of a standard curve does not tell us anything about the effect of the matrix of the diagnostic sample. Laboratory materials, such as EDTA and Ficoll reagents used to extract buffy coats from whole blood, are complex and may contain inhibitory substances that were not present in the purified plasmid template used to generate the standard curve. Variation was expected because absolute qRT-PCR tests suffer from inter-tube variations and qRT-PCR data should be interpreted as semi-quantitative (MacKay et al., 2002).

Although all calves were from the same farm, of similar age, and shed a virus that was identical over a portion of the 5' UTR, calves 1 and 2 had an approximately 1 $\log_{10}$ lower mean RNA levels in the ear notch and nasal swab supernatant than the 5 other

calves. The reason for lower ear notch supernatant and buffy coat extract in calves 1 and 2 is not known, but one could speculate that the time of fetal infection and fetal immunocompetence may impact viral persistence. If the time of fetal infection and immunocompetence influence viral load in PI calves, we would expect all samples collected from PI calves to be lower. Yet, the quantity of viral RNA in serum and nasal swab supernatant was similar across all calves.

In addition to lower RNA in the ear notch supernant and buffy coat extract in calves 1 and 2, the quantity of RNA in ear notch supernatant and buffy coat extract decreased in all calves over the 8 month sampling period. The drop in detectable virus in these two samples was small (0.05 and 0.10 $\log_{10}$ RNA) and has no clear explanation at this time. Nevertheless, the samples collected from these PI calves routinely produced high levels of detectable RNA. When compared to a few other studies, the quantity of BVDV in serum collected from PI cattle was similar using $CCID_{50}$ virus isolation. In one study of 7 PI cattle, the quantity of serum virus titers ranged from 10^3 - 10^5 / ml (Brock et al., 1998). Variation in viral serum concentration has also been reported in other studies and ranged from of 10^4 – 10^6 $CCID_{50}$/ml (Brock et al., 1990; Palfi et al., 1993; Paton et al., 1994). To the author's knowledge, this is the only study that quantified detectable RNA in four routinely collected diagnostic samples obtained from a cohort of naturally exposed PI calves shedding a similar virus.

In summary, buffy coats, serum, ear notch and nasal swab supernatant contains, on average, 10^4 to 10^5 BVDV RNA copies / ml and is much higher than the minimal

detection limit of this qRT-PCR assay (10^1 RNA copies /ml). The buffy coat, ear notch and nasal swab samples are heterogeneous diagnostic samples and contain endogenous and exogenous substances that appear to produce wider variations in quantitative results when compared to serum. Nevertheless, the seven PI calves in this study consistently produced high levels of RNA in routine diagnostic samples that can be consistently detected individually and when pooled.

CHAPTER 7

COMPARISON OF TWO EAR NOTCHING DEVICES FOR THE DETECTION OF *BOVINE VIRAL DIARRHEA VIRUS* RNA BY QUANTITATIVE RT-PCR

Presented as a poster at the 4th U.S. BVDV Symposium:
BVDV Variability: Impact on Virulence, Host Range and Control

Phoenix, Arizona, January 2009

7.1 Introduction:

Skin from an ear notch is a convenient tissue for detection of BVDV PI cattle because it is easy to collect and requires minimal supplies and equipment. Pooling ear notch phosphate-buffered saline (PBS) supernatant for RT-PCR testing is a popular method to screen groups of animals at a reduced cost. The pooling method involves soaking the ear notch in PBS, pooling the supernatant, and testing the pooled supernatant for BVDV by RT-PCR (Kennedy et al., 2006). If the pool is positive, the originally submitted samples can be tested individually to determine the PI animal.

While ear notches have become the sample of choice for PI testing, there is minimal information on the quantity of viral RNA in skin (ear notches) and the PBS supernatant that contains the soaking sample. Pooling procedures have not been extensively validated and are noted to be different among laboratories. The purpose of this study was to compare two sampling devices and quantify BVDV RNA in the PBS supernatant containing the skin sample. All samples were also individually tested by antigen-capture ELISA (ACE).

