Changes in the Open Reading Frame of Bovine Viral Diarrhea Virus during Serial Infection of Pregnant Cattle, Sheep, and Swine

by

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Abstract

Bovine viral diarrhea virus (BVDV) is an economically important pathogen that is endemic in cattle populations of many countries. This virus can also infect pigs, camelids, and a wide range of ruminants including sheep, goats, and wild ruminants. BVDV infections are often subclinical, but can also be associated with severe reproductive losses and signs of respiratory or gastrointestinal disease.

BVDV isolates circulating in animal populations are genetically and antigenically highly diverse. Two species of BVDV are currently recognized within the genus *Pestivirus*, BVDV-1 and BVDV-2. Furthermore, at least 21 BVDV-1 subgenotypes (1a-1u) and four BVDV-2 subgenotypes (2a-2d) have been described to date. The accumulation of mutations during viral replication due to the minimal proofreading activity of the BVDV RNA polymerase is believed to be the main driving force for the generation of altered genomic sequences.

Previous studies have demonstrated that great numbers of nucleotide and amino substitutions occurred during acute BVDV infections in pregnant cattle. As this virus does not possess strict host specificity, cross-species infections provide a further opportunity for viral diversification. However, only limited information exists regarding genomic changes arising during BVDV infections in species other than cattle. The purpose of this research was thus to determine the changes introduced in the open reading frame (ORF) of the BVDV genome during serial infection of pregnant cattle, sheep, and swine.

Serial experimental inoculations were performed in six pregnant heifers, six pregnant

ewes, and six pregnant gilts using the BVDV-1b isolate AU526 in the first dam of each species and serum from the preceding acutely infected dam thereafter. The complete ORF sequences of AU526 and 36 isolates from acutely infected pregnant dams and their congenitally infected offspring were then determined. These sequences were compared to determine the timing, number, location, and type of substitutions introduced during serial infection of pregnant cattle, sheep, and swine.

Greater numbers of nucleotide and amino acid substitutions occurred during serial infection of pregnant sheep and swine than of pregnant cattle. Furthermore, multiple identical amino acid changes were detected in viral isolates obtained from animals of the same species. These changes were more abundant and more rapidly introduced in sheep and swine, and occurred primarily in the E2 and E^{rns} coding regions of the viral genome. Furthermore, most substitutions involved the same amino acid residues in sheep and swine. In contrast, changes repeatedly identified during serial infection of pregnant cattle were not observed in isolates from pregnant sheep and swine serially infected with AU526. Altogether these results suggest that BVDV infections in species other than cattle may serve as a significant source of viral genetic diversity and may be associated with adaptive changes.

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List of Abbreviations

ACE	Antigen-capture ELISA
AEC	Aminoethyl carbazole
APOBEC	Apolipoprotein B-editing catalytic polypeptide
APPV	Atypical porcine pestivirus
BDV	Border disease virus
BHV-1	Bovine herpesvirus-1
BVDV	Bovine viral diarrhea virus
BVD-MD	Bovine viral diarrhea-mucosal disease
C	Core
CO1	Cytochrome c oxidase subunit 1
CP	Cytopathic
Cq	Quantification cycle
CSFV	Classical swine fever virus
ds	Double-stranded
ELISA	Enzyme-linked immunosorbent assay
FMDV	Foot-and-mouth disease virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus
ICTV	International committee on taxonomy of viruses

IFN	Interferon
IHC	Immunohistochemistry
IPMA	Immunoperoxidase monolayer assay
IRES	Internal ribosome entry site
Jiv	J-domain protein interacting with viral protein
LV	Linda virus
MAb	Monoclonal antibody
MD	Mucosal disease
MDBK	Madin-Darby bovine kidney
MEM	Minimum essential medium
NCP	Noncytopathic
ORF	Open reading frame
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PI	Persistently infected
pi	Postinoculation
RFU	Relative fluorescence unit value
RT-PCR	Reverse transcriptase PCR
RT-nPCR	Reverse transcriptase nested PCR
RT-qPCR	Reverse transcriptase quantitative PCR
RNA	Ribonucleic acid

RNAse	Ribonuclease
$TCID_{50}$	50% tissue culture infective dose
USA	United States of America
UTR	Untranslated region
VD	Virus diarrhea
VI	Virus isolation
VN	Virus neutralization

Chapter 1: Literature Review

History

In 1946, Olafson et al. described an "apparently new transmissible disease" with high morbidity (33 to 88%) and low mortality (4 to 8%) affecting six cattle herds in the state of New York, USA (Olafson et al., 1946). This "acute, infectious and contagious disease" was characterized by anorexia, depression, increased rectal temperature, ocular and nasal discharges, diarrhea, leukopenia, and ulceration of the oral, pharyngeal, and esophageal mucosa. Pregnant animals were also frequently found to abort following clinical or subclinical infection. This disease could be reproduced experimentally by drenching susceptible animals with feces from affected cattle or by injecting under the skin blood or splenic emulsion from affected cattle. Given the absence of visible organisms in infectious materials, a viral etiology was suspected and this new disease was named virus diarrhea (VD) (Olafson & Rickard, 1947). In the same year, Childs reported similar clinical findings in young cattle from the province of Saskatchewan, Canada (Childs, 1946). Two clinical presentations were described: an acute form leading to death within a few days and a subacute form associated with milder clinical signs and progressive weight loss. In contrast to previous reports, this disease of unknown origin was associated with high mortality and low morbidity (5 to 20%). A similar disease was later described in young cattle from Iowa and surrounding states (Ramsey & Chivers, 1953). This highly fatal disease was characterized by extensive erosive and ulcerative lesions of the alimentary tract and thus was named mucosal disease (MD).

In 1957, a noncytopathic viral isolate from cattle affected with VD was carried for 20 consecutive passages in skin-muscle tissue culture and 15 additional passages in bovine kidney

cells (Lee & Gillespie, 1957). Calves inoculated with fluid from the 11th, 20th, 25th, 30th, and 35th passage in tissue culture showed signs of illness consistent with VD. For the first time, an isolate of "VD virus" was successfully passaged in cultured cells, while retaining its virulence. In the same year, a cytopathic virus was isolated in bovine kidney cells from tissues of cattle affected with MD (Underdahl et al., 1957).

Subsequent in vitro studies demonstrated that antibodies raised against an isolate of VD virus (Oregon C24V) were able to reduce the growth of a virus isolated from cattle with MD (Gillespie et al., 1961). Similarly, antibodies from animals exposed to isolates of MD virus were found to neutralize the Oregon C24V isolate (Kniazeff & Pritchard, 1960; Thomson & Savan, 1963). These results revealed that viruses from cattle with VD and MD were antigenically related and suggested that these two diseases were caused by the same agent, namely bovine viral diarrhea virus (BVDV). The term bovine viral diarrhea-mucosal disease (BVD-MD) has also been used to refer to the disease caused by BVDV (Kennedy et al., 1968).

Taxonomy

In 1960, Darbyshire demonstrated a serological relationship between BVDV and classical swine fever virus (CSFV) (Darbyshire, 1960). These two viruses were then grouped together as belonging to the *Togaviridae* family (Horzinek et al., 1971). In 1973, Horzinek introduced the term pestivirus to congregate these antigenically related viral species (Horzinek, 1973). BVDV and CSFV were later found to be also related to border disease virus (BDV) (Plant et al., 1973). In 1975, the term pestivirus was adopted by the International Committee on Taxonomy of Viruses (ICTV) and pestiviruses were assigned generic status in the *Togaviridae* family, with BVDV as the prototype. Pestiviruses were subsequently found to have greater similarities in genomic organization and replication and expression strategies to flaviviruses than to togaviruses

(Collett et al., 1988a). They were thus reclassified in the *Flaviviridae* family in 1990 (Francki et al., 1991).

Immunofluorescence, neutralization, and binding assays performed with polyclonal and monoclonal antibodies demonstrated high antigenic variation among BVDV isolates and distinct groups of viruses were identified (Bolin et al., 1988; Edwards et al., 1988; Magar et al., 1988). In 1994, phylogenetic analysis segregated BVDV isolates into two genetic groups, BVDV-1 and BVDV-2 based on sequences from the 5' untranslated region (UTR) of the viral genome (Pellerin et al., 1994; Ridpath et al., 1994). Although the term genotype has been used to refer to these two genetic groups, these were later recognized by the ICTV as two different species within the genus *Pestivirus* (Fauquet et al., 2005).

Genomic sequence relatedness is deemed an important criterion for species demarcation and a divergence threshold of 25% has been used to distinguish between pestivirus species (Simmonds et al., 2017). Species demarcation also considered the antigenic relatedness of viral isolates. For instance, polyclonal antisera generated against BVDV-1 viruses usually showed a several-fold greater neutralizing titer against viruses of the same species than against viruses from another species in cross-neutralization tests (Pellerin et al., 1994).

In 1993, new BVDV-2 isolates associated with severe hemorrhagic disease were isolated from veal calves in Quebec. These BVDV-2 outbreaks killed about 25% of the veal calves in Quebec that year (Pellerin et al., 1994). However, BVDV-2 isolates vary in virulence and most isolates do not cause clinically severe disease, similar to what has been described for BVDV-1 isolates (Ridpath et al., 2000).

Besides the two BVDV species, phylogenetic studies showed the existence of large numbers of distinct genetic groups within each species, named subgenotypes. Different genomic

regions have been used for phylogenetic analysis of BVDV isolates including the 5' UTR, N^{pro}, E2, NS2-3, and NS5B-3'UTR regions. Partial 5' UTR sequences have been most frequently used, whereas complete N^{pro} and E2 coding sequences provide high confidence levels for allocation of BVDV isolates into subgenotypes (Becher et al., 1999; Becher et al., 1997). To date, at least 21 BVDV-1 subgenotypes (BVDV-1a to -1u) and four BVDV-2 subgenotypes (BVDV-2a to -2d) have been described (M. Deng et al., 2015; Flores et al., 2002; S. Gao et al., 2013; Giammarioli et al., 2015; Giangaspero & Harasawa, 2004; Jackova et al., 2008; Nagai et al., 2008; Vilcek et al., 2004; Vilcek et al., 2001; F. Xue et al., 2010).

In 1999, subsequent genetic and serologic analysis of viruses isolated from diverse host species suggested the existence of one additional pestivirus species represented by two isolates from a giraffe in Kenya and a bovine cell culture originating from Kenya (Becher et al., 2014; Becher et al., 1999).

In 2004, a new atypical pestivirus (D32/00_'HoBi') isolated from a batch of fetal bovine serum collected in Brazil was described (Schirrmeier et al., 2004). This isolate was genetically and antigenically markedly different from all known pestiviruses. It was proposed that this isolate may be a member of a novel pestivirus species. Other isolates were then found to be closely related to this new pestivirus and named 'HoBi'-like viruses. These viruses were isolated from a buffalo in Brazil, a calf in Thailand, and a cell culture contaminant, possibly originating from a batch of fetal bovine serum produced in South America (Stahl et al., 2007; Stalder et al., 2005).

In 2005, a new divergent pestivirus was isolated from a young blind pronghorn antelope that was sent to the Wyoming State Veterinary Laboratory (Vilcek et al., 2005). Phylogenetic analysis of the 5' UTR and N^{pro} and E2 coding regions suggested that this pronghorn virus may

represent a new pestivirus species. In the same year, Thabti et al. reported nine isolates from different batches of a contaminated Tunisian sheep pox vaccine and one isolate from sheep (Thabti et al., 2005). These viruses were originally assigned to a novel subgroup within the BDV species, but subsequent phylogenetic analysis revealed that these isolates were more closely related to CSFV than to BDV (Liu et al., 2009). These Tunisian isolates were thus proposed as a novel pestivirus species named Tunisian sheep virus. Additional pestiviruses with high similarity to Tunisian isolates have been recently isolated from sheep and goats in France and Italy (Ciulli et al., 2017; Martin et al., 2015).

In 2007, a novel pestivirus was isolated from tissues of pigs with multifocal non-suppurative myocarditis in Australia (Kirkland et al., 2007). These pigs originated from a farm in New South Wales that had experienced an outbreak of stillbirths and sudden deaths in 3- to 4-week-old piglets. This pestivirus was named Bungowannah virus after the location where the first cases were identified. Experimental infection of pregnant sows with Bungowannah virus was associated with a wide range of clinical presentations, depending on the stage at which infection had occurred (Finlaison et al., 2010). Following infection at 35 days of gestation, a high incidence of stillbirth, fetal mummification, and persistent fetal infection was described. Furthermore, chronically infected piglets that were viremic for up to seven months and showed growth retardation were reported after infection at 55 days of gestation. Myocardial lesions were observed in fetuses following infection at 90 days of gestation. Cattle and sheep were also shown to become infected after experimental infection, but no signs of illness were observed (Kirkland et al., 2015). Infection of pregnant cattle between 53 and 65 days of gestation resulted in maternal and fetal infection, but all fetuses mounted an antibody response to the virus.

In 2009, two new isolates from a lamb and a goat kid were described in Turkey (Oguzoglu et al., 2009). These animals originated from flocks in the provinces of Aydin and Burdur, in which abortions and the birth of offspring with poor viability, abnormal fleece, and neurologic signs were observed. Clinical and histopathological findings in the lamb included hindlimb paralysis, abnormal fleece, thymic hypoplasia, and non-suppurative meningoencephalomyelitis with hypomyelination. These findings were similar to those described in small ruminants infected with pestiviruses (Pratelli et al., 1999). Subsequent genetic and antigenic analysis suggested that these two isolates may be members of a new pestivirus species that was closely related to CSFV (Postel et al., 2015). These new isolates, named Aydin-like pestiviruses, may thus interfere with serological diagnosis of CSFV infection.

In 2012, a partial genome sequence (5,130 nucleotides) of a novel pestivirus (RaPestV-1) was identified in the bat species *Rhinolophus affinis* of Hainan, which showed 46.5% to 47.3% nucleotide sequence identities to known pestiviruses (Wu et al., 2012). For the first time, a pestivirus was found in animals that did not belong to the order *Artiodactyla*. However, infectious virus could not be isolated from this host species.

In 2013, a calf persistently infected (PI) with 'HoBi'-like virus was reported (Decaro et al., 2013). This calf originated from a herd in Southern Italy, in which repeated outbreaks of respiratory diseases and abortions had occurred (Decaro et al., 2008; Decaro et al., 2012). The close relationship between 'HoBi'-like viruses and BVDV, and their capacity to establish persistent infection in cattle led some researchers to suggest that these viruses should be recognized as a new genotype of BVDV, namely BVDV-3 (Liu et al., 2009).

In 2014, a novel pestivirus (NrPV) was detected in six commensal Norway rats in New York City (Firth et al., 2014). The polyprotein sequence of this highly divergent isolate shared a

maximum amino acid identity of 60% with known pestivirus polyproteins and was predicted to encode the 12 conserved pestivirus proteins. Interestingly, coinfection with more than one flavivirus was common in infected rats. Viral RNA was detected in serum, oral swab, and urine samples from rats infected with NrPV, but infectious virus could not be isolated.

In 2015, a novel atypical porcine pestivirus (APPV) was isolated from porcine serum collected in the USA (Hause et al., 2015). Additional positive samples were identified in serum samples originating from five states and cross-reactive antibodies were detected in 94% of a collection of porcine serum samples, suggesting widespread distribution of APPV in swine herds in the USA. The polyprotein sequence of this isolate showed 68% identity to the bat pestivirus (RaPestV-1) polyprotein and approximately 25 to 28% identity to other known pestivirus polyproteins. Phylogenetic analysis revealed that the divergence between APPV and RaPestV-1 was similar or greater than between BVDV, CSFV, and BDV. These two isolates were thus proposed as novel pestivirus species. Several studies recently demonstrated that APPV is a prominent cause of congenital tremors in newborn piglets (Arruda et al., 2016; Postel et al., 2016).

In 2017, Lamp et al. described a novel pestivirus named Linda virus (LV), which was isolated from serum samples of piglets with congenital tremors (Lamp et al., 2017). These animals originated from a farm in Southeastern Austria that had reported major piglet losses from congenital tremors. Comparison of the polyprotein sequences yielded an amino acid identity of 69% between LV and Bungowannah virus and of less than 54% with all known pestiviruses. In contrast to APPV, which hardly infects cultured cells, LV could be easily propagated on porcine cells without the need for adaptation, similar to what has been reported for Bungowannah virus. Furthermore, the identification of a strong reactivity against LV-infected

cells when using a BVDV E2-specific monoclonal antibody (6A5) indicated the existence of cross-reactivity between these two species.

In summary, the ICTV currently recognized four species within the genus *Pestivirus*: BVDV-1, BVDV-2, CSFV, and BDV (Simmonds et al., 2017). Additionally, a growing number of related, but as yet unclassified, viruses isolated from various domestic and wild animal species has been described including pronghorn virus, Bungowannah virus, giraffe pestivirus, 'HoBi'-like pestiviruses, Aydin-like pestiviruses, rat pestivirus, bat pestivirus, atypical porcine pestivirus, Tunisian sheep pestiviruses, and Linda virus.

Viral Genome

BVDV has a single strand of positive-sense nonpolyadenylated RNA, which is approximately 12.3 kb in length and contains a single large open reading frame (ORF) encoding about 3,900 amino acids and two UTRs at the 5' and 3' ends (Collett et al., 1988c). The 5' UTR is a highly conserved sequence (370-390 nucleotides), which contains an internal ribosome entry site (IRES) that mediates cap-independent translation of the viral polyprotein (Poole et al., 1995). The 3' UTR (185-270 nucleotides) comprises single-stranded regions and stem-loop structures that play essential roles during viral RNA replication (Yu et al., 1999). In addition, the UTRs can serve as binding determinants for a set of cellular proteins and are believed to coordinate the switch between viral translation and replication (Isken et al., 2003). Translation of the ORF yields a polyprotein that is cleaved by cellular and virus-encoded proteases into 12 mature structural (C, E^{rns}, E1, E2) and nonstructural (N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) viral proteins (Collett et al., 1988b). The organization of protein-coding regions along the genomes of BVDV and flaviviruses were shown to be very much alike (Collett et al., 1988a). Furthermore, comparison of the hydropathic profiles of the viral polyproteins revealed

pronounced similarities between BVDV and members of the *Flaviviridae* family (Collett et al., 1988a). However, two viral proteins, N^{pro} and E^{rns}, were found to be unique to the genus *Pestivirus*. These proteins are implicated in inhibition of the host innate immune response and thus participate in establishment and maintenance of persistent infection (Tautz et al., 2015). When administered to pregnant cows, BVDV isolates missing both proteins replicated only at low levels and had lost the capacity to cause persistent fetal infection (Meyers et al., 2007).

Viral Proteins

The first protein in the polyprotein, N^{pro} (23 kDa), is a nonstructural autoprotease producing its own C-terminus and thereby the N-terminus of the core (C) protein (Wiskerchen et al., 1991). The N^{pro} protein has been shown to be dispensable for viral replication but plays an important role in establishment and maintenance of viral persistence, most likely due to its capacity to interfere with the innate immune system of the host (Meyers et al., 2007; Tratschin et al., 1998). The N^{pro} protein has been reported to block the host cellular type I interferon (IFN) response to different stimuli, including virus infection and treatment with double-stranded (ds) RNA (Gil et al., 2006; Ruggli et al., 2005).

The nucleocapsid C protein (14 kDa) and the three envelope glycoproteins E^{rns} (gp44/48), E1 (gp33), and E2 (gp55) represent the structural proteins of BVDV. All three glycoproteins form disulfide-linked complexes and are involved in virus attachment and entry into target cells. The E^{rns} protein forms a homodimer, whereas E2 forms a homodimer and a heterodimer with E1. The E1-E2 heterodimers are essential for viral entry and infectivity (Ronecker et al., 2008). The E1 (25-33 kDa) and E2 (53-55 kDa) proteins contain transmembrane domains, whereas the E^{rns} protein binds to cell surface glycosaminoglycans and is secreted in considerable amounts into the extracellular space (Iqbal et al., 2000; Rumenapf et al., 1993). The C protein is flexible in length

(86-89 amino acids) and its sequence can be modified in several regions (Riedel et al., 2010). The E^{rns} protein (42-48 kDa) has the unique feature of possessing intrinsic ribonuclease (RNase) activity that can inhibit the production of type I IFN and assist in establishment of persistent infection (Iqbal et al., 2004). The E2 protein is a highly variable, immunologically-dominant glycoprotein and the main target of neutralizing antibodies and cytotoxic T-lymphocytes (Weiland et al., 1990). The E^{rns} protein also induces a neutralizing antibody response (Weiland et al., 1992). The E2 protein contains several domains arranged in a linear fashion (El Omari et al., 2013). Most antigenic regions map to two membrane domains, which are most distal from the viral membrane and thus are likely to be the most exposed on the virus surface (El Omari et al., 2013; Li et al., 2013). The E2 protein is also responsible for species tropism (Liang et al., 2003).

Most nonstructural proteins (NS3-NS5B) are required for viral replication, but these proteins are also involved in virion morphogenesis. The p7 protein (7 kDa) is cleaved inefficiently from the C-terminus of E2 by host signal peptidase (Harada et al., 2000). This small protein is essential for virion assembly and forms a leader sequence to properly orient NS2 in the membrane (Agapov et al., 2004). The NS2 protein (40 kDa) encompasses a cysteine protease domain that catalyzes the cleavage between NS2 and NS3 (Lackner et al., 2004). The NS3 protein (80 kDa) is a multifunctional enzyme with helicase, serine protease, and NTPase activities that is essential for RNA replication (Wiskerchen & Collett, 1991). This serine protease and its cofactor NS4A (10 kDa) are responsible for all cleavages downstream of NS3 (Xu et al., 1997). The uncleaved NS2-3 (120 kDa) polypeptide cannot functionally replace NS3, but plays a critical role in virion morphogenesis (Agapov et al., 2004). No function has been ascribed to NS4B (35 kDa). The NS5A protein (58 kDa) is a phosphoprotein with RNA binding activity, which has an important, but not completely clarified, function in viral RNA replication (Isken et

al., 2014). The NS5B protein (77 kDa) is the viral RNA-dependent RNA polymerase, which catalyzes the asymmetric accumulation of positive-strand RNA over negative-strand RNA (Zhong et al., 1998).

Viral Biotype

A remarkable and unique feature of pestiviruses is the existence of two biotypes, which can be differentiated according to their effect on cultured cells. Cytopathic (CP) viruses produce cytoplasmic vacuolization and cell death via apoptosis within two to three days of inoculation while noncytopathic (NCP) viruses do not induce macroscopic morphological changes (Lee & Gillespie, 1957; Underdahl et al., 1957). Biotype does not correlate with virulence in vivo as BVDV-2 viruses associated with severe hemorrhagic syndrome are NCP. Furthermore, most acute BVDV infections with CP and NCP isolates are associated with mild clinical symptoms.

Acute infection with a NCP virus during the first trimester of pregnancy may lead to the establishment of persistent fetal infection (Kendrick, 1971). Effector cells directed against BVDV are believed to be removed from the fetus when infection occurs at this stage of pregnancy, prior to full establishment of immunocompetence. Importantly, viral persistence is restricted to NCP viruses and there appears to be a mechanism within the fetus that is effective at preventing persistent infection with CP viruses (Brownlie et al., 1989; Charleston et al., 2001).

The CP biotype was shown to strictly correlate with the appearance of large amounts of free NS3 protein in cultured cells. These high levels of NS3 result from the introduction of a cleavage site into the NS2-3 polypeptide or a second copy of the NS3 coding sequence (Meyers et al., 1992; Tautz et al., 1993). The overexpression of NS3, which is regarded as molecular marker for CP viruses, allows efficient RNA amplification and leads to deregulated viral replication. It has been suggested that accumulation of viral RNA in CP infected cells could

activate the ds RNA-activated protein kinase and initiate apoptosis (Vassilev & Donis, 2000). In addition, the serine protease activity of NS3, but not the one of NS2-3, was linked to apoptosis induction (Gamlen et al., 2010).

In contrast, the NS2-3 coding region is largely expressed in NCP isolates as an uncleaved polypeptide. Binding of cellular protein Jiv to uncleaved NS2-3 was found to be a prerequisite for NS3 release (Lackner et al., 2005). Because very low levels of Jiv are available in cultured cells, a strong decline in NS3 expression is observed in NCP infected cells as early as nine hours after infection (Lackner et al., 2004). This results in massive downregulation of viral replication, which is crucial for viral persistence.

The NCP viruses were shown to inhibit cell death and IFN synthesis induced by ds RNA or infection with other viruses (Diderholm & Dinter, 1966; Schweizer & Peterhans, 2001). In contrast, CP viruses induce strong type I IFN response in fetuses and cultured cells, suggesting that CP viruses have lost the capacity to control the host innate immune response (Adler et al., 1997; Charleston et al., 2001). However, it remains unknown why strong type I and type II IFN responses were observed in calves acutely infected with NCP viruses (Charleston et al., 2002).

In summary, key parameters for viral persistence include evasion of the adaptive immune response due to fetal infection during the first trimester of pregnancy, inhibition of the innate immune response by N^{pro} and E^{rns}, and limited RNA replication of NCP viruses.

Viral Replication

Viral replication starts four to six hours after infection of the cell and is initiated by receptor-mediated endocytosis involving the viral glycoproteins E^{rns} and E2, and more than one cell surface molecule. The glycoprotein CD46, which belongs to the regulators of complement activation protein family, has been shown to function as a cellular receptor for BVDV (Maurer et

al., 2004). Viral replication takes place in contact with membranes of the endoplasmic reticulum and most viral proteins, except for N^{pro}, are believed to be associated with intracellular membranes either directly or indirectly via viral membrane-bound interaction partners. Similar to other RNA viruses, most nonstructural proteins serve central functions in viral replication and the minimal part of the viral polyprotein required for autonomous RNA replication encompasses the NS3-NS5B region (Behrens et al., 1998). The genomic sequence immediately downstream of the IRES and the N-terminus of NS3 are also critical for viral replication (Tautz & Thiel, 2003).

Infection studies suggested that BVDV follows a replication strategy similar to what has been described for other positive-strand RNA viruses. Following synthesis and maturation, the viral nonstructural proteins associate with RNA genome and host factors to form replication complexes (Y. H. Gong et al., 1996). Concomitant with the translation of the viral genome, these complexes catalyze the transcription of complementary negative-strand RNA molecules, which act as templates for the synthesis of novel positive-strand RNA molecules. In addition to its function as messenger RNA, the viral genome acts as a template for RNA replication. Viral replication occurs exclusively in cytoplasm and proceeds in an asymmetric manner. The synthesis of positive-strand RNA increases rapidly after infection, whereas the synthesis of negative-strand RNA remains constant (Y. H. Gong et al., 1996).

It has been suggested that the initial site of viral replication is within the oronasal mucosa, particularly the palatine tonsil, and from there the spread is systemic either as free virus in serum or via infected leukocytes (Brownlie, 1991). It is also possible that BVDV spreads from tonsils and gut-associated lymphoid tissue and mucosa by lymphatics (Liebler-Tenorio et al., 1997). Although BVDV can infect a wide range of cell types, there is an apparent predilection for

lymphocytes, mononuclear phagocytes, and epithelial cells (Ohmann, 1983). Cytopathic viruses also appear to have a particular tropism for gut-associated lymphoid tissue (Liebler et al., 1991).

Virion

Pestiviruses are amongst the smallest enveloped animal RNA viruses, displaying a spherical shape and measuring 40 to 60 nm in diameter with a capsid of about 25 nm in diameter (Gray & Nettleton, 1987; Laude, 1979). Pestiviruses share these traits with other flaviviruses. The sedimentation coefficient and buoyant density in sucrose of pestiviruses were estimated to be between 140 and 150S and 1.10 and 1.15 g/ml, respectively. Virion infectivity is stable over a relatively broad pH range, but temperatures greater than 21°C and organic solvents and detergents rapidly inactivate these viruses (Depner et al., 1992).

Pestivirus virions are thought to arise by budding of the C protein and associated genomic RNA into the lumen of modified endoplasmic reticulum structures that are studded with viral structural proteins (Gray & Nettleton, 1987). After budding, pestivirus virions are transported by the host cell secretory pathway from the Golgi compartment to the cell surface, where they are released by exocytosis (Schmeiser et al., 2014). The nonstructural proteins p7, NS2-3, and NS5B were also found to be involved in virion assembly (Ansari et al., 2004; Harada et al., 2000). Therefore, structural and nonstructural viral proteins participate in pestivirus virion morphogenesis as described for other flaviviruses (Murray et al., 2008). Pestivirus virion morphogenesis remains poorly understood and it is currently unknown how genomic RNA is incorporated into virions and how nonstructural proteins contribute to virion assembly.

Clinical Manifestations

BVDV infections may range from clinically inapparent to severe systemic disease. Acute infection with avirulent BVDV usually results in fever, anorexia, and leukopenia. In contrast,

some highly virulent isolates cause a peracute disease termed hemorrhagic syndrome, which is characterized by fever, diarrhea, hemorrhage, leukopenia, thrombocytopenia, and death (Corapi et al., 1990b). However, viral virulence may not be the only factor affecting the outcome of BVDV infections. For instance, the presence of secondary pathogens and concurrent environmental stress may alter the clinical presentation. Clinical signs in acutely infected calves were also found to decrease as the levels of maternal antibodies increased (Bolin & Ridpath, 1995). Similarly, high maternal antibody titers appear able to the prevent transplacental transmission of BVDV. In contrast, infection of naïve pregnant cattle with BVDV usually results in fetal infection (Gillespie et al., 1967; Scott et al., 1972).

BVDV has been associated with teratogenic and lethal effects on the bovine fetus including early embryonic death, fetal mummification, congenital defects, growth retardation, stillbirth, and abortion (Kahrs et al., 1970; Ward et al., 1969). The clinical outcome of intrauterine BVDV infections is strongly influenced by the gestational age of the fetus. Early embryonic death with complete resorption may occur after viral infection in the very early stages of gestation, before the skeleton has fully developed. A prolonged decrease in conception rate has been described following introduction of BVDV in some herds (Houe & Meyling, 1991). Abortion is usually the consequence of infection during the first two trimesters of pregnancy. The period that elapses between viral exposure and abortion is variable, but the minimum period is usually three weeks (Van Oirschot, 1983). Aborted fetuses are often autolyzed, which makes the presence of virus or lesions difficult to confirm. Occasionally, edema of the subcutaneous tissues, excessive fluid accumulation in the body cavities, and reticuloendothelial hyperplasia of the spleen and lymph nodes have been described in aborted fetuses (Casaro et al., 1971; Gillespie et al., 1967). Fetuses, which die in utero in early gestation, may become mummified due to the

resorption of fluid. Stillbirth is usually the consequence of fetal death in the second and third trimesters of pregnancy. Fetal death can occur secondary to viral invasion, but damage to the maternal vascular endothelium may also contribute by disrupting the vascular supply of nutrients (Barlow, 1972). When the fetus survives the initial infection a whole spectrum of abnormalities can develop including malformations, growth retardation, and pathological lesions. Bovine fetuses exposed to BVDV between three and six months of gestation often show central nervous system and ocular malformations, whereas infections after the sixth gestational month usually do not result in fetal abnormalities (Brown et al., 1973; Casaro et al., 1971; Kendrick, 1971; Scott et al., 1973). Cerebellar hypoplasia, hydranencephaly, and hypomyelination of the brain and spinal cord have been frequently described in congenital pestivirus infections (Done et al., 1980). The most common ocular defects are retinopathy and cataracts (Scott et al., 1973). Congenital pestivirus infections can also cause thymic hypoplasia, brachygnathia, and alopecia (Done et al., 1980; Kendrick, 1971).

