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STUDIES ON THE EFFECT OF
BOVINE VIRAL DIARRHEA VIRUS ON THE
BOVINE OVARY

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate
School of The Ohio State University

By

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*****

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ABSTRACT

Bovine viral diarrhea virus (BVDV) is recognized as one of the most important viral pathogens of cattle. The biology of the virus and a complex disease pathogenesis has made the control and prevention of BVDV challenging. The reproductive consequences resulting from BVDV infection are of economic significance. Many studies have been conducted to further understand the reproductive pathogenesis of BVDV infections so that effective control and prevention strategies can be implemented. The objective of this research was to study the effects that BVDV has on the bovine ovary.

Morphological comparisons were made between ovaries from cows persistently infected with BVDV and cows not persistently infected with BVDV. In persistently infected cattle, a significant reduction in the number of antral follicles was observed suggesting that follicle formation is changed in these animals. Additionally, immunohistochemistry was used to localize viral antigen to luteal cells, endothelial cells, stromal cells, and macrophage-like cells of ovaries from cows persistently infected with BVDV.

Ovaries from heifers were sequentially removed by ovariectomy following acute infection with BVDV. Bovine viral diarrhea virus was isolated from ovaries at day 6-8 post infection while antigen was detected using immunohistochemistry for up to 60 days.
post infection. BVDV antigen was detected in stromal and macrophage-like cells located primarily in the ovarian cortex. Concurrent lymphocytic oophoritis of varying severity was also evident for up to 60 days post infection. Attempts to detect BVDV RNA in the ovary using *in situ* hybridization was unsuccessful.

Changes in ovarian function were monitored using ultrasonography and progesterone and estradiol assays. Five post pubertal heifers were followed daily for two estrous cycles before and two estrous cycles after acute infection with BVDV. No significant changes in progesterone or estradiol levels were observed for two estrous cycles after acute BVDV infection. A significant reduction was observed following BVDV infection in dominant follicle size, growth rate and associated subordinate follicles.

Five post pubertal heifers and four mature cows were immunized with a commercially available modified live BVDV vaccine. Cytopathic BVDV was isolated from the ovaries of four animals at 6, 8, 10, and 12 days post immunization. Using immunohistochemistry, BVDV antigen was detected up to 45 days post vaccination.

These studies provide evidence that BVDV infection of the bovine ovary can cause pathology and changes in ovarian function. These changes may be responsible in part for reproductive inefficiency in cattle undergoing acute infection with BVDV.
Dedicated to my wife Donna and daughters Kirsten and Emma.
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BOVINE VIRAL DIARRHEA VIRUS

Bovine viral diarrhea virus (BVDV) has emerged as one of the most important infectious disease agents in cattle (Duffel and Harkness, 1985; Baker, 1987). The insidious nature of BVDV has led to substantial economic losses in both the dairy and beef industry on a worldwide level (Duffel et al., 1986; Houe et al., 1993a). The virus biology and disease pathogenesis has made the control and prevention of BVDV challenging.

"An apparent new transmissible disease of cattle" was first described in 1946 as a gastro-enteritis with severe diarrhea (Olafson et al., 1946). Outbreaks of this disease occurred in several New York herds in which clinical signs included pyrexia, depression, anorexia, leukopenia, and ulcers of the nose, mouth, and muzzle. Weight loss, decreased milk production and abortions were also associated with this new disease. Mortality in these outbreaks ranged from 4 to 8 percent. A viral etiology was established and differentiated from other viral causes of acute gastro-enteritis (Olafson et al., 1946; Walker, 1947). In 1953, mucosal disease, a sporadically occurring, highly fatal disorder
was first described (Ramsey and Shivers, 1953). This disease was later shown to also be caused by BVDV (Gillespie et al., 1961). Initial isolates of BVDV were characteristically noncytopathic in cell culture (Lee and Gillespie, 1957). Isolates of BVDV causing cytopathology in cell culture were identified later (Underdahl et al., 1957; Gillespie et al., 1960). This established the in vitro characteristics of BVDV referred to as biotypes. Persistent infection with BVDV was first recognized in 1978 (Coria and McClurkin, 1978). Further early work established close serological relationships between BVDV, hog cholera virus (Darbyshire, 1960; Dinter, 1963) and border disease virus of sheep (Plant et al., 1973).

The Virus

Bovine viral diarrhea virus, along with hog cholera virus and border disease virus, were initially grouped in a unique genus called Pestivirus within the Togaviridae family of viruses (Horzinek, 1973). However, with advances in the understanding of the molecular biology of Pestiviruses, they are now classified as a separate genus within the family Flaviviridae (Collett et al., 1989). Other members of the Flaviviridae family include the genus Flavivirus of which yellow fever virus is the prototype and hepatitis C virus which is assigned to a genus of its own (Horzinek, 1991). All of the viruses within the Flaviviridae family have strikingly similar structural and genomic organizations (Collett et al., 1989).

Two biotypes of BVDV exist: noncytopathic BVDV and cytopathic BVDV. The biotype is established by the in vitro action of the virus in cell culture. Noncytopathic
BVDV causes no gross cytopathology when cultured with cells in vitro. Cytopathic
BVDV causes characteristic cytopathology when cultured with cells in vitro.
Noncytopathic BVDV is the predominant biotype in nature.

Bovine viral diarrhea virus is a small, single stranded, positive sense, RNA virus.
The virion is made up of genomic RNA encased within a capsid protein C and surrounded
by a lipid membrane. Three glycoproteins, E\textsuperscript{\textit{m}}, E1, and E2, constitute the envelope of the
virion. Using gel electrophoresis, early work estimated the genomic length of Osloss and
NADL strains of BVDV at 12.5 kb (Renard \textit{et al.}, 1985; Collett \textit{et al.}, 1988). Nucleotide
sequencing of cDNA has shown that noncytopathic BVDV is 12,308 nucleotides in
length (Deng and Brock, 1992). Sequencing of several cytopathic BVDV strains has
revealed various insertions, duplications and deletions of genomic regions which makes
their genomic length variable (Collet \textit{et al.}, 1988; Meyers \textit{et al.}, 1991; Meyers \textit{et al.},
length is flanked by untranslated regions (UTR) of 385 nucleotides at the 5' end of the
genome and 226 nucleotides at the 3' end (Deng and Brock, 1993). Several other open
reading frames have been identified but are not translated (Deng and Brock, 1992).
Unlike eukaryotic mRNA, the BVDV genome lacks a 5' cap structure (Brock \textit{et al.}, 1992)
which is necessary for ribosomal binding during the initiation of RNA translation. Instead,
it is thought that the extensive and conserved secondary structure within the 5' UTR acts
as an internal ribosome entry site (Deng and Brock, 1992; Poole \textit{et al.}, 1995).

The positive sense viral RNA is transcribed into a single poly protein which is then
cleaved by both cellular and viral proteases into four structural and eight to nine non-
structural viral components. The N-terminal end of the BVDV polyprotein yields the nonstructural protein N

. This protein has protease activity that cleaves intramolecularly at its C-terminus (Wiskerchen et al., 1991). Other functions of this protein have not been identified. Other flaviviruses do not contain this protein (Donis, 1995). The capsid or C protein is encoded next. This protein functions in packaging the genomic RNA and forming the virion lipid envelope (Thiel et al., 1991). Unlike the capsid protein of hepatitis C virus, the BVDV capsid protein is poorly immunogenic (Donis and Dubovi, 1987b; Khudyakov et al., 1993). The next three proteins are structural glycoproteins E

, E1, and E2. These proteins are cleaved by signalases within the endoplasmic reticulum (Rumenapf et al., 1993). E

 is highly conserved between pestiviruses (Donis et al., 1991). A lack of hydrophobic domains suggest that it forms a loose interaction with the viral envelope (Silva-Krott et al., 1994). High levels of antibodies are produced to this protein, however they have minimal virus neutralizing activity (Boulanger et al., 1991; Xue et al., 1990). E1 is anchored in the virion lipid membrane by two hydrophobic domains. Significant levels of antibody to this protein are not produced (Donis and Dubovi, 1987). E2 is highly immunogenic and contains the major BVDV neutralizing epitopes (Bolin et al., 1988; Bolin et al., 1989; Donis et al., 1988; Donis et al., 1991). One domain of the E2 protein is coded for by a hypervariable region of the viral genome (Deng and Brock, 1992). Limited structural constraints in this area may allow selective immunological pressures to act on this hypervariable region of the genome leading to antigenic variability in the neutralizing epitopes (Donis, 1995). The next protein, NS2-3, is a large nonstructural protein with a variety of domains suggesting many different
functions. At the N-terminus of this protein, a cysteine rich zinc finger is located in close proximity to a very hydrophobic region (De Moerlooze et al., 1990; Deng and Brock, 1992). Functioning as an RNA binding site, the zinc finger, in conjunction with the adjacent hydrophobic domain, may be important in stabilizing the viral replication complex (De Moerlooze et al., 1990). The next important domain has chymotrypsin-like serine protease activity thought to be important in further intra and inter molecular cleavages of the BVDV nonstructural proteins (Bazan and Fletterick, 1989; Wiskerchen and Collet, 1991). The final domain which has been identified has homology to eukaryotic helicases (Gorbalenya et al., 1989). Its exact function in BVDV replication is unknown. Changes in the genomic region of cytopathic BVDV coding for the NS2-3 protein result in the de novo production of NS3 as well as NS2-3 (Donis and Dubovi, 1987a; Pocock et al., 1987). Several changes have been described and include insertion of host cell ubiquitin RNA (Meyers et al., 1991), duplication and rearrangement of viral sequences coding for NS2-3 (Greiser-Wilke et al., 1993a; Meyers et al., 1992), and deletion of viral sequences (Tautz et al., 1994). NS3 is a marker for cytopathic BVDV. It encompasses the C-terminus of NS2-3 and contains the protease and helicase domains of this protein (Donis, 1995). NS3 is an immunodominant yet conserved protein among all pestiviruses suggesting that its conformation is extremely important in its function (Donis and Dubovi, 1987b; Dubovi, 1992). NS2 is a cleavage product of the N-terminus of NS2-3 that is found in conjunction with some but not all cytopathic BVDV isolates (Greiser-Wilke et al., 1993a; Meyers et al., 1992). NS4A and NS4B are encoded downstream of NS2-3. NS4A and NS4B are conserved among pestiviruses, but little is known about their
functions. NS4B may play a role in modulating the protease activity of NS3 (Wiskercher et al., 1991). NS5A and NS5B are the final two proteins produced from the nascent polyprotein. NS5A is believed to be involved in RNA synthesis. NS5B is the putative viral RNA-dependent-RNA polymerase as evidenced by the glycine-aspartate-aspartate motif which is found in other positive sense RNA virus RNA-polymerases (Donis, 1995).

The replication of BVDV takes place entirely within the cytoplasm of infected cells. The virus enters a cell by receptor mediated endocytosis (Boulanger et al., 1992; Schelp et al., 1995). Current evidence suggests that interaction between E2 and a 50 kD cellular receptor are necessary for this process to occur (Xu and Minocha, 1993). This receptor appears to be the same for all Pestiviruses (Flores and Donis, 1995; Flores et al., 1996). Following endocytosis, acidification of the endosome results in release of the viral genome (Boulanger et al., 1992). Once in the cytosol, the viral RNA comes in contact with cellular ribosomes. This process is thought to be mediated by the 5' UTR serving as an internal ribosome entry site. Recognition of the internal ribosome entry site results in translation of the BVDV open reading frame into a single polyprotein (Poole et al., 1995). Processing of the polyprotein proceeds using both cellular and virus derived enzymes. Several precursor proteins have been identified with the resulting production of 4 structural and 7 to 8 nonstructural proteins. Processing of the proteins take place both in the cytosol and within the endoplasmic reticulum. After mature RNA-dependent-RNA polymerase (NS5B) is produced, the BVDV genome can be replicated. This involves making a negative strand copy of the genomic RNA as a template for the synthesis of positive strand genomic RNA. With synthesis of viral RNA completed, virions are
thought to be assembled in the endoplasmic reticulum or golgi apparatus (Bielefeldt-Ohman et al., 1987). A lipid envelope is obtained by budding into vesicular compartments which then release mature virions into the extracellular compartment by exocytosis (Ritchie and Femelius, 1969; Ward and Kaeberle, 1984). Extracellular virions have been detected within 10 hours post infection (Donis and Dubovi, 1987a).

Antigenic variation among BVDV isolates poses significant challenges to the control and prevention of BVDV. Early in vivo cross protection studies showed that different BVDV isolates could protect against heterologous challenge by preventing signs of acute BVDV (Baker et al., 1954). However, there is significant evidence that immunity to one isolate of BVDV may not protect the fetus from infection following maternal challenge with a distinctive heterologous isolate (Harkness, 1987; Meyling et al., 1987; Bolin et al., 1991). Several laboratory methods have been used to demonstrate antigenic differences. Early studies using cross neutralization tests suggested that antigenic diversity between BVDV isolates exist (Femelius et al., 1971). This was confirmed by several investigators who have shown differences in cross neutralization of up to 100 fold (Castrucci et al., 1975, Itoh et al., 1984, Howard et al., 1987). However, since some degree of neutralizing cross reactivity appears to exist between different viral isolates, no distinct BVDV serotypes are recognized (Dubovi 1992). Antigenic diversity has been further confirmed by differences in reactivity of BVDV isolates to monoclonal antibodies (Bolin 1988, Corapi et al., 1990b; Xue et al., 1990). Monoclonal antibody binding assays have mapped the major neutralizing epitopes of BVDV to the E2 glycoprotein (Bolin, 1988; Paton et al., 1991) and minor neutralizing epitopes to the $E^ma$ glycoprotein
Based on studies that compare different BVDV isolates to panels of monoclonal antibodies directed against the E2 viral protein, it is clear that the compliment of neutralizing epitopes varies between isolates (Paton et al., 1991; Corapi 1990).

Antigenic differences among BVDV isolates are supported by recent nucleotide sequence information. Disregarding insertions in cytopathic BVDV, nucleotide sequence data has shown a difference in homology between BVDV isolates as great as 22% over the entire genome (Collett, 1992; Deng and Brock, 1992). The most conserved areas of the genome are the 5' UTR and the coding region for the NS3 protein. The areas of lowest sequence homology are those which code for the major E2 glycoprotein, and the NS2 and NS5A nonstructural proteins (Deng and Brock, 1992).

Recently, two separate groups of BVDV have been identified based on comparison of the nucleotide sequence of the 5' UTR and reactivity of E2 specific monoclonal antibodies. These groups have been termed BVDV genotype I and II (Ridpath et al., 1994). Sequence homology of the 5' UTR between type I and type II is 75% while homology within groups is greater than 90% (Pellerin et al., 1994; Ridpath et al., 1994). The entire nucleotide sequence of one type II isolate of BVDV has been determined. The deduced amino acid sequence homology with various BVDV type I isolates is 74% or less (Ridpath and Bolin, 1995). The genetic differences found in the 5' UTR are correlated with antigenic differences based on monoclonal antibody analysis of the glycoprotein E2 (Pellerin et al., 1994; Ridpath et al., 1994). There may also be pathogenic differences between type I and II BVDV, as type II BVDV has been associated with outbreaks of
higher than expected mortality, especially as a result of hemorrhagic syndrome (Ridpath et al., 1994, Pellerin et al., 1994).

**Epidemiology**

BVDV appears to be a problem in cattle on a worldwide level. Because of the complicated pathogenesis and insidious nature of the disease, the impact of BVDV infection has not been fully appreciated.

Information on the economic impact of BVDV is limited. On a national level, the annual losses due to BVDV infections in England have been calculated at $185 million (Spedding et al., 1985), $73 million (Bennett and Done, 1986) and $32 million (Harkness, 1987). In Denmark, annual losses have been calculated at $20 million per one million calvings (Houe et al., 1993a). On a herd level, economic losses can be extremely variable depending on the clinical nature of the disease in that herd. In a 67 cow dairy herd in England, economic losses associated with BVDV were calculated at between $2600 and $6400 over the course of one year (Duffel et al., 1986). Annual losses from BVDV infections were calculated at an average of $77 per cow on 14 Dutch dairy farms (Wentink and Dijkhuizen, 1990). Losses from mucosal disease outbreaks on 8 Danish dairy herds was estimated at between $743 to $5188 (Houe et al., 1994).

The prevalence of BVDV has been estimated using both serological and virus isolation. Serological data must be interpreted with caution as an indication of infection because of the use of BVDV vaccines in some countries. In the United States, a study of 66 selected herds containing 3157 cattle reported a 1.7% prevalence of persistently
infected cattle and 89% prevalence of antibody positive cattle (Bolin et al., 1985b). In two Michigan counties, testing of all cattle on 20 randomly selected dairy farms showed a 0.13% prevalence of persistently infected cattle (Houe et al., 1995). In the United Kingdom, the prevalence of antibody positive cattle in two separate survey studies was 62.5% (Harkness et al., 1978) and 64.9% (Edwards et al., 1987). In Denmark, a study of 19 representative herds showed a BVDV antibody positive rate of 64% and a persistent infection prevalence of 1.1% (Houe and Meyling, 1991b). In two Swedish studies, 41% and 46% of animals tested were BVDV antibody positive (Alenius et al., 1986; Niskanen et al., 1993). A Norwegian study estimated the prevalence of antibody positive cattle in Norway at 19%. (Loken et al., 1991a). Serological surveys in various South American countries have shown BVDV antibody positive prevalence rates ranging from 42% to 62% (Rweyemamu et al., 1990). In Russia, the prevalence of BVDV antibody positive cattle ranged from 20 to 80% depending on the climate and husbandry practices (Zhidkov and Khalenev, 1990). Based on serological evidence, the prevalence of cattle herds infected with BVDV is much greater. In the United States, 4 of 5 dairy herds not vaccinated for BVDV had serological evidence of previous infection (Houe et al., 1995). Similarly, studies in the United Kingdom (Edwards et al., 1987), Sweden (Niskanen et al., 1991), and Denmark (Houe and Meyling, 1991b) detected antibody positive cows in 95%, 73%, and 100% of the herds tested, respectively. From these studies, it is evident that the prevalence of antibody positive cattle varies in different regions of the world, ranging from approximately 20 to 90%. Herd prevalence of antibody positive cows is much
higher indicating that most herds have been exposed to BVDV whereas the prevalence of cattle persistently infected with BVDV appears to be less than 2%.

The major reservoir of BVDV is persistently infected cattle (Duffel and Harkness, 1985; Baker 1987; Houe, 1995). Their continuous ability to excrete large amounts of virus in milk, urine, feces, semen, saliva, tears and nasal mucous makes them extremely efficient at providing exposure to susceptible herdmates. Cattle acutely infected with BVDV can also spread the virus, but much less efficiently (Meyling and Jensen, 1988; Meyling et al., 1990). Acutely infected cattle shed lower amounts of virus during the viremic stage of infection, which normally occurs between day 4 and 10 post infection. Evidence of BVDV infection has been documented in other species of both domestic, captive and free living animals including swine (Fernelius et al., 1973), sheep (French et al., 1974; Loken, 1995a), goats (Nettleton, 1990; Loken, 1995b), Scottish red deer (Nettleton et al., 1980), elands, antelope, wildebeest and mountain goats (Hamblin et al., 1979; Doyle and Heuschele, 1983). However, experimental transmission has only been documented between sheep and cattle (French et al., 1974; Gibbons et al., 1974). In addition, vaccines have been implicated as a potential source of BVDV infection. Abortions, congenital defects or persistently infected calves have occurred following the use of live vaccines (Liess et al., 1984, Trautwein et al., 1986). Vaccines may also contain adventitious BVDV as a result of contaminated cell lines or fetal calf serum used to manufacture the vaccines (Lohr et al., 1983; Loken et al., 1991b; Bolin et al., 1994).

The transmission of BVDV can occur both vertically and horizontally. Horizontal transmission occurs most commonly by direct contact between an infected animal and
susceptible herdmates. Spread by aerosolization is probably distance dependent. BVDV can spread rapidly in a confined herd exposed to persistently infected cattle whereas separation reduced the spread significantly (Hartley and Richards, 1988; Wentink et al., 1991a). Horizontal spread can also occur by venereal transmission originating from persistently infected bulls (Paton et al., 1990). Although probably of minor importance, indirect transmission of BVDV is possible. BVDV has been isolated from biting and non biting flies following exposure to a persistently infected cow (Gunn, 1993; Tarry et al., 1991). Under experimental conditions, biting flies were able to transmit BVDV from a persistently infected cow to susceptible cows (Tarry et al., 1991). Hypodermic needles and nasal tongues used on a persistently infected cow and then immediately used on susceptible cows have resulted in viral transmission (Gunn 1993). BVDV has also been transmitted following the use of a common palpation sleeve during rectal examinations of persistently infected cows and susceptible cows (Lang-Ree et al., 1994). Survival of BVDV outside of the host is largely dependent on environmental conditions. In the laboratory, BVDV remains stable below 10 C at a pH of 3-9 (Duffel and Harkness, 1985). BVDV has been isolated from manure slurries for up to 3 hours at 37 C and 3 weeks at 5 C (Bendixen, 1993).

