Immunopathogenesis, Prevention and Control of Bovine Viral Diarrhea Virus

by

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Abstract

Bovine viral diarrhea virus (BVDV) is an important infectious agent that affects cattle worldwide (Baker, 1995; Houe, 1999). Acute infections with some strains of BVDV may cause immunosuppression and clinical signs of gastrointestinal, reproductive, and respiratory disease (Fulton et al., 2005a; Makoschey et al., 2001). Strategies to prevent and control BVDV include quarantine and other biosecurity measures to control the spread of infection within and between herds, identification and slaughter of persistently infected cattle, and vaccination (Brock, 2004; Kelling et al., 2000; Reber et al., 2006). An important factor in the evaluation of BVDV vaccine efficacy is rapidity in eliciting an adequate immune response to protect cattle from negative outcomes when exposed to the virus within a few days after vaccination. It was hypothesized that a single dose of a commercial modified-live virus (MLV) vaccine (containing BVDV type 1a and 2 strains) would protect cattle from acute BVDV infection when experimentally inoculated with a NY-1 BVDV soon after vaccination (3, 5, or 7 days post-vaccination). Our results indicated that modified-live BVDV vaccine administered 5 or 7 days before challenge prevented fever, viremia, and leukopenia in calves inoculated with NY-1 BVDV. However, a high proportion of calves vaccinated 3 days before challenge shed BVDV after inoculation.

Infection of pregnant cattle with BVDV may result in abortions, stillbirths, or the birth of calves with congenital defects or persistently infected with BVDV (Baker, 1995). Persistent infections occur if a susceptible pregnant cow is infected with a noncytopathic (ncp) BVDV strain at 30 to 125 days of gestation (Brock et al., 2005). At this time of gestation the fetal immune system is not completely developed and not able to recognize BVDV as a foreign

antigen; thus the fetus becomes immunotolerant of the infecting BVDV strain. Such calves continually shed large amounts of BVDV, representing a risk to susceptible herdmates (McClurkin et al., 1984). The efficacy of modified-live BVDV vaccines to prevent fetal infections and the development of PI animals has been previously evaluated, reporting values between 57.9 and 100% (Brock and Cortese, 2001; Brock et al., 2006; Cortese et al., 1998a; Dean et al., 2003; Fulton, 2005; Leyh et al., 2011; Rodning et al., 2010a). A major concern of MLV BVDV vaccines is the potential risk for contamination with foreign virulent strains becoming a source of spread of BVDV infections (Nuttall et al., 1977). In the second study of this investigation we showed evidence of abortions and BVDV fetal persistent infections following off-label immunization of pregnant heifers with a contaminated modified-live ncp BVDV vaccine. Even though the contaminated vaccine contained both ncp BVDV-1 and BVDV-2, *q*RT-PCR and nucleotide sequencing analysis revealed that vaccinated heifers developed only BVDV-2 PI fetuses. Furthermore, BVDV was apparently shed to unvaccinated heifers causing fetal infections from which only BVDV-1 was detected.

The virulence of the BVDV strain has been correlated with the ability of the virus to cause a decrease in lymphocyte counts (Kelling et al., 2002b; Liebler-Tenorio et al., 2003a; Liebler-Tenorio et al., 2003b) and impair leukocyte function. Thus, highly virulent BVDV induce a significantly more severe and longer lymphopenia and lymphoid tissue depletion than less virulent BVDV (Kelling et al., 2002b; Liebler-Tenorio et al., 2002; Liebler-Tenorio et al., 2003b). The third and fourth studies of this research were focused on the evaluation of the early immune response (through the mRNA gene expression) following inoculation in beef calves with high or low virulence BVDV strains. These studies were performed to determine a possible association between BVDV virulence and the mechanisms by which this virus causes

immunosuppression in susceptible cattle. The results demonstrated an up-regulation of type I interferon-induced antiviral state in spleen and tracheo-bronchial lymph nodes of calves inoculated with high and low virulence BVDV strains. A significant up-regulation of caspase-8 and -9 was observed in tracheo-bronchial lymph nodes in the calves inoculated with the low virulence BVDV (*P*=0.01), but not in those inoculated with the highly virulent BVDV-2 1373. There was a differential expression of some interferon-induced genes (OAS-1 and ISG-15) and pro-apoptosis markers based on BVDV virulence and genotype. In addition, experimental inoculation with BVDV-2 1373 stimulated a significant mRNA expression of both pro-inflammatory (TNF-α, IL-1β, IFN-γ, IL-2, IL-12, IL-15), and anti-inflammatory (IL-4, IL-10, TGF-β) cytokines. However, inoculation with BVDV-1 SD-1 only resulted in up-regulation of IL-12 and IL-15 mRNA, which are associated with activation of macrophages and NK cells during the innate immune response. The observed differential expression of early immune response after infection with low or high virulence BVDV might reflect differences in viral pathogenesis and could possibly determine the clinical outcome. The analysis of cytokine expression during the early events of immune response to BVDV could have important implications for selection of BVDV strains to be used for new vaccine production, since the efficacy of a vaccine will depend on how efficiently it stimulates the innate and early adaptive immune response.

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List of Abbreviations

ABD	Average breeding day
AF	Amniotic fluid
BVDV	Bovine viral diarrhea virus
$CCID_{50}$	Cell culture infectious dose 50%
GM	Geometric mean
IHC	Immunohistochemistry
HB	Heart blood
MDBK	Madin-Darby bovine kidney
MLV	Modified-live virus
NS	Nasal swab
QRT-PCR	Quantitative reverse transcription polymerase chain reaction
USG	Ultrasonography
VI	Virus isolation
VN	Virus neutralization

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I. Literature review

1. History of Bovine Viral Diarrhea Virus

In 1946, Olafson, MacCallum and Fox from Cornell University described a new transmissible disease in cattle observed in Ithaca, New York, caused by an emergent virus. The disease was characterized by fever, depression, leukopenia, anorexia, diarrhea, dehydration, salivation, nasal discharge, gastrointestinal erosions, and hemorrhages in various tissues (Olafson et al., 1946). The morbidity and mortality of the initial outbreaks were 33-88% and 4-8%, respectively. Productive consequences observed during these outbreaks included decreased milk production and the occurrence of abortions and respiratory disease in cows. The diseased animals developed a significant leukopenia concurrent with clinical signs of illness, which was considered to be indicative of a viral infection. Some other animals also showed severe leukopenia without any clinical signs of disease (Fox, 1996). The lesions of bovine viral diarrhea virus (BVDV) were similar to those of rinderpest, an exotic disease for the U.S. However, because US cattle were highly susceptible to rinderpest, its occurrence would have presented with a much more devastating clinical picture, including a higher mortality rate. Additionally, experimental serological and clinical studies demonstrated that sera from cattle that recovered from this disease did not neutralize rinderpest virus, nor did the diseased animals demonstrate resistance to infection with rinderpest virus (Walker and Olafson, 1947).

In 1946, Childs reported a similar but more severe clinical disease occurring in Ontario, Canada (Childs, 1946). This disease was characterized by fever, anorexia, depression, profuse salivation, nasal discharge, gastrointestinal hemorrhages, severe diarrhea with watery feces and sometimes

mixed with blood, oral erosions and ulcers. Few animals in the herd were affected but case fatality rate was high. In 1953, (Ramsey, 1953) reported MD in US and gave the disease its name. However, Childs' report was later considered the first description of mucosal disease (MD) in cattle (Pritchard, 1963).

(Lee and Gillespie, 1957a) demonstrated that the pathogen isolated from clinical cases of disease was bovine viral diarrhea virus (BVDV) (isolate NY-1) did not cause cytopathology in cell cultures. On the other hand, the virus isolated from MD did produce cytopathic effects (Underdahl et al., 1957). The discovery of cytopathic strains permitted the development of virus neutralization assays for antibody titer detection (Gillespie et al., 1961). At this point the neutralization assays indicated that viral agents isolated from BVD and MD in North America and Europe were the same, and that BVD and MD were different disease manifestations caused by the same pathogen (Gillespie et al., 1961).

Pritchard (1963) stated that BVD occurs in three different clinical forms: severe acute BVD, mild acute BVD and BVD MD. A field study showed that seven animals with MD were viremic and serologically negative to the virus (Thomson and Savan, 1963) suggesting that diseased animals were unable to develop an immune response. One mechanism proposed to explain persistent infection and the failure of cattle with MD to produce antibodies to BVDV was the development of BVDV-specific immune tolerance during intrauterine infection (Malmquist, 1968). An alternative mechanism proposed by Malmquist was the destruction of immunological competent cells by the virus (Malmquist, 1968). The hypothesis of immunotolerance and intrauterine infection was later proven in the 1980s.

Abortion outbreaks were also associated with BVDV infections (Olafson et al., 1946). Evidence of an etiologic role was the isolation of noncytopathic BVDV from aborted fetuses

(Baker et al., 1954; Gillespie et al., 1967; Olafson et al., 1946). Congenital defects, such as cerebellar hypoplasia, cataracts, and retinal degeneration, were evidenced in calves born to dams infected with BVDV during pregnancy (Ward et al., 1969). Kahrs et al. (1970) reported that infection occurred around 134-183 days of gestation in 3 of the calves with cerebellar hypoplasia. (Kahrs et al., 1970)

Darbyshire (1962) stated that BVDV was antigenically related to hog cholera virus, more commonly known as classical swine fever virus (CSFV). This encouraged the idea of swine immunization using BVDV to elicit protection from CSFV infection. However, the US Department of Agriculture was against the use of BVDV for this purpose, based on the fact that BVDV was isolated from naturally infected swine (Fernelius et al., 1973).

2. Classification and molecular biology

2.1. Taxonomy

Bovine viral diarrhea virus is a single stranded positive sense RNA virus belonging to the *Pestivirus* genus and the *Flaviviridae* family (Lindenbach and Rice, 2001; Ridpath, 2003). It is an enveloped virus, which makes it susceptible to inactivation by solvent and detergents. (Ridpath, 2005b). The genome consists of a single open reading frame (ORF) flanked by 5' and 3' untranslated regions (UTR) (Collett et al., 1988a; Collett et al., 1988b; Deng and Brock, 1992). During translation, the single ORF produces a single polyprotein that is cleaved by viral and host proteases into individual viral proteins. This polyprotein produces structural and nonstructural proteins. The nucleotide sequence that codes for the nonstructural proteins is localized near the 3' end, whereas the structural protein sequence is located near the 5' end of the genome (Collett et al., 1988b; Ridpath, 2003).

In contrast to other members of the Flaviviridae family such as hepatitis C virus, pestiviruses encode for two unique proteins: nonstructural protein N^{pro} and E^{rns}. The first is an auto-protease, which cleaves itself from the original polyprotein. The nonstructural protein E^{rns} is an envelope glycoprotein with RNase activity (Collett et al., 1988a; Ridpath, 2005a).

The genus *Pestivirus* includes four species: BVDV-1, BVDV-2, border disease virus (BDV) and classical swine fever virus (CSFV). This species differentiation has been accurately performed based on phylogenetic analysis of genomic sequences (Harasawa, 1996; Harasawa and Giangaspero, 1998; Hofmann et al., 1994; Ridpath and Bolin, 1997; Sullivan et al., 1997). The parameter most commonly used to differentiate *Pestivirus* species is the degree of sequence homology in the 5' UTR region (Falcone et al., 2001; Ridpath and Bolin, 1997; Ridpath and Neill, 2000a; Ridpath et al., 2000). However, differences between BVDV-1 and BVDV-2 are found throughout the genome sequence.

Pestiviruses have the ability to antigenically cross-react (despite antigenic differences between species). Thus, previous efforts to classify *Pestivirus* into species based on serological monoclonal antibody binding assay had limited success (Bolin et al., 1988; Edwards et al., 1991; Hess et al., 1988; Zhou et al., 1989).

2.2. BVDV Genotypes

As mentioned, BVDV has been classified into two genotypes BVDV-1 and BVDV-2, based on differences in genomic sequences. The first published classification of BVDV strains into two different genotypes was based on comparison of the 5' UTR (Ridpath et al., 1994), a highly conserved region, compared with other portions of the genome (Lewis et al., 1991;

Ridpath and Bolin, 1991). However, further nucleotide sequencing analysis revealed that differences between genotypes can be found throughout the genome (Ridpath and Bolin, 1995b).

Early studies for genotype classification of BVDV allowed the identification of a BVDV strain isolated from animals suffering from a hemorrhagic syndrome as a different genotype from those commonly used for vaccine production, diagnostic tests and research (Pellerin et al., 1994). This new group of BVDV was classified as BVDV genotype 2; whereas the strains used for research, diagnosis and vaccines was designated as BVDV genotype 1. Both genotypes have similar rates of variation in the 5' UTR, suggesting that the two genotypes have been evolving for approximately the same time span. This indicates that the BVDV-2 genotype is not a new emergent virus, but was recognized after the BVDV-1 genotype. An early study emphasized that not all BVDV-2 strains cause severe acute clinical disease and hemorrhagic syndrome (Ridpath et al., 1994). In that survey only 32 out of 76 BVDV-2 strains were associated with severe clinical disease (Ridpath et al., 1994). The same authors stated that the majority of BVDV-2 strains present in North America seem to be no more virulent than BVDV-1 strains (Ridpath, 2005a). However, it is apparently clear that only strains from the BVDV-2 genotype are associated with severe acute disease (Bolin and Ridpath, 1992; Liebler-Tenorio et al., 2002).

Strains of the BVDV-1 genotype can be classified in two subgenotypes, BVDV-1a and BVDV-1b (Pellerin et al., 1994), which can be differentiated by monoclonal antibodies (Bolin and Ridpath, 1998) and RT-PCR analysis (Ridpath and Bolin, 1998). Studies have identified that BVDV-1b strains are more prevalent than BVDV-1a strains, and are more frequently isolated from calves that die with gross lesions of pneumonia (Fulton et al., 2003a; Fulton et al., 2005b). On the other hand, BVDV-1a strains may predominate in BVDV fetal infections that occur in late gestation (Evermann and Ridpath, 2002). Similarly, BVDV-2 strains have been segregated

into BVDV2a and BVDV 2b (Flores et al., 2002). However, this genotypic classification has not been officially accepted.

2.3. BVDV Biotypes

BVDV strains are classified into two different biotypes cytopathic (cp) and noncytopathic (ncp), based on the effects of virus on cell cultures (Lee and Guillespie, 1957). Both biotypes code for the nonstructural protein NS2-3. Cytopathic strains have the ability to cleave the NS2-3 to an NS2 and an NS3 protein. Therefore, cp strains of BVDV evolve from ncp BVDV by insertion, recombination, mutation, duplications, or deletion (Ridpath and Bolin, 1995b; Tautz and Thiel, 2003). The production of NS3 is associated with insertion of nucleotide sequences into the viral genome at the carboxy-terminus flanked by the aminoacid position 1535 (position A) or 1589 (position B) (Ridpath, 2005a).

Different strategies have been hypothesized for cleavage of NS2/NS3 after the genomic modification. These include: formation of new cleavage sites, introduction of autocatalytic sequences, introduction of conformational changes signaling cellular proteases, or activation of latent protease activity directly encoded by NS2 (Meyers and Thiel, 1996). Specifically, the insertion at the amino acid position B may introduce a new cleavage site at the carboxy-terminus or add an autocatalytic activity at the carboxy-terminus of the insertion, producing separation of NS3. Insertion at position A also results in cleavage of amino acid position 1589, since it might induce conformational changes that permit cleavage via a cryptic mechanism at amino acid 1589 (Meyers and Thiel, 1996). Most of the cp BVDV strains that have been characterized in the literature seem to be the result of genomic insertion of host cell genomic sequences or

duplication of viral sequences and only a small number appear to be associated with recombination between BVDV strains (Hietala and Crossley, 2005).

2.4. Pestivirus virion structure

Pestivirus virions are spherical particles, enveloped between 40 and 60 nm in diameter. The central capsid is composed of C protein and genome RNA, which are surrounded by a lipid bilayer (Ridpath, 2005a). Three virus encoded proteins (E^{rns}, E1 and E2) are recognized to be associated with envelop (Ridpath, 2005a).

The Pestivirus virions are stable within a pH range of 5.7 to 9.3 (Hafez and Liess, 1972). Similar to other enveloped viruses, pestiviruses are susceptible to inactivation by organic solvents and detergents, trypsin treatment (Liess, 1990), electron beam irradiation (Preuss et al., 1997), and gamma irradiation (Lee et al., 1998).

2.5. Pestivirus Genome

The Pestivirus genome has an approximate length of 12.3 Kb (Deng and Brock, 1992; Ridpath and Bolin, 1995b; Ridpath and Bolin, 1997). The genome corresponds with a single ORF of approximately 4000 codons localized between the 5' and 3' UTR. There is no cap structure at the 5' end (Deng and Brock, 1992) nor a poly (A) tract at the 3' end (Ridpath, 2005a). In contrast, the genome ends in a 3' poly (C) tract. The 5'UTR contain multiple initiation codons upstream from the translation initiation site.

Similar to other pestiviruses, the 5' UTR is relatively long, measuring approximately 385 nucleotides. Pestiviruses show a high nucleic acid identity in the 5' UTR. However, there are two short regions that vary significantly among pestiviruses, one of which is located between

nucleotides 208 and 223 and the other between nucleotides 294 and 323 (Ridpath, 2005a). These sequence differences have been used to differentiate BVDV genotypes through PCR and nucleotide sequencing analysis (Ridpath and Bolin, 1998).

The 5' UTR contains tertiary structures which functions as internal ribosomal entry site (IRES), mediating the initiation of translation (Deng and Brock, 1992). The tertiary structure forms pseudoknots identified as A, B, C, D1, D2, D4 and D5 (Deng and Brock, 1993). The tertiary structures forming the IRES mediate internal attachment of ribosome to the translation initiation codon (Hietala and Crossley, 2005) to start translation in a cap-independent manner. It has been hypothesized that the pseudoknots serve a structural role in the placement of ribosomes over the initiation codon AUG (Lemon and Honda, 1997). The 3' UTR also contain primary and secondary structures. There are two hairpin loops that serve to direct initiation of negative strand, being essential for replication (Fields et al., 2001).

The pestivirus genome encodes for a polyprotein that contains individual viral proteins, in the following order: N^{pro}- C - E^{rns} - E1- E2 - p7 - NS2/3 - NS4A - NS4B - NS5A - NS5B. The viral-encoded proteins Npro, NS3 (serine protease, ATPase, and RNA helicase), NS4A (co-factor of NS3), NS4B, NS5A (serine phosphoprotein) and NS5B (RNA-dependent RNA polymerase), constitute the protein replication complex.

2.6. Replication Cycle

Pestivirus infection is a multistep process of binding and entry, initiated by receptor-attachment or interaction of viral proteins (E^{rns} and E2) with specific host cell receptors [e.g. low density lipoprotein receptor (Baranowski et al., 2003); or CD46 (Zezafoun et al., 2011)]; which

subsequently promotes entry by internalization and pH dependent fusion of the viral envelope and cell membrane (Xue et al., 1997).

Recent studies have demonstrated the presence of two important peptides (66EQIV69 and 82GQVLAL87) for the attachment platform (through mapping of the BVD-virion-binding site). In the same study, a complex mRNA splicing pattern was also evidenced for bovine CD46, generating three different serine-threonine-proline segments and five different cytoplasmic domains associated with 10 bovine CD46 isoforms, which render cells permissive to BVDV. This study implied that virus binding generates a cytoplasmic-tail-dependent outside-in signal that determines cell permissivity to BVDV (Zezafoun et al., 2011).

After entry, the genomic RNA is uncoated and released into the host cell cytoplasm, allowing thye initiation of viral protein synthesis (Hietala and Crossley, 2005). Translation is carried out using the IRES located within the 5' UTR (cap-independent mechanism), as described above (Ridpath, 2005a). This 5' UTR secondary structure contains 7 domains (A, B, C, D1, D2, D4, D5) some of which are essential for translation (Deng and Brock, 1993).

2.7. Viral proteins

The synthesized polyprotein is cleaved both cotranslationally and posttranslationally, by cellular and viral proteases. The nonstructural protein N^{pro} serves as an auto-protease promoting its own cleavage from the polyprotein. The C protein forms the nucleocapsid of the virion. The structural glycoprotein E^{rns} is an envelope-associated ribonuclease. The structural proteins E1 and E2 are envelope-associated glycoproteins. Specifically, E2 is an immunodominant structural protein which has the ability to elicit the production of specific neutralizing antibodies. The role of the next nonstructural protein, p7 is unknown. Some researchers hypothesize that this protein

is required for production of infectious virus but not for RNA replication (Harada et al., 2000). The nonstructural proteins NS2/3 and NS3 contain RNA helicase and N-terminal serine protease domains which cleave themselves and remaining nonstructural proteins from the viral polyprotein. This is an immunodominant nonstructural protein. Purified BVDV NS3 also has RNA-stimulated NTPase activities (Tamura et al., 1993; Warrener and Collett, 1995). The function of NS2 is unknown, as it is not required for RNA replication and its cleavage from NS2/3 does not affect the serine protease activity (Behrens et al., 1998). NS4A is a nonstructural protein with a role as a cofactor for the serine protease NS2-3 and NS3. NS4B and NS5A have been hypothesized to be replicase complex components (Ridpath, 2005a). The role of NS5B is as RNA polymerase (Lai et al., 1999).

2.8. Translation and processing of the polyprotein

After translation of the poly-protein the first nonstructural protein, N^{pro} an auto-protease, cleaves itself from the poly-protein. Cleavage between C and E^{rns}, E1 and E2, and E2 and p7 are subsequently performed by host proteases (Rumenapf et al., 1993; Stark et al., 1993). NS2/3 may be further cleaved into NS2 and NS3 in cytopathic BVDV strains; whereas it remains intact in the noncytopathic biotype. These nonstructural proteins act as serine proteases that process the remaining downstream proteins (NS4A, NS4B, NS5A and NS5B) from the polyprotein (Ridpath, 2005a). This protease activity is facilitated by the cofactor NS4A.

2.9. Viral replication

The genomic RNA serves initially as a transcript for translation of the polyprotein and then as a template for viral replication. A secondary structure motif (A and B) within the 5’UTR

contributes to the switch from translation to template replication (Yu et al., 2000). Moreover, accumulation of viral proteins (NS5A and NS5B) inhibits IRES-dependent translation, which may represent another mechanism for the translation-replication switch. *Pestivirus* replication is performed through a viral replication complex, made up of several viral nonstructural proteins and viral RNA (Ridpath, 2005a). After protein synthesis, nonstructural proteins assemble into a functional replicase complex at the 3' terminus of the genome to catalyze the transcription of positive-sense RNA into full-length complementary negative-sense RNA strand. Next, the negative strands are used as templates to synthesize genomic positive strands via a semi-conservative mechanism (Gong et al., 1998). A recent study suggested that the replication cycle occurs in the cytoplasmic side of the endoplasmic reticulum (Zhang et al., 2003).

After replication, viral assembly and maturation are carried out in intracellular vesicles in the Golgi apparatus or endoplasmic reticulum where the lipid envelop is obtained through budding into the vesicle. Finally, virions are released as early as 8 hours postinfection (Nuttall, 1980) by exocytosis (Grummer et al., 2001) since viral proteins are not detected on the membrane of the infected cells (Ridpath, 2005a).

A strategy that BVDV uses to favor viral replication and survival is to stimulate the expression of host genes involved in protein synthesis and post-translational processing, as well as down-regulate cellular genes encoding proteins involved in energy production and cell structure (Neill and Ridpath, 2003). Replication of BVDV occurs as an RNA-dependent RNA synthesis, which has been described using a semi-conservative asymmetric model. After infection of cell culture, BVDV has been detected within 4-6 hours, with a peak titer at12-24 hours post-infection (Gong et al., 1996).

Pestiviruses use the single-stranded positive sense RNA genome as a template for both translation and replication. As previously discussed, after protein synthesis begins, BVDV uses host and viral coded proteins in cotranslation and post-translation proteolytic processing in order to obtain 11 individual viral proteins from a single polyprotein.

Along with other Pestiviruses BVDV has shown a high rate of genetic insertion and recombination during the replication process (Fields et al., 2001). Recombination may occur when virions with different genomes co-infect the same host cell, allowing genetic crossover, potentially resulting in a hybrid BVDV strain (Becher et al., 1999). Previous studies have shown the existence of genomic hot-spots for viral recombination at the border of NS2 and NS3, important for the generation of cytopathic BVDV strains (nucleotide positions 1535 and 1589; (Fields et al., 2001; Ridpath and Neill, 2000b). Genetic recombinations have been previously reported between cytopathic and noncytopathic BVDV; BVDV-1 and BVDV-2 genotypes, BVDV persistent infection strains and vaccine strains, and BVDV and host RNA (Ridpath and Bolin, 1995a). Insertions within the viral genome may occur as the result of duplicated viral sequences, host ubiquitin, ribosomal ubiquitin gene fusion protein, host mRNA encoding a DnaJ or J-domain-regulatory proteins, and multiple microtubule-associated proteins (Hietala and Crossley, 2005).

The RNA-dependent RNA polymerase (NS5B) responsible for RNA replication has a very low proofreading activity, which results in a high mutation rate (Moya et al., 2000). This feature contributes with the production of a non-homogeneous clone, collectively identified as quasispecies (Moya et al., 2000). Generation of quasispecies might be associated with ability of BVDV to effectively evade the host humoral and cell mediated immune responses (Bolin et al., 1991).

3. Transmission

Shedding and transmission of BVDV can be carried out by two patterns: transiently during acute infections of susceptible animals and persistently, when the fetus is infected between 40 and 125 days of gestation (Thurmond, 2005). There are five main factors affecting BVDV transmission including infectivity (exposure dosage and route of infection), number of adequate contacts (nose-to-nose), duration of the infectious period and prevalence, and the presence of susceptible animals which depends on the herd immunity (Haloran, 1998). As BVDV transmission occurs within a herd, infected animals become immune and the number of susceptible animals decline, decreasing the risk that an infectious animal will come in contact with a susceptible animal (Thurmond, 2005).

3.1. Horizontal and vertical transmission.

Transmission of BVDV can take place vertically causing fetal congenital infection or horizontally after birth or postnatal transmission (Thurmond, 2005). During gestation, fetuses that survive infections with ncp strains between days 30 and 125 of gestation develop immunotolerance and become persistently infected (PI). These animals serve as reservoirs, disseminating the virus for life (Brock, 2005). There are two ways by which a fetus can become persistently infected. One is by transmission of the virus from a PI cow to her fetus. The other way is acute infection of the dam with a ncp BVDV strain before 125 days of gestation, transmitting the infection to the fetus (Brock, 2005).

In utero transmission of BVDV after 120-150 days of gestation, when the fetus has become immune-competent, might result in abortion, stillbirth, congenital defects, or the birth of a live normal-appearing calf (Thurmond, 2005). Congenital transmission after 150 days of

gestation can be demonstrated by the presence of pre-colostral BVDV-neutralizing antibodies in calves at birth. Congenitally infected calves have been reported to have a high risk for the development of severe diseases in the first 4-5 months of life and to experience fertility problems as heifers (Muñoz-Zanzi et al., 2003). Horizontal transmission results in acute infections, with severity varying from mild or inapparent to fatal disease. Acutely infected animals shed the virus transiently for approximately one week. Routes of horizontal transmission have been well documented including nose-to-nose contact, BVDV-contaminated fomites, and vaccination with a MLV vaccine (Radostits et al., 1999).

3.2. Routes and means of transmission

BVDV can be shed by acutely infected and PI animals (Houe, 1995) in excretions and secretions such as milk (Thurmond, 2005), tears, saliva, urine, feces, nasal discharge, and semen (Brock et al., 1991). Transmission of BVDV via semen can result in acute and persistent infection. A previous study showed that 12 seronegative heifers that were artificially inseminated with semen from a PI bull developed acute BVDV infection and seroconverted (Meyling and Mikel Jensen, 1988). In the same study, one of the 12 heifers gave birth to a PI calf (8%), showing a significant potential not only for acute transmission of BVDV from PI bulls but also for the development of persistent infections (Meyling and Mikel Jensen, 1988).

Embryo transfer has been recognized as an important means of BVDV transmission (Thurmond, 2005). The virus can be transmitted to the embryo or fetus by the donor, the recipient or if the fetal calf serum used during the flushing process contained BVDV (Brock et al., 1991). In addition, previous studies have shown that BVDV might be associated with single transferable *in vivo*–derived bovine embryos despite washing and trypsin treatment (Thurmond,

2005). Gard et al. (2009) evaluated the potential of BVDV to be transmitted via the intrauterine route at the time of embryo transfer. In this study, after collection and washing, embryos were placed into transfer media containing BVDV and then nonsurgically transferred into the uterus. At 30 d after embryo transfer, 6 of 10 heifers in the treatment group were pregnant; however, 30 d later, only one was still pregnant. This fetus was nonviable and was positive for BVDV. The authors concluded that BVDV associated with bovine embryos after in vitro exposure can result in viremia and seroconversion of seronegative recipients after transfer into the uterus during diestrus (Gard et al., 2009).

A previous study demonstrated that BVDV can be transmitted to seronegative cattle by transrectal palpation of the reproductive tract using the same palpation sleeve that had been previously used to palpate a PI animal (Lang-Ree et al., 1994).

It has been reported that BVDV can survive in a cool, protected environment for several days to weeks (Houe, 1995). Therefore, fomite or iatrogenic transmission might occur if susceptible cattle are exposed to equipment previously used with BVDV infected animals (Houe, 1995). Aerosol transmission of BVDV from coughing calves with respiratory disease caused by BVDV1b has been reported in cattle managed under crowded environmental conditions (Baule et al., 2001). Additionally, BVDV has been isolated from face flies (*Musca autumnalis*) feeding on a PI animal (Gunn, 1993). Another study showed that stable flies (*Stomoxys calcitrans*), horseflies (*Maemapota pluvialis*) and head flies (*Hydrotaea irritans*) were able to transmit infection to susceptible animals (Tarry et al., 1991). However, a more recent study revealed that BVDV may be detected in horn flies collected from PI cattle, but horn flies do not appear to be an important vector for BVDV transmission (Chamorro et al., 2011).

Transmission of BVDV can also occur if susceptible cattle are vaccinated with a MLV vaccine during pregnancy, representing a risk for abortion, congenital defects, stillborns and development of PI calves (Liess et al., 1984). Moreover, transient shedding of BVDV by animals vaccinated with a MLV vaccine may represent a risk for BVDV transmission to commingled susceptible pregnant cattle (Thurmond, 2005).

3.3. Transmission from persistently infected animals

Persistently infected animals shed high amounts of BVDV in secretions and excretions. However, some PI animals have been shown to have intermittent viremic episodes and shedding (Brock et al., 1991). These animals remain undetected in the herd, representing a risk for prolonged and continued transmission (Moerman et al., 1993). Pregnant PI cows invariably will give birth to PI calves. This concept can be used to identify PI maternal lines by testing the dam, daughter and sons of PI females (Brock et al., 1991).

3.4. Transmission from acutely infected animals

Transiently infected animals shed BVDV in body secretions and excretions for a short period of time, commonly a few days (Niskanen et al., 2000). However, the duration of shedding and the amount of virus shed by acutely infected animals will depend on the virulence, the ability of the viral strain to replicate and the presence of cross-protecting neutralizing antibodies developed from previous exposure including vaccination (Bolin et al., 1991; Bolin and Ridpath, 1992). It has been reported that acute infections with highly virulent BVDV strains might result in severe clinical disease and shed the virus for over a week, compared with infections with low virulence strains (Kelling et al., 2002b).

Acute BVDV infection of young bulls approximately at 10 months of age, when the blood-testicle barrier is being developed, might display prolonged shedding of virus in semen for at least 11 months thereafter (Thurmond, 2005). In these cases the virus cannot be detected in serum, buffy coat, or other organs except the testicles; with high serum neutralizing antibody titers maintained to the homologous virus (Voges et al., 1998). Therefore, it is recommended to routinely test bull semen for BVDV as part of a screening program.

As mentioned previously, passive or acquired immunity can affect BVDV transmission by decreasing the amount and duration of BVDV shedding. A shortened duration of shedding will enhance herd immunity by reducing the risk of susceptible animals to be infected if they are exposed to acutely infected animals (Thurmond, 2005).

4. Viral Pathogenesis

4.1. BVDV-induced immune organ dysfunction

BVDV has the ability to infect cells in the bone marrow including myeloid cells and megakaryocytes, affecting their functions (Spagnuolo et al., 1997). Infection with BVDV significantly decreases thymocyte function (Marshall et al., 1994). Normally these cells are clonally selected to mature as a result of negative selection in the thymus with the remaining lymphocytes undergoing deletion by apoptosis. Similar to other lymphoid tissues, there is remarkable lymphocyte depletion in the thymus after BVDV infection; however, viral antigen is not detected except in vascular walls (Liebler-Tenorio et al., 2002). Previous studies have shown a remarkable alteration of T-cells in Peyer's patches after BVDV infection, characterized by depletion of lymphocytes. Similarly, cattle suffering from mucosal disease show extensive loss of lymphocytes in gut associated lymphoid tissue. This lymphoid depletion is characterized by

decreased numbers of B cells and $CD4^+$ T cells in the lymphoid follicles, as well as decreased $CD4^+$ T cells in the interfollicular areas (Liebler et al., 1995). Both cp and ncp BVDV are able to infect spleen cells. Early in infection, viral antigen is present, but lesions are not detected. However, lesions develop later in infection by approximately 2 weeks, and the virus may or may not be present depending on the virulence of the infecting BVDV strain (Liebler-Tenorio et al., 2003b).

4.2. Virulence

Most acute BVDV infections (70-90%) are subclinical; however, some BVDV strains may cause severe clinical presentations, such as respiratory disease, which cause substantial economical losses to the cattle industry worldwide, especially in feedlots systems (Fulton et al., 2005b). Even though the majority of the transient infections with ncp BVDV are clinically inapparent and self-limiting, there is evidence that they result in immunosuppression (Charleston et al., 2002). Respiratory disease during acute BVDV infections has been associated with secondary infections as a consequence of increased host susceptibility due to immunosuppression (Kapil et al., 2005) or exacerbated pathogenicity of co-infecting organisms (Evermann and Barrington, 2005). Co-infection and synergistic effects between BVDV and *Mannheimia haemolytica*, *Pasteurella*, bovine herpes virus type 1 (BHV-1), bovine respiratory sintitial virus (BRSV), Parainfluenza-3 (PI_3) virus, *Mycoplasma* (Potgieter et al., 1984a; Potgieter et al., 1984b) have been well documented. Additionally, concurrent infections with BVDV and *Salmonella spp*, *Escherichia coli*, bovine popular stomatitis virus, rotavirus or coronavirus have been also reported (Grooms, 1999). Similarly, it has been reported that

attenuated virus in MLV vaccines also induces immunosuppression, which is a serious drawback to the vaccine, potentially decreasing its efficacy in protecting the animals (Potgieter, 1995).

Experimental and field evidence have demonstrated that *in vitro* BVDV biotype cytopathology does not correlate with the BVDV virulence *in vivo* (Ridpath et al., 2006); that is, the most clinically severe form of acute BVDV infection has been associated with ncp BVDV-2 strains (Carman et al., 1998). However, not all BVDV-2 are highly virulent (Ridpath et al., 2000). Thus, the wide range of clinical presentations following acute BVDV infections depends on the viral strain and immune status of the animal (Ridpath et al., 2006). Noncytopathic BVDV has had a wider distribution in the host compared to homologous cp viruses (Spagnuolo-Weaver et al., 1997).

Clinically severe disease in susceptible animals infected with BVDV-2 has been associated with a significant decrease of circulating lymphocytes and platelets, accompanied by an increased body temperature (Liebler-Tenorio et al., 2003b). In contrast, experimental inoculation of calves with low-virulence BVDV-1 and BVDV-2 strains did not result in severe clinical disease (Bolin and Ridpath, 1992).

After infection, low virulence BVDV strains replicate in the tonsils and nasal mucosa (Bruschke et al., 1998). Liebler-Tenorio et al. (2003a) reported that viral antigen can be found initially in tonsils, lymph nodes, and Peyer's patches, and subsequently in spleen and thymus (Liebler-Tenorio et al., 2003a). Viral antigen is predominantly detected within lymphoid follicles and thymic cortex associated with lymphocytes and stromal cells (Liebler-Tenorio, 2005). In that study the presence of viral antigen was not associated with tissue lesions (Liebler-Tenorio et al., 2003a). At day 6 post-infection, BVDV antigen was widely distributed and in high amount,

followed by a rapid clearance accompanied by lymphoid depletion of the lymphoid follicles and thymic cortex (Liebler-Tenorio, 2005).

Infection with highly virulent BVDV results in severe clinical signs and significant leukopenia, with a high morbidity and mortality (Bolin and Ridpath, 1992; Liebler-Tenorio et al., 2002). Some infected animals develop severe haemorrhage as a result of significant thrombocytopenia (hemorrhagic syndrome), although this does not occur frequently (Bolin and Ridpath, 1992; Liebler-Tenorio et al., 2002). The dissemination and distribution of viral antigen is initially similar to that of low virulence BVDV strains. During the initial stage of the infection viral antigen is only found within the tonsils and lymphoid tissues, but the amount of virus rapidly increases to levels higher than those detected after infection with low virulence BVDV. In contrast to low virulence strains, in which the viral antigens are restricted to lymphoid follicles and the virus is cleared readily, viral antigens of highly virulent strains are widely distributed in lymphoid tissues including the T cell zones, and multiple other organs (Stoffregen et al., 2000). Thus, viral antigen can be widely detected in lymphoid tissue and mucosa of the gastrointestinal and respiratory tract as well as the interstitium, vascular walls, and parenchymal cells suggesting haematogenous dissemination. However, the presence of histological lesions is restricted. Lesions associated with loss of lymphocytes are observed in lymphoid tissues. Infection with high virulence BVDV strains is also associated with severe necrosis of lymphoid tissues and the mucosa of the respiratory and digestive tract evidenced by erosion and ulceration (Stoffregen et al., 2000).

An inconsistency between the detection of viral antigen and the presence of lesions is evident in the initial stages of the disease (Liebler-Tenorio et al., 2002). During the initial stages of infection, large numbers of cells positive for viral antigen can be detected in lymphoid tissues

with absence of morphological lesions, which subsequently appear during later stages of infection.

Therefore, infections with low and high virulence BVDV have similarities during initial stages of infection and spread. However, there are remarkable differences in the amount of virus in tissues and spread during the advanced stages of infection (Liebler-Tenorio et al., 2003b), showing a widespread distribution after infection with high virulence BVDV compared to early clearance of low virulence strain.

Kelling *et al.* (2002) and Liebler-Tenorio *et al*. (2003) reported that the virulence of the BVDV strain is correlated with the capacity of the virus to cause a decrease in lymphocyte counts (Kelling et al., 2002a; Liebler-Tenorio et al., 2003b). These authors evaluated different isolates of BVDV-2 and concluded that even though lymphoid depletion was noted in calves infected with both low and high virulence BVDV strains, the highly virulent isolates induced a significantly more severe and longer lymphopenia (> 80% reduction in leukocyte numbers) and lymphoid depletion compared to the less virulent isolates (<50% reduction in leukocyte numbers) (Liebler-Tenorio et al., 2003b). Additionally, the duration of the leukopenia, degree of viremia and severity of the clinical signs has been also associated with the virulence of the BVDV strain (Walz et al., 2001). The reduction in the total leukocyte count during BVDV infections might occur as a consequence of cell trafficking from blood into tissue, a decrease in leukogenesis or an outright death of leukocytes (Ridpath et al., 2006).

In contrast to acutely infected animals, PI animals show normal total leukocyte counts (Bolin et al., 1985), but the proportions of lymphocyte subpopulations is altered (decreased proportion of B-cells and increased number of null cells), and leukocyte function might be impaired (Brown et al., 1991).

A recent *in vitro* study to elucidate the effects of different BVDV strains (of distinct biotype and virulence) on the leukocyte counts indicated that animals infected with a highly virulent BVDV (strain 1373) showed a decrease in total leukocytes with a high percentage of cell death (Ridpath et al., 2006). The authors reported that infection with both ncp (strain 1373) and cp (strain 296) BVDV-2 caused the death of bovine lymphoid cells (BL-3 cell line). However, the interval between infection and cell death and the mechanisms involved in cell death were different (Ridpath et al., 2006). In this study, the infection with both high and low virulence BVDV-2 strains caused a decrease of leukocytes. In addition, there was an increase in the percentage of apoptotic and necrotic circulating leukocytes in all infected animals, with a significantly higher level observed in animals infected with BVDV-2 (strain 1373) on day 4, 6 and 9 post inoculations (Ridpath et al., 2006). These results suggest a correlation between infection with highly virulent BVDV and death of circulating leukocytes (Ridpath et al., 2006).

In addition, by day 5 post-infection there was a lower growth rate in cells infected with a highly virulent type 2 BVDV (strain 1373) than in either control cells or cells infected with less virulent type 2 BVDV (strain 28508-5). Moreover, by day 7, most of the cells infected with BVDV (strain 1373) were dead (Ridpath et al., 2006). However, the analysis of the caspase-3 activity indicated that there was no evidence of apoptosis in cells infected with ncp BVDV strains (of high and low virulence). In contrast, there was extensive apoptosis in cells infected with the cp BVDV (strain 296), which was in agreement with previous experiments showing programmed cell death of bovine cells infected with cytopathic strains of BVDV (Hoff and Donis, 1997). Even though the mechanisms of cell death caused by highly virulent ncp BVDV-2 were not explained in that study, it was clear that they are different from those involved in apoptosis of cells infected with cytopathic BVDV (Ridpath et al., 2006).

Brock et al. (2007) reported that experimental challenge with a highly virulent BVDV strain 1373 caused severe clinical disease in 100% of nonvaccinated calves. The authors concluded that this strain induced more severe clinical disease with a higher level of mortality than seen with other BVDV infections (Brock et al., 2007). In contrast, a subsequent study with a similar design using a less virulent BVDV strain, revealed no severe clinical signs in any of the animals (Palomares et al., 2012). In both studies, a significant leukopenia was evidenced in the nonvaccinated animals, characteristic of acute BVDV infection. However, the nonvaccinated animals infected with the highly virulent strain had a lower value of total leukocyte counts (approximately 2,400 WBC/μL on day 6 post-infection) in comparison to animals challenged with the less virulent strain NY-1 (approximately 4,900 WBC/μL on day 6 post-infection). The evidence of these related studies suggest a possible association between the BVDV virulence and the mechanisms by which BVDV cause immunosuppression in susceptible cattle. Virulence factors associated with BVDV have not been clearly identified on an antigenic or genetic level (Liebler-Tenorio, 2005).

Another consequence regularly observed after infection with highly virulent BVDV strains is a significant thrombocytopenia. However, the reduction in platelets does not always result in marked hemorrhage (Liebler-Tenorio et al., 2002) with bleeding occurring when platelet numbers have reached a very low level (Liebler-Tenorio, 2005). Several factors have been hypothesized to contribute to the marked thrombocytopenia, including decreased production, increased consumption, or functional defects of thrombocytes (Walz et al., 2001). The occurrence of thrombocytopenia has been associated with the presence of BVDV antigen in the bone marrow and infection of the megakaryocytes (Liebler-Tenorio et al., 2002).

4.3. Immunity and immunosuppression

Oro-nasal infection is the natural route of BVDV transmission, by contact with suspended droplets or mucus. BVDV replicates in the nasal mucosa and tonsils and with viral spreading throughout the body via leukocytes (Bruschke et al., 1998). Acute BVDV infection results in lymphoid depletion, leukopenia and consequently immunosuppression, predisposing the animals to other respiratory pathogens (Kapil et al., 2005). Both lymphocytes and macrophages are targets for BVDV infection (Bruschke et al., 1998). Decreased B-lymphocytes, $CD4^+$ and $CD8^+$ T-lymphocytes and polymorphonuclear phagocytes have been reported after BVDV infection (Bolin et al., 1985). Acute infections with BVDV result in immunosuppression not only by decreasing the number of circulating leukocytes through necrosis and apoptosis, but also by impairing leukocyte function (Kapil et al., 2005).

