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VETERINARY MICROBIOLOGY - RESEARCH PAPER

Diseases associated with bovine viral diarrhea virus subtypes 1a and 2b in beef and dairy cattle in Uruguay

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Abstract

Bovine viral diarrhea virus (BVDV, *Pestivirus*) causes significant economic losses to the livestock industry worldwide. Although serological surveys show that BVDV exposure is widespread in cattle in Uruguay, BVDV-associated diseases are greatly underreported. The aim of this work is to describe the epidemiological, clinical, pathological, and virological findings from spontaneous outbreaks of BVDV-associated diseases in cattle in Uruguay. Diagnostic investigations were performed during 6 spontaneous disease outbreaks on beef and dairy cattle farms in the departments of Colonia, Rio Negro, and Soriano between November 2016 and April 2018. Carcasses of 8 naturally deceased cattle from these outbreaks were necropsied and subjected to histological examination and immunohistochemistry to detect BVDV antigen in the tissues. Reverse transcription real-time PCR and genomic sequencing were also performed to identify BVDV at the species and subtype levels. Other ancillary diagnostic tests, including bacterial cultures, were performed on a case-by-case basis to rule in/out differential diagnoses based on initial clinicopathological presumptive diagnoses. BVDV-associated conditions that were diagnosed in the 8 cases included mucosal disease, transient postnatal BVDV infections associated with digestive/septicemic salmonellosis by *Salmonella* serovar typhimurium, *Histophilus somni* bronchopneumonia, urinary tract coinfections with *Escherichia coli* and *Streptococcus* sp., enteric coinfection with coccidia, and transplacental fetal infections and abortions with *Neospora caninum* coinfection. BVDV-1a and BVDV-2b were each identified in four of the eight cases. We conclude that BVDV-1a and BVDV-2b contribute significantly to disease and mortality in cattle in Uruguay. Future research should estimate the economic impact of BVDV in the Uruguayan livestock sector.

Keywords Bovine viral diarrhea virus · Infectious diseases · Livestock · *Pestivirus* · South America

Introduction

Bovine viral diarrhea virus (BVDV) is an enveloped virus with a single-stranded RNA genome, which belongs to the genus *Pestivirus*, family Flaviviridae, and is distributed worldwide [1]. BVDV causes significant economic losses to the dairy and beef livestock industries, which are attributed to morbidity, mortality, growth retardation, reduced milk production, premature culling, reduced reproductive performance, and increased occurrences of other diseases [2, 3]. The two currently recognized BVDV species are BVDV-1 and BVDV-2, and they were recently renamed *Pestivirus* A and B, respectively [4, 5]. BVDV-1 is the most genetically diverse, with 21 subtypes (BVDV-1a through u) currently recognized, while 4 subtypes (a–d) have been described for BVDV-2 [6–9]. More recently, the *Pestivirus* HoBi-like virus has been proposed as a

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new BVDV species (BVDV-3 or *Pestivirus* H) based on antigenic and genetic similarities [4, 5, 10, 11].

Clinical outcomes of BVDV infection include (a) transient or acute infection with subclinical, respiratory, and/or severe digestive clinical manifestation characterized by high morbidity and variable mortality and generally associated with noncytopathic (NCP) viral strains; (b) reproductive infections, including oocyte/sperm infections that negatively affect fertility, or transplacental/congenital transmission that may result in embryonic or fetal death; mummification; abortion; congenital anomalies; stillbirths; or, if the fetus survives, the birth of persistently infected (PI) calves, particularly when the fetuses are infected by NCP strains before 4 months of gestation; and (c) mucosal disease (MD) characterized by low morbidity and very high lethality in PI animals, usually before 2 years of age. MD is associated with superinfection with a cytopathic (CP) biotype that can arise through mutation, recombination, or genomic rearrangements of the NCP viral strain that infects PI cattle [12, 13].

As viruses with worldwide distribution [9], BVDV-1 and BVDV-2 have been recognized for many years in South American countries, including Brazil [14], Argentina [15], Colombia [16], Peru, Chile [17], and Uruguay [18], while the *Pestivirus* HoBi-like virus has currently only been identified in Argentina [19] and Brazil [20] on this subcontinent. In Uruguay, the first evidence of BVDV circulation dates from 1996 [21]. A serological study revealed that BVDV exposure is widespread in beef cattle throughout Uruguay [22]. More recently, active BVDV infections and circulating species and subtypes were explored in cattle herds with reproductive problems, and BVDV-1a was revealed as the predominant species/subtype, followed by BVDV-1i and BVDV-2b [18].

Clinicopathological descriptions of BVDV-associated diseases in Uruguay and the impact of these diseases on bovine production systems in the country are lacking in the scientific literature. Recognizing and identifying these diseases in spontaneous field outbreaks is essential for establishing control programs to reduce their economic impacts at the herd and national levels. This work describes the epidemiological, clinical, pathological, and virological findings in spontaneous disease outbreaks associated with BVDV infections in cattle in Uruguay.

Materials and methods

Case selection

Eight natural cases of BVDV-associated diseases (cases 1–8) during six outbreaks (outbreaks 1–6) in commercial beef and dairy herds in Uruguay are described. Cases were diagnosed between November 2016 and April 2018 at INIA's Veterinary Diagnostic Laboratory (Animal Health Platform) in La Estanzuela, Colonia Department, Uruguay. Carcasses of the deceased cattle in cases 1–8 were provided for necropsy by veterinary practitioners and farmers. Additionally, in cases 1 and 6, serum samples collected prior to death by the veterinary practitioners were made available for testing. Epidemiological and clinical information was gathered for each outbreak when available.

Necropsy, histology, and immunohistochemistry

All 8 cattle died spontaneously at commercial farms and were subsequently necropsied. Tissue samples were collected, preserved frozen at – 20 °C for virology, and fixed in 10% neutral buffered formalin for 48 h. Fixed tissues were dehydrated, embedded in paraffin, sectioned at 4–5 μm, mounted on glass slides, and stained with hematoxylin and eosin for routine histological examination under an optic microscope (AxioScope.A1, Carl-Zeiss, Germany).

