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Genetic diversity of bovine viral diarrhea viruses from the Galicia region of Spain

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ABSTRACT

This study examined the frequency and diversity of bovine viral diarrhoea viruses (BVDVs) infecting cattle in Galicia (northwestern Spain). A total of 86 BVDV strains were typed in samples of serum from 79 persistently infected animals and 3 viraemic animals and of abomasal fluid from 4 fetuses. Samples came from 73 farms participating in a voluntary BVDV control programme. Typing was based on a 288-bp sequence from the 5′ untranslated region amplified using primers 324 and 326. Of the 86 strains, 85 (98.8 per cent) belonged to species BVDV-1 and 1 (1.2 per cent) belonged to BVDV-2; 73 strains (84.9 per cent) were typed as BVDV-1b, 2 as BVDV-1e and 6 as BVDV-1d. One strain each was typed as belonging to 1a, 1h, 1k and 1l. The sole BVDV-2 strain was classified as 2a. These results identify BVDV-1b as the predominant species, and they indicate the presence of viral types not previously described anywhere in Spain. This is also the first report of BVDV-2 in Galicia and only the second report of BVDV-2 in Spain.

INTRODUCTION

Bovine viral diarrhea virus (BVDV), a member of the genus *Pestivirus* in the family *Flaviviridae*, causes great economic losses because of reduced milk production, increased mortality of young animals and reproductive, respiratory and intestinal problems. Infected animals are also more susceptible to other diseases (Greiser-Wilke and others 2003, Houe 2003, Diéguez and others 2009).

The virus contains a single–stranded RNA genome (approximately 12.3 kb) with one open reading frame (ORF) of about 4,000 codons (Tautz and others 1997), which encompasses most of the genome. This ORF is flanked on either side by 5′ and 3′ untranslated regions (UTRs). Genetic and antigenic typing of BVDV isolates has led to the definition of two species, BVDV-1 and BVDV-2. Both cause acute and persistent infections and show a similar profile of disease manifestations, except that certain BVDV-2 strains have been associated with severe hemorrhagic syndrome and high mortality (Carman and others 1998, Gethmann and others 2015).

To date at least 21 genetic types of BVDV-1 (BVDV-1a to BVDV-1u) and 4 types of BVDV-2 (BVDV-2a to BVDV-2d) have been described (Alpay and Yeşilbağ 2015; Deng and others 2015; Giammarioli and others 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). Viruses within the same pestiviral type show similar sequence variations in the 5′ UTR, making these variations useful for PCR-based classification of *Pestivirus* into species and types (Sandvik and others 1997).

Molecular epidemiology studies of BVDV 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. Cattle trading is increasing BVDV genetic diversity in most European countries (Jackova and others 2008, Luzzago and others 2012, Booth and others 2013), and correlating animal movements with phylogenetic analyses can help identify potential sources and routes of infection (Booth and others 2013). Such analyses can also help identify and track highly virulent strains. This is particularly important in the case of BVDV: since BVDV-1 and BVDV-2 species are sufficiently different at the antigenic level, a vaccine that protects against one may not protect against the other (Ridpath and others 1994). For this reason, Ridpath and others (2011) have even proposed using a BVDV vaccine that contains

antigens from all types circulating in the target region.

The aim of this study was to investigate the frequency and diversity of BVDV infecting cattle in Galicia (NW Spain) in order to support the design of optimized control programs.

MATERIALS AND METHODS

Study area and animal survey

This study was carried out between 2013 and 2015 in the region of Galicia in northwestern Spain. Galicia is the major dairy cattle-farming region of the country, accounting for 40% of national milk production. Galicia was the first region in Spain to establish a voluntary BVDV control program, which began in 2004 and remains active. Farms wishing to join this program are required to undertake additional BVDV serological testing on all cattle older than 1 year, in addition to the compulsory annual testing for tuberculosis, brucellosis and enzootic bovine leucosis. For those farms participating in the voluntary BVDV control scheme, serum samples are analzsed with a commercial BVDV ELISA, which detects antibodies against the p80 protein (ELISA BVD/BVD-MD p80 Ab, Institut Pourquier, Montpellier, France). The same ELISA is used to monitor herds through semi-annual testing of bulk tank milk and testing of sera from selected heifers older than 9 months.

When results indicate the possible presence of persistent infection, serum or ear-notch samples from individual animals are tested using antigen-capture ELISA that detects the BVDV E^{rns} protein (Antigen serum plus BVD test kit, IDEXX Europe, Hoofddorp, The Netherlands). Two samples positive for BVDV from the same animal taken 3–4 weeks apart are considered to confirm persistent infection.

