DISSERTATION

CONTROL AND ERADICATION METHODS FOR BOVINE VIRAL DIARRHEA VIRUS

Submitted by

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WE HEREBY RECOMMEND THAT THE DISSERTATION PREPARED UNDER OUR SUPERVISION BY SUSAN MICHELLE CLEVELAND ENTITLED CONTROL AND ERADICATION METHODS FOR BOVINE VIRAL DIARRHEA VIRUS BE ACCEPTED AS FULLFILLING IN PART REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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ABSTRACT OF DISSERTATION

CONTROL AND ERADICATION METHODS FOR BOVINE VIRAL DIARRHEA VIRUS

The objectives of this research were 1) to determine the efficacy of an antigen-capture (AC)-ELISA and a micro-titer virus isolation (MTVI)-ELISA in identifying persistently infected (PI) animals from pooled samples; 2) evaluate the economic benefit of pooling samples for PI animal identification; 3) examine the merit of a diagnostic assay able to identify PI animals *in utero*; and 4) to develop an indirect capture ELISA capable of identifying PI animals *in utero*.

To reduce the cost of whole herd screening for BVDV PI animals, the sensitivity and specificity of an AC-ELISA and a MTVI-ELISA using saline from ear notch samples or pooled serum was determined. Pooled saline from ear notch samples, assayed by AC-ELISA, gave a sensitivity and specificity of 98% and 94%, respectively for pools containing two samples and 72% and 100%, for pools of five. The sensitivity of pooled ear notch or serum samples for bovine viral diarrhea virus detection by MTVI-ELISA (sensitivity <5%) or serum samples for detection by AC-ELISA (sensitivity <15%) was found to be too low for use in whole herd screening. Pooling saline from ear notch samples from two animals tested by antigen-capture ELISA, however, could provide a less expensive, reliable method for whole herd screening for bovine viral diarrhea virus.

To assess the economic benefit of using pooled saline from ear notch samples for AC-ELISA, a simulation model (BTMSim$) was used to determine the cost per cow for

whole herd screening and time to BVDV eradication. Identification and removal of PI animals was simulated for years 1, 2 or 3 after BVDV introduction. Simulation results indicate the time to BVDV eradication could increase by one year when using pools of 2 or 3 and may never be achieved using pools of 4 or 5. Simulation herds where BVDV infection was becoming endemic, i.e. 3 years after initial introduction of BVDV, resulted in significantly lower testing costs when pooling samples than when testing individual animals.

Persistently infected animals can transmit BVDV as soon as they are born. Therefore, detection of PI animals *in utero* could greatly benefit cattle producers. To evaluate the impact of PI animal identification *in utero* a BVDV transmission model, BTMSim, was modified to examine the efficacy of a diagnostic test able to identify PI animals *in utero*. Simulation results from BTMSim_inUtero indicate that identification of PI animals *in utero* does not decrease the number of years required to test for PI animals in order to eliminate BVDV from a herd, nor does earlier detection decrease the median number of PI animals born. While use of an *in utero* diagnostic assay may not result in elimination of BVDV transmission sooner than using assay methods currently available, identification of replacement heifers carrying PI calves, prior to introduction to a herd would still be useful in preventing the spread of BVDV.

Currently, there are no diagnostic assays able to identify PI animals *in utero*. An indirect capture ELISA to detect anti-BVDV IgA in the nasal secretions of cows was developed in an attempt to identify BVDV PI fetuses *in utero*. The concentration of IgA, based on optical density (OD) values, in nasal secretion samples from BVDV unexposed and BVDV exposed animals was determined. Using ROC curve analysis, a threshold OD

of 0.12 gave an optimal sensitivity of 65% and a specificity of 80%. To determine if IgA levels in the nasal secretions of cows carrying BVDV persistently infected (PI) fetuses were greater than those of cows not carrying PI fetuses, the concentration of anti-BVDV IgA present in the nasal secretions of 143 cows at 7 to 8 months gestation was obtained. Of the 143 cows, 19 had IgA concentrations 2-fold greater than the positive control. Included in those 19, was a cow carrying a PI fetus.

This research shows that there are varying methods for identifying PI animals to eradicate BVDV from a herd. In addition to current methods of identification, the research identifies opportunities for future development of more efficacious BVDV diagnostic tools.

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DEDICATION

I dedicate this dissertation to the two most important people in my life, my daughter Maisie, who inspires me everyday and my husband Matthew who is my greatest source of joy, peace and love.

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CHAPTER I

REVIEW OF LITERATURE

Prevalence and economic impact of BVD in the United States

The first description of bovine viral diarrhea (BVD) was published over 50 years ago in North America when outbreaks of cattle suffering from diarrhea and erosive lesions of the digestive tract were observed (Olafson et al., 1946). The first cases were identified in a large, recently assembled dairy herd in New York State. Initially, feed contaminants or winter dysentery (infection with an enteric coronavirus resulting in watery diarrhea) were thought to be the cause of disease in the cattle (Olafson et al., 1946). After further examination of the animals and feed, it was decided that the disease was caused by a previously unidentified agent; possibly a virus. Olafson et al., (1946) were successful in reproducing disease in susceptible animals exposed to feces, blood and splenic emulsions from infected animals.

In the 1950s, a "new" disease of cattle was observed in Iowa termed mucosal disease (MD) (Ramsey et al., 1953; Goens, 2002). In 1957 a cytopathic virus was isolated from a case of MD in tissue culture (Underdahl et al., 1957), which resulted in clinical signs similar to that reported by Olafson, et al., (1946), when susceptible animals were exposed to the virus That same year a noncytopathic virus was isolated from a case of viral diarrhea in a cow. By the end of the 1970s, the two viruses were determined to

be very similar and were grouped as the bovine viral diarrhea-mucosal disease complex (BVD-MD) (Goens, 2002) and later renamed bovine viral diarrhea virus (BVDV).

Since its initial discovery, BVDV has been isolated from infected cattle worldwide and represents one of the most important pathogens of ruminants (Mainar-Jaime et al., 2001; Brownlie et al., 1990). Cattle are the primary reservoir for BVDV, however there have been documented cases of BVDV in sheep and other ruminants, including a wide variety of wild animal species (International Symposium: BVDV a 50 Year Review, 1996; Paton et al., 1997; Brownlie et al., 1990; Houe, 1999). BVDV seroprevalence in non-vaccinated cattle differs among regions and countries, ranging from 20-90%. Differences in seroprevalence may be due to differences in cattle density, herd size and other managerial factors (Houe, 1995; Houe, H., 1996; Mainar-Jaime et al., 2001). BVDV infection tends to be endemic in many herds, reaching a maximum level of 1-2% of cattle being persistently infected (PI), with 60-85% of animals in the herd having antibodies to BVDV (Houe, H., 1996).

It is estimated that annual economic losses due to BVDV infection are $20-57 million dollars per one million calves born. Monetary losses can be the result of milk production decreases, infertility, mortality and reproductive losses such as abortion, stillbirths and neonatal calf mortality (Houe, 1999; McGowan et al., 1993; Chi et al., 2002a).

For beef herds, decreased pregnancy percentage as well as decreased reproductive efficiency (measured as weaning percentage; calculated as the total number of calves weaned in each herd divided by the total number of cows bred) that can result from BVDV infection, can lead to significant economic losses (Larson et al., 2002; Sheldon et

al., 2003). The extent of those losses can vary between herds and is dependent on factors such as herd immunity and the virulence of the BVDV strain. It has been estimated that up to 90% of US beef cow/calf operations and over 68% of animals have been exposed to BVDV, either by vaccination or natural exposure (Paisley et al., 1996; Chase et al., 2003). BVDV associated disease has been identified in multiple, vaccinated beef herds; resulting in mortality rates from 2-16% and morbidity rates up to 10% (Van Campen et al., 2000; Kelling et al., 1990; Cleveland et al., 2004). Seroconversion of approximately 50% of calves within 5 months has been documented and demonstrates just how quickly BVDV can spread through a susceptible population (Cleveland et al., 2004).

Many beef calves are shipped to feedlots after weaning. However, there is little information available as to the economic impact BVDV may have in a feedlot, however the risk of a BVDV seronegative animal seroconverting within three months of entering a feedlot was determined to range from 27-40% (Taylor et al., 1995). Therefore, it is reasonable to assume there is a potential for increased morbidity and mortality when BVDV is present and actively being transmitted to susceptible animals (Larson et al., 2002). Seroconversion in the feedlot during the first 28 days has also been associated with decreased weight gain, which can result in further economic losses to the producer (Martin et al., 1999). Additional losses occur due to decreased weight gain in BVDV PI animals (Kelling et al., 1990); which average 0.1-2% of the animals in infected beef herds (Kelling et al., 1990; Paisley et al., 1996).

BVDV is considered a member of the bovine respiratory disease complex, which is considered a major cause of economic losses in the cattle industry (Baker, 1995), and has often been associated with bovine herpesvirus type 1 (BHV-1), bovine respiratory

syncytial virus (BRSV), and parainfluenza virus type 3 (PIV-3) infections (Martin et al., 1999; Martin et al., 1989; Richer et al., 1988). Bovine respiratory disease is thought to result from a cascade of events initiated by stress, which predisposes animals to viral infections; leading to rapid bacterial invasion. While not always associated with BVDV, higher serum neutralization titers to BVDV upon arrival to feedlots has been shown to be protective against bovine respiratory disease, while seroconversion during the first 28 days after arriving in a feedlot is associated with increased risk of developing bovine respiratory disease (Martin et al., 1999; Martin et al., 1989).

Etiology

BVD is an infectious and contagious disease of dairy and beef cattle that is caused by bovine viral diarrhea virus (BVDV), a *pestivirus* in the family *Flaviviridae*, composed of a single-stranded, positive-sense RNA genome of approximately 12.5kb (van Regenmortel, M. H. V. et al., 2000). Based on the effect of the virus in tissue culture BVDV has been divided into two biotypes, noncytopathic (ncp-BVDV) and cytopathic (cp-BVDV).

BVDV has also been divided into two genotypes (BVDV-I and BVDV-II) based on differences in the nucleotide sequences of the 5' untranslated region of the viral genome (Harpin et al., 1995; Pellerin et al., 1994; Bhudevi et al., 2001; Ridpath et al., 1994). BVDV-I has been further subdivided into BVDV-Ia and Ib (Ridpath et al., 1998). BVDV-I represents the classical strains of BVDV, while BVDV-II was isolated in the early 1990s from severe cases of acute BVD, causing hemorrhagic syndrome (Hamers et al., 2001; Ridpath et al., 1994). Both genotypes exist as cp- and ncp- biotypes (Houe, H., 1996).

CD46 is a potential cellular receptor for BVDV; which is thought to bind to CD46 in clathrin coated pits via the E2 envelope protein, and enters the cell via receptor-mediated endocytosis. Low density lipoprotein (LDL) receptor and insulin receptor are also found in coated pits and may serve as BVDV receptors (Grummer et al., 2004; Maurer et al., 2004; Liang et al., 2003).

Upon entry into a cell the genome of BVDV is translated into a single polyprotein: N^{pro}-C-E^{rns}-E1-E2-p7-NS23-NS4A-NS4B-NS5A-NS5B; which is subsequently cleaved by viral and cellular proteases (Collett et al., 1988; Donis, 1995). Structural and nonstructural proteins are C, E^{rns}, E1, E2, p7 and N^{pro}, NS23, NS4A, NS4B, NS5A, NS5B, respectively.

N^{pro}

N^{pro}(p20) is a cis-acting papain-like protease (cysteine endopeptidase) that cleaves intramolecularly at its own C-terminus to release itself from the polyprotein and is responsible for cleavage resulting in the formation of the N-terminus of the capsid protein. No similar proteins have been isolated from other members of the *Flaviviridae* (Donis, 1995).

Capsid protein (C)

The capsid protein or C(p14) is cleaved at its N-terminus by the autocatalytic action of N^{pro}. C(p14) packages the genomic RNA and provides the necessary interactions for the formation of the enveloped virion (Donis, 1995).

E^{rns}

E^{rns}(gp48) contains a signal sequence for translocation into the endoplasmic reticulum at the N-terminus. The protein is cleaved in the ER lumen producing a

hydrophilic mature protein that forms homodimers within the lumen of the ER. The function of E^{rns} is unclear, but the glycoprotein does display ribonuclease activity; catalyzing the hydrolysis of ribonucleic acid (Choi et al., 2004; Grummer et al., 2004; Schneider et al., 1993).

E1

E1(gp25) is an external envelope glycoprotein that contains two hydrophobic domains that anchor the protein in the membrane and initiate translocation of the adjacent polypeptide, E2 (Choi et al., 2004). E1 is found covalently linked to E2 in virions (Donis, 1995).

E2

E2(gp53) forms homodimers and heterodimers with E1 in the virion envelope. E2 is probably involved in the initial binding of BVDV to cells and is therefore a determinant of cell tropism (Grummer et al., 2004; Liang et al., 2003).

p7

The p7 protein is located between E2 and NS23, but is not a major constituent of the virion and is not incorporated into viral particles (Elbers et al., 1996; Agapov et al., 2004). p7 may have a role in glycoprotein maturation and/or virus morphogenesis and may also interact with the capsid protein to initiate budding (Elbers et al., 1996). p7 has been shown to aid in the proper orientation of NS2 in the cell membrane (Agapov et al., 2004).

NS23

NS23(p125) is believed to have helicase-like properties and is able to catalyze the ATP-dependent strand separation of RNA duplexes thought to be required for translation

initiation and RNA replication. A region of the protein is believed to participate in the cleavage of the polyprotein to generate nonstructural polypeptides by a series of intramolecular and intermolecular cleavages (Donis, 1995; Agapov et al., 2004). NS23 may also function to recruit virion components to the site of nucleocapsid assembly.

In cytopathic viruses, NS23 is cleaved into two separate proteins: NS2(p54) and NS3(p80). A cell infected with a cp-BVDV often produces NS2, NS3 and uncleaved NS23 because complete cleavage of NS23 prevents the production of infectious virus (Agapov et al., 2004). NS2 is a hydrophobic protein that contains a zinc finger motif and has no known function (Agapov et al., 2004; Donis, 1995). NS3 is considered the marker for cp-BVDV and contains the viral serine protease and helicase domains of NS23 (Donis, 1995; Choi et al., 2004). BVDV NS3 is homologous to NS3 proteins of other flaviviruses, which are responsible for the cleavage of nonstructural proteins downstream of the protein as well as its own C-terminus (Agapov et al., 2004; Donis, 1995).

NS4A

NS4A(p10) is required in cis for optimal function of the NS3 protein (Agapov et al., 2004). There is little information available concerning NS4A other than the protein is conserved among pestiviruses and is a co-factor for NS3 protease activity (Donis, 1995; Agapov et al., 2004).

NS4B

NS4B(p32) may modulate the protease activity of NS23 in the generation of NS5B. NS4B is one of the two most conserved nonstructural proteins among pestiviruses and accumulates in infected cells in the later stages of infection with cp-BVDV (Donis, 1995; van Olphen et al., 1997).

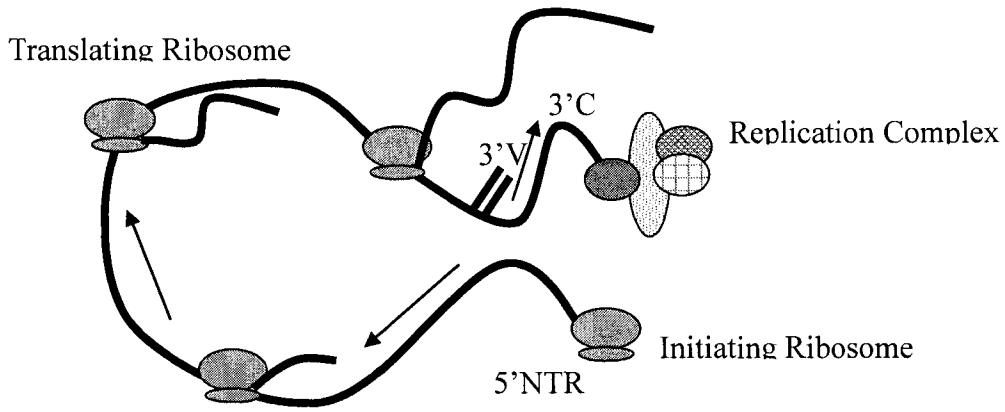
NS5A / NS5B

Both NS5A(p58) and NS5B(p75) have RNA polymerase activity. NS5B is the putative RNA-dependent-RNA polymerase; which is required for viral transcription (Donis, 1995). NS5B is also required to couple RNA replication with virion formation (Ansari et al., 2004).

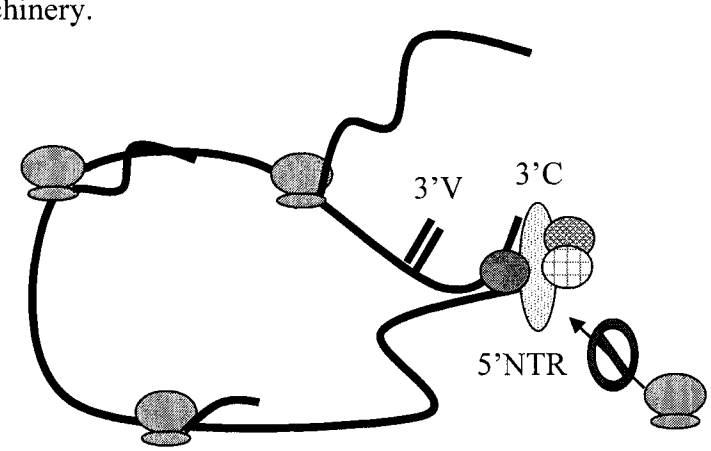
Replication Complex

The BVDV replication complex is composed of NS23, NS4B and NS5A and NS5B and cellular proteins. NS23 may contain an RNA-binding domain that holds the viral replication complex in close proximity to the membrane. The 3' NTR of the genome may have a significant role in the assembly of the replication complex as well as the coordination of translation of the polyprotein and RNA replication (Isken et al., 2004). The 3' NTR of all pestiviruses contain a conserved region (3'C) and a variable region (3'V), located downstream of the translational stop codon. The 3'V region encodes two unstable stem-loop structures and is involved in the displacement of ribosomes.

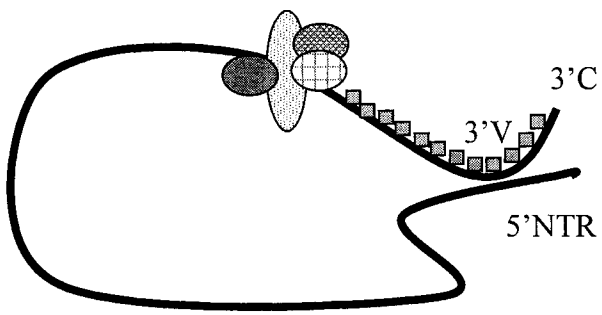
The replication complex is thought to support an interaction between the 5' and 3' termini of the viral RNA (Isken et al., 2004). The 5' NTR codes for an internal ribosomal entry site (IRES); which mediates cap-independent translation of the polyprotein and may be stimulated by the presence of the 3'V region (Isken et al., 2004; Haiying et al., 2000). Conformational changes of the 3'V stem loops may result in translation inhibition, signaling the beginning of viral genome replication (Figure 1).



