

The Role of White-tailed Deer (*Odocoileus virginianus*) in the Epidemiology of Bovine Viral Diarrhea Virus

by

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Abstract

Bovine viral diarrhea virus (BVDV) is the prototypic member of the genus *Pestivirus* in the family *Flaviviridae*. Infections with BVDV cause substantial economic losses to the cattle industries, prompting the introduction of organized control programs in several countries. In North America, these control programs are mainly focused on the identification and removal of persistently infected (PI) cattle, the main reservoir for BVDV. Further measures include the enhancement of BVDV-specific immunity through vaccination and the implementation of biosecure farming practices. For the successful execution of these control measures, knowledge of the epidemiology of BVDV must be complete, including the recognition of other potential sources of the virus. Bovine viral diarrhea virus does not possess strict host-specificity and infections have been demonstrated in over 50 species in the mammalian order Artiodactyla.

In North America, the white-tailed deer (*Odocoileus virginianus*) is the most abundant free-ranging ruminant, and evidence of BVDV infections was described in serological surveys and a single experimental inoculation of fawns. However, susceptibility of white-tailed deer to BVDV infection does not alone imply or prove a role in the epidemiology of the virus or prove the existence of a wildlife reservoir. Additional criteria including shedding and maintenance of BVDV and sufficient contact to cause spill-back infections to cattle must be met to cause concern for developing BVDV control measures. The overall goal of this research was to examine whether white-tailed deer could be a wildlife reservoir for BVDV, and studies were

developed on the basis of the necessary criteria for wildlife reservoirs. For each experiment, free-ranging white-tailed deer were captured by cannon-net or dart-gun and translocated to a captive research facility. Upon capture, all animals were determined to be free from BVDV and antibodies against BVDV.

Persistently infected cattle are the most efficient source of BVDV and constitute the major source of transmission of the virus within and among cattle herds. If the phenomenon of persistent infection could occur in white-tailed deer, these PI animals could shed and maintain BVDV, and pose a potential threat to BVDV control and eradication programs. To assess the outcome of fetal infection in white-tailed deer, in our first experiment, nine pregnant does were inoculated intranasally with 10^6 CCID₅₀ each of a BVDV 1 (BJ) and BVDV 2 (PA131) strain. In this study, only one doe gave birth and delivered a live fawn and a mummified fetus. The live fawn was determined to be PI with BVDV 2 by serial virus isolation from serum, buffy coat, and nasal swabs; immunohistochemistry on skin samples; and RT-PCR on serum. Analogous to PI cattle, the fawn continuously shed BVDV, as indicated by BVDV positive nasal swab samples.

Central to the development of a wildlife reservoir is interspecific contact and transmission of BVDV, which were the focus of our second experiment. At approximately 50 days of pregnancy, seven female and one male white-tailed deer were cohabitated with two PI cattle. In a pen of approximately two acres, both species shared feed and water sources for a period of 60 days. Cohabitation of species resulted in the transmission of BVDV from PI cattle to deer, as indicated by seroconversion in all adult deer. Transplacental transmission of BVDV was demonstrated in four of seven pregnancies, as two stillborn twin fetuses and three live singletons were infected with BVDV at birth. All singlet fawns were demonstrated to be PI by immunohistochemistry and ELISA on skin samples, and RT-PCR and virus isolation procedures

on blood and tissues samples. These findings demonstrate that BVDV may efficiently cross the species barrier to provide a potential alternative niche to ensure viral survival and propagation.

Shedding of BVDV as demonstrated in the PI fawns may result in maintenance of the virus within populations of white-tailed deer, and this would be central to perpetuation of a wildlife reservoir. To evaluate the potential for intraspecific transmission of BVDV and the potential for BVDV maintenance in white-tailed deer, six pregnant does were cohabitated with a PI fawn during the first trimester of pregnancy. All does gave birth to live fawns and no reproductive losses were observed. At birth, evidence of BVDV infection was identified in two singlet fawns, of which one was determined to be PI by repeated serum RT-nPCR, whole blood virus isolation and immunohistochemistry. The birth of a PI fawn in this study strengthens the possibility of continuous intrapopulational propagation of BVDV and the potential for indefinite maintenance of the virus in deer. While the experiment was designed to emulate a wild population, the size of the captive deer facility limits possible extrapolations to free-ranging populations in which various factors may influence the transmission and maintenance of BVDV.

We therefore studied free-ranging white-tailed deer in Alabama for evidence of BVDV infection by assessing serum and skin samples collected from hunter-harvested deer. Upon site visits to deer processing units, 165 serum samples and 406 skin biopsies were collected and analyzed by virus neutralization and immunohistochemistry, respectively. Two serum samples contained virus neutralizing antibodies, and one skin sample was positive on immunohistochemistry. The viral antigen distribution in the skin sample was similar to that of PI cattle, indicating the existence of free-ranging PI white-tailed deer in Alabama.

A central question to control of BVDV in US cattle populations is whether the virus can be transmitted from PI white-tailed deer to susceptible cattle. Our research indicated that survival

of PI white-tailed deer appears to be reduced, prompting the research question whether clinically ill or dead PI deer may be a source of BVDV and hamper current control programs. We therefore evaluated the transmission of BVDV to cattle exposed to carcasses of PI white-tailed deer. In two trials, steers were exposed to the carcass of PI fawn A (BVDV 2) or PI fawn B (BVDV 1). One steer from each of two groups was separated into a pen with the carcass. We chose to expose only one steer from each group to the carcass to simulate the effects of bovine contact networks and social hierarchies on the transmission of BVDV. Following 8 hours, the single steer from each trial was commingled with four other steers for 28 days. Subsequently, one steer was inoculated intranasally with spleen homogenate from fawn A, and two steers were inoculated intranasally or intravenously with spleen homogenate from fawn B as infection controls. Steers in both trials made contact with the carcass, but BVDV transmission did not occur. The intranasally inoculated control for trial A and the intravenously inoculated control for trial B became viremic and seroconverted. Although both PI fawn carcasses were potentially infectious, this study indicated exposure of a single inquisitive bovid to a PI fawn carcass did not result in transmission of BVDV.

The results of our research indicate that BVDV infections in white-tailed deer share many features with infections in cattle, indicating a potential for the development of a wildlife reservoir in this species. This development, however, would be influenced by various endogenous and exogenous factors and further research is needed to aid in the successful control of BVDV.

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Chapter 1: Review of the Literature

Introduction and History of Bovine Viral Diarrhea Virus

In 1946, two North American research groups reported on outbreaks of a disease in cattle herds in the state of New York, USA and the province of Saskatchewan, Canada that had not been previously described. This new disease was termed “X disease” and subsequently “virus diarrhea” (Childs 1946; Olafson et al. 1946). The clinical signs associated with this disease primarily involved the gastrointestinal and respiratory systems, but one report also described the occurrence of abortions in pregnant animals that were observed within 10 days to 3 months after the initial infections (Olafson et al. 1946). Affected cattle showed clinical signs of systemic illness, including elevations in body temperature, depression, dehydration, increased respiratory and heart rates, decreases in milk production, and leukopenia. The two earliest publications on Bovine viral diarrhea virus (BVDV) described a disease that was of severe acute nature, but less severe, subacute cases were also observed (Childs 1946; Olafson et al. 1946). Olafson suspected the causative agent of this disease to be a viral pathogen (Olafson et al. 1946), but the viral etiology of this transmissible disease was not demonstrated until eight years later (Baker et al. 1954).

Viral diarrhea was described as “insidious and unspectacular in nature” but causing serious losses over a period of several months (Childs 1946). In contrast to the characteristics of high morbidity and low mortality of virus diarrhea, a second new disease with very low morbidity but high case fatality was first described in 1953 and termed mucosal disease (Ramsey et al. 1953). Mucosal disease had several clinical similarities with virus diarrhea, but caused much more severe clinical signs including watery diarrhea that sometimes contained blood, gastrointestinal hemorrhages, profuse salivation, and ulcers and erosions on epithelial surfaces. These clinical differences and the apparent inability to transmit the agent of mucosal disease

experimentally prompted researchers to conclude that two distinct agents caused viral diarrhea and mucosal disease (Ramsey et al. 1953).

In 1957, the viral agents of virus diarrhea and mucosal disease were isolated from clinical cases and their different effects on cell-culture were first described (Lee et al. 1957; Underdahl et al. 1957). While the agent of virus diarrhea did not cause cytopathic effects in tissue cultures, cytopathogenicity was noted with viruses isolated from cases of mucosal disease. This discovery later led to the classification of BVDV isolates into non-cytopathic or cytopathic biotypes. The isolation of cytopathic viruses made possible the development of serum neutralization and plaque neutralization assays, the development of vaccines after attenuation of cytopathic BVDV strain Oregon C24V, and the determination that the viral agents of viral diarrhea and mucosal disease were, in fact, the same. In the early 1960's virus neutralization experiments using strain Oregon C24V were performed, and neutralization of this strain by sera from clinical cases of virus diarrhea and mucosal disease identified etiological identity (Gillespie et al. 1961; Thomson et al. 1963). As a result of this discovery, the disease syndrome was later named bovine viral diarrhea-mucosal disease (BVD-MD) (Kennedy et al. 1968).

Only incompletely understood even today, the pathophysiology of persistent infection and mucosal disease was not elucidated the early 1980's (McClurkin et al. 1984; Bolin et al. 1985b). Earlier work had identified that cattle infected *in utero* were often unthrifty, persistently viremic with non-cytopathic BVDV, and unable to produce antibodies in response to vaccination against BVDV despite the ability to seroconvert to IBR virus (Peter et al. 1967; Coria et al. 1978; McClurkin et al. 1979). In 1984, McClurkin and colleagues experimentally infected pregnant cattle with one of four non-cytopathic or one cytopathic strain of BVDV (McClurkin et al. 1984). Infections resulted in pregnancy loss, still-births, and persistently infected calves; and persistent

infections were only induced in developing fetuses infected with non-cytopathic BVDV (McClurkin et al. 1984). Later experiments corroborated that persistent infections are induced only by non-cytopathic biotypes (Brownlie et al. 1989), and this fact is still accepted today.

In the decades following their original descriptions, infections with BVDV in immunocompetent animals were reported to be mainly subclinical, and 70-90% of acutely infected animals were estimated to not display clinical signs (Ames 1986). The emergence of a new genotype of BVDV in the early to mid-1990s changed the perception of the virus as it caused severe losses in North American cattle herds. Two clinical syndromes, the hemorrhagic syndrome and severe acute BVD, were described and caused severe clinical signs that were similar to those of mucosal disease, with mortality rates of up to 100% (Pellerin et al. 1994; Carman et al. 1998). In 1993, 25% of the veal crop in the province of Quebec was lost due to BVDV 2 infection (Pellerin et al. 1994). Genetic sequencing of these new BVDV isolates detected a dissimilarity of greater than 30% between the two BVDV genotypes and recent pestiviral classification contains two species of BVDV (1 and 2) (Ridpath et al. 1994; Ridpath 2005). Retrospective genetic typing of isolates from Ontario revealed that BVDV 2 had been present in Ontario as early as 1981 without causing severe disease symptoms (Carman et al. 1998).

Although it is difficult to determine the validity of all accounts, it is interesting to note that early reports of disease and pathologic findings in heterologous species were associated with BVDV and published within a few years following the first descriptions in cattle. In 1956, Richards and colleagues published a manuscript entitled “Mucosal Disease of Deer” that detailed clinical and pathological findings of deer with inflammation and ulceration of mucosal surfaces (Richards et al. 1956), but these lesions were most likely caused by epizootic hemorrhagic

disease of deer rather than BVDV (Van Campen et al. 2001a). Suspicions of BVDV-related mortalities were also expressed in a report that detailed the loss of 28 ruminants with hemorrhagic diarrhea, pyrexia, and ulcerative enteritis in a zoological collection in 1964, and the authors concluded that “based on pathological-anatomical findings, one can assume with all but certainty that in all affected or perished animals an infection with the pathogen of “mucosal disease –viral diarrhea” (MD-VD)-complex was present” (Brass et al. 1966). This illustrates the common difficulties caused by lack of suitable virological assays for use in heterologous species; however, early reports with convincing evidence of BVDV infection in heterologous species do exist (Romvary 1965; Neumann et al. 1980; Weber et al. 1982).

The antigenic relationship of BVDV and classical swine fever virus was first described in 1960 (Darbyshire 1960) and prompted research efforts aimed at using BVDV for vaccination of swine (Beckenhauer et al. 1961; Tamoglia et al. 1965; Simonyi et al. 1967). Although this idea was later abandoned when shedding of BVDV from vaccinated swine was demonstrated (Fernelius et al. 1973), BVDV infections of swine have caused problems with CSFV diagnostics into recent years (de Smit et al. 1999). Similarly, the antigenic relationship of border disease virus and BVDV was discovered in 1973 and reports of diagnostic difficulties exist (Giangaspero et al. 1999).

Pestivirus: Virus Taxonomy and Characteristics

Bovine viral diarrhea virus is an RNA virus and is the prototypic member of the genus *Pestivirus* within the family *Flaviviridae*. Currently, the genus pestivirus contains four recognized species: *Bovine viral diarrhea virus* 1 and 2, *Border disease virus*, and *Classical swine fever virus* (Ridpath 2005). Other pestiviruses have also been described, but have not yet

been officially classified. These include the genetically distinct genotypes Pronghorn pestivirus, Giraffe pestivirus, strain V60 (Reindeer-1), and Ho_Bi strain that have been isolated from non-bovine hosts or tissue cultures and may prompt further rearrangements of classification (Dekker et al. 1995; Avalos-Ramirez et al. 2001; Becher et al. 2003; Schirrneier et al. 2004; Vilcek 2005). Traditional classification of pestiviruses was based on the host from which each strain was isolated, with BVDV, border disease virus, and classical swine fever virus originating from cattle, sheep, or swine, respectively. The lack of strict host specificity and antigenic relatedness of pestiviruses has caused difficulties with this traditional method of classification, and more accurate taxonomic information is now based on monoclonal antibody binding assays and phylogenetic analysis of genomic sequences (Hofmann et al. 1994; Ridpath et al. 1994; Harasawa 1996; Vilcek et al. 1999; Vilcek et al. 2005a; Giangaspero et al. 2007). Although sequence variations of pestivirus isolates are located throughout the entire genome, the 5'UTR region is most commonly used for the differentiation of pestiviral species (Ridpath et al. 1994; Harasawa et al. 1995; Harasawa 1996; Ridpath et al. 1997; Falcone et al. 2001; Park et al. 2004; Ridpath 2005; Giangaspero et al. 2007). Further division of pestiviral isolates into subgroups is more preferably performed according to the genes encoding the N^{pro} or E2 proteins (van Rijn et al. 1997; Vilcek et al. 1997; Becher et al. 1999).

Bovine viral diarrhea virus isolates are either non-cytopathic or cytopathic and this classification is based on the effects of an isolate on cultured cells. Cytopathic isolates cause cytoplasmic vacuolation and death of tissue cells, while infections of cell cultures with non-cytopathic isolates are inapparent (Lee et al. 1957; Underdahl et al. 1957). The categorization into biotype is not associated with the virulence of a BVDV isolate, and mild or severe disease can be observed with either biotype. The non-cytopathic biotypes are more prevalent in nature

and 60 – 90 % of isolates from specimens at diagnostic laboratories are non-cytopathic (Fulton et al. 2000b; Fulton et al. 2005). Only non-cytopathic BVDV strains can induce persistent infections, and these non-cytopathic strains are believed to be the most common source for cytopathic BVDV strains following homologous or heterologous recombination in the NS2-3 genomic region (Bolin et al. 2004). A third biotype of BVDV, the lympho-cytopathic biotype, has been proposed, and refers to BVDV isolates that have cytopathic effects only on cultured lymphoid but not epithelial cells (Ridpath et al. 2006a). Lympho-cytopathic isolates are BVDV 2 strains that were associated with severe clinical disease (Ridpath et al. 2006a).

The single-stranded pestiviral genome is approximately 12.3 kilobases in length and contains a single large open reading frame that is flanked by untranslated regions at the 5' and 3' termini. The open reading frame is translated into a single polyprotein that is processed cotranslationally and posttranslationally to give rise to the structural and non-structural proteins (Rumenapf et al. 1993). The final protein products are contained in the initial polyprotein in the order: N^{pro} – C – E^{ms} – E1 – E2 – p7 – NS2/3 – NS4A – NS5A – NS5B. Pestiviruses are enveloped viruses and the structural proteins C, E^{ms}, E1, and E2 in conjunction with the host-derived lipid envelope contain the genome, forming the mature virion. The highly conserved nucleocapsid protein (core, C) is 90 amino acids in length and created from the initial polyprotein by three enzymatic cleavages of which the amino-terminal end of C protein is cleaved by a co-translational autoproteolytic event of N^{pro} (Stark et al. 1993; Heimann et al. 2006). Subsequently, C protein is cleaved from E^{ms} in the host cell's endoplasmic reticulum and becomes associated with a lipid membrane that is derived from the endoplasmic reticulum and the envelope glycoproteins E^{ms}, E1, and E2 (Ronecker et al. 2008). Binding of C protein to the viral RNA genome is associated with this protein's intrinsically disordered and basic character

(Murray et al. 2008). Following RNA binding and combination of the envelope components with the core, newly formed virions are trafficked from the endoplasmic reticulum to the extracellular space via cellular secretory pathways.

Complete BVDV virions carry the envelope proteins (E^{ms} , E1, and E2), which are glycosylated and have important roles in virus binding and cell entry, as well as for immunologic recognition by the host, and success of vaccination. The E^{ms} protein is detectable on released virions but is also secreted from infected cells due to a lack of a typical transmembrane anchor (Fetzer et al. 2005). Unlike E1 and E2, E^{ms} is dispensable for cellular entry of BVDV, but may play a role in the initial binding to permissible cells (Iqbal et al. 2000; Ronecker et al. 2008). The E^{ms} protein has been demonstrated to bind to and inactivate dsRNA-dependent signaling events, ultimately limiting the antiviral effects of alpha/ beta interferon (Iqbal et al. 2004). The envelope protein E1 is assumed to function as a membrane anchor for E2, and the formation of E1-E2 heterodimers appears to be essential for cell entry of BVDV (Rumenapf et al. 1993; Ronecker et al. 2008). As the immunodominant protein, neutralizing epitopes of E2 are important targets of vaccination efforts and E2 subunit vaccines have shown promise in research studies (Donofrio et al. 2006; Chimeno Zoth et al. 2007; Couvreur et al. 2007; Kohl et al. 2007; Marzocca et al. 2007; van Rijn 2007; Thomas et al. 2009). Antigenic variability in the E2 protein is important in determination of cell tropism of pestiviral isolates and contributes to vaccination failure after immunization with heterologous vaccine strains (Van Campen et al. 2000; Liang et al. 2003; Ridpath 2005; Tang et al. 2007).

Of the nonstructural proteins, N^{pro} is the first protein to be translated from the pestiviral open reading frame. N^{pro} is an autoprotease that cleaves itself from C and targets interferon regulatory factor 3 to block the type I IFN induction (Baigent et al. 2002; Seago et al. 2007).

Following the structural proteins, the initial polypeptide contains p7, a protein that has been demonstrated to be essential in the late phase of the replication of BVDV and other flaviviruses, and has been proposed to be a suitable target for antiviral therapy (Harada et al. 2000; Tscherné et al. 2008; Luscombe et al.). P7 is not associated with virus particles (Harada et al. 2000). The NS2-3 protein follows p7 in the initial polyprotein. While NS2-3 is associated with non-cytopathic BVDV, in cytopathic BVDV strains this protein is cleaved into NS2 and NS3, and NS3 is considered a marker of infection with cytopathic BVDV (Kameyama et al. 2008). Cleaved NS3 is also present early in infections with noncytopathic BVDV and continuously in persistently infected animals without evidence of cytopathic BVDV infection (Kameyama et al. 2008). The NS2-3 and NS3 proteins are serine-proteases that cleave nonstructural proteins from the polyprotein (Tautz et al. 1997). The uncleaved NS2-3 is essential for the formation of infectious particles, but cannot substitute for cleaved NS3, which is essential for genome replication (Agapov et al. 2004; Lackner et al. 2004). The non-structural protein 4A associates with cleaved NS3 to act as a cofactor for its serine protease activity (Xu et al. 1997), and NS4A is indispensable for NS2-3 in the formation of infectious particles (Moulin et al. 2007). Although the mechanism has not been elucidated, NS4B appears to be associated with cytopathogenicity (Qu et al. 2001). The non-structural protein 5A is part of the replication complex and associates with bovine translation elongation factor 1A (Johnson et al. 2001). Whether uncleaved NS5A is functionally important is currently unknown, but cleaved NS5A represents the viral RNA-dependent RNA polymerase (Steffens et al. 1999).

Prevalence in Cattle Populations

Following the initial description in North America, BVDV has been detected in cattle populations worldwide. The prevalence of seropositive animals depends largely on the management of cattle including type of husbandry, use of vaccination protocols, addition of new herd mates, and presence of PI animals (Houe 1995; Houe et al. 1995). Cattle herds that do not use vaccination program or only use killed vaccines and do not have PI animals have fewer numbers of seropositive cattle than herds containing PI cattle (Houe et al. 1995). In North America, serosurveys demonstrated seropositive rates between 40% to 90% (Bolin et al. 1985a; Durham et al. 1990). The individual seroprevalence among unvaccinated dairy heifers in Canada for BVDV 1 and BVDV 2 was 28.4% and 8.9%, respectively, and the herd-level prevalence was 53.4% for BVDV 1 and 19.7 % for BVDV 2 (Scott et al. 2006). In feedlot cattle, seroconversion to respiratory pathogens including *Pasteurella hemolytica*, *Mycoplasma spp.*, and BVDV following arrival occurred in 40% of cattle (Martin et al. 1989; Taylor et al. 1995), but varied between 0 – 100 % of cattle in 11 pens (Taylor et al. 1995). Similar rates of seroprevalence have been detected in cattle herds worldwide, but the observed range varied widely among countries (13 – 90 %) and was largely influenced by the examined population, potentially reflecting global differences in cattle management (Rossmanith et al. 1998; Ferrari et al. 1999; Grom et al. 1999; Houe 1999; Luzzago et al. 1999; Nuotio et al. 1999; Obando et al. 1999a; Obando et al. 1999b; Sudharshana et al. 1999; Mainar-Jaime et al. 2001; Stahl et al. 2002; Vilcek et al. 2003; Kampa et al. 2004; Niza-Ribeiro et al. 2005; Solis-Calderon et al. 2005; Duong et al. 2008; Brulisauer et al. 2009).

In contrast to seroprevalence rates, the prevalence of PI cattle is considerably lower and is generally believed to be less than 1% of all cattle. Persistently infected cattle may be found in

clusters within groups of cattle, elevating the prevalence within populations (Houe 1992). The prevalence of PI calves arriving at feedlots has been demonstrated to be between 0.1 and 0.3%, and this rate is similar to the reported rate of 0.17% for beef cow-calf operations in the United States (Taylor et al. 1995; Wittum et al. 2001; Loneragan et al. 2005; O'Connor et al. 2005).

Clinical Disease Syndromes

Infections with BVDV may result in a wide variety of clinical manifestations as the outcome of infection depends on the triad of host-associated factors, environmental stressors, and viral characteristics (Baker 1995). Host-associated factors include: a) the immune status and immunocompetence, b) pregnancy status, and c) the gestational age of the fetus at the time of infection. Viral characteristics that influence the outcome of an infection include the biotype of the infecting strain and genotypic variation that determine virulence and antigenicity. Infections with both species of BVDV may result in a broad spectrum of clinical manifestations, but severe acute BVD, thrombocytopenia, and hemorrhagic syndrome, as reported in North America, were associated only with BVDV 2 (Corapi et al. 1990; Pellerin et al. 1994; Carman et al. 1998).

Infections in Immunocompetent Cattle

Postnatal infections in immunocompetent cattle that are able to immunologically respond to the BVDV infection are referred to as 'acute' or 'transient' infections, and the severity of clinical signs depend on the infecting BVDV strain. After infection of susceptible cattle the tonsils and respiratory tract are the first sites of BVDV replication. Subsequently the pathogen disseminates to many other epithelial and lymphoid tissues, resulting in infection of gastrointestinal, integumentary, and respiratory tissues (Ohmann 1982; Ohmann 1983).

The majority of BVDV infections in immunocompetent, seronegative cattle proceed subclinically (Ames 1986). However, closer observation of infected animals usually reveals mild signs including hyperthermia, leukopenia, and decreased milk production. Affected animals develop neutralizing antibodies (Moerman et al. 1994). Symptomatic infections are possible and are most commonly observed in 6 – 24 month-old-cattle following waning of the maternal immunity, colostrum-deprived calves, or in seropositive cattle as a result of infection with a heterologous BVDV strain (Evermann et al. 2005). Clinical signs in affected animals include pyrexia, lethargy, leukopenia, ocular and nasal discharge, oral erosions and ulcers, blunting of oral papillae, diarrhea, and decreased milk production. More virulent BVDV strains can also cause epithelial erosions at the interdigital spaces, coronary bands, teats, or vulva. Although BVDV-associated immunosuppression can result in pneumonia, the tachypnea observed in acutely infected animals is likely the result of hyperthermia and other non-pulmonary factors (Evermann et al. 2005).

In the 1990's, severe acute BVD was described in cattle herds in the United States and Canada (Corapi et al. 1990; Pellerin et al. 1994). The outbreaks were characterized by a peracute course and caused unusually high rates of morbidity and mortality in all ages of cattle. The described clinical signs and post-mortem findings were principally those of mucosal disease, with severe diarrhea, fever, and ulcers and erosions of the upper alimentary tract (Carman et al. 1998). Histopathological findings included a dramatic lymphocytolysis and lymphoid depletion of Peyer's patches, necrosis of intestinal crypt epithelium, and diffuse ulcerative lesions in the upper alimentary tract. Sudden death without premonitory clinical signs, abortions, and pneumonia were also prominent findings in some affected herds. Interestingly, the gross and histopathological postmortem changes were more pronounced and more likely to be observed in

older animals, which is in contrast to most previous descriptions of transient BVDV infections (Carman et al. 1998). The viral isolates from all reports of severe acute BVD were determined to be different from traditional BVDV isolates in genotype and monoclonal antibody binding patterns, prompting the designation BVDV 2 (Pellerin et al. 1994; Ridpath et al. 1994; Ridpath et al. 1998).