For the study, 86 BVDV strains were typed in serum samples from 79 persistently infected (PI) animals and 3 viraemic animals, and in abomasal fluid from 4 fetuses. The samples were gathered during the period 2013–2015 from 73 dairy farms taking part in the voluntary BVDV control program.

Sample analysis

RNA was isolated from 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 and others 1994). Amplified DNA fragments were purified by ExoSAP-IT treatment (USB Corporation, OH, USA) and sequenced at the Sequencing and Fragment Analysis Unit of Santiago de Compostela University using a 3730xl genetic analyzer (Applied Biosystems, CA, USA).

Phylogenetic analysis

Sequences were converted to FASTA format using Chromas Lite 2.1.1 and imported into MEGA 6. Phylogenetic trees were constructed using the neighbour-joining method and validated using bootstrap analysis with 1000 replicates. Evolutionary distances were estimated using the Kimura two-parameter method.

RESULTS

86 BVDV strains were typed, of which 85 (98.8%) belonged to species BVDV-1 and 1 (1.2%) to BVDV-2. Of the 86 strains, 73 (84.9%) were typed as BVDV-1b based on 5′ UTR analysis and comparison with reference. These 73 viral isolates were obtained from 68 PI animals, 1 viraemic animal and the 4 fetuses. The virus in two samples from PI animals was identified as BVDV-1e, while the viruses in six other isolates from PI animals were most closely related to BVDV-1d. One isolate each from PI animals was assigned to types 1a and 1h, while one isolate each from viraemic animals was assigned to types 1k and 1l strains (see online supplementary Table S1).

The only sample of BVDV-2, classified as 2a, came from a PI cow that was 31 months old when diagnosed and that had already calved once. Analysis of the calf (male) was impossible because it was slaughtered soon after birth. The PI cow had been born on the same farm where she was sampled, while her mother had been purchased from another farm in Galicia. The mother was seropositive and virus negative (by antigen ELISA), so it is unlikely that she had persistent infection. Thrombocytopenia, hemorrhage or other clinical signs attributed to highly virulent strains of BVDV-2 were not observed.

In 61 of 73 herds, BVDV was detected in only one animal; in 10 herds, virus was detected in two animals; and on the remaining 2 farms, it was detected in three animals. Multiple strains from the same farm were always identical.

A simplified phylogenetic tree (including one strain per observed species and types) obtained from sequence analysis is shown in Fig 1. The complete tree is provided as online supplementary Fig S1. Reference strains are presented with their GenBank accession numbers.

The 86 sequences from the present paper have been deposited in GenBank under accession numbers KU310543 and KU351559-KU351643.

DISCUSSION

To the authors' our knowledge, this is the largest BVDV typing study ever reported for Spain. Our results of the present study identified eight BVDV types in Galicia, including one BVDV-2a strain. Understanding this diversity will be important for improving clinical diagnosis and vaccination programs (Hamers and others 2001).

The most prevalent BVDV type in the study area was 1b, consistent with previous reports of BVDV isolates

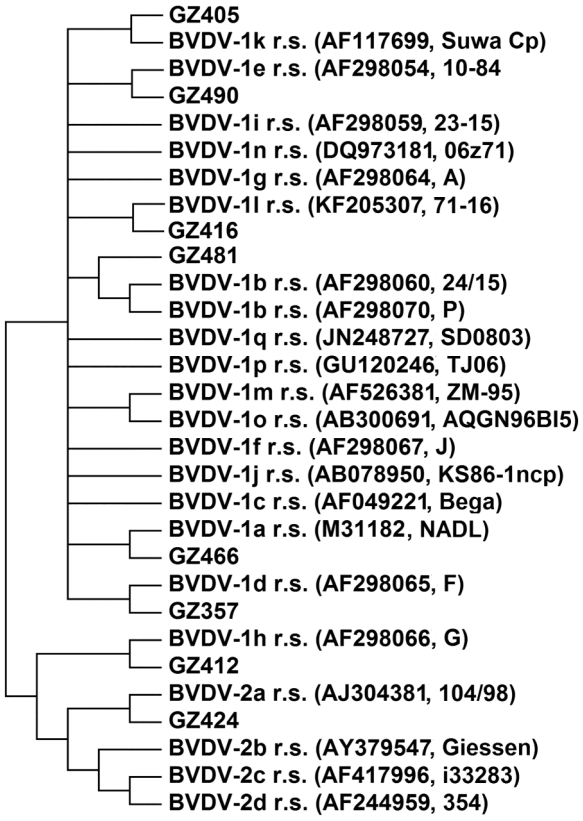

FIG 1: Simplified phylogenetic analysis of 5′ untranslated region sequences of bovine viral diarrhoea virus isolates

