Trop Anim Health Prod (2009) 41:1563–1567
DOI 10.1007/s11250-009-9347-2

ORIGINAL PAPER

Frequency of BCoV detection by a semi-nested PCR assay in faeces of calves from Brazilian cattle herds

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Accepted: 27 March 2009 / Published online: 16 April 2009

Abstract Bovine coronavirus (BCoV) is one of the main causes of neonatal calf diarrhoea. Several diagnostic assays have been employed to detect the presence of the virus in stool samples from calves. Despite this, the frequency of BCoV infection among Brazilian and even South American cattle herds has yet to be well characterised. This study describes the occurrence of BCoV infection among calves from dairy and beef herds in four Brazilian states. A total of 282 stool samples from 1 to 60-day-old calves were evaluated for the presence of BCoV by a semi-nested (SN) PCR assay. The animals were from herds (n=23) located in three geographical regions in Brazil (south, southeast, and center-west). The specific BCoV amplicon was detected in 15.6% (44/282) of the faecal specimens examined, of which 95.4% (42/44) were from diarrhoeic and 4.6% (2/44) from asymptomatic calves. The specificity of the SN-PCR amplicons was evaluated by restriction fragment length polymorphism (RFLP) analysis. The results show that the BCoV is widespread, mainly among calves from 16 to 30-days-old (p=0.0023), and verify the association between BCoV infection and clinical signs of diarrhoea (p=0.005). These findings emphasise the importance of this virus in enteric infections of Brazilian cattle herds.

Keywords Cattle herds · Calves · Diarrhoea · Bovine coronavirus · Semi-nested PCR

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Introduction

Diarrhoea is one of the most important diseases in young calves worldwide, causing considerable economic losses as a consequence of the high resulting morbidity and, in some cases, mortality. Several factors, either singly or in combination, may cause neonatal diarrhoea including environmental, managemental, nutritional, and infectious. Enteric infections may be caused by protozoa, bacteria and/or viruses. In calves, the viruses most commonly associated with neonatal diarrhoea are the group A rotavirus and coronavirus; it is not unusual for both viruses to concomitantly infect calves. Mixed infections might also occur between viruses and bacteria and/or protozoan (Alfieri et al. 2006; Oliveira Filho et al. 2007).

Coronavirus genus belongs to the *Coronaviridae* family and is divided into three genetically distinct groups. Bovine coronavirus (BCoV) is classified in group 2 and is a pneumoenteric virus that causes diarrhoea and respiratory disease in calves, and winter dysentery in adult cattle. The virus has an envelope of

120 to 160 nm in diameter and a helical nucleocapsid. The genome is a single-stranded, positive-sense RNA molecule of 27 to 32 kb. From the 13 open reading frames (ORFs) of the genome, five major structural proteins are encoded, including the nucleocapsid protein (N) (ICTVdB 2008).

In order to elucidate the frequency of infection in neonatal calves, the occurrence of BCoV has been studied in herds from several countries. The frequency of infection shows notable variation between different geographical regions, the production type (dairy or beef) of the evaluated herds, and diagnostic techniques. The highest rate of BCoV infection was reported in calves from Brazil (39%) and Ethiopia (38.9%) using HA/HI and ELISA assays, respectively (Abraham et al. 1992; Jerez et al. 2002). In other countries from America, Asia and Europe, the frequency ranged from 4 to 15.6%, based mostly on the results of PCR assays (Reynolds et al. 1986; Snodgrass et al. 1986; De La Fuente et al. 1998; Khalili et al. 2006; Park et al. 2007). In Brazil, only a few studies have evaluated the BCoV occurrence in stool samples from calves (Jerez et al. 2002; Takiuchi et al. 2006; Oliveira Filho et al. 2007). The rates of infection measured ranges from 16 to 39%; however, the occurrence of BCoV infection has not been well characterised, since the number of evaluated faecal samples examined was relatively low in all these studies. Furthermore, the stool samples were collected from only one region and the animal management conditions were also different, making it difficult to establish a comparison and to draw conclusions about the overall BCoV infection frequency among Brazilian cattle herds.

A few different methods exist to show the presence of BCoV, such as electron microscopy (EM), isolation in tissue cultures, hemagglutination (HA) / inhibition (HI) test, and ELISA, although most of them are not useful for diagnosis because of the long time requirements or low specificity. A reverse transcription-polymerase chain reaction (RT-PCR) assay for BCoV detection in calf faecal samples reportedly shows high sensitivity and specificity when compared to other techniques (Cho et al. 2001). Additionally, a semi-nested (SN) PCR assay was recently developed to detect the N gene of BCoV in fresh or frozen faecal samples from naturally infected calves (Takiuchi et al. 2006).

The aim of this study was to describe the rate of BCoV infection in calves from beef and dairy cattle herds in different Brazilian geographical regions.

