EFFECTIVE DETECTION OF EPIDEMIOLOGICALLY SIGNIFICANT PERSISTENT

INFECTIONS OF BOVINE VIRAL DIARRHEA VIRUS

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Misty Sue Abrams

Certificate of Approval:

Paul H. Walz Assistant Professor Clinical Sciences	M. Daniel Givens, Chair Associate Professor Pathobiology
Julie A. Gard Assistant Professor Clinical Sciences	Robert L. Carson, Jr. Professor Clinical Sciences
David A. Stringfellow Professor Pathobiology	Stephen L. McFarland Acting Dean Graduate School

EFFECTIVE DETECTION OF EPIDEMIOLOGICALLY SIGNIFICANT PERSISTENT INFECTIONS OF BOVINE VIRAL DIARRHEA VIRUS

Misty Sue Abrams

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EFFECTIVE DETECTION OF EPIDEMIOLOGICALLY SIGNIFICANT PERSISTENT INFECTIONS OF BOVINE VIRAL DIARRHEA VIRUS

Misty Sue Abrams

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Signature of Author

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Date of Graduation

VITA

Misty Sue Abrams, daughter of John Author Abrams and Priscilla Woodall Abrams, was born in Montgomery, Alabama on April 25th, 1975. She received her high school diploma from Elmore County High School in Eclectic, Alabama, graduating in May, 1993. Subsequently, she attended Auburn University, earning a Bachelor of Science in microbiology in June 1997 and a Doctor of Veterinary Medicine in May, 2001. After graduation from veterinary school, she practiced veterinary medicine on large and small domestic animals in Lanett and Trussville, Alabama. In June, 2003, Misty Abrams returned to Auburn University's College of Veterinary Medicine as a Theriogenology Resident in the Department of Clinical Sciences and enrolled in graduate school. Diplomate status in the American College of Theriogenologists was conferred to her on August 9, 2005. She will be married to Jason Lee Edmondson on June 17th, 2006.

THESIS ABSTRACT

EFFECTIVE DETECTION OF EPIDEMIOLOGICALLY SIGNIFICANT PERSISTENT INFECTIONS OF BOVINE VIRAL DIARRHEA VIRUS

Misty Sue Abrams

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Prior research has shown that virus cannot be isolated from the serum of some cattle which are persistently infected (PI) with bovine viral diarrhea virus (BVDV). However, the ability of these atypical PI animals to infect susceptible herdmates in a normal pasture environment remains to be determined. The accurate identification of PI cattle is a major factor in the control and prevention of BVDV. Furthermore, a lack of uniform standardization or validation of testing methods between diagnostic laboratories could compromise the ability to consistently and accurately diagnose PI cattle. The

current level of agreement among laboratories and the accuracy of tests to identify animals PI with BVDV have not been fully evaluated.

This research investigated the potential for an atypical PI animal to transmit BVDV to susceptible herdmates in a normal pasture setting. Groups of four naive calves were exposed to either a negative control (BVDV negative), a positive control (PI), or a test animal (PI that previously lacked easily isolated virus in serum by virus isolation). Blood was collected from each animal and tested by virus isolation and serum neutralization. Calves exposed to the negative control did not become viremic or seroconvert, but all calves exposed to either the positive control or the test animal became viremic and seroconverted to BVDV. The outcome of this study indicates that PI cattle that previously lacked easily isolated virus in serum by virus isolation are still capable of transmitting BVDV to susceptible herdmates.

The diagnostic proficiency of various methods for detecting cattle PI with BVDV using intra- and inter-laboratory comparisons was also investigated. Blood and skin biopsies were collected from 2 BVDV negative animals, a PI animal, and a PI animal that previously lacked easily isolated virus in serum. Blind samples were submitted for detection of BVDV to 23 participating laboratories. The level of agreement between laboratories for each diagnostic test ranged from perfect to less than expected by random chance. The results from this research show that there is considerable variation among tests and laboratories and demonstrate the need for standardization of tests used to detect BVDV.

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INTRODUCTION

Bovine viral diarrhea virus (BVDV) causes gastrointestinal, respiratory, and reproductive disease in cattle and is considered one of the most important viral pathogens of cattle in the United States.[129] Due to the negative economic and health impacts of BVDV and the failure of vaccination to provide consistent protection, there is now informed consent for the prevention and control of BVDV in this country.[1] Persistently infected (PI) cattle are the natural reservoir of BVDV. It has been reported that the virus cannot be detected in the serum of some adult PI cattle even when it is present in their white blood cells.[38] It is believed that virus cannot be detected from the serum of these animals due to their ability to produce serum neutralizing antibodies.[113] Given the fact that most PI animals have a very high and persistent viremia and shed BVDV throughout life from almost all secretions and excretions, it is important to determine if animals PI with BVDV that lack easily isolated virus in their serum by virus isolation are also capable of transmitting virus to susceptible herdmates. Such cattle could be misdiagnosed when using serum-based BVDV screening methods which could severely compromise prevention and control efforts.

Control and eradication of this pathogen requires that these animals are accurately identified and removed. Currently, a variety of tests are used for this purpose. These

include virus isolation (VI) in cell culture, reverse transcriptase-polymerase chain reaction (RT-PCR), antigen capture enzyme linked immunosorbent assay (ACE), and immunohistochemical (IHC) tests. The research presented in this thesis determines the epidemiologic importance of PI cattle that lack easily isolated virus in their serum by virus isolation. Further, the proficiency of various diagnostic methods for detecting PI cattle using intra- and inter-laboratory comparisons is evaluated.

LITERATURE REVIEW

Bovine Viral Diarrhea Virus

Introduction. Bovine viral diarrhea virus (BVDV) was first identified as the causative agent of acute diarrhea in cattle in New York state in 1946.[163,164] The early appearance of the disease was associated with high morbidity, low mortality, and clinical signs including fever, anorexia, depression, salivation, nasal discharge, diarrhea, abortion, and ulcers of the mouth, muzzle, and nose.[11,209] However by 1962, there was quite a change in the clinical manifestations of the disease. The early form of the disease was seldom observed, but BVDV was commonly associated with a different syndrome termed mucosal disease.[100,170] Mucosal disease was more sporadic but highly fatal. Clinical signs seen with mucosal disease included fever, erosions of the gastrointestinal tract, and severe diarrhea. In 1993, the clinical appearance of BVDV again changed and was similar to the clinical observations in 1946 with an increase in abortion rates and mortality.[53,62] The more severe presentation of this disease was credited to a novel species of BVDV.[17,165]

Taxonomy. Bovine viral diarrhea virus belongs to the genus *Pestivirus*. The *Pestivirus* genus includes the following species: bovine viral diarrhea virus genotype I (BVDV-1), bovine viral diarrhea virus genotype II (BVDV-2), classical swine fever virus,

border disease virus, and tentatively, giraffe-1 pestivirus and reindeer-1 pestivirus.[9] The genus *Pestivirus* is a member of the *Flaviviridae* family. Other members of the *Flaviviridae* family include the genera *Flavivirus* and *Hepacivirus*.[158] Members of the genus *Flavivirus* include West Nile virus, St. Louis encephalitis virus, yellow fever virus, dengue virus, looping ill virus, and Japanese encephalitis virus. The sole member of the *Hepacivirus* genus is the hepatitis C virus.[158,203]

The viruses that make up the pestivirus genus have several characteristics that help differentiate them from other members of the *Flaviviridae* family. The pestiviruses encode for two proteins that are unique to their genus, N^{pro} and E^{rns}.[171] The N^{pro} is a nonstructural protein whose only known function is to cleave itself from the viral polypeptide.[171] The N^{pro} protein is also the first protein encoded by the pestivirus open reading frame (ORF). The E^{rns} is an envelope glycoprotein that has intrinsic RNase activity.[171]

Biotype. Each of the species in the genus *Pestivirus* are divided into two biotypes, cytopathic or noncytopathic, based upon the ability of the virus to cause cytopathic effects and death in cultured epithelial cells. Most field isolates of BVDV are noncytopathic. Cultured cells infected with the noncytopathic biotype have no microscopic evidence of infection. However, cytopathic isolates of BVDV induce cytoplasmic vacuolization and death by apoptosis of cultured cells within a few days of infection.[116,173] Cells infected with a noncytopathic isolate of BVDV and later superinfected with a cytopathic isolate are refractive to the cytopathic effects of the cytopathic isolate.[173]

Biotypes differ not only in their ability to initiate cell death but also in their ability to biochemically alter the host cell. Noncytopathic biotypes are capable of enhancing the production of reactive nitrogen intermediates in bovine-bone-marrow-derived macrophages. The increase in production of reactive nitrogen intermediates may inhibit macrophage activity and have a role in the immunosuppression associated with BVDV-infected cattle.[2] Also, biotypes vary in regard to tropism as demonstrated by the ability of noncytopathic biotypes to induce thrombocytopenia while cytopathic biotypes are not capable of the same effect.[19]

Based on serological and sequence comparison studies, the cytopathic pestiviruses may develop from the mutation of noncytopathic viruses.[142] Cytopathic BVDV is thought to be produced through the recombination of ribonucleic acid (RNA) by template switching during RNA replication of noncytopathic viruses.[142] However, cytopathic viruses may also develop from the introduction of a set point of mutations within the NS2 gene.[142] It has been shown that these point mutations are responsible for the expression of NS3 and for the cytopathic phenotype.[142] The noncytopathic strains of BVDV predominate over cytopathic strains in nature, and cytopathic strains are usually isolated from mucosal disease outbreaks or post-vaccination disease outbreaks.[27]

Biotype does not correlate to virulence in acute infections with BVDV.[172] Severe acute infections with BVDV are consistently noncytopathic and result in depletion of lymphoid cells.[172] A study by Ridpath et al. demonstrated that infection with a highly virulent BVDV strain was associated with a noticeable reduction of circulating white

blood cells and an increase in the number of apoptotic and necrotic circulating white blood cells.[172]

While BVDV strains are segregated into two biotypes based on their activity in cultured epithelial cells, this study indicates that biotypes of BVDV may be segregated into three biotypes based on their activity in cultured lymphoid cells.[172] These three biotypes are noncytopathogenic (no effects on viability of either cultured epithelial or lymphoid cells), cytopathogenic (cytopathic effect on both cultured epithelial and lymphoid cells within 48 hours of infection), and lymphocytopathogenic (no effect on cultured epithelial cells but cell death in cultured lymphoid cells with 5 days of infection).[172] Also, cell death caused by the lymphocytopathogenic biotype was not associated with changes in the processing of the NS2/3 protein that are typically seen with cytopathic viruses.[172]

Genotype. Genetic analysis of BVDV has led to the identification of two genotypes, type 1 (BVDV-1) and type 2 (BVDV-2). Both BVDV-1 and BVDV-2 genotypes contain noncytopathic and cytopathic biotypes. Bovine viral diarrhea virus-1 strains from North America have been subdivided into subgenotypes 1a and 1b.[165] These two subgenotypes can be distinguished by monoclonal antibody binding and reverse transcription polymerase chain reaction (RT-PCR).[28,174] Bovine viral diarrhea virus-1a represents most of the classical strains of BVDV originating from North America. Bovine viral diarrhea virus-1b, representing most European isolates, also represents the majority of the common reference strains of BVDV such as NADL, SD-1, Oregon, Singer, Osloss, and NY-1.[98,118] It is thought that BVDV-1a strains may predominate in

fetal infections occurring later than 100 days in gestation while epidemiological analyses suggest that BVDV-1b strains may predominate in respiratory cases.[81,91] European BVDV-1 strains appear to be more variable than the North American BVDV-1 strains with the European BVDV-1 strains now being separated into at least 11 subgenotypes.[204]

Bovine viral diarrhea virus-2 is known to cause severe thrombocytopenia and hemorrhage in cattle.[27,62] Following further genetic analysis, BVDV-2 strains from North and South America have been segregated into 2 subgenotypes, 2a and 2b.[86] In South America, the prevalence of BVDV-2a and BVDV-2b is similar.[86] However in North America, BVDV-2b strains are relatively rare.[86]

Genomic and viral structure. Bovine viral diarrhea virus is a single-stranded, positive-sense, enveloped RNA virus that is approximately 12.5 kilobases (kb) in length.[171] The long ORF, approximately 4,000 codons, is flanked by relatively large 5' (360-390 bases) and 3' (200-240 bases) untranslated regions (UTR).[171] The 5' terminus lacks a cap structure, and no polyadenine tail is present on the 3' end.[171] However, the 5' UTR is likely the functional equivalent of the 5' 7-methylguanosine cap and involved in the initiation of translation. The 3' UTR is likely the equivalent of the 3' polyadenine tail and involved in stabilizing the RNA.[37,58,185] Bovine viral diarrhea virus is able to undergo cap-independent translation initiation due to an internal ribosome entry site (IRES) in its genomes.[179] The 5' UTR is divided into 4 domains: A, B, C, and D.[44,67,120] The IRES, which is thought to be a stem-loop structure formed from the 3' two thirds of the 5' UTR, mediates internal attachment of ribosomes to the translation initiation codon.[68,120,179]

The polyprotein that is produced is co- and post-translationally processed by both viral and cellular proteases to form 11 to 12 mature viral proteins.[36] These viral proteins include both structural and nonstructural proteins. Sequentially from the carboxy terminus, the viral proteins of BVDV are the amino-terminal autoprotease (N^{pro}), the nucleocapsid protein (C), the ribonuclease soluble envelope glycoprotein (E^{rns}), the primary envelope glycoprotein (E1), the secondary envelope glycoprotein (E2), a protein of unknown function (p7), the fused second and third nonstructural proteins (NS2-3), the amino-terminal portion of the fourth nonstructural protein (NS4A), the carboxy-terminal portion of the fourth nonstructural protein (NS4B), the amino-terminal portion of the fifth nonstructural protein (NS5A), and the carboxy-terminal portion of the fifth nonstructural protein (NS5B).[197]

Amino-terminal protease (N^{pro}, p20). The amino-terminal autoprotease, N^{pro} (p20), is the first product of translation of the BVDV open reading frame.[179] This protein cleaves itself from the nascent polyprotein and, thus, generates the amino-terminus of the adjacent viral nucleocapsid protein C.[179]

Nucleocapsid protein (C, p14). The nucleocapsid protein is presumably located in the cytoplasm of infected cells.[72] The protein functions to package the viral RNA and to provide necessary interactions for the formation of the enveloped virion.[72]

Ribonuclease soluble envelope glycoprotein (E^{rns}, gp48, formerly E0). The glycoprotein E^{rns} is an envelope ribonuclease that is secreted into the extracellular environment but remains a component of the virion despite the absence of a hydrophobic membrane anchor region.[132] The absence of this membrane anchor region suggests a

loose interaction with the envelope.[132] This glycoprotein demonstrates RNase activity and binds to cell surface aminoglycans.[132,188] Because compounds that inhibited E^{rns} binding to cell surface aminoglycans also inhibited viral replication in tissue culture, the binding of E^{rns} is thought to play an important role in viral infections.[132] The E^{rns} glycoprotein is an immunogenic protein that results in the induction of antibodies and is believed to play a role in viral replication.[143] In addition, the E^{rns} protein exhibits cytotoxic effects on lymphocytes and strongly inhibits the protein synthesis of lymphocytes.[49] The E^{rns} protein is also thought to be involved in apoptosis of lymphocytes which results in leukopenia and immunosuppression.[49]

Primary envelope glycoprotein (E1, gp25). The E1 protein contains two hydrophobic domains that serve to anchor the glycoprotein in the lipid membrane and initiate translocation of the adjacent E2 polypeptide.[180] Through disulfide bonds, E1 covalently links to E2 within the BVDV envelope. The E1 glycoprotein is a unique immunogenic envelope protein because it is not capable of inducing neutralizing antibodies.[73]

Secondary envelope glycoprotein (E2, gp53). The E2 glycoprotein exists in two forms, a short form and a long form. The longer form of E2, which has been shown with classical swine fever virus to be present only inside infected cells, has the small p7 protein still attached at the carboxy terminus.[200,278] However, the E2 (gp53) envelope glycoprotein can elicit virus-neutralizing antibodies.[118,200]

Protein of unknown function (p7). The function of the p7 protein, a 7 kDa protein, is not fully understood. The p7 protein has been detected fused to the E2 glycoprotein

within cells, but others believe that the p7 functions as a signal sequence required for correct processing and membrane translocation of NS2.[79,197,200,217] The p7 protein is not required for replication of subgenomic viral RNA within cells.[79,197]

Fused second and third nonstructural proteins (NS2-3, p125). The NS2-3 protein is a nonstructural protein that consists of two proteins NS2 and NS3. The amino-terminal region of the NS2-3 protein is homologous to the NS2 (p54), and the carboxy-terminal region of the NS2-3 protein is homologous to the NS3 protein.[72] Both noncytopathic and cytopathic strains code for the nonstructural protein NS2-3. The NS2-3 protein remains fused in noncytopathic strains of BVDV. However, cytopathic strains further process the NS2-3 to an NS2 (p54) and an NS3 (p80) protein.[203] Therefore, NS3 can only be detected after infection with a cytopathic BVDV.[15] The detection of NS3 correlates with the appearance of cytopathic effect; therefore, NS3 is regarded as the marker protein for cytopathic strains.[15] There are four important domains present in the NS2-3 protein which include a hydrophobic domain, a zinc finger, a serine protease, and an RNA helicase.[72] If this protein is cleaved into NS2 and NS3, the hydrophobic domain and the zinc finger are associated with the NS2 domain while the serine protease and the RNA helicase are found in the NS3 domain.

Second nonstructural protein (NS2, p54). The NS2 is a highly variable nonstructural protein that contains a hydrophobic region that integrates the polypeptide into the membrane of the endoplasmic reticulum and a zinc finger.[72,141] NS2 is not thought to be necessary for the basic replication of viral RNA.[72,141] The attachment of the NS2 protein to the membrane of the endoplasmic reticulum may act as a chaperone to

assist in the maturation of structural glycoproteins, virion assembly, or virion release.[141] The zinc finger of NS2 is thought to be responsible for holding the viral replication complex in close proximity to the membrane of the endoplasmic reticulum.[72] This protein is found exclusively in some, but not all cytopathic isolates of BVDV.[141]

Third nonstructural protein (NS3, p80). The NS-3 protein is the most conserved protein and serves as a marker for cytopathic strains. The NS-3 protein is immunogenic and stimulates cellular apoptosis resulting in cytopathic effects. The NS-3 protein also contains the serine protease and RNA helicase domains. The serine protease, which is responsible for cleaving the polyprotein at nonstructural protein sites 3/4A, 4A/4B, 4B/5A, and 5A/5B, is essential for pestivirus replication.[217] The helicase functions to catalyze ATP-dependent strand separation of RNA duplexes and is necessary for synthesis of the negative-sense RNA strand.[72,117] Therefore, the RNA helicase of NS3 may function during initiation of negative-sense strand synthesis by unwinding the secondary structures of the viral genome. The RNA helicase could also help release nascent positive-sense strands from the negative-sense template.[117]

Nonstructural 4A protein (NS4A, p10). The NS4A nonstructural protein is a NS3 serine protease cofactor that is required for cleavage at the 4B/5A and 5A/5B sites.[194,217] Although the sequence of NS4A is highly conserved among pestiviruses, the exact function of this protein is not understood.

Nonstructural 4B protein (NS4B, p32) . The NS4B is a phosphorylated protein that is thought to contain a replicase component.[217] The sequence of NS4B is highly conserved.

