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Lorenzetti, Elis; de Arruda Leme, Raquel; Ribeiro, Juliane; Rodrigues Almeida de Souza, Vilma; Fernandes Alfieri, Alice; Alcindo Alfieri, Amauri
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Neonatal diarrhea by bovine coronavirus (BCoV) in beef cattle herds

Diarreia neonatal por coronavírus bovino (BCoV) em rebanhos bovinos de corte

Elis Lorenzetti¹; Raquel de Arruda Leme¹; Juliane Ribeiro¹;
Vilma Rodrigues Almeida de Souza¹; Alice Fernandes Alfieri²;
Amauri Alcindo Alfieri^{2*}

Abstract

Bovine coronavirus (BCoV) is the second most important viral agent involved in neonatal diarrhea in calves worldwide. The reports on the frequency of BCoV infection in beef cattle herds under extensive management are uncommon in Brazil. The present study analyzed 93 diarrheic fecal samples of calves up to 60 days of age from 13 commercial beef cattle herds located in the states of Mato Grosso, Mato Grosso do Sul, Minas Gerais, Paraná, and Rondônia. The fecal samples were collected during 2009-2012 and were previously analyzed for the presence of bovine rotavirus group A (BoRVA), with negative results. The presence of BCoV in the fecal samples was evaluated by the partial amplification of the N gene by using the semi-nested PCR technique. The expected products of 251 bp length were amplified 33.3% (31/93) of the analyzed diarrheic fecal samples. The results revealed that coronaviruses has important participation in the neonatal diarrhea complex of beef cattle herds reared extensively from the different geographical regions of Brazil.

Key words: Calf, coronaviruses, enteric virus, semi-nested PCR

Resumo

O coronavírus bovino (BCoV) é o segundo mais importante agente etiológico viral envolvido em diarreias neonatais em bezerros de todo o mundo. Os relatos sobre a frequência da infecção por BCoV em rebanhos bovinos de corte criados extensivamente são incomuns no Brasil. Este estudo analisou 93 amostras fecais diarreicas de bezerros de corte com até 60 dias de idade, provenientes de 13 rebanhos comerciais distribuídos nos estados de Mato Grosso, Mato Grosso do Sul, Minas Gerais, Paraná e Rondônia. As amostras fecais foram colhidas no período de 2009-2012 e haviam sido previamente analisadas quanto à presença de rotavírus bovino grupo A (BoRVA), com resultados negativos. A presença do BCoV nas amostras fecais foi avaliada por meio da amplificação parcial do gene N pela técnica da *semi-nested* PCR. Em 33,3% (31/93) das amostras analisadas foi possível a amplificação de produtos com tamanho esperado de 251 pb para o BCoV. Os resultados deste estudo demonstraram que a coronavirose tem importante participação no complexo diarreia neonatal em rebanhos bovinos de corte criados extensivamente em várias regiões brasileiras.

Palavras-chave: Bezerro, coronaviroses, vírus entérico, *semi-nested* PCR

¹ Discentes de pós-graduação *Stricto Sensu* em Ciência Animal, área de concentração Sanidade Animal, Laboratório de Virologia Animal, Deptº de Medicina Veterinária Preventiva, Universidade Estadual de Londrina, UEL, Londrina, PR, Brasil. E-mail: lorenzettielis@hotmail.com; quel-arruda@hotmail.com; julianeribeiro@outlook.com; wrvninha@hotmail.com

² Profs. Drs. de Microbiologia Veterinária e Doenças Infecciosas, Deptº de Medicina Veterinária Preventiva, UEL, Londrina, PR, Brasil. E-mail: aalfieri@uel.br; alfieri@uel.br

* Author for correspondence

Introduction

Diarrhea is one of the most economically important syndromes affecting dairy and beef cattle herds worldwide. Neonatal enteric disease is the leading health problem of calves and is the result of interaction of several factors (management, nutrition, environment, and infectious) (ALFIERI et al., 2006).

