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First detection and molecular diversity of Brazilian bovine torovirus (BToV) strains from young and adult cattle

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ABSTRACT

Bovine torovirus (BToV) is an established enteric pathogen of cattle, but its occurrence in Brazilian cattle had not been reported until now. This article describes a survey on BToV in Brazil carried out on 80 fecal samples from diarrheic young and adult cattle, using a nested-RT-PCR targeting the nucleocapsid (N) gene. BToV was detected in 6.25% (5/80) of stool samples from three different geographic regions. Sequences analysis showed that Brazilian BToVs have a high degree of identity with European and Japanese BToVs and a lower degree of identity with North American Breda 1 strain. These results show that, albeit its low frequency and the scarce number of research on the field, BToV is still present amongst cattle populations.

1. Introduction

Diarrhea is an important disease affecting cattle worldwide. Bovine torovirus (BToV), a member of the Coronaviridae family, was first detected in the USA during an outbreak of diarrhea in cattle (Woode et al., 1982). Since then, epidemiological studies have demonstrated the presence of BToV in fecal samples from diarrheic animals in several countries (Lamouliatte, et al., 1987; Koopmans et al., 1991; Liebler et al., 1992; Scott et al., 1996; Duckmanton et al., 1998; Pérez et al., 1998; Matiz et al., 2002; Hoet et al., 2003; Haschek et al., 2006; Ito et al., 2007; Park et al., 2008), but there are no reports of BToV detection in cattle in the Southern hemisphere.

In order to assess the occurrence and the molecular diversity of BToVs in Brazilian cattle, a study was conducted with 80 fecal samples from diarrheic young and adult cattle, from beef and dairy herds. Samples were obtained from 15 farms located in three Brazilian states: Mato Grosso, Rio Grande do Sul and São Paulo (Central, Southern and Southeastern regions of Brazil, respectively), between 2001 and 2010. Fecal samples were tested for parasite eggs and oocysts by Sheater's flotation method (Dubey et al., 1990), for rotavirus by polyacrylamide gel electrophoresis (Herring et al., 1982) and for bovine coronavirus by a nested-PCR assay (Brandão et al., 2005) before torovirus examination.

Samples were prepared as 20% suspensions (v/v) using ultrapure water pre-treated with 0.1% diethyl-pyrocarbonate (DEPC water). Suspensions were clarified at 12,000g for 15 min at 4 °C and only the supernatants were used in the assays. Total RNA was extracted from the supernatants using TRIzol Reagent™ (Invitrogen, Carlsbad, CA, USA) as per manufacturer's instructions in a final volume of 25 μL using cycles as described by Ito et al. (2007). Nested-PCR followed these same procedures but using 2.5 μL of first round product and primers P3 and P4 instead. In order to check for cross-contamination, nested-PCR was carried out using a negative control (DEPC water) as a mock sample after every three test samples.

The amplification products were analyzed using 1.5% agarose gel electrophoresis stained with 0.5 g/mL of ethidium bromide. The amplification mix containing primers P1 and P2. Amplification was carried out with Platinum Taq DNA Polymerase™ (Invitrogen, Carlsbad, CA, USA) as per manufacturer’s instructions in a final volume of 25 μL, using cycles as described by Ito et al. (2007). Nested-PCR products were purified by GFX™ PCR DNA and Gel Band Purification Kit (GE Healthcare, Buckinghamshire, UK) and cloned using InStaClone™ PCR Cloning Kit (Fermentas). Two clones of each sample were selected and both strands were sequenced with BigDye 3.1™ and an ABI 3500 automatic DNA sequencer (Applied Biosystems, Carlsbad, CA, USA). Chromatograms were
submitted for Phred analysis at <http://asparagin.cenargen.embrapa.br/phph/> and only positions with a score of greater than 20 were used. For each sample, a final consensus sequence was assembled with CAP-Contig in Bioedit 7.0.9.0 (Hall, 1999) and submitted to BLAST/n at <http://blast.ncbi.nlm.nih.gov/> to confirm the identity of the amplicons. The nucleotide and deduced amino acids sequences of the partial BToV N gene were aligned with homologous sequences available at GenBank using CLUSTAL/W in Bioedit 7.0.9.0 (Hall, 1999) and a distance neighbor-joining tree was built using the maximum composite likelihood model with 1000 bootstrap replicates with MEGA 5 (Tamura et al., 2011).