Nonstructural 5A protein (NS5A, p58). The NS5A is the most variable nonstructural protein among divergent BVDV isolates and contains a replicase component.[67,133] This protein is hydrophobic, phosphorylated, and relatively stable within infected cells.[133] The NS5A is thought to interact with the α-subunit of bovine translation elongation factor 1A (eEF1A).[133] The eEF1A is required for cell viability due to its role in the formation of peptide bonds during protein translation, the binding and bundling of actin, microtubule severing, protein degradation mediated through ubiquitin-dependent pathways, and association with ribonucleoprotein pathways.[133]

Nonstructural 5B protein (NS5B, p75). The NS5B protein contains the conserved amino acid motif glycine-aspartate-aspartate that is characteristic of RNA-dependent RNA polymerases and has the shortest half-life of all mature BVDV proteins.[72,217] The NS5B protein was shown to have RNA-dependent RNA polymerase activity by synthesizing a double-stranded, covalently-linked molecule from the input template RNA via a copy-back mechanism.[220] The NS5B protein also demonstrates nucleotide-nonspecific and template-independent terminal nucleotidyl transferase activity.[54]

Viral structure. The BVD virion is 40-60 nanometers (nm) in diameter and consists of a central capsid surrounded by a lipid bilayer.[72,179] The capsid is approximately 30 nm in diameter and is composed of viral RNA and the nucleocapsid protein C.[72,179] The lipid bilayer has two glycoproteins, E1 and E2, anchored in it. A third glycoprotein, E^{rns}, is loosely attached to the envelope, and there appear to be 10-12 nm ring-like subunits on the surface of the envelope.[72,171] Because of the infectious nature

of the naked RNA of BVDV, no proteins necessary for catalyzing RNA replication are found packaged within the virion.[72]

Viral replication. RNA viruses, including BVDV, replicate and survive by relying on the biology and biochemistry of their host. Infection of host cells by BVDV results in modifications in gene expression of the host cell that are beneficial to virus replication and survival.[125] The replication strategy of BVDV is thought to be similar to that of other positive-stranded RNA viruses, such as poliovirus.[219] Concurrent with the translation of the viral genome, the nascent viral proteins associate with the RNA genome and host factors to form replication complexes which in turn catalyze the transcription of complementary negative-stranded RNA molecules.[219] These negative-stranded RNA molecules act as templates for the synthesis of novel genomic RNA molecules.[219] Replication of BVDV occurs efficiently in cells derived from Artiodactyla (bovids, camelids, cervids, etc.).[29] Bovine viral diarrhea virus has also been shown to adapt, although with difficulty, to replicate in rabbit, swine, and domestic cats.[11,29] In addition, virus has been found in human cell lines that have been cultured in media containing bovine serum albumin contaminated with BVDV.[4,98]

Replication of BVDV occurs entirely in the cytoplasm of the cell.[72] Viral replication occurs with the synthesis of excess positive-strand RNA compared to the negative-strand RNA intermediate.[219] The peak release of viral progeny occurs 12 to 14 hours after initiation of infection.[72] Each infected cell can release 100 to 1,000 infectious virions.[19]

Bovine viral diarrhea virus gains entry into the cell by receptor-mediated endocytosis. The low-density lipoprotein (LDL) receptor is necessary for endocytosis of the virus. Research has shown that a mutant Madin-Darby bovine kidney cell line (CRID) is resistant to infection by BVDV due to a defect in the expression of LDL receptors.[4,72] Therefore, the LDL receptors must provide a crucial mechanism for the entry of virus into cells.[72]

The structural viral proteins also play a critical role in viral introduction, adherence, and uptake into cells. The ribonuclease soluble envelope glycoprotein, E^{rns}, binds directly to the glycosaminoglycans (GAGs) on the surface of the cell.[132] The envelope glycoproteins, E^{rns} and E2, are located on the surface of infected cells and induce viral-neutralizing antibodies which elicit protective immunity.[132] The E2 envelope glycoprotein is thought to initiate endocytosis by binding to a 50 kD cellular receptor.[72] After endocytosis, acidification of the endosome initiates fusion of the viral envelope with the cellular membrane and release of the viral genome into the cytosol.[72]

Once the virus has reached the cytosol of the cell, the nucleocapsid protein, C, is removed from the viral RNA to allow translation to occur. The internal ribosome entry site in domain D of the 5' UTR binds ribosomes and begins translation of viral RNA into a polyprotein.[72]

After processing the nonstructural proteins involved in replication of viral RNA, an RNA-dependent RNA polymerase of NS5B creates an RNA negative-sense template copy (cRNA) of the positive-sense viral RNA through a copy-back mechanism.[220] Exonuclease activity by the RNA polymerase is lacking which results in a high frequency

of errors during replication. After production of the negative-sense copy of RNA (cRNA), the cRNA template is used to make positive-sense viral RNA. Assembly of BVDV takes place in the endoplasmic reticulum or Golgi.[72,220] The newly formed virions then acquire the lipid envelope. As soon as 8 hours post-infection, the infective virions are released by budding into the cisternae of the endoplasmic reticulum followed by exocytosis.[72,125]

Viral adaptation. The lack of an efficient exonuclease activity of the RNA-dependent RNA polymerase prohibits correction of mutations resulting from base substitutions. This lack of proofreading ability results in approximately 1 error for every 10,000 nucleotides or 1.25 nucleotide changes due to base substitutions during each replication of the genome.[25,72] Therefore, the ability to create new viral mutants or strains is tremendous. Despite the ability of BVDV to produce such genomic diversity, BVDV isolates within a herd seem to remain conserved between persistently infected animals. These "herd specific strains" are present because replication of the resident strain is not immunologically interrupted because emerging mutants of the resident strain are eliminated within the persistently infected animal.[119] The ability of BVDV to constantly create mutants allows this virus to produce genomes that are more resilient and capable of adapting to host immune responses.[25] Therefore, BVDV is not a homogeneous clone but rather a swarm of viral mutants clustered around the most frequent viral sequences.[125] These related but nonhomogeneous populations have been referred to as quasispecies.[125] The presence of quasispecies among BVDV strains is a result of the high replication rate of the virus and the lack of proofreading capacity by the RNA-dependent RNA

polymerase.[125] The results of a high mutation rate and selection pressure from the environment create unique challenges for controlling this virus.

Viral host range. In addition to infecting cattle, BVDV can infect sheep, goats, camelids, wild ruminants, and pigs. The virus has been shown to be a pathogen of sheep and goats, and transmission has been demonstrated from cattle to small ruminants and from small ruminants to cattle.[95,129,189] The pathogenesis and clinical signs associated with BVDV-1 and BVDV-2 infections in pregnant sheep (fetal loss, abortions, stillbirths, and persistently infected lambs) are similar to those observed in cattle.[186] However, clinical signs have not been associated with infections of rabbits and pigs.[11,21] When inoculated with a classical strain of BVDV (NY-1), dogs, cats, and mice did not exhibit clinical signs.[11] Pestivirus isolates that are similar to BVDV have also been found in bison, bongo, deer, giraffe, and reindeer.[17] A report that found a higher prevalence of anti-BVDV antibodies in free-living reindeer than in cattle in Norway implies that wild ruminants may serve as a reservoir for the virus.[129] It has also been reported that BVDV has been isolated from a 1-week-old foal.[187]

On a molecular level, BVDV was able to bind to the LDL receptors on human cells and was endocytosed, yet replication of BVDV was not detected.[4] However, BVDV and a subgenomic replicon of BVDV were able to replicate in human cancer cells and human hepatocyte cells.[18,84] Despite the fact that BVDV has not been identified as a human pathogen, specific anti-BVDV antibodies have been reported in humans.[215] One report indicated that 15.3% (195/1272) of human serum samples contained anti-BVDV antibodies, ranging from 14.2% of samples from human immunodeficiency virus (HIV)

negative persons to 23.3% of samples from HIV positive persons that were exhibiting signs related to acquired immunodeficiency syndrome (AIDS).[97] Another study found that none of the 259 mothers of normal infants exhibited anti-BVDV antibodies, but 1.6% (2/129) of mothers of microcephalic infants exhibited antibodies.[169] A strain of pestivirus that appears very similar to BVDV (Europa genotype Ic) has been isolated from 2 clinically healthy persons.[95,121]

Pestiviruses, specifically BVDV or a previously unknown human pestivirus, have been implicated as possible causes of white matter damage, anopthalmia, schizophrenia, microcephaly, mononeuritis (specifically Personage Turner syndrome), gastroenteritis, and respiratory disease.[22,32,35,57,79] The role of a human pestivirus in white matter damage and anophthalmia in neonates has been proposed but does not have any scientific basis.[65,99] Support for a pestivirus as a cause of schizophrenia, microcephaly, and Personage Turner syndrome (a rare form of mononeuritis) has been based on serological evidence.[96,169] A patient involved with a virulent outbreak of BVDV in cattle developed Personage Turner syndrome following the incident.[96] In a study looking at children under 2 years of age who were experiencing gastroenteritis with concurrent respiratory disease of unknown etiology, pestivirus antigens were found in feces using a monoclonal-antibody-based immunoassay in 24% (30/128) of the patients.[218]

Human exposure to BVDV might occur by exposure to infected animals, treatment with contaminated biologicals, or vaccination with contaminated vaccines.[51,122,123,185] Contamination of live biologicals and vaccines exists due to the use of contaminated fetal bovine serum in culture medium.[40,177] Research indicates that samples

of live vaccine for human use were positive for RNA of BVDV by reverse transcription nested polymerase chain reaction assay (RT-nPCR).[122,123,185] However, when using a single-closed-tube RT-nPCR, Vilcek, et al. reported that none of the 30 samples of live vaccines for human use were positive for RNA of BVDV.[205] The RT-nPCR method was developed to reduce the number of false positives while maintaining the level of sensitivity.[152,153] The role of pestivirus as an etiological agent of human disease still needs further study, but human biologicals or vaccines contaminated with pestiviruses should not be used in humans, especially those with compromised immune systems.[97,121]

Transmission. Direct transmission of BVDV between animals, primarily through inhalation or ingestion of the virus after direct contact, is required for the virus to be maintained.[12,47,69,128,201] The quantitiy of virus necessary to result in an infection depends upon the animal's immune status and the portal of viral entry. Research comparing the route of entry (subcutaneous injection, intranasal inoculation, and conjunctival inoculation) has shown that the infecting dose varies from 2 to 10,000 times 50% of the cell culture infective doses ($CCID_{50}$), depending on the route of infection with intravenous route requiring the lowest infective dose.[60,129]

Acutely infected animals are an important temporary source of BVDV, but persistently infected (PI) animals are the primary reservoir of the virus. As a reservoir, the PI animal efficiently sheds large quantities of virus in saliva, tears, nasal mucous, milk, feces, urine, vaginal mucous, and semen.[129] Houe demonstrated that direct contact with a persistently infected animal for an hour was enough to transmit virus to seronegative cattle.[129] The acutely infected animal sheds low levels of virus transiently

for 1 to 2 days.[157] Acutely infected animals usually are not efficient transmitters of the virus because of the smaller quantities of virus that are shed and because of the transient shedding of the virus. The inefficiency of transmission of BVDV from acutely infected animals was demonstrated when 14 calves failed to seroconvert following 14 days of direct contact with acutely infected calves.[129] However, Ridpath et al. demonstrated that outbreaks of severe acute BVDV observed in North America in 1993 were due to a single strain of BVDV that spread explosively following acute infections.[176] Therefore, outbreaks of severe, acute BVDV should be considered as a significant, potential source for the spread of BVDV.[176]

Venereal transmission of BVDV due to contaminated semen can also occur. Kirkland et al. showed that PI bulls shed large quantities of virus in seminal fluid with $10^{7.6}$ $CCID_{50}$/mL found in extended semen.[139] An acutely infected bull with a competent immune system sheds virus transiently with much less virus present in the semen (5 to 75 $CCID_{50}$/mL) when compared to PI bulls. The immune system of most acutely infected bulls eventually terminates viral replication in the reproductive tract, resulting in semen that is free of virus. However, the quantitiy of BVDV in semen from acutely and persistently infected bulls has been to be enough virus to cause infection with BVDV.[137,157,166] Research has shown that insemination of twelve heifers with extended semen ($10^{5.0}$ to $10^{7.5}$ $CCID_{50}$/mL) from a PI bull resulted in seroconversion of all heifers within two weeks of insemination.[157] All twelve heifers in this study gave birth to clinically normal calves, but one of the calves sired by the PI bull was persistently infected with BVDV as determined by virus isolation from buffy coat and pre-colostral

serum blood samples.[157] Another study demonstrated that seronegative cows also become infected and seroconvert when inseminated with semen from a PI bull, and 2 of 61 calves sired by a PI bull were themselves persistently infected with BVDV.[137] Studies by Kirkland and Meyling demonstrated titers to BVDV in the seminal fluid to be greater than titers to BVDV in serum.[139,157] Due to the fact that the titers to BVDV were higher in the seminal fluid compared to serum, these studies suggest that the seminal vesicles may be a site for viral replication.[139,157] Because both acutely and PI bulls are capable of transmitting BVDV in their semen, it is important that PI bulls are not allowed to enter artificial insemination (AI) centers and bulls being collected at these AI facilities not undergo an acute infection. The ability to prevent bulls with either acute or persistent infections from entering AI centers was not thought to be a real problem until 1998 when a bull with a localized persistent infection of BVDV was found at an AI center.[208] The bull was allowed to enter the AI facility based upon negative results from attempts to isolate BVDV from blood samples.[102,208] This bull had high concentrations of circulating antibodies specific to the viral strain that was being persistently shed in the semen.[102,208] The concentration of virus in the bull's semen was only 2×10^3 $CCID_{50}$/mL, which is lower than previously reported for PI bulls (10^4 to 10^7 $CCID_{50}$/mL) but still much higher than reported for bulls with acute infections (5 to 75 $CCID_{50}$/mL).[102,139,206,208] A seronegative heifer that was inseminated with semen from this bull seroconverted.[102,208] Thus, it was recommended to test semen from bulls entering AI facilities for BVDV.[102,208]

Bovine viral diarrhea virus may also be transmitted by blood-feeding flies, recently contaminated (within 2 hours) pens, and contaminated vaccines and fomites,

such as needles, nose tongs, or sleeves from rectal palpation.[55,63,64,68,88] This virus is susceptible to disinfectants, such as aldehydes, chlorhexidene, hypochlorite, iodophores, and phenol compounds; therefore, transmission of the virus by fomites is easily prevented.[78]

Clinical disease. Bovine viral diarrhea virus has the ability to affect multiple organ systems and produces a wide spectrum of clinical signs. The clinical outcomes of infection with BVDV include reproductive losses, birth defects, retarded fetal growth, weak neonates, pneumonia, diarrhea, mucosal ulceration, and hemorrhagic enteritis. The clinical response to infection with BVDV is complex and depends on several host and agent factors. Host factors that influence the clinical outcome include immunocompetence, pregnancy status, gestational age of the fetus, and level of environmental stress.[82] Agent or viral factors that influence the clinical outcome include the virulence of the strain as well as the genomic and antigenic uniqueness of the virus.[82] Differences in virulence among strains of BVDV have been recognized for many years.[27,82] According to studies by Evermann and Ridpath, the incidence of infection of specific genotypes, along with the clinical outcome after infection, have changed over time.[81] An increase in BVDV-2 and fetal infections was reported between 1980 and 2000.[81]

Manifestations of BVDV infections may range from subclinical, acute infections with low-virulent strains to highly fatal mucosal disease. Ames estimated that 70-90% of BVDV infections in immunocompetent, seronegative cattle occur as subclinical disease.[6]

Subclinical infections typically result in mild fever, a transient leukopenia, and the development of neutralizing antibodies.[47] The presence of high levels of neutralizing antibodies in some cattle populations is probably attributable to subclinical infections.[13,47] The clinical manifestations due to BVDV can be categorized as diseases resulting from 1) acute infections, 2) persistent infections, and 3) reproductive infections.

Acute infections. Acute infections with BVDV occur when seronegative, immunocompetent cattle become infected with the virus.[13] After infection with BVDV, the virus incubates for 5 to 7 days which is followed by a viremia. The viremia, which is associated with a transient fever and leukopenia, lasts for 1 to 2 days but may persist for up to 15 days.[78] Clinical signs include fever, anorexia, lethargy, ocular and nasal discharge, oral erosions and ulcers, blunting and hemorrhage of the oral papilla, diarrhea, and decreased milk production in lactating cows.[82] Epithelial erosions of the interdigital space, teats, and vulva may also be present.[82]

In the 1990s, an atypical and more severe form of BVDV infection was identified in North American cattle.[53,61,166] This outbreak was characterized by high rates of morbidity and mortality in all age groups of cattle.[53,61,165] In a Canadian outbreak, the mean abortion rate was 44% with a mortality rate among all cattle of 25% and a mortality rate of cattle less than 2 years of age at 53%.[53] The viral strains from these severe outbreaks were characterized as genotype II strains of BVDV (BVDV-2).[82]

Hemorrhagic syndrome is a form of severe, acute BVDV that appears to be associated with a noncytopathic, type 2 strain of BVDV. Hemorrhagic syndrome was first seen in Eastern Canada, the Northeastern United States, and England during the mid

1990s.[54] This syndrome is characterized by fever, severe thrombocytopenia, which results in bloody diarrhea, petechial and ecchymotic hemorrhages on mucosal surfaces, bleeding from injection sites or trauma, epistaxis, and hyphema.[82] The clinical signs seen during an outbreak of severe, acute BVDV are quite diverse with only a minority of animals developing hemorrhagic syndrome. All cattle with hemorrhagic syndrome suffer from severe, acute BVDV, but not all cattle with severe, acute BVDV will develop hemorrhagic syndrome.[82] The mechanisms associated with the severe thrombocytopenia and hemorrhagic syndrome are not fully understood, but viral antigen has been associated with both platelets and megakaryocytes.[82] Walz et al. also demonstrated altered platelet function with BVDV-2 infections but not with a BVDV-1 infection.[211] Although virus could be isolated from both BVDV-1 and BVDV-2, platelet dysfunction was only observed with BVDV-1 infections.[211] Thus, a direct BVDV-platelet interaction is not the likely cause of platelet dysfunction.[211] Altered platelet function may be due to BVDV infection of megakaryocytes, the platelet precursors, and a resulting population of aged platelets.[211]

Another common result of infection with BVDV is immunosuppression of infected cattle. The mechanisms by which BVDV causes immunosuppression are complex and multifactorial but include 1) suppression of interferon production, 2) decreased responsiveness of peripheral lymphocytes to mitogens, 3) decreased numbers of circulating B and T lymphocytes, 4) impairment of humoral antibody production, 5) depression of monocyte chemotaxis, 6) alteration in polymorphonuclear cell function, 7) impairment of immunoglobulin secretion by peripheral lymphocytes, 8) facilitation of

bacteremia, and 9) impairment of bacterial clearance from the blood.[2,12,13,47,62,71,72,146] Immunosuppression caused by BVDV has also been associated with other pathogens, such as parainfluenza virus type 3, infectious bovine rhinotracheitis virus, *Pasteurella multocida*, and *Manheimia hemolytica,* as causative agents of the bovine respiratory disease complex.[52]

Persistent Infections. Persistent infection with BVDV occurs when a fetus becomes exposed to a noncytopathic strain of BVDV prior to the development of a competent fetal immune system. This occurs at approximately 125 days of gestation. Infection of the fetus with BVDV during the development of immunocompetence causes the development of immunotolerance to the specific strain of virus that causes the infection. Persistently infected (PI) animals serve as the major reservoir and source for BVDV through continuous shedding of high levels of virus into the environments of susceptible cattle.[147]

Persistently infected cattle have a wide distribution of BVDV throughout their organs, no virus-associated morphological lesions, and no immune response to the persisting BVDV strain.[147] The immune tolerance is restricted to the specific strain of virus to which the animal was exposed in utero. Infections with other strains of BVDV will induce an immune response and, thus, the production of antibodies.[94,147] Houe et al. demonstrated that PI animals have normal cellular and humoral responses to other antigens.[131] Immunotolerance of BVDV by PI animals is thought to be maintained by non-reactive CD4+ T-lymphocytes, B-lymphocytes, and antigen presenting.[88,104,147] Calves that are born PI with BVDV usually fall behind their cohorts in growth rate, and

most PI animals either die or are culled due to poor performance before 2 years of age.[13,78,89] Immunosuppression is thought to be the reason for poor performance by PI animals.[12,14,47]

The prevalence of PI animals in the general population of cattle has been estimated to range between 0.13% and 2.0%.[144,216] One study of randomly selected herds in the United States revealed that 4% of the herds had PI calves, and the majority of these PI calves survived until weaning.[217] Studies have estimated that 10-50% of herds contain PI animals with differences in prevalence due to geographic location (low- and high-prevalence areas).[130,216] According to one report, when acute infections reach 34% within a population, total annual losses on a per calving basis are about $20 with low-virulent strains and $57 with high virulent strains.[129]

Persistently infected cattle can be clinically normal and can become productive adults.[137,150] Because of the persistent viremia present in PI animals, all offspring of a PI cow are immunotolerant to BVDV and are persistently infected. However, transfer of in-vivo-derived embryos from PI cows have produced BVDV-free calves.[10,39,214] Calves that are born following insemination of seronegative cows with contaminated semen may also be persistently infected.[157]

"Atypical" persistent infections. Evidence exists that some PI animals are capable of producing neutralizing antibodies to the persisting or resident strain of BVDV.[30,31,38,113] In general, PI animals have detectable levels of the resident virus in serum following the decline of passively acquired maternal antibodies. Brock et al. discovered a PI cow with an absence of detectable virus in serum and spontaneous

production of virus neutralizing antibody.[38] Although BVDV could not be detected in the serum, virus was isolated from white blood cells from this "atypical" PI cow.[38] It is likely that the development of BVDV-neutralizing antibodies in this cow interfered with the ability to detect BVDV by virus isolation.

The reason for this apparent break in immunotolerance is not fully understood. The antibody response to the resident virus may be due to the PI animal being exposed to a different antigenic strain of field virus and then developing cross-reactive antibodies capable of removing cell-free virus from the serum.[38] Another proposed theory is the loss of immunotolerance to the resident strain of virus and subsequent stimulation of the immune system.[38]

One study analyzed the viral genomes of 2 animals PI with BVDV and revealed quasispecies distribution due to mutations in the sequences encoding for the E2 glycoprotein.[59] The number of E2 mutant clones increased with the age of the animals and neutralizing antibodies were produced.[59] In the same study, 4 of 21 PI animals were found to have produced serum neutralizing antibodies.[59] It is likely that some of the variant viruses generated within these "atypical" PI animals are antigenically different from the persisting virus and, therefore, may illicit an immune response.[38,59]

Grooms and colleagues demonstrated that an "atypical" PI animal could cause seroconversion of susceptible herdmates.[113] In this study, an "atypical" PI cow was placed in a 196 ft^2 isolation room and exposed to a naive steer for 14 days.[113] After 14 days of exposure to the PI cow, the steer was removed and serum and blood samples collected for virus isolation and serological assays.[113] Virus could not be isolated from

the steer on days 14 and 28 of the study, but by day 28 the steer did seroconvert.[113] This study also determined that the prevalence of cattle infected with BVDV that lack detectable virus in their serum is probably extremely low because among 1,952 cattle tested, only 5 cows (0.26%) were PI and all 5 cows had detectable levels of virus in their serum and white blood cell preparations.[113] However, in a study by Collins et al., 4 out of 21 animals PI with BVDV produced serum neutralizing antibodies.[59]

Screening and control programs for BVDV rely on the accurate identification and removal of PI animals from susceptible herds. Because virus is not easily isolated from serum of these "atypical" animals, it is possible that these animals could be missed by routine screening techniques that attempt to isolate virus from serum, such as immunoperoxidase monolayer assay (IPMA). Therefore, these "atypical" PI animals may pose a risk to control and prevention programs aimed at removing PI animals.