There appears to be no difference between cp and ncp BVDV with respect to the production of leukopenia (Kapil et al., 2005). Several studies have reported significant decreases in the total white blood cells after cp BVDV infection (approximately from 7,850 to 5,050 cells/ul) (Bolin et al., 1985). Similarly, experimental infection with ncp BVDV caused a reduction in the total leukocyte number observed on day 3, 5, and 7 after infection (Palomares et al., 2012; Walz et al., 2001). The typical time frame for a decrease in total leukocyte number is between 3 and 12 days after infection (Bolin and Ridpath, 1992; Kelling et al., 2002a).

4.3.1. Innate immunity:

BVDV can specifically infect cells of the innate immune response (neutrophils, monocytes, macrophages, and dendritic cells) and affect their function (Potgieter, 1995). Several mechanisms have been hypothesized to be involved in immunosuppression caused by BVDV

during the innate immune response. Inhibition of the type-I IFN production and the associated molecular pathways (Baigent et al., 2004; Baigent SJ, 2004; Charleston B, 2001; Charleston et al., 2001a; Schweizer et al., 2006); decreased production of tumor necrosis factor alpha (TNF-α) (Potgieter, 1995), and interleukin 1 (IL-1) inhibitors (Jensen and Schultz, 1991); apoptosis of monocytes and lymphocytes in lymphoid tissues, with resultant leukopenia (Glew et al., 2003). Other additional mechanisms that contribute to immunosuppression include failure in chemotaxis, migration, phagocytosis, microbicidal action, and antibody-dependent cell mediated cytotoxicity of neutrophils (Jensen and Schultz, 1991; Potgieter, 1995); reduced capacity of monocytes to present antigens (Glew et al., 2003), down regulation of IL-2 transcription, impaired lymphocyte memory response (Lamontagne et al., 1989), increased production of TGF-β (Charleston et al., 2002). Additionally, BVDV infection results in a reduction of the nonspecific innate immune response of the respiratory tract (Potgieter, 1997). Several functional defects have been observed in alveolar macrophages, including impaired expression of FC and complement receptors, reduced microbicidal activity and chemotactic factors (Liu et al., 1999; Welsh et al., 1995). Moreover, infection of alveolar macrophages with BVDV results in decreased superoxide anion and TNF-α production, enhanced nitric oxide (NO) synthesis in response to LPS (Potgieter, 1995), and stimulation of prostaglandin E2 synthesis (Van Reeth and Adair, 1997).

Type I Interferon is one of the most important innate defense antiviral cytokines. Interferon alpha and beta (IFN-α/β) are structurally different cytokines that bind the same receptors and induce similar biological responses. IFN-α is produced by mononuclear phagocytes, whereas IFN-β is produced by many cell types, particularly fibroblasts. Type-I IFN secretion is stimulated by ds RNA produced during viral replication (Abbas and Lithman, 2005).

The molecular mechanisms by which type-I IFN inhibits viral replication include the synthesis of proteins (Mx, PKR, OAS-1, etc) that interfere with transcription of RNA, which protects the neighboring cells that have not yet been infected (antiviral state), as well as inducing apoptosis of virus-infected cells (Lenschow et al., 2007). Apoptosis is the process of programmed cell death, which effectively eliminates BVDV-infected cells. It has been demonstrated that infection with BVDV is responsible for increased apoptosis of leukocytes contributing to observed leukopenia and lymphoid depletion, especially in infections with highly virulent strains (Liebler-Tenorio et al., 2003b). Additionally, type-I IFN increases the expression of class I MHC molecules on infected cells, stimulates the development of Th1 cells, and increases the cytolytic activity of NK cells (in humans) (Abbas and Lithman, 2005).

It has been reported that cp BVDV induces a strong IFN α/β response in host cells (Adler et al., 1997). On the other hand, *in vivo* and *in vitro* studies have shown that infections by ncp BVDV strains inhibit the induction of a type-I IFN response (Charleston et al., 2001a; Perler et al., 2000; Perler L, 2000; Peterhans et al., 2003). However, recent *in vivo* studies concluded that acute ncp BVDV infection caused an increased type-I IFN response in challenged calves (Brackenbury et al., 2005; Charleston et al., 2002; Müller-Doblies et al., 2004). Smirnova et al. (2008) also found a significant transient up-regulation of the interferon stimulated gene 15 KD-protein (ISG15) mRNA in leukocytes of heifers during acute ncp BVDV infection, compared with control heifers (Smirnova et al., 2008). Similarly, Yamane et al. (2008) reported an upregulation of the type-I IFN and apoptosis-related genes (Mx1, OAS-1, PKR, and TNF-α) in the spleens of PI cattle regardless of their age. In addition, the induction of apoptosis was also upregulated in the spleens of PI cattle compared to those of non-PI cattle (Yamane et al., 2008).

The results of these studies suggest that immunosuppression caused by ncp BVDV is not associated with inhibition of an interferon response (Charleston et al., 2002).

Weiner et al. (2012) using bovine peripheral blood mononuclear cells (PBMC) from ncp BVDV-naïve cattle, demonstrated that BVDV infection activates chemokine receptor 4 (CXCR4), CXCL12, IFN-I, ISGs and selected immune cell marker (CD4, CD8, CD14) mRNAs (Weiner et al., 2012). They also observed that acute response to viral infection was reflected in PBMC cultured with serum from heifers carrying fetuses persistently infected (PI) with ncp BVDV. These researchers concluded that *in vitro* treatment of PBMC with ncp BVDV or uterine vein serum from acutely infected pregnant heifers activates chemokine, ISG and immune cell responses.

It is known that during pathogen recognition, activated macrophages release different primary inflammatory mediators, the most important being IL-1, IL-6, and TNF-α (Müller-Doblies et al., 2004). These cytokines attract additional leukocytes to the site of inflammation, where they release further pro-inflammatory cytokines. An *in vitro* study demonstrated that cp and ncp BVDV strains significantly down-regulated the gene expression of pro-inflammatory cytokines (TNF-α, IL-1β and IL-6) 24 hours post infection (Lee et al., 2008). Chase et al. (2004) reported that down-regulation of TNF-α production could be caused by Th2 type cytokine secretion (Chase et al., 2004). However, Lee et al. (2008) confirmed that expression of the Th2 type cytokine genes for IL-10 and IL-15 was also significantly down-regulated in BVDV-infected monocytes, ruling out this hypothesis (Lee et al., 2008).

It has been demonstrated that infections with both cp and ncp BVDV strains in general show similar effects on the Toll-like receptors (TLR), pro-inflammatory, type-I IFN, Th1/Th2 cytokines, and co-stimulatory molecules gene expression in monocytes (Lee et al., 2008).

However, the authors stated that some significant differences in the gene expression could be due to biological and genetic variations between cp and ncp BVDV strains (Lee et al., 2008). These researchers also hypothesized that both cp and ncp BVDV might evade the innate immune response by regulating the expression of the aforementioned genes (Lee et al., 2008).

4.3.2. Adaptive immunity

After BVDV infection, an antibody response can be detected within 2-3 weeks, and may plateau in approximately 10-12 weeks post-infection (Howard et al., 1992). There are three major proteins that stimulate the humoral immune response against BVDV; E^{rns} (gp48), E1 (gp25), and E2 (gp53) (the later being the immunodominant) (Bolin and Ridpath, 1990). The BVDV non-structural N-terminal protease (Npro) acts as an interferon antagonist and subverts the host innate immunity; however, little is known about its immunogenicity (Mishra et al., 2010). In this context, Mishra et al. (2010), expressed a recombinant BVDV Npro–His fusion protein (28 kDa) in *E. coli* and determined the humoral immune response generated against it in rabbits. When rabbits were immunized with the Npro protein, a humoral immune response was evident by 4 weeks and persisted until 10 weeks post immunization. Despite Npro-specific antibodies being undetectable in 80 serum samples from BVDV-infected sheep and goats, BVDV hyperimmune sera along with some of the field cattle, sheep and goat sera with high BVDV neutralizing antibody titers were found positive for Npro antibodies. These results provided evidence that despite the low immunogenicity of the BVDV Npro protein, a humoral immune response is induced in cattle, sheep and goats with repeated BVDV exposure only (Mishra et al., 2010).

Antibodies against BVDV can be also obtained passively from colostrum. Calves receive immunoglobulins by absorption through the gastrointestinal intestinal tract. The absorption of antibodies occurs during the first 24-48 hours of life, when the gastrointestinal epithelium is permissive to the transfer of these molecules across the epithelium. The high concentration of colostral antibodies in the calf may limit the induction of active B-cell immune response to BVDV vaccination (Kapil et al., 2005). This suggests that vaccination should be started when maternal antibodies are decaying (Ellis et al., 2001). Colostral antibodies of half of the calves decayed to <1:16 by about 110 days of age for BVDV-1 and 80 days of age for BVDV-2 (Muñoz-Zanzi et al., 2002). It has been demonstrated that SN antibody titers between 1:16 and 1:32 are protective against clinical disease after BVDV infection (Bolin and Ridpath, 1992).

Cows (producing colostrum) should be vaccinated against BVDV-1 and BVDV-2 to provide protective levels of colostrum-derived immunoglobulins in calves. As mentioned above, since BVDV maternal neutralizing antibodies can neutralize the BVDV in a MLV vaccine, decreasing vaccine efficacy (Ellis et al., 2001), it is necessary to know at what age the colostral antibodies decay to <1:16. This is the time when calves become more susceptible to BVDV and therefore immunization with a MLV should be done. However, due to the high variability of the age (>1 month) when the maternal antibodies decay, it is recommended to provide multiple vaccination. In dairy calves the transmission of BVDV can begin at about 3 months of age and increase until 7 months of age (Rush et al., 2001).

Passive immunity even in the presence of an optimal antibody titer is not fail safe. Some factors including dose of challenge virus, poor nutrition, exposure to harsh weather, poor ventilation, transportation stress, and increased stocking density may affect the outcome of infection. Moreover, antigenic variation among BVDV strains may result in an incomplete level

of protection. Vaccination of pregnant cows during the last month of gestation with killed vaccines will increase the amount of antibodies against BVDV in colostrum. Vaccination using MLV vaccine during the last stage of gestation also will increase the level of antibodies, although this strategy is only recommended if animals that have been vaccinated before breeding, and the dam has preexisting antibodies (Kapil et al., 2005).

All types of immune cells are infected by BVDV, resulting in significant impairment of their function (Kapil et al., 2005). As stated above, the strain virulence is an important factor determining the level of lymphopenia after acute BVDV infection (Archambault et al., 2000). $CD8^+$ T cells are affected more than $CD4^+$ T cells after acute BVDV infection (Ellis et al., 1988). Depletion of CD4+ T cells increases the period and level of BVDV shedding, suggesting that these cells play a crucial role in a cell mediated immune response early in infection (Kapil et al., 2005). Helper $CD4^+$ T cells are responses mainly lead to the NS3 and E2 proteins. There is also a specific immune response against C, Erns, Npro and NS2-3 proteins (Collen et al., 2002). Proliferation assays have been used to determine the cell immune response against BVDV. The Th1 cells produce IL-2 and IFN- γ but not IL-4 or B cell stimulatory activity. On the other hand, Th2 cells express high levels of IL-4 and low levels of IL-2 and IFN- γ (Rhodes et al., 1999). Proliferating $CD8^+$ T cells (CTL) produce IL-2 and IFN-γ implying a type 1 memory response in BVDV seropositive cattle (Howard et al., 1992; Rhodes et al., 1999).

It has been reported that ncp BVDV tend to polarize the immune response toward a Th2 immunity (Rhodes et al., 1999) and decreased IFN-γ expression(Charleston et al., 2001b). In contrast, cp BVDV has been associated with a higher cell mediated immune response toward a Th1 immunity, characterized by increased IL2 and IL-2 receptor expression (Rhodes et al., 1999).

Antigen presenting cells internalize the viral antigen and present it to T-helper cells with contribution of IFN-γ and IL-12 (Kapil et al., 2005). Production of IFN-γ is a good indicator of T helper 1 type immune response, which is crucial in resistance to viral infections (Platt et al., 2009a). A recent *in vitro* study demonstrated that the production of IFN-γ by peripheral blood mononuclear cells (stimulated with BVDV) was higher in vaccinated animals than in the control group (Platt et al., 2009a). Lee et al. (2007) demonstrated that cp and ncp BVDV strains did not affect the IFN-γ mRNA levels by 24 h post-infection. However, IL-12 transcription (which stimulates IFN-γ secretion) was significantly up-regulated 1 hour after ncp BVDV infection (Lee et al., 2008).

IL-2 plays a major role in the clonal expansion and differentiation of T lymphocytes by interacting with its specific cell surface receptor. Several studies have summarized the ability of IL-2 to induce both humoral and cellular immune responses to viral antigens, through stimulation of Th0 and Th1 cells (Chow et al., 1997). Ghram et al. (1989) showed that IL-2 activity was increased in cultures of mononuclear cells infected with BVDV, BHV-1, and PI_3, when compared with cultures of non-infected cells (Ghram et al., 1989). Moreover, Endsley et al. (2002) reported that BVDV MLV vaccine caused a significant increase in the IL2 receptor α chain following the *in vitro* exposure of lymphocytes to BVDV after vaccination compared to nonvaccinated animals (Endsley et al., 2002). An experiment to determine the effect of cp and ncp BVDV on the cellular metabolic activity and activation status of bovine peripheral blood mononuclear cells revealed that the expression of the IL-2 receptor was preserved in virus-infected cells; however, the DNA and protein synthesis was suppressed, suggesting a novel mechanism for virus-induced immunosuppression, in which the activation signals are maintained (Hou et al., 1998).

4.3.3. Consequences of immunosuppression by BVDV

Experimental studies regarding infection with low virulence BVDV isolates have shown that even in absence of clinical signs of disease after infection, there is transient but significant immunosuppression (Liebler-Tenorio et al., 2003a; Liebler-Tenorio et al., 2003b). This phenomenon has been evidenced by several reports on acute infections with BVDV that potentiate the pathogenesis of several pathogens and impair efficacy of treatment (Liebler-Tenorio, 2005). Severity of infections with rotavirus and coronavirus (Kelling et al., 2002a), BHV-1, BRSV, *Mannheimia haemolytica* (Brodersen and Kelling, 1998; Potgieter et al., 1984a) and *Salmonella spp* (Wray and Roeder, 1987) is increased with coinfection of BVDV. The increased susceptibility to other pathogens after BVDV infection has been associated with impaired memory T cell response to BVDV and other pathogens (Lamontagne et al., 1989). The bovine respiratory disease complex is an example of a multiple etiology disease process in feedlot animals and intensively housed calves. It is not completely understood whether BVDV-induced immunosuppression or primary infection of the respiratory tract plays a major role in this bovine respiratory disease complex. This disease complex represents an important cause of morbidity and mortality in feedlot systems (Kapil et al., 2005). BVDV is frequently isolated from pneumonic lungs of cattle, supporting a primary role in bovine respiratory disease (Greig et al., 1981). Moreover, there is evidence of the existence of pneumotropism for some BVDV strains (Potgieter, 1997). Other infectious agents that have been isolated from cases of cattle with pneumonia including BHV-1 (Biuk-Rudan et al., 1999), PI-3 (Fulton et al., 2000), BRSV (Brodersen and Kelling, 1999), *Pasteurella multocida* (Fulton et al., 2002), *Mannheimia hemolytica* (Fulton et al., 2002), *Mycoplasma bovis* (Shahriar et al., 2002) and *Histophilus sumnus*. Synergism is the ability of two or more infectious agents to potentiate the pathogenicity

of each other and become more virulent. Combined infection of bovine alveolar macrophages with BVDV and BRSV causes a synergistic depression of the cell function (Liu et al., 1999), which might result in a more severe respiratory disease and higher level of viral shedding through nasal secretions (Brodersen and Kelling, 1998). Experimental co-infection with BVDV and BHV-1 was associated with severe clinical disease, and spread of BHV-1 into nonrespiratory tissues compared to calves inoculated with BHV-1 alone (Greig et al., 1981; Potgieter et al., 1984b). This type of synergism has also been observed between BVDV and enteric infectious agents, such as rotavirus, inducing a more severe clinical disease (Kelling et al., 2002a).

Raaperi et al. (2012) studied the associations between BHV-1 status of a herd and BRD occurrence as well as reproductive performance in pregnant heifers and cows. In that study, serum samples collected from cows and young stock from 103 dairy cattle herds were analyzed for antibodies against BHV-1, bovine respiratory syncytial virus (BRSV), bovine viral diarrhoea virus (BVDV), and *Mycoplasma bovis*. A low to moderate prevalence (1-49%) of BRSV antibodies among young stock was associated with a high occurrence of respiratory disease (OR = 6.2) in cows and in-calf heifers. Larger herd size, loose-housing of cows, housing young stock separately from cows until pregnancy, and purchasing new animals were factors possibly related to a high occurrence of respiratory disease signs in pregnant heifers and cows. BHV-1 was not associated with acute respiratory disease in adult dairy cattle, however it was significantly related to reproductive performance. The authors also stated that BRSV possesses the main role in respiratory disease complex in adult dairy cattle (Raaperi et al., 2012).

Burciaga-Robles et al. (2010) determined the effects of an intratracheal *Mannheimia haemolytica* challenge after 72-h exposure to BVDV-1b PI calves on serum antibody production, white blood cell count, cytokine concentrations, and blood gases in feedlot steers. Coinfection of

Mannheimia haemolytica and BVDV resulted in increased rectal temperatures during the initial 24 h after the *M. haemolytica* challenge. WBC count at 36 h post *M. haemolytica* challenge increased compared with the control group. Interleukin 1β, IL-6, and tumor necrosis factor α (TNFα) concentrations were greater in steers exposed to BVDV than steers not exposed to BVDV, and interferon γ, IL-1β, and TNFα levels were greater in steers challenged with *M. haemolytica* than in steers not challenged with *M. haemolytica*. This co-infecting (BVDV- *M. haemolytica*) challenge model was successful at inducing bovine respiratory disease (BRD) (Burciaga-Robles et al., 2010).

Immunization of cattle with modified-live BVDV vaccines has been associated with immunosuppression. Other researchers reported that vaccination with a BVDV MLV impaired the immune response against *Mycobacterium paratuberculosis* (Thoen and Waite, 1990).

4.4. Pathogenesis and clinical outcomes

As mentioned, there are five forms of clinical BVDV including acute BVDV infection, severe acute BVDV infection, hemorrhagic BVDV infection, acute BVDV infection-bovine respiratory disease, and acute BVDV infection-immunosuppression (Grooms et al., 2002). Most of the acute BVDV infections are subclinical (70-90%) and transient. However, regardless of the ability of the BVDV strain to cause clinical disease in the infected animal, there is a period of virus shedding during which BVDV can be transmitted and disseminated within the herd (Evermann and Barrington, 2005). Contact between the infected animal with susceptible pregnant cattle during this period might result in early embryonic death, abortion, persistent infection or congenital defects (Muñoz-Zanzi et al., 2003).

Acute infections with BVDV can result in a broad range of clinical manifestations, ranging from subclinical infections to fatal disease (Baker, 1995). Several host factors influence the clinical outcome after BVDV infection, including immunocompetence (immunocompetent or immunotolerant), immune status (depending on passive or acquired immunity), reproductive status (pregnant or nonpregnant), stage of gestation (< 30 days, 30-125 days, 100-175 days, >150 days), and level of environmental stress (Baker, 1995). Furthermore, viral factors also affect the clinical outcome, such as antigenic and genomic diversity (Archambault et al., 2000).

4.4.1. Subclinical infection

Experimental and field studies have shown that most acute infections are subclinical and self-limiting (Ames, 1986). Infected animals might show mild fever, leukopenia, along with the development of neutralizing antibodies. In dairy cows, a decrease in milk production has been associated with subclinical infections (Moerman et al., 1994).

4.4.2. Clinical BVDV infection

Acute clinical BVDV infections occur in immunocompetent seronegative susceptible animals. Additionally, the disease might occur in seropositive cattle exposed to a BVDV strain antigenically heterologous to the one that caused seroconversion, if neutralizing BVDV antibody titers have decreased enough to increase susceptibility to BVDV, or if there is a high infectious pressure in the herd (Liebler-Tenorio, 2005).

The most important determinant of the clinical outcome during BVDV acute infection is the virulence of the infecting strain (Liebler-Tenorio, 2005). Acute BVDV infection can result in clinical signs such as fever, anorexia, lethargy, tachypnea, leukopenia, ocular and nasal

discharge, increased abnormal lung sounds and coughing, oral erosions and ulcers, oral papilla blunting and hemorrhage, enteritis and diarrhea, and decrease in milk production in lactating cows (Evermann and Barrington, 2005). Respiratory and gastrointestinal signs are associated with damage to the epithelial surfaces of GI and respiratory systems (Blowey and Weaver, 2003) and immunosuppression with subsequent infection by opportunistic bacterial flora.

4.4.3. BVDV in susceptible calves

Experimental acute BVDV infection in colostrum-deprived calves resulted in diphasic pyrexia, leukopenia, anorexia, and diarrhea (Mills and Luginbuhl, 1968). BVDV was isolated from different tissues including spleen, thymus, lymph nodes and respiratory tract during a long time period (25-56 days). Acute BVDV infection in 6-month-old seronegative calves resulted in minimal clinical signs, which included mild bilateral serous nasal discharge at 12 days post-infection (Wilhelmsen et al., 1990). However, a significant leukopenia was observed for up to 12 days. The authors observed a more significant involvement of the lymphoid tissue in the gastrointestinal tract compared with the findings observed in the colostrum-deprived calves. Such differences might be due to differences in age and maturation of the immune system, strain virulence or both (Pellerin et al., 1994).

4.4.4. Severe acute BVDV infection

A severe peracute form of BVDV infection in cattle has been reported in outbreaks in Quebec and Ontario, Canada with high morbidity and mortality. Clinical signs included fever, pneumonia, abortions, and sudden death (Carman et al., 1998; Pellerin et al., 1994). These disease outbreaks were caused by a BVDV strain characterized and identified by nucleotide

sequencing as BVDV-2. However, it is important to emphasize that not all BVDV-2 strains cause serve clinical disease. It is possible for BVDV-1 strains to also cause severe disease (Evermann and Barrington, 2005). In 2008, a northwest Texas feedlot experienced an outbreak of BVDV causing high morbidity and mortality in two lots of calves. Severe mucosal surface lesions were observed grossly in the oral cavity, larynx, and esophagus. Mucosal lesions varied from small (1–3 mm) infrequent mucosal ulcerations to large (5 mm to 1 cm) and coalescing ulcerations (Hessman et al., 2012). A calf persistently infected with BVDV arrived with one lot and the isolated virus was genotyped as BVDV-1b. Identical BVDV-1b strains were isolated from 2 other mortalities. A BVDV-2 genotype was also isolated in this outbreak. This genotype was identical to all BVDV-2 strains isolated in both lots. Seropositivity of calves ranged from zero percent for calicivirus to 100% positive to *Pseudocowpox virus*. At the end of the feeding period, the morbidity and mortality for the two lots involved was 76.2 and 30.8%, respectively. Differential diagnoses included vesicular stomatitis viruses, *Bovine popular stomatitis virus*, and *Foot-and-mouth disease virus* (Hessman et al., 2012).

4.4.5. Hemorrhagic syndrome

Hemorrhagic syndrome is a form of severe acute BVDV infection that appears to be associated with BVDV-2. It is characterized by thrombocytopenia, petechial and ecchymotic hemorrhages of mucosal surfaces, epistaxis, bloody diarrhea, bleeding from injection sites or trauma, fever, leukopenia, and death (Corapi et al., 1990). During an outbreak, due to individual animal variation, it is common for only a few animals develop the fatal hemorrhagic crisis, while most animals do not show clinical manifestation of hemorrhage (Evermann and Barrington, 2005).

4.4.6. Mucosal Disease

Mucosal disease is a sporadic condition, occurring in less than 5 % of cattle herds, with a case fatality rate of near 100%. Affected animals usually die within two weeks after onset of clinical symptoms. Mucosal disease (MD) occurs when a persistently infected animal is super-infected with a cp BVDV strain that is antigenically related (homologous) to the persistent ncp strain (Liebler-Tenorio, 2005). The development of MD may occur within 2-3 weeks or up to months after inoculation of the cp BVDV(Brownlie and Clarke, 1993). The course of infection is influenced by the degree of homology between the persisting ncp BVDV and the cp BVDV (Brownlie and Clarke, 1993).

Experimental studies to induce mucosal disease in BVDV PI cattle have shown an incubation period of 7-14 days after MLV vaccination or inoculation of a cp BVDV strain homologous to the PI strain (Grooms et al., 2002). This condition has been reported as early onset mucosal disease (Liebler-Tenorio, 2005). The reisolated cp BVDV strain was identical to the strain the animal was exposed to(Liebler-Tenorio, 2005). Additionally, early onset MD has also been reported to occur as a mutation of the ncp BVDV until cp BVDV originates (Liebler-Tenorio, 2005). The incubation period until MD appears is unknown. The ncp/cp BVDV virus pair from the animal with MD has shown identical E2 (Liebler-Tenorio, 2005). However, most cases of mucosal disease are assumed to occur months or years after experimental BVDV exposure, as a result of a *de novo* mutation of the ncp BVDV strain in the PI cattle, to give rise to a homologous cp BVDV strain. This condition has been reported as late onset mucosal disease (Liebler-Tenorio, 2005). There might be an initial transient acute BVDV infection in a persistently infected animal with a cp BVDV strain, which is followed by a long phase without clinical signs, but characterized by the production of neutralizing antiodies to the cp BVDV.

Finally, there is a sudden onset of signs of MD (Loehr et al., 1998). Moennig et al. (1993) demonstrated that the reisolated cp BVDV is a recombination between the persisting ncp BVDV and the original cp BVDV (Moennig et al., 1993).

Clinical signs commonly observed in animals with mucosal disease include fever, anorexia, tachycardia, polypnea, decreased milk production, and profuse watery diarrhea with mucosal shreds, fibrinous casts, blood and foul odor. Additionally, erosions and ulcers might be observed on the tongue, gingiva, palate, teats, vulva, prepuce, and in the interdigital space (Evermann and Barrington, 2005). Animals also have leukopenia and thrombocytopenia. Common findings during necropsy include ulceration and erosion of the mucosal surface of the gastrointestinal and respiratory tract, as well as necrosis of the lymphoid organs (Evermann and Barrington, 2005). These pathological changes are associated with the presence of the cp BVDV in the sites of tissue destruction (Liebler et al., 1991). In contrast, ncp BVDV is widely distributed in several organs and is not associated with lesions in an animal with MD (Bielefeldt, 1988).

The spread of cp BVDV in mucosal disease is similar to that observed in acute BVDV infection. Following intranasal inoculation, the cp BVDV replicates in tonsilar epithelium. The virus then spreads to regional lymph nodes and is later detected in Peyer's patches, mucosal associated lymphoid follicles in the large intestines, and lymphoid follicles of the lung and nasal mucosa. This pattern of viral dissemination implies a hematogenous spread to the gastrointestinal and respiratory tract mucosa (Liebler-Tenorio, 2005). It has been reported to a lesser degree to spread to the peripheral lymph nodes, thymus and spleen (Liebler-Tenorio, 2005).

4.4.7. Reproductive consequences

A major economic impact of BVDV infections is intrauterine infections that result in reproductive dysfunction (Dubovi, 1994). There are several host and viral factors that affect the outcome of transplacental/intrauterine infection, including time of gestation, fetal immunocompetence, stage of organogenesis, BVDV biotype, strain virulence, and host immune status, (Liebler-Tenorio, 2005). There exist multiple reproductive consequences of BVDV infection, as subsequently discussed:

BVDV infection during the preovulatory period

Acute infection during the preovulatory period may result in viremia at ovulation causing a decrease in conception rate (Liebler-Tenorio, 2005). Both cp and ncp BVDV have been reported to infect the ovaries after experimental infection of cattle (Grooms et al., 1998b en Liebler-Tenorio). In that study, viral antigen was detected in granulosa and stromal cells between 8 and 30 days postinfection resulting in inflammation and necrosis of follicles (Grooms et al., 1998b, c). Acute BVDV infection can affect follicular dynamics, reducing the growth rate and diameter of the dominant and subordinate follicles (Grooms et al., 1998b).

It has been shown that BVDV causes a decrease in the secretion of GnRH and LH by the hypothalamus and pituitary gland (Fray et al., 2002; Fray et al., 2000a). This condition will negatively affect the estrus and ovulation, as well as the production of estradiol and progesterone by follicles and the *corpus luteum*, respectively (Fray et al., 2000a; Grooms et al., 1998a). Moreover, BVDV-induced leukopenia might result in deficient ovarian leukocyte populations, which are crucial for follicular dynamics, ovulation, and luteolysis (Evermann and Barrington,

2005). In addition, the virus has the ability to replicate in the ovaries of infected cows causing oophoritis, which affects oocyte quality and the CL steroidogenic ability.

Kale et al. (2011) evaluated 400 apparently healthy Holstein heifers for the presence of BVDV antigen (Ag) and antibodies (Ab) against BVDV in blood serum and leukocyte samples and the effect on fertility. There were no BVDV (Ag+/Ab+) pregnant heifers, the differences in average conception rate between BVDV (Ag+/Ab+) pregnant heifers and BVDV (Ag–/Ab–) heifers was found to be statistically different, demonstrating that fertility is decreased in heifers that have been infected with BVDV (Ag–/Ab+, Ag+/Ab– and Ag+/Ab+) (Kale et al., 2011).

BVDV infection at < 40 days of gestation

BVDV infections with cp or ncp strains before implantation result in decreased fertility and conception rate due to failure of fertilization and early embryonic death (Bolin, 1990). Thus, BVDV infections during early stages of gestation may affect the secretion of releasing factors, gonadotropins and sex steroids at the level of the hypothalamus, pituitary and ovaries, respectively. Hormonal imbalance due to BVDV infection has been associated with low conception rate (Fray et al., 2000a).

In addition to oophoritis, acute transient infection with cp or ncp strains can also cause salpingitis, impaired oocyte fertilization and early embryonic development. Moreover, BVDV affects the uterine environment, causing endometritis and impairment of implantation. Therefore, all major organs of the reproductive system are permissive to BVDV and the distribution is similar between acutely infected and persistently infected cows (Fray et al., 2000a). Grooms et al. reported the occurrence of ovarian hypoplasia in PI cows (Grooms et al.,

1996), whereas diffuse interstitial oophoritis has been reported 61 days after acute BVDV infection (Ssentongo et al., 1980).

In vitro fertilization of oocytes in the presence of BVDV resulted in reduced fertilization rates (Kafi et al., 2002). BVDV has been demonstrated to have a direct negative effect on embryonic cells. Once fertilization has occurred there is no uptake of BVDV by embryos after *in vitro* exposure (Potter et al., 1984). During early stages of embryo development (morula and blastocyst) the embryos are protected by the zona pellucida. This membrane prevents viral entry, protecting the embryo against BVDV infection (Singh et al., 1982). However, the virus attaches to the zona and during later stages of embryonic development (>8 dpi) after hatching, the embryo becomes more susceptible to cp BVDV infection resulting in embryonic death (Vanroose et al., 1998). These results demonstrate that BVDV can infect bovine embryos early in their development.

Tsuboi et al. (2011) investigated the effect of BVDV infection on early pregnant cows (n=4) before placenta formation, at day 26 of pregnancy. Two treated cows and one control cow were euthanized on day 3 post-infection and the remaining animals were euthanized on day 6. BVDV was isolated from maternal tissues such as lymphoid or reproductive tissues of treated animals on days 3 and 6 post-infection. Additionally, one treated cow euthanized on day 6 post-infection had evidence of infectious BVDV in the allantoic membranes, allantoic fluid and embryos. In three treated cows, a significant decline in progesterone concentration was also observed post-infection, while in control cows progesterone levels remained constant. Therefore, this study demonstrated that BVDV can infect bovine embryos before placenta formation and may affect progesterone profiles in cows during early pregnancy (Tsuboi et al., 2011).

BVDV infection between 30 and 125 days of gestation

The fetus is susceptible to transplacental infection at approximately 30 days of gestation, with the efficiency of infection increasing from conception to day 30 post-conception (Kirkland et al., 1993). Experimental studies have demonstrated that BVDV crosses the placenta with near 100% efficiency (Evermann and Barrington, 2005). Thus, the efficiency of the infection will be determined by the development of the placenta with formation of placentomes, formation of trophoblasts, and development of fetal tissues (Kirkland et al., 1993).

It is important to emphasize that the absence of clinical signs in the dam during an acute BVDV infection does not imply fetal protection or reduce the risk for transplacental infection (Evermann and Barrington, 2005). After trans-placental infection, the outcome will depend on the biotype, virulence and time of gestation. BVDV can cross the placenta causing vasculitis which allows access to the fetal circulation (Grooms et al., 1998c). After experimental inoculation, BVDV has been isolated in the fetus 7 dpi. Infections at this time point with cp or ncp strains can cause embryonic death and abortion (Bolin, 1990). Fetal infection with a cp BVDV strain may cause abortion or the birth of a healthy seropositive calf if the fetus is immune-competent at the time of intrauterine infection (Brownlie et al., 1989).

Acute ncp BVDV infection between 40 and 125 days of gestation can result in fetal death, abortion, mummification, birth of PI calves, and few cases of teratogenesis (Evermann and Barrington, 2005). Abortion occurs most commonly in the early stages of gestation (<125 days of gestation), although it can occur during late gestation (Liebler-Tenorio, 2005). The fetal expulsion occurs weeks or months after infection (Liebler-Tenorio, 2005). It is accompanied by unremarkable nonspecific lesions of the placenta and placentomes (Murray, 1991). However, Baszler et al (1995) reported a case of necrotizing placentitis.

It is estimated that BVDV causes about 6-10% of diagnosed infectious abortions in cattle (Dubovi, 1994). Although these outcomes represent significant economical losses, the predominant form of infection with BVDV is the congenital infection.

During gestation, fetuses that survive infections with ncp strains between days 30 and 125 of gestation develop immunotolerance and become persistently infected, with BVDV widely spread throughout multiple organs (Frederiksen et al., 1999), disseminating the virus for the rest of their lives. Before 125 days of gestation, the fetal immune system is not completely developed and not able to recognize BVDV as a foreign antigen (Liebler-Tenorio, 2005). Therefore, during lymphocyte maturation at the fetal thymus, any lymphocyte that recognizes BVDV as a foreign antigen is clonally deleted through a process called negative selection. Thus, the fetus becomes immunotolerant of the infecting BVDV strain. Immune tolerance is maintained by nonreactive $CD4^+$ T lymphocytes and B lymphocytes; however, antigen presenting cells are normally reactive (Fray et al., 2000b). Such calves continually shed large amounts of BVDV, representing the most important source of virus and a risk to susceptible herdmates, even though their reported prevalence is less than 1% (Houe et al., 1995; Liebler-Tenorio, 2005; Loneragan et al., 2005; McClurkin et al., 1984; Wittum et al., 2001).

Persistently infected calves may be clinically normal, apparently healthy calves with different functional defects, or calves with more overt defects (Liebler-Tenorio, 2005). Most PI calves have been characterized as poor doers, being stillborn or dying within few hours or days of life (Liebler-Tenorio, 2005). The calves that survive may have growth retardation. Duffel and Harkness (1985) reported up to 50% higher death rate during the first year of life in PI calves compared with BVDV-free calves (Duffell and Harkness, 1985). This higher mortality might be associated with defects of the immune system causing immune suppression (Roth et al., 1986;

Roth et al., 1981). Following birth the PI calf is susceptible to develop mucosal disease (Evermann and Barrington, 2005).

PI cows will always produce PI calves, establishing PI family lines by continuous vertical transmission (Straver et al., 1983). These cows may have decreased reproductive performance as a consequence of critical morphological changes in the ovaries affecting the follicular maturation (Grooms et al., 1996). BVDV can be transmitted by oocytes; however, since approximately 20 % of the follicles from PI cows are BVDV positive (Fray et al., 1998), it is possible that BVDV-free calves can be produced after embryo transfer from a PI donor (Smith and Grimmer, 2000).

Persistently infected calves generally show a wide distribution of BVDV throughout the organs, no virus-associated morphological lesions and no immune response to the persisting BVDV strain. This immune tolerance is limited to the particular infecting BVDV strain responsible for the intrauterine infection. Therefore, infection with different (antigenically or genetically) BVDV strains might elicit an immune response (Fulton et al., 2003c).

BVDV infection between 125 and 175 days of gestation

Around 4-6 months of gestation the immune system is developed in the calf. However, organogenesis is not complete (especially the CNS), so that BVDV infection can cause congenital defects, approaching 100% of cases under experimental conditions (Baker, 1995) due to direct effects on fetal development as well as the effects of the immune response against the virus-infected cells which cause tissue damage. Thus, the fetus seems to be the most susceptible to teratogenic effects of BVDV when the immune competence begins to develop and organ formation is not completed (Duffell and Harkness, 1985).These effects have been suggested to

occur through inhibition of cell growth and differentiation resulting in growth retardation and reduced maturation or direct cell lysis (Castrucci et al., 1990).

Reported fetal abnormalities include: cerebellar hypoplasia, hydrocephalus internus, hydroencephaly, hypomyelogenesis, microencephaly, porencephaly with neurological signs and death (evermann), microphthalmia, retinal atrophy, retinal dysplasia, reduced pigmentation of the retina, optic neuritis, cataracts, hypotrichosis, alopecia, thymic hypoplasia, growth retardation with arrested bone development, pulmonary hypoplasia, brachygnatism, and arthrogryposis (Baker, 1995; Evermann and Barrington, 2005; Trautwein et al., 1986). Neurological signs of tremor ataxis, torticollis, or opisthotonus may be observed in newborn calves (Liess et al., 1984). The non-PI calves with BVDV-related congenital defects are commonly BVDV negative. However, in a few cases BVDV has been detected in the brain or CSF (Liess et al., 1987). Some newborns with BVDV-related malformations are seropositive at birth (Liess et al., 1984).

A recent report showed that BVDV-1b was isolated from premature Holstein calves born to first-calf heifers from a dairy herd that experienced an outbreak of premature births, late-term abortions, brachygnathism, growth retardation, malformations of the brain and cranium, and rare extracranial skeletal malformations. Experimental inoculation of 3 colostrum-deprived calves, aged 2–4 months old, with this BVDV isolate resulted in thrombocytopenia, lymphopenia, and leukopenia. Outbreaks of brachygnathism are rarely associated with BVDV, and thrombocytopenia is rarely associated with BVDV-1 strains (Blanchard et al., 2010).

BVDV infection between 175 days and end of gestation

BVDV infections during the last trimester of gestation when immunocompetence and organogenesis are generally complete, can cause abortion, birth of a weak calf or the birth of a

normal seropositive calf which was able to clear the viral infection, mounting an effective immune response (Bolin, 1990).

5. Diagnosis

Important aspects for the establishment of a diagnostic program include the availability of rapid, economical, and simple methods that are highly sensitive and specific (Goyal, 2005). The diagnosis of BVDV can be performed based on the history and clinical signs of BVDV infection (Goyal, 2005). However, as discussed above, clinical signs of BVDV infection are variable, depending on the virulence, age, immune status, reproductive status, and co-infection with other pathogens.

In order to establish an effective diagnostic plan, it is crucial for the producer, veterinarian and laboratory diagnostician to agree and understand the reasons for testing, since not all available BVDV diagnostic tests are applicable to all situations. The most common reasons for requesting BVDV testing include the detection of acute infections during clinical disease, reproductive failure, and/or abortion outbreaks, and the detection and elimination of persistently infected animals and testing for vaccine efficacy (presence of neutralizing antibodies) (Saliki and Dubovi, 2004). Other reasons for BVDV diagnostic testing include quality control of biologic products (to detect BVDV contamination, e.g. commercial fetal bovine serum), BVDV genotyping to establish the genotype of a BVDV isolate obtained in any given situation (e.g. severe clinical disease), for epidemiologic reasons, and vaccination-challenge research (Saliki and Dubovi, 2004).

5.1. Direct Antigen Detection

Methods of BVDV antigen detection from submitted diagnostic specimens are quicker and cheaper than virus isolation (Goyal, 2005; Saliki and Dubovi, 2004). However, these methods have a lower sensitivity and reliability than virus isolation, and may not serve as the final rule-in/rule-out test. If the goal is to diagnose acute BVDV infections, methods of antigen detection should generally be used as screening methods (Saliki and Dubovi, 2004).

Diagnostic methods for antigen detection include antigen capture ELISAs and immuno-histochemical (IHC) staining. A number of antigen capture ELISAs (ACEs) have been described and at least two are currently available as commercial test kits (Graham et al., 1998; Saliki et al., 2000). Antigen capture ELISA is primarily useful in screening for PI cattle, and may not be reliable for diagnosis of acute BVDV infections (Saliki and Dubovi, 2004). In order to produce consistent results, most of the ACEs require cell-containing samples (e.g. buffy coat cells or tissue extracts). The need to extract white blood cells or disrupt tissues before testing for antigen limits the applicability of these tests for a large number of samples as would be necessary in whole-herd PI animal screening programs (Saliki and Dubovi, 2004). However, a commercial kit that consistently detects BVDV antigen in serum samples from PI cattle has been reported (Plavsic and Prodafikas, 2001; Saliki et al., 2000). Antigen detection in frozen tissue sections by FA staining is frequently used by diagnostic laboratories as a screening test. Furthermore, IHC staining on fixed tissue samples has been broadly used for diagnosis of BVDV acute and persistent infections (Haines et al., 1992). Additionally, IHC staining on skin biopsies (‘‘ear notch’’ test) has gained extensive application in screening for PI cattle (Grooms and Keilen, 2002; Njaa et al., 2000; Ridpath et al., 2002).

5.2. Virus isolation

Isolation of BVDV via cell culture and identification is the ‘‘gold standard’’ diagnostic technique (Goyal, 2005). The virus effectively replicates in many cell lines from several animal species. The most commonly used cell lines for virus isolation are bovine turbinate (BT), bovine testicle (Btest), and Madin Darby Bovine Kidney (MDBK) (Saliki and Dubovi, 2004).

Another aspect that affects the ability to culture BVDV is the inoculation method. The tested sample may be inoculated by dropping the inoculum into culture media overlying a cell monolayer. This technique is less sensitive due to the ‘‘depth of column’’ effect. The distance that the virions need to travel to infect the cells is a limiting factor. Therefore, inoculations in smaller vessels (96-well and 24-well plates), with a higher fluid column on the cell monolayer, are less efficient than inoculations in 25 cm^2 flask. A practical way to inoculate the cell culture is by adding the inoculum directly on the cell monolayer, incubate the flask for 1 hour and then add more cell culture medium (Goyal, 2005).

Whole blood from which white blood cells are extracted is the best sample for BVDV isolation. However, if the sample needs to be taken during necropsy (e.g. aborted fetus or dead calf), the best samples are lymphoid organs, such as mesenteric and tracheo-bronchial lymph nodes, spleen, Peyer’s patches from the small intestine, and thymus. Specimens such as nasal swabs, serum, feces, semen, and various tissues may not be adequate for BVDV isolation during acute infections due to the possible interference by neutralizing antibodies and the transient presence of the virus during the course of the infection. In contrast, in PI animals the amount of circulating virus is so high that almost any secretion, excretions or tissue samples will be optimal for BVDV isolation (Goyal, 2005; Saliki and Dubovi, 2004).

After 3 days of incubation in 5% CO_2 at 37°C, flasks containing the cell culture monolayer inoculated with BVDV are frozen and thawed; 50 µL of the cell suspension from each flask is then transferred into 3 wells of a 96-well plate seeded with cells (Palomares et al., 2012).