Another form of severe acute BVD associated with BVDV 2 is the hemorrhagic syndrome, which is characterized by marked thrombocytopenia. Hemorrhagic syndrome is observed only in a minority of cattle with severe acute BVD, contributing to a diverse clinical picture in an outbreak (Evermann et al. 2005). Clinical signs in affected cattle include petechiation and ecchymoses of mucosal surfaces, epistaxis, bloody diarrhea, bleeding from injection sites or trauma, fever, and death (Corapi et al. 1990). The marked thrombocytopenia and leukopenia of affected animals is accompanied by altered function of platelets, thus quantitative and qualitative platelet defects contribute to the observed hemorrhagic diathesis (Walz et al. 1999b).

In addition to direct effects on the host organism, BVDV suppresses the number and function of various innate and adaptive immune components, further damaging tissues by enabling infections with secondary pathogens. BVDV induced immunosuppression is especially important in polymicrobial diseases such as the bovine respiratory disease complex of feedlot cattle and dairy calves. Infection of cells of the innate immune system may result in the impairment of function and decreases in the number of circulating leukocytes. The microbicidal, chemotactic, and antibody-dependent cell-mediated cytotoxicity was impaired in neutrophils that were infected with BVDV (Potgieter 1995). Infected monocytes may undergo apoptosis, and a reduction of 30-70% in the number of monocytes has been observed after BVDV inoculation of

calves (Lambot et al. 1997; Archambault et al. 2000; Glew et al. 2003). Infection with BVDV diminishes the ability of antigen-presenting cells to present antigen to T helper cells by reduction of Fc and C3 receptor expression and downregulation of MHC II and B7 molecules (Welsh et al. 1995; Adler et al. 1997; Archambault et al. 2000; Chase et al. 2004a). A strain-dependent inhibition of function with reduced ability to kill bacterial and fungal pathogens was observed following BVDV infection of cultured macrophages; however, significant functional inhibition was induced only by virulent strains of BVDV (Chase et al. 2004a). In addition to virulence, the biotype of an infecting isolate appears to influence the outcome of macrophage infection. While macrophages infected with cytopathic BVDV release soluble factors that induce apoptosis in uninfected macrophages, these factors may not be released by macrophages infected with non-cytopathic isolates (Adler et al. 1997; Chase et al. 2004a). Similarly, the interferon response is affected differently according to biotype. While cytopathic strains induce an interferon release and apoptosis, non-cytopathic strains inhibit the production of interferon in infected cells, which may be involved in the pathophysiology of persistent infections (Schweizer et al. 2001; Schweizer et al. 2006).

Similar to innate immunity, adaptive immune responses are affected quantitatively and qualitatively by BVDV infections. Upon infection with BVDV, circulating lymphocytes are reduced and lymphocytes in lymphoid tissues are depleted, and this depletion is strain dependent (Brodersen et al. 1999; Archambault et al. 2000). Of the T-lymphocyte sub-populations, CD8⁺ cytotoxic T-lymphocytes are most dramatically depleted, followed by CD4⁺ T-helper cells, with little reduction of circulating γ/δ T-cells (Ellis et al. 1988). In addition to lymphocyte depletion, functional alterations of CD4⁺ are pronounced, and non-cytopathic BVDV infections result in a shift toward the Th2 response, with reduced cell-mediated but high levels of humoral immunity

(Collen et al. 2000b; Waldvogel et al. 2000; Collen et al. 2002). Virulent BVDV isolates can cause reductions of the MHC I expression of infected cells, thus affecting the cytotoxic CD8⁺ function; however, this reduction appears to be strain dependent (Chase et al. 2004a).

The humoral immunity to BVDV infection depends largely on the quantity of neutralizing antibodies and the antigenic relatedness of the infecting strain with that of the original exposure. Although an amount of cross-reactive protection is conferred by vaccination against heterologous strains, and even between BVDV 1 and 2, superior protection from infection appears to result from homologous exposure (Fulton et al. 2000a; Fulton et al. 2003).

Infections of the Reproductive Organs

Both the male and female gonads and the entire female reproductive system are permissible for replication of BVDV, and BVDV infections can have deleterious effects on pregnant cattle. Although the mechanisms are incompletely understood, reduced fertility has been reported in cattle infected prior to insemination, and reduced conception rates were attributed to failure of fertilization or early embryonic death (McGowan et al. 1995). Possible mechanisms for the impairment of fertility include the infection of ovaries, from which BVDV can be localized for prolonged periods of time (Grooms et al. 1998c; Grooms et al. 1998b). Reduced follicular growth, altered ovarian hormone secretion, and oophoritis have been demonstrated to be results of BVDV infection in seronegative cows and can impair fertility (Archbald et al. 1977; Grooms et al. 1998a; Fray et al. 2000).

BVDV is highly efficient in crossing the placental barrier and fetal infections are common with either biotype (Duffell et al. 1985). The outcome of fetal infection depends on the viral virulence and biotype, and the gestational age. Fetal death and abortions can occur with either biotype at any gestational age, but are most common during the first trimester (Grooms

2004). Fetal death in early pregnancies may result in fetal resorption or mummification, and expulsion of the fetus may occur up to 50 days following exposure to BVDV (Murray 1991). While infections in the pre-implantation phase (before day 40 of pregnancy) result in large numbers of pregnancy losses, fetuses that survive infection with non-cytopathic BVDV of low to moderated virulence from 40 – 125 days become persistently infected (Grooms 2004). Experimental inoculation of seronegative cattle between 18 and 100 days of gestation results in persistent infection in up to 100% of fetuses (Brock et al. 2000; Brock et al. 2001; Grooms 2004). The pathophysiology of this phenomenon is incompletely understood, but persistent infection occurs in fetuses before the completion of the immune system development, when viral antigens are recognized as self-antigens, resulting in negative selection of BVDV-specific lymphocytes (Grooms 2004). The negative selection of reacting CD4⁺ lymphocytes is highly specific and the exchange of a single amino acid in the BVDV antigen is sufficient to stimulate immune responses in CD4⁺ lymphocytes from PI cattle (Collen et al. 2000a). The lack of immune responses results in widespread distribution of BVDV throughout the host's tissues, and PI cattle shed BVDV in multiple secretions and excretions, including nasal discharge, urine, semen, colostrum, milk, and feces (Baker 1987).

Fetal infections during the period of organogenesis (100 – 150 days of gestation) may result in congenital malformations, which include cerebellar hypoplasia, hypomyelinogenesis, hydranencephaly, alopecia, cataracts, optic neuritis, brachygnathism, hydrocephalus, microencephaly, thymic aplasia, hypotrichosis, pulmonary hypoplasia, and growth retardation (Grooms 2004). Following the completion of immunocompetence and organogenesis, fetuses are able to clear the infection with BVDV and mount an effective immune response. These calves are born with precolostral serum antibody titers and usually appear normal at birth (Grooms

2004). Despite their normal appearance, congenitally infected calves are more likely to develop severe illness during the first month of life, indicating that late gestational infections with BVDV are not benign (Munoz-Zanzi et al. 2003).

Both persistently and acutely infected bulls shed BVDV in semen, and the virus can be transmitted to susceptible cows by natural or artificial insemination (Paton et al. 1990; Kirkland et al. 1991). Although normal semen quality has been reported in some PI bulls, the use of semen from PI bulls negatively affects conception rates (Barlow et al. 1986; Kirkland et al. 1994). This is likely due to a combination of factors, including lower quality semen, ill-thrift of affected bulls, and effects of BVDV on the reproductive tract of exposed cows (Grooms 2004). Although cleared from most organ systems of acutely infected animals, BVDV may reside in the testes of bulls following transient infection and viremia. This phenomenon has been detected after natural and experimental infections and is referred to as prolonged testicular infection (Givens et al. 2009). Prolonged testicular infection with BVDV was first identified in the testes of a seropositive, nonviremic bull at an artificial insemination center (Voges et al. 1998). Despite the absence of viremia and the presence of consistently high concentrations of circulating serum antibodies, semen from this bull contained infectious BVDV throughout the animal's life. Localized, prolonged testicular infections with BVDV have also been experimentally produced following acute infection of peri-pubertal bulls with BVDV (Givens et al. 2003; Givens et al. 2007). Viral RNA was detected in semen for 2.75 years following BVDV exposure, and infectious BVDV was isolated from testicular tissue for up to 12.5 months after BVDV exposure (Givens et al. 2009).

Infections in Persistently Infected Animals (Mucosal Disease)

Although PI calves may survive to reproductive age, many are born weak, stunted, and die shortly after birth (Baker 1995). Persistently infected cattle have a 50% greater risk of leaving the herd before the first year of age and dying or being culled due to ill thrift as compared to normal cohorts, which partly may be due to a poor immune response and susceptibility to opportunistic infections (Roberts et al. 1988; Houe 1993). An additional risk for PI cattle is the development of fatal mucosal disease, which develops when cattle that are PI with a non-cytopathic BVDV become superinfected with a cytopathic strain. The severity of the developing clinical signs depends on the homology between strains, and mucosal disease can be classified as acute, chronic, or delayed onset mucosal disease (Bolin 1995).

Acute mucosal disease occurs when the superinfecting cytopathic strain is in close antigenic homology with the PI strain, and most commonly is the result of mutational events that alter the PI strain's biotype (Tautz et al. 1994). Due to the close homology of both strains, acute mucosal disease results in case fatality rates that approach 100% (Evermann et al. 2005). Because acute mucosal disease affects only PI cattle, the disease is characterized by low morbidity but very high mortality (Evermann et al. 2005). However, in groups of cattle with multiple PI cattle that are infected with similar BVDV strains, outbreaks of mucosal disease have been reported (Odeón et al. 2003). Clinical signs of acute mucosal disease include fever, anorexia, tachycardia, tachypnea, nasal discharges, and profuse, foul smelling diarrhea with mucosal shreds, fibrin casts, and blood. Erosions and ulcers are commonly present on the alimentary epithelia, coronary bands, prepuce, and vulva (Evermann et al. 2005).

Superinfections with heterologous cytopathic strains may result in chronic forms of mucosal disease that are not fatal but result in chronic clinical signs. The source for heterologous

cytopathic strains is commonly vaccine-strains in modified-live vaccines (Bolin 1995). Clinical signs include anorexia, weight loss, diarrhea, bloat, lameness, and erosive and ulcerative lesions on epithelial surfaces (Bolin 1995). Sufficient heterology between the PI strain and the cytopathic strain may result in an immune response that clears the superinfection, enabling the animal's recovery from mucosal disease, but not persistent infection (Bolin 1995).

Under experimental conditions, the incubation period of acute fatal mucosal disease is 7-14 days (Fritzemeier et al. 1997). In contrast, delayed-onset mucosal disease occurs in PI cattle several weeks to months after inoculation with the cytopathic strain (Westenbrink et al. 1989). As for chronic mucosal disease, delayed-onset mucosal disease is caused by a heterologous cytopathic strain from an external source, but clinical signs of mucosal disease occur after RNA recombination events between the PI strain and the heterologous cytopathic strain. This RNA recombination creates a homologous cytopathic strain and subsequent fatal mucosal disease (Ridpath et al. 1995).

Transmission of Bovine Viral Diarrhea Virus

Various direct and indirect routes exist for transmission of BVDV and its introduction into susceptible herds, and this may result in the generation of new PI animals and further propagation of the virus within and among cattle herds (Thurmond 2005; Kadohira et al.). The PI animal is generally accepted to be the most important source for BVDV, and contact of susceptible cattle with PI herd-mates results in many more new infections than contact with acutely infected animals (Thurmond 2005). Shedding of BVDV from PI cattle occurs in most secretions and excretions, resulting in direct transmission to other animals and contamination of housing areas (Lindberg et al. 2004). While believed to be nearly continuous, shedding of BVDV

from PI cattle may be influenced by serum antibodies such as passively acquired colostral immunoglobulins, and the level of antibodies may hamper the detection of viremia by virus isolation (Brock et al. 1998; Thurmond 2005). Some PI cattle may not be identified by the serum immunoperoxidase microtiter assay, complicating their timely removal from herds, but this is rare and screening by the serum immunoperoxidase microtiter assay is considered reliable (Grooms et al. 2001).

Although the prevalence of herds that contain one or more PI animals is estimated to be below 4% in the US, different management strategies that are currently employed by beef and dairy producers aid in the maintenance and dispersal of BVDV (Van Campen 2010). Risk of infection is greater in herds with high numbers of animals and those that purchase new additions (Mockeliuniene et al. 2004; Segura-Correa et al. 2010; Smith et al. 2010). In the United States a trend toward fewer and larger herds exists, frequently prompting the purchase of pregnant replacement heifers from facilities that commingle thousands of cattle (Van Campen 2010). According to epidemiological models, the greatest risk of introducing the virus into BVDV-free dairy herds results from the acquisition of pregnant PI heifers, followed by the birth of a PI calf, and the introduction of transiently infected animals (Ezanno et al. 2008). These heifers, when exposed to BVDV in rearing facilities, may carry a PI fetus that can efficiently introduce the virus into susceptible herds (Van Campen 2010). Because BVDV is associated with reduced fertility and infertile cows are often culled, a vicious cycle may develop that prompts further purchases of at-risk animals from heifer rearing facilities (Van Campen 2010).

Adequate measures of biosecurity are as important for preventing the introduction of BVDV into herds as for preventing the transmission between animals within herds. The birth of PI calves contaminates calving areas and can lead to infection of other neonatal calves by contact

with fetal and uterine fluids and proximity to PI offspring (Lindberg et al. 2004). Segregation of individual age groups, and in particular, the separation of young-stock from lactating cows decreases the risk of intra-herd transmission of BVDV (Ezanno et al. 2008). Although the risk for new infections and births of PI calves is reduced with increased herd immunity, the introduction of a single PI calf into a BVDV-free dairy herd renders the possibility of viral clearance from the herd unlikely (Innocent et al. 1997). Mathematical models based on the framework of susceptible, infectious, and removed animals emphasize the importance of improving herd immunity through vaccination and testing and removal of PI animals (Cherry et al. 1998; Smith et al. 2010). While the prevention of reproductive losses in herds without PI animals necessitates immunity of only 57% of herd members, the presence of a PI calf requires 97% of animals to be vaccinated, and best preventive successes result from removal of PI calves within 5 days (Cherry et al. 1998). Effective clearance of BVDV from herds can be achieved by the removal of PI animals and vaccination, and although each individual effort may remove BVDV from smaller herds, clearance of BVDV from larger herds requires the combination of both strategies (Smith et al. 2010).

While PI animals are central to introduction of BVDV into herds, their absence from herds does not entirely prevent new infections, which is cause for concern for control programs that are mainly focused on the removal of PI calves (Moen et al. 2005; Bodmer et al. 2008). The birth of non-surviving PI offspring that are not available for testing, contact with wildlife, use of contaminated semen or vaccines, airborne transmission, or contact with contaminated personnel or fomites are among the explanations offered for new infections in the absence of PI animals (Niskanen et al. 2002; Simpson 2002; Niskanen et al. 2003; Moen et al. 2005). The persistence of BVDV in immunocompetent cattle following an acute infection may further explain new

infections in biosecure herds that are free from PI cattle. Reports of prolonged infections of the ovaries and testes, semen, and white blood cells demonstrate that BVDV is able to persist in animals that mounted a neutralizing immunity following acute infection (Grooms et al. 1998b; Givens et al. 2003; Collins et al. 2009; Givens et al. 2009). Viremia and seroconversion was observed in cattle that were transfused with whole blood from seropositive animals that had been acutely infected 98 days earlier, indicating the persistence of BVDV in white blood cells of acutely infected animals (Collins et al. 2009). In ovarian tissues, BVDV antigen was detected 60 days following inoculation (Grooms et al. 1998b). Detection of BVDV RNA was possible for seven months from semen of acutely infected bulls, and calves intravenously inoculated with semen collected five months after the initial inoculation became viremic and seroconverted (Givens et al. 2003). While the possibility of BVDV transmission from semen of acutely infected bulls has been established experimentally and under field conditions (Kirkland et al. 1997; Rikula et al. 2008), the potential of viral transmission from bulls with prolonged testicular infections appears to be low, despite evidence of testicular viral RNA and infectious virus for up to 2.75 years and 12.5 months, respectively (Givens et al. 2009).

In addition to BVDV infections by contact with shedding animals or their tissues and secretions, BVDV can be transmitted by indirect routes, such as people and fomites. Without disinfection, BVDV remains infectious for at least one week when surface-dried, and when the virus is dried in plasma the efficacy of disinfectants is inhibited (Terpstra et al. 2007).

Translocation of susceptible cattle into areas that have housed PI cattle immediately after their removal and without proper cleaning may result in BVDV infection (Niskanen et al. 2003; Lindberg et al. 2004). Experimentally, BVDV was not transmitted to calves that were translocated four days after removal of PI cattle (Niskanen et al. 2003). The transmission of

BVDV can also occur by aerosolization, and experimentally, calves became infected when housed in proximity to PI cattle at distances of 1.5 and 10 meters (Niskanen et al. 2003).

Similarly, housing of calves in separate rooms with unidirectional air-flow from PI cattle to susceptible calves resulted in BVDV transmission (Mars et al. 1999).

The role of insects in transmission of BVDV is incompletely understood, but two studies evaluated the potential of biting and non-biting insects to transmit BVDV between PI animals and seronegative animals. The transmission of BVDV by the stable fly (*Stomoxys calcitrans*), the horse fly (*Haematopota pluvialis*), and the head fly (*Hydrotea irritans*) was demonstrated experimentally from a PI animal to calves and ewes (Tarry et al. 1991). In addition, inanimate fomites such as contaminated hypodermic needles, rubber stoppers of vaccine vials, and nose-tongs may be sources of BVDV transmission (Gunn 1993; Lindberg et al. 2004).

Diagnosis

Various diagnostic tests are available to detect infection with BVDV; however, not all available diagnostic tests are applicable to each clinical problem and lack of rational use hampers efforts in solving production problems caused by BVDV (Saliki et al. 2004). Principally, all available diagnostic assays can be grouped into methods that culture BVDV in tissue cells, detect antigen in diagnostic specimens, amplify the RNA genome of BVDV, or detect the presence of antibodies in serum or milk (Saliki et al. 2004).

Virus Isolation

The isolation of BVDV from various specimens, including serum, buffy coat, semen, nasal swabs, and tissue samples remains the “gold standard” of diagnosis (Bruschke et al. 1998; Givens et al. 2003; Saliki et al. 2004). For BVDV diagnostics on live animals, white-blood cells

are the preferred sample for virus isolation as there is no interference by serum neutralizing antibodies and the virus is highly cell-associated. Tissues of the lymphoid system including spleen, thymus, and lymph-nodes are the preferred sample from post-mortem examinations or aborted fetuses (Saliki et al. 2004). Bovine viral diarrhoea virus grows readily in various cell lines, including those of other species, and this may result in difficulties when the virus contaminates cell cultures (Rossi et al. 1980; Wessman et al. 1999; Makoschey et al. 2003).

The most commonly used cell lines for routine BVDV diagnostics are the bovine turbinate (BT), Madine Darby Bovine Kidney (MDBK), and bovine testicle (Btest) cell lines. Successful culture of cytopathic BVDV strains is indicated by vacuolation and cell death within a few days of virus inoculation. For non-cytopathic strains, immunofluorescence or immunoperoxidase staining techniques are utilized to visualize cellular infection with BVDV (Smith et al. 1988; Saliki et al. 1997). Because of the depth-of-column effect, analytical sensitivity is less when small culture vessels are used; however, virus isolation may also be used as a herd screening test in the form of the immunoperoxidase monolayer assay. Although economical, this test uses serum as the diagnostic sample and is not ideal for detection of acute infections or the testing of cattle below three months of age that have acquired colostral immunity. Failure to detect PI cattle with the immunoperoxidase monolayer assay has been reported, but the rate of detection error was extremely low (Grooms et al. 2001).

Antigen Detection

Direct detection of BVDV antigen allows the more rapid and economical testing of samples compared to virus isolation. Antigen detection can be performed by the immunofluorescence, immunohistochemistry, and ELISA techniques. In general, the analytical sensitivity and specificity of antigen detection assays are less than those of virus isolation, which

accommodates the detection of PI cattle rather than of acute infections. In contrast to the traditional laboratory identification of PI cattle using two positive virus isolations performed three weeks apart, the commercially available ELISA kits and immunohistochemistry are used to identify PI cattle with a single sample (Njaa et al. 2000). Several studies have demonstrated excellent results for antigen capture ELISA and immunohistochemistry for use as screening tests for PI cattle (Thur et al. 1996; Edmondson et al. 2007; Hilbe et al. 2007; Fulton et al. 2009). False positive results due to acute infections have been reported to be rare, and the antigen capture ELISA and immunohistochemistry tests can be utilized to test calves before the waning of colostral immunity, which interferes with other assays (Brodersen 2004; Saliki et al. 2004). Antigen detection is most frequently performed on ear-skin biopsies, but various samples including tissues, whole blood, serum, milk, and nasal swabs are suitable after appropriate sample preparation (Saliki et al. 2004).

Reverse Transcription – Polymerase Chain Reaction

The description of the genomic sequence of BVDV and the development of suitable probes and commercially available kits has enabled the widespread use of RT-PCR as a routine diagnostic method (Driskell et al. 2006). The technique is highly sensitive and can be utilized for various diagnostic samples including milk, urine, tissues, serum, whole blood, semen, and embryos (Hamel et al. 1995; Drew et al. 1999; Givens et al. 2001; Kim et al. 2003; Kennedy et al. 2006; Tajima et al. 2008). The importance of validation of an assay for use with different samples has been emphasized as the assay sensitivity depends not only on primer sets but also on the RNA extraction procedure (Saliki et al. 2004). Although RT-PCR techniques can be used on diagnostic specimens that are fixed in formalin, RNA fragmentation occurs and reduces the assay sensitivity (Finke et al. 1993).

The high sensitivity of RT-PCR exceeds that of virus isolation, and the assay can detect the viral RNA of a single viremic sample in a pool of 100 negative sera (Weinstock et al. 2001). The ability to pool samples such as milk, serum, or whole blood offset the initial higher cost of the assay and allows an economical screening for PI animals. Pooling strategies using skin biopsies or blood samples rather than bulk-tank milk do not abolish the need to collect a sample from each animal in the herd (Kennedy et al. 2006). In milk, BVDV was detected by RT-PCR in samples that were serially diluted to 1:640 (Radwan et al. 1995). The number of samples that is optimal for pooling has been controversial and pools of 30 serum samples to 100 samples have been recommended (Kennedy 2006; Smith et al. 2008). The economic benefits of sample pooling decrease when greater prevalence rates exist in populations, and the competitive benefit of pooling is diminished when prevalence rates exceed 3% (Munoz-Zanzi et al. 2000).

Serology

The detection of serum antibodies that are directed against BVDV can be performed by various assays but the ELISA and serum neutralization tests predominate and have replaced indirect immunoperoxidase or indirect immunofluorescence techniques (Muvavarirwa et al. 1995). With appropriate application, serological tests allow the assessment of vaccine efficacy and compliance with established vaccination protocols, the detection of herd exposures to BVDV, and the association of clinical signs with BVDV infection. Difficulties exist in determining the etiology of detected antibodies, as seroconversion may be the result of acute infections, vaccination, or transfer of maternal immunity through colostrum. Commercial blocking ELISA assays that detect antibodies directed against the NS3 protein of BVDV are unable to differentiate antibodies derived from natural infection or vaccination, and marker vaccines are currently unavailable for BVDV (Raue et al. 2010).

Serologic testing of previously unexposed sentinel animals can be utilized to detect the presence of PI cattle in herds (Pillars et al. 2002). However, the analysis and comparison of serological titers does not allow a clear distinction between PI-free herds from those with PI cattle (Waldner et al. 2005). Variations in the results of serological tests are common and exist between different ELISA and serum neutralization tests, as well as different laboratories. Only minimal agreement ($\kappa = 0.15$) between ELISA and serum neutralization assays for the prediction of seroconversion existed in one report (Taylor et al. 1995). In addition to differences between ELISA and serum neutralization test, the antigenic differences among BVDV strains and the lack of a reference strain can result in large variations among determined antibody titers. The use of different BVDV strains can result in 10- to 100-fold differences in antibody titers, and therefore monoclonal antibody-binding assay are used for differentiation of isolates (Wensvoort et al. 1989; Dekker et al. 1995; Saliki et al. 2004).

Chapter 2: Journal article:

Bovine Viral Diarrhea Virus Infections in Heterologous Species

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Review Article

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Abstract

Infections with *Bovine viral diarrhoea virus* (BVDV) are not limited to cattle, but may be detected in various species in the mammalian order Artiodactyla. Despite epidemiological evidence of BVDV infections in species other than cattle, current knowledge regarding the impact of BVDV on heterologous species is incomplete. In heterologous hosts, BVDV infections with clinical signs analogous to those in cattle have been described and include disease of multiple organ systems, most notably the reproductive tract and immune system. Clinical infections may negatively impact the health and well-being of heterologous species, including camelids and captive and free-ranging wildlife. Of additional importance are BVDV infections in small ruminants and swine where difficulties arise in laboratory testing for *Border disease virus* and *Classical swine fever virus*, respectively. Pestiviruses are antigenically closely related and their cross-reactivity requires additional efforts in virological testing. In cattle populations, persistently infected animals are considered the main source of BVDV transmission. This phenomenon has also been detected in heterologous species, which could facilitate reservoirs for BVDV that may be of great importance where control programs are in progress. This review summarizes the current epidemiological and clinical knowledge on heterologous BVDV infections and discusses their implications.

Introduction

Pestiviruses of the family *Flaviviridae* are small enveloped RNA viruses that have substantial impact on the livestock industries. Pestivirus isolates are genetically and antigenically related, which in the past has provided difficulties with classification. Traditional classification of viral isolates according to mammalian host of origin may not always be accurate, as pestiviruses do not demonstrate strict host specificity (Nettleton 1990). More recent molecular techniques have allowed classification of Pestivirus species according to genotypic diversity rather than animal host (Giangaspero et al. 2007). Currently, the genus Pestivirus is comprised of *Bovine viral diarrhea virus* (BVDV) 1, BVDV 2, *Border disease virus*, and *Classical swine fever virus* (CSFV). Recent description of genetically distinct pestiviruses such as the genotypes Pronghorn pestivirus, Giraffe pestivirus, strain V60 (Reindeer-1), Ho_Bi strain, and others may lead to further rearrangements of classification (Dekker et al. 1995; Fischer et al. 1998; Avalos-Ramirez et al. 2001; Becher et al. 2003; Schirrneier et al. 2004; Vilcek et al. 2005b).