Vertical transmission occurs through *in utero* infection of the developing fetus. Calves born to persistently infected dams are themselves persistently infected with BVDV, a phenomenon which can be repeated over generations resulting in families of persistently infected cattle (Radostitis and Littlejohns, 1988; Houe, 1995).
Spread of virus within a susceptible herd is dependent on the source of the virus. Several studies have shown that in susceptible herds where the only source of virus is acutely infected animals, virus can circulate for up to 2.5 years (Barber and Nettleton, 1993; Barber et al., 1985; Moerman et al., 1993). Exposure to persistently infected animals usually result in rapid spread of virus among susceptible herdmates (Roeder and Harkness, 1986; Houe and Meyling, 1991; Taylor et al., 1994). In one study where 65 BVDV seronegative cattle were retested 6 months after being grouped with a persistently infected cow, 63 (97%) had seroconverted (Houe and Meyling, 1991). Similarly, 59 of 60 (98%) yearling heifers seroconverted within 3 months following the introduction of a persistently infected calf (Moerman et al., 1993).

Despite the complex pathogenesis of BVDV infections, the epidemiological course of the disease in a susceptible herd follows a relatively predictable pattern. Knowledge of this pattern helps with the diagnosis and control of BVDV infections (Houe, 1995). As described earlier, if the source of infection is a persistently infected animal, most susceptible herdmates become infected quickly. Acute infections are generally subclinical although this may vary with the virulence of the virus. Following the acute infection, the next phase of BVDV infection involves reproduction. This is often the first problem that is noticed when BVDV infects a herd. Problems noted include poor conception rates and abortions. These arise from the virus causing reproductive and fetal pathology early in gestation following acute infection. An increased abortion rate often continues for up to a year or more following the initial BVDV outbreak. The next pattern involves the birth of poor performing calves 4 to 10 months after the initial outbreak. The calves born first
may be afflicted with some type of congenital defect. This is consistent with second trimester fetal infection which is the stage of gestation that these calves were in during the initial disease outbreak. Following the calves with congenital defects, persistently infected calves are generally born resulting from exposure \textit{in utero} before the fourth month of gestation. Most calves born with congenital defects or persistently infected with BVDV die soon after birth. The final phase may involve the development of mucosal disease in those persistently infected calves that survive. This may occur at any time following birth, but usually occurs before 2 years of age. Using this information, a time line can be constructed which can be used to help diagnose BVDV infections or predict the outcome of BVDV infection so that control measures can be implemented (Houe, 1995).

**Clinical Disease**

The clinical manifestations of infection with BVDV are extensive. They range from inapparent infection to a variety of reproductive consequences to fatal mucosal disease. Many factors influence the clinical outcome of BVDV infection. These are best viewed within the scope of the classical host-agent-environment triad. Host factors which are important include age, immune status including immunocompetence or immunotolerance to BVDV, pregnancy status and concurrent fetal age. Environmental factors which influence the clinical outcomes of BVDV infection include those which precipitate stress and increase exposure to secondary pathogens. Many agent factors are important. These include antigenic and genomic heterogeneity among BVDV isolates, biotype, and differences in virulence. The clinical diseases associated with BVDV
infection can be classified into three broad groups: Acute infections, reproductive consequences, and persistent infections.

**Acute Infection**

Acute infection may be defined as the infection of an immunocompetent animal with BVDV. Following infection with BVDV, a short incubation period of 5-7 days is followed by viremia, which generally lasts for 1-2 day but may persist for 15 days (Nuttal et al., 1980; Duffel and Harkness, 1985; Wilhelmsen et al., 1990). The severity of clinical diseases observed following acute infection can vary from subclinical to highly fatal. The majority of acute infections are subclinical in nature (Duffel and Harkness, 1985; Baker, 1987). A mild, biphasic elevation in body temperature and leukopenia may be evident (Duffel and Harkness, 1985; Bolin, 1990a). Lactating cattle may have an associated decrease in milk production (Moerman et al., 1994). In some susceptible animals, more severe clinical disease may be manifested by diarrhea, depression, and anorexia.

Outbreaks of diarrhea and pneumonia in neonatal calves have been associated with BVDV infection (Stott et al., 1980; Lambert and Fernelius, 1968; Lambert et al., 1974; Thomas et al., 1977). However, passively acquired antibodies appear to be protective against these outcomes in neonates (Howard et al., 1989; Shope et al., 1976). Recently, outbreaks of a severe form of BVDV associated disease has been reported in the United Kingdom, Canada, and the United States (Hibberd and Turkington, 1993; David et al., 1994; Drake et al., 1994). These outbreaks have been characterized by relatively high
morbidity and mortality rates. The clinical signs included acute diarrhea, elevated
temperatures, anorexia, agalactia, and the development of oral ulcers. In Canada and the
United States, most of the viral isolates from these outbreaks have been classified as Type
II BVDV.

A hemorrhagic syndrome resulting from thrombocytopenia in association with
acute BVDV infection has been described in the United States (Rebhun et al., 1989) and
more recently in Belgium (Broes et al., 1992). The disease has been reproduced
experimentally using isolates from cattle naturally infected with this clinical form of
BVDV infection (Corapi et al., 1989). Type II BVDV has been the only genotype
associated with this clinical manifestation (Pellerin et al., 1994). The disease has been
described in veal calves (Corapi et al., 1990a) and adult cattle. Characteristics of the
severe thrombocytopenia include bloody diarrhea, petechial and ecchymotic hemorrhages
on mucosal surfaces, epistaxis, and hyphema (Rebhun et al., 1989). The exact cause of
BVDV induced thrombocytopenia is unknown, however an immunoglobulin mediated
mechanism as the result of virus interaction with platelets has been proposed (Corapi et
al., 1990a).

There is substantial evidence that BVDV can result in immunosuppression
following acute infection (Potgeiter et al., 1984; Edwards et al., 1986; Howard, 1990).
As a result, secondary infections caused by opportunistic pathogens are often associated
with concurrent BVDV infection. BVDV has most often been implicated as an
immunosuppressive component of the bovine respiratory disease complex (Dinter and
Bakos, 1961; Greig et al., 1981; Reggiardo, 1979). BVDV is frequently isolated from
pneumonic lungs along with *Pasturella haemolytica* and bovine herpes virus 1 (Greig *et al.*, 1981; Potgeiter, 1977; Reggiardo, 1979). Experimentally, *P. haemolytica* and BVDV have been shown to have synergistic effects in causing respiratory disease (Potgeiter *et al.*, 1984a) and to inhibit the ability of calves to clear bovine herpes virus 1 (Potgeiter *et al.*, 1984b). Different strains of BVDV have been shown to vary in their ability to potentiate bovine respiratory disease (Potgeiter *et al.*, 1985). BVDV infection has also been associated with concurrent *Salmonella* (Wray and Roeder 1987; Penny *et al.*, 1996), *E. coli* (Lambert and Fernelius, 1968), bovine papular stomatitis (Bocha and Yates, 1980) and rotavirus and coronavirus infections (Van Opdenbosch *et al.*, 1981).

The mechanisms of immunosuppression probably involve several aspects of the immune system. Lymphocytes and macrophages are specific targets of BVDV (Truitt and Shechmeister, 1973). Acute infection with BVDV results in a transient leukopenia with lymphoid depletion often noted (Bolin *et al.*, 1985a; Tyler and Ramsey, 1965). *In vitro* studies have suggested different causes of immunosuppression. These include a decreased responsiveness of infected lymphocytes to mitogen stimulation (Muscoplat *et al.*, 1973; Posposil *et al.*, 1977), decrease in interferon production (Diderholm and Dinter, 1966), reduction in monocyte interleukin-1, interleukin-2 and tumor necrosis factor-alpha production (Jensen and Schultz, 1991; Atluru *et al.*, 1990; Adler *et al.*, 1996) and decrease in the chemotactic responses by monocytes (Ketlesen *et al.*, 1979). Regardless of the mechanism, host, agent, and environmental factors undoubtedly influence the degree of immunosuppression that occurs following acute infection with BVDV.
Reproductive Consequences

Although mucosal disease and some of the more serious syndromes associated with acute infections are quite dramatic clinically, they may be relatively unimportant on an economic level because of their rare occurrence. However, the losses associated with poor reproduction following BVDV infection can be devastating. BVDV has been associated with disruption of several phases of reproduction. The specific outcome is largely dependent on the time of infection.

In susceptible cattle exposed to BVDV during breeding, conception rates are significantly lower when compared to cattle seropositive to BVDV (Virakul et al., 1987; McGowan et al., 1992, McGowan et al., 1993; Houe et al., 1993b). The mechanism for the decrease in conception rate has not been fully described. The virus could act at several stages to prevent normal conception. Within the ovary, virus has been isolated from homogenized ovarian tissue for up to 21 days following acute infection with cytopathic BVDV (Ssentongo et al., 1980). BVDV has also been isolated from follicular fluid collected from slaughter house ovaries (Bielanski et al., 1993). Following acute infection with cytopathic BVDV, an interstitial ovaritis has been described with lesions lasting up to 60 days (Ssentongo et al., 1980). With this evidence, it is possible that decreases in conception rate during active infection with BVDV could be a result of direct ovarian malfunction. Significant long term oophoritis could result in ovarian malfunction with subsequent poor conception rates. Limited information is available on ovarian function following BVDV infection. In a study of cattle being superovulated while undergoing experimental challenge with BVDV, the number of palpable corpora lutea and
recovered embryos was significantly reduced (Kafi, et al., 1994). Similarly, in cows persistently infected with BVDV, the number of ovarian antral follicles was significantly reduced when compared to the ovaries from cattle not persistently infected with BVDV (Grooms et al., 1996). These studies suggest that follicular dynamics may be changed in cattle infected with BVDV.

Because of its essential role in fertilization, changes in the oviductal environment could have a detrimental effect on conception rate. BVDV has been detected in oviductal cells (Bielenski et al., 1993; Booth et al., 1995). Archbald et al. isolated BVDV from oviductal tissue and evidence of salpingitis was detected for up to 21 days post intrauterine infusion with cytopathic BVDV (Archbald et al., 1973). Similar findings have not been reported with noncytopathic BVDV.

Early studies suggested that interruption of normal fertilization or embryonic death may be the mechanism for decreases in conception rates associated with acute BVDV infection. Grahn et al. suggested that BVDV interferes with fertilization. This was concluded following the finding that infusion of cytopathic BVDV into the uterus at insemination of superovulated cows resulted in a significant reduction in the number of fertilized ova found at slaughter 3 and 13 days later (Grahn et al., 1984). Similarly, reduction in pregnancy rates has been reported in cattle infused with cytopathic BVDV at insemination. In cattle infused with cytopathic BVDV at 7 days post insemination, a significant reduction in conception rates was observed suggesting that the virus had resulted in embryonic death (Archbald et al., 1979). Although it is felt that BVDV has a direct effect on the developing embryo, inflammatory changes in the uterus following
infusion of cytopathic BVDV may result in an incompatible environment for embryo development (Archibald et al., 1973). Several in vitro studies have been undertaken to elucidate the effects that BVDV may play on early reproductive function. Following ovulation, ovum exposed to BVDV in vitro or collected from follicles have been shown to have virus particles attached to the zona pellucida (Gillespie et al., 1990). However, in vitro studies have shown that the intact zona pellucida protects the developing embryonic cells from BVDV infection allowing normal development to continue (Singh et al., 1982; Potter et al., 1984). In morula and blastocyst stage bovine embryos with the zona pellucida intact or damaged, no cytopathic effects were seen for 48 hours following exposure to cytopathic BVDV (Bielanski and Hare, 1988). Similarly, zona pellucida intact embryos exposed to noncytopathic BVDV infected bovine oviductal epithelial cells for 7 days showed no adverse effects in their rates of development (Zurovac et al., 1994). In contrast, blastocysts hatched from the zona pellucida (day 8 of gestation) have been shown to have decreased survivability when exposed to cytopathic BVDV in vitro. In the same study, noncytopathic BVDV did not decrease blastocyst survivability (Brock and Stringfellow, 1993). These studies suggest that the zona pellucida protects the developing embryo from direct effects of BVDV. However, following removal of the zona pellucida, cytopathic BVDV may have detrimental effects on survivability of blastocysts. No evidence has shown noncytopathic BVDV to have the same effects. As noncytopathic BVDV is the most common virus isolated in acute outbreaks of BVDV and has been the biotype associated with reported decreases in conception rates, further characterization of noncytopathic BVDV effects on the early stages of the developing embryo is necessary.
Interestingly, at day 14 post-hatching, BVDV antigen has been detected in embryos inoculated with noncytopathic BVDV at hatching (Brock and Stringfellow, 1993). In contrast to cytopathic BVDV, it is possible that the effect of exposure of embryos to noncytopathic BVDV may not be evident for some period of time after infection.

Following implantation, transplacental infection of the developing fetus can occur in susceptible cows with either biotype of BVDV. The outcome of the infection is largely dependent on the timing of the infection and the virus biotype involved. Abortions associated with BVDV infection were first described in 1946 (Olafson et al., 1946). Experimental transplacental fetal infection with BVDV was first demonstrated in 1969 (Ward et al., 1969). Fetal death following BVDV infection of susceptible dams can occur at any point during gestation, although they are most common during the first trimester (Cassaro et al., 1971; Done et al., 1980; Duffel and Harkness, 1985; Kahrs, 1968; Kendrick, 1971; Roeder et al., 1986; Sprecher et al., 1991). Depending on the time of infection, fetal reabsorption, mummification or expulsion can occur (Done et al., 1980; Cassaro et al., 1971). Under experimental conditions, both cytopathic BVDV (Brownlie et al., 1989) and noncytopathic BVDV (Done et al., 1980; Duffell et al., 1984; Liess et al., 1984) can cause fetal death following infection of seronegative dams. The rate of abortion in these studies ranged from 19 to 40%. In a field investigation where BVDV was introduced into a susceptible herd as a point source, an abortion rate of 21% occurred during the subsequent 6 months (Roeder and Harkness, 1986). In diagnostic lab surveys in the United States, BVDV has been isolated from between 0.1% (Yamini et al., 1990), 1.5% (Anderson et al., 1990), 4.54% (Kirkbride, 1992) and 27.2% (Woodard,
of submitted abortion cases. In the United Kingdom, BVDV was isolated from 27% of examined abortion cases (Murray, 1990; Murray, 1993). Fetal death usually follows 10-27 days post exposure with expulsion of the fetus occurring up to 50 days later (Murray, 1993). Because of the delay between fetal death and subsequent diagnosis of abortion, fetal and placental lesions seen are usually non-diagnostic and BVDV virus isolation is not always successful (Baker, 1987). Under experimental conditions or when aborted fetuses are expelled soon after death, lesions observed include conjunctivitis, peribronchiolar and interalveolar pneumonia, and non-specific myocarditis. Placental lesions consist mainly of vasculitis, edema, congestion, and hemorrhage with some degeneration and necrosis (Murray, 1993; Jubb et al., 1985). It has been suggested that many BVDV abortions may be the result of placentitis (Murray, 1993).

Fetuses that survive infection with noncytopathic BVDV between 18 and 125 days of gestation invariably develop immunotolerance to the virus and subsequently become persistently infected with BVDV. This phenomenon was first described in an apparently healthy bull (Coria and McClurkin, 1978) and subsequently reproduced experimentally (McClurkin et al., 1984, Liess et al., 1984). Although the exact mechanism of immunotolerance is unclear, it is generally felt that circulation of virus during the period of gestation when immunocompetence is developing (90-120 days) is a prerequisite for persistence. Viral proteins are recognized as self antigens with resulting negative selection of BVDV specific B and T lymphocytes during their ontogeny in the thymus. Persistent BVDV infection in cattle appears to arise from specific B- and T-lymphocyte immunotolerance (Coria and McClurkin, 1978, McClurkin et al., 1984) which results in an
absence of neutralizing and non-neutralizing antibodies to the persistent virus (Donis and Dubovi, 1987b). It is not clear when the exact stage of fetal genesis is during which infection must occur to cause immunotolerance. Under experimental conditions, persistence occurred in 86% and 100%, of calves derived from cows infected with BVDV at day 18 and 30 of gestation, respectively (Kirkland et al., 1993). Persistent infections are rare following fetal infection after day one-hundred, but have been reported up to day one-hundred twenty-five of gestation (Baker, 1985). Noncytopathic BVDV is the only biotype that has been observed or been able to experimentally produce persistence (Brownlie et al., 1984; Bolin et al., 1985, Brownlie et al., 1989). Experimental infections with cytopathic BVDV have failed to produce persistently infected calves (Cassaro et al., 1971; McClurkin et al., 1984; Brownlie et al., 1989).

Fetal infection between one-hundred and one-hundred fifty days of gestation often results in the development of a variety of congenital defects. During this stage of gestation, organogenesis is being completed and the immune system is becoming fully functional. Although not clear, the combination of direct cellular damage by virus and inflammatory response to virus have been proposed as pathogenic mechanisms (Castrucci et al., 1990).

Congenital anomalies involving the central nervous system are most common following fetal infection with BVDV. These include microencephalopathy, hydrocephalus, hydranencephaly (Badman et al., 1981), porencephaly (Hewicker-Trautwein and Trautwein, 1994), cerebellar hypoplasia (Kahrs et al., 1970; Scott et al., 1973) and hypomyelination (Binkhorst et al., 1983). Cerebellar hypoplasia was the first
teratogenic effect of BVDV that was recognized (Ward et al., 1969). At birth, calves have extreme difficulty in becoming ambulatory. Those that can stand are ataxic, resulting in tremors, wide based stance and stumbling gait. The defects are usually severe enough that compensation does not occur and the calves either die or are euthanized (Baker, 1987). The changes in the cerebellum have been characterized as a reduction in the number of molecular layer cells and granular layer cells (Brown et al., 1973; Brown et al., 1974; Bielesfeldt-Ohman, 1984; Done et al., 1980). Purkinje cell numbers are also reduced and often displaced (Brown et al., 1973). Fetal cerebellar effects have been seen following infection as early as the seventy-ninth day and as late as the one-hundred fiftieth day of gestation (Brown et al., 1973). Severity of lesions increase with the age of the fetus at infection up to one-hundred fifty days of gestation (Brown et al., 1973).

Other teratogenic effects that have been associated with BVDV infection include cataracts (Bielfeldt-Ohmann, 1984; Wohrmann et al., 1992), microphthalmia (Brown et al., 1975; Scott et al., 1973; Kahrs et al., 1970), optic neuritis (Bielfeldt-Ohmann, 1984), retinal degeneration (Scott et al., 1973), thymic hypoplasia (Done et al., 1980), hypotrichosis/alopecia (Kendrick, 1971; Baker, 1987), curly hair coat (Larsson et al., 1991), hyena disease (Espinasse et al., 1986), deranged osteogenesis (Constable et al., 1993), and growth retardation (Baker, 1987; Done et al., 1980).

Following the development of immunocompetence during the last trimester of gestation, fetal infection with BVDV is usually cleared with no effects on the developing fetus. These calves are usually normal at birth and have neutralizing antibodies to BVDV
Virus has been isolated from the semen of bulls persistently infected (Coria and McClurkin, 1978; Barlow et al., 1986; Meyling and Jensen, 1988; Revell et al., 1988; Kirkland et al., 1994) and acutely infected (Whitmore et al., 1977; Paton et al., 1989; Kirkland et al., 1991; Kommisurud et al., 1996) with BVDV. In bulls experimentally infected with noncytopathic BVDV, virus was isolated from the semen between 7 and 14 days post infection at a titer ranging from 5 to 75 cell culture infectious doses/ml (CCID/ml) (Kirkland et al., 1991). Immunohistochemical studies of tissues collected from these same bulls at 19 days post infection suggested that virus replication was confined to the seminal vesicles and prostate glands. No changes in semen quality were observed. A virus titer range of $10^4$ to $10^7$ CCID/ml of semen has been reported in persistently infected BVDV bulls (Barlow et al., 1986; Kirkland et al., 1991). BVDV isolation from raw semen may be less successful than from extended semen (Revell et al., 1988). This is presumably due to the documented virucidal effects of semen (Kahrs et al., 1980). However, the semen remains infective which is evident by the demonstration that susceptible cows can become infected following artificial (Paton et al., 1990; Kirkland et al., 1994) or natural insemination (McClurkin et al., 1979; Wentink et al., 1989).