BVDV is classified into two biotypes, cytopathic (CP) and noncytopathic (NCP) strains, depending on whether or not they cause visual cytopathic effects in cell cultures (Gillespie et al., 1960). In the laboratory, 70–90% of BVDV isolates are of the ncp biotype (Fulton et al., 2005b). Therefore, after completion of cell culture for virus isolation, they need to be further tested to detect the presence of ncp BVDV. The cell culture vessels (24-well or 96-well plates) may be fixed with acetone or formaldehyde and tested for BVDV antigen by use of fluorescent antibody (FA) staining using monoclonal or polyclonal primary antibodies, ELISA or immunoperoxidase monolayer assay (IPMA) (Saliki et al., 1997). Using serum samples for virus isolation results are very accurate and reliable for testing of PI animals. However, this method is not sensitive enough for diagnosis of acute BVDV infections (Saliki and Dubovi, 2004).

The main drawback of IPMA in PI testing is its inadequacy to be used on sera from animals younger than 3 months old. Maternal antibodies in young calves can interfere with growth of BVDV in cell cultures. It has been shown that very few PI cattle can be BVDV negative by virus isolation on the serum but BVDV can still be isolated from the white blood cells (Grooms et al., 2001). However, IPMA is still widely accepted as a consistent test for detecting PI cattle of 3 months or older.

5.3. Nucleic acid detection

Polymerase chain reaction is a technique that involves the binding of specific DNA oligonucleotides (primers of around 23 nucleotides) to cDNA target sequences. After several

cycles of melting, annealing and amplification it produces size-specific DNA fragments that are detectable by gel electrophoresis (conventional PCR) or by a specific fluorescent signal above the baseline (real time PCR) . Currently some laboratories use reverse-transcription PCR (RT-PCR) amplification as a routine diagnostic method for BVDV (Goyal, 2005; Ridpath et al., 2002). One of the advantages that RT-PCR offers is the ability to detect and differentiate all three BVDV genotypes (1a, 1b, and 2).

Diagnosis of BVDV can be performed using RT-PCR on all animal specimens (milk, urine, tissues, serum, whole blood, swabs, skin, formalin-fixed tissue). However, the efficiency of viral RNA extraction and isolation can vary depending on the specimen and the specific method. Thus the diagnostic sensitivity of the RT-PCR method will be affected by the effect of the primer set, as well as the ability to successfully isolate RNA from diagnostic specimens (Ridpath et al., 1993).

Before using RT-PCR as a laboratory BVDV diagnostic technique it is strongly recommended to validate it to be specific for various types of animal specimens, including: fluids, swabs, serum, milk, whole blood, buffy coat cells, skin, fresh and formalin-fixed tissues. Additionally, an internal positive control either as a separate reaction or a multiplexed reaction, is strongly recommended to detect false negative due to sample preparation (Saliki and Dubovi, 2004).

Due to the high sensitivity of RT-PCR, it is possible to pool samples to reduce unit test cost. Pooling is appropriate for persistent infection testing wherein one positive specimen can be detected in a pool of about 20 samples (Goyal, 2005; Saliki and Dubovi, 2004). Two convenient uses of pooled sample PCR are bulk tank milk testing (Renshaw et al., 2000) and serum samples (Muñoz-Zanzi et al., 2000) for PI BVDV testing. After the PCR reaction, nucleic acid products

are commonly detected by electrophoresis in ethithium bromide-treated agarose gels, followed by photography. Additionally, fluorescent staining such as SYBR green and the use of fluorogenic probes can be used to make real time visualization possible (Mahlum et al., 2002).

Zhang et al. (2011) developed a quantitative one-step SYBR Green I RT-PCR assay to detect levels of BVDV type-1 in cell culture. The assay had a detection limit as low as 100 copies/ml of BVDV RNA, a reaction efficiency of 103.2%, a correlation coefficient (R2) of 0.995, and a maximum intra-assay CV of 2.63% (Zhang et al., 2011). It was 10-fold more sensitive than conventional RT-PCR and could quantitatively detect BVDV RNA levels from 10-fold serial dilutions of titrated viruses containing a titer from 10-1 to 10-5 $TCID_{50}$, without nonspecific amplification. Melting curve analysis showed no primer-dimers and non-specific products. This one-step SYBR Green I RT-PCR strategy may be further optimized as a reliable assay for diagnosing and monitoring BVDV infection in animals (Zhang et al., 2011).

5.4. Serology

These methods measure the antibody responses of an animal exposed to BVDV, either by a natural exposure or vaccination. These tests have been limited to ELISA tests or serum (virus) neutralization (SN) assays (Goyal, 2005).

One disadvantage of the serological tests is the poor agreement between results obtained by ELISA and SN by the same laboratory, with an agreement quotient (kappa) of 0.15 as previously reported (Taylor et al., 1995). Significant differences have also been found for SN results among different laboratories. Two possible reasons for these differences in the SN test are the strain of virus in the assay, and the cell line used. Differences of 10- to 100-fold can be observed in the SN titer by using a different BVDV strain in the assay (Saliki and Dubovi, 2004).

The viral strain in the SN assay may be an important factor in the significance of titers in order to determine the success of vaccination or when nonvaccinated calves are being screened to detect the presence of BVDV in a herd. The test cells have been proven to significantly influence the apparent titer of a test serum. For example, antibody titers specific for BHV-1 are much higher if RK-13 cells are used rather than MDBK cells (Saliki and Dubovi, 2004).

Regardless of the serological test used, it is important to take into account the herd context when interpreting test results (previous exposure, immunization protocol, and biosecurity program. When correctly used, serology tests can help to assess vaccine efficacy, determine efficacy of a vaccination protocol, evaluate herd status as to exposure to BVDV, and associate BVDV with clinical signs (Goyal, 2005).

In some European countries, differentiation between infected and vaccinated animals is one of the key challenges facing BVDV eradication campaigns. Raue et al. (2011) compared the ability of commercial ELISA kits to differentiate antibodies generated following vaccination with four different commercial inactivated BVDV vaccines from antibodies generated following challenge with virulent BVDV. Although none of the tested vaccine–ELISA combinations were able to differentiate an infected from a vaccinated animal at the individual animal level, p80 blocking ELISAs, in combination with inactivated BVDV vaccines, might have value under certain circumstances at the herd level. In most cases, antibody responses to BVDV vaccines cannot clearly be distinguished from responses seen in the early phase of natural infection. No commercial BVD vaccine showed true marker qualities for differentiating an infected from a vaccinated animal using p80 blocking ELISAs (Raue et al., 2011).

A recent study in Europe investigated the efficacy of sentinel cattle to detect BVDV infection (Corbett et al., 2011). Forty-seven cattle management groups from 36 herds were

selected to evaluate serology as a tool to detect herd infection with BVDV. Serum samples were obtained from 5 non-vaccinated sentinel calves ≥ 6 months old in each management group and virus neutralizing (VN) antibody titers against BVDV genotypes 1 and 2 were determined. In 1 management group from 1 herd (n = 24), 3 sentinel calves had VN antibody titers ≥ 128. Three ear notch samples from that herd were positive for BVDV on RT-PCR assay. All other management groups were negative for BVDV. In that study, the herd sensitivity of sentinel serology was 100% (95% confidence interval [CI]: 0.05–1.0) and herd specificity was 100% (95% CI: 0.90–1.0). The κ value for agreement between sentinel serology and RT-PCR was 1.0 (95% CI: 1.0–1.0). Preliminary results suggest that sentinel animal serology can be utilized in a BVDV eradication program to provide an accurate and efficient evaluation of herd status (Corbett et al., 2011).

5.5. Laboratory application of diagnostic methods and interpretation of results

In order to prevent and control BVDV infections in commercial herds it is necessary to establish a program that includes biosecurity, vaccination and identification of BVDV infected animals. Diagnostic testing can be a valuable aid in monitoring the effectiveness of control programs at all levels. It is also crucial that diagnostic test programs match the management programs of the producers in their area. Therefore, practitioners also have the responsibility to know the tests available in order to make the best selection for their client (Saliki and Dubovi, 2004).

5.5.1. Acute infections (acute onset of signs)

It is crucial to identify if acute disease is occurring in a single or several animals. In the first case it could represent the beginnings of mucosal disease. On the other hand, several

animals showing similar signs could be the recent introduction of the virus into a susceptible herd (Saliki and Dubovi, 2004). The test selected should be able to cover both cases. Test and sample selection is also dependent on whether the animal is dead or alive. Multiple types of samples collected for various types of tests are always the best approach.

For live animals, the best sample for detecting virus is whole blood. For acute infections, virus remains detectable in mononuclear cells for a longer time than in serum. To be successful using serum from an acutely infected animal, the sample needs to be collected 3 to 8 days post-infection, which is difficult since the date of infection is rarely known (Goyal, 2005). Nasal swabs can also be appropriate samples for detecting BVDV particularly early in the acute infection. On the other hand, fecal samples should never be considered the primary sample for BVDV(Saliki and Dubovi, 2004).

A skin biopsy (ear notch) specimen for immunohistochemistry should be collected from all affected animals to help define if the infection is acute or persistent (Goyal, 2005). In most cases, acutely infected animals will not have a significant amount of antigen in the skin tissue. Serological tests to determine antibody responses to BVDV should always be considered for documenting an acute infection. Paired serum samples for measuring neutralizing antibodies titer is the preffered choice. Additional testing should be performed on non-affected herdmates, particularly when no previous testing in the herd has been done (Saliki and Dubovi, 2004). Acute BVDV infections in pregnant cattle might result in abortions (Baker, 1995). Sample collection at the time of the abortion might be unproductive, because the abortions can occur several weeks after the acute infection. During the abortion outbreak, tissue samples from the aborted fetus and placenta should be collected to rule out all causes of abortion. Sampling should include formalin-fixed and fresh tissues. Fetal blood and paired serum samples from the

dam should be collected for detection of antibody. Additionally, collection of tissue samples (spleen, kidneys, liver, thymus, lymph nodes, gut, lungs, etc) is always recommended from dead animals including fetuses (Goyal, 2005; Saliki and Dubovi, 2004).

Several tests can be used to detect acute BVDV infections, including virus isolation, antigen capture ELISA, RT-PCR, and immunohistochemical staining of tissue samples. The validity of the test will depend on the inclusion of proper controls to detect false negatives as well as false positives. To confirm acute infections and rule-out persistent infections, it is necessary to retest BVDV positive animals 3 weeks later by VI, ACE or PCR. If the second sample yields a positive result, the animal is diagnosed as PI. On the other hand, if the second sample results are negative it indicates that the immune system cleared the virus after the acute infection (Goyal, 2005).

RT-PCR is able to detect BVDV even in the presence of neutralizing antibody that can be present in samples from late stage acute infections. Virus isolation is the gold standard for BVDV diagnosis and commonly used. Virus isolation from whole blood is best achieved using the buffy coat (white blood cells without freezing). The presence of neutralizing antibody can generate a negative virus isolation test result. Antigen detection is best achieved using immunohistochemistry on formalin-fixed tissues (Saliki and Dubovi, 2004).

5.5.2. Persistent infections and herd screening

Preventing and controlling BVDV mainly depends on detecting and removing PI animals from herds, and establishing strategies to prevent their introduction into the farm. These animals represent less than 1% of the bovine population (Houe, 1995). There are numerous diagnostic protocols to screen herds for PIs. The testing protocol to be implemented will depend on the management practices of the particular farm.

One option to start a BVDV diagnostic program by collecting whole blood samples from all animals in the herd, including calves. The samples can be pooled (e.g. 20 samples per pool) and a highly sensitive RT-PCR can be performed. This strategy may reduce diagnostic costs (Goyal, 2005).

Several tests (virus isolation, antigen-capture ELISA, or RT-PCR) can be applied to serum on all animals over 3 months of age. With respect to serum testing in young animals, colostral antibodies can interfere with the test or eliminate detectable virus from the fluid fraction of blood for some variable period of time, resulting in potential false negative. However, virus in mononuclear cells is unaffected by colostral antibodies (Saliki and Dubovi, 2004).

Skin biopsies (ear notches) from calves, heifers, cows without calves, and bulls represent a practical and accurate strategy to identify PI animals in the herd. The tests of choice for this sample type are immunohistochemistry on formalin-fixed tissue or antigen-capture ELISA using fresh tissue samples (Njaa et al., 2000). The determination that the calf is not PI automatically defines the status of the dam as not being PI. With this method, ongoing surveillance is maintained with a single test defining the status of two animals. If the calf is PI, then it is necessary to test the dam. For dairy herds, bull calves must be tested as well as the heifer calves to achieve a complete herd screening program (Njaa et al., 2000; Saliki and Dubovi, 2004).

For dairy herds, collect bulk tank milk samples to screen the herd by using virus isolation, antigen-capture ELISA, or RT-PCR. Somatic cells from the milk are screened for BVDV by RT-PCR or virus isolation (Radwan et al., 1995; Renshaw et al., 2000). The number of cows represented by a bulk tank sample should be less than 400, although dilution studies indicate detection levels of one PI in 600.

Every testing scenario has certain limitations in interpretation of the results. For instance, virus isolation can detect PI animals and acutely infected animals. Skin biopsy testing (IHC or ELISA) detects PI animals but rarely acutely infected animals. In contrast, RT-PCR testing can detect BVDV from PI animals, acutely infected animals, and from MLV vaccinated animals (Goyal, 2005).

Regardless of the test used, it is recommended to retest positive animals if possible, as no test is absolutely PI specific. In order to increase accuracy of the tests to detect acutely infected animals it is recommended to use the buffy coats (white blood cells) since a modest immune response can eliminate virus in the fluid phase of blood, but the mononuclear cells remain infected (Brock et al., 1998). Special attention should be paid to the BVDV status of bulls. Several reports have demonstrated the existence of bulls with BVDV positive semen without viremia (Voges et al., 1998). Semen from these bulls can contain infectious virus for extended periods of time (Niskanen et al., 2002).

Hansen et al. (2010) observed that gene expression of immunologic markers differ in maternal blood cells in the presence of PI versus uninfected fetuses. PI adversely affected fetal development and antiviral responses, despite protective immune responses in the dam. Therefore this study demonstrated that fetal PI with BVDV alters maternal immune function, compromises fetal growth and immune responses, and results in expression of maternal blood biomarkers that can be used in future diagnostic tests to identify cows carrying PI fetuses (Hansen et al., 2010).

A recent study revealed that manually plucked hairs might serve as an alternative sample for a quantitative real time polymerase chain reaction (qRT-PCR) testing. In that study 23, BVDV-unvaccinated vaccinated calves (1-3 week old), known to be positive for BVDV by immunohistochemical staining, were selected and hairs were manually plucked from the ear. All

23 animals were positive for the virus by qRT-PCR performed on the whole blood and when samples of more than 30 hairs were assayed (Singh et al., 2011).

As previously mentioned, to determine whether a BVDV infection is persistent, two samples should be taken at 3–4 week intervals and tested for the viral antigen. Bedekovic et al. (2011) developed an indirect immunofluorescence (IF) method using ear notch tissue samples for diagnosis of cattle infected persistently. This study showed that IF is a good alternative to RT-PCR and antigen ELISA and can be a quick and accurate method in diagnosis of BVDV in cattle infected persistently with this virus (Bedekovic et al., 2011).

6. Vaccination

In order to prevent and control BVDV, the herd health program needs to be focused on establishing biosecurity measures to avoid the introduction and dissemination of BVDV within and between herds, eliminating PI carrier cattle and by enhancing immunity through vaccination. Vaccination against BVDV should protect against viremia to avoid dissemination of virus throughout the host following infection. Additionally, an effective vaccination should block infection of target cells of the reproductive and lymphatic systems to prevent occurrence of fetal infection and immunosuppression, respectively (Kelling, 2004).

Antibodies present in the systemic circulation effectively neutralize infecting virions, promote clearance of virus, and prevent fetal infections. The main purpose of immunization is to stimulate both the B and T cell arms of the immune system; in order to inactivate free virus (via neutralizing antibodies) and to eliminate infected cells that have potential to release infectious virus (via T cells). Immunoglobulins also have the ability to aggregate BVDV enhancing

clearance (Murphy and Chanock, 2001). Cell-mediated immunity ($CD4^+$ and $CD8^+$ T cells), is important for the resolution of acute infection with noncytopathic BVDV (Rhodes et al., 1999). There are two types of BVDV commercially available vaccines in the USA: modified-live and inactivated. For many years, most vaccines contained BVDV-1 strains. However, due to antigenic diversity between type 1 and type 2 strains, modified-live and inactivated vaccines containing both type 1 and type 2 BVDV have been developed and are commercially available (Fulton, 2005).

The major advantage of modified-live virus vaccines, in general, is activation of all phases of the immune system yielding a balanced systemic and local immune response, and a balanced humoral and cell-mediated response. The major advantages of inactivated BVDV vaccines are that they are neither immunosuppressive nor fetopathogenic(Fulton, 2005). Disadvantages of inactivated BVDV vaccines include a weaker neutralizing antibody response and shorter duration of protection (which indicates a need for increased frequency of administration). Also, inactivated vaccines may have a disadvantage in relation to modified-live vaccine with respect to limited cytotoxic T-cell response, which plays an important role in recovery from and resistance to disease (Murphy and Chanock, 2001).

Several studies have demonstrated the efficacy of MLV and inactivated vaccines regarding protection against both BVDV-1 and BVDV-2 infections as well as providing fetal protection (Bolin and Ridpath, 1989; Cortese et al., 1998b). When evaluating the efficacy of a vaccine it is important to take into account several aspects such as: level of immune response (humoral and cell mediated), crossreactivity, fetal protection, rapidity of protection, duration of immunity, immunosuppression, reversion to virulence, effect of maternal antibody on immune responses, and purity (Kelling, 2004).

6.1. Immune response to bovine viral diarrhea virus vaccines

Numerous vaccination-challenge studies have been performed to demonstrate the protective responses induced by BVDV vaccines, by evaluating clinical and serologic responses. Thus, vaccine efficacy can be evaluated based on its ability to reduce clinical disease within a population of vaccinated animals compared with a similar group of nonvaccinated animals (Brock and Cortese, 2001). However, due to the limitations in eliciting reproducible and measurable clinical disease by challenge exposure, vaccine efficacy studies have mainly focused on measurement of neutralizing antibody responses (Potgieter, 1995). Modified-live virus vaccines have the advantage of stimulating production of high concentrations of viral neutralizing antibodies (Kelling, 2004).

Additionally, replication of the vaccine virus in the vaccinated animal amplifies the antigens and stimulates the cell mediated immune response. On the other hand, inactivated BVDV vaccines have the major advantage that they are safe, but they generally induce a weaker neutralizing antibody response and a shorter duration of protection (Kelling, 2004).

6.2. Crossreactivity

Since antigenic variation is an important feature of BVDV isolates commonly circulating in the cattle population, crossreactivity of BVDV vaccine represents an essential property to be evaluated. Both inactivated and modified-live BVDV vaccines stimulate production of antibodies that crossreact with a range of antigenically diverse strains of BVDV isolates. In addition, protection against challenge with heterologous virulent BVDV isolates has also been demonstrated for both types of vaccines (Fulton et al., 2003a; Kelling, 2004; Xue et al., 2010b).

Previous studies have demonstrated crossreactivity of the antibody response induced by modified-live BVDV vaccines using *in vitro* neutralization assays. Vaccinated cattle produced antibodies by three weeks that neutralized 10-20 strains of BVDV-1 and BVDV-2 *in vitro* (Bolin and Ridpath, 1989; Cortese et al., 1998b). These results suggest that immunization against most BVDV strains can be expected by 3 weeks after vaccination (Bolin and Ridpath, 1989). Experimental vaccination-challenge experiments have confirmed that modified-live vaccines containing BVDV-1 have the ability to crossprotect calves from experimental infection with virulent BVDV-2 (Dean and Leyh, 1999; Ellis et al., 2001). Similar to MLV vaccines, inactivated BVDV vaccines also have the ability to produce crossreactive antibodies against heterologous BVDV-2 isolates *in vitro* and protect against experimental challenge with heterologous isolates (Fulton and Burge, 2000).

Recent studies revealed that type 1b is the predominant BVDV-1 subgenotype, representing more than 75% of field isolates of BVDV-1 (Fulton et al., 2003a; Fulton et al., 2002). However, nearly all commercially available BVDV type 1 vaccines contain BVDV-1a strains. Previous studies have indicated that anti-BVDV sera, induced by BVDV-1a viruses, show less neutralization activity to BVDV-1b isolates than type 1a. Therefore, it is critically important to evaluate BVDV-1a vaccines for their ability to prevent BVDV-1b infection in calves. In a recent study, calves were vaccinated subcutaneously, intradermally or intranasally with a single dose of a multivalent, modified-live viral vaccine containing a BVDV-1a strain, and were challenged with differing BVDV-1b strains to determine the efficacy and duration of immunity of the vaccine against these heterologous virus strains. Vaccinated calves, in all administration routes, were protected from respiratory disease caused by the BVDV-1b viruses, as indicated by significantly fewer clinical signs, lower rectal temperatures, reduced viral

shedding and greater white blood cell counts than non-vaccinated control animals. The BVDV-1a vaccine elicited efficacious protection against each BVDV-1b challenge strain, with a duration of immunity of at least 6 months (Xue et al., 2011; Xue et al., 2010b).

Xue et al. (2010) evaluated the immunogenicity of a single dose of an intranasally-administered MLV vaccine in 3-8 day old calves against BVDV types 1 and 2, IBR virus, PI-3 virus and BRSV. Calves were challenged with one of the respective viruses three to four weeks post-vaccination in five separate studies. There was significant sparing of disease in calves intranasally vaccinated with the MLV vaccine, as indicated by significantly fewer clinical signs, lower rectal temperatures, reduced viral shedding, greater white blood cell and platelet counts, and less severe pulmonary lesions than control animals. This was the first MLV combination vaccine to demonstrate efficacy against BVDV types 1 and 2, IBR, PI-3 and BRSV in calves 3-8 days of age (Xue et al., 2010a).

While MLV vaccines elicit an immune response with a broader range and a longer duration of immunity, killed vaccines are considered to be safer. One way to improve the performance of killed vaccines is to develop new adjuvants. Ridpath et al. (2010) evaluated new adjuvants, consisting of combinations of Quil A cholesterol and dimethyldioctadecylammonium (DDA) bromide, for use in killed vaccines. Responses to three novel killed vaccines, using combinations of Quil A and DDA as adjuvants, were compared to responses to a commercial modified live and a commercial killed vaccine. All three novel vaccines were efficacious based on reduction in virus isolation, pyrexia, and depression. Compared to a commercial killed vaccine, the three novel vaccines elicited higher VN levels and reduced injection site inflammation (Ridpath et al., 2010).

6.3. Fetal protection

Several field and experimental studies have evaluated the efficacy of vaccines on fetal protection from BVDV infection (Oirschot et al., 1999; Van Campen et al., 1998). In this regard, other reports have questioned the efficacy of BVDV vaccines on fetal protection, since PI calves were born in herds in which dams were vaccinated (Kelling CL, 1990; Van Campen H, 2000). These results have demonstrated significant, although incomplete, protection against fetal infection using modified-live BVDV vaccines (Cortese et al., 1998a) and partial protection using inactivated BVDV vaccines (Ellsworth et al., 1993). One of these experimental studies showed that 10 of 12 dams vaccinated prebreeding with modified-live BVDV type 1 vaccine and experimentally challenged around 75 days of gestation with ncp BVDV type 1, were protected against giving birth to PI calves (Cortese et al., 1998a).

Similarly, in another study, heifers vaccinated with a ncp modified live BVDV-1 (strain WRL) vaccine 21 days before breeding, were challenge-exposed at 55 to 100 days of gestation by intravenous administration of BVDV type 1 (strain 7443). In that study, 92% of calves born to vaccinated heifers were not persistently infected with BVDV (Dean et al., 2003). In contrast, in another study in which cattle were vaccinated prebreeding with inactivated vaccine and experimentally challenge-exposed with BVDV between 80 and 90 days of gestation, only 36% of fetuses were protected against BVDV infection (Ellsworth et al., 1993).

Early experimental studies have used the combination of both an inactivated BVDV vaccine followed by a modified-live BVDV vaccine in heifers. Animals were intranasally challenged four weeks after the second vaccination (between 30 and 120 days of gestation) with a mixture of type 1 and type 2 BVDV isolates (Frey et al., 2002). All nine vaccinated heifers delivered clinically healthy seronegative and BVDV-free calves. On the other hand, all

nonvaccinated control cattle developed viremia and their offspring were PI. Therefore, vaccination can provide significant fetal protection reducing the occurrence of fetal infection. However, several authors have concluded that vaccination is not 100% effective preventing fetal infection, emphasizing the establishment of a biosecurity measures and diagnostic tests for identification and elimination of PI cattle (Frey et al., 2002; Fulton and Burge, 2000; Fulton et al., 2003a).

Xue et al. (2009) evaluated the efficacy of a modified-live virus (MLV) vaccine in protecting fetuses from infection with BVDV-1 or BVDV-2 when 83 pregnant heifers (vaccinated 4 weeks before breeding) were challenged with BVDV-1 at approximately 170 d of gestation with noncytopathic field isolates. Fetuses were collected 60 d after BVDV-2 challenge, and newborn calves were collected before colostrum intake. In this study protection was determined by measuring the serum neutralizing antibody response in the fetus or calf and by virus isolation from thymus, lung, spleen, and kidney tissue samples. There was a measurable SN antibody response to BVDV in all the fetuses and calves of the control heifers, which had received a placebo vaccine (Xue et al., 2009). However, only 4 of 22 calves and 7 of the 28 fetuses of the MLV-vaccinated heifers demonstrated SN antibody after BVDV challenge. BVDV-1was isolated from tissue samples of 5 of the 12 calves of control heifers and none of 22 calves of the MLV-vaccinated heifers challenged with BVDV-1. Type 2 BVDV was isolated from tissue samples of 17 of the 18 fetuses of the control heifers and 2 of the 28 fetuses of the MLV-vaccinated heifers challenged with type 2 BVDV. The results of this study demonstrated that the MLV vaccine reduces the fetal infection rate by at least 82% for BVDV type 1 and by 75% for BVDV type 2 when heifers are exposed to highly fetotrophic BVDV-1 at 170 d of gestation (Xue et al., 2009).

In another study, 80 crossbred beef heifers were used to evaluate the efficacy of three commercially available multivalent BVDV vaccines in preventing development of BVDV PI calves. Heifers were bred by AI and subsequently exposed to two bulls. At 61 d after AI, 70 heifers were pregnant. Three cattle persistently infected with BVDV were commingled with the pregnant heifers (in an isolated pasture) from 68 to 126 d after AI. Resulting calves were assessed for persistent infection using serum PCR, ear notch antigen capture-ELISA, and immunohistochemistry. Persistently infected calves were only produced in the control group (10/10) and the group receiving the inactivated vaccine (2/18). This study demonstrated that commercial vaccines provided effective fetal protection despite prolonged natural exposure to BVDV. Given that viremias were detected in 11 vaccinated heifers after BVDV exposure, and two vaccinated heifers gave birth to persistently infected calves, there is a continued need for biosecurity and diagnostic surveillance, in addition to vaccination, to ensure effective BVDV control (Rodning et al., 2010b).

A modified-live vaccine has been shown to prevent fetal infection with BVDV-2 and, to some extent BVDV-1, when used in association with an inactivated vaccine, in a two-step vaccination protocol. In one study, a modified-live vaccine used alone was able to protect 13 heifers between 49 and 96 days of gestation at challenge from the development leukopenia and virus replication, and for a 4-month period to prevent fetal infection. The efficacy of the BVDV-1 challenge was demonstrated by virus isolation from the fetuses of all nine non-vaccinated control heifers. However, the small number of heifers tested meant that the vaccination failure rate could be as high as 10% in the field (Meyer et al., 2012).

Leyh et al. (2011) evaluated the efficacy of a MLV vaccine containing BVDV-1a and 2 against fetal infection in 50 pregnant heifers exposed on day 64 to 89 of gestation to cattle PI

with BVDV-1b. Eleven fetuses (8 control heifers and 1 IM and 2 SC vaccinates) were positive for BVDV via VI and for BVDV antigen via IHC analysis in multiple tissues. Vaccination against BVDV provided fetal protection in IM vaccinated (17/20) and SC vaccinated (17/20) heifers, but all control heifers (10/10) were considered infected. In this study a single dose of a BVDV-1a and BVDV-2 MLV vaccine administered SC or IM prior to breeding helped (although not 100% effective) protection against fetal infection in pregnant heifers exposed to cattle PI with BVDV-1b (Leyh et al., 2011).

Similarly, Xue et al. (2011) evaluated a MLV containing BVDV-1a and BVDV-2 strains for its efficacy in prevention of persistent infection of fetuses against BVDV-1b strain, when the heifers were vaccinated prior to breeding. The pregnant heifers were challenged with a non-cytopathic BVDV-1b strain at approximately 80 days of gestation. Vaccinated heifers were protected from clinical disease and viremia caused by the BVDV-1b virus. BVDV was isolated from 100% of the fetuses in the non-vaccinated control group, and from only one fetus (4.3%) from the vaccinated group. Results of this study demonstrated that the MLV containing BVDV-1a and BVDV-2 strains provided 96% protection from fetal persistent infection caused by the BVDV-1b strain (Xue et al., 2011).

6.4. Duration of immunity

Duration of immunity after vaccination is an important aspect in the evaluation of a BVDV vaccine efficacy in a typical cattle herd system, particulerly in beef cow-calf operations where animals are handled infrequently (Kelling, 2004). Modified-live virus vaccines generally produce a longer immunity than inactivated vaccines, because they tend to induce a stronger neutralizing antibody response and require fewer administrations of vaccine (Kelling, 2004).

Immunization of BVDV-seronegative calves at 10–14 days of age with a single dose of a modified-live BVDV-1 vaccine provided protection against development of severe disease when challenge with a virulent type 2 BVDV was performed 4 months after vaccination (Ellis et al., 2001).

In one study, neutralizing antibody response was still detectable 18 months after vaccination with modified-live BVDV vaccine in seronegative cows (Cortese et al., 1998b). In contrast, in another study, a more rapid decline of antibody titers by 4.7 months was noted following vaccination (Fulton and Burge, 2000). Similarly, in another study using a single dose of a modified-live BVDV vaccine, antibody titers declined after 3.5 months, and protected against type 2 challenge inoculation (Dean and Leyh, 1999).

6.5. Reversion to virulence

The potential risk for shedding of vaccine virus from animals vaccinated with modified-live BVDV vaccines, followed by transmission to susceptible contact animals and reversion to virulence has been studied. Calves vaccinated with modified-live BVDV vaccines develop transient viremia (Fulton et al., 2003b; Hunsaker et al., 2002). Vaccine virus was present in nasal secretions for several days, corresponding to the time period during which the vaccinated calves were viremic (Hunsaker et al., 2002). In contrast, another study showed that vaccinal BVDV was not shed from calves receiving the modified-live BVDV vaccine to susceptible cohort calves (Fulton et al., 2003b).

6.6. Effect of maternal antibodies on immune response

Colostral antibodies begin to decline around 3 months of age. Vaccination prior to antibody decay in neonatal calves that have fed colostrum (containing a high concentration of

antibodies to BVDV) with a modified-live BVDV-1 vaccine, resulted in a decreased immune response and low level of protection against BVDV challenge (Ellis et al., 2001). Thus, high concentrations of maternally derived BVDV specific antibodies interfere with the development of an immune response to vaccination with a modified-live BVDV vaccine.

6.7. Purity

Contamination of modified-live virus vaccines with adventitious pestivirus, particularly virulent BVDV, during the manufacturing process represents a significant risk factor in the manufacturing process, as previously reported (Barkema et al., 2001). Commercial vaccines contaminated with extraneous BVDV strains could cause severe clinical consequences in inoculated herds. In Europe, severe outbreaks of BVDV-2 infection (≥70% morbidity) have been associated with the use of batches of modified live Bovine Herpes Virus-1 (BHV-1) marker vaccine contaminated with BVDV (Barkema et al., 2001; Falcone et al., 1999; Jagodzinski et al., 2002).

The main source of adventitious BVDV is fetal bovine serum which is used as a supplement in cell culture medium used to grow vaccine virus in cell cultures (Rossi et al., 1980). A BVDV contamination rate in bovine sera has been estimated to be between 10 and 75% (Bolin, 1991; Rossi et al., 1980). Conversely, Audet et al. (2000) investigated possible contamination of 38 lots of viral vaccines using RT-PCR for the presence of BVDV (Audet et al., 2000). The study demonstrated that all vaccines were negative for contaminating BVDV RNA, suggesting that currently US licensed viral vaccines are unlikely to be contaminated with pestiviruses (Audet et al., 2000). However, it is recommended that quality of bovine sera and cell lines to be used for cell cultures are screened for adventitious BVDV by using improved reagents (eg, monoclonal antibodies) or new technology (eg, RT-PCR) with improved sensitivities.

Additionally, fetal bovine sera that pass rigorous testing procedures and are negative for virus may be irradiated or chemically treated as a further preventive measure before being used in cell culture to grow vaccine virus (Wessman and Levings, 1999).

Xia et al. (2011) investigated 33 batches of fetal bovine serum (FBS) obtained from ten suppliers for the presence of both recognized and the atypical bovine pestiviruses contamination. All 33 batches of FBS were positive by real-time RT-PCR assays for at least one species of bovine pestiviruses. According to the certificate of analysis that the suppliers claimed for each batch of FBS, BVDV-1 was detected in all 11 countries and BVDV-2 was detected exclusively in the America continent. The atypical pestiviruses were detected in 13 batches claimed to originate from five countries. This study demonstrated that commercial FBS batches from different geographic origins are contaminated not only with the recognized species BVDV-1 and BVDV-2, but also with the emerging atypical bovine pestiviruses (Xia et al., 2011).

6.8. Bovine viral diarrhea virus vaccination programs

The main objective of a vaccination program is to induce maximal protective responses when the risk and consequences of BVDV infection are greatest during the production cycle. The prebreeding and preweaning stages represent the most critical times when immunity should be high in order to protect cattle against reproductive losses and respiratory tract disease, respectively (Kelling, 2004).

6.8.1. Beef cow–calf herd vaccination

Prevention and control of BVDV infections cannot rely solely upon vaccination to protect against fetal infection, since vaccines do not give complete fetal protection (Oirschot et al., 1999). In addition to strategic vaccination, herd management practices must focus on

biosecurity measures, as well as identification and elimination of PI carriers in order to avoid exposure to BVDV infection (Audet et al., 2000). It is important to emphasize that modified-live vaccines have certain benefits versus inactivated vaccines, including more complete protection against fetal infection (Fulton, 2005).

Several field and experimental studies have recommended vaccination of unstressed, healthy heifers, separated from pregnant cows, with modified-live BVDV vaccine (Cortese et al., 1998b). Vaccination of all replacement heifers should be performed twice before breeding. It is recommended to begin this vaccination protocol three estrous cycles (ie, 2 months) before breeding (Fulton, 2005). Cows should be revaccinated annually, 2-4 weeks before breeding. If inactivated vaccines are used, administration of vaccine should be timed so that maximal responses are achieved at the time of breeding. Following the vaccine manufacturer's recommendation about the dose, timing of booster vaccinations and number of boosters is also essential (Kelling, 2004).

6.8.2. Dairy cow herd vaccination

Cows in a dairy herd are normally at various reproductive stages. This limits selection of vaccines to those that are safe to use in pregnant animals. As discussed for beef cow–calf herds, similar vaccination strategies might be recommended in dairy herds for control of BVDV infection. It is also a crucial in the dairy herd heath program to avoid addition of PI replacement heifers, remove PI carrier cattle, and establish a vaccination program using both modified-live and inactivated BVDV vaccines (Fulton, 2005; Kelling, 2004).

Healthy replacement heifers should be vaccinated twice with modified-live BVDV vaccine before breeding. These animals need to be isolated from pregnant cows during and after

vaccination. Vaccination should be scheduled so that maximal protection is achieved during the critical first 4 months of gestation to maximize the potential for adequate duration of immunity and enhance protection against fetal infection. Replacement heifers should be vaccinated with modified-live BVDV vaccines 60 days before breeding (Kelling, 2004).

If inactivated vaccines are administered, vaccination of heifers before breeding should be timed so that maximal responses are afforded by first 4 months of gestation to maximize protection against fetal infection. Thus, the second booster should be given 2 weeks before breeding. Cows should be revaccinated annually, 2 weeks before breeding (Brownlie et al., 1995; Kelling, 2004).

6.8.3. Beef calf vaccination

It is a common management practice in cow–calf operations to vaccinate young calves in late spring when processed at branding time. At this time maternal antibody titers are still elevated, which may interfere with the immune response to vaccination. Previous studies have shown that high concentrations of maternally derived BVDV specific antibodies blocked induction of protective immune responses to vaccination with a inactivated or modified-live BVDV vaccine, with vaccinated calves developing severe disease when challenged 4 months later (Ellis et al., 2001). Because colostral antibody inhibits replication of modified-live vaccine and results in decreased immune responses, calves vaccinated at an early age should be revaccinated a few months later when maternal antibody titers have declined (Kelling, 2004).

Beef calves, weaned around 7 months of age, are normally seronegative or have low titers of maternally derived antibody (Kelling et al., 1990). Weaning is a management practice that results in stress, and represents a critical time when high risk of infection exists. Since antibody

titers wane by 6 weeks after vaccination (Grooms and Coe, 2002), calves should be immunized a few weeks before weaning so that they achieve maximal protection against BVDV at weaning.

The modified-live BVDV vaccine selected for immunization of calves suckling pregnant cows should be safe regarding the fetopathic potential. The choice of vaccines will be limited to specific products designated for this purpose. An effective strategy for optimizing virus neutralizing antibody titers in a preconditioning program is the use of an inactivated vaccine given to calves nursing pregnant cows followed by a modified-live BVDV vaccine at weaning (Grooms and Coe, 2002).

6.8.4. Dairy calf vaccination

The vaccination program of the dairy calf will depend on the management practices as well as the quantity and quality of colostrum consumed during the first hours of life. Neonatal dairy calves, are separated from the dam at birth or shortly later. Thus, titers of maternally derived BVDV antibody varies widely depending on the quality and quantity of the colostrum consumed, as well as the efficiency of immunoglobulins' intestinal absorption. Consequently, a high percentage of neonatal dairy calves have a failure of immunoglobulin transfer (McGuire et al., 1976). Therefore, the decreased immunity in calves with high concentrations of maternally derived BVDV specific antibodies following vaccination, observed in beef calves, is not a very common and practical consequence in these dairy calves. This management condition suggests that dairy calves should be vaccinated as soon as possible (around 3-4 months) (Bolin and Ridpath, 1995).

7. Antiviral treatments

Currently, no specific therapy exists to treat BVDV PI animals. Newcomer et al. (2012) initiated evaluation of the pharmacokinetic and safety data of a novel antiviral agent in BVDV-free calves and to assess the antiviral efficacy of the same agent in PI calves. One BVDV-free calf was treated with 2-(2-benzimidazolyl)-5-[4-(2-imidazolino)phenyl]furan dihydrochloride (DB772) once at a dose of 1.6 mg/kg intravenously and one BVDV-free calf was treated three times a day for 6 days at 9.5 mg/kg intravenously (Newcomer et al., 2012). Subsequently, four PI calves were treated intravenously with 12 mg/kg DB772 three times a day for 6 days and two PI control calves were treated with an equivalent volume of diluent only. Prior to antiviral treatment, the virus isolated from each calf was susceptible to DB772 *in vitro*. The antiviral treatment effectively inhibited virus for 14 days in one calf and at least 3 days in three calves. Subsequent virus isolated from the three calves was resistant to DB772 *in vitro*. No adverse effects of DB772 administration were detected. This study demonstrated that DB772 administration is safe and exhibits antiviral properties in PI calves while facilitating the rapid development of viral resistance to this novel therapeutic agent (Newcomer et al., 2012).

In another study, 26 9-aminoacridine derivatives were evaluated *in vitro* in cell-based assays for cytotoxicity and antiviral activity against a panel of 10 RNA and DNA viruses. Seventeen compounds exhibited a marked specific activity against BVDV. Some compounds, particularly those bearing a quinolizidinylalkyl side chain, displayed pronounced cytotoxicity (Tonelli et al., 2011).

II. Evaluation of the onset of protection induced by a modified-live virus vaccine in calves challenge inoculated with type 1b bovine viral diarrhea virus

Abstract

The objective of this study was to evaluate the onset of protection induced by a modified-live bovine viral diarrhea virus (BVDV) vaccine administered 7, 5, or 3 days before challenge with type 1b BVDV. Forty beef calves were randomly assigned to 1 of 4 groups: unvaccinated control group or a group vaccinated with a modified-live virus vaccine containing BVDV types 1a and 2, BHV-1, PI3, and BRSV at 7, 5, or 3 days, respectively, before experimental challenge with NY-1 BVDV. Blood samples were collected for total leukocyte counts, serum virus neutralization, and virus isolation (VI); nasal swab (NS) specimens were obtained for VI; and rectal temperatures were monitored for 14 days post-challenge. No significant differences in leukocyte counts or rectal temperatures were detected after BVDV inoculation in vaccinated calves. Vaccinated calves had significantly reduced viremia and viral shedding post-challenge, compared with those for unvaccinated calves. On day 5 post-challenge, a higher proportion of calves vaccinated 3 days before challenge (5/9) had positive NS VI results, compared the results for calves vaccinated 5 (1/10) and 7 (0/10) days before challenge. Unvaccinated calves had leukopenia on days 3, 5, and 6 after inoculation and significantly higher rectal temperatures on days 7 and 8 post-challenge. All unvaccinated calves had ≥ 1 positive NS VI result 3-11 days post-challenge, and 4 became viremic. Modified-live BVDV vaccine prevented fever, viremia, and leukopenia in calves inoculated with NY-1 BVDV. However, a high proportion of calves vaccinated 3 days before challenge shed BVDV after inoculation.

Keywords: Bovine viral diarrhea virus, Modified-live virus vaccine, protection, calves.

1. Introduction

Bovine viral diarrhea virus is an important infectious agent that affects cattle worldwide (Baker, 1995; Houe, 1999). Infection of pregnant cattle with BVDV may result in abortions, stillbirths, or the birth of calves with congenital defects or persistently infected with BVDV (Baker, 1995). Acute infections with some strains of BVDV may cause immunosuppression and clinical signs of gastrointestinal, reproductive, and respiratory disease (Fulton et al., 2005a; Makoschey et al., 2001). Strategies to prevent and control BVDV include quarantine and other biosecurity measures to control the spread of infection within and between herds, identification and slaughter of persistently infected cattle, and vaccination (Brock, 2004; Kelling et al., 2000; Reber et al., 2006).

The efficacy of a MLV vaccine to protect against BVDV infection can be indirectly measured by the vaccine's ability to induce high neutralizing antibody concentrations and a strong cell-mediated immune response. An important factor in the evaluation of BVDV vaccine efficacy is its rapidity in eliciting an adequate immune response to protect cattle from negative outcomes when exposed to the virus within a few days after vaccination. This feature is particularly relevant in certain production systems such as feedlots, where frequent introduction of cattle with unknown BVDV status increases the risk of BVDV infection, which could result in severe respiratory disease and a high death rate in affected cattle and substantial economic losses for producers.

A study to characterize the onset of protection elicited by an MLV BVDV vaccine in calves challenge inoculated with a virulent type 2 strain of BVDV revealed that calves vaccinated 5 or 7 days before experimentally induced infection did not develop clinical signs of BVDV infection, whereas unvaccinated control calves developed severe clinical disease and had a higher death rate (Brock et al., 2007). Vaccination of calves 3 days before challenge exposure with the same type 2 strain elicited some protection, as evidenced by a decreased magnitude of viremia and leukopenia after experimental inoculation, compared with that for unvaccinated control calves (Brock et al., 2007). The authors of that study speculated that such protection may not have been detected if a less-virulent BVDV strain had been used for the challenge inoculation (Brock et al., 2007).