Pestiviruses employ various strategies that ensure their survival and successful propagation in mammalian hosts, including suppression of the host's immune system, transmission by various direct and indirect routes, and, perhaps most importantly, induction of persistently infected hosts that shed and transmit BVDV more efficiently than other sources. Accumulating evidence suggests that pestiviruses are able to infect various mammalian host species in the order Artiodactyla, which may play an important role in the viral survival strategy (Vilcek et al. 2006). The implications of heterologous pestivirus infections are twofold. First, infection of hosts other than cattle may drastically handicap the successful implementation of

BVDV control strategies if these hosts become reservoirs; and second, clinical disease in other mammalian hosts such as zoo animal and wildlife species may be observed.

Among pestiviruses, BVDV has been the species most intensely studied as an infectious agent of heterologous hosts. Evidence of BVDV infection in species other than cattle has been detected mainly by seropositivity, especially in wildlife species. Although the presence of antibodies may provide only limited information as to the role of a species in the ecology of BVDV, high seroprevalence rates within populations suggest the existence of endemic cycles (Van Campen et al. 2001b). Bovine viral diarrhea virus infections have been documented in domestic small ruminants and swine; free-ranging and captive wildlife; and in camelids, and the epidemiology of BVDV infection may revolve around these proposed host clusters (Evermann 2006). Within these proposed host clusters intra-host maintenance of BVDV occurs but the inter-cluster transmission requires optimal conditions or may be negligible (Evermann 2006). This review will summarize BVDV infections in each host cluster as well as swine and discuss their implications.

Bovine viral diarrhea virus in domestic small ruminants and swine

Goats and Sheep

Among Artiodactyls, sheep and goats may have a unique role in the ecology of BVDV. Sheep and goats are phylogenetically more closely related to cattle than most other species considered in this review; therefore, the chance to establish permissive infections is high (DeFillipis et al. 2000). Furthermore, sheep and goats may have close contact with cattle under certain management conditions, such as subsistence agriculture, alpine farming, or hobby farming, thus contributing to BVDV infections in small ruminants. Although one report did not

identify a positive correlation between seropositivity of sheep flocks and presence of cattle (Graham et al. 2001), other studies reported significantly greater seroprevalence rates in sheep on farms with cattle (Tegtmeier et al. 2000; Krametter-Frotscher et al. 2007), especially where communal alpine pasturing of sheep, goats, and cattle was practiced (Krametter-Frotscher et al. 2007).

Several serological surveys have demonstrated the occurrence of BVDV in small ruminants (Table 2.1); however, cross-reactivity of pestivirus antibodies warrants caution when evaluating the results of such surveys. Surprisingly, in recent reports on pestiviral seroprevalence in sheep, cross-neutralization assays identified greatest reactivity against BVDV 1 rather than BDV (Graham et al. 2001; Schleiner et al. 2006; Krametter-Frotscher et al. 2007; Mishra et al. 2009). In Argentina, which is considered free from BDV, 79.7% (43/54 sheep) of samples were seropositive to BVDV 1 or BVDV 2, indicating BVDV circulation in Patagonian populations (Julia, et al. 2009). This is contrasted by another recent report where the majority of seropositive sheep resulted from BDV infection, while the infecting pestivirus in goats remained largely unresolved, as indicated by cross-neutralization assays (Danuser et al. 2009). When antibodies against pestivirus are detected in small ruminants, the distinction of pestivirus species may hold critical information on epidemiology, prevention and control (Konig et al. 2003).

From reports on pestiviral infections in small ruminants prior to the 1990's, determination of whether BDV or BVDV was the infecting species may be difficult. Of 53 viral strains designated as BDV in the nucleotide sequence databases, 22 were revealed to be BVDV by genotyping based on palindromic nucleotide substitutions (Giangaspero et al. 1999). Infections with both species of BVDV have been reported in domestic small ruminants worldwide (Paton et al. 1995; Pratelli et al. 2001; Yadav et al. 2004; Kim et al. 2006;

Willoughby et al. 2006; Krametter-Frotscher et al. 2007; Mishra et al. 2007; Mishra et al. 2008). The inverse, infection and clinical signs of pestivirus infection in cattle associated with BDV appears to be rare (Schirrmeyer et al. 2008). Therefore, determination of which pestivirus species is cause for infection in small ruminants is especially important where BVDV control programs for cattle are in progress.

Infections with BVDV in domestic small ruminants result in clinical signs of Border disease. Postnatal infections commonly cause mild clinical signs, including pyrexia and leukopenia (Taylor et al. 1977). Experimental inoculation of juvenile sheep by the intranasal, intratracheal, and intrabronchial routes resulted in anorexia, tachycardia, pyrexia, and lung lesions especially associated with the pulmonary vasculature (Meehan et al. 1998). Strain-dependent severity of BVDV-associated pathology was observed in 62 gnotobiotic lambs infected with one of ten BVDV strains, and pyrexia, pneumonia, myocarditis, and encephalitis were observed (Jewett et al. 1990). Infections with BVDV in pregnant small ruminants may result in *uteroplacental* pathology and pregnancy loss by fetal resorption or abortions (Loken et al. 1991a). The outcome of transplacental infection depends on the length of gestation and biotype of infecting BVDV strain. Fetal mummification, stillbirth, premature birth, and birth of offspring with congenital disorders may result from *in utero* BVDV infection. Congenital disorders of affected fetuses commonly involve the central nervous system, and neuropathogenicity may be more severe with cytopathic biotypes (Gruber et al. 1995; Hewicker-Trautwein et al. 1995). Although fetal death and non-viability of lambs are common sequelae of transplacental BVDV in sheep, reports of viable PI offspring exist (Carlsson et al. 1994; Scherer et al. 2001). In contrast to cattle and sheep, viable PI kids appear to be a rare result of *in utero* BVDV infection in goats and reproductive failure or severe gross and histological pathology of

affected fetuses are the likely result of infection (Loken et al. 1991a; Kim et al. 2006; Broaddus et al. 2009).

Swine

The close antigenic relationship between ruminant pestiviruses and CSFV has important implications for diagnostic testing and control of CSFV. Currently, examination of blood samples for CSFV antibodies relies on a combination of ELISA and virus neutralization techniques, and considerable efforts are necessary in establishing a CSFV-specific diagnosis (de Smit et al. 1999). During the CSFV outbreak in the Netherlands in 1997/1998, 26.5% of CSFV ELISA positive samples were demonstrated to be caused by presence of antibodies to ruminant pestiviruses (de Smit et al. 1999). The presence of antibodies against ruminant pestivirus in swine resulting in suspicion of CSFV infection has prompted the unnecessary depopulation of herds, further emphasizing the impact of porcine infections with ruminant pestiviruses (Snowdon et al. 1968; Oguzoglu et al. 2001).

Comparative cross-neutralization experiments or monoclonal antibodies have been commonly used to differentiate the causative viral species in seropositive samples (Liess et al. 1990). However, CSFV challenge in swine with prior BVDV infection resulted in higher titers against BVDV than CSFV according to the neutralization peroxidase linked assay, indicating that prior BVDV infection could result in false-negative CSFV diagnostics (Wieringa-Jelsma et al. 2006). Furthermore, a recently developed E2 subunit CSFV marker has been used in combination with an antibody-ELISA against the E^{RNS} to differentiate vaccinated pigs from pigs exposed naturally to CSFV; however, BVDV infection and subsequent seroconversion may result in false positive CSFV serologic testing, complicating the use of this vaccination and testing strategy in a control scenario (Blome et al. 2006; Loeffen et al. 2009).

After the first description of an antigenic relationship between CSFV and BVDV in 1960 (Darbyshire 1960), scientific interest has revolved around the use of BVDV to immunize swine against CSFV (Beckenhauer et al. 1961; Simonyi et al. 1967; Baker et al. 1969; Liou et al. 1975). Difficulties arise when comparing these studies, as various BVDV isolates or vaccine titers were used for immunization, which may explain some of the discordant results. In extensive studies using a BVDV strain Oregon C24V vaccine prior to challenge with different CSFV laboratory and field isolates, Baker and colleagues demonstrated protection rates of up to 95% in challenged vaccinates (Baker et al. 1969). Furthermore, BVDV vaccination was safe, provided protection from clinical CSFV infection for over two years, and BVDV was not spread to in-contact pigs or cattle (Baker et al. 1969). In contrast, all but one pig immunized with BVDV C24V died after challenge with CSFV (Simonyi et al. 1967). When immunizing swine with one of four strains of BVDV and subsequent challenge with CSFV, no clinical signs were observed after immunization with BVDV strain Tobias, while mild clinical signs of disease were observed in swine immunized with BVDV strains NY-1 or VJGM, and typical signs of classical swine fever were observed in swine immunized with BVDV strain Egan (Liou et al. 1975). In a comparison of vaccination efficacy of a BVDV strain NY-1 vaccine with a modified live CSFV vaccine, BVDV vaccinated swine were not as well protected as CSFV vaccinates when challenged with one of ten CSFV isolates (Tamoglia et al. 1965). In that study, transmission of vaccine virus was observed in CSFV vaccinates, but not in BVDV vaccinates (Tamoglia et al. 1965). Recovery of BVDV was possible from the respiratory tract of swine vaccinated with strain CL10, indicating that BVDV may be transmitted by vaccinates (Phillip et al. 1972). More recently, the BVDV strain St. Oedenrode, which had been isolated from a naturally infected pig, was used to immunize swine prior to CSFV challenge (Wieringa-Jelsma et al. 2006). Immunization with this

strain resulted in a self-limiting BVDV infection ($R = 0.2$) and protected vaccinates from clinical disease and CSFV transmission upon challenge (Wieringa-Jelsma et al. 2006).

Only a limited number of serosurveys have been performed in domestic swine and reported seroprevalence rates are relatively low when compared to other domestic livestock. Risk factors associated with seropositivity of domestic swine include the presence of cattle on the same farm, high density of small ruminants near swine populations, vaccination with BVDV contaminated vaccines, and age of tested swine (Lenihan et al. 1977; Vannier et al. 1988; Liess et al. 1990; Loeffen et al. 2009). In an Irish serosurvey, 27.8% of swine in contact with cattle possessed neutralizing antibodies to BVDV, as compared to 4.0% in swine without contact with cattle (Lenihan et al. 1977). The feeding of bovine offal was also described as a source of BVDV infection in swine (Stewart et al. 1971). Increasing specialization of farms resulting in decreased chances for interspecific contacts and improved quality control in vaccine production were suggested to be reasons for a decrease in seroprevalence rates in Dutch swine, where 20.0% of slaughterhouse samples from sows and boars were positive in 1991 (Terpstra et al. 1991), as compared to 2.5% in sows and 0.42% in finishing pigs, and 11.0% and 3.2% at the respective herd level in 2009 (Loeffen et al. 2009). Only one of 660 serum samples was determined to be positive for BVDV antibodies in a study in Northern Ireland, and the authors suggested that use of a Border disease virus ELISA rather than BVDV ELISA for initial serosurveillance may have confounded this comparatively low result (Graham et al. 2001). Other reports found seroprevalence rates of 3.01% in Canada, 6.4% in Denmark, 3.2% in Ireland, and 2.2% in Norway (Jensen 1985; Afshar et al. 1989; Loken et al. 1991b; O'Connor et al. 1991).

Studies on postnatal BVDV infections in non-pregnant swine have rarely described the occurrence of clinical signs based on observation of infected animals (Beckenhauer et al. 1961;

Carbrey et al. 1976; Liess et al. 1990; Dahle et al. 1993; Walz et al. 1999a; Woods et al. 1999; Makoschey et al. 2002). When evaluated, an elevation of the rectal temperature after BVDV inoculation was not detected in most studies despite evidence of viremia and seroconversion (Beckenhauer et al. 1961; Carbrey et al. 1976; Walz et al. 1999a). Experimental infection of six to ten-week-old pigs with a BVDV 2 inoculum containing non-cytopathic and cytopathic biotypes, resulted in slight pyrexia, leukopenia, and thrombocytopenia (Makoschey et al. 2002), which were less pronounced than had been reported in cattle experimentally inoculated using a very similar protocol (Makoschey et al. 2001). Significant, but less severe than observed with homologous pestivirus infections, decreases in lymphocyte populations were detected in intranasally inoculated pigs and a more marked decrease in CD8⁺ lymphocytes was noticed (Rypula 2003). Experimental infection of eight-week-old pigs with either BVDV 1 or BVDV 2 did not result in clinical signs or increases in body temperature in any group, despite the use of a BVDV 2 isolate that was demonstrated to be virulent for cattle (Walz et al. 1999a). In contrast, inoculation of three-day-old piglets with either BVDV strain NY-1 or a cell-culture contaminant, BVDV-C, resulted in loss of appetite, diarrhea, and intestinal pathology for one to four days (Woods et al. 1999). Importantly, the co-infection of either BVDV NY-1 or BVDV-C with attenuated Transmissible gastroenteritis virus resulted in severe clinical signs that were worse with the cell-culture contaminant BVDV-C (Woods et al. 1999). That report underscored the potential impact of cell culture contamination with BVDV, which may result in enhanced pathogenicity of attenuated vaccines (Woods et al. 1999).

Carbrey and colleagues suspected BVDV infection as the cause of reproductive failure, including poor conception, small litter size, and abortions after antibody titers against BVDV were determined to be greater than against CSFV in breeding sows (Carbrey et al. 1976).

Experimental infection of pregnant gilts with one of four BVDV isolates resulted in reproductive disease including intrauterine infection, pregnancy loss, and reduction in litter size (Stewart et al. 1980). In that study, fetal infection was established in one of twenty infected gilts (Stewart et al. 1980). Similarly, experimental transplacental infection was documented in only one of 43 fetuses after intranasal inoculation of four pregnant gilts, indicating an inconsistent ability of BVDV to cross the blood-placental barrier in swine (Walz et al. 2004). Seroconversion, failure to thrive, and premature death have been reported in transplacentally infected swine (Carbrey et al. 1976; Paton et al. 1992). Petechiation and hemorrhages, enlarged lymph nodes, meningitis, myocarditis, nephritis and hepatic necrosis were observed in piglets born to a pregnant sow that was exposed to a persistently infected calf (Paton et al. 1992). Contamination of a CSFV vaccine with BVDV resulted in dead or mummified fetuses, and many living fetuses from vaccinated sows were BVDV positive and showed alopecia, congenital tremors, lymphadenopathy, multifocal petechiation, and ascites. Experimental infection with one of four strains of BVDV between 28 to 55 days of gestation resulted in fetal infection in only one of 20 gilts (Stewart et al. 1980). The authors suggest that passages in porcine PK-15 cells may have resulted in a loss of virulence and the inability to cause transplacental infection in the other BVDV strains evaluated in the experiment (Stewart et al. 1980). In the transplacentally infected 13 fetuses, low antibody titers (<1:4 – 1:16) and histopathological lesions within leptomeninges and choroid plexus were detected (Stewart et al. 1980). In contrast, antibodies against BVDV did not develop in a persistently viremic pig until slaughter at 26 months and in a littermate, seroconversion was not detected until six to eight months of age (Terpstra et al. 1997). In the affected litter of 13, ten piglets died or were euthanized within weeks after birth and multiple clinical signs, including thymic atrophy, were detected. Consistent shedding of BVDV until slaughter was detected only

in the seronegative PI pig and ceased in its littermates upon seroconversion (Terpstra et al. 1997).

Bovine viral diarrhea virus in New and Old World Camelids

Bovine viral diarrhea virus infections were identified through detection of antibodies as early as 1975 in Old World camelids (OWC) and 1983 in New World Camelids (NWC) (Burgemeister et al. 1975; Doyle et al. 1983). In different countries, seroepidemiologic studies identified BVDV antibodies in zero to 19.7% of tested OWC (Burgemeister et al. 1975; Hedger et al. 1980; Bornstein et al. 1987; Bohrmann et al. 1988; Wernery et al. 1990), but a much greater seroprevalence (52.5%) was reported from two regions in Egypt (Zaghawa 1998). Wernery and Wernery explained a higher seroprevalence rate in breeding camels of 9.2% as compared to 3.6% in racing camels by close contact with cattle herds, more intensive management, and large herd sizes in breeding camels (Wernery et al. 1990). While seroprevalence was not different between male and female dromedary camels, older age was associated with an increased rate of seropositivity (Raofi, et al., 2009).

In an outbreak of reproductive disease among nine pregnant dromedary camels, abortion, still births, weak calves, early neonatal death, and neonatal hemorrhagic disease were observed (Hegazy et al. 1996). Gross and histopathological lesions included congenital cataracts, mild cerebellar hypoplasia, lymphoid depletion, vasculitis, optic neuritis, and underpopulated cerebellar molecular layer. Cytopathic BVDV was isolated from two stillborn and one live-born camel calves (Hegazy et al. 1996). Viral isolates were further characterized later by the same laboratory and were typed as BVDV 1 and BVDV 2 (Yousif et al. 2004). Noncytopathic BVDV

has also been isolated from tissues of camels in which histopathological changes were consistent with a BVDV infection (Wahab et al. 2005).

In the past, seroepidemiologic and experimental infection studies suggested that BVDV may cause infections without presenting a serious risk to NWC. Low seroprevalence rates, infections with mild or lack of clinical signs, and limited viral replication within buffy coat cells supported the conclusion that BVDV may not be a significant pathogen in NWC (Mattson 1994; Evermann 2006). Experimental inoculation of four pregnant llamas with a noncytopathic isolate from an aborted llama fetus did not result in clinical signs or fetal infection (Wentz et al. 2003). Reported seroprevalence rates in South American domestic NWC are 11.1% in Peruvian alpacas; 2.05% in Argentinean llamas; and, 10.8% and 14% in Chilean alpacas and llamas, respectively (Rivera et al. 1987; Puntel et al. 1999; Celedon et al. 2001). Seropositivity was associated with presence of other domestic livestock, and domestic NWC without exposure to other livestock or free-ranging NWC were seronegative (Karesh et al. 1998; Celedon et al. 2001). In North America, a seroprevalence rate of 4.4% was reported for llamas in Oregon (Picton 1993). In southern California, 18.6% of tested alpacas were seropositive, and antibody titers were greater against BVDV 1 than BVDV 2 in most cases (Shimeld 2009). The greatest titers were detected on farms on which PI crias were present (Shimeld 2009). Antibodies against BVDV were detected in crias from 16 of 63 (25.4%) herds in the United States (Topliff et al. 2009).

Reports of BVDV isolation and identification of persistent infections in alpacas have prompted increasing attention by industry and the scientific community, and the virus is now considered an emerging pathogen of NWC (Byers et al. 2009). Preceding the descriptions of persistent infections in alpacas, a cytopathic BVDV isolate was identified in kidney, liver, lung, and spleen samples from a stillborn llama (Belknap et al. 2000). In the same report, BVDV was

detected in tissues of a late pregnant adult llama and an emaciated juvenile llama. Non-cytopathic BVDV 1b isolates were detected in tissues of a stillborn alpaca (lung, liver, kidney, and spleen) and tissues from a seven-month-old alpaca (lung, liver, and kidney) with ill-thrift (Goyal et al. 2002; Foster et al. 2005). The first description of a persistent infection in alpacas was made in Canada following natural exposure of a pregnant alpaca to a chronically ill cria (Carman et al. 2005). From the persistently infected cria, a BVDV 1b strain was isolated from buffy coat cells on different occasions before euthanasia of this animal. Several cases of PI alpacas have since been reported in North America and Great Britain (Mattson et al. 2006; Foster et al. 2007; Barnett et al. 2008; Byers et al. 2009; Kim et al. 2009). Persistently infected alpacas may survive for several months but are affected by low birth weight, failure to thrive, inappetence, lethargy, chronic diarrhea, and chronic recurrent infections especially of the respiratory tract. In PI alpacas, BVDV antigen may be detected in epithelial and immune cells of many tissues, including skin, brain, thyroid gland, parotid salivary gland, testis, prostate, esophagus, gastric compartments, kidneys, bone marrow, liver, lung, thymus, and lymph nodes, and skin staining patterns are consistent with those in cattle (Carman et al. 2005; Foster et al. 2007; Byers et al. 2009).

From NWC in Chile, both BVDV 1 and BVDV 2 have been isolated (Celedon, et al., 2006). In contrast, in reports on BVDV isolations from alpacas in North America and the United Kingdom in which the subgenotype of the infecting isolate was determined, all isolates belonged to BVDV 1b (Carman et al. 2005; Foster et al. 2005; Foster et al. 2007; Byers et al. 2009). A recent study analyzed 46 BVDV isolates from over 12,000 North American alpacas and classified all isolates as non-cytopathic strains of subgenotype 1b. Nucleotide identity of $\geq 99\%$ was demonstrated in 45 of 46 isolates using the highly conserved 5'-UTR, indicating an unusual

association of the 1b genotype with BVDV infections in North American alpacas (Kim et al. 2009). Kim and colleagues offer two explanations for this peculiarity, firstly that exposures of alpacas to BVDV are rare and spread of the existing BVDV 1b strain is by extensive movement of a few PI animals or secondly, only unique 1b subgenotypes are able to establish transplacental infections in alpacas (Kim et al. 2009). Interestingly, the simultaneous inoculation of pregnant alpacas with two BVDV 1b isolates of cattle or alpaca origin, respectively and a BVDV 2 strain of cattle origin, resulted in birth of crias PI with 1b of cattle or alpaca origin, but not BVDV 2 (Edmondson et al. 2009). This may further support a unique role of BVDV 1b in alpacas; however, viremia, nasal shedding, and seroconversion were observed when alpacas were inoculated with BVDV 1b or BVDV 2 strains (Johnson et al. 2009). Movement of alpacas (including dams with cria by foot) between farms, mainly for breeding purposes, are common practice and were described in reports of reproductive disease and birth of PI offspring, highlighting the importance of sound biosecurity practices (Carman et al. 2005; Foster et al. 2007; Topliff et al. 2007; Barnett et al. 2008).

Bovine viral diarrhea virus in Wildlife

In addition to the ability to induce persistent infections, the large number of wildlife species in which infections with BVDV have been demonstrated, emphasizes the potential for another important survival strategy of BVDV, the ability to cross species-barriers. With few exceptions, infections with BVDV appear to be limited to species in the order Artiodactyla. Antibodies were detected in 2 of 44 Bennett's wallaby (*Macropus rufogriseus*), however, the titers against BVDV C24V were at dilutions of less than 1:3 (Munday 1972). Antibodies against

BVDV were also detected in free-ranging rabbits (*Oryctolagus cuniculus*), but virus isolation and detection of viral genome from spleen samples were not successful (Frolich et al. 1998).

The mammalian order Artiodactyla is comprised of ten families and 240 species (Grubb 2005), and evidence of BVDV infection exists in the seven families Antilocapridae, Bovidae, Camelidae, Cervidae, Giraffidae, Suidae, and Tragulidae, including over 50 species (Nettleton 1990; Van Campen et al. 2001b; Grondahl et al. 2003). In most species, evidence of BVDV infection mainly has been by the identification of antibodies, therefore it may be difficult to estimate the implications on health of affected species or on BVDV control programs; however, identification of high seroprevalence rates may suggest the presence of endemic cycles (Van Campen et al. 2001b). Several seroepidemiological studies in free-ranging wildlife have been performed globally and large variations of seroprevalence status exist among the studied populations (Table 2.2). The scale, both of the number of samples collected and the size of the sampled habitat, and the employed sampling strategies vary tremendously among serosurveys, perhaps partially explaining the observed variations in seroprevalence.

Although sources of BVDV infections in free-ranging wildlife are unknown, a likely source is contact with cattle, as has been suggested by various authors (Riemann et al. 1979; Stauber et al. 1980; Kocan et al. 1986; Nielsen et al. 2000). This is further supported by the absence of antibodies in deer that were without contact with cattle for over 50 years (Sadi et al. 1991). Significantly greater seroprevalence rates were found in white-tailed deer on ranches where cattle were present as compared to ranches without cattle (Cantu et al. 2008). In contrast, an association between BVDV seroprevalence and cattle density was not found in another study from Germany (Frolich 1995). Similar findings were described in a recent report from Minnesota where a greater percentage of deer were seropositive in a region with lower cattle density (Wolf

et al. 2008). The authors of that report concluded that cattle use and management (i.e. dairy or beef) may have an important impact on interspecific BVDV transmission, as there is likely less wildlife contact with housed dairy cattle as compared to pastured beef cattle (Wolf et al. 2008). This factor was not evaluated in the earlier publication from Germany where the majority of cattle operations are dairies (Anonymous 2008). Various factors likely influence transmission of BVDV between cattle and wildlife as has been described for other pathogens such as bovine tuberculosis (Schmitt et al. 2002). With presence of suitable environmental and management factors, transmission of BVDV from cattle to deer is possible as has been demonstrated recently in a cohabitation experiment (Passler et al. 2009c). In that study, BVDV was efficiently transmitted from PI cattle to white-tailed deer and resulted in seroconversion in all adult deer and PI offspring in four of seven pregnancies (Passler et al. 2009c).

Maintenance of BVDV within a cervid population without presence of cattle was suggested by the presence of antibodies in over 60% of caribou that had not been in contact with domestic ruminants for over 25 years (ElAzhary et al. 1981). The presence of endemic infections with BVDV as indicated by high seroprevalence rates was also suggested in reindeer in Norway and US cervid populations (Stuen et al. 1993; Aguirre et al. 1995; Van Campen et al. 2001b; Lillehaug et al. 2003). This view is further supported by identification of high seroprevalence rates in eland populations of Zimbabwe, in which a PI and a virus-isolation-positive eland were identified (Anderson et al. 1998). In a follow-up study, the non-cytopathic strains isolated from elands were further characterized with monoclonal antibodies and partial genetic sequencing and were demonstrated to be very similar to the BVDV 1a strains NADL and SD1, indicating that cattle had originally introduced BVDV into the eland population (Vilcek et al. 2000).

