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REGULAR ARTICLES

Prevalence and risk factors for agents causing diarrhea (Coronavirus, Rotavirus, *Cryptosporidium* spp., *Eimeria* spp., and nematodes helminthes) according to age in dairy calves from Brazil

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Abstract

The present study attempted to verify the prevalence of and risk factors for diarrhea-causing agents in dairy calves from Brazil. Additionally, ages with a higher risk of occurrence for each agent were verified by means of the receiver operating characteristic (ROC) curve. The collections were performed on 39 farms, belonging to 29 municipalities located in eight states of Brazil. It was possible to conclude that the prevalence of Coronavirus, Rotavirus, *Cryptosporidium* spp., *Eimeria* spp., and nematodes was 7.20% (95% CI 4.54–9.78), 6.37% (95% CI 3.85–8.89), 51.52% (95% CI 45.26–55.57), 3.46% (95% CI 2.24–4.67), and 3.46% (95% CI 2.24–4.67), respectively. Ages with higher probabilities of occurrence of these diseases in calves were < 10, > 8, > 6, > 37, and > 36 days, respectively. Diarrhea occurred more significantly ($P < 0.0001$) in animals less than 21 days old and mainly on those receiving milk through automatic feeders ($P < 0.001$). *Cryptosporidium* spp. were a risk factor for the occurrence of Rotavirus, and vice versa ($P = 0.0039$) and presented a positive correlation with Coronavirus ($P = 0.0089$). Calves that drink water from rivers, streams, and ponds had a higher chance of being infected by *Eimeria* spp. ($P < 0.0001$), as well as developing infection by nematodes ($P < 0.0001$). The results found in this study highlight the importance of studying the agents of diarrhea together, once they act as coinfection where the losses triggered for the owners will involve some of these agents simultaneously.

Keywords Coronavirus · *Cryptosporidium* spp. · Diarrhea · *Eimeria* spp. · Nematodes · Rotavirus

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Introduction

Among the main hindrances to bovine production, gastrointestinal illnesses stand out, especially in relation to younger animals. The impacts of these diseases on production usually consist of delayed growth, diarrhea, and even mortality in some cases (Felippelli et al. 2014; Cruvinel et al. 2018a). Among all major agents causing gastrointestinal symptoms in young bovines, viral (Coronavirus and Rotavirus) and parasitic (*Cryptosporidium* spp., *Eimeria* spp., and gastrointestinal nematodes) agents are notable.

Rotavirus is usually associated with Coronavirus, and both agents are one of the main enteropathogens responsible for neonatal diarrhea syndrome in calves (Coura et al. 2015;

Hayashi et al. 2017; Almeida et al. 2018; Bok et al. 2018; Fritzen et al. 2019).

Cryptosporidium is a protozoan that possesses a direct cycle and is monoxenous and affects bovines, mainly animals aged between 1 and 3 weeks (Murakoshi et al. 2013; Thomson et al. 2017; Masatani et al. 2018). *Eimeria* is another protozoan that contains thirteen species that can parasitize intestinal cells of cattle (Lucas et al. 2014; Passafaro et al. 2015) with the potential to cause losses of productivity in herds around the world, *E. bovis* and *E. zuernii*, depending on animal category and degree of infection (Taubert et al. 2008; Bruhn et al. 2011). Regarding gastrointestinal nematodes, Souza et al. (2008) described that the main impact of infection on livestock production is delayed growth and even mortality, especially young animals.

All previously mentioned agents have been described in an isolated manner in most clinical cases diagnosed; however, on most farms, it is possible that more than one agent may be involved simultaneously. This possibility may prevent researchers and animal owners from achieving a better understanding of the damage caused collectively by these agents in milk-feeding calves aged only a few days, which motivated this research. For this reason, the present study aimed to verify the prevalence and risk factors for agents causing diarrhea (Coronavirus, Rotavirus, *Cryptosporidium* spp., *Eimeria* spp., and gastrointestinal nematodes) in dairy calves from Brazil. In addition, the age with higher risk for occurrence of each agent was verified utilizing the receiver operating characteristic (ROC) curve.

Materials and methods

Selection of farms and animals and collection of fecal samples

The present study was conducted between December 2016 and March 2017 on farms belonging located in the states of Rio Grande do Sul, Paraná, Santa Catarina, Rio de Janeiro, São Paulo, Minas Gerais, Goiás, and Ceará that have a focus on milk production (Fig. 1). These states were chosen because they represent 80% of Brazil's total milk production (IBGE 2017). Initially, 872 farms located in these states were contacted, and only 195 did not administer any vaccine or specific drug against the five agents involved in this study. Among these 195 farms, 39 farms (20%) belonging to 29 municipalities were randomly chosen for conducting the study. In some states, such as Minas Gerais, several farms were selected, while in others, such as Goiás, only one or two were selected. On these 39 farms, fecal samples were collected from 868 female calves. Samples were obtained from animals aged between 1 and 135 days, all of which were consuming milk. All these samples were collected directly from the rectum of animals and, after individual identification (using the animal's ID number), were stored in isothermal containers filled with ice. The samples were subsequently sent to the Veterinary Parasitology Center of the Veterinary and Animal Husbandry School, Federal University of Goiás (Centro de Parasitologia Veterinária da Escola de Veterinária e Zootecnia da Universidade Federal de Goiás, EVZ/UFG), for processing.