Selected formalin-fixed paraffin-embedded (FFPE) sections of various tissues from all cases were processed for immunohistochemistry (IHC) to detect *Pestivirus* antigen using a standard operating procedure kindly provided by Jan Shivers from the University of Minnesota Veterinary Diagnostic Laboratory. Briefly, heat-induced antigen retrieval was performed by placing the deparaffinized sections in a decloaking chamber (Biocare Medical) at 110 °C for 30 s. A commercially available anti-BVDV monoclonal antibody isotype IgG_{2a} produced in mice (catalogue D89, VMRD, Pullman, WA, USA) was applied as the primary antibody for 45 min at a 1:20 dilution. An anti-mouse horseradish peroxidase (HRP)–labeled polymer (EnVision+ HRP goat anti-mouse IgG, K4001, Dako) was used as the detection system, with 3-amino-9-ethylcarbazole (AEC no. 3469, Dako) as the substrate chromogen. A FFPE section of archived intestine from a naturally infected calf that tested positive for BVDV by RT-PCR and IHC was used as a positive control. For the negative controls, serial sections of all tested tissues were processed in parallel as described above, but the primary antibody was replaced with normal mouse serum (NC499L, Biocare Medical) at the same dilution.

Real-time PCR

Nucleic acids were extracted from frozen serum or tissue samples in all cases using the QIAamp® cador® Pathogen Mini Kit (QIAGEN®, Germany), following the manufacturer's instructions. Reverse transcription (RT) was performed using random primers and Super-Script II enzyme (Invitrogen®, USA). Real-time PCR assays targeting a 207-bp fragment of the 5′UTR region of the BVDV were performed using the primers BVDV190F and V326, and the Taq-Man® probe TQ-Pesti as described by Hoffman et al. [23] and Gaede et al. [24], respectively, and later modified by Maya et al.

[18], to detect BVDV-1, BVDV-2, and the HoBi-like *Pestivirus*. All real-time PCRs were performed using the SensiMixTM II Probe Kit (Bioline Reagents Ltd.) and a Rotor-Gene Q instrument (Qiagen®) following the manufacturer's recommendations.

Conventional PCR, sequencing, and phylogenetic analysis

On all real-time PCR-positive samples, a 207-bp fragment of the 5′UTR was amplified by conventional PCR, using the same primer pair used for the real-time PCR. The amplicons were sequenced at Macrogen, Inc. (Seoul, South Korea) in an ABI3730XL Genetic Analyzer (Applied Biosystems, CA, USA). The sequences were edited using SeqMan software (DNAstar Lasergene) and deposited in the GenBank database. Species and subtype assignment were done by phylogenetic analysis of Uruguayan 5′UTR sequences, along with representative BVDV-1, BVDV-2, and HoBi-like *Pestivirus* strains retrieved from the GenBank. Nucleotide sequences were aligned using Clustal W implemented in MEGA 6.06 software [25]. The model of nucleotide substitution that best fit the dataset (Kimura 2 parameters + gamma) was selected using the jModelTest program according to the Akaike Information Criterion (AIC; Akaike, 1974) [26]. A phylogenetic tree was constructed by the neighbor-joining (NJ) method, and statistical significance Bootstrap method was carried out (1000 replicates) using Mega 6.06 version [25].

Ancillary diagnostic testing

Diagnostic investigations in each case also involved performing specific bacteriological, virological, serological, and molecular laboratory tests to assess for other pathogens, based on the presumptive clinical and pathological diagnoses in each case. Online Resource 1 summarizes the ancillary diagnostic tests performed on each of the 8 necropsied cattle.

Results

Table 1 summarizes the information on the disease diagnoses, date of diagnosis confirmation, herd geographic location, breed, production class, age, and the BVDV species/subtypes identified in all 8 cases, and whether cattle from other farms had been recently introduced to the affected herds. Additional epidemiological, clinical, and pathological information on each case/outbreak and interpretation of the ancillary and diagnostic test results (Online Resource 1) are provided below.

Outbreak 1 Outbreak 1 occurred in a herd of 340 rotationally grazing Aberdeen Angus heifers in a commercial breeding herd. Twelve heifers died sporadically from September to November 2016 (mortality 3.5%). Clinical examination of an 18-month-old heifer (case 1) revealed tachypnea (120 breaths/minute), tachycardia (48 beats/minute), a rectal temperature of 39.5 °C (normothermia), ruminal atony, foul-smelling diarrhea, dehydration, and weight loss. The animal died spontaneously after a 96-h clinical course.

The necropsy revealed multiple irregular, well-defined erosions and nonperforating ulcers up to 0.5 cm in the mucosa of the thoracic region of the esophagus (Fig. 1a), petechiae, and ecchymosis in the jejunal serosa, with brown, bloody, fluid, foul-smelling contents in the jejunum, cecum, and colon. Diffusely, the colonic mucosa was markedly reddened, with brown viscous contents and clotted blood adhered to the surface (hemorrhagic colitis) (Fig. 1b).

Microscopically, there was multifocal necrotizing ulcerative esophagitis (Fig. 1c) with microthrombosis in the submucosa and individual keratinocyte necrosis/apoptosis, multifocal superficial rumenitis and reticulitis with swelling and hydropic keratinocyte degeneration, and neutrophil transmigration in the epithelium. Severe extensive necrotizing enterotyphlocolitis with submucosal edema, microthrombosis in the lamina propria, and neutrophilic cryptitis with necrotic enterocytes was also observed. IHC for BVDV antigen detection revealed abundant, strong and frequent intralesional, finely granular, and homogeneous immunolabeling in the cytoplasm of the epithelial (keratinocytes and enterocytes) and inflammatory cells in the esophagus (Fig. 1d), small intestine, and colon.

The BVDV genome was detected in the serum via RT-qPCR (ct 27.70), and BVDV-1a was further identified by sequencing the 5′UTR genomic region (GenBank MN159214). Mucosal disease was diagnosed based on these results (Table 1), while other causes of enterocolitis in cattle, including *Salmonella* spp. and bovine coronavirus, were ruled out by specific testing (see Online Resource 1).