A: The 5' and 3' ends of the genome work together to promote the efficient IRES-meditated translation of the viral polyprotein. 3'V prohibits interference of the translation machinery.



B: At an unknown stage, translation is inhibited or terminated by 3'V. The replication complex may change the conformation of 3'V, affecting the 5'NTR; leading to translation inhibition.



C: Clearance of translating ribosomes enables initiation of the replication cycle; yielding complementary negative-strand intermediates that act as templates for the production of progeny positive-strand RNA genomes.

Figure 1.1: Hypothesized mechanism for BVDV transcription and translation regulation (Adapted from Isken, et al., 2004)

Transmission

Transmission of BVDV occurs by two methods: horizontally from PI animal to susceptible animal and vertically by transplacental transfer from dam to fetus. Ingestion of virus from infected saliva, oculonasal discharge, urine and feces are principle routes of infection. Semen, uterine secretions, amniotic fluid and placenta containing virus are other sources of virus infection along with mechanical vectors such as clothing, veterinary instruments and biting flies (Houe, 1995).

The pathogenesis of BVDV is unique in that vertical transmission resulting in fetal infection with a ncp-BVDV during the first trimester can result in the birth of a PI calf (Harding et al., 2002). Because fetuses are infected before the development of their immune system, the virus is seen as 'self' and does not elicit an immune response (Hamers et al., 2001). Although PI cattle are immunotolerant to the BVDV strain to which they are infected, the animals are immunocompetent with respect to other antigens (Baker, 1995). Persistently infected animals are chronic shedders of BVDV and are the main source of exposure leading to infection. They are generally unthrifty, but may survive for many years.

There is debate as to whether transiently infected animals can spread infectious virus during a brief viremic stage (Niskanen et al., 2000; Niskanen et al., 2002). Acutely infected animals shed low amounts of virus, with viral titers of 1.41 to 1.67 log $TCID_{50}$ per milliliter of serum (McGowan et al., 1993), for short durations (4-10 days), so their role in maintenance and spread of the disease within herds appears to be minimal (Houe, 1995; Kirkland et al., 1991; Mainar-Jaime et al., 2001; Brownlie et al., 1990; Sandvik, 1999). Persistently infected animals shed larger quantities of virus, with virus titers of

4.1 to 5.0 log $TCID_{50}$ per milliliter of nasal secretions (Mars et al., 1999), and are considered the primary source of BVDV infection (Houe, 1995). Female PI animals will produce calves that are also persistently infected (Lindberg, 2001; Baker, 1995). The introduction of a non-persistently infected dam carrying a PI fetus can also serve as a source of BVDV infection to herds (Houe, 1999; Bitsch et al., 1995).

Signs of disease

Clinical outcome of BVDV infection is influenced by many host factors; including immunocompetence or immunosuppression, pregnancy status, gestational age of the fetus, previous infection or vaccination and the concurrent level of environmental stress of the host. Seventy to ninety percent of BVDV infections in immunocompetent cattle are subclinical and typically result in the production of serum neutralizing antibodies (Baker, 1987; Baker, 1995). Infection resulting in clinical disease generally occurs in animals six months to two years in age with more severe infection most often occurring in neonates. The incubation period is five to seven days and is followed by transient fever, leukopenia and viremia for up to 15 days and sometimes anorexia, dehydration and formation of oral ulcers (Baker, 1987; Houe, H., 1996; Hamers et al., 2001). Antibodies develop within two weeks and titers may remain high for years and often for the life of the animal (Kennedy, 2004). Susceptible herds may experience high morbidity, but low to zero mortality (Houe, H., 1996; Baker, 1987).

In the early 1990s, BVD viruses causing acute, severe disease were isolated from infected cattle in North America. Outbreaks resulted in high mortalities, particularly in calves (Hamers et al., 2001). Clinical signs of hypervirulent BVDV infection include thrombocytopenia with extensive hemorrhaging or severe respiratory distress. Severity

of disease has been correlated to the degree of BVDV viremia. The more severe the clinical signs, the greater the quantity of virus in serum, making the degree of viremia induced during BVDV infection an important virulence factor (Walz et al., 2001).

Highly virulent stains of BVDV type II have higher translational efficiencies than low virulent strains in tissue culture systems leading to the hypothesis that virulence was affected by the IRES structure and translation efficiency of the strain. Hypervirulent strains of BVDV type II have been shown to have significantly more stable secondary folding at IRES (Pellerin et al., 1994; Topliff et al., 2005). While BVDV type II genotypes have been isolated from all cases involving a hypervirulent BVDV strain in North America, not all type II genotypes are hypervirulent (Hamers et al., 2001; Ridpath et al., 1994).

Despite the low mortality caused by most BVDV strains, there is potential for more serious disease due to the immunosuppressive effects of the virus (Baker, 1987; Hamers et al., 2001). The virus may enhance the pathogenicity of co-infecting microorganisms (e.g., parainfluenza virus type 3 [PI-3], infectious bovine rhinotracheitis [IBR] virus, coronavirus or rotavirus) (Baker, 1987). Virus from modified-live (MLV) BVDV vaccines has also been shown to be immunosuppressive (Roth et al., 1983). The mechanisms by which BVDV causes immunosuppression have been hypothesized to include suppression of interferon production (Coria et al., 1978; Schweizer et al., 2001), decreased responsiveness of peripheral lymphocytes to a variety of mitogens (Muscoplat et al., 1973) and decreases in the absolute number of circulating neutrophils and B and T lymphocytes (Roth et al., 1983; Bolin et al., 1985a).

In utero BVDV infection can result in embryonic death, abortion, stillbirths or the birth of a PI calf that is immunotolerant to the virus. Possible mechanisms of cellular damage to the fetus, due to viral infection include inhibition of cellular growth, cell differentiation or direct cellular lysis (Castrucci et al., 1990). Infection of the fetus between approximately 100 to 150 days of gestation can result in a variety of congenital defects, particularly of the brain. Congenital defects caused by BVDV include microencephalopathy, cerebellar hypoplasia, hydrocephalus, optic nerve neuritis, retinal dysplasia and thymic hypoplasia (Done et al., 1980; Baker, 1995; Hamers et al., 2001). Calves infected with BVDV during later stages of gestation rarely suffer from congenital defects. These calves may be born normal and are seropositive to BVDV (Baker, 1995; Done et al., 1980). Due to the immunosuppressive effects of BVDV, PI animals can be predisposed to infections with other microorganisms, often resulting in pneumonia and enteritis (Baker, 1995; Hamers et al., 2001).

Mucosal disease (MD) is a sporadically occurring, highly fatal, enteric disease, which usually occurs in PI cattle that are six months to two years of age. Persistently infected animals develop MD as a result of simultaneous infection with both ncp- and cp-BVDV strains. Disease can be acute, leading to death in a few days, or chronic, persisting for weeks to months before the animal dies (Bolin, S. R., 1995b). Mucosal disease is characterized by similar clinical signs as BVD, but includes nasal discharge, salivation and persistent ocular discharge (Baker, 1995). Morbidity is generally low for both acute and chronic MD, but the case mortality rate for both is high, nearing 100%. At necropsy, an animal that dies from MD has mucosal erosions and ulcerations throughout the alimentary tract. Some cattle survive the initial bout of disease and

continue to live for months or years before succumbing to a recurrence of disease (Bolin, S. R., 1995b).

Both non-cp and cp-BVDV biotypes are isolated from animals with mucosal disease. Genomic rearrangement, deletions, insertions or point mutations involving the NS23 gene in ncp-BVDV in a PI calf may result in a cp-BVDV biotype, leading to the occurrence of MD (Becher et al., 1999; Meyers et al., 1992; Fricke et al., 2001). In one PI animal multiple viral sequences can result from mutations in the BVDV RNA (Fricke et al., 2001).

It is hypothesized that the immune system of the PI animal does not recognize the cp-BVDV because the structural proteins of the virus are antigenically similar to the original ncp-biotype (Fricke et al., 2001; Bolin, S. R., 1995b). A PI animal may also become infected through exposure to another PI animal shedding a cp-BVDV or through vaccination with a modified-live vaccine containing a cp-BVDV (Fray et al., 1998; Bolin, S. R., 1995b).

Immune response to BVDV

The production of BVDV specific antibodies is an important component of the immune response to infection with BVDV (Howard, 1990; Shope et al., 1976). An antibody response follows natural exposure or vaccination with either ncp- or cp-BVDV and confers immunity to re-infection. Typically, the majority of antibody produced is of the IgG1 and IgG2 isotypes (Howard, 1990; Howard et al., 1985). Antibody can be detected in serum three weeks post-infection, however the titer will continue to rise for 10 to 12 weeks (Collen et al., 2000). The importance of the humoral immune response was demonstrated by the protection of calves from BVDV challenge, that received

passively transferred antibodies, either when the T-cell response was inhibited by dexamethasone (Shope et al., 1976) or when the T-cell response was not disrupted (Howard et al., 1989).

Animals naturally infected with BVDV produce antibodies specific to the E^{rns}, E1 and E2 envelope glycoproteins (Kwang et al., 1992; Bolin, 1993). Large concentrations of virus neutralizing antibodies are generated against the E2 envelope glycoprotein. While E^{rns} does induce considerable levels of antibodies in infected cattle, the antibody has limited ability to neutralize virus (Boulanger et al., 1991). E1 elicits only small amounts of non-neutralizing antibody as well (Donis et al., 1987b). Non-neutralizing antibodies are produced against the nonstructural proteins NS23 and NS3 (Bolin, 1993; Donis et al., 1987b; Bolin et al., 1989).

Cytopathic strains elicit antibodies against both NS23 and NS3, while noncytopathic strains elicit antibodies against NS23 only (Bolin, 1993). No antibodies to the C-protein, p7, NS4A, NS4B, NS5A or NS5B are elicited in animals naturally exposed to BVDV. Cattle that receive a killed vaccine can elicit a limited antibody response to some non-structural proteins (Donis et al., 1987a; Donis, 1995).

In T-cell depletion studies CD4+ T-cells have been shown to play a critical role in viral clearance (Howard et al., 1992). While both CD4+ and CD8+ T-cells aid in the clearance of virus, the depletion of CD8+ T-cells has little affect on the length of BVDV viremia or viral titer in serum; indicating a limited affect of CD8+ T-cells in BVDV clearance (Howard et al., 1992). The CD4+ T-cells establish a Th-2 response (humoral immunity), by producing IL-4 and low levels of IL-2 and IFN-γ (Rhodes et al., 1999). CD4+ T-cells are specific for the E^{rns} and E2 envelope glycoproteins and the

nonstructural NS23 protein. There is also some reactivity with N^{pro} and capsid proteins (Collen et al., 2000).

Uterine Immunology

Mucosal immunity in the female reproductive tract is unique in that while protecting against foreign pathogens the system is selectively neutral to allogenic spermatozoa and can support a fetal placental unit that is immunologically distinct from the female (Wira, C. R. et al., 1996). Potential mechanisms of immune system evasion have been suggested, including: (1) little if any expression of major histocompatibility complex (MHC) on the placenta; (2) separation of the fetal and maternal systems; and (3) immunosuppression at the conceptus/mother interface (Hansen, 1995; Davies et al., 2004).

The uterus is not an immunopriveledged site, as local antibody can be induced by intrauterine vaccination or the introduction of foreign pathogens (Wira et al., 1989; Prabhala et al., 1991). IgA is characteristically produced in mucosal epithelia and is present in uterine secretions. Antigen specific antibodies can be present in the uterine and cervico-vacinal secretions after distal and local immunization (Wira, C. R. et al., 1996). In addition, antigen specific antibodies can also be found in the Peyer's Patches of the intestinal tract after intrauterine immunization; showing the interaction between different sections of the mucosal immune system (Wira, C. R. et al., 1996). Also, intrauterine immunization can also lead to increased mitogen-stimulated B and T-lymphocyte proliferation in the spleen of immunized animals (Wira, C. R. et al., 1996; Prabhala et al., 1991).

IFN-τ suppresses the proliferation of lymphocytes and may play a role in maternal immune tolerance (Leung et al., 2000), as shown by a decrease in the number of intraepithelial lymphocytes at the time of conceptus attachment, which accompanies an increase in the concentration of IFN-τ (Hansen, 1995). By mid-gestation almost no lymphocytes or macrophages are present in the caruncular enodmetrium; which are the sites in which the fetal chorion comes into close contact with the maternal endometrium and provides a large surface area for nutrient transfer (Senger, P. L., 1999; Leung et al., 2000).

Cows have epitheliochorial placentas (Figure 2) which are the least intimate among animal species (Senger, P. L., 1999). Bovine placentas are also syndesmochorial meaning the endometrial epithelium erodes and then regrows periodically throughout gestation. The erosion of the epithelium allows intermittent exposure of maternal capillaries to the chorion epithelium.

The mechanism(s) of transplacental transfer of BVDV is unknown and little information regarding the protective mechanisms that are important for the prevention of fetal infection is available (Brock et al., 2001). A role for binucleated giant cells (BNGC) of the allontochorion has been proposed. BVDV has been identified in multiple cell types of the chorion epithelium of fetuses from PI dams; in particular BNGC (Fredriksen et al., 1999a).

Binucleated giant cells are derived from trophoblast cells and are formed throughout gestation. BNGC can migrate from the chorion to the endometrial epithelium in ruminants and are thought to secrete lactogen and pregnancy specific protein B (Senger, P. L., 1999). Lactogen stimulates milk production and the function of

pregnancy specific protein B is not clear, but may play an important role in maintenance of pregnancy. After BVDV establishes infection in the dam, the virus spreads within the allantoic and amniotic membranes and then infects the fetus (Swasdipan et al., 2002). The spread of BVDV within the fetus appears to be governed by unknown developmental factors or events (Swasdipan et al., 2002).

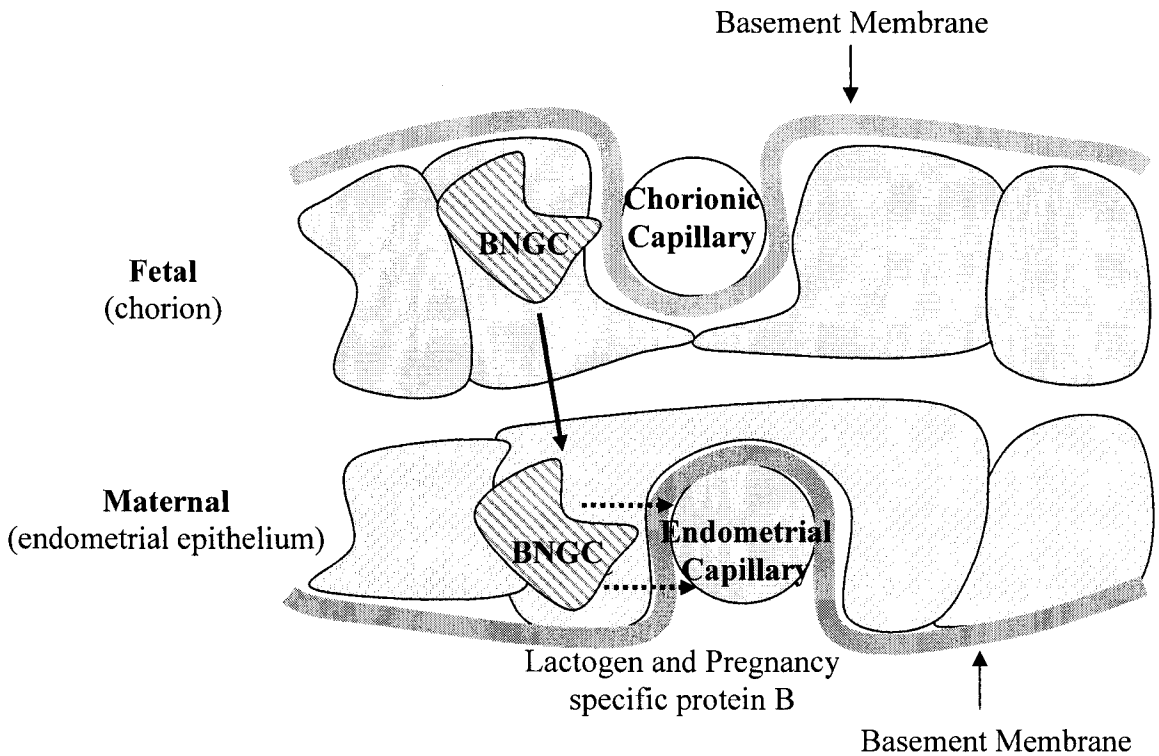


Figure 1.2: Bovine placenta fetal/maternal interface
(Adapted from Hansen, 1995)

Control of BVDV

Effective BVDV control methods include eliminating or reducing stress, identification and removal of PI animals, identification of cows pregnant with PI fetuses and use of BVDV vaccines to prevent severe acute disease and transplacental infections (Baker, 1987; Sandvik, 1999). Currently, management practices such as identifying and

removing PI animals from a herd, are used to reduce the risk of BVDV spread (Bitsch et al., 1995). Prophylactic vaccination programs are also in place to prevent disease outbreaks. There are over 150 federally licensed vaccines in the United States. The efficacy of these vaccines under field conditions, with regard to fetal protection, is questionable and to date, none have been conclusively reported to prevent fetal infection with BVDV (Van Campen et al., 2000; Hamers et al., 2001).

Management practices such as vaccination have been shown to reduce the occurrence of several bovine infectious diseases, such as BVD and enzootic bovine leukosis in Canadian dairy herds (Chi et al., 2002b). Culling of weak and underweight animals often reduces the number of PI cattle in a herd, reducing the risk of infection to susceptible animals (Kelling et al., 1990). However, not all PI animals can be identified based on body weight or thriftiness. Therefore, laboratory tests are required to identify all PI animals in a herd (Kelling et al., 1990). Screening newly purchased animals for BVDV persistent infection before introducing them to a herd will also help decrease the probability of infection. Exposure resulting in infection can also be decreased by not grazing-in-common with infected herds (Houe, 1999). Unfortunately, situations such as sharing fence lines with BVDV infected cattle or contact with wild ruminants are difficult transmission factors to control. It is also important that individuals do not knowingly sell PI animals to other producers to prevent introduction of BVDV to naive herds (Houe, 1999).

Diagnostic Assays

Diagnosis of BVDV infection in a herd requires diagnostic laboratory support as well as a full understanding of the spectrum of disease that can be manifested in a herd.

A complete herd history should be compiled covering the preceding two years with detailed information on all ages and groupings of cattle (Baker, 1987). The introduction of new cattle should receive particular attention, as they are often the source of virus. Clinical and physical examination should be performed on a sample population of the herd to determine the presence of clinical signs compatible with BVDV infection. A definitive diagnosis is dependent on laboratory confirmation, which is dependent on the quality of sample, determined by proper collection, handling and transport (Baker, 1987).

Persistently infected animals are considered the primary transmitters of BVDV and the introduction of just one PI animal can cause an outbreak of disease in an entire herd (Pillars et al., 2002). Therefore early detection of PI animals would be a significant benefit to cattle producers who wish to eliminate BVDV from a herd. Antibody titers of cattle in herds with PI animals are generally higher than those without PI animals, even if the cattle have been vaccinated (Houe et al., 1995b). If BVDV infection is suspected in a herd, high serum neutralization antibody titers can be used to predict the presence of a PI animal (Houe et al., 1995b; Pillars et al., 2002; Van Campen et al., 1998).