Studies have identified that type 1b BVDV strains are equally or more prevalent than type 1a BVDV strains and are isolated more frequently from calves that die with gross lesions of pneumonia (Fulton et al., 2003a; Fulton et al., 2005b). Most commercial vaccines marketed in the United States include type 1a and/or type 2 BVDV strains, which might affect their efficacy because cattle are commonly exposed to a different subgenotype, such as type 1b (Fulton et al., 2005b). The objective of the study reported here was to evaluate the onset of protection in cattle vaccinated with an MLV BVDV vaccine 7, 5, or 3 days before experimental inoculation with a type 1b BVDV (strain NY-1). Our hypothesis was that a single dose of a commercial MLV combination viral vaccine (containing BVDV type 1a and 2 strains) would protect cattle from acute BVDV infection when experimentally inoculated with a NY-1 BVDV soon after vaccination.

2. Methods

2.1. Animals

Forty clinically normal beef calves (36 males and 4 females), 7 to 9 months old, and weighing 191 to 213 kg were used in the study. Calves were seronegative (antibody titer < 1:5) for BVDV as determined by serum VN and had negative results for BVDV via immunohistochemical analysis of ear notch specimens and VI of blood samples. Calves were obtained from a privately owned herd that had an optimal biosecurity program in place and to which new cattle were not introduced (closed herd).

2.2. Experimental design and sample size calculation

The study was designed as a randomized, controlled trial. Sample size was calculated using a power procedure (PROC POWER, SAS, version 9.1, SAS Institute Inc, Cary, NC) on the basis of the expected means (7,000, 5,000, and 3,000 cells/μL) of the total leukocyte count for each vaccinated group, the SDs (1,200 cells/μL) of the means, and a statistical power of 99% (Brock et al., 2007). The calculated sample size was 6 calves/group, but we chose to enroll 10 calves/group to ensure that an adequate number of calves were maintained in each group throughout the study period. Calves were stratified by sex and assigned by use of a random number generator (Research randomizer, version 3.0, Social Psychology Network, Middletown, Conn) to 1 of 4 treatment groups: calves vaccinated 7 days before BVDV challenge inoculation, calves vaccinated 5 days before BVDV challenge inoculation, calves vaccinated 3 days before BVDV challenge inoculation, and control calves not vaccinated before BVDV challenge inoculation.

All 40 calves were housed together at the farm of origin prior to vaccination. As treatment groups were vaccinated (7, 5, or 3 days before BVDV challenge inoculation), they were separated and transported to the Auburn University BVDV isolation farm. At the BVDV isolation farm, vaccinated calves were housed together in the same pasture and allowed to eat and drink from the same feeders and water troughs. The unvaccinated control calves were transported to the BVDV isolation farm on the day of BVDV challenge inoculation (day 0) to prevent any exposure of nonvaccinated calves to BVDV before inoculation. All calves were inoculated with BVDV on the same day and housed together from that time point to the completion of the study. All calf protocols were reviewed and approved by the Institutional Animal Care and Use Committee of Auburn University.

2.3. Vaccine

The vaccine used was a USDA-licensed stock material approved for commercial sale (Express 5, Boehringer Ingelheim, Ridgefield, Conn). The vaccine was an MLV combination vaccine containing type 1a (Singer strain) and type 2 (296 strain) BVDV, bovine herpes virus type 1, parainfluenza virus type 3, and bovine respiratory syncytial virus. A single 2-mL dose of the vaccine was administered IM with a sterile 18-gauge, 1-inch needle used for each calf in accordance with the manufacturer's recommendations.

2.4. BVDV challenge inoculation

Calves were experimentally inoculated with a noncytopathic type 1b BVDV isolate (strain NY-1). The NY-1 BVDV strain was originally isolated by researchers at Cornell University from dairy cattle in the United States (Lee and Gillespie, 1957b). The virus has been

in continuous propagation since 1957 and is available as a challenge BVDV strain from the USDA National Veterinary Services Laboratories (Clifford, 2006). The stock strain of NY-1 BVDV used in the present study was biologically cloned via successive passages by use of limiting dilutions with subsequent minimal propagation to produce an adequate amount of stock virus for characterization and animal challenge exposure studies. The inoculum used consisted of cell culture supernatants containing 1.36 X 10^6 $CCID_{50}$/mL of inoculum. The inoculation was performed by intranasal aerosolization of 5 mL of inoculum by use of an aerosolizer (Aerosolizer deVilbiss, Somerset, Pa) and a vacuum pump.

2.5. Sample and data collection

From each calf, blood samples were collected on days 0, 3, 5, 6, 8, 11, and 14 after BVDV challenge inoculation in blood tubes containing an anticoagulant for determination of the leukocyte count. Blood samples were collected on days 0, 3, 5, 6, 7, 8, 9, 11, and 14 after BVDV challenge inoculation in blood tubes without an anticoagulant to obtain serum for VN and VI. Nasal swab specimens for VI were also obtained and body temperature measured by use of a rectal thermometer on days 0, 3, 5, 6, 7, 8, 9, 11, and 14 after BVDV challenge inoculation.

One swab was used to scrape the nasal mucosa of each calf and was then placed in a tube containing 3 mL of Dulbecco modified Eagle medium supplemented with 10% equine serum, L-alanyl, and L-glutamine. Tubes containing nasal swabs were transported on ice until processing. For processing, tubes were mixed by use of a vortex; swabs were removed and discarded, and the remaining medium was stored at -80°C.

For each calf, immediately prior to and for 14 days after BVDV challenge inoculation, clinical signs of disease and clinical scores evaluating signs of depression, appetite,

conjunctivitis, diarrhea, nasal discharge, coughing, and dyspnea were assessed and recorded daily. Clinical signs were assessed on a scale from 0 to 3, with 0 representing a lack of clinical signs and 3 representing severe clinical signs . Investigators who assessed clinical signs, collected samples, measured temperatures, and assessed the outcomes were unaware of the group assignment for each calf (O'Connor et al., 2010).

2.6. Serum VN

Virus neutralization was performed at serial 2-fold dilutions (1:2 through 1:256) on heat-inactivated serum by use of 100 $CCID_{50}$ of NY-1 BVDV and type 2 (strain 1373). Samples were incubated for 1 hour at 37°C before the addition of MDBK cells (1.5 X 10^5 cells/mL). Cultures were incubated for 72 hours in 5% CO_2 at 37°C. The results were expressed as the relative concentration of antibody necessary to inhibit viral infection as determined by an immunoperoxidase staining technique that used a BVDV-specific polyclonal antibody (B-224).

2.7. Virus isolation

Serum samples and nasal swab specimens were stored frozen at –80°C. For VI, 250 µL of each sample was added to individual 25-cm^2 tissue culture flasks containing a monolayer of MDBK cells. For cell culture, Dulbecco modified Eagle medium supplemented with 10% equine serum, L-alanyl, and L-glutamine was used. After 3 days of incubation in 5% CO_2 at 37°C, flasks were frozen and thawed; 50 µL of the cell suspension from each flask was then transferred into 3 wells of a 96-well plate seeded with MDBK cells (first plate). The inoculated 96-well plate was incubated for 3 days, and the culture medium from each well was transferred to the corresponding wells of a new 96-well plate seeded with MDBK cells (second plate). Both 96-

well plates (first and second) were tested for BVDV antigen by use of an immunoperoxidase staining technique.

2.8. Leukocyte count

Unclotted blood samples were submitted to the clinical pathology service laboratory at the Auburn University College of Veterinary Medicine for leukocyte analysis. The total leukocyte count for each sample was determined by an automatic cell counter (Coulter. Corp., Miami, FL, USA).

2.9. Statistical analysis

To detect changes in leukocyte counts over time, the mean leukocyte count on day 0 was compared with the mean leukocyte count on days 3, 5, 6, 8, 11, and 14 by the use of a repeated measures analysis for a mixed generalized linear model[e] defined by $y_{ij} = \mu + \tau_i + \beta_j + \varepsilon_{ij}$; where y_{ij} represents the response (leukocyte count) of subject j on day i for each treatment group, μ represents the mean, τ_i represents the effect of day i after BVDV inoculation (day 3, 5, 6, 8, 11, or 14), compared with the effect on day 0, β_j represents a variable associated with subject j on day 0 (baseline), and ε_{ij} represents the error. We assumed that days after inoculation were fixed (so $\Sigma i = 1 \cdot \tau i = 0$) and that the calves used were a random sample of calves from a larger population of calves. Thus, the calves collectively represented a random effect, so we assumed that the mean of β_j was 0 and that the variance of β_j was σ^2_{β}. Because the term β_j was common to all days after inoculation on the same calf, the covariance between y_{ij} and $y_{i'j}$ was not 0, but constant across all days and subjects. Changes in rectal temperature over time were analyzed by

use of a statistical model similar to the model used for detecting changes in leukocyte counts over time.

An ANOVA was performed to compare means of temperature, leukocyte counts, and antibody titers, respectively, among the treatment groups by the use of a generalized linear model (PROC GLM, SAS, version 9.1, SAS Institute Inc, Cary, NC) with the Scheffe method used to adjust for multiple comparisons. The model was defined as $y_{i(y0 - y14)} = \mu + \tau_i + \varepsilon_{i.}$; where y_i represents the respective response (leukocyte count, rectal temperature, or antibody titers) of subject j to treatment i (control or vaccination 7, 5, or 3 days before BVDV challenge inoculation) at 0, 3, 5, 6, 8, 11, and 14 days after BVDV challenge inoculation, μ represents the mean, τ_i represents the effect of treatment i (control or vaccination 7, 5, or 3 days before BVDVD challenge inoculation), and ε_{ij} represents the random effect error. The value for R^2 was used to estimate the goodness of model fit. In the present study, R^2 was > 54% for all the continuous variables that were considered.

Virus isolation results from serum samples and nasal swab specimens were analyzed by use of a frequency procedure (PROC FREQ, SAS, version 9.1, SAS Institute Inc, Cary, NC) and compared with a χ^2 test. A logarithmic base 2 transformation was applied to VN values before calculation and comparison of the GMs. Back-transformed GMs for each viral strain tested were calculated for each group at day 0 and 14 days after BVDV challenge inoculation. For all analyses, values of $P < 0.05$ were considered significant.

3. Results

3.1. Clinical signs of BVDV infection

One calf that was vaccinated 3 days before BVDV inoculation was removed from the study because of an injury not associated with vaccination or inoculation. Clinical disease attributable to BVDV was not observed in any calf during the period between vaccination and BVDV inoculation. Clinical signs of severe BVDV infection (clinical score = 3) were not observed in any calf during the 14 days after BVDV inoculation. Mild coughing, nasal discharge, and loose feces were sporadically observed in a few vaccinated calves (4/10, 2/10, and 2/10 for calves vaccinated 7, 5, and 3 days before BVDV inoculation, respectively) during the 14 days after BVDV inoculation. Of the unvaccinated control calves, 7 of 10 developed mild watery nasal discharge during the course of the experiment; 3 of those 7 also had loose feces. Of the remaining 3 calves in the unvaccinated control group, 2 had loose feces as the only clinical sign of BVDV infection after inoculation, and 1 did not develop any clinical signs associated with BVDV infection.

3.2. Rectal temperature

A significant increase in mean rectal temperature was detected in the unvaccinated control group on days 7 ($P = 0.003$) and 8 ($P = 0.001$) after BVDV inoculation, compared with the mean rectal temperature for the group on day 0 (Table 1). The mean rectal temperature for the unvaccinated control group was also significantly higher on days 7 ($P = 0.015$) and 8 ($P < 0.001$) , compared with the respective mean rectal temperatures on those days for the vaccinated groups. In contrast, there was no increase detected in the mean rectal temperatures for calves in the vaccinated groups, compared with the respective mean rectal temperatures for each group on

day 0. There was also no difference in the mean rectal temperatures among any of the vaccinated groups on any specific day during the observation period.

3.3. Serum VN

Most calves were seronegative for BVDV type 1 and 2 on the day of vaccination (antibody titer < 1:5). However, 5 of 40 calves had low antibody titers (1:8 to 1:32) against BVDV type1b and 2 at the beginning of the study, which were attributed to maternally derived antibodies and not to prior exposure to BVDV. Seropositive calves were evenly distributed among the 4 treatment groups and did not affect the outcomes of interest.

Serum VN antibody titers against NY-1 BVDV were significantly ($P < 0.001$) increased on day 14 after BVDV inoculation in all the vaccinated groups, compared with the NY-1 BVDV antibody titers on day 0. At the end of the study period, the GM antibody titer values were 12.69, 10.07, and 11.75 for calves that were vaccinated 7, 5, and 3 days before BVDV inoculation, respectively (Table 2). No differences were found among the vaccinated groups. Interestingly, unvaccinated calves developed very high VN antibody titers (GM, 73.51) against NY-1 BVDV 14 days after the challenge inoculation, and this value was significantly ($P < 0.001$) greater than those detected in the vaccinated groups 14 days after challenge inoculation. Eight of 10 unvaccinated calves had a ≥ 4-fold increase in antibody titers against NY-1 BVDV, which was indicative of an active infection. The 4 unvaccinated calves that became viremic had antibody titers ≥ 64. In contrast, there was no increase in the GM antibody titers against type 2 BVDV from day 0 to day 14 after BVDV inoculation for any of the vaccinated groups or the control group.

3.4. Virus isolation

Six days after BVDV inoculation, BVDV was detected in the serum of 6 calves (4 from the unvaccinated control group, and 1 each from the groups vaccinated 7 and 3 days before BVDV inoculation). The number of calves with viremia in the unvaccinated control group was significantly ($P = 0.04$) higher than the number of calves (n = 0) with viremia in the group vaccinated 5 days before BVDV inoculation, but was not significantly different from the number of calves with viremia in the other 2 vaccinated groups, respectively (Figure 1) .
Viremia was not detected in any of the study calves beginning 9 days after BVDV inoculation and for the remaining 5 days of the observation period.

All calves in the unvaccinated group had positive results for BVDV on ≥ 1 nasal swab specimen by day 9 after BVDV inoculation (Figure 2). On days 7, 8, and 9 after BVDV inoculation, the proportion of calves with positive results for BVDV on nasal swab specimens was significantly higher for the unvaccinated group (Figure 1), compared with the proportion of calves with positive results for BVDV on nasal swab specimens in the group vaccinated 7 days before challenge inoculation ($P = 0.003$), the group vaccinated 5 days before challenge inoculation ($P = 0.003$), or the group vaccinated 3 days before challenge inoculation ($P = 0.04$). In contrast, on days 7, 8, and 9 after BVDV inoculation, respectively, the percentage of calves with positive results for BVDV on nasal swabs did not differ among the vaccinated groups.

Five days after BVDV inoculation, a higher proportion of calves that were vaccinated 3 days before challenge inoculation had positive results for BVDV on nasal swab specimens, compared with the proportion of calves with positive results for BVDV on nasal swab specimens in the groups vaccinated 5 ($P = 0.04$) or 7 ($P = 0.0075$) days before challenge inoculations. Of the calves that were vaccinated 3 days before BVDV inoculation, 6 of 9 had positive results for

BVDV on ≥ 1 nasal swab specimens between 3 and 9 days after BVDV inoculation (Figure 2). There was no significant difference in the cumulative number of calves that had positive results for BVDV on nasal swab specimens between the group vaccinated 3 days before BVDV inoculation and the unvaccinated control group (Figure 2).

3.5. Leukocyte count

Leukopenia was detected in unvaccinated calves on days 3, 5, and 6 after BVDV challenge inoculation; and the mean leukocyte count was significantly ($P < 0.001$) lower on each of those days, compared with that on day 0 (Table 3). This decrease in leukocyte count was associated with the period during which calves in the unvaccinated group became viremic. On days 3, 5, and 6 after BVDV inoculation, the mean leukocyte count was significantly lower for the unvaccinated group compared with the mean leukocyte count on those days in the group vaccinated 7 days before challenge inoculation (P=0.0005, P=0.0007, P=0.032; for days 3, 5 and 6 after inoculation, respectively) the group vaccinated 5 days before challenge inoculation (P=0.016, P=0.032, P=0.0003; for days 3, 5 and 6 after inoculation, respectively) and the group vaccinated 3 days before challenge inoculation (P=0.005, P=0.032, P=0.001; for days 3, 5 and 6 after inoculation, respectively). Significant variations in the mean leukocyte count were not detected within or among the vaccinated groups throughout the observation period.

4. Discussion

In the present study, challenge inoculation with NY-1 BVDV did not cause severe clinical signs of BVDV infection in any of the calves. In another study in which investigators used a similar protocol, experimental challenge exposure with type 2 BVDV (strain 1373) caused

severe clinical disease as evidenced by a high death rate (60%) in unvaccinated calves (Brock et al., 2007); and those results were consistent with results of other studies that involved the use of the same BVDV challenge-exposure strain (Liebler-Tenorio, 2004; Ridpath et al., 2006; Stoffregen et al., 2000). One possible reason for the contrast in clinical outcomes between the present study and the cited studies may be variations in the amount of virus used in the challenge-exposure dose or an increased virulence of the type 2 BVDV challenge strain.

Acute BVDV infection in unvaccinated calves was not accompanied by severe clinical signs of disease in the present study. This observation is similar to those reported from field investigations. However, the significant increase in rectal temperature detected for the unvaccinated group indicates a deviation from clinically normal. Calves that were vaccinated did not have a significant change in rectal temperatures during the course of the experiment, which is comparable with results of another study that included the use of a type 2 BVDV challenge inoculation (Kelling et al., 2007). Ridpath et al. (2007) demonstrated that cattle inoculated with the same NY-1 BVDV strain that was used in the present study developed mild clinical signs of BVDV infection that lasted for a shorter duration, compared with the clinical signs and duration of clinical signs associated with type 2 BVDV strains (Ridpath et al., 2007). Similar to infections caused by the NY-1 strain, acute infections with other BVDV field strains often do not cause severe clinical disease (Baker, 1995). However, irrespective of the ability of a particular strain of BVDV to cause clinical disease, the deleterious effects BVDV has on the immune and reproductive health of cattle in general, along with the potential for the birth of calves persistently infected with BVDV, create the need for effective control measures to prevent the dissemination of BVDV within and between herds.

The GM antibody titers against the NY-1 BVDV challenge strain used in the present study were significantly increased at day 14 after the challenge inoculation, compared with the GM antibody titers on day 0 for all groups of calves. However, the GM antibody titer for the unvaccinated group on day 14 was significantly higher than that for each of the vaccinated groups. It is possible the lower antibody titers in the vaccinated groups could have been caused by early immune responses (production of type I interferon and neutralizing antibodies and stimulation of cell-mediated immunity in the form of natural killer cells and macrophages) elicited by the vaccine that inhibited antigenic stimulation of the immune system following the challenge inoculation and that prevented viral replication in the respiratory tract . The significant difference in antibody titers between the calves that were unvaccinated and vaccinated in the present study could also be attributed to the fact that unvaccinated calves had a primary humoral immune response that focused on the challenge BVDV strain, but vaccinated calves had a primary humoral response initiated by the heterologous BVDV strains included in the vaccine.

The results of VI from serum samples and nasal swab specimens indicate that vaccination 5 or 7 days before challenge inoculation was effective in protecting calves from developing clinical signs associated with BVDV infection and reducing the amount of viral shedding. This is in accordance with other studies in which BVDV was isolated from nasal swab specimens from all calves in an unvaccinated group but not calves in MLV-vaccinated groups (Kelling et al., 2007). Fulton et al (2006) used 2 doses (vaccination 30 and 17 days before exposure to calves persistently infected with BVDV) of the same MLV vaccine (containing BVDV types 1a and 2) used in the present study and determined that vaccination prevented viremia, whereas BVDV was isolated from nasal secretions and leukocytes from unvaccinated control calves on days 6 and 13 after challenge inoculation (Fulton et al., 2006). Although BVDV has been isolated from

the serum of cattle on day 7 after receiving an MLV vaccine containing strains Singer or NADL (Bolin and Ridpath, 1989), vaccinated calves in the present study seldom had BVDV isolated from the serum, and it was never isolated ≤ 5 days after challenge inoculation.

In the present study, calves in the vaccinated groups did not have a significant decrease in mean leukocyte counts, compared with the leukocyte count for calves in the unvaccinated group, which had marked leukopenia during the observation period. This is consistent with results from other studies in which unvaccinated calves experimentally inoculated with a type 2 BVDV strain all developed leukopenia between 3 and 7 days after challenge inoculation (Brock et al., 2007; Kelling et al., 2007).

The ability of vaccination to reduce the frequency of viremia and viral shedding in nasal secretions in calves exposed to BVDV within days after initial vaccination can reduce the spread of BVDV in commingled cattle, especially in production systems (ie, feedlots) with cattle from multiple sources with unknown BVDV status. Vaccination of high-risk cattle with a single dose of an MLV vaccine 5 or 7 days before exposure may immediately decrease BVDV transmission in cattle at risk of becoming infected because of commingling or exposure to cattle persistently infected with BVDV (Brock et al., 2007). However, it must be emphasized that the present study represents a single vaccination–challenge-exposure, the results of which cannot be extrapolated to all cattle populations and production systems. The fact that a high proportion (6/9) of calves vaccinated 3 days before challenge inoculation had BVDV isolated from at least 1 nasal swab specimen between 3 and 9 days after BVDV inoculation indicated vaccination does not prevent viral shedding if BVDV infection develops ≤ 3 days after vaccination. However, vaccination, even within 3 days of inoculation, resulted in an immune response that decreased the frequency of viremia and viral shedding in nasal secretions, compared with the frequency of viremia and

viral shedding in unvaccinated calves. Vaccination also mitigated the severity of the infection as evidenced by no significant variation in mean leukocyte count or rectal temperature in vaccinated calves throughout the observation period. The results of the present study confirm those of another study in which vaccination 3 days before challenge inoculation provided some but not total protection against BVDV infection by decreasing the degree of viremia and the development of leukopenia after inoculation (Brock et al., 2007). However, in that study, as in the present study, viral shedding in nasal secretions was detected in 4 of 10 calves vaccinated 3 days before challenge inoculation, but the amount of shedding was reduced, compared with that of the unvaccinated control group. Fulton et al (2005) reported that calves receiving an MLV vaccine containing BVDV types 1a and 2a 3 days before exposure to persistently infected cattle were not completely protected because a substantial proportion of those calves developed viremia.

Modified-live virus vaccines cause a limited infection at the site of injection that emulates a natural infection and triggers a cell-mediated immune response characterized by the proliferation and differentiation of helper and cytotoxic T cells (Reber et al., 2006). Antibody production against viral protein antigens requires interaction between helper T cells and B cells. Several days are required for proliferation and differentiation of naïve lymphocytes into effector T cells and antibody-producing B cells to effectively control viral infection and replication (Janeway et al., 2005). On the basis of the results from the present study, the immune response induced by a BVDV MLV vaccine in calves vaccinated 3 days before challenge inoculation was insufficient to control BVDV replication in the upper portion of the respiratory tract, as evidenced by BVDV shedding in nasal secretions. It is speculated that under natural conditions, BVDV infection within 3 days after MLV vaccination will result in viral shedding for

approximately 7 days after exposure. Any virus shed by infected cattle puts other susceptible cattle at risk of becoming infected. However, the reduced frequency of BVDV isolation from the nasal secretions of calves vaccinated 5 and 7 days before challenge inoculation indicates that even 2 additional days of immune stimulation can result in a considerable difference in the physiologic immune response against BVDV infection.

Elements of innate immunity, such as type I interferon, might have played a role in the protection of calves that were vaccinated 5 or 7 days before challenge inoculation by minimizing the clinical signs associated with acute BVDV infection and the shedding of BVDV in nasal secretions (Chase et al., 2004). The molecular mechanisms by which type I interferon inhibits viral replication include the synthesis of proteins (eg, Myxovirus resistance factor, dsRNA dependent protein kinase, and 2'5' -oligoadenylate synthetase 1) that interfere with RNA transcription, protection of adjacent cells that have not yet been infected (antiviral state), and the induction of apoptosis of virus-infected cells, all of which impair BVDV viral replication (Lenschow et al., 2007).

A single dose of an MLV BVDV vaccine induced protection against type 1b BVDV infection in calves that were challenge inoculated with the virus soon (3 to 7 days) after vaccination as evidenced by a lack of fever and leukopenia, as well as a reduced frequency of viremia and viral shedding throughout the observation period. However, a high proportion of calves inoculated with BVDV 3 days after vaccination shed the virus in nasal secretions between days 3 and 9 after BVDV challenge inoculation.

III. Bovine Viral Diarrhea Virus Fetal Persistent Infection following Immunization with a Contaminated Modified-live Virus Vaccine.

Abstract

The aim of this study was to determine whether a multivalent modified-live virus (MLV) vaccine containing noncytopathic (ncp) bovine viral diarrhea virus (BVDV) administered off-label to pregnant cattle can result in persistently infected (PI) fetuses and assess whether vaccinal strains can be shed to unvaccinated pregnant cattle commingling with vaccinates. Nineteen BVDV-naïve pregnant heifers were randomly assigned to two groups: cattle vaccinated near day 77 of gestation with MLV vaccine containing BVDV-1a (WRL strain), BHV1, PI_3, and BRSV (Vx; n=10) or control unvaccinated cattle (Co; n=9). During the course of the study a voluntary stop-sale/recall was conducted by the manufacturer due to the presence of BVDV contaminant in the vaccine. At day 175 of gestation, fetuses were removed by caesarean section and fetal tissues were submitted for virus isolation, and *q*RT-PCR using BVDV-1- and BVDV-2-specific probes. Nucleotide sequencing of viral RNA was performed for *q*RT-PCR positive samples. Two vaccinated and two control heifers aborted, but their fetuses were unavailable for BVDV testing. BVDV was isolated from all 8 fetuses in Vx heifers and from 2 of 7 fetuses in Co heifers. Only BVDV-2 was detected in fetuses from vaccinates, whereas only BVDV-1 was detected in the 2 fetuses from controls. Both BVDV-1 and BVDV-2 were detected in the vaccine. In conclusion, vaccination of pregnant heifers with a contaminated modified-live BVDV vaccine resulted in development of BVDV-2 PI fetuses in all the tested vaccinated animals. BVDV was apparently shed to unvaccinated heifers causing fetal infections from which only BVDV-1 was detected.

Keywords: Bovine viral diarrhea virus, Modified-live virus vaccine, Persistent infection, Vaccine contamination.

1. Introduction

Bovine viral diarrhea virus (BVDV) is a positive-sense, single stranded RNA virus belonging to the genus *Pestivirus* within the *Flaviviridae* family (Lindenbach and Rice, 2001). The BVDV genome consists of a single open reading frame (ORF) that is flanked by 5' and 3' untranslated regions (UTRs) (Collett et al., 1988a; Collett et al., 1988b; Deng and Brock, 1992). The 5'UTR role is an internal ribosome entry site that promotes cap-independent translation initiation (Pestova and Hellen, 1999; Poole et al., 1995). The ORF encodes a polyprotein (4000 amino acids) that is co- and post-translationally cleaved by viral and cellular proteases into mature viral proteins including Npro, C, Erns, E1, E2, p7, NS2-3, NS4A,NS4B, NS5A, NS5B (Rice, 1996).

There are two BVDV biotypes, cytopathic (cp) and noncytopathic (ncp), based on their capacity to cause pathogenic effects on cell cultures (Hamers et al., 2001). Two different genotypes (BVDV-1 and BVDV-2) have been identified with remarkable differences in genomic sequences of the 5´ UTR, N-pro and E2 regions (Ridpath, 2003). Bovine viral diarrhea virus is a major infectious agent that affects cattle production worldwide (Baker, 1995; Houe, 1999). Acute infections with some strains may cause clinical presentations of gastrointestinal, reproductive, respiratory disease, and immunosuppression (Fulton et al., 2005a; Makoschey et al., 2001).

Infection of pregnant cattle with BVDV may result in abortions, stillbirths, congenital defects and the birth of persistently infected (PI) calves. Persistent infections occur if a susceptible pregnant cow is infected with a ncp BVDV strain at 30 to 125 days of gestation (Brock et al., 2005). At this time of gestation, the fetal immune system is not completely developed and not able to recognize BVDV as a foreign antigen; thus, the fetus becomes immunotolerant of the infecting BVDV strain. Such calves continually shed large amounts of BVDV, representing a risk to susceptible herdmates (McClurkin et al., 1984). Consequently, while their prevalence is less than 1%, these PI cattle have been recognized as the main source for spread of BVDV within and among cattle herds (Houe et al., 1995; Loneragan et al., 2005; Wittum et al., 2001).

Strategies to prevent and control BVDV include biosecurity measures to avoid introduction of infected animals in the herd, quarantine of animals to control spread between herds, identification and slaughter of PI animals, and vaccination (Brock, 2004; Kelling et al., 2000; Reber et al., 2006). Both inactivated and modified live BVDV vaccines are available. Modified-live virus (MLV) vaccines have the ability to induce high neutralizing antibody levels (Brock et al., 2006; Dean and Leyh, 1999) and a strong cell mediated immunity (Platt et al., 2009b). The efficacy of modified-live BVDV vaccines to prevent fetal infections and the development of PI animals has been evaluated in several studies, reporting values between 57.9 and 100% (Brock and Cortese, 2001; Brock et al., 2006; Cortese et al., 1998a; Dean et al., 2003; Fulton, 2005; Leyh et al., 2011; Rodning et al., 2010a). However, a remarkable disadvantage of MLV vaccines is that these vaccines contain limited antigen mass, requiring viral replication in the host in order to develop an optimal immunity (Bálint et al., 2005).

During the replication cycle, a live virus could recombine or mutate and occasionally revert to virulence and be shed to other susceptible individuals, resulting in severe clinical consequences (Lancaster and Pfeiffer, 2011). Additionally, live BVDV could cross the placental barrier and infect the fetus causing abortion, stillbirth and developmental defects (Liess et al., 1984; Orban et al., 1983). A major concern of ML BVDV vaccines is the occurrence of mucosal disease (MD) following vaccination (Fulton, 2005). Postvaccinal MD could result when a PI animal is exposed to the cp BVDV strain contained in the vaccine (Fulton, 2005). A further disadvantage of MLV BVDV vaccines is their immunosuppressive effect on leukocyte function, resulting in increased susceptibility to other infections (Roth and Kaeberle, 1983). Furthermore, MLV vaccines have the potential risk for contamination with adventitious virulent strains becoming a source of spread of BVDV infections (Nuttall et al., 1977).

Most of the US commercial vaccines contain cp BVDV strains (Nuttall et al., 1977; Ridpath, 2005b); however, there are some MLV vaccines containing ncp BVDV isolates, which might represent a risk for the development of PI animals if these vaccines are administered off-label during gestation. Additional risk exists if susceptible pregnant cows are exposed to recently vaccinated animals a few days following immunization. Therefore, the use of ncp BVDV strains in MLV vaccines still generates safety concerns for veterinarians and cattle producers about the risk of persistent infections.

The initial aim of this study was to determine whether a multivalent modified-live virus (MLV) vaccine containing ncp BVDV could result in PI fetuses when administered off-label to seronegative pregnant cattle at approximately 77 days of gestation and assess whether vaccinal strains can be shed and infect unvaccinated pregnant cattle.

During the course of this study a voluntary stop sale/recall was conducted by the manufacturer due to the presence of an extraneous contaminant strain in some vaccine lots (written communication by Intervet, Schering Plough Animal Health®). This unexpected incident redirected the aims of our study toward the determination of the effects of the contaminated MLV vaccine on BVDV-naive pregnant heifers. In the present study we show evidence of BVDV fetal persistent infections following off-label immunization of pregnant heifers with a contaminated MLV BVDV vaccine. Even though the contaminated vaccine contained both BVDV-1 and BVDV-2, *q*RT-PCR and nucleotide sequencing analysis revealed that vaccinated heifers developed only BVDV-2 PI fetuses. Furthermore, BVDV was apparently shed to unvaccinated heifers causing fetal infections from which only BVDV-1 was detected.

2. Methods

2.1. Animals

A total of 25 nonpregnant beef heifers were enrolled in this study. All heifers were clinically normal, free of BVDV based on virus isolation, and seronegative to both BVDV-1 and BVDV-2 based on serum virus neutralization assays performed at serum dilution of 1:2. The bulls for breeding were previously confirmed free of persistent BVDV infection by immuno-histochemistry of ear notch samples.

2.2. Synchronization and breeding

Heifers were synchronized for estrus using 2.0 ml of gonadotropin releasing hormone (GnRH, Cystorelin, Merial, Duluth, GA) IM. Seven days later, 5 ml of $PGF_2\alpha$ (Dynoprost tromethamine; Lutalyse, Pfizer, Kalamazoo, MI) IM were administered to eliminate any luteal

structure present in the ovaries. On the same day, two healthy bulls of recognized fertility, BVDV seronegative and virus isolation negative were placed with the heifers for breeding. The day that the bulls were introduced was considered the start breeding day. Bulls remained with the heifers for 24 days. Day 12 after $PGF_2\alpha$ injection was considered the average breeding day (day 0). Pregnancy diagnosis was performed by transrectal palpation 58 days after the average breeding day by an experienced veterinarian (Figure 3). Nineteen heifers became pregnant with a gestational age between 46 and 70 days. On day 77 after the average breeding day, fetal viability was confirmed in 19 pregnant heifers using transrectal ultrasonography, by evidencing the fetal heart beat.

2.3. Vaccination

On day 77, Nineteen heifers confirmed to be pregnant were randomly assigned, (using the Research Randomizer, www.randomizer.org), into two experimental groups: Vaccine group (Vx, n=10); and control unvaccinated group (Co, n=9). The vaccine was a USDA-licensed stock material released for sale. This vaccine is a multivalent modified-live combination viral vaccine containing BVDV-1a (strain WRL), infectious bovine rhinotracheitis (IBR) virus, parainfluenza 3 (PI_3) virus, and bovine respiratory syncytial virus (BRSV). The BVDV-1a strain WRL was attenuated by serial passage in swine kidney cells (Dean and Leyh, 1999). A single 2-ml dose of the vaccine was administered subcutaneously off-label to pregnant cattle in the neck region using a sterile 18-gauge x 1-inch needle for each animal at 77 ± 12 days of gestation (65-89 days of gestation). All nineteen heifers were housed in the same field and allowed to eat and drink from the same water troughs and feeders.

2.4. Blood sample collection

Blood samples were collected at the time of vaccination (approximately 65–89 days of gestation) and 45 days post-vaccination (122 days of gestation). Sera were separated from the blood and were used to determine virus neutralizing (VN) antibody titers to both BVDV-1a and BVDV-2.

2.5. Virus neutralizing antibody analysis

Serum virus neutralization was performed at serial two-fold dilutions of heat-inactivated serum starting at 1:5 through 1:640 using 100 $CCID_{50}$ of noncytopathic BVDV-1a (SD-1) and BVDV-2 (PA131) and incubated for 1 hour at room temperature before the addition of MDBK cells (1.5 x 10^5 cells / ml). Cultures were incubated for 72 hours at 37° C in 5% CO_2. The VN antibody titer of each sample was determined using the Reed and Muench's method. The endpoint dilutions reflected the highest dilution of serum that inhibited the replication of virus in cell culture. This was determined by immunoperoxidase (IP) staining using a bovine BVDV specific polyclonal antibody (B-224).

2.6. Fetal harvest and tissue collection

Vaccination with the MLV ncp BVDV strain was performed at approximately 65–89 days of gestation, when the fetuses did not have a fully developed immune system. In order to determine if the fetuses did become PI, pregnant heifers were submitted for caesarean sections at 100 days post-vaccination [175 ± 12 days of gestation (range of gestation length: 163-187 days)], for fetal sample collection. Before performing the cesarean section heifers were palpated transrectally to confirm the presence of the fetus.

During fetal extraction, heart blood (2 ml) and amniotic fluid (2 ml) were obtained for BVDV isolation. Fetuses were identified by placing a tag in one of the legs with a number which corresponded with the dam's identification number. Fetuses were placed in individual sterile bags and transported to the Auburn University pathology necropsy room. Tissue samples (approximately 2 cm^2 of spleen, thymus, lung, liver and kidney) were collected from each fetus using appropriately disinfected surgical instruments. Tissue samples were placed in individual sterile bags and stored at -80° C to be processed for BVDV isolation.

2.7. Virus isolation from fetal tissues

Fetal tissue samples were macerated and homogenized manually for approximately 5 minutes within the sterile bags adding 2 ml of Dulbecco's Modified Eagle's Medium (DMEM) containing antibiotics, and supplemented with 10% equine serum, L-Alanyl and L-glutamine. For virus isolation 250 µl of the homogenized tissue fluids, amniotic fluid and heart blood was added to 25 cm^2 tissue culture flask containing a monolayer of Madin-Darby bovine kidney (MDBK) cells. For cell culture, DMEM supplemented with 10% equine serum, L-Alanyl and L-glutamine was also used. After three days incubation at 37° C and 5% CO_2, flasks were frozen and upon thawing, 50 µl of the cell suspension from each flask were transferred to three wells of a 96 well plate seeded with MDBK cells (first plate). The inoculated 96 well plate was incubated 3 days and the culture medium from each well was transferred to the corresponding wells of a new 96-well plate previously seeded with MDBK cells (second plate). Positive (BVDV) and negative (cells only) control wells were included on each plate. Both 96-well plates (first and second) were tested for BVDV antigen by using IP staining.

2.8. Quantitative reverse transcription PCR (qRT-PCR)

Total RNA was extracted from the vaccine and fetal tissues homogenates (spleen and thymus) using RNeasy Mini Kit (Qiagen Inc, Valencia, CA) according to the manufacturer's directions. Differential detection of BVDV-1 and BVDV-2 5' UTR was done by a two-step *qRT-PCR* with specific primers and probes (Table 4) (Marley et al., 2008). Bovine viral diarrhea virus-1 was monitored by the amplification of a 137 bp fragment, and BVDV-2 was detected with a 134 bp fragment.

The primers BVDu3 (5' CATGCCCAAAGCACATCTTA 3') and BVDl1 (5'TGCCATGTACAGCAGAGATTT3') were used (Studer et al., 2002). The 5' probe (5'AYGRAYACAGCCTGATAGGGTGY3') was labeled with carboxyfluorescein (6-FAM) at the 3' end. The 2nd, 6th, and 23rd bp contained a Y (C+T) and the 4th bp contained an R (A+G) so that it could detect both BVDV-1 and BVDV-2. Two 3' probes were used, one for detection of BVDV-1 and one for detection of BVDV-2. The 3' probe for detection of BVDV-1 (5' CAGAGGCCCACTGTATTGCTACTAAA 3') was labeled with Bodipy® 630 at the 5' end and a phosphate at the 3' end. The 3' probe for detection of BVDV-2 (5' CAGAGACCTGCTATTCCGCTAGTAAA 3') was labeled with Bodipy® TR-X at the 5' end and a phosphate at the 3' end. Primers and probes for detection of BVDV were synthesized by Qiagen (Table 4).

The Roche LightCycler 2.0 (Indianapolis, IN, USA) has the ability to detect multiple reporter dyes in the same capillary. However, each reporter dye can be detected by more than one detection channel. This was corrected by creating a color compensation file that was applied to each analysis.

Reverse transcription (RT) was performed separately from the qPCR. A master mix for reverse transcription was prepared containing 2 μL RNase-free water, 2 μL buffer RT (10X, Qiagen), 2 μL dNTP (Qiagen), 1 μL RNasin® RNase inhibitor (40 U/ μL, Promega, Madison, WI, USA), 1 μL Omniscript RT (Qiagen), and 2 μL BVDV primer mix (10 μM BVDu3 and l1). Ten μL of master mix was added to each tube followed by 10 μL of sample. The tubes were incubated in a heating block set at 37 °C for 1 h. Following reverse transcription, the qPCR was performed.

An oligonucleotide mix was prepared. The mix for detection of BVDV-1 and BVDV-2 contained 5 μM of each primer and 1 μM of each probe. Before running each reaction, a master mix was prepared. The qPCR master mix contained 4 μL of the oligonucleotide mix, 10 μL QuantiTect Multiplex PCR NoRox Master Mix (Qiagen), 2.8 μL RNase-free water, and 0.2 μL uracil-N-glycosalase (Roche; UNG; 1 unit/μL). The QuantiTect Multiplex PCR NoRox Master Mix consisted of HotStarTaq® DNA Polymerase, QuantiTect Multiplex PCR Buffer, dNTP mix (deoxyribonucleotide triphosphate), and 11 mM $MgCl_2$. The dNTP mix contained dATP, dCTP, dGTP, and dTTP/dUTP.

Reactions were performed in 20 μL capillaries. Seventeen μL of the qPCR master mix was added to each capillary, followed by 3 μL of reverse transcribed sample. The capillaries were centrifuged and then placed in the Roche LightCycler 2.0. The following program was performed with a 20 °C/s ramp unless otherwise indicated: UNG carry-over prevention at 50 °C for 2 min; PCR initial activation step at 95 °C for 15 min; 50 cycles of three step cycling consisting of denaturation at 94 °C for 1 min, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s at 2 °C/s; melting curve at 95 °C, 50 °C for 10 s, and then heating to 80 °C at 0.1 °C/s; and cooling to 40 °C for 1 min.

To allow quantitation of PCR products, external standards were constructed. The complementary DNA from BVDV RNA was amplified and subsequently purified by high-resolution agarose gel electrophoresis and filtration. The DNA was quantified using the PicoGreen® DNA fluorescence assay (Molecular Probes, Eugene, OR, USA) and used at 10^7, 10^4, 10^3, 10^2, and 10 genome copies per 5 µL in a background of 100 ng purified salmon sperm DNA in TE buffer (10 mM Tris–HCL and 0.1 mM EDTA). The fluorescence channels used were 640 nm for detection of BVDV-1 and 610 nm for detection of BVDV-2. The fluorescence data acquisition was quantified during the annealing phase. The Roche LightCycler Software Version 4.0 was used.

2.9. Sequence analysis of cDNA generated by RT-PCR from BVDV isolates

Sequence analysis of cDNA from BVDV 5'-UTR (product of RT-PCR using BVDV-100 and BVDV-345 primers) was performed for the vaccine and BVDV-positive fetal spleen samples in triplicates. Amplified cDNA was purified using the QIAquick PCR purification kit (QIAGEN) and submitted to Massachusetts General Hospital DNA core facility for sequencing using the primer BVDV-345 for the 5' UTR (10 ng/µl) (Table 1). Thus, a sequence of approximately 245 nucleotides of the 5' UTR was obtained. Sequences were aligned with published BVDV 5'-UTR sequences obtained from the National Center of Biotechnology Information GenBank (NCBI, Bethesda, MD, USA) using the Basic Local Alignment Search Tool (BLAST, http://www.ncbi.nlm.nih.gov/blast/Blast.cgi, verified November 2011).

Sequences were assembled and aligned using Mac Vector 10.6.0 software (Mac Vector Inc., Cary, NC). Sequence chromatograms were carefully examined to identify nucleotide positions with more than one nucleotide (evident major and minor nucleotide peaks in the same

nucleotide position), indicating the presence of relevant levels of another viral sequence, as previously described by other researchers (McKinley et al., 2008; Van Santen and Toro, 2008). Thus, we evaluated the sequences and identified nucleotide positions with minor nucleotides comprising > 10% of the peak height of the major nucleotide (Van Santen and Toro, 2008). For sequences showing detectable levels of more than one BVDV population, the sequences were called principal or contaminant viral sequences, based on the major or minor nucleotide peaks, respectively. Additional alignments were done between BVDV 5'-UTR sequences from fetal spleen samples and BVDV 5'-UTR vaccinal sequences.

2.10. Statistical analysis

Data were analyzed using the Statistical Analysis System version 9.1 (SAS Institute, Cary, NC). The frequency of virus isolation from fetal tissues for each group was analyzed using PROC FREQ and compared through the chi-square test. A log base 2 transformation was applied to virus neutralizing titers before calculation and comparison of the means. Back-transformed geometric means for each viral strain tested were presented for each group at the time of vaccination, as well as 45 days post-vaccination. A paired t-test was used to compare the geometric mean antibody titer between day 0 and day 45 for each group. Seroconversion was considered if there was a 4-fold or greater rise in the antibody titer from vaccination to day 45 post-vaccination. Values of $P < 0.05$ were considered statistically significant.

