Genetic identification of pestiviruses from beef cattle in Southern Brazil

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Abstract

Bovine pestiviruses, e.g., bovine viral diarrhea virus types 1 (BVDV-1 or *Pestivirus A*), BVDV-2 (*Pestivirus B*), and HoBi-like pestiviruses (HoBiPeV or *Pestivirus H*), have been shown to circulate in Brazilian cattle in varied proportions. In this study, we identified genetically pestiviruses circulating in beef cattle in Rio Grande do Sul, the southern most Brazilian state. Screening of serum of 15,584 beef calves destined to be export by an antigen capture ELISA and, subsequently, by reverse-transcription polymerase chain reaction (RT-PCR), revealed 135 containing pestivirus RNA. Genetic typing of these viruses based on nucleotide sequencing and phylogenetic analysis of the 5' untranslated region (5' UTR) of the viral genome allowed for the identification of 90 different viruses, being 38 BVDV-1 (42.2%), 31 BVDV-2 (34.4%), and 21 HoBiPeV (23.4%). Among BVDV-1, only subtypes BVDV-1a ($n = 28$, 31.1%) and BVDV-1b ($n = 10$, 11.1%) were identified. All 31 BVDV-2 isolates belonged to BVDV-2b subtype and the 21 HoBiPeV viruses clustered to subgroup 3a. Thus, this study provides an approximate genetic profile of pestiviruses circulating in beef cattle in a traditional Brazilian beef cattle-raising state.

Keywords Beef cattle · Pestivirus · Phylogeny

Introduction

Pestiviruses comprise a genus within the family *Flaviviridae*; they are small (40–50 nm), enveloped, single-stranded positive-sense RNA viruses [1]. Bovine pestiviruses include the prototype species, bovine viral diarrhea virus 1 (BVDV-1) and 2 (BVDV-2), and a third species classified as HoBi-like pestiviruses (HoBiPeV) [1, 2]. BVDV-1, BVDV-2, and HoBi-like viruses have also been classified as *Pestivirus A*, *B*, and *H*, respectively [3, 4]. The pestivirus genome is a 12.3-kb RNA molecule that contains a long open reading frame (ORF) flanked by two untranslated regions, 5' and 3' UTRs. The ORF encodes a long polyprotein that is cleaved by viral and host proteases into 11–12 mature viral polypeptides: N^{pro},

C, E^{ms}, E1, E2, p7, NS2-3 (NS2, NS3), NS4A, NS4B, NS5A, and NS5B [5].

Pestivirus species are determined by criteria such as comparisons of the complete nucleotide (nt) sequences, cross-neutralization titer assays, and the strain's host of origin [1, 4, 6]. Nucleotide sequencing and comparison of the highly conserved 5' UTR, N^{pro}, and E2 have also served for the classification and phylogenetic analysis of *Pestivirus* into subtypes or subgenotypes [6–11]. At least 22 subgenotypes (1a–1u) have been identified for BVDV-1, whereas four have been described for BVDV-2 (2a–2d). Considering only published sequences, the number of isolates described for BVDV-1 is much higher than those described for BVDV-2 (88.2% versus 11.8%); yet the relative proportions for each virus vary largely depending on the country or region of origin [11].

BVDV-1 and BVDV-2 are among the most important globally distributed cattle pathogens. These viruses have been associated with a variety of clinical manifestations in their hosts [12]. HoBiPeV were initially identified as contaminants of fetal bovine serum (FBS) of Brazilian origin [13] and were subsequently linked to several livestock diseases in South America [14, 15], Italy [16–18], and Asian countries [19–21]. BVDV-1 and BVDV-2 subgenotypes have been shown to circulate in Brazilian cattle in varied proportions,

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yet most genetic studies have been based on a single or few isolates or have included samples scattered over time and from different geographical origins or clinical backgrounds [7, 14, 22–28]. The first evidence of circulation of HoBiPeV in Brazil dates from around 2002 and a number of subsequent studies indicate that these viruses are endemic among Brazilian cattle [7, 14, 15, 24, 25, 27, 29–31].

In this study, we identified genetically 90 bovine pestivirus genomes detected in the sera of beef calves that were destined to be exported from Rio Grande do Sul (RS), the southern most Brazilian state. Pestivirus-containing sera were detected by antigen capture ELISA and then submitted for RT-PCR for nucleotide sequencing of the amplicons. In contrast with most studies performed to date that have examined few samples from different origins and scattered over time, this study provides an approximate profile of bovine pestivirus species and subtypes circulating in beef cattle of Rio Grande do Sul, Brazil.

