Outbreak of neonatal diarrhea caused by multiple genotypes of rotavirus A in a beef calves herd[1]

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ABSTRACT.- Rondelli A.L.H., Rondelli L.A.S., Monteiro B.R.G., Lorenzetti E., Tineli T.R., Dutra V., Alfieri A.A & Pescador C.A. 2018. **Outbreak of neonatal diarrhea caused by multiple genotypes of rotavirus A in a beef calves herd.** *Pesquisa Veterinária Brasileira 38(10):1890-1895.* Laboratório de Patologia Veterinária, Faculdade de Medicina Veterinária, Universidade Federal de Mato Grosso, Av. Fernando Corrêa da Costa 2367, Cuiabá, MT 78060-900, Brazil. E-mail: carolpescador@yahoo.com.br

Calf diarrhea causes substantial economic losses to beef cattle production worldwide. It is a complex multifactorial pathological condition influenced by infectious, nutritional and environmental factors. The present study focused on analyzing the pathological and molecular characterization of bovine rotavirus A (BoRVA) during a diarrhea outbreak in a beef cattle herd located in the state of Mato Grosso, central-western region, Brazil. The outbreak caused high morbidity (80%) and mortality (12%) among 1,100 calves up to 30 days of age. The BoRVA was identified in 53.3% (16/30) of the diarrheic fecal samples analyzed using the silver-stained polyacrylamide gel electrophoresis (ss-PAGE) technique. The nucleotide sequence analysis of VP7 (G genotype) and VP4 (P genotype) via RT-PCR from eight BoRVA-positive fecal samples showed the genotypes G6P[5] (*n* = 6), G6P[11] (*n* = 1) and G6P[X] (*n* = 1). Three calves were necropsied and the gross findings included edema and thickened, wrinkled bowel mucosa in the small intestine. Microscopic lesions were confined to the villi of the small intestine, characterized mainly by villus fusion and moderate multifocal lymphoplasmacytic enteritis. Immunohistochemical examination of three cases was positive for BoRVA. The 53.3% of the diarrheic fecal samples that were positive for BoRVA in this study suggested that RV was the etiological agent involved in this neonatal calf diarrhea outbreak.

INDEX TERMS: Neonatal, diarrhea, genotypes, rotavirus A, beef calves, enteric disease, rotavirus, gross lesion, genotypes, cattle, pathology.

RESUMO.- [**Surto de diarreia neonatal por múltiplos genótipos de rotavírus A em um rebanho de bovinos de corte.**] A diarreia neonatal provoca perdas econômicas substanciais na produção de bovinos em todo o mundo. É uma condição patológica multifatorial complexa influenciada por fatores infecciosos, nutricionais e ambientais. O presente estudo teve por objetivo caracterizar o rotavírus tipo A (BoRVA) através da análise patológica e molecular durante um surto de diarreia em um rebanho bovino localizado no estado de Mato Grosso, região centro-oeste, no Brasil. O surto causou alta morbidade (80%) e letalidade (12%) em um rebanho composto 1.100 bezerros até 30 dias de idade. O BoRVA foi identificado em 53,3% (16/30) das amostras fecais diarreicas analisadas usando a técnica de eletroforese em gel de poliacrilamida corada com prata (ss-PAGE). A análise da sequência nucleotídica de VP7 (genótipo G) e VP4 (genótipo P) via RT-PCR a partir de oito amostras fecais BoRVA-positivas mostrou os genótipos G6P [5] (n = 6), G6P [11] (n = 1) e G6P [X] (n = 1). Três bezerros foram submetidos à necropsia e os achados macroscópicos incluíram edema e espessamento

da mucosa do intestino delgado. As lesões microscópicas foram observadas nas vilosidades do intestino delgado, sendo caracterizadas principalmente por fusiosamento de vilosidades e enterite linfoplasmocitária multifocal moderada. O exame imunohistoquímico dos três casos foram positivos para o BoRVA. As 53,3% das amostras fecais diarreicas positivas para o BoRVA sugeriram que o rotavírus é o agente etiológico envolvido neste surto de diarreia neonatal em bezerros.