Outbreak 2 Outbreak 2 occurred at a dairy herd of 1222 Holstein cattle, including 580 milking cows, 40 dry cows, 200 heifers, 400 calves, and 2 bulls. Of 533 pregnant cows/heifers aged 2 to 7 years, 16% aborted between 4 and 8 months of gestation. A male fetus (case 2) that had been aborted at approximately 240 gestational days by a 5.5-year-old cow was necropsied.

The only macroscopic lesion was mild pleural petechiation in the right lung. Histology revealed multifocal moderate necrotizing lymphocytic/histiocytic encephalitis with gliosis, multifocal moderate-to-severe lymphocytic and histiocytic myocarditis, and multifocal minimal lymphocytic and histiocytic glossitis. As shown in Online Resource 1, an IHC procedure for detecting *Neospora caninum* antigen revealed intralesional immunolabeling in the fetal encephalon and heart. The protozoan's genome was detected by PCR on a

Table 1 Disease diagnosis, date, herd geographic location, breed/production class, age, BVDV species/subtype identified, and immunohistochemical results for 8 BVDV infection cases

Outbreak no./ geographic location/ date	Case no.	Disease diagnosis	Breed, production class, age	BVDV species/ subtype	BVDV IHC result	Recent cattle introductions from other farms
1/Colonia/Nov 2016	1	Mucosal disease	Aberdeen Angus heifer, beef production on pasture, 18 months old	BVDV-1a	Positive	ND
2/Río Negro/Feb 2017	2	Abortion, acute/transient BVDV infection, coinfection with *N. caninum*	Dairy, aborted fetus, 240–270 gestational days	BVDV-1a	Negative	ND
3/Colonia/May 2017	3	Acute/transient BVDV infection, coinfection with *S. enterica* serotype typhimurium (enteric/septicemic salmonellosis)	Dairy heifer	BVDV-2b	Positive	Yes
	4	Acute/transient BVDV infection, coinfection with *H. somni* (bronchopneumonia)	Dairy heifer	BVDV-2b	Positive	
4/Colonia/June 2017	5	Acute/transient BVDV infection, urinary tract coinfection with *Streptococcus* sp. and *E. coli*	~ 2-year-old beef steer, feed lot	BVDV-1a	Positive	Yes
5/Soriano/April 2018	6	Possible mucosal disease or severe transient infection, enteric coinfection with *Eimeria* sp.	Holstein steer, beef production, 4 years	BVDV-1a	Positive	Yes
6/Colonia/Dec 2017 and Jan 2018	7	Abortion, acute/transient BVDV infection	Dairy, aborted fetus, 180–210 gestational days	BVDV-2b	Negative	ND
	8	Abortion, acute/transient BVDV infection	Dairy, aborted fetus, 240–270 gestational days	BVDV-2b	Negative	ND

IHC immunohistochemistry, *ND* not determined

frozen brain sample. Based on these results, an etiological diagnosis of encephalitis and myocarditis caused by *N. caninum* was reached. However, the BVDV genome was detected by RT-qPCR (ct 27.37) from a pool of fetal tissues and further identified as BVDV-1a (GenBank MN159211). IHC immunolabeling for BVDV antigen was negative for all fetal tissues analyzed using this technique (brain, kidney, lung, spleen, and liver), suggesting an acute/transient BVDV infection rather than a PI fetus (Table 1).

Outbreak 3 Outbreak 3 occurred on a farm dedicated to rearing dairy calves/heifers brought from various farms in different departments of Uruguay. A respiratory disease outbreak was registered in a group of approximately 200 heifers aged 3 to 4 months that had been brought to the farm approximately 30 days earlier. The respiratory disease outbreak started in 20 calves (10%) 15 days after admission, and 5 days later, approximately 80 animals (40%) were affected. Morbidity and mortality rates were 80% and 7.5% (15 heifers), respectively.

Two heifers were necropsied (cases 3 and 4). The pathological findings in case 3 included diffuse moderate fibrinous histiocytic and neutrophilic interstitial pneumonia with necrotizing bronchiolitis, multifocal neutrophilic alveolitis, and microthrombosis with multifocal pleuritis. Multifocally, the lungs and kidneys showed segmental lymphohistiocytic and necrotizing vasculitis affecting the medium-sized arterioles (Fig. 2a). Additionally, there was moderate multifocal erosive typhlocolitis with necrotizing cryptitis, with inflammatory infiltrate extended to the submucosa, along with fibrinous mesenteric lymphadenitis and moderate multifocal lymphocytic and neutrophilic abomasitis. Mild diffuse portal lymphocytic, histiocytic, and neutrophilic hepatitis with cholestasis in the bile ducts and minimal multifocal random necrotizing histiocytic and neutrophilic hepatitis throughout the parenchyma were also observed.

Salmonella enterica serotype typhimurium was isolated from the mesenteric lymph node and intestines, while bovine coronavirus and several viruses causing pneumonia were ruled out (see Online Resource 1). BVDV was identified via RT-qPCR (ct 27.35) from a pool of frozen tissues and characterized as BVDV-2b by sequencing (GenBank MN159205). IHC for BVDV antigen detection in the lung revealed finely granular, homogenous, and focal immunolabeling in the cytoplasm of smooth muscle and endothelial cells from an arteriole affected by arteriolitis and infiltrating macrophages (Fig. 2b). No immunolabeling occurred in the kidney, small intestine, colon, or liver tissues. Based on these results, we diagnosed septicemic salmonellosis in coinfection with BVDV-2b (Table 1), which was detected by IHC within the arteriolar lesions in the lung and was thus suspected to have caused the arteriolitis.