Persistently infected bulls can successfully sire calves, however their breeding efficiency is generally low (Meyling and Jensen 1988; Wentink et al., 1989; Paton et al., 1990; Kirkland et al., 1994). Semen quality is variable, ranging from acceptable (Barlow et al., 1986; Wentink et al., 1989) to containing various defects predominantly involving
the head of the spermatozoa (McClurkin et al., 1979, Revell et al., 1988). Poor reproductive efficiency following the use of bulls persistently infected with BVDV is probably due to a combination of low quality semen, ill thrift often evident in persistently infected cattle, and viral effects on the reproductive tract and conceptus in susceptible females.

**Persistent Infection With BVDV**

As described earlier, cattle persistently infected with BVDV are created when fetal infection with noncytopathic BVDV occurs between 18 and 125 days of gestation resulting in a specific immunotolerance to the infecting virus. The resulting cattle continuously shed virus for their entire life. The immunotolerance is specific to the infecting virus. Persistently infected cattle are able to mount an immune response to infection with a heterologous strain of BVDV (Bolin, 1985c).

Calves born persistently infected with BVDV typically do not perform well. Mortality rates greater than 50% have been reported during the first year of life (Duffel and Harkness, 1985; Houe, 1993). Persistently infected calves that survive often lag behind their cohorts in growth rate. Most either die or are culled because of poor performance before they reach 2 years of age (Baker, 1995). The reasons for ill thrift are not well understood. Immunosuppression with resulting secondary infections is thought to be a major cause of poor performance and death of persistently infected cattle (Johnson and Muscoplat, 1973; Barber et al., 1985). This may be a result of defects in neutrophil and lymphocyte functions (Muscoplat et al., 1973; Roth, et al., 1986). Other
abnormalities reported in persistently infected cattle include subclinical glomerulonephritis and encephalitis (Cutlip, et al., 1980) and congenital anomalies. Persistently infected cattle can be normal grossly and can become productive adults (Coria and McClurkin, 1978; McClurkin et al., 1979; Cutlip et al., 1980). This has important implications in that persistently infected cows produce persistently infected calves, thus maintaining a source of virus in a herd across generations (McClurkin et al., 1979).

The most dramatic clinical manifestation of BVDV infection is mucosal disease. This highly fatal, sporadic form of BVDV occurs only in persistently infected cattle. The proposed pathogenesis of mucosal disease involves concurrent infection with both noncytopathic and cytopathic BVDV (Brownlie et al., 1993). It was first established that mucosal disease required a congenital persistent infection with noncytopathic BVDV which was followed by superinfection with a cytopathic BVDV (Bolin et al., 1985d; Brownlie et al., 1984). However, it was observed that not all persistently infected cattle developed mucosal disease following infection with cytopathic BVDV (Bolin 1985c). This led to the suggestion and subsequent support that noncytopathic and cytopathic BVDV pairs isolated from cattle that died from mucosal disease are antigenically homologous (Corapi et al., 1988; Howard et al., 1987). It was hypothesized that the most likely source of the superinfecting cytopathic BVDV was a mutation of the persistent noncytopathic BVDV which affects the biotype of the virus, but not the antigen makeup. Molecular characterizations of viral pairs isolated from fatal mucosal disease cases confirmed this hypothesis (Meyers et al., 1991; Greiser-Wilke et al., 1993a; Meyers et al., 1992; Tautz et al., 1994). Since the superinfecting cytopathic BVDV is antigenically
homologous to the persistent noncytopathic BVDV, no specific immune response arises against it. This allows the cytopathic BVDV to replicate and spread in the host unabated. However, the pathogenesis of mucosal disease has become more complex with evidence that antigenically heterologous viral pairs can also cause mucosal disease (Bolin et al., 1985c; Bolin et al., 1985d; Corapi et al., 1988; Ridpath et al., 1991). This is most clearly illustrated in the case of modified live vaccine induced mucosal disease where differences in 10 of 29 viral neutralizing monoclonal antibodies were identified between the isolated cytopathic and noncytopathic BVDV (Bolin, 1995a). Further complexity is added in the case of delayed onset mucosal disease. Several investigators noted that following infection of persistently infected cattle with a heterologous cytopathic BVDV isolate, mucosal disease did not occur within a 2 to 4 week period which is normally expected (Brownlie, 1993; Moennig et al., 1990; Westenbrink et al., 1989). Instead, mucosal disease developed months later. Concurrently, neutralizing antibodies developed in the host to the challenge cytopathic BVDV, but not the cytopathic BVDV isolated at post mortem. The proposed mechanism for this phenomenon is a recombinational event between the resident noncytopathic BVDV and the challenge cytopathic BVDV which results in a new cytopathic BVDV which is antigenically homologous to the noncytopathic BVDV (Bolin, 1995a; Ridpath and Bolin, 1996). The challenge cytopathic BVDV is cleared by normal immune mechanisms, however the new recombinant cytopathic BVDV is not cleared and goes on to produce mucosal disease. The replication of the single recombinant cytopathic BVDV to a level sufficient to cause mucosal disease is reflected in the delayed onset of the syndrome.
Mucosal disease presents in two clinical forms: acute and chronic. The clinical and pathological findings of mucosal disease have been described extensively (Ames, 1986; Blood et al., 1986; Baker, 1990; Brownlie, 1985; Duffel and Harkness, 1985; Jubb et., 1985; Radostitis and Liittlejohns, 1988; Perdrizet et al., 1987). Experimentally, acute mucosal disease usually develops within 2-4 weeks following superinfection with cytopathic BVDV. Characteristic signs include an elevated temperature, anorexia, depression and weakness. Within 2-3 days, a severe watery diarrhea develops that is often hemorrhagic. Erosive lesions of the oral cavity including the muzzle, lips, tongue, dental pad, and hard palate are common. Mucopurulent nasal discharge, and hypersalivation may subsequently develop. Corneal edema, conjunctivitis, and excessive lacrimation are at times evident. A severe leukopenia develops early in the disease. Death usually occurs within 10 days after the onset of signs as a result of dehydration, severe metabolic and hematologic disturbances and secondary bacterial infections. On post mortem, erosions involving the esophagus, rumen, abomasum, small intestine, cecum and colon may be noted. The predilection of BVDV for lymphatic tissue is evident in severe necrosis and hemorrhage of the peyers patches, ilium, and proximal colon. Other gross lesions may include edema of the lymph nodes, seborrhea-like dermatitis, and erosive lesions of the perineum, vulva, teats, interdigital cleft and cornet.

The clinical signs of chronic mucosal disease are often similar to the acute form, but on a less severe scale (Baker, 1990; McClurkin et al., 1985). Progressive emaciation, lethargy and anorexia are characteristic signs. Diarrhea may be continual or intermittent. Afflicted cattle may survive for up to 18 months. Death usually results from secondary
infections or severe debilitation. Similar to acute mucosal disease, both noncytopathic BVDV and cytopathic BVDV can be isolated from cattle suffering from chronic mucosal disease (McClurkin et al., 1985). Although the pathogenic mechanism of chronic mucosal disease is not known, there is evidence that it may result from superinfection of a persistently infected animal with a partially homologous/ heterologous cytopathic BVDV strain resulting in a chronically progressive disease (Brownlie, 1993). This would be in contrast to the two extremes -- superinfection with a homologous cytopathic BVDV strain, which would result in acute mucosal disease, or superinfection with a heterologous cytopathic BVDV which results in seroconversion and removal of the virus. Others have suggested that chronic mucosal disease may result from recombinational events between the persisting noncytopathic virus and a cytopathic virus of attenuated vaccine origin (Bolin, 1995a; Ridpath and Bolin, 1996).

**Diagnosis of BVDV**

The control of BVDV is largely dependent on the ability to identify cattle infected with the virus, especially those persistently infected. Because of the complex and insidious nature of BVDV infections, a presumptive diagnosis based on clinical signs is often inadequate. This makes laboratory diagnosis a critical component in the control of BVDV. The sensitivity and specificity of laboratory diagnosis is dependent on many factors. Most important are the type of samples submitted, the timing of sample submission, and the diagnostic test employed. Accurate diagnosis demands a broad
understanding of the pathogenesis of BVDV so that proper samples can be submitted, results interpreted, and control measures implemented.

Virus isolation is most commonly used to identify BVDV (Dubovi, 1990; Edwards, 1990; Brock, 1995). Isolation is commonly done in cell culture using primary bovine testicular or turbinate cells. Noncytopathic BVDV is diagnosed in cell culture by identifying BVDV specific antigens in infected cells using immunoenzyme or immunofluorescent staining techniques (Smith et al., 1988; Afshar et al., 1991; Ward and Kaeberle, 1994). Cytopathic BVDV is identified by visual observation of characteristic cytopathic effects on cells, which is usually evident within 48 hours, and can be confirmed by immunostaining methods (Dubovi, 1990; Underdahl et al., 1957). A microtiter immunoperoxidase assay for the detection of virus is commonly used in diagnostic labs to facilitate the testing of a large number of samples economically. Comparison of this test with fluorescent antibody detection of BVDV in bull semen resulted in complete agreement of the two tests (Afshar et al., 1991). Because of the antigenic diversity of different BVDV isolates, the sensitivity of monoclonal based immunoenzyme tests is of concern. To improve sensitivity, the use of pooled monoclonal antibodies with broad reactivity is preferred (Dereg et al., 1990; Brock, 1995).

Virus isolation can be accomplished from a variety of samples. Ante mortem detection of BVDV is most commonly done from serum, buffy coats, or nasal swabs (Brock, 1995). BVDV can be isolated from semen (Afshar et al., 1991). However, the sensitivity of isolation may be reduced in raw semen presumably because of the antiviral
effects of seminal fluids (Revell et al., 1988). Lymphatic tissues are the preferred samples for BVDV isolation post mortem. These include the spleen, thymus and Peyer's patches (Brock, 1995). In aborted fetuses, the thymus may yield positive results more often than other lymphoid tissues (Grooms, unpublished data).

Several antigen capture ELISA assays have been developed to identify BVDV in serum, white blood cells, blood clots, or tissue homogenates (Fenton et al., 1991; Shannon et al., 1991; Greiser-Wilke et al., 1993b). In two studies, the sensitivity of an antigen capture ELISA was 96% (Fenton et al., 1991) and 97% (Greiser-Wilke et al., 1993b) when compared to virus isolation.

Detection of BVDV antigen in tissues using immunoenzyme or immunofluorescent staining is used commonly (Dubovi, 1990). These techniques have several advantages over virus isolation (Haines and Clark, 1991). Often, viral antigen can be detected in tissues even though efforts to isolate viable virus fail. This may be especially useful in the diagnosis of abortion. In a study of aborted fetuses submitted to a diagnostic lab, the detection rate of BVDV using immunohistochemistry and conventional virus isolation was 97% and 83% respectively (Ellis et al., 1995). Similarly, in cases of known BVDV enteric disease where samples were limited to gastrointestinal tissue, immunohistochemistry was positive in 100% of the cases compared to virus isolation which was positive in only 65% of the cases (Baszler et al., 1995). Immunostaining can be performed rapidly with results obtained in as little as 24 hours. This may be important where timely identification of BVDV is critical in controlling an outbreak. The quality of samples may be less variable when formalin fixed tissues are submitted by practitioners,
especially when there is a significant time lag between sample collection and arrival at the diagnostic lab. Finally, lesions and viral antigen detection can be correlated which may be relevant to the nature of the disease in an infected herd. Currently, the monoclonal antibody 15C5 is commonly used in immunohistochemical procedures on formalin fixed, paraffin embedded tissues (Haines et al., 1992). This monoclonal is directed against the E\textsuperscript{m} glycoprotein, which is highly conserved among BVDV isolates (Corapi, et al., 1990b).

Serology to detect specific antibodies can be a valuable tool in determining the infection status of an animal or herd. However, interpretation of results requires a broad knowledge of BVDV pathogenesis and a clear history of the tested animal or herd. The most commonly used serological test for BVDV is the virus neutralization assay (Dubovi, 1990; Edwards, 1990; Brock, 1995). The lack of a standard BVDV reference strain for use in neutralization tests and variation in laboratory techniques makes the comparison of results from different labs difficult (Edwards, 1990). In a study comparing cytopathic BVDV strains NADL and Singer as references strains, the Singer strain detected more positive samples with higher titers than the NADL strain (Deregt et al., 1992). Similarly, when noncytopathic stains SD-1 and BJ were compared, no correlations between the results could be made (Brock, 1995). ELISA assays have been developed to detect BVDV antibodies. Good correlations between ELISA assays and virus neutralization assays have been shown (Cho et al., 1991; Chu et al., 1985; Durham and Hassard, 1990; Paton et al., 1991). Other assays which have been used for serological assessment of BVDV include the agar gel immunodiffusion test (Harkness et al., 1978), compliment
Several methods have been developed to detect the genomic RNA of BVDV. These tests have the advantage of being able to detect very small amounts of viral RNA thus potentially making them very sensitive. However, sensitivity may also be limited in the diagnosis of BVDV because of the genetic variation evident in different BVDV isolates. Nucleic acid hybridization probes have been developed to different regions of the BVDV genome (Brock, 1991; Brock et al., 1993; Kwang et al., 1991; Lewis et al., 1991; Ridpath et al., 1993; Roberts et al., 1991). Most probes have been directed against the 5' UTR and the coding region of the NS2-3 protein. Both of these regions have been identified as being highly conserved (Brock et al., 1993; Kwang et al., 1991). Using a hybridization probe made from the 5' untranslated region of noncytopathic BVDV strain SD-1, 100% of viral isolates were detected (Brock et al., 1993). In comparison, 63%, 84% and 68% of the same viral isolates were detected by three different probes made from the NS2-3 region of noncytopathic BVDV strain SD-1 and cytopathic BVDV strains NY-1 and NADL respectively (Brock et al., 1993). In situ hybridization has been used to detect BVDV genomic RNA in formalin fixed tissues (Desport et al., 1994). However, because of technical demands, this method is not readily available for diagnostic applications. The use of reverse transcription and polymerase chain reaction (RT-PCR) amplification of genomic RNA can be a valuable tool in the diagnosis of BVDV. The amplification and subsequent detection of specific BVDV genetic material has been demonstrated (Belak and Ballagipordany, 1991; Hertig et al., 1991; Lopez et al., 1991;
Ward and Misera, 1991; Hamel et al., 1995). The polymerase chain reaction has been estimated to be up to 1000 times more sensitive than virus isolation in detecting BVDV (Hertig et al., 1991; Lopez et al., 1991). This technology has been adapted to detect BVDV in several different samples from which RNA can be extracted including serum (Brock, 1991), whole blood/white blood cells (Hamel et al., 1995), homogenized tissues (Belak and Ballagi-Pordany, 1991), nasal swabs (Hamel et al., 1995), formalin fixed tissues (Gruber et al., 1993), and milk somatic cells (Brock and Radwan, 1992).

Because of the complicated nature of BVDV, the accurate laboratory diagnosis of BVDV is important for the control of this virus. A broad understanding of the full spectrum of BVDV infections is necessary in order to select the appropriate diagnostic samples and tests and then to interpret the results so that timely control measures can be recommended. Diagnosis of BVDV should begin with a complete history (Duffel and Harkness, 1985; Baker, 1987). Special attention should be paid to issues of biosecurity, reproduction, neonatal health, and overall herd health. Evaluation of reproductive data is often of value in detecting patterns consistent with BVDV infection (Houe and Meyling, 1991a). Physical and/or postmortem examinations of cattle suspected of being acutely or persistently infected with BVDV should be performed. Signs compatible with BVDV infection should be noted. A final diagnosis of BVDV is dependent on laboratory confirmation (Duffel, 1985; Baker, 1987). Accurate laboratory diagnosis of BVDV depends on the selection of the appropriate samples and tests. Isolation of virus from cattle acutely infected with BVDV is often frustrating because of the short period of viremia. Virus can be isolated from white blood cells for a longer period of time than it
can from serum. Therefore, whole blood is recommended for the detection of acute infections (Brock, 1995). Nasal swabs or postmortem derived lymphoid tissue are also appropriate. Acute and convalescent serum samples collected 30 days apart are also useful for detecting acute infection.

Due to a delay between fetal death and expulsion, isolation of BVDV from aborted fetuses is often difficult (Baker, 1987). Similarly, maternal seroconversion frequently happens before fetal expulsion occurs. This makes serology of less value in diagnosing BVDV associated abortions. Immunohistochemistry has been reported to be more sensitive than virus isolation in diagnosing BVDV abortions (Ellis et al., 1995). Because of the difficulty in diagnosing abortions, submission of fresh lymphoid tissue for virus isolation, formalin fixed tissue for immunohistochemistry, and maternal acute and convalescent serum should be submitted for BVDV diagnosis.

Several methods have been developed to screen herds for the presence of BVDV. Testing of bulk tank somatic cells by RT-PCR has been described (Brock and Radwan, 1992). This screening test may be very sensitive, but only samples lactating cattle in the herd. A bulk tank antibody test has been developed that can predict the likelihood of the presence of cows persistently infected with BVDV by the antibody titer (Niskanen et al., 1991). This test is most useful in unvaccinated herds and is currently being used in BVDV eradication programs in Norway (Loken and Krogsrud, 1992). Monitoring of herds for characteristic reproductive and calf health patterns has been discussed as a tool for detecting BVDV infected herds (Houe and Meyling, 1991a). Serological screening of unvaccinated cattle between 6 and 18 months of age has been used to estimate the
probability of cattle persistently infected with BVDV being present in a herd (Houe, 1994; Houe et al., 1995).

The definitive identification of cattle persistently infected with BVDV requires the isolation of virus from serial samples taken at least 14 days apart. Because of the high level of virus found in the blood of persistently infected cattle, serum samples are generally adequate for virus isolation. There is evidence that some persistently infected cattle can mount a neutralizing immune response which reduces the serum level of BVDV to an undetectable level even though virus can be routinely isolated from white blood cells (Brock and Grooms, 1994). The frequency of this phenomenon is unknown. In neonates, colostrum derived maternal antibodies will reduce the level of free virus in serum to undetectable levels for up to 8 weeks after ingestion (Palfi et al., 1993).

Therefore, the diagnosis of persistently infected calves less than 3 months of age should be performed using white blood cells from whole blood samples (Palfi et al., 1993; Brock, 1995). Historically, serology has been used to screen for cattle seronegative to BVDV on the assumption that persistently infected cattle are immunotolerant to BVDV. However, it is now known that persistently infected cattle can mount a neutralizing antibody response to BVDV and would be missed using this method (Bolin, 1985c; Bolin, 1988).

Control and Prevention of BVDV

It is generally accepted that the control of BVDV infections is challenging. Several factors account for this difficulty including the complex variety and pathogenesis of infections, antigenic variability between BVDV strains, and management practices that
circumvent biosecurity. A broad understanding of these issues is needed to develop sound BVDV control and prevention programs. With the current understanding of BVDV, control and prevention of BVDV should revolve around three facets: 1) elimination of the viral source, 2) vaccination, and 3) biosecurity.

Cattle persistently infected with BVDV are the major source of infection for susceptible herdmates (Bolin 1990b). Not only do they shed large amounts of virus in their body secretions and excretions, but their offspring are invariably persistently infected calves, thus potentially creating families of persistently infected cattle. Elimination of these cattle is critical to the control of BVDV. Most reports of BVDV outbreaks can be linked to the identification of a persistently infected animal (Barber et al., 1985; Duffel and Harkness, 1986; Perdrizet et al., 1987; Roeder and Drew, 1984; Bezek and Mechor, 1992; Taylor et al., 1994). Since persistently infected cattle can not reliably be identified by clinical examination, laboratory identification is necessary (Bolin, 1990b). This requires the testing of serum or white blood cells from all cattle on the farm in question. Following the detection of persistently infected cattle, they should be eliminated from the farm. Since fetal infection is the source of BVDV persistence, all newborn calves need to be tested for at least 9 months after the initial removal of persistently infected animals.

The use of vaccines for the control and prevention of BVDV is controversial. Much of the debate centers around the use of modified live or killed vaccines and their ability to provide broad protection given the antigenic variability of BVDV.