TERMOS DE INDEXAÇÃO: Diarreia, neonatal, genótipos, rotavírus A, bovinos de corte, bezerros, doença entérica, rotavírus, lesões macroscópicas, genótipos, patologia.

INTRODUCTION

Neonatal calf diarrhea (NCD) is one of the major health challenges in beef cattle herds around the world. More than 20% of beef cattle owners consider that calf diarrhea has a significant impact on their economic productivity (Foster & Smith 2009). In several countries, such as Belgium, Sweden, Italy, Switzerland and Brazil, the prevalence and incidence of risk factors for NCD have been reported to be 19.1 and 33.7%, respectively (Papp et al. 2013, Collins et al. 2014). NCD is a pathological condition with complex multifactorial etiology that is influenced by infectious, nutritional and environmental factors, as well as by management practices (Martella et al. 2010, Izzo et al. 2011, Blanchard 2012). Currently, enterotoxigenic *Escherichia coli* (ETEC), *Clostridium perfringens, Cryptosporidium parvum*, rotavirus and coronavirus appear to be the most significant infectious causes of calf diarrhea. Simultaneous or consecutive presence of more than one of these pathogens often causes increased morbidity and mortality rates (Blanchard 2012, Meganck et al. 2015).

In Brazil, epidemiological studies conducted to date have shown that, in calves, bovine rotavirus A (BoRVA) is the virus most commonly associated with neonatal diarrhea (Alfieri et al. 2004, 2006, Silva et al. 2012, Medeiros et al. 2014). Rotaviruses belong to the Reoviridae family and have a triple-layered icosahedral protein capsid. Its genome is composed of 11 double-stranded RNA segments (dsRNA) that can be used to identify the RV groups or species. To date, there are eight groups or species of RV (A to H). RVA strains are classified as G and P-genotypes according to two type-specific outer capsid proteins, VP7 (glycoprotein) and VP4 (protease sensitive), respectively (Estes & Kapikian 2007). The most frequent BoRVA G genotypes detected in bovine feces are G6, G8 and G10. Among the P genotypes, P[1], P[5] and P[11] are found in RVA strains of bovine origin. The G and P genotype combinations most commonly found in bovine RVA strains are G6P[1], G6P[5], G6P[11], G8P[1] and G10P[11] (Alfieri et al. 2004, Caruzo et al. 2010, Martella et al. 2010, Freitas et al. 2011, Badaracco et al. 2012, Papp et al. 2013, Collins et al. 2014, Medeiros et al. 2014). RVs cause malabsorption and toxin-mediated secretory diarrhea, especially in calves less than 3 weeks old (Foster & Smith 2009). Recently, in Brazilian cattle herds, an emergent bovine RVA genotype combination G6P[11] was identified as a cause of a severe neonatal diarrhea outbreak in a beef cattle herd (Medeiros et al. 2014).

The aim of this study was to report the pathological and molecular features of RVA strains recovered from calves, during a diarrhea outbreak in a beef cattle herd.

MATERIALS AND METHODS

This study was approved by the University Animal Care Committee and was conducted in 2014 between October and November months in a farm located in the central-western geographical region of Brazil. The herd was composed of 2,000 Caracu (*Bos Taurus Taurus*) cows that were reared extensively in Diamantino, state of Mato Grosso, Brazil. The reproductive management included a natural breeding period from November to February (4 months). All calves were born in a maternity pen and remain with their dams, feeding on milk ad libitum for 5 to 7 months. After birth, the calves were kept in the maternity pen for about 15 to 30 days and then were moved to another pen with their dams, forming batches of 70 to 150 calves. The dams were vaccinated against rabies, clostridiosis and leptospirosis and were maintained on improved pastures (*Urochloa* spp.). Vaccination against RV to control neonatal diarrhea was not used among the pregnancy cows. No vaccine was used in the newborns.

Since 2013, the farm had been experiencing outbreaks of diarrhea affecting calves that were up to 30 days of age, with a clinical course of 5 to 10 days. According to the owner, a total of 1,100 calves, about 880 had diarrhea and 132 had died, thus resulting in elevated morbidity rates (80%) and lethality rates (12%). All diarrheic calves were medicated with broad-spectrum antibiotics parenterally, but their response to treatment was inconsistent.