Material and methods

A total of 15,584 samples of beef cattle sera were taken from calves destined for export. These samples were originated from over 100 herds in approximately 30 municipalities in Southern RS state, a traditional beef cattle-raising region. Serum samples were examined by an antigen capture ELISA (Idexx Inc.), and 135 were found to be positive for BVDV antigens. Positive samples for BVDV/pestivirus antigens were then submitted to PCR, and different sets of primers were used to detect BVDV-1, BVDV-2, and HoBiPeV. The amplicons were submitted for nucleotide sequencing and phylogenetic analysis. Amplicons with a 100% nt identity were considered as belonging to the same virus, and only one representative sample was included in the genetic analysis. Under this criterion, 90 different pestivirus genomes were identified.

Aliquots of 150 µL of the 135 ELISA-positive serum samples were submitted for RNA extraction using TRIzol® Reagent (Life Technologies, Carlsbad, CA), followed by complementary DNA (cDNA) synthesis using Super Script III Reverse Transcriptase (Life Technologies, Carlsbad, CA). The resulting cDNAs were used to run three PCR assays, each using different sets of primers targeting the 5' UTR. Information regarding the primers used are presented in Table 1. PCR products were resolved in a 2% agarose gel stained by Gel Red® (Biotium, Hayward, CA, USA) and visualized under UV light after electrophoresis (60 V, 40 min). Total RNA extracted from Madin-Darby bovine kidney (MDBK) cells infected with BVDV-1 Singer (cp), BVDV-2890 (ncp), and HoBiPeV LV04/12 [31] were used as controls. Ultrapure water was used as negative control. Pestivirus-free MDBK cells were grown in minimal essential

medium (MEM) with 10% equine serum and supplemented with antibiotics.

For nucleotide sequencing, PCR products were purified using PureLink® Quick Gel Extraction and PCR Purification Combo Kit (Life Technologies, Carlsbad, CA). Positive samples were sequenced in duplicate using the BigDye kit. Results were analyzed by the Staden program [32] to obtain a consensus sequence from each duplicate. The alignment of sequences with those from GenBank was performed using the BioEdit Sequence Alignment Editor Software suite, version 7.0.5.3 (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>). Phylogenetic analysis was conducted by the Molecular Evolutionary Genetics Analysis (MEGA) software 6.0 [33], using the neighbor-joining method, and the evolutionary distances were computed using the Kimura 2-parameter model. The *bootstrap* values were calculated using 1000 replicates.

Results

As previously stated, among the 135 positive sera detected by the BVDV antigen capture ELISA, 90 different viral genomes were identified by nucleotide sequencing and analysis of the 5' UTR amplicons. From these, we identified 38 different genomes of BVDV-1 (42.2%), 31 of BVDV-2 (34.4%), and 21 of HoBiPeV (23.4%) (Table 1). Among BVDV-1, only subgenotypes BVDV-1a ($n = 28$, 31.1%) and BVDV-1b ($n = 10$, 11.1%) were found, while all 31 BVDV-2 isolates belonged to the subgenotype BVDV-2b and the 21 HoBiPeV clustered to subgroup 3a. Forty-five genomes turned out to be duplicate/triplicates of the same virus/genomes and were not included in the analysis.

The nucleotide identity among the identified BVDV-1a genomes and between them and GenBank sequences ranged from 83.4 to 99.3%. The nucleotide identity among BVDV-1b genomes was from 92.1 to 96.9%, and it ranged from 92.7 to 98.1% when compared to GenBank sequences. BVDV-2b strains shared an 89.9%–99.2% identity among them and an 88.4 to 99.2% identity with deposited BVDV-2b sequences. Finally, HoBiPeV nucleotide identity varied from 82.4 to 98.2%, including the identified viruses and GenBank sequences.

Phylogenetic analysis of the 38 5' UTR sequences identified as BVDV-1 showed that they belong to two major genetic groups, subgenotypes BVDV-1a and BVDV-1b (Fig. 1). These sequences were grouped together with other BVDV-1a and BVDV-1b strains from Brazil and other parts of the world and were separated from BVDV-1c, BVDV-1d, BVDV-1e, and BVDV-1i subgenotypes, which have also been previously isolated in Brazil [27, 34]. Attempts to further group these 38 sequences into additional BVDV-1 subgroups/subgenotypes by phylogenetic analysis based on N^{pro}

Table 1 Primers used for 5' UTR amplification, sequencing, and phylogenetic analysis of pestiviruses in beef calves in Southern Brazil