Processing of samples

Coronavirus and Rotavirus

For Coronavirus analysis, fecal suspensions at 10–20% (w/v) were prepared, and nucleic acid extraction was performed as described by Alfieri et al. (2006). The RNA of enteric viruses in fecal samples was investigated by molecular techniques, such as reverse transcription-polymerase chain reaction (RT-PCR) and semi-nested PCR (SN-PCR), for partial amplification of the BCoV N gene (Takiuchi et al. 2006), bovine RVAVP4 and VP7 genes (Gentsch et al. 1992; Gouvea et al. 1990), bovine rotavirus B (RVB) NSP2 gene (Gouvea et al. 1991), and bovine rotavirus C (RVC) VP6 gene (Alfieri et al. 1999). The RT-PCR was performed using the oligonucleotide primers upstream 5′-GCCGATCAGTCCGACCAATC-3′(nt79–98) and downstream 5′-AGAATGTCAGCCGG GGTAT-3′(nt 467–485) that amplify a 407 base pair (bp) fragment of the N gene of BCoV. The technique was carried out as described by Tsunemitsu et al. (1999). The reverse transcriptase reaction was conducted as follows. In the tube, 10 μl of RNA sample was added to 2 μl of the downstream primer (50 pmol). The tube was incubated at 100 °C for 2 min and then quenched on ice for 5 min. Subsequently, 4 μl of 5 × RT buffer [250 mM tris-HCl (pH 8.3), 375 mM KCl, 15 mM MgCl2], 1 μl of 0.1 M dithiothreitol, 2 μl of 10 mM dNTPs, 0.5 μl of RNAsin (Promega Corporation), and 0.5 μl of AMV RT (Promega Corporation) were added and incubated at 37 °C for 60 min. Then 10 μl of the RT reaction samples was added to 40 μl of the PCR mixture. The PCR mixture consisted of 5 μl of 10 × buffer [100 mM Tris-HCl (pH 8.3), 500 mM KCl, 15 mM MgCl2, 0.01% gelatin], 1 μl of 10 mM dNTPs, 0.5 μl of the upstream primer (50 pmol), 0.5 μl of the downstream primer (50 pmol), 32.5 μl of water, and 0.5 μl of Taq polymerase (Promega Corporation) (5 U/μl). The mixture was overlaid with mineral oil and then subjected to 5 min of preheating at 94 °C, 35 cycles of 1 min at 94 °C, 1 min at 58 °C, 2 min at 72 °C, and a final 7-min incubation at 72 °C. 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 μg/ml), and visualized under UV light.

Regarding Rotavirus, nucleic acid extraction from fecal samples was performed using the phenol/chloroform/isoamyl alcohol (Malik et al. 2012; Silva et al. 2012) and silica/

Fig. 1 Spatial distribution of the 39 farms belonging to 29 municipalities located in eight states of Brazil analyzed in this study

guanidinium isothiocyanate nucleic acid extraction methods (Alfieri et al. 2006). Samples were screened for RVA by silver staining after polyacrylamide gel electrophoresis (PAGE) (Herring et al. 1982) modified by Pereira et al. (1983).

In the present study, only samples from bovines less than 21 days old were analyzed for the presence of Coronavirus and Rotavirus. Therefore, from 868 collected samples, 361 were evaluated for the presence of Coronavirus and Rotavirus. This protocol was limited due to costs and was also based on the results obtained from previous studies conducted by Jerez et al. (2002), and Takiuchi et al. (2006), all of which emphasized the importance of these agents in calves up to 3 weeks old.

Cryptosporidium spp.

Each sample was homogenized, filtered with the aid of plastic sieves, and submitted to centrifuge washing with deionized water with 0.02% Tween 20. Later, the purification of samples was performed according to the methodology by Meloni and Thompson (1996), as follows: 32 ml of solution containing the fecal samples, diluted in deionized water/Tween 20, was added to a 50-ml tube together with 8 ml of ether. Next, tubes were agitated vigorously in a vortex for 30 s and then centrifuged at 2000*g* for 8 min.

After purification, the samples were washed twice by centrifugation with the water/Tween 20 solution, and the final pellet was resuspended in phosphate buffered saline solution (PBS). Obtained sediments were washed twice through centrifugation, utilizing distilled water/Tween 20 diluted in phosphate buffered saline solution (PBS). The presence of *Cryptosporidium* spp. oocysts was determined through a negative coloration technique using malachite green (Elliot et al. 1999). Approximately 15 μl of the sample and 15 μl of malachite green were added and homogenized over a microscope slide, and assembly was finished with a cover slip. Oocyst research was performed with the aid of light microscopy at magnifications of × 400 and × 1000.

Similar to Coronavirus and Rotavirus, only samples belonging to animals aged 21 days or less were analyzed for the presence of *Cryptosporidium* spp. Therefore, from all 868 collected samples, 361 were analyzed for the presence of the aforementioned parasite. This experimental protocol was based on previous studies performed by Garcia and Lima (1994), Langoni et al. (2004), Cardoso et al. (2008), Feitosa et al. (2008), and Meireles (2010), in which the authors demonstrated that this disease occurs most frequently and causes the most severe symptoms in milk-feeding calves aged less than 3 weeks.

Eimeria spp. and gastrointestinal nematodes

From each of the 868 fecal samples collected, an aliquot was taken for the quantification of *Eimeria* spp. oocysts per gram (OoPG) of feces and nematodes eggs per gram (EPG) of feces (Gordon and Whitlock 1939, as modified by Ueno and Gonçalves 1998). Each oocyst or egg found corresponded to 50 OoPG or EPG, respectively.

For each farm, all samples that presented OoPG counts of greater than or equal to 50 were placed together in pools. In this case, 5 g of feces was used from each animal to form the pools. For example, in farm X, if there were 100 bovines with OoPG counts of greater than or equal to 50, 5 g of each of these 100 animals was used to compose the pool. On farm Y, if there were 20 animals with OoPG counts of greater than or equal to 50, 5 g of each of these 20 animals was used to compose the pool. These pools were then processed using the method of centrifugal flotation in sugar solution. Later, samples were filtered using sieves with folded gauze. A 2% potassium dichromate ($K_2Cr_2O_7$) solution was added to the results from this filtration with a volume-by-volume approach, and this mixture was kept at 24 °C for 14 days under oxygenation (using aquarium oxygenator pumps) to stimulate oocyst sporulation under laboratory conditions.

Oocysts were recovered by centrifugation in a 60% saturated sugar solution, and approximately 100 oocysts per pool from each farm were identified under an optical microscope coupled to a computerized system (LAS, Leica®). This process of recovering 100 oocysts per pool for the identification of *Eimeria* species was performed in triplicate. Differentiation among species was performed according to the phenotypic characteristics of oocysts, such as color, presence or absence of micropyle, and length and width (Daugschies and Najdrowski 2005).

Data analysis

Values regarding the prevalence (%) of diarrhea, Coronavirus, Rotavirus, *Cryptosporidium* spp., *Eimeria* spp., and gastrointestinal nematodes were classified in increasing order for municipalities and states. For the smallest prevalence, an odds ratio (OR) of one was attributed, with the remaining ORs being calculated in relation to this value using the *Z* test to verify significance ($P \leq 0.05$).

