

## Standard Article

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## Detection of Bovine Coronavirus in Healthy and Diarrheic Dairy Calves

D.E. Gomez , L.G. Arroyo, Z. Poljak, L. Viel, and J.S. Weese**Background:** BCoV is identified in both healthy and diarrheic calves, complicating its assessment as a primary pathogen.**Objectives:** To investigate the detection rates of bovine coronavirus (BCoV) in feces of healthy and diarrheic calves and to describe the usefulness of a pancoronavirus reverse transcriptase (RT) PCR (PanCoV-RT-PCR) assay to identify BCoV in samples of diarrheic calves.**Animals:** Two hundred and eighty-six calves <21 days. Calves with liquid or semiliquid feces, temperature >39.5°C, and inappetence were considered as cases, and those that had pasty or firm feces and normal physical examination were designated as controls.**Methods:** Prospective case-control study. A specific BCoV-RT-PCR assay was used to detect BCoV in fecal samples. Association between BCoV and health status was evaluated by exact and random effect logistic regression. Fecal (n = 28) and nasal (n = 8) samples from diarrheic calves were tested for the presence of BCoV by both the PanCoV-RT-PCR and a specific BCoV-RT-PCR assays. A Kappa coefficient test was used to assess the level of agreement of both assays.**Results:** BCoV was detected in 55% (157/286) of calves; 46% (66/143), and 64% (91/143) of healthy and diarrheic calves, respectively. Diarrheic calves had higher odds of BCoV presence than healthy calves (OR: 2.16, 95% CI: 1.26 to 3.83, *P* = 0.004). A good agreement between PanCoV-RT-PCR and BCoV-RT-PCR to detect BCoV was identified ( $\kappa$  = 0.68, 95% CI: 0.392 to 0.967; *P* < 0.001).**Conclusions and Clinical Importance:** BCoV was more likely to be detected in diarrheic than healthy calves. The PanCoV-RT-PCR assay can be a useful tool to detect CoV samples from diarrheic calves.**Key words:** Emerging; Epidemiology; Infectious diseases; Pancoronavirus; Prevalence; Re-emerging.

Neonatal diarrhea is the most important cause of disease in calves worldwide,<sup>1,2</sup> leading to large economic losses to cattle herds.<sup>3</sup> Common etiologic agents involved in calf diarrhea include *Escherichia coli*, *Cryptosporidium* spp, bovine rotavirus (BRoV), and bovine coronavirus (BCoV). Most of the studies investigating the etiology of calf diarrhea have focused on sick calves.<sup>4–8</sup> However, some agents, especially viruses, could be frequently detected in healthy calves.<sup>9–11</sup> For instance, BCoV has been identified in both healthy and diarrheic calves,<sup>10,11</sup> complicating assessment of its role as a primary pathogen. BCoV is the causative agent of diarrhea in newborn calves,<sup>12</sup> winter dysentery in adult cows,<sup>13</sup> and respiratory tract illness in calves and adults.<sup>14,15</sup> However, some studies have identified numerical but not statistical association between BCoV in fecal or nasal samples and clinical signs.<sup>16–18</sup> Cases of BCoV enteritis occur in both dairy and beef herds, with clinical signs

**Abbreviations:**

BCoV	bovine coronavirus
BRoV	bovine rotavirus
ELISA	enzyme-linked immunosorbent assay
PanCoV	pancoronavirus
PCR	polymerase chain reaction
RT	reverse transcription

developing between 5 and 30 days of life and a peak of incidence between days 7 and 10.<sup>19</sup> Transmission from calf-to-calf is mainly by the fecal-oral route but respiratory transmission can be possible.<sup>16,19</sup> Infected calves can excrete high levels of virus within 48 h of experimental infection, and this can continue up to 14 days.<sup>19</sup> The mechanisms of diarrhea due to BCoV infection are still unclear, but malabsorption and hypersecretion appear to play important roles.<sup>20</sup> Clinically recovered calves can continue to shed low levels of virus for weeks.<sup>21,22</sup>

Diagnosis of BCoV enteritis can be achieved by viral culture, antigen-capture ELISA, hemagglutination assay using mouse erythrocytes, and PCR.<sup>19</sup> Recently, a pancoronavirus reverse transcription (RT) PCR assay (PanCoV-RT-PCR) was described to identify human CoV from samples of people with respiratory diseases,<sup>23</sup> with primers that should amplify a 251-bp fragment of the polymerase gene of all known coronaviruses.<sup>24</sup> The utility of PanCoV-RT-PCR to detect BCoV in samples of animals with clinical diseases is yet to be determined.