Mucosal disease. Mucosal disease is a sporadic form of BVDV with fatalities at nearly 100% and most animals dying within 2 weeks after the onset of clinical signs.[12] Mucosal disease usually occurs in cattle between 6 months and 2 years of age with usually less than 5% of the herd affected.[12] Occasionally, epizootics may involve as much as 25% of the herd.[12] The term mucosal disease was first used by Ramsey and Chivers in 1953 to describe the clinical signs associated with a viral infection in cattle.[170] This virus caused severe erosions, ulcerations, and hemorrhages of the mucosal surfaces of the muzzle, oral cavity, esophagus, forestomachs, abomasum, and the small and large intestines.[170] Severe depletion of the spleen, lymph nodes, and thymus were also noted in the same cattle.[170]

The pathogenesis of mucosal disease involves an animal PI with a noncytopathic strain of BVDV that becomes superinfected with an antigenically similar cytopathic strain.[48] The source of the cytopathic strain responsible for the superinfection may be exogenous or endogenous in origin. An endogenous source of the cytopathic strain of virus may be the result of mutation of the noncytopathic strain.[23] Mucosal disease has been reported following vaccination of PI cattle with a modified-live vaccine because most vaccines are derived from cytopathogenic strains of BVDV.[47] However, the required homology between the noncytopathic and cytopathic strains favors the idea of mutation of a noncytopathic virus as the primary cause of mucosal disease.[47] Studies indicate that nonhomologous RNA recombination during RNA replication may be a possible mechanism of production of a cytopathic virus from a noncytopathic virus.[15,141,142] Other studies have demonstrated point mutations occurring in the NS2 gene that lead to viral cytopathogenicity.[141]

Mucosal disease can occur as an acute or a chronic form. Acute mucosal disease is characterized by depression, weakness, pyrexia, oral and nasal erosions, laminitis, coronitis, and profuse, putrid, watery diarrhea with fresh or clotted blood and fibrinous intestinal casts.[12,48] Death usually occurs within 3 to 10 days after the onset of clinical signs.[12] This acute form of mucosal disease is thought to occur when the cytopathic strain of virus demonstrates perfect antigenic homology to the resident, noncytopathic strain.[147]

Chronic mucosal disease is associated with inappetence, weight loss resulting in emaciation, chronic bloat, intermittent or continuous diarrhea, with some cases

developing chronic oral and interdigital lesions.[147] Animals that suffer from chronic mucosal disease may live for up to 18 months only to succumb to severe debilitation.[23] It is thought that this chronic form of mucosal disease is either due to a partially homologous cytopathic strain of BVDV or due to genetic recombination of exogenous cytopathic strains with a resident noncytopathic strain.[23,105] A delayed onset of mucosal disease has also been reported to occur weeks to months after a PI animal is challenged with a vaccine containing a cytopathic strain of BVDV.[147]

In utero infections. Although clinical signs associated with acute infections of BVDV can be quite dramatic and economically significant, the subtle reproductive consequences of BVDV infections can be much more economically devastating. The outcome of an intrauterine infection with BVDV is dependent upon the stage of gestation when infection occurs as well as the biotype, virulence, and cells targeted by the infecting viral strain. Uterine infections caused by BVDV are usually characterized by the stage of gestation when fetal infection occurs.[109]

(a) *Infection before conception through the embryonic stage (-9 to 45 days of gestation)*. Bovine viral diarrhea virus can have a significant impact on early reproductive performance. In a report by Virakul et al., a group of seronegative cattle accidentally exposed to a PI cow experienced a decline in conception rates (78.6% to 22.2%).[109,207] In another study looking at BVDV infection around breeding, conception rates in heifers infected intranasally 9 days before insemination were 44% compared with 79% for the control group.[154] This decline in conception rates was likely due to either fertilization failure or early embryonic death.[154]

Bovine viral diarrhea virus has been localized in ovarian tissue for prolonged periods of time after acute infection with both cytopathic and noncytopathic virus.[111,112,193] Isolation of BVDV has also been demonstrated from the follicular fluid collected from slaughterhouse ovaries.[21] Exposure of the developing oocytes to BVDV could cause a reduction in survivability either through direct cell damage or indirectly through changes in the local environment of the oocyte.[109] Interstitial oophoritis has also been demonstrated with lesions lasting for up to 60 days following an acute infection with a cytopathic strain of BVDV.[111,192] Several studies have shown significant alterations in ovarian function following acute infections with BVDV. Kafi et al. reported that cattle being superovulated, while undergoing experimental challenge with BVDV, experienced a decease in the number of palpable corpora lutea and the number of recovered embryos was significantly reduced when compared to non-infected controls.[134] Infection with BVDV and subsequent viremia during the pre-ovulatory phase have also been shown to cause a reduction in the follicular growth rate.[87,110] Persistently infected cattle also demonstrated hypoplastic ovaries and a reduction in the number of ovarian antral follicles when compared to cattle not PI with BVDV.[115] These studies indicate that changes in ovarian dynamics of cattle infected with BVDV may lead to transient or long-term reductions in fertility. Bovine viral diarrhea virus may also have detrimental effects on the oviducts. Archbald et al. isolated BVDV from oviductal tissue and detected evidence of salpingitis for up to 21 days post-intrauterine infusion with cytopathic BVDV.[8] Similar findings have also been reported with noncytopathic strains of BVDV.

Archbald et al. provided evidence that BVDV may also interfere with early embryonic development. In this study, BVDV was infused into one horn of superovulated cows with a dramatic reduction in the quality of embryos collected from the infected horn when compared to those collected from the non-infected horn.[7] Another study demonstrated that following intrauterine infusion of cytopathic BVDV, histological changes in both the uterus and the oviduct were present from 6 to 21 days post-infection.[8] Although BVDV may have a direct effect on the developing embryo, inflammatory changes within the uterus following infection may result in an environment that is not conducive to embryo development.

In vitro studies have shown that ova exposed to BVDV can have virus particles attached to the zona pellucida.[101] However, other studies indicate that the intact zona pellucida protects the developing embryo from infection with both cytopathic and noncytopathic strains of BVDV and allows normal development of the embryo to continue.[20,169,191,222] In contrast, blastocysts hatched from the zona pellucida at day 8 of gestation showed decreased viability when exposed to cytopathic strains of BVDV, but viability was not decreased when exposed to noncytopathic strains of virus.[42] These studies indicate that the zona pellucida offers protection to the developing embryo from the effects of BVDV.

Infection after the embryo stage (45 to 175 days of gestation). After implantation, transplacental infection of the developing fetus may occur with either biotype of BVDV.[109] Again, the outcome of the infection depends on the stage of gestation of the fetus or level of immunocompetence, the virus biotype, and the virulence of the virus.[109]

Abortions caused by BVDV usually occur in the early stages of gestation (less than 125 days) but can occur at any stage of gestation. Depending on the time of infection with BVDV, fetal resorption, mummification, or expulsion may occur.[69] Expulsion of the fetus usually does not occur immediately after infection but weeks to months later.[147]

Although the exact mechanism of fetal infection is unknown, BVDV probably crosses the placenta and causes a vasculitis within the caruncle which allows the virus access to the fetus.[89] Fetal death usually occurs 10 to 27 days after infection with expulsion of the fetus occurring up to 50 days later.[161] Because of the delay between fetal death and diagnosis of abortion, fetal and placental lesions are usually non-diagnostic, and isolation of BVDV is not always successful.[12] In surveys of diagnostic laboratories in the United States, BVDV has been isolated from 0.1% to 27.2% of submitted cases of abortion.[109] In the United Kingdom, BVDV was isolated from 27% of submitted cases of abortion.[160] Bovine viral diarrhea virus was also isolated from 4.1% of cases of abortion submitted to the Ontario Ministry of Agriculture.[5]

Most studies have reported unremarkable, nonspecific lesions of the placenta and placentomes of aborted calves.[55,71,161] However, Baszler et al. found necrotizing placentitis associated with the presence of viral antigens.[16] These mild placental lesions may allow for opportunistic infections to cross the placenta.[147] The presence of BVDV antigen was detected in various tissues of aborted fetuses, and infiltrates of mononuclear cells were found in various tissues including the lung and myocardium.[16,71,161] Lesions that have been seen with BVDV abortions include conjunctivitis, peribronchiolar and interalveolar pneumonia, and nonspecific myocarditis.[109] Placental lesions include

vasculitis, edema, congestion, hemorrhage, degeneration, and necrosis.[109] These findings indicate that damage to the fetus may be an important factor for initiation of abortion.

Abortion is not the only outcome of infection occurring during the first trimester of gestation. If an embryo or fetus survives an infection with a noncytopathic biotype of BVDV that was initiated between 18 and 125 days of gestation, the fetus will develop immunotolerance and, thus, become PI with BVDV. The exact mechanism of immunotolerance is unknown, but circulation of virus during the period of gestation when immunocompetence is developing (90-120 days) is thought to cause the fetus to recognize viral proteins as self-antigens which results in negative selection of BVDV specific B and T lymphocyte precursors.[151] The specific immunotolerance of B and T lymphocytes to the virus results in the absence of neutralizing antibodies to the resident virus.[74] The exact time in gestation at which infection must occur to produce PI animals in unclear. Kirkland et al. reported that cows infected with BVDV at day 18 of gestation had 86% persistence of virus in their offspring while 100% of the offspring of cows infected at day 30 of gestation were PI with BVDV.[138] Persistent infections were also induced in cows that were infected with BVDV at 75 days of gestation.[35,36,64] Persistent infections resulting from BVDV infections occurring after day 100 of gestation are considered rare, but PI calves have been reported with infection occurring up to day 125.[13]

Fetal infection with BVDV that occurs between 100 and 150 days of gestation often results in the development of a variety of congenital defects. During this stage of gestation, fetal organogenesis is being completed and the immune system is becoming

fully developed. Although the exact mechanism is not fully understood, the combination of direct cellular damage by the virus and inflammatory responses to the virus has been proposed as a possible explanation.[56]

Congenital defects involving the central nervous system are the most common following fetal infection with BVDV. These defects include the following: cerebellar hypoplasia, microencephalopathy, hydrocephalus, hydranencephaly, porencephaly, and hypomyelination.[12,76,195,201] Cerebellar hypoplasia was one of the first recognized teratogenic effects of BVDV. Calves that have cerebellar hypoplasia show extreme difficulty in becoming ambulatory, and those that can stand are ataxic which results in tremors, wide-based stance, and stumbling gait. These defects are usually severe enough that the calves either die or are euthanized. The effects of BVDV on the cerebellum have been characterized by a reduction in the number of molecular layer cells and granular layer cells.[22,46,45,71] Purkinje cell numbers are also reduced and often displaced.[46] Fetal cerebellar defects have been seen following infection with BVDV as early as day 79 of gestation and as late as day 150 of gestation, and the severity of cerebellar lesions increases with the age of the fetus at the time of infection.[46] Other teratogenic effects associated with BVDV include cataracts, microopthalmia, retinal degeneration, optic neuritis, thymic hypoplasia, hypotrichosis/alopecia, curly hair coat, hyena disease, deranged osteogenesis, mandibular brachygnathism, and growth retardation.

Infection late in gestation (175 to 283 days of gestation). During the later stages of gestation, less dramatic pathological effects are seen because fetal immunocompetence and organogenesis are usually complete. Although abortions and birth of weak calves

have been reported following exposure to BVDV in the last trimester, the immunocompetent fetus can mount an immune response and effectively clear the virus. Calves exposed to the virus at this stage of gestation are usually normal at birth and have serum neutralizing antibodies to BVDV.[76] However, Munoz-Zanzi et al. showed that calves born with BVDV neutralizing antibody titers were twice as likely to experience a severe illness within their first 10 months of life when compared to calves born without BVDV neutralizing antibody titers.[158] Therefore, BVDV may have more detrimental, long-term effects in these calves than previously realized.

In conclusion, BVDV can produce a variety of clinical manifestations including acute infections, persistent infections, and reproductive infections with the outcome of disease depending upon several host and viral factors. Host factors such as the presence of colostral antibodies as well as viral factors such as genotype and biotype can have a significant impact on the ability to accurately diagnose cattle PI with BVDV.

Diagnosis of Bovine Viral Diarrhea Virus

Introduction. Because of the wide range of clinical presentations associated with BVDV infections, only a presumptive diagnosis can be made based on history, clinical signs, or post-mortem findings. Therefore, definitive diagnoses of BVDV infections depends upon laboratory diagnosis.[106] Accurate and rapid diagnostic tests for BVDV are necessary for understanding the prognosis and epidemiology of BVDV. These tests are essential for surveillance and control programs as well as preventing contamination of biologicals.[106] The ability to identify both acutely and PI animals and discriminate between the two is crucial. Especially important to the prevention and control of BVDV is the accurate identification and removal of PI animals. Numerous diagnostic tests are currently available for the detection of BVDV. These tests include methods for isolating virus and for detection of antigen, nucleic acid, and antibodies.

Virus isolation. Virus isolation has been the most reliable method for the detection of cattle PI with BVDV and still remains the "gold standard" diagnostic technique.[77,182] The definitive identification of cattle PI with BVDV requires the isolation of virus from serial samples taken at least 2 weeks apart.[33] The virus readily grows in many cell lines from several animal species. However, three cell lines are most commonly used in diagnostic laboratories for BVDV isolation: bovine turbinate (BT), bovine testicle (Btest) and Madin Darby bovine kidney (MDBK). There is some evidence that BT and Btest cells are more susceptible to BVDV infection than MDBK and other cell lines.[182] However, Givens et al. demonstrated that primary cultures of Btest cells

were less susceptible than MDBK or BT cells to infection with BVDV from contaminated semen.[103] This research indicates that assays using MDBK cells provide acceptable analytical sensitivity for detection of BVDV in semen.[103] Another factor, other than the sensitivity of cell cultures, that affects the ability to culture BVDV involves the method of inoculation of cells.[182] It appears that methods for inoculation that involve dropping the inoculum into culture media that is overlying a cell monolayer are less sensitive because of the "depth of column" effect.[182] Based on this effect, it appears that sensitivity is inversely proportional to the distance that virus particles have to travel before meeting cells.[182] Therefore, inoculations in smaller culture vessels (96-well and 24-well plates) which have a higher fluid column sitting in the cell monolayer are less sensitive than inoculations in larger vessels (25 cm^2 flask). This problem can be avoided by absorbing the inoculum directly on the cell monolayer for 1 to 2 hours before adding more cell culture medium.[182] Another method used in smaller culture vessels (96-well plates) to eliminate this concern involves allowing cells in culture to drop through the inoculum. This is performed by adding cells in culture to plates that were previously inoculated with virus. This method allows maximum contact between culture cells and the virus.

In the live animal, the best sample for isolation of BVDV is whole blood from which the buffy coat or mononuclear cells are extracted and used as the inoculum. Virus may be neutralized in specimens from animals that have circulating neutralizing antibodies which may prevent the isolation of virus. These antibodies may be the result of a humoral immune response or passive transfer. When trying to isolate BVDV from a

PI calf which is less than 3 to 4 months of age, the mononuclear cells may be the only sample from which virus can be isolated. Therefore, samples from calves tested by virus isolation should be collected prior to ingestion of colostrum or after 4 months of age. Other samples such as serum and nasal swabs may also be used for detection of BVDV, but serum neutralizing antibodies may also interfere with virus isolation when using these samples.[106]

Bovine viral diarrhea virus appears to be a very stable virus. Therefore, shipping samples with ice packs and storage of samples in the refrigerator for 1 to 2 days after receipt in the laboratory does not compromise the ability to culture virus.[182] However, freezing buffy coat samples may reduce sensitivity as a result of release of membrane-bound immunoglobulins that bind to the virus and prevent its entry into cells.

An incubation period of 4 to 5 days is usually sufficient for isolation of BVDV, but some may require a second passage. This subculturing may be done by either freeze-thawing or trypsinization methods. Cytopathic strains cause cellular vacuolization and lysis of susceptible cells within 48 hours (h) of inoculation. Because most field strains of BVDV are noncytopathic, cell cultures must be tested further by immunofluorescence or immunoenzyme staining.[93] Immunofluorescent antibody staining may be used with either monoclonal or polyclonal anti-BVDV antibodies. Polyclonal anti-BVDV sera or FITC-conjugates are broadly reactive but may produce nonspecific background staining that could result in false positive results. Monoclonal antibodies produce cleaner fluorescent antibody (FA) stains that are easier to interpret, but care must be taken to ensure that these monoclonal antibodies are broadly reactive against all strains of BVDV to reduce

the chance of false negative results.[182] With the use of smaller culture vessels (96-well and 24-well plates) for isolation, immunologic staining can be performed by fixing the cell monolayers on the plates and performing a standard indirect enzyme-linked immunosorbent assay (ELISA) or immunoperoxidase assay.[182]

A virus isolation method that has been useful for handling large numbers of samples, such as in whole herd screening for PI cattle, is the immunoperoxidase monolayer assay (IPMA) with serum as the diagnostic specimen. For the IPMA, serum is inoculated onto cells for growth of BVDV in 96-well plates. After incubating for 4 days, the plates are fixed with 20% acetone, dried, and the presence of BVDV is determined by ELISA or immunoperoxidase staining.[182] Although this test is considered very accurate and reliable for testing PI animals, it is not sensitive enough to diagnose acute BVDV infections.[182] The major limitation of the IPMA for PI testing is the inability to use the test on sera from animals less than 3 months of age where maternal antibodies can interfere with the growth of BVDV in cell culture. The IPMA would not be able to diagnose the "atypical" PI cattle that produce serum neutralizing antibodies either.

Antigen detection. Detection of BVDV antigen is a rapid and less expensive technique when compared to other methods. However, some methods used for antigen detection lack sensitivity. The available methods fall into two categories: antigen capture ELISAs (ACE) and immunological staining of fresh and formalin-fixed paraffin-embedded tissue sections.

Many antigen capture ELISAs have been described with some available as commercial test kits. The antigen capture ELISA is primarily used to detect PI cattle and

is not reliable for the diagnosis of acute infections. Most ACEs require samples that contain cells such as buffy coat and tissue extracts. The need to extract buffy coat cells or process tissues prior to testing limits the applicability of these tests on large numbers of samples as required with whole-herd screening.[182] However, one of the commercially available test kits can reliably detect BVDV antigen from serum and ear-notch (nonformalin-fixed) samples of PI animals.[167,181] In the past ACEs were directed at the NS2-3 protein, but new ACEs for the detection of BVDV are designed to detect the E^{rns} structural protein. Kuhne et al. tested 11 PI calves before receiving colostrum and up to 5 weeks after receiving colostrum using the E^{rns} ACE on skin biopsies and serum.[140] All 11 calves were clearly positive by ACE on ear notch samples with some influence of colostral antibodies noted.[140] All serum samples collected from the 11 PI calves were negative up to four days after the ingestion of colostrum.[140] However 35 days after the ingestion of colostrum, all 11 PI calves were positive by ACE.[140] This study demonstrates that antibodies can interfere with the detection of BVDV using serum samples for ACE, while ACE using skin biopsies as the sample does not seem to be affected by the presence of circulating antibodies.[140]

Antigen detection in frozen tissue sections by fluorescent antibody (FA) staining is a screening test that is commonly used by diagnostic laboratories. When performed properly, a positive FA test confirms the presence of BVDV. However, negative results may not rule out BVDV because of the relative lack of sensitivity associated with the FA test.[182]

The detection of BVDV antigen in formalin-fixed paraffin-embedded tissues by immunohistochemical staining (IHC) is a widely used diagnostic test for the detection of persistent infections with BVDV.[182] Although studies indicate that IHC of skin biopsies is not a reliable method for detecting acute infections with BVDV, IHC has been occasionally used for the detection of acute infections.[175,182] Immunohistochemical staining is considered to be more reliable than fluorescent antibody staining because the antigen signal is amplified by an enzymatic reaction. This test utilizes the monoclonal antibody, 15c5, which is very specific for the highly conserved E^{rns} (gp48) glycoprotein of BVDV. The use of the 15c5 monoclonal antibody allows this test to recognize virtually all isolates of BVDV.[43] Immunohistochemical staining of skin biopsies does not appear to be affected by colostral antibodies and, thus, is considered a reliable method to detect neonatal calves PI with BVDV.[114] In a study by Grooms and Keilen, six neonatal calves were found to be PI with BVDV by IHC and VI.[114] Results from two calves were not in agreement with one calf positive on IHC and negative by VI and the other calf positive by VI and negative by IHC.[114] Both calves were found to be acutely infected with BVDV.[114] In this study, IHC demonstrated a sensitivity of 100% and a specificity of 99.69% with a positive predictive value of 100% and a negative predictive value of 85.71%.[114]

Animals that are PI with BVDV have diffuse staining of the epidermis and adnexal structures as well as staining of dendritic cells in the dermis and, occasionally, chondrocytes of aural cartilage. However, the distribution of BVDV antigen can be less widely distributed in the epidermis and adnexal structures which makes differentiation between PI animals and acutely infected animals difficult.[43] Immunohistochemical

staining patterns in the skin of persistently infected animals compared to acutely infected animals is somewhat different and may be subject to interpretation.[43] Skin biopsies from animals that are acutely infected with BVDV have IHC staining distribution that is multifocal and often limited to the epidermis and infundibulae of hair follicles.[162]

When animals are acutely infected with BVDV or vaccinated with modified live BVDV vaccine, the probability of detecting viral antigen in skin biopsy by IHC is rare.[50,148,162] BuBois et al. showed that calves vaccinated with a modified live vaccine were negative for IHC staining of repeated skin biopsies which demonstrated that modified live vaccines do not produce false positive staining.[50]

Nucleic acid detection. Many molecular-based diagnostic techniques have been developed to detect the RNA of BVDV. These methods include hybridization probes, reverse transcription-polymerase chain reaction (RT-PCR), and nested RT-PCR (RT-nPCR). Nucleic acid probes have been used that detect the highly conserved sequences for the 5' UTR and the NS2-3 gene.[41] In situ hybridization is a technique that is used to detect viral RNA in formalin-fixed tissues in which labeled riboprobes detect the p125 nonstructural protein.[70] Although a sensitive technique for the detection of viral RNA, in situ hybridization has not been adapted for routine use by diagnostic laboratories.[182]

Reverse transcription-polymerase chain reaction is another reliable method for the detection of BVDV. The RT-PCR assay involves the amplification of viral RNA genome by binding of specific DNA oligonucleotides to cDNA target sequences which results in the amplification of size-specific DNA fragments that are detectable by gel electrophoresis.[182] The RT-PCR assay is thought to be more sensitive than virus

isolation.[124,126] However, extreme care must be used to prevent false positive reactions due to cross-contamination in the laboratory. Other aspects that may affect the sensitivity of RT-PCR assays are the primer set and the ability to isolate primer sets from the samples.[182]

Although the cost of RT-PCR prohibits its use for individual animal testing in most cases, the high analytical sensitivity of RT-PCR allows for pooling of samples to reduce the per unit test cost.[182] The RT-PCR assay has proven to be a sensitive and economical method to screen pooled serum samples and bulk milk from which viral RNA is extracted from somatic cells.[182] Serum and milk samples are commonly pooled for RT-PCR when looking for PI animals.[182] The RT-PCR assay is fifteen fold more sensitive than virus isolation, and the presence of antibodies does not interfere with the test.[106] Weinstock et al. found that a single viremic serum sample could be detected in up to 100 pooled serum samples.[213] Using bulk milk samples, Drew et al. were able to detect one PI cow in a herd of 162 lactating animals.[75]

