Dual natural infection with bovine viral diarrhea virus -1 and -2 in a stillborn calf: tissue distribution and molecular characterization

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Abstract

Dual infections with both bovine viral diarrhea virus (BVDV)-1 and -2 seem to be unusual. The aim of this study was to describe an infection with both BVDV genotypes in a stillborn calf. Virus isolation and phylogenetic analyses of the 5´UTR and NS5B regions confirmed the presence of BVDV-1b and -2b in spleen and lung, whereas BVDV-2b was also detected in brain, heart, liver, kidney and, fluid of cavities. These results confirm that dual infections with both BVDV-1 and BVDV-2 species can occur naturally and their tissue distribution can be different.

Keywords: Bovine viral diarrhea virus, Co-infection, Phylogenetic analysis.

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Introduction

Bovine viral diarrhea virus (BVDV) is a significant cause of economic losses in the cattle industry worldwide. BVDV (family: *Flaviviridae*, genus: *Pestivirus*) can be divided into two species, BVDV-1 and BVDV-2 (Ridpath *et al*., 1994). BVDV-1 and -2 are present in Argentinean bovine herds (Jones *et al*., 2001; Odeón *et al*., 2003; Pecora *et al*., 2014), being BVDV-1b the predominant specie (Pecora *et al*., 2014). The genetic typing of BVDV strains can help in the investigation of BVDV outbreaks (Vilcek *et al*., 2005) and the classification into species and subgenotypes is widely based on the 5´UTR, Npro, and E2 genomic regions (Nagai *et al*., 2004; Vilcek *et al*., 2005).

Infection in pregnant females with BVDV results in a variety of reproductive losses, such as infertility, embryonic death, fetal malformations, abortions, stillbirths, and the birth of weak calves (Baker, 1995). Transplacental infections with the non cytophatic (ncp) BVDV strain between days 40 and 120 of gestation causes the birth of persistently infected (PI) animals (Grooms, 2004). These PI animals are the main source for BVDV transmission in cattle population because they shed large amounts of virus during their lifetime (Grooms, 2004).

Dual infections with more than one BVDV species have been described in fetuses and postnatal cattle either in experimental studies (Broke and Chase, 2000; Arenhart *et al*., 2010; Makoschey and Janssen, 2011; Larska *et al*., 2012) or in natural conditions (Fulton *et al*., 2000; Evermann and Ridpath, 2002; Ahn *et al*., 2005), but only Broke and Chase (2000) detailed the tissue distribution of both species. Despite this background, fetal infections with both BVDV-1 and BVDV-2 appear to be uncommon (Makoschey and Janssen, 2011) or rarely described.

The aim of this study was to report a natural dual infection with both BVDV-1 and BVDV-2 in a stillborn beef calf that occurred in Argentina. Moreover, the tissue distribution and the genetic variability of both strains were described.

Material and Methods

An Aberdeen Angus stillborn calf was submitted for necropsy and laboratory diagnosis of abortion in 2010. The calf belonged to a beef farm located in the county of General Guido, Buenos Aires Province, Argentina. A ncp BVDV strain was initially isolated from the spleen after four blind passages on MDBK cells, whereas other relevant reproductive pathogens (*Campylobacter fetus*, *Tritrichomonas foetus*, *Brucella abortus*, *Leptospira* spp., *Neospora caninum*, bovine herpesvirus) were ruled out. Different tissues samples, fluids of cavities, and the cell culture supernatant of the spleen were preserved at -80ºC for additional studies. Further attempts of virus isolation were performed in 2017 from the brain, heart, lung, spleen, liver, and kidney samples. Also, total RNA was extracted from all the tissue samples, fluids of cavities, the respective cell

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culture supernatants, and the cell culture supernatant of the spleen from the year 2010 by using TRIzol® reagent (Invitrogen, USA) and following the manufacturer`s instructions.

A nested multiplex RT-PCR (Gilbert *et al*., 1999), with modifications, was performed to amplify specific fragments of the NS5B genomic region. For cDNA synthesis, 8 µl of RNA, 0.6 µl (10 µM) of each external primer and 0.8 µl of DMSO were denatured 5 min at 65ºC.

