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RESEARCH ARTICLE

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Phylogenetic analysis and spatial distribution of bovine viral diarrhea virus (BVDV) in dairy cattle from Galicia (NW Spain)

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Abstract

Aim of study: To examine the frequency and diversity of bovine viral diarrhea viruses infecting dairy cattle.

Area of study: The study was carried out in Galicia (NW Spain), the main dairy cattle area of Spain.

Material and methods: A total of 157 BVDV isolates (from 140 dairy herds) were typed. Typing was based on a 288-bp sequence from the 5′ untranslated region of viral RNA genome. Subsequently, to investigate whether the presence of herds diagnosed with a particular strain was higher in some areas or during some specific time period, data were tested using a Bernouille approach.

Main results: Of the 157 isolates, 137 (87.3%) were typed as BVDV-1b, 10 (6.4%%) as 1d, 7 (4.4%) as 1e and 2 (1.3%) as 1f. One isolate was assigned to type 1p. Three of the strains found in the study (the three belonging to type 1b) showed significant spatial clustering.

Research highlights: This report indicates that BVDV-1b was the predominant species, although there was an important genetic diversity in the study population. Spatial analysis indicated important drawbacks in the application of biosecurity measures, especially as regards purchase of cattle or after the reintroduction of animals from cattle concentration points.

Additional keywords: dairy cows; PCR; pestivirus; spatial and temporal clustering; viral typing.

Abbreviations used: ADSG (*Agrupación de Defensa Sanitaria Ganadera*= animal health defense group); BVDV (Bovine viral diarrhea virus); REMO (*Registro de Movimientos* = official register on animal movements); RR (relative risk).

Authors' contributions: Conception and design, acquisition, analysis and interpretation the data: CE, FJD. Drafting the manuscript: CE, FJD, IA. Critical revision of the manuscript: MC, EY.

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Introduction

Bovine viral diarrhea virus (BVDV) is a member of the genus *Pestivirus*, which also comprises border disease virus and classical swine fever virus. The genera Pestivirus are included into the family *Flaviviridae*. BVDV cause bovine viral diarrhea, one of the most important diseases in cattle worldwide. It causes considerable economic losses in cattle, mainly attributable to reduced milk production, reduced reproductive performance, delayed growth, increased susceptibility to other diseases, early culling and increased mortality among young stock (Houe, 2003).

As a consequence of their RNA genome, *Pestiviruses* display high mutation rates, which, in some cases, may lead to the emergence of new virus lineages. Thereby, genetic typing of BVDV isolates distinguishes two recognized species: BVDV-1 and BVDV-2, based on antigenic and genetic properties (Becher *et al.*, 1997). Both are capable of causing acute and persistent infections. Moreover, to the date at least 21 genetic types of BVDV-1 (named BVDV-1a to BVDV-1r) and four types in BVDV-2 (BVDV-2a to BVDV-2d) have

been described (Deng *et al.*, 2015; Giammarioli *et al.*, 2015).

Genetic typing of BVDV isolates can provide valuable information about the diversity of viral strains present in a population and thereby guide control programs, vaccine development and identification of likely infection sources. Such information is therefore important at both local and regional levels.

The aim of the present paper was to investigate the diversity of BVDV species and types infecting cattle in Galicia (NW Spain) and to assess if there were spatial patterns.

Material and methods

Study area and sample collection

For the study, a total of 157 BVDV isolates from the Galicia region (NW Spain) were typed. Isolates were obtained from serum samples of persistently infected animals (n=144) and viremic animals (n=13). These were identified between 2015 and 2017 on 140 infected farms participating in a voluntary BVDV control program.

Galicia is the major dairy cattle-farming region of the country, accounting for approximately 40% of national milk production (1.9% of the milk produced in the European Union). BVDV control programs in Galicia are voluntary, as mentioned, and conducted through an organization established to improve livestock health – Agrupaciones de Defensa Sanitaria Ganadera (ADSG) – which has been working since 2004. In 2004, 4.6% of the dairy herds and 13% of the dairy cows were involved in the program. Nowadays, the percentages have increased to 55.2% and 65%, respectively.