7.2 Materials and Methods:

A quantitative RT-PCR (qrt-PCR) was developed. Briefly, a DNA product of approximately 199 base pairs was generated from a BVDV type 1a isolate (NADL) from a single-step RT-PCR reaction (Mahlum et al., 2002). The purified RT-PCR product was

then cloned to a plasmid vector according to manufacturer specifications (TOPO-TA cloning kit for sequencing; Invitrogen). After incubation, propagation, and purification, a copy number of the purified plasmid was calculated from the concentration of extracted DNA by spectrophotometry (NanoDrop). A standard curve was calculated from 10-fold serial dilutions of plasmid DNA in an estimated range of 10^{-1} to 10^{6} copies/ul. The minimum detection limit using the plasmid template was defined as the lowest plasmid copy number producing a threshold cycle significantly different from reactions with no template DNA.

Ear notches were collected from seven PI calves on three dates, every three weeks (Figure 7.1). The circular skin sample (Figure 7.2) was harvested from the upper ear margin and placed in 0.5 ml of PBS. The triangular skin sample was harvested from the lower ear margin and placed in a sterile glass tube containing 2 ml PBS. The circumference and length of fresh cut skin for the circular and triangular notch were 1.99 cm and 2.60 cm, respectively. The ratio of fresh cut skin to PBS in the circular and triangular notch were 3.99 and 1.3 cm of fresh cut in one ml of PBS, respectively. The skin samples were soaked in PBS for 24 hours after which 200 ul of PBS was extracted and analyzed by qrt-PCR.

Figure 7.1: Image of skin samples collected from one of seven BVDV PI calves. The circular Prosampler sample was taken from the upper ear margin and the triangular sample was taken from the lower ear margin on the right ear of each calf.