Currently, diagnosis of BVDV infection in a single animal is dependent on the detection of infectious virus or viral components in serum or whole blood. Animals are confirmed as persistently infected using virus isolation techniques that are often performed on white blood cells (WBC). PI animals shed virus in most secretions and excretions and can be detected in serum, WBC preparations, and nasal swab specimens (Grooms et al., 2001). However, little is known about the variability or stability of viremia in PI animals over time. Studies have shown that BVD viral concentrations in PI animals can be variable (Grooms et al., 2001). This variation can make detection and

identification of a PI animal difficult. Adult PI cattle have been shown to develop neutralizing antibodies against BVDV that lead to the clearance of virus from the serum, but not from the WBC (Grooms et al., 2001). In addition to variability in viremia, passively acquired colostral antibody in PI calves can decrease the accuracy of some virus detection assays (Brock et al., 1998; Kelling et al., 1990).

Enzyme-Linked Immunosorbent Assays (ELISA) and immunofluorescent staining for antigen are used for screening animals for BVDV infection (Bezek et al., 1988; Durham et al., 1990; Canal et al., 1998; Graham et al., 1997; Howard et al., 1987; Saliki et al., 2000; Sandvik, 1999; Straver et al., 1987). Detection of BVDV antigen by ELISA and other antigen detection assays is less sensitive than virus isolation techniques (Houe, H., 1996; Allcock, 2001). Virus propagated in tissue culture as well as virus extracted from tissue collected from infected animals can be used for reverse-transcription polymerase chain reaction (RT-PCR), which has been shown to be a sensitive and rapid technique for detecting and genotyping BVDV RNA (Bhudevi et al., 2001; Givens et al., 2001).

Detection of BVDV in the blood via virus isolation and/or RT-PCR can be used to identify PI animals, however virus can also be isolated from animals in the viremic phase of an acute infection. Therefore, it is necessary to test suspected PI animals twice, at least three weeks apart, to allow an acutely infected animal time to clear virus from the blood (Bolin et al., 1985b; Sandvik, 1999). PI animals can be identified using blood and tissue samples for virus isolation, RT-PCR and ELISA, but these methods are costly for screening entire herds and may give false negative results due to a lack of virus in serum or neutralization of virus by colostral antibodies (Brock et al., 1998; Grooms et al., 2001).

Other methods of detection, such as immunohistochemical staining of ear-notches are available, but may also produce false negative results (Grooms et al., 2002).

Screening of replacement heifers and other purchased, pregnant cows is necessary to prevent the introduction of a PI animal (Bitsch et al., 1995; Houe, 1999). PI-carriers (PI-Cs) are seropositive and virus-negative (unless the dam is persistently infected), which makes them indistinguishable from other seropositive cattle (Brownlie et al., 1998; Wittum et al., 2001).

Gaps in Current Diagnostic Methods

Despite the numerous tests available to test for PI animals, there are no existing diagnostic tests to detect the presence of PI fetuses *in utero*. Unless the dam is persistently infected, PI-Cs will be seropositive and virus-negative by standard testing methods (Brownlie et al., 1998; Wittum et al., 2001). Studies have shown that virus can be isolated from fetal fluid obtained by amniocentesis as a PI-C detection method. However, the method is costly and the safety of the procedure has not been fully explored (Callan et al., 2002).

An enzyme-linked immunosorbent assay (ELISA) for the detection of total BVDV-specific antibodies has been developed in Europe as a diagnostic tool to screen for PI-Cs (Lindberg, 2001; Brownlie et al., 1998). PI-Cs generally have markedly higher antibody titers to BVDV than cows carrying uninfected fetuses, presumably due to continuous immunological challenge induced by the PI fetus (Houe, 1999; Brownlie et al., 1998). However, the assay is not 100% reliable in that some overlap of antibody titers between PI carriers and non-carriers occurs (Brownlie et al., 1998). The ELISA's sensitivity and specificity in detecting PI-Cs was 94%-100%, and 39%-67% respectively

using data from the Swedish BVD database (Lindberg, 2001); however, the cattle tested did not include vaccinated animals. In the United States, the majority of cattle are vaccinated for BVDV and have antibody titers that overlap those of naturally exposed cattle (Van Campen et al., 1998); therefore, an ELISA assay that measures total serum antibody to BVDV is unlikely to accurately detect PI-Cs in this population.

To detect PI animals whole herds are screened for BVDV. This process can be expensive for many producers who would need to test anywhere from ten to thousands of cattle. Pooled-sample testing, for herds with PI animal prevalences of 0.5% to 3.0%, using a sensitive diagnostic test may provide a cost-effective method for identifying PI animals in a cattle herd (Munoz-Zanzi et al., 2000; Weinstock et al., 2001). Pooling serum samples to test for BVDV by RT-PCR has been shown to have a high sensitivity (>=99%) (Weinstock et al., 2001). Serum is often used in BVDV diagnostic assays, however testing serum or plasma from an animal before three months of age can result in interference by maternal antibodies (Saliki et al., 1997). An alternative to collecting blood samples is collecting ear notch samples; which are quick and easy to collect, require less skill than blood collection and there is a low chance that maternal antibodies can interfere with viral antigen detection.

Vaccination

Vaccination of cattle against BVDV is used to minimize mortality and severity of clinical disease in animals that become infected with BVDV. Experimental BVDV challenge studies have shown that vaccination can reduce the duration of virus shedding in acutely infected animals, possibly reducing a population's overall exposure rate (Thurmond et al., 2001). If animals are vaccinated while maternal antibody is still

present, the humoral response can be blocked or reduced. However, the T-cell response to BVDV is not inhibited (Endsley et al., 2003).

A neutralizing antibody titer of 1:256 to BVDV is considered protective, meaning acute or severe disease is prevented in vaccinated animals (Makoschey et al., 2001). A general concern is whether immunity to one strain can elicit a protective immunity against other strains, and particularly against strains of other antigenic groups (Hamers et al., 2002). Antibodies to glycoproteins can cross react with other strains of BVDV, however different antibody titers may be obtained when one strain is tested against another, meaning infection or vaccination with one strain of BVDV may or may not provide protection against another strain (Sandvik, 1999).

Vaccinated and naturally infected animals will develop serum neutralizing antibodies to the infecting BVDV and other virus strains of the same genotype. In general, animals respond with higher titers to the BVDV strain to which they were vaccinated or naturally infected. Lower cross-reactive antibody titers to heterologous viruses have been observed in vaccinated animals (Brock et al., 2001; Makoschey et al., 2001; Hamers et al., 2002). Hamers, et al., (2002) showed that colostrum-deprived calves vaccinated with two inactivated North American BVDV type-I strains, one a cytopathic and the other a noncytopathic strain, produced antibody titers able to neutralize all conventional European strains *in vitro*; showing that vaccinated calves could produce antibodies against a wide range of antigenically diverse BVDV strains (Hamers et al., 2002).

Despite the apparent benefits of vaccination, if calves are vaccinated too early, maternal antibodies may inhibit the development of an immune response. Calves that are

vaccinated within two weeks of birth and have high maternal antibody concentrations at the time of vaccination respond poorly when challenged with BVDV at four months of age, developing moderate to severe clinical disease (Ellis et al., 2001).

Modified-live (MLV) and inactivated vaccines are currently available for use in the United States. Some products contain a combination of both type I and type II genotypes and/or ncp- and cp- biotypes (International Symposium: BVDV a 50 Year Review, 1996). MLV vaccines have the advantage of producing a more effective immune response in the form of both humoral and cell mediated immunity, without the need for a second vaccination when compared to inactivated vaccines (Bolin, S. R., 1995a; Baker, 1987; Howard et al., 1986). However, the vaccine can be immunosuppressive resulting in increased mortality of calves due to respiratory disease (Baker, 1987; Howard et al., 1986). There is also a risk of inducing MD in PI animals vaccinated with a MLV vaccine containing a cp-BVDV (Baker, 1987; Fray et al., 1998).

Inactivated vaccines do not cause disease or immunosuppression, are safe to use in pregnant animals and are also available with combinations of BVDV genotypes and biotypes. An inactivated vaccine primarily elicits a humoral response, which may not be as protective as the immune response induced by a MLV vaccine (Fredriksen et al., 1999b). Unlike the MLV vaccine, animals given an inactivated vaccine require a second vaccination to obtain sufficient immunity to provide full protection against acute BVD. Repeated intramuscular (IM) vaccination with inactivated BVDV does not enhance the level of antibody production and it is uncertain whether the IM route of inoculation is as efficient as a natural infection for providing protection against transplacental infection with BVDV (Fredriksen et al., 1999b).

Despite its common use as a control method, vaccination can result in negative side effects. Most devastating is the development of MD in PI animals vaccinated with a MLV containing a cp-BVDV (Baker, 1987). MD is normally seen in PI animals whose ncp-BVDV has undergone a mutation or deletion resulting in a cytopathic biotype. Therefore, the two virus strains are antigenically related (Bolin, S. R., 1995b). Immunotolerance and antigenic similarity between virus biotypes are not required for development of MD (Fray et al., 1998).

The biotypes isolated from multiple postvaccinal MD cases have been shown to be antigenically different from one another and the cp-BVDV biotype was shown to have originated from the vaccine (Bolin, S. R., 1995b; Fray et al., 1998). Delayed onset of MD has also been seen in experimental systems. PI animals injected with an antigenically distinct cp-BVDV did not develop MD in the typical incubation period of two to four weeks, but developed disease weeks to months later (Fritzemeier et al., 1997). Antibodies against cp-BVDV were initially produced in animals with delayed onset MD, but selective pressure is thought to have fostered the development of antigenic mutants the antibody was not effective in neutralizing (Fritzemeier et al., 1997).

Use of models to determine efficient BVDV control strategies in US beef herds

Factors that contribute to BVDV transmission under natural field conditions include passive immunity from colostral antibody, active immunity after vaccination, the likelihood of virus shedding from acutely and persistently infected animals and factors that promote or prevent infectious contact such as herd density (Thurmond et al., 2001). All of these factors can influence herd susceptibility and herd immunity to BVDV.

Most of the published BVDV simulation models describe virus transmission in dairy herds and have proven useful in expanding the understanding of BVDV epidemiology (Innocent et al., 1997a; Viet et al., 2004; Innocent et al., 1997b; Cherry et al., 1998). Cherry et al., (1998) has analysed the impact of PI animal removal on BVDV transmission and control in dairy herds. However, there are important temporal and spatial management differences between dairy and beef herds that might impact BVDV spread and infection, making the development of a model specific to United States beef cattle operations necessary. Direct or close contact with a PI animal is required for an animal to become infected with BVDV, and presumably, the most efficient route of transmission is by nose-to-nose contact (Houe, 1995). Therefore, the proximity of susceptible cattle to PI animals is an important factor in transmission of virus.

There are three important differences in the management practices of dairy and Western United States beef operations that affect BVDV transmission: 1. Dairy cattle are normally confined within close proximity of one another and gathered once or twice a day for milking, which would allow for more contacts between susceptible and PI cattle, possibly increasing the rate of BVDV infection. Beef cattle are generally grazed on large pastures where contact with other animals might be less frequent, thus slowing the spread of BVDV (Wentink et al., 1991). 2. Dairy cattle in the United States are typically bred year-round (USDA, 2002), allowing PI animals to be introduced to the herd continuously, increasing exposure to PI animals throughout a year, but potentially decreasing the daily exposure rates to PI animals, since fewer calves are born at the same time. Beef cattle in the western United States are often bred synchronously, therefore there is a limited time period when exposure to BVDV can result in a PI fetus. Cows are often bred before

weaning, giving a PI animal sufficient opportunity to expose a susceptible dam and its fetus to BVDV in the first trimester of gestation. For this reason, there is a limited amount of time when removal of PI animals would be an effective control strategy. 3. Newborn dairy calves are usually taken from heifers before nursing (28%) or within 24 hours of birth (89.5%) (USDA, 2005). Beef calves are left to graze with the dams until weaning.

Multiple studies have demonstrated the prevalence of PI animals and BVDV antibody titers in dairy herds (Sorensen et al., 1994; Bolin et al., 1985b; Mainar-Jaime et al., 2001). In contrast, there is little information regarding the prevalence of infection or the health and monetary consequences of BVDV infection in United States beef herds. Sorensen, et al., (1994) showed transmission of BVDV can proceed at a rate of 20% of animals per week within naïve dairy herds if a PI animal is present. In vaccinated and unvaccinated BVDV endemic dairy herds, the PI animal prevalence was found to range from 1-2% with a seroprevalence of 60-90% (Houe et al., 1991; Houe et al., 1995a). In studies examining both vaccinated and unvaccinated beef herds, PI animal prevalence ranged from 0.1-2% (Houe, 1999). The presence of one PI animal can result in 15%-80% of animals in beef herds developing antibodies to BVDV (Paisley et al., 1996; Kelling et al., 1990; Houe, 1999; Grooms et al., 2001).

It is possible that BVDV transmission in beef herds could be eliminated by management practices such as culling and testing of replacement heifers for BVDV persistent infection without the need for expensive screening for PI animals. Although PI animal screening and removal has been recognized as a useful strategy to eradicate

BVDV from a herd (Ferrari et al., 1999; Cleveland et al., 2004; Cleveland, SM, 2003), the cost of implementing such a control program can be too great for some producers

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CHAPTER II

ASSESSMENT OF A BOVINE VIRAL DIARRHEA VIRUS (BVDV) ANTIGEN CAPTURE ELISA AND A MICRO-TITER VIRUS ISOLATION ELISA USING POOLED EAR NOTCH AND SERUM SAMPLES

Abstract

To reduce the cost of whole herd screening for bovine viral diarrhea virus persistently infected animals, the sensitivity and specificity of an antigen-capture (AC)-ELISA and a microtiter virus isolation (MTVI)-ELISA using pooled serum or saline from ear notch samples was determined. Pooled saline from ear notch samples, assayed by AC-ELISA, gave a sensitivity and specificity of 98% and 94%, respectively for pools containing two samples and 72% and 100%, for pools of five. The sensitivity of pooled ear notch or serum samples for bovine viral diarrhea virus detection by MTVI-ELISA (sensitivity <5%) or serum samples for detection by AC-ELISA (sensitivity <15%) is too low to be utilized for whole herd screening. Pooling of saline from ear notch samples, however, could provide a faster, less expensive, reliable method for whole herd screening for bovine viral diarrhea virus.

Introduction

Infection with bovine viral diarrhea virus (BVDV) can lead to the birth of persistently infected (PI) calves; which are the primary transmitters of BVDV. Identification and removal of PI animals is critical to controlling the spread of this economically important disease (Houe, 1999). In order to combat the spread of BVDV

the state of Colorado has recently begun a voluntary eradication program involving the screening of whole herds for BVDV PI animals (Kennedy, 2004). Currently there are a variety of diagnostic tests used to detect PI animals including virus isolation from blood or tissues, immunohistochemical (IHC) staining of skin samples, reverse transcriptase-polymerase chain reaction (RT-PCR), microtiter virus isolation (MTVI)-ELISA and antigen-capture ELISA (AC-ELISA) (Saliki et al., 1997; Saliki et al., 2000; Njaa et al., 2000; Radwan et al., 1995). The cost of whole herd screening using any of these methods can be too costly for some cattle operations (Grooms et al., 2002). However, pooled-sample testing, for herds with PI animal prevalences of 0.5% to 3.0%, using a sensitive diagnostic test may provide a cost-effective method for identifying PI animals in a cattle herd (Munoz-Zanzi et al., 2000; Larson et al., 2005; Weinstock et al., 2001). The cost of pooled sample testing in herds with a PI animal prevalence above 3% increases to where there is no benefit to pooling samples for testing (Munoz-Zanzi et al., 2000).

Pooling up to 100 serum samples to test for BVDV by RT-PCR has been shown to have high sensitivity (Weinstock et al., 2001). However, RT-PCR is a more expensive diagnostic test than AC-ELISA or MTVI-ELISA and can take longer to complete. If less expensive methods, such as AC-ELISA or MTVI-ELISA could be utilized to assay pooled samples, the overall cost of whole herd screening may be decreased. When testing for PI animals, MTVI-ELISA and AC-ELISA are both 100% sensitive (Saliki et al., 2000; Cornish et al., 2005; Saliki et al., 1997), however MTVI-ELISA requires several days to complete while AC-ELISA can be completed in one day. The sensitivity and specificity of AC-ELISA and MTVI-ELISA have not been determined using pooled samples.

Serum is often used in BVDV diagnostic assays, however maternal antibodies can interfere with testing when an animal is less than three months of age (Saliki et al., 1997). Ear notch samples are quick and easy to collect, require less skill than blood collection and there is a low chance that maternal antibodies can interfere with viral antigen detection. The aim of this study was to assess the performance of an AC-ELISA and MTVI-ELISA using pooled serum or saline from ear notch samples. The BVDV infection status of assayed animals was determined by single-sample testing by AC-ELISA and virus isolation in order to calculate the sensitivity and specificity of the AC-ELISA and MTVI-ELISA for pooled samples.

Materials and Methods

Samples.

Ear-notches and blood samples from known PI animals and BVDV-free animals were collected from diagnostic accessions submitted to Colorado State University Veterinary Diagnostic Laboratory. All ear notch samples were tested individually by AC-ELISA to confirm BVDV status and persistent infection was confirmed by virus isolation from serum samples taken one month later. Serum samples, positive by AC-ELISA, were analyzed by RT-PCR to confirm viremia as previously described (Ridpath et al., 1998).

Virus Stocks.

BVDV stocks of strains NewYork-1 (type I ncp-BVDV) (National Veterinary Services Laboratory, Ames, IA), Singer (type I cp-BVDV) (National Veterinary Services Laboratory, Ames, IA) and strain 125 (type II cp-BVDV) (National Veterinary Services Laboratory, Ames, IA) were propagated on bovine turbinate (BT) (ATCC, Manassas,

VA) cells in Eagle's minimum essential medium (MEM) (Irvine Scientific, Santa Ana, CA) with non-essential amino acids containing 2% horse serum (Sigma, St. Louis, MO), 5% glutamine-penicillin-streptomycin (Irvine Scientific, Santa Ana, CA), 5% HEPES buffer (Irvine Scientific, Santa Ana, CA) and 2.5% amphotericin B (Fisher Scientific, Fair Lawn, NJ). Flasks ($75cm^2$) (Nunc, Inc. Naperville, IL) were frozen at –70C for 24 hours, thawed and clarified at 15,000 rpm for 10 minutes.

Dilution of viral stocks and PI animal serum and ear notches.

BVDV strains Singer ($10^{6.5}$ $TCID_{50}$/ml), 125 ($10^{6.5}$ $TCID_{50}$/ml) and NY-1 ($10^{7.25}$ $TCID_{50}$/ml), were diluted in sterile 1XPBS as shown in Tables 2.2 and 2.3. NY-1 was also diluted in MEM with non-essential amino acids containing 2% horse serum, 5% glutamine-penicillin-streptomycin, 5% HEPES buffer and 2.5% amphotericin B. Ear notch samples from two known PI animals were incubated for 10 minutes in sterile 1XPBS and diluted as shown in Table 2.3.