Risk factors (RF) associated with the occurrence of each agent and diarrhea were associated with sex, race, age, breeding system (isolated housing or tied in chains; paddocks; compost barn), milk offer (bucket/bottle; direct from cows; automatic feeders), weaning age, herd size, if different categories (ages) are kept together, if different animal species are kept together, water source (artesian well; rivers, lakes, and ponds), location where animals are fed (through; directly on the ground), if herd reposition is through acquisition of animals from other farms (if yes, do they quarantine animals?).

Frequency of occurrences of diarrhea, Coronavirus, Rotavirus, *Cryptosporidium* spp., *Eimeria* spp., and nematodes was computed inside each age level, for which posterior correlations were determined using the Spearman post-correlation.

All data manipulation procedures were performed on the Epi Info software, version 7.1.5.2 (WHO, 2015), version 12 (StatSoft, Inc. 2014), and Microsoft Excel® 2016.

The ROC curve was used to establish for each animal age (in days) with the highest combined sensitivity and specificity for the occurrence and detection of the respective agents, as well as diarrhea (Galpasoro and Fernandez 1998).

Results

Coronavirus, Rotavirus, *Cryptosporidium* spp., and diarrhea

Of the 361 fecal samples examined, 26 (7.2%, CI 95% 4.54–9.87) were positive for Coronavirus, 23 (6.37%, CI 95% 3.85–8.89) were positive for Rotavirus, and 182 (50.41%, CI 95% 45.26–55.57) were positive for *Cryptosporidium* spp. (Table 1).

Analyzing these results makes it possible to observe that in all 29 municipalities from which fecal samples were obtained, Coronavirus, Rotavirus, and *Cryptosporidium* spp. were found in 13, 12, and 25 cities, respectively. In this case, the prevalence found for Coronavirus varied between 0.0 and 45.45% (CI 95% 16.03–74.88), with the municipalities of Limoeiro do Norte (Ceará state) and Itutinga (Minas Gerais state) presenting a higher probability of calves being infected by Coronavirus (with OR > 1 and CI 95% > 1). For Rotavirus, such values were between 0.0 and 50.0% (Ampere, Paraná state CI 95% 0.0–119.3). However, no cities were found with elevated probabilities of detecting Rotavirus parasitizing animals. For *Cryptosporidium* spp., prevalence varied from 0.0 to 85.71% (CI 95% 59.79–116.64), and some municipalities on the states of Minas Gerais (Paraopeba, Curvelo, Prata,

Table 1 Analysis of association between the citys of different States of Brazil, referring to the prevalence of different agensts diagnosed in the dairy calves

State	City	Agent	Total	Negative	Positive	Prevalence	Odds ratio					
							Value	95 % CI			z statistic	Significance level
MG	Baependi	Coronavirus	2	2	0	0.00%						
MG	Cruzalia		3	3	0	0.00%						
MG	Curvelo		15	15	0	0.00%						
MG	Pará de Minas		18	18	0	0.00%						
MG	Pouso Alto		6	6	0	0.00%						
MG	Prata		12	12	0	0.00%						
MG	Sete Lagoas		11	11	0	0.00%						
MG	Três Corações		5	5	0	0.00%						
PR	Ampere		2	2	0	0.00%						
PR	Chopinho		6	6	0	0.00%						
PR	Francisco Beltrão		1	1	0	0.00%						
PR	Marechal Candido Rondon		2	2	0	0.00%						
RJ	Barra Mansa		7	7	0	0.00%						
RS	Saldanha Marinho		5	5	0	0.00%						
SC	Xanxerê		14	14	0	0.00%						
SP	Areias		17	17	0	0.00%						
MG	Uberlandia		42	41	1	2.38%	1.0000					
MG	Paraopeba		27	26	1	3.70%	1.5769	0.0945	to	26.3258	0.317	0.7511
GO	Morrinhos		22	21	1	4.55%	1.9524	0.1151	to	33.1132	0.463	0.6432
MG	Inhauma		56	51	5	8.93%	4.0196	0.4425	to	36.5097	1.236	0.2165
PR	Nova Cantu		9	8	1	11.11%	5.1250	0.5280	to	49.7441	1.409	0.1588
CE	Umirim		14	12	2	14.29%	6.8333	0.5273	to	88.5525	1.470	0.1415
RS	Capão do Leão		14	12	2	14.29%	6.8333	0.8226	to	56.7613	1.779	0.0752
CE	Limoeiro do Norte		19	16	3	15.79%	7.6875	1.1053	to	53.4674	2.061	0.0393
RS	Condor		5	4	1	20.00%	10.2500	0.8293	to	126.6924	1.814	0.0697
PR	Palmital		9	7	2	22.22%	11.7143	0.7900	to	173.7121	1.789	0.0737
MG	Virgínia		4	3	1	25.00%	13.6667	0.8691	to	214.9208	1.860	0.0629
RS	Almirante do Sul		3	2	1	33.33%	20.5000	0.7567	to	555.3489	1.794	0.0727
MG	Itutinga		11	6	5	45.45%	**34.1667**	**2.3476**	**to**	**497.2636**	**2.585**	**0.0097**
Total		-	361	335	26	7.20%	-	-		-	-	-
GO	Morrinhos	Rotavirus	22	22	0	0.00%						
MG	Baependi		2	2	0	0.00%						
MG	Cruzalia		3	3	0	0.00%						
MG	Curvelo		15	15	0	0.00%						

Table 1 (continued)