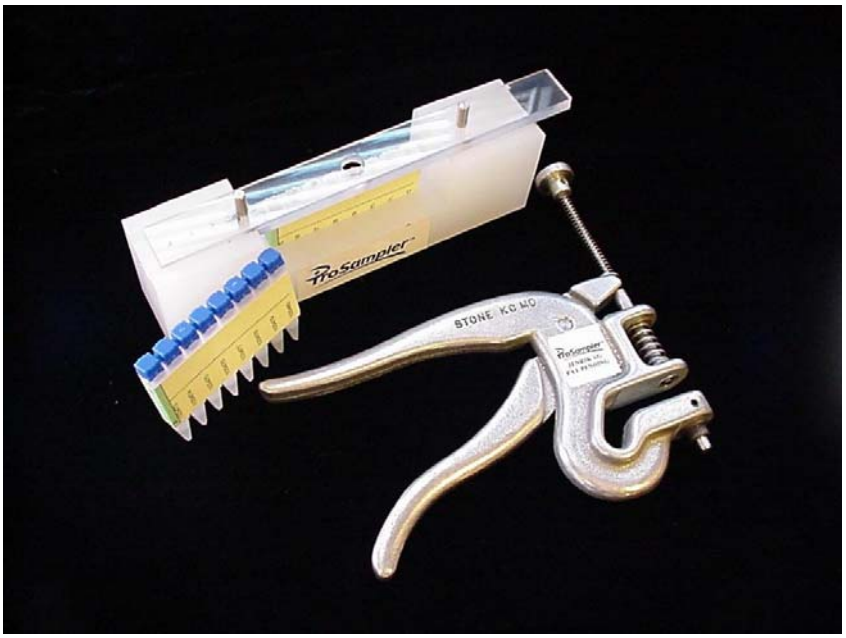


Figure 7.2: Prosampler pliers and collection vials (clip) containing 0.5 mls PBS

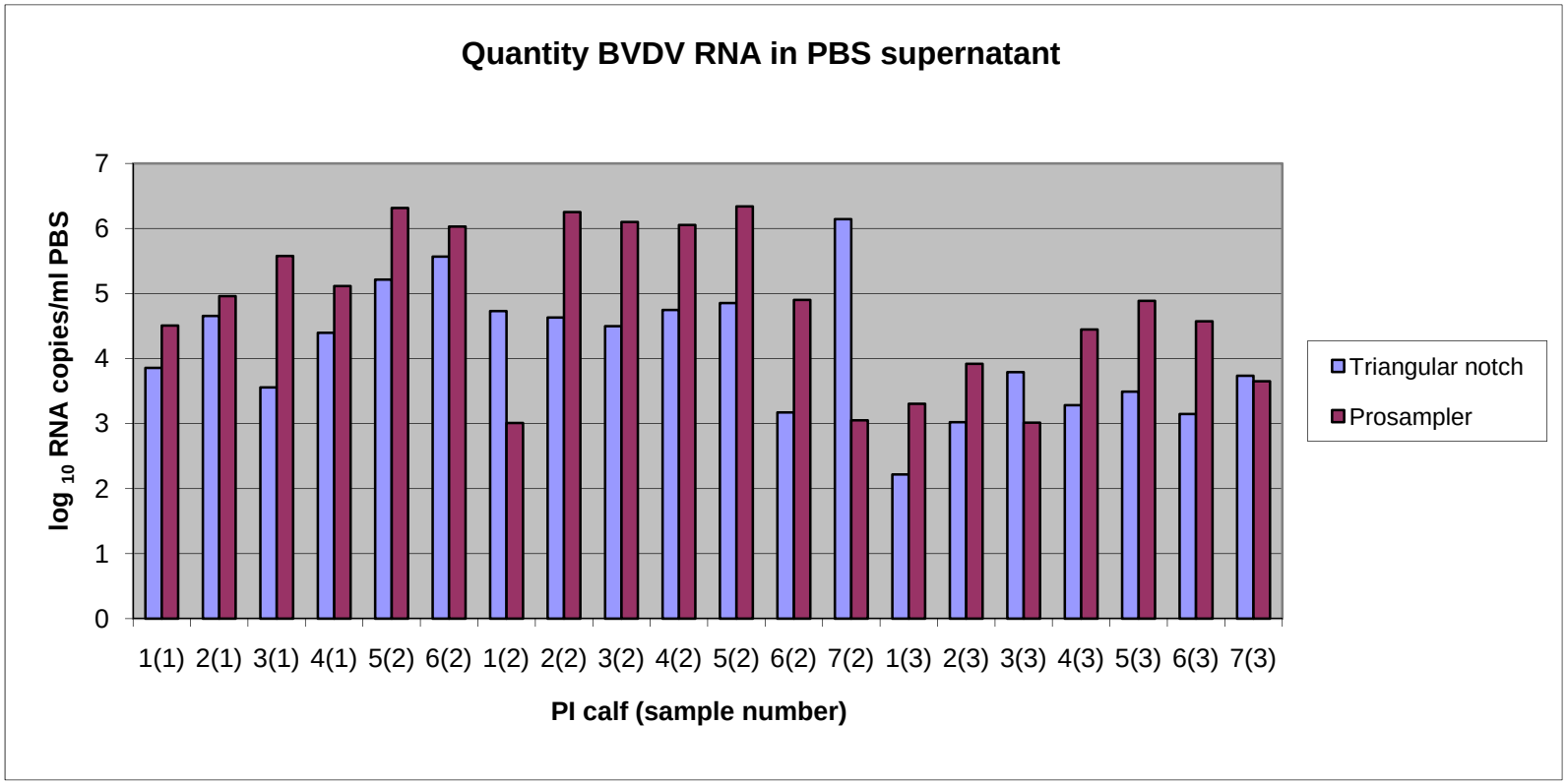


Figure 7.3: Quantity of BVDV RNA in a circular ear sample (Prosampler) and a triangular notch in PI calves as determined by quantitative RT-PCR

7.3 Results and Conclusion

The quantity (log_{10}) of BVDV RNA in the PBS collected from the circular notch (Prosampler) was approximately one log_{10} higher than the PBS collected from the triangular notch (Figure 7.3). The average log_{10} RNA in the PBS containing the circular notch and triangular notch were 4.8 and 4.1, respectively ($p=0.015$). All samples from both sampling devices tested positive by ACE (Bovine Virus Diarrhea Antigen Test Kit®, IDEXX Laboratories, Westbrook, ME). These data suggest that sampling location and the ratio of cut skin to PBS influences the amount of detectable RNA as determined by quantitative RT-PCR.