Only a limited number of studies have surveyed free-ranging wildlife populations for the presence of BVDV or BVDV antigen. Anderson and Rowe utilized an antigen capture ELISA to detect BVDV in a subset of 303 seronegative animals during their serosurvey in Zimbabwe and detected two virus positive eland (Anderson et al. 1998). In Germany, cytopathic BVDV was detected in spleen samples from two of 203 deer, and both animals were seronegative roe deer (Frolich et al. 1995). These isolates were further characterized by monoclonal antibody typing and sequencing of the 5'UTR, and results indicated that distinct BVDV strains may circulate within the sampled roe deer population (Fischer et al. 1998). Recently, three surveys utilizing IHC or ELISA techniques investigated the occurrence of BVDV in free-ranging cervids in the US and results suggest that PI cervids exist in wildlife populations (Duncan et al. 2008b; Passler et al. 2008; Pogranichniy et al. 2008). In Alabama, one of 406 skin samples from white-tailed deer was positive on IHC and the antigen distribution resembled that of PI cattle (Passler et al. 2008). Similarly, the skin sample of one of 5597 deer in Colorado was positive on IHC and this result was confirmed by detection of viral RNA in skin and lymph node samples (Duncan et al. 2008b). In Indiana, two of 745 white-tailed deer were positive for BVDV by antigen capture ELISA with subsequent isolation of cytopathic and non-cytopathic BVDV (Pogranichniy et al. 2008). To date, the validation of BVDV assays for their use in wildlife has not been performed, and this may be critical as considerable variations were observed among the IHC and antigen capture ELISA on skin samples of white-tailed deer (Passler and Walz, unpublished observations). In addition to surveys, isolation of BVDV was successful in free-ranging roe deer in Hungary, a mule deer in Wyoming, and two white-tailed deer in South Dakota (Romvary 1965; Van Campen et al. 2001b; Chase et al. 2008). In these reports, clinical illness including

emaciation, weakness, and death prompted further investigations leading to the isolation of BVDV from tissues.

In addition to free-ranging populations, BVDV has been identified in captive herds and zoo collections, and contact with other ruminant species was described in some reports (Nettleton et al. 1980; Neumann et al. 1980; Weber et al. 1982; Doyle et al. 1983; Grondahl et al. 2003; Nelson et al. 2008). Although the role of BVDV as a pathogen of wildlife is largely unclear, isolation of BVDV from clinically ill wildlife emphasizes the potential for BVDV to induce disease in zoo collections. The isolation of BVDV from deceased captive deer submitted for necropsy was successful in different reports; however whether clinical BVDV infection was cause for the fatalities was mostly uncertain (Neumann et al. 1980; Weber et al. 1982; Diaz et al. 1988). Severe mucosal disease-like lesions were observed in a nilgai, an axis deer, and a barasingha deer that were co-infected with BVDV and the virus of malignant catarrhal fever, suggesting a possible immunosuppressive effect of BVDV in wildlife (Doyle et al. 1983). The close vicinity of ruminant species in zoo collections increases the potential for disease transmission and prompted the euthanasia of six PI mousedeer, emphasizing the threat of BVDV for captive endangered species (Grondahl et al. 2003; Uttenthal et al. 2006). In addition to the aforementioned species, the presence of persistent infection was recently demonstrated in a captive mountain goat (Nelson et al. 2008).

The BVDV antigen distribution in two PI white-tailed deer was similar to that of cattle with broad tissue distribution, most notably in epithelium and vascular endothelium (Duncan et al. 2008a). The role that PI wildlife may have in the epidemiology of BVDV, including frequency of occurrence, transmission potential, and survivability is largely unknown. Seroconversion and viral RNA were detected in a calf exposed to a PI Lesser Malayan

mousedeer by indirect and direct routes; however BVDV infection was not evident in a second calf exposed by the same routes (Uttenthal et al. 2006). The maternal line of PI mousedeer remained without clinical signs and was reproductively sound, giving birth to PI offspring (Uttenthal et al. 2006). Experimental inoculation of white-tailed deer fawns did not result in clinical signs, but BVDV was identified by RT-PCR on nasal and rectal swab samples, indicating the potential of transmission (Raizman, et al., 2009). In a PI white-tailed deer, virus isolation titers from nasal swab samples were equivalent to those in PI cattle (Passler et al. 2007). The cohabitation of pregnant white-tailed deer with a PI white-tailed deer resulted in seroconversion of all exposed animals and birth of a PI fawn, indicating that maintenance of BVDV in cervid populations is possible when a PI animal is present (Passler et al. 2009a). Superinfection of a PI mousedeer with a cytopathic BVDV strain of partial antigenic homology did not result in clinical signs of mucosal disease (Semrau et al. 2008). In contrast to PI Lesser Malayan mousedeer, persistent infection may result in decreased survivability in white-tailed deer (Passler et al. 2009a). This may have implications on the risk of transmission, but would also affect the ability to detect PI white-tailed deer in hunter-harvested samples that are usually collected from adult deer.

Few reports on the clinical picture and outcome of BVDV infection in wildlife exist, but reported clinical signs are similar to BVDV infections in cattle. Experimental inoculation with BVDV NY-1 did not result in clinical signs in four mule deer and one white-tailed deer fawns, despite evidence of viremia and nasal shedding (VanCampen et al. 1997). Similar findings were made in yearling elk that were inoculated either with BVDV 1 Singer or BVDV 2 24515 (Tessaro et al. 1999). In two young reindeer, loose, bloody, and mucoid feces, transient laminitis, or coronitis were observed after inoculation with BVDV Singer and mild lesions were detected at

necropsy (Morton et al. 1990). Naïve white-tailed deer fawns developed moderate pyrexia and marked to moderate decreases in lymphocyte populations in response to either BVDV 1 or BVDV 2; and lethargy or coughing was observed in individual fawns (Mark et al. 2005; Ridpath et al. 2007c). Similar to other species, BVDV infections in wildlife may have the most important implications on reproductive health. Experimental inoculation of pregnant white-tailed deer may result in reproductive failure, including fetal resorption, fetal mummification, stillbirth and abortion (Passler et al. 2007; Ridpath et al. 2008).

Summary

Bovine viral diarrhea virus is one of the most important infectious agents affecting the cattle industry worldwide; however BVDV is not host-specific to cattle as numerous wild and domestic species have been reported susceptible to BVDV infection. Infection with BVDV in heterologous species, with the potential for persistently infected offspring may have great implications for BVDV control in cattle populations, as these species may become reservoirs and source of infection for cattle populations that are free from BVDV. In addition, BVDV disease and mortality in heterologous species, including rare and endangered livestock or zoo animal species threatens natural resources and complicates laboratory testing strategies. Further research, including studies on interspecific interactions and BVDV transmission are warranted to fully understand the importance of BVDV in heterologous species.

Table 2.1 Reported prevalence rates for antibodies directed against BVDV in domestic small ruminants.

Percent Seropositive		Country	Reference
Sheep	Goats		
34.9 / 29.4	11.5	Austria	(Krametter-Froetscher et al. 2006; Schiefer et al. 2006; Krametter-Frotscher et al. 2007)
27.5	31.4	Egypt	(Zaghawa 1998)
6.7	Not given	Germany	(Bechmann 1997)
23.4	16.9	India	(Mishra et al. 2009)
14.0	4.6	Namibia	(Depner et al. 1991)
12.7	4.5	Nigeria	(Taylor et al. 1977)
	3.6	Norway	(Loken 1990)
16.1	25.4	Switzerland	(Danuser et al. 2009)
32.1	24.9	Tanzania	(Hyera et al. 1991)
32.1		Turkey	(Yesilbag et al. 2009)

Table 2.2 Reported prevalence rates for antibodies directed against *Bovine viral diarrhea virus* in free-ranging wildlife.

Species	Rate of Seropositives	Reference
5 species in Germany, hunter-harvested ^(a)	5.9 – 6.6%	(Weber et al. 1978)
5 species of deer in Great Britain, random sampling between 1961 to 1973 ^(b)	0 – 16%	(Lawman et al. 1978)
2 species of exotic deer at Point Reyes National Seashore, hunter-harvested ^(c)	0 – 4%	(Riemann et al. 1979)
15 species in 9 African countries/ regions, collected between 1963 and 1977 ^(d)	6.8 – 100%	(Hamblin et al. 1979)
4 species in Alaska, captured animals at various sites ^(e)	0 – 3%	(Zarnke 1983)
2 species of deer, harvested at 2 locations in Maryland and Virginia, US ^(f)	0/5 – 2/5	(Davidson et al. 1983)
4 species in Germany, hunter-harvested ^(g)	0 – 1.3%	(Dedek et al. 1987)
10 species in Namibia, hunter-harvested ^(h)	0 – 57%	(Depner et al. 1991)
3 species in Northern Tanzania, various locations in two national parks ⁽ⁱ⁾	33% - 49%	(Hyera et al. 1992)
2 species of deer from 8 National Parks in Western US, convenience sampling ^(j)	55 – 59 %	(Aguirre et al. 1995)
3 species of deer from Germany, also includes other species of captive deer, convenience sampling ^(k)	14 of 180 (7.7%)	(Frolich 1995)
16 species in Zimbabwe, convenience sampling at various sites ^(l)	<1.0 – 75%	(Anderson et al. 1998)
4 species of deer in Denmark, hunter-harvested in 8 districts ^(m)	0.6	(Nielsen et al. 2000)
4 species of deer in Norway, hunter-harvested at various sites ⁽ⁿ⁾	1.1 – 12.3%	(Lillehaug et al. 2003)
4 species in southern Austria, hunter-harvested ^(o)	1/145 (0.7%)	(Krametter et al. 2004)
4 species in High Valley of Susa, northwest Italy ^(p)	0 – 25.5 %	(Olde Riekerink et al. 2005)
3 species of deer in 6 German National Parks ^(q)	0/164 (0%)	(Frolich et al. 2006)
Caribou (<i>Rangifer tarandus caribou</i>), hunter-harvested in Georges River area, Northern Quebec, Canada	38/58 (60.7 – 69.3%)	(ElAzhary et al. 1979), (ElAzhary et al. 1981)
Caribou in Saskatchewan, Canada	0%	(Jordan et al. 2003)
European bison (<i>Bison bonasus</i>), hunter-harvested diseased bison in Bialowieza Primeval Forest	29.5%	(Salwa et al. 2007)
European rabbit (<i>Oryctolagus cuniculus</i>), hunter-harvested in North-western Germany	40/100 (40%)	(Frolich et al. 1998)
Fallow deer (<i>Dama dama</i>), hunter-harvested or trapped in Tasmania, Australia	11/76 (14.5%)	(Munday 1972)

Fallow deer , captured in south-eastern New South Wales, Australia	1/86 (1.2%)	(English 1982)
Fallow deer , from San Rossore Preserve, Tuscany, Italy, hunter-harvested	25/43 (58%)	(Giovannini et al. 1988)
Grey brocket deer (<i>Mazama gouazoubira</i>), hunter-harvested in the Gran Chaco, Bolivia	0/17 (0%)	(Deem et al. 2004)
Guanaco (<i>Lama guanicoe</i>), captured in Cabo Dos Bahias Provincial Reserve, Argentina	0/20 (0%)	(Karesh et al. 1998)
Guanaco and Vicuña (<i>Vicugna vicugna</i>) several regions in Chile	0/82 (0%)	(Celedon et al. 2001)
Moose (<i>Alces alces</i>), hunter-harvested in Cypress Hills Park, Southeastern Alberta	3/22 (13.6%)	(Thorsen et al. 1971)
Moose , seven areas in Alaska	13/110 (12%)	(Kocan et al. 1986)
Mule deer (<i>Odocoileus hemionus</i>), trapped in south-central Idaho, US	17/25 (adults) 7/57 (fawns)	(Stauber et al. 1977)
Mule deer , hunter-harvested in south-central New Mexico, US	26/76 (34%)	(Couvillion et al. 1980)
Mule deer , captured at Pinedale, WY, USA	74/124 (60%)	(Van Campen et al. 2001b)
Pampas deer (<i>Ozotoceros bezoarticus celer</i>), captured in Campos del Tuyu Reserve, Argentina	0/14 (0%)	(Uhart et al. 2003)
Pronghorn (<i>Antilocapra americana</i>), live-trapped at 4 locations in Alberta and Saskatchewan, Canada	3/84	(Barrett et al. 1975)
Pronghorn , live-trapped at 3 locations in southeastern Idaho, US	2% (adults) 0% (fawns)	(Stauber et al. 1980)
Pronghorn , hunter-harvested in 4 areas of Arizona, US	7/128 (5%)	(Dubay et al. 2006)
Red deer (<i>Cervus elaphus</i>), hunter-harvested, stratified sampling	4/234 (1.7%)	(Koppel et al. 2007)
Reindeer (<i>Rangifer tarandus</i>), from Northern Norway and Svalbard	41% (adults) 6% (calves)	(Stuen et al. 1993)
Wapiti (<i>Cervus elaphus</i>), hunter-harvested in southwestern Alberta, Canada	52% of 28	(Kingscote et al. 1987)
White-tailed deer (<i>Odocoileus virginianus</i>); random sampling in New York state	3%	(Kahrs et al. 1964)
White-tailed deer , hunter-harvested from 2 herds	5.75%	(Friend et al. 1967)
White-tailed deer , hunter-harvested on Anticosti Island, Quebec, Canada	0%	(Sadi et al. 1991)
White-tailed deer , captured, on 15 ranches in northeastern Mexico	331/521 (63.5%)	(Cantu et al. 2008)
White-tailed deer , convenience sampling, from 23 facilities across Alabama, US	2/165 (1.2%)	(Passler et al. 2008)
White-tailed deer , captured at multiple locations in Southern Minnesota, US	25 % (SE) 41% (SW)	(Wolf et al. 2008)

- a. Seropositive: Roe deer, Red deer; Seronegative: Fallow deer, Wild boar, and European mouflon
- b. Seropositive: Fallow deer, Red deer, Chinese water deer, Sika deer; Seronegative: Roe deer
- c. Seropositive: Axis deer; Seronegative: Fallow deer
- d. Seropositive: African buffalo, Nyala, Kudu, Eland, Waterbuck, Defassa waterbuck, Lechwe, Reedbuck, Sable antelope, Oryx, Tsessebe, Hartebeest, Wildebeest, Impala, Springbok, Duiker, Giraffe, Wart hog; Seronegative: Bush buck, Puku, Kob, Roan antelope, Topi, Blesbok, Grant's gazelle, Klipspringer, Oribi, Steinbok, Grysbok, Hippopotamus, Elephant, Bush pig, Zebra
- e. Seropositive: Caribou; Seronegative: Dall sheep, Moose, American bison
- f. Seropositive: Sika deer, White-tail deer
- g. Seropositive: Roe deer, Red deer; Seronegative: Fallow deer, European mouflon
- h. Seropositive: Giraffe, Gemsbok, Roan antelope, Blue wildebeest, Eland, Kudu, Sable antelope; Seronegative: Red hartebeest, Black wildebeest, Springbok
- i. Seropositive: African buffalo, Wildebeest, Topi
- j. Seropositive: Mule deer, Wapiti
- k. Seropositive: Fallow deer, Roe deer, Red deer
- l. Seropositive: Nyala, Eland, Bushbuck, Lichtenstein's hartebeest, Kudu, Reedbuck, African buffalo, Sable antelope, Impala, Wildebeest, Waterbuck, Tsessebe, Giraffe; Seronegative: Warthog, White Rhinoceros
- m. Seropositive: Red deer; Seronegative: Roe deer, Fallow deer, Sika deer
- n. Seropositive: Roe deer, Reindeer, Moose, Red deer
- o. Seropositive: Red deer; Seronegative: Roe deer, Fallow deer, Chamois
- p. Seropositive: Red deer, Wild boar, Chamois, Seronegative: Roe deer
- q. Seronegative: Red deer, Roe deer, Fallow deer

Chapter 3: Journal article:

Experimental Persistent Infection with Bovine Viral Diarrhea Virus in White-tailed Deer

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Short Communication

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Abstract

Bovine viral diarrhea virus (BVDV) infections cause substantial economic losses to the cattle industries. Persistently infected (PI) cattle are the most important reservoir for BVDV. White-tailed deer (*Odocoileus virginianus*) are the most abundant species of wild ruminants in the United States and contact between cattle and deer is common. If the outcome of fetal infection of white-tailed deer is similar to cattle, PI white-tailed deer may pose a threat to BVDV control programs. The objective of this study was to determine if experimental infection of pregnant white-tailed deer with BVDV would result in the birth of PI offspring. Nine female and 1 male white-tailed deer were captured and housed at a captive deer isolation facility. After natural mating had occurred, all does were inoculated intranasally at approximately 50 days of pregnancy with 10^6 CCID₅₀ each of a BVDV 1 (BJ) and BVDV 2 (PA131) strain. Although no clinical signs of BVDV infection were observed or abortions detected, only one pregnancy advanced to term. On day 167 post-inoculation, one doe delivered a live fawn and a mummified fetus. The fawn was translocated to an isolation facility to be hand-raised. The fawn was determined to be PI with BVDV 2 by serial virus isolation from serum and white blood cells, immunohistochemistry on skin biopsy, and RT-PCR. This is the first report of persistent infection of white-tailed deer with BVDV. Further research is needed to assess the impact of PI white-tailed deer on BVDV control programs in cattle.

Author Keywords

Bovine viral diarrhea virus, cattle virus, livestock-wildlife interface, *Odocoileus virginianus*, persistent infection, white-tailed deer

Introduction

Infections with bovine viral diarrhea virus (BVDV) occur globally and are cause for substantial economic and genetic losses to the beef and dairy industries. Two distinct BVDV species exist, BVDV 1 and BVDV 2, which are referred to as the genotype. In addition, BVDV occurs in two biotypes, non-cytopathic (ncp) and cytopathic (cp), which is determined by the effects of a strain on cells in culture. Most BVDV infections occurring naturally are caused by ncp strains of BVDV.

There are two forms of infection associated with BVDV: acute or transient infection and persistent infection. Acute infections are postnatal infections in an immunocompetent host. In contrast, persistent infection only occurs by *in utero* infection of the developing fetus with a ncp BVDV prior to the development of immunocompetence (Brownlie et al. 1987). The virus is recognized as a self-antigen, and the animal is considered immunotolerant and persistently infected (PI) with BVDV. In the epidemiology of BVDV, PI animals constitute the major source of transmission of the virus within and among cattle herds, and maintain the virus in a population (Wittum et al. 2001). Until recently, reports of persistent infections occurring in species other than cattle have been limited to traditional domestic animals, such as sheep, goats, and swine. Within the past three years, persistent BVDV infections in other heterologous species have been reported. Identification of PI alpacas (*Lama pacos*) and lesser Malayan mousedeer (*Tragulus javanicus*) has indicated there may be reservoirs of BVDV in species other than cattle (Uttenthal et al. 2005; Mattson et al. 2006). Simpson (2002) suggested wildlife reservoirs of BVDV may be responsible for failures of BVDV eradication programs that cannot be traced to introduction of or contact with PI cattle (Simpson 2002).

The white-tailed deer (*Odocoileus virginianus*) is the most abundant wild ruminant species in many regions of North America. Estimates of total number, while widely variable, suggest there are at least 15 million, but possibly up to 30 million animals in the United States (Curtis et al. 2001). Considering the abundance of white-tailed deer, contact between cattle and deer is common. Over the last 50 years, a number of virological and serological studies have been conducted to assess BVDV infection of white-tailed deer, and results suggest that this species can be infected and possibly maintain the virus in the population (Richards et al. 1956; Kahrs et al. 1964; Friend et al. 1967; Sadi et al. 1991; Van Campen et al. 1997; Chase et al. 2004b). To date, BVDV infection of pregnant white-tailed deer resulting in the birth of a PI fawn has not been reported; however, there have been indications this may be possible (Van Campen et al.; Van Campen 2002; Chase et al. 2004b). The objective of this study was to determine if experimental infection of pregnant white-tailed deer with two genotypes of BVDV would result in the birth of PI offspring. In cattle, the epidemiological importance of PI animals is well recognized. Identification of persistent BVDV infection in white-tailed deer may be influential when planning for BVDV control or eradication programs.

Materials and Methods

Experimental inoculation of deer with BVDV

The work reported herein was performed under the approval of the Institutional Animal Care and Use Committee of Auburn University (2005-0909). In December 2005, nine female and one male white-tailed deer were captured from the wild. All animals were determined to be negative for BVDV on virus isolation and serum neutralization at initial capture. Natural mating occurred in captivity with peak breeding season estimated to be centered around January 20th,

2006. On March 11, 2006, the nine does were sedated and examined ultrasonographically, either transrectally and/or transabdominally, to determine pregnancy status and gestation length. When possible, the number of fetuses per dam and the fetal crown to rump length to estimate fetal ages were determined according to previously described methods (Hamilton et al. 1985). All pregnant white-tailed deer were inoculated with the ncp BVDV 1 strain BJ and the ncp BVDV 2 strain PA131. The BVDV strains were propagated in Madin-Darby bovine kidney (MDBK) cells in Eagle's minimum essential medium (EMEM), supplemented with 10% equine serum, L-glutamine, penicillin G (100 units/ml), and streptomycin (100µg/ml). Virus was harvested from cells by a single freeze-thaw method, titered, and stored (-80°C) until the day of inoculation. Each doe was inoculated by intranasal administration with 10⁶ cell culture infective doses (50% endpoint) (CCID₅₀) of the BVDV 1 BJ and BVDV 2 PA131 strains diluted in 2 mL of EMEM (1 mL per nostril).

Following inoculation, pregnancies were allowed to advance without interference until parturition, which was expected to occur from the middle of August until the beginning of September, 2006. Once born, the fawn was moved to an isolation facility and housed individually while BVDV testing was performed. Blood was collected for virus isolation, virus neutralization and RT-PCR procedures, nasal swabs were collected for virus isolation, and skin was collected for immunohistochemistry. With exception of immunohistochemistry on skin biopsies, samples collections and virological procedures were repeated on days 31 and 60 of life.

Approximately 30 days after the last doe had been expected to give birth (206 days post inoculation), all adult deer were sedated. Blood samples were collected for virus isolation and virus neutralization procedures, and all deer were euthanized using a lethal injection of

barbiturate. Gross pathologic examinations were performed, and samples of spleen, mesenteric lymph node and uterus were collected for virus isolation procedures.

Virus isolation

Virus isolation procedures were performed on serum, whole blood, nasal swabs, and tissues collected at postmortem examination. Samples were assayed for BVDV by passage through Madin Darby bovine kidney (MDBK) cells, according to previously described methods (Givens et al. 2003). Following passage, staining for BVDV antigen by an immunoperoxidase monolayer assay using BVDV-specific monoclonal antibodies D89 and 20.10.6 was performed (Givens et al. 2003).

Skin biopsy immunohistochemistry

Immunohistochemical (IHC) detection of BVDV antigen was performed on formalin-fixed paraffin-embedded skin biopsies using a monoclonal antibody, 15C5 (Brodersen 2004). The 15C5 monoclonal antibody reacts with an epitope of the E^{RNS} of BVDV that is shared by diverse BVDV isolates and therefore is a suitable target for the detection of a wide variety of isolates of BVDV, including BJ and PA131.

Virus neutralization

A standard virus neutralization microtiter assay was used for the detection and quantification of antibodies in serum (Givens et al. 2003). Sera obtained at initial capture were tested for neutralizing antibodies to the cp BVDV 1 strain NADL and the cp BVDV 2 strain 125C. The sera collected from adult deer at the time of inoculation and postmortem examination, and from the fawn were tested for neutralizing antibodies to the ncp BVDV 1 BJ and ncp BVDV

2 PA131 strains with which the deer had been experimentally inoculated. MDBK cells were used as the indicator cells. Each test included a back titration of the virus and a positive and negative serum control. Following a 5 day incubation period, antibody titer was defined as the inverse of the highest dilution with complete inhibition of cytopathic effect (sera collected at initial capture) or complete inhibition of staining by the immunoperoxidase test (sera collected from adult deer at inoculation and pathologic examination, and from the fawn).

Reverse transcriptase polymerase chain reaction and sequencing of virus

The BVDV was detected by a two-round rapid-cycle PCR assay on whole blood samples obtained from the fawn. This reverse transcription nested PCR (RT-nPCR) has been previously described in detail (Givens et al. 2001). All steps of the RT-nPCR were performed in a single-tube reaction. In the first round, the outer primers, BVD 100 (5'-GGCTAGCCATGCCCTTAG-3') and HCV 368 (5'-CCATGTGCCATGTACAG-3') amplified a 290 base pair sequence of the 5' untranslated region of the viral genome. In the second round of the reaction, the inner primers BVD 180 (5'-CCTGAGTACAGGGDAGTCGTCA-3') and HCV 368 amplified a 213 base pair sequence within the first amplicon. After completion of the PCR cycle, 5 µl of the RT-nPCR products were separated by 1.5% agarose gel electrophoresis. Ethidium bromide staining allowed visualization of the RT-nPCR using an ultraviolet transilluminator.

Sequence analysis was performed on aliquots of the RT-nPCR products carried out in triplicate. If positive for BVDV on agar gel electrophoresis, samples were purified and sequenced by automated dye terminator nucleotide sequencing using both the 5' and 3' primers (BVD 180 and HCV 368, respectively). Consensus sequences were determined for each sample

using Align X® computer software (Vector NTI Suite 7.1, InforMax, Inc., Bethesda, MD, USA) and compared to nucleotide sequences of the challenge strains of BVDV.

Results

On the day of inoculation, ultrasound examination revealed that all does were pregnant with 1-2 fetuses (mean: 1.625). Gestational age of fetuses was estimated to be between 30 and 80 days (mean: 52.9). Stress from capture and inoculation caused mortality in 5 does, which were found on post-inoculation day 1. Necropsies and tissue collections for virological procedures were performed on all does. No gross lesions were noted in the deceased animals. Virus isolation from tissue samples (spleen, lung, liver, kidney, placenta, and embryo/fetus) did not yield positive results.

On August 25th, 2006 a male fawn was found and immediately removed from the captive deer pen. The fawn was dry, appeared bright and alert, and was able to ambulate. The fawn was estimated to be approximately 12 hours old, and colostrum intake from the dam was assumed. The birth weight of the fawn was 1.5 kg (3.3 lbs) which is at the low end of bodyweights reported previously for neonatal white-tailed deer in Alabama (Haugen 1959). A mummified fetus was found in immediate proximity of the fawn, lying in the same birthing area as the fawn. A crown-to-rump length measurement of the mummified fetus indicated that the fetus had died at approximately 94 days of pregnancy (Hamilton et al. 1985), approximately, 50 days after inoculation.

At the time when no further parturitions were expected to occur, all adult deer were sedated and humanely euthanized. All does were determined to be non-pregnant at this time and no further mummified fetuses were found. Serum virus neutralization was positive in all adult

animals, including the buck that had not been inoculated (Table 3.1). Virus was not isolated from serum or tissue samples collected after euthanasia.