State	City	Agent	Total	Negative	Positive	Prevalence	Odds ratio					
							Value	95 % CI			z statistic	Significance level
MG	Itutinga		11	11	0	0.00%						
MG	Pará de Minas		18	18	0	0.00%						
MG	Sete Lagoas		11	11	0	0.00%						
MG	Três Corações		5	5	0	0.00%						
MG	Virgínia		4	4	0	0.00%						
PR	Chopinho		6	6	0	0.00%						
PR	Francisco Beltrão		1	1	0	0.00%						
PR	Marechal Candido Rondon		2	2	0	0.00%						
PR	Nova Cantu		9	9	0	0.00%						
PR	Palmital		9	9	0	0.00%						
RS	Almirante do Sul		3	3	0	0.00%						
RS	Condor		5	5	0	0.00%						
RS	Saldanha Marinho		5	5	0	0.00%						
CE	Limoeiro do Norte		19	18	1	5.26%	1.0000					
SP	Areias		17	16	1	5.88%	1.1250	0.0649	to	19.4973	0.081	0.9355
CE	Umirim		14	13	1	7.14%	1.3846	0.0788	to	24.3436	0.222	0.8239
RS	Capão do Leão		14	13	1	7.14%	1.3846	0.0780	to	24.5796	0.222	0.8245
SC	Xanxerê		14	13	1	7.14%	1.3846	0.0780	to	24.5796	0.222	0.8245
MG	Prata		12	11	1	8.33%	1.6364	0.0913	to	29.3207	0.334	0.7380
MG	Inhauma		56	51	5	8.93%	1.7647	0.1872	to	16.6390	0.496	0.6198
MG	Paraopeba		27	24	3	11.11%	2.2500	0.4964	to	10.1991	1.052	0.2930
MG	Uberlandia		42	37	5	11.90%	2.4324	0.5316	to	11.1302	1.146	0.2520
MG	Pouso Alto		6	5	1	16.67%	3.6000	0.3463	to	37.4241	1.072	0.2836
RJ	Barra Mansa		7	5	2	28.57%	7.2000	0.4831	to	107.3135	1.432	0.1521
PR	Ampere		2	1	1	50.00%	18.0000	0.7188	to	450.7804	1.759	0.0786
Total		-	361	338	23	6.37%	-	-		-	-	-
RS	Condor	Cryptosporidium spp	5	5	0	0.00%	0.0000					
MG	Cruzalia		3	3	0	0.00%	0.0000					
PR	Francisco Beltrão		1	1	0	0.00%	0.0000					
PR	Palmital		9	9	0	0.00%	0.0000					
RS	Saldanha Marinho		5	4	1	20.00%	1.0000					
CE	Limoeiro do Norte		19	14	5	26.32%	1.4286	0.1273	to	16.0268	0.289	0.7725
MG	Sete Lagoas		11	7	4	36.36%	2.2857	0.4626	to	11.2926	1.014	0.3104
MG	Três Corações		5	3	2	40.00%	2.6667	0.3044	to	23.3642	0.886	0.3757

Table 1 (continued)

State	City	Agent	Total	Negative	Positive	Prevalence	Odds ratio					
							Value	95 % CI			z statistic	Significance level
MG	Uberlandia		42	25	17	40.48%	2.7200	0.4100	to	18.0470	1.036	0.3000
MG	Paraopeba		27	15	12	44.44%	3.2000	1.2038	to	8.5066	2.332	0.0197
GO	Morrinhos		22	12	10	45.45%	3.3333	1.0750	to	10.3354	2.085	0.0370
SP	Areias		17	9	8	47.06%	3.5556	0.9991	to	12.6530	1.959	0.0502
PR	Ampere		2	1	1	50.00%	4.0000	0.2134	to	74.9789	0.927	0.3539
PR	Marechal Candido Rondon		2	1	1	50.00%	4,0000	0.0794	to	201.6018	0.693	0.4882
MG	Pará de Minas		18	9	9	50.00%	4.0000	0.2153	to	74.2984	0.930	0.3524
MG	Baependi		2	1	1	50.00%	4.0000	0.2153	to	74.2984	0.930	0.3524
SC	Xanxerê		14	7	7	50.00%	4.0000	0.2066	to	77.4425	0.917	0.3592
MG	Curvelo		15	7	8	53.33%	4.5714	1.0635	to	19.6507	2.043	0.0411
PR	Nova Cantu		9	4	5	55.56%	5.0000	0.9501	to	26.3133	1.900	0.0575
RS	Capão do Leão		14	6	8	57.14%	5.3333	0.9861	to	28.8448	1.944	0.0519
MG	Prata		12	5	7	58.33%	5.6000	**1.1753**	**to**	**26.6835**	**2.163**	**0.0306**
CE	Umirim		14	5	9	64.29%	7.2000	**1.4756**	**to**	**35.1316**	**2.441**	**0.0146**
MG	Inhauma		56	19	37	66.07%	7.7895	**2.2877**	**to**	**26.5227**	**3.284**	**0.0010**
RS	Almirante do Sul		3	1	2	66.67%	8.0000	**0.6811**	**to**	**93.9589**	**1.654**	**0.0980**
MG	Virgínia		4	1	3	75.00%	12.0000	**0.4430**	**to**	**325.0823**	**1.476**	**0.1399**
MG	Itutinga		11	2	9	81.82%	18.0000	**1.1703**	**to**	**276.8459**	**2.073**	**0.0382**
PR	Chopinho		6	1	5	83.33%	20.0000	**1.4305**	**to**	**279.6257**	**2.226**	**0.0260**
MG	Pouso Alto		6	1	5	83.33%	20.0000	**0.9601**	**to**	**416.6103**	**1.934**	**0.0531**
RJ	Barra Mansa		7	1	6	85.71%	24.0000	**1.1768**	**to**	**489.4649**	**2.066**	**0.0388**
Total		-	361	179	182	50.41%	-	-		-	-	-
RS	Almirante do Sul	Eimeria spp	22	22	0	0.00%						
PR	Ampere		4	4	0	0.00%						
SP	Areias		26	26	0	0.00%						
MG	Baependi		9	9	0	0.00%						
PR	Chopinho		17	17	0	0.00%						
MG	Cruzalia		9	9	0	0.00%						
MG	Curvelo		42	42	0	0.00%						
PR	Francisco Beltrão		5	5	0	0.00%						
MG	Inhaúma		61	61	0	0.00%						
MG	Itutinga		16	16	0	0.00%						
CE	Limoeiro do Norte		41	41	0	0.00%						
PR	Marechal Candido Rondon		17	17	0	0.00%						

Table 1 (continued)