Case 4 from the same outbreak had pathological findings including severe extensive fibrinosuppurative

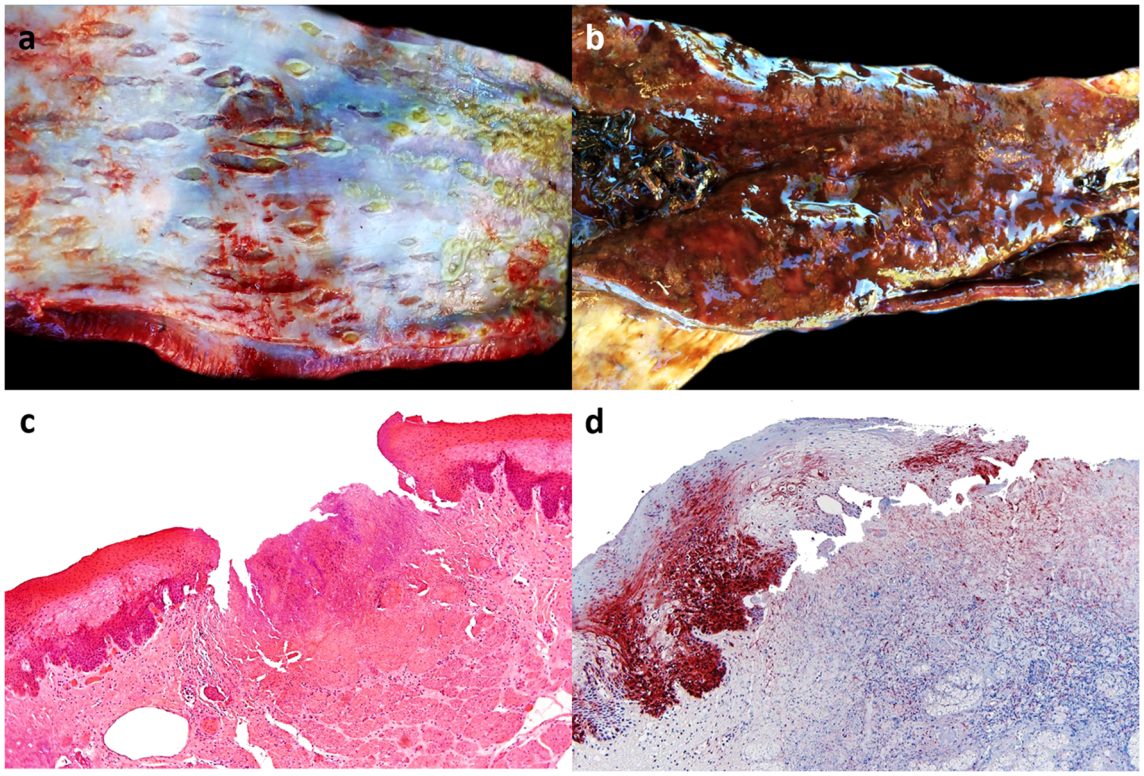

Fig. 1 Pathological findings in an Aberdeen Angus heifer with mucosal disease caused by BVDV-1a (case 1). **a** The mucosa of the esophagus shows multiple, coalescing, irregular, well-defined erosions and nonperforating ulcers (ulcerative esophagitis), some of which have a hemorrhagic halo. **b** The colonic mucosa is markedly reddened, necrotic, and ulcerated and contains scant brown-tinged fluid and few blood clots (necro-hemorrhagic colitis). **c** Low-magnification micrograph of the esophageal mucosa depicting one of the ulcers shown in **a**, with underlying necrosis of the submucosa, and hydropic degeneration of keratinocytes in the adjacent epithelium, H&E stain. **d** BVDV immunohistochemistry in a serial section of the lesion shown in **c** denoting abundant antigen immunolabeling in the esophageal mucosa adjacent to the ulcer bed

bronchopneumonia involving approximately 70% of the pulmonary parenchyma and fibrinous pleuritis. The tongue showed multifocal necrosis of the basal keratinocyte layer in the mucosa and multifocal apoptosis of individual keratinocytes in the esophagus and rumen.

Histophilus somni was isolated from the lung, and viral causes of pneumonia were ruled out (Online Resource 1). BVDV was identified in a frozen sample of pooled tissues by RT-qPCR (ct 26.08), and genome sequencing revealed the same subtype as in case 3 of this outbreak (GenBank MN159204). IHC for BVDV antigen revealed focal intralesional immunolabeling in the basal mucosal epithelium of the tongue, in the cytoplasm of the endothelial and muscular cells of blood vessels in the lungs and in macrophages infiltrating the pleura and interstitium. No immunolabeling was found in the kidney, lymph node, heart, intestinal, or rumen tissues. *H. somni* bronchopneumonia was diagnosed (Table 1) in coinfection with BVDV-2b that was detected by IHC in the pulmonary and lingual lesions.

Outbreak 4 Outbreak 4 occurred in a feedlot with approximately 300 beef cattle from different origins. An approximately 2-year-old beef steer of undetermined breed (case 5) presented inappetence and stopped drinking water. Antibiotic treatment (penicillin G procaine and dihydrostreptomycin) was administered for 5 days; however, the steer died after a 10-day clinical course. Within 6 months, two other steers with similar clinical presentations died, yielding a 1% mortality rate.

A partial necropsy was performed by the veterinary practitioner, and some organs, including the kidney, heart, liver, urinary bladder, and small and large intestines, were submitted to the diagnostic laboratory. Pathological findings in the kidneys included extensive cortical coagulative necrosis (renal infarcts) (Fig. 3a) and occasional necrotizing segmental arteriolitis with thrombosis (Fig. 3b). Severe acute/subacute transmural necrotizing, fibrinosuppurative, and hemorrhagic urocystitis with thrombosis and individual necrosis of arteriolar leiomyocytes was also present. In addition, focal acute mild colitis with necrotizing cryptitis was observed.

