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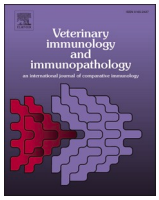

Short communication

Long-term acute infections during a bovine viral diarrhea virus (BVDV) outbreak in dairy farm from Galicia (NW Spain)

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ABSTRACT

An observational study describes an outbreak of bovine viral diarrhea virus (BVDV) in a dairy herd in Spain. The herd was subjected to a voluntary control program. In a sampling carried out in June 2020, bulk tank milk antibody levels increased compared to the previous sampling. Additionally, serum samples from 4 young heifers also tested positive for antibodies. Since the results were consistent with a recent infection, we proceeded to detect possible persistently infected (PI) animals using antigen ELISA (on serum/ear-notch samples), following the program guidelines. From this moment on, 42 animals tested positive for BVDV antigen, of which 17 were under typical acute infection (AI), 13 were deemed as PI, and eight died early on the farm before having information to determine their status. The remaining 4 showed intriguing test results consistent with a long-term AI since they tested BVDV positive in at least two antigen tests more than 3 weeks apart. Thus, one animal was positive until 80 days of age in serum, and others even for longer periods in ear-notch samples, until they finally tested negative for BVDV. Based on these results, longer follow-up may be necessary in BVDV positive animals to accurately confirm persistent infection.

1. Introduction

Bovine viral diarrhea virus (BVDV) is one of the most important viral pathogens among cattle worldwide. BVDV is a positive single-stranded RNA virus, which belongs to the genus *Pestivirus*, in the family *Flaviviridae*, which also includes Border Disease Virus and Classical Swine Fever Virus (Hamers et al., 2001; Bauermann et al., 2013; Mirosław and Polak, 2019).

BVDV is currently grouped into two different species (BVDV-1 and BVDV-2) based on antigenic and genetic properties (Bauermann and Ridpath, 2015). Moreover, to the date at least 21 genetic types of BVDV-1 (named BVDV-1a to BVDV-1r) and 4 types in BVDV-2 (BVDV-2a to BVDV-2d) have been described, based mainly on analysis of the partial 5′UTR sequence, followed by analyses of other genomic regions, such as N^{pro} (Deng et al., 2015; Giammarioli et al., 2015). In addition, HoBi-like viruses, also referred to as bovine viral diarrhea virus 3 (BVDV-3) or atypical *Pestivirus*, have been proposed as a new putative bovine *Pestivirus* species (Bauermann and Ridpath, 2015). Until 2015 BVDV-2 was not detected in Spain (Aduriz et al., 2015), being currently BVDV-1b the predominant type in the country (Factor et al., 2016; Eiras et al., 2019).

The disease, bovine viral diarrhea (BVD) causes considerable economic losses in cattle herds, mainly attributable to reduced milk production, reduced reproductive performance, delayed growth, increased susceptibility to other diseases, early culling and increased mortality among young stock (Santman-Berends et al., 2015; Richter et al., 2017; Yarnall and Thrusfield, 2017). BVD is associated with a wide range of clinical manifestations in cattle, depending mainly on virus strain, age and immune-physiological status of the animal when first infected (Discontools, 2018). In pregnant cows, consequences include embryonic death, abortion, stillbirths or the birth of immunotolerant persistently infected (PI) calves. PI are animals infected in the uterus, mainly during the first four months of pregnancy. PI animals continuously excrete viruses into the environment throughout their lifetime and, in that way, they are a major source of BVDV. They rarely develop any antibody response against the virus, whereas the decline of passively derived

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BVDV antibodies is considerably faster compared to immunocompetent calves (Palfi et al., 1993). Thereby, the identification and removal of PI animals from the herd is a key measure in BVDV control (WOHA, 2021). Although PI animals often show various degrees of growth retardation and unthrifty appearance, a significant proportion is clinically normal (Walz et al., 2020).