Serum from two known PI animals was pooled with either serum neutralization (SN) negative or SN positive serum (titer ≥2048 for both type I and type II BVDV) and with each other to determine if the presence of anti-BVDV neutralizing antibodies could affect the results of AC-ELISA or MTVI-ELISA. All samples were assayed by AC-ELISA (Bovine Viral Diarrhea Antigen Test Kit, Syracuse Bioanalytical, Inc., Ithica, NY) per manufacturer's instructions and MTVI-ELISA as previously described (Saliki et al., 1997).

Pooling.

Individual ear notch samples were incubated in two milliliters 1XPBS per manufacturer's instructions. The samples were kept in separate tubes to better facilitate

the testing of individual samples from positive pools. Serum and saline from ear notch samples were randomly assigned into pools containing 2, 3, 4 or 5 samples using the Microsoft Excel (Microsoft Corp., Redmond, WA) random number generator. A total of 265 pools were prepared for each pool size; 212 contained serum or saline from one known positive sample; all other samples in the pool were from BVDV negative animals, and 53 pools contained serum or saline from known negative samples. Pools contained 100μl serum or saline from each individual sample. All pools were assayed for BVDV by AC-ELISA and MTVI-ELISA.

The sensitivity of the AC-ELISA and MTVI-ELISA for each pool size was calculated by dividing the number of pools correctly identified as containing BVDV (a) by the total number of pools that did contain a BVDV positive sample (a+c), as determined by viral isolation or AC-ELISA. The specificity was calculated by dividing the number of pools correctly identified as not containing a BVDV positive sample (d) by the total number of pools that did not contain a BVDV positive sample (b+d), as determined by viral isolation or AC-ELISA (Table 2.1).

Table 2.1: Sensitivity and Specificity

	Condition according to individual testing	
Pooled test result	Positive	Negative
Positive	a = true positive	b = false positive
Negative	c = false negative	d = true negatives
Total	a+c	b+d
	Sensitivity = a / (a+c)	Specificity = d / (b+d)

Statistical Analysis.

McNemar's test with exact binomials was used to determine the sample size required to test the hypothesis that the assays being compared are similar at an α=0.05 with 0.9 power. Z-proportion tests, adjusting for multiple comparisons, were used to

determine if there were differences in the sensitivity and specificity between pool sizes for either the AC-ELISA or MTVI-ELISA. McNemar's chi-square test was used to compare the sensitivity and specificity between the AC-ELISA and MTVI-ELISA.

Results and Discussion

Pooled Ear Notch Samples.

The sensitivity and specificity of the AC-ELISA and MTVI-ELISA for pooled saline from ear notch samples are provided in Table 2.2. The sensitivity and specificity of AC-ELISA ranged from 98% to 72% and 94% to 100%, respectively using pools of 2 to 5. For all pool sizes the sensitivity of the MTVI-ELISA was less than 3% and the specificity was greater than 98%. Due to the low sensitivity of the MTVI-ELISA using pools of two, the sensitivity and specificity for pools of 3 or 4 were not determined. The sensitivity of the AC-ELISA was significantly different than the MTVI-ELISA for pools of two and five. Except for pools of two and three samples, the sensitivity results from all pool sizes using the AC-ELISA were significantly different from each other. No significant differences in the specificity between or within assays were observed.

Dilution of virus stocks and PI animal ear notches.

Saline from two PI animal ear notches, and virus stocks of NY-1, Singer and strain 125 were diluted in 1XPBS to determine if the decrease in AC-ELISA sensitivity, as pool size increased, was due to the dilution of viral antigen. For all viruses the AC-ELISA gave a negative result with a 1:10 dilution of the original starting material (Table 2.3).

Table 2.2: Sensitivity and Specificity of Pools of 2, 3, 4 or 5 using AC-ELISA or MTVI

A

Ear Notches - AC-ELISA				
Pool Size	Sensitivity	95% CI	Specificity	95% CI
2	98.58%[a]	95.58%, 99.63%	94.34%[a]	83.37%, 98.53%
3	94.81%[a]	90.66%, 97.25%	94.34%[a]	83.37%, 98.53%
4	84.43%[b]	78.69%, 88.90%	98.11%[a]	88.60%, 99.90%
5	72.17%[c]	65.54%, 77.98%	100.00%[a]	91.58%, 100.0%

B

Serum - AC-ELISA				
Pool Size	Sensitivity	95% CI	Specificity	95% CI
2	14.29%[a]	5.94%, 29.23%	100.00%[a]	78.12%, 100%
3	nd	nd	nd	Nd
4	nd	nd	nd	Nd
5	1.42%[b]	0.37%, 4.42%	100.00%[a]	91.58%, 100%

C

Ear Notches – MTVI				
Pool Size	Sensitivity	95% CI	Specificity	95% CI
2	2.83%[a]	1.16%, 6.35%	100.00%[a]	91.58%, 100%
3	nd	nd	nd	Nd
4	nd	nd	nd	Nd
5	2.83%[a]	1.16%, 6.35%	98.11%[a]	98.11%, 99.9%

D

Serum – MTVI				
Pool Size	Sensitivity	95% CI	Specificity	95% CI
2	4.76%[a]	0.83%, 17.42%	100.00%[a]	78.12%, 100%
3	nd	nd	nd	Nd
4	nd	nd	nd	Nd
5	0.47%[b]	0.02%, 0.3%	98.11%[a]	98.11%, 99.9%

A-D: Values with different letters among the column are significantly different using a p-value of 0.05. A: Bonferoni multiple comparison adjustment (p=0.008). nd = not done. 95% CI: 95% confidence intervals.

Table 2.3: AC-ELISA BVDV dilution results

	Singer	Strain 125	NY-1 in PBS	NY-1 in Media	Ear notch 1	Ear notch 2
No dilution	+	+	+	+	+	+
1:2	+	+	+	+	+	+
1:4	+	+	+	+	+	+
1:8	+	-	-	-	+	+
1:10	-	-	-	-	+	+
1:16	-	-	-	-	+	+
1:32	nd	nd	nd	nd	+	+
1:100	-	-	-	-	-	+
1:1000	-	-	-	-	-	-
1:10,000	-	-	-	-	-	-
1:100,000	-	-	-	-	-	-
1:1,000,000	-	-	-	-	-	-

Singer = cytopathic type I BVDV, Strain 125 = cytopathic type II BVDV, NY-1 = non-cytopathic type I BVDV. nd = not done. Purified BVD viruses were diluted in 1XPBS from an initial viral titer of $10^{6.5}$ (Singer, Strain 125) or $10^{7.25}$ (NY-1) $TCID_{50}$ per ml.

Ear notches from confirmed PI calves gave positive AC-ELISA results at a dilution of 1:32 (ear notch 1) or 1:100 (ear notch 2). Diluting cultured virus and saline from ear notch samples is not necessarily equivalent because the ELISA detects antigen not infectious virus. The concentration of antigen in ear notch samples may not be the same as that seen in culture virus stocks.

To determine if the inability to propagate virus from PI ear notches in the MTVI-ELISA was due to 1XPBS, the ability of BVDV diluted in 1XPBS to infect BT cells was observed. NY-1 was diluted in 1XPBS or MEM with 2% horse serum and assayed by MTVI-ELISA (Table 2.4). NY-1 was able to infect and replicate in BT cells regardless of dilution buffer.

Table 2.4: MTVI-ELISA BVDV dilution results

	NY-1 in PBS	NY-1 in Media
No dilution	+	+
1:10	+	+
1:100	+	+
1:1000	+	+
1:10,000	+	-
1:100,000	-	-
1:1,000,000	-	-

Purified BVD virus was diluted in 1XPBS from an initial viral titer of $10^{7.25}$ $TCID_{50}$ per ml.

Dilution of purified BVDV I (strain NY-1) showed 1XPBS had no apparent affect on the ability of the virus to infect bovine turbinate cells (Table 2.4). It is possible that there is some inhibitory substance present in the ear, preventing infection of bovine turbinate cells by BVDV. Another potential explanation for the decrease in sensitivity is a lack of infectious virus present in the ear notch. The results from these studies indicate that in at least some samples, infectious virus is present. For the majority of ear notch samples assayed, however, no replicating virus was detected by MTVI-ELISA. Virus stocks diluted in 1XPBS could be detected by MTVI-ELISA to a titer of $10^{3.25}$ $TCID_{50}$ per milliliter (1:10,000 dilution). It is possible that the titer of infectious virus leached out of the ear into the 1XPBS is below the detection limit of the MTVI-ELISA. Interference by neutralizing antibody is unlikely due to the sampling method.

Pooled Serum Samples.

The sensitivity for pools of two serum samples was 4.76% for MTVI and 14.29% for AC-ELISA. Due to the low sensitivity values for both AC-ELISA and MTVI-ELISA using pools containing two serum samples (Table 2.2), the sensitivity and specificity for pools of 3 or 4 were not determined.

Pooling of PI animal serum with SN negative or positive serum.

To determine if the low sensitivity using pooled serum samples was due to the presence of neutralizing antibody, PI animal serum was pooled with SN negative or SN positive BVDV negative serum. For two different PI animals, pooling of SN antibody positive serum with serum from a known PI animal, resulted in negative AC-ELISA and MTVI-ELISA results. When PI serum was pooled with SN antibody negative serum, the AC-ELISA and MTVI-ELISA were positive for BVDV (Data not shown); indicating that neutralizing antibody is interfering with the assays.

The presence of neutralizing antibodies in BVD virus negative animals prevents infectious virus in PI animal serum from infecting and replicating in tissue culture; giving the MTVI-ELISA a sensitivity too low to be used for screening pools of serum. The AC-ELISA used in this study identifies the E^{rns} envelope glycoprotein of BVDV which induces the production of virus neutralizing antibodies in infected animals (Kwang et al., 1992). These antibodies probably bind any E^{rns} antigen present in the pooled serum, preventing the AC-ELISA from recognizing the antigen.

Conclusions

Screening for PI animals is an important control method used to prevent the spread of BVDV in cattle populations. Unfortunately, the cost of whole herd testing may not be economically feasible for every cattle operation. Pooling individual samples for BVDV testing would provide a less expensive alternative for screening whole herds (Weinstock et al., 2001). Using computer simulation, the cost of pooled-sample testing for herd screening using RT-PCR, where PI animal prevalence ranged from 0.5%-3%, was determined to be lower than the cost incurred by testing individual animals by RT-

PCR (Munoz-Zanzi et al., 2000). Using RT-PCR would be more time consuming than other BVDV diagnostic assays and might result in higher costs than if AC-ELISA was used on pooled saline from ear notch samples.

The AC-ELISA and MTVI-ELISA have been used extensively to identify PI animals. Pooling serum samples for testing by AC-ELISA or MTVI-ELISA proved to be inadequate for identification of PI animals. The sensitivity of the MTVI-ELISA using pooled saline from ear notch samples is also too low to be used for whole herd screening.

Despite a lack of sensitivity with the MTVI-ELISA, pooling saline from ear notch samples does appear to be a reliable method to detect PI animals using AC-ELISA. The ability of the AC-ELISA to detect antigen in saline from a PI animal ear notch indicates that the concentration of E^{rns} antigen is sufficient to be detected, regardless of whether infectious virus is present. The AC-ELISA may have a lower threshold of detection than the MTVI-ELISA and therefore less virus or antigen is necessary for identification. E^{rns} is an envelope glycoprotein and may be more readily released into the incubation buffer than whole virus.

Dilution of saline from two PI animal ear notches indicates that the concentration of antigen present in the ears of individual animals may vary (Table 2.3). The decrease in assay sensitivity with increased pool size may be the result of individual variation between animals.

While the sensitivity of the AC-ELISA is less than 100% using pools of two, it may still be an effective method for reducing the costs of whole herd screening. For large herds decreasing the cost of testing by 50%, may be worth the risk of using a less sensitive test. Previous work has shown BVDV can be eradicated from endemically

infected herds even if a PI animal is not correctly identified (Cleveland et al., 2004). For situations where the prevalence of PI animals is potentially high (>3%), testing samples individually is likely to be more cost effective than pooling samples for AC-ELISA or RT-PCR.

A combination of the two methods, RT-PCR and AC-ELISA, may be more cost effective in herds where PI animal prevalence is expected to be below 3%. Initial screening of pooled saline from ear notch samples by RT-PCR, followed by AC-ELISA for individual animals within positive pools, may result in lower cost per cow estimates.

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CHAPTER III

COST OF TESTING BY AC-ELISA FOR PI ANIMALS USING POOLED SAMPLES

Abstract

Pooling ear notch samples has been shown to be a sensitive method for identifying persistently infected (PI) animals (Cleveland et al., 2006). Using a simulation model (BTMSim$) the cost per cow for whole herd screening and time to BVDV eradication using pooled saline from ear notch samples for AC-ELISA was compared between pools of 1, 2, 3, 4 or 5 samples. Identification and removal of PI animals was simulated in years 1, 2 or 3 after BVDV introduction. Simulation results indicate the time to BVDV eradication could increase by one year when using pools of 2 or 3 and may never be achieved using pools of 4 or 5. Simulation herds where BVDV infection was becoming endemic, i.e. 3 years after initial introduction of BVDV, resulted in significantly lower costs when pooling samples than when testing individual animals. In endemically infected herds the increased herd immunity to BVDV results in a lower prevalence of PI animals being generated each year. The lower prevalence of PI animals results in lower testing costs when samples are pooled as compared to individual testing.

Introduction

Bovine viral diarrhea virus (BVDV) is an economically important virus causing milk production losses, infertility, reproductive losses and neonatal death in affected herds (Houe, 1999). The spread of BVDV within and between herds is accomplished by

the introduction of persistently infected (PI) animals. PI animals are chronic shedders of BVDV and are considered the primary transmitters of the virus (Baker, 1995). Due to the generally low prevalence of PI animals in infected herds (0.5%-3%) it is necessary to test each individual animal within a herd for persistent infection. Testing each individual animal can be costly for some producers.

An alternative to testing animals individually is pooling samples to be assayed for BVDV. Antigen-capture (AC)-ELISA has been shown to be a sensitive method for identifying PI animals in pooled saline from ear notch samples (Cleveland et al., 2006). However, pooling ear notch samples for AC-ELISA is not as sensitive as testing individual animals (100%) and the affects of decreased sensitivity have not been investigated. Previous work with RT-PCR, has shown that in herds with low PI animal prevalence (0.5%-3%) the cost of pooling samples can be lower than testing individual animals (Munoz-Zanzi et al., 2000).

In this study the cost of whole herd screening versus pooling saline from ear notch samples for AC-ELISA was simulated using a BVDV transmission model (BTMSim$). In addition, the time to BVDV eradication as measured by the generation of PI animals was compared between pool sizes of 1, 2, 3, 4 or 5.

Materials and Methods

Model Description.

BTMSim (Cleveland, SM, 2003) was modified to simulate the cost of whole herd screening for PI animals in a hypothetical 500-cow beef ranch in the western United States, herein referred to as BTMSim$ (Cleveland, SM, 2003). Briefly, the model is a state transition model based on the Reed-Frost equation, incorporating multiple stochastic

parameters, including the sensitivity of the assay used to identify PI animals and the survival rate of PI fetuses and calves. The model assumes that cattle are bred once a year in the summer months and calved in early spring. Transmission in a 100% susceptible herd was simulated for ten years after the introduction of one PI animal to the simulated herd.

The cost of whole herd screening was calculated by multiplying the number of pools by the cost of the assay. For each pool that was positive for BVDV, an additional cost for testing each individual animal within the pool was added. Each positive pool was assumed to contain saline from one PI animal. All animals were tested the first year identification was used as a control method; calves and purchased heifers only were tested in all subsequent years.

Because ranchers are not always aware of BVDV transmission upon initial introduction of a PI calf, the cost of testing was simulated at the initial time of introduction as well as two and three years after the PI animal was introduced. The effect of transmission of BVDV from acutely infected to susceptible animals was examined, however it was assumed that the primary source of BVDV transmission was PI calves (Houe, 1999). There is debate as to whether acutely infected animals can effectively transmit BVDV and therefore the effect of transient transmission was assumed to be nominal (Niskanen et al., 2002; Niskanen et al., 2000). Transmission of BVDV between wildlife and cattle was not considered due to the low likelihood of contact between species and the lack of literature to estimate contact rates. Four disease states were modeled: naturally immune, transiently infected, persistently infected and susceptible.

Parameter Estimates.

BTMSim$ was created in @RISK (Palisades Corp., Newfield, NY), a commercially available spreadsheet program with a simulation add-in for Monte Carlo sampling. The infectious contact rate (k) was optimized based on serological analysis of field data (Cleveland et al., 2004) using the Goal Seek function in the @Risk program, which uses a computer algorithm based on the Ridders' method (Ridders, 1979). Seven infectious contacts per PI animal per day resulted in approximately 50% of the population in a susceptible herd seroconverting within the first year, which is similar to what was seen in the field data (Cleveland, et al., 2004) and was therefore considered the optimal value for k.

All susceptible cows infected during the first trimester of gestation were assumed to be PI-carriers (PI-Cs). The immune response due to vaccination was assumed to be inadequate for preventing fetal infection because it has been shown that dams that were vaccinated and had no natural exposure to BVDV were unable to protect the fetus from infection (Hamers et al., 2001; Van Campen et al., 2000). However, natural exposure results in long-term immunity to BVDV that may protect a fetus from BVDV exposure *in utero* (Potgieter, 1995; Cortese et al., 1998). Therefore, it was assumed that once a cow had been naturally exposed to BVDV any future offspring were protected from the virus *in utero*.

The total number of cows infected during the first trimester was predicted and the number of PI animals generated by those cows was stochastically determined using a binomial distribution with a probability of 80% that a PI fetus would survive pregnancy (Taylor et al., 1997; Baker, 1995). The probability of a PI calf surviving each month

after birth was assumed to be 95% (Baker, 1995) and modeled stochastically using a binomial density function.

It was assumed that approximately 60% of the total PI fetuses were born into the herd during the first and last month of calving and approximately 40% were born in the second month of calving. Once a cow was infected with BVDV it was assumed the animal had life-long immunity, sufficient to prevent fetal infection (Saliki et al., 2000).

Each year the culling and replacement of 10% of the total cow population was simulated. It was assumed that the culled cattle were older animals and would be immune to BVDV infection due to the increased likelihood of exposure to PI cattle (Houe, 1995; Van Campen et al., 1998). Replacement cattle were assumed to be younger cattle with a lower likelihood of infectious contact and therefore the replacement population was considered 100% susceptible to infection.

The total cost to screen a herd for BVDV is a function of the herd size, pool size, prevalence of BVDV PI animals and the cost of the assay. The AC-ELISA was given a cost of $7.00 a head based on Colorado State University Veterinary Teaching Hospital diagnostic lab costs. The sensitivity of the AC-ELISA for pools of saline from ear notch samples was varied depending on the pool size used and was based on pooled sample testing results (Cleveland et al., 2006). A sensitivity of 100% was used for pools of 1, 98% for pools of 2, 94% for pools of 3, 84% for pools of 4 and 72% for pools of 5.