The objectives of this study were to investigate the detection rates of BCoV in feces of healthy and diarrheic dairy calves and to describe the usefulness of a PanCoV-RT-PCR assay to identify BCoV in nasal and fecal samples of a group of calves from a dairy farm suffering an outbreak of diarrhea.

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## Material and Methods

### *Detection of BCoV in Healthy and Diarrheic Dairy Calves from Southern Ontario*

#### *Sample Size Calculation*

A prospective case-control study approach was used, and the sample size was calculated based on an estimated 30% BCoV detection rate in healthy calves. The set parameters to detect an odds ratio of 2, including equal number of cases and controls, with a 95% confidence and a power of 0.8, yielded a total of a 138 calves in each group.

#### *Study Design, Definitions, and Sample Collection*

Calves from six dairy farms selected from a convenience sample of commercial dairy farms within a 120-km radius from the University of Guelph (Guelph, ON) were sampled between the fall of 2014 and the fall of 2016. The number of diarrheic calves enrolled from each farm (approximately 5% of the total number of calves raised at each farm per year), the year and season in which the samples were collected, the management practices as well as the total duration the farm was enrolled in the study are presented in Table 1. Cases (diarrheic) and control (healthy) calves were farm-, season-, year-, and age-matched. Cases and controls were defined on the basis of the physical examination and fecal consistency (liquid, semiliquid, pasty, or firm). Calves that had liquid or semiliquid feces, fever (temperature >39.5°C) and decreased appetite were considered as diarrheic (i.e., cases), and those that had pasty or firm feces and normal physical examination were designated as controls. Once a diarrheic calf was identified an age-matched ( $\pm 2$  days), control calf was enrolled on each farm. Calves were not included in the study if they had a previous incident of diarrhea since birth (only the first case of diarrhea was included in the study). All samples were screened for the presence of BCoV by the BCoV-RT-PCR assay.

### *Bovine Coronavirus RT-PCR Assay*

Briefly, RNA was extracted with a commercial kit<sup>a</sup> according to the manufacturer's instructions. Amplification of an 81 bp fragment of the BCoV nucleocapsid protein was performed with the following primer set: Cor-FW (5'-CTAGTAACCAGGCTGATGTCAAT ACC-3') and Cor-RV (5'-GGCGGAAACCTAGTCGGAATA-3'). These one-step RT-PCR assays (One-Step RT-PCR kit; QIAGEN) were undertaken in a 50  $\mu$ L reaction volume containing 10  $\mu$ L RNA-extract, 10  $\mu$ L 5 $\times$  QIAGEN One-Step RT-PCR Buffer, 2  $\mu$ L dNTP mix (final concentration of 400  $\mu$ M of each dNTP), 1.8  $\mu$ L One-Step RT-PCR Enzyme Mix (a combination of Omniscript and Sensiscript reverse transcriptase and Hot-StarTaq DNA polymerase)<sup>b</sup>, 4  $\mu$ M of each primer, and RNase-free water to 50  $\mu$ L. The reaction was carried out with an initial reverse transcription step at 50°C for 30 minutes, followed by PCR activation at 95°C for 10 minutes, 40 cycles of amplification (30 seconds at 94°C; 45 seconds at 60°C; 1 minute at 72°C), and a final extension step at 72°C for 10 min in a GeneAmp PCR system 9600 thermal cyclers.<sup>c</sup> A BCoV isolate recovered from a cow suffering from winter dysentery was used as a positive control. Specific methods to assess for the presence of nonspecific inhibitors of PCR were not performed.

### *Use of Pancoronavirus Assay to Detect CoV in Fecal and Nasal Samples of Diarrheic Calves from an Outbreak*

#### *Sampling*

A large dairy farm located in Southern Ontario, Canada, experienced a sudden increase in the incidence of diarrhea and death attributable to diarrhea in calves younger <30 days of age that was sustained over a 2-month period. At the time of the outbreak, approximately 200 preweaned calves were maintained in the calf barn. The calculated incidence of diarrhea increased from (calculated from the month before the outbreak) 36% to 57% (54/94)

**Table 1.** Farms characteristics, number, and demographic data of the calves tested for the presence of BCoV, year, and season of sampling.