When infection with a NCP isolate occurs during the first trimester of pregnancy, the immature fetal immune system may develop a highly specific immunotolerance to the infecting virus, resulting in life-long viral persistence in this animal (Kendrick, 1971). The virus can be incorporated into the repertoire of host antigens because the fetal immune system is usually not fully developed at this stage of pregnancy. Therefore, PI animals do not exhibit detectable antibody or T-cell responses to the infecting NCP virus (Collen et al., 2000). However, these animals are immunocompetent because they can resolve infections with antigenically dissimilar BVDV isolates or other viruses including bovine herpesvirus-1 (BHV-1) and parainfluenza-3 virus (Bolin et al., 1985b; McClurkin et al., 1984). PI animals seem to be predisposed to respiratory and enteric diseases and have an increased risk of dying or being slaughtered due to

unthriftiness. A cohort study in ten Danish dairy herds revealed that 29 of 34 PI animals (85%) had died or had been slaughtered before the age of two years (Houe, 1993).

After efficient viral replication for about one to two years, PI animals may develop MD. These animals typically suffer from severe fibrinous enteritis resulting in death within two to three weeks (Ohmann, 1983). Pathological findings are characterized by lymphoid depletion and severe erosive to ulcerative lesions of the alimentary tract. The trigger for the onset of this highly fatal disease is the appearance of an antigenically similar or identical CP viral variant (Brownlie et al., 1984). Although superinfection with a CP virus antigenically similar to the infecting NCP virus can induce MD, CP viruses usually emerge de novo in PI animals by RNA recombination of the infecting NCP virus (Howard et al., 1987). RNA recombination between the infecting NCP virus and a vaccine CP virus has also been reported and can be associated with a delayed onset of MD (Ridpath & Bolin, 1995).

Serological and virological studies have demonstrated that BVDV is commonly associated with respiratory disease in young calves and feedlot cattle (Fulton et al., 2000; Loneragan et al., 2005; Potgieter et al., 1984b; Richer et al., 1988; Rosenquist & Dobson, 1974; Wilhelmsen et al., 1990). Pulmonary lesions resulting from acute experimental infections consisted of mild interstitial pneumonia and tracheitis. Acute BVDV infections have been shown to cause a transient decrease in the absolute number of circulating B- and T-cells and in the percentage of circulating T-cells (Bolin et al., 1985a). BVDV was also found to impair lymphocyte and polymorphonuclear leukocyte function and to suppress IFN production and monocyte chemotactic response (Diderholm & Dinter, 1966; Ketelsen et al., 1979; Muscoplat et al., 1973; Roth et al., 1981). This immunosuppression is thought to impair bacterial clearance from the respiratory tract and to enhance disease caused by other viral pathogens including

BHV-1 and bovine respiratory syncytial virus (Pollreisz et al., 1997; Potgieter et al., 1984a; Potgieter et al., 1984b).

BVDV can also infect ovarian and testicular tissues and can be isolated from semen of acutely and persistently infected bulls (Coria & McClurkin, 1978; Whitmore et al., 1978). Interestingly, an immunocompetent postpubertal bull was found to be persistently shedding BVDV in semen over a period of 11 months, while demonstrating no viremia (Voges et al., 1998). A subsequent study revealed that the virus can be shed in the semen of postpubertal bulls for up to five months after initial exposure to BVDV (Givens et al., 2003).

Host Range

Although the natural host of BVDV is cattle, it can infect and be isolated from pigs and diverse domestic and wild ruminant species, including alpacas, bongos, Canadian bisons, buffaloes, Bactrian camels, dromedaries, mouse deer, mule deer, red deer, reindeer, roe deer, sika deer, white-tailed deer, elands, elks, domestic goats, mountain goats, llamas, pudus, bighorn sheep, domestic sheep, water buffaloes, wildebeests, and yaks (Anderson & Rowe, 1998; Becher et al., 1999; Craig et al., 2008; Deregt et al., 2005; Doyle & Heuschele, 1983; Fernelius et al., 1973b; Fraser et al., 1981; S. Gao et al., 2013; Y. Gao et al., 2011; X. Gong et al., 2014; Goyal et al., 2002; Grondahl et al., 2003; Martucciello et al., 2009; Motha & Tham, 1992; Nelson et al., 2015; Nettleton et al., 1980; Paton et al., 1992; Pizarro-Lucero et al., 2005; Romvary, 1964; Tessaro et al., 1999; Van Campen et al., 2001; Van Campen et al., 1997; Wolff et al., 2016; Yousif et al., 2004).

Experimental studies demonstrated that acutely infected pigs, camelids, deer, and small ruminants typically do not show obvious signs of illness (Johnson et al., 2010; Loken & Bjerkas, 1991; Stewart et al., 1980; Van Campen et al., 1997; Ward, 1971; Wentz et al., 2003). However,

abortion, fetal mummification, stillbirth, and fetal malformations have been reported in pregnant camelids, goats, sheep, and swine infected with BVDV (Belknap et al., 2000; Goyal et al., 2002; Loken & Bjerkas, 1991; Plant et al., 1976; Wensvoort & Terpstra, 1988). Congenital persistent infections have been described in alpaca, mouse deer, mule deer, white-tailed deer, eland, domestic goats, mountain goats, sheep, and swine (Carman et al., 2005; Duncan et al., 2008; Gard et al., 1976; Grondahl et al., 2003; Loken & Bjerkas, 1991; Nelson et al., 2008; Passler et al., 2007; Terpstra & Wensvoort, 1997; Vilcek et al., 2000). Diarrhea, pneumonia, weight loss, failure to thrive, and poor viability have been reported in these PI animals (Belknap et al., 2000; Chase et al., 2008; Gard et al., 1976; Loken & Bjerkas, 1991; Mattson et al., 2006; Nelson et al., 2008; Terpstra & Wensvoort, 1997).

Natural infection of swine with BVDV was first reported in Australia in 1964, but BVDV was first isolated from a naturally infected sow in 1973 (Fernelius et al., 1973a; French & Snowdon, 1964). The sow originated from a farm in Iowa, in which pigs were kept in close proximity to cattle. This BVDV isolate was shown to be extremely virulent for neonatal calves and four serial passages in swine did not attenuate its virulence for cattle (Fernelius et al., 1973a). Virus was isolated from nasal secretions and feces of experimentally infected pigs, suggesting that acute infections in swine may contribute to virus transmission.

Pregnant sows exposed to BVDV were found to give birth to completely or partially-infected litters (Paton & Done, 1994). These results suggested that not all fetuses were infected simultaneously. It is possible that one or more fetuses may become initially infected with subsequent spread to adjacent fetuses, which has been reported with porcine parvovirus (Bachmann et al., 1975; Redman et al., 1974). Interestingly, infected piglets were found to be either chronically or persistently infected. Chronically infected piglets were viremic for several

weeks, but ultimately seroconverted. This was associated with an immediate clearance of the virus from serum, but delayed clearance from the tissues. Similar findings have been reported in pregnant sows exposed to CSFV (Van Oirschot, 1979). The earlier the sows were infected with CSFV during pregnancy, the more infected piglets were born. Furthermore, the earlier the infection occurred, the greater was the number of PI piglets born in infected litters. Lambs congenitally infected with BDV that seroconverted and cleared the virus later in life have also been described (Nettleton et al., 1992; Westbury et al., 1979).

Antigenic Variability

While CSFV isolates were found to be antigenically closely related, high antigenic heterogeneity was demonstrated among BVDV isolates using neutralization and binding assays (Bolin et al., 1988; Corapi et al., 1990a; Deregt et al., 1998a; Deregt et al., 1998b; Edwards et al., 1988; Paton et al., 1995). Bolin et al. demonstrated that ten BVDV-1 isolates could be segregated into four distinct groups using nine monoclonal antibodies (MAbs) generated against the E2 protein (Bolin et al., 1988). Using a panel of 40 MAbs, Corapi et al. (1990a) identified 32 distinct binding patterns among 70 BVDV isolates. These studies also revealed that most neutralizing antibodies raised against the E2 protein bind to a single immunodominant domain for BVDV-1 isolates, whereas neutralizing epitopes mapped to three antigenic domains for BVDV-2 isolates (Deregt et al., 1998a; Deregt et al., 1998b).

Furthermore, large antigenic differences and distinct MAb binding patterns were demonstrated between BVDV-1 and BVDV-2 isolates (Pellerin et al., 1994; Ridpath et al., 1994; Ridpath et al., 2000). Ridpath et al. demonstrated that most MAbs generated against the E2 protein of BVDV-1 isolates were unable to bind BVDV-2 isolates and vice versa (Ridpath et al.,

1994; Ridpath et al., 2000). These studies also revealed that MAb binding patterns were more heterogeneous among BVDV-1 isolates than among BVDV-2 isolates.

Following an acute infection with a BVDV-1 isolate, neutralizing antibody titers raised against other BVDV-1 isolates are typically several-fold greater than against BVDV-2 isolates. Isolation of BVDV-2 viruses from PI calves born to dams vaccinated against BVDV-1 suggested that these antigenic differences were clinically relevant (Bolin et al., 1991; Ridpath et al., 1994; Van Campen et al., 2000). Similarly, modified live BVDV-1 vaccines were demonstrated to provide better fetal protection in pregnant cattle exposed to BVDV-1 (83%) compared to BVDV-2 (58%) (Brock & Cortese, 2001; Cortese et al., 1998). Furthermore, modified-live BVDV-1a vaccines were found to induce lesser neutralizing antibody titers against BVDV-1b isolates than against other BVDV-1a isolates (Fulton et al., 2003; L. Jones et al., 2001). These lesser titers may also result in reduced fetal protection against BVDV-1b infections.

Genetic Variability

As mentioned previously, BVDV isolates circulating in cattle populations worldwide are genetically highly diverse and at least 21 BVDV-1 subgenotypes (BVDV-1a to -1u) and four BVDV-2 subgenotypes (BVDV-2a to -2d) have been described to date (M. Deng et al., 2015; Flores et al., 2002; S. Gao et al., 2013; Giammarioli et al., 2015; Giangaspero & Harasawa, 2004; Jackova et al., 2008; Nagai et al., 2008; Vilcek et al., 2004; Vilcek et al., 2001; F. Xue et al., 2010). This variability is unevenly distributed throughout the viral genome. The 5' UTR and NS3 coding region are highly conserved regions, whereas the E2 and NS2 coding regions are the most variable sequences of the viral genome (R. Deng & Brock, 1992).

Genetic variability in RNA viruses result from three different processes: (i) accumulation of point mutations resulting from the error-prone nature of the viral RNA-dependent RNA polymerase, (ii) nonhomologous RNA recombination, and (iii) homologous RNA recombination.

Assuming a mutation rate around 10^{-4} substitutions per site per replication cycle, similar to what has been reported for other RNA viruses, one mutation is estimated to occur per BVDV replication cycle. This high mutation rate results in the generation of multiple distinct, but closely related, genetic viral variants. The term quasispecies is used to refer to this swarm of mutants clustered around the base sequence of the parent virus (Domingo et al., 2012). This ability to mutate rapidly allows the viral population to quickly adapt to the host immune response and produce variants that replicate and infect the host cells more efficiently than the rest of the viral population. Only variants with a selective advantage and without deleterious mutations will ultimately progress during the course of an infection.

Two main mechanisms have been proposed for viral RNA recombination: (i) replicative template-switching, also known as copy-choice, and (ii) non-replicative breakage and rejoining. The first model predicts that during replication the viral RNA polymerase pauses RNA synthesis and eventually jumps to a different region of the same template or to another template to continue RNA synthesis. Experimental studies have demonstrated that the BVDV RNA polymerase can switch templates during RNA synthesis (Kim & Kao, 2001). The existence of viral replication-independent RNA recombination has also been described (Gallei et al., 2004). Analysis of the viral recombinants obtained in this study revealed that the crossover sites were unique and thus did not result from sequence-specific reactions.

The molecular analysis of CP isolates has revealed a broad spectrum of genomic alterations including deletions and duplications of viral sequences and insertions of viral and

cellular mRNA fragments (Meyers et al., 1991; Meyers et al., 1992). Importantly, almost all these genomic rearrangements resulted from nonhomologous RNA recombination events. Furthermore, the identification of a large variety of recombined viral RNAs within PI cattle showed that RNA recombination represents a continuous process in these animals (Desport et al., 1998). Even in individual PI calves with MD, a variety of related CP viruses could be identified, resulting from different duplications or deletions of viral sequences (Fricke et al., 2001). Homologous RNA recombination has also been described between BVDV-1 and BVDV-2 viruses (Ridpath & Bolin, 1995). A recent study identified five recombinants among 62 BVDV-1 isolates and confirmed that homologous recombination events were frequent in the evolution of BVDV and occurred between viruses of the same or different subgenotypes (Kovago et al., 2016).

It is well known for other RNA viruses that viral persistence is associated with genetic variability, including hepatitis C virus (HCV), foot-and-mouth disease virus (FMDV), and human immunodeficiency virus (HIV) (Coffin, 1995; Enomoto et al., 1996; Gebauer et al., 1988). Initially, the comparison of partial sequences of viruses isolated from PI cattle at different time points failed to detect any nucleotide substitutions (Hertig et al., 1995; Paton et al., 1994). Subsequently, multiple amino acid differences in the E2 coding region were identified between viruses isolated from two PI calves at 11- and 18-month intervals (Collins et al., 1999). In this study, amino acid variation was also observed between individual PCR clones obtained from the same sample, suggesting the existence of a viral quasispecies in PI animals. The interclonal variation was greater in the E2 coding region compared to the NS3 coding region. Interestingly, this variation also increased with age with seven positions affected by substitutions in the E2 coding region in the earlier samples compared to 15 positions in the later samples. Furthermore,

the existence of a 5' UTR quasispecies was later demonstrated in a BVDV isolate obtained from multiple tissues of an infected bovine fetus (L. R. Jones et al., 2002).

A recent study confirmed the presence of minor differences among consensus sequences of viruses that were isolated from 20 PI cattle at different time points (Ridpath et al., 2015). This study also revealed marked differences in the level of variation at each amino acid position between viral populations circulating in serum of PI cattle. Similarly, different levels of intrahost viral variability have been reported in PI cattle (Chernick et al., 2018; Dow et al., 2015). The greatest variation was observed in genomic regions that were encoding structural proteins or were not associated with known vital functions. Interestingly, the overall pattern of variation was consistent between cattle infected with the same subgenotype and a higher level of variation was observed among cattle infected with BVDV-1b than BVDV-1a (Chernick et al., 2018). Furthermore, one of these studies demonstrated the tissue compartmentalization of the BVDV genome in PI cattle, as previously reported in patients infected with HCV (Dow et al., 2015).

Acute BVDV infections are believed to favor survival of viral variants that can escape the host immune response and thus to contribute to the genetic variability of BVDV. Using MAb binding assays, epitope changes have been detected in calves acutely infected with BVDV (Bolin & Ridpath, 1992). Sequence comparison of isolates from 15 PI calves that were born to cows exposed to the same inoculum revealed that up to four nucleotide substitutions occurred in the E2 coding region during the establishment of persistent infections (Stokstad et al., 2004). A subsequent study showed that the majority of the changes observed in isolates from PI calves could be detected in isolates from their acutely infected dams, suggesting that these changes were established before the virus crossed the placenta (Neill et al., 2012). In this study, most amino acid substitutions occurred in genomic regions encoding structural viral proteins,

suggesting that infection of pregnant cattle with BVDV may play an important role in the generation of antigenic variation. Furthermore, greater numbers of substitutions were detected during acute infections of pregnant cattle than of non-pregnant cattle. Altogether, these results suggested than infection of pregnant cattle with BVDV may serve as a significant source of viral genetic and antigenic variability.

As BVDV does not possess strict host specificity, cross-species infections provide a further opportunity for viral diversification. Amino acid substitutions have been detected in regions encoding structural and nonstructural viral proteins during the establishment of persistent infections in goats (Bachofen et al., 2013; Passler et al., 2014a). Furthermore, one or more host-specific antigenic changes have been observed during serial infection of pregnant cattle and sheep with BVDV (Gunn et al., 1992; Paton et al., 1997). Surprisingly, these changes sometimes required several months of virus replication in PI lambs to be detected. Antigenic differences have also been detected between BVDV isolates from pigs and cattle that were kept on the same farm (Paton et al., 1992).

Transmission

BVDV can be transmitted continuously by PI animals or transiently by acutely infected animals. PI animals are considered the most important source of infection because they shed large amounts of virus in most excretions and secretions, including nasal secretions, saliva, urine, feces, tears, milk, and semen (Coria & McClurkin, 1978). In acutely infected animals, virus is usually isolated from blood and nasal secretions for up to ten days after exposure (Brock, 1995).

Horizontal transmission can occur by direct or indirect contact. Nose-to-nose contact between susceptible and PI animals is the most efficient means of transmission, one hour of direct contact being sufficient for transmission to occur (Traven et al., 1991). Direct contact with

acutely infected animals can also transmit the virus, but less efficiently (Niskanen et al., 2000; Sarrazin et al., 2014). This is likely due to the shorter duration of infection and the intermittent shedding of relatively lesser amounts of virus. Indirect transmission has also been attributed to the use of BVDV-contaminated vaccines or fomites including hypodermic needles, nose tongs, and rectal sleeves (Lang-Ree et al., 1994; Lohr et al., 1983; Roeder, 1994). Calves housed in a pen directly after removal of a PI calf, but not those that entered the pen four days later, were demonstrated to become infected with BVDV (Niskanen & Lindberg, 2003). In fact, BVDV was shown to survive in slurry for three weeks at 5°C and for three days at 20°C under controlled conditions (Botner & Belsham, 2012). Transmission of BVDV by biting insects has been demonstrated in cattle (Tarry et al., 1991). However, hematophagous flies did not appear to be an important vector for transmission in a recent study (Chamorro et al., 2011).

Vertical transmission can occur in pregnant dams acutely or persistently infected with BVDV. Interestingly, calves delivered from PI dams have always been demonstrated to be PI (McClurkin et al., 1979; Straver et al., 1983). Vertical transmission via semen from PI bulls has also been reported (Meyling & Jensen, 1988). In this study, all heifers became pregnant and gave birth to clinically normal calves, of which one was persistently infected with BVDV. Embryo transfer can constitute an additional means of transmission to valuable breeding stock (Houe, 1995). The virus can be transmitted to the fetus if the donor is acutely or persistently infected with BVDV and the embryo is not adequately washed. Vertical transmission can also occur if the recipient is infected with BVDV or if fetal bovine serum used to wash the embryo is contaminated with BVDV.

Transmission of BVDV to species other than cattle is believed to be caused by close contact with PI cattle. For instance, seven pregnant white-tailed deer housed with two PI cattle in

a 0.8 ha pen for 60 days became infected with BVDV, resulting in the birth of PI fawns (Passler et al., 2009). Several studies have also demonstrated an association between the seroprevalence of BVDV in swine and small ruminants and the presence of cattle. In a study, sow herds with cattle on the premises had 3.4 higher odds of having animals seropositive to BVDV compared to herds without cattle (Loeffen et al., 2009). Another study revealed that 27% of sheep housed with cattle were seropositive to BVDV, whereas only 14% of sheep kept separate from cattle were seropositive to BVDV (Braun et al., 2013).

Experimental studies have demonstrated that acutely infected alpaca, elk, mule deer, white-tailed deer, sheep, and swine can shed virus in nasal or oral secretions and/or feces (Johnson et al., 2010; Negron et al., 2012; Tessaro et al., 1999; Van Campen et al., 1997; Wieringa-Jelsma et al., 2006). These animals may thus participate in intra- and interspecies transmission of BVDV. Indirect transmission between acutely infected white-tailed deer fawns and naïve calves has been reported (Ridpath et al., 2009). However, transmission was not shown to occur between acutely infected sheep and naïve sheep that were kept in the same paddock (Evans et al., 2017b). Transmission to cattle has also been shown to occur from mouse deer, goats, and sheep that were persistently infected with BVDV (Gunn et al., 1992; Loken, 1994; Passler et al., 2014b; Uttenthal et al., 2006). PI animals are also likely to participate in intraspecies transmission of BVDV as previously described in alpaca, mouse deer, white-tailed deer, goats, sheep, and swine (Bachofen et al., 2013; Byers et al., 2011; Evans et al., 2018; Passler et al., 2010; Paton & Done, 1994; Plant et al., 1977; Uttenthal et al., 2006).

The existence of mature PI animals able to reproduce and generate PI offspring has been reported in mouse deer at a zoo in Denmark (Uttenthal et al., 2006). PI pigs have also been reported to live for more than two years (Paton & Done, 1994; Terpstra & Wensvoort, 1997).

However, in most species, the survival rate of PI animals has been found to be low under natural and experimental conditions (Carman et al., 2005; Evans et al., 2015; Loken, 1994). Therefore, it has been suggested that PI cattle are the main reservoir for BVDV in these heterologous species (Loken, 1995).

Geographical Distribution and Economic Importance

BVDV is endemic in cattle populations of many countries worldwide, with 60 to 85% of cattle being antibody positive (Houe, 1999). Epidemiological studies have shown that various BVDV subgenotypes predominate in different countries. Recent studies suggested that BVDV-1b is the predominant subgenotype worldwide, followed by BVDV-1a and BVDV-1c (Yesilbag et al., 2017). The extensive genetic diversity of BVDV is reflected by the high number of subgenotypes circulating in China, Turkey, and several European countries.

BVDV is causing major economic losses to the cattle industry worldwide. Direct losses due to BVDV infections include increased morbidity and mortality due to immunosuppression, increased reproductive losses, and reduced milk yield (McGowan et al., 1993; Niskanen et al., 1995). Most estimations of total annual losses at the national level ranged between $10 and $40 million per million calvings (Houe, 2003). Direct losses due to BVDV in 15 countries varied considerably, ranging from $0.5 to $688 per animal (Richter et al., 2017). Direct losses per naïve dairy cow were on average $25 higher than per beef cow.

Diagnosis

The wide spectrum of clinical manifestations associated with BVDV infections complicates diagnosis and may result in infections being overlooked because of the lack of pathognomonic signs or lesions. When an infection is suspected, virological or serological tests

are necessary to confirm a clinical or pathologic diagnosis. Once a definitive diagnosis is made, a complete epidemiologic investigation can be performed to identify the source of infection.

Virological tests include virus isolation (VI) in susceptible cultured cells and various antigen-capture enzyme-linked immunosorbent assays (ACEs). Approved samples for ACE include serum, plasma, milk, and skin biopsy samples. Rapid identification of BVDV antigen in fixed or frozen tissues can also be performed using immunohistochemistry (IHC) or immunofluorescence. The E^{rns} and NS3 proteins are highly conserved among BVDV isolates and thus monoclonal antibodies targeting either or both proteins are commonly used in ACE and IHC procedures.

Following acute BVDV infection, virus is usually only isolated from blood and nasal secretions for three to ten days (Brock, 1995). In contrast, samples used to test PI animals may be taken from a wide range of tissues or organs. In cattle, optimum samples for VI are blood samples from which viable mononuclear cells can be harvested and lymphoid tissues including thymus, spleen, tonsils, mesenteric lymph nodes, and Peyer's patches.

Large amounts of BVDV antigen were detected in epidermis and hair follicles of PI animals and testing of skin biopsy samples by IHC or ACE was demonstrated to be an accurate and relatively inexpensive method for identifying PI animals (Kuhne et al., 2005; Njaa et al., 2000; Thur et al., 1996). Although VI has been the gold standard for the diagnosis of BVDV infections, this method typically requires four days of cell culture and subsequent viral identification by immunocytochemistry or immunofluorescence. Therefore, antigen-based methods are now routinely used for detection of PI cattle.

The presence of antibodies to BVDV in serum samples, which commonly occurs in young calves with high levels of colostrum-acquired antibodies, can cause a significant reduction

in serum viremia levels or block antigen detection (Brock et al., 1998; Palfi et al., 1993). Serum testing by VI or ACE is thus not recommended in animals less than three months of age.

BVDV antigen distribution in PI alpacas, mountain goats, sheep, and white-tailed deer has been demonstrated to be largely equivalent to that of PI cattle (Henningson et al., 2013; Nelson et al., 2008; Passler et al., 2012; Scherer et al., 2001). BVDV antigen was broadly distributed in many organs with greatest antigen staining in epithelial tissues. However, antigen staining was found to be less frequent and less intense in lymphatic and alimentary tissues in PI white-tailed deer than in PI cattle (Passler et al., 2012). Therefore, the collection of skin, liver, and reproductive organs appears most appropriate for BVDV diagnosis in this species. Similar findings have been reported in PI alpacas with significantly less extensive antigen distribution in nervous and alimentary tissues (Henningson et al., 2013). Therefore, skin, thyroid, thymus, lung, and brain appear to be useful diagnostic samples in alpacas. Furthermore, placenta, heart, thymus, and brain were shown to be reliable tissues for BVDV antigen detection in aborted goat fetuses (Lamm et al., 2009). Placenta, kidney, thymus, lung, and brain were also found to be suitable for BVDV diagnosis in lambs (Scherer et al., 2001).

Reverse transcriptase polymerase chain reaction (RT-PCR) assays are available to detect targeted region(s) of the BVDV genome. Virtually any sample obtained from an infected animal can be tested, including blood, milk, skin, saliva, and tissue samples. However, a single positive result does not define the clinical status of an animal because these assays can detect acutely and persistently infected animals as well as animals recently vaccinated with modified-live BVDV vaccines (Bhudevi & Weinstock, 2001). RT-PCR is a highly sensitive method that allows the use of pooled-sample testing as a herd-screening tool for detection of PI cattle (Munoz-Zanzi et al.,

2000). RT-PCR can also be used on bulk tank milk samples to detect the presence of PI animals within the milking herd (Radman et al., 1995).

A combined strategy including testing of skin biopsy samples by ACE or IHC and testing of whole blood by RT-PCR has the greatest likelihood of identifying animals that are acutely or persistently infected with BVDV. The current standard for definitive determination of PI status in cattle requires one of two criteria: (i) identification of BVDV in two separate serum or buffy coat samples with a minimum of 21 days between samples and (ii) identification of BVDV antigen by IHC on skin biopsy specimens, preferably repeated after 30 days. Large amounts of virus in serum and the lack of antibodies to BVDV and other pestiviruses have been suggested as additional criteria.

Seroconversion to BVDV generally occurs two to four weeks after acute infection and antibodies to BVDV typically persist for life (Fredriksen et al., 1999). Paired samples collected three to four weeks apart are thus required to identify a 4-fold increase in serum antibody titers. Serological tests can also be used to determine whether an animal has been properly immunized or whether an animal has been exposed to BVDV in utero. Virus neutralization (VN) is the most common serological method used for determining levels of antibodies to BVDV. Unfortunately, different reference strains can be used and thus VN titers may differ from laboratory to laboratory. Various ELISAs have been developed to evaluate antibody levels in serum and milk samples. The use of commercially available ELISA kits has reduced variation between laboratories because standard reagents and protocols are used.

The high level of antigenic cross-reactivity between pestivirus species can complicate the interpretation of serological test results. Most importantly, the presence of antibodies to BVDV in swine may affect serological surveillance of CSFV. In many CSFV-free countries, the

diagnosis of CSFV infections relies on combined interpretation of up to three VN test results. Neutralizing antibody titers against CSFV after an acute CSFV infection are typically at least 3-fold greater than against BVDV or BDV. However, pigs that have been previously exposed to BVDV were found to have much higher titers to BVDV than CSFV after an acute CSFV infection (Wieringa-Jelsma et al., 2006). These results suggested that CSFV infections may be misinterpreted as BVDV infections in pigs that have been exposed to BVDV.

The presence of antibodies to BVDV may also hamper CSFV control programs that involve vaccination with CSFV marker vaccines. These vaccines only elicit antibodies against the E2 protein of CSFV and thus natural CSFV infections are diagnosed when antibodies against the E^{rns} protein of CSFV are detected using an ELISA kit. However, the presence of antibodies to BVDV were found to give false-positive results when this kit was used (Loeffen et al., 2009).

Antigenic variability of BVDV can impact the diagnosis of BVDV infections. Gripshover et al. (2007) described a BVDV isolate with a unique mutation in the E^{rns} coding region that escaped detection by IHC and ACE. This isolate was detected using VI and RT-PCR, but remained undetectable using these two antigen-based methods. Indeed, these assays relied on a single Mab binding to the E^{rns} protein of BVDV. The results of this study suggested that multiple testing methods may be necessary to detect all PI animals. Similarly, amino acid substitutions in the E2 coding region were shown to be associated with failure to detect BVDV antigen by direct fluorescent antibody test in tissue samples from two cattle infected with BVDV (Yan et al., 2016).

The lack of species-specific reagents hampers the diagnosis of BVDV infections in species other than cattle. For instance, cell cultures derived from domestic species may not work for the propagation of viruses that are adapted to wild ruminants. Furthermore, most diagnostic

tests have not been validated in heterologous species and false-positive results with commercially available ACE kits have been reported in camelids (Kapil et al., 2009). When compared to VN, an ELISA kit used to detect antibodies against the NS3 protein was also found to have lower specificity in pigs (97.3%) than in cattle (99%) (Loeffen et al., 2009). However, a BVDV antibody ELISA kit has been reported to have 100% agreement with VN in white-tailed deer (Kirchgessner et al., 2013). A recent study also demonstrated that two ELISA kits can be successfully used in sheep for detection of antibodies to BVDV (Evans et al., 2017a). However, sensitivity and specificity values greater than 95% were only obtained when the manufacturer's cutoff thresholds were altered. These results confirmed that validated methods are needed to accurately identify BVDV infections in species other cattle.

Control and Eradication

Identification and elimination of PI animals, together with the implementation of biosecurity measures, are crucial for prevention and control of BVDV. Vaccination can represent an accompanying tool to prevent the birth of PI calves and limit clinical disease and viral transmission. However, its efficacy as a sole method of control is compromised by the lack of complete fetal protection and the antigenic heterogeneity among BVDV isolates. Vaccination is typically used when the risk of BVDV reintroduction is high.

Inactivated and modified-live virus vaccines are commercially available in the USA for prevention of clinical disease and/or fetal infection after exposure to BVDV. Onset of immunity is usually delayed four to six weeks from the initial administration of an inactivated vaccine. In contrast, reduced viremia and nasal shedding were demonstrated in calves challenged with BVDV five days after the administration of a modified-live virus vaccine (Brock et al., 2007; Palomares et al., 2012). Although the level of neutralizing antibodies required for fetal protection

is unknown, severity and duration of clinical disease were shown to decrease in calves with a titer of 16 or greater, and systemic viral spread was prevented in calves with a titer greater than 256 (Bolin & Ridpath, 1995). Recent studies have demonstrated fetal protection rates ranging from 85 to 100% following administration of modified-live BVDV vaccines (Givens et al., 2012; Leyh et al., 2011; W. Xue et al., 2011). In contrast, inactivated vaccines were found to provide significant, but variable levels of fetal protection (Grooms et al., 2007; Rodning et al., 2010). Interestingly, annual revaccination of pregnant cattle with an inactivated BVDV vaccine following two pre-breeding doses of modified-live virus vaccine has been demonstrated to provide greater fetal protection than annual revaccination with a modified-live virus vaccine (Walz et al., 2017).