For each pool size one simulation using a k of 7 and a 10% replacement rate, with 2500 iterations was run. Output for each scenario was the median number of PI animals at the end of calving each year and the median cost for testing each year, for ten years.

Statistical Analysis.

Differences in the median number of PI animals born and the median cost of testing for PI animals (over a 10 year period of time) between pool sizes were determined using the Wilcoxon two-sample test. We used a 5% significance level for all the statistical analyses performed in this study with a Bonferoni multiple comparison adjustment (α=0.005).

Results and Discussion

No Identification and Removal of PI Animals.

Bovine viral diarrhea virus transmission, measured by the median number of PI animals generated, and simulated by BTMSim$, without any PI animal identification and removal is shown in Figure 3.1. The simulated herd is 100% susceptible to BVDV infection and culled animals are replaced with 100% susceptible animals. As seen previously (Cleveland, SM, 2003), the median number of PI animals born spikes in year 2 and decreases with increased herd immunity. The practice of replacing 10% of the herd with susceptible animals allows the continued spread of BVDV within the herd resulting in an endemically infected herd.

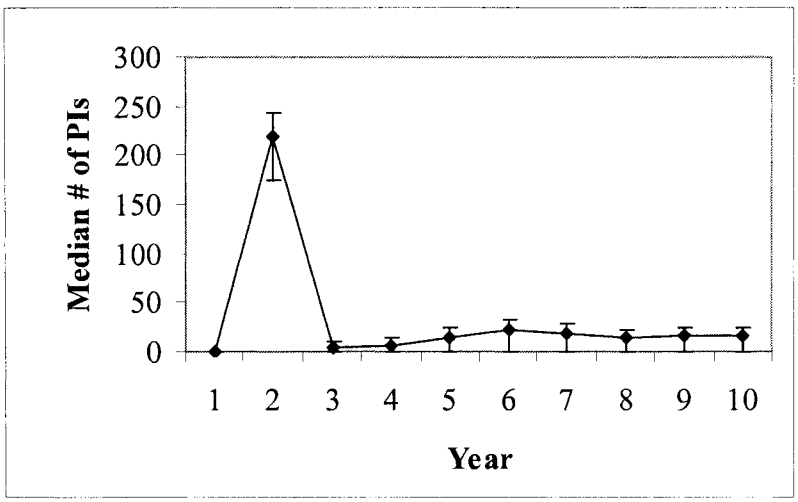

Figure 3.1: Median number of PI animals born over ten years
Bars indicate 5% lower bound and 95% upper bound.

Herds can remain endemically infected as long as PI animals are present and susceptible animals are introduced to the herd. The identification and removal of PI animals can result in reduced transmission of BVDV as well as eventual eradication of the virus from a herd (Cleveland, SM, 2003).

Identification and Removal of PI Animals

Previously, sensitivity analysis of BTMSim showed the median number of PI animals born each year is not affected by the assay sensitivity of the test used to identify PI animals (Cleveland, SM, 2003); indicating that in this study the decrease in AC-ELISA sensitivity with increased pool size would not affect the median number of PI animals born each year. Analysis of the median number of PI animals born, for ten years, following initial PI animal introduction, simulated in BTMSim$, confirmed this assessment. There were no significant differences in the median number of PI animals born between pool sizes. Pooling samples for PI animal identification and removal does not appear to affect the number of animals born into a herd. However, the number of years required to test for PI animals can be affected.

If typical BVDV control methods are applied, i.e., all animals introduced to a herd are tested for BVDV prior to introduction to the herd, whole herd screening for BVDV can be stopped the first year no PI animals are detected. Table 3.1 shows the number of years testing for BVDV PI animals is required when pooling samples.

Table 3.1: Median years of required BVDV PI animal testing

Pool Size	LB	**Year 1**	UB	LB	**Year 2**	UB	LB	**Year 3**	UB
1	3	3	4	2	2	2	1	2	2
2	3	3	4	2	2	3	1	2	2
3	3	3	4	2	2	3	1	2	3
4	3	4	NE	2	3	7	1	2	NE
5	3	4	NE	2	3	NE	1	2	NE

NE: not eliminated. LB: 5%, lower bound. UB: 95%, upper bound

The median cost per animal simulated by BTMSim$ over a ten year period for identification and removal of PI animals after the first year of BVDV introduction is shown in Figure 3.2.

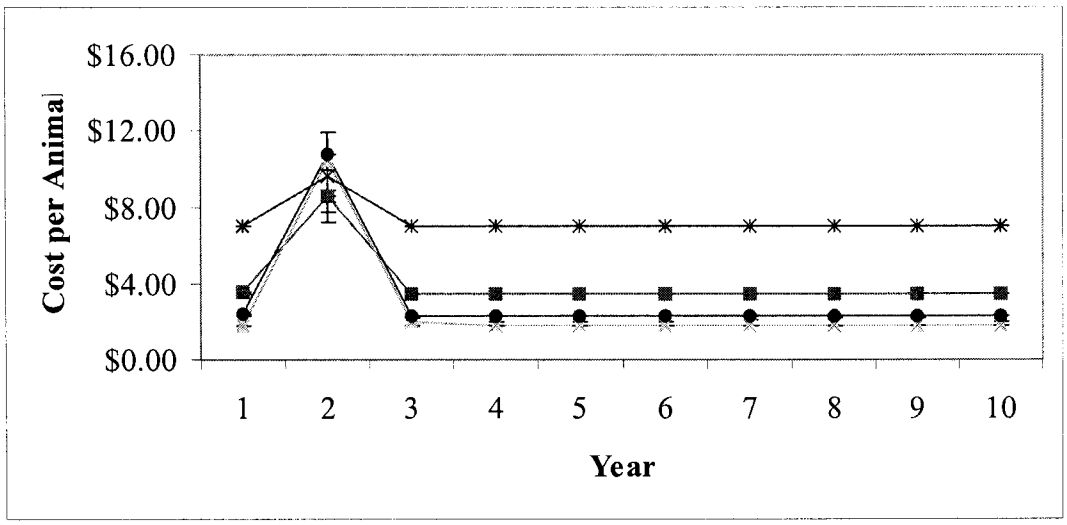

Figure 3.2: Median cost per cow estimates for identification and removal of PI animals in year 1 of BVDV introduction

(✱) = pools of 1 (100%), (■) = pools of 2 (98%), (◆) = pools of 3 (94%), (▲) = pools of 4 (84%), (×) = pools of 5 (72%). Bars indicate 5% lower bound and 95% upper bound.

The cost of testing for all pool sizes increases at year two because of the increased prevalence of PI animals. As shown in Figure 3.1, the PI animal prevalence decreases in year 3; which results in lower costs per animal tested when using pooled samples. The median cost per animal tested is significantly lower when using pool sizes of 2, 3, 4 or 5 than when individual samples are assayed. Pools of 4 and 5 result in a significantly lower cost per animal than pools of 2. However, the risk of prolonged transmission due to failure to eradicate BVDV is greater using pools of 4 or 5 (Table 3.1).

When PI animals are identified and removed the first year a PI animal is introduced to the herd, the number of years testing is required to identify and remove PI

animals is not affected when using pool sizes of 2 or 3. The sensitivity of the assays is high enough to eliminate BVDV transmission in the same time frame as testing animals individually. However for pool sizes of 4 or 5 there is a risk that eradication can take more time or may not be achieved at all. An assay with a sensitivity of 84% or 72% is not sufficient to eliminate the spread of BVDV.

One PI animal may not be detected upon initial introduction to a herd, so the cost per animal for testing for BVDV was also simulated beginning the second year of BVDV introduction (Figure 3.3). Because the initial PI animal is not identified, the animal stays in the herd longer and more PI calves are generated in year two. Increased PI animal prevalence results in higher test costs in year 2 than those seen when identification and removal began in year 1. With increased pool size the cost per animal is significantly lower.

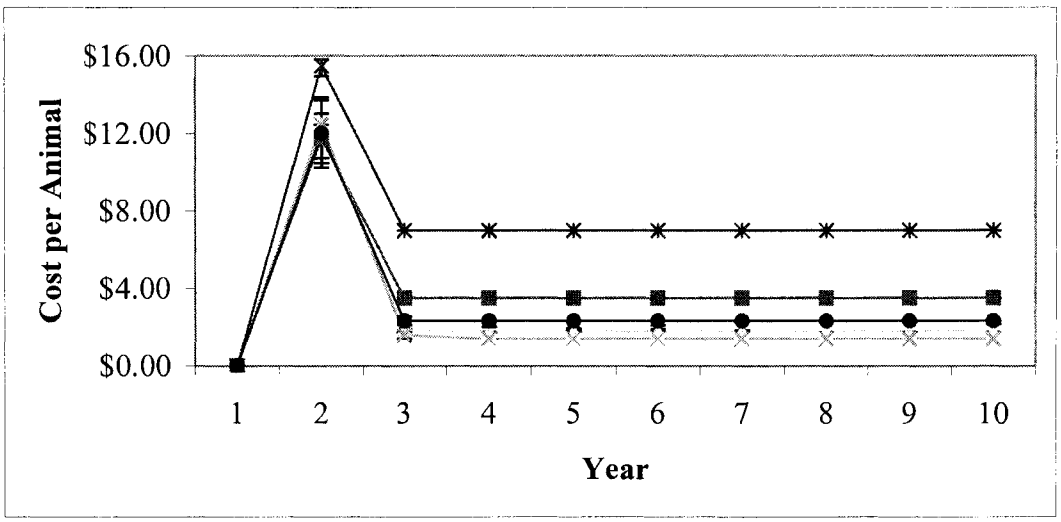

Figure 3.3: Median cost per cow estimates for identification and removal of PI animals in year 2 of BVDV introduction

(✱) = pools of 1 (100%), (■) = pools of 2 (98%), (◆) = pools of 3 (94%), (▲) = pools of 4 (84%), (×) = pools of 5 (72%). Bars indicate 5% lower bound and 95% upper bound.

The number of years testing for PI animals is required increases by no more than one year for pools of 2 or 3, when compared to individual testing. With decreased sensitivity, as seen for pools of 4 or 5, the time required to eliminate BVDV increases; with the worst case scenario being PI animals are not fully eliminated by year ten. Again, the risk in using pools of 4 or 5 is therefore greater than pools of 2 or 3 when looking at how many years are required to eliminate BVDV from a herd.

If testing does not begin until year 3, the cost of pooling samples for PI animal identification is significantly lower with increased pool size (Figure 3.4). The high level of herd immunity results in decreased PI animal prevalence in year three. Because PI animal prevalence is low, to test individual animals would cost more than to pool samples; indicating that with lower PI animal prevalence, pooling samples is a more economical choice.

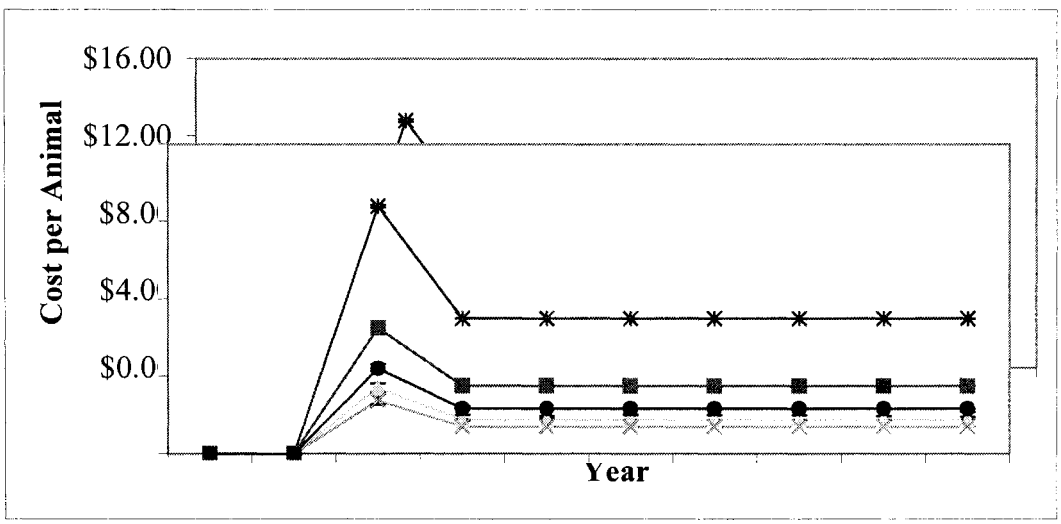

Figure 3.4: Median cost per cow estimates for identification and removal of PI animals in year 3 of BVDV introduction

(✱) = pools of 1 (100%), (■) = pools of 2 (98%), (◆) = pools of 3 (94%), (▲) = pools of 4 (84%), (×) = pools of 5 (72%). Bars indicate 5% lower bound and 95% upper bound.

Further evidence for the decreased cost with deceased PI animal prevalence using pooled samples was shown using computer simulation for herd screening using RT-PCR. When PI animal prevalence ranged from 0.5%-3% the cost incurred by testing individual animals by RT-PCR was determined to be higher than pooling samples (Munoz-Zanzi et al., 2000). The cost of testing per cow increased as the prevalence of PI animals increased.

Herd immunity and PI animal prevalence more resembles that of an endemically infected herd in year 3. The prevelance of PI animals in year 3 is low and the number of susceptible animals that become infected decreases. Therefore eradication of BVDV as measured by the generation of PI animals can be achieved with fewer years of testing than when PI prevalence is higher and the number of susceptible animals that become infected with BVDV is higher as seen when testing begins in years 1 or 2 (Table 3.1). As seen in years 1 and 2 there is an increased risk of not eliminating BVDV transmission when using pools of 4 or 5 samples.

Conclusions

This model was designed to assess the cost of testing only. The model does not account for financial losses due to animal death or treatment for infection. While simulation results show that pooling saline from ear notch samples can decrease the cost of testing and result in eradication of BVDV in the same time as testing individual animals, the affect on overall herd health is not assessed in this model. Using less sensitive diagnostic methods may cause PI animals to remain in a herd, resulting in increased infection rates among herd animals; which may result in higher costs due to the expense of treatment or decreased profits due to decreased herd health.

Simulation results indicate that even with a 100% sensitivity for pools of one, elimination of PI animals can take up to three years. Infection of pregnant cows and the production of PI animals can continue despite identifying 100% of PI animals because of the time when animals are typically tested for BVDV. Often calves are not tested until cows have already been bred. Bred cows can then be exposed to virus resulting in the birth of a PI calf. If PI animals could be detected *in utero*, testing for one year may be all that would be required to eliminate BVDV from a herd.

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CHAPTER IV

SIMULATION RESULTS FOR A DIAGNOSTIC ASSAY TO DETECT PI ANIMALS *IN UTERO*

Abstract

A bovine viral diarrhea (BVDV) transmission model, BTMSim was modified to examine the efficacy of a diagnostic test able to identify persistently infected (PI) animals *in utero* during the later months of gestation. Simulation results from BTMSim_inUtero indicate that identification of PI animals *in utero* does not decrease the number of years required to test for PI animals in order to eliminate BVDV from a herd, nor does earlier detection decrease the median number of PI animals born.

Introduction

Bovine viral diarrhea virus is a highly infectious respiratory virus of cattle. The primary transmitters of BVDV are persistently infected (PI) animals (Harding et al., 2002). These animals were infected in the first trimester of gestation and shed large quantities of virus their whole lives. One PI calf can potentially infect 50% of a susceptible herd in approximately six months (Cleveland et al., 2004). An important source of infection for beef herds is the introduction of female cattle carrying a PI fetus (Bitsch et al., 1995). A common practice in beef herds that wish to introduce new genetics or to expand production is the purchase of bred replacement heifers. The majority of cows who have PI calves are immunocompetent animals that have been

exposed to BVDV during early pregnancy (Wittum et al., 2001). Unless the dam is persistently infected herself, cows bearing PI fetuses (PI-Cs) will be seropositive and virus-negative by standard testing methods (Brownlie 1998; Wittum 2001).

The introduction of just one PI animal can cause an outbreak in an entire herd, therefore early detection of persistently infected animals would be a significant benefit to cattle producers who wish to implement BVDV control programs. Current testing methods include virus isolation or antigen-capture ELISA using serum from calves. Unfortunately interference with maternal antibodies prevents testing via serum samples until calves are four to six months of age (Saliki et al., 1997) and in beef cattle, managed under typical United States management practices, this time frame allows bred cows to be exposed to BVDV in the first trimester; potentially resulting in the birth of a PI calf. Recently an antigen capture ELISA for the detection of the BVDV E^{rns} glycoprotein using ear notch samples has been developed and can be used on calves at birth. However this method can be difficult for large herds to implement where animals can be spread over large areas. Also calving season may last for several months and some producers may decide to wait to test until all calves are born.

The spread of BVDV and generation of PI calves may be decreased with the implementation of a diagnostic assay able to detect PI animals *in utero*. The goal of this study was to determine if identification of PI animals *in utero* would decrease the spread of BVDV as measured by the generation of PI animals.

Materials and Methods

Model Description

A previously published model BTMSim (Cleveland, SM, 2003) was adapted to include the testing of cows at seven to nine months gestation for PI fetuses. All model parameters and parameter estimates remained the same as presented in BTMSim with the exception of when PI animals were identified and removed from the herd. In BTMSim calves are identified and removed before breeding. In the adapted version of BTMSim, heretofore referred to as BTMSim_InUtero, identification and removal of PI animals occurred after calving the first year the presence of PI animals was suspected (year 1, 2 or 3 post-introduction) and during gestation thereafter. It was assumed that any potential PI-Cs were separated from the herd, calved separately and the calf tested for BVDV persistent infection prior to introduction to the herd. If the calf tested positive the animal was not allowed to join the rest of the herd.

Briefly, for simulation BTMSim_InUtero uses a commercially available spreadsheet with a simulation add-in for Monte Carlo sampling called @RISK (Palisades Corp., Newfield, NY). The infectious contact rate (k) was optimized based on serological analysis of field data (Cleveland et al., 2004) using the Goal Seek function in the @Risk program, which uses a computer algorithm based on the Ridders' method (Ridders, 1979). Seven infectious contacts per PI animal per day resulted in approximately 50% of the population in a susceptible herd seroconverting within the first year, which is similar to what was seen in the field data and was therefore considered the optimal value for k.

All susceptible cows infected during the first trimester of gestation were assumed to be PI-Cs. The total number of cows infected during the first trimester was predicted

and the number of PI animals generated by those cows was stochastically determined using a binomial distribution with a probability of 80% that a PI fetus would survive pregnancy (Taylor et al., 1997; Baker, 1995). The probability of a PI calf surviving each month after birth was assumed to be 95% (Baker, 1995) and modeled stochastically using a binomial density function. It was assumed that approximately 60% of the total PI fetuses were born into the herd during the first and last month of calving and approximately 40% were born in the second month of calving.

Once a cow was infected with BVDV it was assumed the animal had life-long immunity, sufficient to prevent fetal infection (Saliki et al., 2000). The immune response due to vaccination was assumed to be inadequate for preventing fetal infection because it has been shown that dams that were vaccinated and had no natural exposure to BVDV were unable to protect the fetus from infection (Hamers et al., 2001; Van Campen et al., 2000).