In 2014, during a visit to the farm, three affected calves were euthanized. Tissue samples, including fragments from all intestinal segments, were collected at the necropsies, fixed in 10% buffered formalin, and processed following standard procedures for histopathological analysis, processed by immunohistochemistry (IHC) or stained by hematoxylin and eosin (HE). A total of 30 diarrheic fecal samples collected directly from the rectum in plastic tubes were kept refrigerated at 4°C until they were processed in a laboratory. Culturing was performed as described by Quinn et al. (1994). Afterwards, 2mL of fecal samples from each animal were kept frozen at -20°C until the silver-stained polyacrylamide gel electrophoresis (ss-PAGE) and reverse transcriptase - polymerase chain reaction (RT-PCR) techniques were performed.

Additionally, to evaluate rotavirus-antigen, segments of small intestine tissue were embedded in paraffin and were sent to the Veterinary Pathology Laboratory of the University of Nebraska-Lincoln (UNL), USA, for immunohistochemistry procedures. Before analysis for bovine RV, the diarrheic fecal samples were evaluated by means of the modified Ziehl-Neelsen technique for *Cryptosporidium* spp. in accordance with Henriksen & Pohlenz (1982).

For rotavirus and coronavirus detection fecal suspensions (20% w/v) in Tris/Ca^{++} buffer (50mM Tris-HCl; 10mM NaCl; 1.5mM 2-mercaptoethanol; 3mM CaCl$_2$) were centrifuged at 3,000x g for 10min at 4°C. Aliquots of 500μL of supernatant were treated with sodium dodecyl sulfate (1% final concentration) and incubated for 30min at 56°C. A combination of the phenol/chloroform/isoamyl alcohol and silica/guanidine isothiocyanate techniques (Alfieri et al. 2006) was used for nucleic acid extraction. The nucleic acid was eluted in 50μL of ultrapure sterile water treated with diethyl pyrocarbonate (Invitrogen Life Technologies, Carlsbad, CA, USA). The cell culture-adapted RVA NCDV strain (G6P[1]) and aliquots of Tris/Ca^{++} buffer were included in all RNA extraction and RT-PCR procedures as positive and negative controls respectively.

The presence of dsRNA of RV in the diarrheic fecal samples was evaluated using the 7.5% ss-PAGE technique (Pereira et al. 1983) followed by silver staining as described by Herring et al. (1982).

All 30 samples were subjected to RT-PCR assays using previously described consensus primers for amplification of the RVA genes VP7

(G genotype) and VP4 (VP8*, P genotype) (Gouvea et al. 1990, 1993, Gentsch et al. 1992, Isegawa et al. 1993, Martella et al. 2006).

Presence of bovine coronavirus (BCoV) RNA in the diarrheic fecal samples was evaluated using a RT-PCR assay followed by a semi-nested PCR assay (SN-PCR), as described by Takiuchi et al. (2006). The BCoV cell culture-adapted Mebus strain was used as a positive control and sterile water was used as a negative control.

Aliquots of 5µL from the RT-PCR and SN-PCR products were subjected to electrophoresis on 2% agarose gel in TEB buffer (89mM Tris, 89mM boric acid, 2mM EDTA), at pH 8.4, under constant voltage (100 V) for 60min. The product was stained using ethidium bromide (0.5µg/mL) and viewed under UV light.

For sequencing analyses, the RT-PCR products from the genes VP7 and VP4 (VP8*) were purified using the PureLink Quick Gel Extraction and PCR Purification combo kit (Invitrogen Life Technologies, Carlsbad, CA, USA) and were quantified by means of a Qubit fluorometer (Invitrogen Life Technologies, Eugene, OR, USA). The DNA sequences were obtained using the BigDye® Terminator v3.1 cycle sequencing kit (Applied Biosystems, Foster City, CA, USA) with the ABI3500 Genetic Analyzer sequencer (Applied Biosystems,

Foster City, CA, USA), using the forward and reverse primers used in the RT-PCR assay. Sequence quality analyses and contig assembly were performed using PHRED and CAP3 software (http://asparagin. cenargen.embrapa.br/phph/), respectively. Similarity searches were performed with sequences deposited in GenBank using the Basic Local Alignment Search Tool (BLAST) software (http://blast.ncbi. nlm.nih.gov/Blast.cgi). The G and P genotypes were determined using the RotaC $^{2.0}$ genotyping tool (Maes et al. 2009).