Acutely infected (AI) animals show a short-term viremia (often 4–7 days) and shed virus in all body secretions including nasal discharge, saliva, semen, and faeces (Müller-Doblies et al., 2004). The length of transmission following acute infection may vary based on health, stress level, age and presence of other pathogens. They include respiratory pathogens (bovine herpesvirus 1, parainfluenza virus 3, bovine respiratory syncytial virus, and *Mannheimia haemolytica*), enteric pathogens (bovine rotavirus and *Salmonella enterica* serovar Typhimurium) or reproductive pathogens (*Leptospira borgpetersenii* serovar hardjo, *Coxiella burnetii, Campylobacter fetus, and Neospora caninum)* (Bolin, 2002; Discontools, 2018). Furthermore, neutralizing antibodies might be detected at 2–3 weeks post-infection (Chase et al., 2015). However, in AI animals, BVDV has demonstrated the potential to be maintained as a chronic infection within immunoprivileged sites. Immunoprivileged sites within cattle that can support chronic infections with BVDV include seminiferous tubules within testicular tissue, ovaries, and to a lesser extent, tissues of the central nervous system, and circulating white blood cells (Givens and Marley, 2013). Moreover, recently, it has been described a field case in which AI animals (in this case with a BVDV-2 strain) showed long-term viral presence in serum samples, with test results similar to those of PI cattle (Goto et al., 2021a). This could pose a new difficulty in BVDV control.

The aim of the present study was to describe a new field case with long-term detection of a BVDV-1d strain in AI animals on a dairy cattle farm from Galicia (NW Spain).

2. Materials and methods

2.1. Area description

The studied herd was in Galicia (northwestern Spain). Galicia is the main dairy cattle area of the country accounting for 55% of farms and 38% of milk production. The mean herd size is 43 cows. Farms are still predominantly family owned and managed.

An official voluntary BVDV control program has been implemented since 2004 in Galicia. The control program is based on the regular monitoring of herds to detect farms with possible active infection and potential presence of PI animals, and the compulsory control of purchased cattle. BVDV positive animals can only leave the farm for slaughter. Most farms cull PI animals immediately upon confirmation. Vaccination is at voluntary basis (Xunta de Galicia, 2019).

Briefly, in non-vaccinated herds or those using inactivated vaccines, bulk tank milk (BTM) samples are collected, at least, every 6 months from each herd and tested for antibodies against the p80 protein of the BVDV using a commercial blocking ELISA. At the same time as the BTM collections, blood samples are taken from a selection of young heifers (9–24 months of age) and tested for BVDV antibodies using the same ELISA kit. If tested samples indicate a possible PI on the farm (*e.g.* a positive ELISA antibody result in a young heifer or a significant increase in the level of antibodies compared to the previous sample), this is confirmed by a commercial antigen ELISA on serum or ear notch samples, including the monitoring of all new born calves. According to the program, ear tissue is the sample indicated for animals under three months of age. In older animals, both ear tissue and serum are accepted. Two samples tested positive for BVDV from the same animal taken 3–4 weeks apart are considered to confirm persistent infection (Xunta de Galicia, 2019).

2.2. Description of the herd

The herd comprised 400 adult dairy cows, all Holstein-Friesian breed, under a voluntary BVDV control program. The farm did not use BVDV vaccines.

In a sampling carried out in June 2020, the level of antibodies against the p80 protein in BTM significantly increased compared to that in the previous sampling, and serum samples from 4 young heifers tested positive for antibodies. Thus, according to the control program guidelines, we proceeded to detect possible PI animals on the farm (including testing of all newborns) using an antigen ELISA (based on the detection of the E^{rns} protein of virus) on serum or ear-north samples following control program guidelines (Xunta de Galicia, 2019). Blood was collected from the coccygeal vein using anticoagulant free vacutainer tubes; after coagulation, serum was separated by centrifugation (5000 g, 5 min) and aspiration. Skin biopsy samples (ear notches) measuring approximately 2–3 mm in diameter, were obtained from the dorsal pinna margin of each calf using a commercial ear notcher (Tissue-sampling ear tags, Caisley International GMBH, Germany).