The success of BVDV programs in Norway, Sweden, Finland, and Denmark demonstrated that eradication may be possible at a national level and has led to the development of several regional or national control programs in Lower Austria, France, Germany, Italy, Ireland, the Netherlands, Scotland, Slovenia, Switzerland, and the USA (Bitsch et al., 2000; Ferrari et al., 1999; Graham et al., 2014; Grom & Barlic-Maganja, 1999; Grooms et al., 2013; Hult & Lindberg, 2005; Joly et al., 2005; Loken & Nyberg, 2013; Mars & Van Maanen, 2005; Presi & Heim, 2010; Rikula et al., 2005; Rossmanith et al., 2010; Synge et al., 1999; Voas, 2012; Wernike et al., 2017).

Eradication of BVDV is considered feasible in areas where cattle density is low and vaccination is not common practice (Greiser-Wilke et al., 2003). Mandatory eradication programs typically rely on segregation of cattle population into non-infected and infected herds, removal of PI animals from infected herds, continuous monitoring of non-infected herds, and biosecurity measures to avoid reintroduction of the virus. In contrast, the aim of voluntary

BVDV programs in high density areas with high seroprevalence is to minimize economic losses by reducing virus circulation. These programs typically rely on identification and removal of PI animals from participating herds, biosecurity measures, and vaccination.

Free-living wild ruminants share range, feed, and water sources with range cattle and thus successful control programs rely on knowing whether these species can serve as reservoirs for BVDV. However, there is limited evidence to date demonstrating the introduction of BVDV into naïve cattle herds by exposure to wild ruminants. In the USA, the apparent prevalence of persistent BVDV infections in wild cervid populations ranged from 0.02 to 0.3% (Duncan et al., 2008; Passler et al., 2008; Pogranichniy et al., 2008). High seroprevalence rates in caribou herds that had no direct contact with domestic ruminants suggested the existence of an endemic cycle in wildlife in Canada (Elazhary et al., 1981). A recent study revealed no spatial overlap between one cluster of BVDV antigen-positive cattle herds and two clusters of BVDV antibody-positive deer in New York State, USA (Kirchgessner et al., 2013). These results supported the existence of an endemic cycle in white-tailed deer in the USA. BVDV infection was also demonstrated in three species of sympatric wild ruminants on adjacent mountain ranges in Nevada, USA (Wolff et al., 2016). In this study, BVDV-1 was isolated from three bighorn sheep in 2009 and one mountain goat in 2011, whereas BVDV-2 was isolated from two mule deer in 2013. Serosurveillance from 2011 to 2015 also revealed high seroprevalence rates against BVDV-1 and BVDV-2 in all three species. Furthermore, temporal and spatial overlap between domestic and wild ruminants was found to occur in these mountain ranges.

Chapter 2: Identification of Conserved Amino Acid Substitutions during Serial Infection of Pregnant Cattle and Sheep with Bovine Viral Diarrhea Virus

Abstract

Bovine viral diarrhea virus (BVDV) is an economically important pathogen of cattle that can also infect a wide range of domestic and wild species including sheep, goats, deer, camelids, and pigs. BVDV isolates are genetically highly diverse and previous work demonstrated that many substitutions were introduced in the viral genome during acute infections in cattle. In contrast, only limited information exists regarding changes occurring during BVDV infections in species other than cattle. The purpose of this study was to determine the changes introduced in the open reading frame (ORF) of the BVDV genome during serial infection of pregnant cattle and sheep with an isolate of bovine origin. Serial experimental inoculations were performed in six pregnant heifers and six pregnant ewes using BVDV-1b isolate AU526 in the first heifer and ewe, and serum from the preceding acutely infected dam thereafter. Complete ORF sequences were determined for 23 BVDV-1b isolates including AU526, one isolate from each pregnant dam, and one isolate from each BVDV-positive offspring born to these dams. Sequence comparison revealed that greater numbers of substitutions occurred during serial infection of pregnant sheep than of pregnant cattle. Furthermore, multiple host-specific amino acid changes were gradually introduced and conserved. These changes were more abundant in ovine isolates and occurred primarily in the E2 coding region. These results suggest that BVDV infections in heterologous species may serve as a significant source of viral genetic diversity and may be associated with adaptive changes.

Introduction

Bovine viral diarrhea virus (BVDV) is an economically important pathogen of cattle worldwide, producing a wide range of clinical manifestations, including gastrointestinal, reproductive, and respiratory disease (Baker, 1995; Houe, 1999). BVDV is the prototypic member of the genus *Pestivirus* within the family *Flaviviridae*. Similar to other pestiviruses, BVDV has an enveloped, positive-sense, single-stranded RNA genome that is approximately 12.3 kb in length and contains a single open reading frame (ORF) encoding about 3,900 amino acids and two untranslated regions at the 5' and 3' ends (Collett et al., 1988c). Translation of the ORF generates a large polyprotein that is co- and post-translationally processed to yield four structural (C, E^{rns}, E1, E2) and eight nonstructural (N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) viral proteins (Collett et al., 1988b). BVDV isolates are genetically and antigenically highly diverse and can be segregated into two different species, BVDV-1 and BVDV-2 (Pellerin et al., 1994; Ridpath et al., 1994). Phylogenetic studies have demonstrated the existence of at least 21 BVDV-1 subgenotypes (1a-1u) and four BVDV-2 subgenotypes (2a-2d) (Giammarioli et al., 2015; Yesilbag et al., 2017). RNA viruses are characterized by high mutation rates and the lack of proofreading activity of RNA-dependent RNA polymerases is believed to be the main driving force for the generation of altered genomic sequences (Sanjuán et al., 2010; Steinhauer et al., 1992).

The clinical outcome of BVDV infection depends on various host and viral factors including reproductive status, gestational age, vaccination status, concurrent infections, viral virulence, and viral biotype (Baker, 1995). For instance, exposure of a naïve pregnant cow to BVDV causes an acute systemic infection that usually results in transplacental infection of the fetus (Gillespie et al., 1967). A remarkable feature of BVDV is the existence of two biotypes,

cytopathic and noncytopathic, according to whether or not the virus causes cell death in cultured cells (Lee & Gillespie, 1957; Underdahl et al., 1957). Although viral biotype does not correlate with virulence in vivo, the establishment of persistent infection is restricted to noncytopathic viruses (Brownlie et al., 1989). When infection occurs in early pregnancy with a noncytopathic virus, the fetal immune system may develop a highly specific immunotolerance resulting in lifelong viral persistence in this animal (Kendrick, 1971). Persistently infected (PI) animals shed large amounts of virus in most excretions and secretions, and therefore are the primary means of BVDV transmission in cattle populations (Coria & McClurkin, 1978; Houe, 1999).

Acute BVDV infections in pregnant cattle were found to result in 2.3 to 8 times more nucleotide substitutions than in non-pregnant cattle (Neill et al., 2012). Furthermore, most genetic changes introduced during the establishment of a persistent infection were shown to arise during the acute infection of the pregnant dam (Neill et al., 2012). BVDV infections in naïve pregnant cows are thus believed to be an important source of viral genetic diversity.

Although its natural host is cattle, BVDV can infect various species of domestic and wild ungulates. The establishment of persistent infection has been reported in seven species including alpaca, goats, sheep, swine, and white-tailed deer (Carman et al., 2005; Gard et al., 1976; Loken & Bjerkas, 1991; Passler et al., 2007; Terpstra & Wensvoort, 1997). It has been suggested that these species may serve as reservoirs for BVDV and as an additional source of viral diversity. Only limited information exists regarding genetic changes occurring during BVDV infections in species other than cattle. Multiple amino acid changes in the E2 coding region have been reported during the establishment of persistent infections in goats (Bachofen et al., 2013; Passler et al., 2014a). Host-specific changes in the E2 and N^{pro} coding regions were also introduced during serial infection of pregnant cattle and sheep (Paton et al., 1997). Unfortunately, genetic

analysis was often limited in these previous studies to the E2 coding region and parts of the N^{pro} and C coding regions of the viral genome.

The purpose of this study was to determine the changes introduced in the ORF of the BVDV genome during serial infection of pregnant cattle and sheep with an isolate of bovine origin. To infect a heterologous host, a virus must be able to efficiently bind a cell receptor, express its genes, replicate its genome, and produce infectious virions. However, there are several host barriers and defense mechanisms that may restrict virus infection including innate immune response and intercellular junctions (Bergelson, 2009; Kawai & Akira, 2006). One or more changes in the viral genome would be required to overcome each of these obstacles and thus we hypothesized that the number of viral genomic changes would be greater in sheep than in cattle during serial infection with a BVDV isolate of bovine origin.

Materials and Methods

Animals

All experimental procedures were performed with approval and under the guidelines of the Institutional Animal Care and Use Committee of Auburn University (No. 2015-2604 and 2015-2706). Six Angus-cross pregnant beef heifers were acquired from Auburn University Animal Health Research and six Suffolk-cross pregnant nulliparous ewes were acquired from a local commercial flock. The sheep experiment started in November 2014 and ended in May 2015, and the cattle experiment started in June 2015 and ended in September 2016.Prior to inclusion, animals were confirmed to be pregnant by transrectal or transabdominal ultrasonography, negative for BVDV by virus isolation (VI), and seronegative to BVDV by virus neutralization (VN). Animals were transported to the North Auburn BVDV unit at Auburn University and

housed in isolated pens. At the time of inoculation, pregnant heifers and ewes were at 75 to 80 and 30 to 60 days of gestation, respectively. Animals were visually inspected daily for signs of illness and evidence of reproductive losses throughout the study period.

Virus inoculation and sample collection

On day 0, a physical examination was performed, pregnancy was confirmed by ultrasonography, and blood was collected for VI and VN prior to inoculation. Subsequently, the first heifer and the first ewe were each inoculated intravenously with approximately 1.0×10^6 50% tissue culture infective dose ($TCID_{50}$) of BVDV-1b AU526. The noncytopathic BVDV-1b AU526 virus had been isolated from the serum of a PI cow, which was part of a research herd at Auburn University Animal Health Research. This isolate was shown to cause persistent infections in goats and white-tailed deer (Passler et al., 2014a; Passler et al., 2007). Virus stock had been passaged twice in Madin-Darby bovine kidney (MDBK) cells. Virus inoculum was prepared by adding 20 µl of virus stock at a titer of 5.0×10^7 $TCID_{50}$/ml to 980 µl of culture medium. Residual inoculum was stored at -80°C for estimation of received dose by virus titration.

On days 5 and 7 postinoculation (pi), a physical examination was performed and blood was collected for VI and reverse transcriptase quantitative PCR (RT-qPCR). Nasal swabs were also collected from the ewes for VI. The second, third, fourth, fifth, and sixth heifers were inoculated intravenously with 1 ml of BVDV-positive serum obtained on day 7 pi from the first, second, third, fourth, and fifth heifers, respectively. Analogously, the second ewe was inoculated intravenously with 1 ml of BVDV-positive serum obtained from the first ewe on day 7 pi. When serum samples obtained from pregnant ewes on days 5 and 7 pi were both positive for BVDV by RT-qPCR, the sample with the greatest relative fluorescence unit value was chosen as inoculum.

The third and sixth ewes were thus inoculated with serum obtained on day 5 pi from the second and fifth ewes, whereas the fourth and fifth ewes were inoculated with serum obtained on day 7 pi from the third and fourth ewes, respectively. In summary, serial experimental inoculations were performed in six pregnant heifers and six pregnant ewes using BVDV-1b isolate AU526 in the first heifer and ewe, and serum from the preceding acutely infected pregnant dam thereafter.

Pregnant dams were commingled following inoculation and pregnancies were allowed to proceed to term. Blood was collected every 28 days for VN until term. Ultrasound examinations were performed to assess pregnancy viability at the same intervals.

At the time of parturition or abortion, blood and skin biopsy samples were collected from offspring for VI and VN or antigen-capture enzyme-linked immunosorbent assay (ACE), respectively. Blood was also collected from dams for VN. Postmortem examinations were performed on aborted and stillborn fetuses as well as deceased offspring. Representative sections of placental and fetal tissues were collected for VI including liver, spleen, thymus, lymph nodes, kidney, lung, heart, gonad, small intestine, and brain. Additional blood samples were collected every six weeks from viable offspring for VI and VN until six months of age.

Sample processing

Blood collected in plain tubes was allowed to clot at room temperature for at least 30 min. Following centrifugation at 200 × *g* for 20 min, serum was harvested and stored at -80°C or immediately used in VI, VN, and RT-qPCR procedures. Blood collected in EDTA-containing tubes was processed to yield buffy coat. Following centrifugation at 700 × *g* for 30 min, buffy coat was removed using a sterile Pasteur pipette. Lysis of red blood cells was performed using 10 ml of 0.15 M ammonium chloride (NH_4Cl). Buffy coat was washed in 10 ml of culture medium. Following centrifugation at 700 × *g* for 10 min, buffy coat was resuspended in 1 ml of culture

medium to be used in VI procedures. Nasal swabs were placed in tubes containing culture medium to be used in VI procedures. Skin biopsy samples were immediately placed in tubes containing phosphate-buffered saline (PBS) to be used in ACE procedures. Sections of placental and fetal tissues were placed in stomacher bags containing 3 ml of culture medium and homogenized for 5 min with a Tekmar Stomacher® laboratory blender (Tekmar, Co., Cincinnati, OH, USA) to be used in VI procedures.

Cells

MDBK cells were purchased from the American Type Cell Culture Collection (CCL-22™) and confirmed to be of bovine origin through amplification and sequencing of mitochondrial cytochrome c oxidase subunit 1 (CO1) gene (CellCheck™, IDEXX Laboratories, Inc., Westbrook, ME, USA). PCR assays also demonstrated the absence of *Mycoplasma* and mammalian interspecies contamination (STAT-Myco™, IDEXX Laboratories, Inc.). Cells were grown in minimum essential medium (MEM) with Earle's salts supplemented with 10% equine serum, L-glutamine (0.02 mM), sodium bicarbonate (0.75 mg/ml), penicillin (100 U/ml) and streptomycin (100 µg/ml).

Virus isolation

Serum, buffy coat, and nasal swab samples as well as placental and fetal tissue samples were assayed for BVDV by passage through MDBK cells for 4 days. An immunoperoxidase monolayer assay (IPMA) was subsequently used to identify BVDV-positive cells.

Briefly, serum samples were assayed by adding 768 µl of serum to 192 µl of culture medium on 9.6-cm^2 wells of a 6-well culture plate that had been seeded 24 h earlier with MDBK cells. The plates were incubated at 37°C for 1 h in humidified air containing 5% CO_2. Subsequently, 3 ml of culture medium was added to each well. The plates were then incubated

for 4 days. Following a single freeze-thaw cycle to release intracellular virus, cell lysates were assayed in triplicate by adding 10 µl of cell lysate to 90 µl of culture medium and 50 µl of culture medium containing MDBK cells on 0.32-cm^2 wells of a 96-well culture plate. The plates were then incubated for 3 days. Following fixation, 50 µl of a mixture of two monoclonal antibodies, D89 and 20.10.6, was added to each well. These antibodies are specific for the envelope protein E2 and the nonstructural protein NS3 of BVDV, respectively. The plates were incubated at 37°C for 1 h to allow antibody binding. Following washing with PBS containing 0.05% Tween 20 to remove unbound antibodies, 50 µl of diluted peroxidase-conjugated rabbit anti-mouse IgG was added to each well and culture plates were incubated at 37°C for 1 h. Following washing with PBS containing 0.05% Tween 20, 50 µl of aminoethyl carbazole substrate was added and culture plates were incubated at room temperature for 15 min. This enzyme substrate produced a reddish-brown color when oxidized by horseradish peroxidase. Color change was examined by use of light microscopy and compared with that of positive- and negative-control samples included on each culture plate.

Buffy coat and nasal swab samples as well as placental and fetal tissue samples were assayed by adding 1 ml of sample suspension on 9.6-cm^2 wells of a 6-well culture plate that had been seeded 24 h earlier with MDBK cells. The plates were processed and cell lysates were subsequently assayed for BVDV in triplicate using an IPMA as described above.

Virus titration

Virus titration was performed on the initial inoculum, VI-positive serum samples, and VI-positive fetal tissue samples. The quantity of BVDV was determined by multiple 10-fold dilutions of samples using the statistical method of Reed and Muench (1938). Briefly, each sample was assayed by adding 10 µl of sample to 90 µl of culture medium on each of three 0.32-

cm^2 wells of a 96-well culture plate that had been seeded 24 h earlier with MDBK cells. Serial 10-fold dilutions were made in culture medium, retaining 90 µl of each dilution per well. The plates were incubated at 37°C for 3 days in humidified air containing 5% CO_2. The IPMA described above was subsequently used to identify BVDV-positive cells.

Virus neutralization

A standard VN microtiter assay was used to detect and quantify neutralizing antibodies to BVDV-1b AU526 in serum samples obtained from dams and their offspring. Serum samples were heat inactivated by incubation at 56°C for 30 min. Each sample was assayed in triplicate by adding 50 µl of sample to 50 µl of culture medium on each of three 0.32-cm^2 wells of a 96-well culture plate that had been seeded 24 h earlier with MDBK cells. Serial 2-fold dilutions were made in culture medium, retaining 50 µl of each dilution per well. An equal volume of culture medium containing 200 $TCID_{50}$ of BVDV-1b AU526 was added to each well. The plates were incubated at 37°C for 1 h in humidified air containing 5% CO_2 and 50 µl of culture medium containing MDBK cells was then added to each well. The plates were incubated for 3 days and subsequently assayed for BVDV using the IPMA described above. Antibody titer was defined as the reciprocal of the highest dilution at which 2 out of 3 wells were free of staining.

Antigen-capture enzyme-linked immunosorbent assay

Skin biopsy samples obtained from calves were assayed for BVDV using the BVDV PI X2 Test kit (IDEXX Laboratories, Inc.) according to the manufacturer's instructions. This test relies on monoclonal antibodies directed against the to capture the viral antigen and detects antigen-antibody complexes with enzyme-conjugated antibody by spectrophotometry. The sample to positive (S/P) ratio was calculated for each sample and a ratio of 0.3 or greater was considered positive.

Skin biopsy samples obtained from lambs were assayed for BVDV using the SNAP® BVDV Antigen Test kit (IDEXX Laboratories, Inc.) according to the manufacturer's instructions. This assay uses the same monoclonal antibodies against the E^{rns} protein of BVDV.

Reverse transcriptase quantitative PCR

Serum samples obtained from pregnant ewes on days 5 and 7 pi were assayed for BVDV by RT-qPCR. Viral RNA was isolated from serum samples using the High Pure PCR Template Preparation Kit (Roche Diagnostics Corporation, Indianapolis, IN, USA). Briefly, 200 µl of serum was added to 40 µl of proteinase K with 200 µl of binding buffer into a 1.5-ml microcentrifuge tube. Following incubation at 70°C for 10 min, 100 µl of isopropanol was added and the solution was centrifuged through a filter tube containing glass fibers for 1 min at 11,000 × *g*. Following centrifugation, 500 µl of inhibitor removal buffer was added and the filter tube was subsequently centrifuged at 11,000 × *g* for 1 min. Following centrifugation, the filter tube was washed twice with 450 µl of wash buffer and centrifuged at 11,000 × *g* for 1 min. Viral RNA on the glass fibers was then eluted in 60 µl of elution buffer by centrifuging at 8,000 × *g* for 1 min.

The RT-qPCR assay was performed using the iTaq™ Universal SYBR® Green One-Step kit (Bio-Rad Laboratories, Inc., Hercules, CA, USA) on the Bio-Rad CFX96 Touch™ real-time PCR instrument (Bio-Rad Laboratories, Inc.). Briefly, 5 µl of viral RNA was added on the wells of a PCR plate containing 10 µl of iTaq™ Universal SYBR® Green Supermix, 0.25 µl of iScript™ reverse transcriptase, 0.3 µl of each forward and reverse primer (20 µM) and 4.15 µl of ultra-pure water. The PCR primers BVDV_F (5'-TAGCCATGCCCTTAGTAGGAC-3') and BVDV_R (5'-GACGACTACCCTGTACTCAGG-3') were amplifying a 290-bp sequence of the 5' untranslated region of the BVDV genome. Thermal cycling protocol included 10 min of

reverse transcription at 50°C and 1 min of polymerase activation and DNA denaturation at 95°C followed by 40 cycles of amplification with denaturation at 95°C for 10 s and primer annealing and extension at 56.5°C for 30 s. Following the final amplification cycle, a melting curve was obtained by cooling to 65°C and then slowly heating to 95°C at 0.1°C/s while continuously monitoring fluorescence. Data were analyzed using the Bio-Rad CFX Manager software (Bio-Rad Laboratories, Inc.).

Viral genome sequencing

VI-positive serum, buffy coat, and fetal tissue samples containing at least 10^4 $TCID_{50}$/ml of virus were directly used in genome sequencing procedures. VI-positive samples with a lower virus titer were propagated in MDBK cells. Growth in cell culture was limited to one passage to minimize the introduction of artifactual changes in the viral genome. Virus was harvested by freezing and thawing the infected cells and samples were stored at -80°C.

Host DNA and RNA were removed by adding 180 µl of sample to 20 µl of 10× DNase I buffer (100 mM Tris-HCl, 25 mM $MgCl_2$, 5 mM $CaCl_2$, pH 7.6) and a cocktail of nucleases consisting of 5 U of DNase I (Thermo Fisher Scientific, Inc., Waltham, MA, USA), 10 U of Turbo-DNase (Thermo Fisher Scientific, Inc.), 5 U of Base-line Zero™ DNase (Epicentre, Madison, WI, USA), 25 U of benzonase (EMD Millipore, Billerica, MA, USA), 4 U of RNase One™ Ribonuclease (Promega Corporation, Madison, WI, USA), 2 µg of RNase A (Thermo Fisher Scientific, Inc.), and 5 U of RNase T1 (Thermo Fisher Scientific, Inc.). Following incubation at 37°C for 2 h, viral RNA was extracted using the QIAamp® MinElute® Virus Spin Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions.

Briefly, 200 µl of sample was added to 25 µl of QIAGEN protease and 200 µl of buffer AL containing linear acrylamide (10 ng/µl) in a 1.5-ml microcentrifuge tube. Following

incubation at 56°C for 15 min, 250 µl of ethanol was added and the solution was incubated at room temperature for 5 min. The solution was then centrifuged through a spin column containing a silica membrane at 6,000 × *g* for 30 s. Following centrifugation, the spin column was washed with 500 µl of buffer AW1 and centrifuged as before. Following centrifugation, 500 µl of buffer AW2 was added and the spin column was centrifuged at 20,000 × *g* for 3 min. Following centrifugation, 500 µl of ethanol was added and the spin column was centrifuged at 20,000 × *g* for 30 s. Following an additional centrifugation at 20,000 × *g* for 3 min, viral RNA was eluted by adding 23 µl of ultra-pure water, incubating at room temperature for 1 min and then centrifuging at 20,000 × *g* for 30 s. Viral RNA was stored at -80°C or immediately used.

A random primed, barcoded library technique utilizing primers composed of 20 bases of known sequence with 8 random bases at the 3' end was used. These 28-mer primers conferred sequence independence by random priming both first and second strand cDNA synthesis.

In the first step, 20 µl of viral RNA was added to 1 µl of 28-mer random primer (100 nM) in PCR tubes. Following incubation at 75°C for 5 min, 6.5 µl of RT MasterMix containing 0.5 mM of dNTPs, 4 U of RNAsin, 2.5 µl of 5× SuperScript™ III RT buffer (Thermo Fisher Scientific, Inc.) and 200 U of SuperScript™ III reverse transcriptase (Thermo Fisher Scientific, Inc.) was added to each PCR tube. First strand reactions were incubated at 42°C for 1 h, heated to 95°C for 2 min, and immediately placed on ice.

The second strand cDNA was synthesized in the same tube using Sequenase Version 2.0 DNA polymerase (Affymetrix, Inc., Cleveland, OH, USA) and the nucleotide and primers still present after first strand reaction. Five microliters of 5× Sequenase reaction buffer with 4 U of Sequenase was added to each tube on ice. Reaction tubes were placed in a thermocycler and were ramped from 4°C to 37°C at 0.1°C/s. Following incubation at 37°C for 10 min, tubes were

heated to 94°C for 2 min and immediately placed on ice. Four units of Sequenase 2.0 was then added to each tube and the ramping and incubation were repeated with the exception that the 37°C incubation was extended to 30 min.

The double-stranded cDNA was purified using the QIAamp® MinElute® Virus Spin Kit (Qiagen) according to the manufacturer's instructions. The DNA was subsequently amplified by PCR using primers consisting of the first 20 known bases of the 28-mer primers used in the cDNA synthesis. Each PCR reaction mixture contained 10 µl of cDNA, 5 U of TaKaRa EX Taq™ polymerase (Takara Bio USA, Inc., Mountain View, CA, USA), 10 µl of 5× TaKaRa EX Taq buffer (Takara Bio USA, Inc.), 400 µM of dNTPs, and 1 µl of 20-mer primer (100 nM). PCR reaction tubes were incubated at 95°C for 5 min and then subjected to 35 thermal cycles at 95°C for 10 s, 40°C for 30 s, and 72°C for 1 min. Following a final elongation step at 72°C for 5 min, reactions were maintained at 4°C.

PCR reactions were transferred to 96-well culture plates and the DNA was size-fractionated and purified using paramagnetic beads (Agencourt® AMPure® XP, Beckman Coulter, Indianapolis, IN, USA) at a DNA-to-bead ratio of 1:0.8. This resulted in the removal of DNA fragments of less than 300 bp including adaptor dimers and unligated adaptors. The DNA was prepared for sequencing using the Nextera™ DNA Library Preparation Kit (Illumina, Inc., San Diego, CA, USA) according to the manufacturer's instructions. The use of indexed barcoded PCR primers allowed multiplexing of libraries. The DNA was subjected to sequence analysis with the MiSeq™ platform using the MiSeq Reagent kit v2 (Illumina, Inc.) for 2 × 150 base paired-end sequencing.

Genomic sequences were assembled and edited using the Lasergene SeqMan NGen software (DNASTAR, Inc., Madison, WI, USA) and the genomic sequence of AU526 as the

assembly reference. Sequences were further edited using the CodonCode Aligner software (Codoncode Corporation, Centerville, MA, USA). Numbering of nucleotides started at the ATG initiation codon of the ORF. Isolates were named using the animal's identification number. Dams were identified using a letter for the species (B, bovine; O, ovine) and the order number in the inoculation series. Offspring were identified using the dam's identification number and a letter for the birth order (A, first; B, second).

Data analysis

Pairwise comparisons of genomic sequences were performed using the MEGA software (version 7.0.18 available at http://www.megasoftware.net). Ambiguous nucleotides were not considered to be substitutions and thus not included in the results of pairwise comparisons. Statistical significance of the difference between the median number of substitutions was evaluated using the Wilcoxon rank sum test. The number of positions affected by substitutions was compared using the Pearson's Chi-squared test. The number of substitutions in isolates from offspring that were not detected in isolates from their dams was also compared using the Pearson's Chi-squared test. The ratio of the number of observed substitutions to the number of expected substitutions assuming a random distribution of substitutions across the viral genome was calculated for each protein-coding region.

Accession numbers

The viral genomic sequences determined in this study have been deposited in GenBank under accession numbers MG950344 to MG950366.

Results

Clinical, virological, and serological findings

All heifers and ewes were confirmed to be seronegative to BVDV and free of BVDV and clinical disease prior to inoculation (Table 1.1). Virus titration of residual inoculum demonstrated that the viral dose received was 6.2×10^5 $TCID_{50}$ for the first heifer and 2.0×10^6 $TCID_{50}$ for the first ewe. Infection was confirmed in all dams by positive VI results on day 5 or 7 pi and seroconversion by day 28 pi (Table 1.1). Clinical signs of disease were not observed in any of the heifers nor in two ewes (O3 and O6). In the remaining ewes, inappetence, lethargy, and rectal temperature above 39.5°C were noted at least once during the first week pi.

All heifers carried the pregnancy to term and delivered one stillborn calf and five live calves that were viable and nursed shortly after birth (Table 1.1). Persistent infection with BVDV was demonstrated in these five calves by repeated VI from blood samples obtained at 21, 42, 84, and 168 days of age (Table 1.2). The stillborn calf (B1A) was of normal size and appearance. Transplacental infection with BVDV was confirmed in this calf by VI from a composite of tissue samples including liver, spleen, thymus, kidney, lung, and heart (Table 1.2).

Two ewes (O5 and O6) aborted on days 46 and 70 pi at approximately 80 and 100 days of gestation, respectively (Table 1.1). The aborted fetus (O6A) had brachygnathia superior. Uterine contents were not recovered from the other ewe. The remaining ewes gave birth to three live non-viable lambs, two stillborn lambs, and one mummified fetus (Table 1.1). Two live lambs (O1A and O3A) were weak, underweight, and unable to stand or nurse at birth. These lambs were fed colostrum by oroesophageal tubing but were ultimately euthanized within 24 h of birth. Lamb O1A had brachygnathia inferior and postmortem examination revealed thymic atrophy in this lamb. The third live lamb (O4A) was observed to nurse after birth. However, its

condition deteriorated rapidly and it was ultimately euthanized 24 h after birth. The stillborn lambs (O2A and O2B) were of normal size and appearance. The mummified fetus (O1B) was 39.5-cm long and fetal death was estimated to have occurred 70 days pi at 120 days of gestation.

Transplacental infection with BVDV was confirmed in two non-viable lambs and two stillborn lambs by VI from blood and tissue samples and by detection of BVDV antigen in skin biopsy samples (Table 1.3). The presence of live virus and the absence of precolostral antibodies to BVDV suggested that these lambs were born persistently infected with BVDV. Transplacental infection was confirmed in the third non-viable lamb (O3A) by a high antibody titer in precolostral serum. Virus was not recovered from this lamb.

Genomic sequence analysis

Complete ORF sequences were determined for 23 BVDV-1b isolates including AU526, one isolate from each acutely infected dam, and one isolate from each VI-positive offspring born to these dams. For all but one dam, serum samples obtained on day 5 or 7 pi were used in genome sequencing procedures. A buffy coat sample obtained on day 7 pi was used for the sixth heifer (B6). Serum or tissue samples were used for offspring. The first VI-positive serum sample collected after birth was used for PI calves. Five sequences contained ambiguous nucleotides with a median of 1 nucleotide (range, 1-2).

When compared to AU526, a median of 23 nucleotide substitutions (range, 9-26) was observed in isolates from acutely infected pregnant heifers (Table 1.4). In contrast, there was a median of 46 nucleotide differences (range, 0-51) between AU526 and isolates from pregnant ewes. Similar to their dams, a median of 27 substitutions (range, 23-34) was observed in isolates from calves and a median of 50 substitutions (range, 49-52) was observed in isolates from lambs. The difference between the median number of nucleotide changes observed in bovine and ovine

isolates was statistically significant (P = 0.002).

Most substitutions were transitions with a median transition-to-transversion ratio of 4.9 in isolates from pregnant heifers and 4.4 in isolates from pregnant ewes (Table 1.4). A median transition-to-transversion ratio of 4.8 and 4.6 was observed in isolates from calves and lambs, respectively. In bovine isolates, nucleotide substitutions were more prevalent in nonstructural protein-coding regions with a median of 5 changes in the NS5A coding region and a median of 3 changes in the NS3 and NS5B coding regions. Similarly, a median of 11 and 9 changes occurred in ovine isolates in the NS5B and NS3 coding regions, respectively. Furthermore, a median of 4 and 6 changes was found in the E2 coding region in bovine and ovine isolates, respectively. A median of 7 changes was also identified in the E^{rns} coding region in ovine isolates. Analysis of the number of changes per protein-coding region revealed that nucleotide substitutions were essentially randomly distributed (Table 1.5).