After translocation of the fawn to the isolation facility, blood samples and a skin biopsy were taken for virological examinations. Neutralizing antibodies were not detected by standard virus neutralization. Serum and white blood cell fractions tested positive for BVDV on virus isolation. The IHC on the skin biopsy also yielded a positive result, indicated by uptake of immunoperoxidase stain by skin tissues (fig. 1). The IHC staining pattern from the fawn resembled skin biopsies obtained from PI calves. Nested RT-PCR was positive on serum and whole blood of the fawn, corroborating the results of virus isolation (fig. 2). The PCR product of the nested RT-nPCR was submitted for sequencing, and analysis of the sequence revealed BVDV 2. Furthermore, sequencing demonstrated the BVDV isolate from the fawn was the BVDV 2 strain PA131 that had been used at inoculation. With exception of the skin biopsy IHC, all virological examinations were repeated on samples taken from the fawn on days 31 and 60 post partum. Virus isolation from whole blood, serum and nasal swabs and RT-nPCR tested positive for BVDV validating the PI status of the fawn. The titer of BVDV neutralizing antibodies remained <1:2. At the time of submission of this publication (day 94 of life), the fawn has remained free from clinical signs of disease and has developed normally.

Discussion

Our work demonstrates that experimental infection of pregnant white-tailed deer (*Odocoileus virginianus*) with BVDV may result in the birth of PI offspring. This is the first published report of experimentally induced persistent BVDV infection in this species; however, previous reports indicate that natural infection with BVDV may cause persistent infection in

white-tailed deer (Chase et al. 2004b). Interestingly, like the BVDV isolate causing persistent infection in our study, a BVDV isolate obtained from a wild white-tailed deer was also determined to belong to genotype 2 (Chase et al. 2004b). These findings contrast recent reports of persistent infections in other heterologous species, such as mousedeer and alpacas, where BVDV 1 was the PI strain (Grondahl et al. 2003; Carman et al. 2005; Mattson et al. 2006). In our study, pregnant does were inoculated with strains of BVDV from both genotypes in order to determine which genotype of BVDV is more suitable to result in PI offspring. The inoculation procedure used in this study was based on a protocol developed for the evaluation of BVDV vaccines in cattle using a multiple BVDV strain challenge model (Brock et al. 2000). Unlike in that protocol, where both genotypes of BVDV were isolated from whole blood samples of PI fetuses, only BVDV 2 was isolated from blood samples of the PI fawn. In the study by Brock and Chase (2000), the BVDV 2 strain was isolated more consistently from tissues samples when compared to BVDV 1, indicating a more global replication of BVDV 2 and possibly a better host adaptation of this strain (Brock et al. 2000). This observation may be emphasized by the markedly greater antibody titer against BVDV 2 compared to BVDV 1 that was detected in all adult animals at time of euthanasia. Furthermore, the time of virological examination may contribute to the observed differences. While samples were examined 60 days post-inoculation in the cattle study (Brock et al. 2000), samples from the fawn were examined 167 days post-inoculation. Further research is needed to determine the significance of BVDV genotype in causing the PI state in white-tailed deer.

Persistent infection of white-tailed deer may need consideration, when planning BVDV eradication or control strategies in the United States, as has been suggested by the Academy of Veterinary Consultants and later by the National Cattlemen's Beef Association. White-tailed

deer have been recognized as a wildlife reservoir host for different pathogens, including *Ehrlichia chaffeensis* and *Mycobacterium bovis* (Lockhart et al. 1997; Schmitt et al. 2002). The identification of *M. bovis* in white-tailed deer in Michigan is believed to be the result of a spill-over infection from cattle (Schmitt et al. 2002), and this demonstrates how increasing numbers of white-tailed deer in conjunction with changing management factors of wildlife and cattle populations can affect the dynamics of an infectious disease (O'Brien et al. 2002). Infectibility with a pathogen alone does not cause a species to be a wildlife reservoir. Other factors, such as sufficient transmission as a result of consistent shedding of the infectious agent, sufficient contact between species, and maintenance of the infectious agent within a wildlife population are necessary to constitute a reservoir species. Whether these factors are present to make white-tailed deer a wildlife reservoir for BVDV is not known. The central role of PI cattle in the epidemiology of BVDV and the findings of this study make it plausible that white-tailed deer may constitute a wildlife reservoir for BVDV.

Reproductive deficiency, including reduced conception rates, early embryonic deaths, and abortions are commonly associated with BVDV infection of pregnant animals (Grooms 2004). The cause of pregnancy loss in most of the experimentally infected does can only be speculated because the use of wild, captive white-tailed deer for this study precluded close observation. Potential causes for fetal loss following capture and inoculation include hypoxemia associated with capture and administration of sedatives, or BVDV infection. Hypoxemia and hyperthermia are common side effects of chemical immobilization of deer (Read et al. 2001).

By our calculations, the dam of the PI fawn and mummified fetus was inoculated on day 43 of gestation. To our knowledge, the fetal age when the development of the immune system in white-tailed deer occurs is unknown; therefore, determining the gestational age at which BVDV

may cause persistent infection in white-tailed deer is difficult to predict. Immune system competence of bovine fetuses occurs prior to day 125-150 of gestation. Experimental inoculation of pregnant heifers with the same strains of BVDV 1 and BVDV 2 used in this study on day 75 (± 5) of gestation induced the PI state in all fetuses (Brock et al. 2000). Extrapolation of these data was the basis for the mean day of gestation chosen for experimental inoculations of white-tailed deer. The gestation length for white-tailed deer is approximately 200-205 days. The gestational timeframe at which persistent infection in white-tailed deer can occur is likely to be similar to cattle, where fetuses in the first and early second trimester are most susceptible to becoming PI. Further research is needed to determine the gestational age when PI infection in fetal white-tailed deer may occur.

Figure 3.1 Positive immunohistochemical staining for BVDV antigen in epidermal and hair follicle epithelium from a fawn persistently infected with BVDV (note reddish-brown staining).

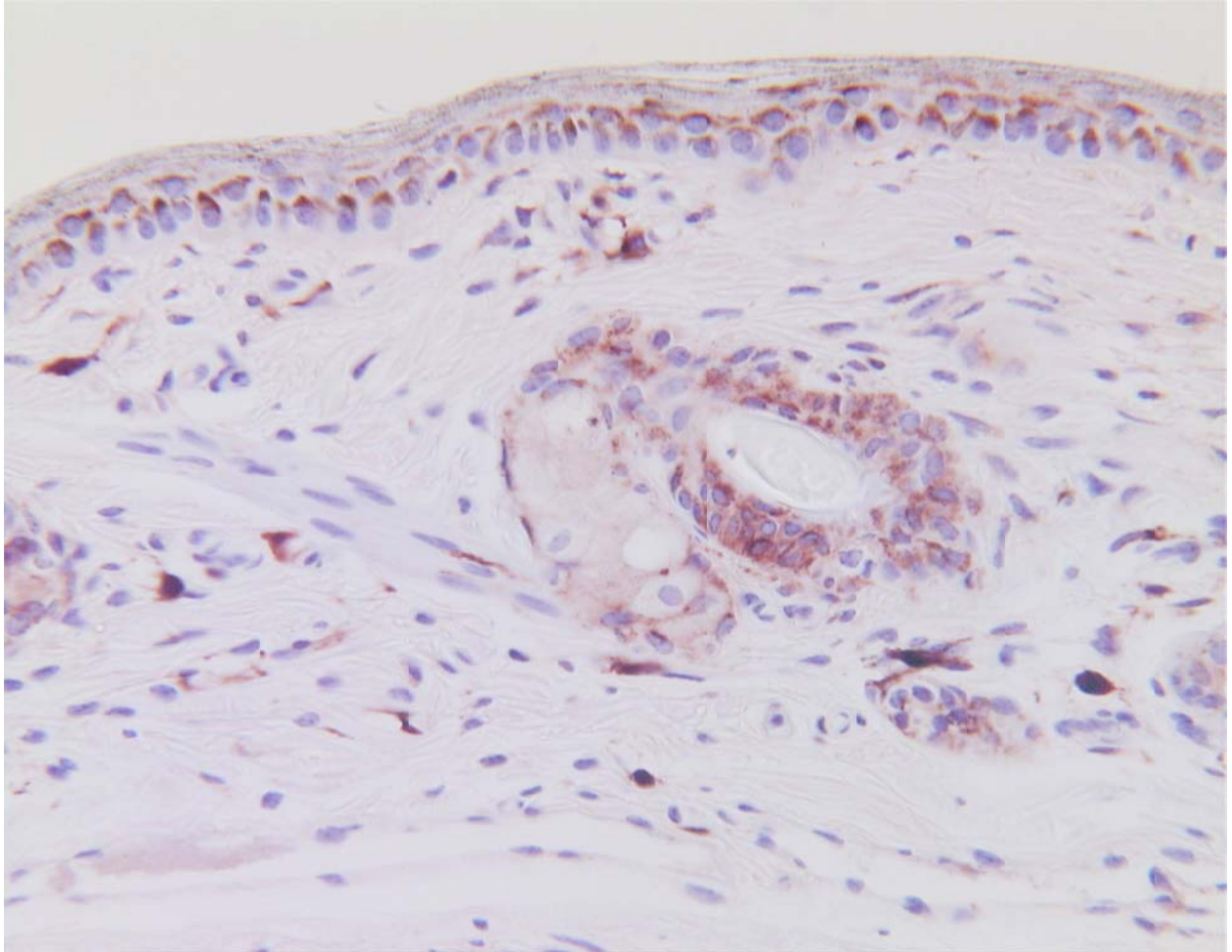


Figure 3.2 Agarose gel electrophoresis of the amplification products generated from a persistently infected white-tailed deer using primers specific for bovine viral diarrhea virus. A: Serum from fawn in duplicate; B: Whole blood from fawn in duplicate; C: Negative and positive control for RNA extraction; D: Negative and positive control for RT-nPCR.

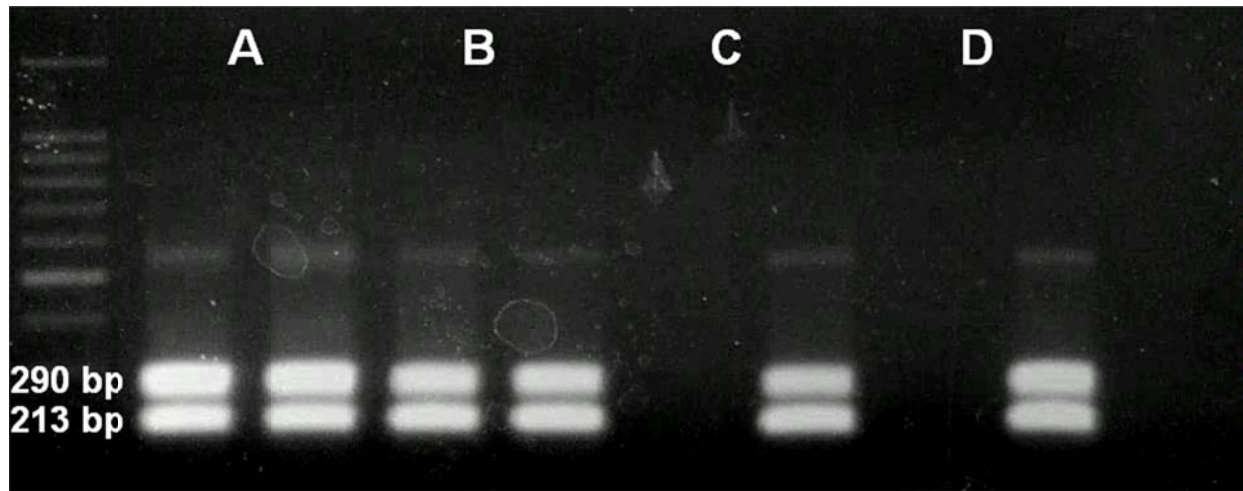


Table 3.1 Serum antibody titers of adult deer on day of inoculation and 206 days thereafter.

	BVDV 1 (BJ)		BVDV 2 (PA131)	
	Day 0	Day 206	Day 0	Day 206
Doe 1	<1:2	1:128	1:2	1:1024
Doe 3	<1:2	1:64	<1:2	1:1024
Doe 5	<1:2	1:128	<1:2	1:2048
Doe 6	<1:2	1:64	<1:2	1:512
Buck	<1:2	1:16	<1:2	1:512

Chapter 4: Journal article:

Cohabitation of Pregnant White-tailed Deer and Cattle Persistently Infected with Bovine Viral
Diarrhea Virus Results in Persistently Infected Fawns

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Short Communication

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Abstract

Economic losses due to infection with bovine viral diarrhea virus (BVDV) have prompted introduction of organized control programs. These programs primarily focus on removal of persistently infected (PI) animals, the main source of BVDV transmission. Recently, persistent BVDV infection was demonstrated experimentally in white-tailed deer, the most abundant wild ruminant in North America. Contact of cattle and white-tailed deer may result in interspecific BVDV transmission and birth of persistently infected offspring that could be a threat to control programs. The objective of this study was to assess the potential for interspecific BVDV transmission from persistently infected cattle cohabitated with pregnant white-tailed deer. Seven female and one male white-tailed deer were captured and bred in captivity. At approximately 50 days of gestation, two cattle persistently infected with BVDV 1 were cohabitated with the deer. In a pen of approximately 0.8 hectares, both species shared food and water sources for a period of 60 days. Transmission of BVDV as indicated by seroconversion was demonstrated in all exposed adult deer. Of the seven pregnancies, four resulted in offspring that were infected with BVDV. Persistent infection was demonstrated in three singlet fawns by immunohistochemistry and ELISA on skin samples, PCR, and virus isolation procedures. Furthermore, two stillborn fetuses were apparently persistently infected. This is the first report of BVDV transmission from cattle to white-tailed deer using a model of natural challenge. Under appropriate circumstances, BVDV may efficiently cross the species barrier to cause transplacental infection and persistently infected offspring in a wildlife species.

Key words

Bovine viral diarrhea virus, interspecific transmission, livestock-wildlife interface, *Odocoileus virginianus*, persistent infection, white-tailed deer

Introduction

Bovine viral diarrhea virus (BVDV), a pestivirus in the family Flaviviridae, is capable of infecting various mammalian host species in the order Artiodactyla (Vilcek et al. 2006). Cattle persistently infected (PI) with BVDV are considered the most important source of virus, however PI wildlife including white-tailed deer (*Odocoileus virginianus*) have been reported (Vilcek et al. 2000; Passler et al. 2007). Identification of heterologous PI hosts may have important implications for the epidemiology of BVDV, as these non-bovid PI animals may serve as reservoirs for BVDV. The livestock-wildlife interface is of great concern in the control of the related pestivirus classical swine fever virus (CSFV), where great measures are taken to prevent contact of domestic swine and wild boars, which are considered a reservoir of CSFV (Ruiz-Fons et al. 2008). Survival of PI wildlife to adulthood has been demonstrated in an eland and in surveys of hunter-harvested deer, and this may contribute to interspecific transmission (Vilcek et al. 2000; Passler et al. 2007). Reintroduction of BVDV into BVDV-free cattle herds by wildlife reservoirs has been speculated and certainly could present a serious threat to BVDV control efforts that are currently evolving in the United States (Simpson 2002; Sandvik 2004).

Experimental intranasal inoculation of a pregnant white-tailed deer with a BVDV 2 isolate resulted in the birth of a PI fawn and a mummified fetus (Passler et al. 2007). This PI fawn consistently shed high titers of BVDV, as indicated by repeated nasal swab virus isolations, thus demonstrating that PI white-tailed deer may be an efficient source of BVDV (Passler et al.

2007). The purpose of this study was to evaluate the potential for interspecific BVDV transmission using a natural model of transmission through cohabitation of PI cattle and in-contact pregnant white-tailed deer. White-tailed deer are the most abundant free-ranging ruminant in North America and home ranges commonly overlap with those of pastured cattle.

Materials and Methods

Animals

In December 2006, 7 female and 1 male free-ranging white-tailed deer were captured by cannon net, as previously described (Hawkins et al. 1968). All animals were determined to be free from BVDV and BVDV antibodies by whole blood virus isolation and serum virus neutralization, respectively. Following sample collections, captured deer were translocated to a pen at the Captive Deer Research Facility. The 0.8 ha deer pen contained a source of free-choice water and two wooden covered livestock feeders. Daily, a commercially available 16% protein, high fiber ration at approximately 1.8 kg per deer and free choice grass hay was fed. Natural mating occurred in captivity with peak breeding season in Alabama centered around January 20th.

On March 8th, 2007, when gestation length of deer was estimated to be approximately 50 days, two female cattle known to be PI with BVDV were cohabitated with captive deer. On the day of translocation, blood, nasal swab, and skin biopsies (ear notch) samples were obtained from cattle to reconfirm their status as PI animals. In addition, RT-nPCR products from both cattle were further characterized by sequence analysis and determined that both cattle were PI with BVDV 1b strains (designated AU526 and KY16). Nasal swab virus isolation titers at the beginning of cohabitation were 3.5×10^5 CCID₅₀ for both BVDV strains and serum virus

isolation titers were 6.2×10^3 CCID₅₀/ml for BVDV KY16 and 3.5×10^4 CCID₅₀/ml for BVDV AU526.

During cohabitation, PI cattle and deer shared feed and water sources as may occur in a North American farm setting. Amount of contact between cattle and deer was also observed visually and by intermittent time lapse videography. Captive deer were observed daily from a distance for clinical signs of BVDV infection. Following 60 days of cohabitation, PI cattle were removed from the captive deer pen. Upon removal of cattle, nasal swab virus isolation titers were 1.1×10^5 CCID₅₀/ml for BVDV KY16 and 6.2×10^4 CCID₅₀/ml for BVDV AU526. Serum virus isolation titers were determined to be 6.2×10^3 CCID₅₀/ml for BVDV KY16 and 2.0×10^4 CCID₅₀/ml for BVDV AU526. Following removal of PI cattle, pregnancies of deer were allowed to advance without interference.

Parturitions were expected to begin at the end of July 2007, when daily inspections for neonatal fawns were performed. Blood, skin biopsy, and nasal swab samples were collected for virus isolation, virus neutralization, RT-nPCR, and immunohistochemistry (IHC) procedures. Following sample acquisition, fawns remained at the captive deer pen for an additional 24 hours to ensure colostrum intake and then were translocated to an isolation room to be hand-raised.

Hand-raised fawns were fed a commercially available, multi-species milk replacer at 15% body weight, and hay and water were available ad libitum. Postmortem examinations were performed on all fawns that died or at time of euthanasia. At necropsy, tissues (lymph nodes, spleen, thymus) were collected from fawns that had been identified to harbor BVDV at birth and virus isolation and IHC procedures were performed. Approximately five months after parturition all adult deer were euthanized. At time of euthanasia, serum was collected for virus isolation and virus neutralization procedures.

Maternity testing

A skin biopsy sample was collected from all adult and neonatal deer and stored at -80°C until genetic analysis to verify the maternity of fawns was performed as previously described (Anderson et al. 2002; DeYoung et al. 2003). Information of maternity testing was used to assign fawn identification according to doe number and twin fawns were designated by assignment of letters A and B.

Virus isolation

Virus isolations were performed on sera, whole blood and nasal swab samples from fawns at birth; whole blood samples from adult deer at capture and euthanasia; and tissues (lymph nodes, spleen, thymus) collected from fawns at postmortem examination. Samples were assayed for BVDV by passage through MDBK cells, as has been described previously (Givens et al. 2003).

Skin biopsy immunohistochemistry

Immunohistochemical (IHC) detection of BVDV antigen was performed on formalin-fixed paraffin-embedded skin biopsies using a monoclonal antibody, 3.12F1. Furthermore, at post-mortem examination, tissue samples (lymph node, spleen, and thymus) were collected from fawns that had been identified to harbor BVDV at birth and processed for IHC using the 3.12F1 monoclonal antibody. Antigen distribution in white-tailed deer, persistently infected with BVDV is analogous to that detected in PI cattle (Passler et al. 2007; Duncan et al. 2008a).

Antigen capture ELISA

The BVDV antigen detection in skin biopsy samples was performed using a commercially available kit (IDEXX Laboratories, Westbrook, ME 04092, USA) developed for BVDV detection in bovine samples, according to the manufacturer's instructions. Presence or absence of BVDV within samples and classification as negative, suspect, or positive sample was established by using sample to positive (S/P) ratios of <0.20 , $0.20 - 0.39$, or >0.39 , respectively.

Virus neutralization

A standard virus neutralization microtiter assay was used for the detection and quantification of antibodies in serum of adult deer at capture and euthanasia, and from those fawns that had not been determined to harbor BVDV at birth. Sera were tested for neutralizing antibodies to BVDV AU526 and KY16, as previously described (Givens et al. 2003). Antibody titer was defined as the inverse of the greatest dilution with complete inhibition of staining by the immunoperoxidase test.

Reverse transcriptase polymerase chain reaction and sequencing

BVDV was detected by a two-round rapid-cycle PCR assay on whole blood samples, serum, and tissues from fawns. This reverse transcription nested PCR (RT-nPCR) is characterized by an increased sensitivity when compared with conventional RT-PCR and has been previously described in detail (Givens et al. 2001).

RT-nPCR positive samples were purified using the QIAquick® PCR purification kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's specifications and sequenced by automated dye terminator nucleotide sequencing using both the 5' and 3' primers (BVD 180

and HCV 368, respectively). Consensus sequences were determined for each sample using Align X® computer software (Vector NTI Suite 7.1, InforMax, Inc., Bethesda, MD, USA) and compared to nucleotide sequences of the challenge strains of BVDV.

Results

During cohabitation, both species were observed to favor a common area within the pen when resting, and close interspecific contact appeared possible in this area. Time lapse videography demonstrated that white-tailed deer and cattle did not feed at the same time. Rather, when feed troughs were filled, PI cattle ate first, followed by the deer within minutes thereafter. At observation from a distance, clinical signs of BVDV infection were not detected in the captive deer during or after exposure to PI cattle.

Nine live fawns (six twins and three singlets) and two twin stillborn fetuses were born within 30 days beginning on July 30th, 2007. Pertinent data on fawns including results of virological assessment are presented in Table 4.1. All live fawns appeared healthy and vigorous when found. The fawns were determined to be full-term and body weights at birth were in the normal range (Haugen 1959). The twin stillborn fetuses weighed below normal range and appeared to be pre-term according to dental eruption and appearance of hair-coat. Following a 24 hour period at the captive deer pen to ensure colostrum intake, fawns were translocated to an isolation room and quickly adapted to bottle-feeding. By the end of August 2007 three of the eight hand-raised fawns (19A, 23A, and 25A) had died, prompting the decision to leave any fawns born thereafter with their dams. This decision was made to remove any artificial factors of hand-raising that may have been involved in fawn mortality. Only one fawn (17A) was born after

this decision had been made. At post-mortem examination and histopathology, pyelonephritis was diagnosed in one of the deceased fawns (25A).

Evidence of BVDV infection was detected in the three singlet fawns (17A, 23A, 25A) and both stillborn fetuses (18A and 18B). All twin fawns were negative for BVDV infection according to virological assessment. From the samples collected on the first day of life, BVDV was isolated from the buffy coat of two fawns and the nasal swab sample of one fawn. Serum virus isolation was negative in all live-born fawns. In the stillborn fetuses, virus was isolated from four of five tissues, including lungs, spleen, thymus and lymph node. Ileal samples of these fetuses were microbiologically contaminated and BVDV was not isolated.

Diffuse antigen distribution was detected with IHC on ear notch samples in the three singlet fawns. The IHC staining pattern resembled those from PI cattle and a previously reported PI white-tailed deer (Passler et al. 2007). Ear notch samples from stillborn fetuses also were positive on IHC. In the skin samples from twin fawns, BVDV antigen was not detected. Tissue samples collected from singlet fawns and stillborn fetuses at post-mortem examination were positive for BVDV antigen, with multifocal to diffuse distribution within tissues sections.

To substantiate results from IHC, ear notch biopsies from all fawns with the exception of the fawn that remained with its dam (17A) were processed for antigen detection by ELISA. Bovine viral diarrhea virus antigen was detected in two fawns, of which one fawn was classified as positive and the second as suspect, according to S/P ratio.

RT-nPCR was performed on sera of all live-born fawns at birth and demonstrated presence of viral RNA in the same two fawns that were positive on WBC virus isolation. At death, tissue samples were collected from fawns and positive RT-nPCR results were detected in the thymus and lymph nodes of one fawn, and spleen and thymus of a second fawn. In both

stillborn fetuses, samples from lungs, spleen, lymph node, and thymus were positive on RT-nPCR. Genotyping of RT-nPCR products was performed by sequence analysis and demonstrated that the three singlet fawns were infected with BVDV strain AU526, while the stillborn fetuses were infected with BVDV strain KY16.

Virus neutralization was performed on serum samples from those fawns that did not harbor BVDV at birth and BVDV antibodies were detected in all samples. Antibody titers varied amongst fawns and were appreciably greater in a pair of twins (21A and 21B). Comparison of virus neutralization results from serum of adult deer at time of capture and time of euthanasia demonstrated seroconversion in all adult animals (Table 4.2). Virus was not isolated from adult deer at capture or time of euthanasia.

Discussion

This study demonstrates that cohabitation of naïve pregnant white-tailed deer with cattle PI with BVDV results in interspecific transmission of BVDV and birth of PI white-tailed deer. This is the first report of BVDV transmission from cattle to white-tailed deer using a model of natural challenge. Identification of seropositivity in all adult deer and birth of PI offspring in four of seven pregnancies emphasizes that BVDV may efficiently cross the species barrier to cause PI offspring in a wildlife species. The results of the present and two previous studies indicate that both species of BVDV are capable of inducing persistent infection in white-tailed deer (Passler et al. 2007; Duncan et al. 2008a).

Interestingly, all PI fawns in the present study were singlets, while non-PI fawns were twins. This observation may be the result of coincidence but raises the question of BVDV effects on gestation in polytocous species. In a previous study, a viable PI white-tailed deer fawn was

found in the same birthing area as a mummified fetus indicating that outcome of transplacental BVDV infection may vary among twin deer fetuses (Passler et al. 2007). The pathogenesis of transplacental BVDV infection is incompletely understood. Fetal infection with pestivirus may be by a stochastic mechanism and varying outcome of infection is a common finding among fetuses in twin pregnancies (Scherer et al. 2001; Swasdipan et al. 2002). In twin sheep and goat fetuses, different combinations of virological and clinical findings have been reported, including viropositive with vironegative fetuses; viable with stillborn or autolyzed fetuses; or stillborn with autolyzed fetuses (Loken et al. 1991a; Scherer et al. 2001; Swasdipan et al. 2002). The causes for these findings are unknown but infection of twin fetuses at different times or different fetal responses to infection have been suggested (Scherer et al. 2001). Further research comparing the factors that are involved in the varying outcome of fetal BVDV infection in polytocous species may contribute valuable information to understanding the pathogenesis of transplacental viral infections.

