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High frequency of bovine viral diarrhea virus type 2 in Southern Brazil

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ABSTRACT

Ruminant pestiviruses can infect cattle populations worldwide and cause significant economic losses due to their impact on productivity and health. Knowledge of pestivirus diversity is important for control programs and vaccine development and for determining probable sources of infection. In this paper, we describe a search for ruminant pestiviruses with RT-PCR in sera of 9078 calves from 6 to 12 months of age. The calves were first analyzed in pools and then analyzed individually. Thirty-three RT-PCR positive animals were detected (0.36%) from 6.9% (24) of the 346 herds. The sequencing analysis of the 5′ non-coding region and N terminal autoprotease showed the presence of BVDV-1a (15 isolates), -1b (3), -1d (1) and -2b (14), with a higher frequency (42.4%) of BVDV-2 in comparison with other countries. The presence of sheep was significantly associated with BVDV infection. Our results also suggested that a BVDV control program based only on the investigation of cattle would not be successful, especially in regions with farms harboring multiple animal species. This study may also serve as a reference for future control programs in Southern Brazil because it reports the prevalence of cattle with active infections and the genetic background of the circulating strains.

1. Introduction

The genus *Pestivirus* of the family *Flaviviridae* consists of four recognized species: *Bovine viral diarrhea virus 1* (BVDV-1), BVDV-2, *Border disease virus* (BDV) and *Classical swine fever virus* (CSFV) (Simmonds et al., 2011). Moreover, an atypical group of pestiviruses, initially detected in fetal calf serum and putatively named 'HoBi'-like virus (Schirrmeier et al., 2004) has been associated with clinical disease in cattle (Decaro et al., 2011; Weber et al., 2014).

Ruminant pestiviruses (BVDV-1, BVDV-2, BDV and 'HoBi'-like viruses) can cause acute or persistent infections in cattle (MacLachlan and Dubovi, 2011; Weber et al., 2014). Acute infections are generally not apparent; when they are symptomatic, the principal clinical manifestations are mild diarrhea, fever and respiratory signs that are terminated by a vigorous immune response. Persistent infection occurs due to the ability of the virus to cross the placenta and infect non-immunocompetent fetuses, thus generating persistently infected (PI) calves that show retarded growth and excrete variable amounts of virus throughout their lives, spreading the infection in the herd. PI calves usually die during the first two years of life from mucosal disease or due to other diseases, most likely as a consequence of virus-induced immune depression (Baker, 1995; MacLachlan and Dubovi, 2011).

Pestiviruses have a single-stranded positive-sense RNA genome that contains one open reading frame, flanked by non-coding regions (NCR) at the 5′ and 3′ ends, that encodes a polyprotein that is processed into 12 polypeptides (Simmonds et al., 2011). The 5′NCR and N terminal autoprotease (N^{pro}) are widely used to characterize the genus, species and subtypes of new strains using phylogenetic approaches (Vilcek et al., 2001; Mahony et al., 2005; Pizarro-Lucero et al., 2006; Xue et al., 2010; Deng et al., 2012; Strong et al., 2013). In addition, they can be used to divide BVDV-1 into at least 17 subtypes (1a through 1q) (Vilcek et al., 2001; Stalder et al., 2005; Deng et al., 2012) and BVDV-2 into two subtypes (2a and 2b) (Flores et al., 2002). Knowledge of the circulating genetic variants in the genus *Pestivirus* has significance for establishing correct diagnostic tools and control programs because there are reports of the failure of commonly used detection techniques at the species level (Schirrmeier et al., 2004; Weber et al., 2014) and because significant antigenic changes at the species and subtype levels have been

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shown by cross-neutralization (Pizarro-Lucero et al., 2006; Ridpath et al., 2010).

A few studies have investigated BVDV risk factors (Valle et al., 1999; Presi et al., 2011; Machado et al., 2014). The principal risk factors detected to date are related to biosecurity measures (Humphry et al., 2012), reproduction management (Houe, 1999; Humphry et al., 2012), herd size (Presi et al., 2011), animal introduction (Houe, 1999; Valle et al., 1999; Presi et al., 2011), direct contact with other animals (from the same species or not) (Lindberg and Alenius, 1999; Valle et al., 1999), communal grazing (Valle et al., 1999; Presi et al., 2011), or the age of the animals (Presi et al., 2011).

The analysis of pooled sera allows the simultaneous testing of a large number of samples and has been proposed as a rapid and cost-efficient approach for the detection of pestivirus in cattle (Weinstock et al., 2001; Hanon et al., 2012). Thus, the goal of the present study was to investigate the herd prevalence of active infections by pestivirus in calves up to one year old from farms located in regions with high livestock movement in Southern Brazil, to determine the genetic diversity of the current viruses and to assess the risk factors for infection based on RT-PCR results at the herd level.

2. Materials and methods

2.1. Study area, target population and sample size

Located in Brazil's Southern region, the state of Rio Grande do Sul (RS) has an area of 268,781.896 km^2 (3.16% of the country) and is bordered by Argentina and Uruguay. It is divided into seven regions. The regions are subdivisions of Brazilian states and group together various counties by proximity and according to common agro-ecological characteristics (Fig. 1). According to official data from the State Veterinary Office (SEAPA-RS), the State has more than 13 million cattle distributed on approximately 346 thousand farms. The majority of the bovine population consists of females whose age is greater than 36 months. The predominant activity of the farms extends from breeding to fattening. Beef cattle are predominant, although these characteristics change depending on the region of the State. Statistics on the number of cattle per property show that 88% of the farms have up to 50 bovines and that approximately 1% of the properties have more than 500 animals.