BVDV was detected by RT-qPCR (ct 34.04) from the frozen tissue pool and further identified as BVDV-1a via sequencing (GenBank MN159221). IHC for BVDV antigen detection showed multifocal and infrequent cytoplasmic immunolabeling in macrophages infiltrating the urinary bladder mucosa; no labeling was seen in any other analyzed tissues

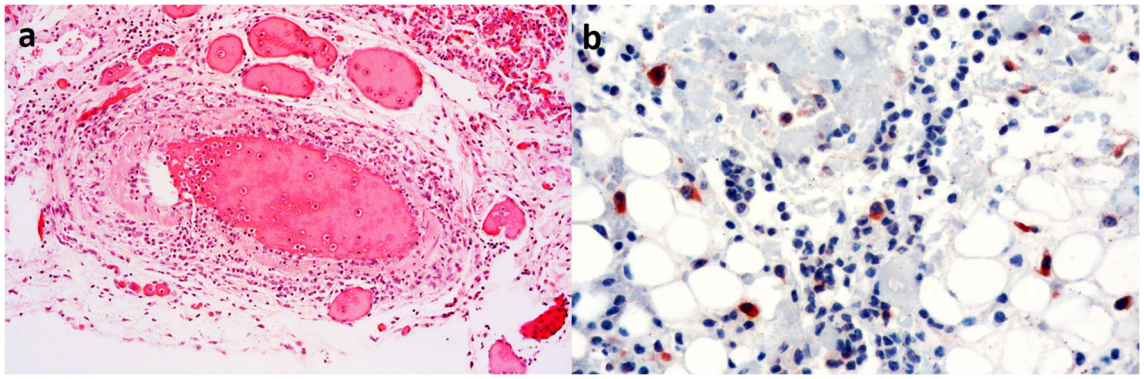

Fig. 2 Microscopic lesions in the lung of a Holstein heifer with transient BVDV-2b infection (case 3). **a** Inflammatory cells infiltrate the tunica media of a medium-sized arteriole in the pulmonary interstitium (arteriolitis), H&E stain. **b** BVDV immunohistochemistry revealing strong granular intracytoplasmic immunolabeling in macrophages infiltrating the pulmonary interstitium (pleura)

(liver, kidney, spleen, lung, and colon). Additionally, in this case, *Streptococcus* sp. was isolated from the kidney, and *Escherichia coli* was isolated from the kidney, urinary bladder, and liver (Online Resource 1). These were considered potential opportunistic secondary pathogens. Because BVDV IHC was negative in multiple tissues except the bladder, an acute/transient BVDV-1a infection was suspected.

Outbreak 5 Outbreak 5 occurred at a calf-rearing and steer-fattening operation, with 708 cattle of different breeds and origins. Between January and April 2018, 12 Holstein steers (1.7%) presented emaciation, anorexia, isolation, and diarrhea and died after a 1-month clinical course. An approximately 4-year-old Holstein steer (case 6) in poor-body condition was necropsied.

The pathological examination revealed a chronic enteropathy affecting the ileum and colon, characterized by depletion of intestinal glands/crypts in the mucosa, occasional neutrophilic/necrotizing cryptitis, and infrequent intraepithelial coccidia, morphologically compatible with *Eimeira* spp. Additionally, multifocal, moderate, chronic, lymphocytic interstitial nephritis was observed as well as moderate pleuritis with pleural fibrosis and fibrosing interstitial pneumonia and moderate suppurative lymphadenitis of undetermined etiology.

IHC for BVDV revealed strong and abundant finely granular and homogeneous intracytoplasmic immunolabeling in the epithelial cells, which was diffuse throughout all examined tissues (lung, intestines, liver, and kidney). BVDV was detected in the serum by RT-qPCR (ct 28.75), and BVDV-1a was identified by sequencing (GenBank MN186041). Other causes of enterocolitis were ruled out, including *Salmonella* spp., *Mycobacterium avium paratuberculosis*, bovine coronavirus, and group A rotavirus (Online Resource 1). Based on the overall results, this was likely either a PI calf with BVDV-1a or a severe transient infection.

Outbreak 6 Outbreak 6 occurred at a dairy farm of unknown herd size, where an increased abortion rate was registered. Two aborted Holstein fetuses (cases 7 and 8) of approximately 180–210 gestational days were necropsied. For case 7, the diagnostic investigation did not allow determining the cause of the abortion because the fetus presented no lesions, and all ancillary diagnostic tests were negative (Online Resource 1) except the RT-qPCR for BVDV (ct 32.17), which was positive from a pool of fetal tissues.

In case 8, the histological examination revealed moderate multifocal lymphohistiocytic necrotizing encephalitis, severe random multifocal necrotizing hepatitis, moderate multifocal lymphocytic myocarditis, and multifocal lymphohistiocytic placentitis with trophoblast necrosis. All lesions were highly compatible with neosporosis. *Neospora caninum* was detected intralesionally by IHC in the brain and by PCR in the same tissue (Online Resource 1). BVDV was detected by RT-qPCR (ct 33.90) from the pool of fetal tissues.

BVDV-2b was identified in both fetuses by sequencing (GenBank MN186039 and MN186040). IHC for BVDV antigen detection was negative in tissues from both fetuses, including the brain, lung, and spleen in case 7 and the brain, kidney, liver, and spleen in case 8. Based on these results, acute/transient BVDV infections, rather than PI, were suspected in both cases (Table 1).

Phylogenetic analysis

As shown in Fig. 4, the phylogenetic analysis revealed that 4 of the strains were BVDV-1a and the other 4 BVDV-2b with bootstrap values of 71% and 63%, respectively.

