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SHORT COMMUNICATIONS

Molecular detection of bovine coronavirus in a diarrhea outbreak in pasture-feeding Nellore steers in southern Brazil

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Abstract Worldwide diarrhea outbreaks in cattle herds are more frequently detected in calves being that diarrhea outbreaks in adult cattle are not common. Winter dysentery (WD) is a bovine coronavirus (BCoV) enteric infection that is more reported in Northern hemisphere. Seasonal outbreaks of WD in adult cattle occur mainly in dairy cows. WD has not been described in beef cattle herds of tropical countries. This study describes the molecular detection of BCoV in a diarrhea outbreak in beef cattle steers (Nellore) raised on pasture in Parana, southern Brazil. During the outbreak, the farm had about 600 fattening steers. Watery and bloody diarrhea unresponsive to systemic broad-spectrum antibiotic therapy reveals a morbidity rate of approximately 15 %. The BCoV N gene was identified in 42.9 % (6/14) of the diarrheic fecal samples evaluated by semi-nested polymerase chain reaction (SN-PCR) technique. Other enteric microorganisms occasionally identified in adult cattle and evaluated in this study such as bovine groups A, B, and C rotavirus, bovine viral diarrhea virus, bovine torovirus, aichivirus B, and *Eimeria* sp. were not identified in the fecal samples. To the best knowledge of the authors, this is the first description of the BCoV diagnosis in fecal samples collected in a diarrhea outbreak in adult beef cattle grazing in the grass in a tropical country.

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Introduction

The bovine coronavirus (BCoV) is a RNA virus that belongs to the *Betacoronavirus* 1 species in the *Betacoronavirus* genus of the *Coronaviridae* family. The BCoV is a RNA virus, non-enveloped, diameter of 120 nm, single-stranded (ssRNA) positive-sense, and non-segmented with 27–32 Kb size (ICTV 2015).

The genome of the coronavirus consists of five structural proteins; nucleocapsid (N), transmembrane (M), hemagglutinin esterase (HE), spike (S), and small membrane (E) proteins. The S protein comprises two subunits (S1 and S2), and shows an important role during the virus-host cell interaction acting in viral pathogenesis. The N protein is considered best target for detection of genomic RNA to represent the most conserved region among strains and more abundant on infected cells (ICTV 2015).

The BCoV infections are associated with severe diarrhea in newborn calves (Boileau and Kapil 2010), respiratory infections in calves and feedlot cattle (Fulton et al. 2013), and winter dysentery (WD) in adult cattle (Ko et al. 2006; Natsuaki et al. 2007).

Diarrhea outbreaks in adult cattle are uncommon. The BCoV was characterized as agent of acute diarrhea outbreaks in dairy cows in the winter season known as WD (Jeong et al. 2005). The infection is characterized by affect mainly dairy cattle displaying dark watery diarrhea, which is accompanied by variable depression and anorexia (Valle et al. 2006). This disease is also marked by high morbidity (100 %) with spontaneous recovery and rarely death cases are recorded in affected animals (Natsuaki et al. 2007).

The occurrence of BCoV infection causing WD in dairy cows has already been reported in countries of Europe (Decaro et al. 2008), Asia (Park et al. 2006; Natsuaki et al. 2007), and America (Valle et al. 2006; Takiuchi et al. 2009).

The BCoV is more stable in cold temperatures favoring viral spread in the herd (Takiuchi et al. 2009). The WD is described mainly in winter in the Northern hemisphere countries (Decaro et al. 2008; Boileau and Kapil 2010). However, this disease has already been reported in dairy cattle herds in warmer seasons (Park et al. 2006) and in tropical country (Takiuchi et al. 2009).