Currently, modified live vaccines contain cytopathic BVDV that has been attenuated by serial passage through cell culture (Bolin, 1995b). Several advantages of
modified live vaccines have been suggested. Protective immunity can be obtained within 3 weeks following a single dose of vaccine thus reducing the handling of cattle (Bolin and Ridpath, 1989; Bolin, 1995b). Because modified virus replicates in the animal, antigen is amplified to high levels \textit{in vivo}. Thus only small amounts of virus are needed which reduces the cost of the vaccines (Kahrs, 1966; Bolin, 1995b). Finally, modified live vaccines appear to induce immunity to BVDV in the face of colostral antibodies although the response is variable (Brar et al., 1978; Menanteau-Horta et al., 1985). The major disadvantage of modified live vaccines is their ability to cross the placenta and cause fetal pathology (Liess et al., 1984; Orban et al., 1983). These vaccines have also been reported to cause immunosuppression (Roth et al., 1983) which can result in secondary infections by other pathogens (Baker, 1987). Post vaccination mucosal disease has been initiated following modified live vaccination (McKercher et al., 1968; Peter et al., 1967; Rosner, 1968). This most likely occurs only in cattle who are persistently infected with BVDV and then immunized with a modified live vaccine that contains cytopathic BVDV (Bolin, 1990a). Another disadvantage is the potential for virus mutation resulting in a return to virulence (Bolin, 1995b).

The major advantage of killed vaccines is their safety. The inactivation process destroys their ability to replicate. Because of this, the possibility for fetal infection, immunosuppression, post vaccination mucosal disease or return to virulence is eliminated (Baker, 1987; Bolin, 1995b). The major disadvantage of killed vaccines is the need for an initial 2 doses of vaccine for primary immunization (Baker, 1987; Bolin, 1995b). Because killed vaccines do not replicate \textit{in vivo}, a higher antigenic mass is needed to stimulate the
immune system which ultimately makes them more costly (Harkness, 1987; Bolin, 1995).

A final concern is the duration and extent of protection induced by killed vaccines, especially with respect to fetal infection (Baker, 1987; Bolin, 1995). Calves persistently infected with BVDV have been born to cows both experimentally and naturally infected with BVDV following immunization with a killed BVDV vaccine (Bolin et al., 1991; Harkness, 1987; Meyling et al., 1987; Roeder and Harkness, 1986). Based on these studies, it is clear that fetal protection may be incomplete following vaccination with killed BVDV products and is dependent on the time of infection following vaccination and the antigenic differences between the vaccine and challenge virus strains.

The final key to BVDV control and prevention is biosecurity. Cattle persistently infected with BVDV are the major source of virus for susceptible animals (Houe, 1995). Because of this, all cattle added to a herd should be tested for viral persistence. Special attention should be paid to the fetus, which is often overlooked. Although persistently infected cattle are the major source of viral introduction, others sources must be considered when developing control programs. Cattle brought back to a farm following exposure to cattle of unknown background may be at risk of acute infection and virus shedding. Isolation of these cattle for 3 weeks is recommended before reintroduction to the herd. Again, special attention should be paid to the fetus of any gestating animal since exposure to BVDV may result in viral persistence. BVDV can be spread through semen from either acutely or persistently infected bulls (Whitmore et al., 1977; Kommisrud et al., 1996). Because of this, semen should be obtained from reputable bull studs where rigorous screening for BVDV and isolation is done. Herd bulls should be tested for
BVDV infection before introduction to the breeding herd. Information on the spread of
BVDV through embryos is limited. BVDV has not been isolated from zona pellucida
intact embryos derived from persistently infected cows which have undergone washing
procedures as recommended by the International Embryo Transfer Society (Potter et el.,
1984; Singh et al., 1982; Stringfellow et al., 1991). Calves born after embryo transfer
from persistently infected cows to uninfected cows have all shown no evidence of BVDV
infection, although the reports involve limited numbers (Wentink et al., 1991b; Bak et al.,
1992; Brock et al., 1997). The role of fomites in the spread of BVDV is not clear.
BVDV can survive outside of the host for a limited time (Bendixen, 1993) and has shown
to be transmitted experimentally using contaminated hypodermic needles and common
palpation sleeves (Gunn, 1993; Lang-Ree et al., 1994). Therefore, reasonable
precautions should be taken to prevent the spread of the virus by fomites.

BOVINE OVARY

The bovine ovary is located in the pelvic cavity lateral to the nonpregnant uterus
(Miller and Campbell, 1978). The ovary is composed of an outer cortex and inner
medulla. The cortex contains the active ovarian tissues, the most important being follicles
and corpora lutea (Miller and Campbell, 1978). Within follicles, oocytes are found either
in a quiescent or developing stage (Allen et al., 1976). Cortical interstitial cells are
derived from granulosa cells of atretic primary follicles and theca interna cells of atretic
antral follicles (Guraya, 1968). The cortex is supported by stromal connective tissue, blood vessels, nerves and lymphatics. The medulla consists mainly of dense connective tissue and large blood vessels (Miller and Campbell, 1978).

The Estrous Cycle

The nature, control and regulation of the bovine estrous cycle has been extensively studied. The estrous cycle is composed of cyclical alterations in behavior, ovarian morphology and function and uterine morphology and function. These changes are primarily controlled by a complex neuroendocrine relationship between the hypothalamus, pituitary gland and ovary. In domestic cattle, the estrous cycle length is 21 days with a range of 17 to 25 days considered as normal (McDonald, 1980). Unlike other domestic animals, seasonal changes in the estrous cycle attributable to changing photoperiod do not occur (Knickerbocker et al., 1986). For purposes of describing ovarian dynamics during the estrous cycle, Hansel and Convey divided the estrous cycle into three phases: pregonadotropin surge phase, postgonadatropin surge phase and luteal phase (Hansel and Coney, 1983).

During the pregonadotropin surge phase, luteal regression and the final stages of maturation of the ovulatory follicle occur. With luteal regression, luteinizing hormone (LH) concentrations increase as a consequence of removal of the negative feedback effect of progesterone on the hypothalamus and anterior pituitary gland (Chenault et al., 1975). Pulsatile release of LH during this period occur characteristically at a higher frequency but lower amplitude (Rahe et al., 1980). With growth of the ovulatory follicle, peripheral
estradiol levels increase and reach a peak at estrus (Baird and Scaramuzzi, 1976). Both LH and follicle stimulating hormone (FSH) are released just prior to the onset of behavioral estrus constituting the gonadotropin surge (Akbar et al., 1974). The gonadotropin surge is stimulated by peak estradiol production from the developing ovulatory follicle (Beck and Convey, 1977; Martin et al., 1978). However, exogenous progesterone can block the estradiol-induced gonadotropin surge indicating that luteal regression is a prerequisite to this event (Kesner et al., 1981). Evidence suggests that estradiol triggers the gonadotropin surge by increasing pulsatile release of gonadotropin releasing hormone (GnRH) from the hypothalamus and sensitizing pituitary responsiveness to GnRH (Kesner et al., 1983; Hansel and Convey, 1983). The gonadotropin surge occurs near the onset of behavioral estrus and lasts for approximately 8 to 10 hours (Knickerbocker et al., 1986). It is terminated following the development of refractoriness of the anterior pituitary gland to GnRH (Kesner and Convey, 1982). Behavioral estrus lasts for about 12 to 16 hours and is the result of high concentrations of estradiol acting on the central nervous system (McDonald, 1980).

Immediately following the gonadotropin surge, peripheral estradiol concentrations decrease rapidly while peripheral progesterone, LH, and FSH remain at baseline levels (Hendricks et al., 1972). Intrafollicular concentrations of estradiol also decrease while follicular progesterone levels increase (Ireland and Roche, 1982). Ovulation occurs approximately 24 to 30 hours after the gonadotropin surge and 12 to 15 hours after the end of behavioral estrus (McDonald, 1980).
Following ovulation, the luteal phase of the estrous cycle begins. The corpus luteum is rapidly formed, reaching mature size by 7 days post ovulation (Donaldson and Hansel, 1965). A concurrent increase in the peripheral progesterone concentration also occurs peaking at 10 days post ovulation (Hansel et al., 1973). During the early luteal phase, mild increases in estradiol occur which correspond to the growth of nonovulatory follicles (Holst et al., 1972). Peripheral concentrations of LH remain low early in the luteal phase due to depletion of pituitary stores (Convey et al., 1977) and subsequently due to negative feedback from rising concentrations of progesterone (Rahe et al., 1980). Late in the luteal phase, a dominant preovulatory follicle begins to grow with a subsequent rise in estradiol concentrations. Estradiol is thought to induce luteal regression by stimulating uterine prostaglandin $F_{2\alpha}$ production (Peterson et al., 1975; Knickerbocker et al., 1986). This is supported by studies which have shown that administration of exogenous estrogen can initiate luteolysis (Eley et al., 1979) while removal of ovarian follicles extends luteal life (Villa-Goody et al., 1981). Surgical manipulation of the utero-ovarian vascular system provided evidence that the luteolytic signal is transferred from the uterus to the ovary by counter-current exchange (Ginther and Del Campo, 1974). Simultaneous ovarian production of oxytocin is felt to enhance uterine prostaglandin $F_{2\alpha}$ (Flint and Scheldrick, 1982). Simultaneous to the detection of increasing levels of peripheral prostaglandin $F_{2\alpha}$ metabolites, estradiol levels continue to rise while progesterone levels decline thus initiating another pregonadotropin surge phase of the estrous cycle (Chenault et al., 1975; Peterson et al., 1975).
The Follicular System

The follicular system of the bovine ovary has two main functions; oocyte maturation and production of ovarian steroids (Lunenfield et al., 1975). Development of follicles can be subdivided into three parts. First, primordial follicles are transformed into secondary follicles. Secondary follicles consist of two or more layers of granulosa cells surrounding the developing oocyte (Allen et al., 1976). Tertiary or antral follicles are formed from secondary follicles. Tertiary follicles consist of an antrum filled with follicular fluid and a follicular wall consisting of three distinct cellular layers: the granulosa cell layer which lines the antrum, the theca interna and theca externa (Asdell, 1960; Dellman and Brown, 1976). The oocyte is surrounded by the zona pellucida and a layer of granulosa cells called the corona radiata. The oocyte and its surrounding cells project from the granulosa cell layer into the antrum on the cumulus oophorus (Dellman and Brown, 1976).

The growth and atresia of follicles during the course of the estrous cycle is known as follicular dynamics (Lucy et al., 1992). Early studies noted that during the normal bovine estrous cycle, cyclic changes take place within the follicular system (Rajakoski, 1960; Choudary et al., 1968; Dufour et al., 1972). Using morphological analysis, conflicting results emerged as to the nature of this cyclic change. Based on the study of over 700 ovaries, Matton et al. concluded that the growth of follicles was continuous and constant during the estrous cycle (Matton et al., 1981). Other studies concluded that at least two periods of follicular growth occurred during the course of the estrous cycle (Rajakoski, 1960; Matton et al., 1981; Ireland and Roche, 1983). With the development
of transrectal ultrasonography, follicular dynamics could be observed in vivo for the entire length of the estrous cycle. It was established that one to four waves of follicular growth and development occur during the course of one estrous cycle with the ovulatory follicle arising from the last wave (Savio et al., 1988; Siros and Fortune, 1988; Ginther et al., 1989). Early in the estrous cycle, a group of small antral follicles begins to develop. Although unclear, increases in FSH following ovulation may stimulate the emergence of these follicles (Walters and Schallenberger, 1984). From this cohort of follicles, a single dominant follicle is selected and continues to grow while the rest of the group regress in size. The mechanism for selecting the dominant follicle has not been identified. The dominant follicle of the first follicular wave remains active until the middle of the estrous cycle. The active life span of the dominant follicle is generally five to seven days (Lucy et al., 1992). During this period, no new follicles greater than 5 mm in diameter can be visualized (Ginther et al., 1989). In the majority of estrous cycles, the first dominant follicle regresses. Subsequently, a second follicular wave arises followed by the emergence of a new dominant follicle (Savio et al., 1988; Siros and Fortune, 1988). In cows with two wave cycles, this follicle ovulates after luteolysis. Alternatively, in cows with three wave cycles, this process is repeated a third time with the emergence of a third dominant follicle (Siros and Fortune, 1988). In general, cows with three wave of follicles have a longer estrous cycle (Taylor and Rajamahendran, 1991).

The control of follicular dynamics involves both local and neuroendocrine regulatory factors. FSH appears to be crucial in the emergence of each follicular wave. Increases in plasma FSH have been shown to occur two to four days prior to the
emergence of each follicular wave (Adams et al., 1992; Sunderland et al., 1994). With the emergence of the dominant follicle, FSH levels are suppressed (Sunderland et al., 1994). The control of FSH levels is probably directly related to factors produced by the dominant follicle. It has been shown that the infusion of proteinaceous fractions found in follicular fluid can inhibit the emergence of follicular cohorts during the first five days of the estrous cycle (Kastelic et al., 1990; Adams et al., 1992). Subsequent experiments showed that the presence of the dominant follicle was directly related to the regression of subordinate follicles and suppression of the emergence of the next follicular wave (Ko et al., 1991).

Two follicular products, inhibin and estradiol, can control the release of FSH in a negative fashion (Price and Webb, 1988; Beard et al., 1990), although inhibin may be of greater importance (Sunderland et al., 1994; Glencross et al., 1994). The turnover of dominant follicles appears to be regulated by luteal levels of progesterone. Luteal levels of progesterone suppress the pulsatile release of LH from the pituitary gland (Walters et al., 1984; Rahe et al., 1984). Because LH stimulates androgen biosynthesis in follicles (Hansel and Convey, 1983), it is hypothesized that low levels of LH may result in limited androgen availability for estradiol synthesis resulting in their eventual atresia (Lucy et al., 1992). This hypothesis has been supported by studies that have shown that sub-luteal levels of progesterone result in increased LH pulsatile release with subsequent higher estradiol levels and maintenance of the dominant follicle for a prolonged period of time (Siros and Fortune, 1990; Stock and Fortune, 1993). Thus the following scenario can be hypothesized. FSH stimulates the emergence of a cohort of follicles at the beginning of each follicular wave. A dominant follicle is selected by unknown mechanisms. With
maturity, the dominant follicle produces inhibin and estradiol, which provide negative feedback to the pituitary gland leading to suppression of FSH secretion. Without adequate FSH, subordinate follicles regress and new follicular waves are suppressed. With luteal levels of progesterone suppressing LH release, follicle estradiol production is minimized resulting in the initiation of atresia. As the dominant follicle begins to regress, FSH levels rise and the emergence of a new follicular wave begins.

**Cytokines and Ovarian Function**

In addition to their role in the regulation and control of the immune system, cytokines have been postulated to play an important role as local regulators of ovarian function (Adashi, 1990). Macrophages, known to be a major source of cytokines (Takemura et al., 1984), have been demonstrated to be a major constituent of the interstitial ovarian compartment (Hume et al., 1984) and the corpus luteum (Bagavandoss et al., 1988; Paavola, 1977). Therefore, resident ovarian macrophages have been postulated as important local regulators of ovarian function (Adashi, 1990). Interferons, interleukins and tumor necrosis factors have received the most attention with respects to ovarian function.

Interleukin-1 has been shown to suppress the functional and morphological luteinization of cultured porcine and murine granulosa cells (Fukuoka et al., 1988; Gottschall et al., 1987; Gottschall et al., 1989; Yasuda et al., 1990). Interleukin-1 also has been shown to inhibit the number of FSH induced LH receptors on cultured granulosa cells (Gottschall et al., 1988). High levels of interleukin-1 have been detected in ovarian
follicular fluid, but the exact cellular source is unknown (Khan et al., 1988). It has been suggested that the antigonadotropic effect of interleukin-1 could serve as a suppressor of premature follicular luteinization (Adashi, 1990).

Intraovarian sources of tumor necrosis factor alpha include luteal cells (Roby et al., 1990; Ji et al., 1991), granulosa cells (Roby and Terranova, 1989; Zolti et al., 1990) and macrophages (Bagavandoss et al., 1988; Nakamura et al., 1987). Experiments have shown that tumor necrosis factor alpha can attenuate the differentiation of cultured granulosa cells by blocking the stimulatory effects of FSH (Emoto and Baird, 1988; Adashi et al., 1989; Darbon et al., 1989). Tumor necrosis factor alpha has also been shown to cause increased prostaglandin production and a corresponding decrease in progesterone levels in cultured luteal cells (Fairchild Benyo and Pate, 1992). Tumor necrosis factor appears to play a role in the processes of follicular atresia and luteolysis.

Interferon gamma has been shown to inhibit granulosa cell differentiation and to have potent inhibitory effects on FSH induced estradiol production in cultured granulosa cells collected from small follicles (Gorospe et al., 1988; Spicer and Alpizar, 1994). In cultured luteal cells, increased levels of interferon gamma has been shown to enhance prostaglandin production, inhibit luteal cell steroidogenesis (Fairchild and Pate, 1991) and to induce class II major histocompatibility antigens (Fairchild and Pate, 1989).
Oophoritis

Oophoritis or inflammation of the ovary, has rarely been described in cattle (Miller and Campbell, 1978). In a group of dairy cattle, 65 cases of oophoritis were diagnosed but no etiology was suggested (Zemjanis et al., 1966). Summers et al. described mild interstitial oophoritis consisting of perivascular lymphocytic infiltrates in a study of infertile beef cows. No etiological agent was identified, but an infectious or immunologic process was suspected (Summers and Campbell, 1974). Miller found an incidence of 85% interstitial oophoritis in 168 non-pregnant cattle and 72% in 25 pregnant cattle which was less severe (Miller and Campbell, 1978). Again, an etiological cause of the oophoritis was not identified. Oophoritis following experimental acute infection with cytopathic BVDV has been described (Ssentongo et al., 1980). In this study, interstitial lymphocytic infiltrates of varying severity were first observed starting at day 8 post infection and remained evident up to day 60 post infection. A severe necrotizing oophoritis primarily involving the corpus luteum, has been described following infection with infectious bovine rhinotracheitis virus (Miller and Van Der Maaten, 1984; Van Der Maaten and Miller, 1984). *Mycoplasma agalactiae* var. *bovis* has been shown to cause oophoritis following insemination with infected semen (Hirth et al., 1966). Other causes of oophoritis usually result from hematogenous spread or ascending infection from the uterus of pyogenic organisms (Jubb et al., 1985).
REFERENCES


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ABSTRACT

Bovine viral diarrhea virus (BVDV) is a significant cause of reproductive failure in cattle. Previous observations indicate that ovarian activity in cattle persistently infected with BVDV is reduced. In this study, ovaries from cows persistently infected with BVDV and noninfected cattle were compared using morphological and immunohistochemical analysis. There was a significant reduction in the number of tertiary follicles, graafian follicles, atretic follicles and corpora hemorrhagica/lutea/albicans in ovaries from cows persistently infected with BVDV (P < 0.01). No difference in the number of primordial and secondary follicles was observed. Immunostaining of BVDV antigen was detected in luteal cells, macrophage-like cells and endothelial cells in the corpus luteum and macrophage-like cells and stromal cells in the ovarian cortex. Cattle
persistently infected with BVDV have a significant reduction in the number of antral follicles and corpora lutea/hemorrhagica/albicans when compared to cattle not persistently infected with BVDV. These changes suggest a reduction in normal ovarian activity which may reduce reproductive efficiency. Furthermore, BVDV antigen can be detected in specific ovarian cell types in cattle persistently infected with BVDV. Virus replication in these cells may have direct or indirect effects on ovarian function.

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is recognized as a cause of substantial worldwide economic losses in the cattle industry. Its complicated pathogenesis and involvement in various disease syndromes are continuing to emerge.

The effect of BVDV on bovine reproduction may be the most important consequence of infection. Many effects on reproduction have been documented, including infertility, early embryo death, abortions, and congenital defects (Baker, 1987). Additionally, infection of the fetus at a critical time in the development of the immune system can lead to the birth of calves that are persistently infected with BVDV (McClurkin et al., 1984) These cattle are important in the epidemiology of the disease; they act as constant sources of virus for susceptible cattle.

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Cattle acutely infected with BVDV have been documented to have poor conception rates (McGowan et al., 1992; Houe et al., 1993). To the authors' knowledge, the cause of poor fertility associated with BVDV infections has not been identified. In addition, studies have not been done to characterize fertility of adult cattle persistently infected with BVDV.