Discussion

The diagnoses of BVDV infection in 8 cases reported here were based on viral genome detection by RT-qPCR along with detailed pathological examinations to characterize the lesions and IHC to identify BVDV antigen in the tissues of the

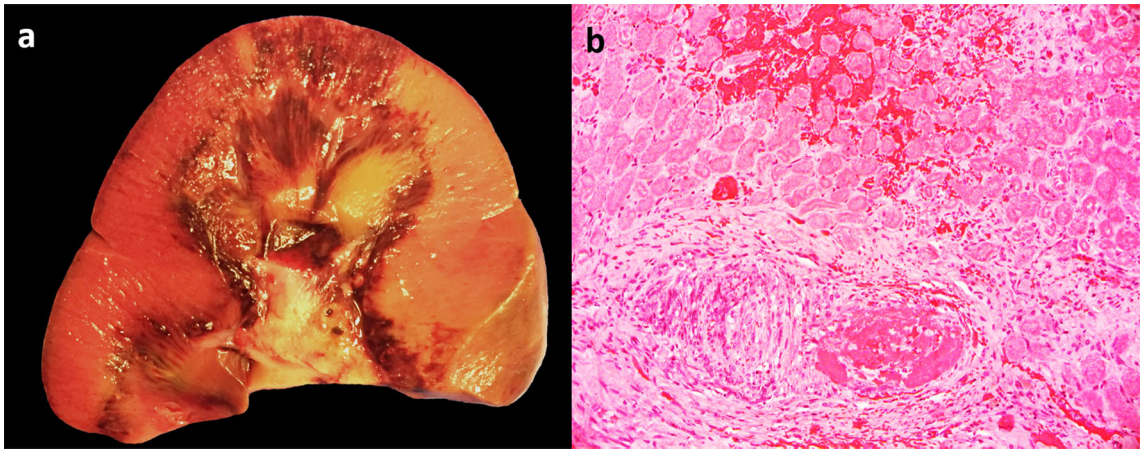

Fig. 3 Renal lesions in a beef steer infected with BVDV-1a (case 5). **a** A section of kidney shows multifocal cortical renal infarcts. **b** Micrograph of the affected kidney depicting severe arteriolitis with thrombosis (bottom) and extensive coagulative necrosis of the adjacent cortical renal tubules with interstitial hemorrhage (acute infarction), H&E stain

deceased cattle. The BVDV subtype involved in all cases was identified by sequencing the 5′UTR genomic region. Ancillary tests were performed to identify coinfections and to rule in/out differential diagnoses on a case-by-case basis (Online Resource 1). Diagnostic investigations in the 8 cases were compatible with different BVDV infection outcomes, including MD (case 1 and eventually case 6), transplacental/congenital transmission with potential transient/acute fetal infection (cases 2, 7, and 8), transient/acute postnatal infections associated with coinfections by pathogenic bacteria, such as *Salmonella* typhimurium (case 3) and *Histophilus somni* (case 4), or opportunistic bacteria, such as *Streptococcus* sp. and *E. coli* (case 5), or eventually severely transient infection associated with enteric coccidia (case 6).

The clinical signs and pathological findings in the heifer from outbreak 1 were those typically described in the literature for MD cases [13, 27]. Detection of the viral genome and the colocalization of BVDV antigen intralesionally by IHC confirmed the etiological diagnosis, while other causes of necrotizing colitis, such as *Salmonella* spp. and bovine coronavirus, were ruled out [13].

The occurrence of MD requires the presence of PI animals congenitally infected with BVDV, which are the main source of infections in the herds. PI cattle are viremic, lifelong viral shedders, and immunotolerant to the NCP strain with which they are infected because their immune systems do not recognize the BVDV antigens as foreign, thus leading to extensive viral replication in the target cells [13, 27]. In our case, viremia was confirmed by identifying BVDV-1a in the serum, while IHC allowed detecting abundant viral antigen in the alimentary tract tissues (esophagus, small, and large intestines), which showed typical MD lesions. MD occurs in PI animals when the NCP BVDV strain is converted to the cytopathic biotype via mutation, recombination, and/or genomic rearrangements. Generally, the NS2/3 viral protease containing the NCP strain is cleaved to NS2 and NS3 in the cytopathic strains [12, 28]. This results in a devastating infection that destroys the host cells, to which the animal's immune system cannot respond [13].

PI neonate calves may be clinically normal, weak, born smaller than normal, or show growth retardation; however, most die of MD before 2 years of age [12, 13], which is the same age range of the heifer with MD in case 1. The 4-year-old steer in outbreak 5 (case 6) presented severe intestinal histological lesions compatible with MD, although no macroscopic lesions typical of MD were observed in the digestive tract. This reinforces the idea that PI animals can remain asymptomatic in the herds for more than 2 years, perpetuating infection and disease. In BVDV PI cattle, IHC should show strong positive immunolabeling in all or most tissues/organs, since infection in these animals is disseminated/multisystemic, and the tissues usually contain high viral loads [29]. The strong widespread intra- and extralesional immunolabeling in the lungs, intestines, liver, and kidneys of case 6, along with the viral genome detected in the serum, indicated that this steer was viremic and most likely represented a PI animal.

Fetal responses to BVDV infection are influenced mainly by gestational age, fetal immune response, and viral biotype and subtype [29, 30]. Abortions at 6–8 gestational months identified as cases 2 and 8 in this work were most likely caused by *N. caninum* given the nature of the fetal lesions, which were typical of this protozoan, and its intralesional detection via IHC in the brain/myocardium as well as by PCR in the brain in both cases. However, coinfections with BVDV-1a (case 2) and BVDV-2b (case 8) were identified by RT-qPCR/sequencing, indicating a congenital/transplacental circulation and transmission of both viral species/subtypes in these fetuses. Because fetuses infected with NCP BVDV strains before the 4th gestational month are generally immunotolerant to