Reverse transcription nested polymerase chain reaction (RT-nPCR) for BVDV that is commonly used is a rapid, closed-tube method of viral detection that allows the detection of small quantities of virus.[126] This new method was very sensitive and less prone to giving false positive results compared to nested PCR carried out in separate reaction tubes.[152] The use of RT-nPCR limits cross-contamination and allows detection of virus that is inactive due to sample mishandling or virus neutralized by antibody.[127,212]

Antibody detection. The detection of BVDV antibodies is still a widely used method of determining the status of a herd. The most popular methods for detecting

antibodies to BVDV have been ELISA tests and serum (virus) neutralization (SN) assays. Agreement appears to be poor between ELISA and SN as evidenced by a study that found that the agreement quotient, kappa, was 0.15.[198] In North America, ELISA has not been widely used because the heavy use of both killed and modified-live vaccines with different antigenic combinations makes this test relatively useless.[182]

Serum neutralization is considered the gold standard test for detecting antibodies to BVDV.[178] Serum neutralization is performed by incubating serial dilutions of serum and a constant amount of virus and then finding the highest dilution of serum that inhibits virally induced cytopathic effects. Although considered the gold standard for serology, the results from SN tests can vary greatly among various laboratories.[182] This variation among SN assays may be due to differences in the strain of virus in the assay or the type of cells used in the assay.[182] Differences of 10- to 100- fold can be found in the SN titer just by using a different strain of virus in the assay.[182] Because of the variation in BVDV antibody titers, care should be used when interpreting titers without adequate herd information (exposure to BVDV, vaccination protocols, biosecurity programs).[182] Again, the widespread use of BVDV vaccines makes antibody status of an animal useless as a screening method for animals PI with BVDV. Serology tests, when properly used, can still offer useful information such as 1) assessing vaccine efficacy, 2) assessing vaccine protocol compliance, 3) assessing herd exposure to BVDV, and 4) associating BVDV with clinical signs.[182]

Comparison of tests. Because the identification and removal of PI animals is critical to the control of BVDV and because there are so many diagnostic tests available,

it is important to determine the best tests available for this purpose. Several methods are available to describe the performance of diagnostic tests. The diagnostic or epidemiological sensitivity of a test is defined as the percentage of true positives which test positively with the test or the probability that an animal having the disease will actually test positive.[183] The diagnostic or epidemiological specificity is the percentage of true negatives recognized as such by the test or the probability that an animal that does not have the disease will actually test negative.[183] The sensitivity and specificity should be as high as possible, but in practice they are usually inversely related.[183] The predicitve values of a test result describe the probability that animals that are diagnosed as positive or negative by a test actually have or do not have the disease.[183] The positive and negative predictive values also depend on the sensitivity and specificity of the test as well as the prevalence of the disease in the tested population.[183]

In a 1996 study using specimens collected from 15 PI animals, there was complete agreement between IHC and VI while only 13 samples were positive by antigen-ELISA.[199] This study showed that of the 104 cattle that tested negative by parallel tests, there was complete agreement among the tests which indicates 100% sensitivity when comparing IHC to VI.[199] Njaa et al. reported 97.5% agreement between IHC on skin biopsies and VI on peripheral blood leukocytes.[162] All 45 animals that tested negative by VI were also negative by IHC.[162] In this study, 41 of 42 animals were positive by IHC with discordant results on one animal.[162] It has also been reported that there is very good agreement between IHC and IPMA.[43] Brodersen compared IHC, VI, and IPMA on 109 samples.[43] Immunohistochemical tests were reported to be very close

to 100% sensitivity and a slightly lower specificity (as acute infections may transiently affect skin). While virus isolation resulted in 91.2% sensitivity and 92.3% specificity, and IPMA yielded a sensitivity of 80.7% and a specificity of 100%.[43] In a study comparing IHC, VI and immunofluorescence on specimens from aborted and neonatal calves, IHC had a sensitivity of 97% and a specificity of 97%.[80] This is compared to VI with a sensitivity of 83% and a specificity of 100% and immunofluorescence with a sensitivity of 77% and a specificity of 88%.[80] Immunohistochemical staining appears to be a reliable diagnostic test for the detection of PI cattle.

A study by Cornish et al. compared VI, IHC and ACE for detection of calves PI with BVDV.[63] Sixty-seven of 559 calves tested positive at initial screening by IHC, ACE or VI.[63] Of the calves positive at initial screening 59 of the 67 were determined to be PI and 8 were determined to be acutely infected.[63] Both IHC and ACE detected 100% of the PI calves, but these tests also detected 6 and 8 acutely infected animals, respectively, at initial screening and 3 and 4 acutely infected calves, respectively, 3 months after initial screening.[63] Although considered to be a rare occurrence, 3 acutely infected calves had IHC staining indistinguishable from PI calves at initial screening.[63] It appears that both IHC and ACE accurately detect animals PI with BVDV. However, both tests may also detect some animals acutely infected with BVDV.

Because there are many diagnostic tests available for the detection of cattle PI with BVDV, it is important to remember that each method has its advantages, disadvantages, and applicability. The sensitivity and specificity of laboratory tests are dependent upon the type of sample, the timing of sample submission, and the individual

diagnostic test. Additional factors that can affect the efficiency of a particular diagnostic test include antigenic and/or genetic diversity of the virus, variation in virus load, and antibody interference. In order to facilitate control of BVDV by diagnostic testing and removal of PI animals, diagnostic tests must be economically feasible for whole herd screening and must reliably identify cattle PI with BVDV.

Prevention and Control of Bovine Viral Diarrhea Virus

Introduction. Prevention and control of BVDV has become more important due to a greater understanding of the economic consequences of the virus. As stated previously, according to a report by Houe et al., when acute infections reach 34% within a population, total annual losses on a per calving basis were estimated to be $20 with low-virulent strains and $57 with high-virulent strains.[129] Another study estimated an annual loss of $1,525 among 50-cow dairy herds.[57] Bovine viral diarrhea virus also has an important impact in the feedlot. Although direct economic losses attributable to BVDV have not been well documented, the role of BVDV as an immunosuppressive agent and as a potentiator for other diseases or disease complexes, particularly bovine respiratory disease (BRD), has been well documented.[52] It is estimated that BRD accounts for $458 to $624 million in annual losses in feedlots, which would account for approximately 7% of all production costs.[191] Thus, it is important to remember that the true economic impact of BVDV is underestimated due to many subtle losses attributable to immunosuppression and subfertility.[81,87,135] Because of the economic impact of BVDV, control and prevention of BVDV is becoming increasingly popular. Prevention of BVDV involves the following steps: elimination of the viral source, increasing herd immunity through vaccination, and biosecurity to prevent reintroduction of virus.

Elimination of viral source. As previously discussed, the major source of BVDV within a herd is the PI animal. Therefore, a mandatory objective of any BVDV control program is the culling or removal of animals PI with BVDV. Because of vertical

transmission of BVDV from viremic dams to their fetuses, PI animals must be removed prior to the beginning of the breeding season which is defined or controlled in beef herds and removed as soon as possible from direct contact with the breeding cows in dairy herds.[133] The removal of these PI animals requires the application of reliable diagnostic assays for surveillance programs. These assays include VI from whole blood (buffy coats) or serum, IHC staining of viral antigen in skin biopsies, ACE from skin biopsies or serum, and PCR methods from whole blood or serum.[145] Although PI cattle are usually seronegative to BVDV, an immune response can be elicited to a heterologous strain of the virus.[38] It is thought that the immune response can be due to either vaccine or natural exposure. In addition, some cattle in both vaccinated and unvaccinated herds are seronegative which make serology alone an unsuitable method for the identification of PI animals.[145]

Several factors influence which diagnostic tests are suitable for mass testing of animals for BVDV control programs. These factors include the type of sample to be tested, cost, investigation time and the applicability of a technically reliable test that can be applied to large scale testing for control programs. In most whole-herd sampling situations, IHC on skin samples is currently the test of choice because it can accurately detect PI animals of any age without the interference of neutralizing antibodies.[145] Virus isolation and testing using PCR both require a second test 3 weeks following any positive results to differentiate between acute and persistent infections.[145] When an animal is identified as PI, it should be euthanized or removed for slaughter and the dam should be tested.

Biosecurity. Biosecurity to prevent herd exposure to PI or acutely infected cattle is a critical step in the control and prevention of BVDV. The maintenance of biosecurity is especially important after the removal of PI cattle. Therefore, all replacement heifers and bulls that enter a breeding herd, whether they are raised or purchased, should be tested prior to the start of the breeding season to ensure that they are not PI. If a pregnant animal is purchased, it should be segregated from the breeding herd until both the dam and the calf are confirmed to be BVDV negative.[145] Fence-line contact should be managed so that during early gestation the breeding herd is not adjacent to neighboring stocker cattle or other herds with questionable biosecurity and vaccination programs.[145]

Cattle that are exposed to animals of unknown BVDV status, such as at shows and sales, should be isolated for 3 to 4 weeks before reintroduction into the herd. In addition, cows pregnant less than 125 days of gestation should not be exposed to animals of an unknown health status. Because BVDV can be transmitted through semen, all semen used for artificial insemination should be purchased from reputable bull studs where testing for BVDV and stringent biosecurity practices, such as isolating bulls on arrival, are employed. Equipment such as nose tongs, balling guns, and speculums should be cleaned and disinfected to prevent introduction and spread of virus. Biosecurity also involves the application of a vaccination protocol to reduce the risk of fetal infection in the event that the breeding herd is exposed to infectious virus.[145]

Biosecurity issues should also be considered with current and emerging reproductive technologies. First generation embryo technologies include *in vivo* embryo production systems involving superovulation of donors, non-surgical embryo collection,

embryo cryopreservation, and non-surgical transfer of embryos to synchronized recipients. Second generation embryo technologies include *in vitro* embryo production systems involving transvaginal aspiration and *in vitro* maturation of oocytes, *in vitro* capacitation of spermatozoa, *in vitro* fertilization and *in vitro* culture of embryos to blastocysts. The third generation of embryo technologies includes somatic cell cloning and transgenics. These new technologies represent environmental changes in which semen, cell lines, and reagents used in cell culture, such as fetal calf serum, can serve as sources of BVDV. Exposure to virus could lead to infection of the embryo with BVDV.

Vaccination. While elimination of PI animals is the priority for controlling BVDV, the prevalence of the virus and the potential for reintroduction into a herd create the need to vaccinate cattle in North America. Vaccination may provide some protection in the face of an outbreak due to a virulent strain and may assist in the prevention of persistent fetal infections.[156,202] Bovine viral diarrhea virus vaccines have been available in the United States for over 40 years with more than 180 licensed vaccines currently available.[136] Both modified-live and inactivated (killed) vaccines are available with each type of vaccine exhibiting advantages and disadvantages.

Modified-live virus (MLV) vaccines contain mostly cytopathic strains of BVDV that have been attenuated by serial passage through cell culture or by chemically induced mutations.[24] Following administration of a MLV vaccine, the BVDV vaccine strains replicate in the animal and produce a viremia that lasts for 3 to 7 days after which the animals clear the virus and produce antibodies.[90] The MLV vaccines require smaller amounts of virus than killed vaccines and usually require only one dose for initial

immunization.[90] Another advantage of MLV vaccines is that these vaccines can produce immunity in the presence of colostral antibodies.[32] Modified-live virus vaccines also provide greater cross-reactivity among the different strains of BVDV-1 and BVDV-2.[66,202] One disadvantage of MLV vaccines is the requirement for more stringent handling procedures because the vaccine is susceptible to inactivation by chemicals and exposure to high temperatures.[90] These vaccines may also cause immunosuppression of the vaccinate due to decreased leukocyte function.[90] Another disadvantage of MLV vaccines involves the effects of the MLV on the reproductive tract. MLV has been detected in the ovaries of heifers following vaccination.[90] A major concern for using a MLV vaccine for BVDV has been the observation that MLV strains exhibit the potential to undergo genetic recombination. This recombination may result in reversion of the vaccine strain to a virulent form that may cause mucosal disease in persistently infected vaccinates.[24,90,93,155] The use of MLV vaccines of BVDV has been contraindicated in pregnant cattle due to the potential for the vaccine virus to cross the placenta and cause abortions, stillbirths, or developmental defects of the fetus.[90] Another disadvantage of MLV vaccines is the potential for contamination of a MLV vaccine with noncytopathic biotype which could induce a PI calf if the fetus were exposed between days 42-125 of gestation.[90] Although MLV BVDV may replicate in the vaccinate and produce a viremia, sufficient levels of virus needed to cause transmission to susceptible animals is not thought to occur. In a study by Fulton et al., calves vaccinated with three BVDV 1a MLV vaccines developed a transient viremia that was cleared after the induction of antibodies and there was no transmission of virus to susceptible herdmates.[92]

Inactivated or killed vaccines, which include cytopathic and noncytopathic strains of BVDV, are grown to high titers and then chemically treated to render them non-infectious.[202] Killed vaccines are more expensive to produce compared to MLV vaccines because larger amounts of virus are required to prepare each dose of the vaccine as well as the added cost of an adjuvant.[90] Because the virus has been killed, the killed vaccine strain of virus is not capable of 1) reverting to a virulent strain, 2) undergoing recombination with other strains, 3) causing post-vaccination outbreaks of disease, 4) causing fetal disease, or 5) causing immunosuppression in the vaccinate.[202] However, killed vaccines require 2 doses for the initial immunization and have a greater lag phase before protective immunity is established. Both MLV and killed vaccines induce antibodies to a wide range of BVDV subtypes but usually induce higher antibody titers to the specific strains of BVDV found in the vaccine.[90] There are concerns that the duration of immunity produced by killed vaccines may be less than one year.[26,24,202] Studies have shown that both MLV and killed BVDV vaccines offer protection of the vaccinate to prevent clinical symptoms associated with acute infection, but neither type of vaccine completely protects the fetus from in utero infection.[202] In order to completely eliminate the birth of PI calves, a goal of future vaccine development should be the creation of vaccines that confer complete protection to pregnant heifers and cows against viremia. It is also important to remember that vaccination alone does not prevent introduction of BVDV infection. Therefore, vaccination is only a tool that can help prevent acute and some persistent infections and must be used in combination with biosecurity to ensure protection.

Eradication programs. Because of the negative impact of BVDV on animal health and the inconsistencies with which vaccines have been able to control infection, several countries and territories have initiated eradication programs; these include Denmark, Finland, Norway, Sweden, the Shetland Islands, Austria, Belgium, Greece, and Slovenia.[85,108,129,149,196] In 1993, Sweden was one of the first countries to design and implement an eradication program. The Swedish program began as a volunteer program run by farmers' organizations with advisory input from animal health authorities. Upon joining the program, herd managers agreed to follow guidelines restricting the movement of animals of unknown BVDV status and hygienic measures designed to prevent spread of the virus.[184] This program, largely funded by the farmers, allowed regular screening to determine the incidence of infections with BVDV in herds and identification of PI animals.[184] When certified free of BVDV, these herds were allowed to engage in livestock trade with all other certified herds.[184] In 1993 only 35% of Swedish herds of cattle were BVDV-free, but by March 2004 about 96% of all herds were declared officially free of BVDV.[107,193]

In the United States, prevention and control efforts have been instituted on a per farm basis, and eradication programs have been viewed with skepticism. This skepticism is based on the presence of non-cattle reservoirs and the ease of viral transmission. However, the enormous impact of BVDV on the livestock industry has led some specialty groups such as the Academy of Veterinary Consultants to endorse the formation of national prevention, control, and eventual eradication programs.[1] Implementation of BVDV control programs in Europe has focused on the presence of anti-BVDV antibodies

to determine exposure and prevalence of the virus. These serology-based control programs would not be suitable for cattle herds in North America due to the use of BVDV vaccines.

In conclusion, BVDV is an economically important pathogen that causes significant disease in cattle. Prevention and control programs in North America must rely upon the implementation of strict biosecurity procedures and the use of efficacious BVDV vaccines but only after the accurate identification and removal of PI animals. Although numerous diagnostic assays are now readily available, standardization of methods and validation of testing methods between laboratories are lacking.[34] To efficiently prevent and control BVDV, laboratory results must be consistent allowing for confidence in the culling of PI animals.

III. STATEMENT OF RESEARCH OBJECTIVES

The work presented in this thesis examined methods for effective detection of epidemiologically significant persistent infections (PI) with bovine viral diarrhea virus (BVDV). The first objective was to determine the natural transmission potential of a PI animal that previously lacked detectable virus in serum to transmit virus to susceptible herdmates in a natural pasture environment. The second objective was to use intra- and inter-laboratory comparisons to investigate the diagnostic proficiency of various methods for detecting cattle PI with BVDV.

IV. EPIDEMIOLOGICAL SIGNIFICANCE OF CATTLE PERSISTENTLY INFECTED WITH BOVINE VIRAL DIARRHEA VIRUS THAT PREVIOUSLY LACKED EASILY ISOLATED VIRUS IN SERUM

ABSTRACT

Bovine viral diarrhea virus (BVDV) is one of the most important viral pathogens of cattle.[12] Cows persistently infected (PI) with BVDV are the primary reservoirs and major sources for transmission of this virus to susceptible animals.[12,129] However, some animals PI with BVDV lack easily isolated virus in serum (atypical PI) by virus isolation. These atypical PI animals could be misdiagnosed based on serum-based screening methods. The ability of atypical PI animals to transmit virus to susceptible herdmates in a normal pastural environment has not been evaluated. In this infectivity study, groups of four naive calves were exposed for 28 days to either an animal PI with BVDV (positive control group), a BVDV negative animal (negative control group), or an atypical PI animal (experimental group). Serum and whole blood samples for isolation of BVDV were collected from all animals on days 0, 6, 7, 8, 9, 10, 14, and 28 and for serum neutralization assays on days 0 and 28 (exposure = day 0). All calves in the experimental group became viremic during the exposure period and had seroconverted by the end of

the exposure period. Therefore, the atypical PI animal was capable of transmitting BVDV to susceptible herdmates in a typical pasture environment.

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is a significant pathogen which has been associated with gastrointestinal, respiratory, and reproductive diseases of cattle worldwide.[129] Persistent infection with BVDV occurs when a conceptus is infected with a noncytopathic strain of BVDV prior to the development of a competent fetal immune system (about gestation day 125). Persistently infected (PI) cattle are capable of shedding large quantities of virus throughout their lives and are considered the primary reservoirs for BVDV.

Evidence exists that some PI animals are capable of producing neutralizing antibodies to the persisting or resident strain of BVDV.[30,31,38,113] In general, PI animals have easily isolated quantities of the resident virus in serum following the decline of passively acquired maternal antibodies. Brock et al. discovered a PI cow that was characterized by an absence of readily isolated virus in serum and spontaneous production of virus neutralizing antibody.[38] Although BVDV could not be isolated from the serum, virus was recovered from the buffy coat samples of this "atypical" PI cow.[38] It is likely that the development of BVDV-neutralizing antibodies in this cow interfered with the ability to detect BVDV by virus isolation. The reason for this apparent break in immunotolerance is not fully understood. In one study, a PI cow that lacked isolatable

virus in serum demonstrated the ability to transmit virus to a naive steer when housed in a confined space (196 ft^2) for 14 days.[113] Despite the prolonged inability to isolate BVDV from serum that is usually observed in atypical PI animals, virus had been transiently isolated from the serum of this atypical PI cow for less than 200 days after which virus was again no longer detectable by virus isolation (VI) from serum.[113] Stress due to close confinement may have led to the reappearance of virus in serum from this atypical PI animal.[113] Although the prevalence of PI animals that lack easily isolated virus in their serum is thought to be low, such animals could be missed by serum-based BVDV screening tests and, thus, compromise prevention and control efforts.[113]

Because most PI animals have a very high and persistent viremia and shed BVDV from almost all secretions and excretions throughout their life, it is important to determine if these atypical PI animals are capable of infecting susceptible herdmates in a normal pastural environment. Thus, the objective of this study was to determine the potential for an atypical PI animal to transmit virus to susceptible herdmates in a natural environment.

MATERIALS AND METHODS

Animals. Three groups of animals, each composed of five hybrid beef calves greater than 7 months of age, were used in this study. Calves were randomly assigned to these groups which consisted of four naive bull calves and either a positive control animal (heifer PI with BVDV), a negative control animal (BVDV negative bull), or an

experimental animal (atypical PI). Immunohistochemical staining of skin biopsies (ear notches) and VI from serum and buffy coat samples were performed to confirm persistence of infection with BVDV in both PI animals. The naive bull calves were screened using serum neutralization assays and virus isolation of buffy coat samples and found to be free of anti-BVDV antibodies and virus.

The positive control and experimental groups were maintained on separate pastures at a BVDV-isolation facility. The negative control group was maintained on pasture at a separate but nearby location. Each group of animals was pastured on approximately 2.5 acres that was separated from adjacent cattle by a minimum of 200 ft. Each pasture contained a small holding pen (30 ft x 25 ft) with a chute to restrain animals for sample collection. Animals within each group shared common feed and water troughs with the feed trough located in the small holding pen to ensure regular and adequate contact among animals within the group.

Atypical PI Heifer. A PI heifer that previously lacked easily isolated virus in serum was first identified as being PI with BVDV by IHC staining of an ear notch sample taken at approximately five months of age. Further testing revealed that this heifer was unusual because virus could be isolated on VI by immunoperoxidase monolayer assay (IPMA) from buffy coat samples, but virus was undetectable on virus isolation by IPMA from serum. This heifer has been referred to as atypical because of the lack of detectable virus in serum. Results from RT-nPCR on both serum and buffy coat samples revealed weak positives for BVDV. In addition, serum neutralization (SN) assays for detecting

antibodies to the resident strain of virus (134F) in the atypical PI heifer revealed the presence of serum neutralizing antibodies.