3. Results

3.1. Serological response

All heifers were seronegative to both BVDV-1a (SD-1) and BVDV-2 (PA131) at the time of vaccination (antibody titer <1:5). Serum VN antibody titers to BVDV-1a and BVDV-2 were dramatically increased in all the vaccinated heifers on day 45 following vaccination (Table 5), with geometric mean titers (GMT) of 1:130 and 1:149, for BVDV-1 and BVDV-2, respectively (Table 6). Therefore, all vaccinated heifers showed a significant seroconversion against both BVDV genotypes 45 days after vaccination ($P < 0.05$). Interestingly, four unvaccinated heifers seroconverted against BVDV-1a (465, 476, 509, 542), showing significantly high VN antibody titers on day 45 following vaccination ($P< 0.05$ Table 6). These control heifers had a GMT against BVDV-1a of 1:211 (Table 6). Only one heifer (465) in the control group developed high VN antibody titers against BVDV-2 (1:160; Table 5).

3.2. Virus isolation from fetal tissues. Fetal BVDV persistent infection.

Two out of 10 vaccinated heifers (20.0%) aborted before the day of the cesarean section and fetal collection; therefore, those fetuses were not available to be collected for virus isolation. All fetuses recovered from vaccinated heifers (8/8, 100%) were BVDV positive (lungs, spleen, kidney and thymus), indicating that the fetuses were PI (Tables 7 and 8). In the control group, 2 out of 9 heifers (22.2%) aborted before the time of fetal harvest. These aborted fetuses were not available for virus isolation. Virus isolation test was performed on tissues from seven fetuses in the unvaccinated group. Bovine Viral Diarrhea Virus was isolated in two out of seven fetuses (28.5%) collected from the unvaccinated heifers (Tables 7 and 8). Conversely, BVDV was not consistently isolated from fetal fluids (amniotic fluid and heart blood) in both vaccinated and

control groups (Table 7). As expected the frequency of virus isolation was significantly higher in the vaccinated group compared with the control group ($P < 0.05$; Figure 4). However, BVDV caused infection in some fetuses carried by unvaccinated heifers commingling with the immunized animals.

3.3. Quantitative Reverse Transcription PCR (qRT-PCR).

Viral RNA extracted from fetal spleen and thymus was assayed for differential detection of BVDV-1 and BVDV-2 by *qRT-PCR* with specific primers and probes (Marley et al., 2008). The *qRT*-PCR system was previously evaluated for detection of positive controls [BVDV-1 (one BVDV-1a strain and four BVDV-1b strains) and BVDV-2 (AU501 and 134F strains)]. All of these BVDV strains were detected in the appropriate detection channels (Marley et al., 2008). Bovine viral diarrhea virus was detected in all spleen and thymus samples derived from vaccinated animals (n=8). Only two fetuses in the control group (509 and 542) were BVDV positive by *qRT-PCR*.

3.3.1. Specific detection of BVDV-1 (LightCycler channel 640).

Bovine viral diarrhea virus-1 was not detected in any of the fetal spleen and thymus samples collected from the vaccinated heifers. In contrast, BVDV-1 was detected in the two BVDV- positive fetuses from the control group (509 and 542; Table 9).

3.3.2. Specific detection of BVDV-2 (LightCycler channel 610).

All the spleen and thymus samples from vaccinated animals (n=8) were positive for BVDV-2. On the other hand, BVDV-2 was not detected in any fetal sample from the control

group (Table 9). The concentration (number of viral copies/μl) of BVDV-1 and BVDV-2 in fetal spleen and thymus samples from vaccinated and control groups is documented in Table 9.

3.3.3. Detection of BVDV-1 and BVDV-2 in the vaccine

Both BVDV-1 and BVDV-2 were detected in the vaccine using the previously described *q*RT-PCR system with specific primers and probes.

3.4. Sequencing analysis of cDNA generated by RT-PCR from BVDV

A portion of BVDV 5' UTR corresponding to base positions 100 to 345 was consistently amplified and sequenced from the vaccinal and BVDV-positive fetal spleen samples (8 from the vaccinated group and 2 from the control group). Sequence chromatograms of the vaccine RT-PCR products were consistent with the presence of major and minor peaks at the same nucleotide positions, indicating the presence of two BVDV sequences in the vaccine (Figure 5). This finding confirmed the manufacturer's recall about the presence of a BVDV contaminant in the vaccine. These two sequences were read separately and subsequently classified as principal and contaminant vaccinal BVDV populations, based on the major or minor nucleotide peaks, respectively (Table 12). In contrast, chromatograms of the 10 sequences from spleen RT-PCR products showed an evident single peak at any nucleotide position, suggesting the presence of only a major BVDV population.

Alignment analysis using BLAST is shown in Tables 10 and 11. Alignment on the vaccine principal BVDV sequence revealed 95 to 99% identity with 5'UTR of several BVDV-1 strains (e.g. NADL, SD1 and Oregon CV-24). On the other hand, the sequence obtained from minor peaks (contaminant sequence) showed 95-98% homology with the 5'UTR of diverse

BVDV-2 strains (e.g. NY-93, 1373 and 890; Table 11). Alignment of sequences from fetal spleen samples of the vaccinated group revealed high identity with several published BVDV-2 5'UTR sequences. In contrast, sequences from spleen samples 509 and 542 (control group) showed 98% identity with previously published 5'UTR BVDV-1 nucleotide sequences (e.g. NY-1 and Osloss; Table 10). All the samples in the vaccinated group exhibited a highly conserved 5' UTR BVDV sequence, which had 98.9% homology (172 out of 174 nucleotides were identical) with the contaminant sequence found in the vaccine (Table 12). Consistently, there was only one nucleotide mismatch between sequences in the vaccinated group and the vaccinal contaminant sequence. However, such sequences of the vaccinated group did not align at all with the principal vaccinal BVDV sequence. Conversely, alignment of the BVDV sequences obtained from control fetal spleens (509 and 542) showed a moderate-high identity (88.8 and 89.3%) with the principal vaccinal BVDV sequence. No homology was found between these two sequences and the vaccine contaminant sequence (Table 12).

4. Discussion

In the present study, abortions and BVDV fetal persistent infections occurred following off-label vaccination of BVDV-naïve pregnant heifers with a contaminated MLV vaccine. Additionally, findings suggest that BVDV was shed from vaccinates to herdmate control heifers which also resulted in fetal infections. All fetuses recovered from vaccinated heifers developed BVDV persistent infections, which were consistently detected by VI from spleen, thymus, lungs and kidneys. Interestingly, two of the fetuses harvested from the control group were BVDV-positive. Infection with a ncp BVDV strain between 30 and 125 days of gestation may result in fetal death and abortion (Brock et al., 2005). Fetuses that survive this infection develop BVDV

specific immunotolerance and persistent infection (Brock et al., 2005). Circulation of ncp BVDV during the period when fetal immunocompetence is developing (90-120 days of gestation) is the main mechanism leading to persistent infection. Therefore, the fetal immune system accepts the viral antigens as part of the self-antigen repertoire (Peterhans et al., 2003; Potgieter, 1995), with a resulting negative selection of BVDV specific B and T lymphocytes (Donis and Dubovi, 1987). Consequently, BVDV immunotolerance results in absence of humoral and cell mediated immunity to the virus and failure to clear the infection and a persistent viremia (Hansen et al., 2010).

Modified-live virus vaccines develop a limited infection at the site of entry emulating a natural infection, aimed to trigger both humoral and cell mediated immune responses (Reber et al., 2006). Currently, in the United States some licensed MLV vaccines containing cp BVDV are approved to be administered to pregnant cattle or calves nursing pregnant cows, provided the heifers or cows have been vaccinated prior to breeding or/and within the past 12 months. Moreover, the use of MLV vaccines containing ncp BVDV strains to prevent fetal persistent infections has been proven to be efficacious and safe when administered prior to breeding under the manufacture's recommendations (Brock et al., 2006; Dean et al., 2003; Dean and Leyh, 1999). The vaccine used in the present study was labeled with a caution for not being used in pregnant cows or calves nursing pregnant cows. However, there is a potential risk for transplacental fetal infection and development of PI fetus if susceptible pregnant animals have an occasional contact with recently vaccinated animals.

All vaccinated heifers showed a significant seroconversion against both BVDV-1 and BVDV-2 by day 45 following vaccination. Recent studies have shown that immunization with BVDV-1a ML vaccines can confer an adequate antibody response, and cross-protection against

clinical disease associated with both BVDV-1b and BVDV-2 (Dean and Leyh, 1999; Kelling et al., 2007; Xue et al., 2010b). In contrast, other field studies suggest that vaccination with BVDV-1 can elicit certain but not complete immune response and fetal protection against heterologous BVDV-2 challenge (Bolin et al., 1991; Kelling et al., 1990; Van Campen et al., 2000). Thus, the effect of antigenic diversity on fetal protection is evident by different rates of protection provided by monovalent BVDV-1 vaccines when the challenge is performed with BVDV-1 and BVDV-2 (Brock et al., 2005). It is possible to infer that the BVDV-2 contaminant in the vaccine could have been responsible for eliciting a high virus neutralizing antibody response against BVDV-2 in the vaccinated group. Four heifers in the control group seroconverted against BVDV-1 (465, 476, 509 and 542), and only one against BVDV-2 (465), indicating that unvaccinated animals commingling with vaccinated heifers were exposed to BVDV. Two of these heifers aborted (447 and 465) and the other two developed PI fetuses (509 and 542) detected by VI and *q*RT-PCT. These findings suggest that BVDV was apparently shed by the vaccinated heifers resulting in fetal infections in the control group.

Quantitative RT-PCR and nucleotide sequencing analysis revealed the presence of both BVDV-1 and BVDV-2 in the vaccine, confirming the vaccine contamination reported by the manufacturer. The vaccine contained similar concentration of BVDV-1 (principal strain, 1.65 x 10^5 viral copies/µl) and BVDV-2 (contaminant strain, 1.50 x 10^5 viral copies/µl). Several reports have been published about contamination of experimental and commercially available live vaccines with exogenous BVDV strains (Bálint et al., 2005; Barkema et al., 2001; Falcone et al., 2003; Falcone et al., 1999; Jagodzinski et al., 2002; Vilcek et al., 1998). Commercial vaccines contaminated with extraneous BVDV strains could cause severe clinical consequences in inoculated herds. In Europe, severe outbreaks of BVDV-2 infection (≥70% morbidity) have

been associated with the use of batches of modified live bovine herpes virus-1 (BHV-1) marker vaccine contaminated with BVDV (Barkema et al., 2001; Falcone et al., 1999; Jagodzinski et al., 2002). In contrast, a concurrent report showed that vaccination of calves with BHV-1 marker vaccine batches contaminated with BVDV-1 did not result in clinical BVDV infections (Antonis et al., 2001). In the present study, clinical signs of disease were not monitored during the experimental period. Common sources of BVDV contamination include permanent cell lines used for viral replication-attenuation and fetal calf serum batches (Jagodzinski et al., 2002; Nuttall et al., 1977; Pastoret, 2010). Yanagi et al. (1996), in an attempt to establish cell cultures for hepatitis C virus demonstrated that a significant number of commercially available bovine sera are contaminated with BVDV (Yanagi et al., 1996). A BVDV contamination rate in bovine sera has been estimated between 10 and 75% (Bolin, 1991; Rossi et al., 1980). Conversely, Audet et al. (2000) investigated possible contamination of 38 lots of viral vaccines using RT-PCR for the presence of BVDV (Audet et al., 2000). The study demonstrated that all vaccines were negative for contaminating BVDV RNA, suggesting that currently US licensed viral vaccines are unlikely to be contaminated with pestiviruses (Audet et al., 2000). However, it is recommended that bovine sera and cell lines be screened for adventitious BVDV by RT-PCR when used for cell cultures. This strategy would help to prevent contamination before and during vaccine production and increase the product's safety (Barkema et al., 2001; Yanagi et al., 1996).

Only BVDV-2 was consistently detected in all fetuses recovered from vaccinated heifers, whereas BVDV-1 but not BVDV-2 was detected in the two fetuses from the control group. This result is consistent with previous reports where only one BVDV isolate was detected in most or all calves born to dams that had been inoculated with a mixture of two or more BVDV strains (Brock and Chase, 2000; Frey et al., 2002; Zimmer et al., 2002). All BVDV genotypes (1a, 1b

and 2) are capable of causing persistent infections (Brock et al., 2005). The simultaneous experimental challenge of pregnant cattle with both BVDV-1 and BVDV-2 has resulted in dual persistent infections with the two BVDV genotypes (Brock and Chase, 2000). In that study, dual BVDV persistent infections were consistently detected in fetal peripheral blood leukocytes but not in fetal tissues (Brock and Chase, 2000). Furthermore, RT-PCR amplification of RNA extracted directly from tissues revealed that BVDV-2 was identified in a greater number of tissues than BVDV-1 (Brock and Chase, 2000). The authors hypothesized that there could be differences in adaptation, level of replication, pathogenesis and/or tissue tropism between BVDV-1 and BVDV-2 during the fetal infection (Brock and Chase, 2000; Zimmer et al., 2002).

Recent studies performed in pregnant heifers challenged separately with both BVDV-1 and BVDV-2 in either nostril, revealed that dual transplacental infection with both viruses was not consistently reproduced (Makoschey and Janssen, 2011). On the other hand, only BVDV-2 was detected in all the PI calves born to inoculated dams (Makoschey and Janssen, 2011); being consistent with the results of the present study. Previous experiments have shown that an inoculated ncp BVDV-2 strain had apparently a slightly higher rate of transplacental infection of fetuses than BVDV-1b (Bielefeldt-Ohmann et al., 2008; Hansen et al., 2010). However, it is unknown whether this difference in ability to cross the placenta between the two genotypes is generalized or is a characteristic only of the strains used in that study (Bielefeldt-Ohmann et al., 2008). Additionally, further experimental assays to determine viral titers in cell culture following an inoculation with a 0.5 multiplicity of infection showed that BVDV-2 generated a 10-fold higher $CCID_{50}$ titer compared with BVDV-1 (Brock and Chase, 2000).

Viral homologous interference or superinfection exclusion has been previously described for pestiviruses (Mittelholzer et al., 1998). Superinfection exclusion is the ability of an

established viral infection to block or interfere with a homologous superinfecting virus. An *in vitro* experiment demonstrated that MDBK cells acutely infected with BVDV were protected from superinfection by homologous BVDV strains but not by heterologous vesicular stomatitis virus (Lee et al., 2005). A dual molecular mechanism for BVDV superinfection interference was demonstrated to occur at the level of viral entry and replication (Lee et al., 2005).

Only BVDV-1 but not BVDV-2 was detected in the two fetuses from the control group. It might be possible that following immunization with the contaminated vaccine, the BVDV-1 principal strain replicated more efficiently in the respiratory tract of the vaccinated group than the BVDV-2 contaminant strain. Therefore, the BVDV-1 strain could have been selectively shed via respiratory secretions being able to infect the control group causing the development of PI fetuses. Biologic and antigenic variability of BVDV have been well documented and some isolates could be more pneumotropic than others (Brock et al., 1988).

The alignment of the amplified BVDV 5' UTR sequences from fetal samples of the vaccinated group with BVDV-2 vaccine contaminant strain demonstrated high homology (98.9%). On the other hand, the BVDV 5' UTR sequences from spleen samples 509 and 542 (control group) did not align to the contaminant nucleotide sequence. These two sequences showed similar variation pattern when aligned with the vaccinal BVDV-1 principal sequence (88.8 and 89.3% homology). Differences in percentage of homology between the vaccinal strains and the respective BVDV sequences from fetal samples (98.9% for BVDV-2 and 88.8% for BVDV-1) may imply that the contaminant BVDV-2 strain was less attenuated than the BVDV-1 strain; since only two nucleotide changes were consistently detected in the BVDV-2 sequences amplified from the infected fetuses. The extent of changes in the nucleotide sequences in samples 509 and 542 might suggest continual selection of a more fit quasispecies over the

inoculated BVDV-1 population (due to host-virus interaction), as previously reported to occur after BVDV fetal infection (Jones et al., 2002) and vaccination with other single stranded RNA viruses (Gallardo et al., 2010; McKinley et al., 2008). Moreover, mutations or recombination leading to significant nucleotide changes could have also occurred during the animal passages (Becher et al., 1999; Jones et al., 2002).

In conclusion, vaccination of pregnant heifers with a contaminated modified-live BVDV vaccine containing both BVDV-1 and BVDV-2 resulted in the development of BVDV-2 PI fetuses in all the tested vaccinated animals. BVDV was apparently shed to unvaccinated heifers causing fetal infections from which only BVDV-1 was detected.

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IV. Expression of Type I Interferon-induced Antiviral State and Pro-apoptosis markers during Experimental Infection with Low and High Virulence Bovine Viral Diarrhea Virus in Beef Calves.

Abstract

The objective of this study was to compare the mRNA expression of host genes involved in type-I interferon-induced antiviral state (IFN-α, IFN-β, Mx-1, PKR, OAS-1 and ISG-15), and apoptosis (Caspase-3, -8, and -9), after experimental infection of beef calves with low or high virulence noncytopathic (ncp) bovine viral diarrhea virus (BVDV) strains. Thirty BVDV-naïve, clinically normal calves were randomly assigned to three groups. Calves were intranasally inoculated with low (LV; n= 10, strain SD-1) or high (HV; n= 10, strain 1373) virulence ncp BVDV or BVDV-free cell culture medium (Control, n= 10). Quantitative RT-PCR was used to determine the target gene expression in tracheo-bronchial lymph nodes and spleen 5 days after infection. Interferon-α and -β mRNA levels were up-regulated in tracheo-bronchial lymph nodes ($P<0.05$) in the HV group, but not in the LV group, compared with the control group. There was an up-regulation of type I interferon-induced genes in spleen and tracheo-bronchial lymph nodes of HV and LV groups, compared with the control group ($P<0.01$). mRNA levels of OAS-1 and ISG-15 were significantly higher in LV than HV calves ($P<0.05$). A significant up-regulation of caspase-8 and -9 was observed in tracheo-bronchial lymph nodes in the LV group ($P=0.01$), but not in the HV group. In conclusion, experimental infection with high or low virulence BVDV strains induced a significant expression of the type I interferon-induced antiviral state in beef

calves. There was a differential expression of some interferon-induced genes (OAS-1 and ISG-15) and pro-apoptosis markers based on BVDV virulence and genotype.

Key words: BVDV, Gene expression, Cytokines, Interferon, Antiviral state.

1. Introduction

Bovine viral diarrhea virus (BVDV) is an important infectious agent that affects cattle worldwide (Baker, 1995; Houe, 1999). BVDV is a positive-sense single stranded RNA virus within the genus *Pestivirus* and the *Flaviviridae* family (Lindenbach and Rice, 2001). Two different genotypes (BVDV-1 and BVDV-2) have been identified, with remarkable differences in genomic sequences of the 5´ UTR, N-pro and E2 regions (Ridpath, 2003).There are two virus biotypes cytopathic (cp) and noncytopathic (ncp), based on their ability to cause pathogenic effects on cell cultures (Hamers et al., 2001). Most BVDV acute infections are subclinical, transient and self-limiting. However, BVDV is considered a potentiator of secondary infections causing a clinical illness known as bovine respiratory disease complex (Baker, 1995). The increased susceptibility to secondary infections is a consequence of the ability of BVDV to cause immunosuppression, enhancing the pathogenesis of other infectious agents and impairing the response to treatments (Kapil et al., 2005). Infections with some virulent BVDV-2 strains may result in severe clinical disease and high mortality rate (Brock et al., 2007; Liebler-Tenorio et al., 2003b). Thus, the viral strain virulence and immune status of the animal are major factors determining the wide range of clinical presentations following BVDV acute infections (Ridpath et al., 2006).

It has been reported that the virulence of the BVDV strain is correlated with the ability of the virus to cause a decrease in lymphocyte counts (Kelling et al., 2002b; Liebler-Tenorio et al., 2003a; Liebler-Tenorio et al., 2003b). Thus, highly virulent BVDV induce a significantly more severe and longer lymphopenia and lymphoid tissue depletion than less virulent BVDV (Kelling et al., 2002b; Liebler-Tenorio et al., 2002; Liebler-Tenorio et al., 2003b). These evidences suggest a possible association between the BVDV virulence and the mechanisms by which this virus cause immunosuppression in susceptible cattle. In addition, BVDV may also cause immunosuppression by impairing the function of cells associated with both innate and acquired immune systems (Baigent et al., 2004; Charleston et al., 2001a; Glew et al., 2003; Jensen and Schultz, 1991; Lamontagne et al., 1989; Liu et al., 1999; Potgieter, 1995; Schweizer et al., 2006).

An important mechanism that has been implicated in the immunosuppression by BVDV is a decrease in the type-I IFN production and the associated molecular pathways (Baigent et al., 2004; Charleston et al., 2001a; Schweizer et al., 2006). Type-I IFNs (α and β) are structurally different cytokines produced in response to viral infections that bind the same receptors and inhibit viral replication (Abbas and Lithman, 2005). The molecular mechanisms by which type I IFN blocks viral replication include the synthesis of proteins such as myxovirus resistance factor (Mx-1), dsRNA dependent protein kinase (PKR), 2'5'-oligoadenylate synthetase-1 (OAS-1), and IFN-stimulated gene 15 kDa (ISG-15) (Sadler and Williams, 2008). These proteins serve a variety of roles that contribute to the immune defense including anti-proliferative and apoptotic effects, known as "antiviral state" (Shoemaker et al., 2009). During the antiviral state the IFN induced proteins interfere with transcription of RNA, protecting the neighbouring cells that have not been infected yet, and inducing apoptosis of the virus-infected cells (Lenschow et al., 2007).

The Mx proteins are GTPases that bind viral nucleocapsids or other viral components to degrade them (Sadler and Williams, 2008). In addition, PKR blocks viral replication and the production of virion progeny by inhibiting protein synthesis through the inactivation of eIF-2α (ribosomal initiation factor of protein synthesis) and upregulation of the cyclin-dependant kinase inhibitor $p21^{WAF1}$ (Kronfeld-Kinar et al., 1999). The OAS proteins activate the latent form of RNase L, which mediates RNA degradation (Sadler and Williams, 2008), interfering with transcription of viral RNA or DNA and viral replication. Moreover, RNase L also inhibits the host's cell transcription machinery which results in apoptosis of the virus-infected cells (Müller-Doblies et al., 2004). The ubiquitin-like protein ISG-15 has the ability to conjugate to a variety of intracellular proteins and thus influence their activity through posttranslational modification and enhancement of the antiviral response, including regulation of IRF3 and PKR (Zhao et al., 2005).

It has been reported that cp BVDV induces a strong IFN α/β response in host cells (Adler et al., 1997). On the other hand, *in vivo* and *in vitro* studies have shown that infections by ncp BVDV strains inhibited the induction of type-I IFN response (Baigent et al., 2002; Charleston et al., 2001a; Glew et al., 2003; Perler et al., 2000; Peterhans et al., 2003). More recent *in vivo* studies have concluded that acute ncp BVDV infection caused an increased type-I IFN response in the challenged calves (Brackenbury et al., 2005; Charleston et al., 2002; Müller-Doblies et al., 2004; Smirnova et al., 2008). The results of these *in vivo* studies suggest that immunosuppression caused by ncp BVDV is not associated with inhibition of type I-interferon response (Charleston et al., 2002). However, since BVDV strains show a wide range of genetic diversity and virulence, the mechanisms involved in immunosuppression as well as the level of immunosuppression might not be identical for all the BVDV strains existing in nature. It was

hypothesized that BVDV strains of distinct virulence and genotype may induce differently the mRNA expression of IFN-α and -β, type I IFN-induced genes, and pro-apoptosis related genes during experimental acute infections of beef calves. The objective of this study was to compare the mRNA expression of host genes involved in the type I interferon-induced antiviral state (IFN-α, IFN-β, Mx-1, PKR, OAS-1 and ISG-15), and the pro-apoptosis related genes Caspase-3, -8, and -9 in lymphoid tissues after experimental infection with low (strain SD-1) and high (strain 1373) virulence BVDV.

2. Methods

2.1. Animals

A total of thirty beef calves (7 months of age) were enrolled in this study. All calves were clinically normal, free of BVDV based on virus isolation from serum and immuno-histochemistry of ear notch biopsies, and seronegative to both BVDV-1 and BVDV-2 based on serum virus neutralization assays performed at serum dilution of 1:2. Animals were obtained from a farm owned by Auburn University, located in Huntsville, AL. This farm is characterized by an optimal biosecurity program in which new animal introduction is not permitted (closed system).

2.2. Experimental design and sample size calculation

The study was designed as a randomized, controlled trial. Sample size was calculated using Proc Power (SAS) on the basis of the expected means of target gene expression ($\geq$ 1.5 fold-regulation of mRNA level; normalized to the housekeeping gene and relative to the control

group) for each experimental group, the SDs (± 0.2 fold-regulation) of the means, and a statistical power of 99%.

Calves were randomly assigned by use of a random number generator (Research Randomizer, www.randomizer.org), to 1 of 3 treatment groups:

LV (n= 10): Animals challenged with a low virulence type 1a ncp BVDV (strain SD-1).

HV (n= 10): Animals challenged with a high virulence type 2 ncp BVDV (strain 1373).

Control (n= 10): Animals inoculated with BVDV-free cell culture medium.

Animals were transported from the farm of origin to the Auburn University BVDV-free farm three days before the beginning of the study. All 30 calves were housed together at the BVDV-free farm prior to inoculation. As treatment groups were inoculated, they were separated and transported to the Auburn University BVDV isolation farm, located at 1.6 miles away from the BVDV-free farm, except for the control group which stayed in the BVDV-free farm throughout the experimental period.

Additionally, in order to avoid shedding and exchange of BVDV among groups and undesired BVDV infection of the control group due to commingling with the BVDV inoculated calves, the study was performed during three different rounds of viral inoculation and necropsy, which were spatially and temporally separated. During the first week, 10 calves assigned to the LV group were separated and brought to the BVDV-isolation unit. After inoculation, LV calves stayed in the BVDV isolation farm until day 5, when they were euthanized and transported to the necropsy room of the Department of Pathobiology, College of Veterinary Medicine, Auburn University.

During the second week, 10 animals assigned to the control group were separated, transported for approximately 5 minutes around the same geographic area and returned to the

BVDV-free farm. This transportation was performed in order to provide similar management conditions to all groups. At the BVDV-free farm, these animals were separated from the rest of the calves and maintained in a different pasture. The control group was not brought to the BVDV-isolation farm in order to prevent any exposure to BVDV during the experimental period. On day 5, calves in the control group were euthanized and transported to the necropsy room.

During the second, third and fourth week, there were no BVDV- infected calves on the BVDV isolation farm. This resting time was included in the experimental protocol to favor viral clearance from the environment, in an attempt to prevent the presence of BVDV strain SD-1 on the farm by the time the HV group were brought to the BVDV isolation farm. It has been reported that BVDV can survive in a cool protected environment for several days (Houe, 1995). In addition, after departure of the LV group and before arrival of the HV group, concrete floors, chutes and pens at the BVDV isolation farm were disinfected using bleach (sodium hypochlorite, NaOCl 5.25 %). Finally, on the fifth week, 10 animals assigned to the HV group were separated and transported to the BVDV-isolation farm, where they were experimentally inoculated with BVDV-2 1373. On day 5, HV calves were euthanized and transported to the necropsy room.

At both farms, calves within each group (LV, HV or control) were housed together in the same pasture and allowed to eat and drink from the same feeders and water troughs. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Auburn University.

2.3. BVDV challenge inoculation

Calves were experimentally inoculated with a low (strain SD-1) or high (strain 1373) virulence ncp BVDV isolates. The strain BVDV1a SD-1 was originally isolated from a

persistently infected heifer (Deng and Brock, 1992). The strain BVDV-2 1373 was originally isolated from a severe acute BVDV outbreak in Ontario, Canada (Carman et al., 1998). The stock BVDV strains used in the present study were biologically cloned via successive passages in Madin-Darby bovine kidney (MDBK) cells by use of limiting dilutions with subsequent minimal propagation by incubation of the MDBK monolayer for 48 hours at 37 °C and 5% CO2 to produce an adequate amount of stock virus with the desired CCID50/mL for characterization and animal challenge exposure studies. The inocula used consisted of cell culture supernatants containing 1.3 X 10^5 CCID50/mL of BVDV-1a strain SD-1 and 1.3 x 10^5 CCID50/mL of BVDV-2 strain 1373 of inoculum in Dulbecco's Modification of eagle's medium (DMEM) supplemented with 10% equine serum, L-Alanyl and L-glutamine. The inocula were obtained following freeze-thaw cycles to disrupt the cells and release the viral particles. Inoculum cell culture supernatant was stored at -80 °C until the day of inoculation. After freezing, an aliquot of the BVDV inoculum was titrated by using the Reed-Muench method to determine the final CCID50/mL for inoculation. The inocula were aliquoted (5 mL) in individual tubes within 1 hour of inoculation and stored on ice until use. The inoculation was performed by intranasal aerosolization of 5 mL of inoculum by use of an aerosolizer and a vacuum pump. One sample of each inoculum was transported on ice and returned to the lab to determine the CCID50/mL after inoculation.

2.4. Sampling

From each calf, blood samples were collected on days 0, 2, 4, and 5 after BVDV challenge inoculation in blood tubes containing an anticoagulant for determination of the total leukocyte count. On day 5 post challenge, animals were euthanized using a captive bolt device

and transported to the necropsy room at the Department of Pathobiology, College of Veterinary Medicine, Auburn University. Necropsy examination was performed on all calves. Tissue samples were collected from spleen and tracheo-bronchial lymph nodes. Immediately after tissue collection, fresh samples were submerged in 5 ml of RNA*later* reagent (RNeasy protect kit, QIAGEN®), in order to be protected from RNA degradation by RNAse. The stabilized samples were stored at -80° C as recommended by the manufacturer until processing.

2.5. Clinical Pathology

Unclotted blood samples collected on days 0, 4 and 5 post-challenge were submitted to the clinical pathology service laboratory at the Auburn University College of Veterinary Medicine for leukocyte analysis. The total leukocyte count for each sample was determined by an automatic cell counter (Coulter. Corp., Miami, FL, USA).

2.6. Virus isolation

Virus isolation was performed on days 0 and 5 post challenge from serum samples and from spleen samples on day 5 post challenge to confirm BVDV infection and viremia in the inoculated animals. For virus isolation, 250 µl of each sample was added to individual 25 cm^2 tissue culture flasks containing a monolayer of MDBK cells. For cell culture, Dulbecco's Modification of eagle's medium (DMEM) supplemented with 10% equine serum, L-Alanyl and L-glutamine was used. After three days incubation at 37° C and 5% CO_2, flasks were frozen and upon thawing, 50 µl of the cell suspension from each flask was transferred to three wells of a 96 well plate seeded with MDBK cells (first plate). The inoculated 96-well plate was incubated 3 days and the culture medium from each well was transferred to the corresponding well of a new

96-well plate previously seeded with MDBK cells (second plate). Both 96-well plates (first and second) were tested for BVDV antigen by using IP staining, using a bovine BVDV specific polyclonal antibody (B-224).

2.7. mRNA Gene expression analysis

2.7.1. RNA extraction and purification

Spleen and tracheo-bronchial lymph node tissue samples (approximately 30 mg) were disrupted and homogenized in buffer containing guanidine isothiocyanate using a bullet blender machine (Next Advance® Averill Park, NY, USA) and 1 mm glass beads. Total RNA for quantitative real time RT-PCR (*q*RT-PCR) was extracted and purified from spleen and tracheo-bronchial lymph node samples using an RNeasy mini kit (QIAGEN®, Valencia, CA USA), according to the manufacturer's protocol.

2.7.2. Reverse transcription

Synthesis of cDNA was performed from 1µg of total RNA template using a First cDNA strand reverse transcription kit (Roche®, Indianapolis, IN USA) according to the manufacturer´s protocol. After reverse transcription, final cDNA products had a concentration of approximately 2 µg/µl in a total volume of 20 µl. Synthesized cDNA was diluted 10-fold with RNAse free water to reach a template concentration of approximately 200 ng/µl to be used for *q*RT-PCR. Four aliquots were prepared from each cDNA sample in order to avoid consecutive freezing-thawing events that could affect cDNA integrity and concentration.

2.7.3. Quantitative Real Time RT-PCR

Quantitative real time PCR was performed in 20 µL reactions to amplify the target gene sequences and determine the mRNA expression using a LightCycler (Roche®, Indianapolis, IN USA) and DNA Master^{plus} SYBR I green kit (Roche®, Indianapolis, IN USA). The PCR mix (15 µL) was prepared using 4 µL of the master mix, 9 µL of dd H_2O and 1 µL of each primer in a final concentration of 1 µM. Finally, 5 µL of cDNA template (200 ng/ µL) was added. The PCR reaction was conducted at 95 ◦C for 10 min (pre-incubation), followed by 45 cycles of 95˚C for 15 seconds (denaturation), 60-71˚C (primer dependent) for 10 seconds (annealing) and 72˚C for 10 seconds (extension). Upon completion of *q*RTPCR amplification, melting curve analysis was performed to evaluate the specificity and quality of the amplification. Melting curve analysis was done by incubation of the *q*RT-PCR products for 60 seconds at each step with temperature gradually increasing by 0.1˚C/sec from 65 to 95 ˚C. All samples were analyzed in duplicate to increase the accuracy of the results and the average of both runs was used to calculate the relative amount of mRNA. Non-template negative controls were concurrently run with the samples, replacing the template DNA with PCR-grade water.

2.7.4. Normalization and relative quantification of target genes

*q*RT-PCR procedure yielded crossing points or threshold cycle (CT) as the fundamental quantitative units, corresponding to the PCR cycle wherein the amount of DNA of the amplified gene generates a fluorescent signal significantly higher than the baseline. The CT value therefore correlates negatively to the amount of target mRNA, i.e. the higher the amount of mRNA, the sooner the threshold is reached and the lower the CT value obtained. Results for gene expression were analyzed with the comparative $^{\triangle\triangle}$Ct method (Livak and Schmittgen, 2001). Gene

expression analysis was performed through the relative quantification of the mRNA level or fold regulation of target genes normalized to a housekeeping gene (unaffected by the studied conditions) and compared with the uninfected control group (Livak and Schmittgen, 2001).

The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous reference gene. The efficiency of the *q*RT-PCR (necessary for the comparative $^{\triangle\triangle}$Ct method), was determined for each target gene by creating a standard amplification curve using 1µg, 100 ng, 10 ng, 1 ng and 0.1 ng per reaction of a cDNA template mix. The cDNA template mix was created by mixing equal amounts of cDNA solution from 3 tracheo-bronchial lymph node samples from each experimental group. Target genes and specific primer sequences are documented in Table 13.

2.8. *Viral RNA levels*

To allow quantitation of BVDV viral RNA levels, external standards were constructed. For the external standards, BVDV RNA from cell culture lysate was extracted using the QIAamp viral RNA kit (Qiagen, Valencia, CA, USA) to yield 60 mL of sample. The complementary DNA from BVDV RNA was amplified and subsequently purified by high-resolution agarose gel electrophoresis and filtration. The DNA was quantified using DNA Master^{plus} SYBR I green kit (Roche®, Indianapolis, IN USA) and used at 10^7, 10^6, 10^5, 10^4, 10^3, 10^2, and 10 genome copies per 5 mL in a background of 100 ng purified salmon sperm DNA in TE buffer (10 mM Tris–HCl and 0.1 mM EDTA). The Roche LightCycler Software Version 4.0 was used. Viral RNA was extracted using the QIAamp viral RNA kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. Levels of BVDV RNA were determined in tracheobronchial lymph node tissue samples using specific primers for 5' untranslated region.

2.9. Immunohistochemistry

Immunohistochemistry was performed on formalin-fixed paraffin-embedded sections of tissue samples (spleen, tracheobronchial and mesenteric lymph nodes, lungs and tonsils). Following fixation, tissues were processed routinely and embedded in paraffin wax for routine histopathology and immunohistochemistry (IHC). Sections (4 μm) were cut on silane-coated slides and dried. Sections were then dewaxed and rehydrated by sequential immersion in xylene followed by graded concentrations of ethanol, then tap water. IHC was performed with a commercial autostainer (DakoNorth America Inc., Carpinteria, California,USA). Blocking of endogenous peroxidase activity was performed with 3% H_20_2 and sections were pretreated with proteinase K prior to application of primary antibody. The monoclonal antibody (MAb) 15C5 (Syracuse Bioanalytical, East Syracuse, New York, USA) was utilized, for detection of BVDV antigen. Following incubation with the primary antibody, BVDV antigen was detected using a biotinylated link antibody followed by peroxidase labeled streptavidin (Dako). The substrate was NovaRED (Vector Laboratories, Burlingame, California, USA). The sections were counterstained with haematoxylin and coverslipped under non-aqueous mounting medium. Each BVDV-labelled tissue section was accompanied by a negative control slide in which BVDV antibody was replaced with primary antibody diluent. The location and intensity of antigen distribution were evaluated and compared among groups.

2.10. Statistical analysis

Data were analyzed using the Statistical Analysis System (SAS® version 9.1 SAS Institute, Cary, NC). Statistical parametric assumptions of normality and constant variance were tested through Shapiro Wilk's and Levene's tests, respectively. Additionally, normal probability,

residuals and quartile-quartile plots were constructed and analyzed to confirm any significant departure from normality and constant variability of the data. Since relative mRNA expression data were not normally distributed (heavily skewed), and had a heterogeneous variance, for the mean comparison analysis gene expression data were transformed using a Box-Cox power transformation, indexed with a power number suggested by data themselves, which maximized the parameter λ. The outcomes produced by the transformation were carefully examined for possible violations of the tentative assumption of normality.

The general linear model procedure (Proc GLM) was used for the analysis of variance to compare the relative mRNA level of target genes among groups (using the Least Significant difference, LSD Test). The general linear model procedure (Proc GLM) was also used for the analysis of variance to compare mean leukocyte counts among the groups (through the LSD Test). Mean comparisons between day 0 and subsequent days (4 and 5) were accomplished through the repeated measures analysis of variance for each group. Pearson correlation analysis was performed to identify correlated gene expression. Differences with *p*-values less than 0.05 were considered statistically significant.

3. Results

Two calves in the LV group were euthanized due to reasons not related with the BVDV infection (e.g. bone fracture due to aggresive temperament).

3.1. Clinical Pathology

A significant decrease in total leukocyte count was observed in the calves challenged with HV BVDV strain 1373 on days 4 and 5 compared with those in animals inoculated with LV

BVDV strain SD-1 and control group (*P*< 0.01). Significant differences were not found between LV and control groups (Figure 6).

3.2. Diagnosis of BVDV acute infection

All calves were BVDV negative at the day of experimental inoculation (day 0). BVDV was isolated from spleen samples in all the animals of LV (8/8) and HV (10/10) groups on day 5 post challenge, demonstrating the BVDV acute infection in all the inoculated calves. In contrast, spleen samples of all the animals in the control group were BVDV negative in spleen samples at the end of the experimental period (Table 14).

3.3. Gene expression analysis

3.3.1. Type I Interferon alpha and beta

Type I Interferon (α and β) mRNA expression was up-regulated in tracheo-bronchial lymph nodes ($P < 0.05$) of calves inoculated with high virulence BVDV compared with the control group (Figure 7). Calves experimentally challenged with low virulence BVDV did not show any significant increase in type I interferon mRNA levels in spleen and tracheo-bronchial lymph nodes on day 5 post inoculation. The IFN-α and -β mRNA levels were significantly higher in tracheo-bronchial lymph nodes of calves inoculated with the high virulence BVDV strain 1373 compared with those in calves challenged with the low virulence BVDV ($P < 0.05$).

3.3.2. Type I Interferon-induced genes

The mRNA levels of all type I interferon induced genes (Mx-1, PKR, OAS-1 and ISG-15) were found to be significantly up-regulated in spleen of both BVDV inoculated groups,

compared with the control group ($P < 0.001$, Figure 8A). Moreover, mRNA expression of type I interferon induced genes was similar in spleen of calves inoculated with low and high virulence BVDV ($P > 0.05$).

A dramatic up-regulation of Mx-1 mRNA (24-fold for calves in LV and 21.5-fold for calves in HV) was observed on day 5 post inoculation in tracheo-bronchial lymph nodes of both groups challenged with BVDV when compared with the control calves ($P < 0.001$). No significant differences were found in the mRNA expression level of Mx1 in tracheo-bronchial lymph nodes between calves inoculated with low and high virulence BVDV. The expression of the other interferon induced genes (PKR, OAS-1 and ISG-15) was also significantly increased in tracheo-bronchial lymph nodes of both challenged groups compared with the uninfected group (Figure 8B $P < 0.001$), indicating an extensive activation of type I IFN response.

OAS-1 and ISG-15 mRNA levels in tracheo-bronchial lymph nodes were up-regulated differentially following exposure to BVDV SD-1 (LV) versus BVDV 1373 (HV). Calves inoculated with SD-1 showed a 2-fold higher OAS-1 mRNA level than calves inoculated with the strain 1373 ($P = 0.04$ Figure 8). Similarly, expression of ISG-15 mRNA in tracheo-bronchial lymph nodes was significantly higher in calves infected with SD-1 compared with calves infected with 1373 ($P = 0.02$; 14.37 vs 8.61 fold increase in ISG-15 mRNA level relative to the control group; Figure 8B).

3.3.3. Pro-apoptosis related genes. Expression of Caspase-3, -8 and -9

The mRNA transcription levels of caspase-3, -8 and -9 genes in spleen of BVDV-inoculated calves were not significantly different from those in the uninfected control calves, suggesting that these pro-apoptosis pathways were not activated in spleen by day 5 post-

infection ($P > 0.05$). Interestingly, there was a significant up-regulation of caspase-8 and -9 mRNA levels in tracheo-bronchial lymph nodes in calves inoculated with low virulence BVDV ($P = 0.01$, Figure 9), but not in calves inoculated with the high virulence BVDV strain, compared with the control group.

Relative mRNA levels of caspase-9 in tracheo-bronchial lymph nodes were significantly higher for calves inoculated with low virulence BVDV than those for calves inoculated with high virulence BVDV ($P = 0.005$). Additionally, there was a tendency (0.5 fold, although not significant, $P = 0.15$) for higher expression of caspase-8 mRNA levels in tracheo-bronchial lymph nodes for the calves experimentally challenged with low virulence BVDV strain SD-1 compared with calves inoculated with high virulence BVDV strain 1373. mRNA levels of caspase-3 in tracheo-bronchial lymph nodes were similar among the three experimental groups.

3.4. Viral RNA levels

Viral RNA levels were similar on day 5 post inoculation in tracheo-bronchial lymph nodes of calves experimentally challenged with high and low virulence BVDV ($P > 0.05$, Figure 10).

3.5. Correlation analysis of the genes involved in the interferon induced antiviral state

The multivariate statistical analysis showed a significantly high positive correlation between IFN-α and IFN-β ($R = 0.98$, $P < 0.001$). Type I interferon (α and β) gene expression was negatively correlated with Mx-1 and ISG-15, in both spleen and tracheo-bronchial lymph nodes ($P < 0.05$; Tables 15, 16). Additionally, significant negative correlations were found between the transcription levels of type I interferon and the studied pro-apoptosis related genes (caspase-3, -8

and -9) in spleen of the experimentally inoculated calves ($P < 0.001$). Conversely, gene expression analysis of type I interferon in tracheo-bronchial lymph nodes only showed negative correlation with mRNA expression levels of caspase-8 ($P \leq 0.01$).

There were significant and strong positive correlations among the mRNA levels of Mx-1, PKR, OAS-1 and ISG-15 in both spleen and tracheo-bronchial lymph nodes of calves experimentally challenged with low and high virulence BVDV ($P < 0.01$; Tables 15, 16). Moreover, mRNA levels of type I interferon-stimulated genes were significantly correlated with the expression of caspases in spleen and TB lymph nodes of BVDV infected calves.

3.6. Immunohistochemistry

BVDV antigen was similarly distributed in multiple tissues (spleen, lymph nodes, lungs and tonsils) and cell types in both groups of BVDV inoculated calves. Although variable in extent and distribution the most pronounced antigen labeling was found in bronchiolar mucosa and alveolar epithelium of lungs. Similar evidence of mild to moderate lymphoid depletion was present in spleen and lymph nodes of calves inoculated with low and high virulence BVDV isolates (Figure 11).