	Farm 1	Farm 2	Farm 3	Farm 4	Farm 5	Farm 6
Farms characteristics						
Breed	Holstein	Holstein	Holstein	Holstein	Holstein	Jersey
Calves born per year	800	1000	350	330	150	80
Housing	Group pen	Individual	Group pen	Hutches	Hutches	Group pen
Bedding	Saw dust	Shavings	Straw	Straw	Straw	Straw
Colostrum (pooled)	4L first 6 h	6L first 6 h	6L first 4 h	5L first 6 h	5L first 6 h	5L first 6 h
Diet (up to 12 wks.)	Pasteurized milk	Milk replacer	Pasteurized milk	Pasteurized milk	Pasteurized milk	Pasteurized milk
Feeding method	Robot machine	Individual bucket	Robot machine	Individual bucket	Individual bucket/ Robot machine	Bottle
Vaccination of pregnant cows <sup>f</sup>	Yes	Yes	Yes	Yes	Yes	Yes
Sampled calves						
Calves enrolled	84	94	48	30	20	10
Sex (n,%)						
Female	60 (71%)	65 (69%)	39 (81%)	30 (100%)	20 (100%)	8 (80%)
Male	24 (29%)	30 (31%)	9 (19)	0 (0%)	0 (0%)	2 (20%)
Age (mean $\pm$ SD)	10 $\pm$ 4.7	8.9 $\pm$ 2.2	7.7 $\pm$ 3.4	8.5 $\pm$ 2.1	10.5 $\pm$ 1.7	9.5 $\pm$ 2.7
Sampling year and season						
	F-2015	F-2014	F-2014	W-2015	Sp-2016	Su-2016
	W-2016	W-2015	W-2015	Sp-2015	Su-2016	F-2016
	Sp-2016	Sp-2015	Sp-2015	Su-2015		
	Su-2016	Su-2015				

wks, weeks; F, fall; W, winter; Sp, spring; Su, summer.

<sup>f</sup>In all farms, cows were vaccinated against bovine rotavirus and coronavirus 8 and 4 weeks before calving using a vaccine containing attenuated strains of bovine rotavirus and coronavirus.

during the outbreak period in calves <30 days. The case fatality increased from 5% to 17% (9/54). Eight severely affected diarrheic calves were referred to the Ontario Veterinary College Health Sciences Centre (OVC-HSC) for diagnosis and treatment of diarrhea. Rectal swabs or fecal samples and nasal swabs were collected at admission. Fecal samples were tested by culture for *Salmonella* spp using Rappaport-Vassiliadis and tetrathionate broths and inoculation onto brilliant green sulphur and Hektoen agars. *Escherichia coli* was isolated on blood and MacConkey's agars, and isolates were serotyped using antiserum against F4 (K88) and F5 (K99) fimbriae for detection of ETEC. Sucrose wet mount assay was used for detection of *Cryptosporidium* spp and *Giardia* Spp, and real-time polymerase chain reaction was performed to detect ruminant rotavirus A and B. PanCoV-RT-PCR and BCoV-RT-PCR assays were used to screen fecal samples and nasal swabs for the presence of BCoV. Additionally, a farm visit took place 3 days after presentation of the eight affected calves. Fecal samples were collected from 20 diarrheic calves for PanCoV-RT-PCR and BCoV-RT-PCR.

### Pancoronavirus RT-PCR Assay

Amplification of a 251-bp fragment of the BCoV polymerase gene was performed with the following primer set: Cor-FW (5'-ACWCARHTVAAYYTNAARTAYGC-3') and Cor-RV (5'-TCRCAYTTDGGRTARTCCCA-3'). These one-step RT-PCR assays were undertaken similarly than those described for the BCoV-RT assay. The reaction was carried out with an initial reverse transcription step at 50°C for 30 minutes, followed by PCR activation at 95°C for 15 minutes, 50 cycles of amplification (30 seconds at 94°C; 30 seconds at 48°C; 1 minutes at 72°C), and a final extension step at 72°C for 10 minutes in a GeneAmp PCR system 9600 thermal cycler.<sup>c</sup> Specific methods to assess for the presence of nonspecific inhibitors of PCR were not performed. A BCoV isolate recovered from a cow suffering from winter dysentery was used as a positive control. All samples were further tested with specific BCoV primers.