from Galicia. Diéguez and others (2008) analyzed 15 viral isolates from PI animals in Galicia using the same primers 324 and 326 as in the present study. They found that 9 isolates (60%) belonged to BVDV-1b, 3 to type 1d, 2 to type 1f and 1 to type 1a. Arias and others (2003) determined the N^{pro} gene sequence of 24 BVDV isolates from Galicia and the border regions of Asturias and Castilla-León. They found that 21 isolates belonged to type 1b, in addition to two BVDV-1c isolates and one BVDV-1h isolate. Hurtado and others (2003) analyzed 41 BVDV strains from northern Spain, of which 35 were 1b and 6 were 1e. Thus the present study identified several BVDV types not previously detected in Galicia or surrounding regions, suggesting that the virus may show greater genetic variability than previously suspected, at least in this part of Spain.

The present results are consistent with studies in several European countries, including Germany, France and Italy, in which the most prevalent BVDV types were reported to be 1b, 1d and 1e (Tajima and others 2001, Jankova and others 2008, Luzzago and others 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 and others 2006). In the Portuguese study, all BVDV-2a or 2b strains came from animals showing clinical signs of hemorrhagic disease.

The present paper is the first report of BVDV-2 in Galicia and the second report of BVDV-2 in Spain, following the detection of BVDV-2a in two samples from northern Spain outside Galicia (Aduriz and others 2015). The present BVDV-2 isolate and the two from the previous Spanish study show 100% identity to strain 104/98 detected in Germany (Tajima and others 2001). In the present study and the previous two (Tajima and others 2001, Aduriz and others 2015), the presence of this BVDV-2 strain did not correlate with severe clinical signs. BVDV-2 not only encompasses not only strains of high virulence but also those of moderate and low virulence (Ridpath and others 2000).

In any case, vigilance against spread of virulent BVDV-2 types in Europe remains important. In 2012 and 2013, Germany and the Netherlands reported outbreaks of hemorrhagic syndrome with high mortality in calves and cows caused by an unusually virulent BVDV-2c strain (Gethmann and others 2015), which may have caused disease recurrence in 2014.

BVDV is usually introduced into herds via incorporation of infected animals or contact with infected animals in another herd (Lindberg and Alenius 1999). The dam of the PI animal from which we isolated BVDV-2a was 8 days old when she arrived at the farm, and no other animals were brought onto the farm until her calf was diagnosed with persistent infection. Other 4 nearby farms (with high within herd prevalance but none vaccinated against BVDV) imported cattle from France and the Netherlands (including a short stay in a cattle trading farm, already in Spain) during the period in which the infection occurred, which may be the likely source of the BVDV-2a in our study. Contact with animals from these neighboring farms through pasture fences was possible.

Nevertheless, on these herds only dry cows and heifers came out to pasture for a few hours during the day; besides the meadows were annexed to the farms so the risk of contact with potentially carrier wildlife would be low. Moreover, these farms had no contact with other domestic species and their animals never went to cattle concentration points.

Contaminated semen and embryos were another two possible sources of the virus (Lindberg 2003). However, in the area where BVDV-2 was found, the semen which was used came in every case from BVDV-free artificial insemination facilities and none of the farms performed embryo transfer, so these routes are rather unlikely.

The need for vigilance against BVDV, particularly BVDV-2, highlights one drawback of the BVDV control program in Galicia: it is voluntary. As a result, it covers only 60% of cows and 40% of herds in the region. Farms not in the program frequently do not test purchased animals; with their BVDV status unknown, these animals pose a risk to the rest of their herd and to neighboring herds as well.

This report, the first study of BVDV genetic diversity in Galicia, indicates that BVDV-1b is the predominant species, as in other European countries. This report also identifies several BVDV types not yet described anywhere

in Spain, and it shows for the first time that BVDV-2 is present in Galicia. In fact, this potentially more virulent virus is likely to have been present in Galicia for at least 36 months, based on the age of the animal in which we identified the BVDV-2a strain (31 months) and the fact that the infection had to occur in the early months of pregnancy. We speculate that this strain entered the study area when infected animals were imported from other countries onto farms near those in our study area. Those farms lacked sanitary programs and did not test the incorporations.

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Data sharing statement Sequences presented in the paper are available on GenBank. GenBank accession numbers are available in the paper. Data sheet available at the corresponding author's request.

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