Thereafter, 5 µl of RT Buffer, 1 µl of dNTPs (10 mM each, Promega, USA), 1 µl (200U) of M-MLV (Promega, USA), and 3 µl of dH$_2$O was incubated at 37ºC for 50 min and 70ºC for 15 min. The first round of PCR was conducted in a total volume of 50 µl containing 2.5 µl of cDNA, 5 µl of MgCl$_2$ (25mM), 10 µl of 5X GoTaq® Buffer, 1 µl of dNTPs (10 mM each), 1.5 µl of each external primer (10 µM), 0.5 µl of GoTaq® DNA Polymerase (Promega, USA) and 28 µl of dH$_2$O.

Cycling conditions were as follows: 1 min 94ºC; 25 cycles of 1 min 94ºC, 1 min 50ºC, 2 min 72ºC; and a final step of 15 min 72ºC. An aliquot (5 µl) was used as template in the second round of PCR, which was carried out in the same manner as the first round of PCR, but 1.5 µl of each internal primer (10 µM) were included in the mix. The sizes of the expected amplicons were 369 bp (BVDV-1) and 615 bp (BVDV-2).

Also, the second round of PCR was performed in the same conditions but only BVDV-1 specific primers were included. Additionally, a fragment from the 5´UTR genomic region was amplified from the RNA of all tissue samples and fluids of cavities using the primers 324/326 (Vilcek *et al*., 1994).

PCR products were resolved by electrophoresis in 2% agarose gels stained with SYBR® Green (Invitrogen, USA). The nested PCR products from the NS5B genomic region (spleen, lung, and brain tissues), and the PCR products from the 5´UTR genomic region (brain tissue and fluids of cavities) were purified with Illustra™ ExoProStar™ (GE Healthcare Life Sciences, UK). These amplicons were sequenced by the dideoxy chain termination method on an automated ABI PRISM 377 DNA Sequencer (Applied Biosystems Group).

Sequencing reactions were conducted in both directions using the genotype-specific primers (Gilbert *et al*., 1999) and the primers pair 324/326 (Vilcek *et al*., 1994). Consensus nucleotide sequences were assembled using the BioEdit software v7.2.5 (Hall, 1999) and the nucleotide identities of the NS5B and 5´UTR fragments were compared with cognate sequences from GenBank using the BLAST software. Phylogenetic analyses were conducted using MEGA7 software (Kumar *et al*., 2016).

Genetic distances were calculated using the Kimura-2 correction parameter and the phylogenetic trees were constructed using the neighbor-joining method with 1000 bootstrap replicates.

Nucleotide sequences of the 5´UTR and NS5B fragments were deposited in the GenBank database (accession numbers MH294527 to MH294529).

Results and Discussion

All tissue samples from the stillborn calf were positive for the isolation of a ncp-BVDV, even after long term (84 months) storage at -80ºC (Table 1).

The nested multiplex RT-PCR revealed that the lung tissue and the cell culture supernatant of the spleen (from 2010) contained two amplicons (369 bp and 615 bp), indicating a mixed infection by both BVDV-1 and BVDV-2, whereas the rest of the samples only contained an amplicon of 615 bp expected for BVDV-2 (Table 1).

Moreover, the nested RT-PCR using only BVDV-1 specific primers revealed an amplicon of 369 bp in the lung and spleen tissues, whereas this amplicon was not observed in the rest of the samples (brain, heart, liver, kidney, and fluid of cavities) (Table 1).

The nested RT-PCR performed on cell culture supernatants were only positive for BVDV-2, either when specific primers for BVDV-1 and BVDV-2 or only for BVDV-1 were included (Table 1).

Nucleotide sequence analyses of the NS5B fragments also confirmed the presence of both BVDV-1 and BVDV-2 in the different samples. Sequence similarities of the NS5B fragments, obtained using only BVDV-1 specific primers, revealed 100% identity between the amplicons from lung and spleen tissues, so this BVDV-1 strain was designated as 10-636 spleen/lung. Also, sequences similarities of the 5´UTR fragments showed 100% identity between the amplicons from brain tissue and fluids of cavities, so this BVDV-2 strain was designated as 10-636 brain/fc.

Table 1. Isolation and genotyping of BVDV from tissue samples and cell culture supernatants.

Sample	VI[a]	Nested RT-PCR on tissue		Nested RT-PCR on supernatants	
		BVDV-1 and -2 SP[b]	BVDV-1 SP[b]	BVDV-1 and -2 SP[b]	BVDV-1 SP[b]
Brain	+	2	-	2	-
Heart	+	2	-	2	-
Lung	+	1 + 2	1	2	-
Spleen	+	2	1	2	-
Liver	+	2	-	2	-
Kidney	+	2	-	2	-
Fluid of cavities	*	2	-	*	*

(a): Virus isolation; (b): Gilbert *et al*. (1999); (SP): Specific Primers; (*): Not performed.