### Statistical Analysis

The primary exposure of interest for development of diarrhea in this study was the presence of BCoV, and sex of animals was

considered as a possible confounder. The matching variables were as follows: age, farm, and time of diarrhea cases. We, therefore, summarized primary exposure in case and control animals by simple cross-tabulations, and additionally examined frequency of primary exposure in case and control animals across variables representing the matched variables including age, farm, calendar year, and season. Statistical analysis was conducted by two approaches: conditional exact logistic regression accounting for the matched pair as strata and by random intercept logistic regression where the matched pair designation represented a random effect on the intercept. After the univariable analysis, difference in the effect of the BCoV across each of the matched factors was evaluated by evaluating statistical significance of interaction between BCoV with each of the matching variables. The final models (Exact logistic regression and Random effect logistic regression) considered inclusion of all variables that were statistically significant at liberal *P*-value of <0.20. Interaction between exposure of interest and sex was also tested. Choice of the final model was based on the combination of the achieved level of statistical significance and the AIC obtained in the random effect model. Analysis was performed by a statistical software.<sup>d</sup>

To test whether the PanCoV-RT-PCR and BCoV-RT-PCR assays were equally likely in detecting CoV in samples from sick calves, the McNemar's test was used. The level of agreement between both assays in detecting BCoV was assessed by Kappa coefficient test. Analysis was performed by the statistical software JMP.<sup>e</sup>

## Results

### Detection of BCoV in Healthy and Diarrheic calves from Farms of Ontario

A total of 143 diarrheic calves between 3 and 21 days of age and 143 healthy farm- and age-matched control calves were enrolled (Table 1). None of the healthy calves developed diarrhea within one week after sampling. Cases were distributed across six herds (Fig 1), all four seasons (Fig 2), and over a period of 3 years (Fig 3). BCoV was detected in 157/286 (55%) of calves;

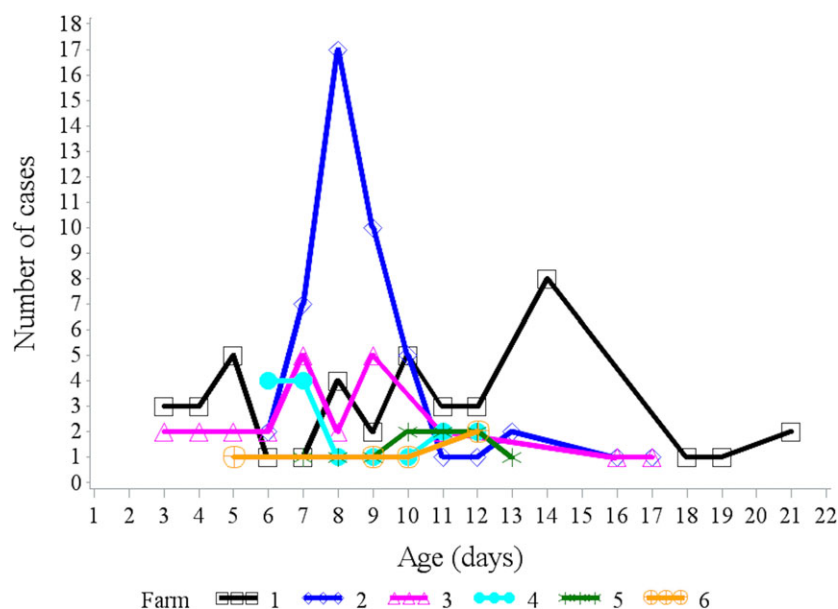
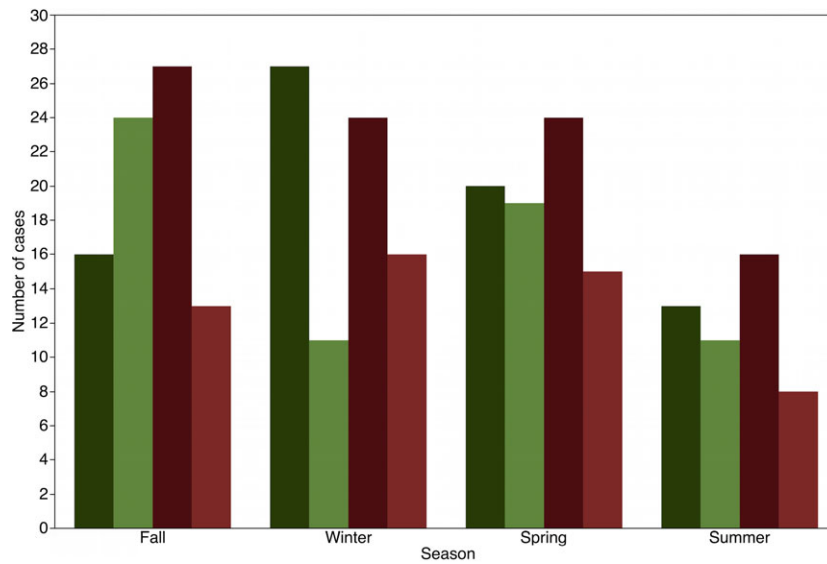
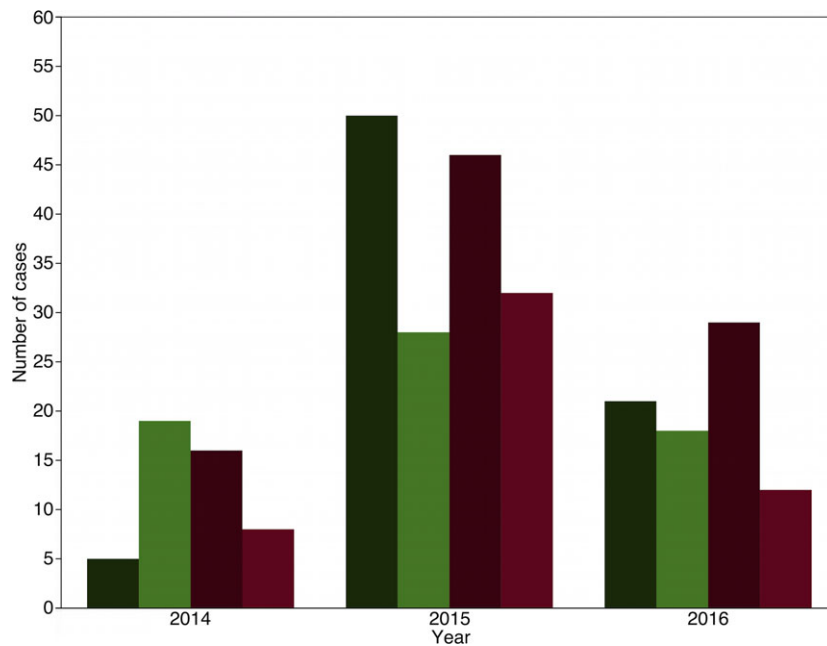