RESULTS

Since the 2013 birth season, calves in this herd had been presenting clinic signs of profuse diarrhea (Fig.1A), dehydration and loss weight, which usually lasted 5-7 days. The diarrhea could also be prolonged, lasting up to 10 days. The color of their feces varied from yellow or brownish-grey to light green and seldom contained blood or mucus.

During the visit, at least 66 calves (47.1%) of a batch of 140 animals were presenting similar signs of diarrhea. BoRVA was detected in 53.3% (16/30) of the diarrheic fecal samples
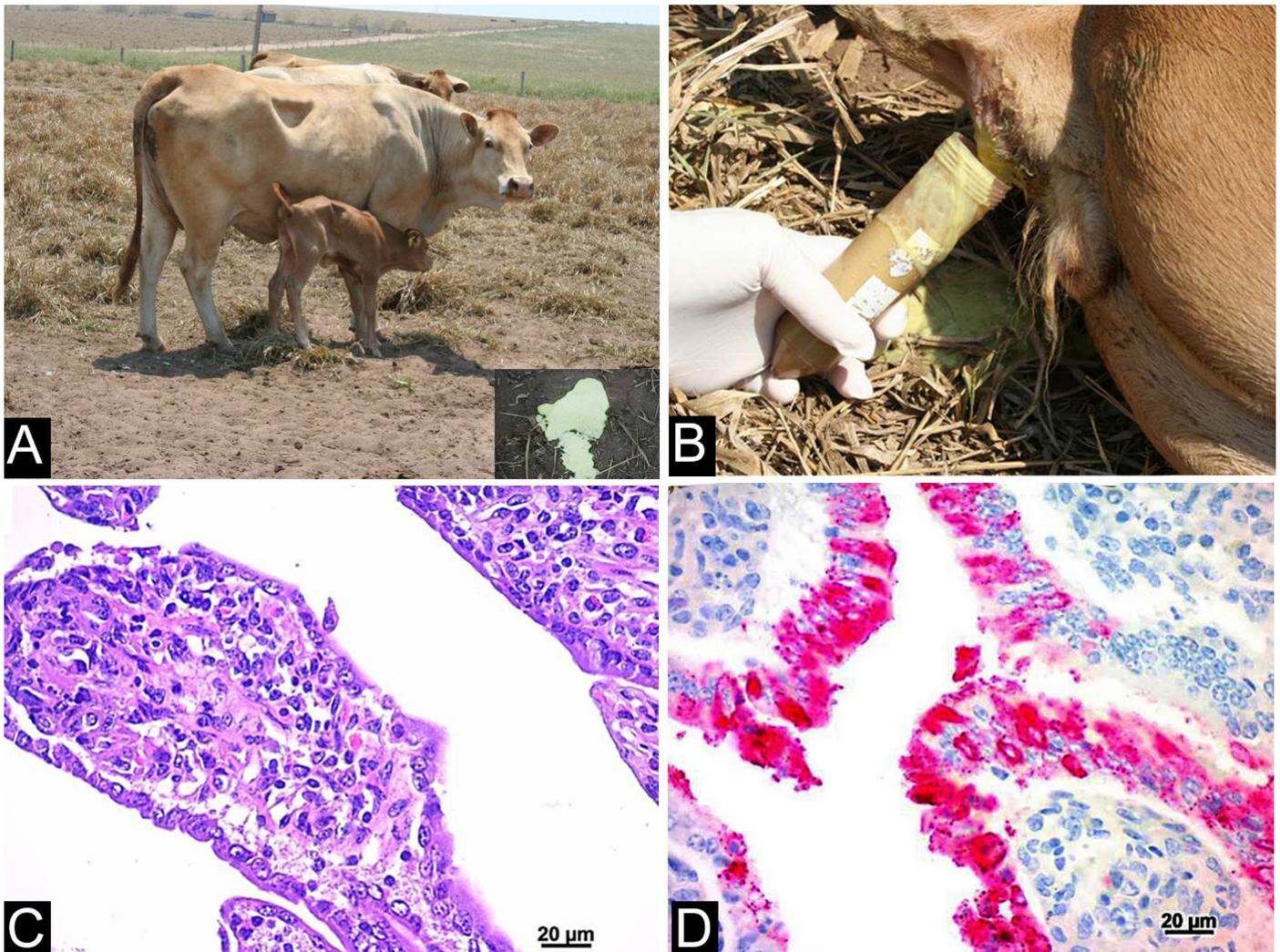