Each year the culling and replacement of 10% of the total cow population was simulated. It was assumed that the culled cattle were older animals and would be immune to BVDV infection due to the increased likelihood of exposure to PI cattle (Houe, 1995; Van Campen et al., 1998). Replacement cattle were assumed to be younger cattle with a lower likelihood of infectious contact and therefore the replacement population was considered 100% susceptible to infection. Because BVDV infection may not be apparent at the initial introduction of one PI animal, the efficacy of PI animal identification and removal beginning in the first, second and third year of BVDV introduction was simulated with 2500 iterations per run. Output for each scenario was the median number of PI animals at the end of calving each year.

The antigen capture ELISA using ear notch samples is 99% sensitive and 100% specific for detection of BVDV persistently infected animals. An *in utero* diagnostic test would also need to be highly sensitive to detect all potential PI calves. The median number of PI animals born and the time to eliminate BVDV from a herd when testing calves prior to breeding was compared to the median number of PI animals generated when using an *in utero* diagnostic assay at 7-9 months gestation that was 90%, 95%, 99% and 100% sensitive.

Statistical Analysis.

Differences in the median number of PI animals born over a 10 year period between identification methods was determined using the Wilcoxon two-sample test. We used a 5% significance level for all the statistical analyses performed in this study. A Bonferoni multiple comparison adjustment gave an $\alpha = 0.0023$.

Results and Discussion

The median number of PI animals born over ten years for testing beginning in year 1, 2 or 3 post PI animal introduction is provided in Figures 4.1, 4.2 and 4.3. When PI animals are not identified and removed there is a spike in the median number of PI animals in year two and then continuous generation of PI animals over the ten years. Testing cows for PI-carrier status the same year one PI animal is introduced to a herd would occur if a producer/veterinarian suspects BVDV in a herd because of decreased animal health resulting from BVDV infection, i.e. diarrhea, coughing, death. Although not statistically significant, the identification and separation of potential PI-Cs results in a decreased median number of PI animals born as assay sensitivity is increased. If the *in*

utero assay is 99% or 100% sensitive all PI-Cs are identified and transmission does not proceed past year 1 (Table 4.1).

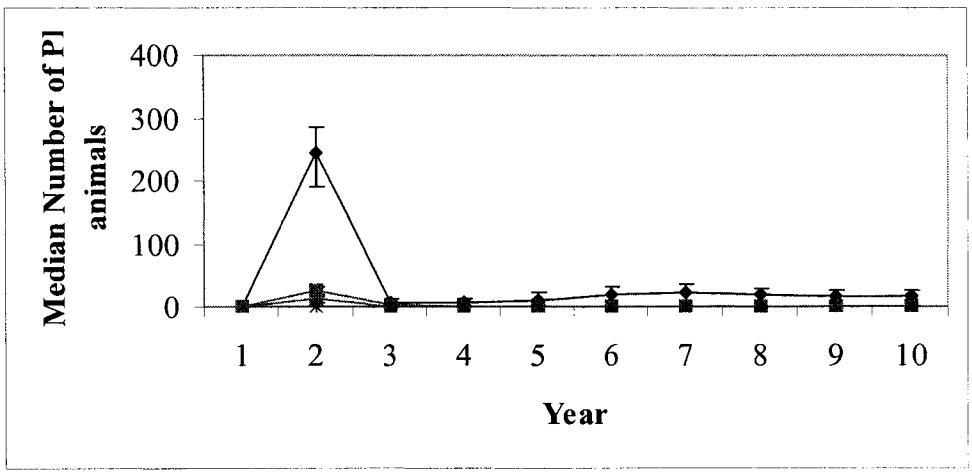

Figure 4.1: Median number of PI animals when testing begins the first year a PI animal is introduced

(◆) = No ID and removal, (■) = 90% assay sensitivity, (▲) = 95% assay sensitivity, (×) = 99% assay sensitivity, (✱) = 100% assay sensitivity. Bars indicate 5% lower bound and 95% upper bound.

If BVDV is not suspected in a herd the first year of PI animal introduction, decreased fertility or weaning rates may cause a producer/veterinarian to suspect BVDV as a potential cause. Testing for PI-Cs in the second year post-PI animal introduction (Figure 4.2) results in elimination of BVDV spread in one year regardless if assay sensitivity is 90%, 95%, 99% or 100% (Table 4.1).

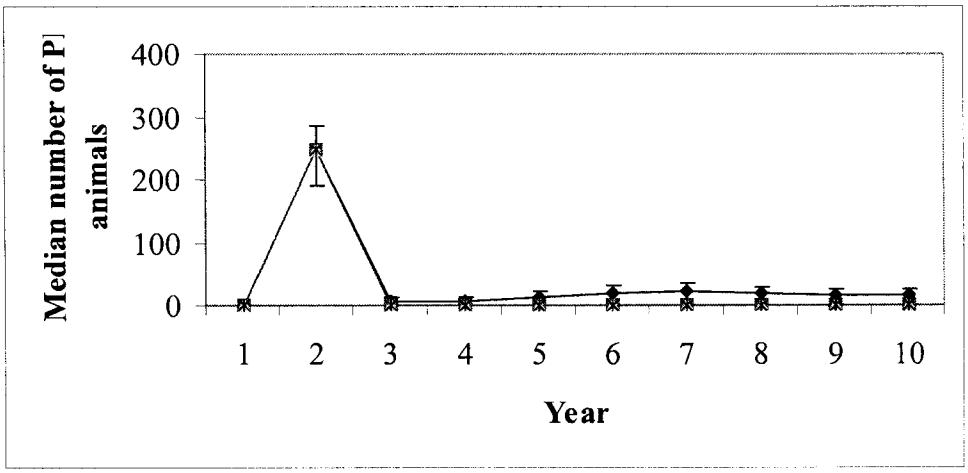

Figure 4.2: Median number of PI animals when testing begins the second year post PI animal introduction

(◆) = No ID and removal, (■) = 90% assay sensitivity, (▲) = 95% assay sensitivity, (×) = 99% assay sensitivity, (✳) = 100% assay sensitivity. Bars indicate 5% lower bound and 95% upper bound.

When testing for PI-Cs does not occur until three years post-PI animal introduction, BVDV transmission, measured by the generation of PI animals can be eliminated after one year of testing (Table 4.1).

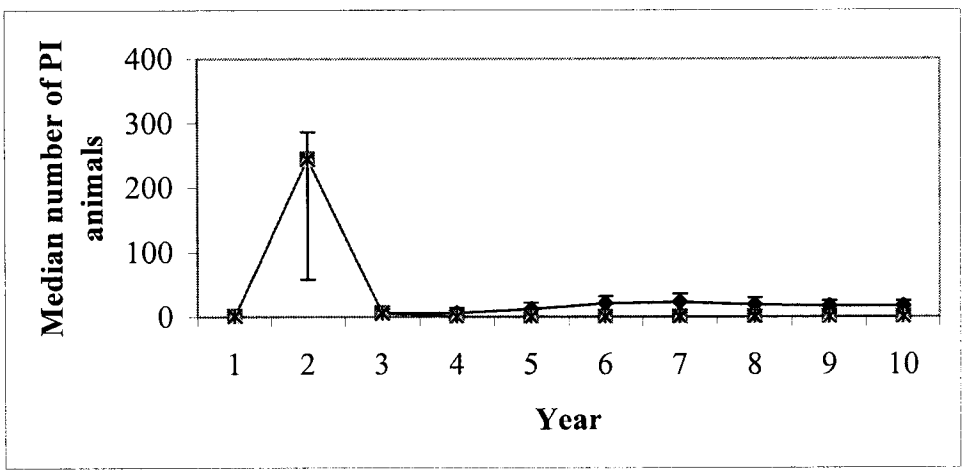

Figure 4.3: Median number of PI animals when testing begins the third year post PI animal introduction

(◆) = No ID and removal, (■) = 90% assay sensitivity, (▲) = 95% assay sensitivity, (×) = 99% assay sensitivity, (✳) = 100% assay sensitivity. Bars indicate 5% lower bound and 95% upper bound.

If typical BVDV PI animal identification and removal strategies are implemented, testing for PI animals can stop the first year no PI-Cs are identified. The number of years testing is required to eliminate BVDV is shown in Table 4.1.

Table 4.1: Median years of required BVDV PI animal testing

Assay Sensitivity	LB	**Year 1**	UB	LB	**Year 2**	UB	LB	**Year 3**	UB
In Utero									
0%	3	NE	NE	2	NE	NE	1	NE	NE
90%	3	4	4	2	2	2	1	2	2
95%	3	3	4	2	2	2	1	2	2
99%	2	2	3	2	2	2	1	2	2
100%	2	2	2	2	2	2	1	2	2
Normal testing									
99%	3	3	4	2	2	3	1	2	2
100%	3	3	4	2	2	2	1	2	2

NE: not eliminated. LB: 5%, lower bound. UB: 95%, upper bound

The number of years testing is required to eliminate BVDV is not affected when comparing current testing strategies to using an *in utero* assay, with the exception of testing the first year BVDV is introduced. Testing the first year BVDV is introduced decreases the number of years testing is required by 1-2 years. Identification of PI animals *in utero* prevents the spread of BVDV after they are born. Current control strategies do not involve the testing of calves until approximately six weeks old, allowing some animals to become infected and the generation of PI animals to continue. Even if calves are tested at birth the results are not known right away and a PI animal may stay in a herd for two weeks before it is removed.

A limitation of BTMSim_inUtero is that all naturally infected animals are considered immune for life and able to prevent fetal infection. While there is evidence to support this assumption (Saliki et al., 2000), it is possible that a PI calf could be generated from infection of a previously exposed animal, thereby increasing the number of susceptible animals and the prevalence of BVDV. The immune response of individual

animals varies and infection with BVDV may not elicit as strong a response in some animals.

The model also assumes that 80% of PI fetuses will survive each month and 50% of PI animals will die within the first year. This level may vary depending on the strain of BVDV with which the animals are infected. Some strains cause more severe disease than others (Bolin et al., 1992); resulting in increased death rates among PI animals as well as other animals within the herd. Changes in PI animal prevalence and the number of susceptible animals would affect the transmission curve simulated by BTMSim_inUtero.

Conclusions

With the exception of testing the first year BVDV is introduced to a herd the time required to eradicate BVDV is not affected. The transmission of BVDV is such that identifying PI animals *in utero* does not decrease the spread of BVDV when compared to testing after animals are born.

While these results indicate that development of an *in utero* diagnostic test may not be necessary for eradicating BVDV from a beef herd, an *in utero* assay may be useful when buying replacement heifers. Knowing the PI-carrier status of a replacement heifer would prevent the introduction of a PI animal to a BVDV free herd.

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CHAPTER V

A POTENTIAL MECHANISM FOR INCREASED IMMUNOGLOBULIN A LEVELS IN NASAL SECRETIONS DUE TO CONTINUOUS EXPOSURE TO BVDV VIA A PI FETUS

Introduction

Bovine viral diarrhea virus is an economically important pathogen of cattle worldwide. The transmission of BVDV vertically from dam to fetus and horizontally from animal to animal compounds the difficulty of controlling of these infections. While infection of a cow with BVDV is often subclinical, infection of a fetus can result in abortion, stillbirth, congenital malformations or the birth of a persistently infected (PI) calf. Persistently infected fetuses do not recognize BVDV antigen as different from self and do not elicit an immune response to the virus. These animals are chronic shedders of BVDV and are considered the primary transmitters of the virus. Identification and removal of PI animals has been shown to be an efficacious method for eradication of BVDV from a herd (Cleveland et al., 2004;Synge et al., 1999;Lindberg et al., 1999;Ferrari et al., 1999).

Bovine viral diarrhea virus (BVDV) can be introduced to a herd by a dam carrying a PI fetus (PI-C). When the PI animal is born, BVDV can be transmitted to any animal in a herd. Currently there are assays able to identify PI animals after birth, but there are no tests available to detect PI calves *in utero*, which would prevent the

introduction of BVDV into a herd altogether. Identification of dams carrying PI fetuses would be a valuable tool in the control of BVDV.

PI-Cs are virus negative, seropositive cows that have been shown to have higher serum neutralizing antibody titers than non-PI-C counterparts (Brownlie et al., 1998;Lindberg, 2001;Stokstad et al., 2003). The increase in serum antibody is thought to be the result of continuous immunological challenge by the PI fetus (Lindberg, 2001;Brownlie et al., 1998).

PI-Cs can potentially be identified by measuring the level of serum neutralizing antibodies present in the cow, with higher titers indicating the presence of a PI fetus. However, testing must occur when the cow is between 7 and 9 months gestation (Stokstad et al., 2003). In addition, the sensitivity (94%-100%) and specificity (39%-67%) of the ELISA assay used to detect PI-Cs is not sufficient to correctly identify PI fetuses *in utero* and has never been analyzed for use with vaccinated animals (Brownlie et al., 1998). Animals in the United States are generally vaccinated against BVDV and may have artificially higher serum neutralization antibody titers than non-vaccinated animals.

Increased serum neutralization titers to BVDV in PI-Cs suggests that continuous exposure of the immune system to the virus occurs via the PI fetus. To distinguish between increases in antibody titers due to vaccination versus continuous exposure, examining the antibody response in mucosal secretions may prove beneficial. Unlike serum antibody, intramuscular vaccination does no lead to increased antibody titers in mucosal secretions. Continuous exposure may also elicit increased anti-BVDV IgA titers in the nasal secretions of PI-Cs compared to non-PI-Cs. Upon initial exposure to an

antigen, the intranasal IgA response peaks and declines rapidly, reaching low levels within 4-6 weeks (Todd, 1973;Kimman et al., 1987;Saif, 1987). However, titers increase quickly (3-5 days) following re-exposure and remain high up to five months post-infection (Todd, 1973). Continuous exposure of the dam to BVDV through an infected fetus may result in higher concentrations of nasal anti-BVDV IgA titers in PI-Cs than non-PI-C animals and could possibly be used to identify PI-Cs.

Animals are initially exposed to BVDV via the intranasal route where the virus can elicit an immune response in the mucosal-associated lymphoid tissue (MALT). MALT is a major inductive site for B- and T-cells which continuously supply mucosal sites with IgA producing plasma cells and effector T-cells (Sminia, T. et al., 1999;Kiyono et al., 1992). MALT is composed of subepithelial lymphoid follicles covered with glandular mucosal secretions (Bienenstock, J. et al., 1999;Ham, A. W., 1969). Mucosal lymphoid follicles in different systems of the body have some common features and have been linked to what is referred to as the common mucosal immune system; which includes the gut-associated lymphoid tissue (GALT), bronchial-associated lymphoid tissue (BALT) and nasal-associated lymphoid tissue (NALT). All follicles have efferent lymphatics draining to regional lymph nodes. Mucosal lymphoid follicles have an overlying specialized epithelium containing M (microfold) cells (Bienenstock, J. et al., 1999;Owen, 1977).

M cells are responsible for regulating the access of microorganisms and antigens to areas of the MALT equipped to generate an immune response. M cells internalize luminal antigen either by phagocytosis (large particles and bacteria) or endocytosis via clathrin-coated vesicles (small particles and viruses). The internalized material is

transported in endocytic vesicles to endosomal compartments where antigen is delivered to the underlying lymphoid tissue by exocytosis through the basolateral membrane (Man et al., 2004). Present outside the basolateral membrane of M cells are lymphocytes which migrate to the M cell from lymphoid tissue. Thus, M cell "pockets" facilitate contact between incoming antigens and the immune system (Man et al., 2004).

When an animal is exposed to BVDV via the intranasal route, virus can be absorbed by endocytosis through the epithelium by M cells and presented to immunoregulatory cells such as B-cells and T-cells (Figure 5.1) (Sminia, T. et al., 1999;Tilney, 1971).

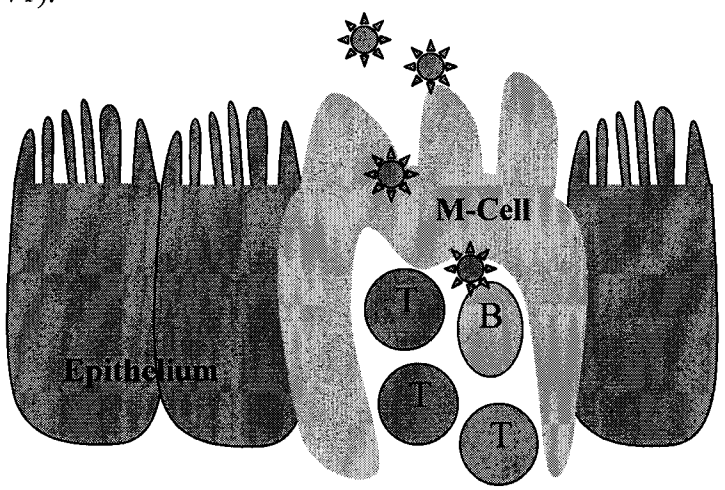

Figure 5.1: Absorbtion of BVDV by M-cells for presentation to immunoregulatory cells

The local stimulation of the mucosal immune system by BVDV induces local immune responses, specifically the production of secretory IgA and IgM (SIgA and SIgM) (Sminia, T. et al., 1999). Initial virus replication is thought to occur in the oronasal mucosa and the tonsils (Bruschke et al., 1998). Immune cells, such as antigen presenting cells (APCs), and B and T-cells will be exposed to BVDV antigen and will

drain to the posterior cervical lymph nodes where the cells will differentiate into effector and memory lymphocytes (Bruschke et al., 1998).

From the lymph nodes, the cells will migrate to other areas of the infected animal's body by way of the lymphatics (Corbeil, L.B. et al., 2001). The greatest accumulation of IgA producing plasma cells is at the site of initial introduction of the antigen; however, some cells move to other mucosal sites such as the lung, gut, mammary glands and reproductive tissues (Corbeil, L.B. et al., 2001). The cellular and humoral immune responses are adequate for clearance of the virus in an otherwise healthy animal (Corbeil, L.B. et al., 2001).

However, the clearance of BVD virus can take up to 17 days in a primary exposure to BVDV (Silflow et al., 2005;Howard, 1990). From the oronasal cavity, the virus can spread via the lymphatic vessels to the regional lymph node. It has been shown that by day two post-inoculation, virus can travel to the spleen and jejunum of infected animals, presumably via blood (Bruschke et al., 1998). Also, BVDV can cause leukopenia leading to increased susceptibility of the animal to secondary infections.

It is the immunosuppressive effects of BVDV that are thought to aid in the continued spread of the virus despite the local mucosal response. It has been shown that infection of macrophages with BVDV decreases the stimulation of T-cell proliferation; causing a decrease in the time to antibody production (Glew et al., 2003). Antibody to BVDV can be detected in serum by three weeks post-infection with titers continuing to increase for 10 to 12 weeks (Howard, 1990). Also, BVDV can prevent the production of interferon in infected cells (Charleston et al., 2001;Schweizer et al., 2001). Interferon is produced in response to virus infection and induces surrounding cells to produce proteins

that degrade mRNA, preventing neighboring cells from becoming infected with BVDV. By preventing the production of interferon, BVDV can freely spread throughout the animal.

A second exposure to BVDV results in a more rapid immune response and clearance of the virus (approximately 7 days). Quick clearance of BVDV upon secondary exposure is thought to be the result of a rapid humoral immune response (Silflow et al., 2005).