The infectious etiology of neonatal diarrhea in calves is complex and involves different microorganisms, such as bacteria, protozoa, and virus. Worldwide, bovine rotavirus group A (BoRVA) is the most common viral agent associated with diarrhea in calves (ALFIERI et al., 2006; GARAICOECHEA et al., 2006; MARTELLA et al., 2010; MAYAMEEI et al., 2010), followed by bovine coronavirus (BCoV) (STIPP et al., 2009; MAYAMEEI et al., 2010). The co-infection of these two viral agents is not unusual and mixed infections with viruses, bacteria and/or protozoa is frequent (BRANDÃO et al., 2007; OLIVEIRA FILHO et al., 2007; BARRY et al., 2009; CHO et al., 2010; SAIF, 2011).

BCoV is member of *Nidovirales* order, *Coronaviridae* family and is classified as *Betacoronavirus 1* species in the genus *Betacoronavirus*. The virus has an envelope of 120-160 nm and a single-stranded RNA (ssRNA) genome of positive polarity, which has 27 to 32 Kb of length (ICTV, 2011).

The 13 open reading frames (ORFs) of the BCoV genome encode five major structural proteins, which are the nucleocapsid (N), the transmembrane (M), the hemagglutinin esterase (HE), the spike (S), and the small membrane (E) proteins. The N gene has been considered a good target for BCoV RNA amplification not only because it is a highly conserved region among BCoV strains, but is also the most abundant antigen in coronavirus-infected cells, enhancing the sensitivity of diagnostic methods (TAKIUCHI et al., 2006).

The implication of BCoV in neonatal diarrhea is known to be most frequent in calves up to 30 days of age (TAKIUCHI et al., 2006; STIPP et al., 2009). The virus is also responsible for enteric infections in adult cattle (winter dysentery) and respiratory disease in cattle of all ages (CHO et al., 2001; TAKIUCHI et al., 2009).

The frequency of BCoV infection vary among different geographic regions, the cattle production type (dairy or beef herds), and the diagnostic techniques used. In Brazil, the studies that have evaluated the occurrence of BCoV using molecular diagnostic methods in stool samples from calves presented rates of infection varying from 14 to 39% (TAKIUCHI et al., 2006; BRANDÃO et al., 2007; OLIVEIRA FILHO et al., 2007; BARRY et al., 2009; STIPP et al., 2009). However, these results of BCoV infection have been associated with both dairy and beef calves, and some of the studies have reported the BCoV in co-infection with BoRVA. The frequency of BCoV infection as single enteric viral agent specifically in extensively managed beef calves is poorly studied.

In Brazil, the extensive breeding system is prevalent within beef cattle-producing farms. The Midwest region of Brazil possesses the major national cattle herd (34.1%), followed by North (20.3%) and Southeast (18.5%) regions. The smaller national cattle herd is located in the South region of Brazil (13.1%). The ten main Brazilian producer states hold 81.1% of the national cattle herd (IBGE, 2011)

The aim of this study was to evaluate the BCoV presence in diarrheic fecal samples, previously known as negative for BoRVA, of beef calves managed extensively in different geographical regions of Brazil.

Materials and Methods

Ninety-three diarrheic fecal samples from calves were included in this study. The samples were

derived from a collection of feces obtained from 2009 to 2012, and were stored at 4°C. All fecal samples were chosen according to consistency, age of the animals, cattle breed, previous results for BoRVA infection by polyacrylamide gel electrophoresis (PAGE) (PEREIRA et al., 1983), and the state of origin. First, fecal samples from calves up to 60 days of age with clinical signs of acute diarrhea were selected. The fecal samples from beef cattle breeds were chosen. Then, it was selected the fecal samples that were negative for BoRVA by PAGE technique. To complete, only fecal samples from the states that are among the ten major cattle producers were evaluated.

The Brazilian states that had calf diarrheic fecal samples evaluated were Minas Gerais (MG, $n=23$), representing the Southeast region of Brazil; Mato Grosso do Sul (MS, $n=27$) and Mato Grosso (MT, $n=5$), from the Midwest Brazilian region; Paraná (PR, $n=17$) from the South Brazilian region; and Rondônia (RO, $n=21$) from the North region of Brazil. A total of 13 beef cattle herds were evaluated. The number of herds according to the states was MG, $n=2$; MS, $n=5$; MT, $n=1$; PR, $n=2$; and RO, $n=3$. The herds included in this study were not vaccinated against the BCoV.

