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

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;
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 in fecal samples in 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'-GCCGATCAGTCCGACCAATCT-3'(nt79–98) and downstream 5'-AGAATGTCAGCCGGGTAT-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, 1 μl of 0.1 M dithiothreitol, 2 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/
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 2000g 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 (K₂Cr₂O₇) 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 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,
| 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     | Uberlândia      |          | 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 Canta      |          | 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     | Limeheiro 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     | Virginia        |          | 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%      |            |            |            |        |        |
| 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    | Virginia |       | 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 | 0.00 to 19.4973 | 0.081            | 0.9355            |
| CE    | Umirim |       | 14    | 13       | 1        | 7.14%      | 1.3846     | 0.0788 | 0.00 to 24.3436 | 0.222            | 0.8239            |
| RS    | Capão do Leão |     | 14    | 13       | 1        | 7.14%      | 1.3846     | 0.0780 | 0.00 to 24.5796 | 0.222            | 0.8245            |
| SC    | Xanxeré |       | 14    | 13       | 1        | 7.14%      | 1.3846     | 0.0780 | 0.00 to 24.5796 | 0.222            | 0.8245            |
| MG    | Prata |       | 12    | 11       | 1        | 8.33%      | 1.6364     | 0.0913 | 0.00 to 29.3207 | 0.334            | 0.7380            |
| MG    | Inhauma |       | 56    | 51       | 5        | 8.93%      | 1.7647     | 0.1872 | 0.00 to 16.6390 | 0.496            | 0.6198            |
| MG    | Paraopeba |     | 27    | 24       | 3        | 11.11%     | 2.2500     | 0.4964 | 0.00 to 10.1991 | 1.052            | 0.2930            |
| MG    | Uberlandia |    | 42    | 37       | 5        | 11.90%     | 2.4324     | 0.5316 | 0.00 to 11.1302 | 1.146            | 0.2520            |
| MG    | Pouso Alto |     | 6     | 5        | 1        | 16.67%     | 3.6000     | 0.3463 | 0.00 to 37.4241 | 1.072            | 0.2836            |
| RJ    | Barra Mansa |   | 7     | 5        | 2        | 28.57%     | 7.2000     | 0.4831 | 0.00 to 107.3135 | 1.432            | 0.1521            |
| PR    | Ampere |       | 2     | 1        | 1        | 50.00%     | 18.0000    | 0.7188 | 0.00 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    | Cruzeiro |       | 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 | 0.00 to 16.0268 | 0.289            | 0.7725            |
| MG    | Sete Lagoas |     | 11    | 7        | 4        | 36.36%     | 2.2857     | 0.4626 | 0.00 to 11.2926 | 1.014            | 0.3104            |
| MG    | Três Corações | | 5     | 3        | 2        | 40.00%     | 2.6667     | 0.3044 | 0.00 to 23.3642 | 0.886            | 0.3757            |
| State | City              | Agent       | Total | Negative | Positive | Prevalence | Odds ratio |
|-------|------------------|-------------|-------|----------|----------|------------|------------|
|       |                  |             |       |          |          |            |            |
| MG    | Uberlandia       |             | 42    | 25       | 17       | 40.48%     | 2.7200     |
| MG    | Paraopeba        |             | 27    | 15       | 12       | 44.44%     | 3.2000     |
| GO    | Morinhas         |             | 22    | 12       | 10       | 45.45%     | 3.3333     |
| SP    | Areias           |             | 17    | 9        | 8        | 47.06%     | 3.5556     |
| PR    | Ampere           |             | 2     | 1        | 1        | 50.00%     | 4.0000     |
| PR    | Marechal Candido Rondon | | 2 | 1 | 1 | 50.00% | 4.0000 | |
| MG    | Pará de Minas    |             | 18    | 9        | 9        | 50.00%     | 4.0000     |
| MG    | Baependi         |             | 2     | 1        | 1        | 50.00%     | 4.0000     |
| SC    | Xanxerê          |             | 14    | 7        | 7        | 50.00%     | 4.0000     |
| MG    | Curvelo          |             | 15    | 7        | 8        | 53.33%     | 4.5714     |
| PR    | Nova Canta       |             | 9     | 4        | 5        | 55.56%     | 5.0000     |
| RS    | Capão do Leão    |             | 14    | 6        | 8        | 57.14%     | 5.3333     |
| MG    | Prata            |             | 12    | 5        | 7        | 58.33%     | 5.6000     |
| CE    | Umirim           |             | 14    | 5        | 9        | 64.29%     | 7.2000     |
| MG    | Inhauma          |             | 56    | 19       | 37       | 66.07%     | 7.7895     |
| RS    | Almirante do Sul |            | 3     | 1        | 2        | 66.67%     | 8.0000     |
| MG    | Virginia         |             | 4     | 1        | 3        | 75.00%     | 12.0000    |
| MG    | Itutinga         |             | 11    | 2        | 9        | 81.82%     | 18.0000    |
| PR    | Chopinho         |             | 6     | 1        | 5        | 83.33%     | 20.0000    |
| MG    | Pouso Alto       |             | 6     | 1        | 5        | 83.33%     | 20.0000    |
| RJ    | Barra Mansa      |             | 7     | 1        | 6        | 85.71%     | 24.0000    |
| Total |                  |             | 361   | 179      | 182      | 50.41%     | -          |