Fig.1. Calf rotavirus infection. (**A**) Calf with clinical signs of profuse diarrhea and hind legs with feces. Small picture shows light green diarrheic feces. (**B**) Fecal sample collected from a diarrheal calf. (**C**) Histological section through small intestine, showing moderate villus fusion with a moderate proprial infiltrate composed by lymphocytes and plasmocytes. Villi are covered by flattened surface epithelium. HE, obj.20x; bar = 20µm. (**D**) Small intestine showing positive reactions characterized by large quantities of stained epithelial cell cytoplasm. IHC, permanent red, obj.20x; bar = 20µm.

analyzed by means of the ss-PAGE technique. Out of the total of 16 RVA-positive samples, molecular characterization to determine the G and P genotypes was done on eight of them. Regarding VP4 (VP8*), two distinct P genotypes were identified: P[5] and P[11]. Regarding the VP7 gene, just one G genotype (G6) was identified. In one sample, it was not possible to determine the P genotype (P[X]). The genotype combinations G6P[5] (*n* = 6), G6P[11] (*n* = 1) and G6P[X] (*n* = 1) were detected in the eight RVA strains analyzed in this study.

In the necropsied calves, the characteristic gross findings included presence of diarrheic feces on the perineum and hind legs. High levels of yellowish to brownish-grey fluid content with mucus and fibrin filaments were observed in the small intestinal lumen. There was also edema and thickened, wrinkled bowel mucosa in the small intestine (Fig.1B). The microscopic findings were similar in all the necropsied calves. The main findings were largely confined to the villi of the small intestine, which was characterized by multifocal severe fusion affecting the villi. Brush-bordered columnar epithelial cells were absent and were replaced by cuboidal cells from the crypts. Multifocal, severely inflammatory cells consisting of lymphocytes and plasmocytes infiltrated the intestinal lamina propria of the mucosa and the submucosa (Fig.1C). Immunohistochemical examination of these three cases revealed clearly positive reactions, characterized by large quantities of stained epithelial cell cytoplasm in the small intestine (Fig.1D).

Pure cultures of *Escherichia coli* were obtained from the intestinal content of the necropsied calves and from the fecal specimens (*n* = 30) that were collected directly from the rectum. Bacteriological detection of *Salmonella* spp. was negative in the intestines of all the animals studied. Modified Ziehl-Neelsen staining for *Cryptosporidium* spp. was negative in smears from all the diarrheic fecal samples. All of these samples were negative for bovine coronavirus, via SN-PCR assay.

DISCUSSION

In Brazil, as in other countries, diarrhea outbreaks caused by RVA continue to be the most common cause of losses of calves less than one month (Freitas et al. 2011, Blanchard 2012). The 53.3% (16/30) of the diarrheic fecal samples that were positive for RVA in the present study suggested that this enteric virus was the major etiological agent associated with the neonatal diarrhea outbreak described. Additionally, all the diarrheic fecal samples were negative for common enteric enteropathogens such as bovine coronavirus and *Cryptosporidium* spp. Regarding the ages of the calves in this study, the frequency of infection was highest among calves aged 8 to 14 days (73%). This range of calves has been reported to be the one most susceptible to infection by RV (Foster & Smith 2009). The main microscopic lesion was atrophy of villi, which results in diarrhea due to maldigestion and malabsorption. Maldigestion results in undigested food in the colon, which leads to bacterial overgrowth and osmotic pressure, thereby exacerbating the diarrhea (Foster & Smith 2009, Martella et al. 2010, Blanchard 2012).

The RVA genotype combinations G6P[5] and G10P[11] are the most frequently reported in calves with diarrhea, in several studies around the world (Alfieri et al. 2004, Freitas et al. 2011, Badaracco et al. 2012, Medeiros et al. 2015).