In a previous report by Brock (Brock et al., 1997), cattle persistently infected with BVDV were used as embryo donors to determine the likelihood of transmitting the virus during embryo transfer. It was observed during that study that cattle persistently infected with BVDV responded poorly to typical superovulation regimens. The ovaries did not respond with multiple follicle formation, as usually develops after superovulation of healthy cows, and subsequent collection of embryos was generally poor. These findings were especially evident after multiple attempts at superovulation, and suggested that animals persistently infected with BVDV may have reduced ovarian function, possibly attributable to persistent viral infection. The objective of the study reported here was to use morphological and histological parameters to compare the ovaries of cattle persistently infected with BVDV with those of cattle that were BVDV negative.
MATERIALS AND METHODS

Specimens - The ovaries from 6 adult cows persistently infected with BVDV were collected at necropsy. Each cow was previously determined to be persistently infected with BVDV by isolation of the virus from serially obtained serum samples taken at least 3 weeks apart. The ovaries from 6 postpubertal cows with grossly normal looking, nonpregnant reproductive tracts were randomly selected from a pool of ovaries collected from a local abattoir. These cows were considered to be free of persistent infection with BVDV after attempted virus isolation from at least 2 specimens of ovarian tissue and immunohistochemical evaluation of formalin-fixed ovarian sections. Ovaries were placed in 10% zinc-formalin and prepared for light microscopy within 48 hours. A cross section of each ovary representing the medial pole, middle portion, and lateral pole were taken and processed by dehydration in graded ethanol, then were embedded in paraffin. Four micron sections of each tissue were cut and stained with Mayer's H & E.

Morphologic analysis - Sections from each ovary representing the medial pole, middle portion, and lateral pole were analyzed. The number of histological structures on each section corresponding to the following categories was counted: primordial follicles, secondary follicles, tertiary follicles, graafian follicles, atretic follicles, and corpus hemorrhagicum/luteum/albicans. Structures were categorized using accepted ovarian morphological standards (Priedkalns, 1976). The number of primordial follicles was determined as the mean number of primordial follicles counted in at least 4 linear fields (1
linear field = 5 mm). All other structures were counted as the absolute number observed in each section. Other histological abnormalities also were noted. Each section was observed without knowledge of the cows' BVDV infection status.

Statistical analysis, comparing the number of observed histological ovarian structures between BVDV persistently infected and BVDV negative cows, was performed, using the 2-sample t-test (Devore and Peck, 1986).

**Immunohistochemistry** - Sections of ovaries from BVDV persistently infected cows, identified as PI-1, PI-2, and PI-3, and all 6 control cows were stained for BVDV antigen, using a procedure similar to that described (Haines et al., 1992). Briefly, sections were deparaffinized in Hemo-De\(^*\) (Fisher Scientific, Pittsburgh, PA) and then rehydrated in graded concentrations of ethanol to distilled water. Endogenous tissue peroxidase was blocked by immersion of sections in freshly prepared 3% hydrogen peroxide in absolute methanol for 10 minutes. A 0.1% solution of protease XIV (Sigma Chemical Co., St. Louis, MO) was used to digest the tissue sections for 10 minutes at 37 C. Nonspecific binding of proteins to tissue was blocked by incubating sections with 5% normal horse serum for 30 minutes at 37 C. Sections were then immersed for 30 minutes at 37 C with the BVDV monoclonal antibody 15c5 (Provided by Dr. E.J. Dubovi, Cornell University, Ithaca, NY) diluted 1:1,000 with 0.05 M Tris buffer containing 5% normal horse serum. After the incubation period, tissues were rinsed 10 times with washing buffer. Samples were then incubated for 20 minutes at 37 C with biotin labeled horse anti mouse IgG (Vector Laboratories, South San Francisco, CA) diluted with Tris buffer containing 5%
normal horse serum. Tissues were rinsed again with wash buffer 10 times. An avidin-biotin-complex (Vector Laboratories, South San Francisco, CA) was diluted in Tris buffer as recommended by the manufacturer and applied to the tissues for 20 minutes at 37 C. Following incubation, tissues were rinsed 10 times with wash buffer and exposed to the chromagen 3-amino-9-ethylcarbazole (Zymed Laboratories Inc., Burlingam, CA) for 5 minutes at 22 C. Slides were stained with Mayer's hematoxylin and cover slipped with an aqueous mounting medium.

RESULTS

The ovaries of the BVDV persistently infected cows were grossly smaller and had fewer follicles and corpora lutea compared with the ovaries from cows not infected with BVDV (Figure 2.1). Histologically, the ovaries of 5 of the 6 BVDV persistently infected cows were determined to have follicular hypoplasia. Other appreciable changes were not observed. The ovaries of 1 BVDV persistently infected cow and those of all control cows were considered histologically normal.

There were no significant differences in the number of primordial and secondary follicles counted within sections of ovaries from cows persistently infected with BVDV and those not infected with BVDV. However, the number of tertiary follicles, graafian follicles, atretic follicles, and corpus hemorrhagicum/luteum/albicans were significantly (P<0.01) lower in the ovaries from cows persistently infected with BVDV (Table 2.1).
Using immunohistochemistry, BVDV antigen was identified in the ovaries of the 3 persistently infected cows examined, but not in the control cows (Figure 2.2). Immunostaining of BVDV antigen was most intense in luteal tissues. In cow PI-2, cells within the corpus luteum that were stained included macrophage-like cells, luteal cells, and endothelial and smooth muscle cells associated with small arterioles. Macrophage-like cell aggregates located in corpora albicans of PI-1 and PI-3 also were heavily immunostained. Staining of scattered macrophage-like cells also was evident in the outer cortex of ovaries from all BVDV persistently infected cows.

**DISCUSSION**

In normal cycling post pubertal cows, the ovary undergoes constant changes (McDonald, 1980). These changes consist of continuous maturation and atresia of primary, secondary, tertiary, and graafian follicles (follicular waves), with eventual emergence of an ovulatory follicle. The corpus hemorrhagicum, corpus luteum, and corpus albicans are sequential stages of a transitory ovarian endocrine gland that follow ovulation during the estrous cycle. In healthy adult cows with normal functioning ovaries, follicles in different stages of maturation and degeneration can be observed histologically. Primordial follicles are the reservoir of arrested oogonia, awaiting an appropriate signal to progress toward graafian follicles. Graafian follicles are large fluid-filled structures encasing a maturing oocyte. Given the proper signal, graafian follicles undergo final
maturation, resulting in ovulation. Follicles can undergo atresia at any stage of
development. Characteristic morphologic cellular changes can be used to differentiate
atretic follicles from progressively maturing follicles (Priedkalns, 1976).

Bovine viral diarrhea virus has been isolated from bovine ovarian tissue and
follicular aspirates (Ssentongo et al., 1980; Bielanski et al., 1993). Associated oophoritis
has been documented after acute infection with cytopathic BVDV (Ssentongo et al.,
1980) However, the importance of isolating BVDV from ovarian tissue has not been
determined. Furthermore, no studies have reported on the morphologic or functional
changes in the bovine ovary when it is infected with BVDV.

It is suggested that the morphologic appearance of ovaries from cattle persistently
infected with BVDV is significantly different from that of cattle not infected with BVDV.
The significantly fewer number of tertiary follicles, graafian follicles, atretic follicles, and
corpus hemorrhagicum/luteum/albicans suggests that the normal structural changes that
occur in the bovine ovary may be altered. Using real-time ultrasonography, one of the
authors (DLG) analyzed the dynamic changes of the ovary in cattle persistently infected
with BVDV; results suggested a reduction in the number of follicular waves and follicles
per wave. Although cattle persistently infected with BVDV manifest estrus activity and
can become pregnant, this reduction in normal ovarian activity may reduce reproductive
performance.

The immunohistochemical results indicate that several ovarian cell types may
become infected with BVDV. It is not surprising that one of the most prevalent cell types
infected with BVDV in the bovine ovary is macrophage-like cells. Lymphocytes and
macrophages are one of the major target cells for BVDV (Bolin et al., 1990). In the ovary, macrophages are thought to have a role, perhaps via production of cytokines, in the dynamic changes that occur, especially in the corpus luteum (Fairchild and Pate, 1991; Tabibzadeh, 1994). It is possible that infection of these macrophages may disrupt their normal role in ovarian function. Infection of luteal cells may also lead to changes in ovarian activity by disrupting any one of their many metabolic activities, such as progesterone production. To the authors' knowledge, studies of ovarian function by hormone analysis in BVDV persistently infected cattle have not been done.

Prevalence of BVDV persistently infected cattle is low (Houe et al., 1990). From an individual standpoint, their contribution to herd reproductive performance is insignificant. However, in herds where BVDV is actively circulating and susceptible cattle are being exposed constantly to infective doses of the virus, effects of BVDV on reproductive performance may be substantial. Ovarian changes may also occur in cattle acutely infected with BVDV and may help to explain poor conception rates that have been described in herds undergoing infection with BVDV. Further studies are needed to determine whether ovarian morphology and function are changed in cattle acutely infected with BVDV.

Salvage of valuable genetics from cows persistently infected with BVDV is of interest to some cattle producers. This study provides evidence that the ovaries of these BVDV persistently infected cows may not be functioning properly, thus potentially reducing the chance for successful superovulation and embryo recovery.
Results of this study further document the types of cells in ovarian tissue that are infected with BVDV and provide evidence of marked morphologic differences that may indicate altered ovarian function in cattle persistently infected with BVDV. More work is necessary to further characterize the ovarian changes that occur in BVDV persistently infected cattle and to determine whether similar events occur in cattle acutely infected with BVDV.
Figure 2.1: Gross representative sections of ovaries from cows not persistently infected with bovine viral diarrhea virus (A) and cows persistently infected with bovine viral diarrhea virus (B).
Figure 2.2: Photomicrograph of BVDV immunostaining in ovaries from a cow persistently infected with BVDV (arrows). A) Arteriole with in corpus luteum, B) Luteal cells. Bar = A) 12.5 μm, B) 6.0 μm.
<table>
<thead>
<tr>
<th>Structure</th>
<th>BVDV Negative</th>
<th>BVDV Persistent</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Primordial follicles</td>
<td>12.7 +/- 1.1</td>
<td>9.7 +/- 1.0</td>
<td>0.049</td>
</tr>
<tr>
<td>Secondary follicles</td>
<td>9.8 +/- 1.0</td>
<td>13.1 +/- 1.2</td>
<td>0.037</td>
</tr>
<tr>
<td>Tertiary follicles</td>
<td>5.1 +/- 0.5</td>
<td>0.9 +/- 0.2</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Graafian follicles</td>
<td>0.8 +/- 0.1</td>
<td>0.02 +/- 0.02</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Atretic follicles</td>
<td>9.3 +/- 0.8</td>
<td>3.1 +/- 0.5</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Corpus luteum, hemorrhagicum, albicans</td>
<td>1.1 +/- 0.1</td>
<td>0.5 +/- 0.1</td>
<td>0.0001*</td>
</tr>
</tbody>
</table>

*Significant at P ≤ 0.01.

Table 2.1: Mean number of ovarian structures observed in ovaries removed from cows persistently infected with bovine viral diarrhea virus (BVDV) and in cows that were test-negative for BVDV. Data expressed as mean +/- standard error of mean.
LIST OF REFERENCES


CHAPTER 3

DETECTION OF BOVINE VIRAL DIARRHEA VIRUS IN THE OVARIES OF CATTLE ACUTELY INFECTED WITH BOVINE VIRAL DIARRHEA VIRUS

ABSTRACT

Bovine viral diarrhea virus (BVDV) is recognized world wide as a major cause of economic loss in cattle. Infection with BVDV can result in several different clinical outcomes. However, the reproductive consequences may be the most important. Infertility, early embryonic death, abortion, and congenital anomalies have all been reported following acute infection with BVDV. The cause of infertility following acute BVDV infection is not known. Bovine viral diarrhea virus has been isolated from the bovine ovary and associated with chronic oophoritis. The purpose of this study was to identify the ovarian cell types infected with BVDV following acute infection. Twelve heifers were acutely infected with noncytopathic BVDV and ovariectomies were performed between 6 and 60 days post infection. Bovine viral diarrhea virus was isolated at days 6 and 8 post infection. Viral antigen was detected in macrophage-like cells and
stromal cells in the ovarian cortex from 6 to 60 days post infection. Oophoritis was evident from day 6 to 60 days post infection. Based on these findings, acute infection with BVDV may result in changes in ovarian function that could lead to reduced fertility.

INTRODUCTION

Infection of cattle with bovine viral diarrhea virus (BVDV) can result in many clinical manifestations (Baker, 1995). Reproductive effects may be the most important consequence following acute infection of susceptible cattle. Reduced fertility, early embryonic death, abortions and various congenital anomalies have been reported to occur following acute BVDV infection (Moennig and Liess, 1995). Reduced fertility in cattle undergoing infection with BVDV has been reported and is often a significant complaint in herds where BVDV has been identified (Houe et al., 1993; McGowan et al., 1993). Although the mechanism of this reduced fertility has not been determined, several suggested explanations include failure of fertilization (Grahn et al., 1984), early embryonic death (Archbald et al., 1979), and ovarian dysfunction (Kafi et al., 1994). Herd fertility problems have also been associated with poor quality semen originating from bulls persistently infected with BVDV (Kirkland et al., 1994).

Bovine viral diarrhea virus has been isolated from the ovaries of cattle experimentally infected with BVDV and ovaries collected from abattoirs (Ssentongo et al., 1980; Bielanski et al., 1993). Chronic oophoritis has also been described following
acute infection with BVDV (Ssentongo et al., 1980). These findings could lead to changes in ovarian function resulting in a reduction in fertility. Previous studies have suggested that ovarian function may be changed in cattle acutely or persistently infected with BVDV (Kafi et al., 1994; Grooms et al., 1996). The purpose of this study was to identify cell types infected with BVDV following acute infection using immunohistochemistry. With an understanding of the cell types involved in BVDV infection of the ovary, hypotheses can be made as to potential causes of infertility manifested at the level of the ovary.

MATERIAL AND METHODS

Twelve post pubertal BVDV seronegative and virus negative Angus heifers were infected intranasally with $10^5$ CCID$_{50}$ of noncytopathic BVDV strain 1088. Unilateral ovariectomies were performed on two heifers by laparotomy at days 4, 6, 10, 12, 14, and 16 post infection. A second unilateral ovariectomy was performed on the same heifers at days 18, 25, 45, and 60 post infection. Whole blood and serum samples were collected daily for two weeks and then weekly to for virus isolation and serology. From each ovary, 1 ml of follicular fluid and a 1 gram section of corpus luteum were placed into 1 ml and 4 ml of Dulbecco's modified eagle medium (DMEM) (GIBCO Laboratories, Grand Island, NY), respectively, containing penicillin (100 units/ml), streptomycin (100
μg/ml) and fungizone (2.5 μg/ml). The remaining portion of ovary was fixed in zinc formalin.

Luteal tissue and follicular fluid was processed for virus isolation by first homogenizing and then passing the resulting supernate through a 0.45 μm syringe tip filter. Duplicate 200 μl samples were inoculated onto low passage bovine turbinate cells in 24 well cell culture plates. Supernate was passed three times. White blood cells were isolated from whole blood samples following hypotonic lysis of red blood cells with a 0.83% solution of ammonium chloride in distilled water. The cells were washed once with 10 ml of DMEM and then resuspended in 1 ml of DMEM. Following freezing at -70 C to lyse the white blood cells, 50 μl was inoculated onto bovine turbinate cells in 96 well plates and passed once. Cells were observed daily for cytopathology typical of cytopathic BVDV. A pooled monoclonal antibody-based immunoperoxidase test was performed on each passage to detect noncytopathic BVDV (Afshar et al., 1989).

Zinc formalin fixed ovaries were processed 48 hours following collection. Three separate sections from each ovary were embedded in paraffin. Sections were processed for routine histological staining with haematoxylin and eosin. Immunohistochemistry was done using a modification of methods previously described (Haines et al., 1992). In brief, 5 micron sections were mounted on silane treated slides and baked for 1 hour at 56 C. Sections were deparafinized in Hemo-De (Fischer Scientific, Pittsburgh, PA) for 15 minutes and then rehydrated through graded ethanol to distilled water. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide in absolute methanol for 5 minutes. Tissues were digested for 15 minutes at 37 C in 0.1% Protease
XIV (Sigma Chemical Co., St. Louis, MO). Following digestion, slides were washed in distilled water and then incubated with 5% horse serum in 0.05 M Tris buffer for 30 minutes at 37 C to block nonspecific antibody binding. Detection of BVDV antigen was performed using the BVDV monoclonal antibody 15C5 (Provided by Dr. E.J. Dubovi, Cornell University, Ithaca, NY) in conjunction with an indirect avidin-biotin-peroxidase (ABC) detection system (Vector Labs Inc, South San Francisco, CA). The BVDV monoclonal antibody 15C5 was diluted 1:1500 in blocker and applied to the tissues for 30 minutes at 37 C. After washing to remove unbound primary antibody, tissues were incubated with horse anti-mouse IgG for 20 minutes at 37 C. Following washing to remove unbound secondary antibody, the ABC reagent was prepared as recommended by the manufacturer and incubated with the tissues for 20 minutes at 37 C. Removal of unbound ABC was followed by the substrate 3-amino-9-ethyl carbazole (AEC) (Zymed Laboratories Inc., Burlingam, CA) for 10 minutes at room temperature. Sections were counter stained with Mayer's haematoxylin and cover slipped using an aqueous mounting media. A heterologous monoclonal antibody directed against infectious bursal disease virus (Provided by Dr. Y.M. Saif, Ohio Agricultural Research and Development Center, Wooster OH) was substituted for the primary antibody to detect nonspecific binding. Sections of ovary from cows persistently infected with BVDV and cows not infected with BVDV as determined by virus isolation and serology, were used as positive and negative controls.
RESULTS

Bovine viral diarrhea virus was isolated from the buffy coats from 10/12 heifers between days 6 and 10 post infection. One animal died due to post surgical complications. All heifers had a fourfold or greater increase in serum neutralizing antibodies at day 45 post infection (geometric mean titer 1:75, range 1:40 to 1:320). Bovine viral diarrhea virus was isolated from the corpus luteum of two animals at day 6 and 8 post infection. In both cases, BVDV was also isolated from white blood cells at the same time. No virus was isolated from the follicular fluid collected from any of the animals. Histologically, scattered accumulations of interstitial lymphocytes were evident at day 8 post infection (Figure 3.1). Lymphocytic infiltrates increased and became more widespread between days 12 and 18 post infection. Scattered lymphocytic infiltrates remained evident at day 60 post infection. Occasional eosinophils were observed in association with the lymphocytic infiltrates from day 14 to 25 post infection. Vacuolation of the ovarian stroma was observed starting at day 8 post infection. Vacuolation was most prominent between days 14 to 25 post infection, but remained evident to day 60. Bovine viral diarrhea virus antigen was first detected at day 6 post infection and remained evident in ovaries removed at day 60 (Figure 3.2). Positive staining was located exclusively in the cellular cytoplasm and was characteristically granular to mottled in appearance. The greatest amount of BVDV antigen immunostaining occurred in ovaries removed between days 8 and 25 post infection. Bovine viral diarrhea virus specific antigen containing cells were found predominantly in the stroma of the ovarian cortex.
These include interstitial stromal cells and macrophage-like cells. Positive cells were found in association with primary follicles, secondary follicles, antral follicles, corpus luteum, and corpus albicans. Staining of luteal, theca, or granulosa cells was not observed.
Figure 3.1: Representative histological changes observed in ovaries following acute infection with BVDV. A) Perivascular lymphocytic infiltrate day 14 post infection (arrow). B) Vacuolation with in the cortical region of the ovary at day 16 post infection. Bar = A) 25\mu m, B) 40\mu m.
Figure 3.2: Immunohistochemical detection of BVDV antigen in ovaries following acute infection with BVDV. Positive cells are identified by arrows. A) 18 days post infection. B) 60 days post infection. Bar = A) 25 μm, B) 10 μm.
DISCUSSION

The isolation of BVDV from the corpus luteum during the period of viremia following acute infection is consistent with the vascular nature of this tissue. In this study, BVDV was not isolated from any follicular fluid samples. Previous investigators have reported isolation of virus from follicular fluid taken from abattoir derived ovaries (Bielanski, et al., 1993). Since the BVDV status of the animals from which the ovaries were collected was not determined by Bielanski et al., these findings may reflect the presence of persistent infection with BVDV. Detection of BVDV in granulosa cells from cows persistently infected with BVDV has been reported (Booth et al., 1992). There are several possible explanations for the inability to isolate BVDV from the ovary after the period of viremia even though antigen was detected. First, immunohistochemistry localized BVDV infected cells almost exclusively to the stroma of the ovarian cortex, an area different from that was sampled. The tissue to be sampled in this study was determined based on previous reports of BVDV detection in follicular fluid and luteal tissue. No previous studies had localized BVDV to the stromal tissue of the ovary. Second, neutralization of virus activity may have occurred through exposure to BVDV specific antibodies in the processed tissue homogenates. Bovine viral diarrhea virus specific immunoglobulins in the reproductive tract of cattle, including follicular fluid, have been detected and quantified (Whitmor and Archbald, 1977). Finally, the detected BVDV antigen may represent antigen-antibody complexes persisting in resident ovarian macrophages.
Chronic lymphocytic oophoritis following experimentally induced acute BVDV infection was evident in the samples collected from day 8 to 60 post infection. These findings are similar to those reported by Ssentongo et al. following acute infection with cytopathic BVDV (Ssentongo et al., 1980). This inflammation correlates with the extended period of time which BVDV antigen was detected in cells within the ovarian stroma. Antigen was detected in cortical interstitial cells and macrophage-like cells within the ovarian cortex. This antigen may serve as a source of chronic immune stimulation resulting in prolonged oophoritis. Interestingly, no BVDV antigen was detected in macrophages associated with regressing corpora lutea or atretic follicles. It is not clear if the detected antigen is associated with viable virus.