Phylogenetic analyses of the nucleotide sequences corresponding to the NS5B and 5´UTR fragments revealed that the strain 10-636 brain/fc (BVDV-2) was clustered within the subtype 2b, whereas analysis of the nucleotide sequence of the NS5B fragment showed that the strain 10-636 spleen/lung (BVDV-1) was clustered within the subtype 1b (Fig 1). The 5´UTR fragment of the BVDV-1 strain could not be sequenced.

The current study provides further evidence that infections with both BVDV species can occur in natural conditions. However, to the authors´ knowledge, this study reports for the first time a dual natural infection with BVDV-1 and BVDV-2 in a stillborn calf and their tissue distribution based on RT-PCR amplification. In a retrospective analysis of BVDV positive samples, Ahn *et al.* (2005) described the occurrence of dual natural infections in two bovine aborted fetuses (4 to 6 months of gestation).

Also, infections with both BVDV species had been reported in 2% (2/103), 4% (2/54) and 11% (20/117) of the positive samples from the United States (Fulton *et al.*, 2000; Evermann and Ridpath, 2002; Ahn *et al.*, 2005). Few studies, under experimental conditions, have shown evidence that dual infections with BVDV-1/BVDV-2 (Brock and Chase, 2000; Arenhart *et al.*, 2010; Makoschey and Janssen, 2011) and BVDV-1/Hobi-like (Larska *et al.*, 2012) can occur, but this kind of infections could not be achieved consistently (Arenhart *et al.*, 2010; Makoschey and Janssen 2011).

In the present report, BVDV-2 was identified in more tissue samples than BVDV-1, which was only detected in lung and spleen. Furthermore, BVDV-1 could be detected in the spleen only when the primer for BVDV-2 was excluded from the reaction mix. Previously, Brock and Chase (2000) characterized the tissue distribution of both species in four fetuses infected under experimental conditions. These authors demonstrated that not all tissues (spleen, thymus, and small intestine) had both species by RT-PCR analyses. Moreover, Frey *et al.* (2002) could only detect BVDV-2 after the experimental challenge of pregnant heifers with BVDV-1 and BVDV-2 strains. Additionally, in that study BVDV-2 was also detected in all six calves born from the inoculated heifers.

It has been proposed that differences in the level of replication, as well as the tissue tropism, may exist between BVDV-1 and BVDV-2 (Brock and Chase, 2000; Bielefeldt-Ohmann *et al.*, 2008). Moreover, BVDV-2 has been isolated more frequently in fetuses/calves delivered from dams inoculated with both species (Brock and Chase, 2000; Frey *et al.*, 2002; Makoschey and Janssen, 2011).

Consequently, it has been suggested that BVDV-2 is more able to cross the placenta and infect the fetus in comparison to BVDV-1 (Bielefeldt-Ohmann *et al.*, 2008; Passler *et al.*, 2014).
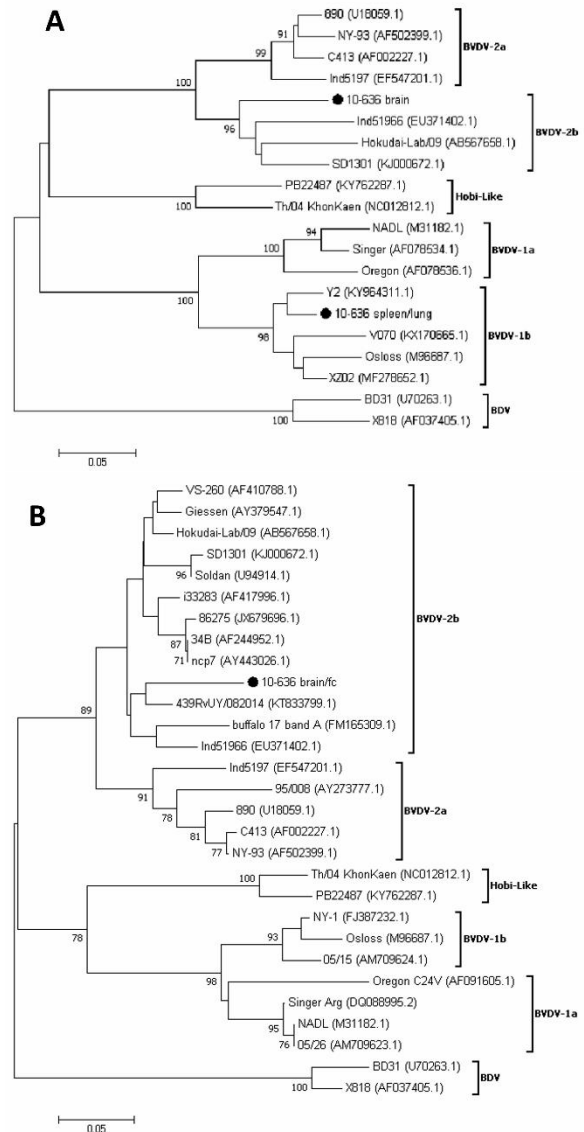