Primer	Position (nt)	Target (bp)	Sequence (5'-3')	Identified subgenotypes (n)
HCV90-368 [9]	107–389 ^a	283	F-CATGCCCATAGTAGGAC R-CCATGTGCCATGTACAG	BVDV-1a (28) BVDV-1b (10)
BVDV-2#3 [24]	136–358 ^b	223	F-GCGGTAGCAGTGAGTTATTGG R-TTACTAGCGGGATAGCAGGTC	BVDV-2b (31)
N2-R5 [2]	186–335 ^c	150	F-TCGACGCATCAAGGAATGCCT R-TAGCAGGTCTCTGCAACACCCTAT	HoBiPeV subgroup <i>a</i> (21)

^a Position based on BVDV-1 sequence NADL strain (M31182)

^b Position based on BVDV-2 sequence USMARC-60765 strain (KT832818)

^c Position based on HoBiPeV sequence SV478/07 (KY767958)

sequences²⁷ failed, resulting in a grouping similar to that based on 5' UTR sequences (not shown). Thus, only BVDV-1a and BVDV-1b subgroups were detected among the BVDV-1 viruses present in the studied population.

Phylogenetic analysis of BVDV-2 sequences amplified by primers 90-368 [9] and BVDV2#3 [24] grouped them in the BVDV-2b subgenotype (Fig. 2). Given that no other BVDV-2 subgenotype was identified among the viruses circulating in the population tested, our data confirm the predominance—and nearly exclusive—circulation of the BVDV-2b genotype in Brazil [7, 27, 28, 35]. Finally, phylogenetic analysis of all 21 HoBiPeV genomes, amplified by 5' UTR primers N2-R5, grouped them together and in the same genetic group of several previously described Brazilian sequences, subgroup 3a (Fig. 3)[24, 27, 29, 31].

Discussion

The high number of identical viruses identified among the positive samples, 45 out of 135, was likely due, at least in part, to virus transmission among animals during the 21-day quarantine period in which they were housed together for the completion of sanitary exams and requirements for export. During this time, opportunities for virus transmission likely abounded, mainly considering that hundreds of calves from different origins or herds were brought and housed together in collective pens. Additionally, different calves belonging from the same herd or farm would possibly harbor the same virus strain, resulting in duplicated or triplicated sampling of such viruses.

A number of studies have genetically characterized bovine pestiviruses from Brazil. Canal et al. [22] and Gil et al. [36] demonstrated that both BVDV-1a and BVDV-2 were circulating in Southern and Southeastern Brazil by 1998, with a clear predominance of BVDV-1 (83%), mainly the subtypes BVDV-1a and BVDV-1b, over BVDV-2 (17%). Subsequently, phylogenetic analysis demonstrated that Brazilian BVDV-2 isolates clustered to a genetic group distinct from North American isolates, thus representing a

distinct subgenotype, BVDV-2b [37]. Genetic characterization of 19 BVDV isolates from aborted fetuses from different geographic regions and previously associated with varied clinical backgrounds identified 11 isolates as BVDV-1 (from which 8 were BVDV-1a), 6 as BVDV-2b, and 2 as “atypical” pestiviruses examined the 5' UTR of BVDV isolates both from clinical cases and from cattle sera from Southern Brazil and found a high frequency (45%) of BVDV-2 (3 BVDV-2a and 6 BVDV-2b), in addition to BVDV-1a ($n = 7$) and BVDV-1b ($n = 1$). Three isolates classified as “atypical pestiviruses” and subsequently classified as HoBiPeV were also identified. Weber et al. [15] also used the 5' UTR and the N^{pro} to determine the pestivirus strains present in Southern Brazil and found a high frequency of BVDV-2 (42.3%, exclusively BVDV-2b), in addition to a variety of BVDV-1 subtypes, predominantly BVDV-1a but also BVDV-1b and BVDV-1d.

Identification of BVDV-1 subgenotypes BVDV-1a, BVDV-1b, and BVDV-1d in persistently infected (PI) animals of a vaccinated dairy herd from Parana state, Southern Brazil, called attention to vaccine failure. [26] Subsequently, Silveira et al. [31] characterized 85 pestiviruses obtained from 1985 to 2014 from different Brazilian sources, including clinical cases, follicular fluid, PI animals, FBS, and contaminated cell lines. This study revealed the presence of BVDV-1, BVDV-2, and HoBiPeV, in a proportion of 53.9%, 33.7%, and 12.3%, respectively, with BVDV-1a (35.9% of all sequences) and BVDV-2b (31.4%) as the predominant subgenotypes, followed by BVDV-1b (10.1%) and BVDV-1d (6.7%). Most BVDV-1d isolates were obtained from contaminated cell cultures. BVDV-1e (1.1%) and BVDV-2c (2.2%) were also identified; however, the latter was detected as a contaminant of the cell culture, and thus, its geographical origin is uncertain. Weber et al. [15] identified HoBiPeV associated with an outbreak of a gastro enteric condition resembling mucosal disease (MD). Mosena et al. [25, 34] reported the complete genome sequence of a BVDV-1i isolated from a cow in Southern Brazil and the complete genomic sequences of two Brazilian HoBiPeV isolates, respectively. Bianchi et al. [29] described the isolation and genetic identification of 22 strains involved in an unusual outbreak of mucosal disease (MD). These