Fig 1. Age distribution of diarrheic cases from 6 different farms that tested positive for bovine coronavirus in feces.



**Fig 2.** Frequency distribution of healthy (green) and diarrheic (red) calves that tested positive (dark) and negative (light) for bovine coronavirus (BCoV) in feces during the different sampling seasons. (%) represents the percentage of the total number of cases.



**Fig 3.** Frequency distribution of healthy (green) and diarrheic (red) calves that tested positive (dark) and negative (light) for bovine coronavirus (BCoV) in feces during the different sampling years. (%) represents the percentage of the total number of cases.

46% (66/143) of healthy calves, and 64% (91/143) of diarrheic calves (Table 2). Overall, diarrhea cases had higher odds of BCoV presence than the age-matched control animals, although the numerical estimates varied slightly between the estimates based on exact logistic regression and random intercept logistic regression (Table 3). Sex was also associated with the presence of BCoV, with male calves at increased risk (Table 3). Based on the nonsignificant interaction between BCoV and matching factors (age, farm, season, and year), the association of BCoV with diarrhea did not appear to

vary according to age of the animals, season, and farm (Tables 3 and 5, Figs 2 and 3). In exact logistic regression, interaction of exposure with year of sampling was close to statistical significance and indicated that in year 2015, the effect of BCoV could have been different than in the other 2 years (Table 3). The effect of year in 2015 could not be identified as statistically significant in the random effect model for the year of 2015 (Fig 3). The final model (exact logistic regression and random effect logistic regression) contained covariates for BCoV and sex. Their interaction was not statistically significant,

**Table 2.** Frequency distribution of bovine coronavirus positivity status stratified by clinical status and sex of 286 calves from six dairy farms.