This is the first report on the detection of torovirus infection in cattle in Brazil. Five out of 80 samples were positive for BToV and valid sequences were obtained from three positive samples. The newly determined sequences were deposited in the GenBank database under accession numbers JQ619485, JQ619486 and JQ619487 (Table 1).

The percentage of BToV infections in the present study (6.25%) is comparatively higher than that reported in South Korea – 2.9% – (Park et al., 2008). However, it is lower than those reported in other studies with symptomatic animals: 8.4% in Japan (Ito et al., 2007), 9.7% in the USA (Hoet et al., 2003), 36.4% in Canada (Duckmanton et al., 1998). Despite such differences, one cannot make a reliable comparison amongst these frequencies due to the lack of a sample size design in the present and in other studies.

Although bovine torovirus is recognized as an enteric pathogen of calves, the role of BToV in cases of diarrhea in adult cattle (Koopmans et al., 1991; Scott et al., 1996; Aita et al., 2012) was also suggested in the present study as, from the five positive samples, three were from adult animals.

The already suggested association between BToV and outbreaks of winter dysentery (Koopmans et al., 1991) is supported by the fact that two samples positive for BToV (E-729 and E-766) were obtained from adult cattle during an episode of winter dysentery.

Concerning other enteric agents, two positive samples for BToV were also positive for Cryptosporidium spp. and two samples were also positive for bovine coronavirus. One sample, from an adult cow with severe diarrhea, was positive for BToV alone (Table 1).

Table 1
General characteristics of the BToV-positive samples.

| Sample name | Accession number | Location (Brazil) | Age  | Sampling year | BToV Nested PCR results | Other enteric pathogens detected |
|-------------|------------------|-------------------|------|---------------|-------------------------|---------------------------------|
| E-729       | –                | Rio Grande do Sul| Adult| 2007          | +                       | bovine coronavirus              |
| E-766       | –                | Rio Grande do Sul| Adult| 2007          | +                       | bovine coronavirus              |
| H1          | JQ619485         | Mato Grosso       | Young| 2009          | +                       | Cryptosporidium spp.            |
| O1          | JQ619486         | Mato Grosso       | Young| 2009          | +                       | Cryptosporidium spp.            |
| HV          | JQ619487         | São Paulo         | Adult| 2010          | +                       |                                  |

Fig. 1. Neighbor-joining phylogenetic tree, based on nucleotide sequences of the N gene, showing the relationships among BToV from different countries. Labels indicate accession number, strain (in bold), country and year of isolation. The number on each branch shows the percent occurrence in 1000 bootstrap replicates and values over 50% are shown. * Complete genome. ** Partial sequence.
These findings suggest the potential of this virus as a primary enteric pathogen in cattle, although it can also play a synergistic role in mixed infections.

The three Brazilian BToVs showed a high degree of nucleotide identity (95–99.3%) and amino acid identity (96–100%). Comparing Brazilian sequences with those from Europe and Japan, we found a high degree of sequence identity (94.3–99.3% nucleotide identity and 94–100% amino acid identity).

We also compared Brazilian sequences with the partial and complete sequences of the Breda 1 strain, the first BToV detected in the USA in 1982 (Woode et al., 1982). A moderate degree of identity was shown between Brazilian sequences and the North American Breda 1 strain (65–67.3% nucleotide identity and 63.1–65% amino acid identity) This fact, also reported by Kuwabara et al. (2007), might reflect the temporal distance between the detection of the Breda 1 strain and the present study.

Phylogenetic data of nucleotides and amino acids sequences of partial N gene revealed that BToV isolates from Brazil, Europe and Japan are closely related and clustered together. The sequences related to American Breda 1 strain clustered on a separate branch. This data supports the hypothesis that Brazilian sequences of BToV N gene are more closely related to sequences from Japan and Europe than to the Breda 1 strain (Fig. 1).

As a conclusion, close phylogenetic relationship exists amongst Brazilian and Japanese and European BToV strain, with a role in both young and adult cattle diarrhea. Overall, BToV must be regarded as a relevant pathogen for cattle.

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