In white-tailed deer, the gestational age of fetuses at which persistent infection may occur is unknown; however, in a previous report a PI fawn resulted from intranasal inoculation of the doe on day 52 of pregnancy (Passler et al. 2007). A pair of twin PI fawns were born to a white-tailed deer that was experimentally inoculated at approximately 42 to 49 days of gestation (Duncan et al. 2008a). In the present study, a cohabitation time of 60 days was chosen to allow for contact between deer and PI cattle at an estimated pregnancy stage of 40 to 100 days based on extrapolation of knowledge from cattle. In a recent study on vaccinal fetal protection in pregnant cattle, a cohabitation time of 98 days was chosen, resulting in transplacental infection of 14/14 bovine fetuses (Grooms et al. 2007). The amount of contact between PI cattle and

white-tailed deer necessary to cause interspecific BVDV transmission is important epidemiologic knowledge that is needed to establish measures of biocontainment.

In cattle, BVDV commonly causes reproductive failure including infertility, embryonic resorption, fetal mummification, or abortion. The outcome of BVDV infection in pregnant animals is largely dependent on time of infection and infecting strain of BVDV (Grooms 2004). Similarly, fetal resorption, mummification and abortions have been reported in white-tailed deer experimentally challenged with BVDV strains of bovine or cervine origin, respectively (Passler et al. 2007; Ridpath et al. 2007a). All does in the present study carried their pregnancies to term and embryonic resorption or abortions were not observed. However, birth of two stillborn fetuses with low birth weights should be considered evidence of in-utero effects of BVDV. In contrast to PI fawns that were infected with BVDV AU526, the BVDV KY16 was isolated from both stillborn fetuses possibly indicating different strain-dependent effects on fetuses.

Transmission of BVDV in populations of white-tailed deer is likely influenced by human management of livestock and wildlife. Considering the pathogenesis of BVDV and the importance of PI hosts in the epidemiology of BVDV, winter-feeding may be an especially important factor of interspecific transmission of the virus. This study and a previous study indicate that a gestation length of approximately 50 days is suitable for induction of persistent infection in white-tailed deer, which coincides with the coldest months of the year when most intense winter feeding is likely practiced. A recent study performed in Minnesota reported seroprevalence rates of 41% and 25% in sampled white-tailed deer (Wolf et al. 2008). This is in contrast to a study performed in Alabama, where only 1.2% of animals were seropositive for BVDV (Passler et al. 2008). Longitudinal variations in management practices and climate may

explain the different seroprevalence rates, as dependence of white-tailed deer on winter feeding is likely less important in more temperate climates.

Bovine viral diarrhea virus control programs are mainly focused on removal of PI cattle from herds, thereby eradicating the most important source of BVDV infection for susceptible animals. With ongoing success of these programs, a main focus will have to be the prevention of BVDV reintroduction into BVDV-free herds. In conclusion, the present study emphasizes that BVDV is able to successfully cross species barriers and under appropriate circumstances may have the ability to establish wildlife reservoirs.

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Table 4.1 Pertinent data on fawns.

ID	Sex	Birth weight in kg	Day of birth	Exposure [‡]	Virus isolation				RT-nPCR		ELISA	IHC			Virus neutralization	
					Serum	WBC	Nasal swab	Tissues	Serum	Tissues	Ear notch	Ear notch	Tissues	AU526	KY16	
17 A	M	ND	8/28	32	-	+	-	ND	+	ND	ND	+	ND	ND	ND	
18 A	M	1.22	7/31†	62†	-	ND	-	+	ND	+	ND	+	+	ND	ND	
18 B	F	1.34	7/31†	62†	-	ND	-	+	ND	+	ND	+	+	ND	ND	
19 A	M	2.65	7/30	61	-	-	-	-	-	ND	-	-	ND	16	16	
19 B	M	3.06	7/30	61	-	-	-	ND	-	ND	-	-	ND	128	128	
21 A	F	2.69	7/30	61	-	-	-	ND	-	ND	-	-	ND	32,768	65,536	
21 B	F	2.50	7/30	61	-	-	-	ND	-	ND	-	-	ND	4,096	131,072	
23 A	M	2.40	8/21	39	-	+	+	-	+	+	+	+	+	ND	ND	
24 A	M	2.13	8/26	34	-	-	-	ND	-	ND	-	-	ND	64	256	
24 B	F	2.22	8/26	34	-	-	-	-	-	ND	-	-	ND	128	256	
25 A	F	3.03	8/4	56	-	-	-	-	-	+	+	+	+	ND	ND	

Key: ND – not determined; †stillborn; ‡ Gestational age at which exposure to PI cattle first occurred.

Table 4.2 Serum antibody titers of adult deer at capture and at euthanasia.

	BVDV AU526		BVDV KY 16	
	Capture	Euthanasia	Capture	Euthanasia
Buck	<1:4	1:128	<1:4	1:256
Doe 17	<1:4	1:1024	<1:4	1:32
Doe 18	<1:4	1:256	<1:4	1:256
Doe 19	<1:4	1:64	<1:4	1:16
Doe 21	<1:4	1:256	<1:4	1:256
Doe 23	<1:4	1:1024	<1:4	1:128
Doe 24	<1:4	1:256	<1:4	1:512
Doe 25	<1:4	1:512	<1:4	1:64

Chapter 5: Journal article:

Transmission of Bovine Viral Diarrhea Virus Among White-tailed Deer (*Odocoileus virginianus*)

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Abstract

Cattle persistently infected (PI) with bovine viral diarrhea virus (BVDV), a pestivirus in the family *Flaviviridae*, are an important source of viral transmission to susceptible hosts. Persistent BVDV infections have been identified in white-tailed deer (*Odocoileus virginianus*), the most abundant free-ranging ruminant in North America. As PI deer shed BVDV similarly to PI cattle, maintenance of BVDV within white-tailed deer populations may be possible. To date, intraspecific transmission of BVDV in white-tailed deer has not been evaluated, which prompted this study. Six pregnant white-tailed deer were captured in the first trimester of pregnancy and cohabitated with a PI white-tailed deer. Cohabitation with the PI deer resulted in BVDV infection in all does, as indicated by seroconversion. All does gave birth to live fawns and no reproductive losses were observed. At birth, evidence of BVDV infection was identified in two singlet fawns, of which one was determined to be PI by repeated serum RT-nPCR, whole blood virus isolation and immunohistochemistry. This study demonstrates for the first time that BVDV transmission may occur among white-tailed deer. The birth of a PI fawn through contact to a PI white-tailed deer indicates that under appropriate circumstances, BVDV may be maintained in white-tailed deer by congenital infection.

Key words

Bovine viral diarrhea virus / BVDV transmission / *Odocoileus virginianus* / persistent infection / white-tailed deer

Introduction

Bovine viral diarrhea virus (BVDV), a pestivirus in the family *Flaviviridae*, employs different strategies to ensure survival and successful propagation in the mammalian host population. These strategies include the modification of the host's immune response, multiple direct and indirect routes of transmission, and establishment of persistently infected (PI) carrier animals following transplacental infections in the first trimester of pregnancy (Thurmond 2005; Schweizer et al. 2006). Persistently infected cattle generally shed BVDV throughout their life and at much greater levels than acutely infected animals, resulting in a larger coefficient of infectiousness (Thurmond 2005). In addition, BVDV may efficiently cross species barriers and infect various mammalian hosts in the order Artiodactyla, which may be important in the pathogen's survival strategy (Vilcek et al. 2006).

The mammalian order Artiodactyla consists of ten families, and evidence of infection with BVDV has been reported in the families Antilocapridae, Bovidae, Camelidae, Cervidae, Giraffidae, Suidae, and Tragulidae, including over 50 species (Nettleton 1990; Van Campen et al. 2001a; Grondahl et al. 2003). Epidemiologically intriguing, persistent BVDV infections have been described in different species other than cattle, including domestic small ruminants, swine, alpacas, eland (*Taurotragus oryx*), lesser Malayan mouse-deer (*Tragulus javanicus*), and white-tailed deer (*Odocoileus virginianus*) (Loken 1995; Anderson et al. 1998; Grondahl et al. 2003; Carman et al. 2005; Passler et al. 2007). However, little knowledge exists regarding the role of PI heterologous species in the epidemiology of BVDV. The virus was transmitted horizontally to two calves and vertically within a maternal line by PI lesser Malayan mouse-deer, demonstrating transmission of BVDV among heterologous PI hosts (Uttenthal et al. 2006). High seroprevalence rates were reported in Zimbabwean eland populations, in which a PI eland was found, suggesting

endemic viral circulation (Anderson et al. 1998). In a PI white-tailed deer, levels of BVDV shedding from the nasal passages were similar to those found in PI cattle, suggesting that, given sufficient contact to susceptible animals, BVDV may be efficiently transmitted (Passler et al. 2007).

In North America, white-tailed deer are the most abundant free-ranging ruminant and home ranges extend from southern Canada to northern South America (Baker 1984). In this species, infections with BVDV following experimental and natural infections have been described (Passler et al. 2007; Chase et al. 2008). Bovine viral diarrhea infections in white-tailed deer have resulted in subclinical signs, pyrexia, lymphopenia, and reproductive disease (Van Campen et al. 1997; Ridpath et al. 2007b; Ridpath et al. 2008). Reproductive disease following experimental BVDV infections included abortion, embryonic resorption, fetal mummification, and stillbirth (Passler et al. 2007; Ridpath et al. 2008). In contrast, all white-tailed deer in a recent study carried their pregnancies to term after infection with BVDV by cohabitation with PI cattle in the first trimester of gestation (Passler et al. 2009c). In that study, four of seven pregnancies resulted in BVDV positive offspring, of which three were PI with BVDV indicating efficient interspecific transmission (Passler et al. 2009c).

Understanding the ecology of BVDV including host range and potential for transmission among different species is vital to control programs in domestic and wild animal populations. In a North American pastoral environment, frequent contact of cattle and white-tailed deer is likely; however the risk of white-tailed deer to be a reservoir for BVDV also depends on maintenance of BVDV within deer populations. To date, knowledge about BVDV maintenance in white-tailed deer does not exist, prompting the present study, in which pregnant white-tailed deer were cohabitated with a PI fawn.

Materials and Methods

Animals

The research described herein was performed under the approval of the Institutional Animal Care and Use Committee of Auburn University (2008-1340). Between March 7th and April 11th, 2008, 6 female white-tailed deer were captured by cannon-net or dart-gun similarly to previously described methods (Hawkins et al. 1968; Saalfeld et al. 2007). For sedation after capture by cannon-net, 120 mg of xylazine HCL (AnaSed®, Lloyd Laboratories, Shenandoah, IA) and 150 mg of tiletamine HCl/zolazepam HCl (Telazol, Ford Dodge Animal Health, Fort Dodge, IA) were administered intramuscularly. Alternatively, 200 mg of xylazine HCl and 250 mg of tiletamine HCl/ zolazepam HCl were administered by dart gun. Under sedation, blood and skin samples were collected to determine the deer to be free from BVDV or BVDV antibodies by serum and whole-blood virus isolation, RT-nPCR, immunohistochemistry, and virus neutralization. Pregnancy examinations were performed by transrectal and/or transabdominal ultrasound. When possible, placentome or vesicle sizes, and/or fetal crown-to-rump lengths were recorded to assist in estimating fetal ages at exposure. The deer were transported to a 2.0 ha deer pen at the Captive Deer Research Facility at Auburn University in which two additional white-tailed deer were housed. Of these two deer, one was PI with a BVDV 1b strain (designated AU526) as result of the cohabitation of its dam with two PI cattle (Passler et al. 2009c). At the beginning of cohabitation, the PI deer was approximately 6.5 months old. Beginning in July, 2008, the deer pen was searched for fawns daily. When a fawn was found, it was evaluated and whole blood, serum, skin biopsy (ear notch) and nasal swab samples were collected for virus isolation, RT-nPCR, ELISA, IHC, and virus neutralization. Fawns remained with their dams

until all deer had given birth. When a fawn was positive for BVDV at initial testing or appeared unthrifty, further samples were collected approximately 3 weeks after birth. Following the cessation of parturitions, all adult deer and fawns that were negative for BVDV were euthanized. At euthanasia, blood samples were collected for virus isolation and virus neutralization procedures. When a fawn was found dead, additional blood and tissue samples were collected for virus isolation and IHC.

Maternity testing

A skin biopsy sample was collected from all adult and neonatal deer and stored at -80°C until testing was performed as previously described (Anderson et al. 2002; DeYoung et al. 2003). Briefly, DNA was isolated using a commercial kit (Qiagen DNeasy, Qiagen Genomics Inc., Bothell, Washington) and PCR was used to amplify 13 microsatellite DNA loci from a panel optimized for use in white-tailed deer (BL25, BM6438, BM848, O, BM4208, BM6506, D, P, Cervid1, ILSTS011, INRA011, N, Q). The PCR products were loaded on an ABI 3130 automated DNA sequencer (Applied Biosystems, Foster City, California) and multilocus genotypes were constructed for all individuals using GENEMAPPER software (Applied Biosystems, Foster City, California). Maternity was assigned for all fawns using a likelihood ratio method in the computer program CERVUS 3.0, available at www.fieldgenetics.com.

Virus isolation

From adult deer, serum and whole blood samples obtained at the time of capture and euthanasia were tested by virus isolation. From fawns, virus isolations were performed on whole blood and nasal swab samples at birth; serum and whole blood samples at retesting; and serum and whole blood samples at euthanasia, as well as tissues (lymph nodes, spleen, thymus)

collected at postmortem examination. Samples were assayed for BVDV by passage through MDBK cells, as has been described previously (Givens et al. 2003).

Skin biopsy immunohistochemistry

Immunohistochemical (IHC) detection of BVDV antigen was performed on formalin-fixed paraffin-embedded skin biopsies collected at birth and retesting using the monoclonal antibody, 3.12F1. The 3.12F1 monoclonal antibody reacts with an epitope of the E^{RNS} of BVDV that is shared by diverse BVDV isolates and therefore is a suitable target for the detection of a wide variety of isolates of BVDV. The immunohistochemical stain distribution in animals PI with BVDV is represented by diffuse staining in the epidermis and hair follicle epithelium; stain may also be present in the dermis and adnexal structures surrounding hair follicles (Brodersen 2004). Antigen distribution in white-tailed deer, persistently infected with BVDV is analogous to that detected in PI cattle (Passler et al. 2007; Duncan et al. 2008a).

Antigen capture ELISA

The BVDV antigen detection in skin biopsy samples was performed using a commercially available kit (IDEXX Laboratories, Westbrook, ME 04092, USA) developed for BVDV detection in bovine samples, according to the manufacturer's instructions. Presence or absence of BVDV within samples and classification as negative, suspect, or positive sample was established by using sample to positive (S/P) ratios of <0.20, 0.20 – 0.39, or >0.39, respectively.

Virus neutralization

A standard virus neutralization microtiter assay was used for the detection and quantification of antibodies in serum of adult deer at capture and euthanasia, and from fawns at

birth, retesting, and euthanasia. Sera were tested for neutralizing antibodies as previously described (Givens et al. 2003). The test isolate used in the serum neutralization assay was BVDV AU526 which had been isolated from the PI fawn that exposed the pregnant does. The antibody titer was defined as the inverse of the highest dilution with complete inhibition of staining by the immunoperoxidase test.

Reverse transcriptase polymerase chain reaction and sequencing

Viral RNA was detected by a two-round, rapid-cycle PCR assay on whole blood samples, serum, and tissues from fawns; and serum samples from adult deer at capture and euthanasia. This reverse transcription nested PCR (RT-nPCR) is characterized by increased sensitivity as compared to conventional RT-PCR and has been previously described in detail (Givens et al. 2001). Briefly, RNA was isolated from samples using the QIAamp® viral RNA mini kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. All steps of the RT-nPCR were performed in a single-tube reaction. In the first round, the outer primers, BVD 100 (5'-GGCTAGCCATGCCCTTAG-3') and HCV 368 (5'-CCATGTGCCATGTACAG-3') amplified a 290 base pair sequence of the 5' untranslated region of the viral genome. In the second round of the reaction, the inner primers BVD 180 (5'-CCTGAGTACAGGGDAGTCGTCA-3') and HCV 368 amplified a 213 base pair sequence within the first amplicon. After completion of the PCR cycle, 5 µl of the RT-nPCR products were separated by 1.5% agarose gel electrophoresis. Ethidium bromide staining allowed visualization of the RT-nPCR using an ultraviolet transilluminator. On RT-nPCR positive samples, sequence analysis was performed on aliquots of the RT-nPCR products carried out in triplicate.

If positive for BVDV on agar gel electrophoresis, samples were purified using the QIAquick® PCR purification kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's specifications and sequenced by automated dye terminator nucleotide sequencing using both the 5' and 3' primers (BVD 180 and HCV 368, respectively). Consensus sequences were determined for each sample using Align X® computer software (Vector NTI Suite 7.1, InforMax, Inc., Bethesda, MD, USA) and compared to nucleotide sequences of BVDV AU526.

Results

In June, 2008 the PI deer used for exposure died at approximately ten months of age and BVDV antigen was detected by IHC in skin sample biopsies. A total of ten fawns (two singlets and eight twins) were born to the six does, which is in agreement with previously reported rates of reproductive success in white-tailed deer (Ozoga 1987; Verme 1989). All fawns were born between July 4th and August 16th, 2008, which corroborated the estimated days of birth based on initial ultrasound examinations of the captured does. All twin fawns were adequate in weight and appearance; however, both singlet fawns were below the normal weight reported for white-tailed deer fawns in Alabama (Haugen 1959).

At initial testing, evidence of BVDV infection was detected in two fawns, one a singlet (Fawn No. 21) and the other a twin (No. 19) (Table 5.1). Serum from both fawns was positive by RT-nPCR assay and BVDV was isolated from the buffy coat of the singlet (No. 21). While all fawns were negative on nasal swab virus isolation and ELISA, the singlet fawn was also positive on skin sample IHC. An additional singlet fawn (No. 22) appeared small at birth and had an ill-thrifty appearance. From this fawn and the two fawns positive for BVDV at birth (Nos. 19 and 21), additional samples were collected to assess their status as PI. These samples mirrored the

initial results as the RT-nPCR was positive in 2 fawns, the virus isolation from the buffy coat was positive in 1 fawn (No. 21), and the ill-thrifty fawn (No. 22) was negative for BVDV. Both singlet fawns (Nos. 21 and 22) died within 1 month of birth, and further evidence of BVDV infection was identified in fawn No. 21 as indicated by IHC. According to the repeated isolation of BVDV and positive IHC results, fawn No. 21 was determined to be PI with BVDV. However, as virus was not isolated from the RT-nPCR positive fawn (No. 19), its status of infection was considered uncertain. Therefore, additional samples were collected from fawn No.19 at approximately 5 months of age, which resulted in negative results using virus isolation and RT-nPCR procedures.

Based on pregnancy ultrasound examinations and day of birth, first exposure of the doe that gave birth to the PI fawn (No. 21) was at approximately 41 days of gestation. For all does that did not give birth to PI offspring, first cohabitation with the PI deer occurred from day 63 or later. Virus was not detected in adult deer at capture or euthanasia, or the remaining fawns at euthanasia. Cohabitation of the pregnant does with the PI deer resulted in seroconversion in all adult deer with antibody titers from 1:128 to 1:512 at euthanasia; approximately 6-8 month after the PI deer had died (Table 5.2). The greatest antibody titers were detected in the dams of the two singlet fawns, of which one was determined to be PI.

Discussion

This study demonstrates for the first time that BVDV may be transmitted among white-tailed deer and result in the birth of PI offspring. Despite evidence of persistent infection in only one fawn (No. 21), seroconversion in all exposed does indicates that efficient transmission of BVDV occurred as a result of exposure to a PI deer during pregnancy. Furthermore, the study

emphasizes that, despite low survival in PI white-tailed deer and substantially reduced life expectancy, a fawn from a previous year may survive long enough to be present during the early gestational period of the following year.

Maintenance of BVDV within free-ranging white-tailed deer populations would depend on the presence of BVDV shedders, such as PI deer during the critical gestational period. According to the rose-petal hypothesis, female offspring establish home ranges that tend to overlap that of their mother (Porter et al. 1991). As successive generations of female offspring are born, the spatial distribution of a group of related individuals will continue to expand like the petals of a rose. These cohesive social groups consisting of related females may be particularly susceptible to BVDV infection once exposed. Female white-tailed deer typically exhibit very low rates (<5%) of dispersal (Dusek et al. 1989), suggesting that female PI offspring have strong potential to transmit BVDV to related pregnant females within their home range. Within these matrilineal groups, there is frequent close contact among deer, as was emulated in this study, and BVDV transmission may be similarly efficient. Intramatrilineal BVDV maintenance in white-tailed deer may be comparable to the transmission and maintenance of BVDV in domesticated alpacas, where PI crias remain with their dam during the subsequent breeding period, therefore efficiently infecting other susceptible females (Kim et al. 2009). The efficiency of transfer among matrilineal groups will ultimately be a function of the BVDV immune status of the population, the social organization and genetic relatedness within the population, and the management of the population by harvest. Deer populations that are subjected to high rates of antlerless harvest may exhibit reduced spatial and genetic structuring (Comer et al. 2005), which could reduce the potential for maintenance of BVDV within a population. In contrast to females, most male white-tailed deer disperse between 12 and 18 months of age. Dispersal distances are

normally between 5 and 10 km, but can be as great as 40 km (Long et al. 2005; McCoy et al. 2005). This high rate of dispersal suggests that male PI fawns that survive to dispersal age have greater potential to transmit BVDV to unrelated groups of deer across a greater geographical area than females; however, orphaned male fawns exhibit much lower rates of dispersal than their unorphaned counterparts (Holzenbein et al. 1992). Theoretically, an increase in the number of orphaned fawns by antlerless harvest would reduce the probability of a male PI fawn dispersing to a new area.

Results of this and other studies demonstrate the potential for maintenance of BVDV in white-tailed deer populations as indicated by viral shedding from PI deer, which is similar to cattle, and transmission among white-tailed deer (Passler et al. 2007). However, there likely are regional variations in the extent of BVDV maintenance that depend on population density and its influence on contact rates among deer. Increasing population densities result in greater chances for contact among deer and therefore increased transmission potential. Supplemental winter feeding may be an important factor in maintenance of BVDV in white-tailed deer, as this artificial commingling of deer occurs at a time when fetuses may be susceptible to BVDV and become PI. Any deer management practices that increase deer densities or rates of contact among deer have the potential to increase the potential for maintenance of BVDV within a population, as has been described for maintenance of bovine tuberculosis in white-tailed deer populations (Schmitt et al. 2002).

To date, information on the susceptible gestational age for persistent infections in white-tailed deer is unknown. According to the estimated gestational age based on ultrasound examination and the date of birth of the PI fawn (No. 21), transplacental infection occurred at 41 days of gestation based upon a gestational length of 200 days for white-tailed deer in Alabama.

This is corroborated by previous studies, where PI fawns were born to white-tailed deer intranasally inoculated on days 43 or 42 – 49, respectively (Passler et al. 2007; Ridpath et al. 2008). Based on the shorter gestation length in white-tailed deer as compared to cattle, a gestational age below 67 days was suspected to be the most susceptible time at which white-tailed deer fetuses may become PI (Ridpath et al. 2008). Further research is necessary to substantiate these extrapolations, as the critical gestational age is important epidemiological information that likely influences BVDV maintenance in white-tailed deer.

In a recent study, experimental inoculation of pregnant white-tailed deer with a strain of BVDV isolated from white-tailed deer resulted in severe reproductive disease (Ridpath et al. 2008). In contrast, all does in this study maintained pregnancies and gave birth to live fawns. Similar observations were made in another study, where only one of seven pregnancies resulted in birth of stillborn fawns, and live fawns were born to the remaining six does after cohabitation with PI cattle during the first trimester of pregnancy (Passler et al. 2009c). Differences in these findings may be due to viral isolates, as BVDV strains of bovine rather than cervine origin were used in studies by this group. Furthermore, in the study by Ridpath and colleagues (Ridpath et al. 2008), the intranasal route of infection was used which likely resulted in viral challenge dynamics much different from exposures to PI deer, as was used in the present study.

The findings of this study add to a body of knowledge that indicates that white-tailed deer have the potential to become a reservoir for BVDV. However, most of the current knowledge on BVDV in white-tailed deer is from experimental research rather than observations in free-ranging populations. Regional variations in deer management and varying population dynamics emphasize the need for further systematic evaluation of BVDV in deer. Infections with BVDV in

white-tailed deer populations may have a negative impact on health and welfare, but should also be considered where BVDV control programs are planned.

Acknowledgements

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Table 5.1 Results of virological assays on fawns.

Fawn ID		13	14	15	16	17	18	19	20	21	22		
Birth	Serum	VN	4096	4096	2048	4096	2048	8192	2048	4096	16384	4096	
		nPCR	-	-	-	-	-	-	+	-	+	-	
	Nasal swab	VI	-	-	-	-	-	-	-	-	-	-	
	Buffy coat	VI	-	-	-	-	-	-	-	-	+	-	
	Skin	IHC	-	-	-	-	-	-	-	-	-	+	-
		ELISA	-	-	-	-	-	-	-	-	-	-	-
Re-test	Serum	VN							512		4096	1024	
		VI							-		-	-	
		nPCR							+		+	-	
	Buffy coat	VI							-		+	-	
	Skin	IHC							-		+	-	
Euthanasia	Serum	VN	32	256	1024	2048	256	2048	1024	512			
		VI	-	-	-	-	-	-	-	-			
		nPCR	-	-	-	-	-	-	-	-	-		
	Buffy coat	VI	-	-	-	-	-	-	-	-			
	Tissues	VI	-	-	-	-	-	-	-	-			

Table 5.2 Results of virological assays on does.

ID of Doe	BVDV serotiter at capture	BVDV serotiter at re-test	Gestational age at exposure^a	ID of offspring	Status of offspring
31	< 4	512	41 days	21	Persistently infected
32	< 4	NA	66 days	18 and 19	Seropositive, 19 RT-nPCR positive at birth
33	< 4	128	75 days	17 and 20	Seropositive
34	< 4	512	63 days	22	Seropositive
35	< 4	128	104 days	13 and 14	Seropositive
36	< 4	128	114 days	15 and 16	Seropositive

Chapter 6: Journal article:

Evaluation of Hunter-harvested White-tailed Deer for Evidence of Bovine Viral Diarrhea Virus
Infection in Alabama

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Abstract

Bovine viral diarrhea virus (BVDV) is one of the most relevant pathogens affecting today's cattle industries. Although great strides have been made in understanding this virus in cattle, little is known about the role of wildlife in the epidemiology of BVDV. While persistently infected (PI) cattle are the most important reservoir, free-ranging ungulates may become infected with BVDV as demonstrated by serosurveys and experimental infections. Therefore, free-ranging wildlife may maintain BVDV as the result of an independent cycle and serve as a reservoir for the virus. Systematic studies on prevalence of BVDV-specific antibodies or frequency of persistent BVDV infection in North American wildlife are sparse and no information is available from the southeastern United States. The objective of this study was to evaluate blood and skin samples from hunter-killed white-tailed deer (*Odocoileus virginianus*) for evidence of BVDV infection. Virus neutralizing antibodies were detected in 2/165 serum samples. Skin biopsy immunohistochemistry (IHC) was performed on samples from 406 deer using a BVDV-specific monoclonal antibody (MAb) (15c5) and BVDV antigen was detected in one sample. A similar IHC staining pattern was obtained using a second BVDV MAb (3.12F1). Viral antigen distribution in the skin sample of this deer resembled that found in persistently infected cattle and a previously described persistently infected white-tailed deer, thus the deer was presumed to be persistently infected. Evidence of BVDV infection in free-ranging white-tailed deer should encourage further systematic investigation of the prevalence of BVDV in wildlife.