BCoV	Farm												Sex				
	1		2		3		4		5		6			All farms			
	H	D	H	D	H	D	H	D	H	D	H	D	H	D	M	F	
-	14 (33%)	10 (24%)	24 (51%)	15 (32%)	19 (79%)	3 (60%)	8 (53%)	7 (70%)	4 (40%)	4 (80%)	3 (60%)	4 (80%)	4 (80%)	3 (60%)	77 (54%)	52 (36%)	25 (27%)
+	28 (67%)	32 (76%)	23 (49%)	32 (68%)	5 (21%)	2 (40%)	7 (47%)	3 (30%)	6 (60%)	1 (20%)	2 (40%)	6 (60%)	1 (20%)	2 (40%)	66 (46%)	91 (64%)	39 (18%)
Total	42	42	47	47	24	5	15	10	10	5	5	10	5	5	143	143	64
																	222

BCoV, Bovine Coronavirus; +, positive; -, negative; H, healthy (controls); D, diarrhea (cases); M, male; F, female.

and the coefficients for both BCoV and sex changed by very small quantity once both variables were in the model. This suggests that sex failed to act as confounder for the association between BCoV and diarrhea in this dataset (Table 3).

**PanCoV-RT-PCR for Detection of CoV in Fecal and Nasal Samples**

By PanCoV-RT-PCR, the CoV RNA was detected in 8/8 fecal samples and 6/8 nasal swabs from the calves presented to OVC-HSC (Table 4). All but one sample that was positive for CoV by the PanCoV-RT-PCR were also positive on the BCoV-RT-PCR. Thirteen out of 20 (65%) diarrheic calves from the same farm tested positive for CoV by PanCoV-RT-PCR assay. All of the samples that tested positive in the PanCoV-RT-PCR assay were also positive when screening by BCoV-RT-PCR assay, but three samples positive for BCoV in the BCoV-RT-PCR assay were negative on the PanCoV-RT-PCR assay (Table 5). McNemar’s test demonstrated that both tests were equally likely to detect CoV in samples from calves ( $P = 0.67$ ). Kappa agreement test revealed a good agreement between both tests for detection of BCoV in fecal and nasal samples from diarrheic calves ( $\kappa = 0.68$ , 95% CI: 0.392 to 0.967;  $P < 0.001$ ). The results of the bacterial, viral, and protozoal analyses performed in feces of the calves presented to the OVC-HSC are presented in Table 5. All 20 fecal samples were negative for *Salmonella*.

**Discussion**

The results of this study demonstrated a positive association between BCoV and diarrhea in dairy calves as detection rates of this agent were higher in diarrheic calves than in farm-, season-, aged-matched nondiarrheic calves. Similar results were found in one case-control study involving 380 calves.<sup>25</sup> However, studies including small numbers of calves ( $n < 100$ ) identified numerical but not statistical association between BCoV in fecal or nasal samples and clinical signs.<sup>16-18</sup> Discrepancies between studies can be explained, at least in part, by differences in the source population, methodology, especially, the samples size used in the different investigations.<sup>16,25</sup>

Detection of BCoV, either in diarrheic and in nondiarrheic calves, was higher in comparison with previous studies from the same geographic area,<sup>10,26</sup> other studies from different regions worldwide<sup>6,7,25</sup> and more in line with recent studies from Europe.<sup>4</sup> Earlier studies (1980 to 2007) investigating the presence of infectious agents in feces of healthy and diarrheic calves reported a prevalence of BCoV in healthy calves between 0 and 8.2% and in diarrheic calves between 3 and 40%.<sup>10,26</sup> More recent studies (2008 to 2012) reported prevalence of BCoV in healthy calves of 7.4% and in diarrheic calves between 3.4 and 40%.<sup>6,7</sup> Interestingly, a recent report from Europe identified a prevalence of BCoV of 79% of diarrheic calves;<sup>4</sup> however, this study failed to investigate healthy controls. The prevalence of



**Table 3.** Evaluation of the association between BCoV, diarrhea, and matched variables (age, farm, season, and year).

		Analysis of single factors of interests							
		Exact logistic regression				Random effect logistic regression			
		OR	95% CI		P-value	OR	95% CI		P-value
BCoV (+ve)	+ve	2.19	1.28	3.86	0.031	2.04	1.27	3.29	0.0037
Sex (Male)	Male	1.87	0.99	3.68	0.054	1.77	0.99	3.14	0.050
BCov*age	–	–	–	–	0.92	–	–	–	0.97
BCov*season	–	–	–	–	0.47	–	–	–	0.86
BCov*year	–	–	–	–	0.03	–	–	–	0.17
	2014*BCoV	2.51	1.13	+inf		4.74	1.62	13.9	
	2015*BCoV	0.43	–inf	0.83		1.67	0.95	2.91	
	2016*BCoV	3.36	1.85	+inf		2.11	1.09	4.11	
BCoV*farm	–	–	–	–	0.76	–	–	–	0.84

		Final model							
		Exact logistic regression				Random effect logistic regression			
		OR	95% CI		P-value	OR	95% CI		P-value
BCoV	+ve	2.16	1.26	3.83	0.004	1.96	1.21	3.17	0.006
Sex	Male	1.84	0.95	2.95	0.072	1.63	0.91	2.92	0.097

BCoV, Bovine coronavirus; +ve, positive; OR, odds ratio, CI, confidence interval.

