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Persistent bovine viral diarrhea virus subgenotype 1d infection in dairy heifer calves born within 38 days interval

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Abstract

Bovine viral diarrhea virus (BVDV) causes a significant economic impact on the beef and dairy industries worldwide. Fetal infection with a non-cytopathic strain may lead to the birth of persistently infected (PI) offspring, the main event in the epidemiological chain of BVDV infection. This report describes the birth of 10 PI dairy calves from Brazil and the infecting BVDV subgenotype. Serum and blood samples were collected from 10 cows and 10 calves; all 10 calves were previously deemed BVDV positive by the ear notch rapid test. Serum samples were used in the virus neutralization technique to detect anti-BVDV antibody. Blood samples were used to detect BVDV RNA using the reverse transcription polymerase chain reaction (RT-PCR) assay of the 5' UTR and N^{pro} genes. All 10 cows were negative for BVDV RT-PCR, while all 10 calves were RT-PCR positive. Phylogenetic analyses were performed and the strain was classified as BVDV-1d. High titers of BVDV-specific antibodies in the serum of cows indicated recent circulation of BVDV in the dairy herd, whereas calves presented intermediate, low, or no anti-BVDV-1a antibody titers. The monitoring of circulating BVDV subgenotypes and the detection of PI animals is of great importance in disease control, and regular vaccination alone is insufficient to prevent BVDV infection.

Introduction

Bovine viral diarrhea virus (BVDV) is considered an important pathogen in cattle because it can cause severe economic losses in the beef and dairy industries and decreased fertility and milk production worldwide (Khodakaram-Tafti and Farjanikish 2017). BVDV infection can result in subclinical infections or a wide range of distinct clinical signs, such as diarrhea, respiratory signs, and reproductive dysfunctions, including infertility, abortion, malformations, and persistently infected (PI) offspring (Timurkan and Aydin 2019).

BVDV is an enveloped positive-sense single-stranded RNA virus that belongs to the genus *Pestivirus* within the family *Flaviviridae*. According to the International Committee on Taxonomy of Viruses, pestiviruses have been classified into 11 species (ICTV 2019). *Pestivirus A* (BVDV-1), *Pestivirus B* (BVDV-2), and *Pestivirus H* (HoBi-*like Pestivirus*) are the major pestiviruses infecting bovine species (ICTV 2019). Furthermore, there are at least 23 subgenotypes of BVDV-1 (1a-1w) and four subgenotypes of BVDV-2 (2a-2d) (Deng et al. 2020, Hou et al. 2019). BVDV can exist in two different biotypes based on its effects on infected cell cultures: cytopathic (cp) and non-cytopathic (ncp) (Baker 1995).

Reproductive failures are the most important impact caused by BVDV infections and occur according to the gestation period of infection, biotype (cp/ncp), and viral strain (Ridpath 2010). Fetal infection by ncp viruses between 40 and 120 days of gestation may lead to the birth of immunotolerant and PI calves. During this period, the fetal immune system is still immature and incapable of differentiating between viral proteins and self-proteins (Martin et al. 2016).

PI animals are the major source of BVDV transmission in cattle herds because they eliminate the virus at high titers in all their secretions and excretions (Timurkan and Aydin 2019). PI females might reach

reproductive age and give birth to new PI animals (Browlie 1990).

The control of this infectious disease relies on eliminating the reservoir of the pathogen and preventing transmission from infected to susceptible animals. However, the identification and elimination of PI cattle can be costly and frustrating, as less than 1% of the cattle population is PI with BVDV (Newcomer et al. 2017). In addition, cows should receive two doses of vaccines annually and immediately before the reproductive season to ensure optimal antibody response and reproductive results during conception (Alfieri and Alfieri 2017, Aono et al. 2013).

To date, BVDV isolates from several countries have shown distinct subgenotype predominance according to geographic location (Ridpath 2010). According to published data, in the Americas, Asia, and Europe, BVDV-1b is the predominant subgenotype (Yesilbag et al. 2017). In South America, BVDV-1a is the most prevalent subgenotype in Colombia and Uruguay (Barbosa et al. 2019, Maya et al. 2016), whereas BVDV-1b predominates in Argentina and Chile (Pecora et al. 2014, Pizarro-Lucero et al. 2006). In Brazil, BVDV-1a is the most frequently detected BVDV subgenotype, followed by BVDV-2b and HoBi-*like* (Flores et al. 2018).