The diarrhea in adult bovine may also be occasionally caused by other enteric viral agents such as bovine rotavirus groups A (BoRVA) (Masuda et al. 2014), B (BoRVB) (Chang et al. 1997), and C (BoRVC) (Mawatari et al. 2004), bovine torovirus (BToV) (Ito et al. 2007), aichivirus B (Ribeiro et al. 2014), and bovine viral diarrhea virus (BVDV) (Lunardi et al. 2008).

The diarrhea is considered a multifactorial and multietiologic syndrome (Takiuchi et al. 2006). Although, the production of beef cattle created extensively in Brazil has received improvements in breeding techniques. Practices to prevent and control diseases should be based on good practices of creation of the animals (vaccination, deworming, water, and good quality pasture), herd size, presence of pathogens, and environmental factors (Cho and Yoon 2014).

Around the world, there are no reports of diarrhea outbreaks caused by BCoV affecting adult beef cattle in extensive management in a tropical country. The present study reports a diarrhea outbreak in a beef cattle grazing in the grass in a Brazilian herd.

Materials and methods

Steers management

The farm contains 600 Nellore breed steers created in an extensive management (pasture feeding), located in northern of the Parana state (23° 17′ 34″ S; 51° 10′ 24″ W), in southern Brazil. The steers are routinely vaccinated against foot and mouth disease and clostridiosis; received regular treatment against endo- and ectoparasites, maintained predominantly on green pastures with predominant *Brachiaria brizantha*, and supplemented with commercial mineral salt (protein salt during winter). The good quality water was given ad libitum.

Fecal samples

During June of 2013 occurred a diarrhea outbreak in this herd affecting 15 % of the steers with watery and bloody diarrhea with 5 days of duration of the clinic signs.

The animals had between 24 and 36 months old and demonstrated clinical signs of enteric disorders associated with weight loss, besides depression, anorexia, and diarrhea aqueous dark of sudden onset in which four diarrheic steers died.

Preliminary exams performed with 10 fecal samples for the diagnosis of *Eimeria* sp. by flotation technique and examined microscopically using 400× showed that all diarrheic fecal samples evaluated to coccidiosis were negative (data not shown).

The steers showing clinical signs of diarrhea were unresponsive to systemic and oral broad-spectrum antibiotic therapy (oxytetracycline). Therefore, the virological diagnosis was conducted by collecting 14 fecal samples from steers with clinical signs of diarrhea. The samples were transported at 4 °C and stored at −80 °C until processing.

Nucleic acid extraction

Fecal suspensions were prepared at 10–20 % (*w*/*v*) with 0.01 M phosphate-buffered (PBS) pH 7.2 (137 mM NaCl; 3 mM KCl; 8 mM Na_2HPO_4; 14 mM KH_2PO_4) and centrifuged at 3000 × *g* for 5 min. Aliquots of 400 μl of supernatant were treated with SDS (1 % final concentration) and incubated for 30 min at 56 °C. The nucleic acid extraction was realized using a combination of phenol/chloroform/isoamyl alcohol (25:24:1) and silica/guanidinium isothiocyanate methods (Alfieri et al. 2006). The nucleic acid was eluted in 50 μl of ultrapure RNase-free diethylpyrocarbonate (DEPC)-treated sterile water. Negative control (sterile water) was included in all nucleic acid extraction procedures.

Virological analysis

The RNA of enteric viruses in the diarrheic fecal samples was investigated by molecular techniques (RT-PCR and SN-PCR) for amplification of 251 bp length of BCoV N gene (Takiuchi et al. 2006); 876 bp and 1062 bp of BoRVA VP4 and VP7 genes, respectively (Gouvea et al. 1990; Gentsch et al. 1992); 434 bp of BoRVB NSP2 gene (Gouvea et al. 1991); 270 bp of BoRVC VP6 gene (Alfieri et al. 1999); 471 bp of BToV N gene (Ito et al. 2007); 216 bp of aichivirus B RdRp gene (Reuter et al. 2009); and 288 bp of BVDV 5′UTR region gene (Vilcek et al. 1994).