Outbreak follow-up is maintained for at least 9 months from the date of birth of the last BVDV positive animal.

In this particular herd, some male and crossbreed female calves were fattened in the farm itself but in a separate shed, even if confirmed as PI. This type of management is patchy in dairy farming in Galicia (most farms cull PI animals immediately upon confirmation), but in this case, it allowed the monitoring of these animals, which were subjected to additional antigen tests.

2.3. Laboratory analysis

Commercial ELISA kits used were “BVDV p80 Ab, Idexx/ ID Screen® BVD p80, IdeVet” to detect antibodies against BVDV both in BTM and serum samples, and “BVDV Ag/Serum Plus, Idexx” to detect BVDV both in serum and ear notch samples.

Additionally, BVDV was typed from two animals who tested positive to antigen ELISA. For the phylogenetic analysis, RNA was extracted from serum samples using the QIAamp Viral RNA Mini Kit® (Qiagen, Manchester, UK). cDNA was synthesized from template RNA (1 ng–5 μg) using the AffinityScript Multiple Temperature cDNA Synthesis kit® (Agilent Technologies, CA, USA) according to the manufacturer’s instructions. Then a 288-bp DNA product from the 5′ UTR region was PCR-amplified using primers 324 and 326 as described (Vilček et al., 1994). Amplified DNA fragments were purified by ExoSAP-IT treatment (USB Corporation, OH, USA) and sequenced in both directions using the mentioned PCR primers. Sequencing was performed at the “Sequencing and Fragment Analysis Unit” of Santiago de Compostela University using a 3730xl genetic analyzer (Applied Biosystems, CA, USA). Sequences were converted to FASTA format using Chromas Lite 2.1.1 and imported into MEGA 7. Phylogenetic trees were constructed using the neighbor-joining method and validated using bootstrap analysis with 1000 replicates. Pairwise distance matrices were conducted with the Kimura 2-Parameter.

3. Results and discussion

This study describes a field case of BVDV infection in animals with long-term AI. The World Organization for Animal Health (WOAH) indicated in the Manual of Diagnostic Tests and Vaccines for Terrestrial Animals that persistence of virus infection should be confirmed by resampling the animals after an interval of at least 3 weeks, when, in PI animals, virus would again be detected (WOHA, 2021). This criterion was followed by the BVDV control program in Galicia, and according to regulations, animals with a BVDV positive result can only leave the farm for the slaughterhouse. Exceptionally, the described dairy cattle farm routinely kept some male and crossbreed female calves in the herd until sacrifice in order to be fattened, regardless of their health status.

Table 1
Summary of test results of BVDV antigen ELISA from ear notch/serum and BVDV p-80 antibody ELISA from serum on animals tested positive both to antigen and antibody ELISA over time.

Calf No	Age of initial BVDV detection (days)	Timing of sample collection (age at sampling in days)	Days from the 1st BVDV positive test	p-80 antibody ELISA	Antigen ELISA ear-notch	Titers[a]	Antigen ELISA serum	Titers[a]
6444	23	1st (23)	0		Positive	2.5		
		2nd (51)	28		Positive	2.3		
		3rd (78)	55	Positive			Positive	3.9
5988	2	1st (2)	0		Positive	3.3		
		2nd (30)	28		Positive	4		
		3rd (78)	76	Positive	Positive	3.7	Positive	5.9
5986	19	1st (19)	0		Positive	3.9	Positive	4
		2nd (70)	51	Positive				
		3rd (74)	55		Positive	3.9		
5991	30	1st (30)	0		Positive	4		
		2nd (37)	7		Positive	3.6		
		3rd (81)	51		Positive	3.9		
		4th (85)	55	Positive			Positive	4.5
5937	16	1st (16)	0				Positive	3.8
		2nd (18)	2	Positive	Positive	3.6		
		3rd (72)	56	Positive	Positive	3.4	Positive	5.9

[a] Expressed as corrected optical density (OD) calculated as follows: OD value of the negative control- OD value of the sample. Values $\geq$ 0.3 are considered positive

Table 2
Summary of test results of BVDV antigen ELISA from ear notch/serum and BVDV p-80 antibody ELISA from serum on animals tested BVDV positive that reverted to negative over time.