In a meta-analysis, Scharnböck et al. (2018) predicted a decrease of PI animals in Europe and an increase of PI animals in North America. In the USA, Peddireddi et al. (2018) reported on 10 PI cattle — two with BVDV-1a, six with – 1b, and two with – 2a — and Cornish et al. (2005) detected 56 PI calves with BVDV-1 and – 2. In Italy, Nogarol et al. (2017) confirmed the presence of five PI animals carrying a BVDV-2a strain. Valdez et al. (2018) described PI animals with BVDV-1 in Peru, and Barbosa et al. (2019) detected BVDV-1a in Colombia. In a case-control study conducted by Kadohira and Tajima (2010) in Japan, 41 PI cows were detected in 23 different farms between 2006 and 2007, without subgenotype determination.

PI animals infected with BVDV have been described in a few reports in Brazil. Otonel et al. (2014) and Bianchi et al. (2017) reported BVDV-1d infection in PI cows in vaccinated and non-vaccinated herds, respectively.

The aim of this report was to describe the birth and BVDV subgenotypes of 10 PI heifer calves, born within a short period (38 days), in a high-production dairy cattle herd in Paraná State, Brazil.

Materials And Methods

Herd and biological sample collection

Serum and whole blood samples were collected from 10 calves of 32–70 days of age that were born within 38 days. All 10 calves, at two days of age, had already been identified as BVDV positive in the rapid SNAP® BVDV Antigen (ear notch) Test (IDEXX Laboratories, Inc., Westbrook, MA, USA). Serum and whole blood samples were also collected from their respective mothers. The animals came from a Holstein cattle herd with high milk production (average 29.2 liters/cow/day), located in Paraná State, southern Brazil. The herd, with approximately 1,500 lactating cows, is considered an open dairy cattle

herd because of the occasional introduction of female cattle (heifers and cows) into the herd for maintenance of milk production. All cows were regularly vaccinated against reproductive diseases, including BoHV-1, BVDV-1, and BVDV-2 strains. The biological samples were stored at −80 °C until processing.

Detection of anti-BVDV and anti-BoHV-1 antibodies

The presence of antibodies against BVDV and BoHV-1 in calf and cow serum samples was evaluated by the virus neutralization test (VN). After inactivation of the serum samples at 56 °C for 30 min, the VN test was performed in Madin–Darby bovine kidney (MDBK) cells, and 100 tissue culture infective doses of 50% of the cell culture-adapted BVDV-1a (Singer strain) and BoHV-1 (Los Angeles strain), according to the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (OIE 2018). The neutralizing antibody titer was considered the reciprocal of the highest serum dilution capable of neutralizing viral replication. Serum samples with neutralizing activity at a final dilution of ≥1:10 and ≥1:2 were considered positive for BVDV and BoHV-1, respectively (OIE 2018).

Nucleic acid extraction

Nucleic acid was extracted from whole blood samples using TRIzol™ reagent (Invitrogen™ Life Technology, Carlsbad, CA, USA) according to the manufacturer's instructions. The nucleic acid was then eluted with diethylpyrocarbonate-treated water (Invitrogen™ Life Technology, Carlsbad, CA, USA) and stored at −80 °C until molecular analyses were performed. Aliquots of ultrapure sterile water were used as the negative controls.

Reverse transcription polymerase chain reaction (RT-PCR) assay

The RT-PCR assay was performed using 324/326 primers to amplify the 5' UTR BVDV genomic region (Vilcek et al. 1994) and BD1/BD3 primers to amplify a fragment of the N^{pro} gene (Vilcek et al. 2001). The cell culture-adapted Singer strain (BVDV-1a cp) was used as the positive control. The amplified product was analyzed by electrophoresis on a 2% agarose gel containing 0.5 µg/mL ethidium bromide in Tris-borate-EDTA buffer (89 mM Tris, 89 mM boric acid, and 2 mM EDTA) at pH 8.4, and was visualized under ultraviolet light.

Sequencing, phylogenetic analysis, and genotyping

Two RT-PCR amplicons from 32- and 70-day old calves for each gene (5' UTR and N^{pro}) were selected for nucleotide (nt) sequencing and phylogenetic analysis. The RT-PCR products were purified using PureLink® Quick Gel Extraction and PCR Purification Combo (Invitrogen Life Technologies, Carlsbad, CA, USA) and quantified using the Qubit™ Fluorometer with the Quant-iT dsDNA BR Assay kit (Invitrogen Life Technologies, Eugene, OR, USA) according to the manufacturer's instructions.