Fecal suspensions were prepared at 10 to 20%

(w/v) in 0.01 M phosphate-buffered saline (PBS), pH 7.2, and centrifuged at 5,000 x g for 3 min. The supernatants were used for nucleic acid extraction.

To determine the presence of BCoV, the viral ssRNA was extracted using a combination of phenol/chloroform/isoamyl alcohol (25:24:1) and silica/guanidinium isothiocyanate nucleic acid extraction methods (ALFIERI et al., 2006).

The semi-nested (SN) polymerase chain reaction (PCR) assay was performed using specific primers targeting the N gene of the BCoV RNA genome, and the technique was carried out according to Takiuchi et al. (2006). The amplified products were analyzed by electrophoresis on 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 g/ml) and visualized under UV light. The expected size of the amplified SN-PCR products was 251 bp.

Results and Discussion

Of the 93 fecal samples analyzed, 31 (33.3%) were positive for BCoV. Within the 13 herds evaluated, 8 (61.5%) of these were infected by BCoV. Positive results for BCoV were observed throughout the period evaluated (2009-2012). The results of BCoV detection are summarized in Table 1.

Table 1. Detection of BCoV by SN-PCR assay from fecal samples of diarrheic beef calves according to the herds and Brazilian regions/states evaluated.

Region	State	Herds		Samples	
		Evaluated	Positive	Evaluated	Positive (%)
Southeast	MG	2	2	23	8 (34.8)
Midwest	MS	5	4	27	13 (48.1)
	MT	1	0	5	0
South	PR	2	1	17	7 (41.2)
North	RO	3	1	21	3 (14.3)
TOTAL	5	13	8 (61.5%)	93	31 (33.3)

MG (Minas Gerais), MS (Mato Grosso do Sul), MT (Mato Grosso), PR (Paraná), RO (Rondônia).

Source: Elaboration of the authors.

The positive impact of the beef cattle production for the Brazilian agribusiness and economy highlights the importance of permanent surveillance of infections that may compromise the health and productivity of cattle herds. The present study represents the first survey conducted for the detection of BCoV specifically in BoRVA-negative fecal samples of diarrheic beef calves from Brazil. The frequency (33.3%) of BCoV positive samples observed was considered high, since this was a cross-sectional study, and reveals that the virus has disseminated in different beef cattle-producing regions of Brazil.

Herds from all the geographical regions evaluated were positive for BCoV infection. There was only one state (MT) where BCoV was not detected. A more detailed evaluation of this state would be useful; however, the inclusion criteria of samples adopted for this study made this impossible. Considering that MT comprises the major cattle herd of Brazil (IBGE, 2011) and that the results presented herein refers to a small number ($n=5$) of fecal samples from a single herd, suggest that the virus is circulating within beef cattle herds from this state.

The clinical signs of diarrhea in calves depend on different factors, such as enteropathogens involvement and virulence of strains, immunological profile of herds, and the management practices adopted in calf production farms (OLIVEIRA FILHO et al., 2007).

The findings of BCoV in diarrheic calves suggests that the virus had some participation on the development of the syndrome, since coronavirus in cattle affects the mature absorptive cells lining the intestinal villi and mucosal surface in the small intestine, leading to villus atrophy and consequent osmotic diarrhea (SAIF, 2011).

Studies have evaluated the presence of BCoV in dairy and beef calves in Brazil, but no differentiation in the rates of detection based on the type of production has been realized (TAKIUCHI et al., 2006; BARRY et al., 2009; STIPP et al., 2009).

Detections of BCoV specifically in beef calves from Brazil have been reported, but in co-infection with other enteropathogens (BRANDÃO et al., 2007; OLIVEIRA FILHO et al., 2007). The BCoV presence exclusively in extensive cattle herds has been poorly evaluated. The results obtained in this study reveal that beef calves managed extensively are important hosts for BCoV infection.