Keywords

BVDV; Immunohistochemistry; Seroprevalence; *Odocoileus virginianus*; white-tailed deer

Infections with bovine viral diarrhea virus (BVDV) are cause for substantial losses to cattle industries worldwide. The virus may result in respiratory, gastrointestinal, and reproductive tract disease of varying severity, ranging from subclinical to fatal disease (Baker 1995). There are two forms of infection with BVDV, transient and persistent. Transient infections (TI) occur in postnatal, immunocompetent animals and initiate formation of BVDV-specific immunity that results in clearance of the virus. In contrast, persistent infection (PI) is the result of in utero exposure of the developing fetus to a noncytopathic strain of BVDV prior to the development of immunocompetence. The virus is recognized as self, does not result in antibody formation and continues a life-long replication in most tissues of the host (Bolin 1995).

Infections with BVDV are not limited to cattle, but can occur in a variety of species of the order Artiodactyla, as has recently been reviewed (Van Campen et al. 2001a). White-tailed deer (*Odocoileus virginianus*) are the most abundant species of wild ruminants in the United States, and over the last 50 years, the occurrence of BVDV infection in this species has been evaluated by different researchers. Previous work has demonstrated that white-tailed deer are susceptible to BVDV infection and may develop clinical disease (Van Campen et al. 1997; Ridpath et al. 2006b). A recent report demonstrated that experimental infection of pregnant white-tailed deer may result in the birth of PI offspring (Passler et al. 2007). Despite these published reports, only three studies have evaluated BVDV infection in free-ranging white-tailed deer. In contrast to high BVDV prevalence rates detected in other Cervidae, BVDV seroprevalence in free-ranging white-tailed deer from Anticosti Island, Quebec, and New York State were reported to be 0, 3, and 5.75%, respectively (Kahrs et al. 1964; Friend et al. 1967; Sadi et al. 1991). In light of the extensive distribution of white-tailed deer, ranging from southern

Canada to South-America, application of data from these serosurveys to other geographic areas may not be appropriate. Additional studies were performed in captive or locally confined white-tailed deer and cannot provide information on the risk of BVDV transmission from free-ranging white-tailed deer to cattle populations (Davidson et al. 1983; Brooks et al. 2007). Because BVDV eradication and control programs are being developed and implemented by veterinary and producer organizations in different states, it is necessary to investigate the occurrence of BVDV in wildlife species. Populations of free-ranging wildlife may harbor BVDV and could be a reservoir for the virus (ElAzhary et al. 1981; Van Campen et al. 2001b). Therefore, the objective of this study was to evaluate free-ranging white-tailed deer in Alabama for evidence of BVDV infection.

Samples were collected during the 2005 - 2006 Alabama white-tailed deer hunting season. Site visits were made to 23 deer processing units across the state of Alabama. In order to increase the diversity of sample origin, a maximum of 40 skin and 20 serum samples were collected from each deer processing facility. Blood and skin biopsy samples were collected from all deer arriving during a visit. When possible, blood samples were collected from the opened heart or major vessels. If a deer had been field-dressed, blood was collected from the thoracic cavity. Additional skin samples were taken from all deer stored in refrigeration that had been processed earlier. Blood was collected into 10 ml serum tubes and placed on ice for transportation to the laboratory. Skin biopsies (25 mm²) were harvested using commercially available ear notch pliers from the ventral aspect of the ear and placed into 10% neutral buffered formalin. All samples were delivered to Auburn University within 24 hours of collection for further processing.

A standard microtiter virus neutralization assay was used for detection and quantification of antibodies in serum (Edwards 1990). Sera were tested for neutralizing antibodies to the cytopathic BVDV 1 strain NADL and the cytopathic BVDV 2 strain 125c. Sera were inactivated for 30 minutes in a 56°C water bath. Serial two-fold dilutions, ranging from 1:4 to 1:4096, were made for each serum sample in 50 µL of minimum essential medium with Earle's salts^a supplemented with 10% (vol:vol) equine serum^b, sodium bicarbonate^b (0.75 mg/dL), L-glutamine^c (0.29 mg/mL), penicillin G^d (100 U/mL), streptomycin^d (100 U/mL), and amphotericin B^d (0.25 µg/mL). For each dilution, 3 wells of a 96-well microtiter plate were inoculated with 50 µL of culture media containing 100 cell culture infectious doses (CCID50) of the respective BVDV strain. The plates were incubated for 1 h at 37°C in humidified air containing 5% CO₂. Madin Darby bovine kidney (MDBK) cells were used as the indicator cells by adding 50 µL of media containing 15,000 cells per well. Each test included a back titration of the virus and a positive and negative serum control. Following a 5-day incubation period, antibody titer was defined as the reciprocal of the highest dilution with complete inhibition of cytopathic effect.

Immunohistochemical (IHC) detection of BVDV antigen was performed on formalin-fixed paraffin-embedded skin biopsies using a monoclonal antibody (MAb), 15c5^e (Haines et al. 1992). The BVD MAb 15c5 reacts with an epitope of the ERNS protein of BVDV that is shared by and therefore is suitable for detection of a wide variety of BVDV strains. The immunohistochemical stain distribution in animals persistently infected with BVDV is represented by diffuse staining in the epidermis and hair follicle epithelium; stain may also be present in the dermis and adnexal structures surrounding hair follicles (Brodersen 2004).

In Alabama, the white-tailed deer population is estimated at approximately 1.7 million animals. Paired serum and skin samples were collected from a total of 165 animals. Additionally, 241 skin samples were harvested from deer that had already been stored in refrigeration. The majority of samples were obtained from deer harvested in the central, eastern-central, and northern regions of the state. Two of 165 (1.21%; 95% confidence interval: 0 – 2.88%) samples were positive by virus neutralization. One sample was positive for BVDV 1 (NADL) and BVDV 2 (125c), exhibiting a greater titer against the former (1:128 for NADL; 1:16 for 125c). The second sample only contained neutralizing antibodies against BVDV 2 (strain 125c) with a titer of 1:4 but did not react positive for BVDV1 (<1:4). Bovine viral diarrhea virus antigen was detected by IHC in only one of the 406 skin biopsy samples (0.2%; 95% confidence interval: 0 – 0.6%), and this IHC-positive deer was a doe sampled from central Alabama (Fig 1). Positive IHC staining was distributed diffusely in epidermal cells and multifocally in hair follicle epithelium and apocrine gland cells. To reevaluate and ascertain this result, another IHC was performed using a different monoclonal antibody, BVDV MAb 3.12F1^f, as described previously (Blas-Machado et al. 2004). Antigen was similarly distributed with BVDV MAb 3.12F1 as detected with BVDV MAb 15c5.

The VN results indicated that BVDV infection had occurred in 2 of the 165 sampled deer. The low titer (1:8) found in one of the samples may have indicated a false positive VN and increasing the cut-off for positive samples to a higher VN titer may increase the specificity of the test, while decreasing its sensitivity. Virus neutralization (VN) is a common serologic method to determine BVDV specific antibodies and allows quantification of serum-neutralizing antibody titers. In samples obtained from heterologous species, it may be difficult to decide which strain to use for VN as there exists considerable antigenic heterogeneity amongst strains of BVDV in

cattle, and this may be exacerbated by passage in a different host. Additionally, there is no universally accepted reference strain for BVDV VN, further complicating the choice of the strain used for this procedure. Virus neutralization cannot detect acute infections before formation of anti-BVDV antibodies has occurred, and the low titer found in one of the serum samples could have resulted from a very recent exposure. A low antibody titer could furthermore result from a transient infection that had occurred long before VN testing.

Surveys for BVDV specific antibodies have been performed in a number of countries and many species of wildlife, identifying a highly variable BVDV seroprevalence in different free-ranging wildlife populations. Similarly, serosurveys conducted in North America also demonstrate variable results. Bovine viral diarrhea antibody prevalence in North American ungulates was reported to be 0% on Anticosti Island, Quebec (Sadi et al. 1991), 3% in Alaska (Zarnke 1983), 3% in New York State (Kahrs et al. 1964), 4% in Alberta and Saskatchewan (Barrett et al. 1975), 34% in New Mexico (Couvillion et al. 1980), 59% in selected national parks of the western United States (Aguirre et al. 1995), 60% in Wyoming (Van Campen et al. 2001b), and 60.7% / 69.3% in Quebec (ElAzhary et al. 1979). Reasons for this variation of reported seroprevalences can only be speculated. It is plausible that increased contact with cattle causes a greater BVDV seroprevalence in wildlife; however, this was not true in a study performed by Fröhlich (1995), comparing the BVDV antibody prevalence of wild Cervidae from areas of different cattle densities (Frolich 1995). Much of the known data on BVDV seroprevalence stems from distinct and often isolated areas and populations of wildlife, such as from wildlife refuges and national parks. Exact epidemiologic implications for state-wide or national BVDV-control programs and the role of wildlife in BVDV maintenance and transmission may therefore be difficult to extrapolate from these studies. However, evidence of

BVDV occurring in free-ranging wildlife and high prevalence rates reported for some wildlife populations, indicating intrapopulation maintenance of BVDV (Van Campen et al. 2001b), should encourage further systematic prevalence surveys.

The importance of PI animals in the epidemiology of BVDV has prompted the development and evaluation of different assays for antigen detection. Immunohistochemistry (IHC) on skin biopsies (i.e., ear notch) has been successfully used since the 1990s to identify PI cattle. The test shows high agreement with results of microtiter virus isolation (Brodersen 2004). Immunohistochemical detection of animals PI with BVDV is not affected by colostral antibodies, and transient infections (TI) only rarely cause false positive results (Brodersen 2004). In a recent study, IHC was performed on a skin biopsy collected from a white-tailed deer fawn, known to be PI with BVDV (Passler et al. 2007). Immunohistochemical stain distribution in the skin sample from this PI fawn resembled that found in PI cattle. Viral antigen was identified in only one skin biopsy in the present study and the stain distribution was similar to that found in PI cattle and the PI white-tailed deer. Unfortunately, a blood sample was not available from the deer from which the ear notch had been taken, making further testing by VN or virus isolation impossible. However, given the usefulness of IHC for identification of PI and its agreement with microtiter virus isolation in cattle, it is presumed that this white-tailed deer was PI with BVDV. In cattle, TI may rarely cause positive antigen staining in ear notch samples (Brodersen 2004). In samples from TI cattle, BVDV antigen is distributed multifocally in the epidermis and infundibulae of hair follicles (Njaa et al. 2000), rather than diffusely as found in this deer. Experimental TI in calves did not result in positive IHC in one study (Ridpath et al. 2002). The likelihood of positive IHC in TI cattle increases when high doses of BVDV inoculum are administered to calves experimentally (Njaa et al. 2000), or when a large number of PI cattle are

in a herd (Brodersen 2004). The exposure of the IHC positive deer to high titers of BVDV is unlikely, indicating PI rather than TI BVDV in this animal.

To assess the possibility of cross reactivity of MAb 15c5 with non-BVDV antigen, resulting in a false positive result, another BVDV MAb (3.12F1) was used in a second IHC on the suspect ear notch. Interestingly, this MAb resulted in identification of the same antigen distribution within the suspect specimen, underscoring the correct identification of BVDV antigen by MAb 15c5. Further research is needed, evaluating BVDV antigen distribution in skin of white-tailed deer and the value of IHC for identification of PI animals in heterologous species.

In cattle herds, presence of a PI animal results in seroconversion in a large percentage of in-contact herd mates. In white-tailed deer, a similarly high seroprevalence would be expected when a PI animal is present within a sampled population. In contrast to the present study, Van Campen and colleagues (2001) detected a 60% BVDV seroprevalence rate in mule deer sampled from the same geographic area as a clinically-ill BVDV isolation positive mule deer (Van Campen et al. 2001b). The low seroprevalence rate detected in the present study may be the result of more widespread sampling from across the state of Alabama rather than sampling within a limited population containing a BVDV-positive animal. It is unlikely that multiple deer from the same group were presented to the deer processor on the same sampling day. No other samples obtained from deer at the location where the IHC positive sample was collected were positive for BVDV; therefore, the IHC positive deer may represent a locally contained infection that has not spread to other populations of white-tailed deer within the region.

Although it has been demonstrated that white-tailed deer can be infected with BVDV, the main route of transmission in free-ranging animals has not been elucidated (Van Campen et al. 1997; Passler et al. 2007). While close proximity to cattle was presumed to be the cause for high

seroprevalence rates in wildlife by some authors (Barrett et al. 1975; Aguirre et al. 1995), others hypothesized that an independent cycle is responsible for intrapopulation persistence (ElAzhary et al. 1981; Frolich 1995). Transmission of BVDV among individuals of a white-tailed deer population may be dependent on a variety of factors, including amount and degree of individual contact, human interaction in the form of wildlife management, and survivorship of persistently infected animals. With seasonal variation, a female white-tailed deer normally has limited contact with other deer in her home range except for those within her matrilineal group (Hawkins et al. 1970; Aycrigg et al. 1997), which may preclude BVDV transmission to a large number of other animals. Recently, white-tailed deer in Michigan have been identified as a reservoir for *Mycobacterium bovis* (Schmitt et al. 2002). The maintenance of tuberculosis in free-ranging white-tailed deer resulted largely from increasing deer densities in the state of Michigan and the practice of baiting and winter feeding for hunting purposes which resulted in closer contact of animals than in populations where supplemental feeding is less prevalent (Schmitt et al. 2002). In Alabama, supplemental feeding during the hunting season is illegal, and the harsh winters that force deer to commingle around limited feeding areas in northern regions are not as influential. Therefore, BVDV may be contained within distinct social groups in white-tailed deer in Alabama.

The low BVDV seroprevalence rate detected in white-tailed deer in Alabama corroborates previous work in this species but contrasts reports of high anti-BVDV antibody rates detected in other wildlife species. Therefore, determination of the PI prevalence rather than seroprevalence may more accurately assess the role of white-tailed deer in BVDV control or eradication programs.

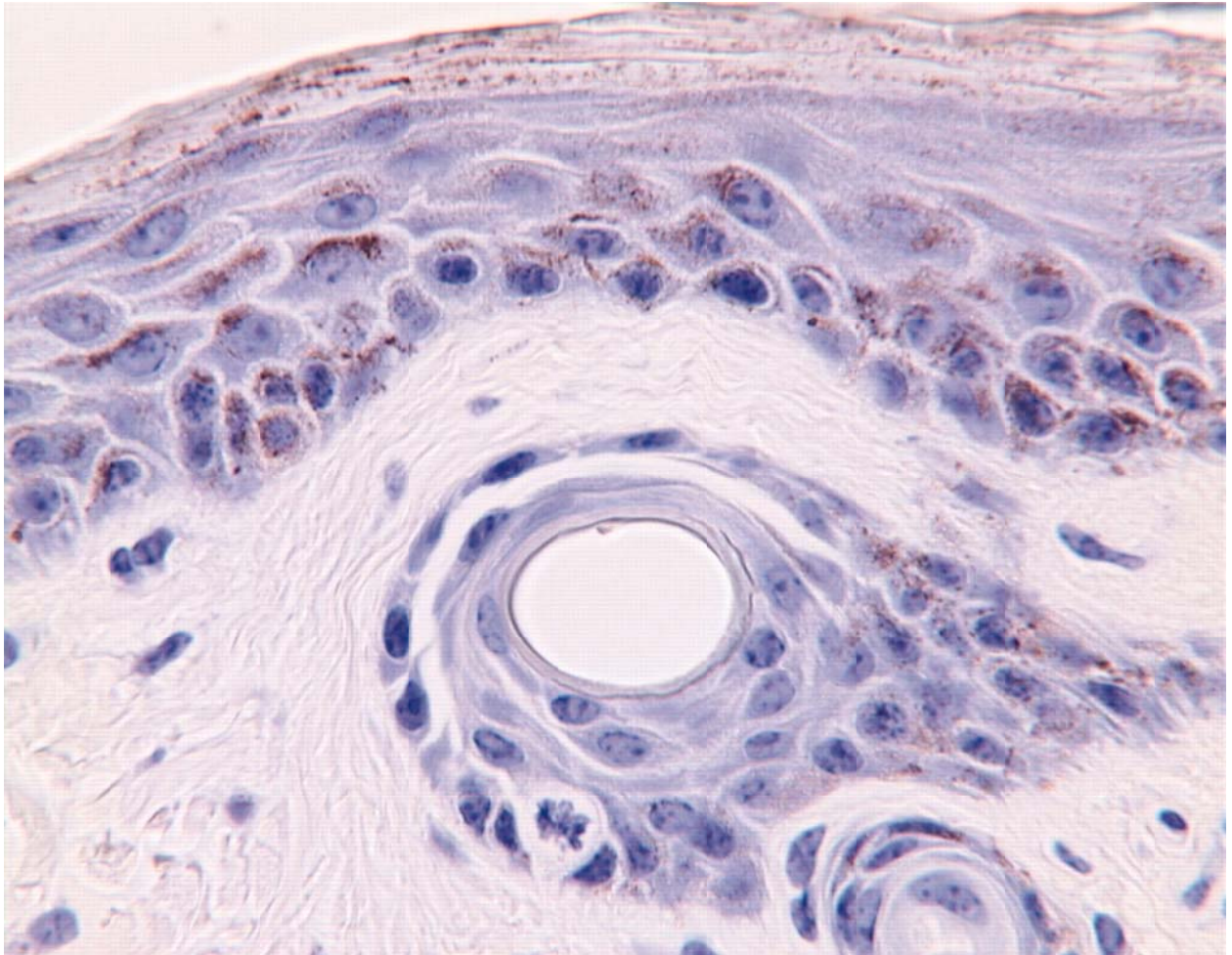
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Sources and manufacturers

- a. Minimum Essential Medium, Sigma Chemical Co, St. Louis, MO
- b. Equine Serum, Sigma Chemical Co, St. Louis, MO
- c. L-glutamine (100X), Gibco BRL, Life Technologies, Grand Island, NY
- d. Penicillin-streptomycin solution (100X), Sigma Chemical Co, St. Louis, MO
- e. BVD MAb 15c5, Syracuse Bioanalytical, East Syracuse, NY
- f. BVDV MAb 3.12F1, Oklahoma Animal Disease Diagnostic Laboratory, Stillwater, OK

Figure 6.1 Skin sample from a white-tailed deer positive for bovine viral diarrhea virus antigen by immunohistochemical testing.



Chapter 7:

Evaluating Transmission of Bovine Viral Diarrhea Virus to Cattle by Exposure to Carcasses of
Persistently Infected White-tailed deer (*Odocoileus virginianus*)

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Abstract

Infections with bovine viral diarrhea virus (BVDV) that are clinically analogous to cattle have been described in the white-tailed deer (*Odocoileus virginianus*), but the epidemiologic role of persistently infected (PI) white-tailed deer is unknown. Persistently infected white-tailed deer shed BVDV, transmitting and maintaining BVDV in groups of deer. Survival of PI white-tailed deer appears to be reduced, and clinically ill or dead PI deer may be a source of BVDV. This study sought to determine if BVDV transmission could occur when cattle come in contact with carcasses of PI white-tailed deer. In two trials, steers were exposed to the carcass of PI fawn A (BVDV 2) or PI fawn B (BVDV 1). One steer from each of two groups was separated into a pen with the carcass. The number of contacts with the carcass was monitored. Following 8 hours, the single steer from each trial was commingled with four other steers for 28 days. Animals were tested for BVDV and BVDV antibodies. Controls included one steer inoculated intranasally with spleen homogenate from fawn A, and two steers inoculated intranasally or intravenously with spleen homogenate from fawn B. Steers in both trials made contact with the carcass, but BVDV transmission did not occur. The intranasally inoculated control for trial A and the intravenously inoculated control for trial B became viremic and seroconverted. Although both PI fawn carcasses were potentially infectious, this study indicates exposure of a single inquisitive bovid to a PI fawn carcass did not result in transmission of BVDV.

Keywords

Bovine viral diarrhea virus / carrion transmission / *Odocoileus virginianus* / persistent infection / white-tailed deer

Introduction

Bovine viral diarrhea virus (BVDV) is the prototypic member of the genus *Pestivirus* in the family *Flaviviridae*. As implied by name, BVDV was first isolated from cattle and is considered a bovine pathogen (Olafson et al. 1946). However, the virus lacks strict host specificity and infections have been described in many species (Passler et al. 2009b). The implications of BVDV infections in heterologous species are multifaceted. In addition to causing disease in affected species, infections of heterologous hosts may initiate the development of heterologous reservoirs and hamper BVDV control programs in cattle (Passler et al. 2009b). Infection of free-ranging or captive wild ruminants with BVDV may threaten natural resources, including rare and endangered species. Contact of infected wildlife with susceptible cattle populations could present a risk to ongoing BVDV control and eradication programs (Van Campen 2010). The risk of BVDV transmission between wildlife and cattle is currently unknown, but because wildlife and cattle commonly share habitat, this could be a critical, yet poorly understood, aspect of BVDV control programs in the United States (Ridpath et al. 2009).

In white-tailed deer (*Odocoileus virginianus*), the most abundant free-ranging ruminant in North America, BVDV infections with clinical signs analogous to cattle have been described (Van Campen et al. 1997; Ridpath et al. 2007b; Chase et al. 2008; Ridpath et al. 2008). The virus may cause reproductive losses in white-tailed deer, but survival of fetuses and birth of PI fawns is described (Passler et al. 2007; Ridpath et al. 2008; Passler et al. 2009c). Persistently infected white-tailed deer can shed BVDV at similar levels to PI cattle (Passler et al. 2007), and contact with pregnant does results in efficient transmission of BVDV and subsequent birth of PI fawns (Passler et al. 2009a). In the United States, evidence of persistent infection in free-ranging white-tailed deer has been demonstrated, and reported prevalence rates for PI white-tailed deer were

0.1 – 0.3% (Chase et al. 2004b; Duncan et al. 2008b; Passler et al. 2008; Pogranichniy et al. 2008). These surveys utilized samples collected from adult hunter-harvested deer, which could result in biased sampling as poor survival of PI deer may result in few infected deer matriculating into sampled populations. Persistently infected white-tailed deer resulting from experimental infections had decreased survival and died before 10 months of age (Passler et al. 2007; Ridpath et al. 2008; Passler et al. 2009a; Passler et al. 2009c). While likely to affect epidemiological surveys, the early death of PI deer would reduce their potential to transmit BVDV to susceptible populations by nose-to-nose contact. However, exposure of susceptible cattle to BVDV by contact with a dying or dead deer may be possible and was the subject of this investigation.

Materials and methods

Experimental design and animals

This research was performed under the approval of the Institutional Animal Care and Use Committee of Auburn University (2009-1659). The study was designed to emulate the natural exposure of a susceptible steer to BVDV as result of the presence of a deceased PI deer in the grazing area, and sought to evaluate direct carcass-to-steer transmission with subsequent transmission to susceptible herd mates. This study consisted of two separate trials that each used one of two carcasses of PI white-tailed deer fawns. Both fawns were born to dams experimentally exposed to either BVDV 2 strain PA131 (Fawn A) or BVDV 1b strain AU526 (Fawn B) during gestation (Passler et al. 2009c). The diagnosis of persistent infection was confirmed on samples collected at birth and repeated sampling at the time of death (Table 1). A

post-mortem examination was performed on each carcass. Prior to inclusion in the study, the carcasses had been frozen at -20 °C for approximately two years.

Two groups, each containing five Holstein steers, were established for the carcass exposure experiments. Three additional steers were inoculated with spleen homogenates from each fawn to confirm infectivity of the carcasses. All steers were seronegative and BVDV-negative prior to inclusion in the study. Each group of steers was maintained in a two-hectare pasture. The general health of each steer was assessed once daily. For each trial, the carcass of a PI fawn was thawed at 5 °C for 12 hours prior to placement into a research pen of approximately 125 m². One steer was separated from its group and placed in the pen for a total of eight hours for potential exposure to the carcass (Day 0). Every two hours, the size of the pen was decreased by approximately half, and six hours after first exposure, feed was placed in the direct vicinity of the carcass. A contact with the carcass was defined as the muzzle of the steer approaching the carcass at a distance of less than 20 cm. A separate event of contact was defined as being at least 15 minutes subsequent to a prior event. The number of contacts of the steer with the carcass was monitored by time lapse photography. To limit behavioral alterations by presence of personnel, visual observations were not performed, with the exception of the times of sample collection from the carcasses. Following eight hours of exposure, the steer was commingled with the four other steers for 28 days. Samples were collected from each animal to monitor viremia and antibody production against BVDV.

The steers that served as infection controls were infected after the conclusion of both trials and did not have contact with principal animals. For trial A, one steer was intranasally inoculated with 2 ml of spleen homogenate in minimal essential medium (MEM) containing 1x10⁴ CCID₅₀/ ml of BVDV from fawn A. While consistently positive by RT-PCR, virus

isolation (VI) procedures on samples from carcass B were negative at death and subsequent retesting. Therefore, quantification of BVDV by virus titration was impossible on this carcass. As controls for trial B, two steers were inoculated with 2 ml of inoculum containing 0.25 ml of spleen homogenate from fawn B, either by the intranasal or intravenous route.

Sample collection

On day 0 of the trials, the carcasses were swabbed with a Dacron polyester swab at 0, 2, 4, 6 and 8 hours at two consistent sites. Muscle samples were collected at 0 and 8 hours. All carcass samples were processed on the day of collection. The muscle samples were processed by homogenization with a Tekmar Stomacher (Model 80, Tekmar Co, Cincinnati, OH, USA) and resuspended with 3mL of MEM prior to assay. Blood samples were collected from steers on days 0, 4, 6 - 11, 14 - 18, 21, 22 and 28. White blood cells and sera were refrigerated for ≤ 72 hours before VI procedures were performed. Sera were removed and stored at -80° C for virus neutralization (VN) assays. To obtain the buffy coat, white blood cell samples were processed as described previously (Walz et al. 2008) except that samples were resuspended in 1 mL of MEM.