Fig. 1. Phylogenetic trees based on the partial nucleotide sequences of the genomic regions NS5B (A) and 5´UTR (B). Genetic distances were calculated using the Kimura-2 correction parameter and phylogenetic trees were constructed using the neighbor-joining method. Bootstrap values (1000 replicates) above 70% are shown. Nucleotide sequences from Border disease virus (bDV) were included for out-group rooting. BVDV strains reported in the current study are highlighted by dots. The GenBank accession number of each strain is indicated in brackets.

This experimental evidence supports our finding that BVDV-2 was more widely distributed in the tissues of the stillborn calf. In contrast, Paton *et al.* (1999) observed that BVDV-1 appeared to be more efficient than BVDV-2 at infecting the fetuses of seronegative ewes under experimental conditions. In the present study, whether differences in tissue distribution between both species is specific to these strains or is a general feature of BVDV-1 and BVDV-2 is uncertain.

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Broke and Chase (2000) have detected dual infections by RT-PCR from cell culture supernatants after viral isolation but not directly from tissue samples. Based on those results, in the current study viral isolation was attempted from all tissues after storage for 84 months at -80ºC. Only BVDV-2 could be detected by nested multiplex RT-PCR from the cell culture supernatants (Table 1). Since both species were identified from lung, spleen and the cell culture supernatant of the spleen from the year 2010, it can be suggested that BVDV-1 was inactivated during storage but its RNA remains preserved. Interestingly, those findings also demonstrated that the viral-RNA could be stable in tissue samples for at least 84 months at -80ºC. This fact was addressed by Ridpath *et al.* (2014) who have shown that BVDV nucleic acid was preserved in archived tissue samples maintained at -80ºC for at least 36 months under different storage conditions.

Phylogenetic analysis of the NS5B genomic region revealed the presence of subgenotypes BVDV-1b and BVDV-2b in the lung and spleen tissues from the stillborn calf. These results confirm the usefulness of the nested multiplex RT-PCR (Gilbert *el al.*, 1999) for genotyping purposes. The NS5B genomic region has also been used for phylogenetic analyses of BVDV-1 and BVDV-2 strains (Nagai *et al.*, 2004; Mishra *et al.*, 2007, 2008), but in Argentina there are no previous reports on the phylogenetic study of the NS5B region among BVDV circulating strains. Most researches have characterized the 5´UTR and E2 regions (Jones *et al.*, 2001; Odeón *et al.*, 2003; Pecora *et al.*, 2014). Nevertheless, the results of the phylogenetic analyses conducted with NS5B and 5´UTR regions agreed and confirmed that strain 10-636 brain/fc (BVDV-2) belonged to subgenotype 2b. This finding is quite remarkable since previous studies indicate that BVDV-1a and -1b are the predominant subgenotypes of Argentinean strains infecting ruminants (Jones *et al.*, 2001; Odeón *et al.*, 2003; Pecora *et al.*, 2014), while BVDV-2 is less frequently report in this country. In bovine fetal and neonatal samples from Argentina, only a BVDV-2a strain recovered from an aborted fetus (Vilcek *et al.*, 2004) and two BVDV-2b strains isolated from apparently healthy fetuses (Jones *et al.*, 2001) were identified. This information indicates that no BVDV-2b was isolated previously from a stillborn calf in this country.

The detection of both BVDV-1 and BVDV-2 in the stillborn calf provides further evidence that dual infections can occur naturally. Viral co-infections are important as they may be a source of natural recombination between viruses, which can facilitate rapid genetic diversification, increased virulence or evasion of vaccine protection. While it was not investigated in this study, viral recombination has been reported for BVDV (Weber *et al.*, 2015).

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