Brazilian dairy calves frequently suffer from diarrhea, and the environmental and management are considered as two important factors that are direct and indirectly implicated in the occurrence of this syndrome (BOTTEON et al., 2008). The intensive and semi-intensive bovine production systems are commonly used in dairy herds. The high number of animals per herd was suggested to favor the occurrence of diarrhea due to the management difficulties, the need of more farm workers, and the increase of common sources of infection, which is directly related to calf mortality, especially in the first months of life. Furthermore, the type of installations used (individual or collective), although frequently underestimated by farmers, can also affect the incidence of diseases in dairy calves (MACHADO NETO et al., 2004; BOTTEON et al., 2008).

Beef cattle in Brazil are commonly created under extensive breeding system. A few years ago, neonatal diarrhea in herds of beef cattle breeds within extensive management was an uncommon event. However, nowadays it has been observed with increasing frequency and intensity of diarrheic episodes in Brazilian beef calves raised extensively.

To enhance the productive performance the commercial beef cattle herds are being submitted to a restructuration of the management practices, which includes breeding techniques and the adoption of shortened breeding season (3 to 4 months). However, these management practices also increase the risk factors for the health of the herds. The use of fixed-time artificial insemination has as its main advantages the improvement of herd fertility and productivity. Consequently, there has

been concentration of parturitions, intensive use of calving areas, the abundance and accumulation of young animals, which are potential hosts for enteropathogens. The early weaning and mixing of calves at different ages are other practices that, together with environmental contamination, increase the challenges and early exposition of calves to microorganisms. The differentiated management system of the extensively raised cattle herds might be related to the sanitary problems recently observed in Brazilian beef herds.

The absence of vaccination against BCoV is another factor favoring the viral dissemination, since the pathogenic potential of BCoV has been underestimated by farmers, who do not usually immunize their cows. It has been suggested that the viral shedding and development of clinical signs of disease is less likely in cattle with high level of BCoV antibodies (SAIF, 2011). Considering the high frequency of BCoV in calves up to 3 weeks of age, passive immunity is relevant for preventing BCoV infection and dam vaccination is essential to provide colostrum rich in anti-BCoV antibodies.

Worldwide, neonatal diarrhea is closely related to BoRVA infection. A comparison of the detection rates of BCoV (15.9% to 24%) by molecular diagnostic methods (TAKIUCHI et al., 2006; BRANDÃO et al., 2007; OLIVEIRA FILHO et al., 2007; BARRY et al., 2009; STIPP et al., 2009) with BoRVA (9.9% to 27.7%) using enzyme immunoassay (EIA) and/or PAGE (JEREZ et al., 2002; BARREIROS et al., 2004; ALFIERI et al., 2006; OLIVEIRA FILHO et al., 2007; BUZINARO et al., 2009; CARUZO et al., 2010; FREITAS et al., 2011) from diarrheic fecal samples of calves up to 90 days of age revealed that the rates of BCoV infection reported herein and in other studies performed in different geographical regions of Brazil are not distant from the rates of BoRVA detections. Interestingly, a high number of studies that have evaluated the BoRVA infections has been performed, while the investigations of BCoV are not frequently realized, probably leading to inadequate interpretation of available

epidemiological data. These observations strongly indicate that coronavirus infections are neglected by both field veterinaries and farmers.

In conclusion, the BCoV detection from fecal samples of diarrheic calves demonstrates that the viral infection has been frequent in extensively managed beef cattle over the last 4 years (2009-2012). The results revealed that coronaviruses has important participation in the neonatal diarrhea complex of beef cattle raised under extensive breeding system in different geographical regions of Brazil. Specific animal health programs and the orientation of professionals and farm workers should be established in an attempt to prevent productive and economic losses.