Concluding Summary:

Rapid consolidation of the dairy industry will increase the odds that a dairy farm, or farms, will purchase BVDV PI cattle. One challenge for bovine veterinarians and livestock producers is that there is a lack of simple, cost-effective strategies to determine endemic BVDV infections in large dairy herds that routinely vaccinate for BVDV. Attempting to demonstrate seroconversion in a subset of cattle by testing paired sera (acute and convalescent) is confounded by vaccination. Non-vaccinated sentinel calves have been used with marginal success and require that a every animal on the farm have direct contact with the nonvaccinated sentinel to detect infection. Using highly sensitive antigen or nucleic acid detection tests to detect PI cattle can be used to determine herd status, but many cattle would have to be tested to achieve a high level of confidence that the herd is free of BVDV infections and PI cattle because such animals often represent less that 1% of the cattle in a the herd. Newborn calves can serve as useful sentinels when sampled for BVDV antibodies prior to colostrum feeding and the data in chapter 2 suggests that there are approximately 8 calves seropositive for BVDV as compared to those that are BVDV PI. Screening newborn calves and documenting fetal seroconversion is a much more efficient method than screening for PI when attempting to determine herd status.

Chapter 3 describes natural BVDV infections, transmission and test performance in a large commercial dairy herd. This chapter is only one of a few case reports that described the use of precolostral screening, RT-PCR, and nucleotide sequences to

describe and document natural BVDV infections in a large commercial dairy herd. Detection and characterization of viral infections in this herd were consistent with previous research describing BVDV pathogenesis and control. This chapter was found to be a useful example of BVDV detection and control for veterinarians and producers who have implemented, or are considering, BVDV control programs.

An apparent increase in stillborn dairy calves throughout the dairy industry prompted an investigation into the stillborn calves obtained from the dairy herd described in chapter 3. Although endemic BVDV infections and PI cattle were documented in the herd, fetal infections were not overrepresented in the aborted fetuses or stillborn calves. A majority of the stillborn calves in this herd had features of anoxia (aspirated amniotic fluid, meconium staining, etc). In this herd, endemic BVDV infections did not result in increased fetal loss.

Like most RNA viruses, BVDV lacks a proof-reading mechanism and mutations and recombination of the viral genome is expected. Tracking the changes is important for BVDV control and eradication programs. In the Midwest, most of the BVDV PI calves are shedding a subgenotype similar to the Draper 1b reference strain and most PI calves are the offspring of heifers indicating that BVDV infections appear more common in heifer youngstock populations than adult lactating cow populations. Investigating the possible inclusion of a 1b subgenotype stain in a vaccination program appears justified.

Because PI cattle are rare and the odds of finding a PI animal in a group of cattle is unlikely, the fees associated with testing are often not justified by producers and veterinarians. One method to reduce fees is to pool samples and test the pool for BVDV

with RT-PCR. Although a few studies have shown that pooling will efficiently detected PI cattle, there is a lack of information on the quantity of viral RNA in clinical samples. The data reported in chapter 6 quantified and described the amount of detectable RNA in a cohort of seven, naturally exposed PI cattle. In that group of PI calves, ear notch skin, buffy coats, serum and nasal swabs generate large amounts of RNA that was easily detected by the qRT-PCR test when tested individually and pooled.

In summary, the bovine industry has many methods and assays to detected BVDV infections and BVDV PI cattle. When used corrected, they appear highly effective and appear to complement each other. As farm consolidation continues, we will need these diagnostic tools and methods to control, and potentially eliminate, BVDV from the North American cattle herd.

SECTION F: REFERENCES

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