State	City	Agent	Total	Negative	Positive	Prevalence	Odds ratio					
							Value	95 % CI			z statistic	Significance level
PR	Nova Cantu		9	9	0	0.00%						
PR	Palmital		16	16	0	0.00%						
MG	Pouso Alto		19	19	0	0.00%						
MG	Prata		36	36	0	0.00%						
PR	Realeza		5	5	0	0.00%						
RS	Saldanha Marinho		12	12	0	0.00%						
MG	Sete Lagoas		42	42	0	0.00%						
MG	Três Corações		11	11	0	0.00%						
MG	Virgínia		12	12	0	0.00%						
SC	Xanxerê		66	66	0	0.00%						
MG	Paraopeba		94	92	2	2.13%	1.0000					
MG	Pará de Minas		37	36	1	2.70%	1.2778	0.1124	to	14.5320	0.198	0.8433
MG	Uberlândia		69	66	3	4.35%	2.0909	0.2098	to	20.8417	0.629	0.5295
RS	Condor		22	21	1	4.55%	2.1905	0.2162	to	22.1978	0.664	0.5069
RS	Capão do Leão		29	27	2	6.90%	3.4074	0.2890	to	40.1756	0.974	0.3301
GO	Morrinhos		37	34	3	8.11%	4.0588	0.6324	to	26.0518	1.477	0.1397
CE	Umirim		32	29	3	9.38%	4.7586	0.8911	to	25.4121	1.825	0.0680
RJ	Barra Mansa		28	25	3	10.71%	5.5200	**1.0212**	**to**	**29.8368**	**1.984**	**0.0472**
MG	Pequi		23	11	12	52.17%	50.1818	**11.7668**	**to**	**214.0096**	**5.292**	**0.0000**
Total		-	868	838	30	3.46%	-	-		-	-	-
PR	Ampere	Gastrointestinal nematodes	4	4	0	0.00%						
SP	Areias		26	26	0	0.00%						
MG	Baependi		9	9	0	0.00%						
RS	Capão do Leão		29	29	0	0.00%						
PR	Chopinho		17	17	0	0.00%						
MG	Cruzalia		9	9	0	0.00%						
MG	Curvelo		42	42	0	0.00%						
PR	Francisco Beltrão		5	5	0	0.00%						
MG	Inhaúma		61	61	0	0.00%						
MG	Itutinga		16	16	0	0.00%						
CE	Limoeiro do Norte		41	41	0	0.00%						
PR	Marechal Candido Rondon		17	17	0	0.00%						
GO	Morrinhos		37	37	0	0.00%						
PR	Nova Cantu		9	9	0	0.00%						

Table 1 (continued)

State	City	Agent	Total	Negative	Positive	Prevalence	Odds ratio					
							Value	95 % CI			z statistic	Significance level
PR	Palmital		16	16	0	0.00%						
MG	Pará de Minas		37	37	0	0.00%						
MG	Paraopeba		94	94	0	0.00%						
MG	Prata		36	36	0	0.00%						
PR	Realeza		5	5	0	0.00%						
RS	Saldanha Marinho		12	12	0	0.00%						
MG	Uberlândia		69	69	0	0.00%						
CE	Umirim		32	32	0	0.00%						
MG	Virgínia		12	12	0	0.00%						
SC	Xanxerê		66	66	0	0.00%						
MG	Sete Lagoas		42	41	1	2.38%	1.0000					
MG	Três Corações		11	10	1	9.09%	4.1000	0.2356	to	71.3604	0.968	0.3330
RS	Almirante do Sul		22	20	2	9.09%	4.1000	0.3307	to	50.8384	1.098	0.2720
RS	Condor		22	20	2	9.09%	4.1000	0.5248	to	32.0293	1.345	0.1785
RJ	Barra Mansa		28	22	6	21.43%	11.1818	2.0202	to	61.8901	2.766	0.0057
MG	Pouso Alto		19	14	5	26.32%	14.6429	**3.7472**	**to**	**57.2194**	**3.860**	**0.0001**
MG	Pequi		23	10	13	56.52%	53.3000	**14.3470**	**to**	**198.0133**	**5.938**	**0.0000**
Total		-	868	838	30	3.46%	-	-		-	-	-

Inhauma, and Itutinga), Goiás (Morrinhos), Ceará (Umirin), Paraná (Chopinho), and Rio de Janeiro (Barra Mansa) presented higher probabilities of animals being infected by this protozoan (OR > 1 and CI 95% > 1).

The prevalence of Coronavirus, Rotavirus, *Cryptosporidium* spp., and diarrhea observed in calves based on the age of animals in days is described in Fig. 2. It is possible to observe that the most prevalence of Coronavirus (4–14%), Rotavirus (5–27%), *Cryptosporidium* spp. (4–14%), and diarrhea (1–15%) occurred on days 4 to 15, 9 to 15, 8 to 15, and 1 to 21 of age respectively (Fig. 2).

Analyses performed by the ROC curve determined the age, in days, associated with a higher occurrence of the investigated agents. Through this analysis, it was possible to verify superior sensitivity and specificity for the occurrence of Coronavirus in cattle ≤ 10 days old. However, for *Cryptosporidium* spp. and Rotavirus, the association criteria for these diseases in animals were determined to be > 6 and > 8 days old, respectively (Table 2). When analyzing diarrhea occurrence by the ROC curve, higher combined sensitivity and specificity for microbe detection with these parameters were observed when the animals were less than 21 days old (Table 2). Importantly, for diarrhea, ROC curve analysis was performed with all 868 fecal samples collected from calves between 1 and 135 days old.

Concerning the results from all 13 risk factors evaluated (Table 3), regarding the ORs of each of these factors influencing animals acquiring any of the three evaluated agents and diarrhea, it was verified that bovines with Rotavirus presented a greater probability of coinfection by *Cryptosporidium* spp. (OR 1.4783; $P = 0.0039$) and diarrhea (OR 1.776; $P < 0.0001$). Similarly, cattle with *Cryptosporidium* spp. presented a higher relative risk of being coinfected by Rotavirus (OR 1.4783; $P = 0.0039$) and of presenting diarrhea (OR 1.6096; $P = < 0.0001$). In addition, raising animals in compost barn systems presented as a risk factor for bovines to be infected with *Cryptosporidium* spp. compared with animals raised isolated in housing or tied on chains (OR 1.1928; $P = 0.0273$).

The occurrence of diarrhea in animals presents a relative risk of occurring with the presence of Rotavirus (OR 6.3423; 95% CI 1.9183–20.9698; $P = 0.0025$) and *Cryptosporidium* spp. (OR 1.6188; 95% CI 1.3042–2.00094; $P < 0.0001$) and when milk is provided for animals through automatic feeders (OR 2.0172; 95% CI 1.7600–2.3120; $P < 0.0001$), as described in Table 3.