Sequencing was performed using the BigDye Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA), with the same primers used in the RT-PCR assay, in the ABI3500 Genetic Analyzer

sequencer (Applied Biosystems, Foster City, CA, USA) according to the manufacturer's instructions. The quality of nucleotide sequences and assembled contigs were obtained using PHRED (CodonCode Corporation, Centerville, MA, USA) and CAP3 software (http://asparagin.cenargen.embrapa.br/phph/), respectively. The similarities of the sequences obtained were compared with those deposited in GenBank using the Basic Local Alignment Search Tool software (http://blast.ncbi.nlm.nih.gov/). The phylogenetic trees were reconstructed using the neighbor-joining method with the Kimura 2-parameter model, based on 1,000 bootstrapped datasets in MEGA 7 software. The nt sequence identity matrices were generated using the BioEdit version 7.2.5.

Results

Fifteen (75%) of the 20 serum samples evaluated were seropositive for BVDV in the VN test. Nine of the 10 cows had high (640–10,240) antibody titers for BVDV, and the remaining cow had a low (10) titer. Five calves had intermediate (40) antibody titers, one1 showed a low (10) titer, and four calves were seronegative.

All cows were negative for BVDV in the RT-PCR assay; however, BVDV RNA was detected in the whole blood samples of all the calves. At two days of age, all 10 calves tested positive for BVDV in the SNAP BVDV Antigen (ear notch) test. The results of RT-PCR, performed between 30 and 68 days, allowed us to classify them as PI calves.

In the phylogenetic analysis, the BVDV field strains described herein were classified as BVDV-1d (Fig. 1A and 1B). Analysis of the 5' UTR region of BVDV revealed that the BVDV wild-type strains (UEL15-BR/18 and UEL16-BR/18) were 100% identical to each other, and that the nt identity ranged from 97–97.8% with the 16/111 and F BVDV-1d prototype strains (Vilcek et al. 2001). The analysis also revealed 99.1–99.5% nt identity with other Brazilian BVDV-1d strains, namely, UEL2-BR/07 (Otonel, et al. 2014), UEL6-BR/11, UEL7-BR/11 (Headley et al. 2014), and UEL11-BR/11 (Oliveira et al. 2020). Based on the nt sequences obtained from the N^{pro} gene, the BVDV wild-type strains exhibited 99.5% nt identity to each other, and 90.2% identity with the F BVDV-1d prototype strain (Vilcek et al. 2001).

Discussion

The high titers (640–10,240) of BVDV antibodies detected in the VN test, using serum samples from cows, indicate a recent circulation of BVDV field strain in the herd. This is possible despite the cows being regularly vaccinated because commercial vaccines do not induce an immune response of this magnitude in antibody titer (Martin et al. 2016).

The PI calves presented low-to-intermediate antibody titers for BVDV, which were possibly acquired from colostral intake. Four PI calves were seronegative, which is consistent with the findings of previous studies that reported that this is a common occurrence in PI animals (Dezen et al. 2013). Despite the

results of the VN test for BVDV, we identified that there was no failure in passive transfer since all 10 calves showed antibody response against BoHV-1, with titers ranging from 32 to 128.

The evaluated herd is an open dairy cattle herd, as heifers and cows from other dairy herds in the neighborhood are acquired for replacement and maintenance of milk production. A retrospective analysis showed that the new females introduced into the herd most likely transmitted BVDV-1d to the cows in the first trimester of gestation, leading to the birth of PI calves. The dairy cattle herd had a large number of cows, and many were in the same stage of gestation when the virus started to spread, which resulted in the birth of 10 PI calves within a short period of 38 days.

Although the cows were regularly vaccinated against BVDV subgenotype 1a in the first trimester of gestation, they must have been naturally infected with a field strain of BVDV subgenotype 1d. In addition, we concluded that the infection of pregnant cows was transient, as all the whole blood samples from mother cows of PI calves were BVDV negative in the RT-PCR assay.

This report emphasizes that regular vaccination alone is insufficient for the prevention and control of BVDV infection in a high-production dairy cattle herd. Open dairy herds are at high risk of introducing different subgenotypes of BVDV, and biosecurity measures, such as quarantine and testing of acquired animals, are fundamental in the identification of both transient and persistent infections.

Figure 1 Phylogenetic trees generated by the neighbor-joining method with the Kimura 2-parameter model based on nucleotide sequences with 240 bp from the 5' UTR region (A) and 400 bp from the N^{pro} gene (B). The numbers adjacent to the nodes represent the percentage of bootstrap support (1,000 replicates) for the clusters. Bootstrap values of less than 50% are not shown. The name of the strain is followed by the GenBank accession number in parentheses. The BVDV strains derived from this study are highlighted with a black circle. BVDV-2 strain was used as the outgroup.

Declarations

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Availability of data and material The datasets in this study are available from the corre-sponding author on reasonable request.

Code availability Not applicable

Authors' contributions NZZ performed the literature research and wrote the manuscript. RPM performed the RNA extraction and RT-PCR assay. JTTF, EL, AAA and AFA supervised and critically reviewed the manuscript. All authors read and approved the final manuscript.