Approximately 30% of substitutions were nonsynonymous, resulting in a median of 6 amino acid differences (range, 4-7) between AU526 and isolates from pregnant heifers and a median of 13 amino acid differences (range, 0-14) between AU526 and isolates from pregnant ewes (Table 1.6). Similar to their dams, a median of 7 substitutions (range, 6-13) was detected in isolates from calves and a median of 15 substitutions (range, 13-17) was observed in isolates from lambs. The difference between the median number of amino acid changes observed in bovine and ovine isolates was statistically significant (P = 0.003). In bovine isolates, amino acid substitutions occurred most frequently in the E2 and N^{pro} coding regions with a median of 3 and 2 changes in these regions, respectively (Table 1.6). In ovine isolates, a median of 5, 3, and 2 substitutions was found in the E2, NS5B, and E^{rns} coding regions, respectively. In all but two bovine isolates, the number of amino acid changes observed in the E2 and N^{pro} coding regions

was at least 2.5 times greater than expected from random substitutions across the viral genome (Table 1.7). In all but four ovine isolates, amino acid changes occurred in the E^{rns} and E2 coding regions at least 2.5 times more frequently than expected from random events. In contrast, there were no amino acid substitutions in the NS4A coding region. Furthermore, there were only four isolates (O2, B6A, O1A, and O2B) with an amino acid change in regions encoding the viral proteins C, p7, and NS5A.

A total of 284 nucleotide substitutions occurred in bovine isolates at 125 different positions, whereas 429 substitutions occurred in ovine isolates at 109 different positions. There were also 84 amino acid substitutions at 44 different positions in bovine isolates and 124 substitutions at 36 different positions in ovine isolates (Figure 1.1). Amino acid residues that were often affected by substitutions in ovine isolates were rarely substituted in bovine isolates and vice versa. Furthermore, the number of nucleotide and amino positions affected by substitutions in bovine isolates was statistically different from that observed in ovine isolates ($P < 0.001$ and $P = 0.025$, respectively). However, there was no statistical evidence that the number of nucleotide and amino acid positions affected by substitutions was different between isolates from pregnant dams and those from their offspring ($P = 0.59$ and $P = 0.87$, respectively).

Multiple nucleotide and amino acid changes introduced during the first and second passages in pregnant dams were found to be conserved in the remaining passages. In cattle, five amino acid substitutions detected in the isolate from the third heifer (B3) were also observed in the isolates from the fourth and sixth heifers and the last four calves (Table 1.8). Two of these changes occurred in both the N^{pro} and E2 coding regions. In sheep, five amino acid changes detected in the isolate from the second ewe (O2) were also observed in the isolates from the last four ewes and their lambs (Table 1.9). Seven additional amino acid changes identified in the

isolate from the third ewe (O3) were also detected in the isolates from the subsequent ewes and their offspring. One additional substitution was shared by the isolates from the lambs of the second ewe (O2A and O2B) and the isolates from the last three ewes. In summary, a total of 13 amino acid substitutions were conserved in the isolates from the last three ewes and their lambs. These changes occurred most frequently in regions encoding the viral proteins E2, NS5B, and E^{rns} with four, three, and two changes in these regions, respectively.

On average 43% of nucleotide and 42% of amino acid differences between AU526 and isolates from calves were observed in isolates from their dams (Table 1.10). Similarly, on average 39% of nucleotide and 40% of amino acid differences between AU526 and isolates from lambs were also observed in isolates from their dams. Of these amino acid differences, an average of 54% and 74% occurred in structural protein-coding regions in isolates from calves and lambs, respectively. In comparison, on average 56% and 63% of changes in isolates from heifers and ewes that were not detected in their offspring occurred in regions encoding nonstructural proteins, respectively (Table 1.11).

Results of all pairwise sequence comparisons as well as the location and type of nucleotide and amino acid substitutions between AU526 and isolates from acutely infected pregnant dams and their offspring are given in the Supplemental Material (Tables S1.1-S1.5). Furthermore, virus titers in samples from pregnant dams and their offspring as well as the results of RT-qPCR analyses of serum samples from pregnant ewes are given in the Supplemental Material (Tables S1.6-S1.8).

Discussion

In this study, the BVDV-1b isolate AU526 was serially passaged in six pregnant heifers and six pregnant ewes. Acute maternal infections resulted in transplacental transmission of BVDV and the birth of six calves and four lambs, from which virus could be isolated. The complete ORF sequences of AU526 and isolates from pregnant dams and their offspring were obtained and compared to determine the timing, number, location, and type of substitutions introduced during serial infection of pregnant cattle and sheep with BVDV.

The median number of nucleotide and amino acid differences between AU526 and isolates from pregnant heifers was similar to the median of 20 nucleotide and 8 amino acid changes observed in isolates from four pregnant cows exposed to calves persistently infected with BVDV (Neill et al., 2012). Compared to cattle, greater numbers of changes were detected between AU526 and isolates from pregnant ewes with a median of 46 nucleotide and 13 amino acid substitutions. Similarly, 10 and 36 nucleotide substitutions were detected in isolates from two pregnant does acutely infected with BVDV (Passler et al., 2014a). When comparing AU526 to isolates from offspring born to these dams, similar findings were observed with more changes in isolates from lambs than in those obtained from calves. Altogether, these results revealed that many substitutions were introduced in the BVDV genome during the establishment of serial persistent infections in sheep and suggest that BVDV infections in pregnant small ruminants may serve as a significant source of viral genetic diversity. Previous studies demonstrated severe reduction in RNA virus populations during vector-borne transmission and when viruses spread within a single host (Dow et al., 2015; Forrester et al., 2012; Navas et al., 1998). There are several host barriers and defense mechanisms that limit virus infection and spread in a heterologous host and therefore cross-infections are likely to impose severe bottlenecks on virus

populations. It is thus possible that serial infection of pregnant sheep with a BVDV isolate of bovine origin was associated with greater numbers of substitutions due to high selection pressure on virus populations.

Although greater numbers of changes occurred during serial infection of pregnant sheep, the number of different nucleotide and amino acid positions affected by substitutions was greater in bovine isolates than in ovine isolates. It is possible that substitutions were more easily tolerated in the host of origin or that more diverse changes were introduced in cattle because host factors affected the activity of the BVDV polymerase complex. Furthermore, acute BVDV infections in pregnant cattle may result in the birth of PI calves. Considering that PI calves consistently shed large amounts of virus and that additional substitutions were detected in isolates from calves, these animals may serve as an important source of viral isolates that are genetically diverse from those infecting their dams. Altogether these results suggest that acute infections in pregnant cattle may greatly contribute to the generation and spread of new BVDV isolates in animal populations.

Nucleotide substitutions occurred randomly throughout the BVDV genome in bovine and ovine isolates. However, there was a bias toward amino acid changes in the E2 and N^{pro} coding regions in bovine isolates and the E2 and E^{rns} in ovine isolates. Previous studies have also demonstrated that amino acid changes occurred primarily in regions encoding the structural proteins E2 and E^{rns} during the establishment of persistent infections in cattle (Neill et al., 2011, 2012). The envelope proteins E2 and E^{rns} are the main targets of neutralizing antibodies in animals infected with BVDV and immune pressure on the immunodominant residues may have contributed to the selection of amino acid changes in these coding regions. Furthermore, amino acid changes in the N^{pro} coding region were anticipated since this region of the viral genome is

relatively variable across pestivirus genomes and within PI cattle (Chernick et al., 2018; Smith et al., 2017).

Detailed examination of the genomic sequences revealed that multiple amino acid substitutions were gradually introduced and conserved during serial infection of pregnant cattle and sheep with BVDV. A total of five amino acid changes were shared by all but one of the isolates from the last four heifers and their calves. Analogously, 12 to 13 identical changes were detected in the isolates from the last four ewes and their lambs. The amino acid residues involved were different from one species to another and these changes were most frequently observed in the E2 coding region. These conserved substitutions may thus be involved in host adaptation. Host-specific amino acid changes in the E2 coding region were described during serial infection of pregnant cattle and sheep (Paton et al., 1997). Conserved amino acid changes in the E2 and E^{rns} coding regions were also reported in BVDV-1b isolates from alpacas (Neill et al., 2015). Analogously, most conserved changes detected in ovine isolates in this study occurred in the same coding regions of the BVDV genome.

An unexpected finding was the presence of some of these conserved changes in isolates from offspring (B2A, B5A, O1A, O2A, and O2B) that were born to dams whose isolates did not have these changes. This finding suggests that similar selection mechanisms may be present in acutely infected pregnant animals and PI offspring. It is currently unknown, and will be the focus of future experiments, if these host-specific changes provided a selective advantage in infected sheep and if similar results would have been obtained if a BVDV isolate belonging to a different subgenotype or species had been serially passaged in sheep.

In contrast to a study conducted in cattle (Neill et al., 2012), most nucleotide and amino acid changes identified in isolates from calves and lambs were not detected in isolates from their

acutely infected dams. However, there was a high degree of variation between offspring. For instance, 96% of nucleotide substitutions identified in the isolate of the calf B3A were detected in the isolate of its dam. Similar findings were reported in goats with 95% of nucleotide substitutions observed in the isolate of a PI goat kid that were detected in the isolate of its dam whereas 60% of changes observed in the isolate of another PI goat kid were not detected in the isolate of its dam (Passler et al., 2014a). Considering the existence of a viral quasispecies in PI cattle, it is possible that the substitutions observed in isolates from offspring might have been first established in pregnant dams in a limited number of viral mutants that were subsequently selected in PI offspring (Dow et al., 2015; Ridpath et al., 2015). Fluctuations in minor variants within the viral quasispecies may partially explain the discrepancies observed between and within studies performed in cattle and other species. This hypothesis could not be confirmed or excluded since our analysis was restricted to the consensus sequence of the BVDV ORF.

The majority of the amino acid substitutions present in isolates from acutely infected dams that were also detected in isolates from their offspring occurred in regions encoding structural viral proteins. In contrast, amino acid changes in isolates from pregnant dams that were not observed in isolates from their offspring occurred primarily in nonstructural protein-coding regions. These results suggest that amino acid substitutions in regions encoding structural viral proteins were positively selected during establishment of persistent infection in offspring. It is possible that these changes were associated with fitness gain in PI offspring.

In conclusion, greater numbers of nucleotide and amino acid substitutions were introduced during serial infection of pregnant sheep than of pregnant cattle. Furthermore, multiple host-specific amino acid changes were gradually introduced and conserved in bovine and ovine isolates. These changes were primarily in the E2 coding region and more abundant in

ovine isolates. These results suggest that BVDV infections in heterologous hosts may serve as a significant source of viral genetic diversity and may be associated with adaptive changes.

Tables

Table 1.1. Virological and serological analysis of serial inoculation of pregnant heifers and ewes with BVDV

		0 dpi		5 dpi	7 dpi	28 dpi	56 dpi	84 dpi	112 dpi	140 dpi	168 dpi	Day of parturition or abortion		
Dam	Fetus[a]	VN	VI[b]	VI[c,d]	VI[c,e]	VN	VN	VN	VN	VN	VN	VN	dpi	Offspring
B1	80	< 2	-/-	+	+/+	256	1,024	4,096	4,096	8,192	8,192	2,048	197	Stillborn BVDV+ calf
B2	80	< 2	-/-	-	+/+	1,024	2,048	4,096	4,096	8,192	8,192	4,096	194	Live viable BVDV+ calf
B3	75	< 2	-/-	+	+/+	512	2,048	2,048	4,096	4,096	8,192	8,192	197	Live viable BVDV+ calf
B4	80	< 2	-/-	+	-/+	1,024	4,096	8,192	4,096	8,192	8,192	4,096	199	Live viable BVDV+ calf
B5	80	< 2	-/-	+	-/+	1,024	1,024	4,096	8,192	8,192	8,192	4,096	192	Live viable BVDV+ calf
B6	80	< 2	-/-	-	-/+	1,024	1,024	2,048	2,048	4,096	8,192	32,768	207	Live viable BVDV+ calf
O1	50	< 2	-/-	+/+/NT	-/+/NT	512	16,384	32,768	NA	NA	NA	32,768	96	Live non-viable BVDV+ lamb / mummified fetus
O2	40	< 2	-/-	+/+/NT	+/+/+	256	2,048	2,048	NA	NA	NA	8,192	106	Two stillborn BVDV+ lambs
O3	60	< 2	-/-	+/+/-	+/+/+	2,048	2,048	NA	NA	NA	NA	1,024	77	Live non-viable BVDV- lamb
O4	40	< 2	-/-	+/+/-	+/+/-	1,024	16,384	16,384	NA	NA	NA	16,384	102	Live non-viable BVDV+ lamb
O5	35	< 2	-/-	+/+/+	+/+/+	256	NA	NA	NA	NA	NA	512	46	Uterine contents not recovered
O6	30	< 2	-/-	+/+/-	+/+/-	512	512	NA	NA	NA	NA	8,192	70	BVDV- aborted fetus

[a] Estimated gestational age at the time of inoculation based on ultrasound findings

[b] Results are given for serum samples followed by those for buffy coat samples

[c] Results are given for serum samples followed by those for buffy coat and nasal swab samples

[d] Only serum samples from heifers were tested at 5 dpi

[e] Only serum and buffy coat samples from heifers were tested at 7 dpi

dpi, day postinoculation; NA, not applicable; NT, not tested; VI, virus isolation; VN, virus neutralization; +, positive; -, negative

Table 1.2. Virological and serological analysis of calves born to heifers infected with BVDV in early pregnancy

	Day of birth					21 doa			42 doa			84 doa			168 doa		
	serum		BC	skin	tissues[a]	serum		BC	serum		BC	serum		BC	serum		BC
Offspring	VN	VI	VI	ACE	VI	VN	VI	VI	VN	VI	VI	VN	VI	VI	VN	VI	VI
B1A	< 2	-	NA	-	+	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA	NA
B2A	32,768	-	-	+	NA	2,048	-	+	8	-	+	4	+	+	< 2	+	+
B3A	< 2	+	+	+	NA	4,096	-	+	4	-	+	4	-	+	8	-	+
B4A	32,768	-	+	-	NA	4,096	-	+	< 2	+	+	< 2	+	+	< 2	+	+
B5A	65,536	-	-	+	NA	128	-	+	< 2	+	+	< 2	+	+	< 2	+	+
B6A	32,768	-	-	-	NA	16,384	-	-	8	+	+	< 2	+	+	< 2	+	+

[a] Composite tissue including liver, spleen, thymus, kidney, lung, and heart

ACE, antigen-capture ELISA; BC, buffy coat; doa, days of age; NA, not applicable; VI, virus isolation; VN, virus neutralization; +, positive; -, negative

Table 1.3. Virological and serological analysis of lambs born to ewes infected with BVDV in early pregnancy

		Day of birth						
		serum		BC	skin	tissues[b]	brain	spleen
Offspring	Fetus[a]	VN	VI	VI	ACE	VI	VI	VI
O1A	50	< 2	+	+	+	-	+	+
O1B	50	NA	NA	NA	-	-	NT	NT
O2A	40	NT	NT	NT	+	+	NT	NT
O2B	40	NT	NT	NT	+	+	NT	NT
O3A	65	8,192	-	-	-	-	NT	NT
O4A	40	< 2	+	+	+	+	NT	NT
O6A	30	NT	NT	NT	-	-	NT	NT

[a] Estimated gestational age at the time of inoculation based on ultrasound findings

[b] Composite tissue including liver, spleen, thymus, kidney, lung and heart

ACE, antigen-capture ELISA; BC, buffy coat; NA, not applicable; NT, not tested; VI, virus isolation; VN, virus neutralization; +, positive; -, negative

Table 1.4. Nucleotide substitutions between AU526 and isolates from acutely infected dams and their offspring

AU526:Dam	Total	Transitions	Transversions	Ratio	N^{pro}	C	E^{rns}	E1	E2	p7	NS2	NS3	NS4A	NS4B	NS5A	NS5B	% S[a]	% NS[b]
AU526:B1	9	8	1	8.0	0	0	1	0	1	0	2	2	0	1	1	1	22.2	77.8
AU526:B2	16	11	5	2.2	2	0	1	0	3	0	1	4	0	2	0	3	25.0	75.0
AU526:B3	25	20	5	4.0	2	1	0	0	4	1	0	3	1	5	5	3	20.0	80.0
AU526:B4	26	21	5	4.2	2	1	1	1	5	1	0	4	1	2	5	3	30.8	69.2
AU526:B5	25	23	2	11.5	1	0	1	0	4	0	2	2	0	4	7	4	20.0	80.0
AU526:B6	20	17	3	5.7	2	1	0	0	4	1	0	2	1	1	5	3	25.0	75.0
Median	23	19	4	4.9	2	1	1	0	4	1	1	3	1	2	5	3	23.6	76.4
Mean	20	17	4	5.9	2	1	1	0	4	1	1	3	1	3	4	3	23.8	76.2
AU526:O1	0	0	0		0	0	0	0	0	0	0	0	0	0	0	0		
AU526:O2	37	28	9	3.1	0	1	6	3	3	0	3	9	0	2	2	8	35.1	64.9
AU526:O3	42	33	9	3.7	1	2	4	2	6	0	4	9	0	3	2	9	33.3	66.7
AU526:O4	49	40	9	4.4	1	3	7	3	6	0	4	9	0	3	2	11	38.8	61.2
AU526:O5	49	40	9	4.4	1	3	7	3	6	0	4	9	0	3	2	11	38.8	61.2
AU526:O6	51	42	9	4.7	1	3	7	3	7	0	4	9	0	3	2	12	39.2	60.8
Median	46	37	9	4.4	1	3	7	3	6	0	4	9	0	3	2	10	38.8	61.2
Mean	38	31	8	4.1	1	2	5	2	5	0	3	8	0	2	2	9	37.0	63.0

AU526:Offspring	Total	Transitions	Transversions	Ratio	N^{pro}	C	E^{rns}	E1	E2	p7	NS2	NS3	NS4A	NS4B	NS5A	NS5B	% S[a]	% NS[b]
AU526:B1A	34	30	4	7.5	0	2	3	4	4	0	4	6	0	5	0	4	69.2	30.8
AU526:B2A	26	21	5	4.2	2	1	0	0	6	1	0	4	1	1	6	4	42.9	57.1
AU526:B3A	23	18	5	3.6	2	1	0	1	4	1	0	4	1	1	5	3	50.0	50.0
AU526:B4A	28	24	4	6.0	2	1	0	1	9	1	0	4	1	1	5	3	70.0	30.0
AU526:B5A	25	21	4	5.3	2	1	0	2	4	1	0	4	1	2	5	3	50.0	50.0
AU526:B6A	27	22	5	4.4	2	1	0	0	5	1	0	6	1	1	6	4	28.6	71.4
Median	27	22	5	4.8	2	1	0	1	5	1	0	4	1	1	5	4	50.0	50.0
Mean	27	23	5	5.2	2	1	1	1	5	1	1	5	1	2	5	4	51.8	48.2
AU526:O1A	49	35	14	2.5	2	1	3	5	3	2	4	13	1	3	4	8	24.5	75.5
AU526:O2A	49	40	9	4.4	1	3	7	3	6	1	4	9	0	3	2	10	38.8	61.2
AU526:O2B	51	42	9	4.7	1	4	8	3	5	1	4	9	0	3	2	11	39.2	60.8
AU526:O4A	52	43	9	4.8	1	3	7	3	7	1	4	9	0	3	2	12	38.5	61.5
Median	50	41	9	4.6	1	3	7	3	6	1	4	9	0	3	2	11	38.6	61.4
Mean	50	40	10	4.1	1	3	6	4	5	1	4	10	0	3	3	10	35.2	64.8

[a] Percentage of substitutions within structural protein-coding regions

[b] Percentage of substitutions within nonstructural protein-coding regions

Table 1.5. Ratio of the number of observed nucleotide substitutions per protein-coding region to the number of expected substitutions assuming a random distribution across the viral genome

AU526:Dam	Total	N^{pro}	C	E^{rns}	E1	E2	p7	NS2	NS3	NS4A	NS4B	NS5A	NS5B
AU526:B1	9	0.00	0.00	1.91	0.00	1.16	0.00	2.14	1.17	0.00	1.25	0.87	0.60
AU526:B2	16	**2.90**	0.00	1.07	0.00	1.95	0.00	0.60	1.32	0.00	1.40	0.00	1.02
AU526:B3	25	1.86	1.53	0.00	0.00	1.67	**2.56**	0.00	0.63	2.44	2.25	1.57	0.65
AU526:B4	26	1.78	1.47	0.66	0.77	2.00	2.46	0.00	0.81	2.34	0.86	1.51	0.63
AU526:B5	25	0.93	0.00	0.69	0.00	1.67	0.00	0.77	0.42	0.00	1.80	2.20	0.87
AU526:B6	20	2.32	1.91	0.00	0.00	2.08	**3.20**	0.00	0.53	**3.05**	0.56	1.96	0.81
Median	23	1.82	0.73	0.67	0.00	1.81	1.23	0.30	0.72	1.17	1.33	1.54	0.73
Mean	20	1.63	0.82	0.72	0.13	1.76	1.37	0.59	0.81	1.30	1.35	1.35	0.76
AU526:O1	0												
AU526:O2	37	0.00	1.03	**2.78**	1.62	0.85	0.00	0.78	1.28	0.00	0.61	0.42	1.17
AU526:O3	42	0.55	1.82	1.64	0.95	1.49	0.00	0.92	1.13	0.00	0.80	0.37	1.16
AU526:O4	49	0.47	2.34	2.45	1.22	1.28	0.00	0.79	0.97	0.00	0.69	0.32	1.22
AU526:O5	49	0.47	2.34	2.45	1.22	1.28	0.00	0.79	0.97	0.00	0.69	0.32	1.22
AU526:O6	51	0.45	2.25	2.36	1.18	1.43	0.00	0.75	0.93	0.00	0.66	0.31	1.28
Median	46	0.47	2.25	2.45	1.22	1.28	0.00	0.79	0.97	0.00	0.69	0.32	1.22
Mean	38	0.39	1.96	2.34	1.24	1.26	0.00	0.80	1.05	0.00	0.69	0.35	1.21
AU526:Offspring	Total	N^{pro}	C	E^{rns}	E1	E2	p7	NS2	NS3	NS4A	NS4B	NS5A	NS5B
AU526:B1A	34	0.00	2.25	1.52	2.35	1.23	0.00	1.13	1.08	0.00	1.65	0.23	0.64
AU526:B2A	26	1.78	1.47	0.00	0.00	2.41	2.46	0.00	0.61	2.34	0.86	1.81	0.83
AU526:B3A	23	2.02	1.66	0.00	0.87	1.81	**2.78**	0.00	0.69	**2.65**	0.98	1.71	0.71
AU526:B4A	28	1.66	1.36	0.00	0.71	**3.35**	2.28	0.00	0.56	2.18	0.80	1.40	0.58
AU526:B5A	25	1.86	1.53	0.00	1.60	1.67	**2.56**	0.00	0.63	2.44	1.35	1.57	0.65
AU526:B6A	27	1.72	1.42	0.00	0.00	1.93	2.37	0.00	0.98	2.26	0.83	1.75	0.80
Median	27	1.75	1.50	0.00	0.79	1.87	2.41	0.00	0.66	2.30	0.92	1.64	0.68
Mean	27	1.51	1.61	0.25	0.92	2.07	2.07	0.19	0.76	1.98	1.08	1.41	0.70
AU526:O1A	49	0.95	0.78	1.05	2.04	0.85	1.30	0.79	1.29	2.49	0.69	0.64	0.89
AU526:O2A	49	0.47	2.34	2.45	1.22	1.49	0.00	0.79	0.97	0.00	0.69	0.32	1.11
AU526:O2B	51	0.45	**3.00**	**2.69**	1.18	1.23	0.00	0.75	0.93	0.00	0.66	0.31	1.17
AU526:O4A	52	0.45	2.20	2.31	1.15	1.60	0.00	0.74	0.91	0.00	0.65	0.30	1.25
Median	50	0.46	2.27	2.38	1.20	1.36	0.00	0.77	0.95	0.00	0.67	0.31	1.14
Mean	50	0.58	2.08	2.13	1.40	1.29	0.33	0.77	1.02	0.62	0.67	0.39	1.10

Ratio values greater than 2.5 are bolded and underlined.

Table 1.6. Amino acid substitutions between AU526 and isolates from acutely infected dams and their offspring

AU526:Dam	Total	N^{pro}	C	E^{rns}	E1	E2	p7	NS2	NS3	NS4A	NS4B	NS5A	NS5B	% S[a]	% NS[b]
AU526:B1	4	0	0	0	0	1	0	2	0	0	1	0	0	25.0	75.0
AU526:B2	7	1	0	1	0	3	0	0	0	0	1	0	1	57.1	42.9
AU526:B3	6	2	0	0	0	2	0	0	1	0	1	0	0	33.3	66.7
AU526:B4	6	2	0	0	1	2	0	0	1	0	0	0	0	50.0	50.0
AU526:B5	7	1	0	1	0	2	0	1	1	0	0	0	1	42.9	57.1
AU526:B6	5	2	0	0	0	2	0	0	1	0	0	0	0	40.0	60.0
Median	6	2	0	0	0	2	0	0	1	0	1	0	0	41.4	58.6
Mean	6	1	0	0	0	2	0	1	1	0	1	0	0	41.4	58.6
AU526:O1	0	0	0	0	0	0	0	0	0	0	0	0	0		
AU526:O2	13	0	0	3	2	2	0	2	0	0	0	1	3	53.8	46.2
AU526:O3	12	0	0	2	1	5	0	1	1	0	0	0	2	66.7	33.3
AU526:O4	13	0	0	2	1	5	0	1	1	0	0	0	3	61.5	38.5
AU526:O5	13	0	0	2	1	5	0	1	1	0	0	0	3	61.5	38.5
AU526:O6	14	0	0	2	1	6	0	1	1	0	0	0	3	64.3	35.7
Median	13	0	0	2	1	5	0	1	1	0	0	0	3	61.5	38.5
Mean	11	0	0	2	1	4	0	1	1	0	0	0	2	61.6	38.4

AU526:Offspring	Total	N^{pro}	C	E^{rns}	E1	E2	p7	NS2	NS3	NS4A	NS4B	NS5A	NS5B	% S[a]	% NS[b]
AU526:B1A	13	0	0	2	3	4	0	1	0	0	2	0	1	69.2	30.8
AU526:B2A	7	2	0	0	0	3	0	0	1	0	0	0	1	42.9	57.1
AU526:B3A	6	2	0	0	1	2	0	0	1	0	0	0	0	50.0	50.0
AU526:B4A	10	2	0	0	1	6	0	0	1	0	0	0	0	70.0	30.0
AU526:B5A	6	2	0	0	1	2	0	0	1	0	0	0	0	50.0	50.0
AU526:B6A	7	2	0	0	0	2	0	0	2	0	0	1	0	28.6	71.4
Median	7	2	0	0	1	3	0	0	1	0	0	0	0	50.0	50.0
Mean	8	2	0	0	1	3	0	0	1	0	0	0	0	51.8	48.2
AU526:O1A	17	1	0	2	3	4	1	3	0	0	1	0	2	52.9	47.1
AU526:O2A	13	0	0	2	1	6	0	1	1	0	0	0	2	69.2	30.8
AU526:O2B	14	0	1	3	1	5	0	1	1	0	0	0	2	71.4	28.6
AU526:O4A	15	0	0	2	1	7	0	1	1	0	0	0	3	66.7	33.3
Median	15	0	0	2	1	6	0	1	1	0	0	0	2	67.9	32.1
Mean	15	0	0	2	2	6	0	2	1	0	0	0	2	65.1	34.9

[a] Percentage of substitutions within structural protein-coding regions

[b] Percentage of substitutions within nonstructural protein-coding regions

Table 1.7. Ratio of the number of observed amino acid substitutions per protein-coding region to the number of expected substitutions assuming a random distribution across the viral genome

AU526:Dam	Total	N^{pro}	C	E^{rns}	E1	E2	p7	NS2	NS3	NS4A	NS4B	NS5A	NS5B
AU526:B1	4	0.00	0.00	0.00	0.00	**2.61**	0.00	**4.81**	0.00	0.00	**2.81**	0.00	0.00
AU526:B2	7	**3.31**	0.00	2.45	0.00	**4.47**	0.00	0.00	0.00	0.00	1.60	0.00	0.77
AU526:B3	6	**7.73**	0.00	0.00	0.00	**3.47**	0.00	0.00	0.88	0.00	1.87	0.00	0.00
AU526:B4	6	**7.73**	0.00	0.00	**3.33**	**3.47**	0.00	0.00	0.88	0.00	0.00	0.00	0.00
AU526:B5	7	**3.31**	0.00	2.45	0.00	**2.98**	0.00	1.37	0.75	0.00	0.00	0.00	0.77
AU526:B6	5	**9.28**	0.00	0.00	0.00	**4.17**	0.00	0.00	1.05	0.00	0.00	0.00	0.00
Median	6	**5.52**	0.00	0.00	0.00	**3.47**	0.00	0.00	0.82	0.00	0.80	0.00	0.00
Mean	6	**5.23**	0.00	0.82	0.56	**3.53**	0.00	1.03	0.59	0.00	1.05	0.00	0.26
AU526:O1	0												
AU526:O2	13	0.00	0.00	**3.96**	**3.08**	1.60	0.00	1.48	0.00	0.00	0.00	0.60	1.25
AU526:O3	12	0.00	0.00	**2.86**	1.67	**4.34**	0.00	0.80	0.44	0.00	0.00	0.00	0.90
AU526:O4	13	0.00	0.00	**2.64**	1.54	**4.01**	0.00	0.74	0.41	0.00	0.00	0.00	1.25
AU526:O5	13	0.00	0.00	**2.64**	1.54	**4.01**	0.00	0.74	0.41	0.00	0.00	0.00	1.25
AU526:O6	14	0.00	0.00	2.45	1.43	**4.47**	0.00	0.69	0.38	0.00	0.00	0.00	1.16
Median	13	0.00	0.00	**2.64**	1.54	**4.01**	0.00	0.74	0.41	0.00	0.00	0.00	1.25
Mean	11	0.00	0.00	**2.91**	1.85	**3.69**	0.00	0.89	0.33	0.00	0.00	0.12	1.16

AU526:Offspring	Total	N^{pro}	C	E^{rns}	E1	E2	p7	NS2	NS3	NS4A	NS4B	NS5A	NS5B
AU526:B1A	13	0.00	0.00	**2.64**	**4.61**	**3.21**	0.00	0.74	0.00	0.00	1.73	0.00	0.42
AU526:B2A	7	**6.63**	0.00	0.00	0.00	**4.47**	0.00	0.00	0.75	0.00	0.00	0.00	0.77
AU526:B3A	6	**7.73**	0.00	0.00	**3.33**	**3.47**	0.00	0.00	0.88	0.00	0.00	0.00	0.00
AU526:B4A	10	**4.64**	0.00	0.00	2.00	**6.25**	0.00	0.00	0.53	0.00	0.00	0.00	0.00
AU526:B5A	6	**7.73**	0.00	0.00	**3.33**	**3.47**	0.00	0.00	0.88	0.00	0.00	0.00	0.00
AU526:B6A	7	**6.63**	0.00	0.00	0.00	**2.98**	0.00	0.00	1.51	0.00	0.00	1.12	0.00
Median	7	**6.63**	0.00	0.00	**2.67**	**3.47**	0.00	0.00	0.82	0.00	0.00	0.00	0.00
Mean	8	**5.56**	0.00	0.44	2.21	**3.98**	0.00	0.12	0.76	0.00	0.29	0.19	0.20
AU526:O1A	17	1.36	0.00	2.02	**3.53**	2.45	**3.76**	1.70	0.00	0.00	0.66	0.00	0.64
AU526:O2A	13	0.00	0.00	**2.64**	1.54	**4.81**	0.00	0.74	0.41	0.00	0.00	0.00	0.83
AU526:O2B	14	0.00	**2.73**	**3.68**	1.43	**3.72**	0.00	0.69	0.38	0.00	0.00	0.00	0.77
AU526:O4A	15	0.00	0.00	2.29	1.33	**4.86**	0.00	0.64	0.35	0.00	0.00	0.00	1.08
Median	15	0.00	0.00	2.47	1.48	**4.27**	0.00	0.71	0.36	0.00	0.00	0.00	0.80
Mean	15	0.34	0.68	**2.66**	1.96	**3.96**	0.94	0.94	0.28	0.00	0.17	0.00	0.83

Ratio values greater than 2.5 are bolded and underlined.