The results of the Spearman correlation analysis show that there was a negative correlation ($P \leq 0.05$) between the age of calves and the presence of Coronavirus, *Cryptosporidium* spp., and diarrhea (Table 4). In addition, positive correlations ($P \leq 0.05$) were demonstrated between Rotavirus and *Cryptosporidium* spp., *Cryptosporidium* spp. and Coronavirus, as well as Coronavirus/Rotavirus/*Cryptosporidium* spp. and the occurrence of diarrhea (Table 4).

Eimeria spp. and gastrointestinal nematodes

Of all 868 fecal samples collected from animals between 1 and 135 days of age, 30 (3.46%, CI 95% 2.24–4.67) were diagnosed as positive for *Eimeria* spp. and gastrointestinal nematodes. In the 29 sampled municipalities, it was possible to

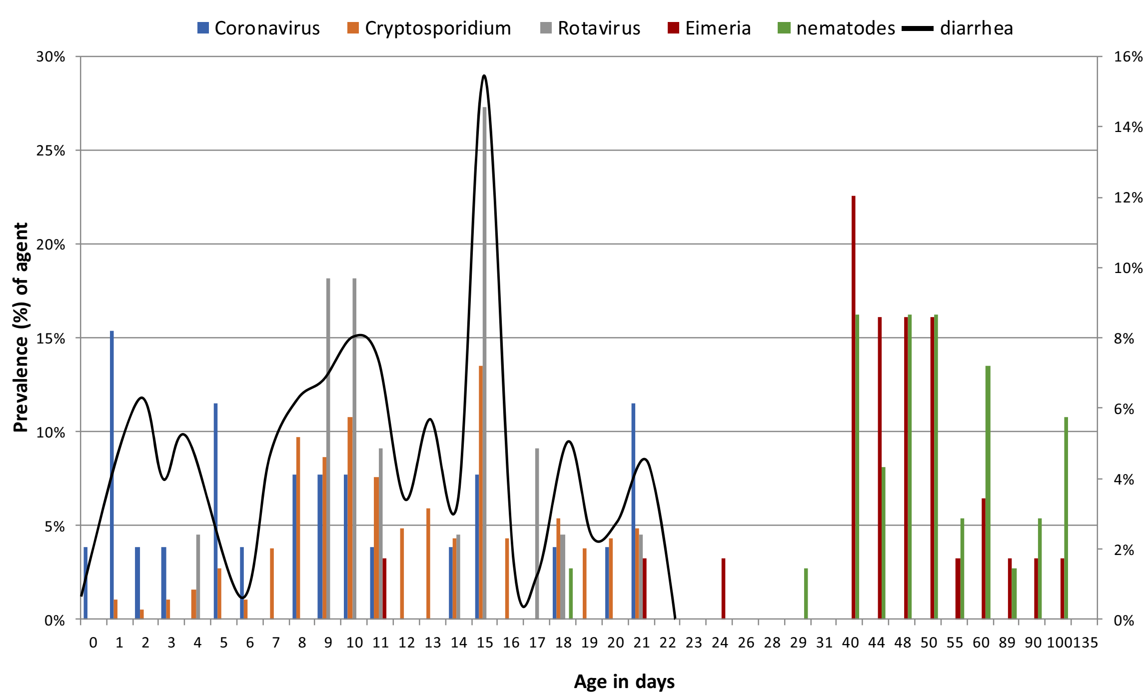

Fig. 2 Results obtained for prevalence of each of the five agents, as well as prevalence of diarrhea observed in calves

Table 2 Calculation of the area under the ROC curve, confidence interval, level of significance, and result of the association criterion for agent occurrence

Variable	Value	Variable	Value
Coronavirus		Rotavirus	
Area under the TOC curve (AUC)	0.645	Area under the TOC curve (AUC)	0.667
95% confidence interval	0.574–0.712	95% confidence interval	0.600–0.730
Significance level *P*	0.0433	Significance level *P*	0.0103
Associated criterion for occurrence in age (days)	≤ 10	Associated criterion for occurrence in age (days)	> 8
Cryptosporidium spp.		*Eimeria* spp.	
Area under the TOC curve (AUC)	0.689	Area under the TOC curve (AUC)	0.654
95% confidence interval	0.623–0.749	95% confidence interval	0.613–0.694
Significance level *P*	< 0.0001	Significance level *P*	0.0007
Associated criterion for occurrence in age (days)	> 6	Associated criterion for occurrence in age (days)	> 37
Gastrointestinal nematodes		Diarrhea	
Area under the TOC curve (AUC)	0.653	Area under the TOC curve (AUC)	0.652
95% confidence interval	0.612–0.692	95% confidence interval	0.614–0.688
Significance level *P*	0.0002	Significance level *P*	< 0.0001
Associated criterion for occurrence in age (days)	> 36	Associated criterion for occurrence in age (days)	≤ 21

detect bovines infected by these agents in nine and seven cities, respectively (Table 1). In locations where *Eimeria* spp. presence was identified, a prevalence of 2.13% (Paraopeba, state of Minas Gerais; CI 95%, 0.0–5.04) to 52.17% (Pequi, state of Minas Gerais; CI 95% 31.56–72.59) was observed. For gastrointestinal nematodes, values of prevalence in places where these agents were found varied between 2.38% (Sete Lagoas, Minas Gerais; CI 95% −2.23–6.99) and 56.52% (Pequi, Minas Gerais; CI 95% 36.26–76.78). The cities Barra Mansa, state of Rio de Janeiro (10.71%; CI 95% 0.0–22.17), and Pequi (52.17%; CI 95% 31.76–72.59) presented an elevated probability of bovines being infected by *Eimeria* spp. (OR > 1 and CI 95% > 1), while both these cities, as well as Pouso Alto, state of Minas Gerais, demonstrated elevated probabilities of animals being infected by gastrointestinal nematodes (Table 1).