4. Discussion

Experimental nasal challenge with both low and high virulence BVDV strains effectively resulted in acute BVDV infections and viremia, demonstrated by virus isolation from serum and spleen samples. Acute infection with the highly virulent BVDV-2 strain 1373 caused a significant leukopenia, evidenced on days 4 and 5 post challenge. BVDV infections are associated with leukopenia, immunosuppression and in some cases thrombocytopenia with

hemorrhages. This significant reduction in the total leukocytes during BVDV-2 infection might have occurred as a consequence of trafficking from blood into tissue, decrease in leukogenesis or outright death of leukocytes (Ridpath et al., 2006).

A strong up-regulation of the type I interferon (α and β) mRNA level was detected by day 5 post challenge in tracheo-bronchial lymph nodes of the calves inoculated with the highly virulent BVDV-2 strain 1373 compared with the control group. After viral infections, viral RNA is recognized by pattern recognition receptors such as Toll-like receptors (TLR-3, -7 and -8; in the cell membrane) and the RNA helicases RIG-I and MDA5 (in the cytosol) which subsequently stimulate the type I interferon transcription (Alexopoulou et al., 2001; Yamane et al., 2009). The regulation of IFN-α and -β synthesis requires the participation of several transcription factor interactions, including nuclear factor –κB (NF- κB), ATF/JUN, TBK1 and IKKε, and especially IFN-regulatory factors (IRFs). After activation of transcription factors, these are transported to the nucleus, where they activate or repress the expression or either IFNs or IFN-regulated genes (Katze et al., 2002). Once the type I IFN pathway is activated, it results in stimulation of a signal transduction cascade that produces an antiviral state in the host cells and elicits the adaptive immune response (Samuel, 2001).

In the present study, a significant increase of type I IFN mRNA level was not evidenced by day 5 post challenge in calves experimentally infected with the low virulence strain SD-1. This lack of up-regulation of IFN-α and –β genes (even in the presence of increased mRNA levels of Mx1, PKR, OAS-1, and ISG-15 observed 5 days after inoculation) might be associated with a temporal regulation of the type I IFNs expression. Accordingly, it might be possible that IFN-α and –β mRNA levels could have been transiently increased before day 5 post inoculation with BVDV SD-1 strain, resulting in expression of the type I IFN-stimulated genes and

activation of the antiviral state. Consequently, potential regulatory mechanisms such as 'negative feedback' might have been activated to turn off the IFN-α/-β expression (Bego et al., 2012; Witwer et al., 2010). It has been demonstrated that following a viral infection IFN responses are generally transient and self-limiting in order to avoid prolonged and uncontrolled IFN effects which can interfere with normal immune response and hematopoiesis (Lin et al., 1998). Several molecules and pathways (e.g. BST2, Dcp2, miRNAs etc) have been reported to provide negative feedback signals to control strength and duration of IFN production by infected cells, preventing a prolonged antiviral state (Bego et al., 2012; Li et al., 2012; Witwer et al., 2010). Ganheim et al. (2003) evaluated the IFN-α concentrations in serial blood samples (on days 1, 8, 10-12, 14 and 16) after experimental infection of calves with BVDV. All the BVDV inoculated calves (n=6) had elevated IFN-α levels on days 4 and 6 post-infection. After day 6, only 2 calves had significant levels of IFN-α (Gånheim et al., 2003). Those results demonstrated that the IFN-α response was short-lasting in the BVDV infected calves, confirming previous results (Tråvén et al., 1991). On the other hand, the higher IFN-α/-β mRNA levels expressed in calves inoculated with BVDV strain 1373 might be due to the higher virulence of this isolate, inducing a strong and sustained type I IFN transcription. Type I IFN mRNA expression was up-regulated in tracheo-bronchial lymph nodes but not in spleen, reflecting a spatial regulation type I IFN transcription. Since experimental challenge was done through nasal aerosolization, the differential expression of type I IFN mRNA in these lymphoid organs may be due to the route of viral infection.

There was a marked activation of the antiviral response in both BVDV-infected groups, evidenced by up-regulation of Mx-1, PKR, OAS-1 and ISG-15 mRNA levels in tissue samples of tracheo-bronchial lymph nodes and spleen. Following transcription of IFN-α and –β during a

viral infection, these proteins bind to a common receptor (IFNAR), which triggers the intracellular IFN signaling pathway JAK-STAT. This signaling cascade results in phosphorylation and nuclear translocation of STAT1 and STAT2, and transcription of several of interferon stimulated genes (ISG) such as Mx-1, PKR, OAS-1, and ISG-15 (Stark et al., 1998). The IFN- induced genes control viral infections through direct antiviral effector functions (Schoggins et al., 2011) and by regulating the adaptive immune response (Colonna et al., 2004). Surprisingly, the expression of Mx-1, PKR, OAS-1 and ISG-15 observed in the LV group, occurred without an apparent expression of type I IFN- α/-β by day 5 post challenge. As mentioned above, a broad range of molecular mechanisms of negative feedback could have shut down the IFN-α/-β signaling around day 5 post challenge in this group (Bego et al., 2012; Li et al., 2012; Witwer et al., 2010). Additionally, it is possible that transcription of the antiviral genes (Mx1, PKR, OAS-1 and ISG-15) have been induced directly and independently of IFN -α and -β by the low virulence BVDV, as previously reported with other RNA viruses (Goetschy et al., 1989; Hug et al., 1988; Ronni et al., 1997; Ronni et al., 1995). Early experiments have shown that diverse IFN-induced genes (e.g ISG15) could be directly activated by virus infection, in absence of IFN production as reported for Newcastle disease virus (Bandyopadhyay et al., 1995; Wathelet et al., 1992). IRF-7 has the potential to induce ISGs independently of IFN signaling pathway as it was previously shown for other genes, including IRF7 itself, IFNB, MXA, CXCL10 and TRAIL (Di Domizio et al., 2009; Nakaya et al., 2001; Ning et al., 2005). In contrast, other authors have reported that Mx expression is stimulated closely and specifically via signaling through the type I IFN receptor (Baigent et al., 2002; von Wussow et al., 1990; Yamane et al., 2008), inferring that up-regulation of Mx gene involves the activation of the interferon-α/-β pathway (Fray et al., 2001; Simon et al., 1991). Correspondingly, Muller-Doblies

et al. (2004) demonstrated that Mx stimulation after BVDV infection was paralleled by endogenous synthesis and signaling of IFN-α/-β (Müller-Doblies et al., 2004). Therefore, the increase in the mRNA expression of several IFN-stimulated genes and the positive correlation between them indicated that this complex and massive up-regulation might be to some extent attributable to the activation of a type I interferon response (Yamane et al., 2008).

OAS-1 and ISG-15 mRNA expression levels in tracheo-bronchial lymph nodes were up-regulated differentially following challenge with BVDV strains SD-1 and 1373. Calves experimentally inoculated with low virulence BVDV showed a higher expression of these genes in tracheo-bronchial lymph nodes by day 5 post-inoculation. A similar tendency (although not significant) was observed for the Mx-1 mRNA expression in the calves inoculated with the low virulence BVDV. However, two calves challenged with BVDV 1373 (# 8042 and # 8081) showed extremely high levels of Mx1 mRNA (outliers), which increased the group mean to a level similar to that observed in the calves inoculated with low virulence BVDV. Since the calves inoculated with the highly virulent BVDV strain showed an apparent sustained expression of IFN-α/-β; and considering that their interferon-induced genes mRNA levels were lower compared with those observed in the calves inoculated with SD-1; it is possible that further transcription of IFN-induced genes could occur subsequently, as previously observed (Müller-Doblies et al., 2004). These differences might reflect a temporal variation in the induction of the antiviral immune response by the two viral strains.

The caspase-8 and -9 mRNA levels indicated an evident activation of both intrinsic and extrinsic pathways of apoptosis in tracheo-bronchial lymph nodes in calves challenged with the low virulence BVDV-1 SD-1. Apoptosis or programmed cell death is considered an essential homeostatic mechanism during viral infections to remove infected cells (Pedrera et al., 2012).

There are two different caspase-dependent pathways of apoptosis: the extrinsic pathway, which is activated by member of the tumor necrosis factor (TNF) family of cytokine receptors, and is associated with the cleavage and activation of caspase-8 (Rasper et al., 1998; Stennicke et al., 1998); and the intrinsic pathway, which can be induced by release of cytochrome-c from mitochondria into the cytosol and the subsequent ATP-dependent activation of the death regulator apoptotic protease-activating factor 1 (Apaf-1), which stimulate the cleavage and activation of the initiator caspase-9 (Li et al., 1997; Slee et al., 1999). Finally, both pathways induce apoptosis via activation of effector caspases such as caspase-3, which once activated leads to irreversible cell death. These results are consistent with a previous study wherein a high expression of caspase-8 was observed in gut-associated lymphoid tissue of the ileum from calves inoculated with ncp BVDV-1 (Pedrera et al., 2012). Moreover, in that study the number of cells expressing caspase-3 was also increased (Pedrera et al., 2012).

The lack of increase in the levels of caspase-8 and -9 mRNA in the calves challenged with the highly virulent BVDV-2, suggests that there might be temporal differences in the expression of pro-apoptotic markers between these two viral strains. Therefore, there could be a delay in the activation of apoptosis after infection with the highly virulent BVDV 1373, as shown in previous in vitro experiments (Bendfeldt et al., 2007). In that study, infection of a bovine lymphoid cell line with the highly virulent BVDV-2 1373 resulted in a delayed (> day 5 post-inoculation) and less pronounced disruption of the mitochondrial transmembrane potential with a weaker activation of the caspase cascade, compared with the apoptotic changes caused by cp BVDV (Bendfeldt et al., 2007). On the other hand, Pedrera et al. (2012) stated that the intrinsic pathway may play a minor role in the induction of lymphocyte apoptosis after experimental infection of calves with ncp BVDV-1, since they observed a reduced number of

cells expressing caspase-9 in the gut associated lymphoid tissue, and over-expression of the anti-apoptotic factor Bcl-2 (Pedrera et al., 2012). Since no significant increase in Caspase-3, -8 and -9 was observed in spleen of any BVDV -inoculated groups, there appears to exist temporal-spatial differences for activation of apoptosis between peripheral lymphoid tissue near the site of infection (tracheo-bronchial lymph nodes) versus central lymphoid organs (spleen). In the present study the mRNA expression of interferon-induced genes was correlated with the caspase-3, -8, -9 mRNA levels, indicating an association between the antiviral state and the induction of apoptosis, being consistent with previous reports (Yamane et al., 2008). Apoptosis of lymphoid cells during BVDV acute infection has been reported to occur by direct action of BVDV on lymphocytes during replication and accumulation of viral RNA (Adler et al., 1997; Grummer et al., 2002; Vassilev and Donis, 2000; Zhang et al., 1996); via binding of dsRNA-to reactive proapoptotic ISG products, PKR and OAS-1,during the antiviral state (Grummer et al., 2002; Jordan et al., 2002; Schweizer and Peterhans, 2001; Yamane et al., 2006) or by an indirect mechanism mediated by pro-apoptotic cytokines produced by macrophages (Gomez-Villamandos et al., 2001; Sanchez-Cordon et al., 2005; Sanchez-Cordon et al., 2002; Sanchez-Cordon et al., 2003) .

In conclusion, experimental infection with low and high virulence BVDV strains induced a significant expression of the interferon-stimulated antiviral state in both spleen and tracheo-bronchial lymph nodes. On day 5 post-inoculation, calves inoculated with BVDV-1a SD-1 showed a significantly higher expression of some interferon-induced genes (OAS-1 and ISG-15) and pro-apoptosis markers (caspase-8 and -9) compared with calves infected with BVDV-2 1373, which could reflect a temporal difference in the transcriptional events during the innate immunity against BVDV strains of different virulence and genotype.

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V. Differential Expression of Pro-inflammatory and Anti-inflammatory Cytokines during Experimental Infection with Low or High Virulence Bovine Viral Diarrhea Virus in Beef Calves.

Abstract

The objective of this study was to compare the mRNA expression of cytokines involved in pro-inflammatory (TNF-α, IL-1β, IFN-γ, IL-2, IL-12, IL-15), and anti-inflammatory (IL-4, IL-10, TGF-β) responses after experimental infection of calves with low or high virulence noncytopathic (ncp) bovine viral diarrhea virus (BVDV) strains. Thirty BVDV-naïve, clinically normal beef calves (seronegative to BVDV) were randomly assigned to one of three groups. Calves were intranasally inoculated with low (LV; n= 10, SD-1) or high (HV; n= 10, 1373) virulence ncp BVDV or with BVDV-free cell culture medium (Control, n= 10). Calves were euthanized on day 5 post-inoculation and tissue samples of tracheo-bronchial lymph nodes and spleen were collected for quantitative-RT-PCR analysis to determine the mRNA level of the target genes. mRNA levels of pro-inflammatory (TNF-α, IL-1β, IL-2, IFN-γ) and anti-inflammatory (IL-4 and IL-10) cytokines were significantly up-regulated in tracheo-bronchial lymph nodes of HV group, but not in LV group, compared to the control group ($P < 0.05$). The IL-12 mRNA level was up-regulated in tracheo-bronchial lymph nodes of both LV and HV groups, compared with the control group ($P \leq 0.05$). A significant up-regulation of IL-15 mRNA was observed in tracheo-bronchial lymph nodes for LV calves ($P < 0.002$), but not for HV calves. Experimental inoculation with BVDV-2 1373 stimulated a significant mRNA expression of both pro-inflammatory and anti-inflammatory cytokines. However, inoculation with BVDV-1

SD-1 only resulted in up-regulation of IL-12 and IL-15 mRNA, which are associated with activation of macrophages and NK cells during the innate immune response.

Key words: BVDV, Gene expression, Cytokines, Proinflammatory, Antinflammatory.

1. Introduction

Bovine viral diarrhea virus (BVDV) is a positive-sense single stranded RNA virus belonging to the genus *Pestivirus* within the *Flaviviridae* family (Lindenbach and Rice, 2001). There are two virus biotypes cytopathic (cp) and noncytopathic (ncp), based on their ability to cause pathogenic effects on cell cultures (Hamers et al., 2001). Two different genotypes (BVDV-1 and BVDV-2) have been identified, with remarkable differences in genomic sequences of the 5´ UTR, N-pro and E2 regions (Ridpath, 2003).

This virus is an important infectious agent that affects cattle worldwide (Baker, 1995; Houe, 1999). Infection of pregnant cattle with BVDV may result in abortions, stillbirths, or the birth of calves with congenital defects or persistently infected with BVDV (Baker, 1995). Most BVDV acute infections are subclinical, transient and self-limiting. However, BVDV is considered a potentiator of secondary infections causing bovine respiratory disease (Baker, 1995), one of the most important infectious diseases in commercial feedlot production. This increased susceptibility to secondary infections is a consequence of the ability of BVDV to cause immunosuppression and synergism with other viral and bacterial agents (Kapil et al., 2005).

Acute infections with some virulent BVDV-2 strains may cause a severe clinical disease characterized by fever, depression, respiratory signs, diarrhea, lymphopenia, thrombocytopenia and high mortality rate (Brock et al., 2007; Liebler-Tenorio et al., 2003b). Thus, the wide range

of clinical presentations following acute BVDV infections depend on the viral strain virulence and immune status of the animal (Ridpath et al., 2006).

Kelling et al. (2002) and Liebler-Tenorio et al. (2002, 2003b) reported that the virulence of the BVDV strain is correlated with the capability of the virus to cause a decrease in lymphocyte counts. Thus, highly virulent BVDV induced a significantly more severe and longer lymphopenia and lymphoid tissue depletion (via apoptosis and necrosis) than less virulent BVDV (Kelling et al., 2002b; Liebler-Tenorio et al., 2002; Liebler-Tenorio et al., 2003b). These findings suggest a possible association between BVDV virulence and the mechanisms by which the virus causes immunosuppression in susceptible cattle. In addition to reduction in leukocyte counts, BVDV may cause immunosuppression by impairing the function of cells such as mononuclear phagocytes and lymphocytes, which are important for both innate and acquired immune systems (Baigent et al., 2004; Charleston et al., 2001a; Glew et al., 2003; Jensen and Schultz, 1991; Lamontagne et al., 1989; Liu et al., 1999; Potgieter, 1995; Schweizer et al., 2006). Macrophages and dendritic cells produce pro-inflammatory cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1) which stimulate leukocyte chemo-attraction, phagocytosis and enhance further cytokine and chemokine production (Krishnadasan et al., 2003; Pfeffer, 2003). Previous reports have shown that BVDV infection results in reduced secretion of these pro-inflammatory cytokines both in vivo and in vitro, causing immunosuppression (Adler et al., 1996; Lee et al., 2008; Pedrera et al., 2009; Risalde et al., 2011; Yamane et al., 2005).

Early in the course of infection, IL-2 is produced by T cells during antigen recognition, stimulating the proliferation of the antigen specific cells (Tizard, 2009). Similarly, IL-15 stimulates the proliferation of natural killer (NK) cells as an innate immune mechanism during

viral infections (Abbas and Lithman, 2005). Subsequently, $CD4^+$ T helper cells proliferate and differentiate into subsets of effector cells (Th1 or Th2) that secrete diverse cytokines with different effector functions (Tizard, 2009). Interferon-γ (IFN-γ) is a Th1 cytokine secreted by $CD4^+$ Th1 cells, $CD8^+$ cytotoxic T cells, and NK cells in response to IL-12. This cytokine stimulates cytotoxicity of T cells, production of immunoglobulin G (IgG) by B cells, activation of macrophages and phagocytosis, thus increasing the immune response against viral infections (Biron and Sen, 2001; Samuel, 2001). In contrast, other groups of cytokines have regulatory functions on the immune response. IL-4 is an anti-inflammatory cytokine produced by Th2 cells that stimulates the generation of more Th2 cells, serving as an autocrine growth factor (Abbas and Lithman, 2005). IL-4 promotes B cell Ig heavy chain isotype switching to produce IgE. Additionally, IL-4 antagonizes the effects of IFN-γ on macrophage activation, inhibiting cell-mediated immune reactions (Abbas and Lithman, 2005). IL-10 represents another regulatory cytokine with anti-inflammatory effects, inhibiting IL-12 production by activated macrophages and dendritic cells, as well as the activities of the pro-inflammatory cytokines, controlling innate immune reactions and cell mediated immunity (Biron and Sen, 2001; Pestka et al., 2004). Similarly, transforming growth factor beta (TGF-β) inhibits activation of macrophages as well as proliferation and differentiation of lymphocytes (Abbas and Lithman, 2005).

A Th1/Th2 polarization, previously described in mice and humans (Abbas and Lithman, 2005) is not well defined in ruminants (Estes and Brown, 2002). Some studies have demonstrated the development of a Th1 immune response after BVDV infection (Charleston et al., 2002; Lee et al., 2008). Conversely, others have shown the establishment of a Th2 type immune response after infection with ncp BVDV, which could inhibit the Th1 response and cause immunosuppression leading to secondary pathogen infections (Rhodes et al., 1999). A

recent study to determine the memory response of cytokine expression in peripheral blood mononuclear cells from cattle after stimulation with ncp BVDV was not consistent for demonstrating that BVDV induces a Th1 or Th2 biased immune response (Waldvogel et al., 2000).

In the present study, it was hypothesized that acute infection with high and low virulence BVDV strains can differentially regulate the expression of pro-inflammatory (Th1 response) and anti-inflammatory (Th2 response) cytokines in BVDV-naïve calves, which would reflect differences in their ability to cause immunosuppression. Specifically, the hypothesis was that high virulence BVDV might cause a higher level of immunosuppression, represented by decreased expression of pro-inflammatory cytokines and increased expression of anti-inflammatory cytokines, compared with the low virulence BVDV. The objective of this study was to compare the mRNA expression of cytokines involved in pro-inflammatory (TNF-α, IL-1β, IFN-γ, IL-2, IL-12, IL-15), and anti-inflammatory (IL-4, IL-10, TGF-β) responses after experimental infection of BVDV-naïve beef calves with low (SD-1 strain) and high (1373 strain) virulence ncp BVDV, to determine if there is an association between BVDV virulence and immunosuppression.

2. Methods

2.1. Animals

A total of thirty beef calves (7 months of age) were enrolled in this study. All calves were clinically normal, free of BVDV based on virus isolation from serum and immuno-histochemistry of ear notch biopsies, and seronegative to both BVDV-1 and BVDV-2 based on serum virus neutralization assays performed at serum dilution of 1:2. Animals were obtained

from a farm owned by Auburn University, located in Huntsville, AL. This farm is characterized by an optimal biosecurity program in which new animal introduction is not permitted (closed system).

2.2. Experimental design and sample size calculation

The study was designed as a randomized, controlled trial. Sample size was calculated using Proc Power (SAS) on the basis of the expected means of target gene expression (≥ 1.5 fold-regulation of mRNA level, normalized to the housekeeping gene and relative to the control group) for each experimental group, the SDs (± 0.2 fold-regulation) of the means, and a statistical power of 99%.

Calves were randomly assigned by use of a random number generator (Research Randomizer, www.randomizer.org) to 1 of 3 treatment groups:

LV (n= 10): Animals challenged with a low virulence type 1a ncp BVDV (strain SD-1).

HV (n= 10): Animals challenged with a high virulence type 2 ncp BVDV (strain 1373).

Control (n= 10): Animals inoculated with BVDV-free cell culture medium.

Animals were transported from the farm of origin to the Auburn University BVDV-free farm three days before the beginning of the study. All 30 calves were housed together at the BVDV-free farm prior to inoculation. As treatment groups were inoculated, they were separated and transported to the Auburn University BVDV isolation farm, located at 1.6 miles away from the BVDV-free farm, except for the control group which stayed in the BVDV-free farm throughout the experimental period.

Additionally, in order to avoid shedding and exchange of BVDV among groups and undesired BVDV infection of the control group due to commingling with the BVDV inoculated

groups, the study was performed during three different rounds of viral inoculation and necropsy, which were spatially and temporally separated. During the first week, 10 calves assigned to the LV group were separated and brought to the BVDV-isolation unit. After inoculation, LV calves stayed in the BVDV isolation farm until day 5, when they were euthanized and necropsies were performed.

During the second week, 10 animals assigned to the control group were separated, transported for approximately 5 minutes around the same geographic area and returned to the BVDV-free farm. This transportation was performed in order to provide similar management conditions to all groups. At the BVDV-free farm, these animals were separated from the rest of the calves and maintained in a different pasture. The control group was not brought to the BVDV-isolation farm in order to prevent any exposure to BVDV during the experimental period. On day 5, calves in the control group were euthanized and necropsies were performed.

During the second, third and fourth week, there were no BVDV- infected calves on the BVDV isolation farm. This resting time was included in the experimental protocol to favor viral clearance from the environment, in an attempt to prevent the presence of BVDV strain SD-1 on the farm by the time the HV group were brought to the BVDV isolation farm. It has been reported that BVDV can survive in a cool protected environment for several days (Houe, 1995). In addition, after departure of the LV group and before arrival of the HV group, concrete floor, chutes and pens at the BVDV isolation farm were disinfected using bleach (sodium hypochlorite, NaOCl 5.25 %). Finally, on the fifth week, 10 animals assigned to the HV group were separated and transported to the BVDV-isolation farm, where they were experimentally inoculated with BVDV-2 1373. On day 5, HV calves were euthanized and transported to the necropsy room.

At both farms, calves within each group (LV, HV or control) were housed together in the same pasture and allowed to eat and drink from the same feeders and water troughs. All animal protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Auburn University.

2.3. BVDV challenge inoculation

Calves were experimentally inoculated with a low (strain SD-1) or high (strain 1373) virulence ncp BVDV isolates. The strain BVDV1a SD-1 was originally isolated from a persistently infected heifer with fatal mucosal disease (Deng and Brock, 1992). The strain BVDV-2 1373 was originally isolated from a severe acute BVDV outbreak in Ontario, Canada (Carman et al., 1998). The stock BVDV strains used in the present study were biologically cloned via successive passages in Madin-Darby bovine kidney (MDBK) cells by use of limiting dilutions with subsequent minimal propagation by incubation of the MDBK monolayer for 48 hours at 37 °C and 5% CO_2 to produce an adequate amount of stock virus with the desired $CCID_{50}$/mL for characterization and animal challenge exposure studies. The inocula used consisted of cell culture supernatants containing 1.3 X 10^5 $CCID_{50}$/mL of BVDV-1a strain SD-1 and 1.3 X 10^5 $CCID_{50}$/mL of BVDV-2 strain 1373 of inoculum in Dulbecco's Modified eagle's medium (DMEM) supplemented with 10% equine serum, L-Alanyl and L-glutamine. The inocula were obtained following freeze-thaw cycles to disrupt the cells and release the viral particles. Inoculum cell culture supernatant was stored at -80 °C until the day of inoculation. After freezing, an aliquot of the BVDV inoculum was titrated by using the Reed-Muench method to determine the final $CCID_{50}$/mL for inoculation. The inocula were aliquoted (5 mL) in individual tubes within 1 hour of inoculation and stored on ice until use. The inoculation was

performed by intranasal aerosolization of 5 mL of inoculum by use of an aerosolizer[d] and a vacuum pump. One sample of each inoculum was transported on ice and returned to the lab to determine the $CCID_{50}$/mL after inoculation.

2.4. Sampling

From each calf, blood samples were collected on days 0, 2, 4, and 5 after BVDV challenge inoculation in blood tubes containing an anticoagulant for determination of the total leukocyte count. Blood samples were collected on the same days in blood tubes without an anticoagulant to obtain serum for VI and RT-PCR for diagnosis of BVDV. Serum samples were aliquoted and stored frozen at -80° C.

On day 5 post challenge, animals were euthanized using a captive bolt device and transported to the necropsy room at the Department of Pathobiology, College of Veterinary Medicine, Auburn University. Necropsy examination was performed on all calves. Tissue samples were collected from spleen and tracheo-bronchial lymph nodes. Immediately after tissue collection, fresh samples were submerged in 5 ml of RNA*later* reagent (RNeasy protect kit, QIAGEN®), in order to be protected from RNA degradation by RNAse. The stabilized samples were stored at -80° C as recommended by the manufacturer until processing.

2.5. Clinical Pathology

Unclotted blood samples collected on days 0, 4 and 5 post-challenge were submitted to the clinical pathology service laboratory at the Auburn University College of Veterinary Medicine for leukocyte analysis. The total leukocyte count for each sample was determined using an automatic cell counter (Coulter. Corp., Miami, FL, USA).

2.6. Diagnosis of BVDV acute infection

2.6.1. Virus isolation

Virus isolation was performed on days 0 and 5 post challenge from serum samples and from spleen samples on day 5 post challenge to confirm BVDV infection and viremia in the inoculated animals.

For virus isolation, 250 µl of each sample was added to individual 25 cm^2 tissue culture flasks containing a monolayer of MDBK cells. For cell culture, Dulbecco's Modification of eagle's medium (DMEM) supplemented with 10% equine serum, L-Alanyl and L-glutamine was used. After three days incubation at 37° C and 5% CO_2, flasks were frozen and upon thawing, 50 µl of the cell suspension from each flask was transferred to three wells of a 96 well plate seeded with MDBK cells (first plate). The inoculated 96-well plate was incubated 3 days and the culture medium from each well was transferred to the corresponding well of a new 96-well plate previously seeded with MDBK cells (second plate). Both 96-well plates (first and second) were tested for BVDV antigen by using IP staining, using a bovine BVDV specific polyclonal antibody (B-224).

2.6.2. Reverse Transcription PCR

Viral RNA was extracted from MDBK cell culture supernatant (upon freeze and thaw), previously inoculated with serum on days 0 and 5 post-challenge and spleen tissue homogenate on day 5 post-challenge as described above for virus isolation. Viral RNA extraction was performed using QIAamp® Viral RNA mini kit (QIAGEN®, Valencia, CA USA). Reverse transcription-PCR was performed to determine the presence of BVDV using a conventional thermocycler (Applied Biosystems®, Carlsbad, Ca USA) and a QIAGEN One Step RT-PCR Kit

(QIAGEN®, Valencia, CA USA) according to the manufactures' recommendations. Specific primers (reverse and forward) were used to amplify a 245-nucleotide BVDV sequence located within the 5'-UTR (between the nucleotides 100 and 345). The presence of BVDV in the PCR product (cDNA) was determined by agarose gel-electrophoresis demonstrating a significant band similar to the BVDV positive control band.

2.7. mRNA Gene expression analysis

2.7.1. RNA extraction and purification

Spleen and tracheo-bronchial lymph node tissue samples (approximately 30 mg) were disrupted and homogenized in buffer containing guanidine isothiocyanate using a bullet blender machine (Next Advance® Averill Park, NY, USA) and 1 mm glass beads. Total RNA for quantitative real time RT-PCR (*q*RT-PCR) was extracted and purified from spleen and tracheo-bronchial lymph node samples using an RNeasy mini kit (QIAGEN®, Valencia, CA USA), according to the manufacturer's protocol.

2.7.2. Reverse transcription

Synthesis of cDNA was performed from 1μg of total RNA template using a First cDNA strand reverse transcription kit (Roche®, Indianapolis, IN USA) according to the manufacturer´s protocol. After reverse transcription, final cDNA products had a concentration of approximately 2 μg/μl in a total volume of 20 μl. Synthesized cDNA was diluted 10-fold with RNAse free water to reach a template concentration of approximately 200 ng/μl to be used for *q*RT-PCR. Four aliquots were prepared from each cDNA sample in order to avoid consecutive freezing-thawing events that could affect cDNA integrity and concentration.

2.7.3. Quantitative Real Time RT-PCR

Quantitative real time PCR was performed in 20 µL reactions to amplify the target gene sequences and determine the mRNA expression using a LightCycler (Roche®, Indianapolis, IN USA) and DNA Master^{plus} SYBR I green kit (Roche®, Indianapolis, IN USA). The PCR mix (15 µL) was prepared using 4 µL of the master mix, 9 µL of dd H_2O and 1 µL of each primer in a final concentration of 1 µM. Finally, 5 µL of cDNA template (200 ng/ µL) was added. The PCR reaction was conducted at 95 ◦C for 10 min (pre-incubation), followed by 45 cycles of 95˚C for 15 seconds (denaturation), 60-71˚C (primer dependent) for 10 seconds (annealing) and 72˚C for 10 seconds (extension). Upon completion of *q*RTPCR amplification, melting curve analysis was performed to evaluate the specificity and quality of the amplification. Melting curve analysis was done by incubation of the *q*RT-PCR products for 60 seconds at each step with temperature gradually increasing by 0.1˚C/sec from 65 to 95 ˚C. All samples were analyzed in duplicates to increase the accuracy of the results and the average of both runs was used to calculate the relative amount of mRNA. Non-template negative controls were concurrently run with the samples, replacing the template DNA with PCR-grade water.

2.7.4. Normalization and relative quantification of target genes

*q*RT-PCR procedure yielded crossing points or threshold cycle (CT) as the fundamental quantitative units, corresponding to the PCR cycle wherein the amount of DNA of the amplified gene generates a fluorescent signal significantly higher than the baseline. The CT value therefore correlates negatively to the amount of target mRNA, i.e. the higher the amount of mRNA, the sooner the threshold is reached and the lower the CT value obtained. Results for gene expression were analyzed with the comparative $^{\triangle\triangle}$Ct method (Livak and Schmittgen, 2001). Therefore,

gene expression analysis was performed through the relative quantification of the mRNA level or fold regulation of target genes normalized to a housekeeping gene (unaffected by the studied conditions) and compared with the uninfected control group (Livak and Schmittgen, 2001).

The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the endogenous reference gene. The efficiency of the *q*RT-PCR (necessary for the comparative $^{\triangle\triangle}$Ct method), was determined for each target gene by creating a standard amplification curve using 1μg, 100 ng, 10 ng, 1 ng and 0.1 ng per reaction of a cDNA template mix. The cDNA template mix was created by mixing equal amounts of cDNA solution from 3 tracheo-bronchial lymph node samples from each experimental group. Target genes and specific primer sequences are documented in Table 17.

2.8. Statistical analysis

Data were analyzed using the Statistical Analysis System (SAS® version 9.1 SAS Institute, Cary, NC). Statistical parametric assumptions of normality and constant variance were tested through Shapiro Wilk's and Levene's tests, respectively. Additionally, normal probability, residuals and quartile-quartile plots were constructed and analyzed to confirm any significant departure from normality and constant variability of the data. Since relative mRNA expression data were not normally distributed (heavily skewed), and had a heterogeneous variance, for the mean comparison analysis gene expression data were transformed using a Box-Cox power transformation, indexed with a power number suggested by data themselves, which maximized the parameter λ. The outcomes produced by the transformation were carefully examined for possible violations of the tentative assumption of normality.

The general linear model procedure (Proc GLM) was used for the analysis of variance to compare the relative mRNA level of target genes among groups (using the Least Significant difference, LSD Test). The general linear model procedure (Proc GLM) was also used for the analysis of variance to compare mean leukocyte counts among the groups (through the LSD Test). Mean comparisons between day 0 and subsequent days (4 and 5) were accomplished through the repeated measures analysis of variance for each group. Pearson correlation analysis was performed to identify correlated gene expression. Differences with *p*-values less than 0.05 were considered statistically significant.

3. Results

Two calves in the LV group were euthanized on day 2 post-challenge due to reasons not related with the BVDV infection.

3.1. BVDV Isolation

All calves were BVDV negative at the day of experimental inoculation (day 0). On day 5 post challenge, virus isolation from serum samples was less frequent in calves inoculated with low virulence BVDV than that in calves inoculated with high virulence BVDV (Table 18). BVDV was detected by virus isolation from spleen samples in all the animals of LV (8/8) and HV (10/10) groups on day 5 post challenge, demonstrating BVDV acute infection in the inoculated calves. In contrast, all spleen samples from the animals in the control group were BVDV negative at the end of the experimental period (Table 18).

3.2. BVDV RT-PCR

All calves were found to be PCR BVDV negative at the day of experimental inoculation (day 0). Viral RNA was amplified from serum on day 5 post-inoculation in 8 and 10 calves in the LV and HV groups, respectively. Similarly, by day 5 post-inoculation viral RNA was detected in spleen in all the calves in both BVDV inoculated groups. In contrast, all the spleen samples in the control group were BVDV PCR negative at the end of the experimental period (Table 18).

3.3. Clinical Pathology

A significant decrease in total leukocyte counts were observed in the calves challenged with high virulence BVDV strain 1373 on days 4 and 5 compared with animals inoculated with low virulence BVDV strain SD-1 and control group during the same days ($P < 0.01$). Significant differences were not found between calves inoculated with low virulence BVDV and calves in the control group (Figure 6).

3.4. Gene Expression Analysis

3.4.1. Pro-inflammatory cytokines

The TNF-α mRNA levels were up-regulated ($P < 0.05$) in tracheo-bronchial lymph nodes and spleen of calves inoculated with high virulence BVDV compared with the control group. Calves experimentally challenged with low virulence BVDV did not show any significant increase in TNF-α mRNA levels in spleen and tracheo-bronchial lymph nodes. There were significant differences for TNF-α mRNA expression in tracheo-bronchial lymph nodes ($P = 0.01$) and spleen ($P = 0.005$) between calves inoculated with high and low virulence BVDV. Similarly, expression of IL-1β was higher in tracheo-bronchial lymph nodes ($P = 0.03$) in calves infected

with the highly virulent BVDV-2 1373 compared with that of the calves inoculated with the low virulence BVDV (Figure 12).

The relative mRNA expression of IL-12 was up-regulated in tracheo-bronchial lymph nodes, but not in spleen of both BVDV inoculated groups compared with the control group ($P < 0.05$). No significant differences were observed for the IL-12 mRNA levels in spleen and tracheo-bronchial lymph nodes between BVDV inoculated groups (Figure 13).

There was a remarkable up-regulation of IFN-γ and IL-2 mRNA levels in tracheo-bronchial lymph nodes of calves experimentally challenged with the highly virulent BVDV (Figure 13). Calves inoculated with the low virulence BVDV did not increase the expression of these Th1 type cytokines; whose levels were significantly different from those observed in the calves inoculated with the high virulence BVDV (P=0.006 and P=0.01 for IFN-γ and IL-2, respectively; Figure 13). No changes in IFN-γ and IL-2 mRNA levels were observed in spleen of any of the groups experimentally challenged with BVDV compared with the control group.

A significant up-regulation of IL-15 mRNA was observed on day 5 post inoculation in tracheo-bronchial lymph nodes of calves inoculated with the low virulence BVDV strain, when compared with the baseline IL-15 mRNA expression level of control calves ($P = 0.002$). Levels of IL-15 mRNA did not change in the calves inoculated with the high virulence BVDV as compared with the baseline. Significant differences were detected in IL-15 expression level between calves inoculated with low and high virulence BVDV ($P = 0.002$).

3.4.2. Anti-inflammatory cytokines

The levels of IL-4 and IL-10 mRNA were up-regulated in tracheo-bronchial lymph nodes following exposure to high virulence BVDV-2 1373, but not after infection with the low

virulence BVDV-1a SD-1 (Figure 14). Accordingly, significant differences were found in the expression of IL4 (P=0.005) and IL-10 (P=0.02) between calves inoculated with high and low virulence BVDV. There was no significant difference in the mRNA expression level of TGF-β in tracheo-bronchial lymph nodes and spleen between calves inoculated with low and high virulence BVDV. Relative mRNA levels for IL-4 and IL-10 did not increase in spleen samples 5 days following inoculation with BVDV-1a SD-1 or BVDV-2 1373.

3.4.3. Correlation analysis of the cytokines mRNA levels

Results of the Pearson correlation analysis are shown in Table 19.The multivariate analysis showed a significantly high positive correlation (R= 0.65 - 0.92) between mRNA levels of all evaluated pro-inflammatory cytokines (TNF-α, IL-1β, IFN-γ, and IL-2; P <0.01, Table 19). Surprisingly, the expression of these pro-inflammatory cytokines was also positively correlated with the mRNA levels of the anti-inflammatory cytokines (IL-4 and IL-10). In contrast, gene expression analysis of the pro-inflammatory cytokines in tracheo-bronchial lymph nodes showed moderate negative correlation with mRNA expression levels of IL-12, IL-15, and TGF-β ($P \leq 0.01$).

4. Discussion

Experimental challenge with both low and high virulence BVDV strains effectively resulted in acute infections and viremia, demonstrated by virus isolation and RT-PCR analysis from serum and spleen samples. During BVDV infection, the virus replicates in tonsils, nasal mucosa and draining lymph nodes and disseminates via circulating leukocytes into the blood, causing viremia (Bruschke et al., 1998; Liebler-Tenorio, 2005). It has been reported that viremia

can be detected as early as 24 hours post-infection (Mills and Luginbuhl, 1968). Typically, acute BVDV infections result in short-term viremia, lasting up to 15 days (Evermann and Barrington, 2005). Acute infection with the highly virulent BVDV-2 strain 1373 caused a significant leukopenia, evidenced on days 4 and 5 post challenge. Infection with highly virulent BVDV is associated with leukopenia, lymphoid depletion, immunosuppression and in some cases thrombocytopenia (Bolin and Ridpath, 1992; Chase et al., 2004; Liebler-Tenorio et al., 2002).

The significant decrease in leukocyte counts leads to a reduction in immune protection, which predisposes the animal to secondary infections and more severe clinical disease (Bolin and Ridpath, 1992; Kapil et al., 2005). This significant reduction in the total number of leukocytes during BVDV-2 infection might occur as a consequence of leukocyte trafficking from blood into tissue to cause inflammation, a decrease in leukogenesis, or the outright death of leukocytes (Ridpath *et al* 2006).

Our data demonstrated a significant up-regulation of the pro-inflammatory cytokines TNF-α and IL-1β mRNA in tracheo-bronchial lymph nodes after infection with the highly virulent BVDV-2 1373. This finding could be associated with the commonly observed severe inflammatory lesions in the respiratory tract following experimental infection with virulent BVDV-2 strains ((Ellis et al., 1998; Liebler-Tenorio, 2005). In previous studies the experimental inoculation of unvaccinated beef calves with BVDV-2 1373 resulted in severe respiratory disease with high morbidity and mortality (Brock et al., 2007). TNF-α and IL-1β are secreted by mononuclear phagocytes after infection, then stimulating expression of integrins on leukocytes (Le and Vilcek, 1987; Van Reeth et al., 1999). This cellular process results in leukocyte recruitment to the site of viral infection with the subsequent activation of the T cell mediated immune response (Ley et al., 2007; Rivera-Rivas et al., 2009); in addition to contributing to viral

multiplication and dissemination, by increasing the number of target cells (Risalde et al., 2011).

The results of the present study are consistent with those of a previous experiment that showed a significant increase in serum concentrations of TNF-α in calves co-infected experimentally with BVDV and bovine herpes virus-1 (BHV-1) (Risalde et al., 2011). In contrast, several *in vitro* studies have shown that infection with ncp BVDV resulted in reduced expression of TNF-α and IL-1β in monocytes and macrophages (Adler et al., 1996; Lee et al., 2008; Raya et al., 2011; Risalde et al., 2011; Yamane et al., 2005) which has been associated with suppression of the host immune response (Lee et al., 2008; Raya et al., 2011). Furthermore, a previous study demonstrated that down-regulation of IL-1 expression induced by BVDV infection occurred at the protein but not mRNA level (Jensen and Schultz, 1991). A subsequent experiment showed that the production of an IL-1 inhibitor following infection with BVDV might interfere with the activation and proliferation of lymphocytes during the early immune response or alternatively work as a negative feedback mechanism, preventing the constant stimulation of T cells by IL-l (Jensen and Schultz, 1991; McCarthy et al., 1989). Down-regulation of TNF-α production observed in most studies has been speculated to be achieved by a Th2 type cytokine response, such as IL-10 (Chase et al., 2004). In the present study TNF-α and IL-1β mRNA levels did not increase by day 5 post challenge in calves experimentally infected with the low virulence BVDV SD-1. Experimental and field studies have revealed that most BVDV infections are transient and self-limiting, resulting in none or mild clinical signs of disease (Ames, 1986; Bolin and Ridpath, 1992). The low degree of inflammation and damage of the respiratory tract and lymphoid tissue commonly evidenced following infection with low virulence BVDV could be related to the observed low expression of the pro-inflammatory cytokines.

The significant up-regulation of IL-12 mRNA in both high and low virulence BVDV inoculated groups suggests an apparent activation of macrophages and dendritic cells in the lymph nodes close to the site of inoculation. IL-12 is secreted by mononuclear phagocytes during the early immune reaction in response to many microbial stimuli, including viral infections (Abbas and Lithman, 2005). In addition, antigen stimulated T helper cells induce production of IL-12 by macrophages and dendritic cells through the interaction of CD40-CD40L and expression of IFN-γ during the induction of cell-mediated immune responses (Abbas and Lithman, 2005). Subsequently, IL-12 promotes the differentiation of $CD4^+$ helper T cells into IFN-γ producing Th1 cells, which in turn activate macrophages to kill phagocytosed microbes, as well as enhancing the cytolytic mechanisms of activated NK cells and $CD8^+$ cytolytic T cells (Abbas and Lithman, 2005). The results of this study agree those of an *in vitro* assay showing that mRNA levels of IL-12 and IFN-γ were significantly up-regulated in monocytes in early stages post infection (1 hour) with ncp BVDV (Lee et al., 2008). In contrast, Risalde et al (2011) observed that the serum concentration of IL-12 of calves co-infected experimentally with BVDV and BHV-1 remained low during most of the experimental period (14 days). However, in that study there was an increase at 4 days post infection, coincident with an increased production of IFN-γ (Risalde et al., 2011).