Calf No	Age of initial BVDV detection (days)	Timing of sample collection (age at sampling in days)	Days from the 1st BVDV positive test	p-80 antibody ELISA	Antigen ELISA ear-notch	Titers[a]	Antigen ELISA serum	Titers[a]
3616	95	1st (95)	0	Positive	Positive	0.8		
		2nd (130)	35		Positive	0.3		
		3rd (165)	70		Negative			
6405	16	1st (16)	0	Negative	Positive	1.6	Positive	5.9
		2nd (44)	28		Positive	0.8		
		3rd (79)	63		Positive	0.5		
		4th (100)	84	Positive	Negative		Negative	
6456	13	1st (13)	0		Positive	0.5		
		2nd (48)	35		Positive	1.4		
		3rd (69)	56	Positive	Positive	0.4	Negative	
		4th (104)	91		Negative			
5925	10	1st (10)	0	Positive	Positive	3.8	Negative	
		2nd (38)	28	Positive	Positive	4.2	Positive	4.6
		3rd (80)	70	Positive	Positive	4	Positive	4
		4th (108)	98	Positive	Positive	4	Negative	

[a] Expressed as corrected optical density (OD) calculated as follows: OD value of the negative control- OD value of the sample. Values $\geq$ 0.3 are considered positive

During a time period of 560 days, 42 animals tested positive for BVDV by antigen ELISA from ear-notch and/or serum samples in the study farm, with the first animal tested positive in September, 2020.

Seventeen out of the 42 animals tested BVDV negative in the following sampling (3–4 weeks apart); therefore, they were classified as having typical BVDV AI. Likewise, 13 other animals were deemed to have a PI. Eight out of these 13 animal tested positive to the antigen test several times until they were slaughtered, whereas accessory antibody tests were negative with the passage of sampling. Remarkably, the remaining 5 animals (nos. 5937, 5980, 5988, 5991 and 6444) consistently tested positive for BVDV, while the antibody test also remained positive (Table 1). In PI calves, it is considered that the level of antibodies that come from the colostrum drops very quickly, dating this drop between 4 and at most 8 weeks until seronegativy (Palfi et al., 1993).

Four out of the 42 BVDV positive animals (nos. 3616, 6405, 6456, 5925) showed intriguing test results consistent with long-term AI (Table 2). Calf 3616 tested BVDV positive on two consecutive ear-notch samples collected 35 days apart; finally, it tested negative 70 days after the first positive antigen test. Animal 6405 tested positive for BVDV for at least 63 days (during which 3 samples were taken from the animal) and become negative in serum and ear samples after 84 days. Animal 6456 was positive for at least 56 days in ear samples, although the serum sample was negative on day 56. Finally, animal 5925 was positive for at least 70 days in ear and serum, and 98 days in ear samples; the animal was always antibody-positive (Table 2). To our knowledge, there is only one described case of long-lasting AI in which animals were BVDV positive for more than 3 weeks that was reported in Japan (Goto et al., 2021a).

Previous data have indicated that pestiviral infections may be significantly prolonged within ovarian tissue (up to 61 days), testicular tissue (up to 7 months), circulating white blood cells (after seroconversion), or even the central nervous system (Givens and Marley, 2013). The role of these infections in maintaining and disseminating BVDV within the cattle population and heterologous host species remains to be fully understood, but in any case, they do not significantly affect diagnosis, since the samples routinely used are serum, milk, and ear notches. However, the present study identified AI animals with long-term presence of BVDV from samples used regularly in control programs, resulting in animals being indistinguishable from PI cattle, even after two tests were conducted more than 3 weeks apart.