The prevalence of *Eimeria* spp. and gastrointestinal nematodes observed in calves based on the age of animals in days is described in Fig. 2. It is possible to observe that the most prevalence of *Eimeria* spp. (16–23%) and gastrointestinal nematodes (8–16%) occurred on days 40 to 50 of age respectively (Fig. 2). In the ROC curve analysis performed for both of these agents, it was possible to verify the association criteria for the significant occurrence of *Eimeria* spp. and gastrointestinal nematodes in bovines aged > 37 and > 36 days, respectively (Table 2). Among the risk factors analyzed, it could be verified that water source was presented as a risk factor for calves to be infected by *Eimeria* spp.; specifically, animals whose water source was lakes, streams, and ponds had an almost eightfold greater probability of infected by this protozoan compared with animals with artesian water sources (OR 7.931; $P < 0.0001$). Additionally, bovines infected by *Eimeria* spp. presented a more than 18-fold greater probability of being coinfected by gastrointestinal nematodes (OR 18.622; $P < 0.0001$), as described in Table 3.

It was possible to identify the following seven *Eimeria* species in decreasing order of frequency of occurrence: *E. bovis* (33.2%), *E. alabamensis* (17.8%), *E. zuernii* (15.2%), *E. cylindrica* (13.9%), *E. ellipsoidalis* (11.6%), *E. wyomingensis* (5.1%), and *E. canadensis* (3.3%).

No risk factors were verified for calves to be infected by gastrointestinal nematodes, and no correlation was found between these two agents (*Eimeria* spp. and gastrointestinal nematodes) and animal age, presence of Coronavirus, Rotavirus, *Cryptosporidium* spp., and diarrhea when analyzed by Spearman's method (Table 4).

Figure 2 summarizes the results obtained for the prevalence of each of the five agents described in this research paper, as well as the prevalence of diarrhea observed in calves based on the age of animals in days.

Discussion

Although published research studies are of high importance to science, from an epidemiological point of view, there is a gap in the body of knowledge, since such works study infectious agents separately or grouping only viral pathogens for example.

The joint occurrence of Coronavirus and Rotavirus was investigated by Jerez et al. (2002) on dairy farms located in several municipalities of São Paulo state, Brazil. In this case, coinfections with Rotavirus and Coronavirus were detected in 3% of all calves. Although these two viral agents were

Table 3 Risk factors associated with infection by different agents and diarrhea in dairy calves of different states in Brazil

Rotavirus		Negative	Positive	Relative risk			
				Valor teste	95% CI	*z* statistic	Significance level
Cryptosporidium spp.	Negative	169	169	1.4783	1.1339 to 1.9271	2.888	0.0039
	Positive	6	17				
Diarrhea	Negative	172	165	1.776	1.4655 to 2.1524	2.632	< 0.0001
	Positive	3	20				
Cryptosporidium spp.		Negative	Positive	Relative risk			
				Valor teste	95% CI	*z* statistic	Significance level
Rotavirus	Negative	169	169	1.4783	1.1339 to 1.9271	2.888	0.0039
	Positive	6	17				
Diarrhea	Negative	106	68	1.6096	1.2971 to 1.9974	4.322	< 0.0001
	Positive	69	117				
Calf rearing	Isolated	132	126	1.1928	1.0199 to 1.3949	2.207	0.0273
	Collective	129	180				
Eimeria spp.		Negative	Postive	Relative risk			
				Valor teste	95% CI	*z* statistic	Significance level
Source water	Artesian well	660	11	7.931	3.7928 to 16.5843	5.502	< 0.0001
	No artesian well	174	23				
Gastrointestinal nematodes	Negative	820	18	18.6222	9.8865 to 350.767	9.052	< 0.0001
	Positive	18	12				
Diarrhea		Negative	Positive	Relative risk			
				Valor teste	95% CI	*z* statistic	Significance level
Cryptosporidium spp.	Negative	107	68	1.6188	1.3042 to 2.0094	4.368	< 0.0001
	Positive	69	117				
Rotavirus	Negative	173	3	6.3423	1.9183 to 20.9698	3.028	0.0025
	Positive	165	20				
Milk supply	Automatic breastfeeding	18	150	2.0172	1.7600 to 2.3120	10.08	< 0.0001
	Bucket/bottle	170	135				

observed simultaneously in animals, it was not possible to determine the occurrence of one agent that was a risk factor for the appearance of either agent ($P > 0.05$). This possibility was confirmed in the present work, as it is possible to observe that these agents appear in different periods and ages, as previously described. Feitosa et al. (2008), analyzing the importance of *Cryptosporidium* spp. as a cause of diarrhea in calves, verified a peak of occurrence in 15-day-old animals and observed that all samples positive for this protozoan were also positive for Coronavirus and Rotavirus. In the present study, which analyzed all of these enteropathogenic agents together, it is possible to verify that Rotavirus was a risk factor for the occurrence of *Cryptosporidium* spp., and this protozoan also showed a significant positive correlation with Coronavirus, with these three agents triggering diarrhea in animals. However, when analyzing specificity and sensitivity to detect an agent obtained by the ROC curve, it is possible to determine that there were interactions between *Cryptosporidium* spp. and Coronavirus from the 6th to the 10th day of age, as well as interactions between *Cryptosporidium* spp. and Rotavirus from the 7th to 21st day of age, with peaks of diarrhea being observed in animals at the 15th day of life, as observed by Feitosa et al. (2008), only for *Cryptosporidium* spp. The results of this study indicate that the diarrhea affecting calves between 6 and 10 days of life occurs mainly by Coronavirus and *Cryptosporium*, whereas the diarrhea described in calves at 10 to 21 days of age occurs mainly by *Cryptosporidium* and Rotavirus. The results obtained in this work demonstrate the importance of conducting studies involving several enteropathogenic agents together in calves of different ages.

Another risk factor that should be emphasized is that calves raised in compost barns were 1.19 times more likely to become infected with *Cryptosporidium* spp. than animals bred in isolated housing or tied in chains ($P = 0.0273$). The results obtained in a study conducted by Waele et al. (2010) support the findings of the present study. In the study by these researchers, the use of halofuginone lactate for preventing cryptosporidiosis in naturally infected neonatal calves was investigated on a dairy farm with a high prevalence of infection, where animals were kept in two different calf rearing systems. These researchers reported that halofuginone lactate use combined with good hygiene measures, such as rearing animals in clean individual pens, was the most effective method to reduce

Table 4 Correlation matrix and significance level of the parameters evaluated by the Spermann method