Serum and buffy coat samples were collected monthly for 5 months for virus isolation by IPMA, RT-nPCR, and SN assays. For approximately one year, virus could not be isolated by VI from the serum of this atypical PI (Table 2). The heifer was also producing serum neutralizing antibodies to the resident strain of virus during this time. However, just prior to and during this study, virus could be consistently isolated from both serum and buffy coat samples by IPMA and RT-nPCR. The atypical PI heifer still has low concentrations of serum neutralizing antibodies to the 134F strain of virus, yet virus can still be isolated by IPMA from serum.

Table 1. Results from preliminary tests to detect and confirm bovine viral diarrhea virus (BVDV) from an animal exhibiting a typical persistent infection (PI). Immunohistochemical (IHC) tests performed on skin biopsies. Reverse transcriptase-nested polymerase chain reaction (RT-nPCR) and virus isolation (VI) assays performed on whole blood (wb) and serum. Age in months (mo.).

Typical PI Heifer:

	Test	IHC	VI wb	VI serum	ACE skin	SN
Age						
7		+	+	+		
8			+	+	+	
9			+	+		
12		+				
13		+				
17				+		
20			+	+		

Table 2. Results from preliminary tests to detect bovine viral diarrhea virus (BVDV) from an animal exhibiting an atypical PI. Immunohistochemical (IHC) tests performed on skin biopsies. Reverse transcriptase-nested polymerase chain reaction (RT-nPCR) and virus isolation (VI) assays performed on whole blood (wb) and serum. Age in months (mo.).

Atypical PI Heifer:

	Test	IHC	VI wb	VI serum	ACE skin	SN
Age						
5		+	+	-		**1:8**
6			-	-		**1:8**
7			+	-		**1:4**
8		+	+	+		**1:8**
9			+	-		**1:4**
10			+	-		**1:8**
11		+	+	-		**1:2**
12			+	-		**1:4**
14			+	+		**1:4**
15			+	+		**1:8**
16			+	-		
17			+	+		
18			+	+		
19			+	-		
20			+	+	+	
21			+	+		
24		+				
25		+				
28				+		
29				+		
32			+	+		

Exposure and sample collection. Whole blood (collected in tubes containing ethylenediaminetetraacetic acid [EDTA]) and serum (obtained from clotted blood samples) samples were collected by jugular venipuncture. Clinical scores were assigned at the time of each sample collection.[83] Samples from each group of animals were obtained by separate personnel to ensure that transmission between groups did not occur. Day 0 samples were collected prior to introduction of the naive calves into their randomly assigned groups. For sample collection, animals were restrained in a head catch and squeeze chute. Following sample collection on day 0, each group was allowed to commingle in their individual holding pens for 4 hours to allow the animals to acclimate to their new environment.

Virus detection. In addition to standard virus isolation (VI) by immunoperoxidase monolayer assay (IPMA) on serum, the serum samples also were passaged to maximize the opportunity for viral replication. For cell culture passage, 768 µl of serum was layered over Madin Darby bovine kidney (MDBK) cells growing in the log phase in wells of a 24-well plate (BD Falcon, Franklin Lakes, NJ). The plate was then incubated for 1 hour at 38.5° C in humidified air and 5% CO_2. Following incubation, 3 ml of supplemented minimum essential medium (MEM) was added to each well and the incubation continued for 5 days. After passage, the plates were frozen at -80° C and thawed to release virus. Samples were then aliquoted and stored at -80° C until being assayed for virus. Buffy coat samples were first prepared for VI by IPMA by centrifuging whole blood samples in EDTA at 2,020 rpm for 30 minutes at 4° C. Using a Pasteur pipette (Fisher Scientific International, Inc., Pittsburgh, PA) the buffy coat or white blood

cells were harvested and placed into a sterile 15 ml centrifuge tube (Fisher Scientific International, Inc., Pittsburgh, PA). Then, 10 ml of 0.1M NH_4CL (Sigma-Aldrich Co., St. Louis, MO) was added to the buffy coat. The sample was then mixed well (by vortex) and centrifuged at 2,020 rpm for 10 minutes at 4° C. The supernatant was then poured off of the buffy coat cells, and the pellet of buffy coat cells mixed well. Next, 10 ml of supplemented MEM was added to the cells and mixed well. The cells were once again centrifuged as previously described for 10 minutes and the supernatant removed. The remaining buffy coat pellet was then resuspended in 0.5 ml of supplemented MEM, mixed, and transferred into a sterile cryogenic vial (Sarstedt Inc., Newton, NC) for VI by IPMA.

Both serum and whole blood samples were assayed for BVDV using virus isolation with virus detected by an IPMA developed by Afshar et al.[3] Using a 96-well plate (BD Falcon, Franklin Lakes, NJ), serum and whole blood samples were assayed in triplicate by adding 90 µl of supplemented MEM and 10 µl of sample per well followed by the addition to each well of 50 µl of MEM containing approximately 2.5 x 10^3 MDBK cells. Subsequently, plates were incubated at 38.5° C in a humidified atmosphere of 5% CO_2 and air for 72 hours. The cells were then fixed as follows: Medium was discarded and cells were allowed to air dry for 1 hour at room temperature. Then, 100 µl of fixative (20 ml acetone, 0.02 ml of bovine serum albumin [Sigma Chemicals, St. Louis, MO], 79.98 ml of NaCl [8.5mg/ml]) was added to each well and incubated for 10 minutes. Next, the fixative was discarded, and the plates were again air dried for 2 hours at room temperature prior to the labeling procedure.

The labeling procedure utilized two anti-BVDV monoclonal antibodies, D89, (Veterinary Medical Research and Development, Pullman, WA) specific for E2 (gp53), a major envelope glycoprotein of BVDV, and 20.10.6 (Dubovi, Cornell University, Ithaca, NY) specific for NS3 (p80), a conserved nonstructural protein.[61,205,221,222] Fifty microliters of each antibody (1:500 of D89 and 1:800 of 20.10.6) was added to each well and incubated for 30 minutes at 38.5° C in a humidified atmosphere of 5% CO_2 and air. Excess antibody was removed by washing with Dulbecco's phosphate buffered saline (PBS) containing calcium chloride and magnesium chloride (Gibco-Invitrogen, Grand Island, NY) and Tween 20 (Gibco-Invitrogen, Grand Island, NY). Next, 50 µl of peroxidase-conjugated rabbit anti-mouse IgG (Jackson Immuno Research Lab, West Grove, PA) was added to each well, and plates were again incubated and washed as previously described. Finally, 50 µl of the enzyme substrate, aminoethyl carbazole (AEC Kit, Zymed Laboratories, Inc., San Francisco, CA) was added to each well. Presence of virus resulted in the production of a reddish-brown color when oxidized by horseradish peroxidase. Color change was visualized using a light microscope and compared to known positive and negative controls.

Serum virus neutralization. The serum samples were heat inactivated by incubation at 56° C for 30 minutes. Then, 50 µl of MEM was added to each well of a 96-well plate followed by the addition of 50 µl of heat-inactivated serum to each well in the top row of the plate. From a starting dilution of 1:2, serial two-fold dilutions were made of the test sera using MEM as diluent, and each dilution was assayed in triplicate. Following dilution of the samples, 50 µl of I-23 BVDV virus (Givens, Auburn

University, Auburn, AL) was diluted in MEM such that the inoculum contained 2 cell culture infective doses with a 50% endpoint ($CCID_{50}$) per microliter for a total of 100 $CCID_{50}$. The plates are then incubated for one hour at 38.5° C in a humidified atmosphere of 5% CO_2 and air. Next, 50 µl of MDBK cells were added to each well, and plates were incubated at 38.5° C in a humidified atmosphere of 5% CO_2 and air for 72 hours. After this incubation, the contents of plates were removed and immunoperoxidase staining performed as previously described to detect the presence of noncytopathic BVDV. An endpoint determination for the serum neutralization assay was determined based on the greatest dilution at which at least 1 of 3 wells was free of detectable virus.

Summary of experimental design. The design of the infectivity study is illustrated in Figure 1. The four naive bull calves in the positive control group were exposed to a heifer PI with BVDV, the four naive bull calves in the negative control group were exposed to a BVDV negative bull calf, and the experimental group was exposed to a PI heifer that previously lacked easily isolatable virus in serum. Animals were maintained in their respective groups and exposed to the positive control, the negative control, or the atypical PI for 28 days. Serum and whole blood samples were collected from all animals for BVDV virus isolation on days 0, 6, 7, 8, 9, 10, 14, and 28 (exposure = day 0). Serum antibody titers were determined by serum neutralization assays on serum samples collected on days 0 and 28.

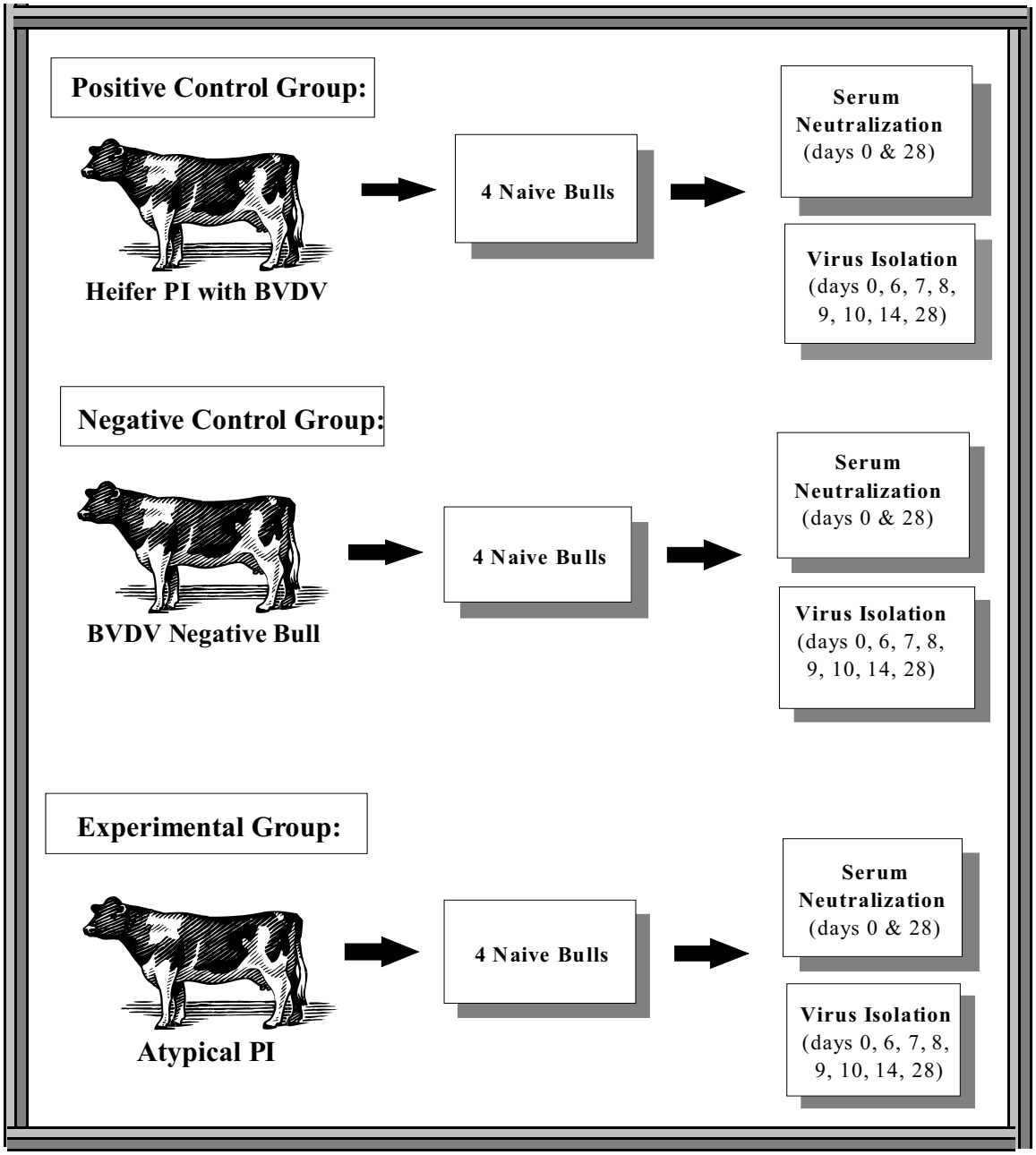

Figure 1. Experimental design of infectivity study.

RESULTS

All naive calves exposed to the BVDV negative control animal were determined to be free of BVDV and anti-BVDV antibodies throughout the study. All calves in the positive control group (typical PI) and the experimental group (atypical PI) became viremic (Table 3) and seroconverted by the end of the 28 day exposure period.

Table 3. Results from virus isolation (VI) assays to detect bovine viral diarrhea virus (BVDV) in whole blood (wb) or serum samples from calves exposed to an animal exhibiting a typical persistent infection (PI) or exposed to an animal exhibiting an atypical PI; positive/total assayed.

Exposure to	Test	Day 0	Day6	Day 7	Day 8	Day 9	Day 10	Day14	Day 28
Typical PI	VI wb	0/4	1/4	2/4	0/4	1/4	0/3*	0/4	0/4
(n=4)	VI serum	0/4	2/4	3/4	2/4	0/4	0/4	0/4	0/4
Atypical PI	VI wb	0/4	0/4	1/4	1/4	1/3*	1/4	0/4	0/4
(n=4)	VI serum	0/4	2/4	4/4	3/4	3/4	0/4	0/4	0/4

* Sample not available from one calf.

DISCUSSION

Studies have shown that virus is not readily isolated from the serum of some animals that are PI with BVDV, yet the virus can be isolated from buffy coat samples.[38] These atypical PI cattle are also capable of producing antibodies in the serum that neutralize the resident strain of virus.[38,113] However, the inability to isolate virus from the serum is thought to be a transient phenomenon in some atypical PI animals.[113] The development of cross-neutralizing serum antibodies likely interferes with the ability to

detect the resident strain of BVDV through virus isolation from serum. The ability of such an atypical PI animal to transmit virus to a susceptible animal in a confined, isolation facility was demonstrated by Grooms et al.[113] However, prior to this study, the ability of an atypical PI animal to transmit virus to susceptible herdmates in a typical pasture environment had not been determined. Therefore, the objective of this study was to determine the ability of such an atypical PI animal to transmit virus to susceptible herdmates in a more spacious environment.

Results from serum neutralization assays and VI from serum and buffy coat samples confirm that the PI animal that previously lacked easily isolated virus in serum is capable of transmitting virus to susceptible herdmates in a typical pasture environment. Transmission of virus from a PI animal that previously lacked easily isolated virus in serum appears to be similar to that reported for most animals PI with BVDV including those PI animals that lack easily isolated virus in serum.[113,129] Immunohistochemical tests and ACE performed on skin biopsy samples and PCR performed on serum (Table 2) are capable of accurately identifying a PI animal that previously lacked easily isolated virus in serum. Therefore, IHC and ACE performed on skin biopsies and PCR on serum would be acceptable assays to detect PI animals in this situation. However, an animal PI with BVDV that lacks detectable virus in serum by virus isolation could be misdiagnosed using routine, serum-based screening protocols, such as IPMA.[113] Virus may be neutralized in specimens from animals that have circulating neutralizing antibodies (humoral immune response or passive transfer) which may prevent the isolation of virus. Maintenance of an atypical PI animal in a herd can lead to further transmission of the

virus to susceptible herdmates. Because BVDV is associated with mononuclear cells, the best sample for isolation of BVDV is whole blood from which the buffy coat or mononuclear cells can be extracted and used as the inoculum.

In summary, an animal PI with BVDV that previously lacked detectable virus in serum is capable of transmitting virus to susceptible herdmates in a typical pasture environment. Because these atypical PI animals are epidemiologically significant to the transmission of BVDV, buffy coat samples should be recommended as the sample of choice for the detection of BVDV for virus isolation from blood.

V. COMPARISON OF ASSAYS FOR DETECTION OF CATTLE PERSISTENTLY INFECTED WITH BOVINE VIRAL DIARRHEA VIRUS

ABSTRACT

Persistently infected (PI) cattle are the natural reservoir of bovine viral diarrhea virus (BVDV). Thus, prevention and control of this virus requires that these animals are accurately identified and removed. Currently, a variety of tests are used for this purpose. These tests include immunohistochemical tests (IHC), antigen capture ELISA (ACE), virus isolation (VI), and reverse transcriptase-polymerase chain reaction (RT-PCR). However, a lack of standardization of methods could compromise the ability to consistently detect PI animals. This study evaluated the diagnostic proficiency of current methods for detecting PI cattle using intra- and inter-laboratory comparisons. Samples were collected on the same day from four animals greater than 7 months of age (two BVDV negative animals, a PI animal, and a PI animal that previously lacked detectable virus in serum as determined by VI). Samples (triplicate samples from PI animals and duplicate samples from negative animals) were submitted blindly to 23 participating diagnostic laboratories utilizing the respective laboratory's standard submission protocol. Samples collected for submission included 1) serum for ACE, RT-PCR, and VI, 2) whole

blood for RT-PCR and VI, and 3) skin biopsies for ACE and IHC. The ACE performed on skin provided the highest diagnostic sensitivity and perfect level of agreement among laboratories. Virus isolation performed on serum yielded the lowest sensitivity and level of agreement. The level of agreement between laboratories for detecting animals PI with BVDV ranged from perfect to less than expected by random chance. The variations between laboratories clearly demonstrate the need for standardization of assays used to detect BVDV.

INTRODUCTION

Bovine viral diarrhea virus (BVDV) is a significant pathogen that is associated with gastrointestinal, respiratory, and reproductive diseases of cattle worldwide.[129] Persistent infection with BVDV occurs when a conceptus is infected with a noncytopathic strain of BVDV prior to the development of a competent fetal immune system (about gestation day 125). Persistently infected (PI) cattle are capable of shedding large quantities of virus throughout their lives and are considered the primary reservoirs for BVDV. Evidence exists that some PI animals are capable of producing antibodies which neutralize the persisting or resident strain of BVDV.[30,31,38,113] Brock et al. discovered a PI cow with an absence of detectable virus in serum and spontaneous production of virus neutralizing antibody.[38] Although BVDV could not be detected in the serum, virus was isolated from the buffy coat samples of this “atypical” PI cow.[38]

Essential to preventing the spread of BVDV is identification and removal of PI animals that serve as the natural reservoirs. Currently, a wide variety of tests are used for this purpose. These tests include virus isolation (VI) in cell culture, reverse transcription-polymerase chain reaction (RT-PCR), antigen capture enzyme linked immunosorbent assay (ACE), and immunohistochemical (IHC) tests. Isolation of BVDV in cell culture followed by identification of the viral isolate by immunofluorescence or immunoperoxidase monolayer assay (IPMA) is one of the most reliable diagnostic techniques. This assay is commonly referred to as the "gold standard" for the detection of BVDV.[106] Pooling of serum and milk samples for the highly sensitive RT-PCR, which detects viral RNA, is becoming a popular screening method for detection of PI cattle. The ACE, a relatively new assay available as a commercial test kit (IDEXX Laboratories, Inc., Westbrook, ME), uses monoclonal antibodies to capture viral antigen (E^{rns}) and detects antigen-antibody complexes with enzyme-conjugated antibody by spectrophotometer.[106] Because of reduced costs and ease of sample collection, IHC staining of formalin-fixed, paraffin-embedded skin biopsies is widely used for the detection of PI animals.[43] This immunohistochemical staining utilizes the 15C5 monoclonal antibody which reacts with the E^{rns} (gp48) protein of BVDV for the detection of diverse isolates of the virus.[43] These tests have the high levels of sensitivity and specificity that are considered adequate for use in screening programs aimed at identifying PI animals.[63,140,181]

Although many tests are available for the detection of cattle PI with BVDV, failure to validate and standardize these tests could lead to variable results between

diagnostic laboratories and compromise our overall ability to accurately identify PI cattle. The purpose of this study was to investigate the diagnostic proficiency of various methods for detecting PI cattle using intra- and inter-laboratory comparisons.

MATERIALS AND METHODS

Animals. This study was approved by the Auburn University Institutional Animal Care and Use Committee (AUIACUC No. 2004-0746). Samples were collected from four hybrid beef calves greater than 7 months of age. Two animals were negative for BVDV, one was PI with BVDV, and one was a PI animal that previously lacked easily isolatable virus in serum by VI. Immunohistochemical staining of multiple skin biopsies (ear notches) and VI of multiple buffy coat samples were initially performed to confirm that both the typical and atypical PI animals were PI with BVDV (Tables 1 and 2). The two BVDV negative calves were confirmed to be free of virus and anti-BVDV antibodies screened using VI on buffy coat and serum neutralization assays. Persistently infected calves were maintained and samples collected at a BVDV isolation facility. Calves negative for BVDV were maintained and samples colleted at a location separate from the BVDV-isolation facility.

Table 1. Results from preliminary tests to detect bovine viral diarrhea virus (BVDV) from an animal exhibiting a typical persistent infection (PI) and an animal exhibiting an atypical PI. Immunohistochemical (IHC) tests performed on skin biopsies. Reverse transcriptase-nested polymerase chain reaction (RT-nPCR) and virus isolation (VI) assays performed on whole blood (wb) and serum. Age in months (mo.).

Typical PI Heifer

	Test	IHC	VI wb	VI serum	ACE skin	SN
Age						
7		+	+	+		
8			+	+	+	
9			+	+		
12		+				
13		+				
17				+		
20			+	+		

Table 2. Results from preliminary tests to detect bovine viral diarrhea virus (BVDV) from an animal exhibiting an atypical PI. Immunohistochemical (IHC) tests performed on skin biopsies. Reverse transcriptase-nested polymerase chain reaction (RT-nPCR) and virus isolation (VI) assays performed on whole blood (wb) and serum. Age in months (mo.).

Atypical PI Heifer

	Test	IHC	VI wb	VI serum	ACE skin	SN
Age						
5		+	+	-		1:8
6			-	-		1:8
7			+	-		1:4
8		+	+	+		1:8
9			+	-		1:4
10			+	-		1:8
11		+	+	-		1:2
12			+	-		1:4
14			+	+		1:4
15			+	+		1:8
16			+	-		
17			+	+		
18			+	+		
19			+	-		
20			+	+	+	
21			+	+		
24		+				
25		+				
28				+		
29				+		
32			+	+		

Atypical PI Heifer. A PI heifer that previously lacked easily isolated virus in serum by VI was first identified as being PI with BVDV by IHC staining of an ear notch sample taken at approximately five months of age. Further testing revealed that this heifer was unusual because virus could be isolated on virus isolation by IPMA from buffy coat samples, but virus was undetectable on virus isolation by IPMA from serum. This heifer has been referred to as atypical because of the lack of easily isolated virus in serum. Results from RT-nPCR on both serum and buffy coat samples revealed weak positives for BVDV. In addition, serum neutralization (SN) assays for detecting antibodies to the resident strain of virus (134F) in the atypical PI heifer revealed the presence of serum neutralizing antibodies.