The RT-PCR and SN-PCR products were analyzed by electrophoresis in a 2 % agarose gel in TBE buffer pH 8.4 (89 mM Tris; 89 mM boric acid; 2 mM EDTA), stained with ethidium bromide (0.5 μg/ml), and visualized under UV light.

Sequencing analysis

The presence of the BCoV in the diarrheic fecal samples was confirmed by sequencing. Two SN-PCR products with better quality were purified using the GFX PCR DNA and Gel Band

Purification Kit (GE Healthcare, Little Chalfont, UK), quantified in a Qubit® Fluorometer (Invitrogen Life Technologies, Eugene, OR, USA), and sequenced in an ABI3500 Genetic Analyzer sequencer with the BigDye® Terminator v3.1 Cycle Sequencing Kit using the forward and reverse primers used in the SN-PCR assay (Applied Biosystems, Foster City, CA, USA). Sequence quality analysis was performed using Phred software and the consensus sequences were assembled using the CAP3 software (http://asparagin.cenargen.embrapa.br/phph/). Sequence similarity searches were performed using the basic local alignment search tool (BLAST) software (http://blast.ncbi.nlm.nih.gov/), to verify the nucleotide similarity with sequences that are deposited in public databases. The phylogenetic tree and the nucleotide identity matrix were realized using the MEGA version 5.10 and Bioedit version 7.1.3.0 software, respectively. The analyses were based on the maximum-likelihood method from the Jukes-Cantor model. Bootstrapping was statistically supported with 1000 replicates. The referenced sequences included in this study were acquired from the National Center for Biotechnology Information, USA (GenBank) (http://www.ncbi.nlm.nih.gov/GenBank/).

Results and discussion

Diarrhea outbreaks affecting animals rearing in pasture frequently are not reported. In this study, the cattle farm had good nutritional and health management; however, several steers negative for coccidial infections and unresponsive to broad-spectrum antibiotic therapy showed diarrhea simultaneously. Therefore, we used the techniques of RT-PCR and SN-PCR that have high specificity and sensitivity to detect the main agents that may be involved in enteric outbreaks of adult cattle.

Amplicons of 251 bp length were amplified by SN-PCR performed for BCoV diagnosis in 6 (42.85 %) of the 14 diarrheic fecal samples included in this study. The SN-PCR amplified products were confirmed as BCoV N gene by nucleotide (nt) sequence analysis. Two nt sequences of better quality selected for phylogenetic analysis exhibited 100 % of nt identity with each other, 98.1 % with other Brazilian BCoV strains (FJ603611 to FJ603613) that were described in a diarrhea outbreak in calves (Stipp et al. 2009), and 100 % with strains related in enteric problems in dairy cows (KJ719308 to KJ719309) (Fig. 1).

Around the world, other viruses have also been described in smaller frequency in cases of diarrhea in adult animals highlighting BoRVA (Masuda et al. 2014), BoRVB (Chang et al. 1997), BoRVC (Mawatari et al. 2004), aichivirus B (Ribeiro et al. 2014), BVDV (Lunardi et al. 2008), and BToV (Ito et al. 2007). In order to assess the type of enteric (single or mixed) infection, these potentially pathogenic viral agents already described in diarrheic fecal samples of adult cattle were included in the differential diagnosis. However, all diarrheic fecal samples analyzed were negative for these six virus evaluated.

The Brazil shows a tropical weather revealing not defined seasons. During the month of June/2013, period of diarrhea outbreak, the Parana state registered maximum temperatures of 22 °C and a minimum of 10 °C. Cold weather and low air relative humidity facilitate the BCoV spread in the environment (Takiuchi et al. 2009)

In a cattle herd, some steers can be with BCoV subclinical infections and are asymptomatic carriers of the virus. For cattle raised on pasture, weather conditions that lead to rapid thermal inversion are certainly a cause of great stress for the animals. Based on the maximum and minimum temperatures registered in Parana state during the period of the outbreak, we believe that thermal stress may have caused the virus circulation in the herd causing the enteric infection and the diarrhea outbreak.