Table 1.8. Conserved amino acid substitutions during serial infection of pregnant heifers with BVDV in early pregnancy

Viral protein	N^{pro}		E2		NS3
AA position	59	146	887	946	1871
AU526	D	K	L	R	D
B1	D	K	L	R	D
B1A	D	K	L	R	D
B2	D	K	L	R	D
B2A	N	R	A	R	E
B3	N	R	V	Q	E
B3A	N	R	V	Q	E
B4	N	R	V	Q	E
B4A	N	R	V	Q	E
B5	D	K	L	Q	D
B5A	N	R	V	Q	E
B6	N	R	V	Q	E
B6A	N	R	V	Q	E

Conserved amino acid (AA) substitutions are shown on a dark grey background.

Table 1.9. Conserved amino acid substitutions during serial infection of pregnant ewes with BVDV in early pregnancy

Viral protein	E^{rns}		E1	E2					NS2	NS3	NS5B		
AA position	439	453	554	728	944	973	993	1065	1146	1607	3284	3457	3611
AU526	H	V	V	G	T	Y	R	S	R	M	R	I	I
O1	H	V	V	G	T	Y	R	S	R	M	R	I	I
O1A	D	V	M	G	T	H	K	L	R	M	K	I	I
O2	D	V	M	G	T	H	K	S	R	M	K	I	I
O2A	D	I	M	R	N	H	K	L	Q	I	K	I	V
O2B	D	I	M	R	N	H	K	L	Q	I	K	I	V
O3	D	I	M	R	N	H	K	L	Q	I	K	V	I
O4	D	I	M	R	N	H	K	L	Q	I	K	V	V
O4A	D	I	M	R	N	H	K	L	Q	I	K	V	V
O5	D	I	M	R	N	H	K	L	Q	I	K	V	V
O6	D	I	M	R	N	H	K	L	Q	I	K	V	V

Conserved amino acid (AA) substitutions are shown on a dark grey background.

Table 1.10. Substitutions between AU526 and isolates from offspring that were detected in isolates from their acutely infected dams

AU526:Offspring	No of nucleotide substitutions	%	% S[a]	% NS[b]	No of amino acid substitutions	%	% S[a]	% NS[b]
AU526:B1A	0	0.0			0	0.0		
AU526:B2A	2	7.7	50.0	50.0	2	28.6	50.0	50.0
AU526:B3A	22	95.7	22.7	77.3	5	83.3	40.0	60.0
AU526:B4A	22	78.6	22.7	77.3	5	50.0	40.0	60.0
AU526:B5A	1	4.0	100.0	0.0	1	16.7	100.0	0.0
AU526:B6A	20	74.1	25.0	75.0	5	71.4	40.0	60.0
Median	11	40.9	25.0	75.0	4	39.3	40.0	60.0
Mean	11	43.3	44.1	55.9	3	41.7	54.0	46.0
AU526:O1A	0	0.0			0	0.0		
AU526:O2A	15	30.6	46.7	53.3	5	38.5	80.0	20.0
AU526:O2B	15	29.4	46.7	53.3	5	35.7	80.0	20.0
AU526:O4A	49	94.2	38.8	61.2	13	86.7	61.5	38.5
Median	15	30.0	46.7	53.3	5	37.1	80.0	20.0
Mean	20	38.6	44.0	56.0	6	40.2	73.8	26.2

[a] Percentage of substitutions within structural protein-coding regions

[b] Percentage of substitutions within nonstructural protein-coding region

Table 1.11. Substitutions between AU526 and isolates from acutely infected dams that were not detected in isolates from their offspring

AU526:Dam	No of nucleotide substitutions	%	% S[a]	% NS[b]	No of amino acid substitutions	%	% S[a]	% NS[b]
AU526:B1	9	100.0			4	100.0	25.0	75.0
AU526:B2	14	87.5	50.0	50.0	5	71.4	60.0	40.0
AU526:B3	3	12.0	22.7	77.3	1	16.7	0.0	100.0
AU526:B4	4	15.4	22.7	77.3	1	16.7	100.0	0.0
AU526:B5	24	96.0	100.0	0.0	6	85.7	33.3	66.7
AU526:B6	0	0.0	25.0	75.0	0	0.0		
Median	7	51.4	25.0	75.0	3	44.0	33.3	66.7
Mean	9	51.8	44.1	55.9	3	48.4	43.7	56.3
AU526:O1	0				0			
AU526:O2[c]	22	59.5	27.3	72.7	8	61.5	37.5	62.5
AU526:O4	0	0.0			0	0.0		
Median	0	29.8	27.3	72.7	0	30.8	37.5	62.5
Mean	7	29.8	27.3	72.7	3	30.8	37.5	62.5

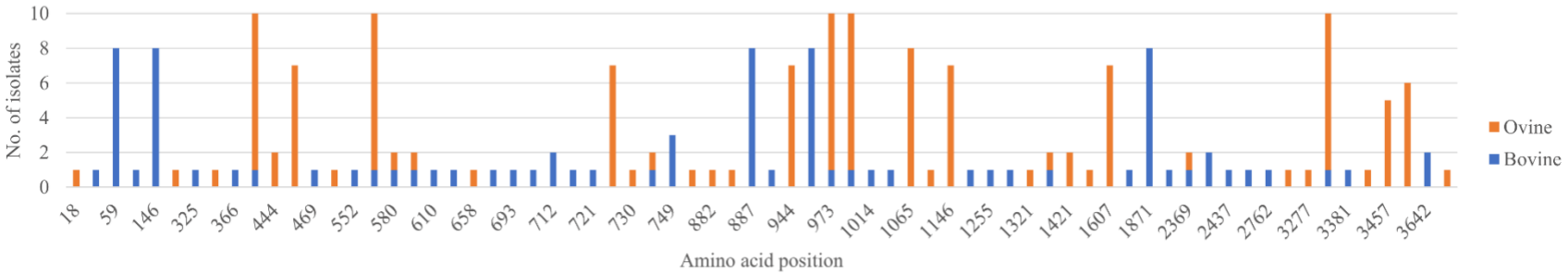
[a] Percentage of substitutions within structural protein-coding regions

[b] Percentage of substitutions within nonstructural protein-coding region

[c] Identical results were obtained for the two lambs of the second ewe (O2A and O2B)

Figures

Figure 1.1. Location of all amino acid substitutions identified in bovine and ovine isolates.



Supplemental Material

Table S1.1. Number of nucleotide differences between AU526 and isolates from acutely infected dams and their offspring

	AU526	B1	B1A	B2	B2A	B3	B3A	B4	B4A	B5	B5A	B6	B6A	O1	O1A	O2	O2A	O2B	O3	O4	O4A	O5
B1	9																					
B1A	34	41																				
B2	16	21	48																			
B2A	26	31	60	38																		
B3	25	32	59	41	9																	
B3A	23	30	57	39	7	4																
B4	26	33	60	42	10	7	5															
B4A	28	35	60	44	12	9	7	10														
B5	25	34	59	41	51	48	46	49	51													
B5A	25	32	59	41	9	6	4	7	9	48												
B6	20	27	54	36	8	5	3	6	8	43	5											
B6A	27	34	61	43	11	8	6	9	11	50	8	7										
O1	0	9	33	16	26	25	23	26	28	25	25	20	27									
O1A	49	58	47	63	75	74	72	73	77	74	74	69	74	48								
O2	37	46	43	51	63	62	60	63	65	62	62	57	64	37	50							
O2A	49	58	57	63	73	72	70	73	75	74	72	67	74	49	64	56						
O2B	51	60	59	65	75	74	72	75	77	76	74	69	76	51	66	58	4					
O3	42	51	52	56	66	65	63	66	68	67	65	60	67	42	59	51	7	9				
O4	49	58	57	63	73	72	70	73	75	74	72	67	74	49	64	56	2	4	5			
O4A	52	61	60	66	76	75	73	76	78	77	75	70	77	52	67	59	5	7	8	3		
O5	49	58	57	63	73	72	70	73	75	74	72	67	74	49	64	56	2	4	5	0	3	
O6	51	60	59	65	75	74	72	75	77	76	74	69	76	51	66	58	4	6	7	2	5	2

Table S1.2. Number of amino acid differences between AU526 and isolates from acutely infected dams and their offspring

	AU526	B1	B1A	B2	B2A	B3	B3A	B4	B4A	B5	B5A	B6	B6A	O1	O1A	O2	O2A	O2B	O3	O4	O4A	O5
B1	4																					
B1A	13	17																				
B2	7	7	20																			
B2A	7	9	20	10																		
B3	6	10	19	13	6																	
B3A	6	10	19	13	6	2																
B4	6	10	19	13	6	2	2															
B4A	10	14	21	17	10	6	6	6														
B5	7	11	20	14	14	11	11	11	15													
B5A	6	10	19	13	6	2	2	2	6	11												
B6	5	9	18	12	5	1	1	1	5	10	1											
B6A	7	11	20	14	7	3	3	3	7	12	3	2										
O1	0	4	12	7	7	6	6	6	10	7	6	5	7									
O1A	17	21	12	24	24	23	23	21	27	24	23	22	24	16								
O2	13	17	16	20	20	19	19	19	23	20	19	18	20	13	16							
O2A	13	17	16	20	20	19	19	19	23	20	19	18	20	13	18	16						
O2B	14	18	17	21	21	20	20	20	24	21	20	19	21	14	19	17	3					
O3	12	16	15	19	19	18	18	18	22	19	18	17	19	12	17	15	3	4				
O4	13	17	16	20	20	19	19	19	23	20	19	18	20	13	18	16	2	3	1			
O4A	15	19	18	22	22	21	21	21	25	22	21	20	22	15	20	18	4	5	3	2		
O5	13	17	16	20	20	19	19	19	23	20	19	18	20	13	18	16	2	3	1	0	2	
O6	14	18	17	21	21	20	20	20	24	21	20	19	21	14	19	17	3	4	2	1	3	1

Table S1.3. Location and type of nucleotide and amino acid substitutions between AU526 and isolates from acutely infected dams

AU526:Dam	Total	Location and type of nucleotide substitutions	Total	Location and type of amino acid substitutions
AU526:B1	9	1017 C:T, **2246 C:T**, **3763 T:C**, **3841 G:C**, 5649 C:T, 5904 T:C, **7120 G:A**, 9138 T:C, 11328 G:A	4	749 T:I, 1255 F:L, 1281 A:P, 2374 E:K
AU526:B2	16	133 C:T, **287 C:A**, **1096 A:G**, **2078 T:C**, **2146 A:G**, **2246 C:T**, 4467 A:G, 5223 C:A, 5733 A:G, 5982 G:A, 6534 C:A, **7120 G:A**, 7788 T:C, 9552 C:A, 10863 G:A, **10924 G:T**	7	96 P:H, 366 K:E, 693 F:S, 716 T:A, 749 T:I, 2374 E:K, 3642 A:S
AU526:B3	25	**175 G:A**, **437 A:G**, 549 G:A, 2208 T:C, 2439 A:G, **2659 C:G**, **2837 G:A**, 3292 C:T, **5613 C:A**, 5712 T:C, 6180 G:A, 6819 C:A, 7047 G:A, 7392 T:A, 7416 A:C, **7559 G:A**, 7746 C:T, 8182 C:T, 8769 A:G, 9024 C:T, 9138 T:C, 9327 T:C, 10044 C:T, 10068 A:G, 10479 G:A	6	59 D:N, 146 K:R, 887 L:V, 946 R:Q, 1871 D:E, 2520 R:K
AU526:B4	26	**175 G:A**, **437 A:G**, 549 G:A, 912 G:A, **1738 T:A**, 2208 T:C, 2439 A:G, 2562 C:T, **2659 C:G**, **2837 G:A**, 3292 C:T, **5613 C:A**, 5712 T:C, 6171 C:T, 6180 G:A, 6819 C:A, 7047 G:A, 7392 T:A, 8182 C:T, 8769 A:G, 9024 C:T, 9138 T:C, 9327 T:C, 10044 C:T, 10068 A:G, 10479 G:A	6	59 D:N, 146 K:R, 580 S:T, 887 L:V, 946 R:Q, 1871 D:E
AU526:B5	25	**83 C:T**, **975 C:A**, 2475 A:G, **2714 C:T**, 2739 C:T, **2837 G:A**, **3494 G:A**, 3789 A:G, **5179 A:G**, 6234 C:T, 7467 T:C, 7623 A:G, 7707 C:T, 8013 A:G, 8073 G:A, 8517 A:G, 8520 T:C, 8736 C:T, 9285 C:T, 9315 C:T, 9369 T:C, **10141 C:A**, 10455 G:A, 10683 T:C, 11577 G:A	7	28 A:V, 325 H:Q, 905 S:F, 946 R:Q, 1165 R:K, 1727 T:A, 3381 H:N
AU526:B6	20	**175 G:A**, **437 A:G**, 549 G:A, 2208 T:C, 2439 A:G, **2659 C:G**, **2837 G:A**, 3292 C:T, **5613 C:A**, 6180 G:A, 6819 C:A, 7047 G:A, 8182 C:T, 8769 A:G, 9024 C:T, 9138 T:C, 9327 T:C, 10044 C:T, 10068 A:G, 10479 G:A	5	59 D:N, 146 K:R, 887 L:V, 946 R:Q, 1871 D:E
AU526:O1	0		0	
AU526:O2	37	783 C:T, **988 G:A**, 1008 T:C, 1203 T:C, 1245 C:A, **1315 C:G**, **1330 G:T**, 1626 A:G, **1660 G:A**, **1973 C:A**, **2917 T:C**, **2978 G:A**, 3174 C:T, 3720 C:T, **3962 A:G**, **4261 A:G**, 4701 A:G, 4881 G:T, 4956 C:T, 5148 G:A, 5181 G:A, 5562 A:G, 5733 A:G, 6009 A:G, 6372 A:G, 7524 T:C, 7836 A:G, 8451 A:C, **9034 G:A**, **9851 G:A**, 10005 T:C, 10245 G:T, **10248 G:T**, 10947 G:A, 11301 C:T, 11469 T:C, **11603 T:A**	13	330 A:T, 439 H:D, 444 V:L, 554 V:M, 658 T:N, 973 Y:H, 993 R:K, 1321 K:R, 1421 T:A, 3012 A:T, 3284 R:K, 3416 E:D, 3868 V:E
AU526:O3	42	247 T:C, 669 A:G, 783 C:T, 846 A:G, 1254 A:G, **1315 C:G**, **1357 G:A**, 1626 A:G, **1660 G:A**, **2182 G:C**, 2451 C:A, **2831 C:A**, **2917 T:C**, **2978 G:A**, **3194 C:T**, **3437 G:A**, 3531 T:A, 3792 G:A, 3846 A:G, 4701 A:G, **4821 G:A**, 4881 G:T, 4959 A:T, 5016 T:C, 5148 G:A, 5562 A:G, 5679 A:G, 5733 A:G, 7524 T:C, 7725 T:A, 7962 A:G, 8461 T:C, 9408 T:C, **9851 G:A**, 10002 C:A, 10044 C:T, **10369 A:G**, 10407 T:C, 10911 A:G, 10947 G:A, 11049 T:C, 11094 T:C	12	439 H:D, 453 V:I, 554 V:M, 728 G:R, 944 T:N, 973 Y:H, 993 R:K, 1065 S:L, 1146 R:Q, 1607 M:I, 3284 R:K, 3457 I:V
AU526:O4	49	247 T:C, 669 A:G, 726 A:G, 783 C:T, 846 A:G, 978 T:C, 1008 T:C, 1254 A:G, **1315 C:G**, **1357 G:A**, 1446 A:G, 1626 A:G, **1660 G:A**, 1812 A:G, **2182 G:C**, 2451 C:A, **2831 C:A**, **2917 T:C**, **2978 G:A**, **3194 C:T**, **3437 G:A**, 3531 T:A, 3792 G:A, 3846 A:G, 4701 A:G, **4821 G:A**, 4881 G:T, 4959 A:T, 5016 T:C, 5148 G:A, 5562 A:G, 5679 A:G, 5733 A:G, 7524 T:C, 7725 T:A, 7962 A:G, 8461 T:C, 9408 T:C, **9851 G:A**, 9927 T:C, 10002 C:A, 10044 C:T, **10369 A:G**, 10407 T:C, **10831 A:G**, 10911 A:G, 10947 G:A, 11049 T:C, 11094 T:C	13	439 H:D, 453 V:I, 554 V:M, 728 G:R, 944 T:N, 973 Y:H, 993 R:K, 1065 S:L, 1146 R:Q, 1607 M:I, 3284 R:K, 3457 I:V, 3611 I:V
AU526:O5	49	247 T:C, 669 A:G, 726 A:G, 783 C:T, 846 A:G, 978 T:C, 1008 T:C, 1254 A:G, **1315 C:G**, **1357 G:A**, 1446 A:G, 1626 A:G, **1660 G:A**, 1812 A:G, **2182 G:C**, 2451 C:A, **2831 C:A**, **2917 T:C**, **2978 G:A**, **3194 C:T**, **3437 G:A**, 3531 T:A, 3792 G:A, 3846 A:G, 4701 A:G, **4821 G:A**, 4881 G:T, 4959 A:T, 5016 T:C, 5148 G:A, 5562 A:G, 5679 A:G, 5733 A:G, 7524 T:C, 7725 T:A, 7962 A:G, 8461 T:C, 9408 T:C, **9851 G:A**, 9927 T:C, 10002 C:A, 10044 C:T, **10369 A:G**, 10407 T:C, **10831 A:G**, 10911 A:G, 10947 G:A, 11049 T:C, 11094 T:C	13	439 H:D, 453 V:I, 554 V:M, 728 G:R, 944 T:N, 973 Y:H, 993 R:K, 1065 S:L, 1146 R:Q, 1607 M:I, 3284 R:K, 3457 I:V, 3611 I:V
AU526:O6	51	247 T:C, 669 A:G, 726 A:G, 783 C:T, 846 A:G, 978 T:C, 1008 T:C, 1254 A:G, **1315 C:G**, **1357 G:A**, 1446 A:G, 1626 A:G, **1660 G:A**, 1812 A:G, **2182 G:C**, **2300 C:T**, 2451 C:A, **2831 C:A**, **2917 T:C**, **2978 G:A**, **3194 C:T**, **3437 G:A**, 3531 T:A, 3792 G:A, 3846 A:G, 4701 A:G, **4821 G:A**, 4881 G:T, 4959 A:T, 5016 T:C, 5148 G:A, 5562 A:G, 5679 A:G, 5733 A:G, 7524 T:C, 7725 T:A, 7962 A:G, 8461 T:C, 9408 T:C, **9851 G:A**, 9927 T:C, 10002 C:A, 10044 C:T, **10369 A:G**, 10407 T:C, **10831 A:G**, 10911 A:G, 10947 G:A, 11049 T:C, 11094 T:C, 11332 T:C	14	439 H:D, 453 V:I, 554 V:M, 728 G:R, 767 P:L, 944 T:N, 973 Y:H, 993 R:K, 1065 S:L, 1146 R:Q, 1607 M:I, 3284 R:K, 3457 I:V, 3611 I:V

Nonsynonymous nucleotide substitutions are bolded.

Table S1.4. Location and type of nucleotide and amino acid substitutions between AU526 and isolates from offspring born to dams infected with BVDV in early pregnancy

AU526:Offspring	Total	Location and type of nucleotide substitutions	Total	Location and type of amino acid substitutions
AU526:B1A	34	774 A:G, 783 C:T, 1008 T:C, **1315 C:G**, **1406 A:G**, **1660 G:A**, 1800 A:G, **1802 A:G**, **2055 A:G**, **2135 C:T**, **2211 A:T**, **2917 T:C**, **2978 G:A**, 3462 T:C, 4017 T:C, 4071 T:C, **4117 A:G**, 4701 A:G, 4881 G:T, 5094 T:C, 5562 A:G, 5733 A:G, 5862 G:A, 6702 T:C, **7105 G:A**, **7309 A:T**, 7362 T:C, 7524 T:C, 7575 C:T, 8079 T:C, **9851 G:A**, 10947 G:A, 11007 A:G, 11301 C:T	13	439 H:D, 469 K:R, 554 V:M, 601 E:G, 685 I:M, 712 P:L, 737 E:D, 973 Y:H, 993 R:K, 1373 I:V, 2369 A:T, 2437 T:S, 3284 R:K
AU526:B2A	26	**175 G:A**, **437 A:G**, 549 G:A, 2208 T:C, **2246 C:T**, 2439 A:G, **2659 C:G**[a], **2660 T:C**[a], **3041 G:A**, 3292 C:T, **5613 C:A**, 5712 T:C, 6180 G:A, 6819 C:A, 7047 G:A, 7392 T:A, 8182 C:T, 8745 A:G, 8769 A:G, 9024 C:T, 9138 T:C, 9327 T:C, 10044 C:T, 10068 A:G, 10479 G:A, **10924 G:T**	7	59 D:N, 146 K:R, 749 T:I, 887 L:A, 1014 G:E, 1871 D:E, 3642 A:S
AU526:B3A	23	**175 G:A**, **437 A:G**, 549 G:A, **1843 T:G**, 2208 T:C, 2439 A:G, **2659 C:G**, **2837 G:A**, 3292 C:T, **5613 C:A**, 5712 T:C, 6180 G:A, 6819 C:A, 7047 G:A, 7392 T:A, 8182 C:T, 8769 A:G, 9024 C:T, 9138 T:C, 9327 T:C, 10044 C:T, 10068 A:G, 10479 G:A	6	59 D:N, 146 K:R, 615 L:V, 887 L:V, 946 R:Q, 1871 D:E
AU526:B4A	28	**175 G:A**, **437 A:G**, 549 G:A, **1654 T:C**, **2102 C:T**, **2135 C:T**, **2161 C:T**[b], **2162 A:G**[b], 2208 T:C, 2439 A:G, **2659 C:G**, **2837 G:A**, **3104 C:T**, 3292 C:T, **5613 C:A**, 5712 T:C, 6180 G:A, 6819 C:A, 7047 G:A, 7392 T:A, 8182 C:T, 8769 A:G, 9024 C:T, 9138 T:C, 9327 T:C, 10044 C:T, 10068 A:G, 10479 G:A	10	59 D:N, 146 K:R, 552 S:P, 701 S:L, 712 P:L, 721 Q:W, 887 L:V, 946 R:Q, 1035 S:F, 1871 D:E
AU526:B5A	25	**175 G:A**, **437 A:G**, 549 G:A, 1689 A:G, **1828 G:A**, 2208 T:C, 2439 A:G, **2659 C:G**, **2837 G:A**, 3292 C:T, **5613 C:A**, 5712 T:C, 6180 G:A, 6819 C:A, 7047 G:A, 7122 A:G, 7392 T:A, 8182 C:T, 8769 A:G, 9024 C:T, 9138 T:C, 9327 T:C, 10044 C:T, 10068 A:G, 10479 G:A	6	59 D:N, 146 K:R, 610 G:R, 887 L:V, 946 R:Q, 1871 D:E
AU526:B6A	27	**175 G:A**, **437 A:G**, 549 G:A, 2208 T:C, 2295 C:A, 2439 A:G, **2659 C:G**, **2837 G:A**, 3292 C:T, **5613 C:A**, 5712 T:C, 6180 G:A, **6301 C:T**, 6645 C:T, 6819 C:A, 7047 G:A, 7392 T:A, 8182 C:T, **8285 A:G**, 8769 A:G, 9024 C:T, 9138 T:C, 9327 T:C, 10044 C:T, 10068 A:G, 10479 G:A, 10647 G:A	7	59 D:N, 146 K:R, 887 L:V, 946 R:Q, 1871 D:E, 2101 P:S, 2762 K:R
AU526:O1A	49	**53 C:T**, 327 A:T, 783 C:T, 1008 T:C, **1315 C:G**, **1330 G:T**, 1626 A:G, **1660 G:A**, **1738 T:A**, **1802 A:G**, 1833 A:G, **2211 A:T**, **2917 T:C**, **2978 G:A**, **3194 C:T**, **3206 T:A**, 3690 T:G, **4117 A:G**, **4261 A:G**, **4378 G:A**, 4701 A:G, 4881 G:T, 4899 A:T, 5148 G:A, 5553 A:G, 5562 A:G, 5679 A:G, 5733 A:G, 5811 T:C, 6114 G:T, 6216 A:T, 6645 C:T, 6858 T:C, 6996 A:G, **7105 G:A**, 7524 T:C, 7692 G:A, 8598 T:C, 8604 G:A, 9195 G:A, 9318 A:T, **9831 G:T**, **9851 G:A**, 10119 A:G, 10542 A:G, 10947 G:A, 11037 A:T, 11082 C:T, 11301 C:T	17	18 S:F, 439 H:D, 444 V:L, 554 V:M, 580 S:T, 601 E:G, 737 E:D, 973 Y:H, 993 R:K, 1065 S:L, 1069 F:Y, 1373 I:V, 1421 T:A, 1460 E:K, 2369 A:T, 3277 K:N, 3284 R:K
AU526:O2A	49	247 T:C, 669 A:G, 726 A:G, 783 C:T, 846 A:G, 978 T:C, 1008 T:C, 1254 A:G, **1315 C:G**, **1357 G:A**, 1446 A:G, 1626 A:G, **1660 G:A**, 1812 A:G, **2182 G:C**, 2451 C:A, **2644 G:A**, **2831 C:A**, **2917 T:C**, **2978 G:A**, **3194 C:T**, **3437 G:A**, 3531 T:A, 3792 G:A, 3846 A:G, 4701 A:G, **4821 G:A**, 4881 G:T, 4959 A:T, 5016 T:C, 5148 G:A, 5562 A:G, 5679 A:G, 5733 A:G, 7524 T:C, 7725 T:A, 7962 A:G, 8461 T:C, 9408 T:C, **9851 G:A**, 9927 T:C, 10002 C:A, 10044 C:T, 10407 T:C, **10831 A:G**, 10911 A:G, 10947 G:A, 11049 T:C, 11094 T:C	13	439 H:D, 453 V:I, 554 V:M, 728 G:R, 882 V:M, 944 T:N, 973 Y:H, 993 R:K, 1065 S:L, 1146 R:Q, 1607 M:I, 3284 R:K, 3611 I:V
AU526:O2B	51	247 T:C, **523 G:A**, 669 A:G, 726 A:G, 783 C:T, 846 A:G, 978 T:C, 1008 T:C, 1254 A:G, **1315 C:G**, **1357 G:A**, **1435 G:A**, 1446 A:G, 1626 A:G, **1660 G:A**, 1812 A:G, **2182 G:C**, 2451 C:A, **2831 C:A**, **2917 T:C**, **2978 G:A**, **3194 C:T**, **3437 G:A**, 3531 T:A, 3792 G:A, 3846 A:G, 4701 A:G, **4821 G:A**, 4881 G:T, 4959 A:T, 5016 T:C, 5148 G:A, 5562 A:G, 5679 A:G, 5733 A:G, 7524 T:C, 7725 T:A, 7962 A:G, 8461 T:C, 9408 T:C, **9851 G:A**, 9927 T:C, 10002 C:A, 10044 C:T, 10407 T:C, 10416 G:A, **10831 A:G**, 10911 A:G, 10947 G:A, 11049 T:C, 11094 T:C	14	175 G:R, 439 H:D, 453 V:I, 479 G:R, 554 V:M, 728 G:R, 944 T:N, 973 Y:H, 993 R:K, 1065 S:L, 1146 R:Q, 1607 M:I, 3284 R:K, 3611 I:V
AU526:O4A	52	247 T:C, 669 A:G, 726 A:G, 783 C:T, 846 A:G, 978 T:C, 1008 T:C, 1254 A:G, **1315 C:G**, **1357 G:A**, 1446 A:G, 1626 A:G, **1660 G:A**, 1812 A:G, **2182 G:C**, **2189 A:G**, 2451 C:A, **2648 C:T**, **2831 C:A**, **2917 T:C**, **2978 G:A**, **3194 C:T**, **3437 G:A**, 3531 T:A, 3792 G:A, 3846 A:G, 4701 A:G, **4821 G:A**, 4881 G:T, 4959 A:T, 5016 T:C, 5148 G:A, 5562 A:G, 5679 A:G, 5733 A:G, 7524 T:C, 7725 T:A, 7962 A:G, 8461 T:C, 9408 T:C, **9851 G:A**, 9927 T:C, 10002 C:A, 10044 C:T, 10137 A:G, **10369 A:G**, 10407 T:C, **10831 A:G**, 10911 A:G, 10947 G:A, 11049 T:C, 11094 T:C	15	439 H:D, 453 V:I, 554 V:M, 728 G:R, 730 Q:R, 883 P:L, 944 T:N, 973 Y:H, 993 R:K, 1065 S:L, 1146 R:Q, 1607 M:I, 3284 R:K, 3457 I:V, 3611 I:V

[a] Nucleotide substitutions associated with the same amino acid substitution (887 L:A)
[b] Nucleotide substitutions associated with the same amino acid substitution (721 Q:W)
Nonsynonymous nucleotide substitutions are bolded.