Sample collection. Calves were restrained in a squeeze chute and head gate for sample collection. Animals were administered butorphanol (0.1mg/kg) intramuscularly in the neck for sedation and sulfadimethoxine sustained release antibiotic boluses (137.5mg/kg) orally for prevention of secondary infections following collection of skin biopsies. Whole blood (collected in EDTA) and serum (collected in 25 ml microvette syringe tubes [Sarstedt Inc., Newton, NC]) were collected by jugular venipuncture. Following blood collection, skin biopsies were harvested from the left and right paralumbar areas of each calf. The paralumbar area was aseptically prepared and local anesthesia provided by performing an inverted-L block with 25cc of 2% lidocaine (Hospira, Inc., Lake Forest, IL). A 10 mm sterile punch biopsy instrument (Acuderm Inc., Ft. Lauderdale, FL) was then used to collect skin biopsy samples. Each biopsy site was then closed with either #1 chromic gut suture (Ethicon, Inc., Somerville, NJ) or #1

supramid suture (S. Jackson, Inc., Alexandria, VA) in a simple interrupted pattern. Skin biopsies were placed into additive-free glass tubes that contained either 5 ml of 10% neutral buffered formalin (Fisher Scientific International, Inc., Pittsburgh, PA) for IHC, 5 ml of Dulbeccos's phosphate-buffered saline (PBS [Gibco-Invitrogen, Grand Island, NY]) for ACE , or no buffer for ACE according to laboratory preferences.

Triplicate samples from animals PI with BVDV and duplicate samples from animals negative for BVDV were collected, processed, and shipped to participating laboratories within 10 hours. A total of 562 ml of blood and 50 skin biopsies were collected from the BVDV negative calves. A total of 843 ml of blood and 75 skin biopsies were collected from each animal PI with BVDV. Samples collected for submission included 1) serum for ACE, RT-PCR, and VI, 2) whole blood for RT-PCR and VI, and 3) skin biopsies for ACE and IHC.

Sample preparation and submission. Once samples were collected, they were placed in a cooler and taken to the laboratory for processing ($\leq$2 hours). Each sample was randomly assigned an identification number based on the type of sample (serum, whole blood, or skin biopsy) and the type of animal (calves negative for BVDV, animal PI with BVDV, and atypical PI animal) in order to blind laboratories to the identification of the samples. Following collection, samples were prepared for submission to each of the 23 participating laboratories. Clotted blood samples were centrifuged at 15,000 rpm for 30 minutes, and serum was aliquoted into 4 ml cryogenic tubes (Corning, Inc., Corning, NY) according to volume requirements for each laboratory. Prior to shipment, serum and whole blood samples were stored at 4° C while skin biopsies were held at

room temperature. Samples were placed into specimen shippers (Saf-T-Pak, Inc., # STP 100, Edmonton, AB, Canada), with or without ice packs, and shipped by overnight mail. Blind samples were packaged and submitted according to each laboratory's standard submission protocol.

Diagnostic laboratories. All laboratories voluntarily participated in the study and were informed of the nature of the research prior to the initiation of the study. Each of the 23 participating laboratories was asked to perform all tests offered for the detection of cattle PI with BVDV.

Summary of experimental design. Blinded samples were collected on the same day from two animals PI with BVDV and two animals negative for BVDV. Samples were then submitted to diagnostic laboratories according to each laboratory's preference. A total of 10 samples, 6 samples from 2 animals PI with BVDV and 4 samples from 2 BVDV negative animals, were submitted for each test performed.

Statistical analysis. Sensitivity, specificity, positive predictive value, and negative predictive value for detecting cattle PI with BVDV were calculated for each of the following diagnostic tests: IHC on skin, ACE on serum, ACE on skin, VI on serum, VI on whole blood, PCR on serum, and PCR on whole blood. The kappa value (κ) was used to determine the level of agreement between any two laboratories for each diagnostic test. A negative κ indicates that the agreement between laboratories is less than expected by random chance, a κ of 0 indicates that the agreement is the same as chance, $\kappa \leq 0.20$ = poor agreement, $0.21 \leq \kappa \geq 0.40$ = fair agreement, $0.41 \leq \kappa \geq 0.60$ = moderate agreement, $0.61 \leq \kappa \geq 0.80$ = substantial agreement, $\kappa \geq 0.80$ = good agreement, and a κ

equal to one indicates perfect agreement. Chi-square statistical comparison was performed to evaluate the number of samples from the typical and atypical PI animals identified positive by each prospective test.

RESULTS

Only 43.5% (10/23) of laboratories accurately identified all blind samples submitted for detection of PI cattle. Analysis of 890 test results for samples submitted to 23 participating diagnostic laboratories is shown in Tables 3 and 4. Chi-square statistical analysis revealed no significant difference in the sensitivity between the typical and atypical PI animals for each assay.

Table 3. Sensitivity (Sn) and specificity (Sp) results for laboratories (A through W) and tests used to detect cattle PI with BVDV. Immunohistochemical (IHC) tests performed on skin biopsies. Reverse transcriptase-nested polymerase chain reaction (RT-nPCR) and virus isolation (VI) assays performed on whole blood (wb) and serum. * denotes statistics for two different VI assays performed by the same laboratory.

Lab	IHC		ACE serum		ACE skin		VI serum		VI wb		PCR serum		PCR wb		Total	
	Sn	Sp	Sn	Sp	Sn	Sp	Sn	Sp	Sn	Sp	Sn	Sp	Sn	Sp	Sn	Sp
A	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100	100
B	100	100	100	100	100	100	100	100			100	100			100	100
C			100	100	100	100	100	100	100	100					100	100
D									100	100			100	100	100	100
E	100	100	100	100											100	100
F							100	100			100	100			100	100
G			100	100	100	100									100	100
H			100	100	100	100									100	100
I	100	100													100	100
J	100	100													100	100
K	83	100	100	100			100	100	100	100					96	100
L	100	100	100	50			100	100	100	100	100	100	100	100	100	92
M	100	100			100	100			75*	100*			83	100	90	100
N			100	100	100	100	83	100	100	100	100	50	100	100	97	92
O	83	75							100	100	100	100	83	100	92	94
P	100	100	100	100					100	100	100	50	67	100	93	90
Q			67	100			100	100	100*	88*					92	94
R	100	100	100	100			17	100	100	100					79	100
S	100	100					67	100	67	100					78	100
T											50	100	100	100	75	100
U	100	100	100	100	100	100	42*	88*	58*	88*					71	93
V			100	100	100	100	17	100	67	100					71	100
W	0	100	0	100	100	100	0	100	83	100	17	100	100	100	43	100
Total	90	98	91	97	100	100	69	98	88	97	85	89	93	100	87	97
Total Minus Lab W	97	93	98	96	100	100	74	93	88	97	94	88	92	100	91	95

Table 4. Analysis of 890 test results for research samples submitted to 23 participating diagnostic laboratories. wb = whole blood; PPV = positive predictive value; NPV = negative predictive value. †Kappa value (κ) $\leq$ 0.20 = poor agreement, 0.21 $\leq \kappa \geq$ 0.40 = fair agreement, 0.41 $\leq \kappa \geq$ 0.60 = moderate agreement, 0.61 $\leq \kappa \geq$ 0.80 = substantial agreement, $\kappa \geq$ 0.80 = good agreement, κ = 0 indicates that the agreement is the same as chance, κ = 1.00 indicates perfect agreement, and a negative κ indicates that the agreement between laboratories is less than expected by random chance.

	Number of Labs Performing Test/ Total Labs	Diagnostic Sensitivity (%)	Diagnostic Specificity (%)	PPV (%)	NPV (%)	Range of Kappa Values †
IHC (skin)	14/23 (60.8%)	76/84 (90%)	55/56 (98%)	84/85 (98%)	56/64 (87%)	-0.2 to 1.0
ACE (serum)	15/23 (65.2%)	82/90 (91%)	58/60 (97%)	90/92 (97%)	60/68 (88%)	-0.2 to 1.0
ACE (skin)	10/23 (43.5%)	60/60 (100%)	40/40 (100%)	60/60 (100%)	40/40 (100%)	1.0
VI (serum)	13/23 (56.5%)	58/84 (69%)	55/56 (98%)	84/85 (98%)	56/82 (68%)	-0.2 to 1.0
VI (wb)	15/23 (65.2%)	95/108 (88%)	70/72 (97%)	108/110 (98%)	72/85 (84%)	-0.2 to 1.0
PCR (serum)	9/23 (39.1%)	46/54 (85%)	32/36 (88%)	54/56 (96%)	36/44 (81%)	0.1 to 1.0
PCR (wb)	9/23 (39.1%)	50/54 (93%)	36/36 (100%)	54/54 (100%)	36/40 (90%)	0.4 to 1.0

DISCUSSION

Many tests are currently available for the accurate detection of animals PI with BVDV. These tests include IHC, ACE, VI, and RT-PCR. However, before this study, the diagnostic proficiency of tests used to detect PI cattle had not been compared using intra- and inter-laboratory assessments. Thus, the objective of this study was to compare the proficiency of IHC, ACE, VI, and RT-PCR at detecting cattle PI with BVDV.

When comparing the results from the atypical and the typical PI animals for each assay, no significant difference in the sensitivity was noted between animals. Therefore, the differences in PI animals did not affect the sensitivity of the assays. Analysis of 890 test results for whole blood, serum, and skin biopsy samples submitted to 23 diagnostic laboratories revealed that the ACE performed on skin biopsy samples was the most repeatable test for detecting cattle PI with BVDV in this study. The ACE performed on skin biopsy samples demonstrated the highest diagnostic sensitivity and level of agreement between laboratories, thus, supporting previous studies that indicated that ACE on skin biopsies was an accurate test for the detection of PI cattle.[63,181] However, VI performed on serum was the least repeatable at detecting PI animals and yielded the lowest sensitivity and level of agreement. These results were unexpected because virus isolation in cell culture has long been considered the most reliable method for the detection of PI cattle and still remains the "gold standard" diagnostic technique.[77,182] These results were contrary to previous studies demonstrating close agreement between IHC and VI. In one of these studies, specimens collected from 15 PI animals demonstrated complete agreement between IHC and VI while only 13 samples were positive by antigen-ELISA.[199] Of the 104 cattle that tested negative by parallel tests, there was complete agreement among the tests which indicates 100% sensitivity when comparing IHC to VI.[199] In another study, 97.5% agreement was reported between IHC on skin biopsies and VI on peripheral blood leukocytes, and all 45 animals that tested negative by VI were also negative by IHC.[162] The discrepancy between our comparisons of IHC, VI, and ACE and results from previous studies may be due to problems regarding

the validation of VI assays for BVDV. In previous studies, VI was validated in order to make positive comparisons between and among samples.[77,162,182,199] Some laboratories participating in this study may not routinely perform VI for the detection of BVDV. Therefore, these laboratories may not routinely validate their assays for VI of BVDV as compared to laboratories in the previous studies where there was strong correlation between IHC, VI, and ACE.[77,162,182,199]

Because there are many diagnostic tests available for the detection of cattle PI with BVDV, it is important to remember that each method has its advantages, disadvantages, and applicability. The ability of different laboratories to accurately detect BVDV by VI may be influenced by susceptibility of cell cultures to infection, the cell culture medium, the serum used to supplement media, the testing procedure, immunological labeling, laboratory contamination of samples, and frequency of assay performance. This study indicates considerable variation among laboratories and tests available for the detection of PI animals.

In summary, the comparison of diagnostic tests for the detection of cattle PI with BVDV revealed considerable variation among tests and between laboratories. This variation between laboratories compromises our overall ability to accurately detect PI cattle thus undermining efforts to prevent or control infections with BVDV. Thus there is a clear need for standardization and validation of testing methods used by different laboratories for the detection of cattle PI with BVDV.

VI. GENERAL SUMMARY AND CONCLUSIONS

Bovine viral diarrhea virus may not be detected in the serum of some adult PI cattle even when it is present in their white blood cells.[38] Virus may not be detected from the serum of these animals due to their ability to produce serum neutralizing antibodies.[113] It is important to determine if animals PI with BVDV that lack easily isolated virus in their serum are also capable of transmitting virus to susceptible herdmates. Control and eradication of BVDV requires that PI animals are accurately identified and removed. The objectives of this research were to determine the epidemiologic importance of cattle PI with BVDV that lack easily isolated virus in their serum and determine the proficiency of various diagnostic methods for detecting PI cattle using intra- and inter-laboratory comparisons.

Results (chapter IV) from serum neutralization and VI from serum and buffy coat samples confirmed that the animal PI with BVDV that previously lacked easily isolated virus in serum is capable of transmitting virus to susceptible herdmates in a typical pasture environment. Because these atypical PI animals are epidemiologically significant to the transmission of BVDV, buffy coat samples should be recommended as the sample of choice for the detection of BVDV from blood.

Results described in chapter V for the comparison of diagnostic tests for the detection of cattle PI with BVDV revealed considerable variation among tests and laboratories. The ACE performed on skin biopsy samples was the most repeatable test for detecting cattle PI with BVDV in this study. Antigen capture ELISA performed on skin biopsies demonstrated the highest diagnostic sensitivity and level of agreement between laboratories. However, VI on serum samples was the least repeatable at detecting PI animals and yielded the lowest sensitivity and level of agreement. This variation between laboratories compromises the ability to accurately detect PI cattle thus undermining efforts to prevent and control infections with BVDV. These findings demonstrate the need for standardization and validation of tests for detection of cattle PI with BVDV.

VII. CUMULATIVE BIBLIOGRAPHY

1. Academy of Veterinary Consultants, Ames, Iowa, United States Department of Agriculture: 2002, Position statement on bovine viral diarrhea virus.

2. Adler H, Frech B, Meier P et al: 1994, Noncytopathic strains of bovine viral diarrhea virus prime bovine bone marrow-derived macrophages for enhanced generation of nitric oxide. Biochem Biophys Res Commun 202:1562-1568.

3. Afshar A, Dulac GC, Dubuc C, Howard TH: 1991, Comparative evaluation of the fluorescent antibody test and microtiter immunoperoxidase assay for detection of bovine viral diarrhea virus from bull semen. Can J Vet Res 55:91-93.

4. Agnello V, Abel G , Elfahal M et al: 1999, Hepatitis C virus and other flaviviridae viruses enter cells via low density lipoprotein receptor. Proc Natl Acad Sci U S A 96:12766-12771.

5. Alves D, McEwen B, Hazlett M et al: 1996, Trends in bovine abortions submitted to the Ontario Ministry of Agriculture, Food and Rural Affairs, 1993-1995.Can Vet J 37:287-288.

6. Ames TR: 1986, The causitive agent of BVD: Its epidemiology and pathogenesis.Vet Med 81:848-869.

7. Archbald LH, Fulton R, Seger C et al: 1979, Effect of the bovine viral diareha virus on preimplantation embryos: a preliminary study. Theriogenology 11:81-89.

8. Archbald LH, Gibson CD, Schultz RH et al: 1973, Effects of intrauterine inoculation of bovine viral diarrhea-mucosal disease virus on uterine tubes and uterus of nonpregnant cows. Am J Vet Res 34(9):1133-1137.

9. Avalos-Ramirez R, Orlich M, Thiel HJ, Becher P: 2001, Evidence for the presence of two novel pestivirus species. Virology 286(2):456-465.

10. Bak A, Callesen A, Meyling A, Greve T: 1992, Calves born after embryo transfer from donors persistently infected with BVD virus. Vet Rec 131:37.

11. Baker JA, York CJ, Gillespie JH, Mitchell GB: 1954, Virus diarrhea in cattle. Am J Vet Res 15:525-531.

12. Baker JC: 1987, Bovine viral diarrhea virus: a review. J Am Vet Med Assoc 190:1449-1458.

13. Baker JC: 1995, The clinical manifestations of bovine viral diarrhea infection. Vet Clin North Am Food Anim Pract 11(3):425-445.

14. Barber DM, Nettleton PF, Herring JA: 1985, Disease in a dairy herd associated with the introduction and spread of bovine virus diarrhoea virus. Vet Rec 117:459-464.

15. Baroth M, Orlich M , Thiel HJ, Becher P: 2000, Insertion of cellular NEDD8 coding sequences in a pestivirus. Virology 278(2):456-466.

16. Baszler TV: 1995, Diagnosis of naturally occurring bovine viral diarrhea virus infections in ruminants using monoclonal antibody-based immunohistochemistry. Vet Pathol 32:609-618.

17. Becher P, Orlich M, Kosmidou A et al: 1999, Genetic diversity of pestiviruses: identification of novel groups and implications for classification. Virology 262:64-71.

18. Behrens SE, Grassmann CW, Thiel HJ et al: 1998, Characterization of an autonomous subgenomic pestivirus RNA replicon. J Virol 72:2364-2372.

19. Bezek DM, Grohn YT, Dubovi EJ: 1999, Effect of acute infection with noncytopathic or cytopathic bovine viral diarrhea virus isolates on bovine platelets. Am J Vet Res 55(8):1115-1119.

20. Bielanski A, Hare WCD: 1988, Effect in vitro of bovine viral diarrhea virus on bovine embryos with the zona pellucida intact, damaged and removed. Veterinary Research Communications 12:19-24.

21. Bielanski A, Loewen KS, Del Campo MR et al: 1993, Isolation of bovine herpesvirus-1 (BHV-1) and bovine viral diarrhea virus (BVDV) in association with the in vitro production of bovine embryos. Theriogenology 40:531-538.

22. Bielefeldt-Ohmann H: 1984, An ocular-cerebellar syndrome caused by congenital viral diarrhea virus-infection.Acta Vet Scand 25:36-49.

23. Bolin SR: 1995, Control of Bovine Viral Diarrhea Infection by Use of Vaccination. Vet Clin North Am Food Anim Pract 11(3):615-625.

24. Bolin SR: 1995, The Pathogenesis of Mucosal Disease. Vet Clin North Am Food Anim Pract 11(3):489-500.

25. Bolin SR, Grooms DL: 2004, Origination and consequences of bovine viral diarrhea virus diversity. Vet Clin North Am Food Anim Pract 20(1):51-68.

26. Bolin SR, Littledike ET, Ridpath JF: 1991, Serologic detection and practical consequences of antigenic diversity among bovine viral diarrhea viruses in a vaccinated herd. Am J Vet Res 52(7):1033-1037.

27. Bolin SR, Ridpath JF: 1992, Differences in virulence between two noncytopathic bovine viral diarrhea viruses in calves. Am J Vet Res 53:2157-2163.

28. Bolin SR, Ridpath JF: 1998, Prevalence of bovine viral diarrhea virus genotypes and antibody against those viral genotypes in fetal bovine serum .J Vet Diagn Invest 10(2):135-139.

29. Bolin SR, Ridpath JF, Black J et al: 1994, Survey of cell lines in the American Type Culture Collection for bovine viral diarrhea virus. J Virol Methods 48:211-221.

30. Bonds MD, Larson LJ, Strause T et al: 1998, Bovine viral diarrhea virus (BVDV) persistently infected (PI) cattle with false negative tests for virus. Proceedings from the 79th Annual Conference of Research Workers in Animal Diseases:75.

31. Bonds MD, Larson LJ, Schultz RD: 2000, Three animals persistently infected with BVDV that have become virus negative. Proceedings from the 81st Annual Meeting of the Conference of Research Workers in Animal Diseases:76.

32. Brar JS, Johnson DW, Muscoplat CC et al: 1978, Maternal immunity to infectious bovine rhinotracheitis and bovine viral diarrhea viruses: duration and effect on vaccination in young calves. Am J Vet Res 39(2):241-244.

33. Brock KV: 1995, Diagnosis of bovine viral diarrhea virus infections. Vet Clin North Am Food Anim Pract 11(3):549-561.

34. Brock KV: 2004, Strategies for the control and prevention of bovine viral diarrhea virus.Vet Clin North Am Food Anim Pract 20(1):171-180.

35. Brock KV, Chase CC: 2000, Development of a fetal challenge method for the evaluation of bovine viral diarrhea virus vaccines.Vet Microbiol 77(1-2):209-214.

36. Brock KV, Cortese V: 2001, Experimental fetal challenge using type II bovine viral diarrhea virus in cattle vaccinated with modified-live virus vaccine. Vet Ther 2:354-360.

37. Brock KV, Deng R, Riblet SM: 1992, Nucleotide sequencing of 5' and 3' termini of bovine viral diarrhea virus by RNA ligation and PCR. J Virol Methods 38:39-46.

38. Brock KV, Grooms DL, Ridpath JF, Bolin SR: 1998, Changes in levels of viremia in cattle persistently infected with bovine viral diarrhea virus. J Vet Diagn Invest 10:22-26.

39. Brock KV, Lapin DR, Skrade DR: 1997, Embryo transfer from donor cattle persistently infected with bovine viral diarrhea virus. Theriogenology 47:837-844.

40. Brock KV, Redman DR, Vickers ML, Irvine NE: 1991, Quantitation of bovine viral diarrhea virus in embryo transfer flush fluids from a persistently infected heifer. J Vet Diagn Invest 3:99-100.

41. Brock KV, Ridpath JF, Deng R: 1993, Comparative hybridization and nucleotide sequence information from two noncytopathic isolates of bovine viral diarrhea virus. Vet Microbiol 36:69-82.

42. Brock KV, Stringfellow DA: 1993, Comparative effects of cytopathic and noncytopathic bovine viral diarrhea virus on bovine blastocysts. Theriogenology 39:196.

43. Brodersen BW: 2004, Immunohistochemistry used as a screening method for persistent bovine diarrhea virus infection. Vet Clin North Am Food Anim Pract 20(1):85-94.