The sera analyzed in this study were collected for the biannual surveillance study performed in Brazil in 2010 to demonstrate the absence of *Foot-and-mouth disease virus* (FMDV) circulation. The target population of the present study included all bovine farms that harbor FMDV-susceptible species enrolled in the national FMDV control and eradication program (http://www.agricultura.gov.br/arq_editor/file/Serological_Monitoring_efficiency_vaccination_FMDfreezone_2010_final_report_2.pdf). The units addressed by sampling were bovines from six to 12 months of age grouped on farms, which were considered the primary units for sampling purposes. The population from which the sample was randomly drawn comprised farms located in counties with high cattle movement according to State Veterinary Office data on livestock movement. To define the sampling process, 93 counties were identified. A frame list containing the farm's identification was provided and a representative sample obtained from each of the identified counties. Subsequently, 346 farms and 9078 cattle aged from 6 to 12 months were randomly sampled.

The sample size needed to detect the disease was calculated. This procedure was performed by the Ministry of Agriculture, Livestock and Food Supply-Epidemiology Division using FreeCalc version 2 (http://www.ausvet.com.au/content.php?page=software#freecalc), recommended by OIE. The methodology applied was that recommended by Cannon and Roe (Cannon and Roe, 1982) and Martin and others (Martin et al., 1992). The statistical and epidemiological parameters applied to determine the sample size were as follows: confidence level, 95%; minimal prevalence detected on affected farms, 1%; and minimal prevalence detected in affected animals on each farm, 10%.

Table 1
Nucleotide sequence of the primers used for RT-PCR.

Primer	Sequence (5′–3′)	Position
PanPesti F	GAG ATG CYA YGT GGA CGA GGG C	227–248[a]
PanPesti R	GYC TCT GCS RCA CCC TAT CAG G	345–324[a]
LV Pesti F	CTG TAC ATG GCA CAT GGA GTT G	373–394[b]
LV Pesti R	AAT CTG TTG TAT ACC CAT TT	861–842[b]

[a] Position in BVDV-1 strain NADL (GenBank accession number: NC_001461.1).
[b] Position in BVDV-2 strain 890 (GenBank accession number: U18059.1).

2.2. Primer design and validation of the RT-PCR

For primer design, 1412 pestivirus sequences of 5′-NCR in GenBank (http://www.ncbi.nlm.nih.gov/genbank/) with sizes ranging between 118 and 424 nucleotides were selected. The sequence alignment was performed using Muscle version 3.8.31, and Genedoc software version 2.7.001 (http://www.genedoc.us) was used to identify the most conserved regions. The primers resulted in an amplification product of 118 bp (Table 1).

The selected primers were used to test representative samples of pestivirus: BVDV-1 (NADL, Singer, Oregon C24V and Osloss), BVDV-2 (Soldan and SV260) BDV (137/4 and BD Weybridge) and 'HoBi'-like viruses (LV01/12, LV02/12, LV03/12 and LV04/12). Furthermore, the bovine herpesvirus type 1 (BoHV-1) strain Los Angeles and BoHV-5 strain EVI88 were tested to assess specificity.

The detection limit was calculated in triplicate by spiking a negative serum with 10-fold dilutions of BVDV-1 strain NADL ($10^{6.8}$ $TCID_{50}$/mL) to reach a dilution of 10^{-8}.

The validation of the test (sensitivity, specificity, predictive values and accuracy) was assessed by comparing the results from the set of primers used in the present study with the results from the classical pair of primers 324 and 326 (Vilcek et al., 1994).

2.3. Sample preparation, RNA isolation and RT-PCR

The blood collected was centrifuged at 2000 × *g* for 10 min, and the sera were stored at −80 °C prior to analysis. To obtain the pools, equal volumes of the 9078 individual sera (30 μL) were mixed in 227 pools of up to 44 samples. The positive pools resulting from this stage of the analysis were further analyzed individually.

Viral RNA was isolated from 250 μL of sample using TRIzol® LS Reagent (Life Technologies, Carlsbad, CA, USA) and was suspended in 50 μL of ultrapure water according to the manufacturer's instructions. The cDNA was synthesized with SuperScript® III Reverse Transcriptase Kit (Life Technologies) using the reverse primer in a total volume of 20 μL following the manufacturer's recommendations.

The amplification of cDNA by PCR was conducted in a total volume of 25 μL containing 1× PCR buffer, 1 mM of $MgCl_2$, 0.5 mM of dNTP mix, 0.24 mM of PanPesti F and PanPesti R and 1 unit of Platinum® Taq DNA Polymerase (Life Technologies). Reactions were performed in a Veriti 60-well Thermal Cycler (Applied Biosystems, Foster City, USA) under the following conditions: 3 min at 95 °C, followed by 35 cycles of 45 s at 95 °C, 45 s at 60 °C and 45 s at 72 °C, with a final extension at 72 °C for 7 min.

2.4. Sequencing and phylogenetic analysis

The amplification of partial sequences of 5′-NCR for sequencing was performed using the primers 324 and 326 (position in BVDV-1