Table S1.5. Location and type of nucleotide and amino acid substitutions between BVDV-1b isolates from acutely infected dams and their offspring

Dam:Offspring	Total	Location and type of nucleotide substitutions	Total	Location and type of amino acid substitutions
B1:B1A	43	774 A:G, 783 C:T, 1008 T:C, 1017 T:C, **1315 C:G**, **1406 A:G**, **1660 G:A**, 1800 A:G, **1802 A:G**, **2055 A:G**, **2135 C:T**, **2211 A:T**, **2246 T:C**, **2917 T:C**, **2978 G:A**, 3462 T:C, **3763 C:T**, **3841 C:G**, 4017 T:C, 4071 T:C, **4117 A:G**, 4701 A:G, 4881 G:T, 5094 T:C, 5562 A:G, 5649 T:C, 5733 A:G, 5862 G:A, 5904 C:T, 6702 T:C, **7105 G:A**, **7120 A:G**, **7309 A:T**, 7362 T:C, 7524 T:C, 7575 C:T, 8079 T:C, 9138 C:T, **9851 G:A**, 10947 G:A, 11007 A:G, 11301 C:T, 11328 A:G	17	439 H:D, 469 K:R, 554 V:M, 601 E:G, 685 I:M, 712 P:L, 737 E:D, 749 I:T, 973 Y:H, 993 R:K, 1255 L:F, 1281 P:A, 1373 I:V, 2369 A:T, 2374 K:E, 2437 T:S, 3284 R:K
B2:B2A	38	133 T:C, **175 G:A**, **287 A:C**, **437 A:G**, 549 G:A, **1096 G:A**, **2078 C:T**, **2146 G:A**, 2208 T:C, 2439 A:G, **2659 C:G**[a], **2660 T:C**[a], **3041 G:A**, 3292 C:T, 4467 G:A, 5223 A:C, **5613 C:A**, 5712 T:C, 5733 G:A, 5982 A:G, 6180 G:A, 6534 A:C, 6819 C:A, 7047 G:A, **7120 A:G**, 7392 T:A, 7788 C:T, 8182 C:T, 8745 A:G, 8769 A:G, 9024 C:T, 9138 T:C, 9327 T:C, 9552 A:C, 10044 C:T, 10068 A:G, 10479 G:A, 10863 A:G	10	59 D:N, 96 H:P, 146 K:R, 366 E:K, 693 S:F, 716 A:T, 887 L:A, 1014 G:E, 1871 D:E, 2374 K:E
B3:B3A	4	**1843 T:G**, 7416 C:A, **7559 A:G**, 7746 T:C	2	615 L:V, 2520 K:R
B4:B4A	10	912 A:G, **1654 T:C**, **1738 A:T**, **2102 C:T**, **2135 C:T**, **2161 C:T**[b], **2162 A:G**[b], 2562 T:C, **3104 C:T**, 6171 T:C	6	552 S:P, 580 T:S, 701 S:L, 712 P:L, 721 Q:W, 1035 S:F
B5:B5A	48	**83 T:C**, **175 G:A**, **437 A:G**, 549 G:A, **975 A:C**, 1689 A:G, **1828 G:A**, 2208 T:C, 2439 A:G, 2475 G:A, **2659 C:G**, **2714 T:C**, 2739 T:C, 3292 C:T, **3494 A:G**, 3789 G:A, **5179 G:A**, **5613 C:A**, 5712 T:C, 6180 G:A, 6234 T:C, 6819 C:A, 7047 G:A, 7122 A:G, 7392 T:A, 7467 C:T, 7623 G:A, 7707 T:C, 8013 G:A, 8073 A:G, 8182 C:T, 8517 G:A, 8520 C:T, 8736 T:C, 8769 A:G, 9024 C:T, 9138 T:C, 9285 T:C, 9315 T:C, 9327 T:C, 9369 C:T, 10044 C:T, 10068 A:G, **10141 A:C**, 10455 A:G, 10479 G:A, 10683 C:T, 11577 A:G	11	28 V:A, 59 D:N, 146 K:R, 325 Q:H, 610 G:R, 887 L:V, 905 F:S, 1165 K:R, 1727 A:T, 1871 D:E, 3381 N:H
B6:B6A	7	2295 C:A, 5712 T:C, **6301 C:T**, 6645 C:T, 7392 T:A, **8285 A:G**, 10647 G:A	2	2101 P:S, 2762 K:R
O1:O1A	49	**53 C:T**, 327 A:T, 783 C:T, 1008 T:C, **1315 C:G**, **1330 G:T**, 1626 A:G, **1660 G:A**, **1738 T:A**, **1802 A:G**, 1833 A:G, **2211 A:T**, **2917 T:C**, **2978 G:A**, **3194 C:T**, **3206 T:A**, 3690 T:G, **4117 A:G**, **4261 A:G**, **4378 G:A**, 4701 A:G, 4881 G:T, 4899 A:T, 5148 G:A, 5553 A:G, 5562 A:G, 5679 A:G, 5733 A:G, 5811 T:C, 6114 G:T, 6216 A:T, 6645 C:T, 6858 T:C, 6996 A:G, **7105 G:A**, 7524 T:C, 7692 G:A, 8598 T:C, 8604 G:A, 9195 G:A, 9318 A:T, **9831 G:T**, **9851 G:A**, 10119 A:G, 10542 A:G, 10947 G:A, 11037 A:T, 11082 C:T, 11301 C:T	17	18 S:F, 439 H:D, 444 V:L, 554 V:M, 580 S:T, 601 E:G, 737 E:D, 973 Y:H, 993 R:K, 1065 S:L, 1069 F:Y, 1373 I:V, 1421 T:A, 1460 E:K, 2369 A:T, 3277 K:N, 3284 R:K
O2:O2A	56	247 T:C, 669 A:G, 726 A:G, 846 A:G, 978 T:C, **988 A:G**, 1203 C:T, 1245 A:C, 1254 A:G, **1330 T:G**, **1357 G:A**, 1446 A:G, 1812 A:G, **1973 A:C**, **2182 G:C**, 2451 C:A, **2644 G:A**, **2831 C:A**, 3174 T:C, **3194 C:T**, **3437 G:A**, 3531 T:A, 3720 T:C, 3792 G:A, 3846 A:G, **3962 G:A**, **4261 G:A**, **4821 G:A**, 4956 T:C, 4959 A:T, 5016 T:C, 5181 A:G, 5679 A:G, 6009 G:A, 6372 G:A, 7725 T:A, 7836 G:A, 7962 A:G, 8451 C:A, 8461 T:C, **9034 A:G**, 9408 T:C, 9927 T:C, 10002 C:A, 10005 C:T, 10044 C:T, 10245 T:G, **10248 T:G**, 10407 T:C, **10831 A:G**, 10911 A:G, 11049 T:C, 11094 T:C, 11301 T:C, 11469 C:T, **11603 A:T**	16	330 T:A, 444 L:V, 453 V:I, 658 N:T, 728 G:R, 882 V:M, 944 T:N, 1065 S:L, 1146 R:Q, 1321 R:K, 1421 A:T, 1607 M:I, 3012 T:A, 3416 D:E, 3611 I:V, 3868 E:V
O2:O2B	58	247 T:C, **523 G:A**, 669 A:G, 726 A:G, 846 A:G, 978 T:C, **988 A:G**, 1203 C:T, 1245 A:C, 1254 A:G, **1330 T:G**, **1357 G:A**, **1435 G:A**, 1446 A:G, 1812 A:G, **1973 A:C**, **2182 G:C**, 2451 C:A, **2831 C:A**, 3174 T:C, **3194 C:T**, **3437 G:A**, 3531 T:A, 3720 T:C, 3792 G:A, 3846 A:G, **3962 G:A**, **4261 G:A**, **4821 G:A**, 4956 T:C, 4959 A:T, 5016 T:C, 5181 A:G, 5679 A:G, 6009 G:A, 6372 G:A, 7725 T:A, 7836 G:A, 7962 A:G, 8451 C:A, 8461 T:C, **9034 A:G**, 9408 T:C, 9927 T:C, 10002 C:A, 10005 C:T, 10044 C:T, 10245 T:G, **10248 T:G**, 10407 T:C, 10416 G:A, **10831 A:G**, 10911 A:G, 11049 T:C, 11094 T:C, 11301 T:C, 11469 C:T, **11603 A:T**	17	175 G:R, 330 T:A, 444 L:V, 453 V:I, 479 G:R, 658 N:T, 728 G:R, 944 T:N, 1065 S:L, 1146 R:Q, 1321 R:K, 1421 A:T, 1607 M:I, 3012 T:A, 3416 D:E, 3611 I:V, 3868 E:V
O4:O4A	3	**2189 A:G**, **2648 C:T**, 10137 A:G	2	730 Q:R, 883 P:L

[a] Nucleotide substitutions associated with the same amino acid substitution (887 L:A)
[b] Nucleotide substitutions associated with the same amino acid substitution (721 Q:W)
Nonsynonymous nucleotide substitutions are bolded.

Table S1.6. Virus titers in passaged serum samples from acutely infected pregnant dams

	Virus titers ($TCID_{50}$/ml)	
Dam	5 dpi	7 dpi
B1	NA	6.2×10^4
B2	NA	2.0×10^6
B3	NA	6.2×10^5
B4	3.5×10^2	NA
B5	2.0×10^4	NA
B6	NA	NA
O1	6.2×10^7	NA
O2	2.0×10^8	2.0×10^7
O3	NA	2.0×10^7
O4	NA	6.2×10^6
O5	3.5×10^6	NA
O6	NA	2.0×10^7

dpi, day postinoculation; NA, not applicable

Table S1.7. Virus titers in passaged serum or tissue samples from offspring born to dams infected with BVDV

	Virus titers ($TCID_{50}$/ml)			
Offspring	day of birth	42 doa	84 doa	168 doa
B1A	3.5×10^4	NA	NA	NA
B2A	NA	NA	2.0×10^4	2.0×10^5
B3A	6.2×10^5	NA	NA	NA
B4A	NA	3.5×10^5	3.5×10^7	3.5×10^7
B5A	NA	6.2×10^6	3.5×10^7	3.5×10^7
B6A	NA	3.5×10^2	2.0×10^5	1.1×10^5
O1A	6.2×10^6	NA	NA	NA
O2A	2.0×10^6	NA	NA	NA
O2B	6.2×10^7	NA	NA	NA
O4A	2.0×10^4	NA	NA	NA

doa, days of age; NA, not applicable

Table S1.8. Results of RT-qPCR analyses of serum samples from acutely infected pregnant ewes

	qRT-PCR[a]	
Dam	5 dpi	7 dpi
O1	+ (400/275/36.18)	- (192/275/37.57)
O2	+ (363/311/28.53)	- (160/311/14.17)
O3	- (7.31/281/0)	- (219/281/37.33)
O4	- (4.46/380/0)	- (73.6/380/37.41)
O5	- (289/434/24.52)	- (74.9/434/6.57)
O6	- (338/503/14.56)	+ (934/503/33.86)

[a] End relative fluorescence unit (RFU) values are given in parentheses followed by RFU cut-off and mean quantification cycle (Cq) values
dpi, day postinoculation; +, positive; -, negative

Chapter 3: Changes in the Open Reading Frame of Bovine Viral Diarrhea Virus during Serial Infection of Pregnant Swine

Abstract

Bovine viral diarrhea virus (BVDV) is an economically important pathogen of cattle, but this virus can also infect pigs, camelids, and a wide range of domestic and wild ruminants. BVDV isolates circulating in animal populations are genetically and antigenically highly diverse. Previous studies demonstrated that many substitutions were introduced in the viral genome during acute BVDV infections in cattle, but little is known about BVDV infections in species other than cattle. Using BVDV-1b isolate of bovine origin (AU526), greater numbers of substitutions occurred during serial infection of pregnant sheep than of pregnant cattle. To our knowledge, genomic changes arising during BVDV infections in swine have not been investigated. The purpose of this study was to determine the changes introduced in the open reading frame (ORF) of the BVDV genome during serial infection of pregnant swine with an isolate of bovine origin. Serial inoculations were performed in six pregnant gilts using BVDV-1b isolate AU526 in the first gilt and serum from the preceding acutely infected gilt thereafter. The complete ORF sequences of 14 BVDV-1b isolates obtained from acutely infected gilts and their congenitally infected piglets were determined. Sequence comparison revealed that great numbers of genomic changes occurred during serial infection of pregnant swine. Furthermore, 12 identical amino acid substitutions were detected in all porcine isolates, with four and two substitutions in the E2 and E^{rns} coding regions, respectively. These results suggest that BVDV infections in swine may serve as a significant source of viral genetic variability and may be associated with adaptive changes.

Introduction

Bovine viral diarrhea virus (BVDV) is an economically important pathogen of cattle, but this virus can also infect pigs, camelids, and a wide range of domestic and wild ruminants, including sheep, goats, and deer (Passler & Walz, 2010). Acute BVDV infections in pigs typically result in mild or no signs of illness (Stewart et al., 1971). However, BVDV may cause severe reproductive losses in pregnant swine, including early embryonic death, fetal mummification, fetal growth retardation, stillbirth, and abortion (Paton & Done, 1994; Terpstra & Wensvoort, 1988). Pregnant sows experimentally exposed to BVDV gave birth to completely or partially-infected litters (Paton & Done, 1994). Piglets congenitally infected with BVDV remained persistently infected (PI) for life or were chronically infected for several weeks, after which they seroconverted and cleared the virus. Similar findings have been reported in piglets born to sows exposed to classical swine fever virus (CSFV) during pregnancy (Van Oirschot, 1979).

BVDV is the prototypic member of the genus *Pestivirus* within the *Flaviviridae* family. Similar to other pestiviruses, BVDV has an enveloped, positive-sense, single-stranded RNA genome that is approximately 12.3 kb in length and contains a single open reading frame (ORF) encoding about 3,900 amino acids and two untranslated regions at the 5' and 3' ends (Collett et al., 1988c). Translation of the ORF generates a large polyprotein that is co- and post-translationally processed to yield 12 mature structural (C, E^{rns}, E1, E2) and nonstructural (N^{pro}, p7, NS2, NS3, NS4A, NS4B, NS5A, NS5B) viral proteins (Collett et al., 1988b). BVDV isolates circulating in animal populations are genetically and antigenically highly diverse. Two BVDV species, BVDV-1 and BVDV-2, are currently recognized and at least 21 BVDV-1 subgenotypes (1a-1u) and four BVDV-2 subgenotypes (2a-2d) have been described to date (Giammarioli et al.,

2015; Simmonds et al., 2017; Yesilbag et al., 2017). The accumulation of point mutations due to the minimal proofreading activity of the viral RNA polymerase is believed to be the main driving force for the generation of altered genomic sequences (Steinhauer et al., 1992).

Acute BVDV infections in pregnant cattle were found to result in 2.3 to 8 times more nucleotide substitutions than in non-pregnant cattle (Neill et al., 2012). Furthermore, most amino acid substitutions occurred in genomic regions encoding structural viral proteins. These results suggested that infections in pregnant cattle may greatly contribute to the genetic and antigenic variability of BVDV. Amino acid substitutions in the E2 coding region have also been identified during infection of pregnant sheep and goats with BVDV (Bachofen et al., 2013; Gunn et al., 1992; Passler et al., 2014a; Paton et al., 1997). Using a BVDV-1b isolate of bovine origin (AU526), greater numbers of substitutions occurred during serial infection of pregnant sheep than of pregnant cattle (Kuca et al., submitted).

To our knowledge, genomic changes arising during BVDV infections in swine have not been investigated. The purpose of this study was to determine the changes introduced in the ORF of the BVDV genome during serial infection of pregnant swine with an isolate of bovine origin. There are many host barriers and defense mechanisms that prevent interspecies transmission of viruses including innate and adaptive immune responses and apolipoprotein B-editing catalytic polypeptide (APOBEC) proteins (Parrish et al., 2008). Genomic changes would likely be required to overcome these obstacles and we thus hypothesized that the number of changes would be greater in swine than in cattle during serial infection with a BVDV isolate of bovine origin.

Materials and Methods

Animals

All experimental procedures were performed with approval and under the guidelines of the Institutional Animal Care and Use Committee of Auburn University (No. 2015-2706). Seven American Yorkshire-cross pregnant gilts were acquired from Auburn University Swine Research and Education Center.

Prior to inclusion, animals were confirmed to be pregnant by transabdominal ultrasonography, free of BVDV by virus isolation (VI), and seronegative to BVDV by virus neutralization (VN). Animals were transported to the Sugg Laboratory Isolation building at Auburn University and housed in isolation rooms. At the time of inoculation, pregnant gilts were at 27 to 39 days of gestation. Animals were visually inspected daily for signs of illness and evidence of reproductive losses throughout the study period.

Virus inoculation and sample collection

On day 0, a physical examination was performed, pregnancy was confirmed by ultrasonography, and blood was collected for VI and VN prior to inoculation. The first gilt was inoculated intravenously with approximately 1.0×10^6 50% tissue culture infective dose ($TCID_{50}$) of BVDV-1b AU526. The noncytopathic BVDV-1b isolate AU526 had been isolated from the serum of a PI cow, which was part of a research herd at Auburn University Animal Health Research. Virus inoculum was prepared by adding 20 µl of virus stock at a titer of 5.0×10^7 $TCID_{50}$/ml to 980 µl of culture medium. Virus stock had been passaged twice in Madin-Darby bovine kidney (MDBK) cells. Residual inoculum was stored at -80°C for estimation of received dose by virus titration. The BVDV-1b isolate AU526 was utilized in this study because of its capacity to establish persistent infection in species other than cattle, including goats and

white-tailed deer (Passler et al., 2014a; Passler et al., 2007). In a previous study, this isolate was serially passaged in six pregnant heifers and six pregnant ewes following a procedure similar to the one described below (Kuca et al., submitted).

On days 5 and 7 postinoculation (pi), a physical examination was performed and blood was collected for VI and reverse transcriptase nested PCR (RT-nPCR). The second, third, and fourth gilts were inoculated with 1 ml of serum obtained on day 5 pi from the first, second, and third gilts, whereas the fifth, sixth, and seventh gilts were inoculated with 1 ml of serum obtained on day 7 pi from the third, fifth, and sixth gilts, respectively.

Following inoculation, pregnant gilts were either housed individually or in pairs and pregnancies were allowed to proceed to term. Blood was collected every 28 days for VN until term. Ultrasound examinations were performed to assess pregnancy viability at the same intervals. One week prior to the anticipated date of farrowing, gilts were housed individually.

At the time of farrowing, skin biopsy samples were collected from piglets for antigen-capture enzyme-linked immunosorbent assay (ACE) and reverse transcriptase PCR (RT-PCR). Blood was also collected from dams for VN. Postmortem examinations were performed on stillborn and deceased piglets. Representative sections of placental and fetal tissues were collected for VI including liver, spleen, thymus, lymph nodes, kidney, lung, heart, gonad, small intestine, and brain. Additional blood samples were collected every six weeks from viable piglets for VI and VN until six months of age.

Sample processing

Blood collected in plain tubes was allowed to clot at room temperature for at least 30 min. Following centrifugation at 200 × *g* for 20 min, serum was harvested and stored at -80°C or immediately used in VI, VN, and RT-nPCR procedures. Blood collected in EDTA-containing

tubes was processed to yield buffy coat. Following centrifugation at 700 × *g* for 30 min, buffy coat was removed using a sterile Pasteur pipette. Lysis of red blood cells was performed using 10 ml of 0.15 M ammonium chloride (NH_4Cl) and the mixture was allowed to stand at room temperature for 1 h. Buffy coat was subsequently washed in 10 ml of culture medium. Following centrifugation at 700 × *g* for 10 min, buffy coat was resuspended in 1 ml of culture medium to be used in VI and RT-nPCR procedures. Nasal swabs were placed in tubes containing culture medium to be used in VI and RT-nPCR procedures. Skin biopsy samples were placed in tubes containing phosphate-buffered saline (PBS) to be used in ACE procedures. Sections of placental and fetal tissues were placed in stomacher bags containing 3 ml of culture medium and homogenized for 5 min with a Tekmar Stomacher® laboratory blender (Tekmar, Co., Cincinnati, OH, USA) to be used in VI procedures.

Cells

MDBK cells were purchased from the American Type Cell Culture Collection (CCL-22™) and confirmed to be of bovine origin through amplification and sequencing of mitochondrial cytochrome c oxidase subunit 1 (CO1) gene (CellCheck™, IDEXX Laboratories, Inc., Westbrook, ME, USA). PCR assays also demonstrated the absence of *Mycoplasma* and mammalian interspecies contamination (STAT-Myco™, IDEXX Laboratories, Inc.). Cells were grown in minimum essential medium (MEM) with Earle's salts supplemented with 10% equine serum, L-glutamine (0.02 mM), sodium bicarbonate (0.75 mg/ml), penicillin (100 U/ml) and streptomycin (100 µg/ml).

Virus isolation

Serum, buffy coat, and nasal swab samples as well as placental and fetal tissue samples were assayed for BVDV by passage through MDBK cells for 4 days. An immunoperoxidase monolayer assay (IPMA) was subsequently used to identify BVDV-positive cells.

Briefly, serum samples were assayed by adding 768 µl of serum to 192 µl of culture medium on 9.6-cm^2 wells of a 6-well culture plate that had been seeded 24 h earlier with MDBK cells, whereas buffy coat, nasal swab, and tissue samples were assayed by adding 1 ml of sample suspension on 9.6-cm^2 wells of a 6-well culture plate previously seeded with MDBK cells. The plates were incubated at 37°C for 1 h in humidified air containing 5% CO_2. Subsequently, 3 ml of culture medium was added to each well. The plates were then incubated for 4 days. Following a single freeze-thaw cycle to release any intracellular virus, cell lysates were assayed in triplicate by adding 10 µl of cell lysate to 90 µl of culture medium and 50 µl of culture medium containing MDBK cells on 0.32-cm^2 wells of a 96-well culture plate. The plates were then incubated for 3 days. Following fixation, 50 µl of a mixture of two monoclonal antibodies, D89 and 20.10.6, was added to each well. These antibodies were specific for the envelope protein E2 and the nonstructural protein NS3 of BVDV, respectively. The plates were incubated at 37°C for 1 h to allow antibody binding. Following washing with PBS containing 0.05% Tween 20 to remove unbound antibodies, 50 µl of diluted peroxidase-conjugated rabbit anti-mouse IgG was added to each well and culture plates were incubated at 37°C for 1 h. Following washing with PBS containing 0.05% Tween 20, 50 µl of aminoethyl carbazole (AEC) substrate was added to each well and culture plates were incubated at room temperature for 15 min. This enzyme substrate produces a reddish-brown color when oxidized by horseradish peroxidase. Color change was

examined by use of light microscopy and compared with that of positive- and negative-control samples included on each culture plate.

Virus titration

Virus titration was performed on the initial inoculum and VI-positive serum, buffy coat, and fetal tissue samples. The quantity of BVDV was determined by multiple 10-fold dilutions of samples using the statistical method of Reed and Muench (1938). Briefly, each sample was assayed by adding 10 µl of sample to 90 µl of culture medium on each of three 0.32-cm^2 wells of a 96-well culture plate that had been seeded 24 h earlier with MDBK cells. Serial 10-fold dilutions were made in culture medium, retaining 90 µl of each dilution per well. The plates were incubated at 37°C for 3 days in humidified air containing 5% CO_2. The IPMA described above was subsequently used to identify BVDV-positive cells.

Virus neutralization

A standard VN microtiter assay was used to detect and quantify neutralizing antibodies to BVDV-1b AU526 in serum samples obtained from gilts and their piglets. Serum samples were heat inactivated by incubation at 56°C for 30 min. Each sample was assayed in triplicate by adding 50 µl of sample to 50 µl of culture medium on each of three 0.32-cm^2 wells of a 96-well culture plate that had been seeded 24 h earlier with MDBK cells. Serial 2-fold dilutions were made in culture medium, retaining 50 µl of each dilution per well. An equal volume of culture medium containing 200 $TCID_{50}$ of AU526 was added to each well. The plates were incubated at 37°C for 1 h in humidified air containing 5% CO_2 and 50 µl of culture medium containing MDBK cells was then added to each well. The plates were incubated for 3 days and subsequently assayed for BVDV using the IPMA described above. Antibody titer was defined as the reciprocal of the highest dilution at which 2 out of 3 wells were free of staining.

Antigen-capture enzyme-linked immunosorbent assay

Skin biopsy samples obtained from piglets were assayed for BVDV using the BVDV PI X2 Test kit (IDEXX Laboratories, Inc.) according to the manufacturer's instructions. This test relies on monoclonal antibodies raised against the E^{rns} protein of BVDV to capture the viral antigen and detects antigen-antibody complexes with enzyme-conjugated antibody by spectrophotometry. The sample to positive (S/P) ratio was calculated for each sample and a ratio of 0.3 or greater was considered positive.

Reverse transcriptase nested PCR

Serum and buffy coat samples obtained from pregnant gilts on days 5 and 7 pi were assayed for BVDV by RT-nPCR. Viral RNA was extracted using the QIAamp® Viral RNA Mini Kit (Qiagen, Inc., Valencia, CA, USA) according to the manufacturer's instructions. Briefly, 140 µl of serum was added to 560 µl of buffer AVL containing carrier RNA into a 1.5-ml microcentrifuge tube. After incubation at room temperature for 10 min, 560 µl of ethanol was added and the solution was centrifuged through a spin column containing a silica membrane at 14,000 × *g* for 1 min. After centrifugation, spin column was washed with 500 µl of buffer AW1 and centrifuged at 14,000 × *g* for 1 min. After centrifugation, 500 µl of buffer AW2 was added and spin column was centrifuged at 14,000 × *g* for 3 min. Viral RNA was eluted by adding 60 µl of buffer AVE, incubating at room temperature for 1 min, and then centrifuging at 14,000 × *g* for 1 min. Viral RNA was frozen at -80°C or immediately used in RT-nPCR procedures.

All steps of the RT-nPCR were performed in a single closed-tube reaction. In the first step, 5 µl of trehalose (22% stock) was added to 0.4 µl of each inner primer BVD 180 and HCV 368 (50 µM) with 0.25 µl of Taq polymerase (5 U/µl) and 1 µl of dNTPs (10 mM) into the cap of a 200-µL tube. The tubes were allowed to dry at room temperature for 2 h before storage.

In the second step, 5 µl of viral RNA was added into PCR tubes containing 0.5 µl of Taq polymerase (5 U/µl), 2 µl of dNTPs (10 mM), 1 µl of each outer primer BVD 100 and HCV 368 (5 µM), 10 µl of 10× buffer, 8 µl of $MgCl_2$ (25 mM), 1 µl of Triton X-100 (10% stock), 0.25 µl of dithiothreitol (100 mM), 0.25 µl of RNAsin (40 U/µl), 0.5 µl of Moloney murine leukemia virus reverse transcriptase (200 U/µl), and 25.5 µl of RNase-free water.

First thermal cycling protocol included 45 min of reverse transcription at 37°C and 5 min of polymerase activation and DNA denaturation at 95°C followed by 20 cycles of amplification with denaturation at 94°C for 1 min and primer annealing at 55°C for 1 min and extension at 72°C for 1 min. After a final elongation step at 72°C for 10 min, reactions were maintained at 4°C. PCR tubes were centrifuged at 14,000 × *g* for 12 s and then subjected to 30 thermal cycles at 94°C for 1 min, 55°C for 1 min, and 72°C for 45 s. After a final elongation step at 72°C for 10 min, reactions were maintained at 4°C. PCR products were separated by 1.5% agarose gel electrophoresis. Agarose gels contained ethidium bromide (0.5 µg/ml) to allow visualization of PCR products using an ultraviolet transilluminator.

The outer PCR primers BVD 100 (5'-GGCTAGCCATGCCCTTAG -3') and HCV 368 (5'-CCATGTGCCATGTACAG-3') were amplifying a 290-bp sequence of the 5' untranslated region (UTR) of the viral genome. The inner PCR primers HCV 368 and BVD 180 (5'-CCTGAGTACAGGGDAGTCGTCA-3') were amplifying a 213-bp sequence within the first amplicon.

Reverse transcriptase PCR

Skin biopsy samples obtained from piglets born to gilts exposed to BVDV were assayed for BVDV by RT-PCR. Viral RNA was extracted using the RNeasy Mini Kit (Qiagen, Inc.) according to the manufacturer's instructions. The RT-PCR assay was performed on the Applied

Biosystems 2720 Thermal Cycler (Thermo Fischer Scientific, Inc., Waltham, MA, USA) using the SuperScript™ II Reverse Transcriptase kit (Thermo Fischer Scientific, Inc.) and the PCR primers described above (BVD 100, HCV 368, and BVD 180).

Viral genome sequencing

VI-positive serum, buffy coat, and fetal tissue samples containing at least 10^4 $TCID_{50}$/ml of virus were directly used in genome sequencing procedures. VI-positive samples with a lower virus titer were propagated in MDBK cells. Growth in cell culture was limited to two passages to minimize the introduction of artifactual changes in the viral genome. Virus was harvested by freezing and thawing the infected cells and samples were stored at -80°C.

Sequencing of BVDV isolates was performed as previously described (Neill et al., 2014). A random primed, barcoded library technique utilizing primers composed of 20 bases of known sequence with 8 random bases at the 3' end was used. These 28-mer primers conferred sequenced independence by random priming both first and second strand cDNA synthesis.

To adapt sequencing to the MiSeq™ platform (Illumina, Inc., San Diego, CA, USA), double-stranded cDNA PCR reactions were transferred to 96-well culture plates and the DNA was size-fractionated and purified using paramagnetic beads (Agencourt® AMPure® XP, Beckman Coulter, Indianapolis, IN, USA) at a DNA-to-bead ratio of 1:0.8. This resulted in the removal of DNA fragments of less than 300 bp including adaptor dimers and unligated adaptors. The DNA was prepared for sequencing using the Nextera™ DNA Library Preparation Kit (Illumina, Inc.) according to the manufacturer's instructions. The use of indexed barcoded PCR primers allowed multiplexing of libraries. The DNA was subjected to sequence analysis with the MiSeq™ platform using the MiSeq Reagent kit v2 (Illumina, Inc.) for 2 × 150 base paired-end sequencing.

Genomic sequences were assembled and edited using the Lasergene SeqMan NGen software (DNASTAR, Inc., Madison, WI, USA) and the genomic sequence of AU526 as the assembly reference. Sequences were further edited using the CodonCode Aligner software (Codoncode Corporation, Centerville, MA, USA). Numbering of nucleotides started at the ATG initiation codon of the ORF. Isolates were named using the animal's identification number. Gilts were identified using the letter P and the order number in the inoculation series. Piglets were identified using the dam's identification number and a letter for the birth order (A, first; B, second; C, third; etc.).

Data analysis

The ORF sequences of isolates from acutely infected gilts and their piglets were compared to AU526 and isolates from acutely infected heifers and ewes and their offspring, which were obtained in a previous study using a similar inoculation procedure (Kuca et al., submitted). Pairwise comparisons were performed using the MEGA software (version 7.0.18 available at http://www.megasoftware.net). Ambiguous nucleotides were not considered to be substitutions and thus not included in the results of pairwise comparisons.

The difference between the median number of nucleotide and amino acid substitutions was evaluated using the Wilcoxon rank sum test. The number of positions affected by substitutions was compared using the Pearson's Chi-squared test. The number of substitutions in isolates from congenitally infected piglets that were not present in isolates from their dams was also compared using the Pearson's Chi-squared test. The ratio of the number of observed substitutions to the number of expected substitutions assuming a random distribution of substitutions across the viral genome was calculated for each protein-coding region.

Monoclonal antibody binding

To investigate antigenic changes associated with serial BVDV infections in pregnant cattle, sheep, and swine, seven monoclonal antibodies (CA1, CA3, CA34, CA82, BZ24, BZ30, and BZ33) that specifically react with the E2 protein of BVDV were used to compare isolates from the first and last gilts and isolates from the first and last heifers and ewes, which were obtained in a previous study using a similar inoculation procedure (Kuca et al., submitted).

Monoclonal antibody binding patterns were determined by IPMA with MDBK cells infected with the different BVDV isolates. Monoclonal antibodies were obtained from hybridoma cell cultures as described by Bolin et al. (Bolin et al., 1988) and were diluted 1:2 to 1:16 in PBS with bovine albumin (0.01%). Standardization of binding procedures was accomplished by using the highest dilution of monoclonal antibody that showed no significant loss of reactivity in IPMA against AU526.

Briefly, culture medium containing 500 $TCID_{50}$ of each isolate was added to each of three 0.32-cm^2 wells of a 96-well culture plate that had been seeded 24 h earlier with MDBK cells. Each culture plate also contained cells infected with AU526 as positive control and mock-infected MDBK cells as negative control. Culture plates were incubated at 37°C for 3 days in humidified air containing 5% CO_2. Following fixation, 50 µl of each diluted monoclonal preparation was added to each well and culture plates were incubated at 37°C for 1 h to allow antibody binding. After washing with PBS containing Tween 20 to remove unbound antibodies, 50 µl of horseradish peroxidase-conjugated rabbit anti-mouse IgG antibody was added to each well and culture plates were incubated at 37°C for 1 h. After washing with PBS containing Tween 20, 50 µl of AEC substrate was added to each well and culture plates were incubated at room temperature for 15 min. This enzyme substrate produces a reddish-brown color when

oxidized by horseradish peroxidase. Color change was examined by use of light microscopy and compared with that of positive- and negative-control samples included on each culture plate.