**Table 4.** Test results for bacterial, viral, and protozoal analyses performed in feces of eight hospitalized diarrheic calves.

Pathogen/Calf	1	2	3	4	5	6	7	8
<i>E. coli</i> (F5/K99)	–	–	–	–	–	–	–	–
<i>Cryptosporidium</i>	+	+	–	+	–	–	–	+
BRoV-A	–	+	–	–	–	–	–	–
BRoV-B	–	+	–	–	–	–	–	–
PanCoV-RT-PCR	+	+	+	+	+	+	+	+
BCoV-RT-PCR	+	+	+	+	+	+	+	+
<i>Salmonella</i> spp.	–	–	–	–	–	–	–	–
<i>Giardia</i> spp.	–	+	–	–	–	+	–	–

F5/K99, *E. coli* F5 (K99) fimbriae attachment factor; BRoV, bovine rotavirus; PanCoV-RT-PCR assay, pancoronavirus reverse transcriptase PCR assay; BCoV-RT-PCR assay, bovine coronavirus reverse transcriptase PCR assay.

infectious agents causing diarrhea in calves was previously investigated in dairy farms from Ontario at three different periods by ELISA assays, 1982,<sup>26</sup> 1990–1991,<sup>27</sup> and 1995–1997.<sup>10</sup> The overall prevalence of BCoV in the 1982's study was 5% (3/56), but this study did not discriminate the prevalence of healthy and diarrheic calves.<sup>26</sup> In the 1992 study, the prevalence of BCoV in diarrheic calves was 17% (54/312), whereas in 1998, the overall prevalence was 10% (16/161), and BCoV was detected in 13% (15/118) and 2.3% (1/43) of diarrheic and healthy calves, respectively. The reasons for these differences in the detection rates of BCoV in calves (healthy and diarrheic) between this study and previous investigations in the same geographic area (Ontario) are unclear. In fact, it is difficult to discern whether the increasing detection of BCoV reflects a true rise in

**Table 5.** Comparison of Coronavirus detection results by Pancoronavirus reverse transcriptase (PanCoV) and BCoV reverse transcriptase (BCoV) assays.

	PanCoV		
	+	–	
BCoV			
+	26	3	29
–	1	6	7
Total	27	9	36

McNemar's test  $P$ -value = 0.67. Kappa agreement test,  $\kappa$  = 0.68, 95% CI: 0.392 to 0.967;  $P$  < 0.001.

prevalence in the course of time or whether it is due to simply to the better detection rate.<sup>28</sup> The differences between studies could be explained, at least in part, by changes in farm management practices overtime (e.g., calf population density, antimicrobial drug use, and management/agricultural practices), differences in the type of assay used for detection of BCoV, and the emergence or re-emergence of BCoV strains over the years.

Regarding the influence that farm management practices can have on the prevalence of infectious diseases, contradictory results have reported.<sup>1,29–31</sup> In one study, the prevalence of salmonellosis in preweaned calves was reported to be higher in group pen calves than those in individual pens.<sup>29</sup> Contrary, some studies failed to identify an effect of management and infrastructure variables on the presence of enteropathogens in calves.<sup>30,31</sup> This study was not designed to evaluate farm-level differences, something that would warrant further investigation.

Differences in the type of assay used for detection of BCoV (e.g., ELISA vs. RT-PCR) also can explain differences in the detection rates of BCoV between studies. One study investigating the rates of BCoV fecal shedding in diarrheic calves found a detection rate of BCoV of 53% when using ELISA and 96% when using RT-PCR<sup>16</sup> suggesting that RT-PCR was a more sensitive method. These results were further confirmed by one study reporting that the sensitivity and specificity of some ELISA assays were lower compared with quantitative RT-PCR when both tests were used to identify the presence of BCoV in feces of diarrheic calves.<sup>32</sup> Furthermore, more sensitive diagnostic assays also could contribute to detect lower levels of BCoV that cannot be detected by other methods.<sup>32</sup>