References

- ALFIERI, A. A.; PARAZZI, M. E.; TAKIUCHI, E.; MEDICI, K. C.; ALFIERI, A. F. Frequency of group A rotavirus in diarrhoeic calves in Brazilian cattle herds, 1998-2002. *Tropical Animal Health and Production*, Edinburgh, v. 38, n. 7-8, p. 521-526, 2006.
- BARREIROS, M. A. B.; ALFIERI, A. F.; MÉDICI, K. C.; LEITE, J. P. G.; ALFIERI, A. A. G and P Genotypes of group a rotavirus from diarrhoeic calves born to cows vaccinated against the NCDV (P[1],G6) Rotavirus Strain. *Journal of Veterinary Medicine, Series B*, Berlín, v. 51, n. 3, p. 104-109, 2004.
- BARRY, A. F.; ALFIERI, A. F.; STIPP, D. T.; ALFIERI, A. A. Bovine coronavirus detection in a collection of diarrheic stool samples positive for group a bovine rotavirus. *Brazilian Archives of Biology and Technology*, Curitiba, v. 52, p. 45-49, 2009.
- BOTTEON, R. D. C. C. M.; BOTTEON, P. D. T. L.; SANTOS JÚNIOR, J. D. C. B.; PINNA, M. H.; LÓSS, Z. G. Frequência de diarreia em bezerros mestiços sob diferentes condições de manejo na região do médio Paraíba - Rio de Janeiro e Minas Gerais. *Brazilian Journal of Veterinary Research and Animal Science*, São Paulo, v. 45, n. 2, p. 153-160, 2008.
- BRANDÃO, P. E.; VILLARREAL, L. Y. B.; SOUZA, S. L. P. de; RICHTZENHAIN, L. J.; JEREZ, J. A. Mixed infections by bovine coronavirus, rotavirus, and *Cryptosporidium parvum* in an outbreak of neonatal diarrhea in beef cattle. *Arquivos do Instituto Biológico*, São Paulo, v. 74, n. 1, p. 33-34, 2007.