For BVDV, program was based on the use of antibody ELISA (against the p80 antigen of the virus) to test serum and milk samples and determine the serological profile of the herds. When tested samples indicated possible persistent infection in the farm (*i.e.*, when a positive result was obtained for a young heifer), this was confirmed by antigen capture ELISA based on detection of the E^{rns} viral protein. Two samples positive for BVDV from the same animal taken 3–4 weeks apart were considered to confirm persistent infection.

Preparation of PCR products

For 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).

Sequencing and phylogenetic analysis

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 6. 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.

Isolates with less than 2% difference (based on pairwise distances) were classified as the same strain (Fujiyuki *et al.*, 2006).

Spatial analysis

To investigate whether the presence of herds diagnosed with a particular strain was higher in some areas, data were tested using a Bernouille approach as implemented in SaTScan 9.5 software. For analysis, a herd positive for one of the strains was defined as the case, and herds positive for any other were defined as non-cases. This method moves a circular or elliptic scanning window (over the study area) and compares the observed and expected cases numbers inside and outside this window in order to detect clusters. Likewise, it produces relative risk for the different clusters (as the ratio observed/expected cases within the cluster, divided by the ratio observed/expected outside the cluster), and a corresponding *p*-value for each cluster, based on the Monte-Carlo simulations. The maximum spatial cluster size varied from zero up to 50 % of the population size (default value in SatScan). After running the analysis, SaTScan creates a shape file that includes cluster locations. This file was opened in Quantum GIS 2.18 to visualize clusters on the map.

Possible connections between farms within a cluster were examined *a posteriori* through the official register on livestock movements (REMO, by its Spanish acronym) and by means of personal interviews with the veterinarians responsible for these farms, during which they were inquired about the main biosecurity risks of the farms.

Results

Of the 157 isolates, 137 (87.3%) were typed as BVDV-1b, 10 (6.4%%) as 1d, 7 (4.4%) as 1e and 2 (1.3%) as 1f. The only BVDV-1p strain came from a cow purchased in 2016 in the neighboring region of Castilla León. This cow was 4 years old when purchased; upon arrival, she tested positive for BVDV antigen (by ELISA) and sent to slaughter.

The 157 sequences have been deposited in GenBank under accession numbers from MG986220 to MG986377. Multiple isolates from the same farm were equal, except in one farm in which two different BVDV-1b strains were found. Information on BVDV species and types found per year in the 140 farms is presented in Table 1.

Fig. 1 summarizes the results from spatial cluster analysis. This indicates that three of the strains found in the study (the three belonging to type 1b and appearing in 12 and 10 and 5 herds, respectively) showed spatial clustering. Regarding the first of these strains, 7 out of the 12 cases were concentrated in an area of 18.7 km radius in the center of the region (centroid: 42.88 N, 7.87 W). Within this cluster, the relative risk (RR) for a herd to have a positive sample for this strain was 7.8 times higher than expected ($p < 0.001$) (cluster 1). About the second, 3 out of the 10 cases appeared in an area of 6.1 km in the north-west (centroid: 43.05 N, 8.81 W). The RR was 8.3 ($p = 0.041$) (cluster 2). In regard to the last strain, 4 out of the 5 cases were found in an area of 11.5 km in the north-east (centroid: 43.31, 7.35 W) (RR = 31.3; $p = 0.011$) (cluster 3).

Discussion

The genetic analysis identified up to five different BVDV types in Galicia during the period 2015-2017. The most prevalent was 1b, and to a lesser extent 1d and 1e. Spatial clustering was detected for three BVDV strains.