The detection of BVDV antigen and an associated oophoritis may explain reduced fertility following acute infection with BVDV. Knowledge of the intricate relationship between ovarian physiology and the immune system is just beginning to evolve (Gill and Repetti, 1979; Adashi, 1990; Tabibzadeh, 1994). Macrophages and several cytokines have been shown to play important roles in ovarian function (Gottschall et al., 1987; Fukuoka et al., 1988; Adashi et al., 1989; Darbon et al., 1989; Adashi, 1990; Tabibzadeh, 1994). Tumor necrosis factor alpha (TNF-α) and interleukin-1 are two macrophage products that have been identified as having important effects on follicular growth and differentiation (Gottschall et al., 1987; Fukuoka et al., 1988; Adashi et al., 1989; Darbon et al., 1989). In vitro studies have identified changes in the function of macrophages following acute BVDV infection (Ketelsen et al., 1979; Atluru et al., 1990; Jensen and Schultz, 1991; Atluru et al., 1992; Adler et al., 1996). Changes in these cytokine
concentrations as a result of inflammation or disruption of normal tissue macrophage function may lead to an interference with normal ovarian dynamics. Function of the ovary following acute infection with BVDV is the subject of ongoing studies.

The possibility that virus is sequestered within ovarian cells for extended periods of time has important implications in the control and prevention of BVDV. Evidence supporting BVDV latency is limited. In early studies, BVDV has been isolated from lymphoid tissue for up to 56 days post infection (Mills and Luginbuhl, 1968) and blood and nasal swabs up to 102 days post infection (Lambert and Femelius, 1968). However, no recent studies have supported these findings. Based on kinetics of the antibody response to acute BVDV infection, it has been suggested that viral latency is a possibility (Brownlie, 1990). Following acute BVDV infection, neutralizing antibody titers rise slowly over a 10-12 week period. It has been argued that this slow rise may be the result of either infectious virus sequestered in immune cells or viral antigen being continuously presented to the immune system (Brownlie, 1990). Finally, other closely related flaviviruses including hepatitis C and hog cholera virus, have been shown to develop chronic infections following postnatal infection (Dahle and Liess, 1992; Nishioka, 1994).

In addition to the chronic oophoritis observed following acute infection with BVDV, the potential consequences of chronically sequestered viruses in the ovary are considerable. If the detected virus is viable, it is possible that selective pressures could allow the virus to mutate and reemerge. If this possibility exists, further considerations must be made when designing control and prevention strategies. Additionally, local immune suppression of the reproductive tract during gestation could result in virus
entering the uterine environment causing fetal infection. Future studies will be needed to determine the potential long term viability of BVDV in the ovary following acute infection.
REFERENCES


CHAPTER 4

CHANGES IN OVARIAN FOLLICLES FOLLOWING ACUTE INFECTION WITH BOVINE VIRAL DIARRHEA VIRUS

ABSTRACT

Bovine viral diarrhea virus (BVDV) has been associated with several different reproductive problems in cattle including poor fertility, early embryonic deaths, abortion, and congenital anomalies. Little is known about the cause of poor fertility in cows acutely infected with BVDV. Isolation of BVDV and chronic oophoritis has been reported following acute infection. No connection between these findings and poor fertility has been made. The purpose of this study was to identify changes in ovarian function following acute infection with noncytopathic BVDV. The ovaries of 5 BVDV seronegative and virus negative pubertal heifers were monitored daily for 4 consecutive estrous cycles. The position and diameter of all follicles (>5mm) and luteal structures were recorded. Daily plasma samples were collected to measure peripheral progesterone and estradiol levels. Each heifer was infected intranasally with noncytopathic BVDV.
following ovulation of the second estrous cycle. The maximum diameter and growth rate of dominant anovulatory and ovulatory follicles were significantly reduced following acute BVDV infection. Similarly, the number of subordinate follicles associated with both the anovulatory and ovulatory follicle was reduced following infection. There were no significant differences in other follicle or luteal dynamic parameters or in peripheral progesterone or estradiol levels. The ovarian follicular growth are different during the first two estrous cycles following acute infection with BVDV. These differences may explain reduced fertility often observed in herds undergoing acute infection with BVDV.

INTRODUCTION

Reproductive efficiency is a critical component in the profitability of both dairy and cow-calf operations. Many factors have been identified that can adversely affect reproduction. Bovine viral diarrhea virus (BVDV) is recognized as one of the most important infectious diseases of cattle world wide (Baker, 1995). The effect that BVDV has on reproduction may be the most important consequence of infection. Early embryonic death, abortion, congenital defects and the birth of calves persistently infected with BVDV have all been reported in cattle following acute infection with BVDV (McGowan and Kirkland, 1995; Moennig and Liess, 1995). A reduction in conception rates in cattle undergoing acute infection with BVDV has been reported and is very often a major complaint in herds where the BVDV is identified (Houe et al., 1993; McGowan et
The cause of reduced fertility in these herds is unknown.

BVDV has been isolated from the ovaries of both experimental and slaughtered cattle (Ssentongo et al., 1980; Bielanski et al., 1993). Following acute infection with noncytopathic BVDV, virus has been isolated for up to 25 days (Ssentongo et al., 1980). Bovine viral diarrhea virus antigen has been detected in the ovaries following acute infection for up to 60 days (Grooms, 1997). Oophoritis of varying severity and lasting for up to 60 days post infection, has been described following acute infection (Ssentongo et al., 1980; Grooms, 1997). In cattle persistently infected with BVDV, a morphological reduction in the number of antral follicles is evident (Grooms et al., 1996). This evidence makes it conceivable that acute infection with BVDV may disrupt normal ovarian function resulting in reduced fertility. The objective of this study was to monitor ovarian function following acute infection with BVDV using ultrasonography and peripheral progesterone and estradiol.

**MATERIALS AND METHODS**

Five pubertal Angus heifers were determined to be BVDV negative by virus isolation from white blood cells and seronegative by virus neutralization. Rectal exams were performed to verify that they had complete reproductive tracts with no evidence of pregnancy or gross pathology and that they were cyclic by evidence normal size ovaries and the presence of luteal and follicular structures. Estrous cycles were synchronized.
using two doses of dinoprost (Lutalyse®, Upjohn, Kalamazoo, MI) fourteen days apart. Following synchronization, the ovaries of each animal were examined daily using transrectal ultrasonography for 4 consecutive estrous cycles. The position and maximum diameter of each luteal structure and follicle greater than 5 mm on both ovaries was recorded. Daily whole blood samples were collected in EDTA-coated tubes. Within one hour of collection, plasma was removed and frozen at -20°C until assayed. Within 48 hours following ovulation of the second estrous cycle, the heifers were infected intranasally with $10^5$ CCID$_{50}$ of noncytopathic BVDV strain 1088. White blood cells from whole blood samples and serum were collected daily for the 14 days following infection to monitor for viremia by virus isolation in cell culture. White blood cells were isolated from whole blood samples following hypotonic lysis of red blood cells with a 0.83% solution of ammonium chloride in distilled water. The cells were washed once with 10 ml of Dulbecco's modified eagle medium (DMEM) (GIBCO Laboratories, Grand Island, NY), resuspended in 1 ml of DMEM and then lysed by freezing at -70°C. Fifty microliters of serum and white-blood cell suspension was inoculated onto bovine turbinate cells in 96 well plates and passed once. Cells were observed daily for cytopathology typical of cytopathic BVDV. A pooled monoclonal antibody-based immunoperoxidase test was performed on each passage to detect noncytopathic BVDV (Afshar et al., 1989). Serum samples were collected weekly to determine the time of seroconversion using a standard virus neutralization assay (Edwards, 1990). The study was conducted in the winter to eliminate the effects that heat stress may have on ovarian
Peripheral progesterone concentrations were measured using a competitive ELISA as previously described (Rasmussen et al., 1996) and validated (Petroff et al., in review) with the following modifications. Ninety-six-well micro titer plates were coated with affinity purified donkey anti-sheep IgG (Sigma Chemical Co., St. Louis MO) at least 48 hours before use and stored at 4 C. The primary antiserum, sheep anti-progesterone-11-BSA #337 (provided by Dr. Gordon Niswender, Colorado State University) was diluted 1:600,000 in assay buffer and incubated in the coated plates for 1 hr at 37 C. After washing, 50 μls of standard and unknown samples were added. Plates were incubated for 1 hr at 27 C and then 50 μl of progesterone-horseradish peroxidase conjugate was added. Following incubation for 2 hrs at 27 C, horseradish peroxidase activity was quantified by adding 3,3',5,5'-tetramethylbenzidine and recording the light absorbency at 450-600 nm using an automated ELISA reader. The intra- and interassay coefficients of variation were 6.9% and 13.2% respectively. The sensitivity of the assay was determined to be 0.9 ng/ml. Peripheral estradiol concentrations were measured using a competitive RIA as previously described (Kojima et al., 1992) and validated (Anderson et al., 1996). The intra- and interassay coefficients of variation were 10.4% and 14.4% respectively. The sensitivity of the assay was determined to be 0.5 pg/ml.

The following criteria were used to define dynamic ovarian structures: Follicles were measured and mapped if equal to or greater than 5 mm in diameter. A follicular wave was defined as a cohort of developing antral follicles from which a dominant follicle
was derived. Dominant anovulatory follicles were those follicles which became dominant during the course of the estrous cycle but regressed without ovulating. Dominant ovulatory follicles were those follicles which became dominant and then subsequently ovulated as evidenced by their rapid disappearance over a 24-hour period and the corresponding formation of a corpus hemorrhagicum. The growth rates of dominant follicles were measured as the difference between the maximum diameter and the diameter when first observed, divided by the total days taken to reach the maximum diameter. All follicles which were identified and measured within three days of the identification of the dominant follicle were classified as subordinate follicles of that specific follicular wave. Values from each animal were averaged together. Statistical analyses, comparing follicular and luteal measurements and progesterone and estradiol concentrations before and after acute infection with BVDV, were performed using the Student's t-test (Devore and Peck, 1986). Daily concentrations of estradiol and progesterone were compared using repeated measures analyses of variance (Devore and Peck, 1986).

RESULTS

Bovine viral diarrhea virus was isolated from the buffy coats of all five heifers between days six and ten post challenge. By 45 days post challenge, all heifers had a 4-fold or greater increase in their serum neutralizing titer to BVDV (geometric mean titer of 1:70 with a range of 1:40 to 1:320). Data obtained for follicular and luteal structures are
presented in Table 4.1. No differences before or after infection with BVDV were seen in the interovulatory intervals, the day of first detection of the dominant anovulatory follicle or dominant ovulatory follicle, or the number of days from first detection of the dominant ovulatory follicle to ovulation (P>0.05). Similarly, no significant differences in the maximum diameter or growth rate of the corpus luteum were measured (P>0.05). Two follicular waves were observed during the course of the estrous cycle in all heifers before and after infection with BVDV. The maximum diameter and growth rate of both the dominant anovulatory follicle and dominant ovulatory follicle were significantly reduced following infection with BVDV (P<0.05). The number of subordinate follicles associated with both the dominant anovulatory follicle or dominant ovulatory follicle were significantly reduced following infection (P<0.05). The maximum diameter of the largest subordinate follicles arising with both the dominant anovulatory follicle or dominant ovulatory follicle was significantly reduced after infection (P<0.05).

Following infection with BVDV, there were no differences in the peripheral estradiol or progesterone patterns associated with the normal bovine estrous cycle. There was no significant differences in daily peripheral estradiol (Figure 4.1) or progesterone (Figure 4.2) concentrations when estrous cycles were matched with respects to day of ovulation (P>0.05). No significant differences in peak progesterone or peak estradiol levels were measured (Figure 4.3). The average peripheral progesterone concentration during the luteal phase of the estrous cycle, as defined by that period when the progesterone concentration was two standard deviations above a baseline of 0.5 ng/ml,
was not significantly different (P>0.05). Likewise, the average estradiol concentration for the 6 days prior to ovulation was not different after infection with BVDV (P>0.05).
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Before Infection</th>
<th>After Infection</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Interovulatory interval (days)</td>
<td>20.3 (0.7)</td>
<td>20.4 (0.7)</td>
<td>0.90</td>
</tr>
<tr>
<td>Dominant Anovulatory Follicle:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Day of Detection (day of cycle)</td>
<td>0.4 (0.5)</td>
<td>0.6 (0.3)</td>
<td>0.69</td>
</tr>
<tr>
<td>Maximum Diameter (mm)</td>
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<td>11.8 (0.5)</td>
<td>0.002</td>
</tr>
<tr>
<td>Growth Rate (mm/day)</td>
<td>1.5 (0.2)</td>
<td>1.0 (0.1)</td>
<td>0.01</td>
</tr>
<tr>
<td>Dominant Ovulatory Follicle:</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Day of Detection (day of cycle)</td>
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<td>11.4 (0.7)</td>
<td>0.55</td>
</tr>
<tr>
<td>Detection to Ovulation (days)</td>
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<td>9.3 (0.6)</td>
<td>0.60</td>
</tr>
<tr>
<td>Maximum Diameter (mm)</td>
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<td>12.6 (0.3)</td>
<td>0.04</td>
</tr>
<tr>
<td>Growth Rate (mm/day)</td>
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<td>0.9 (0.1)</td>
<td>0.02</td>
</tr>
<tr>
<td>Subordinate Follicles Emerging With:</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Dominant Anovulatory Follicle (#)</td>
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<td>2.5 (0.4)</td>
<td>0.02</td>
</tr>
<tr>
<td>Dominant Ovulatory Follicle (#)</td>
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<td>Largest Subordinate Follicle Associated With:</td>
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<td>Dominant Anovulatory Follicle (mm)</td>
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<td>6.3 (0.2)</td>
<td>0.02</td>
</tr>
<tr>
<td>Dominant Ovulatory Follicle (mm)</td>
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<td>7.3 (0.4)</td>
<td>0.04</td>
</tr>
<tr>
<td>Corpus Luteum</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Maximum Diameter</td>
<td>23.9 (0.7)</td>
<td>24.1 (0.9)</td>
<td>0.83</td>
</tr>
<tr>
<td>Growth Rate</td>
<td>1.8 (0.2)</td>
<td>1.5 (0.2)</td>
<td>0.30</td>
</tr>
</tbody>
</table>

Data in bold text is significant at P<0.05.

Table 4.1: Ovarian parameters before and after acute infection with noncytopathic BVDV. Data are presented as the average (+/- standard error of the mean) of five heifers during two consecutive estrous cycles before and after infection with BVDV.
Figure 4.1: Mean plasma estradiol concentration of five heifers for two estrous cycles before and two estrous cycles after infection with bovine viral diarrhea virus (BVDV). Heifers infected within 24 hours of ovulation following the second estrous cycle. Data was adjusted using moving average transformation. There were no significant differences in daily estradiol concentrations between estrous cycles pre and post infection with BVDV (P>0.05).
Figure 4.2: Mean plasma progesterone concentration of five heifers for two estrous cycles before and two estrous cycles after infection with bovine viral diarrhea virus (BVDV). Heifers infected within 24 hours of ovulation following the second estrous cycle. Data was adjusted using moving average transformation. There were no significant differences in daily progesterone concentrations between estrous cycles pre and post infection with BVDV (P>0.05).
Figure 4.3: Comparison of mean peripheral estradiol and progesterone levels for two estrous cycles before and two estrous cycles after acute infection with noncytopathic BVDV. A) peak estradiol level, B) peak progesterone level, C) mean estradiol level for six days prior to ovulation, D) mean progesterone level during the luteal phase of the estrous cycle. No significant differences were detected (P>0.05).
DISCUSSION

Ovarian dysfunction following acute infection with BVDV has been suggested previously (Ssentongo et al., 1980; Kafi et al., 1994). In cattle acutely infected with BVDV 9 days before being inseminated following superovulation, a significant reduction in the number of palpable corpora lutea and recovered embryos was reported (Kafi et al., 1994). This would suggest that formation of follicles did not occur as would be expected with routine superovulation regimens. In cattle persistently infected with BVDV, the number of antral follicles being formed is significantly reduced (Grooms et al., 1996).

Although persistent and acute infection with BVDV cannot be equated, these two studies suggest a link between infection with BVDV and a change in normal follicular dynamics.

In this study, the decrease growth of both the dominant anovulatory follicle and dominant ovulatory follicle following acute infection with BVDV is evidence that follicle formation is altered. Likewise, the decrease in the number of subordinate follicles associated with each follicular wave adds merit to this argument. Unlike the luteolytic nature of bovine herpes virus-1 (Van Der Maaten and Miller, 1984) no measurable effects on the corpus luteum were observed as measured by both peripheral progesterone levels and luteal tissue dynamics. Bovine viral diarrhea virus did not affect ovarian function at a level that changed patterns of either peripheral progesterone or estradiol hormone or estrous cycle lengths. However, with increasing knowledge of the complexity of ovarian physiology, these parameters may be relatively insensitive indicators of normal ovarian functions which are necessary for optimal fertility.
In conducting this study, comparisons were made between the same animals before and after infection with BVDV. Under ideal conditions, uninfected control heifers paralleling the challenged heifers should have been used to exclude confounding factors as causing the observed changes. However, given the fact that an infectious agent was used, duplication of the environmental conditions between two separate groups while retaining their BVDV exposure status would have been difficult. By using the same animals as their own controls, it was intended that environmental conditions could be controlled more closely. Additionally, by using the same animals as their own controls, normal physiological variation between animals could be reduced.

Follicle formation is a dynamic event beginning with primordial follicles which progress through various stages of growth toward becoming a mature ovulatory follicle (Fortune et al., 1988). The control of follicular growth and maturation involves a complex interaction between gonadotropins, systemic hormones and local ovarian mediators (Ross et al., 1979; Richards, 1980). The gonadotropins, FSH and LH, play a dominant role in follicle growth and maturation. However, they are insufficient by themselves to account for the dynamic changes that occur in the ovary. Several intraovarian factors have been identified and characterized which can influence formation of the follicle (Tonetta and diZerega, 1989).

BVDV may act at the level of the hypothalamic-pituitary axis to change the release of gonadotropin, which could then result in a change in follicular maturation. Direct infection of the hypothalamus or pituitary is unlikely as it has not been demonstrated that BVDV can cross the mature blood-brain barrier. However, BVDV could affect the
feedback mechanisms originating from the ovary that help to dictate both FSH and LH release. It has been suggested that an elevation in blood cortisol levels seen during the period of viremia following acute infection can result in suppression of LH release leading to a delay or failure in ovulation (Kafi et al., 1994). In this study, ovulation occurred at normal intervals after infection. However, none of the animals were viremic during the time when ovulation was expected. No studies have reported FSH or LH levels in cattle following acute BVDV infection.

Although not determined in this study, chronic oophoritis and the detection of antigen in macrophage-like cells following acute BVDV infection has been described (Ssentongo et al., 1980; Grooms, 1997). These findings have important implications on ovarian function. Tumor necrosis factor-alpha (TNF-α) and interleukin-1 (IL-1), both are macrophage products that are mediators of inflammation (Beutler and Cerami, 1986; Dinarello, 1989) and have been shown in vitro to play a role in granulosa cell development and differentiation (Adashi, 1990; Tabibzadeh, 1994). Tumor necrosis factor-α has an antigonadotropic effect on cultured granulosa cells leading to attenuation of their differentiation (Emoto and Baird, 1988; Darbon et al., 1989). It has been suggested as a potential intraovarian regulator of follicular atresia. Interleukin-1 has also been associated with the suppression of cultured granulosa cell differentiation and luteinization (Gottschall et al., 1987; Fukuota et al., 1988). The exact source and function of these cytokines in vivo are yet to be determined. Through its affinity for macrophage cells, BVDV may interfere with the normal production of these cytokines leading to a disruption of normal follicle formation. Cytokines have been suggested to be responsible
in part for the disruption of ovarian function during times of chronic inflammation or cachexia, maladies in which cytokines have important roles (Emoto and Baird, 1988).