Theory: Continuous BVDV exposure to a cow via a PI fetus leads to increased anti-BVDV IgA in nasal secretions.

Ruminants have a cotyledonary placenta having 70 to 120 cotyledons, which are placental units of trophoblastic origin consisting of abundant blood vessels and connective tissue. The point of interface between the fetal and maternal tissues is the placentome and has a fetal cotyledon contributed by the chorion and a maternal cotyledon originating from the caruncular regions of the uterus (Senger, P. L., 1999). This interface allows placental transfer of nutrients from the dam and transfer of metabolic wastes from the fetus. The bovine placenta is a syndesmochorial placenta and is considered the least intimate among placental types because there is a complete intact layer of epithelium in both the maternal and fetal components (Figure 5.2) (Senger, P. L., 1999).

However the bovine endometrial epithelium does transiently erode and regrow allowing intermittent exposure of maternal capillaries to the chorionic epithelium (Figure 5.3) (Senger, P. L., 1999). While the endometrial epithelium is eroded, the BVD virus may cross over into the endometrial capillary where the virus may be taken up by APCs.

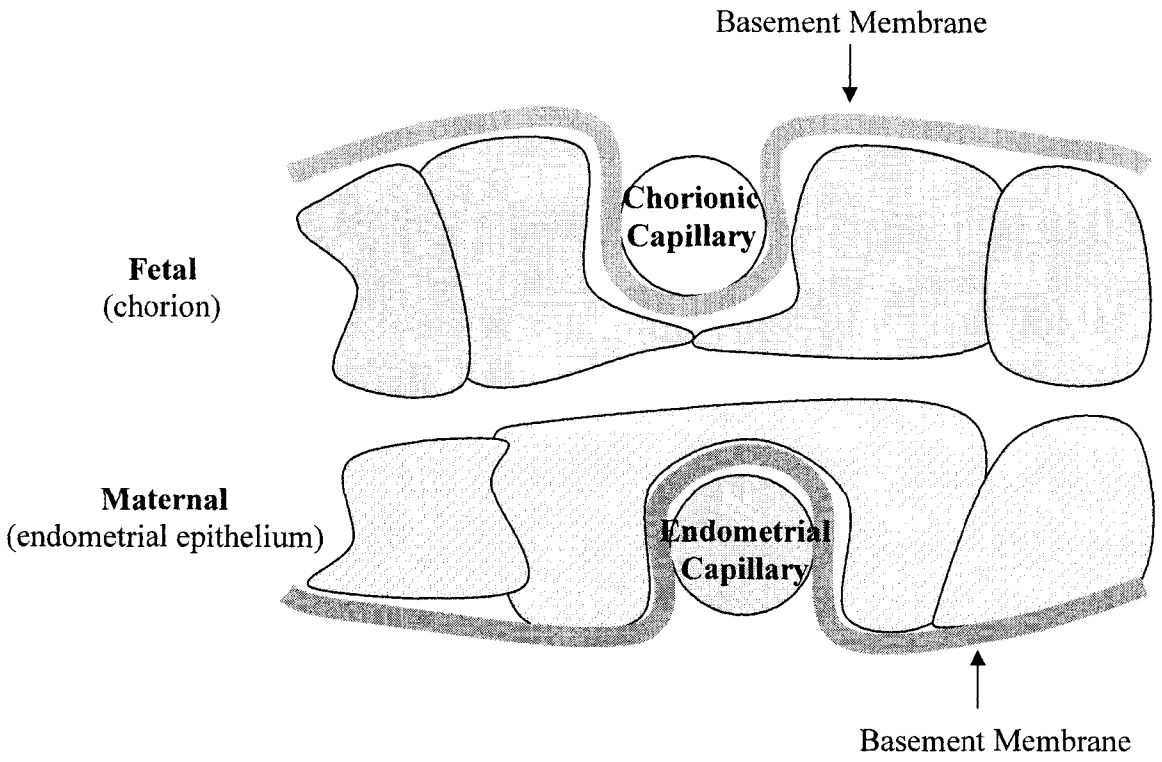

Figure 5.2: Intact bovine syndesmochorial placenta (Adapted from Hansen, 1995)

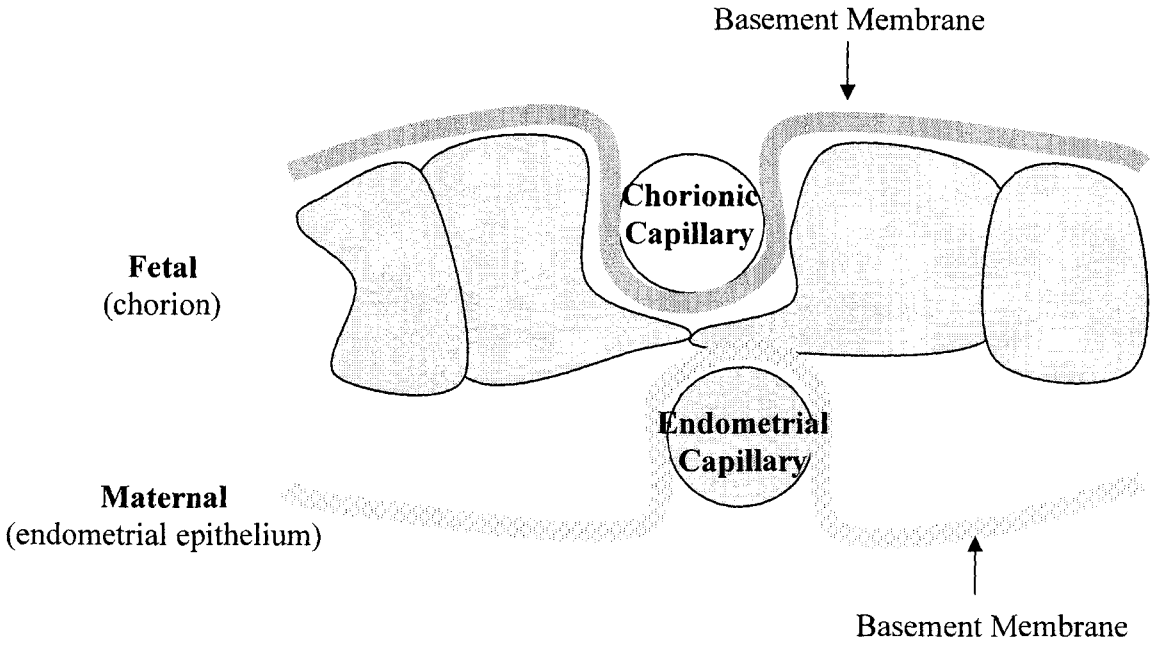

Figure 5.3: Syndesmochorial placenta with eroded endometrial epithelium

A BVDV infected fetus can readily replicate and secrete BVDV into amniotic fluid (Callan et al., 2002), however, only a few cells of the fetal chorion have been shown to stain positive for BVDV; mainly binuclear giant cells (BNGC) (Fredriksen et al., 1999). BNGC are large cells with two nuclei. They are trophoblastic in origin and are believed to form continuously throughout gestation. BNGC constitute around 20% of the fetal placenta (Senger, P. L., 1999;Hoffman et al., 1993). During gestation, BNGC migrate from the chorionic epithelium and invade the endometrial epithelium where the cells secrete placental lactogen and pregnancy specific protein B. BNGC also produce progesterone and estrogen (Senger, P. L., 1999;Hoffman et al., 1993). While in contact with the endometrial epithelium, virus may bud from the BNGC and enter the endometrial epithelium or capillary where the virus can spread through the blood (Figure 5.4). The effect BVDV may have on BNGC is unknown.

Once in circulation, BVDV may encounter APCs; such as macrophage or dendritic cells which will present antigen to B and T-cells in the spleen or other lymph nodes. Antigen specific B-cells will interact with the activated helper T-cells, stimulating the proliferation of BVDV-specific B-cells within the spleen.

Antibody secreting B-cells then migrate throughout the body. Activated B-cells will often return to the location where antigen was first contacted; in the case of BVDV the nasal mucosa. Homing of lymphocytes to specific organs is controlled by high endothelial venules (HEV) and the expression of lymphocyte homing receptors (Sminia, T. et al., 1999;Duijvestijn et al., 1989;Girard et al., 1995). Many lymphocytes that originated in the NALT will return to the site of induction where IgA will be secreted into

mucosal secretions, thus increasing the concentration of anti-BVDV IgA present in nasal secretions.

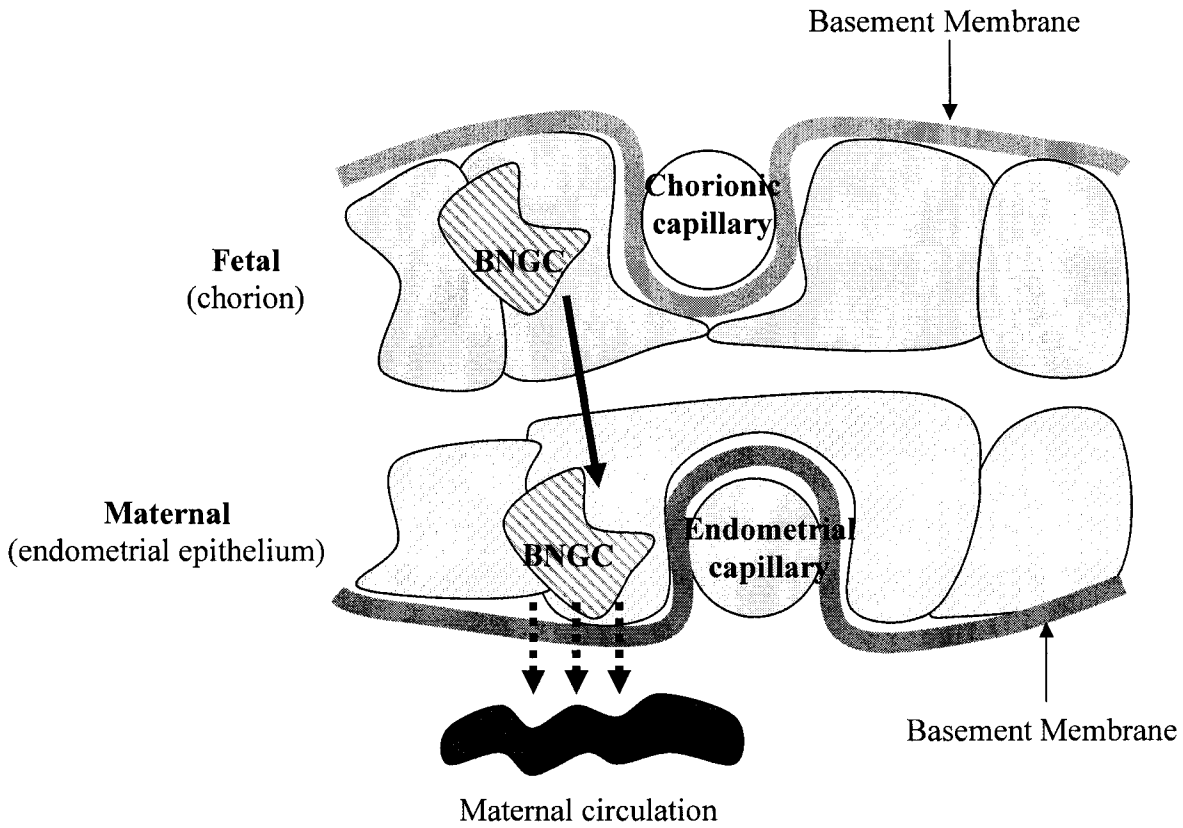

Figure 5.4: Migration of BNGC from chorion to endometrial epithelium (Adapted from Hansen, 1995)

Using an ELISA, the concentration of anti-BVDV IgA present in the nasal secretions of PI-Cs can be measured and compared to non-PI-Cs in an effort to detect PI animals *in utero*.

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CHAPTER VI

AN INDIRECT CAPTURE ELISA TO DETECT ANTI-BVDV IGA IN THE NASAL SECRETIONS OF PREGNANT DAMS: AN ATTEMPT TO IDENTIFY PERSISTENTLY INFECTED FETUSES *IN UTERO*

Abstract

An indirect capture ELISA to detect anti-BVDV IgA in the nasal secretions of cows was developed in an attempt to identify BVDV persistently infected fetuses *in utero*. The concentration of IgA in nasal secretion samples from BVDV unexposed and BVDV exposed animals was determined. Using ROC curve analysis, a threshold optical density of 0.12 gave an optimal sensitivity of 65% and a specificity of 80%. To determine if IgA levels in the nasal secretions of cows carrying BVDV persistently infected (PI) fetuses were greater than those of cows not carrying PI fetuses, the concentration of anti-BVDV IgA present in the nasal secretions of 143 cows at 7 to 8 months gestation was obtained. Of the 143 cows, 19 had IgA concentrations 2-fold greater than the positive control. Included in those 19, was a cow carrying a PI fetus.

Introduction

Bovine viral diarrhea virus (BVDV) persistently infected (PI) animals can be introduced to a herd through the purchase of a non-persistently infected cow carrying a PI fetus (Bitsch et al., 1995). Unless the dam is persistently infected itself, PI-carriers (PI-Cs) are seropositive and virus-negative, which makes them indistinguishable from other

seropositive cattle (Brownlie et al., 1998). To date, there are no assays available for routine detection of PI animals *in utero*.

Studies have shown that virus can be isolated from fetal fluid as a PI-C detection method. However, the method is costly and the safety of the procedure has not been evaluated (Callan et al., 2002). In addition to testing fetal fluid, an enzyme-linked immunosorbant assay (ELISA) for the detection of total BVDV antibody titers has been developed as a diagnostic tool to screen for PI-Cs (Lindberg, 2001;Brownlie et al., 1998).

PI-Cs generally have markedly higher serum antibody titers to BVDV than cows carrying healthy fetuses, presumably due to continuous immunological challenge induced by the PI fetus (Houe, 1999;Brownlie et al., 1998). However, the serologic testing is not 100% reliable in that Brownlie, et al., (1998) reported some overlap of antibody titers between PI carriers and non-carriers. Lindberg, et al., (2001) reported on the ELISA sensitivity (94%-100%) and specificity (39%-67%) using data from the Swedish BVD database. For routine use, the specificity of the assay is too low and was not analyzed against data from animals vaccinated against BVDV. In the United States, many cattle are vaccinated for BVDV and an ELISA assay for total serum antibody may not be as sensitive a test for vaccinated PI-Cs.

When an animal is vaccinated with a modified-live vaccine there is generally both a systemic cellular and humoral immune response while an inactivated vaccine primarily elicits a humoral response. However, a limited mucosal immune response is elicited after vaccination with either vaccine. Studies have shown that the nasal secretory antibody response is notably absent after intramuscular vaccination with either attenuated or inactivated infectious bovine rhinotracheitis or parainfluenza-3 virus, resulting in little, if

any IgA production in nasal secretions. (Todd, 1973;Ogra et al., 1980;Lascelles, A. K. et al., 1986) Intranasally vaccinated or naturally infected animals, on the other hand, demonstrate a more profound and consistent nasal secretory antibody response, specifically IgA (Lascelles, A. K. et al., 1986;Todd, 1973;Ogra et al., 1980).

Continuous immunlogic challenge of a dam by a PI fetus may stimulate increased immunoglobulin concentrations at all mucous membranes, particularly at sites where BVDV was initially introduced to the PI-C, i.e., nasal secretions. The goal of this study was to develop a diagnostic test able to identify PI-Cs; specifically an ELISA for the detection of anti-BVDV IgA in the nasal secretions of pregnant cows.

Materials and Methods

Serum and nasal secretion samples

Nasal secretions collected from 22 vaccinated and unvaccinated cows during a BVDV vaccine trial were available for the study. Nasal secretions were collected using a syringe and were stored in 5ml snap cap tubes at –20°C. All animals used in the study were BVD virus and antibody negative at the beginning of the trial. Cows were vaccinated, bred and challenged with either type I or type II ncp-BVDV by intranasal route on day 80 of gestation. Samples were collected prior to intranasal challenge (d=0) and 3 weeks post-challenge and stored at –20°C.

In addition to samples from the vaccine trial, nasal secretion samples were obtained from 143 cows at 7 to 8 months gestation in a BVDV endemically infected beef herd in the fall of 2004. The cows were vaccinated annually with MLV BVDV vaccines. All calves born in 2005 were tested by antigen-capture ELISA on ear notch samples for persistent infection. One PI calf was identified and confirmed by virus isolation from

serum taken two weeks after the initial test. The mother of the calf was identified and tested negative for persistent infection with BVDV.

Coating Antigen preparation

BVDV stocks of strains Singer, a type I cp-BVDV, (National Veterinary Services Laboratory, Ames, IA) and strain 125, a type II cp-BVDV, (National Veterinary Services Laboratory, Ames, IA) were propagated on bovine turbinate (BT) (ATCC, Manassas, VA) cells in Eagle's minimum essential medium (MEM) (Irvine Scientific, Santa Ana, CA) with non-essential amino acids containing 2% horse serum (Sigma, St. Louis, MO), 5% glutamine-penicillin-streptomycin (Irvine Scientific, Santa Ana, CA), 5% HEPES buffer (Irvine Scientific, Santa Ana, CA) and 2.5% amphotericin B (Fisher Scientific, Fair Lawn, NJ). Flasks ($75cm^2$) (Nunc, Inc. Naperville, IL), containing media and virus, were frozen at –70°C for 24 hours, thawed and clarified at 15,000 rpm for 10 minutes. Virus stocks were further clarified by ultracentrifugation at 35,000 rpm for 30 minutes.

To determine the tissue culture infectious dose affecting 50% of wells ($TCID_{50}$), virus was serially diluted 1:2 in 96-well plates (8 wells per dilution). Virus was incubated with BT cells for 72 hours at 37°C and then wells were examined for cytopathic effect. The Reed Muench method was used to calculate the $TCID_{50}$.

Indirect Capture ELISA

The indirect capture ELISA protocol is outlined in Figure 6.1. The optimal concentration of BVDV antigen to coat Nunc polysorp 96-well flat bottom plates (Nunc, Inc., Naperville, IL) was determined by checkerboard titration to be $10^{5.1}$ $TCID_{50}$ per milliliter. One hundred microliters of BVDV virus diluted to $10^{5.1}$ $TCID_{50}$ per milliliter in carbonate/bicarbonate buffer (Sigma, St. Louis, MO) (pH 9.5) was added to all wells.

Substrate control and conjugate control wells were incubated with 100µl carbonate/bicarbonate buffer. Plates were incubated at 37°C for two hours and at 4°C overnight.