This is the largest BVDV typing study ever reported for Spain and was carried out in the main dairy area of the country and the 9th in the European Union (Eurostat, 2018). One possible limitation of the study was that only one region of the viral genome was amplified. Several previous studies on BVDV typing, as the present paper, used the primers 324 and 326 (Vilček *et al.*, 1994) to obtain a 288-bp DNA product from the 5'UTR of the viral genome. Others amplified the genomic region encoding Npro protein, or sometimes part of the 5'UTR with Npro, C, and part of Erns proteins. In addition, some studies have explored the use of two or even more regions (Booth *et al.*, 2013; Diéguez *et al.*, 2017). A previous paper analyzed a selection of 30 representative viruses in the Npro region that had been previously analyzed in the 5'UTR; all viruses were clustered in the same phylogenetic branches as for the tree based on the 5´UTR, with similar bootstrap values (Vilček *et al.*, 2001). However, another previous paper indicated that ideally, at least two regions of the BVDV genome should be analyzed and agreement sought between them, in order to increase accuracy (Booth *et al.*, 2013).

Data on BVDV typing in Galicia correspond with studies published in several European countries, including Germany, France or Italy indicating that BVDV-1b is the most frequent type (Tajima *et al.*, 2001; Jackova *et al.*, 2008; Luzzago *et al.*, 2012). In neighboring Portugal, most BVDV strains, primarily in cattle, belong to BVDV-1b, with types 1a, 1d, 1e, 2a and 2b also present (Barros *et al.*, 2006).

In Galicia, a similar study carried out between 2013 and 2015 over 87 isolates, indicated that 73 strains (84.9%) were typed as BVDV-1b, 6 (6.9%) as BVDV-1d and 2 (2.3%) as BVDV-1e. One strain each was typed as belonging to 1a, 1h, 1k and 1l. The BVDV-2 species has also been found, in this case the type 2a (Factor *et al.*, 2016).

In the present study − carried out in the same region during a later period − types 1b and 1d were found in percentage similar to that observed by Factor *et al.* (2016), while type 1e was found in greater proportion.

Table 1. Number (percentage) of isolates from each type found in the 140 farms sampled in Galicia (NW Spain) for each year of the period 2015-2017.

Species	Types	2015	2016	2017	Total
BVDV-1	1b	23	67	34	124 (88.6%)
	1d	1	4	2	7 (5.0%)
	1e	0	3	3	6 (4.3%)
	1f	0	1	1	2 (1.4%)
	1p	0	1	0	1 (0.7%)
BVDV-2		0	0	0	
	Total	24 (17.1%)	76 (54.3%)	40 (28.6%)	140 (100%)