In the present study, G6P[5] and G6P[11] were the genotype combinations identified. Alfieri et al. (2004) conducted a survey on herds in four Brazilian states and found that the RVA genotype G6P[5] was the most frequently observed in cattle, 40% (20/50), while the genotype G10P[11] was observed in only 16% (8/50) of the strains. The genotype G6P[11] has often been reported in Brazilian cattle herds, at rates ranging from 2% (1/50) to 34.3% (12/35) of the samples analyzed (Alfieri et al. 2004, Caruzo et al. 2010, Silva et al. 2012). However, Medeiros et al. (2014) reported that the genotype G6P[11] caused a severe neonatal diarrhea outbreak as a single infection in Nelore calves. Additionally, the RVA genotype G6P[11] identified in their study belongs to the G6-III lineage, which contains human and bovine RVA strains that are distant from the most usual G6-IV lineage, which includes G6P[5] (UK strain) and G6P[1] (NCDV-Lincoln strain) prototypes (Medeiros et al. 2014). The genotype G6P[11] has been identified in RVA strains from human hosts in Africa and cattle may constitute a reservoir of this RVA genotype for humans (Martella et al. 2010).

Although these differences may result only from geographical differences, they do seem to show that diffusion of homotypic immunity in these herds against genotypes that were more frequently found in previous years has occured. Consequently, in the present study, the G and P combinations suggest that at the time of the diarrhea outbreak, different RVA strains were circulating. In diarrhea outbreaks among calves that are caused by RVA, only one G and P genotype combination is usually detected (Caruzo et al. 2010, Martella et al. 2010, Badaracco et al. 2012, Papp et al. 2013, Medeiros et al. 2014). However, in the neonatal diarrhea outbreak described here, three different genotype combinations were identified in the herd.

According to Freitas et al. (2011), the high prevalence of bovine RVA in dairy herds in Brazil is directly related to the conditions of the production and management systems adopted. High environmental contamination, herds with large numbers of animals and management in which the immunization rate is very low are factors that favor spreading of the virus in herds. In the present study, the farm owner did not use any RV vaccine on this herd. On the other hand, a study conducted in the state of Mato Grosso do Sul, central-western region of Brazil, showed that the genotype G6P[5] caused a diarrhea outbreak in a herd that had been vaccinated against the genotypes G6P[1] and G10P[11] (Medeiros et al. 2015). Use of broad-spectrum antibiotics and fluid therapy may be helpful, but the results have been inconsistent and time-dependent (Alfieri et al. 2004, Medeiros et al. 2015).

RV preferentially targets the mature villous enterocytes and rarely the crypts, thus affecting the caudal small intestine. In young calves, RV appears to be widely distributed, but is detected less frequently in older calves (Foster & Smith 2009, Blanchard 2012). According to Varshney et al. (1995), immunostained RV antigen was detected in the villi of the small intestine but not in the crypt enterocytes of gnotobiotic calves that had been experimentally infected with a cloned RVA strain (C3-160). However, its presence was three times greater in one-day-old than in ten-day-old calves, thus showing that younger calves are more susceptible to diarrhea. In our study, immunohistochemical examination on the three cases revealed clearly positive reactions that were characterized by

large quantities of stained villus enterocyte cytoplasm in the small intestine, as found by Varshney et al. (1995).

Although *Escherichia coli* was isolated in all the samples of diarrheal feces, K99 fimbria detection tests were not performed. Therefore, it is not possible to implicate this pathogen as the agent responsible for the pathological clinical picture seen in this study. However, we suggest that it is possible that this bacterium may have contributed to the state of dehydration, since neonatal diarrhea is a multifactorial problem.

CONCLUSIONS

Bovine RVA has shown genomic variability and complexity, which makes it difficult to implement efficient control and prophylaxis programs for this enteric infection. In the present study, the genotypes found through molecular characterization of RVA strains suggest a high genotypes diversity involvement in calf diarrhea.

While vaccines are highly likely to protect against the strains causing disease in Brazilian cattle, further epidemiological studies within Brazilian cattle would need to be conducted to monitor vaccine effectiveness.

In the outbreak of the present report, RV was the agent responsible for NCD.

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