Ciência Rural, Santa Maria, v.54:02, e20220472, 2024 http://doi.org/10.1590/0103-8478cr20220472
ISSNe 1678-4596
MICROBIOLOGY

Severe enteritis in dogs associated with single and mixed infections

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ABSTRACT: Infectious enteritis is highly prevalent among dogs worldwide and, in some cases, it can be fatal. This study describes the clinical and laboratorial findings of single and mixed infections associated with severe enteritis in 76 dogs from Southern Brazil. Intestinal segments and/or fecal samples were subjected to histopathology and molecular detection of DNA viruses, bacteria and protozoa. Severe intestinal lesions were observed in most cases. Single infections were detected in 52.6% of cases, double (36.8%) and triple (10.5%) infections were also identified. *Carnivore protoparvovirus* 2 (CPV-2) was the most frequent agent in single infections (36.8%). Coinfection by CPV-2 and *Giardia* spp. was the most common in dual infections (19.7%), followed by CPV-2 and *Cryptosporidium* spp. (10.5%). The most frequent triple infection was CPV-2, *Giardia* sp. and *Cryptosporidium* spp. (6.6%). Our results shown that single and mixed infections are associated with severe enteritis in dogs in southern Brazil, mainly involving CPV-2 and *Giardia* sp.
Key words: gastroenteritis, dogs, bacteria, virus, protozoa, diagnosis.

Enterites severas em cães associadas a infecções simples e mistas

RESUMO: Enterites infecciosas são altamente prevalentes entre cães em todo o mundo e, em alguns casos, podem ser fatais. Este estudo descreve os achados clínicos e laboratoriais de infecções simples e mistas associadas a enterites graves em 76 cães do sul do Brasil. Segmentos intestinais e/ou fezes foram submetidos à análise patológica e detecção molecular de vírus de genoma DNA, bactérias e protozoários. Lesões intestinais severas foram observadas na maioria dos casos. Infecções únicas foram detectadas em 52,6% dos casos, e infecções duplas (36,8%) e triplas (10,5%) também foram identificadas. O *Protoparvovírus carnívoro* 2 (CPV-2) foi o agente mais frequente nas infecções simples (36,8% do total). Coinfecção por CPV-2 e *Giardia* sp. foi a mais comum em infecções duplas (19,7%), seguido por CPV-2 e *Cryptosporidium* spp. (10,5%). A tripla infecção mais frequente foi CPV-2, *Giardia* spp. e *Cryptosporidium* spp. (6,6%). Nossos resultados demostraram que infecções simples e mistas estão associadas a enterites graves em cães no sul do Brasil, envolvendo principalmente CPV-2 e *Giardia* sp.
Palavras-chave: gastroenterite, cães, bactérias, vírus, protozoários, diagnóstico.

INTRODUCTION

Infectious and parasitic agents are commonly associated with gastrointestinal disease in dogs, ranging in severity from mild diarrhea to fatal enteritis. Gastrointestinal infections are distributed worldwide and affect mainly dogs younger than 1 year-old, yet adult infections and disease have also been reported (GIZZI et al., 2014). Protozoan and/or infectious enteritis remain as a major challenge to veterinarians, since many agents may cause similar clinical signs, maiking difficult a definitive diagnosis and the implementation of appropriate therapy (DUIJVESTIJN et al., 2016; GIZZI et al., 2014).

Gastrointestinal dog infections are important both for animal and public health, since some agents are zoonotic and/or produce severe disease, frequently resulting in death (ITOH et al., 2019; MARTINS et al., 2019). These fatal enteritis are frequently associated with *Carnivore protoparvovirus* 2 (CPV-2) infection due to its high virulence. However, other agents may also produce acute enteritis with similar clinical course, including protozoan and bacteria (DUIJVESTIJN et al., 2016;

Received 08.23.22 Approved 04.17.23 Returned by the author 06.22.23
CR-2022-0472.R3
Editor: Rudi Weiblen

GIZZI et al., 2014). Secondary agents are frequently neglected during clinical inspection or laboratory investigation (ELLWANGER & CHIES, 2019), but sometimes could to aggravate the clinical course (DENHOLM et al., 2001; SILVA et al., 2017).

A number of studies aimed to detect/identify viral, bacterial or protozoa agents in intestinal segments or fecal samples of dogs with enteritis (DUIJVESTIJN et al., 2016; GIZZI et al., 2014). However, many studies focused on one or two specific agents, and failed to detect mixed infections caused by another microorganisms (ALVES et al., 2018; DE OLIVEIRA et al., 2018; OSMARI et al., 2021; SILVA et al., 2017). Furthermore, the clinic-epidemiological, molecular and pathological aspects have been only sporadically investigated and/or reported in fatal cases of enteritis in dogs. Thus, the aim of this study was to investigate the etiological agents, the clinical and pathological findings in dogs undergoing a severe course of enteritis caused by single or mixed infections.

MATERIALS AND METHODS

Experimental design

Samples of 76 dogs presenting a history of severe enteritis, mostly evolving death, were included in the study. The animals were submitted to veterinary assistance due to history of diarrhea, emesis/vomit, anorexia and/or apathy. Dogs that died naturally were submitted to necropsy. The material collected at clinical inspection or necropsy was stored at -20 ºC until processing molecular analysis. Nucleic acids extracted from feces and/or intestinal fragments were submitted to molecular detection by PCR for viral, bacterial and protozoa agents. Additionally, histological examination was performed and the clinical, epidemiological and pathological findings were analyzed. The study comprised animals submitted to veterinary clinical and/or pathology services from Santa Maria and Porto Alegre, Rio Grande do Sul, Southern Brazil, between 2015 and 2020.