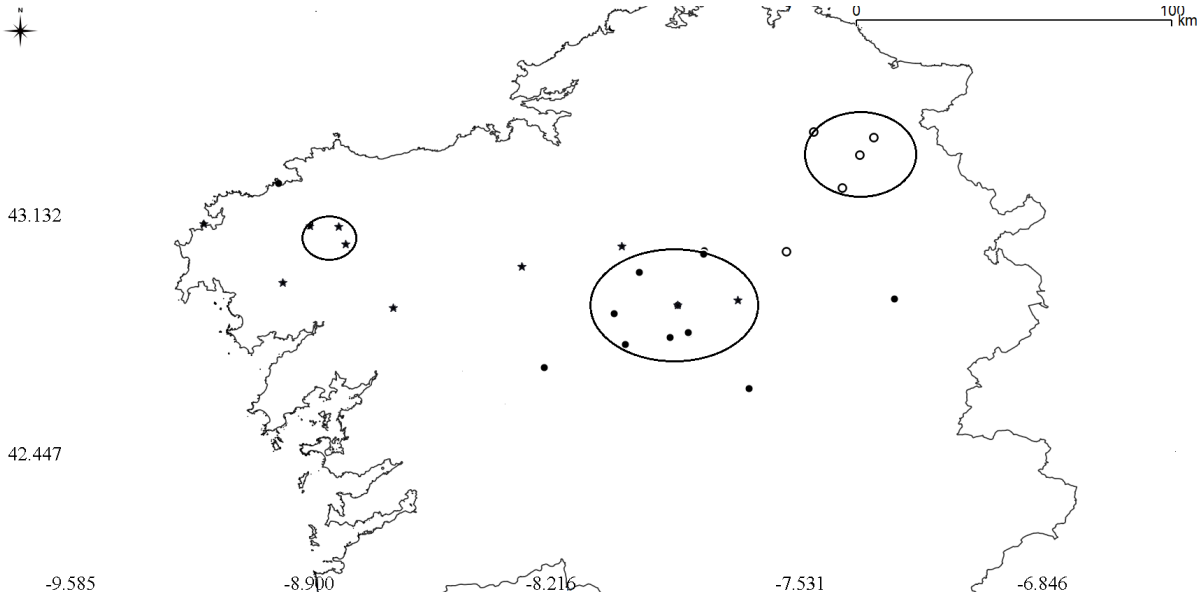

Figure 1. Statistically significant spatial clusters of three BVDV-1b strains (whose cases are represented by circles, solid circles and stars, respectively) in cattle farms from Galicia (NW Spain), based on sampling carried out between 2015 and 2017.

In addition, type 1f was also found on two farms, and, type 1p on one farm. Other types (*i.e.* 1a, 1h, 1k, 1l) and BVDV-2 have not been found yet.

The target population in both studies was enrolled in ADSGs and BVDV circulation was active. Nowadays, 55.2% of the herds and 65.0% of Galician bovine census are involved in ADSGs in Galicia (Arnaiz *et al.*, 2018) and subsequently subjected to BVDV control programs. ADGSs are usually circumscribed to individual councils. However, there are cases in which two or more ADSGs at adjacent councils operate as a unique body. Although Factor *et al.* (2016) included samples from 43 councils and the present paper provides samples from 65 councils (32 of which are common to both studies), the studied farms were exclusive to each study.

In the same region and outbreak of abortions within a sheep herd caused by a BVDV-2d strain was also previously described (Eiras *et al.*, 2017).

Existence of spatial clustering should be reflective of area spread under certain conditions. It was observed that herds in cluster 1 had an intense animal trade between them. In addition, some of these herds shared common animal suppliers that were located in a neighboring region. Evaluating the prevalence of infection in purchased cattle is one important way to reduce the risk of introducing disease on the farm (Amelung *et al.*, 2018; Bezerra *et al.*, 2019). In Galicia, for farms in ADSG is compulsory to test all purchased animals against BVDV (using an established protocol that includes antibody and antigen tests) (DOG, 2017). However, these tests were often performed once the new animals have already been incorporated into the herd.

Data from the REMO also indicated that all farmers from cluster 3 went to the same cattle market. Participation in cattle fairs or markets is uncommon in Galicia; only 2.1% of the herds reported to perform this type of movement (Moya *et al.*, 2018). Remarkably, the four farms within this cluster took cattle to a market located in the vicinity, with the possibility of returning animals to the farm.

No specific connections could be established for herds in cluster 2, but, for the three spatial clusters, the communal veterinary service provider and the concurrence of certain visitors implied certain connection among the farms. Besides, common feed-mixed vehicles were regularly shared among several of the farms. BVDV was capable of surviving in various materials used in livestock production that can act as fomites. There is very little information available regarding the contamination rates of personnel, vehicles, and equipment after visiting BVDV positive farms. However, survival of these viruses even for a short period outside the host suggest that fomites are a potential source of transmission that need further attention (Evans *et al*., 2019).

In conclusion, this report indicates that BVDV-1b is the predominant species, as in other European countries and identifies up to ten different BVDV types in the region. Additionally, spatial analysis emphasizes the importance of biosecurity measures to help prevent

herds becoming infected though purchase of cattle or after the reintroduction of animals from cattle concentration points (such as fairs or markets).

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