44. Brown EA, Zhang H, Ping LH, Lemon SM: 1992, Secondary structure of the 5' nontranslated regions of hepatitis C virus and pestivirus genomic RNAs. Nucleic Acids Res 20:5041-5045.

45. Brown TT, De Lahunte A, Bistner SI et al: 1974, Pathogenetic studies of infection of the bovine fetus with bovine viral diarrhea virus. Vet Pathol 11:486-505.

46. Brown TT, De Lahunte A, Scott FW et al: 1973, Virus induced congenital anomalies of the bovine fetus. Cornell Vet 63:561-578.

47. Brownlie J: 1990, The pathogenesis of bovine virus diarrhoea virus infections. Rev Sci Tech 9:43-59.

48. Brownlie J, Clarke MC: 1993, Experimental and spontaneous mucosal disease of cattle: a validation of Koch's postulates in the definition of pathogenesis. Intervirology 35:51-59.

49. Bruschke CJ, Hulst MM, Moormann RJ et al: 1997, Glycoprotein Erns of pestiviruses induces apoptosis in lymphocytes of several species. J Virol 71:6692-6696.

50. BuBois WR, Cooper VL, Ball RL, Starr Jr. BD: 2000, A preliminary evaluation of the effect of vaccination with modified live bovine viral diarrhea virus (BVDV) on detection of BVDV antigen in skin biopsies using immunohistochemical methods. Bovine Practitioner 34:98-100.

51. Buttner M, Oehmig A, Weiland F et al: 1997, Detection of virus or virus specific nucleic acid in foodstuff or bioproducts-hazards and risk assessment. Arch Virol Suppl 13:57-66.

52. Campbell JR: 2004, Effect of bovine viral diarrhea virus in the feedlot. Vet Clin North Am Food Anim Pract 20(1):39-50.

53. Carman S, van Dreumel T, Ridpath JF et al: 1998, Severe acute bovine viral diarrhea in Ontario, 1993-1995. J Vet Diagn Invest 10:27-35.

54. Carruthers TD, Petrie L: 1996, A survey of vaccination practices against bovine viral diarrhea (BVD) virus in Saskatchewan dairy herds. Can Vet J 37:621-622.

55. Casaro APE, Kendrick JW, Kennedy PC: 1971, Response of the bovine fetus to bovine viral diarrhea-mucosal disease virus. Am J Vet Res 32(10):1543-1562.

56. Castrucci G, Frigeri F, Osburn BI et al: 1990, A study of some pathogenetic aspects of bovine viral diarrhea virus infection. Comp Immunol Microbiol Infect Dis 13:41-49.

57. Chi J, VanLeeuwen J, Weersink A, Keefe G: 2002, Direct production losses and treatment costs from bovine viral diarrhoea virus, bovine leukosis virus, Mycobacterium avium subspecies paratuberculosis, and Neospora caninum. Prev Vet Med 55(2):137.

58. Collett MS, Larson R, Belzer SK, Retzel E: 1988, Proteins encoded by bovine viral diarrhea virus: the genomic organization of a pestivirus. Virology 165:200-208.

59. Collins ME, Desport M, Brownlie J: 1999, Bovine viral diarrhea virus quasispecies during persistent infection. Virology 259:85-98.

60. Cook LG, Littlejohns IR, Jessep TM: 1990, Induced sero-conversion in heifers with a field strain of bovine pestivirus--a comparison of methods and doses. Aust Vet J 67:393-395.

61. Corapi WV, Elliott RD, French TW et al: 1990, Thrombocytopenia and hemorrhages in veal calves infected with bovine viral diarrhea virus. J Am Vet Med Assoc 196(4):590-596.

62. Corapi WV, French TW, Dubovi EJ: 1999, Severe thrombocytopenia in young calves experimentally infected with noncytopathic bovine viral diarrhea virus. J Virol 63:3934-3943.

63. Cornish TE, van Olphen AL, Cavender JL et al: 2005, Comparison of ear notch immunohistochemistry, ear notch antigen-capture ELISA, and buffy coat virus isolation for detction of calves persistently infected with bovine viral diarrhea virus. J Vet Diagn Invest 17:110-117.

64. Cortese VS, Grooms DL, Ellis J et al: 1998, Protection of pregnant cattle and their fetuses against infection with bovine viral diarrhea virustype 1 by use of a modified-live virus vaccine. Am J Vet Res 59:1413.

65. Dammann O, Leviton A: 1998, Is some white matter damage in preterm neonates induced by a human pestivirus? Arch Dis Child Fetal Neonatal Ed 78:F230-F231.

66. Dean HJ, Leyh R: 1999, Cross-protective efficacy of a bovine viral diarrhea virus (BVDV) type 1 vaccine against BVDV type 2 challenge. Vaccine 17:1117-1124.

67. Deng R, Brock KV: 1992, Molecular cloning and nucleotide sequence of a pestivirus genome, noncytopathic bovine viral diarrhea virus strain SD-1. Virology 191:867-879.

68. Deng R, Brock KV: 1993, 5' and 3' untranslated regions of pestivirus genome: Primary and secondary structure analyses. Nucleic Acids Res 21:1949-1957.

69. Depner K, Bauer TH, Liess B: 1992, Thermal and pH stability of pestiviruses. Rev Sci Tech Off Int Epiz 11(3):885-893.

70. Desport M, Collins ME, Brownlie J: 1994, Detection of bovine virus diarrhoea virus RNA by in situ hybridization with digoxigenin-labelled riboprobes. Intervirology 37:269-276.

71. Done JT, Terlecki S, Richardson C et al: 1980, Bovine virus diarrhoea-mucosal disease virus: pathogenicity for the fetal calf following maternal infection. Vet Rec 106:473-479.

72. Donis RO: 1995, Molecular biology of bovine viral diarrhea virus and its interaction with the host. Vet Clin North Am Food Anim Pract 11(3):393-423.

73. Donis RO, Dubovi EJ: 1987, Characterization of bovine viral diarrhoea-mucosal disease virus- specific proteins in bovine cells. J Gen Virol 68:1597-1605.

74. Donis RO, Dubovi EJ: 1987, Molecular specificity of the antibody responses of cattle naturally and experimentally infected with cytopathic and noncytopathic bovine viral diarrhea virus biotypes. Am J Vet Res 48:1549-1554.

75. Drew TW, Yapp F, Paton DJ: 1999, The detection of bovine viral diarrhoea virus in bulk milk samples by the use of a single-tube RT-PCR. Vet Microbiol 64:145-154.

76. Dubovi EJ: 1994, Impact of bovine viral diarrhea virus on reproductive performance in cattle. Vet Clin North Am Food Anim Pract 10(3):503-514.

77. Dubovi: 1996, Laboratory diagnosis of bovine viral diarrhea virus infections. Vet Med 91(9):867-872.

78. Duffell SJ, Harkness JW: 1985, Bovine virus diarrhoea-mucosal disease infection in cattle. Vet Rec 117:240-245.

79. Elbers K, Tautz N, Becher P, Stoll D, Rumenapf T, Thiel HJ: 1996, Processing in the pestivirus E2-NS2 region: identification of proteins p7 and E2p7. J Virol 70:4131-4135.

80. Ellis JA, Martin K, Norman GR, Haines DM: 1995, Comparison of detection methods for bovine viral diarrhea virus in bovine abortions and neonatal death. J Vet Diagn Invest 7:433-436.

81. Evermann J, Ridpath JF: 2002, Clinical and epidemiologic observations of bovine viral diarrhea virus in the northwestern United States. Vet Microbiol 89(2-3):129.

82. Evermann, J F, Barrington GM: 2005, Clinical Features. *In:* Bovine Viral Diarrhea Virus: Diagnosis, Management, and Control, ed. Goyal SM, Ridpath, JF, 1st ed., pp. 105-119. Blackwell Publishing, Ames, IA.

83. Falcone E, Cordioli P, Tarantino M et al: 2003, Experimental infection of calves with bovine viral diarrhoea virus type-2 (BVDV-2) isolated from a contaminated vaccine. Veterinary Research Communications 27:577-589.

84. Fernelius AL, Lambert G, Hemness GJ: 1969, Bovine viral diarrhea virus-host cell interactions: adaptation and growth of virus in cell lines. Am J Vet Res 30(9):1561-1572.

85. Ferrari G, Scicluna MT, Bonvicini D et al: 1999, Bovine virus diarrhoea (BVD) control programme in an area in the Rome province (Italy). Vet Microbiol 64:237-245.

86. Flores EF, Ridpath JF, Weiblen R et al: 2002, Phylogenetic analysis of Brazilian bovine viral diarrhea virus type 2 (BVDV-2) isolates: Evidence for a subgenotype within BVDV-2. Virus Res 87:51-60.

87. Fray MD, Mann GE, Clarke MC, Charleston B: 2000, Bovine viral diarrhoea virus: its effects on ovarian function in the cow. Vet Microbiol 77(1-2):185-194.

88. Fray MD, Supple EA, Morrison WI, Charleston B: 2000, Germinal centre localization of bovine viral diarrhoea virus in persistently infected animals. J Gen Virol 81:1669-1673.

89. Fredriksen B, Press CM, Loken T, Odegaard SA: 1999, Distribution of viral antigen in uterus, placenta and foetus of cattle persistently infected with bovine virus diarrhoea virus. Vet Microbiol 64:109-122.

90. Fulton RW: 2005, Vaccines. *In:* Bovine Viral Diarrhea Virus: Diagnosis, Management, and Control, ed. Goyal SM, Ridpath JF, 1st ed., pp. 209-222. Blackwell Publishing, Ames, IA.

91. Fulton RW, Ridpath JF, Saliki JT et al: 2002, Bovine viral diarrhea virus (BVDV) 1b: predominant BVDV subtype in calves with respiratory disease. Can J Vet Res 66(3):181-190.

92. Fulton RW, Saliki JT, Burge LJ, Payton ME: 2003, Humoral immune response and assessment of vaccine shedding in calves receiving modified live virus vaccines containing bovine herpesvirus-1 and bovine viral diarrhoea virus 1a. J Vet Med B 50:31.

93. Fulton RW, Saliki JT, Confer AW et al: 2000, Bovine viral diarrhea virus cytopathic and noncytopathic biotypes and type 1 and 2 genotypes in diagnostic laboratory accessions: clinical and necropsy samples from cattle. J Vet Diagn Invest 12(1):33-38.

94. Fulton RW, Step DL, Ridpath JF et al: 2003, Response of calves persistently infected with noncytopathic bovine viral diarrhea virus (BVDV) subtype 1b after vaccination with heterologous BVDV strains in modified live virus vaccines and Mannheimia haemolytica bacterin-toxoid. Vaccine 21(21-22):2980-2985.

95. Giangaspero M, Harasawa R: 1999, Ovine pestiviruses: their taxonomic status revealed by palindromic nucleotide substitutions. Vet Microbiol 70:33-39.

96. Giangaspero M, Harasawa R, Verhulst A: 1997, Genotypic analysis of the 5'-untranslated region of a pestivirus strain isolated from human leucocytes. Microbiol Immunol 41:829-834.

97. Giangaspero M, Vacirca G, Buettner M et al: 1993, Serological and antigenical findings indicating pestivirus in man. Arch Virol Suppl 7:53-62.

98. Giangaspero M, Vacirca G, Harasawa R et al: 2001, Genotypes of pestivirus RNA detected in live virus vaccines for human use. J Vet Med Sci 63(7):723-733.

99. Gilbert R: 1993, "Clusters" of anophthalmia in Britain [editorial]. BMJ 307:340-341.

100. Gillespie JH, Coggins L, Thompson J, Baker JA: 1961, Comparison by neutralization tests of strains of virus isolated from virus diarrhea and mucosal disease. Cornell Vet 51:155-159.

101. Gillespie JH, Schlafer DH, Foote RH et al: 1990, Comparison of persistence of seven bovine viruses on bovine embryos following in vitro exposure. Dtsch Tierarztl Wochenschr 97:65-68.

102. Givens MD, Heath AM, Brock KV et al: 2003, Detection of bovine viral diarrhea virus in semen after infection of seronegative, post-pubertal bulls. Am J Vet Res 64(4):428-434.

103. Givens MD, Heath AM, Carson RL et al: 2003, Analytical sensitivity of assays used for detection of bovine viral diarrhea virus in semen samples from the Southeastern United States. Vet Microbiol 96(2):145-155.

104. Glew EJ, Howard CJ: 2001, Antigen-presenting cells from calves persistently infected with bovine viral diarrhoea virus, a member of the Flaviviridae, are not compromised in their ability to present viral antigen. J Gen Virol 82(Pt 7):1677-1685.

105. Gordon I.: 1994, Dispersion of cumulus cells. *In:* Laboratory Production of Cattle Embryos, pp. 173. CAB International, Wallingford.

106. Goyal SM: 2005, Diagnosis. *In:* Bovine Viral Diarrhea Virus: Diagnosis, Management, and Control, ed. Goyal SM, Ridpath JF, 1st ed., pp. 197-208. Blackwell Publishing, Ames, IA.

107. Greiser-Wilke I, Grummer B, Moennig V: 2003, Bovine viral diarrhoea eradication and control programs in Europe. Biologicals 31:113-118.

108. Grom J, Barlic-Maganja D: 1999, Bovine viral diarrhoea (BVD) infections-control and eradication programme in breeding herds in Slovenia. Vet Microbiol 64:259-264.

109. Grooms DL: 2004, Reproductive consequences of infection with bovine viral diarrhea virus. Vet Clin North Am Food Anim Pract 20(1):5-19.

110. Grooms DL, Brock KV, Pate JL, Day ML: 1998, Changes in ovarian follicles following acute infection with bovine viral diarrhea virus. Theriogenology 49:595-605.

111. Grooms DL, Brock KV, Ward LA: 1998, Detection of bovine viral diarrhea virus in the ovaries of cattle acutely infected with bovine viral diarrhea virus. J Vet Diagn Invest 10:125-129.

112. Grooms DL, Brock KV, Ward LA: 1998, Detection of cytopathic bovine viral diarrhea virus in the ovaries of cattle following immunization with a modified live bovine viral diarrhea virus vaccine. J Vet Diagn Invest 10:130-134.

113. Grooms DL, Kaiser L, Walz PH, Baker JC: 2001, Study of cattle persistently infected with bovine viral diarrhea virus that lack detectable virus in serum. J Am Vet Med Assoc 219:629-631.

114. Grooms DL, Keilen ED: 2002, Screening of neonatal calves for persistent infection with bovine viral diarrhea virus by immunohistochemistry on skin biopsy samples. Clin Diagn Lab Immunol 9(4):898-900.

115. Grooms DL, Ward LA, Brock KV: 1996, Morphologic changes and immunohistochemical detection of viral antigen in ovaries from cattle persistently infected with bovine viral diarrhea virus. Am J Vet Res 57:830-833.

116. Grummer B, Beer M, Liebler-Tenorio E, Greiser-Wilke I: 2001, Localization of viral proteins in cells infected with bovine viral diarrhoea virus. J Gen Virol 82(Pt 11):2597-2605.

117. Gu B, Liu C, Lin-Goerke J et al: 2000, The RNA helicase and nucleotide triphosphatase activities of the bovine viral diarrhea virus NS3 protein are essential for viral replication. J Virol 74(4):1794-1800.

118. Hamers C, Dehan P, Couvreur B et al: 2001, Diversity among bovine pestiviruses. Vet J 161(2):112-122.

119. Hamers C, Lecomte C, Kulcsar G et al: 1998, Persistently infected cattle stabilise bovine viral diarrhea virus leading to herd specific strains. Vet Microbiol 61:177-182.

120. Harasawa R: 1999, Comparative analysis of the 5' non-coding region of pestivirus RNA detected from live virus vaccines. J Vet Med Sci 56:961-964.

121. Harasawa R, Mizusawa H: 1995, Demonstration and genotyping of pestivirus RNA from mammalian cell lines. Microbiol Immunol 39:979-985.

122. Harasawa R, Sasaki T: 1995, Sequence analysis of the 5' untranslated region of pestivirus RNA demonstrated in interferons for human use. Biologicals 23:263-269.

123. Harasawa R, Tomiyama T: 1994, Evidence of pestivirus RNA in human virus vaccines. J Clin Microbiol 32:1604-1605.

124. Hertig C, Pauli U, Zanoni R, Peterhans E: 1991, Detection of bovine viral diarrhea (BVD) virus using the polymerase chain reaction. Vet Microbiol 26:65-76.

125. Hietala SK, Crowhurst RC: 2005, Virus replication. *In:* Bovine viral diarrhea virus: diagnosis, management, and control, ed. Goyal SM, Ridpath JF, 1st ed., pp. 81-89. Blackwell Publishing, Ames, Iowa.

126. Hooft van Iddekinge BJ, van Wamel JL, van Gennip HG, Moormann RJ: 1992, Application of the polymerase chain reaction to the detection of bovine viral diarrhea virus infections in cattle. Vet Microbiol 30:21-34.

127. Horner GW, Tham KM, Orr D et al: 1995, Comparison of an antigen capture enzyme-linked assay with reverse transcription--polymerase chain reaction and cell culture immunoperoxidase tests for the diagnosis of ruminant pestivirus infections. Vet Microbiol 43:75-84.

128. Horzinek MC: 1990, Bovine virus diarrhoea virus: an introduction. Rev Sci Tech 9:13-23.

129. Houe H: 1999, Epidemiological features and economical importance of bovine virus diarrhoea virus (BVDV) infections. Vet Microbiol 64:135-144.

130. Houe H: 2005, Risk assessment. *In:* Bovine Viral Diarrhea Virus: Diagnosis, Management, and Control, ed. Goyal SM, Ridpath JF, 1st ed., pp. 35-64. Blackwell, Ames, IA.

131. Houe H, Heron I: 1993, Immune response to other agents of calves persistently infected with bovine virus diarrhea virus (BVDV). Acta Vet Scand 34:305-310.

132. Iqbal M, Flick-Smith H, McCauley JW: 2000, Interactions of bovine viral diarrhoea virus glycoprotein E(rns) with cell surface glycosaminoglycans. J Gen Virol 81(2):451-459.

133. Johnson CM, Perez DR, French R et al: 2001, The NS5A protein of bovine viral diarrhoea virus interacts with the alpha subunit of translation elongation factor-1. J Gen Virol 82(Pt 12):2935-2943.

134. Kafi M, McGowan M, Jillella D: 1994, The effect of bovine viral diarrhea virus (BVDV) during follicular development on the superovulatory response of cattle. Theriogenology 41:223.

135. Kahrs RF: 2001, Bovine viral diarrhea. *In:* Viral Diseases of Cattle, 2nd ed., pp. 113-126. Iowa State University Press, Ames, IA.

136. Kelling CL: 2004, Evolution of bovine viral diarrhea virus vaccines. Vet Clin North Am Food Anim Pract 20(1):115-129.

137. Kirkland PD, Mackintosh SG, Moyle A: 1994, The outcome of widespread use of semen from a bull persistently infected with pestivirus. Vet Rec 135:527-529.

138. Kirkland PD, McGowan MR, Mackintosh SG: 1993, Factors influencing the development of persistent infection of cattle with pestivirus. Proceedings from the 2nd Symposium on Pestiviruses, 117-121.

139. Kirkland PD, Richards SG, Rothwell JT, Stanley DF: 1991, Replication of bovine viral diarrhoea virus in the bovine reproductive tract and excretion of virus in semen during acute and chronic infections. Vet Rec 128:587-590.

140. Kuhne S, Schroeder C, Hollmquist G et al: 2005, Detection of bovine viral diarrhea virus infected cattle - Testing tissue samples derived from ear tagging using Erns capture ELISA. J Vet Med B 52:272-277.

141. Kummerer BM, Meyers G: 2000, Correlation between point mutations in NS2 and the viability and cytopathogenicity of Bovine viral diarrhea virus strain Oregon analyzed with an infectious cDNA clone. J Virol 74(1):390-400.

142. Kummerer BM, Tautz N, Becher P et al: 2000, The genetic basis for cytopathogenicity of pestiviruses. Vet Microbiol 77(1-2):117-128.

143. Lai VC, Kao CC, Ferrari E et al: 1999, Mutational analysis of bovine viral diarrhea virus RNA-dependent RNA polymerase. J Virol 73:10129-10136.

144. Larson R, Pierce VL: 2002, Bovine viral diarrhea virus. Bovine Practitioner 36:106-112.

145. Larson RL: 2005, Management systems and control programs. *In:* Bovine Viral Diarrhea Virus: Diagnosis, Management, and Control, ed. Goyal SM, Ridpath JF, 1st ed., pp. 223-238. Blackwell Publishing, Ames, IA.

146. Liebler EM, Kusters C, Pohlenz JF: 1995, Experimental mucosal disease in cattle: changes of lymphocyte subpopulations in Peyer's patches and in lymphoid nodules of large intestine. Vet Immunol Immunopathol 48:233-248.

147. Liebler-Tenorio EM: 2005, Pathogenesis. *In:* Bovine Viral Diarrhea Virus: Diagnosis, Management, and Control, ed. Goyal SM, Ridpath JF, 1st ed., pp. 121-143. Blackwell Publishing, Ames, IA.

148. Liebler-Tenorio EM, Ridpath JF, Neill JD : 2003, Distribution of viral antigen and development of lesions after experimental infection of calves with a BVDV 2 strain of low virulence. J Vet Diagn Invest 15:221-232.

149. Lindberg AL, Alenius S: 1999, Principles for eradication of bovine viral diarrhoea virus (BVDV) infections in cattle populations. Vet Microbiol 64:197-222.

150. McClurkin AW, Coria MF, Cutlip RC: 1979, Reproductive performance of apparently healthy cattle persistently infected with bovine viral diarrhea virus. J Am Vet Med Assoc 174:1116-1119.

151. McClurkin AW, Littledike ET, Cutlip RC et al: 1984, Production of cattle immunotolerant to bovine viral diarrhea virus. Can J Comp Med 48:156-161.

152. McGoldrick A, Bensaude E, Ibata G et al: 1999, Closed one-tube reverse transcription nested polymerase chain reaction for the detection of pestiviral RNA with fluorescent probes. J Virol Methods 79:85-95.

153. McGoldrick A, Lowings PJ, Ibata G: 1998, A novel approach to the detection of classical swine fever virus by RT-PCR with a fluorogenic probe (Taqman). J Virol Methods 72:125-135.