Variable	-	*Age*	*Coronavirus*	*Cryptosporidium spp*	*Diarrhea*	*Eimeria spp*	*Nematodes*	*Rotavirus*
Age	correlation matrix	1.0000	**-0.5914**	**-0.4657**	**-0.6215**	-0.0996	-0.1439	-0.0238
	value of P	1.0000	**0.0003**	**0.0063**	**0.0001**	0.5812	0.4243	0.8954
Coronavirus	correlation matrix	**-0.5914**	1.0000	**0.5836**	**0.6594**	0.1526	0.2747	0.3147
	value of P	**0.0003**	1.0000	**0.0004**	**0.0000**	0.3965	0.1218	0.0745
Cryptosporidium spp	correlation matrix	**-0.4657**	**0.5836**	1.0000	**0.8893**	0.2725	0.2555	**0.4482**
	value of P	**0.0063**	**0.0004**	1.0000	**0.0000**	0.1249	0.1513	**0.0089**
Diarrhea	correlation matrix	**-0.6215**	**0.6594**	**0.8893**	1.0000	0.3696	0.3808	**0.5137**
	value of P	**0.0001**	**0.0000**	**0.0000**	1.0000	0.3426	0.2879	**0.0022**
Eimeria spp	correlation matrix	-0.0996	0.1526	0.2725	0.3696	1.0000	-0.1000	0.2054
	value of P	0.5812	0.3965	0.1249	0.0634	1.0000	0.5798	0.2515
Nematodes	correlation matrix	-0.1439	0.2747	0.2555	0.3808	-0.1000	1.0000	0.2518
	value of P	0.4243	0.1218	0.1513	0.0788	0.5798	1.0000	0.1575
Rotavirus	correlation matrix	-0.0238	0.3147	**0.4482**	**0.5137**	0.2054	0.2518	1.0000
	value of P	0.8954	0.0745	**0.0089**	**0.0022**	0.2515	0.1575	1.0000

cryptosporidiosis risk among 7–13-day-old calves when comparing these calves with animals bred in collective pens. The latter had easier access to excrement from other calves in comparison with calves reared in isolated housing or tied with chains. In this way, the occurrence of a particular disease tends to be higher in cases in which calves are bred in a collective manner, such as in compost barns.

Another risk factor for diarrhea occurrence that was associated with calves in the present study was the way that milk was offered to animals. Calves that received milk through automatic feeders were 2.01 times more likely to have diarrhea than animals receiving milk in buckets or bottles ($P < 0.0001$). This fact is directly related to the findings described in the previous paragraph, since automatic feeders are used only in calves on collective pens; in addition, such equipment is likely to be cleaned after several calves have used the same feeder compared with buckets or bottles.

Regarding *Eimeria* spp., Feitosa et al. (2008) detected samples of diarrhea from calves aged approximately 15 days and positive for *Cryptosporidium* spp., Rotavirus, and *Eimeria* spp. However, the results found in the present study allow us to infer that the diarrhea found in 15-day-old calves reported by Feitosa et al. (2008) did not occur due to the presence of *Eimeria* spp. In the present study, it was possible to find bovines 11 days old infected with *Eimeria* spp. However, analyzing the ROC curve made it possible to verify the association criteria for *Eimeria* spp. in calves > 37 days old. This period is significantly later than that reported by Feitosa et al. (2008) for *Eimeria* spp. and is later than that found in this work for Coronavirus (< 10 days), Rotavirus (> 8 days), *Cryptosporidium* (> 6 days), and diarrhea (< 21 days) occurrence in calves. A work published by Cruvinel et al. (2018b) reinforces the inference described above for *Eimeria* spp., since these investigators observed an outbreak of *E. zuernii*, exhibiting bloody diarrhea and animal mortality, in beef cattle with an average age of 45 days. It is important to emphasize that in this research, unlike the results found by Cruvinel et al. (2018b), it was not possible to verify a relationship between *Eimeria* spp. and diarrhea in animals. However, the most prevalent species found in the present study was *E. bovis* followed by *E. alabamensis* and *E. zuernii*.

Another risk factor that interfered with *Eimeria* spp. occurrence in calves was the source of animals' water supplies. When water originated from rivers, streams, and ponds, bovines presented 7.931 times higher probability of being infected by *Eimeria* spp. compared with animals that received water from artesian wells ($P < 0.0001$). The previously mentioned research of consecutive outbreaks by *E. zuernii* in calves, published by Cruvinel et al. (2018b), also describes such similarity, indicating that water from rivers, streams, and ponds is more easily contaminated by feces/oocysts of *Eimeria* spp. than water from artesian wells. In addition, calves infected with *Eimeria* spp. were 18.62 times more likely to be infected with gastrointestinal nematodes ($P < 0.0001$). These results demonstrate the importance of conducting future studies to determine the true impact that these two parasites can trigger together in infected animals.

Based on previously discussed results obtained in the present study, it is possible to conclude that the prevalence of Coronavirus, Rotavirus, *Cryptosporidium* spp., *Eimeria* spp., and gastrointestinal nematodes is 7.20% (CI 95% 4.54–9.87), 6.37% (CI 95% 3.85–8.89), 51.52% (CI 95% 45.26–55.57), 3.46% (CI 95% 2.24–4.67), and 3.46% (CI 95% 2.24–4.67), respectively. The ages with the highest probabilities of occurrence of such diseases are < 10, > 8, > 6, > 37, and > 36 days old, respectively. There is a positive correlation ($P \leq 0.05$) between Coronavirus, Rotavirus, *Cryptosporidium* spp., and diarrhea. In addition, diarrhea occurs more significantly

($P < 0.0001$) in bovines less than 21 days old and mainly in those that received milk through automatic feeders ($P < 0.001$). *Cryptosporidium* spp. and Rotavirus are risk factors for each other's occurrence ($P = 0.0039$), and the former also presents a positive correlation with Coronavirus ($P = 0.0089$). In addition, calves bred in compost barns are more easily infected by *Cryptosporidium* spp. than those that are raised in isolated housing or tied to chains ($P = 0.0273$). Young bovines that drink water from rivers, streams, and ponds have a higher chance of being infected by *Eimeria* spp. ($P < 0.0001$) and gastrointestinal nematodes ($P < 0.0001$). The presence of diarrhea in calves was not significantly correlated ($P > 0.05$) with the presence of *Eimeria* spp. and gastrointestinal nematodes in animals.

The results found in this study highlight the importance of studying the agents of diarrhea together, once they act as co-infection where the losses triggered for the owners will involve some of these agents simultaneously.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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