Following acute BVDV infection, chronic oophoritis may be a local reflection of this concept with a resulting change in follicular dynamics. The relationship between follicular dynamics and fertility is not known. Changes in follicular dynamics have been reported in cattle during early lactation in dairy cattle (Lucy et al., 1992a; Lucy et al., 1992b), during the feeding of low energy diets (Murphy et al., 1991; Rhodes et al., 1995) and following heat stress (Badinga et al., 1993). Each of these situations has been associated with reduced fertility. The effect that BVDV may have on fertility through changes in ovarian function may not be fully appreciated until the factors associated with the growth and differentiation of follicles and how changes in follicular dynamics affect fertility are understood.

This study presents evidence that cattle undergoing acute infection with BVDV may exhibit alterations in ovarian function that may lead to reduced fertility. In cattle herds with poor reproductive performance, BVDV should be considered as a potential cause. With an increased understanding of the effect that BVDV has on ovarian function, management schemes can be implemented to improve reproductive efficiency in herds where BVDV is a problem.
REFERENCES


CHAPTER 5

DETECTION OF CYTOPATHIC BOVINE VIRAL DIARRHEA VIRUS (BVDV) IN THE OVARIES OF CATTLE FOLLOWING IMMUNIZATION WITH A MODIFIED LIVE BVDV VACCINE

ABSTRACT

Economic loss from infection with bovine viral diarrhea virus is of worldwide concern. The unique pathogenesis and antigenic variability of BVDV has made it challenging to control. Vaccination programs are a major component of control and prevention strategies. Both killed and modified live vaccines are commercially available. Preference between killed and modified live vaccines is controversial. Of major concern is the safety of modified live vaccines. Little information is available on their tissue tropism and potential for causing pathology, especially with respect to the reproductive system. The objective of this study was to determine if BVDV could be detected in the ovary of cattle following immunization with a modified live BVDV vaccine. In two separate trials, 6 heifers and 4 mature cows were immunized with a modified live BVDV vaccine and ovaries were removed between 7 and 40 days post vaccination. Cytopathic BVDV was
isolated from ovaries removed at days 8, 10 and 12. Bovine viral diarrhea virus antigen was detected using immunohistochemistry at days 10 through 40. These findings are significant in that replication of virus in the ovary could potentially cause ovarian dysfunction resulting in reduced fertility.

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is considered to be one of the most important viral pathogens of cattle (Duffel and Harkness, 1985; Baker, 1995). Its control and prevention are of worldwide concern. Due to its complex pathogenesis and antigenic diversity, control and prevention of BVDV infections has proved to be extremely challenging. Control programs center around elimination of cattle persistently infected with BVDV, biosecurity, and vaccination (Duffel and Harkness, 1985; Baker, 1995).

The role of vaccinations in the prevention of BVDV has become extremely controversial. Much of the debate revolves around the use of modified live or killed BVDV vaccines. Modified live BVDV vaccines have been available since 1961 (Coggins et al., 1961). Currently, these vaccines use cytopathic BVDV which have been attenuated through multiple cell culture passage (Bolin, 1995). It is generally felt that modified live vaccines provide higher levels of protective immunity which are of longer duration (Baker, 1995; Bolin, 1995). However, the safety of these vaccines is of concern (Harkness, 1987; Baker, 1995). Post vaccination mucosal disease (Peter et al., 1967;
McKercher et al., 1968), immunosuppression (Roth and Kaeberle, 1983) and fetal infection resulting in abortion and congenital anomalies (Liess et al., 1984; Trautwein et al., 1986) have been reported following modified live BVDV vaccination. Also, disease resulting from the contamination of modified live vaccines with noncytopathic BVDV strains has been reported (Kreeft et al., 1990, Levings and Wessman, 1990). Finally, with the in vivo replication of the vaccine virus, the potential exists for viral mutations resulting in increased virulence (Bolin, 1995).

Safety is the major advantage of killed BVDV vaccines. However, the need for two initial doses of vaccine to stimulate a primary immune response and the apparent short duration of protective immunity are major disadvantages of killed vaccines (Bolin, 1995).

Reproductive pathology resulting from acute infection with BVDV is well documented. Poor conception rates, early embryonic deaths, abortions, various congenital defects, and the establishment of persistent infection with BVDV in the gestating fetus have all been reported (Moennig and Liess, 1995). The cause of reduced fertility in cattle undergoing acute BVDV infection is unclear. Little is known about the effects that BVDV may have on the ovary following acute infection and the role this may play in reducing fertility. Bovine viral diarrhea virus has been isolated from the ovary following experimental acute infection (Ssentongo et al., 1980) and from ovaries collected from abattoirs (Bielanski et al., 1993). Oophoritis has also been described following acute BVDV infection (Ssentongo et al., 1980). Bovine viral diarrhea virus infection has been suggested as a cause of ovarian dysfunction, but few studies have been conducted to
examine this possibility (McGowan and Kirkland, 1995; Ssentongo et al., 1980). In cattle being superovulated while undergoing acute BVDV infection, a significant reduction in the number of palpable corpus luteums and recovered embryos has been reported (Kafi et al., 1994). In cattle persistently infected with BVDV, a reduction in the number of antral follicles is evident (Grooms et al., 1996). These studies suggest that BVDV infection may affect follicular dynamics. To date, there are no reports of viral isolation or ovarian pathology following immunization with modified live BVDV vaccines. The objective of this study was to determine if BVDV could be isolated or identified in the ovary following modified live BVDV immunization.

MATERIALS AND METHODS

Two separate trials were conducted. In trial A, 4 BVDV seronegative and virus negative mature Holstein cows were immunized with a commercially available multivalent vaccine containing modified live bovine viral diarrhea virus, infectious bovine rhinotracheitis virus, bovine respiratory syncytial virus, and parainfluenza-3 virus. The cattle were isolated for the entire length of the study. Whole blood samples were taken daily to monitor for viremia. Unilateral ovariectomies were performed on two cows at 7 and 10 days post infection. A second ovariectomy was performed on the same cows at day 20 and 30 post infection. Sections of ovary were processed for virus isolation, histology, and immunohistochemistry. In trial B, 6 BVDV seronegative and virus negative
pubertal Angus heifers were vaccinated with the same vaccine as above, but from a
different lot. The heifers were kept isolated for the length of the study. Necropsies were
performed on two animals at day 8, 10, and 12 post vaccination. Ovaries were collected
for virus isolation, histology and immunohistochemistry. In addition, samples of uterus,
oviduct, kidney, Peyers's patch, liver, jejunal and bronchial lymph node, thymus and tonsil
were collected for virus isolation. Whole blood and nasal swab samples were taken daily
to monitor for viremia.

White blood cells were isolated from whole blood samples following hypotonic
lysis of red blood cells with a 0.83% solution of ammonium chloride in distilled water.
The cells were washed once with 10 ml of Dulbecco's modified eagle media (DMEM)
(GIBCO Laboratories, Grand Island, NY), resuspended in 1 ml of DMEM and then froze
at -70 C to lyse the white blood cells. Nasal swabs were placed in 4 ml of DMEM
containing penicillin (100 units/ml), streptomycin (100 μg/ml) and fungizone (2.5 μg/ml)
and then vortexed. One gram of each tissue sample was homogenized in 4 ml of DMEM
containing the same antimicrobials. The homogenate was then centrifuged at 1000 rpm
for 10 minutes and the supernate removed. Virus isolation was performed by inoculating
200 ul samples of white blood cells, nasal swab supernate, or tissue homogenate supernate
onto low passage bovine turbinate cells in 24 well flat bottom cell culture plates and then
passed three times. Plates were observed daily for evidence of cytopathology and then
stained after 5 days using a BVDV specific immunoperoxidase test as previously described
(Afshar et al., 1991). Samples of reconstituted vaccine were also subject to virus isolation
to verify the presence of cytopathic BVDV.
Ovaries were fixed in zinc formalin for 48 hours and then processed for routine paraffin embedding. Sections were processed for routine histological staining with haematoxylin and eosin. Immunohistochemistry was done using a modification of methods previously described (Haines et al., 1992). Briefly, 5 micron sections were mounted on silane treated slides and baked for 1 hour at 56 C. Sections were deparaffinized in Hemo-De (Fischer Scientific, Pittsburgh, PA) for 15 minutes and then rehydrated through graded ethanol to distilled water. Endogenous peroxidase activity was blocked by incubation with 3% hydrogen peroxide in absolute methanol for 5 minutes. Tissues were digested for 15 minutes at 37 C in 0.1% Protease XIV (Sigma Chemical Co., St. Louis, MO). Following digestion, slides were washed in distilled water and then incubated with 5% horse serum in 0.05 M Tris buffer for 30 minutes at 37 C to block nonspecific antibody binding. Detection of BVDV antigen was performed using the BVDV monoclonal antibody 15C5 (Provided by E.J. Dubovi, Cornell University, Ithaca, NY) in conjunction with an indirect avidin-biotin-peroxidase detection system (ABC) (Vector Laboratories Inc., South San Francisco, CA). The BVDV monoclonal antibody 15C5 was diluted 1:1500 in blocker and applied to the tissues for 30 minutes at 37 C. After washing to remove unbound primary antibody, tissues were incubated with horse anti-mouse IgG for 20 minutes at 37 C. Following washing to remove unbound secondary antibody, the ABC reagent was prepared as recommended by the manufacturer and incubated with the tissues for 20 minutes at 37 C. Removal of unbound ABC was followed by the substrate 3-amino-9-ethyl carbazole (AEC) (Zymed Laboratories Inc., Burligame, CA) for 10 minutes at room temperature. Sections were counter stained with
Mayer's haematoxylin and cover slipped using an aqueous mounting media. A heterologous monoclonal antibody directed against infectious bursal disease virus (Provided by Dr. Y.M. Saif, Ohio Agricultural Research and Development Center, Wooster, OH) was substituted for the primary antibody to detect nonspecific binding. Sections of ovary from cows persistently infected with BVDV and cows not infected with BVDV based on virus isolation and serology, were processed as described previously and used as positive and negative controls.

RESULTS

In trial A, no cytopathic or noncytopathic BVDV was isolated from white blood cells following immunization. Cytopathic BVDV was isolated from the ovary of one cow at day 10 post immunization. Bovine viral diarrhea virus antigen was detected in all ovarian sections taken at day 10, 20, and 30 post immunization (Table 5.1). The greatest amount of immunostaining was observed in ovaries taken at day 20. Immunostaining involved stromal and macrophage-like cells located in the cortical region of the ovary (Figure 5.1). Scattered accumulations of interstitial lymphocytes were observed starting at day 7 post immunization and increasing in prominence by day 10 post immunization. Focal lymphocytic infiltrates remained evident at 21 days post immunization and were minimal by day 30. Scattered eosinophils and rare neutrophils were observed in association with the lymphocytic infiltrates. Stromal vacuolation was observed from day 7
to 21, but was most severe at day 10 post immunization. Focal lymphocytic infiltration of the corpus luteum was observed in one ovary removed at day 10 post immunization.

In trial B, cytopathic BVDV was isolated from the white blood cells of 3 of 6 heifers between day 6 to 10 post vaccination (Table 5.2). BVDV was isolated from the blood of only one heifer (#99) at necropsy. No virus was isolated from nasal swabs. Cytopathic BVDV was isolated from the spleen of 1 heifer, the thymus and tonsil of 2 heifers and the Peyers's patch of 3 heifers. Cytopathic BVDV was isolated from the ovaries of three heifers removed at day 8, 10 and 12 post vaccination (Table 5.3). Because of technical problem with tissue fixation, immunohistochemistry and histology were not done.

Samples of reconstituted vaccine from both trials were cytopathic in cell culture and positive for BVDV antigen by immunoperoxidase assay.
Table 5.1: Trial A; Results of virus isolation and immunohistochemical detection of bovine viral diarrhea virus from the ovaries of cattle following immunization with a modified live BVDV vaccine.

<table>
<thead>
<tr>
<th>Animal #</th>
<th>Day Post Vaccination</th>
<th>Virus Isolation</th>
<th>Immunohistochemistry</th>
</tr>
</thead>
<tbody>
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<td>2</td>
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<td>Negative(^b)</td>
<td>Negative</td>
</tr>
<tr>
<td>1</td>
<td>7</td>
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<td>Negative</td>
</tr>
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</tr>
<tr>
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<td>10</td>
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<td>Negative</td>
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</tr>
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</tr>
<tr>
<td>3</td>
<td>30</td>
<td>Negative</td>
<td>Negative</td>
</tr>
</tbody>
</table>

\(^a\)POSITIVE = Isolation of cytopathic bovine viral diarrhea virus, \(^b\)Negative = No BVDV isolated.
Figure 5.1: Photomicrograph showing positive immunostaining for BVDV antigen in the ovarian cortex at 20 days post immunization with a modified live BVDV vaccine. Positive cells are identified with arrows. Bar = 25 μm.
### Day Post Immunization

<table>
<thead>
<tr>
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<th>6</th>
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<th>11</th>
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<td>POS</td>
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</table>

*Neg = Negative for isolation of BVDV, POS = Positive isolation of cytopathic bovine viral diarrhea virus from white blood cells, ND = Test not done.*

Table 5.2: Trial B; Results of virus isolation from white blood cells in heifers immunized with a modified live bovine viral diarrhea virus vaccine.
Table 5.3: Trial B; Results of bovine viral diarrhea virus isolation from bovine tissues following immunization with a modified live BVDV vaccine.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Day PV</th>
<th>Ovary</th>
<th>Uterus</th>
<th>Oviduct</th>
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<th>JLN</th>
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</tr>
<tr>
<td>3</td>
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<td>Neg</td>
<td>Neg</td>
<td>Neg</td>
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<sup>a</sup>PV = Days Post Vaccination, <sup>b</sup>PP = Peyers’s Patch, <sup>c</sup>JLN = Jejunal Lymph Node, <sup>d</sup>Neg = Negative for isolation of BVDV
<sup>e</sup>POS = Positive for isolation of cytopathic bovine viral diarrhea virus.
DISCUSSION

All current commercially available modified live BVDV vaccines are made from attenuated cytopathic BVDV (Bolin, 1995). The attenuation process is aimed at altering the virus so that replication is restricted. By doing this, virulence and shedding of the virus is reduced. It is known that vaccine virus can cross the placenta in gestating cattle resulting in fetal pathology (Liess et al., 1984; Trautwein et al., 1986). For this reason, modified live BVDV vaccines are not recommended for use in pregnant cattle.

Cytopathic BVDV has been isolated from the bovine ovary following experimental infection and has been associated with prolonged oophoritis (Ssentongo et al., 1980). The isolation of BVDV from the ovary following vaccination with a modified live BVDV vaccine has not been reported. In this study, cytopathic BVDV was isolated from the ovaries of four animals between days 8 and 12 following immunization with a modified live BVDV vaccine. Isolation of BVDV during this period of time period is consistent with experimental challenge studies where both cytopathic and noncytopathic BVDV have been isolated between 5 and 15 days post infection (Nuttall et al., 1980; Ssentongo et al., 1980; Wilhelmsen et al., 1990; Baker, 1995). Given the time frame of viral isolation, the cytopathic nature of the virus, and the lack of exposure to other BVDV sources, it is assumed that the isolated virus was of vaccine origin.

In Trial B, cytopathic BVDV was isolated from various lymphoid tissues in the same three heifers that BVDV was found in the ovaries. This is consistent with the lymphoid tissue tropism of BVDV (Brownlie, 1990). In Trial A, BVDV antigen was
detected in ovarian sections by immunohistochemistry up to 30 days post vaccination.

Previous work has shown similar findings following acute infection with noncytopathic BVDV (Grooms, 1997). The histological evidence of oophoritis was similar to that described by Ssentongo et al. following infection with cytopathic BVDV and to that described by Grooms following acute infection with noncytopathic BVDV (Ssentongo et al., 1980; Grooms, 1997). Although the inflammatory changes were similar to those observed following acute infection with noncytopathic BVDV, the cause of the lesions cannot be solely attributed to the modified live cytopathic BVDV because of the presence of modified live infectious bovine rhinotracheitis virus in the vaccine which has been documented to cause oophoritis (Van Der Maaten et al., 1985).

The isolation of BVDV from the ovary following immunization with a modified live vaccine may be of significance if ovarian function is changed. Any functional changes may result in reduced fertility. This is of concern given that commonly recommended BVDV control and prevention strategies often involves the use of modified live BVDV vaccines near the time of breeding. Vaccine derived BVDV located in the ovary may have no effect on fertility. However, even modest reductions in fertility as a result of poor ovarian function may be significant from an economic standpoint. It is known that modified infectious bovine rhinotracheitis virus used in commercial vaccines can cause luteal necrosis and oophoritis which can result in short term changes in ovarian function (Miller et al., 1989). No controlled studies have been conducted looking at the fertility of cattle immediately following vaccination with BVDV. This study was done in seronegative heifers. Viral replication in the ovary following modified live BVDV
vaccination may not occur in seropositive cattle. If cattle are seropositive by the time they reach breeding age, vaccination with modified live vaccines may have no effect on the ovaries of these animals.

This study demonstrates that BVDV can be isolated from the ovaries of seronegative heifers following immunization with a modified live vaccine. The potential for causing ovarian dysfunction and reduced fertility is unknown. However, this possibility should be considered when designing vaccine protocols for breeding age cattle.
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CHAPTER 6

ATTEMPTED DETECTION OF BOVINE VIRAL DIARRHEA VIRUS (BVDV) RNA IN THE OVARIES OF CATTLE ACUTELY INFECTED WITH BVDV

ABSTRACT

Observation of chronic oophoritis and the detection of antigen for up to 60 days in the ovaries of cattle following acute infection with BVDV has raised the possibility of viral latency in the ovary. The objective of this study was to use in situ hybridization to detect BVDV genomic RNA in the same ovaries which BVDV antigen was detected for up to 60 days after acute BVDV infection. A digoxigen labeled riboprobe directed against a conserved nucleotide sequence within the NS2-3 protein coding region of BVDV was constructed. The specificity of the probe was determined using dot blot hybridization and in situ hybridization in BVDV positive and negative control tissues. No BVDV RNA was detected in paraffin embedded ovarian tissues collected from heifers following acute infection with BVDV. These findings would suggest that no viable BVDV is present in the ovary following acute infection. However, because tissues were not collected and
processed in an optimized system for in situ hybridization, negative findings must be interpreted with caution.

INTRODUCTION

Bovine viral diarrhea virus (BVDV) antigen was detected using immunohistochemistry in the ovaries of cattle acutely infected with BVDV for up to 60 days post infection. Virus was isolated from the ovaries at day 6 and 8 post infection. An explanation for this discrepancy has been discussed earlier (see chapter 3).

In this study, in situ hybridization was used in an attempt to detect viral genomic RNA of BVDV in the ovaries of cattle acutely infected with BVDV. In situ hybridization can be used to detect and localize viral nucleic acids within cells without the influence of neutralizing antibodies or other factors that may inhibit the isolation of viable virus (Nuovo, 1994). This study was initiated following the finding of BVDV antigen in ovaries for up to 60 days in cattle acutely infected with BVDV. The objective was to determine if BVDV genomic material could also be detected. Ideally, the most sensitive way to identify viral RNA would be to use the reverse transcription polymerase chain reaction (RT-PCR) assay to amplify a portion of the genome to a detectable level (Nuovo, 1994). RT-PCR has been reported to be up to 1000 fold more sensitive than BVDV virus isolation (Hertig et al., 1991; Lopez et al., 1991). However, because of the retrospective nature of this study, the only samples available were formalin fixed tissues.
MATERIALS AND METHODS

A negative sense digoxigenin labeled riboprobe was designed and constructed. Bovine viral diarrhea virus cDNA from a previously constructed cDNA plasmid library was obtained (Deng and Brock, 1992). Using the restriction endonuclease Bgl II, cDNA corresponding to SD-1 BVDV nucleotide sequence 5764 to 6287 was cut from plasmid A13/B6 and cloned into the Bgl II restriction site within the multicloning region of plasmid pSP72 (Promega, Madison, WI) (Figure 6.1). The correct orientation of the fragment within the plasmid was confirmed by restriction endonuclease analysis (Figure 6.2). A single plasmid clone was selected and denoted as pSP72-3.