Compliance with ethical standards

Competing interests The authors declare no competing interests.

Ethics approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Consent to participate Not applicable

Consent for publication All authors give consent for publication

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Figures

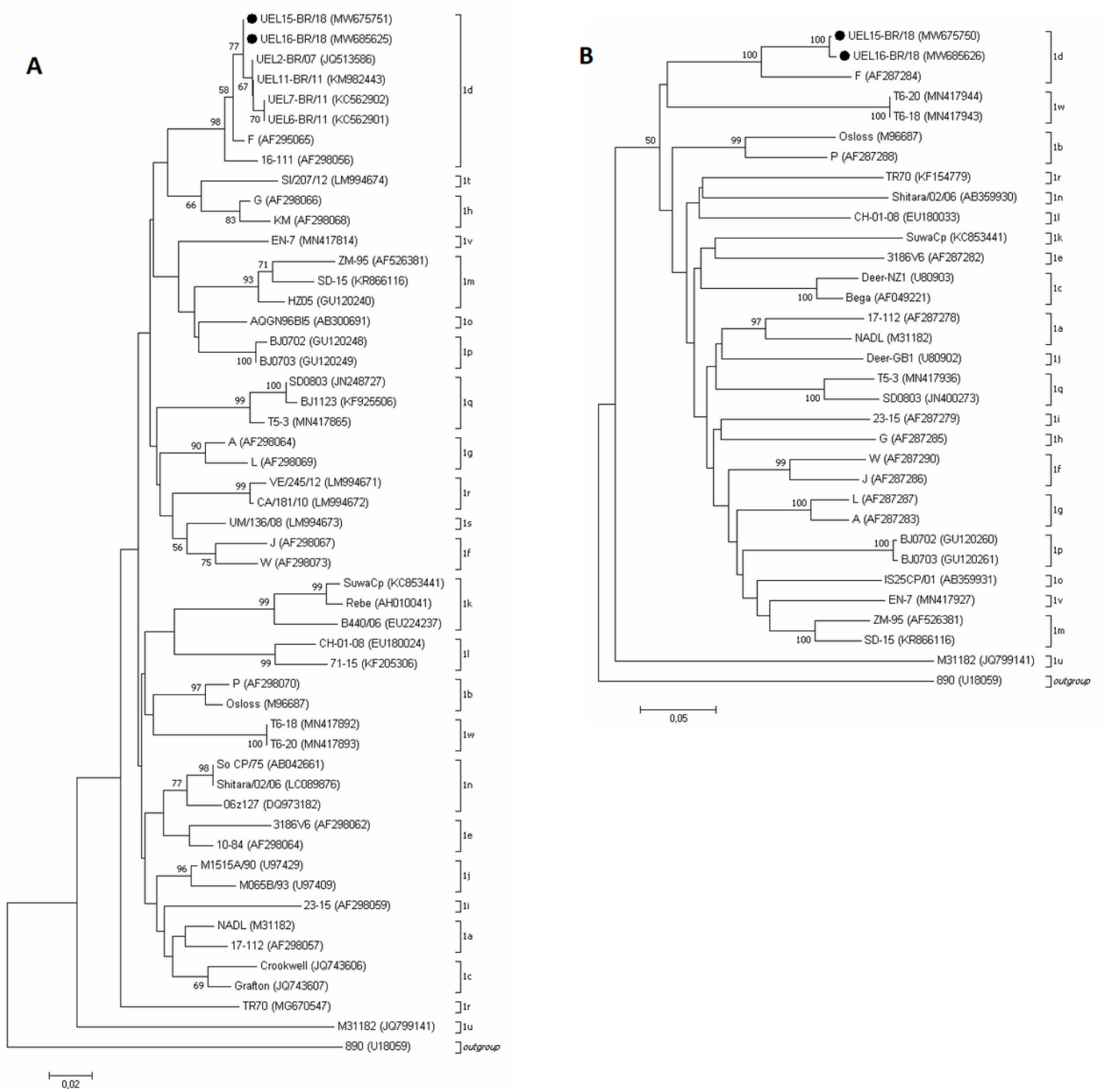

Figure 1

Phylogenetic trees generated by the neighbor-joining method with the Kimura 2-parameter model based on nucleotide sequences with 240 bp from the 5' UTR region (A) and 400 bp from the Npro gene (B). The numbers adjacent to the nodes represent the percentage of bootstrap support (1,000 replicates) for the clusters. Bootstrap values of less than 50% are not shown. The name of the strain is followed by the GenBank accession number in parentheses. The BVDV strains derived from this study are highlighted with a black circle. BVDV-2 strain was used as the outgroup.

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