Results

Clinical, virological, and serological findings

All gilts were confirmed to be free of BVDV and seronegative to BVDV prior to inoculation (Table 2.1). Virus titration of residual inoculum demonstrated that the viral dose received by the first gilt was 2.5×10^4 $TCID_{50}$. The fourth gilt (P4) was inoculated with 1 ml of serum obtained on day 5 from the third gilt (P3) and did not become infected. The gilt P4 was thus excluded from the study and the fifth gilt (P5) was inoculated with 1 ml of serum obtained on day 7 from P3 to continue the serial inoculation. Infection was confirmed in six gilts by positive VI or RT-nPCR results on day 5 or 7 pi and seroconversion by day 28 pi (Table 2.1).

Clinical signs of disease were not observed in any of the infected gilts, which carried the pregnancy to term and delivered 37 live piglets, 16 mummified piglets, and two stillborn piglets (Table 2.1). The median number of live piglets was 7 (range, 2-10) and the median number of mummified piglets was 2 (range, 0-10). Furthermore, live piglets had poor viability with 32% of them dying or being euthanized during the first four weeks of life.

Transplacental transmission of BVDV was confirmed in two gilts (P5 and P7) by positive ACE and RT-PCR results in skin biopsy samples from their offspring (Table 2.2). Five piglets in the litter of the fifth gilt (P5A-P5D and P5F) were shown to be congenitally infected with BVDV as virus could be isolated from serum or buffy coat samples obtained at birth. These five piglets died or were euthanized within 24 hours of birth, whereas the piglet P5E remained apparently healthy until euthanasia at six months of age. Skin biopsy samples obtained at birth and at 42

days of age from P5E were positive by ACE and RT-PCR, but a skin biopsy sample obtained at six months of age tested negative. Furthermore, virus could not be isolated from serum or buffy coat samples obtained every six weeks until six months of age or a composite of tissue samples obtained at six months of age. All piglets in the litter of the seventh gilt (P7A-P7H) were positive for BVDV by ACE and/or RT-PCR at birth. One piglet (P7A) died within 24 hours of birth and virus was isolated from a composite of tissue samples including skin, liver, spleen, thymus, kidney, lung, heart, and brain. Virus was isolated from buffy coat samples obtained at 21 days of age from four of seven piglets (P7C-P7F). These four piglets were demonstrated to be PI with BVDV by repeated VI from buffy coat samples obtained at 21, 42, and 84 days of age (Table 2.2). Of the three piglets that tested negative, one piglet (P7G) died at 26 days of age and tissue samples from this piglet were also negative by VI. Skin biopsy samples obtained at 21 days of age from the other two piglets (P7B and P7H) were also negative by ACE and RT-PCR. These two piglets remained apparently healthy until euthanasia at 84 days of age. Tissue samples obtained from these piglets were also negative by VI.

Genomic sequence analysis

The complete ORF sequences of 14 BVDV isolates obtained from five acutely infected gilts and nine congenitally infected piglets were determined. Buffy coat samples obtained from pregnant gilts on day 5 or 7 pi and serum or buffy coat samples obtained from piglets at birth or at 21 days of age were used in genome sequencing procedures. Three sequences (P6, P7, and P7A) contained one, three, and four ambiguous nucleotides, respectively. Virus could not be isolated from samples collected from the gilt P3 and a complete ORF sequence could not be determined for the piglet P7D.

When compared to AU526, a median of 41 nucleotide substitutions (range, 33-45) was

observed in isolates from acutely infected pregnant gilts (Table 2.3). Similar to their dams, a median of 49 substitutions (range, 44-57) was observed in isolates from congenitally infected piglets. The median number of nucleotide changes in porcine isolates was statistically different from what was observed in bovine isolates ($P < 0.001$).

Most nucleotide substitutions were transitions with a median transition-to-transversion ratio of 5.8 in isolates from pregnant gilts and 8.2 in isolates from piglets (Table 2.3). Substitutions were more prevalent in nonstructural protein-coding regions with a median of 10 and 5 changes in the NS3 and NS5B coding regions, respectively (Table 2.3). However, a median of 7 changes were also found in the E^{rns} and E2 coding regions. Further analysis revealed that nucleotide substitutions occurred in the E^{rns} coding region at least 2.5 times more frequently than expected from random events in all but six porcine isolates (Table 2.4).

Approximately 30% of substitutions were nonsynonymous, resulting in a median of 13 amino acid differences (range, 12-14) between AU526 and isolates from pregnant gilts. Similar to their dams, a median of 14 substitutions (range, 14-16) was detected in isolates from piglets. Similar to nucleotide changes, the median number of amino acid changes in porcine isolates was statistically different from what was observed in bovine isolates ($P < 0.001$). These substitutions occurred most frequently in the E2 coding region with a median of 5 changes in this region (Table 2.5). A median of 2 changes was also observed in the E^{rns} and NS5B coding regions. In all porcine isolates, the number of amino acid substitutions observed in the E2 coding region was at least 2.5 times greater than expected from random substitutions across the viral genome (Table 2.6). In contrast, there were no amino acid substitutions in regions encoding the nonstructural proteins N^{pro}, p7, and NS4a. Furthermore, there was only one isolate (P7F) with an amino acid change in the C coding region.

A total of 647 nucleotide and 201 amino acid substitutions occurred in porcine isolates at 90 and 29 different positions, respectively. The number of positions affected by substitutions in porcine isolates was statistically different from what was observed in bovine and ovine isolates ($P < 0.01$). Furthermore, amino acid residues that were often affected by substitutions in porcine isolates were rarely substituted in bovine isolates and vice versa (Figure 2.1). In contrast, most substitutions occurred at the same amino acid positions in porcine and ovine isolates.

A total of 32 nucleotide and 12 amino acid substitutions were detected in all porcine isolates (Tables 2.7 and 2.8). These changes were established during the acute infection of the first gilt (P1) and were subsequently conserved during five passages in pregnant swine. One additional nucleotide substitution that occurred in the isolate from the fifth gilt (P5) was also observed in isolates from her piglets, the last two gilts, and the piglets of the last gilt. Conserved nucleotide changes were more prevalent in nonstructural protein-coding regions, with eight substitutions occurring in the NS3 coding region. In contrast, conserved amino acid changes were found primarily in regions encoding structural viral proteins, with four and two substitutions in the E2 and E^{rns} coding regions, respectively.

Results of all pairwise sequence comparisons as well as the location and type of nucleotide and amino acid substitutions between AU526 and isolates from acutely infected pregnant gilts and congenitally infected piglets are given in the Supplemental Material (Tables S2.1-S2.5). Furthermore, virus titers in samples from pregnant gilts and their piglets are given in Tables S2.6-S2.7.

Monoclonal antibody binding assays

Two porcine isolates (P1 and P7), two bovine isolates (B1 and B4), and two ovine isolates (O1 and O6) were used in monoclonal antibody binding assays. The isolate from the last

heifer (B6) was not available and thus the isolate from the fourth heifer (B4) was used instead. This isolate was selected because there were only six nucleotide differences between these two isolates, one of which was a synonymous substitution located in the E2 coding region.

Identical binding patterns were obtained for AU526 and isolates from acutely infected pregnant heifers, ewes, and gilts using a panel of seven monoclonal antibodies (data not shown). These results suggest that serial infection of pregnant cattle, sheep, and swine with a BVDV isolate of bovine origin may not be associated with antigenic changes in the E2 protein.

Discussion

In this study, the BVDV-1b isolate AU526 was serially passaged in six pregnant gilts. Transplacental transmission of BVDV was confirmed in two gilts and resulted in the birth of ten congenitally infected piglets, from which virus could be isolated. The complete ORF sequences of 14 isolates from five acutely infected gilts and nine congenitally infected piglets were subsequently obtained and compared to determine the timing, number, location, and type of genomic changes introduced during serial infection of pregnant swine. In a previous study, the isolate AU256 had been serially passaged in six pregnant heifers and six pregnant ewes (Kuca et al., submitted). Six calves and four lambs congenitally infected with BVDV were born to these dams infected in early pregnancy. To investigate differences between host species, the sequences determined in this study were compared to those of isolates from acutely infected heifers and ewes and their offspring generated in that study.

Virus could not be isolated from serum and buffy coat samples obtained on days 5 and 7 pi from P3 and thus the ORF sequence of this isolate could not be determined. The serum sample collected on day 7 pi from P3 was positive by RT-nPCR and the gilt P5 did become infected

after inoculation with this sample. These findings suggested low virus titers on day 7 pi.

The median number of nucleotide and amino acid differences between AU526 and isolates from pregnant gilts was similar to the median of 46 nucleotide and 13 amino acid changes observed in isolates from six pregnant ewes serially infected with the same isolate (Kuca et al., submitted). Smaller numbers of changes were detected in isolates from six pregnant heifers serially infected with AU526, with a median of 23 nucleotide and 6 amino acid substitutions. Similar results were obtained when comparing the isolates obtained from offspring born to these heifers, ewes, and gilts. Altogether, these results revealed that great numbers of substitutions were introduced in the BVDV genome during serial infection of pregnant swine and suggest that these infections may contribute significantly to the genetic variability of BVDV.

Interestingly, similar numbers of genomic changes were introduced during serial infection of pregnant sheep and swine with a BVDV isolate of bovine origin. It is possible that the number of changes observed in these two species corresponds to the maximum number of changes that can be introduced or tolerated in a heterologous host, regardless of its phylogenetic relatedness to cattle.

In a previous study, nucleotide substitutions occurred randomly across the BVDV genome during serial infection of pregnant cattle and sheep (Kuca et al., submitted). In this study, there was a bias toward nucleotide changes in the E^{rns} coding region. Previous studies have demonstrated that amino acid changes occurred primarily in regions encoding the structural proteins E2 and E^{rns} during the establishment of persistent infections in cattle (Neill et al., 2011, 2012). Similar results were obtained during serial infection of pregnant swine with a BVDV isolate of bovine origin. The envelope proteins E2 and E^{rns} are the main targets of neutralizing

antibodies in acutely infected animals and immune pressure on the immunodominant residues may have contributed to the selection of amino acid changes in these genomic regions.

Twelve amino acid substitutions introduced during the acute infection of the first gilt were found to be conserved during five passages in pregnant swine. These substitutions were also detected in congenitally infected piglets born to these gilts. These changes occurred primarily in the E2 and E^{rns} coding regions, with four and two substitutions in these genomic regions, respectively. These two envelope proteins play an important role in cell attachment and viral entry (El Omari et al., 2013; Lazar et al., 2003). Furthermore, the E^{rns} protein can inhibit the production of type I interferon and assist in the establishment of persistent infection (Iqbal et al., 2004). It is thus possible that these conserved changes may be involved in host adaptation. Conserved amino acid substitutions have been described in the same genomic regions during serial infection of pregnant sheep with AU526 (Kuca et al., submitted). Conserved amino acid changes in the E2 and E^{rns} coding regions have also been reported in BVDV-1b isolates from alpacas (Neill et al., 2015). Of the conserved substitutions identified in isolates from pregnant sheep serially infected with AU526, 10 were found to be identical to the ones described in this study. However, the conserved substitutions introduced during serial infection of pregnant cattle with AU526 were found to be distinct from those detected in isolates from pregnant sheep and swine (Kuca et al., submitted). It is currently unknown, and will be the focus of future experiments, if these conserved changes provided a selective advantage in swine and sheep and if these changes would also have occurred with a BVDV isolate belonging to a different subgenotype or species.

In this study, all conserved amino acid changes were detected after one passage in pregnant swine, whereas the 13 conserved changes observed during serial infection of pregnant

sheep were gradually introduced in the first three acutely infected ewes (Kuca et al., submitted). These results suggest that conserved genomic changes are more rapidly introduced during serial BVDV infection in a species less closely related to cattle.

Although up to five amino acid substitutions in the E2 coding region were detected in isolates from dams serially infected with AU526, there were no changes in binding patterns using a panel of seven monoclonal antibodies raised against the E2 protein of BVDV. However, it is possible that antigenic changes that had occurred in additional epitopes were not detected. Cross-neutralization assays with polyclonal antisera may be used in the future to further investigate antigenic changes arising during serial infection of pregnant cattle, sheep, and swine.

In conclusion, great numbers of nucleotide and amino acid substitutions occurred during serial infection of pregnant swine with a BVDV isolate of bovine origin. Furthermore, 12 amino acid changes established during the acute infection of the first gilt were found to be conserved during five passages in pregnant swine. These changes occurred primarily in the E2 and E^{rns} coding regions. These results suggest that BVDV infections in swine may serve as a significant source of viral genetic variability and may be associated with adaptive changes.

Tables

Table 2.1. Virological and serological analysis of serial inoculation of gilts with BVDV in early pregnancy

		0 dpi		5 dpi		7 dpi		28 dpi	56 dpi	Day of farrowing		
Gilt	Fetus[a]	VN	VI[b]	VI[b]	RT-nPCR[b]	VN[b]	RT-nPCR[b]	VN	VN	VN	dpi	Offspring
P1	31	< 2	-/-	-/+	+/+	-/+	+/+	512	2,048	2,048	83	10 live BVDV- piglets and 2 mummified piglets
P2	39	< 2	-/-	-/+	+/-	-/+	+/-	128	1,024	2,048	78	2 live BVDV- piglets, 10 mummified piglets, and 1 stillborn piglet
P3	30	< 2	-/-	-/-	-/-	-/-	+/-	128	1,024	4,096	86	4 live BVDV- piglets and 1 stillborn piglet
P4	39	< 2	-/-	-/-	-/-	-/-	-/-	< 2	< 2	NT	NA	NA
P5	27	< 2	-/-	-/+	+/+	-/+	+/+	256	2,048	4,096	89	6 live BVDV+ piglets and 1 mummified piglet
P6	33	< 2	-/-	-/+	+/+	-/+	+/+	512	4,096	4,096	84	7 live BVDV- piglets and 3 mummified piglets
P7	28	< 2	-/-	-/+	+/+	-/+	+/+	128	2,048	4,096	88	8 live BVDV+ piglets

[a] Estimated gestational age at the time of inoculation based on artificial insemination date

[b] Results are given for serum samples followed by those for buffy coat samples

dpi, day postinoculation; NA, not available; NT, not tested; RT-nPCR, nested reverse transcriptase PCR; VI, virus isolation; VN, virus neutralization; +, positive; -, negative

Table 2.2. Virological analysis of piglets born to gilts infected with BVDV in early pregnancy

		Day of birth			21 doa			42 doa	Day of death or euthanasia	
		skin		tissues[b]	skin		BC	BC		tissues[b]
Gilt	Fetus[a]	ACE	RT-PCR	VI	ACE	RT-PCR	VI	VI	doa	VI
P1	31	0/10	0/10	NA	NT	NT	0/10	NT	NT	NT
P2	39	0/3	0/3	0/1	NA	NA	NA	NA	8,9	0/2
P3	30	0/4	0/4	0/1	NT	NT	NT	NT	5,12,13	0/3
P5	27	6/6	6/6	5/5	1/1	0/1	0/1	0/1	182	0/1
P6	33	0/7	0/7	NA	NT	NT	0/7	NT	NT	NT
P7	28	6/8	8/8	1/1	5/7	3/7	4/7	4/6	26,84,84	0/3

Results are given as the number of positive piglets over the total number of piglets tested

[a] Estimated gestational age at the time of inoculation based on artificial insemination date

[b] Composite of tissue samples including skin, liver, spleen, thymus, kidney, lung, heart and brain

ACE, antigen-capture ELISA; BC, buffy coat; doa, days of age; NA, not applicable; NT, not tested; RT-PCR, reverse transcriptase PCR; VI, virus isolation

Table 2.3. Nucleotide substitutions between AU526 and isolates from acutely infected gilts and their congenitally infected piglets

AU526:Dam	Total	Transitions	Transversions	Ratio	N^{pro}	C	E^{rns}	E1	E2	p7	NS2	NS3	NS4A	NS4B	NS5A	NS5B	% S[a]	% NS[b]
AU526:P1	41	35	6	5.8	1	2	6	3	6	0	4	10	0	2	3	4	41.5	58.5
AU526:P2	42	34	8	4.3	1	2	5	3	7	0	3	11	0	3	2	5	40.5	59.5
AU526:P5	45	40	5	8.0	2	2	7	2	7	0	4	10	0	2	4	5	40.0	60.0
AU526:P6	33	28	5	5.6	1	2	6	2	5	0	2	8	0	1	2	4	45.5	54.5
AU526:P7	35	30	5	6.0	1	2	6	2	7	0	2	8	0	1	2	4	48.6	51.4
Median	41	34	5	5.8	1	2	6	2	7	0	3	10	0	2	2	4	41.5	58.5
Mean	39	33	6	5.9	1	2	6	2	6	0	3	9	0	2	3	4	43.2	56.8
AU526:Offspring	Total	Transitions	Transversions	Ratio	N^{pro}	C	E^{rns}	E1	E2	p7	NS2	NS3	NS4A	NS4B	NS5A	NS5B	% S[a]	% NS[b]
AU526:P5A	44	36	8	4.5	1	2	7	3	7	0	3	11	0	3	2	5	43.2	56.8
AU526:P5B	47	39	8	4.9	1	2	7	3	10	0	3	11	0	3	2	5	46.8	53.2
AU526:P5C	50	45	5	9.0	2	2	7	2	7	0	6	10	0	4	5	5	36.0	64.0
AU526:P5D	45	37	8	4.6	1	2	7	3	7	0	3	12	0	3	2	5	42.2	57.8
AU526:P5F	48	43	5	8.6	2	2	7	2	7	0	6	10	0	3	4	5	37.5	62.5
AU526:P7A	49	44	5	8.8	2	2	7	3	7	0	5	10	0	3	4	6	38.8	61.2
AU526:P7C	56	50	6	8.3	2	2	7	3	10	0	7	10	0	3	4	8	39.3	60.7
AU526:P7E	57	49	8	6.1	2	2	8	3	11	0	5	11	0	3	4	8	42.1	57.9
AU526:P7F	55	49	6	8.2	2	3	8	4	8	0	5	10	0	4	4	7	41.8	58.2
Median	49	44	6	8.2	2	2	7	3	7	0	5	10	0	3	4	5	41.8	58.2
Mean	50	44	7	7.0	2	2	7	3	8	0	5	11	0	3	3	6	40.9	59.1

[a] Percentage of substitutions within structural protein-coding regions

[b] Percentage of substitutions within nonstructural protein-coding regions

Table 2.4. Ratio of the number of observed nucleotide substitutions per protein-coding region to the number of expected substitutions assuming a random distribution across the viral genome

AU526:Dam	Total	N^{pro}	C	E^{rns}	E1	E2	p7	NS2	NS3	NS4A	NS4B	NS5A	NS5B
AU526:P1	41	0.57	1.86	**2.51**	1.46	1.53	0.00	0.94	1.28	0.00	0.55	0.58	0.53
AU526:P2	42	0.55	1.82	2.04	1.43	1.74	0.00	0.69	1.38	0.00	0.80	0.37	0.65
AU526:P5	45	1.03	1.70	**2.67**	0.89	1.62	0.00	0.86	1.17	0.00	0.50	0.70	0.60
AU526:P6	33	0.70	2.32	**3.12**	1.21	1.58	0.00	0.58	1.28	0.00	0.34	0.48	0.66
AU526:P7	35	0.66	2.18	**2.94**	1.14	2.08	0.00	0.55	1.20	0.00	0.32	0.45	0.62
Median	41	0.66	1.86	**2.67**	1.21	1.62	0.00	0.69	1.28	0.00	0.50	0.48	0.62
Mean	39	0.70	1.98	**2.66**	1.23	1.71	0.00	0.72	1.26	0.00	0.50	0.51	0.61
AU526:Offspring	Total	N^{pro}	C	E^{rns}	E1	E2	p7	NS2	NS3	NS4A	NS4B	NS5A	NS5B
AU526:P5A	44	0.53	1.74	**2.73**	1.36	1.66	0.00	0.66	1.32	0.00	0.77	0.36	0.62
AU526:P5B	47	0.49	1.63	**2.56**	1.28	2.22	0.00	0.61	1.23	0.00	0.72	0.33	0.58
AU526:P5C	50	0.93	1.53	2.40	0.80	1.46	0.00	1.15	1.05	0.00	0.90	0.79	0.54
AU526:P5D	45	0.52	1.70	**2.67**	1.33	1.62	0.00	0.64	1.40	0.00	0.75	0.35	0.60
AU526:P5F	48	0.97	1.59	**2.50**	0.83	1.52	0.00	1.20	1.10	0.00	0.70	0.65	0.56
AU526:P7A	49	0.95	1.56	2.45	1.22	1.49	0.00	0.98	1.08	0.00	0.69	0.64	0.66
AU526:P7C	56	0.83	1.36	2.15	1.07	1.86	0.00	1.20	0.94	0.00	0.60	0.56	0.77
AU526:P7E	57	0.81	1.34	2.41	1.05	2.01	0.00	0.84	1.02	0.00	0.59	0.55	0.76
AU526:P7F	55	0.84	2.08	2.50	1.45	1.52	0.00	0.87	0.96	0.00	0.82	0.57	0.69
Median	49	0.83	1.59	2.50	1.22	1.62	0.00	0.87	1.08	0.00	0.72	0.56	0.62
Mean	50	0.76	1.61	2.49	1.16	1.71	0.00	0.91	1.12	0.00	0.73	0.53	0.64

Ratio values greater than 2.5 are bolded and underlined.

Table 2.5. Amino acid substitutions between AU526 and isolates from acutely infected gilts and their congenitally infected piglets

AU526:Dam	Total	N^{pro}	C	E^{rns}	E1	E2	p7	NS2	NS3	NS4A	NS4B	NS5A	NS5B	% S[a]	% NS[b]
AU526:P1	13	0	0	2	1	4	0	1	1	0	1	1	2	53.8	46.2
AU526:P2	14	0	0	2	1	5	0	1	1	0	1	1	2	57.1	42.9
AU526:P5	13	0	0	2	1	4	0	2	1	0	0	1	2	53.8	46.2
AU526:P6	12	0	0	2	1	4	0	1	1	0	0	1	2	58.3	41.7
AU526:P7	12	0	0	2	1	4	0	1	1	0	0	1	2	58.3	41.7
Median	13	0	0	2	1	4	0	1	1	0	0	1	2	57.1	42.9
Mean	13	0	0	2	1	4	0	1	1	0	0	1	2	56.3	43.7
AU526:Offspring	Total	N^{pro}	C	E^{rns}	E1	E2	p7	NS2	NS3	NS4A	NS4B	NS5A	NS5B	% S[a]	% NS[b]
AU526:P5A	14	0	0	2	1	5	0	1	1	0	1	1	2	57.1	42.9
AU526:P5B	16	0	0	2	1	7	0	1	1	0	1	1	2	62.5	37.5
AU526:P5C	14	0	0	2	1	4	0	3	1	0	0	1	2	50.0	50.0
AU526:P5D	14	0	0	2	1	5	0	1	1	0	1	1	2	57.1	42.9
AU526:P5F	14	0	0	2	1	4	0	3	1	0	0	1	2	50.0	50.0
AU526:P7A	13	0	0	2	1	4	0	2	1	0	0	1	2	53.8	46.2
AU526:P7C	17	0	0	2	1	6	0	3	1	0	0	1	3	52.9	47.1
AU526:P7E	18	0	0	3	1	8	0	2	1	0	0	1	2	66.7	33.3
AU526:P7F	17	0	1	3	2	5	0	2	1	0	0	1	2	64.7	35.3
Median	14	0	0	2	1	5	0	2	1	0	0	1	2	57.1	42.9
Mean	15	0	0	2	1	5	0	2	1	0	0	1	2	57.2	42.8

[a] Percentage of substitutions within structural protein-coding regions

[b] Percentage of substitutions within nonstructural protein-coding regions

Table 2.6. Ratio of the number of observed amino acid substitutions per protein-coding region to the number of expected substitutions assuming a random distribution across the viral genome

AU526:Dam	Total	N^{pro}	C	E^{rns}	E1	E2	p7	NS2	NS3	NS4A	NS4B	NS5A	NS5B
AU526:P1	13	0.00	0.00	**2.64**	1.54	**3.21**	0.00	0.74	0.41	0.00	0.86	0.60	0.83
AU526:P2	14	0.00	0.00	2.45	1.43	**3.72**	0.00	0.69	0.38	0.00	0.80	0.56	0.77
AU526:P5	13	0.00	0.00	**2.64**	1.54	**3.21**	0.00	1.48	0.41	0.00	0.00	0.60	0.83
AU526:P6	12	0.00	0.00	**2.86**	1.67	**3.47**	0.00	0.80	0.44	0.00	0.00	0.65	0.90
AU526:P7	12	0.00	0.00	**2.86**	1.67	**3.47**	0.00	0.80	0.44	0.00	0.00	0.65	0.90
Median	13	0.00	0.00	**2.64**	1.54	**3.47**	0.00	0.80	0.41	0.00	0.00	0.60	0.83
Mean	13	0.00	0.00	**2.69**	1.57	**3.42**	0.00	0.90	0.41	0.00	0.33	0.62	0.85
AU526:Offspring	Total	N^{pro}	C	E^{rns}	E1	E2	p7	NS2	NS3	NS4A	NS4B	NS5A	NS5B
AU526:P5A	14	0.00	0.00	2.45	1.43	**3.72**	0.00	0.69	0.38	0.00	0.80	0.56	0.77
AU526:P5B	16	0.00	0.00	2.15	1.25	**4.56**	0.00	0.60	0.33	0.00	0.70	0.49	0.68
AU526:P5C	14	0.00	0.00	2.45	1.43	**2.98**	0.00	2.06	0.38	0.00	0.00	0.56	0.77
AU526:P5D	14	0.00	0.00	2.45	1.43	**3.72**	0.00	0.69	0.38	0.00	0.80	0.56	0.77
AU526:P5F	14	0.00	0.00	2.45	1.43	**2.98**	0.00	2.06	0.38	0.00	0.00	0.56	0.77
AU526:P7A	13	0.00	0.00	**2.64**	1.54	**3.21**	0.00	1.48	0.41	0.00	0.00	0.60	0.83
AU526:P7C	17	0.00	0.00	2.02	1.18	**3.68**	0.00	1.70	0.31	0.00	0.00	0.46	0.96
AU526:P7E	18	0.00	0.00	**2.86**	1.11	**4.63**	0.00	1.07	0.29	0.00	0.00	0.44	0.60
AU526:P7F	17	0.00	2.25	**3.03**	2.35	**3.07**	0.00	1.13	0.31	0.00	0.00	0.46	0.64
Median	14	0.00	0.00	2.45	1.43	**3.68**	0.00	1.13	0.38	0.00	0.00	0.56	0.77
Mean	15	0.00	0.25	**2.50**	1.46	**3.62**	0.00	1.28	0.35	0.00	0.26	0.52	0.76

Ratio values greater than 2.5 are bolded and underlined.

Table 2.7. Conserved nucleotide substitutions during serial infection of pregnant gilts with BVDV

Viral protein	N^{pro}	C		E^{rns}						E1		E2					NS2		NS3								NS4B	NS5A		NS5B			
NT position	247	726	783	978	1008	1125	1254	1315	1342	1626	1660	2136	2182	2917	2978	3194	3437	3792	4701	4821	4881	4959	5148	5562	5679	5733	7524	8461	8611	9851	10831	10947	11904
AU526	T	A	C	T	T	T	A	C	A	A	G	A	G	T	G	C	G	G	A	G	G	A	G	A	A	A	T	T	A	G	A	G	T
P1	C	G	T	T	C	C	G	**G**	**G**	G	**A**	T	**C**	**C**	**A**	**T**	**A**	A	G	**A**	T	T	A	G	G	G	C	C	**G**	**A**	**G**	A	C
P2	C	G	T	T	C	C	G	**G**	**G**	G	**A**	T	**C**	**C**	**A**	**T**	**A**	A	G	**A**	T	T	A	G	G	G	C	C	**G**	**A**	**G**	A	C
P5	C	G	T	C	C	C	G	**G**	**G**	G	**A**	T	**C**	**C**	**A**	**T**	**A**	A	G	**A**	T	T	A	G	G	G	C	C	**G**	**A**	**G**	A	C
P5A	C	G	T	C	C	C	G	**G**	**G**	G	**A**	T	**C**	**C**	**A**	**T**	**A**	A	G	**A**	T	T	A	G	G	G	C	C	**G**	**A**	**G**	A	C
P5B	C	G	T	C	C	C	G	**G**	**G**	G	**A**	T	**C**	**C**	**A**	**T**	**A**	A	G	**A**	T	T	A	G	G	G	C	C	**G**	**A**	**G**	A	C
P5C	C	G	T	C	C	C	G	**G**	**G**	G	**A**	T	**C**	**C**	**A**	**T**	**A**	A	G	**A**	T	T	A	G	G	G	C	C	**G**	**A**	**G**	A	C
P5D	C	G	T	C	C	C	G	**G**	**G**	G	**A**	T	**C**	**C**	**A**	**T**	**A**	A	G	**A**	T	T	A	G	G	G	C	C	**G**	**A**	**G**	A	C
P5F	C	G	T	C	C	C	G	**G**	**G**	G	**A**	T	**C**	**C**	**A**	**T**	**A**	A	G	**A**	T	T	A	G	G	G	C	C	**G**	**A**	**G**	A	C
P6	C	G	T	C	C	C	G	**G**	**G**	G	**A**	T	**C**	**C**	**A**	**T**	**A**	A	G	**A**	T	T	A	G	G	G	C	C	**G**	**A**	**G**	A	C
P7	C	G	T	C	C	C	G	**G**	**G**	G	**A**	T	**C**	**C**	**A**	**T**	**A**	A	G	**A**	T	T	A	G	G	G	C	C	**G**	**A**	**G**	A	C
P7A	C	G	T	C	C	C	G	**G**	**G**	G	**A**	T	**C**	**C**	**A**	**T**	**A**	A	G	**A**	T	T	A	G	G	G	C	C	**G**	**A**	**G**	A	C
P7C	C	G	T	C	C	C	G	**G**	**G**	G	**A**	T	**C**	**C**	**A**	**T**	**A**	A	G	**A**	T	T	A	G	G	G	C	C	**G**	**A**	**G**	A	C
P7E	C	G	T	C	C	C	G	**G**	**G**	G	**A**	T	**C**	**C**	**A**	**T**	**A**	A	G	**A**	T	T	A	G	G	G	C	C	**G**	**A**	**G**	A	C
P7F	C	G	T	C	C	C	G	**G**	**G**	G	**A**	T	**C**	**C**	**A**	**T**	**A**	A	G	**A**	T	T	A	G	G	G	C	C	**G**	**A**	**G**	A	C

Conserved nucleotide (NT) substitutions are shown on a dark grey background and nonsynonymous substitutions are bolded.