The recombinant plasmid was linearized with Spe I and used as a template for antisense RNA transcription. Antisense digoxigenin labeled RNA transcripts were made using a T7 RNA polymerase kit according to the manufacturer's recommendations (Boehringer Manheim, Indianapolis, IN). Following transcription, the concentration of the probe was determined according to the manufacturer's recommendations (Boehringer Manheim, Indianapolis, IN). Dot blots were made of plasmid DNA both with and without the cloned BVDV and RNA extracted from BVDV infected and uninfected cell
Figure 6.1: Restriction endonuclease analysis of pSP72-3: A) 1 KB DNA ladder, B) uncut plasmid, C) cut with Acc I (expect bands at 2757 & 228 bp), D) Spe I (expect bands at 2985), E) uncut plasmid, F) Ava I (expect bands at 2402, 536 & 47), G) Hind III (expect bands at 2608 and 377), and H) 1KB DNA ladder.
Figure 6.2: Schematic representation of riboprobe synthesis. A conserved region of the NS2-3 coding region of BVDV was cloned into the multicloning site of plasmid pSP72. The cloned plasmid, pSP72-3, was linearized with Spe-1. An antisense riboprobe 494 nucleotides in length incorporating digoxigenin-11-UTP was synthesized.
cultures. The probe was tested against these dot blots to confirm its specificity and
determine its sensitivity for BVDV RNA. Detection was done according to the
manufacturer's recommendations (Boehringer Manheim, Indianapolis, IN).

**In situ** hybridization was done using a modification of a previously
reported protocol for detecting BVDV RNA (Desport *et al.*, 1994). All procedures were
done using Rnase free techniques. Five micron sections of paraffin embedded tissues
obtained as previously described (see chapter 3) were cut onto silane coated slides.
Tissues were then dried for 6 hours at 37 C. Sections were deparaffinized for 5 minutes in
Hemo-De (Fisher Scientific, Pittsburgh, PA) and then rehydrated by immersing for 5
minutes each in 95%, 70%, and 50% ethanol and then diethyl pyrocarbonate (DEP) water.
Tissues were then post-fixed in 4% paraformaldehyde for 20 minutes at 4 C, rinsed for 5
minutes in DEP water, immersed in 0.2 N HCL for 5 minutes to denature proteins and
then rinsed again in DEP water for 5 minutes. Tissues were digested for 15 minutes at 37
C with Proteinase K (Sigma Chemical Co, St. Louis, MO) in digestion buffer (10 mM
Tris-HCL, 2 mM CaCl₂) and then rinsed for 5 minutes in two changes of DEP water.
Tissues were then dehydrated by immersing for 5 minutes each in 50%, 75% and 95%
ethanol and then allowed to dry at room temperature.

Tissue sections were prehybridized for 1 hour at 62 C in a humid chamber with
hybridization buffer containing the following: 50% formamide, 10% dextran, 4X SSC (1X
SSC = 0.15 M sodium chloride, 0.015 M sodium citrate), 1X Denharts solution, and
sheared salmon sperm (400 µg/ml). The digoxigenin riboprobe was mixed with fresh
hybridization solution to provide a final probe concentration of 200 ng/ml. The sections were hybridized overnight at 62 C in a humid chamber.

Following hybridization, the slides were washed twice in 4X SSC for 15 minutes, twice in 2X SSC for 15 minutes, and then twice in 0.1X SSC for 15 minutes at 62 C. The digoxigenin probes were detected using a commercial kit as recommended by the manufacturer (Boehringer Mannheim, Indianapolis, IN). In brief, to block nonspecific antibody binding, sections were incubated with blocking solution (1% blocking reagent in buffer 1: 0.1 M maleic acid, 0.15 M sodium chloride, ph 7.5) for 1 hour at room temperature. Alkaline phosphatase labeled polyclonal sheep anti digoxigenin Fab fragments (Boehringer Mannheim, Indianapolis, IN) were diluted 1:500 in blocking solution and incubated with the tissues for 30 minutes at room temperature. Unbound antibody was removed by rinsing the slides twice for 5 minutes in buffer 1. Sections were equilibrated for 5 minutes in detection buffer (100 mM Tris-HCL, 100 mM sodium chloride, 50 mM magnesium chloride, ph 9.5) and then immersed in color substrate (45 μl NBT, 35 μl X phosphate, 10 ml detection buffer) until color development was optimal (4-6 hours). Slides were then rinsed in distilled water and cover slipped using an aqueous mounting media.

The probe was tested on tissues from cattle persistently infected with BVDV and BVDV negative cattle to determine its effectiveness in formalin fixed tissues and to optimize conditions. Controls for nonspecific staining included omitting the probe or substituting a positive sense probe transcribed from the pSP72-3 plasmid using the sp6 promoter.
RESULTS

The concentration of the labeled probe was determined to be 100 ng/ul. The specificity of the probe was confirmed by dot blot hybridization. Positive signals were obtained when the digoxigenin labeled probe was hybridized with the plasmids pSP72-3 and A13/B6 which contained cDNA corresponding to the BVDV genomic region to be probed. Positive signals were also obtained following hybridization with RNA extracted from cell cultures infected with BVDV strains 1088 and SD-1. Negative signals occurred following hybridization with the plasmids pSP72, pSP72-DLG, and uninfected cell culture RNA (Figure 6.3).

Positive hybridization signals were obtained in various formalin fixed, paraffin embedded tissue sections taken from a calf persistently infected with BVDV. The positive in situ hybridization correlated with immunohistochemistry localization of BVDV antigen in the tissues. No hybridization signals were detected in tissues from cattle not exposed to BVDV.

In this study, no BVDV positive hybridization signals were detected in ovaries removed from cows acutely infected with BVDV.
Figure 6.3: Dot blot hybridization of digoxigenin labeled BVDV riboprobe to plasmids containing BVDV cDNA homologous to the probe (pSP72-3 and A13/B6), plasmid with no BVDV cDNA insert (pSP72), heterologous control DNA, and RNA from cell culture infected with BVDV (SD-1 and 1088) and uninfected with BVDV (cell culture RNA).

DISCUSSION

*In situ* hybridization has become an invaluable research tool used in localizing both DNA and RNA within cells. This technique has been used to provide information on the pathogenesis of many infectious viruses including BVDV and hepatitis C virus.
et al., 1994; Johnson and Davis, 1994). The major advantage of *in situ* hybridization as compared to other nucleic acid detection techniques is the maintenance of tissue morphology so that infected cell types can be identified (Boulter and Teo, 1994).

Successful *in situ* hybridization requires optimal conditions (Nuovo, 1994). This is especially true when trying to detect viral nucleic acids. Depending on the agent, genomic copies present in individual cells may vary from 1 to 1000 (Gowans et al., 1994). The ability to detect very low numbers of genomic copies requires optimal conditions (Nuovo, 1994). The most important factors include tissue fixation, tissue digestion, and hybridization stringency (Nuovo, 1994).

In this study, BVDV genomic RNA was not detected in the ovaries of cattle following acute BVDV infection. Detection of BVDV genomic RNA in tissues removed from persistently infected cattle confirmed that the probe was able to detect the intended target. The most obvious explanation for this finding is that BVDV RNA was absent or at a level too low to detect by *in situ* hybridization. Under ideal conditions, the limit of detection for RNA *in situ* hybridization is near 50 copies per cell (Hofler, 1990). In a study of liver biopsies taken from patients chronically infected with hepatitis C, viral genome was detected by *in situ* hybridization in only 30% of the samples (Johnson and Davis, 1994). The only reports of successful *in situ* hybridization for BVDV RNA has involved cattle persistently infected with BVDV where tissue virus levels may be as high as $10^7$ TCID$_{50}$/g (Desport et al., 1994; Booth et al., 1995) As mentioned earlier, the use of RT-PCR would have been a more sensitive assay to detect low levels of virus RNA in the ovarian tissue.
Tissue fixation is one of the most important variables affecting the ability to detect viral genomic material by in situ hybridization. Although different fixatives have been used successfully in conjunction with in situ hybridization, neutral buffered formalin has been shown to provide consistently reproducible results (Nuovo, 1989; Nuovo, 1994). The use of fixatives containing heavy metals such as zinc formalin may reduce the sensitivity of in situ hybridization (Nuovo, 1994). Although unclear, it appears that heavy metals cause a combination of nucleic acid degradation and conformational changes resulting in a reduction in base pair matching necessary for hybridization reactions (Nuovo, 1994). The tissues used in this study were fixed in zinc formalin for 24 - 48 hours. Although optimal for the preservation and subsequent immunohistochemical detection of protein antigens, viral RNA may have been at least partially degraded by this fixative.

In this study, no BVDV genomic RNA was detected using in situ hybridization in the ovaries of cattle following acute infection with BVDV. Given that all conditions were optimized, this would suggest that viral RNA was absent or at a level below the detection limits of in situ hybridization. However, since tissue fixation conditions were not optimal and may have led to degradation of viral RNA, the negative results in this study must be interpreted with caution. Amplification of BVDV RNA by RT-PCR of whole tissue RNA would be the most sensitive assay to determine if viral genomic RNA is present in the bovine ovary for a chronic period of time.
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GENERAL DISCUSSION

Bovine viral diarrhea virus is a significant contributor to economic loss in the cattle industry. It has the potential to cause clinical disease and economic loss at all levels of cattle production. The losses caused by BVDV associated reproductive dysfunction are significant. The reproductive consequences of BVDV infection are important not only because of the economic losses caused by reduced reproductive efficiency, but also because of the key role that the development of viral persistence following fetal infection plays in the maintenance and spread of the virus. A broad understanding of the pathogenesis of BVDV associated reproductive disease is important for purposes of diagnosis, control and prevention of BVDV.

Diagnosis of BVDV is often challenging because of the insidious nature of the virus. In many cases, the only evidence that BVDV may be a problem in individual herds is reduced reproductive efficiency. Information about the pathogenic mechanisms and subsequent clinical outcomes associated with BVDV infection is necessary to facilitate epidemiological and laboratory investigation of potential BVDV infections. With an appreciation for the pathogenic mechanisms, more effective control strategies can be developed based on how the virus is spread and maintained in the population. Once BVDV is introduced into a herd, understanding the viral pathogenesis allows for the
prediction of future outcomes and the implementation of plans to minimize any detrimental effects. In developing effective BVDV prevention plans, information about viral pathogenesis is necessary to determine what programs are most appropriate. Prevention programs are most effective if they are implemented with an understanding of their limitations. With continued study of BVDV pathogenesis, the limits of prevention programs can be recognized and controlled.

Both practitioners and researchers have associated BVDV infection with reduced fertility. However, the pathogenic mechanisms are unclear and the relative significance when compared to other causes of infertility are unknown. The objective of this study was to look for evidence that BVDV can affect ovarian function which may explain in part reduced reproductive efficiency in cattle following infection with BVDV. Diagnostically, evidence that BVDV causes ovarian dysfunction is important in strengthening the association between BVDV infection and reduced reproductive performance. With a strengthened association, more attention can be placed on ruling out BVDV as a cause of poor reproduction on individual farms. By understanding how BVDV affects the ovary and how these effects may cause reduced fertility, more effective control and prevention strategies can be devised. If the specific mechanisms affect ovarian function are known, recommendations can be made to minimize or counteract these effects. Similarly, if mechanisms of ovarian pathology are understood, specific prevention schemes can be developed and implemented if warranted.

The findings from these studies provide evidence that BVDV does cause changes in the bovine ovary which may result in infertility. Based on morphological analysis of
ovaries from cattle persistently infected with BVDV, antral follicle formation is
depressed. A correlation can be made in cattle acutely infected with BVDV where antral
follicle formation is also affected. This is evident by a reduction in dominant follicle size,
growth rate and the number of associated subordinate follicles for the first two estrous
cycles following infection. Interpretation of these similarities should be done with caution
because of the inherent differences between cattle persistently infected with BVDV and
those not persistently infected with BVDV.

The resulting changes in follicular dynamics may be insignificant. However,
alterations in follicular dynamics may also be an indicator of unmeasured changes in the
physiology of developing follicles which could then affect the viability and survivability of
the associated oocyte. Mammalian oocytes are arrested at the diplotene stage of the first
meiotic division (Szollosid, 1993). Prior to ovulation, the oocyte must undergo both
nuclear and cytoplasmic maturation. A complete understanding of the factors necessary
for oocyte maturation is lacking. However some factors known to be important include
the maintained integrity of cumulus cells surrounding the oocyte, proper steroid hormone
balance and proper gonadotropin stimulation (Szollosid, 1993). Measurement of
follicular progesterone/estradiol ratios or gonadotropin dynamics in cattle following
infection with BVDV may help to clarify the effect that BVDV has on follicular formation
and oocyte maturation. As more information is learned about the factors essential for
optimal follicular and oocyte maturation, a more precise effect of BVDV on ovarian
function can be defined.
Further evidence for the argument that BVDV can cause ovarian dysfunction is the finding of chronic oophoritis following acute infection, an observation which has been described previously. The nature of inflammation usually results in some degree of functional change of the affected tissue (Slauson and Cooper, 1990). Depending on the severity of the inflammation and the tissue affected, the functional change may or may not result in a significant clinical problem. The degree of oophoritis observed in this study was not severe, however that should not reduce its significance. As an understanding of ovarian physiology evolves, it is apparent that components of the immune system are integral parts of ovarian function. Examples of these components include macrophages and cytokines such as tumor necrosis alpha, interferon gamma, and interleukin-1. It can be argued that any factor which affects or changes the immune system components of the ovary can indirectly affect ovarian function. Oophoritis as a result of acute BVDV infection invariably will result in some degree of change in these components. It is not known how much disruption or change is needed to significantly affect ovarian function and subsequent reproductive efficiency.

Bovine viral diarrhea virus antigen detection in macrophage-like cells and stromal cells located in the ovarian cortex is consistent with the inflammatory changes observed in the same location. Antigen detection in macrophage-like cells is an important finding because of their potential role in follicular development through production of cytokines. Antigen location in spatial relationship to developing follicles also adds support to the conclusion that BVDV can effect follicular dynamics.
Of added interest is the chronic nature of the inflammation observed. Except in
the case of autoimmune diseases, inflammation invariably results from the immune system
reacting to some type of foreign substance (Tizard, 1992). Chronic inflammation occurs
in response to sustained antigen presentation to the immune system. This study
demonstrates that BVDV antigen can be detected in the ovary for a prolonged period of
time following acute infection. Although no viable virus was isolated after 10 days post
infection, the possibility that infectious virus is present within the ovary can not be ruled
out. These studies were not designed for the purpose of looking for persistent or latent
virus, therefore negative results should be interpreted with caution. It is increasingly
recognized that many viruses can establish persistent infections following acute infection
(Oldstone, 1989; Oldstone, 1991). Examples of viruses known to establish persistent
infections which are of importance to veterinary medicine include bovine herpesvirus-1,
African swine fever virus, foot and mouth disease virus, canine distemper virus, ovine
progressive pneumonia, and bovine leukemia virus (Fenner et al., 1993). Establishing a
persistent infection is advantageous to viruses in helping to ensure their survival. To
establish this type of relationship, the virus must employ a nonlytic strategy of
intracellular survival while at the same time escaping immune recognition. Several
mechanisms of immune system avoidance are known. These include continuous alteration
of the viral neutralizing epitopes, disruption of MHC molecule expression, replication in
cells that lack MHC expression, suppression of immune cell functions and integration of
proviral DNA into the host genome (Oldstone, 1991). In the case of BVDV, it has been
established both in vitro and in vivo that nonlytic intracellular replication can occur,
therefore one criteria for viral persistence has already been met. It is also known that
immunotolerance to BVDV can be established following fetal infection. Although there is
circumstantial evidence suggesting that BVDV can persist following acute infection of
immunocompetent cattle (Brownlie, 1990), no studies have been reported which
conclusively prove or disprove this possibility. Studies designed to look specifically for
viral persistence following acute infection should be done because of the major
implications these finding could have in controlling the spread of BVDV.

The clinical significance of BVDV infection of the ovary may be dependent on
several factors. It is apparent that different BVDV strains have different phenotypic
disease expressions. The molecular basis for these differences are unknown. It is possible
that ovarian changes and resulting clinical outcomes following BVDV infection may vary
significantly with different BVDV strains. Host factors could play a role in how BVDV
affects the ovary. These would include immune status, physiological status and even
genetic factors. Previous immunity to BVDV through natural infection or immunization
would be expected to alter the ability of BVDV to infect the ovary, although this
protection may be incomplete. Altered physiological function, such as those seen in early
lactation, could potentiate or enhance the effects of ovarian infection with BVDV. Little
is known about genetic resistance to BVDV, but it is conceivable that there are breed
differences in susceptibility to BVDV. In addition to host and viral factors, management
factors may affect the outcome of ovarian infection with BVDV. Under optimal
reproductive management conditions, ovarian changes following infection with BVDV
may be insignificant. However, these changes may be magnified if conditions needed for
optimal reproductive efficiency are poor. This may especially be true with conditions
known to cause changes in ovarian dynamics such as poor nutrition or heat stress. As
with most diseases, there is little doubt that the clinical outcome of BVDV infections is
influenced by several factors encompassed by the host-agent-environment triad. Further
understanding of how these factors interact to dictate the outcome of BVDV infection is
necessary for developing effective and cost efficient BVDV control and prevention
programs.

The significance of this research can be viewed from different levels. The
prevalence of mature cattle persistently infected with BVDV is estimated to be less than
2%. The failure of persistently infected cattle to become productive can be related to
many factors. Poor reproductive performance as a result of ovarian dysfunction may be
a significant factor in early culling of cattle persistently infected with BVDV. This is a
benefit in that one key to controlling BVDV at the herd level is to eliminate the virus
source. However, their early demise creates a diagnostic dilemma in herds where BVDV
is suspected. Herd screening procedures are often based on identifying cattle persistently
infected with BVDV. If these methods focus on the productive population, herds
infected with BVDV may be missed because of the small percentage of cows which reach
maturity and remain productive. Voluntary culling of cattle because of poor reproductive
performance resulting from ovarian dysfunction may account for part of the low
prevalence of adult cows persistently infected with BVDV.

Poor reproductive performance as a result of ovarian dysfunction in specific
cohorts of cattle may be a clue to the presence of persistent BVDV infection in that
cohort. Due to the pathogenesis of BVDV upon introduction into a susceptible herd, calves persistently infected with BVDV are often born in groups. With changes in ovarian function as well as other physical problems, reproductive performance may be less than optimal in this group when compared to herdmates not persistently infected with BVDV. Therefore diagnostics should be conducted so that BVDV can be ruled out in cohorts of cattle which have poor reproductive performance.

With the findings that BVDV causes changes in the ovary following acute infection, more evidence is now available that BVDV infection needs to be considered in cattle herds with poor fertility even if other clinical evidence of BVDV is lacking. The findings from this study suggest that the changes in the ovary may last for an extended period of time which could result in prolonged breeding problems. In herds with both acute and chronic fertility problems, BVDV should be high on the differential list of possible causes. How long the observed changes last and the overall significance in reproductive fertility is yet to be determined. Also of interest is the role that prior exposure to BVDV through vaccination or natural infection has in preventing changes in the ovary following subsequent exposure to BVDV. This would have important implications in deciding what areas to emphasize when designing control and prevention programs.

Changes in ovarian function resulting from acute BVDV infection provides further reasons to be diligent in implementing sound prevention programs. Resulting reproductive inefficiency can cause a significant economic drain. The economic losses may not result from dramatic clinical problems such as abortions or mucosal disease, but the subclinical
nature can be just as devastating. Prevention strategies should focus on decreasing the risk of exposure to BVDV by eliminating persistently infected cattle and implementing sound biosecurity measures. Vaccination strategies should be aimed at providing maximum protection during the breeding and gestation period.

With the finding that a vaccine strain of BVDV can be isolated from the ovary, caution should be used when vaccinating with a modified live BVDV vaccine prior to breeding. No functional changes in the ovary have been demonstrated following modified live BVDV vaccination. However, with replication of virus in the ovary comes the possibility of changes similar to those observed following infection with a field strain of BVDV. Until more information is available, the possibility of reduced fertility as a result of vaccine strain BVDV replication in the ovary should be factored into decisions regarding vaccination programs. Programs which utilize modified live BVDV vaccines should build in a reasonable period of time between immunization and the initiation of breeding. This is probably most important with initial immunization of heifers where virus replication may be more extensive in various tissues because of a lack of prior BVDV exposure.

There is little doubt that considerable progress has been made in understanding the complex nature of BVDV. However there still remains much to learn. Continued progress in understanding all aspects of BVDV will allow for the implementation of better control and prevention programs. Although the ultimate goal may be to eradicate BVDV, this may be an impossibility. Therefore the goals of BVDV control programs and the research needed to support these programs should be aimed at minimizing the risk of
infection and decreasing the effects of infection with the ultimate goal of improving producer profitability and providing a safe food source for consumers.
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