154. McGowan MR, Kirkland PD, Richards SG, Littlejohns IR: 1993, Increased reproductive losses in cattle infected with bovine pestivirus around the time of insemination. Vet Rec 133:39-43.

155. Menanteau-Horta AM, Ames TR, Johnson DW, Meiske JC: 1985, Effect of maternal antibody upon vaccination with infectious bovine rhinotracheitis and bovine virus diarrhea vaccines. Can J Comp Med 49:10-14.

156. Meyling A, Houe H, Jensen AM: 1990, Epidemiology of bovine virus diarrhoea virus. Rev Sci Tech 9:75-93.

157. Meyling A, Jensen AM: 1988, Transmission of bovine virus diarrhoea virus (BVDV) by artificial insemination (AI) with semen from a persistently- infected bull. Vet Microbiol 17:97-105.

158. Munoz-Zanzi CA, Hietala SK, Thurmond MC, Johnson WO: 2003, Quantification, risk factors, and health impact of natural congenital infection with bovine viral diarrhea virus in dairy calves.Am J Vet Res 64(3):358-365.

159. Murphy FA, Gibbs EPJ, Horzinek MC, Studdert MJ: 1999, Flaviviridae. *In:* Veterinary Virology, 3rd ed., pp. 555-569. Academic Press, Boston, MA.

160. Murray RD: 1990, A field investigation into causes of bovine abortion in dairy cattle. Vet Rec 127(22):543-547.

161. Murray: 1991, Lesions in aborted bovine fetuses and placenta associated with bovine viral diarrhoea virus infection. Arch Virol Suppl 3:217-224.

162. Njaa BL, Clark EG, Janzen E: 2000, Diagnosis of persistent bovine viral diarrhea virus infection by immunohistochemical staining of formalin-fixed skin biopsy specimens. J Vet Diagn Invest 12(5):393-399.

163. Olafson P, McCallum AD, Fox FH: 1946, An apparently new transmissible disease of cattle. Cornell Vet 36:205-213.

164. Olafson P, Rickard CG: 1947, Further observations on the virus diarrhea (new transmissible disease) of cattle. Cornell Vet 37:104-106.

165. Pellerin C, van den Hurk J, Lecomte J, Tijssen P: 1994, Identification of a new group of bovine viral diarrhea virus strains associated with severe outbreaks and high mortalities. Virology 203:260-268.

166. Philpott M: 1993, The dangers of disease transmission by artificial insemination and embryo transfer. Br Vet J 149:339-369.

167. Plavsic MZ, Prodafikas G: 2001, Evaluation of a new sandwich enzyme-linked immunosorbent assay for detection of bovine viral diarrhea virus in unprocessed fetal bovine serum. J Vet Diagn Invest 13(3):261-262.

168. Potter ML, Corstvet RE, Looney CR: 1984, Evaluation of bovine viral diarrhea virus uptake by preimplantation embryos. Am J Vet Res 45:1778-1780.

169. Potts BJ, Sever JL, Tzan NR: 1987, Possible role of pestiviruses in microcephaly [letter]. Lancet 1:972-973.

170. Ramsey FK, Chivers WH: 1953, Mucosal disease of cattle. North Am Vet 34:629-633.

171. Ridpath, JF: 2005, Classification and molecular biology. *In:* Bovine Viral Diarrhea Virus: Diagnosis, Management, and Control, ed. Goyal SM, Ridpath JF, 1st ed., pp. 65-80. Blackwell Publishing, Ames, IA.

172. Ridpath JF, Bendfeldt S, Neill JD, Liebler-Tenorio E: 2006, Lymphocytopathogenic activity in vitro correlates with high virulence in vivo for BVDV type 2 strains: Criteria for a third biotype of BVDV. Virus Res In Press.

173. Ridpath JF, Bolin SR: 1990, Viral protein production in homogeneous and mixed infections of cytopathic and noncytopathic BVD virus. Arch Virol 111:247-256.

174. Ridpath JF, Bolin SR: 1998, Differentiation of types 1a, 1b and 2 bovine viral diarrhoea virus (BVDV) by PCR. Mol Cell Probes 12:101-106.

175. Ridpath JF, Hietala SK, Sorden S, Neill JD: 2002, Evaluation of the reverse transciption-polymerase chain reaction/probe test of serum samples and immunohistochemistry of skin sections for detection of acute bovine viral diarrhea infections. J Vet Diagn Invest 14(4):303-307.

176. Ridpath JF, Neill JD, Vilcek S, Dubovi EJ, Carman S: 2006, Multiple outbreaks of severe acute BVDV in North America occurring between 1993 and 1995 linked to the same BVDV2 strain. Vet Microbiol In Press.

177. Rossi CR, Bridgman BS, Kiesel GK: 1980, Viral contamination of bovine fetal lung cultures and bovine fetal serum. Am J Vet Res 41(10):1680-1681.

178. Rossi CR, Kiesel GK: 1971, Microtiter tests for detecting antibody in bovine serum to parainfluenza 3 virus, infectious bovine rhinotracheitis virus, and bovine virus diarrhea virus. Appl Microbiol 22:32-36.

179. Rumenapf T, Stark R, Heimann M, Thiel HJ: 1998, N-terminal protease of pestiviruses: Identification of putative catalytic residues by site-directed mutagenesis. J Virol 72(3):2544-2547.

180. Rumenapf T, Unger G, Strauss JH, Thiel HJ: 1993, Processing of the envelope glycoproteins of pestiviruses. J Virol 67:3288-3294.

181. Saliki JT, Huchzermeier R, Dubovi EJ: 2000, Evaluation of a new sandwich ELISA kit that uses serum for detection of cattle persistently infected with BVD virus. Ann NY Acad Sci 916:358-363.

182. Saliki JT, Dubovi EJ: 2004, Laboratory diagnosis of bovine viral diarrhea virus infections. Vet Clin North Am Food Anim Pract 20(1):69-83.

183. Sandvik T: 1999, Laboratory diagnostic investigations for bovine viral diarrhoea virus infections in cattle. Vet Microbiol 64:123-134.

184. Sandvik T: 2004, Progress of control and prevention programs for bovine viral diarrhea virus in Europe. Vet Clin North Am Food Anim Pract 20:151-169.

185. Sasaki T, Harasawa R, Shintani M et al: 1996, Application of PCR for detection of mycoplasma DNA and pestivirus RNA in human live viral vaccines. Biologicals 24:371-375.

186. Scherer CF, Flores EF, Weiblen R et al: 2001, Experimental infection of pregnant ewes with bovine viral diarrhea virus type-2 (BVDV-2): effects on the pregnancy and fetus. Vet Microbiol 79(4):285-299.

187. Schipper IA, Weiss R, Moen RA: 1978, Isolation of a bovine virus diarrhea (BVD) virus from a 1-week-old foal. Vet Med Small Anim Clin 73(6):786-786.

188. Schneider R, Unger G, Stark R et al: 1993, Identification of a structural glycoprotein of an RNA virus as a ribonuclease. Science 261:1169-1171.

189. Seidel Jr GE: 1990, Techniques for freezing mammalian embryos. 1990 short course proceedings, Animal Reproduction and Biotechnology Laboratory, Colorado State University, Fort Collins, Colorado:85-86.

190. Singh EL, Eaglesome MD, Thomas FC et al: 1982, Embryo transfer as a means of controlling the transmission of viral infections. I. The in vitro exposure of preimplantation bovine embryos to akabane, bluetongue and bovine viral diarrhoea viruses. Theriogenology 17(4):437-444.

191. Smith RA: 1996, Introduction. *In:* Bovine Respiratory Disease: Sourcebook for the Veterinary Professional, 5th ed., Veterinary Learning Systems, Trenton, NJ.

192. Ssentongo YK, Johnson RH, Smith JR: 1997, Association of bovine viral diarrhoea-mucosal disease virus with ovaritis in cattle. Australian Veterinary Journal 56:272-273.

193. Stahl K, Baule C, Lindberg A: 2004, Molecular epidemiology and surveillance of BVD during the last phase of the Sewdish BVD-programme. Revista Portuguesa de Ciencias Veterinarias Supl. 127:29-36.

194. Steffens S, Thiel HJ, Behrens SE: 1999, The RNA-dependent RNA polymerases of different members of the family Flaviviridae exhibit similar properties in vitro. J Gen Virol 80:2583-2590.

195. Straver PJ, Journee DL, Binkhorst GJ: 1983, Neurological disorders, virus persistence and hypomyelination in calves due to intra-uterine infections with bovine virus diarrhoea virus. II. Virology and epizootiology. Vet Q 5:156-164.

196. Synge BA, Clark AM, Moar JA et al: 1999, The control of bovine virus diarrhoea virus in Shetland. Vet Microbiol 64:223-229.

197. Tautz N, Harada T, Kaiser A: 1999, Establishment and characterization of cytopathogenic and noncytopathogenic pestivirus replicons. J Virol 73:9422-9432.

198. Taylor LF, Van Donkersgoed J, Dubovi EJ et al: 1995, The prevalence of bovine viral diarrhea virus infection in a population of feedlot calves in western Canada. Can J Vet Res 59:87-93.

199. Thur B, Zlinszky K, Ehrensperger F: 1996, Immunohistochemical detection of bovine viral diarrhea virus in skin biopsies: a reliable and fast diagnostic tool. Zentralbl Veterinarmed B 43(3):163-166.

200. Toth RL, Nettleton PF, McCrae MA: 1999, Expression of the E2 envelope glycoprotein of bovine viral diarrhoea virus (BVDV) elicits virus-type specific neutralising antibodies. Vet Microbiol 65:87-101.

201. Tremblay R: 1996, Transmission of bovine viral diarrhea virus. Vet Med 91(9):858-866.

202. van Oirschot JT, Bruschke CJ, van Rijn PA: 1999, Vaccination of cattle against bovine viral diarrhoea. Vet Microbiol 64:169-183.

203. Vilcek S, Greiser-Wilke I, Nettleton P, Paton DJ: 2000, Cellular insertions in the NS2-3 genome region of cytopathic bovine viral diarrhoea virus (BVDV) isolates. Vet Microbiol 77(1-2):129-136.

204. Vilcek S, Paton DJ, Durkovic B et al: 2001, Bovine viral diarrhoea virus genotype 1 can be separated into at least eleven genetic groups. Arch Virol 146:99-115.

205. Vilcek S, Paton DJ, Minor P, Bentley M: 1999, No confirmation of pestivirus RNA in human virus vaccines [letter; comment]. J Clin Microbiol 37:1653-1653.

206. Vilcek S, Strojny L, Durkovic B et al: 2001, Storage of bovine viral diarrhoea virus samples on filter paper and detection of viral RNA by a RT-PCR method. J Virol Methods 92(1):19-22.

207. Virakul P, Fahning ML, Joo HS, Zemjanis R: 1988, Fertility of cows challenged with a cytopathic strain of bovine viral diarrhea virus during an outbreak of spontaneous infection with a noncytopathic strain. Theriogenology 29(2):441-449.

208. Voges H, Horner GW, Rowe S, Wellenberg GJ: 1998, Persistent bovine pestivirus infection localized in the testes of an immuno-competent, non-viraemic bull. Vet Microbiol 61:165-175.

209. Walker RVL: 1947, Failure of virus diarrhea of cattle to immunize against rinderpest. Cornell Vet 37:107-112.

210. Walz PH, Baker JC, Mullaney TP et al: 1999, Comparison of type I and type II bovine viral diarrhea virus infection in swine. Can J Vet Res 63:119-123.

211. Walz PH, Bell TG, Grooms DL et al: 2001, Platelet aggregation responses and virus isolation from platelets in calves experimentally infected with type I or type II bovine viral diarrhea virus. Can J Vet Res 65:241-247.

212. Ward P, Misra V: 1991, Detection of bovine viral diarrhea virus, using degenerate oligonucleotide primers and the polymerase chain reaction. Am J Vet Res 52:1231-1236.

213. Weinstock D, Bhudevi B, Castro AE: 2001, Single-tube single-enzyme reverse transcriptase PCR assay for detection of bovine viral diarrhea virus in pooled bovine serum. J Clin Microbiol 39(1):343-346.

214. Wentink GH, Aarts T, Mirck MH, van Exsel ACA: 1991, Calf from a persistently infected heifer born after embryo transfer with normal immunity to BVDV. Vet Rec 129:449-450.

215. Wilks CR, Abraham G, Blackmore DK: 1989, Bovine pestivirus and human infection [letter].Lancet 1:107.

216. Wittum TE: 2004, Persistent bovine viral diarrhoea virus infection in US beef herds. Prev Vet Med 49:83-94.

217. Xu J, Mendez E, Caron PR et al: 1997, Bovine viral diarrhea virus NS3 serine proteinase: polyprotein cleavage sites, cofactor requirements, and molecular model of an enzyme essential for pestivirus replication. J Virol 71:5312-5322.

218. Yolken R, Dubovi EJ, Leister F et al: 1989, Infantile gastroenteritis associated with excretion of pestivirus antigens. Lancet 1:517-520.

219. Yu H, Grassmann CW, Behrens SE: 1999, Sequence and structural elements at the 3' terminus of bovine viral diarrhea virus genomic RNA: functional role during RNA replication. J Virol 73:3638-3648.

220. Zhong W, Gutshall LL, Del Vecchio AM: 1998, Identification and characterization of an RNA-dependent RNA polymerase activity within the nonstructural protein 5B region of bovine viral diarrhea virus. J Virol 72(11):9365-9369.

221. Zurovac O, Stringfellow DA, Brock KV et al: 1994, Noncytopathic bovine viral diarrhea virus in a system for in vitro production of bovine embryos. Theriogenology 41:841-853.

VIII. APPENDICES

APPENDIX A

METHODS OF IMMUNOPEROXIDASE ASSAY FOR

BOVINE VIRAL DIARRHEA VIRUS

1. Reagents must be prepared for the immunoperoxidase assay. Required reagents include:
   a) Fixative: for fixation of cells prior to step one
   b) Diluent: for preparation of working solutions of monoclonal antibodies and conjugated antibodies
   c) Washing mixture: for removing unbound antibodies
   d) Preservation medium: for preserving the stained cells

Fixative

Ingredient	Volume
Acetone	20.00 ml
Bovine Serum Albumin (1%)	0.02 ml
NaCl (0.85%)	79.98 ml
Total Volume	**100.00 ml**

Diluent

Ingredient	Volume
Tween 20	0.05 ml
Bovine Serum Albumin (1%)	0.02 ml
Phosphate Buffered Saline (PBS)	99.93 ml
Total Volume	**100.00** ml

Washing Mixture

Ingredient	Volume
Tween 20	0.05 ml
PBS	99.95 ml
Total Volume	**100.00 ml**

Preservation Medium

Ingredient	Volume
Dulbecco's PBS	96.00 ml
Formladehyde	4.00 ml
Total Volume	**100.00 ml**

Note: Basic reagents (fixative, diluent, washing mixture, and preservation medium) can be prepared in advance and stored for several months in the refrigerator at 4°C.

Preparation of Antibodies

1. Working concentrations of anti-BVDV monoclonal antibodies:
   a) D89 anti-BVDV monoclonal antibody was diluted 1:500 in diluent (10 µl:5000 µl for 5 ml/plate)
   b) 20.10.6 anti-BVDV monoclonal antibody was diluted 1:800 in diluent (10 µl:8000 µl)

2. Working concentration of conjugated rabbit anti-mouse IgG:
   a) Rabbit anti-mouse IgG was diluted 1:200 in diluent (30 µl:6000 µl for 6 ml/plate)

CELLS

1. Madin Darby bovine kidney (MDBK) cells, determined to be free of BVDV are used for culture of virus. Cells are grown in monolayers propagated in 25 cm^2 flasks. Cells are maintained in the following culture medium:

Ingredient	Amount
Minimum essential medium	100 ml
Equine serum	10 ml
Sodium bicarbonate ($NaHCO_3$)	1ml (75 mg)
L-glutamine	1 ml (29.2 mg)
Penicillin G	10,000 U*
Streptomycin	10,000 µg*
Amphotericin B	25 µl*

*1ml of penicillin, streptomycin, amphotericin combination

2. Wells of a 96-well cell culture plate (BD Falcon, Franklin Lakes, NJ), containing 100 µl of culture medium are each inoculated with 10 µl of test sample for serial dilution of virus. The samples were tested by adding 10 µl of sample to the wells. Next, 50 µl of MEM with equine serum was added for cell nourishment.

3. Serial dilution (10^{-1} to 10^{-6}) will be performed on the diluted stock virus to determine concentration of virus.

4. A confluent monolayer of MDBK cells is washed 2X with Ca^{++} and Mg^{++} free PBS and trypsinized with 1 ml trypsin-EDTA for 2 minutes.

5. After 3 minutes, the activity of trypsin is stopped with 10 ml culture medium. This mixture is vigorously pipetted to create a single cell suspension and transferred to a sterile trough.

6. With the aid of a multichannel pipettor, 50 µl of suspended cells are placed in each inoculated well of the 96-well cell culture plate. Immediately after attachment cells should be approximately 80% confluent.

7. Plates are incubated for 72 hours at 37°C in a humidified atmosphere of 5% CO_2 in air.

CELL CULTURE PASSAGE OF SERUM SAMPLES

1. Seven hundred sixty-eight microliters of test sera is layered over MDBK cells growing in log phase in wells of a 24-well plate (BD Falcon, Franklin Lakes, NJ).

2. Plates are incubated for 1 hour at 38.5°C in a humidified atmosphere of 5% CO_2 in air.

3. Three milliliters of supplemented MEM are added to each well.

4. Plates are incubated for 5 days at 38.5°C in a humidified atmosphere of 5% CO_2 in air.

5. Following incubation, plates are frozen at -80°C and then thawed to release virus.

6. Samples may be aliquoted and stored at -80°C until being assayed for virus.

ISOLATION OF BUFFY COAT

1. Whole blood samples in ethylenediaminetetraacetic acid (EDTA) are centrifuged at 2,020 rpm for 30 minutes at 4°C.

2 Using a Pasteur pipette (Fischer Scientific International Inc., Pittsburgh, PA), the buffy coat cells are extracted and placed into a sterile 15 ml centrifuge tube (Fischer Scientific International Inc., Pittsburgh, PA).

3. Ten milliliters of 01.M NH_4CL (Sigma-Aldrich Co., St. Louis, MO) is added to the buffy coat. Mix well (by vortex).

4. Centrifuge the sample at 2,020 rpm for 10 minutes at 4°C. Pour supernatant off the buffy coat, and mix the pellet of buffy coat cells well.

5. Add 10 ml of supplemented MEM to the cells and mix well.

6. Centrifuge the cells at 2,020 rpm for 10 minutes at 4°C.

7. Pour supernatant off the buffy coat pellet and resuspend the buffy coat pellet in 0.5 ml of supplemented MEM. Mix well and transfer into a sterile cryogenic vial (Sarstedt Inc., Newton, NC) for virus isolation by immunoperoxidase monolayer assay.

IMMUNOPEROXIDASE TEST

1. After incubation, medium is dumped from all wells and the plate is air dried. It is critical to allow the cells to dry thoroughly (approximately 1 hour) otherwise detachment of cell monolayers could occur during the following steps. One hundred µl of fixative are dispensed into each well and fixation is allowed for 10 minutes at room temperature. After this time, as much fixative as possible is discarded by tapping inverted plates on a double layer of paper towels. Plates are again allowed to air dry (at this point plates could be preserved at -20°C for several weeks).

2. Fifty microliters of the diluted mixture of each anti-BVDV monoclonal antibody is added to each well and incubated for 20 minutes at 37°C. (Antibody mixture contains 10 µl/5 ml of D89 and 10 µl /8 ml of 20.10.6 in diluent for each plate.)

3. Plates are washed 3 times with PBS-T by adding 100 µl of washing mixture to each well with the aid of a multichannel pipettor. Plates are rocked gently (by hand) and the mixture is discarded by inverting the plates and tapping them on a double layer of paper towels. As much as possible of the liquid is discarded.

4. Fifty microliters of diluted, conjugated rabbit anti-mouse IgG (Jackson Immuno Research Lab, West Grove, PA) is dispensed per well and incubated for 20 minutes at 37°C. (30 µl IgG/6 ml of diluent for 1 plate, 60 µl IgG/12 ml of diluent for 2 plates)

5. Plates are washed 3 times (100 µl per well) as described in step 4.

6. Substrate is prepared immediately before use according to the manufacturer's instructions:
   a) Add 5 drops of reagent A and 5 drops of reagent B to 5 ml distilled water.
   b) Mix well.
   c) Add 5 drops of reagent C.
   d) Mix well again.
   e) Dispense 50 µl into each well in the 96-well culture plate.

7. Plates are incubated 10-15 minutes at room temperature.

8. Substrate is replaced with 100 µl per well of preservation medium to restore plates.

9. Plates are read using a light microscope.

10. When wrapped in aluminum foil, plates can be stored in the refrigerator at 4°C for up to several months.

APPENDIX B

METHODS OF SERUM VIRUS NEUTRALIZATION ASSAY FOR BOVINE VIRAL DIARRHEA VIRUS

1. Serum test samples are heat inactivated by incubating the samples at 56°C for 30 minutes.

2. Fifty microliters of supplemented minimum essential medium (MEM) is added to all wells of a 96-well plate (BD Falcon, Franklin Lakes, NJ).

3. Next, 50 µl of heat-inactivated serum is added to the top row of the plate. From a starting dilution of 1:2, serial two-fold dilutions are made of the test sera using MEM as diluent. Each dilution is run in triplicate.

4. Following dilution of the sera, 50 µl of I-23 BVDV virus (Givens, Auburn University, Auburn, AL) is diluted in MEM such that the inoculum contains 2 cell culture infective doses with a 50% endpoint ($CCID_{50}$) per microliter for a total of 100 $CCID_{50}$.

5. Plates are incubated for 1 hour at 38.5°C in a humidified atmosphere of 5% CO_2 and air.

6. Fifty microliters of Madin Darby bovine kidney (MDBK) cells are added to each well.

7. Plates are incubated for 72 hours at 38.5°C in a humidified atmosphere of 5% CO_2 and air.

8. The contents of the plates are discarded. Immunoperoxidase staining is performed as previously described (Appendix A).