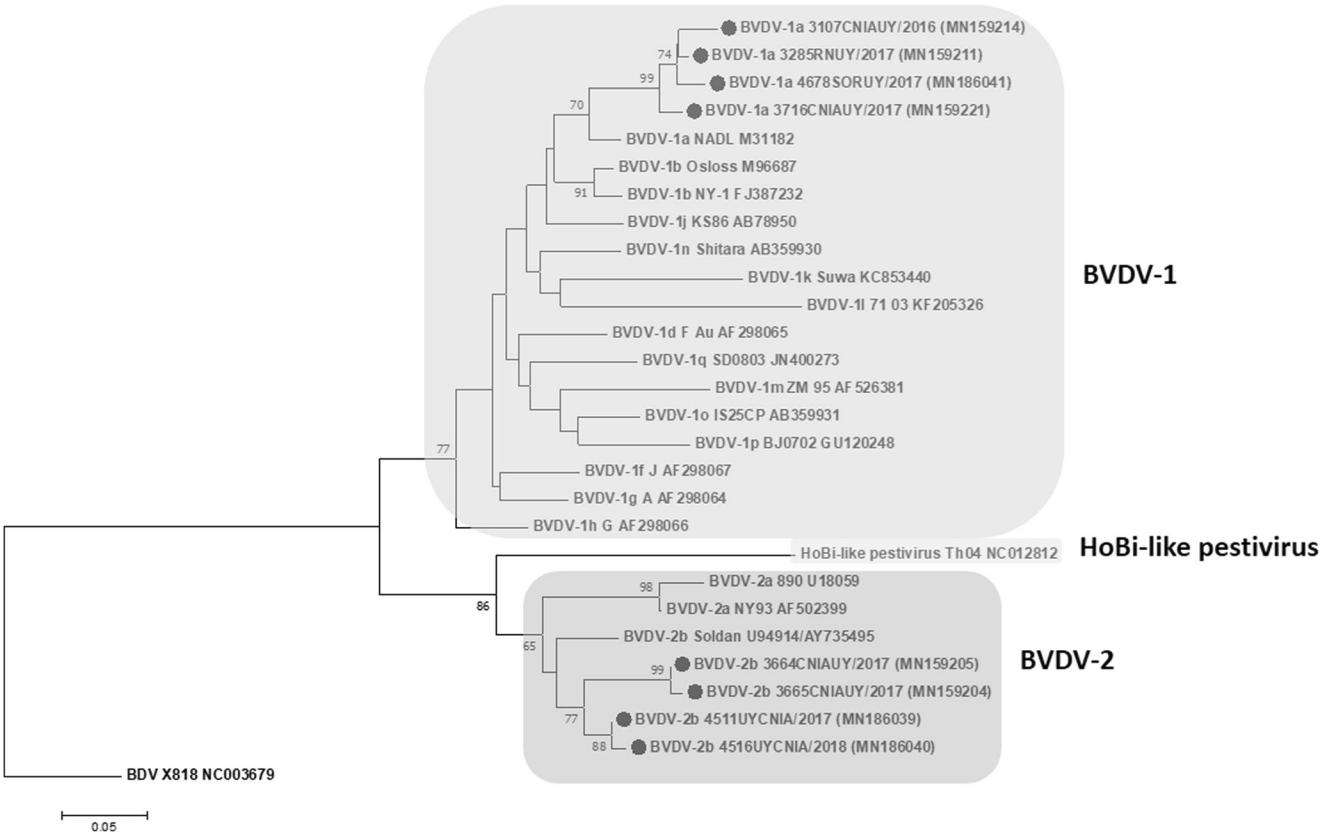

Fig. 4 Phylogenetic tree constructed by the neighbor-joining method using 207 nt of the 5′UTR of the BVDV genome. Uruguayan strains are indicated by black dots. Reference sequences of species BVDV-1, BVDV-2, and HoBi-like *Pestivirus* were retrieved from GenBank for comparison. Labels and sequence names indicating the BVDV species and subtypes. A border disease virus (BDV) sequence was included as an out-group

the virus and therefore develop persistent infections, the viral load in PI fetal tissues is usually high and detectable by IHC [29]. In contrast, in our fetuses (cases 2, 7, and 8), the virus was undetected by IHC, although it was identified by RT-qPCR, suggesting that the viral load may have been below the IHC detection limit and indirectly suggesting that these fetal infections were transient/acute. The roles of BVDV-1a and BVDV-2b infections as causes of abortions in these cases could not be determined. Interestingly, in a study conducted in dairy cattle, BVDV-induced immunosuppression was hypothesized to contribute to the occurrence of abortions due to *N. caninum* [31], although more detailed investigations are needed to confirm the possible synergistic association between these agents [32].

Fetuses that survive NCP BVDV strain infections before day 90, and more rarely up to day 125 of gestation, invariably develop immunotolerance to the virus and are born PI [33]. Immunotolerance is postulated to be mediated by the virus's ability to inhibit type I interferon production in infected fetal cells, allowing the infection to persist [34]. However, depending on the gestational age and viral strain, transplacental infection can also result in embryonic/fetal death (reabsorption, mummification, or abortion), congenital malformations, or birth of clinically healthy calves [30, 35].

BVDV subtyping of the case 2 fetus (outbreak 2) was identified as BVDV-1a, while for the case 7 and 8 fetuses (outbreak 6), subtyping was identified as BVDV-2b. Bielefeldt-Ohmann et al. [29] reported that both BVDV-2 and BVDV-1b have a similar tissue tropism, although BVDV-2 passes through the placenta more quickly. Additionally, antigenic immunolabeling in the fetal tissues was more intense for BVDV-2 than for BVDV-1b [29]. In the 3 fetuses evaluated in our study, BVDV immunolabeling was negative in all analyzed tissues, independent of the infecting viral species/subtype. This finding suggests that the fetuses were not PI, but rather were acutely/transiently infected, which may or may not have contributed to abortion due to *N. caninum* in cases 2 and 8 as discussed previously. When infection occurs in the last third of gestation, the fetal bovine immune system is sufficiently developed to respond to the virus, being comparable with a transient postnatal infection [35, 36]. Serological assays to assess whether these fetuses had developed specific humoral responses to BVDV were not performed in our study.

Transient/acute BVDV infections are largely subclinical or cause mild clinical signs, although they can induce leukopenia (lymphopenia, monocytopenia) and reduce antibody production and neutrophil release and function, resulting in immunosuppression. Such immunosuppression favors secondary bacterial and/or viral infections [13] that can contribute to respiratory, digestive, or septicemic diseases [13, 37]. In the three animals from outbreaks 3 and 4 (cases 3–5), BVDV infection likely contributed to the occurrence of pneumonic histophilosis (case 3), enteric/septicemic salmonellosis (case 4), and septicemia or urinary infection by opportunistic pathogens such as *Streptococcus* sp. and *E. coli* (case 5). These coinfections were confirmed by isolating the bacterial agents involved in each case, and their pathogenic role was established by evaluating the lesions compatible with those caused by these agents in the respective pathological examinations.