Materials and methods

Stool samples

Faecal specimens were collected from March to December of 2004 from 282 calves of up to 60 days of age. The calves were from 23 separate herds comprising beef (n=4), dairy (n=15) and mixed beef and dairy (n=4) cattle herds located in four Brazilian states: Mato Grosso (MT; n=181), Minas Gerais (MG; n=11), Paraná (PR; n=71), and São Paulo (SP; n=19). Sixty-one samples had normal consistency while 221 were diarrhoeic. Stool samples were considered diarrhoeic when the consistency was observed to vary macroscopically from pasty to liquid.

Faecal suspensions were prepared at 10 to 20% in 0.01 M phosphate-buffered saline (PBS) pH 7.2, and centrifuged at 3.000 g for 15 min at 4°C. The supernatants were used for RNA extraction.

Virus and cell culture

The BCoV Kakegawa strain was propagated in HRT-18 (human rectal tumor-18) cells maintained in Dulbecco's Eagle Media (Gibco BRL, USA), supplemented with 10% foetal bovine serum (Gibco BRL, USA), gentamicin (55 μg/ml) (Sigma Co., USA), and amphotericin B (2.5 μg/ml) (Bristol-Meyers Squibb, Brazil) was used as a positive control in the SN-PCR assay.

RNA extraction

Aliquots (400 μl) of the supernatant from the faecal suspension were treated with SDS at a final concentration of 1% (v/v) and kept at 56°C for 30 min. Thereafter, a combination of phenol/chloroform/isoamyl alcohol and silica/guanidinum isothiocyanate methods were performed (Alfieri et al. 2006) to extract the RNA. The RNA was eluted in 50 μl of ultra-pure RNase-free DEPC-treated sterile water and used in the SN-PCR assay. Sterile water was included as a negative control for the RNA extraction.

Semi-nested PCR assay

The SN-PCR assay was performed according to Takiuchi et al. (2006) with the primers BCoV1 sense (5′-CGATGAGGCTATTCCGAC-3′) and BCoV2 antisense (5′-TGTGGGTGCGAGTTCTGC-3′); the latter was also used in the reverse transcription (RT). For the second round of amplification (semi-nested), BCoV3 sense (5′-TTGCTAGTCTTGTTCTGGC-3′) and BCoV2 antisense were used. The primer sequences were based on the highly conserved region of the N gene of the Mebus strain (GenBank accession number U00735), and the reactions were also performed according to Takiuchi et al. (2006). The sizes of predicted PCR and SN products were 454 and 251 bp, respectively.

The SN-PCR products were analysed by electrophoresis on a 2% agarose gel stained with 0.5 μg/ml ethidium bromide, and visualised under UV light.

RFLP analysis

The specificity of the 251 bp fragments from the SN-PCR was confirmed by restriction fragment length polymorphism using *Hae III* (Invitrogen™, USA). The reaction was performed according to the manufacturer's instructions.

Statistical analysis

Either the chi-square (χ^2) or Fisher's exact test with 95% of confidence limits ($p<0.05$) was used to verify the association between the BCoV infection and variables such as faecal characteristics (diarrhoea/normal) and age of the calves. Statistical analysis was performed using the software Epi Info™, version 6.04d (Dean et al. 1998).

Results

From the 282 stool samples analysed by SN-PCR assay, 44 (15.6%) were positive for BCoV, with 42 (14.9%) being from diarrhoeic and 2 (0.7%) from asymptomatic calves (Table 1). The specificity of the amplicons was confirmed by RFLP analysis using the restriction enzyme *Hae III*. The SN-PCR amplicons of 251 bp (second round) yielded 88 and 163 bp fragments.

Table 1 Bovine coronavirus detection by semi-nested PCR assay from diarrhoeic and asymptomatic calves from Brazilian dairy and beef cattle herds

Faecal consistency	Number of samples (%)		
	Positive	Negative	Total
Diarrhoeic	42 (19.0)[a]	179 (81.0)	221
Normal	2 (3.3)	59 (96.7)	61
Total	44 (15.6)	238 (84.4)	282

[a] $p=0.005$

BCoV was present in 19.0% (42/221) of the diarrhoeic stool samples. Only two (3.3%) of the 61 specimens with normal consistency were positive for BCoV as determined by the SN-PCR assay. The statistical analysis revealed an association ($p=0.005$) between the presence of the virus and clinical signs of diarrhoea (Table 1). Positive specimens were detected in the four Brazilian states included in the study, but, since the number of evaluated samples in MG and SP was low, it was not possible to perform statistical analyses.

All the four age-groups into which the calves were divided had at least one faecal sample positive for BCoV. However, 16 to 30-day-old calves showed a higher (29.9%) frequency of BCoV infection compared to the other three age groups (Table 2), although the statistical association ($p=0.0023$) was verified only when comparing the 16 to 30-day-old group with the 31 to 45-day-old group.