Diarrhea outbreaks involving the BCoV usually not cause death of animals. Even not having the opportunity to perform

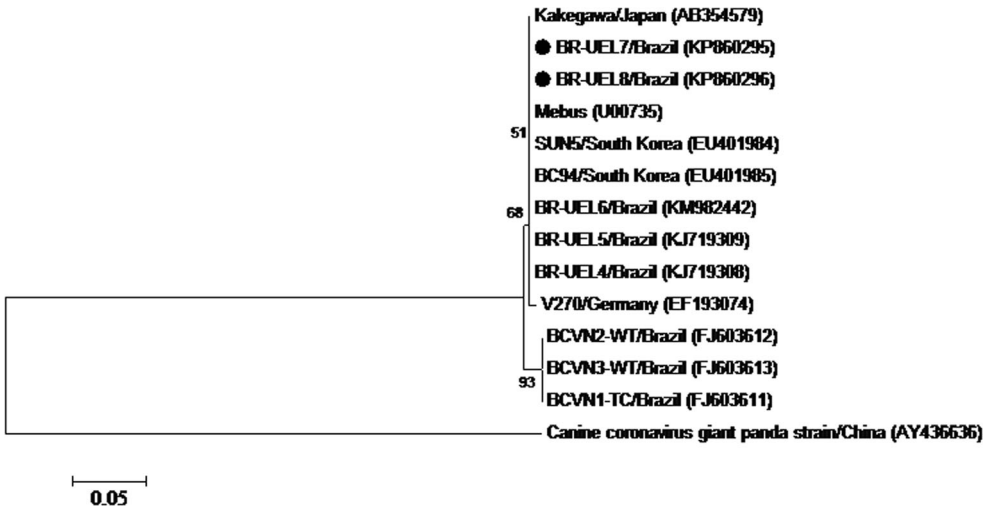

Fig. 1 Phylogenetic analysis of a partial nucleotide sequence (nt 218) of the N gene of BCoV. The tree was generated using the maximum-likelihood method from the Jukes-Cantor as the nucleotide substitution model. Bootstraps values (1000 replicates). The Brazilian BCoV strains described in this study are marked with a *filled circle*. Canine coronavirus strain was used as outgroup

necropsy, all the information regarding the epidemiology, treatment, and the range of negatives etiologic diagnoses suggest that this outbreak can be classified as uncommon because the death of four diarrheic steers occurred.

The BCoV is an important infectious agent that must be investigated in cases of enteric disorders in adult animals (Jeong et al. 2005; Natsuaki et al. 2007). However, this infection is more frequently reported in cows of dairy farms (Decaro et al. 2008; Takiuchi et al. 2009). This study demonstrated that the presence of BCoV in diarrhea outbreak in pasture-feeding Nellore steers was caused by BCoV infection. This is the first description of adult diarrhea outbreak in a beef cattle herd in a tropical country demonstrating the importance of the BCoV diagnosis in diarrhea outbreak in adult beef cattle in extensive management.

Usually, the diarrhea outbreaks related to extensive breeding herds are not investigated. However, with improvements in animal breeding techniques, it is important to research all pathogens that affect livestock. In situations of clinical cases occurrence characterized with sporadic diarrhea, the laboratory diagnosis is rarely realized and is difficult to identify the etiological agent involved. Thus, frequently, the result obtained is not conclusive or rarely exhibited.

This study allowed to identify for the first time that the BCoV is a potential cause of diarrhea in pasture-feeding Nellore steers in a tropical country. With that, the results showed the importance of the inclusion of BCoV in the diagnosis of diarrhea outbreaks in adult cattle regardless the type of herd (dairy or beef), management (intensive or extensive), and season (winter or summer).

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

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