- BUZINARO, M. G.; SAMARA, S. I.; PEREIRA, E. A. S.; FUENTES, D. B.; OLIVEIRA, M. C. S. Ocorrência dos genótipos G e P do rotavírus do grupo A em bezerros de rebanho de corte no estado de São Paulo, Brasil. *Arquivos do Instituto Biológico*, São Paulo, v. 76, n. 1, p. 99-105, 2009.
- CARUZO, T. A. R.; BRITO, W. M. E. D.; MUNFORD, V.; RÁCZ, M. L. Molecular characterization of G and P-types bovine rotavirus strains from Goiás, Brazil: high frequency of mixed P-type infections. *Memorias do Instituto Oswaldo Cruz*, Rio de Janeiro, v. 105, n. 8, p. 1040-1043, 2010.
- CHO, K. O.; HASOKSUZ, M.; NIELSEN, P. R.; CHANG, K. O.; LATHROP, S.; SAIF, L. J. Cross-protection studies between respiratory and calf diarrhea and winter dysentery coronavirus strains in calves and RT-PCR and nested PCR for their detection. *Archives of Virology*, New York, v. 146, n. 12, p. 2401-2419, 2001.
- CHO, Y. I.; KIM, W. I.; LIU, S.; KINYON, J. M.; YOON, K. J. Development of a panel of multiplex real-time polymerase chain reaction assays for simultaneous detection of major agents causing calf diarrhea in feces. *Journal of Veterinary Diagnostic Investigation*, Columbia, v. 22, n. 4, p. 509-517, 2010.
- FREITAS, P. P. S.; UYEMURA, S. A.; SILVA, D. G.; SAMARA, S. I.; BUZINARO, M. G. Rotavírus bovino: fatores de risco, prevalência e caracterização antigênica de amostras em rebanhos leiteiros no estado de São Paulo. *Arquivo Brasileiro de Medicina Veterinária e Zootecnia*, Belo Horizonte, v. 63, n. 4, p. 820-827, 2011.
- GARAICOECHEA, L.; BOK, K.; JONES, L. R.; COMBESSIES, G.; ODEON, A.; FERNANDEZ, F.; PARRENO, V. Molecular characterization of bovine rotavirus circulating in beef and dairy herds in Argentina during a 10-year period (1994-2003). *Veterinary Microbiology*, Amsterdam, v. 118, n. 1-2, p. 1-11, 2006.
- INSTITUTO BRASILEIRO DE GEOGRAFIA E ESTATÍSTICA - IBGE. Produção da pecuária municipal. Rio de Janeiro: IBGE, 2011. Disponível em: <http://ftpftp.ibge.gov.br/Producao_Pecuaria/Producao_da_Pecuaria_Municipal/2011/ppm2011.pdf>. Acesso em: 08 fev. 2012.
- INTERNATIONAL COMMITTEE ON TAXONOMY OF VIRUSES - ICTV. Virus taxonomy: classification and nomenclature of viruses: eight report of the international committee on taxonomy of viruses. San Diego: Elsevier Academic Press, 2011. Disponível em: <http://talk.ictvonline.org/files/ictv_official_taxonomy_updates_since_the_8th_report/m/vertebrate-official/4165.aspx>. Acesso em: 03 nov. 2012.
- JEREZ, J. A.; BRANDÃO, P. E.; BUZINARO, M. G.; GREGORI, F.; ROSALES, C. A. R.; ITO, F. H.; SAKAI, T. Detecção de rotavírus e coronavírus em fezes de bezerros neonatos com diarreia criados em vários municípios do estado de São Paulo, Brasil. *Arquivos do Instituto Biológico*, São Paulo, v. 69, n. 2, p. 19-23, 2002.
- MACHADO NETO, R.; FARONI, C. E.; PAULETTI, P.; BESSI, R. Levantamento do manejo de bovinos leiteiros recém-nascidos: desempenho e aquisição de proteção passiva. *Revista Brasileira de Zootecnia*, Viçosa, v. 33, n. 6, p. 2323-2329, 2004.
- MARTELLA, V.; BÁNYAI, K.; MATTHIJNSSENS, J.; BUONAVOGLIA, C.; CIARLET, M. Zoonotic aspects of rotaviruses. *Veterinary Microbiology*, Amsterdam, v. 140, n. 3-4, p. 246-255, 2010.
- MAYAMEEL, A.; MOHAMMADI, G.; YAVARI, S.; AFSHARI, E.; OMIDI, A. Evaluation of relationship between *Rotavirus* and *Coronavirus* infections with calf diarrhea by capture ELISA. *Comparative Clinical Pathology*, London, v. 19, n. 6, p. 553-557, 2010.
- OLIVEIRA FILHO, J. P.; SILVA, D. P. G.; PACHECO, M. D.; MASCARINI, L. M.; RIBEIRO, M. G.; ALFIERI, A. A.; ALFIERI, A. F.; STIPP, D. T.; BARROS, B. J. P.; BORGES, A. S. Diarréia em bezerros da raça Nelore criados extensivamente: estudo clínico e etiológico. *Pesquisa Veterinária Brasileira*, Rio de Janeiro, v. 27, n. 10, p. 419-424, 2007.
- PEREIRA, H. G.; AZEREDO, R. S.; LEITE, J. P. G.; CANDEIAS, J. A. N.; RÁCZ, M. L.; LINHARES, A. C.; GABBAY, Y. B.; TRABULSI, J. R. Electrophoretic study of the genome of human rotaviruses from Rio de Janeiro, São Paulo and Pará, Brazil. *Journal of Hygiene*, Cambridge, v. 90, n. 1, p. 117-125, 1983.
- SAIF, L. J. Coronaviridae. In: MACLACHLAN, N. J.; DUBOVI, E. J. (Ed.). *Fenner's veterinary virology*. 4.th ed. San Diego: Academic Press, 2011. cap. 24, p. 393-413.
- STIPP, D. T.; BARRY, A. F.; ALFIERI, A. F.; TAKIUCHI, E.; AMUDE, A. M.; ALFIERI, A. A. Frequency of BCoV detection by a semi-nested PCR assay in faeces of calves from Brazilian cattle herds. *Tropical Animal Health and Production*, Edinburgh, v. 41, n. 7, p. 1563-1567, 2009.
- TAKIUCHI, E.; BARRY, A. F.; ALFIERI, A. F.; FILIPPSEN, P.; ALFIERI, A. A. An outbreak of winter dysentery caused by bovine coronavirus in a high-production dairy cattle herd from a tropical country. *Brazilian Archives of Biology and Technology*, Curitiba, v. 52, p. 57-61, 2009. Número Especial.
- TAKIUCHI, E.; STIPP, D. T.; ALFIERI, A. F.; ALFIERI, A. A. Improved detection of bovine coronavirus N gene in faeces of calves infected naturally by a semi-nested PCR assay and an internal control. *Journal of Virological Methods*, Amsterdam, v. 131, n. 2, p. 148-154, 2006.