Figure 1 Phylogenetic tree of 5' UTR sequences amplified of pestivirus detected using primer 90–368. Sequences were analyzed by Neighbor-joining method and the Kimura 2-parameter model. Bootstrap (1000 replicates) values > 50 are indicated at the internal nodes. Brazilian isolates detected in this study are highlighted with symbols (diamond and triangle). The length of each pair of branches represents the distance between sequence pairs and the scale bar represents the percentage of nucleotide differences in the rectangular tree

Fig. 2 Phylogenetic tree of 5' UTR sequences amplified of pestivirus detected using primers 90–368 and BVDV-2#3. Sequences were analyzed by neighbor-joining method and the Kimura 2-parameter model. Bootstrap (1000 replicates) values > 50 are indicated at the internal nodes. Brazilian isolates detected in this study are highlighted with symbols (square). The length of each pair of branches represents the distance between sequence pairs and the scale bar represents the percentage of nucleotide differences in the rectangular tree

isolates or genomes turned out to be the same virus, BVDV-1d. Cortez et al. [30] published the complete genome sequence of a HoBiPeV (strain SV757/15) isolated from a heifer with gastro enteric disease in southeastern Brazil. Genetic characterization of 17 pestivirus genomes detected in pooled cattle sera from two Brazilian northeastern states also revealed the predominance of HoBiPeV. Monteiro et al. [24] analyzed

Fig. 3 Phylogenetic tree of 5' UTR sequences amplified of pestivirus detected using primer N2-R5. Sequences were analyzed by neighbor-joining method and the Kimura 2-parameter model. Bootstrap (1000 replicates) values >50 are indicated at the internal nodes. Brazilian isolates detected in this study are highlighted with symbols (circle). The length of each pair of branches represents the distance between sequence pairs and the scale bar represents the percentage of nucleotide differences in the rectangular tree

73 commercial FBS batches collected in Brazil from 2006 to 2014, in which 39 (53.4%) were positive for pestivirus. Among these, 34 lots (46.6%) contained BVDV-1, with 24 BVDV-1a isolates, four BVDV-1b isolates, and two BVDV-1d isolates. Six lots (8.2%) contained BVDV-2b, and four (5.5%) were contaminated with HoBiPeV.

Altogether, these studies show that BVDV-1, BVDV-2, and HoBiPeV co-circulate in Brazilian cattle with a clear predominance of BVDV-1, followed by BVDV-2 and lastly by HoBiPeV [14, 24, 27–29]. BVDV-1a predominates among BVDV-1 subgenotypes, followed by BVDV-1b and BVDV-1d. BVDV-1c appears at a low frequency, and subgenotypes BVDV-1e and BVDV-1i have been rarely detected [27, 34]. A high prevalence of BVDV-2, in proportions that may reach up to 45%, has also been observed by some studies; most of them belong to the BVDV-2b group, but a few BVDV-2a strains can be found [7, 28]. A BVDV-2c isolate was also found as a contaminant of a cell culture of an uncertain origin²⁷ and BVDV-2a isolates have been rarely identified [7]. To date, there is no description of any BVDV-2d circulation in Brazil. This high BVDV-2 prevalence (mostly BVDV-2b) seems somehow unique to Southern Brazil [7, 28], compared to overall frequencies described elsewhere. Furthermore, our results confirm that there is an important circulation of HoBiPeV among RS cattle, encompassing 23.3% of the detected viruses, higher than previously described [7, 31]. A recent study also suggested that HoBiPeV may even be the predominant bovine pestivirus in some Northeastern Brazilian states [31], yet these findings await confirmation.

Genetic and antigenic characterization of isolates is an ongoing process which has been proven relevant and useful for pestivirus diagnosis and control in Brazil. For instance, the initial identification of BVDV-2 in the country [22, 35, 36] paved the way for the introduction of BVDV-2 strains in current vaccines. The identification of BVDV-2 in Brazil also led to an appraisal and adaptation of molecular and immunodiagnostic tools for pestivirus detection [22, 24, 38]. The identification of HoBiPeV among Brazilian cattle has called attention for the need of an evaluation of the molecular and immunodiagnostic assays for these strains [2, 24, 31, 39]. Whether to include these novel pestiviruses in current BVDV vaccines has also become a subject of debate [39].

Our findings add valuable information about pestivirus epidemiology in Southern Brazil and will certainly contribute to improve both the diagnostic and control techniques against these viruses in the country.

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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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