The mRNA levels of IFN-γ and IL-2 were increased in tracheo-bronchial lymph nodes after inoculation with BVDV-2 1373, suggesting the activation of a Th1 immune response in an attempt to control this highly virulent infection. This response would contribute with a strong inflammatory response in the respiratory tract (cell mediated immunity) with the development of severe clinical signs as previously reported (Risalde et al., 2011). This apparent Th1 immune reaction was not observed in calves experimentally inoculated with BVDV-1 strain SD-1, which

could be related with mild or no clinical signs commonly reported after experimental infections with low virulence BVDV-1 (Ames, 1986; Bolin and Ridpath, 1992). Therefore, it is speculated that the magnitude of the inflammatory response and tissue lesions developed after BVDV infection could be associated with the virulence of the BVDV strain (Liebler-Tenorio et al., 2003b). It could be possible that some mechanisms of the early innate immune response, such as up-regulation of type I IFN-induced antiviral state (data not shown), macrophages and NK cells (increased IL-12 and IL-15 mRNA levels), were apparently sufficient to control this low virulence infection by day 5 post inoculation, resulting in absent or delayed activation of Th1 type adaptive immunity (IL-2 and IFN-γ). However, previous studies have shown that immunization with a MLV vaccine containing low virulence BVDV-1 induced both humoral and cell mediated immune responses 21 days after immunization (Endsley et al., 2002).

IFN-γ is a cytokine produced by NK cells and T cells that activates macrophages to kill phagocytosed microbes, and promotes isotype switching to IgG subclasses (Tizard, 2009). Additionally, it stimulates the differentiation of $CD4^+$ T cells to the Th1 subset, and induces the expression of class I and class II MHC thereby promoting cell-mediated immunity, inflammation and tissue injury (Waldvogel et al., 2000). Activated T cells produce IL-2 which exerts paracrine and autocrine roles, which contribute to T cell and B cell proliferation and differentiation and initiation of the adaptive immune response (Abbas and Lithman, 2005). A recent study showed that IFN-γ mRNA was significantly increased in peripheral blood mononuclear cells of BVDV-seropositive cows after *in vitro* stimulation with ncp BVDV (Waldvogel et al., 2000). Similarly, the development of a Th1 immune response characterized by increased levels of IFN-γ was observed after co-infection with BVDV and BHV-1 (Risalde et al., 2011). Moreover, a previous *in vitro* experiment demonstrated that $CD8^+$ T cells (but not $CD4^+$ T cells) from BVDV

seropositive cattle were responsible for the increased levels of the Th1 cytokines IL-2 and IFN-γ after stimulation with ncp BVDV, suggesting that antiviral T cell responses involve $CD8^+$ T cells as effectors against BVDV-infected cells (Rhodes et al., 1999).

The increased expression of IL-15 in calves inoculated with BVDV-1 SD-1, but not in calves inoculated with BVDV-2 1373, suggests that the highly virulent BVDV strain might have inhibited the transcription of this cytokine; which is essential for NK cell activation during the innate immune response against viruses (Abbas and Lithman, 2005). This could be a potential mechanism by which highly virulent BVDV could cause immunosuppression. Other investigators (Chase et al., 2004; Lee et al., 2008) have demonstrated that IL-15 gene expression was significantly down-regulated after infection with ncp BVDV. During a viral infection, IL-15 is produced by activated monocytes/macrophages and dendritic cells and stimulates the proliferation of NK cells in a similar manner to IL-2 during the initiation of the adaptive immune response (Abbas and Lithman, 2005).

In the present study, calves inoculated with high virulence BVDV strain showed an increased expression of the Th2 cytokines IL-4 and IL-10. During a viral infection, secretion of IL-4 is important to stimulate B cell production of neutralizing antibodies (Waldvogel et al., 2000). Several studies have shown that experimental and natural infections with ncp BVDV stimulate the development of significant titers of neutralizing antibodies essential for prevention BVDV re-infection (House and Manley, 1973; Howard et al., 1989; Shope et al., 1976). Although the observed expression of IL-4 might contribute to the neutralizing antibody response (commonly observed in ncp BVDV-infected animals) to limit the spread of the virus (Doherty et al., 1997); such a Th2 response is not normally associated with antiviral effector functions (Rhodes et al., 1999). On the other hand, IL-4 can stimulate further development of Th2 cells

from naïve CD4^{+} T cells and antagonize the macrophage activating effects of IFN-γ, inhibiting the cell mediated immune response (Abbas and Lithman, 2005). Similarly, IL-10 has anti-inflammatory effects through its ability to inhibit activated macrophages by blocking the production of IL-12 and the expression of costimulators and class II MHC (Abbas and Lithman, 2005; Tizard, 2009). It might be possible that the increased expression of IL-4 and IL-10 mRNA in calves infected with BVDV-2 1373 is a regulatory mechanism to prevent an exacerbated inflammatory reaction associated with the Th1 immune response (Grütz, 2005). Moreover, in the present study a balance between Th1 and Th2 responses (with high positive correlation between Th1 and Th2 cytokines) observed during this early post-infection period might have been generated in an attempt to clear this virulent viral infection and control collateral damage (Waldvogel et al., 2000). An *in vitro* experiment demonstrated that CD4^{+} T cells from BVDV seropositive cattle produced high levels of IL-4 after stimulation with ncp BVDV, suggesting the development of a strongly Th2 biased memory immune response after BVDV infection (Rhodes et al., 1999). However, in the same study the CD8^{+} T cell response appeared to be Th1-like (producing IL-2 and IFN-γ). The Th2 cell immune reaction observed after infection with BVDV might be involved in BVDV-induced immunosuppression and increased susceptibility to secondary infections by interfering with the protective Th1 response (Arneborn and Biberfeld, 1983; Whittle et al., 1978).

A recent study showed that IL-4 mRNA was significantly up-regulated in BVDV seropositive cattle, which was associated with a concurrent increase in IFN-γ mRNA (Waldvogel et al., 2000), as seen in the current study. Moreover, Risalde et al. (2011) observed a significant increase of IL-10 concentration in calves co-infected with ncp BVDV and BHV-1, which was associated with the peak concentration of TNF-α. In that study, the concentration of IL-4 in

serum did not change, maintaining low and constant levels throughout the study (Risalde et al., 2011). In contrast, other researchers have reported that the expression of IL-10 mRNA was significantly decreased in BVDV-infected monocytes 24 h post-infection (Chase et al., 2004; Lee et al., 2008). In the same assay IL-4 gene showed background expression levels in the BVDV infected cells (Lee et al., 2008). The results of the current study indicate that there was no apparent early polarization toward a Th1 or Th2 immune response, since both pro-inflammatory and anti-inflammatory cytokine mRNA levels were up-regulated in the calves inoculated with BVDV 1373. Other authors have observed the existence of Th1 and Th2 subsets in cattle (Brown et al., 1994; Brown et al., 1993; Canals et al., 1997). Differentiation of naive Th cells toward Th1 or Th2 subsets and the expression of cytokines are influenced by several factors, such as the type of antigen presenting cells, cytokine environment (Rissoan et al., 1999), antigen dose, antigen density, presence of particular co-stimulation (Swain, 1999), and hormonal environment (Piccinni et al., 1995). In the present study simultaneous stimulation of different cell populations (Th1 and Th2) may have contributed to the observed cytokines expression without mutual inhibition (Waldvogel et al., 2000). On the other hand, it is possible to hypothesize that Th0 cell type, which express both IFN-γ and IL-4 mRNA, could have been responsible for the symmetrical cytokine expression observed (Brown et al., 1994; Brown et al., 1993; Waldvogel et al., 2000).

Calves inoculated with BVDV-1 SD-1 did not show up-regulation of IL-4 and IL-10 mRNA expression, which was accompanied by an absence of IFN-γ and IL-2 mRNA up-regulation. These results suggest that the experimental acute infection with the low virulence BVDV-1 did not activate the adaptive immune response by day 5 post inoculation, in contrast to infection with BVDV-2 1373, which was characterized by expression of different cytokines

associated with proliferation and differentiation of T lymphocytes. Expression of TGF-β mRNA did not increase in tracheo-bronchial lymph nodes and spleen of any of the BVDV inoculated groups, compared with the baseline expression observed in the control group. The expression of TGF-β has been associated with inhibition of macrophages, and T cell activation and stimulation of tissue repair after the inflammatory reaction has been controlled (Abbas and Lithman, 2005). Therefore, the lack of transcription of this cytokine in tracheo-bronchial lymph nodes and spleen post inoculation with BVDV could have been associated with the prompt time post infection (5 days post-infection) during which none or few inflammatory changes could have occurred in the respiratory tract.

In summary, experimental inoculation with high virulence BVDV-2 1373 stimulated both pro-inflammatory and anti-inflammatory cytokine gene expression, corresponding with activation of the innate and adaptive immune responses. In contrast, infection with the low virulence BVDV-1 SD-1 only resulted in up-regulation of IL-12 and IL-15, which is associated with activation of macrophages and NK cells (innate immunity). In addition to differences in virulence between the two strains, it is possible that the differential cytokine expression might be due to differences in the BVDV sub-genotype. The analysis of cytokine expression during the early events of the immune response to BVDV could have important implications for selection of BVDV strains to be used for new vaccine production, since the efficacy of a vaccine will depend on how efficiently it stimulates the innate and early adaptive immune response.

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VI. General conclusions

The research information presented in this dissertation constitutes a four-part investigation that was conducted to evaluate the immunopathogenesis of low and high virulence bovine viral diarrhea virus strains during acute infections as well as the effectiveness of vaccination protocols to prevent and control this infectious agent. Vaccines used in these studies were commercially available products in the US that were administered to investigate their efficacy and safety eliciting protective responses in susceptible calves and pregnant heifers.

The initial experiment was a vaccination-challenge study performed to evaluate the onset of protection induced by a modified-live BVDV vaccine administered 7, 5, or 3 days before challenge inoculation with a low virulence type 1b BVDV (strain NY-1). The modified-live BVDV vaccine prevented fever, viremia, and leukopenia in calves when they were inoculated with NY-1 BVDV. However, a high proportion of calves vaccinated 3 days before challenge inoculation shed BVDV after inoculation. Unvaccinated calves showed a significant decrease in leukocyte counts and increased temperature after experimental challenge with BVDV indicative of acute infection. In addition, a high proportion of unvaccinated calves showed viremia and disseminated BVDV in nasal secretions after BVDV challenge. This study represents a clinically relevant assay that demonstrates an efficacious strategy to prevent BVDV infection when animals are exposed shortly after vaccination. This strategy is particularly applicable for certain production systems such as feedlots, where frequent introduction of cattle with unknown BVDV status increases the risk of BVDV infection, which could result in severe respiratory disease in affected cattle and substantial economic losses for producers.

In the second part of the investigation a contaminated multivalent MLV vaccine containing ncp BVDV-1 and -2 was administered off-label to pregnant cattle to determine its ability to develop fetal persistent infections and be shed to cattle commingling with vaccinates. In the present study, abortions and BVDV fetal persistent infections occurred following off-label vaccination of BVDV-naïve pregnant heifers with the contaminated MLV vaccine as well as in the unvaccinated herdmates.

Virus isolation, quantitative real time RT-PCR and nucleotide sequencing analysis confirmed that vaccination of pregnant heifers with a contaminated modified-live BVDV vaccine resulted in development of BVDV-2 PI fetuses in all the tested vaccinated animals. BVDV was apparently shed to unvaccinated heifers causing fetal infections from which only BVDV-1 was detected. The hypothesized model of "viral superinfection exclusion" (ability of an established viral infection to block a homologous superinfecting virus) could possibly explain the fact that only BVDV-2 was detected in all PI fetuses from vaccinated heifers. On the other hand, differences in "viral pneumotropism" could be associated with the detection of only BVDV-1 in the 2 PI fetuses obtained from the unvaccinated heifers. It might be possible that following immunization with the contaminated vaccine, the BVDV-1 principal strain replicated more efficiently in the respiratory tract of the vaccinated group than the BVDV-2 contaminant strain. Therefore, the BVDV-1 strain could have been selectively shed via respiratory secretions being able to infect the control group causing the development of PI fetuses.

The vaccine used in the present study was labeled with a caution for not being used in pregnant cows or calves nursing pregnant cows. However, there is a potential risk for transplacental fetal infection and development of PI fetus if susceptible pregnant animals have an occasional contact with recently vaccinated animals. Quantitative RT-PCR and nucleotide

sequencing analysis revealed the presence of both BVDV-1 and BVDV-2 in the vaccine, confirming the vaccine contamination reported by the manufacturer. This study demonstrated that contamination of MLV vaccines with BVDV still represents a risk for bovine herd health programs. Common sources of BVDV contamination include permanent cell lines used for viral replication-attenuation and fetal calf serum batches. Therefore, it is recommended that bovine sera and cell lines be screened for adventitious BVDV by RT-PCR when used for cell cultures. This strategy would help to prevent contamination before and during vaccine production and increase the product's safety

Finally, a BVDV challenge inoculation model was designed for evaluation of the early immune response during acute BVDV infections. This study compared the mRNA expression of host genes involved in type-I interferon-induced antiviral state (IFN-α, IFN-β, Mx-1, PKR, OAS-1 and ISG-15), and apoptosis (Caspase-3, -8, and -9), after experimental infection of beef calves with low or high virulence ncp BVDV strains. The results indicated that experimental infection with high or low virulence BVDV strains induced a significant expression of the type I interferon-induced antiviral state in beef calves. Surprisingly, the expression of Mx-1, PKR, OAS-1 and ISG-15 observed in calves inoculated with the low virulence BVDV occurred without an apparent expression of type I IFN- α/-β by day 5 post challenge. It might be possible that IFN-α and -β mRNA levels could have been transiently increased before day 5 post inoculation with BVDV SD-1 strain, resulting in expression of the type I IFN-stimulated genes and activation of the antiviral state. Subsequently, a broad range of molecular mechanisms of negative feedback could have shut down the IFN-α/-β signaling around day 5 post challenge in this group. In addition, it is possible that transcription of the antiviral genes (Mx1, PKR, OAS-1

and ISG-15) have been induced directly and independently of IFN -α and -β by the low virulence BVDV.

There was a differential expression of some interferon-induced genes (OAS-1 and ISG-15), with mRNA levels that were higher for the calves inoculated with the low virulence BVDV strain. Type I IFN mRNA expression was up-regulated in tracheo-bronchial lymph nodes but not in spleen, reflecting a spatial regulation type I IFN transcription. Since experimental challenge was done through nasal aerosolization, the differential expression of type I IFN mRNA in these lymphoid organs may be due to the route of viral infection. A significant up-regulation of caspase-8 and -9 was observed in tracheo-bronchial lymph nodes of the calves inoculated with low virulence BVDV, but not in those inoculated with the highly virulent BVDV-2 1373.

Finally, the mRNA expression of cytokines involved in pro-inflammatory (TNF-α, IL-1β, IFN-γ, IL-2, IL-12, IL-15), and anti-inflammatory (IL-4, IL-10, TGF-β) responses after experimental infection of beef calves with low or high virulence ncp BVDV strains was also compared. Experimental inoculation with BVDV-2 1373 stimulated a significant mRNA expression of both pro-inflammatory and anti-inflammatory cytokines. However, inoculation with BVDV-1 SD-1 resulted in up-regulation of only IL-12 and IL-15 mRNA, which are associated with activation of macrophages and NK cells during the innate immune response.

The significant expression of the pro-inflammatory cytokines and cytokines involved in the activation of the adaptive cell mediated immune response after infection with the BVDV-2 1373, could be associated with the commonly observed severe inflammatory lesions in the respiratory tract and the development of severe clinical signs following experimental infection with virulent BVDV-2 strains. In contrast, the low expression of these cytokines observed following infection with the low virulence BVDV strain could be related to the low degree of

damage of the respiratory tract and mild or no clinical signs commonly evidenced during most infections with low virulence BVDV-1. It could be possible that some mechanisms of the early innate immune response, such as up-regulation of type I IFN-induced antiviral state, macrophages and NK cells (increased IL-12 and IL-15 mRNA levels), were apparently sufficient to control this low virulence infection by day 5 post inoculation, resulting in absent or delayed activation of Th1 type adaptive immunity (IL-2 and IFN-γ).

It might be also possible that the increased expression of IL-4 and IL-10 mRNA in calves infected with BVDV-2 1373 was a regulatory mechanism to prevent an exacerbated inflammatory reaction associated with the Th1 immune response. The results of the current study indicate that there was no apparent early polarization toward Th1 or Th2 immune response, since both pro-inflammatory and anti-inflammatory cytokine mRNA levels were up-regulated in the calves inoculated with BVDV 1373. Calves inoculated BVDV-1 SD-1 did not show up-regulation of IL-4 and IL-10 mRNA expression, which was accompanied by an absence of IFN-γ and IL-2 mRNA up-regulation. The observed differential expression of early immune response after infection with low or high virulence BVDV might reflect differences in viral pathogenesis and could possibly determine the clinical outcome . The analysis of cytokine expression during the early events of immune response to BVDV could have important implications for selection of BVDV strains to be used for new vaccine production, since the efficacy of a vaccine will depend on how efficiently it stimulates the innate and early adaptive immune responses.

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Appendix I: Tables

Table 1. Mean ± SEM rectal temperature (°C) the day of (day = 0) and for 14 days after challenge inoculation with type 1b BVDV (strain NY-1) for calves vaccinated with an MLV vaccine containing types 1a and 2 BVDV, BHV-1, PI3, and BRSV 7 (n = 10), 5 (10), or 3 (9) days before BVDV inoculation or that were unvaccinated control calves (10).

Day	Calves vaccinated 7 days before inoculation	Calves vaccinated 5 days before inoculation	Calves vaccinated 3 days before inoculation	Unvaccinated control calves
0	39.4 ± 0.15	39.5 ± 0.11	39.1 ± 0.13	38.9 ± 0.14
3	38.6 ± 0.28	38.4 ± 0.18	38.4 ± 0.13	38.8 ± 0.17
5	38.2 ± 0.46	38.2 ± 0.25	38.5 ± 0.16	38.6 ± 0.14
6	38.1 ± 0.44	38.3 ± 0.16	38.8 ± 0.18	38.8 ± 0.15
7	38.8 ± 0.10	38.8 ± 0.13	39.3 ± 0.13	40.2 ± 0.29*†
8	38.9 ± 0.13	38.7 ± 0.09	38.9 ± 0.22	40.1 ± 0.18**‡
9	38.8 ± 0.17	39.0 ± 0.12	38.9 ± 0.08	39.0 ± 0.19
11	39.2 ± 0.15	39.2 ± 0.18	39.2 ± 0.11	39.2 ± 0.08
14	39.3 ± 0.07	39.2 ± 0.09	38.8 ± 0.12	39.2 ± 0.13

*Within a column, value is significantly (*P* = 0.003) different from temperature on day 0. ** Within a column, value is significantly (*P* = 0.001) different from temperature on day 0. †Within a row, value is significantly (*P* = 0.015) different from the value for each of the other treatment groups. ‡ Within a row, value is significantly (*P* < 0.001) different from the value for each of the other treatment groups. SEM: Standard error of the mean.

Table 2. Geometric mean ± SEM antibody titer against NY-1 BVDV (type 1b) the day of (day = 0) and 14 days after challenge inoculation with type 1b BVDV (strain NY-1) for calves vaccinated with an MLV vaccine containing types 1a and 2 BVDV, BHV-1, PI3, and BRSV, 7 (n = 10), 5 (10), or 3 (9) days before BVDV inoculation or that were unvaccinated control calves (10).

Day	Calves vaccinated 7 days before inoculation	Calves vaccinated 5 days before inoculation	Calves vaccinated 3 days before inoculation	Unvaccinated control calves
0	1.78 ± 1.21	2.0 ± 1.18	1.52 ± 1.05	2.46 ± 1.27
14	12.69 ± 1.25*	10.07 ± 1.38*	11.75 ± 1.46*	73.51 ± 1.35*†

*Within a column, values differ significantly ($P < 0.001$) from the value on day 0. †Within a row, value differs significantly ($P < 0.001$) from the value for each of the other treatment groups. SEM: Standard error of the mean.

Table 3. Mean ± SEM leukocyte count on the day of (day = 0) and for 14 days after challenge inoculation with type 1b BVDV (strain NY-1) for calves vaccinated with an MLV vaccine containing types 1a and 2 BVDV, BHV-1, PI3, and BRSV, 7 (n = 10), 5 (10), or 3 (9) days before BVDV inoculation or that were unvaccinated control calves (10).

Day	Calves vaccinated 7 days before inoculation	Calves vaccinated 5 days before inoculation	Calves vaccinated 3 days before inoculation	Unvaccinated control calves
0	6,780 ± 801.6	5,738 ± 341.0	7,106 ± 583.2	8,646 ± 790.8
3	7,554 ± 573.5	7,485 ± 349.2	6,448 ± 516.0	4,941 ± 425.2*†
5	6,900 ± 651.2	6,877 ± 451.7	8,161 ± 877.6	4,978 ± 271.5*†
6	7,069 ± 785.8	6,522 ± 338.4	7,454 ± 370.3	4,958 ± 313.1*†
8	7,048 ± 557.7	7,031 ± 355.5	7,302 ± 234.6	7,874 ± 574.3
11	7,519 ± 774.5	7,177 ± 302.6	6,660 ± 438.0	7,263 ± 530.4
14	7,869 ± 580.7	7,798 ± 576.6	6,680 ± 350.4	7,163 ± 498.3

*Within a column, value differs significantly ($P < 0.001$) from the value on day 0. †Within a row, value differs significantly ($P < 0.05$) from the value for each of the other treatment groups.

Table 4. Primers and probes used for amplification by RT-PCR and nucleotide sequencing of a portion of the 5' untranslated region (5'UTR) of BVDV .

Primer and probes	Name	Sequences (5'- 3')	Reference
BVDV primers (qRT-PCR)	BVDu3	F: CAT GCC CAA AGC ACA TCT TA	(Marley et al., 2008)
	BVD11	R: TGC CAT GTA CAG CAG AGA TTT	
5'-BVDV probe (qRT-PCR)		AYG RAY ACA GCC TGA TAG GGT GY	(Marley et al., 2008)
3'-BVDV-1 probe (qRT-PCR)	BVDV-1	CAG AGG CCC ACT GTA TTG CTA CTA AA	(Marley et al., 2008)
3'-BVDV-2 probe (qRT-PCR)	BVDV-2	CAG AGA CCT GCT ATT CCG CTA GTA AA	(Marley et al., 2008)
BVDV primers (sequencing)	BVDV-100	F: GGC TAG CCA TGC CCT TAG	(Deng and Brock, 1993)
	BVDV-345	R: GCC TCT GCA GCA CCC TAT	

Table 5. Virus neutralizing antibody titers against BVDV-1a (SD-1) and BVDV-2 (PA131) in vaccinated and control heifers the day of (day 0) and 45 days after vaccination (day 45) with a MLV vaccine containing ncp BVDV strain WRL.

Heifer ID	Group	BVDV-1 Antibody titer*		BVDV-2 Antibody titer*	
		day 0	day 45	day 0	day 45
434	Vx	<1:5	≥1:640	<1:5	1:320
444	Vx	<1:5	≥1:640	<1:5	1:320
449	Vx	<1:5	1:160	<1:5	1:160
467	Vx	<1:5	1:40	<1:5	1:80
469	Vx	<1:5	1:80	<1:5	1:160
482	Vx	<1:5	1:40	<1:5	1:160
507	Vx	<1:5	1:80	<1:5	1:80
511	Vx	<1:5	1:160	<1:5	1:80
547	Vx	<1:5	1:80	<1:5	1:160
566	Vx	<1:5	1:160	<1:5	1:160
447	Co	<1:5	<1:5	<1:5	<1:5
465	Co	<1:5	1:320	<1:5	1:160
476	Co	<1:5	≥1:640	<1:5	1:20
502	Co	<1:5	<1:5	<1:5	<1:5
509	Co	<1:5	≥1:640	<1:5	1:20
510	Co	<1:5	<1:5	<1:5	<1:5
512	Co	<1:5	1:10	<1:5	<1:5
520	Co	<1:5	<1:5	<1:5	<1:5
542	Co	<1:5	1:320	<1:5	<1:5

Virus neutralization (VN) was performed at serial two-fold dilutions starting at 1:5 through 1:640.

* The antibody titer of each sample was determined using the Reed and Muench's method. The endpoint dilutions reflected the highest dilution of serum that inhibited the replication of virus in cell culture.

Table 6. Virus neutralizing antibody titers (GMT) and percentage of seroconversion against BVDV-1 and BVDV-2 in vaccinated and control heifers.

Group	BVDV-1				BVDV-2			
	GMT		Seroconversion		GMT		Seroconversion	
	day 0	day 45*	n	%	day 0	day 45*	n	%
Vaccinated	< 5	130 ± 6.5	10/10	100	< 5	149 ± 5.8	10/10	100
Control	< 5	211 ± 10.8	4/9	44.4	< 5	40 ± 10	1/9	11.1

Vaccinated heifers received a single dose of MLV vaccine containing BHV-1, PI3, BRSV and ncp BVDV strain WRL. * Only antibody titer above baseline (>1:5) was considered for calculation of GMT against BVDV-1 and BVDV-2 on day 45 post vaccination.

Table 7. Virus isolation from fetal fluids and tissues collected from vaccinated (Vx) and control (Co) heifers. Vaccinated heifers received a single dose of MLV vaccine containing ncp BVDV WRL strain.

Heifer ID	Group	Fetal Fluids and Tissues						Observations
		AF	HB	Lungs	Spleen	Kidney	Thymus	
434	Vx	NA	NA	NA	NA	NA	NA	Aborted
444	Vx	-	+	+	+	+	+	
449	Vx	-	+	+	+	+	+	
467	Vx	-	-	+	+	+	+	
469	Vx	-	+	+	+	+	+	
482	Vx	-	-	+	+	+	+	
507	Vx	+	-	+	+	+	+	
511	Vx	NA	NA	NA	NA	NA	NA	Aborted
547	Vx	-	+	+	+	+	+	
566	Vx	+	+	+	+	+	+	
447	Co	NA	NA	NA	NA	NA	NA	Aborted
465	Co	NA	NA	NA	NA	NA	NA	Aborted
476	Co	-	-	-	-	-	-	
502	Co	-	-	-	.	-	-	
509	Co	-	-	+	+	+	+	
510	Co	-	-	-	-	-	-	
512	Co	-	-	-	-	-	-	
520	Co	-	-	-	-	-	-	
542	Co	+	+	+	+	+	+	

AF: Amniotic fluid. HB: Heart blood.

Table 8. Virus isolation (VI) from fetal tissues collected from vaccinated and control heifers. Vaccinated heifers received a single dose of MLV vaccine containing ncp BVDV WRL strain.

Group	Aborted fetuses*		Fetuses BVDV-Positive (VI)	
	n	%	n	%
Vaccinated n=10)	2/10	20.0[a]	8/8	100[a]
Control (n=9)	2/ 9	22.2[a]	2/7	28.6[b]

[a,b] Within a column, value differs significantly ($P < 0.05$) between groups.

Table 9. Quantitative RT-PCR analysis on fetal spleen and thymus using specific probes for BVDV-1 and BVDV-2.

		Spleen				Thymus			
		BVDV-1		BVDV-2		BVDV-1		BVDV-2	
Dam ID	Group	(+/-)	copies/μl	(+/-)	copies/μl	(+/-)	copies/μl	(+/-)	copies/μl
444	Vx	-	0	+	4.98×10^5	-	0	+	3.10×10^5
449	Vx	-	0	+	1.29×10^5	-	0	+	1.77×10^5
467	Vx	-	0	+	2.58×10^5	-	0	+	3.84×10^4
469	Vx	-	0	+	1.34×10^4	-	0	+	2.28×10^3
482	Vx	-	0	+	7.46×10^5	-	0	+	8.54×10^4
507	Vx	-	0	+	1.12×10^5	-	0	+	2.16×10^5
547	Vx	-	0	+	1.16×10^5	-	0	+	4.98×10^5
566	Vx	-	0	+	9.92×10^4	-	0	+	8.32×10^4
476	Co	-	0	-	0	-	0	-	0
502	Co	-	0	-	0	-	0	-	0
509	Co	+	4.68×10^5	-	0	+	1.09×10^5	-	0
510	Co	-	0	-	0	-	0	-	0
512	Co	-	0	-	0	-	0	-	0
520	Co	-	0	-	0	-	0	-	0
542	Co	+	3.78×10^6	-	0	+	2.14×10^5	-	0

Table 10. Alignment of BVDV 5'UTR sequences extracted from fetal spleen samples with BVDV sequences published in GenBank using BLAST.

ID	Group	Sequences with Significant alignment	BVDV Strains	maximal identity (%)	Coverage (%)	GenBank Accession number
444 449 467 469 482 507 547 566	Vx Vx Vx Vx Vx Vx Vx Vx	5' UTR BVDV-2	23025	100	99	AF039172
			NY-93	99	99	AF502399.1
			1373	98	99	AF145967.2
			890	97	99	AF039180.1
509 542	Co Co	5' UTR BVDV-1	MS-1	99	95	GU395526.1
			6004306	98	94	FJ387314.1
			NY1	96	96	AF039178
			Osloss	96	93	AY279527.1

Table 11. Alignment of BVDV 5'UTR sequences extracted from the contaminated MLV vaccine with BVDV sequences published in GenBank using BLAST.

Vaccinal Sequences	Sequences with Significant alignment	BVDV Strains	maximal identity (%)	Coverage (%)	GenBank Accession number
Principal (Large peaks)	BVDV-1 5' UTR	221707	99	97	FJ387311.1
		NADL	98	92	AJ133739.1
		SD-1	95	99	M96751.1
		Oregon CV24	95	100	AF091605.1
Contaminant (Small peaks)	BVDV-2 5' UTR	23025	98	100	AF039172
		NY-93	97	100	AF502399.1
		1373	96	100	AF145967.2
		890	95	100	AF039180.1

Table 12. Alignment of the 5' UTR of strains obtained from persistently infected fetuses with the two BVDV sequences present in the contaminated MLV vaccine. Dots indicate identical residues and hyphens illustrate gaps.

Sample	Group	Sequence 5'-3'
		34 … 122
Contaminant		CCCCGCATGGGTTAAGATGTGCCGTGTGCATGCCCTCGTCCACATGGCATCTCGACACTCCTCAACTAATGGAGTGTCGAACCATTGAC
444	Vx	..........................G............................G.................................
449	Vx	..........................G............................G.................................
467	Vx	..........................G............................G.................................
469	Vx	..........................G............................G.................................
482	Vx	..........................G............................G.................................
507	Vx	..........................G............................G.................................
547	Vx	..........................G............................G.................................
566	Vx	..........................G............................G.................................
		20 … 110
Predominant		GTTTTCGCCCGAGCGACCCCTGCTCGGGTTAAGATGTGCTTTGGGCATGCCCTCGTCCACGTGGCATCTCGAGACCTTTATGCCAAGGCGT
509	Co	......A..T..A.......C.TC.A.A............G................................G-....CCC-.G.A....
542	Co	......A..T..A.......C.TC.A..............G..........................A.....G.T.G.CCT....A....

Sample	Group	Sequence 5'-3'
		123 … 207
Contaminant		GACTCCCCTGTACTCAGGGGTTCGGCCATCCAACGAACTCACTGCTACCGCTAGTCCCCTCCTTTTGCTAGTCCTACTAAGGGCA
444	Vx	....
449	Vx	....
467	Vx	....
469	Vx	....
482	Vx	....
507	Vx	....
547	Vx	....
566	Vx	....
		111 … 199
Predominant		CGAACCACTGACGACTACCCTGTACTCAGGGCTTAAGCCATCCAACGAACTCACCACTGGCTACCCCCCTCATTATGCTAGTCCTACTA
509	Co	..................................C...................................T...T..............
542	Co	..................................C...................................T...T..............

Table 13. Primers used for quantitative RT-PCR during the mRNA expression analysis of genes involved in the type I Interferon-induced antiviral state after experimental challenge with low (BVDV-1 strain SD-1) or high (BVDV-2 strain 1373) virulence BVDV.

Genes	GeneID	Description	Primer sequences	Reference
GAPDH	281181	Glyceraldehyde-3-phosphate dehydrogenase (*Bos taurus)*	Fwd: 5´GATTGTCAGCAATGCCTCCT 3´ Rev: 5´GGTCATAAGTCCCTCCACGA3´	(Smirnova et al., 2008)
IFN-α	EU276064	Interferon alpha (*Bos taurus)*	Fwd:5´GTGAGGAAATACTTCCACAGACTCACT3' Rev: 5´TGARGAAGAGAAGGCTCTCATGA3´	(Baldwin, 2008)
IFN-β	EU276065	Interferon, beta 1, fibroblast (*Bos taurus)*	Fwd: 5´ACAGAGTCACCCACCTCACC 3´ Rev: 5´GACCAGGTGTGTGTCAGTCC 3´	(Baldwin, 2008)
Mx-1	280872	Myxovirus resistance factor 1, interferon-inducible protein p78 *(Bos taurus)*	Fwd: 5´ATCTTTCAACACCTGACCGCG3´ Rev: 5´GGAGCACGAAGAACTGGATGAT3´	(Yamane et al., 2008)
PKR	282875	Interferon-inducible RNA-dependent protein kinase	Fwd: 5´GTTGGGATGGGCATGATTATG3´ Rev: 5´AACGTTTGTCTGGCTTCTTGC3´	(Yamane et al., 2008)
OAS-1	347699	2',5'-Oligoadenylate synthetase 1 40/46kDa (*Bos taurus)*	Fwd: 5´AGCCATCGACATCATCTGCAC3´ Rev: 5´CCACCCTTCACAACTTTGGAC3´	(Yamane et al., 2008)
ISG-15	281871	Interferon stimulated gene 15KD, Ubiquitin-like modifier *(Bos taurus)*	Fwd: 5´GGTATCCGAGCTGAAGCAGTT3´ Rev: 5´ACCTCCCTGCTGTCAAGGT3´	(Smirnova et al., 2008)
Casp 3	NM_214131	Bos taurus caspase 3, apoptosis-related cysteine peptidase	Fwd: 5´AACCTCCGTGGATTCAAAATC 3´ Rev: 5´TTCAGGRTAATCCATTTTGTAAC 3´	(Kliem et al., 2009)
Casp 8	NM_001045970	Bos taurus caspase 8, apoptosis-related cysteine peptidase	Fwd: 5´TGTCACAATCGCTTCCAGAG3´ Rev: 5´GAAGTTCAGGCACCTGCTTC3´	(Groebner et al., 2010)
Casp 9	NM_001205504.1	Bos taurus caspase 9, apoptosis-related cysteine peptidase	Fwd: 5´CCTGTGGTGGAGAGCAGAAAG3´ Rev:5´CATCTGGCTCGTCAATGGAA3´	(Norgaard et al., 2008)

Table 14. Virus isolation from serum and spleen samples on days 0 and 5 in beef calves experimentally challenged with low (LV, strain SD-1) and high (HV, strain 1373) virulence BVDV.

		Day 0	Day 5	
		Serum	Serum	Spleen
ID tag	Group	VI	VI	VI
8031	LV	-	+	+
8040	LV	-	-	+
8054	LV	-	+	+
8082	LV	-	+	+
8089	LV	-	-	+
8118	LV	-	+	+
8121	LV	-	-	+
8131	LV	-	+	+
8042	HV	-	+	+
8063	HV	-	+	+
8065	HV	-	+	+
8072	HV	-	+	+
8073	HV	-	+	+
8077	HV	-	+	+
8078	HV	-	+	+
8081	HV	-	-	+
8100	HV	-	+	+
8124	HV	-	+	+
8030	Co	-	-	-
8064	Co	-	-	-
8070	Co	-	-	-
8075	Co	-	-	-
8090	Co	-	-	-
8105	Co	-	-	-
8125	Co	-	-	-
8126	Co	-	-	-
8127	Co	-	-	-
8133	Co	-	-	-

Table 15. Pearson correlation analysis between mRNA expression of cytokines involved in the Type I Interferon induced antiviral state in tracheo-bronchial lymph nodes of calves challenged with low (SD-1) and high (1373) virulence BVDV strains , relative to the control group.

	IFN-α	IFN-β	Mx-1	PKR	OAS-1	ISG-15	Casp-3	Casp-8	Casp-9
IFN-α	1								
IFN-β	0.98 (P < 0.001)	1							
MX-1	-0.56 (P=0.01)	-0.59 (P =0.009)	1						
PKR	NS	NS	0.53 (P=0.02)	1					
OAS-1	NS	NS	NS	0.70 (P =0.001)	1				
ISG-15	-0.59 (P = 0.009)	-0.59 (P=0.009)	0.79 (P < 0.001)	0.66 (P = 0.002)	0.70 (P =0.001)	1			
Casp-3	NS	NS	0.72 (P= 0.0007)	0.61 (P = 0.007)	NS	0.56 (P = 0.01)	1		
Casp-8	-0.58 (P = 0.01)	-0.61 (P =0.007)	0.89 (P < 0.001)	0.64 (P = 0.003)	0.59 (P = 0.009)	0.86 (P < 0.0001)	0.77 (P = 0.0001)	1	
Casp-9	NS	NS	0.48 (P= 0.04)	0.61 (P = 0.007)	0.70 (P =0.001)	0.85 (P < 0.0001)	NS	0.59 P = (0.009)	1

Values of $P < 0.05$ are considered statistically significant.

Table 16. Pearson correlation analysis between mRNA expression of cytokines involved in the Type I Interferon induced antiviral state in spleen of calves challenged with low (SD-1) and high (1373) virulence BVDV strains , relative to the control group.

	IFN-α	**IFN-β**	**Mx-1**	**PKR**	**OAS-1**	**ISG-15**	**Casp-3**	**Casp-8**	**Casp-9**
IFN-α	1								
IFN-β	0.94 (P < 0.001)	1							
MX-1	-0.51 (P=0.02)	-0.52 (P =0.02)	1						
PKR	NS	NS	0.83 (P<0.0001)	1					
OAS-1	NS	NS	0.59 (P = 0.008)	0.68 (P =0.001)	1				
ISG-15	-0.53 (P = 0.02)	-0.56 (P=0.01)	0.86 (P < 0.0001)	0.65 (P = 0.003)	0.61 (P =0.006)	1			
Casp-3	-0.60 (P = 0.007)	-0.62 (P = 0.005)	0.70 (P= 0.001)	0.62 (P = 0.005)	NS	NS	1		
Casp-8	-0.64 (P = 0.004)	-0.71 (P =0.0008)	0.68 (P = 0.001)	0.72 (P = 0.0007)	0.61 (P = 0.007)	NS	0.89 (P < 0.0001)	1	
Casp-9	-0.82 (P < 0.0001)	-0.85 (P < 0.0001)	0.66 (P= 0.002)	0.55 (P = 0.01)	0.78 (P =0.0001)	0.64 (P = 0.004)	0.70 (P= 0.001)	0.79 (P < 0.0001)	1

Values of $P < 0.05$ are considered statistically significant.

Table 17. Primers used for quantitative RT-PCR during the mRNA expression analysis of proinflammatory and antinflammatory cytokines after experimental challenge with low (BVDV-1 strain SD-1) and high (BVDV-2 strain 1373) virulence BVDV strains.

Genes	GeneID	Description	Primer sequences	Reference
BVDV 5'UTR		5' untranslated region between nucleotides 100 and 345.	Fwd: 5´GATTGTCAGCAATGCCTCCT 3´ Rev: 5´GGTCATAAGTCCCTCCACGA3´	(Deng and Brock, 1992)
GAPDH	281181	Glyceraldehyde-3-phosphate dehydrogenase (*Bos taurus)*	Fwd: 5´GATTGTCAGCAATGCCTCCT 3´ Rev: 5´GGTCATAAGTCCCTCCACGA3'	(Smirnova et al., 2008)
TNF-α	280943	Tumor necrosis factor alpha (*Bos taurus*	Fwd : 5´CCTGGTACGAACCCATCTA3´ Rev: 5´ATCCCAAAGTAGACCTGCC3´	(Yamane et al., 2008)
IL-1β	EU438767	Bovine Interleukin 1-beta	Fwd: 5´AAAGCTTCAGGCAGGTGGTG3´ Rev: 5´TGCGTAGGCACTGTTCCTCA3´	(Werling et al., 2002)
IFN-γ	EU276066	Bovine Interferon gamma	Fwd: 5´GTAGCCCTGTGCCTGATTTC3´ Rev: 5´CACATTGTCCCTTCCCAGAG3´	(Baldwin, 2008)
IL-2	M12791	Bovine interleukin 2	Fwd:5´GAAAGTTAAAAATCCTGAGAACCTCAA 3´ Rev: 5´GCGTTAACCTTGGGCACGTA 3´	(Seo et al., 2007)
IL-12	U11815	Bos taurus interleukin 12	Fwd:5´CATCAGGGACATCATCAAACCA3´ Rev: 5'CCTCCACCTGCCGAGAATT´3´	(Seo et al., 2007)
IL-15	EU682380.1	Bovine Interleukin 15 (*Bos taurus*)	Fwd: 5´GCAAGGATCCCCATATTTGAGAAGTACTTCCATCCAG3´ Rev: 5´GCAAGGGCCCAGAAGTGTTGATGAACATTTG 3´	(Baldwin, 2008)
IL-4	M77120	Bovine interleukin 4 (*Bost taurus*)	Fwd 5'AGGAGCCACACGTGCTTGA 3' Rev 5'TTGCCAAGCTGTTGAGATTCC3'	(Seo et al., 2007)
IL-10	U00799	Charolais interleukin-10 (*Bos taurus*)	Fwd 5'TTCTGCCCTGCGAAAACAA3' Rev 5'TCTCTTGGAGCTCACTGAAGACTCT3'	(Seo et al., 2007)
TGF-β	M36271	Bovine transforming growth factor-beta-1	Fwd 5'CATCTGGAGCCTGGATACACAGT 3' Rev 5'GAAGCGCCCGGGTTGT3'	(Seo et al., 2007)

Table 18. Diagnosis of BVDV by virus isolation and RT-PCR from serum and spleen samples in beef calves experimentally challenged with low (LV, strain SD-1) and high (HV, strain 1373) virulence BVDV.

		Serum				Spleen	
		Day 0		Day 5		Day 5	
ID tag	Group	VI	PCR	VI	PCR	VI	PCR
8031	LV	-	-	+	+	+	+
8040	LV	-	-	-	+	+	+
8054	LV	-	-	+	+	+	+
8082	LV	-	-	+	+	+	+
8089	LV	-	-	-	+	+	+
8118	LV	-	-	+	+	+	+
8121	LV	-	-	-	+	+	+
8131	LV	-	-	+	+	+	+
8042	HV	-	-	+	+	+	+
8063	HV	-	-	+	+	+	+
8065	HV	-	-	+	+	+	+
8072	HV	-	-	+	+	+	+
8073	HV	-	-	+	+	+	+
8077	HV	-	-	+	+	+	+
8078	HV	-	-	+	+	+	+
8081	HV	-	-	-	+	+	+
8100	HV	-	-	+	+	+	+
8124	HV	-	-	+	+	+	+
8030	Co	-	-	-	-	-	-
8064	Co	-	-	-	-	-	-
8070	Co	-	-	-	-	-	-
8075	Co	-	-	-	-	-	-
8090	Co	-	-	-	-	-	-
8105	Co	-	-	-	-	-	-
8125	Co	-	-	-	-	-	-
8126	Co	-	-	-	-	-	-
8127	Co	-	-	-	-	-	-
8133	Co	-	-	-	-	-	-

Table 19. Pearson correlation analysis between mRNA expression of cytokines involved in T cell activation and antinflammatory response in tracheo-bronchial lymph nodes of calves challenged with low (SD-1) and high (1373) virulence BVDV strains, relative to the control group.