Table 2 Distribution of semi-nested PCR assay results for BCoV detection in diarrhoeic faecal samples from calves by age groups

Age group (days)	Number of faecal specimens (%)		
	Positive	Negative	Total
1–15	1 (11.1)	8 (88.9)	9
16–30	29 (29.9)[a]	68 (70.1)	97
31–45	9 (10.5)	77 (89.5)	86
46–60	3 (10.3)	26 (89.7)	29
Total	42 (19.0)	179 (81.0)	221

[a] $p=0.0023$

Discussion

Although the coronavirus has been studied in a few countries, in Brazil, only limited studies have been conducted on the involvement of BCoV infection in calf diarrhoea. In the present study, the overall rate of BCoV infection among 1 to 60-day-old calves in four Brazilian states was 15.6% out of a significant number (n=282) of stool samples that were analysed by an SN-PCR assay. This data reveals that BCoV is widespread among Brazilian dairy and beef cattle herds, at least among younger animals, as diarrhoea is among the most important health problems that afflicts calves.

As the technique (SN-PCR) employed for the diagnostic assay was reported to be more sensitive than other assays such as antigen-capture ELISA, the frequency rate presented in this study more accurately reflects the actual infection rate of the Brazilian cattle herds (Cho et al. 2001). Furthermore, the primers used in this study were designed based on a highly conserved region of the N gene, which is the most abundant protein found in infected cells. The N RNA is also abundant and thus diagnosis based on the RNA is sensible as it represents a good marker for BCoV infection (Hiscox et al. 2001; Takiuchi et al. 2006).

The infection rate we measured in diarrhoeic calves (19%) was lower than that determined by a previous study performed in Brazil, in which Jerez et al. (2002) determined 39% of stool samples to be positive for BCoV using HA/HI assay. Since the HA test was performed directly on faecal specimens, the non-specific hemagglutinins present in the faeces may cause false-positive results justifying this higher rate of BCoV infection (Sato et al. 1977). Moreover, in this previous study, only dairy herds (n=16) were included, which does not necessarily reflect the overall rate of BCoV infection among Brazilian cattle herds. Another study performed only on faecal samples from calves with diarrhoea also presented a higher infection rate (24%) than that reported in this study. Since the previous study used the SN-PCR assay with the same primers and conditions, the higher reported frequency may be justified by the low sampling used that did not necessarily reflect the actual infection rate of BCoV among calves from Brazilian herds (Takiuchi et al. 2006).

Only 3.3% (2/61) of the non-diarrhoeic stool samples were determined to be positive by the SN-PCR assay, showing the involvement of and the role played by BCoV in the development of the clinical signs. Furthermore, as the faecal samples were collected only once from each animal, these calves may not have had diarrhoea only at the moment of sampling and possibly presented clinical signs before or after the sampling. Additionally, they may also have been infected but were asymptomatic as a result of viral load, strain virulence, passive immune status, environmental, and management factors (Snodgrass et al. 1986).

A previous study describes that BCoV infection was more frequent among calves of up to 30 days of age (Clark 1993). The present study shows similar results, since 16 to 30-day-old calves showed a significantly higher (p=0.0023) frequency of infection when compared to the other age groups evaluated. Similar results were also described, with a higher rate of viral shedding, in 15 to 18-day-old calves (Jerez et al. 2002). The association between calf age and BCoV infection was probably due to the decreasing levels of antibodies acquired from the colostrum. The frequency of infection among diarrhoeic calves in the other age groups was very similar (Table 2), but higher than that found in the control group (asymptomatic calves), which showed a BCoV infection rate of 3.3%. This result shows that calves of up to 60 days of age, when infected with BCoV, frequently develop the clinical symptoms, even with some passive immunity in the first week of life or natural resistance that acquires significant levels at 30 days of age.

Group A rotavirus is a well-known cause of acute diarrhoea in young calves that is responsible for the majority of gastroenteritis cases worldwide (Flewett and Woode 1978). In Brazil, a recent study, conducted in animals up to 90 days, found 19.4% of diarrhoeic beef and dairy calves to be infected by this virus, but the infection rate in diarrhoeic 1 to 60 day-old animals was 22.5% (Alfieri et al. 2006). The rate of BCoV infection in diarrhoeic calves herein presented (19.0%) was similar. Surprisingly, when comparing only results from stool samples taken from calves of up to 30 days of age from both studies, the BCoV frequency rate of infection was slightly higher than the rate of group A rotavirus infection determined by the previous study (28.3 and 26%, respectively). In this way, the BCoV infection must also be considered an important neonatal enteric disease since it was

present at a similar frequency as rotaviruses and caused diarrhoea in most of the cases. The four Brazilian states included in this study were also evaluated in the rotavirus study, which makes the comparison more accurate.

The data from this study show that the BCoV, like rotaviruses, is an important etiological agent of neonatal calf diarrhoea in Brazilian cattle herds. This study gives a more global view of BCoV infection in cattle herds, since it includes both beef and dairy calves. In this way, the BCoV frequency rate reported here, especially from diarrhoeic calves, reinforces the importance of the adoption of prophylactic measures for the BCoV infection control in cattle herds such as cow vaccination to increase passive immunity.

Acknowledgements We would like to thank to the Brazilian Institutes CNPq, CAPES, FINEP, and Fundação Araucária for financial support. Alfieri, A.A. and Alfieri, A.F. are recipient of CNPq fellowships.

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