It has been suggested that one causative factor of abnormal long term AI term infections could be failure to transfer passive immunity via colostrum to calves (Goto et al., 2021a, 2021b). In addition, BVDV infections in neonates or very young calves might result in prolonged viremia due to an inefficient immune response in these animals (Schweizer et al., 2021). Generally, calves younger than 1 month old could not activate sufficient self-immunity, which might have prolonged their BVDV infections (Houe, 1995). However, in the present case, one

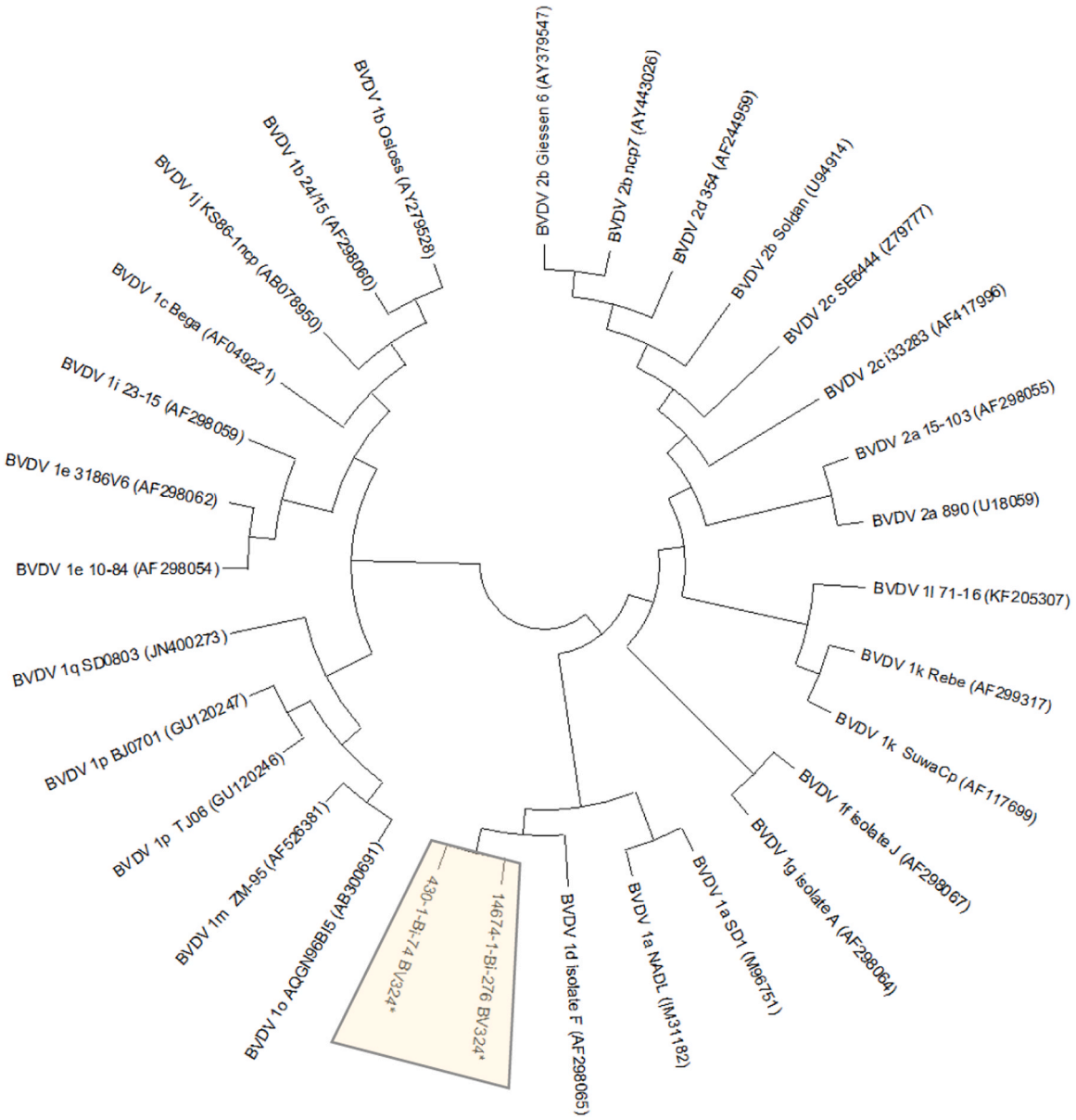

Fig. 1. Phylogenetic analysis of 5′ untranslated region sequences of two bovine viral diarrhea virus isolates obtained from samples collected at the study farm in Galicia in 2020 (marked with an asterisk and highlighted).