**Note:** The table continues with similar entries.
| State | City          | Agent                        | Total | Negative | Positive | Prevalence | Odds ratio | Value | 95 % CI     | z statistic | Significance level |
|-------|---------------|------------------------------|-------|----------|----------|------------|------------|-------|-------------|--------------|-------------------|
| PR    | Nova Cantú    | Gastrointestinal nematodes   | 9     | 9        | 0        | 0.00%      | 1.0000     |       |             |              |                   |
| PR    | Palmital      |                              | 16    | 16       | 0        | 0.00%      | 1.0000     |       |             |              |                   |
| MG    | Pouso Alto    |                              | 19    | 19       | 0        | 0.00%      | 1.0000     |       |             |              |                   |
| MG    | Prata         |                              | 36    | 36       | 0        | 0.00%      | 1.0000     |       |             |              |                   |
| PR    | Realeza       |                              | 5     | 5        | 0        | 0.00%      | 1.0000     |       |             |              |                   |
| RS    | Saldanha Marinho |                          | 12    | 12       | 0        | 0.00%      | 1.0000     |       |             |              |                   |
| MG    | Sete Lagoas   |                              | 42    | 42       | 0        | 0.00%      | 1.0000     |       |             |              |                   |
| MG    | Três Corações |                              | 11    | 11       | 0        | 0.00%      | 1.0000     |       |             |              |                   |
| MG    | Virginia      |                              | 12    | 12       | 0        | 0.00%      | 1.0000     |       |             |              |                   |
| SC    | Xanxerê       |                              | 66    | 66       | 0        | 0.00%      | 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%      | 1.0000     |       |             |              |                   |
| SP    | Areias        |                              | 26    | 26       | 0        | 0.00%      | 1.0000     |       |             |              |                   |
| MG    | Baependi      |                              | 9     | 9        | 0        | 0.00%      | 1.0000     |       |             |              |                   |
| RS    | Capão do Leão |                              | 29    | 29       | 0        | 0.00%      | 1.0000     |       |             |              |                   |
| PR    | Chopinó       |                              | 17    | 17       | 0        | 0.00%      | 1.0000     |       |             |              |                   |
| MG    | Cruzalia      |                              | 9     | 9        | 0        | 0.00%      | 1.0000     |       |             |              |                   |
| MG    | Curvelo       |                              | 42    | 42       | 0        | 0.00%      | 1.0000     |       |             |              |                   |
| PR    | Francisco Beltrão |                      | 5     | 5        | 0        | 0.00%      | 1.0000     |       |             |              |                   |
| MG    | Inhaúma       |                              | 61    | 61       | 0        | 0.00%      | 1.0000     |       |             |              |                   |
| MG    | Itutinga      |                              | 16    | 16       | 0        | 0.00%      | 1.0000     |       |             |              |                   |
| CE    | Limoeiro do Norte |                       | 41    | 41       | 0        | 0.00%      | 1.0000     |       |             |              |                   |
| PR    | Marechal Candido Rondon |                     | 17    | 17       | 0        | 0.00%      | 1.0000     |       |             |              |                   |
| GO    | Morrinhos     |                              | 37    | 37       | 0        | 0.00%      | 1.0000     |       |             |              |                   |
| PR    | Nova Cantú    |                              | 9     | 9        | 0        | 0.00%      | 1.0000     |       |             |              |                   |
| 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    | Virginia       | 12        | 12    | 0        | 0.00%    |             |            |
| SC    | Xanxerê        | 66        | 66    | 0        | 0.00%    |             |            |
| MG    | Sete Lagoas    | 42        | 41    | 1        | 2.38%    | 1.0000     | 1.0000     |
| MG    | Três Corações  | 11        | 10    | 1        | 9.09%    | 4.1000     | 0.2356     |
| RS    | Almirante do Sul | 22   | 20    | 2        | 9.09%    | 4.1000     | 0.3307     |
| RS    | Condor         | 22        | 20    | 2        | 9.09%    | 4.1000     | 0.5248     |
| RJ    | Barra Mansa    | 28        | 22    | 6        | 21.43%   | 11.1818    | 2.0202     |
| MG    | Pouso Alto     | 19        | 14    | 5        | 26.32%   | 14.6429    | 3.7472     |
| MG    | Pequi          | 23        | 10    | 13       | 56.52%   | 53.3000    | 14.3470    |
|       | Total          | 868       | 838   | 30       | 3.46%    | -          | -          |

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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 ≤ 0.05) between the age of calves and the presence of Coronavirus, Cryptosporidium spp., and diarrhea (Table 4). In addition, positive correlations (P ≤ 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](image-url)
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
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 Cryptosporidium, 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 3 Risk factors associated with infection by different agents and diarrhea in dairy calves of different states in Brazil |
|---|
| | Rotavirus | Cryptosporidium spp. | Diarrhea |
| | Negative | Positive | Cryptosporidium spp. | Negative | Positive | Cryptosporidium spp. | Negative | Positive |
| | Valor teste | 95% CI | z statistic | Significance level | Valor teste | 95% CI | z statistic | Significance level | 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 | 172 | 165 | 1.776 | 1.4655 to 2.1524 | 2.632 | < 0.0001 |
| | Positive | 3 | 20 | | | | | |
| Calf rearing | Isolated | 132 | 126 | 1.1928 | 1.0199 to 1.3949 | 2.207 | 0.0273 |
| | Collective | 129 | 180 | | | | | |
| Eimeria spp. | 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 | 107 | 68 | 1.6188 | 1.3042 to 2.0094 | 4.368 | < 0.0001 |
| | Positive | 69 | 117 | | | | | |
| Cryptosporidium spp. | 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 | | | | | |

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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 ≤ 0.05) between Coronavirus, Rotavirus, Cryptosporidium spp., and diarrhea. In addition, diarrhea occurs more significantly

### 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      | 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 | -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 | -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 | -0.6215 | 0.6594 | 0.8893 | 1.0000 | 0.3696 | 0.3808 | 0.5137 |
| value of P | 0.0011 | 0.0000 | 0.0000 | 1.0000 | 0.3426 | 0.2879 | 0.0022 |
| Eimeria spp | -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 | -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 | -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 |
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