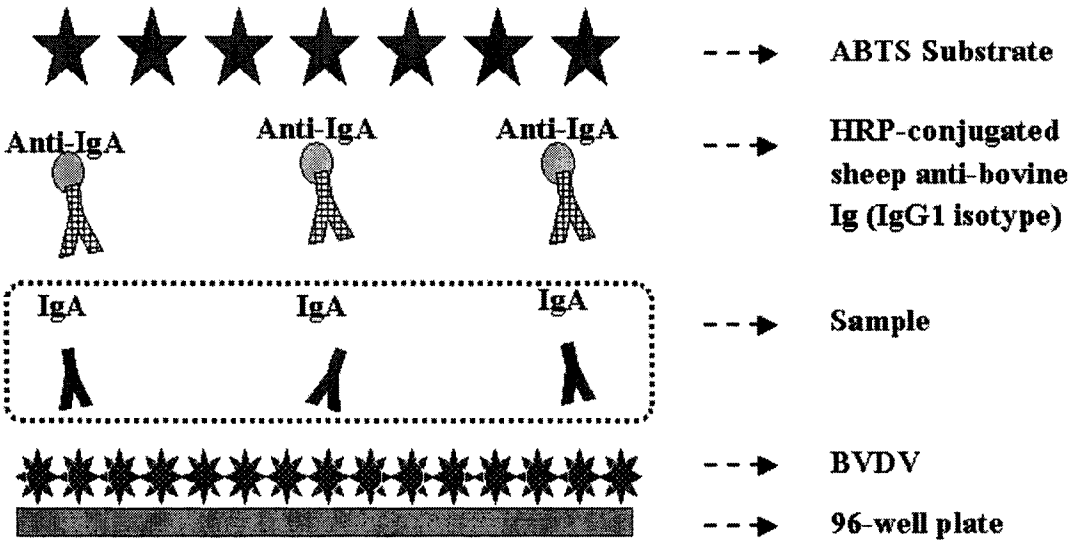

Figure 6.1: A 96-well Nunc-PolySorp plate was coated with a cytopathic BVDV to capture BVDV specific antibody from bovine nasal secretions. After incubation with nasal secretion samples, HRP-conjugated sheep anti-bovine IgA was added to the well. The presence of anti-BVDV IgA antibody was detected by addition of the ABTS substrate. Optical density values were read at 405nm.

Plates were washed four times with 200µl PBS-T20 (1X PBS + 0.05% Tween 20) prior to blocking with 50µl 1% fish gelatin (Sigma, St. Louis, MO) in PBS-T20 (500µl fish gelatin + 50ml PBS-T20). Following a one-hour incubation at 37°C, plates were again washed four times with 200µl PBS-T20. Nasal secretion samples were treated at a 1:1 ratio with DL-Dithiothreitol (DTT) (Sigma, St. Louis, MO) for one hour at 37°C. Samples were diluted to a final concentration of 1:200 in 1% fish gelatin in PBS-T20 and added to plates in triplicate (50µl per well). Blank, conjugate control and substrate control wells were incubated with 50µl PBS-T20. Each ELISA was run using an anti-

BVDV IgA positive sample from a cow with known BVDV exposure. Plates were incubated at 37°C for one hour and then washed four times with 200µl PBS-T20.

Horseradish peroxidase (HRP) conjugated anti-bovine IgA (Bethyl Laboratories, Inc., Montgomery, Texas) was then added to wells (50µl per well). By checkerboard titration, the optimal concentration for the conjugated antibody was determined to be 1:1000 for IgA. Substrate control wells were incubated with 50µl PBS-T20. The plates were incubated for one hour at 37°C. Plates were washed four times with 200µl PBS-T20, followed by the addition of 100µl per well of 2,2-azino-di(3-ethylbenzthiazoline) sulfanic acid (ABTS) (Zymed Laboratories Inc., San Francisco, CA). Plates were incubated for 20 minutes at room temperature in the dark and the optical density was read at 405nm by a MRX Revelation plate reader (Dynex Technologies, Inc., Chantilly VA).

Intra- and Inter-assay Coefficient of Variation

Intra-assay coefficient of variation (CV) measures the repeatability of the assay while inter-assay coefficient of variation measures the reproducibility of the assay. Both calculations measure the error associated with the performance of the assay. The intra- and inter-assay CV was calculated from the formula CV = 100% x Standard Deviation/mean (Kurstak, 1985). The intra-assay CV was calculated using OD values from the anti-BVDV IgA positive sample diluted 1:100 or 1:200 in 24 wells each, on the same plate. The inter-assay CV was similarly calculated, only the positive sample was run in triplicate on seven different plates.

ROC Curve Analysis

The performance of the assay, i.e. sensitivity and specificity, depends on the threshold value chosen to distinguish between positive and negative results. To visualize

test performance, assay results from nasal secretion samples collected from cows in the vaccine trial were used to produce receiver operating characteristic (ROC) curves. Samples collected before BVDV challenge were considered anti-BVDV IgA negative because the animals had never been exposed to the virus by vaccination and were seronegative by serum neutralization. Samples collected three weeks post-inoculation were considered anti-BVDV IgA positive. No virus isolation was performed to confirm BVDV infection, however seroconversion was reported in all animals, indicating exposure to the virus (Fairbanks et al., 2003).

ROC curves are generated by graphing sensitivity by 1- specificity. Sensitivity is defined as the ability of a test to correctly identify all screened individuals who actually have a disease or condition, while specificity is defined as the ability of a test to correctly identify all screened individuals who do not have a specific disease or condition. The sensitivity of the indirect capture ELISA was calculated by dividing the number of nasal secretion samples correctly identified as containing anti-BVDV IgA by the total number of nasal secretion samples that did contain anti-BVDV IgA. The specificity was calculated by dividing the number of nasal secretion samples correctly identified as not containing anti-BVDV IgA by the total number of nasal secretion samples that did not contain anti-BVDV IgA. The sensitivity and specificity of the indirect capture ELISA was determined for several threshold values.

AUC Analysis

The area under the curve (AUC) was calculated to further determine if there were differences between anti-BVDV IgA negative and positive samples. The closer the AUC

is to 1, the more likely the OD value indicates a positive result. The farther away from 1, the more likely the OD value indicates a negative result.

The number of samples with OD values between 0 and 0.05, 0.051 and 0.1, 0.101 and 0.15, 0.151 and 0.2, 0.201 and 0.25, 0.251 and 0.3, 0.301 and 0.35, 0.351 and 0.4, and 0.401 and 0.45 was determined for both groups (Table 6.1).

Table 6.1: Number of samples within OD limits

OD	Negative Samples	Positive Samples
0.05	8	1
0.10	6	4
0.15	4	7
0.20	1	2
0.25	1	5
0.30	0	0
0.35	0	0
0.40	0	0
0.45	0	1

The approximate area under the curve was determined using the Spline method. The Spline method fits cubic polynomials to data points in order to calculate the area under a curve.

Results and Discussion

Validation of Indirect Capture ELISA

The intra-assay and inter-assay coefficient of variation of the indirect capture ELISA for detection of anti-BVDV IgA antibodies are summarized in Table 6.2. The intra-assay CV for dilutions of 1:100 and 1:200 were 8.84% and 9.09%, respectively. The inter-assay CV for dilutions of 1:100 and 1:200 was 23.86% and 32.06%, respectively. Ideally the intra-assay CV would be <=5% and the inter-assay CV would be <=10% (Kurstak, 1985). The inability to better purify the BVDV coating antigen may have caused the intra- and inter-assay CV values to be higher than ideal. Every attempt

was made to limit variation within and between plates by using the same batch of reagents for all experiments, monitoring the incubator and refrigerator for changes in temperature between assays and using a plate washer to standardize the plate washing procedure.

Table 6.2: Intra-assay and Inter-assay coefficient of variation

Dilution	n	Mean OD	SD	CV%
Intra-assay variation				
1:100	24	0.470	0.042	8.84%
1:200	24	0.291	0.026	9.09%
Inter-assay variation				
1:100	21	0.411	0.098	23.86%
1:200	21	0.232	0.074	32.06%

Anti-BVDV IgA positive nasal secretion sample was diluted 1:100 or 1:200.

ROC Curve Analysis

The sensitivity and specificity of an ELISA is affected by the choice of cut-off or threshold value. Changing the threshold value changes the sensitivity and specificity of an assay (Noe, D. A., 1985). To determine the appropriate threshold value, ROC curve analysis, using the sensitivity and specificity of the ELISA was performed using varying threshold values (Table 6.3). A ROC curve was generated; graphing 1-Specficity by the corresponding sensitivity (Figure 6.2).

The sensitivity and specificity of the anti-BVDV IgA indirect capture ELISA was determined using nasal secretion samples from the vaccine trial. All animals were known to be BVDV exposure negative at the time of the first nasal secretion sampling. At the second sampling, all cows had been exposed to BVDV approximately 20 days prior to nasal secretion collection and all animals had seroconverted. All samples were assayed

for anti-BVDV IgA at a dilution of 1:100. Optical density (OD) values were determined and used to develop a ROC curve.

Table 6.3: Receiver Operating Characteristic (ROC) curve analysis

Threshold Value	True Pos	Positive by threshold	Negative by threshold	True Neg	Sensitivity	Specificity
0.10	15	21	19	14	0.750	0.700
0.12	**13**	**16**	**24**	**16**	**0.650**	**0.800**
0.14	9	10	30	18	0.450	0.900
0.16	7	9	31	18	0.350	0.900
0.18	7	8	32	19	0.350	0.950
0.20	6	7	33	19	0.300	0.950
0.22	5	6	34	19	0.250	0.950
0.24	2	3	37	19	0.100	0.950
0.26	1	1	39	20	0.050	1.000
0.28	1	1	39	20	0.050	1.000
0.30	1	1	39	20	0.050	1.000

Pos: Positive, Neg: Negative

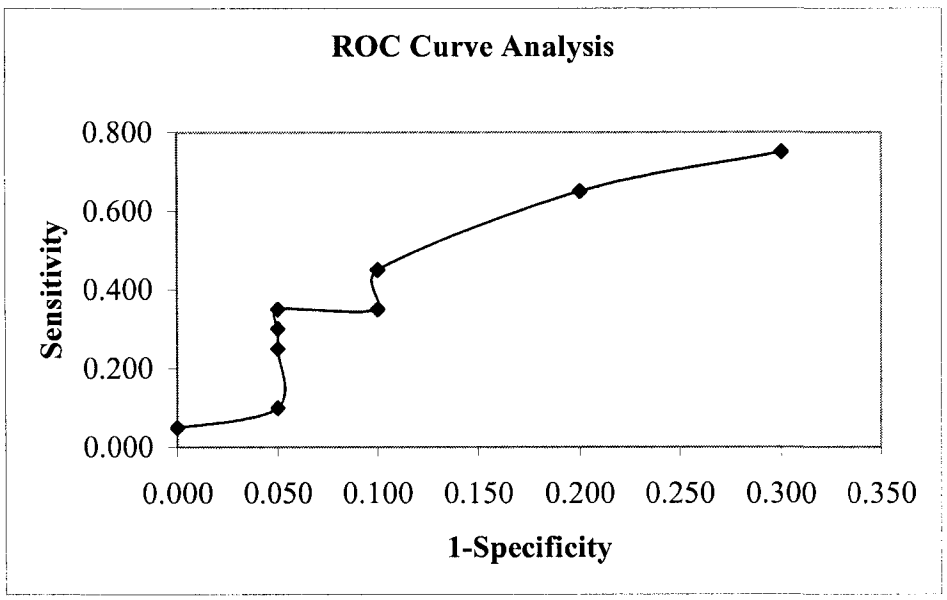

Figure 6.2: ROC curve using vaccine trial nasal secretion samples

For identification of BVDV PI animals a test that is highly sensitive is desired. The prevalence of PI animals in infected herds is generally low (0.5%-2%). However, one PI animal can infect 50% of a herd in less than six months (Cleveland et al., 2004),

therefore it is important to identify every PI animal. Specificity, as related to the proportion of false positive samples identified, is also important because suspect PI animals are often slaughtered, resulting in monetary losses to the producer. The removal of a non-PI animal would result in additional losses to the producer. It is important to identify only those animals that are truly persistently infected to avoid such losses.

While a threshold of 0.1 gives a sensitivity of 75%, the specificity is only 70%. A specificity of 70% would allow too many false positive animals to be identified. The optimal critical value of 0.12 gives a sensitivity of 65% and specificity of 80%.

A sensitivity of 65% is low and would result in the continued exposure of susceptible animals to the unidentified PI animals. However, the calculated sensitivity and specificity of this assay is underestimated. It has been shown that upon initial intranasal introduction of a pathogen the IgA response peaks between days 10 and 14 and diminishes by day 21 (Kimman et al., 1987;Rudin et al., 1998;Saif, 1987;Mohanty et al., 1976;Todd, 1973). In addition, the IgA titers in nasal secretions can vary between animals (Mohanty et al., 1976;Kimman et al., 1987;Saif, 1987). Nasal secretion samples after BVDV challenge were not collected until approximately 20 days post infection. Therefore IgA titers in some animals were probably diminished and not detected by the indirect capture ELISA.

Area Under the Curve

In addition to a ROC curve analysis, the area under the curve (AUC) for BVDV unexposed samples and BVDV exposed samples was determined. The AUC for the unexposed samples was 0.795 and the exposed animals gave an AUC of 0.995. These

data indicate that animals exposed to BVDV are more likely to have higher OD values when being assayed for anti-BVDV IgA than unexposed animals.

Analysis of nasal secretions from cows at 7 to 8 months gestation

Nasal secretions collected from cows at 7 to 8 months gestation were incubated 1:1 in DTT and then analyzed by the anti-IgA indirect capture ELISA. The inter-assay CV, using the positive control samples was calculated to be 13.9%. Of the 143 samples tested, 23 (16%) tested negative (OD < 0.120) and 120 (84%) tested positive (OD < 0.120) for IgA antibodies. It was expected that many of the animals would have detectable titers to BVDV due to repeated exposure to the virus through contact with persistently infected animals and annual injections with a modified live vaccine (Kimman et al., 1987;Rudin et al., 1998;Saif, 1987).

To compare OD values between plates, samples were standardized by dividing the average OD values by the average of the positive control. Of the 143 samples tested, 68 or 47.5% had standardized values less than 1-fold greater than the positive control, 124 or 86.7% had standardized values less than 2-fold greater than the positive control. Nineteen samples (13.3%) had standardized OD values 2-fold greater than the positive control. One of those samples was collected from a cow that had given birth to a PI calf after nasal secretion sampling, i.e., a PI-C.

A hypothesis test was performed to determine if there was a difference in standardized value between the 19 samples. Of the 19 samples with high standardized values, all but two were statistically similar to the PI-C sample. The two different samples were significantly larger than the PI-C sample (Figure 6.3).

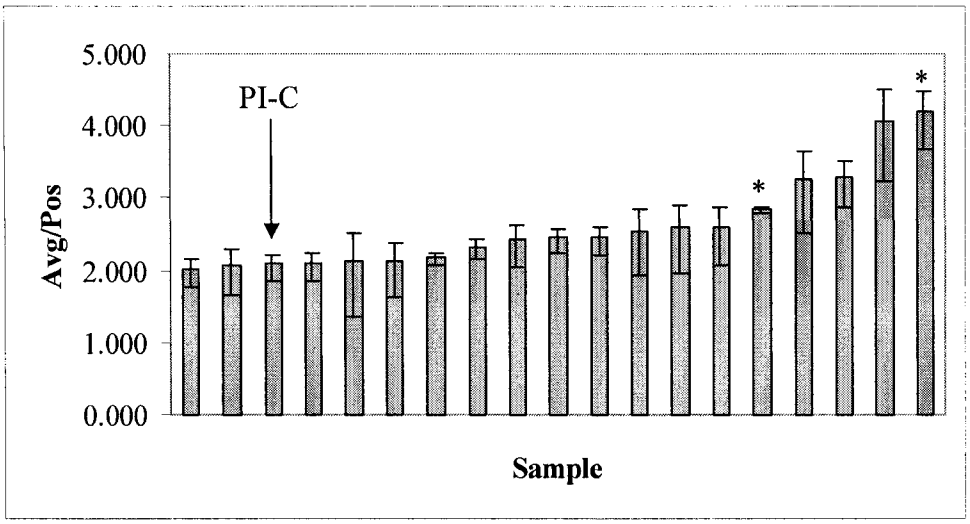

Figure 6.3: Standardized OD values for the 19 samples >=2 fold greater than positive control
* Indicate standardized values significantly different than the PI-C sample

Increased IgA levels to BVDV in the 19 animals can be the result of several biological phenomenon including age, reproductive history, previous PI-C status or individual variations between animals. Older animals would have been exposed more often to the virus, either by vaccination or through contact with a PI animal. Repeated exposure to BVDV could result in increased IgA levels in the nasal secretions of these animals.

At the time of nasal secretion collection, of the 19 cows with high IgA titers two were 10, two were 8, two were 7, three were 6, two were 5, two were 4, four were 3 and two were 2 years of age. The majority of the 19 animals with high IgA concentrations were five years of age or older (58%) while the majority of animals in the herd (53%) were 4 years or younger. A Z-proportion test was used to determine that there was no difference (p=0.2593) in the age of the 19 animals testing positive for high anti-BVDV IgA concentrations compared to the age of all animals in the herd.

If the animals had a history of not being pregnant at a pregnancy check, it may be an indication that the cow's fetus was infected with BVDV and the conceptus was lost. Continuous exposure from a PI fetus, even if present for only a short period of time may cause an increased sensitivity to the virus leading to a more pronounced increase in the level of IgA present in nasal secretions with future exposure to the virus. However, there can be many biological factors other than BVDV that may contribute to a cow not being pregnant at pregnancy check. None of the animals with high concentrations of IgA in nasal secretions had a history of infertility or any other reproductive abnormality; indicating that increased IgA titers were not the result of an aborted or absorbed, BVDV infected fetus.

The nasal secretion samples were retrieved from a BVDV endemically infected herd. It is possible that some of the animals with high levels of IgA were previous PI-Cs. Continuous exposure to BVDV through a PI fetus has been shown to increase overall antibody titers during pregnancy, but the duration of that increase has not been investigated. A history of carrying a PI fetus may result in a more pronounced immune response to vaccinations or other exposures to the virus.

In addition to the cow carrying a PI fetus at the time of nasal secretion sampling, one of the 19 animals with high concentrations of IgA carried a PI fetus the previous year. The continuous exposure to the PI fetus may have made the animals more sensitive to BVDV exposure leading to a more pronounced humoral immune response, upon re-exposure to the virus, specifically the production of IgA in the nasal secretions.

Conclusions

The concentration of anti-BVDV IgA in the nasal secretions of cows is not an indicator of whether or not a cow is a PI-C. While the PI-C in the sampled herd did have high levels of IgA in the nasal secretion sample (> 2-fold of positive control), 18 other cows did as well. The indirect IgA ELISA may be detecting something other than IgA in some samples or some samples may be "stickier" than others. The mucin in nasal secretion samples can cause a sample to bind nonspecifically to a plate leading to false positive results. To alleviate this problem, samples were incubated for one hour in DTT. Perhaps this incubation time was not sufficient to decrease nonspecific binding.

The indirect capture ELISA may be made more sensitive by refining the coating antigen. The overall anti-BVDV IgA concentration in nasal secretions may be high in several animals in a herd, but there may be specific antigens that PI-C animals produce antibodies against that non-PI-C animals do not. The use of immunoprecipitation or protein microarray to determine if there is a specific antigen that PI-C animals develop IgA antibodies to, may aid in making the assay more sensitive. If a specific immunodominant antigen was identified a recombinant antigen could be used to coat the ELISA plates, making the assay more sensitive for identifying PI-Cs.

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