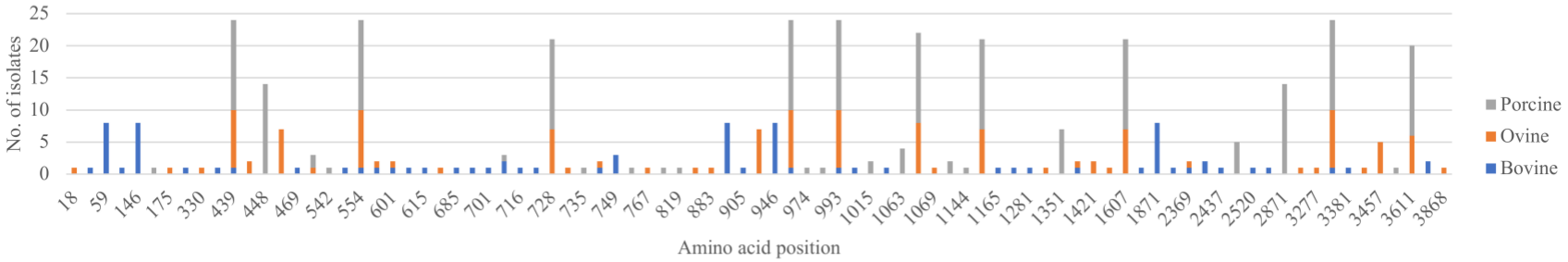
Table 2.8. Conserved amino acid substitutions during serial infection of pregnant gilts with BVDV

Viral protein	E^{rns}		E1	E2				NS2	NS3	NS5A	NS5B	
AA position	439	448	554	728	973	993	1065	1146	1607	2871	3284	3611
AU526	H	T	V	G	Y	R	S	R	M	T	R	I
P1	D	A	M	R	H	K	L	Q	I	A	K	V
P2	D	A	M	R	H	K	L	Q	I	A	K	V
P5	D	A	M	R	H	K	L	Q	I	A	K	V
P5A	D	A	M	R	H	K	L	Q	I	A	K	V
P5B	D	A	M	R	H	K	L	Q	I	A	K	V
P5C	D	A	M	R	H	K	L	Q	I	A	K	V
P5D	D	A	M	R	H	K	L	Q	I	A	K	V
P5F	D	A	M	R	H	K	L	Q	I	A	K	V
P6	D	A	M	R	H	K	L	Q	I	A	K	V
P7	D	A	M	R	H	K	L	Q	I	A	K	V
P7A	D	A	M	R	H	K	L	Q	I	A	K	V
P7C	D	A	M	R	H	K	L	Q	I	A	K	V
P7E	D	A	M	R	H	K	L	Q	I	A	K	V
P7F	D	A	M	R	H	K	L	Q	I	A	K	V

Conserved amino acid (AA) substitutions are shown on a dark grey background.

Figures

Figure 2.1. Location of all amino acid substitutions identified in porcine, ovine, and bovine isolates



Supplemental Material

Table S2.1. Number of nucleotide differences between AU526 and isolates from acutely infected gilts and their piglets

	AU526	P1	P2	P5	P5A	P5B	P5C	P5D	P5F	P6	P7	P7A	P7C	P7E
P1	41													
P2	42	9												
P5	45	22	23											
P5A	44	11	2	23										
P5B	47	14	5	26	3									
P5C	50	27	28	5	28	31								
P5D	45	12	3	24	1	4	29							
P5F	48	25	26	3	26	29	2	27						
P6	33	9	10	12	10	13	17	11	15					
P7	35	9	10	11	9	12	16	10	14	2				
P7A	49	26	27	4	27	28	5	28	3	16	15			
P7C	56	33	34	11	34	35	12	35	10	23	22	6		
P7E	57	34	35	12	35	38	13	36	11	24	23	7	15	
P7F	55	32	33	10	33	36	11	34	9	22	21	6	13	12

Table S2.2. Number of amino acid differences between AU526 and isolates from acutely infected gilts and their piglets

	AU526	P1	P2	P5	P5A	P5B	P5C	P5D	P5F	P6	P7	P7A	P7C	P7E
P1	13													
P2	14	1												
P5	13	2	3											
P5A	14	1	0	3										
P5B	16	3	2	5	2									
P5C	14	3	4	1	4	6								
P5D	14	1	0	3	0	2	4							
P5F	14	3	4	1	4	6	0	4						
P6	12	0	1	1	1	3	2	1	2					
P7	12	1	2	1	2	4	2	2	2	0				
P7A	13	2	3	0	3	3	1	3	1	1	1			
P7C	17	6	7	4	7	8	5	7	5	5	5	3		
P7E	18	7	8	5	8	10	6	8	6	6	6	5	9	
P7F	17	6	7	4	7	9	5	7	5	5	5	4	8	7

Table S2.3. Location and type of nucleotide and amino acid substitutions between AU526 and isolates from gilts infected with BVDV in early pregnancy

AU526:Dam	Total	Location and type of nucleotide substitutions	Total	Location and type of amino acid substitutions
AU526:P1	41	247 T:C, 726 A:G, 783 C:T, 912 G:A, 1008 T:C, 1125 G:A, 1254 A:G, **1315 C:G**, **1342 A:G**, 1626 A:G, **1660 G:A**, 1866 A:G, 2136 A:T, **2182 G:C**, 2526 A:G, **2917 T:C**, **2978 G:A**, **3194 C:T**, **3437 G:A**, 3792 G:A, 4212 T:C, 4230 A:G, 4701 A:G, **4821 G:A**, 4881 G:T, 4959 A:T, 5121 A:G, 5148 G:A, 5562 A:G, 5679 A:G, 5733 A:G, 6588 G:A, 7524 T:C, **7543 A:T**, 8461 T:C, 8541 C:T, **8611 A:G**, **9851 G:A**, **10831 A:G**, 10947 G:A, 11094 T:C	13	439 H:D, 448 T:A, 554 V:M, 728 G:R, 973 Y:H, 993 R:K, 1065 S:L, 1146 R:Q, 1607 M:I, 2515 M:L, 2871 T:A, 3284 R:K, 3611 I:V
AU526:P2	42	247 T:C, 726 A:G, 783 C:T, 1008 T:C, 1125 G:A, 1254 A:G, **1315 C:G**, **1342 A:G**, 1626 A:G, **1660 G:A**, 1866 A:G, 2136 A:T, **2182 G:C**, 2526 A:G, **2917 T:C**, **2978 G:A**, **3188 T:G**, **3194 C:T**, **3437 G:A**, 3792 G:A, 4230 A:G, 4701 A:G, **4821 G:A**, 4881 G:T, 4959 A:T, 5121 A:G, 5148 G:A, 5562 A:G, 5679 A:G, 5733 A:G, 6282 T:C, 6585 T:C, 7524 T:C, **7543 A:T**, 7647 G:A, 8461 T:C, **8611 A:G**, **9851 G:A**, **10831 A:G**, 10947 G:A, 11094 T:C, 11601 A:C	14	439 H:D, 448 T:A, 554 V:M, 728 G:R, 973 Y:H, 993 R:K, 1063 V:G, 1065 S:L, 1146 R:Q, 1607 M:I, 2515 M:L, 2871 T:A, 3284 R:K, 3611 I:V
AU526:P5	45	247 T:C, 345 T:C, 726 A:G, 783 C:T, 840 T:C, 978 T:C, 1008 T:C, 1125 G:A, 1254 A:G, **1315 C:G**, **1342 A:G**, 1626 A:G, **1660 G:A**, 2136 A:T, **2182 G:C**, 2598 T:C, 2682 C:T, **2917 T:C**, **2978 G:A**, **3194 C:T**, **3437 G:A**, 3792 G:A, 3837 A:G, **4052 G:A**, 4653 G:A, 4701 A:G, **4821 G:A**, 4881 G:T, 4959 A:T, 5148 G:A, 5562 A:G, 5679 A:G, 5733 A:G, 6486 G:A, 7524 T:C, 7539 G:A, 8184 A:G, 8461 T:C, 8604 G:A, **8611 A:G**, **9851 G:A**, 10710 G:A, **10831 A:G**, 10947 G:A, 11094 T:C	13	439 H:D, 448 T:A, 554 V:M, 728 G:R, 973 Y:H, 993 R:K, 1065 S:L, 1146 R:Q, 1351 R:K, 1607 M:I, 2871 T:A, 3284 R:K, 3611 I:V
AU526:P6	33	247 T:C, 726 A:G, 783 C:T, 978 T:C, 1008 T:C, 1125 G:A, 1254 A:G, **1315 C:G**, **1342 A:G**, 1626 A:G, **1660 G:A**, 2136 A:T, **2182 G:C**, **2917 T:C**, **2978 G:A**, **3194 C:T**, **3437 G:A**, 3792 G:A, 4701 A:G, **4821 G:A**, 4881 G:T, 4959 A:T, 5148 G:A, 5562 A:G, 5679 A:G, 5733 A:G, 7524 T:C, 8461 T:C, **8611 A:G**, **9851 G:A**, **10831 A:G**, 10947 G:A, 11094 T:C	12	439 H:D, 448 T:A, 554 V:M, 728 G:R, 973 Y:H, 993 R:K, 1065 S:L, 1146 R:Q, 1607 M:I, 2871 T:A, 3284 R:K, 3611 I:V
AU526:P7	35	247 T:C, 726 A:G, 783 C:T, 978 T:C, 1008 T:C, 1125 G:A, 1254 A:G, **1315 C:G**, **1342 A:G**, 1626 A:G, **1660 G:A**, 2136 A:T, **2182 G:C**, 2526 A:G, 2682 C:T, **2917 T:C**, **2978 G:A**, **3194 C:T**, **3437 G:A**, 3792 G:A, 4701 A:G, **4821 G:A**, 4881 G:T, 4959 A:T, 5148 G:A, 5562 A:G, 5679 A:G, 5733 A:G, 7524 T:C, 8461 T:C, **8611 A:G**, **9851 G:A**, **10831 A:G**, 10947 G:A, 11094 T:C	12	439 H:D, 448 T:A, 554 V:M, 728 G:R, 973 Y:H, 993 R:K, 1065 S:L, 1146 R:Q, 1607 M:I, 2871 T:A, 3284 R:K, 3611 I:V

Nonsynonymous nucleotide substitutions are bolded.

Table S2.4. Location and type of nucleotide and amino acid substitutions between AU526 and isolates from piglets born to gilts infected with BVDV in early pregnancy

AU526:Offspring	Total	Location and type of nucleotide substitutions	Total	Location and type of amino acid substitutions
AU526:P5A	44	247 T:C, 726 A:G, 783 C:T, 975 C:T, 978 T:C, 1008 T:C, 1125 G:A, 1254 A:G, **1315 C:G**, **1342 A:G**, 1626 A:G, **1660 G:A**, 1866 A:G, 2136 A:T, **2182 G:C**, 2526 A:G, **2917 T:C**, **2978 G:A**, **3188 T:G**, **3194 C:T**, **3437 G:A**, 3792 G:A, 4230 A:G, 4701 A:G, **4821 G:A**, 4881 G:T, 4959 A:T, 5121 A:G, 5148 G:A, 5562 A:G, 5679 A:G, 5733 A:G, 6282 T:C, 6585 T:C, 7524 T:C, **7543 A:T**, 7647 G:A, 8461 T:C, **8611 A:G**, **9851 G:A**, **10831 A:G**, 10947 G:A, 11094 T:C, 11601 A:C	14	439 H:D, 448 T:A, 554 V:M, 728 G:R, 973 Y:H, 993 R:K, 1063 V:G, 1065 S:L, 1146 R:Q, 1607 M:I, 2515 M:L, 2871 T:A, 3284 R:K, 3611 I:V
AU526:P5B	47	247 T:C, 726 A:G, 783 C:T, 975 C:T, 978 T:C, 1008 T:C, 1125 G:A, 1254 A:G, **1315 C:G**, **1342 A:G**, 1626 A:G, **1660 G:A**, 1866 A:G, 2136 A:T, **2182 G:C**, 2526 A:G, **2917 T:C**, **2920 G:A**, **2978 G:A**, **3043 G:A**[a], **3044 A:G**[a], **3188 T:G**, **3194 C:T**, **3437 G:A**, 3792 G:A, 4230 A:G, 4701 A:G, **4821 G:A**, 4881 G:T, 4959 A:T, 5121 A:G, 5148 G:A, 5562 A:G, 5679 A:G, 5733 A:G, 6282 T:C, 6585 T:C, 7524 T:C, **7543 A:T**, 7647 G:A, 8461 T:C, **8611 A:G**, **9851 G:A**, **10831 A:G**, 10947 G:A, 11094 T:C, 11601 A:C	16	439 H:D, 448 T:A, 554 V:M, 728 G:R, 973 Y:H, 974 E:K, 993 R:K, 1015 E:R, 1063 V:G, 1065 S:L, 1146 R:Q, 1607 M:I, 2515 M:L, 2871 T:A, 3284 R:K, 3611 I:V
AU526:P5C	50	247 T:C, 345 T:C, 726 A:G, 783 C:T, 840 T:C, 978 T:C, 1008 T:C, 1125 G:A, 1254 A:G, **1315 C:G**, **1342 A:G**, 1626 A:G, **1660 G:A**, 2136 A:T, **2182 G:C**, 2598 T:C, 2682 C:T, **2917 T:C**, **2978 G:A**, **3194 C:T**, **3401 T:C**, **3437 G:A**, 3792 G:A, 3837 A:G, **4052 G:A**, 4155 A:G, 4653 G:A, 4701 A:G, **4821 G:A**, 4881 G:T, 4959 A:T, 5148 G:A, 5562 A:G, 5679 A:G, 5733 A:G, 6486 G:A, 7524 T:C, 7539 G:A, 7584 T:C, 7788 T:C, 8181 G:A, 8184 A:G, 8461 T:C, 8604 G:A, **8611 A:G**, **9851 G:A**, 10710 G:A, **10831 A:G**, 10947 G:A, 11094 T:C	14	439 H:D, 448 T:A, 554 V:M, 728 G:R, 973 Y:H, 993 R:K, 1065 S:L, 1134 V:A, 1146 R:Q, 1351 R:K, 1607 M:I, 2871 T:A, 3284 R:K, 3611 I:V
AU526:P5D	45	247 T:C, 726 A:G, 783 C:T, 975 C:T, 978 T:C, 1008 T:C, 1125 G:A, 1254 A:G, **1315 C:G**, **1342 A:G**, 1626 A:G, **1660 G:A**, 1866 A:G, 2136 A:T, **2182 G:C**, 2526 A:G, **2917 T:C**, **2978 G:A**, **3188 T:G**, **3194 C:T**, **3437 G:A**, 3792 G:A, 4230 A:G, 4701 A:G, **4821 G:A**, 4881 G:T, 4959 A:T, 5121 A:G, 5148 G:A, 5166 A:G, 5562 A:G, 5679 A:G, 5733 A:G, 6282 T:C, 6585 T:C, 7524 T:C, **7543 A:T**, 7647 G:A, 8461 T:C, **8611 A:G**, **9851 G:A**, **10831 A:G**, 10947 G:A, 11094 T:C, 11601 A:C	14	439 H:D, 448 T:A, 554 V:M, 728 G:R, 973 Y:H, 993 R:K, 1063 V:G, 1065 S:L, 1146 R:Q, 1607 M:I, 2515 M:L, 2871 T:A, 3284 R:K, 3611 I:V
AU526:P5F	48	247 T:C, 345 T:C, 726 A:G, 783 C:T, 840 T:C, 978 T:C, 1008 T:C, 1125 G:A, 1254 A:G, **1315 C:G**, **1342 A:G**, 1626 A:G, **1660 G:A**, 2136 A:T, **2182 G:C**, 2598 T:C, 2682 C:T, **2917 T:C**, **2978 G:A**, **3194 C:T**, **3401 T:C**, **3437 G:A**, 3792 G:A, 3837 A:G, **4052 G:A**, 4155 A:G, 4653 G:A, 4701 A:G, **4821 G:A**, 4881 G:T, 4959 A:T, 5148 G:A, 5562 A:G, 5679 A:G, 5733 A:G, 6486 G:A, 7524 T:C, 7539 G:A, 7788 T:C, 8184 A:G, 8461 T:C, 8604 G:A, **8611 A:G**, **9851 G:A**, 10710 G:A, **10831 A:G**, 10947 G:A, 11094 T:C	14	439 H:D, 448 T:A, 554 V:M, 728 G:R, 973 Y:H, 993 R:K, 1065 S:L, 1134 V:A, 1146 R:Q, 1351 R:K, 1607 M:I, 2871 T:A, 3284 R:K, 3611 I:V
AU526:P7A	49	247 T:C, 345 T:C, 726 A:G, 783 C:T, 840 T:C, 978 T:C, 1008 T:C, 1125 G:A, 1254 A:G, **1315 C:G**, **1342 A:G**, 1626 A:G, **1660 G:A**, 1695 C:T, 2136 A:T, **2182 G:C**, 2598 T:C, 2682 C:T, **2917 T:C**, **2978 G:A**, **3194 C:T**, **3437 G:A**, 3792 G:A, 3837 A:G, **4052 G:A**, 4155 A:G, 4653 G:A, 4701 A:G, **4821 G:A**, 4881 G:T, 4959 A:T, 5148 G:A, 5562 A:G, 5679 A:G, 5733 A:G, 6486 G:A, 7524 T:C, 7539 G:A, 7788 T:C, 8184 A:G, 8461 T:C, 8604 G:A, **8611 A:G**, **9851 G:A**, 10710 G:A, **10831 A:G**, 10947 G:A, 10989 A:G, 11094 T:C	13	439 H:D, 448 T:A, 554 V:M, 728 G:R, 973 Y:H, 993 R:K, 1065 S:L, 1146 R:Q, 1351 R:K, 1607 M:I, 2871 T:A, 3284 R:K, 3611 I:V
AU526:P7C	56	247 T:C, 345 T:C, 726 A:G, 783 C:T, 840 T:C, 978 T:C, 1008 T:C, 1125 G:A, 1254 A:G, **1315 C:G**, **1342 A:G**, 1626 A:G, **1660 G:A**, 1695 C:T, **2135 C:T**, 2136 A:T, **2182 G:C**, 2562 C:T, 2598 T:C, 2682 C:T, **2917 T:C**, **2978 G:A**, **3043 G:A**, **3194 C:T**, **3431 T:C**, **3437 G:A**, 3792 G:A, 3837 A:G, **4052 G:A**, 4155 A:G, 4194 T:C, 4653 G:A, 4701 A:G, **4821 G:A**, 4881 G:T, 4959 A:T, 5148 G:A, 5562 A:G, 5679 A:G, 5733 A:G, 6486 G:A, 7524 T:C, 7539 G:A, 7788 T:C, 8184 A:G, 8461 T:C, 8604 G:A, **8611 A:G**, **9851 G:A**, **10478 C:A**, 10710 G:A, **10831 A:G**, 10839 T:C, 10947 G:A, 10989 A:G, 11094 T:C	17	439 H:D, 448 T:A, 554 V:M, 712 P:L, 728 G:R, 973 Y:H, 993 R:K, 1015 E:K, 1065 S:L, 1144 L:P, 1146 R:Q, 1351 R:K, 1607 M:I, 2871 T:A, 3284 R:K, 3493 P:Q, 3611 I:V
AU526:P7E	57	247 T:C, 345 T:C, 726 A:G, 783 C:T, 840 T:C, 978 T:C, 1008 T:C, 1125 G:A, 1254 A:G, **1315 C:G**, **1342 A:G**, **1435 G:A**, 1626 A:G, **1660 G:A**, 1695 C:T, 2136 A:T, **2182 G:C**, **2204 C:T**, **2254 G:A**, **2383 C:A**, **2456 A:T**, 2598 T:C, 2682 C:T, **2917 T:C**, **2978 G:A**, **3194 C:T**, **3437 G:A**, 3792 G:A, 3837 A:G, **4052 G:A**, 4155 A:G, 4653 G:A, 4701 A:G, **4821 G:A**, 4881 G:T, 4959 A:T, 5148 G:A, 5562 A:G, 5679 A:G, 5733 A:G, 6178 C:T, 6486 G:A, 7524 T:C, 7539 G:A, 7788 T:C, 8184 A:G, 8461 T:C, 8604 G:A, **8611 A:G**, 9840 C:A, **9851 G:A**, 10464 G:A, 10710 G:A, **10831 A:G**, 10947 G:A, 10989 A:G, 11094 T:C	18	439 H:D, 448 T:A, 479 G:R, 554 V:M, 728 G:R, 735 T:I, 752 E:K, 795 L:I, 819 Q:L, 973 Y:H, 993 R:K, 1065 S:L, 1146 R:Q, 1351 R:K, 1607 M:I, 2871 T:A, 3284 R:K, 3611 I:V
AU526:P7F	55	247 T:C, 345 T:C, **520 G:A**, 726 A:G, 783 C:T, 840 T:C, 978 T:C, 1008 T:C, 1125 G:A, 1254 A:G, **1315 C:G**, **1342 A:G**, **1435 G:A**, **1625 A:T**, 1626 A:G, **1660 G:A**, 1695 C:T, 2136 A:T, **2182 G:C**, 2598 T:C, 2682 C:T, **2917 T:C**, **2947 G:A**, **2978 G:A**, **3194 C:T**, **3437 G:A**, 3792 G:A, 3837 A:G, **4052 G:A**, 4155 A:G, 4653 G:A, 4701 A:G, **4821 G:A**, 4881 G:T, 4959 A:T, 5148 G:A, 5562 A:G, 5679 A:G, 5733 A:G, 6486 G:A, 7365 C:T, 7524 T:C, 7539 G:A, 7788 T:C, 8184 A:G, 8461 T:C, 8604 G:A, **8611 A:G**, **9851 G:A**, 10626 A:G, 10710 G:A, **10831 A:G**, 10947 G:A, 10989 A:G, 11094 T:C	17	174 D:N, 439 H:D, 448 T:A, 479 G:R, 542 K:M, 554 V:M, 728 G:R, 973 Y:H, 983 E:K, 993 R:K, 1065 S:L, 1146 R:Q, 1351 R:K, 1607 M:I, 2871 T:A, 3284 R:K, 3611 I:V

[a] Nucleotide substitutions associated with the same amino acid substitution (1015 E:R)
Nonsynonymous nucleotide substitutions are bolded.

Table S2.5. Location and type of nucleotide and amino acid substitutions between isolates from acutely infected pregnant gilts and their piglets

Dam:Offspring	Total	Location and type of nucleotide substitutions	Total	Location and type of amino acid substitutions
P5:P5A	23	345 C:T, 840 C:T, 975 C:T, 1866 A:G, 2526 A:G, 2598 C:T, 2682 T:C, **3188 T:G**, 3837 G:A, **4052 A:G**, 4230 A:G, 4653 A:G, 5121 A:G, 6282 T:C, 6486 A:G, 6585 T:C, 7539 A:G, **7543 A:T**, 7647 G:A, 8184 G:A, 8604 A:G, 10710 A:G, 11601 A:C	3	1063 V:G, 1351 K:R, 2515 M:L
P5:P5B	26	345 C:T, 840 C:T, 975 C:T, 1866 A:G, 2526 A:G, 2598 C:T, 2682 T:C, **2920 G:A**, **3043 G:A**[a], **3044 A:G**[a], **3188 T:G**, 3837 G:A, **4052 A:G**, 4230 A:G, 4653 A:G, 5121 A:G, 6282 T:C, 6486 A:G, 6585 T:C, 7539 A:G, **7543 A:T**, 7647 G:A, 8184 G:A, 8604 A:G, 10710 A:G, 11601 A:C	5	974 E:K, 1015 E:R, 1063 V:G, 1351 K:R, 2515 M:L
P5:P5C	5	**3401 T:C**, 4155 A:G, 7584 T:C, 7788 T:C, 8181 G:A	1	1134 V:A
P5:P5D	24	345 C:T, 840 C:T, 975 C:T, 1866 A:G, 2526 A:G, 2598 C:T, 2682 T:C, **3188 T:G**, 3837 G:A, **4052 A:G**, 4230 A:G, 4653 A:G, 5121 A:G, 5166 A:G, 6282 T:C, 6486 A:G, 6585 T:C, 7539 A:G, **7543 A:T**, 7647 G:A, 8184 G:A, 8604 A:G, 10710 A:G, 11601 A:C	3	1063 V:G, 1351 K:R, 2515 M:L
P5:P5F	3	**3401 T:C**, 4155 A:G, 7788 T:C	1	1134 V:A
P6:P7A	15	345 T:C, 840 T:C, 1695 C:T, 2526 G:A, 2598 T:C, 3837 A:G, **4052 G:A**, 4155 A:G, 4653 G:A, 6486 G:A, 7539 G:A, 7788 T:C, 8184 A:G, 8604 G:A, 10989 A:G	1	1351 R:K
P6:P7C	22	345 T:C, 840 T:C, 1695 C:T, **2135 C:T**, 2526 G:A, 2562 C:T, 2598 T:C, **3043 G:A**, **3431 T:C**, 3837 A:G, **4052 G:A**, 4155 A:G, 4194 T:C, 4653 G:A, 6486 G:A, 7539 G:A, 7788 T:C, 8184 A:G, 8604 G:A, **10478 C:A**, 10839 T:C, 10989 A:G	5	712 P:L, 1015 E:K, 1144 L:P, 1351 R:K, 3493 P:Q
P6:P7E	23	345 T:C, 840 T:C, **1435 G:A**, 1695 C:T, **2204 C:T**, **2254 G:A**, **2383 C:A**, **2456 A:T**, 2526 G:A, 2598 T:C, 3837 A:G, **4052 G:A**, 4155 A:G, 4653 G:A, 6178 C:T, 6486 G:A, 7539 G:A, 7788 T:C, 8184 A:G, 8604 G:A, 9840 C:A, 10464 G:A, 10989 A:G	6	479 G:R, 735 T:I, 752 E:K, 795 L:I, 819 Q:L, 1351 R:K
P6:P7F	21	345 T:C, **520 G:A**, 840 T:C, **1435 G:A**, **1625 A:T**, 1695 C:T, 2526 G:A, 2598 T:C, **2947 G:A**, 3837 A:G, **4052 G:A**, 4155 A:G, 4653 G:A, 6486 G:A, 7365 C:T, 7539 G:A, 7788 T:C, 8184 A:G, 8604 G:A, 10626 A:G, 10989 A:G	5	174 D:N, 479 G:R, 542 K:M, 983 E:K, 1351 R:K

[a] Nucleotide substitutions associated with the same amino acid substitution (1015 E:R)
Nonsynonymous nucleotide substitutions are bolded.

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Table S2.6. Virus titers in passaged buffy coat samples from acutely infected gilts

	Virus titers ($TCID_{50}$/ml)	
Gilt	5 dpi	7 dpi
P1	3.5×10^4	6.2×10^3
P2	3.5×10^3	3.5×10^3
P5	6.2×10^4	6.2×10^4
P6	2.0×10^6	3.5×10^5
P7	3.5×10^6	6.2×10^6

dpi, day postinoculation

Table S2.7. Virus titers in passaged serum or buffy coat samples from piglets born to gilts infected with BVDV in early pregnancy

	Virus titers ($TCID_{50}$/ml)	
Piglet	day of birth	21 doa
P5A	3.5×10^6	NA
P5B	6.2×10^5	NA
P5C	3.5×10^6	NA
P5D	2.0×10^7	NA
P5F	3.5×10^4	NA
P7A	3.5×10^5	NA
P7C	NT	2.0×10^2
P7D	NT	3.5×10^5
P7E	NT	3.5×10^4
P7F	NT	2.0×10^3

doa, days of age; NA, not applicable; NT, not tested

Chapter 4: Summary and Conclusion

BVDV isolates circulating in animal populations worldwide are genetically and antigenically highly diverse and at least and at least 21 BVDV-1 subgenotypes (1a to 1u) and four BVDV-2 subgenotypes (2a to 2d) have been described to date. RNA viruses are characterized by high mutation rates and the minimal proofreading activity of the RNA polymerases is believed to be the main driving force for the generation of altered genomic sequences. Despite maintaining high levels of viral replication during their entire life, only minor changes at the consensus level have been demonstrated in isolates obtained from PI cattle at different time points (Collins et al., 1999; Ridpath et al., 2015). Therefore, it has been suggested that the specific immune tolerance to the infected strain is accompanied by the elimination of new viral variants. In contrast, genetic and antigenic changes were shown to occur in acutely infected cattle (Bolin & Ridpath, 1992; Neill et al., 2011, 2012). Acute BVDV infections are believed to favor survival of viral variants that can escape the host immune response and thus may greatly contribute to the generation of genetically distinct isolates. Furthermore, greater numbers of substitutions occurred during acute infections of pregnant cattle than of non-pregnant cattle and most amino acid substitutions were detected in genomic regions encoding structural viral proteins (Neill et al., 2012). These results suggest that BVDV infections in pregnant cattle may serve as a significant source of viral genetic and antigenic variability.

Although its natural host is cattle, BVDV can infect pigs, camelids, and a wide range of domestic and wild ruminants, including sheep, goats, and deer. Cross-species infections may thus provide a further opportunity for viral diversification. However, there is limited information regarding genetic and antigenic changes arising during BVDV infections in species other than

cattle. Amino acid substitutions have been reported in the E2 coding region during the establishment of persistent infections in sheep and goats (Bachofen et al., 2013; Passler et al., 2014a; Paton et al., 1997). Genetic analysis was often limited in these previous studies to the E2 coding region and parts of the N^{pro} and C coding regions of the viral genome.

The objective of this research was thus to evaluate the changes introduced in the open reading frame (ORF) of the BVDV genome during serial infection of pregnant cattle, sheep, and swine with an isolate of bovine origin. There are many host barriers and defense mechanisms that prevent interspecies transmission of viruses and several genomic changes would likely be required to successfully infect a heterologous host. Therefore, we hypothesized that the number of genomic changes would be greater in sheep and swine than in cattle.

Serial experimental inoculations were performed in six pregnant heifers, six pregnant ewes, and six pregnant gilts using the noncytopathic BVDV-1b isolate AU526 in the first dam of each species and serum from the preceding acutely infected dam thereafter. This isolate had been isolated from the serum of a PI cow and demonstrated to cause persistent infection in goats and white-tailed deer (Passler et al., 2014a; Passler et al., 2007). Complete ORF sequences were determined for 37 isolates including AU526, 17 isolates from acutely infected dams, and 19 isolates from their congenitally infected offspring. Sequence comparison revealed that greater numbers of substitutions occurred during serial infection of pregnant sheep and swine than of pregnant cattle. Previous studies have demonstrated severe reduction in RNA virus populations during vector-borne transmission and when viruses spread within a single host (Dow et al., 2015; Forrester et al., 2012; Navas et al., 1998). Therefore, cross-species infections are also likely to impose severe bottlenecks on virus populations and it is possible that serial BVDV infections in

sheep and swine were associated with greater numbers of substitutions due to high selection pressure on virus populations.

Furthermore, multiple identical amino acid substitutions were detected in isolates obtained from animals of the same species. These changes were more abundant in ovine and porcine isolates and occurred primarily in the E2 and E^{rns} coding regions. These envelope proteins are the main targets of neutralizing antibodies in animals infected with BVDV and immune pressure on the immunodominant residues may have contributed to the selection of amino acid changes in these coding regions. These two viral proteins also play an important role in cell attachment and viral entry, whereas the E^{rns} protein is implicated in inhibition of the host innate immune response and establishment of persistent infection. It is thus possible that these conserved changes may be involved in host adaptation. Conserved amino acid changes in the E2 and E^{rns} coding regions have been reported in BVDV-1b isolates from alpacas (Neill et al., 2015). Interestingly, most conserved substitutions in ovine and porcine isolates were found to be identical, but different from those observed during serial passage in cattle. It is currently unknown, and will be the focus of future experiments, if these changes provided a selective advantage in sheep and swine and if similar results would have been obtained if a BVDV isolate belonging to a different subgenotype or species had been used.

Altogether these results suggest that BVDV infections in heterologous species may serve as a significant source of viral genetic diversity and may be associated with adaptive changes.

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