An alternative explanation for the changes in detection rates of BCoV in healthy and diarrheic calves over the years in dairy farms from Ontario could be the emerging and re-emerging of BCoV strains. Similar to other RNA viruses, CoVs are believed to mutate frequently that could result in altered antigenicity and pathogenicity of new strains.<sup>33,34</sup> During the past decades, several CoVs have caused serious problems in humans and animals (e.g., severe acute respiratory syndrome coronavirus, SARS-CoV; Middle East respiratory syndrome coronavirus, MERS-CoV, and porcine epidemic diarrhea virus, PEDV).<sup>35</sup> Emerging outbreaks associated with CoV also have been documented in horses worldwide,<sup>36</sup> and emerging canine CoV variants have been associated with systemic infections in the European Union.<sup>37</sup> In an Oklahoma study, a new CoV belonging to a BCoV clade 2 was isolated from nasal secretion and lung fluids of postweaned healthy beef calves and calves with respiratory disease.<sup>38</sup> This new field isolate was antigenically and genetically different to previously isolated BCoV strains including those from neonatal diarrhea, reference strains, and the strain in the current modified live vaccine BCoV licence in United States that belong to CoV clade 1.<sup>38</sup> Similar results have been reported in Europe<sup>4</sup> and Asia.<sup>39</sup> In 2012, the BCoV clade 2 strain was recovered from postweaned calves (6–7 months) suffering of severe enteritis with bloody diarrhea; the authors of that study suggested that this clinical syndrome caused by BCoV clade 2 should be added to the already known syndromes caused by BCoV.<sup>40</sup> The results of these studies suggest that current BCoV strains circulating in worldwide are diverging from the prototype BCoV isolated in earlier studies and used in vaccines.<sup>38</sup> This can result in the emergency of new clinical syndromes,<sup>40</sup> outbreaks of enteric diseases with higher morbidity and case fatality (similar to the one described in this study), increased detection rates of BCoV in nasal and fecal samples of healthy and ill calves,<sup>4,18,41</sup> and the lack of efficacy vaccination protocols.<sup>42</sup> Future studies should focus on investigating the biological, antigenical, and genetical characteristic of BCoV circulating in Canada. It is important to highlight that in this study no other virus, parasites, and bacterial agents were investigated, and the role of BCoV in association with multiple pathogens was not determined which prevent us to clearly identify

the role of BCoV as a primary cause of disease or as a agent associated with coinfection.

This study also demonstrated that a recently described PanCoV-RT-PCR assay can be as useful as the BCoV-RT-PCR assay to identify BCoV in fecal and nasal samples of diarrheic calves, with the added benefit of providing a broader test that would detect novel CoVs. Currently, viral culture is the gold standard for laboratory diagnosis of viral gastrointestinal disorders; however, detection of coronavirus by culture isolation or serological methods can be difficult.<sup>23</sup> To overcome this issue and obtain rapid diagnostic results, several sensitive molecular assays for detection of CoV have been developed, including RT-PCR, nested RT-PCR, and real-time RT-PCR. Recently, a novel PanCoV-RT-PCR assay was designed to allow detection of all known CoV based on an alignment of conserved genome regions of several coronavirus.<sup>43</sup> These results showed a good level of agreement between BCoV-RT-PCR and the novel PanCoV-RT-PCR assays indicating that PanCoV-RT-PCR can be a rapid tool to detect BCoV in samples from cattle. Future studies with a larger sample size are required to corroborate these findings.

## Conclusion

Calves positive to BCoV were more likely to suffer from diarrhea than farm- and aged-matched healthy calves. These results also showed an apparent higher prevalence of BCoV in fecal samples of healthy and diarrheic calves in Ontario, Canada, compared with previous years. A new PanCoV-RT-PCR was useful in identifying the presence of CoV in nasal and fecal samples from diarrheic calves during an outbreak of diarrhea.

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## Footnotes

- <sup>a</sup> QIAamp Viral RNA Mini kit, QIAGEN, Toronto, ON
- <sup>b</sup> One-Step RT-PCR kit; QIAGEN, Toronto, ON
- <sup>c</sup> Applied Biosystems, Foster City, CA
- <sup>d</sup> SAS Institute Inc., Cary, NC
- <sup>e</sup> JMP 12, SAS Institute Inc., Cary, NC
- <sup>f</sup> Calf Guard, Zoetis, Parsippany, NJ

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*Conflict of interest Declaration:* Authors declare no conflict of interest.

*Off-label Antimicrobial Declaration:* Authors declare no off-label use of antimicrobials.

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