long-term AI animal showed positivity until 80 days of age in serum, and other animals showed positivity even for longer periods in ear-notch samples. Moreover, long-term AI animals could also be the result of co-existence with PI animals causing continuous reinfections; however, BVDV-infected cattle usually acquire strong immunity to prevent this occurrence. Gupta et al. (2014), provided evidence that peripheral blood cells from BVDV-immune cattle were susceptible to reinfection with homologous or heterologous BVDV, albeit to a lower extent in the former case. No evidence of this has been proven in serum or ear tissue.

In addition to BVDV-free countries (i.e., Sweden, Norway, Finland, Denmark, Austria, and Switzerland), in Europe, some territories follow mandatory control programs (i.e., Scotland, Ireland, Germany, Belgium, and the Netherlands), whereas others have opted for voluntary or regional programs, as occurs in Spain (Metcalfe, 2019; Yue et al., 2022). The majority are following a similar approach to PI search and removal; therefore, the data presented in the present study provide relevant information for the disease approach.

In Europe, there are two main types of systematic control programmes in place, those allowing and those prohibiting vaccination.

Finally, it was not possible to follow up eight BVDV positive animals because they died before obtaining sufficient information to define their status.

Genetic analysis revealed the presence of BVDV-1 type d on this farm. Two samples were typed and were 100% identical to each other. The phylogenetic tree obtained from sequence analysis is shown in Fig. 1. The previous long-term AI case described in Japan involved a BVDV-2 strain; the infection in this case was subclinical. Phylogenetic analysis revealed that the BVDV-2 strain found was different from the highly pathogenic BVDV-2 types previously described (Goto et al., 2021a). Previous data have reported BVDV-1b as the predominant species in Galicia, followed by BVDV-1e and BVDV-1d, while BVDV-2 has only been detected sporadically (Factor et al., 2016; Eiras et al., 2019). Data on BVDV typing in Galicia correspond with studies published in several European countries, including Germany, France, Italy, and Portugal indicating that BVDV-1b was the most frequent type (Tajima et al., 2001; Jackova et al., 2008; Luzzago et al., 2012, Barros et al., 2006).

Despite the general usefulness and efficacy of BVDV diagnostic tools,

their limitations and challenges still need to be considered. Therefore, in this field case, although seroepidemiological surveillance allowed for the initial detection of the outbreak, the existence of unusual long-term AI in samples routinely used in the control programs was later confirmed. Consequently, longer monitoring times are necessary for BVDV-positive animals, which could imply an excessively prolonged time to confirm PI. This additional time could be valuable for calves of high value, such as females with high expected genetic value.

Ethical statement

No procedure was performed specifically for the preparation of this study. All procedures are limited to those sampling performed routinely in official control programs.

CRediT authorship contribution statement

L. Nodar: Conceptualization, Formal analysis, Visualization, Writing – original draft, Writing – review & editing. **I. Arnaiz:** Data curation, Investigation, Methodology, Project administration, Resources, Supervision, Validation, Visualization, Writing – review & editing. **J.J. Pedreira:** Data curation, Investigation, Visualization, Writing – review & editing. **J. Díez:** Data curation, Investigation, Project administration, Visualization, Writing – review & editing. **C. Calvo:** Resources, Supervision, Validation, Visualization, Writing – review & editing. **J.F. Diéguez:** Conceptualization, Formal analysis, Investigation, Methodology, Project administration, Software, Supervision, Validation, Visualization, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

The author declare they have not competing interest.

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