	TNF-α	IL-1β	IFN-γ	IL-2	IL-12	IL-15	IL-4	IL-10	TGF-β
TNF-α	1								
IL-1β	0.92 (P < 0.0001)	1							
IFN-γ	0.89 (P=0.0001)	0.91 (P < 0.0001)	1						
IL-2	0.65 (P=0.003)	0.62 (P =0.006)	0.82 (P<0.0001)	1					
IL-12	-0.53 (P=0.02)	-0.63 (P =0.004)	-0.48 (P =0.04)	NS	1				
IL-15	-0.58 (P = 0.01)	-0.59 (P=0.009)	-0.62 (P =0.005)	-0.54 (P =0.01)	0.60 (P =0.007)	1			
IL-4	0.93 (P < 0.0001)	0.91 (P < 0.0001)	0.96 (P<0.0001)	0.78 (P =0.0001)	NS	-0.61 (P =0.006)	1		
IL-10	0.64 (P = 0.003)	0.67 (P =0.001)	0.57 (P=0.01)	NS	NS	NS	0.64 (P =0.003)	1	
TGF-β	-0.69 (P = 0.001)	-0.76 (P = 0.0002)	-0.65 (P= 0.002)	NS	0.89 (P <0.0001)	0.67 (P =0.002)	-0.65 (P =0.003)	NS	1

Values of $P < 0.05$ are considered statistically significant.

Appendix II: Figures

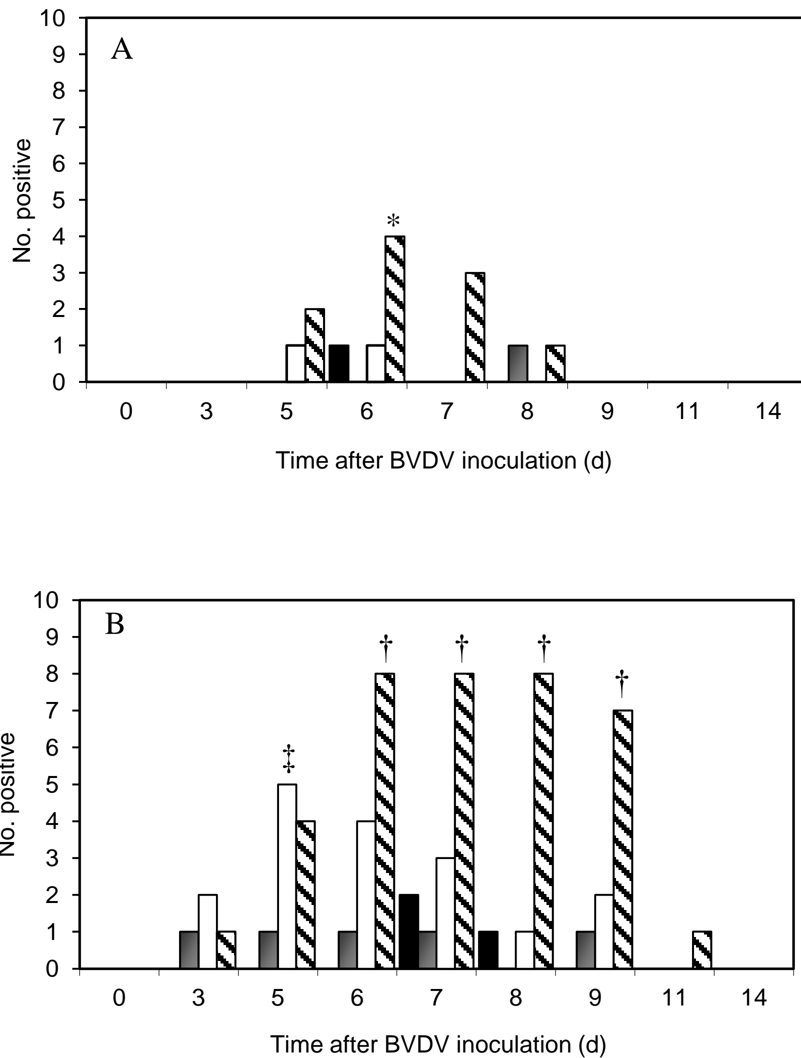

Figure 1. Number of positive results for BVDV via VI from serum samples (A) and nasal swab specimens (B) obtained from calves the day of (day = 0) and for 14 days after challenge inoculation with type 1b BVDV (strain NY-1). Calves were vaccinated with an MLV vaccine containing types 1a and 2 BVDV, BHV-1, PI-3, and BRSV 7 (n = 10; black bars), 5 (10; gray bars), or 3 (9; white bars) days before BVDV inoculation or were unvaccinated control calves (10; diagonal-striped bars). *Value is significantly (*P* = 0.04) higher than that for calves vaccinated 5 days before BVDV inoculation. †Value is significantly (*P* < 0.05) higher than that of each of the other 3 groups. ‡Value is significantly higher than that for calves vaccinated 5 (*P* = 0.04) or 7 (*P* = 0.0075) days before BVDV inoculation.

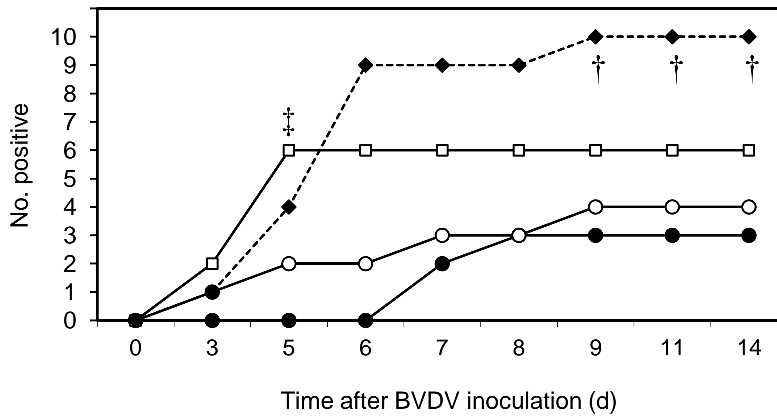

Figure 2. Cumulative number of positive results for BVDV via VI from nasal swab specimens obtained from calves the day of (day = 0) and for 14 days after challenge inoculation with type 1b BVDV (strain NY-1). Calves were vaccinated with an MLV vaccine containing types 1a and 2 BVDV, BHV-1, PI3, BRSV 7 (n = 10; line with closed circles), 5 (10; line with open circles), or 3 (9; line with open squares) days before BVDV inoculation or were unvaccinated control calves (10; dashed line with closed diamond). †Value is significantly higher than that for calves vaccinated 5(P = 0.0043) *or* 7 (P = 0.0024) days before BVDV inoculation . ‡Value is significantly higher than that for calves vaccinated 5 (P = 0.04) or 7 (P = 0.0024) days before BVDV inoculation.

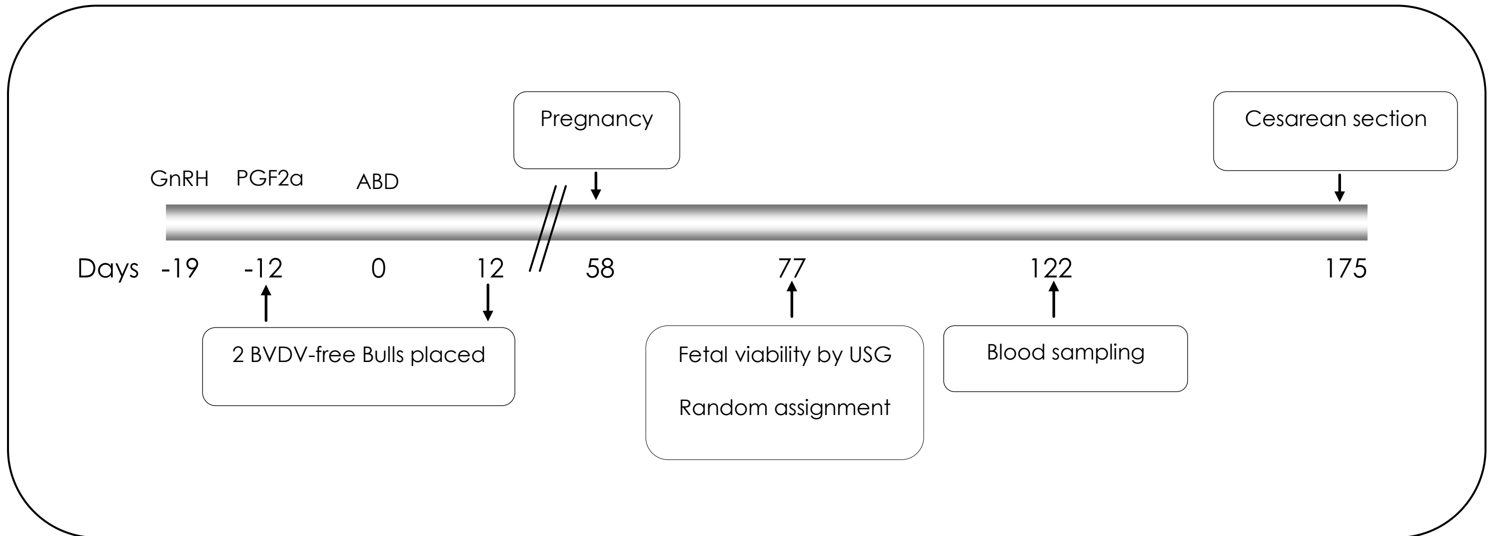

Figure 3. Experimental protocol for estrus synchronization, breeding, pregnancy diagnosis, ultrasonography (USG), blood sampling, caesarean section and fetal harvest. ABD: Average breeding day. USG: ultrasonography.

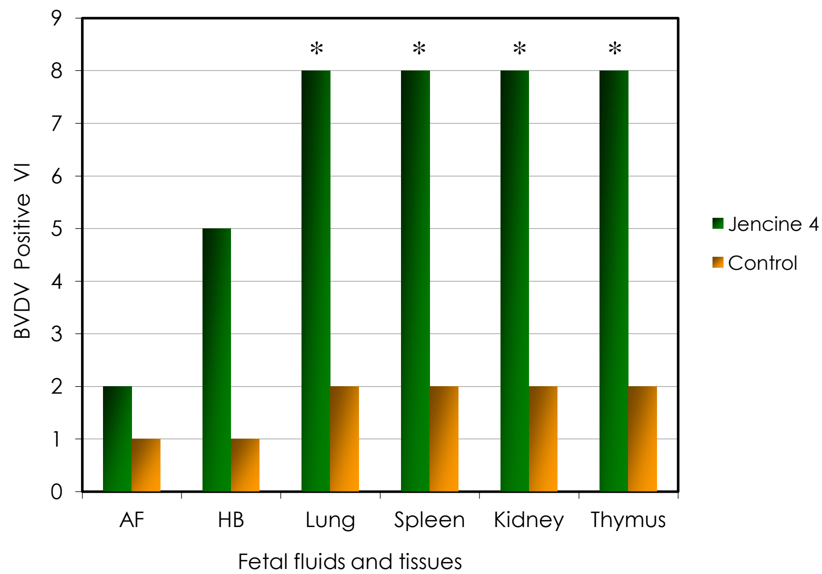

Figure 4. Virus isolation on fetal fluids and tissues recovered from vaccinated and unvaccinated heifers with a contaminated MLV vaccine. *Significant difference between groups ($P < 0.05$). AF: Amniotic fluid. HB: Heart blood.

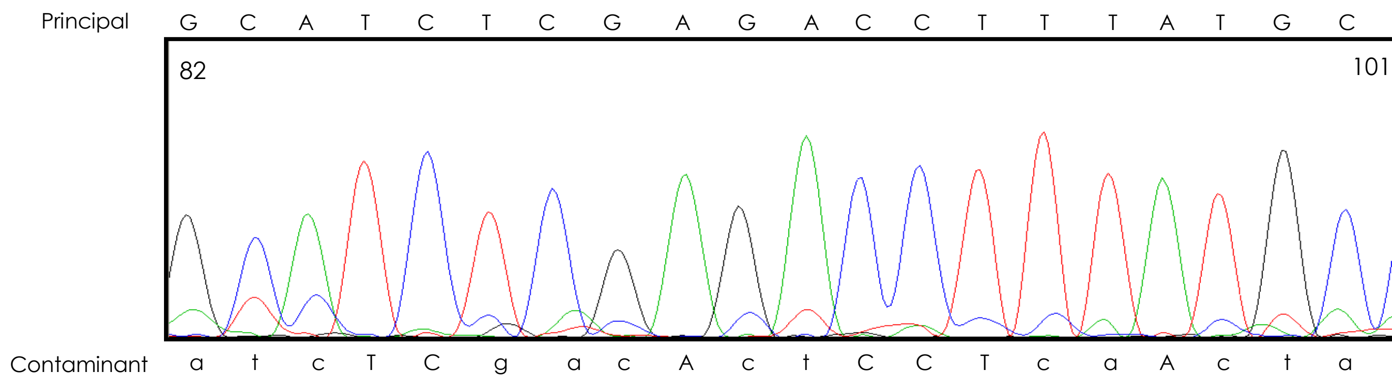

Figure 5. Chromatogram of BVDV 5' UTR sequences present in the vaccine showing major and minor peaks at the same nucleotide positions which indicated the presence of two viral populations, principal (BVDV-1) and contaminant (BVDV-2) strains.

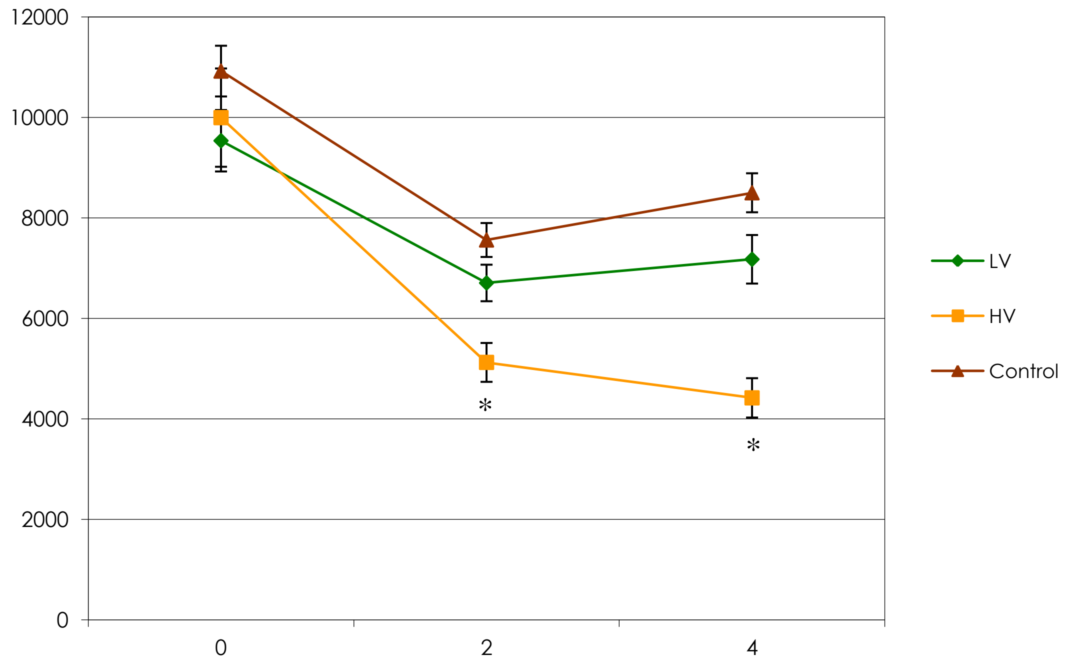

Figure 6. Total leukocyte counts in beef calves 0, 4, and 5 days after challenge with low (LV, SD-1) or high (HV, 1373) virulence BVDV strains. *($P < 0.01$) .

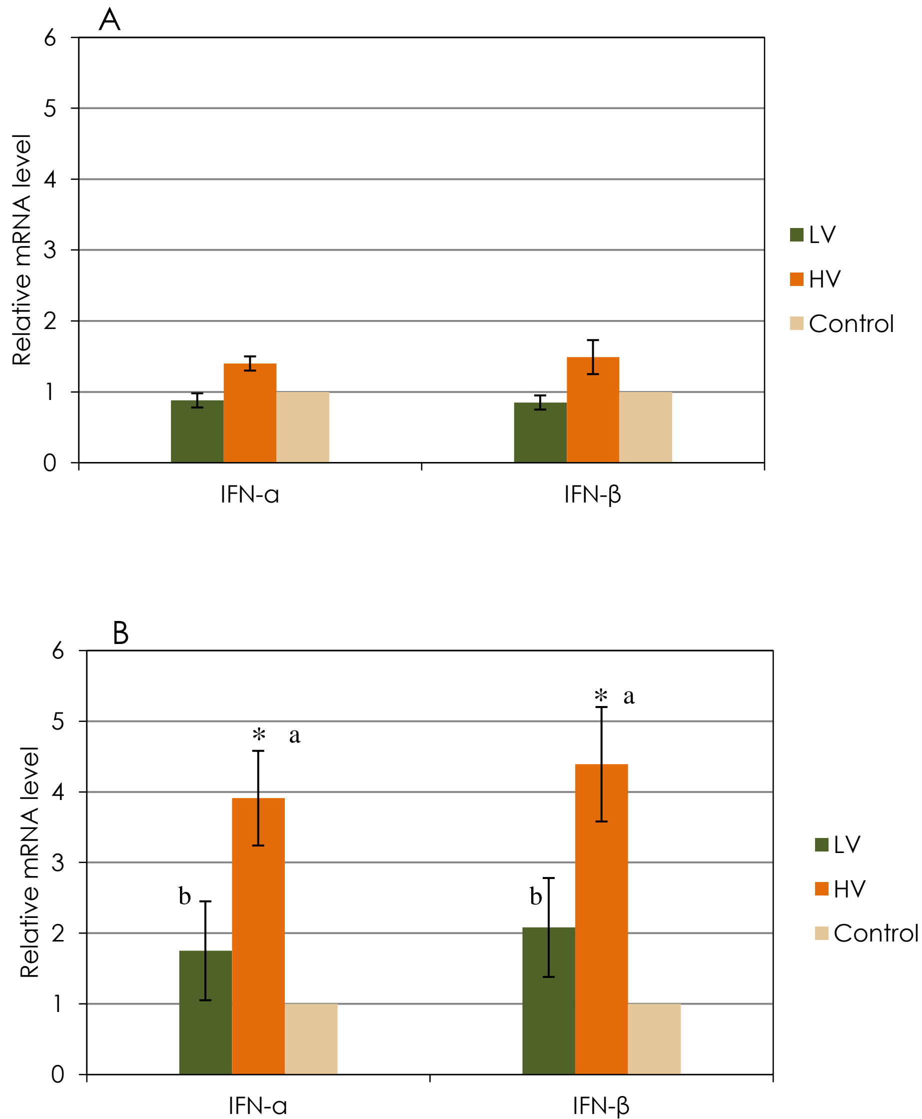

Figure 7. Changes in mRNA expression of Type-I interferon (α and β) in spleen (A) and tracheo-bronchial lymph nodes (B) of calves challenged with low (LV) and high (HV) virulence BVDV strains relative to the control calves. Data are expressed as fold difference of mRNA expression normalized to the housekeeping gene (GAPDH), relative to the values obtained for uninfected calves. *Significant upregulation ($P < 0.05$). [a,b] Significant difference between groups (*$P < 0.05$).

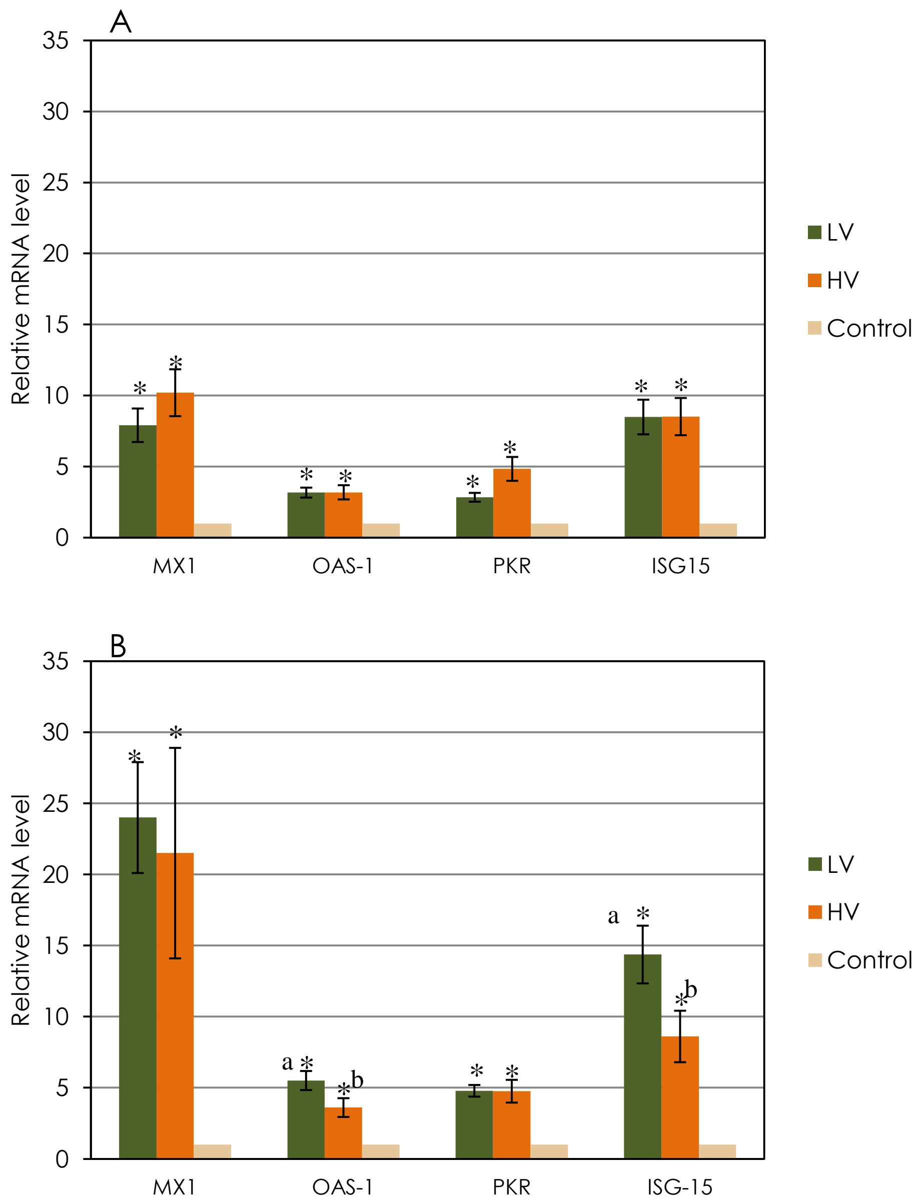

Figure 8. Changes in mRNA expression of Type I interferon-stimulated genes (Mx-1, OAS-1, PKR, and ISG-15) in spleen (A) and tracheo-bronchial lymph nodes (B) of calves challenged with low (LV) and high (HV) virulence BVDV strains relative to the control calves. Data are expressed as fold difference of mRNA expression normalized to the housekeeping gene (GAPDH), relative to the values obtained for uninfected calves. *Significant upregulation ($P < 0.001$). [a,b] Significant difference between groups ($P < 0.05$).

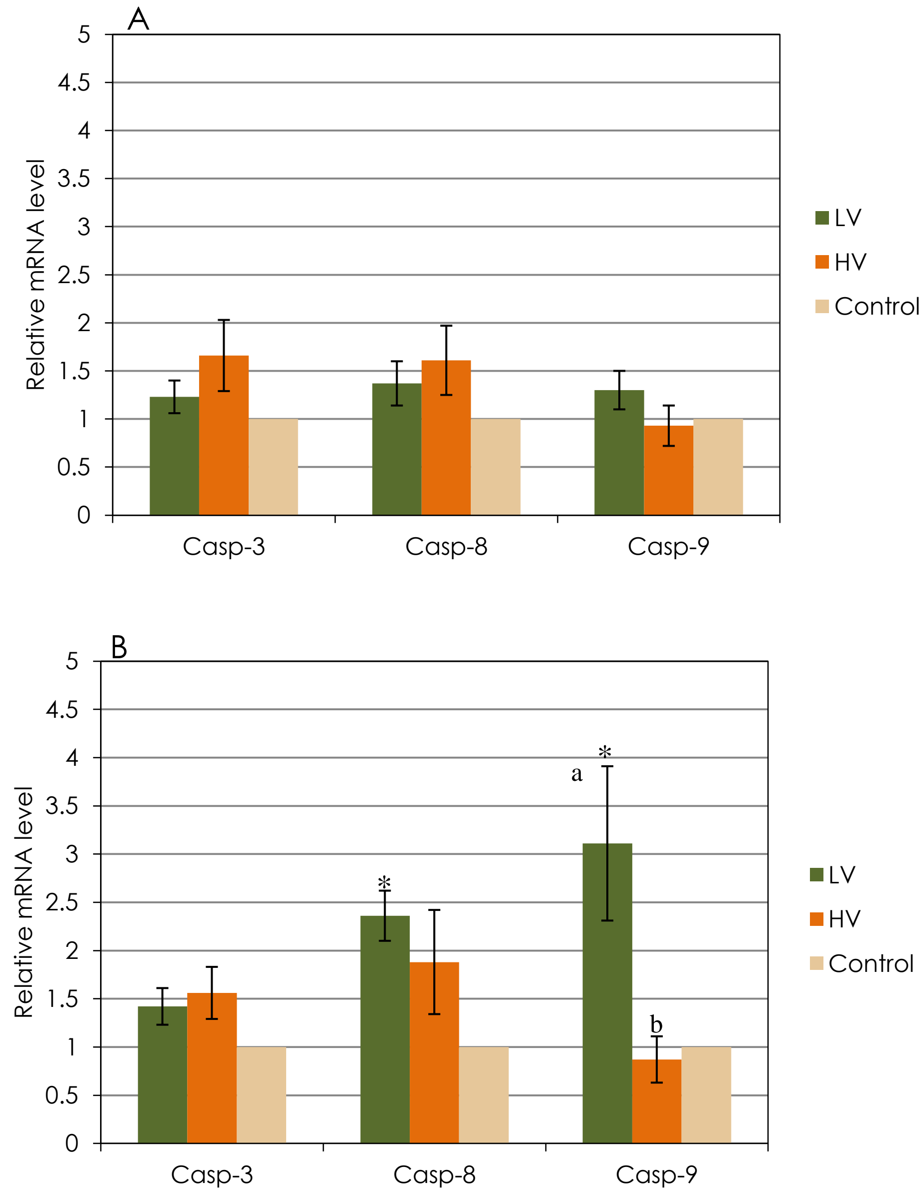

Figure 9. Changes in mRNA expression of pro-apoptosis markers (caspase-3, -8, and -9) in spleen (A) and tracheo-bronchial lymph nodes (B) of calves challenged with low (LV) and high (HV) virulence BVDV strains relative to the control calves. Data are expressed as fold difference of mRNA expression normalized to the housekeeping gene (GAPDH), relative to the values obtained for uninfected calves. *Significant upregulation ($P = 0.01$). [a,b] Significant difference between groups ($P < 0.01$).

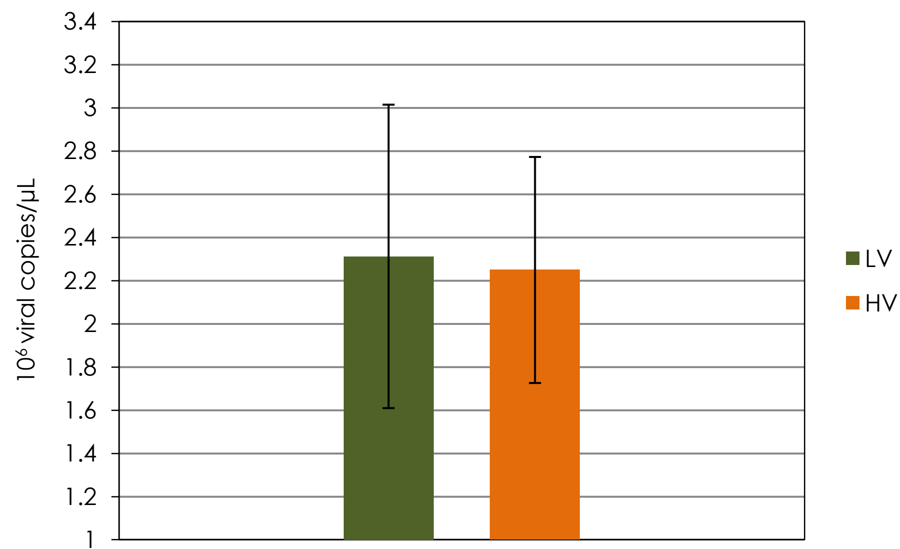

Figure 10. RNA Viral level in tracheo-bronchial lymph nodes of calves challenged with low (LV) or high (HV) virulence BVDV strains. There were no significant differences between the groups.

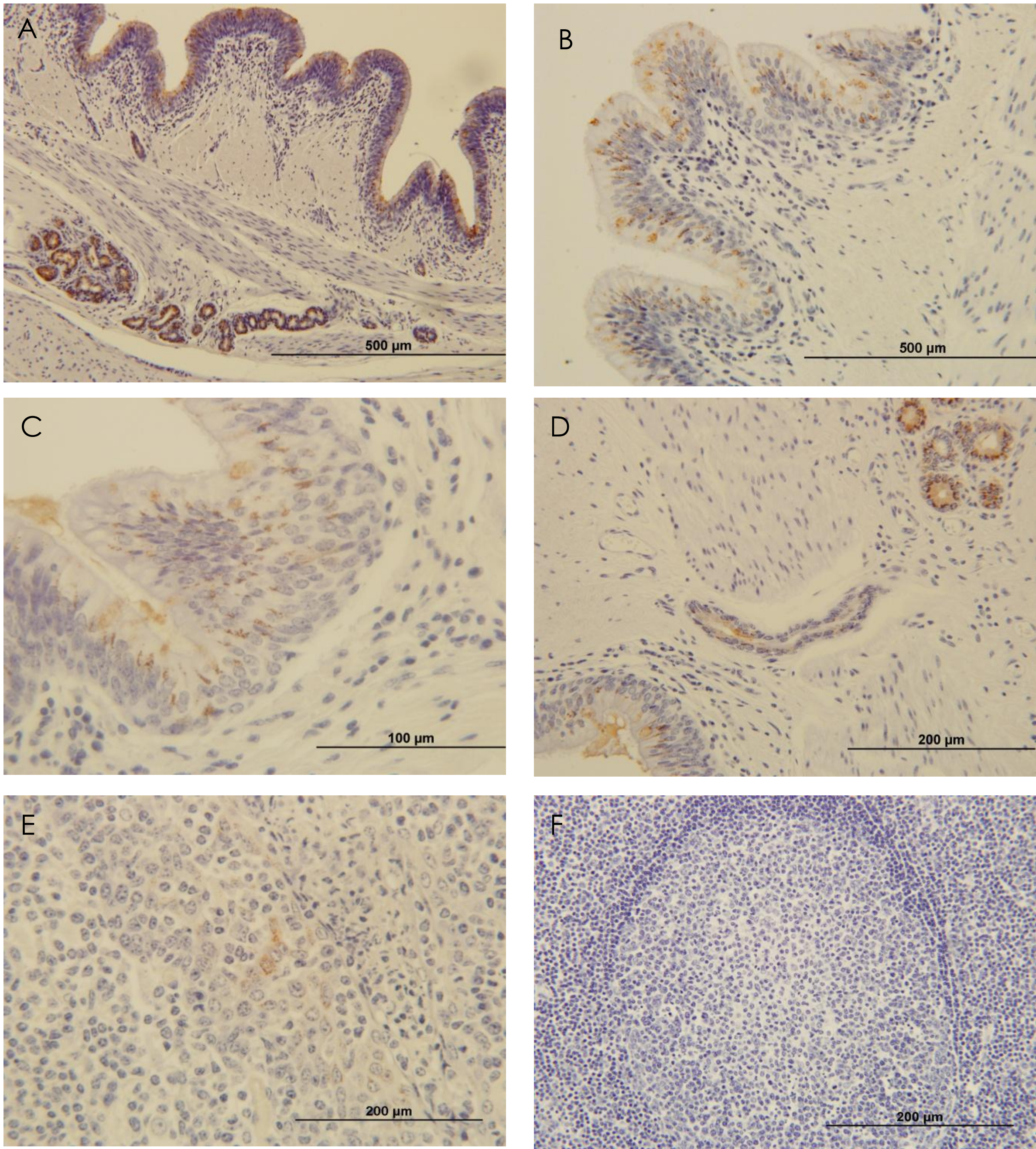

Figure 11. Photomicrograph of BVDV-specific immunohistochemical staining on tissue samples from beef calves 5 days following challenge with low (LV) or high (HV) virulence BVDV strains. Bronchiolar lining of calves inoculated with LV BVDV-1a SD-1(A) and HV BVDV-2 1373 (B,C). BVDV antigen located in bronchiolar submucosa (D) and tonsils (E). Similar evidence of mild to moderate lymphoid depletion was present in spleen and lymph nodes (F) of calves inoculated with low (SD-1) and high (1373) virulence BVDV isolates.

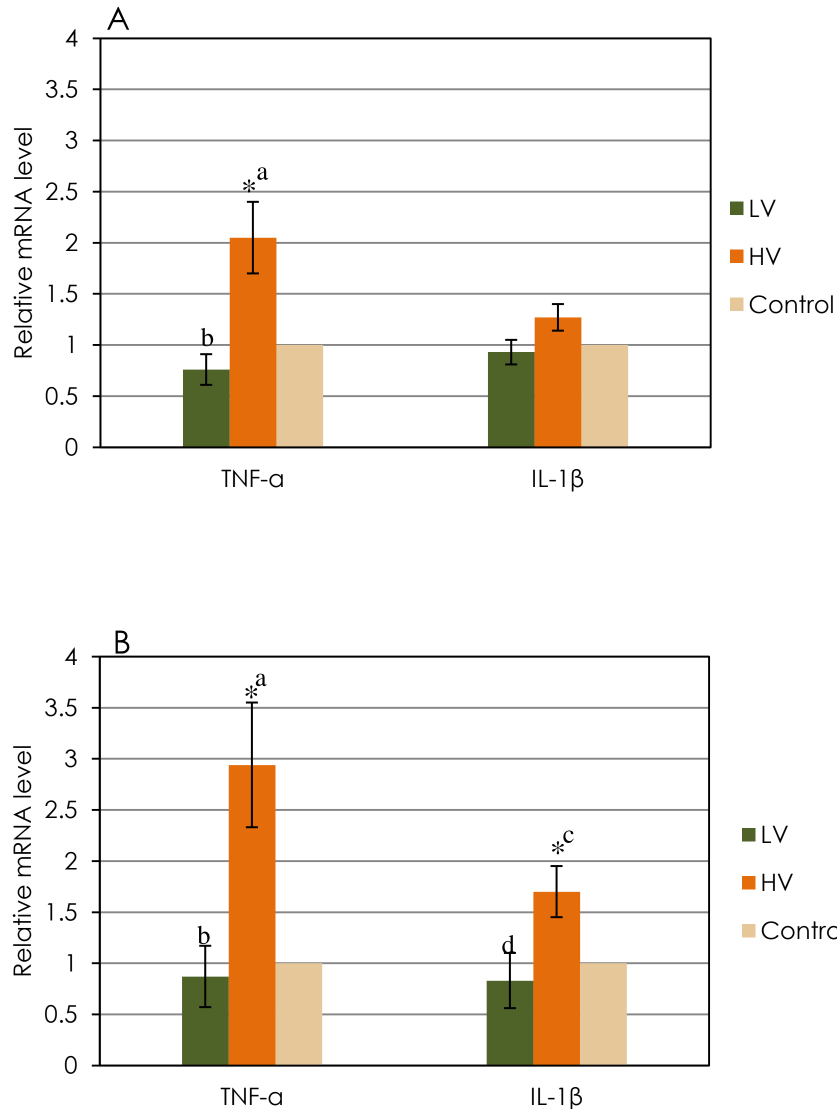

Figure 12. Changes in mRNA expression of pro-inflammatory cytokines (TNF-α and IL-1β) in spleen (A) and tracheo-bronchial lymph nodes (B) of calves challenged with low (LV) and high (HV) virulence BVDV strains relative to the control calves. Data are expressed as fold difference of mRNA expression normalized to the housekeeping gene (GAPDH), relative to the values obtained for uninfected calves. *Significant upregulation ($P < 0.05$). [a,b] Significant difference between groups ($P \leq 0.01$). [c,d] Significant difference between groups ($P < 0.05$).

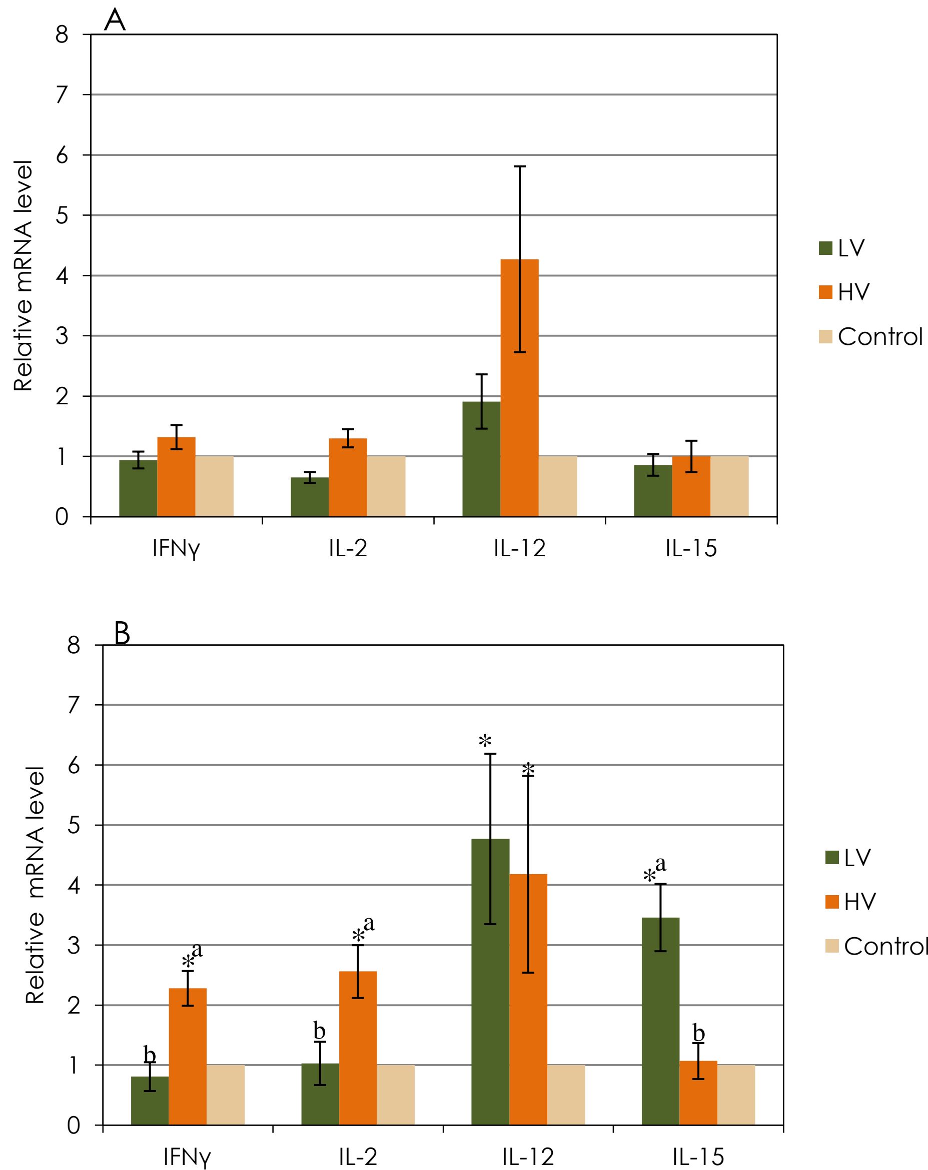

Figure 13. Changes in mRNA expression of cytokines involved in T-cell activation (IFNγ, IL-2, IL-12 and IL-15) in spleen (A) and tracheo-bronchial lymph nodes (B) of calves challenged with low (LV) and high (HV) virulence BVDV strains relative to the control calves. Data are expressed as fold difference of mRNA expression normalized to the housekeeping gene (GAPDH), relative to the values obtained for uninfected calves. *Significant upregulation ($P < 0.05$). [a,b] Significant difference between groups ($P \leq 0.01$).

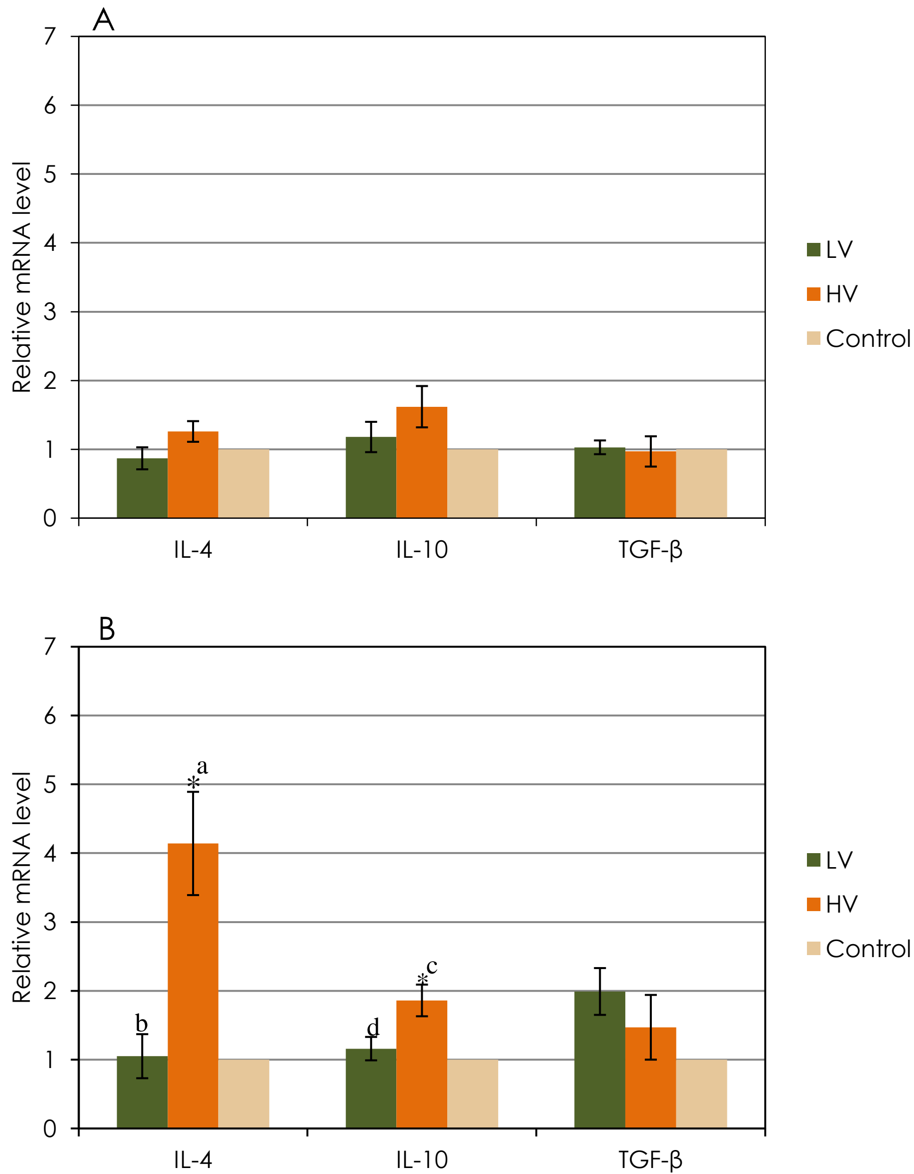

Figure 14. Changes in mRNA expression of antinflammatory cytokines (IL-4, IL-10 and TGF-β) in spleen (A) and tracheo-bronchial lymph nodes (B) of calves challenged with low (LV) and high (HV) virulence BVDV strains relative to the control calves. Data are expressed as fold difference of mRNA expression normalized to the housekeeping gene (GAPDH), relative to the values obtained for uninfected calves. *Significant upregulation (P <0.05). [a,b] Significant difference between groups (P= 0.005).[c,d] Significant difference between groups (P= 0.02).