Virus titration of carcass samples

Virus titration of carcass swab media and carcass muscle samples involved multiple ten-fold dilutions in triplicate and employed the statistical method of Reed and Muench (1938) to determine the quantity of BVDV in samples (Reed et al. 1938). An immunoperoxidase monolayer assay was performed to confirm the presence of non-cytopathic BVDV as previously described (Afshar et al. 1991; Givens et al. 2003).

Virus isolation

Serum, buffy coat, carcass swab media, and carcass muscle samples were passaged in monolayers of Madin Darby bovine kidney (MDBK) cells to isolate BVDV. The procedure was performed as described previously (Walz et al. 2008). The carcass swab samples were assayed as described for buffy coat samples. The carcass muscle samples were assayed as described for serum samples except that after one hour adsorption, samples were removed and the monolayer rinsed twice with PBS. Following four days of incubation, the six-well culture plates (9.6 cm²) underwent a single freeze-thaw cycle to release intracellular virus. Lysates from this procedure were assayed in triplicate by adding 10 µL of lysate sample and 90 µL MEM to wells of a 96-well culture plate followed by the addition of 50 µL of MEM containing MDBK cells. Following three days of incubation, plates underwent the immunoperoxidase monolayer assay as described above.

Virus neutralization

The VN assays were performed on serum samples as described previously except that serum was not initially diluted (Givens et al. 2003). Serial two-fold dilutions of heat inactivated serum were made in 50 µL MEM in triplicate. Each well was then inoculated with 50 µL of MEM containing 50 to 300 CCID₅₀ of BVDV. The virus corresponded to the strains isolated from the carcasses. Following one hour of incubation, 50 µL of MEM containing MDBK cells were added to each well. Plates were incubated for three days and then underwent the immunoperoxidase monolayer assay procedure described above.

Reverse transcriptase polymerase chain reaction and sequencing

A two-round rapid-cycle RT-PCR (RT-nPCR) assay was performed on aliquots of spleen homogenates of fawn carcasses and serum samples from steers used as infection controls. This RT-nPCR assay has been previously described in detail (Givens et al. 2001). RT-nPCR positive samples were purified using the QIAquick® PCR purification kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's specifications and sequenced by automated dye terminator nucleotide sequencing using both the 5' and 3' primers (BVD 180 and HCV 368, respectively). Consensus sequences were determined for each sample using Align X® computer software (Vector NTI Suite 7.1, InforMax, Inc., Bethesda, MD, USA), and results were used to compare the nucleotide sequences of BVDV strains in spleen homogenates of the PI fawns and at the time of viremia in the infection control steers.

Results

In both trials, the individually-exposed cattle made contact with the PI carcass at regular intervals throughout the exposure period. On multiple events, steers in both trials investigated the carcasses at distances less than 5 cm. In trial A, the steer investigated the carcass on 7 separate events with a minimum of 15 minutes and a maximum of 158 minutes between two separate contacts. In the second trial, the steer investigated the carcass on 14 separate events, and the minimum and maximum time spans between events were 16 and 55 minutes, respectively.

The carcasses were assessed for BVDV by VI on muscle biopsy and surface swab samples (Table 2). Isolation of BVDV was successful only from the carcass in trial A, and, with the exception of one viral titration, both muscle biopsies and surface swab samples were positive. In contrast, neither sample type was positive for BVDV from carcass B.

Carcass exposure did not result in direct or indirect transmission of BVDV and steers in both trials were consistently negative on VI and VN. Furthermore, daily clinical examinations including rectal temperature and complete blood counts did not indicate BVDV infection, as clinical parameters remained within reference ranges.

Virus was isolated from whole blood and serum on days 6 to 14 and 8 to 14, respectively from the control steer inoculated intranasally with spleen homogenate from fawn A. Seroconversion was demonstrated on post-inoculation day 28. Only the intravenously inoculated control steer for trial B became viremic. Virus was isolated from whole blood and serum samples from this steer on days 6 to 10 and 7 to 8, respectively. On post-inoculation day 28 this steer had an antibody titer of 1:512. An infection with BVDV could not be demonstrated in the steer that was intranasally inoculated with spleen homogenate from fawn B. Sequence homology was demonstrated between the 5' UTR of BVDV PA131 in carcass A and control steer for trial A, and BVDV AU526 in carcass B and the intravenously-inoculated steer of trial B.

Discussion

Bovine viral diarrhea virus was successfully isolated from muscle biopsies and surface swabs collected from the carcass of a PI fawn after approximately two years in storage at -20° C. Titration of BVDV from surface swabs indicated that BVDV remains infectious on tissues for at least eight hours, despite exposure to environmental conditions. While transmission of BVDV by exposure to carcasses was not observed in this study, the observed frequency and proximity of investigative contacts by steers with the carcasses emphasizes the potential for disease transmission to cattle by this route. The survival of BVDV in tissues of dead PI animals has not been investigated, but in slaughtered PI cattle, BVDV remains infectious in muscle tissues for up

to 60 days at refrigeration temperatures (Givens, M. D., personal communication). The related pestivirus, classical swine fever virus, remains infectious in curing pork products for 126 to 252 days (Mebus et al. 1997). While the virus is viable for up to 85 days in chilled pork, inactivation at 20° C occurs after 4 days (Edwards 2000). The greatest risk of classical swine fever virus transmission through contaminated tissues is by the oral route when contaminated undercooked pork products are fed to swine (Gibbens et al. 2000). Scavenging on carrion is an established route of transmission of bovine tuberculosis to carnivores (Bruning-Fann et al. 2001), and possibly herbivores like the brush-tailed possum (*Trichosurus vulpecula*) (Ragg et al. 2000). Herbivores likely become infected by direct contact with carrion rather than the alimentary route.

Steers kept in the pen containing the carcass made several contacts throughout the 8-hour exposure period. Investigative behaviors, including sniffing, were described when alpacas, sheep, deer, and cattle were presented with possums or ferrets sedated to simulate behavior of terminal tuberculosis (Sauter et al. 1995a; Black et al. 1999). The ruminants came into aerosol transmission distance of <1.5 m or had direct physical contact, and in at least one instance intensely licked the sedated marsupial (Sauter et al. 1995a).

Within cattle herds, specific individuals may have a greater likelihood of making contact with non-bovine species, and may have the potential to act as a hub in the transmission of diseases (Bohm et al. 2009). The present study sought to emulate these herd structures, and by limiting the exposure of a deer carcass to one steer, tried to explore if this animal could introduce BVDV into the remaining herd. Observations of cattle suggest that animals ranked higher in social hierarchies and those with greater rates of intra-herd contacts are more likely to make interspecific contacts (Sauter et al. 1995b; Bohm et al. 2009). Cattle and deer in the highest ranks of the group hierarchy were most likely to investigate and make physical contacts with sedated

badgers, and a significant positive correlation existed between dominance rank and a positive reaction on the tuberculin test (Sauter et al. 1995b).

The failure to isolate BVDV from the spleen of carcass B on MDBK cells despite the successful inoculation of an intravenously inoculated steer with spleen homogenate from this carcass is noteworthy. Tissues collected from fawn B at death were positive only by RT-PCR results but negative by VI on MDBK cells, a result that was corroborated by a second VI procedure. At birth, fawn B was positive on multiple sample types, and ear notch biopsies resembled those of PI cattle and PI white-tailed deer on ELISA and immunohistochemistry procedures. Possible explanations for the negative VI at death and from carcass tissues include the inability to successfully culture BVDV strains obtained from deer in cells of bovine origin, or the clearance of BVDV by fawn B during the time from birth to death. Our group and others have successfully cultured BVDV isolates from deer on bovine cells, including field strains of unknown source that were isolated from sick deer (Chase et al. 2008). The elimination of BVDV following prolonged postnatal viremia was reported in pigs (Paton et al. 1994; Terpstra et al. 1997), and clearance of the infection from blood was associated with seroconversion (Paton et al. 1994). Similar observations were made in two alpacas that were born VI positive after experimental inoculation of pregnant alpacas. In monthly testing, BVDV was consistently isolated from white-blood cells of two crias until four and six month of age. On subsequent VI, BVDV was no longer detected, and apparent clearance of BVDV from blood was associated with the development of high serotiters (Edmondson, M. A., personal communication).

Conclusion

Despite the observed frequency and proximity of investigative contacts between the susceptible steer and an infectious carcass of a PI fawn, the results of the present study suggest that little risk of BVDV transmission occurs in this manner. The true risk for BVDV infection and introduction into susceptible herds by contact with a carcass is complex and is dependent upon various factors, including BVDV survival in tissues, likelihood of contact with the carcass, and contact network structures of cattle herds. The influence of wildlife on the control of BVDV in the United States is currently unknown, and further research is certainly necessary to understand the complex interactions at the wildlife-livestock interface.

Table 7.1 Results of virological assessments of PI fawns at birth and death.

Fawn ID	Time of sampling							
	Birth					Death		
	Virus isolation		RT-PCR	ELISA	IHC	Virus isolation	RT-PCR	IHC
	WBC	Nasal swab	Serum	Ear notch*	Ear notch	Tissues	Tissues	Tissues
A	+	-	+	+	+	+	+	+
B	+	+	+	+	+	-	+	+

* The sample-to-positive-ratios were: Fawn A: 1.518; Fawn B: 1.353, where the established cut-off for positive bovine samples is S/P >0.39 (WBC – white-blood cells; RT-PCR – reverse transcriptase polymerase chain reaction; IHC – immunohistochemistry)

Table 7.2 Results of the virus isolation procedures on samples from carcasses.

Carcass ID	BVDV		0 hr		2 hr	4 hr	6 hr	8 hr	
			Swab	Muscle	Swab	Swab	Swab	Swab	Muscle
A	Type 2 PA131	Passage	+	+	+	+	+	+	+
		Titration	6.2×10^3	2×10^3	0	2×10^2	3.5×10^3	2×10^2	3.5×10^4
B	Type 1 AU526	Passage	-	-	-	-	-	-	-
		Titration	0	0	0	0	0	0	0

Chapter 8:

Summary and Conclusion

Since the first description of bovine viral diarrhea virus by Olafson and colleagues in 1946, a large number of research studies have been performed to understand clinical, epidemiological, and pathophysiological features of the virus. This is reflected upon entering the search term “bovine viral diarrhea virus” that retrieves 2646 research studies in a medical database (www.ncbi.nlm.nih.gov/sites/entrez, accessed on May 13, 2010). Despite tremendous advances in the knowledge on BVDV, central aspects that are important for the success of control strategies and efforts to eradicate the virus from cattle populations are still incompletely understood. The adaptive and variable nature of BVDV, which is in part conferred by the infidelity of RNA genome replication, have created more questions for BVDV researches than anticipated before embarking on the quest to understand and control this virus. Examples of these novel features that were unfamiliar to BVDV researchers and veterinarians until recently include the occurrence of the hemorrhagic syndrome and severe acute BVD in association with BVDV 2 and the discovery of prolonged testicular infections (Carman et al. 1998; Voges et al. 1998; Goens 2002; Givens et al. 2003). Similarly, BVDV infections of heterologous hosts, albeit recognized as being possible in early years of pestiviral research, received only modest attention, and research efforts on the role of wildlife in the epidemiology of BVDV were limited. In recent years, infections with BVDV have been demonstrated in various mammalian hosts, specifically species within the order Artiodactyla, and indicated a lack of host specificity. The ability to infect various species in addition to cattle may be important in the survival strategy and epidemiology of BVDV (Vilcek et al. 2006).

Although BVDV infections of white-tailed deer (*Odocoileus virginianus*) were already recognized by serosurveys in the 1960's (Kahrs et al. 1964; Friend et al. 1967), the abundance and close contact with cattle of white-tailed deer did not prompt further research on this species'

possible role in the epidemiology of BVDV and the potential to become a wildlife reservoir for the virus. The focus of our research was the wildlife-livestock interface. The overall research hypothesis was that white-tailed deer can become infected with BVDV and are an important reservoir for the virus. This hypothesis was, and remains to be, timely because different states were developing control strategies for BVDV when our studies commenced. To test this over-spanning hypothesis, the following sub-hypotheses were established and served as the foundation for the research studies documented in this dissertation:

1. Infection of pregnant white-tailed deer with BVDV results in the birth of PI fawns.
 - a. Experimental inoculation of white-tailed deer results in PI fawns.
 - b. Natural exposure of pregnant white-tailed deer to PI cattle results in PI fawns.
2. Bovine viral diarrhea virus is maintained in a wildlife population.
 - a. Surveillance of hunter-harvested white-tailed deer reveals free-ranging deer PI with BVDV.
 - b. Exposure of pregnant deer to a PI fawn results in birth of PI offspring.
3. Persistently infected white-tailed deer are capable of transmitting BVDV to cattle.
 - a. Exposure of seronegative calves to a PI fawn results in viremia and seroconversion.
 - b. Exposure of pregnant cattle to a PI fawn results in viremia and the birth of PI calves.

The goal of this research was to examine whether white-tailed deer could be a wildlife reservoir for BVDV. To have this potential, different necessary criteria, including the susceptibility to infection, shedding of BVDV, maintenance of the infection within individual hosts and host-populations, and sufficient contact with cattle populations, must be fulfilled. As a

critical community size must be present for a pathogen to persist in a population indefinitely, we hypothesized that the abundance of white-tailed deer in the US may allow this species to become a reservoir host for BVDV (Haydon et al. 2002). In addition, because of the high coefficient of infectiousness of PI cattle and their fundamental role in BVDV transmission within and between cattle herds (Thurmond 2005), we hypothesized that as for cattle, PI white-tailed deer would be central to the maintenance and spread of BVDV.

A prior experimental infection of white-tailed deer had been performed, and the susceptibility to BVDV infection was successfully documented in four mule deer and one white-tailed deer as indicated by virus isolation and seroconversion (Van Campen et al. 1997). In that study, shedding of BVDV was also demonstrated by positive virus isolation on nasal swabs. Although an important addition to the body of knowledge on BVDV in white-tailed deer, Van Campen and colleagues utilized fawns rather than pregnant does for their inoculation study (Van Campen et al. 1997), and therefore were unable to discern whether the epidemiologically central phenomenon of persistent infection would also occur in white-tailed deer.

In contrast to the prior inoculation study by Van Campen and colleagues (Van Campen et al. 1997), we captured nine female and one male white-tailed deer that bred in captivity. The goals of the study were to examine whether intranasal inoculation of pregnant white-tailed deer with BVDV would result in transplacental infection and birth of PI fawns. Secondly, we planned to characterize the PI offspring by serial sample collections and virological assessments. Two BVDV strains (BVDV 1 BJ and BVDV 2 PA131) were used for inoculation. These strains were chosen because they had been isolated from PI cattle and, in cattle, were known to reliably cause PI infections. Although confirmed pregnant at the time of inoculation, only one doe gave birth in this study, while the stress of recapture likely caused pregnancy losses in the other does.

However, pregnancy loss as a result of BVDV infection could not be ruled out. On August 25, 2006, a live fawn and a mummified fetus were detected in the same birthing nest, and the fawn was translocated to an isolation room to be hand-raised. Samples were collected serially from the fawn, and virus isolation, RT-PCR, and immunohistochemistry procedures demonstrated for the first time that BVDV can persistently infect white-tailed deer. The BVDV antigen distribution on immunohistochemistry samples resembled that in PI cattle, with diffuse antigen distribution in the skin tissues (Brodersen 2004). Also similar to PI cattle was the amount of viral shedding as indicated by repeated nasal swab virus isolations and titrations, and the virus isolation and titration of serum samples (Table 8.1) (Fulton et al. 2009). The similarities of the antigen distribution in PI fawns as compared to PI cattle were also demonstrated by another group (Duncan et al. 2008a). That study described a wide distribution of antigen throughout many tissues and cell types, particularly in epithelia and vascular endothelium. In that study, a diffuse depletion of B-lymphocytes was detected, and in contrast to cattle, lymphocytes of the two PI fawns under investigation contained little BVDV antigen (Duncan et al. 2008a).

The interspecific transmission of BVDV would be a central component of BVDV reservoir patterns, and prompted by the identification of a PI fawn by intranasal inoculation, we evaluated the potential of interspecific transmission of BVDV between PI cattle and white-tailed deer. For this study, seven female and one male white-tailed deer were successfully captured and bred in captivity. Because a second capture for the purpose of intranasal inoculation was unnecessary in this study, pregnancy losses as a result of capture-associated stress were not of concern. At an estimated average gestational length of 50 days, two PI cattle infected with BVDV 1 strains were cohabitated with the pregnant deer and shared feed and water sources for a period of 60 days. All adult deer became infected with BVDV and four pregnancies resulted in

infected offspring. Per definition, a diagnosis of persistent infection could not be made on the two stillborn fetuses, but several tissues from both were infected with BVDV. Of the fawns that were born alive, three were demonstrated to be PI, indicating for the first time that BVDV can efficiently cross the species barrier from cattle to deer and cause transplacental infections and PI white-tailed deer. The infection of heterologous species through the presence of PI cattle has not been studied intensively and little is known about the source of BVDV infection in wildlife. In domestic small ruminants, the presence of cattle on mixed livestock farms was associated with an increased seroprevalence (Tegtmeier et al. 2000; Krametter-Frotscher et al. 2007). Pregnancy losses, reproductive disease, and fetal malformations were reported in goats experimentally exposed to PI cattle, but PI goat kids were not born (Broaddus et al. 2009). The close relatedness of the PI strain from eland antelopes (*Taurotragus oryx*) with those of common cattle strains, as indicated by monoclonal antibody and sequencing assays (Vilcek et al. 2000), also corroborated that BVDV transmission from cattle to wildlife is possible. With the exception of our study, transmission of BVDV from cattle to deer has not again been evaluated, but a recent serosurvey indicated that white-tailed deer on ranches where cattle were present were more likely to be seropositive than those on ranches without cattle (Cantu et al. 2008).

Although PI white-tailed deer are likely central to the temporary maintenance of BVDV in white-tailed deer populations, the indefinite persistence of the virus and distribution to other populations would depend on intraspecific spread of the virus. To test the possibility of intraspecific transmission of BVDV in white-tailed deer, a PI deer that was not hand-raised but remained at the Auburn University Captive Deer Facility after birth, was cohabitated with six pregnant does. The animals shared feed and water sources, and after a cohabitation period of approximately 3 months, the PI fawn died in June 2008. Reproductive losses were not observed

and all does gave birth to one or two fawns. Although only one fawn was identified to be PI with BVDV, the virus was efficiently transmitted among deer, as indicated by seroconversion of all adult deer and high titers of BVDV-specific neutralizing antibodies in each fawn. These findings emphasize that analogous to PI cattle, the PI deer was an efficient source of BVDV and caused infections in in-contact deer. The birth of a PI fawn in this study strengthens the possibility of continuous intrapopulation propagation of BVDV and the potential for indefinite maintenance of the virus in deer. The existence of free-ranging populations of white-tailed deer in which BVDV is maintained is difficult to assess and likely requires studies that identify PI deer and serological surveys.

White-tailed deer populations exist in matrilineal groups in which female deer disperse only over small distances according to the rose pedal hypothesis (Porter et al. 1991). Although suitable for screening of multiple, widespread populations, simple random sampling across larger regions, as has been performed in most studies that evaluated BVDV in white-tailed deer (Kahrs et al. 1964; Pogranichniy et al. 2007; Duncan et al. 2008b; Passler et al. 2008), may not adequately acknowledge these social structures and hence miss any evidence of intrapopulation maintenance of BVDV. A recent study demonstrated that a majority of blood samples (63.5%) from captured white-tailed deer were positive for BVDV antibodies (Cantu et al. 2008). Interestingly, the study evaluated individual populations of deer on 15 ranches. Large variations among these populations were detected, and while the seroprevalence was as low as 11% on one farm, 100% of sampled deer were seropositive on another (Cantu et al. 2008). Although the presence of cattle on sampled farms contributed to larger numbers of seropositive deer, a determination could not be made, whether seropositive deer were the result of frequent contact with cattle or intraspecific maintenance of BVDV. In a population of mule deer, in which a

BVDV positive deer was detected, 60% of animals were seropositive (Van Campen et al. 2001b). The authors of that study concluded that the high rate of seroprevalence and the isolation of BVDV suggested the intrapopulation circulation of the virus (Van Campen et al. 2001b).

Further research and particularly suitable surveys of free-ranging populations are necessary to determine the possible impact of BVDV infections of white-tailed deer on BVDV control and eradication in cattle; however, the result of the studies presented in this dissertation indicate that white-tailed deer are capable of becoming infected with BVDV, shed the virus, and maintain BVDV by acute and persistent infection. The characteristics of BVDV infections in white-tailed deer are strikingly analogous to infections in cattle, emphasizing the lack of BVDV host-specificity. In hitherto unpublished work, we evaluated PI white-tailed deer for the distribution of histopathological lesions and BVDV antigen in tissues. The BVDV antigen was distributed widely throughout many cell types and tissues, with the most pronounced viral antigen staining located in epithelial-lined tissues. This distribution is very similar to that of PI cattle, and corroborates a previous report of BVDV antigen distribution in two white-tailed deer fawns (Duncan et al. 2008a). The antigen distribution in skin samples with diffuse staining of epithelia has also been found to be identical to that of PI cattle, and immunohistochemistry has been used for the detection of PI deer (Passler et al. 2007; Duncan et al. 2008b; Passler et al. 2008). However, a formal evaluation of the various BVDV detection assays for use in heterologous species, including white-tailed deer, has not been performed and warrants further research efforts.

Although not a primary research question of the studies documented in this dissertation, indications for the gestational age at which persistent infections can occur in white-tailed deer have become apparent from our work. This window of susceptibility, which in bovine fetuses

most commonly is stated as 40 – 125 days of gestation, likely has important impact on the epidemiology of BVDV in white-tailed deer and the prevention of spill-back infection to cattle. The risk of BVDV infection in a pregnant white-tailed deer carrying a susceptible fetus likely is greatest when nutritional demands encourage feeding from supplemental feed sources, such as winter feeding or feed sources provided by cattle farmers. An example for increased disease transmission at feed sources is provided by the wildlife-livestock mode of maintenance of *Mycobacterium bovis*, where increased contact of deer with shedding hosts has been indentified central to the pathogen's epidemiology (Schmitt et al. 2002).

Extrapolated from the susceptible gestational age of bovine fetuses and adjusted for the shorter duration of pregnancy in white-tailed deer of approximately 200 days, we estimated that the window of susceptibility of cervine fetuses would be around 50 days of gestation. This time frame was used for the experimental exposures documented in these studies, and essentially proved to be accurate (Table 8.2). Although all does were examined by ultrasound for gestational status at capture or exposure to BVDV, the fetal age at infection was calculated based on the date of birth and a gestational length of 200 days. Accurate assessment of gestational age of pregnancy by ultrasound was impossible as research on use of this technique in white-tailed deer has not been published. Therefore, the presented data may be influenced by variations in pregnancy duration and the duration of the time of infection of the doe and fetal infection.

The six PI deer that resulted from this work were infected between 27 and 51 days of gestation. This is earlier than the susceptible fetal age of persistent infection in cattle and likely reflects an earlier maturation of the cervine fetal immune system. The pathophysiology of persistent infection entails the negative selection of BVDV-specific lymphocytes during the maturation of the immune system; however, knowledge on the time of this development in

white-tailed deer does not exist. Further research is necessary to answer these epidemiologically important questions, as managerial influences such as winter feeding may coincide with fetal susceptibility.

In conclusion, infections with BVDV in white-tailed deer are strikingly similar to those in cattle, and encompass the range of clinical features, including persistent infections, as observed in bovine BVDV infections. Our research findings may support the conclusion that white-tailed deer are an important reservoir for BVDV, as stated in the over-spanning research hypothesis. However, many factors including viral features and strain variations; human interactions such as biosecurity measures and wildlife management; and host characteristics such as survival of PI deer are likely to influence BVDV infections in free-ranging populations. Our findings should be considered in the development of BVDV eradication programs in North America, but further research is needed to fill the many gaps of knowledge on BVDV infections in white-tailed deer.

Table 8.1 Titration of BVDV in serum and nasal swabs from a persistently infected fawn.

Day of sample collection	Serum virus isolation	Nasal swab virus isolation
8/25/06	6.2×10^5 CCID ₅₀ /ml	2×10^6 CCID ₅₀ /ml
9/25/06	6.2×10^5 CCID ₅₀ /ml	2×10^6 CCID ₅₀ /ml
10/23/06	6.2×10^5 CCID ₅₀ /ml	6.2×10^5 CCID ₅₀ /ml

Table 8.2 The gestational age of susceptibility for the development of persistent infections in white-tailed deer.

Fawn ID	Infection status	Date of birth	Calculated date of conception	Method of exposure	Calculated age at exposure
GN	Persistently infected	8/25/2006	2/6/2006	Intranasal	33
1	Seropositive at birth	7/30/2007	1/11/2007	PI cattle	56
2	Seropositive at birth	7/30/2007	1/11/2007	PI cattle	56
3	Seropositive at birth	7/30/2007	1/11/2007	PI cattle	56
4	Seropositive at birth	7/30/2007	1/11/2007	PI cattle	56
5	Persistently infected	8/4/2007	1/16/2007	PI cattle	51
6	Persistently infected	8/15/2007	1/27/2007	Intranasal	46
7	Persistently infected	8/21/2007	2/2/2007	PI cattle	34
9	Seropositive at birth	8/26/2007	2/7/2007	PI cattle	29
10	Seropositive at birth	8/26/2007	2/7/2007	PI cattle	29
12	Persistently infected	8/28/2007	2/9/2007	PI cattle	27
13	Seropositive at birth	7/6/2008	12/19/2008	PI deer	104
14	Seropositive at birth	7/6/2008	12/19/2008	PI deer	104
15	Seropositive at birth	7/6/2008	12/19/2008	PI deer	114
16	Seropositive at birth	7/6/2008	12/19/2008	PI deer	114
17	Seropositive at birth	8/1/2008	1/14/2008	PI deer	66

18	Seropositive at birth	8/1/2008	1/14/2008	PI deer	66
19	Seropositive at birth	8/1/2008	1/14/2008	PI deer	75
20	Seropositive at birth	8/1/2008	1/14/2008	PI deer	75
21	Persistently infected	8/13/2008	1/26/2008	PI deer	41
22	Seropositive at birth	8/16/2008	1/29/2008	PI deer	63

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