Acquiring animals without checking their BVDV infection status is an important risk factor for BVDV infection in cattle herds. This is directly related to introducing PI animals that can carry the infection or new viral strains into the herds [38–41]. In 3 of the outbreaks reported here (outbreaks 3–5), the anamnestic information revealed that cattle from different origins were introduced and mixed into the herds without prior BVDV testing, which possibly contributed to disseminating and spreading infection and disease between farms. Detecting and removing infected cattle from the herds is key in controlling BVDV infections.

BVDV-2 is generally considered more virulent, being frequently related to disease and death [36, 42]. However, in a study conducted in the USA, both BVDV-1 and BVDV-2 were diagnosed in cattle with clinical respiratory and digestive manifestations, as well as in necropsied cattle [42], with BVDV-1 being the most common species [42, 43]. BVDV-1a (cases 1, 5, and 6) and BVDV-2b (cases 3 and 4) were identified in heifers/steers in our work, indicating that both species are related to postnatal morbidity and mortality in cattle in Uruguay. In other South American countries, clinical disease has been reported for BVDV-1a, BVDV-1b, BVDV-2a, and BVDV-2b in Argentina [44–46]; BVDV-1b, BVDV-1d, BVDV-1i, and BVDV-2b in Brazil [47–50]; and BVDV-1a, BVDV-1b, and BVDV-1c in Chile and Peru [17, 51].

Although cattle exposed to BVDV in utero constitute a significant portion of clinical presentations, cases of transient/acute postnatal infection may be significant sources of direct losses [1]. Several of the deaths reported in outbreaks 3 and 4 (cases 3–5) were likely due to transient/acute BVDV infections aggravated by secondary bacterial infections. The arteriolitis observed in case 3 is a lesion frequently found in BVDV cases [13], whereas it is not a typical finding in salmonellosis [52], the agent with which this calf was coinfected. This case presented typical lesions of both agents, and although *Salmonella* typhimurium can cause disease and death by itself, it also behaves as an opportunistic pathogen, and thus, in this case, the BVDV may have predisposed to clinical salmonellosis. Additional evidence of transient/acute infection in these cases included detection of intralesional BVDV antigens by IHC in only some of the calf tissues examined by this technique.

The IHC performed in this study used a commercial monoclonal primary antibody against BVDV glycoprotein 55 (envelope glycoprotein E2) made using the BVDV NADL strain. Although this antibody binds to and cross-reacts with most BVDV strains, it does not bind to some strains (such as Oregon C24V used to produce vaccines in the USA) per the manufacturer and published information [53]. The major glycoprotein E2 is the most variable and immunodominant glycoprotein in BVDV, and as such, some strains may not bind to this antibody [54]. Our results indicated that this procedure enabled identifying at least four Uruguayan field strains: three BVDV-1a strains from outbreaks 1, 4, and 5, and one BVDV-2b strain from outbreak 3. However, additional cross-neutralization studies should be performed to assess whether this antibody cross-reacts with other local field BVDV strains, since reduced reactivity of anti-BVDV antibodies has been identified to cause failed detection of field isolates (particularly BVDV-2a) [55].

In Uruguay, a serological study conducted in beef cattle in 2000–2001 indicated that 69% of 6358 animals were seropositive and that 100% of 230 herds had at least one seropositive animal [22]. Even with a high viral circulation in the country's cattle population, only 3% of farmers implement preventive vaccinations [22]. More recently, active BVDV infections in cattle in Uruguay were explored in a study in which 390 serum samples from 14 herds were analyzed to search for viral antigens by capture ELISA and for viral genomes by RT-qPCR. Sixteen (4.1%) of these animals were positive by both techniques [18], suggesting that they were either PI animals and/or acute/transient infections acquired postnatally. Interestingly, the mortality rate was 3.5% in the herd with MD from outbreak 1 in our study, suggesting, hypothetically, that 3.5% of this herd could have been PI animals, a value close to that found by Maya et al. [18]. Although the prevalence of PI animals in a herd is generally < 2%, it can be as high as 25–30% in herds where many cows/heifers were exposed to NCP BVDV strains during early pregnancy [13].

The only published study to explore BVDV genetic diversity in Uruguay found that the main species/subtype was BVDV-1a [18]. Coincidentally, this subtype was found in the MD case (outbreak 1) and in outbreaks 2, 4, and 5 described herein, indicating that, besides being frequent, this subtype is associated with disease and death in cattle, which is unprecedented in Uruguay in the scientific literature. Based on our results, BVDV-2b can also be regarded as a significant cause of disease in Uruguay. In addition, this work expands the geographical distribution (south) and the biotype of

infected cattle (dairy) in Uruguay for BVDV-2b, since this subtype had previously been detected only in beef cattle in the department of Rivera, in northern Uruguay [18].

Vaccines commercially available for BVDV prevention in Uruguay are manufactured with inactivated virus and can only provide partial protection, unlike modified-live vaccines used in other regions [56], including South American countries such as Brazil [57]. Recent investigations in Argentina have led to developing an enhanced BVDV subunit vaccine expressed in a baculovirus, based on a truncated E2 glycoprotein fused to a single-chain antibody that targets antigen-presenting cells [58]. This vaccine's immunogenicity was tested in guinea pigs and cattle, and immunized cattle developed high levels of neutralizing antibodies against BVDV up to 1 year after immunization. Based on these results, the vaccine was scaled up and registered and is being commercialized as the first Argentinean subunit vaccine for cattle [59]. Whether these vaccines protect against Uruguayan BVDV strains remains to be addressed.

Conclusions

BVDV causes pathology and is associated with diseases and mortality in beef and dairy cattle in Uruguay. More extensive and systematic studies are necessary to determine the epidemiology and economic impacts of BVDV for livestock in Uruguay as well as the distribution and frequency of the different species/subtypes involved in clinical settings. Cost-benefit analyses and feasibility studies could help establish preventive and control programs for BVDV at the farm, regional, and national levels.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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