DNA extraction, PCR and nucleotide sequencing

Intestinal fragments and/or feces (100 to 200 mg) were macerated and mixed with 500 µl of TE 1X solution (10mM Tris-HCl, EDTA 0.1mM), shacked by 15 s and submitted to three cycles of thermic shock (37 ºC to -80 ºC) to perform the lysis of parasite cysts or oocysts presents in feces. DNA extraction of 500 µl of each sample was performed using phenol and chloroform protocol (BARKER et al., 1998) with modification in initial step of digestion (samples were incubated with 20mg/ml of proteinase K by 1 h at 56 ºC).

Molecular detection of protozoan, bacteria and DNA viruses was performed by PCR and nested-PCR, using primers and protocols described in table 1. The most common agents associated with intestinal infection in dogs were investigated: *Giardia* spp., *Cryptosporidium* spp., *Sarcocystis* spp., *Neospora caninum*, *Toxoplasma gondii*, *Clostridium difficile*, *Clostridium perfringens* A, *Carnivore protoparvovirus 2* (CPV-2) and *Canine Circovirus* (CanineCV). RNA viruses were not included in the survey, as the samples were stored for a long time at -20 °C until processing, and they were not kept in a special buffer for RNA preservation.

For this, 100 – 200 ng of total DNA, 1.25 mM of $MgCl_2$, 2.5 mM 10x *Taq* buffer, 1 U of Taq DNA polymerase (0.2 µl) (Thermo Fisher Scientific®), 20 µM deoxyribonucleotides (dNTPs), 1 µM each primer and ultrapure water up to 25 µl were used in each reaction. PCR products were resolved in a 1.5 % agarose gel stained by Gel Red (Biotium Inc, Fremont, CA, USA) and visualized under ultraviolet light after electrophorese (60 V, 60 min). CPV-2 classification was based on the analysis of the amino acid residue at position 426 of the VP2 FPLV/CPV-2 gene, as follows: asparagine determines subtype 2a; aspartic acid determines 2b; and glutamic acid determines 2c (BUONAVOGLIA et al., 2001).

Clinical, epidemiological and pathological analysis

Clinical and epidemiological data of all cases were collected and analyzed, including age, clinical signs and the time of clinical evolution. Most dogs included in the present study were submitted to a full necropsy. Tissue fragments were fixed in 10% buffered formalin for 24 to 48 h. After tissue processing, paraffin-embedded samples were cut in 3-µm-thick sections and stained with hematoxylin and eosin for histological examination.

RESULTS

Sixty-eight of the 76 cases presented gastroenteric signs and evolved to death. Eight dogs remained alive (8/76), of which we only had access to feces and clinical history. At least one agent was detected (52.6%), but double (36.8%) and triple infections (10.5%) where frequently reported. The single infection by CPV-2 was the most common associated with severe enteritis in analyzed cases (36.8% of total). Co-infection between *Giardia* spp. and CPV-2 (19.7%) was the most frequent double

Table 1 - Agent, target gene, primer sequences and expected amplicon size of PCR and nested-PCR used to detect DNA virus, bacteria and protozoa agents in feces and intestinal fragments of dogs with severe enteritis.

Agent	Gene	Primers (5'-3')	Product (bp)	Reference
Giardia spp.	β-giardin (bg)	G7: AAGCCCGACGACCTCACCCGCAGTGC G759:GAGGCCGCCCTGGATCTTCGAGACGAC BG-Nst-F: GAACGAACGAGATCGAGGTCCG BG- Nst-R: CTCGACGAGCTTCGTGTT	753[a] 511[b]	(OSMARI et al., 2021)
Cryptosporidium spp.	SSU rRNA	F1: TTCTAGAGCTAATACATGCG R1: CCCATTTCCTTCGAAACAGGA F2: GGAAGGGTTGTATTTATTAGATAAAG R2: AAGGAGTAAGGAACAACCTCCA	1325[a] 819–825[b]	(ALVES et al., 2018)
Sarcocystis spp.	18s rDNA	F: CGCAAATTACCCAATCCTGA R: ATTTCTCATAAGGTGCAGGAG	700	(PORTELLA et al., 2016)
Toxoplasma gondii	B1	F:CGCTGCAGGGAGGAAGACGAAAGTTG R:CGCTGCAGACACAGTGCATCTGGATT	529	(PORTELLA et al., 2016)
Neospora caninum	Nc5	F:CCCAGTGCGTCCAATCCTGTAAC R:CTCGCCAGTCAACCTACGTCTTCT	338	(PORTELLA et al., 2016)
Clostridium perfringens	Toxin A	CPA 5L: AGTCTACGCTTGGGATGGAA CPA 5R: TTTCCTGGGTTGTCCATTTC	900	(BURET, A. et al., 1992)
Clostridium difficile	*tcdA* and *tdB*	NK2:CCCAATAGAAGATTCAATATTAAGCTT NK3: GGAAGAAAAGAACTTCTGGCTCACTCAGGT cdBFor: TAATAGAAAACAGTTAGAAA cdBRev: TCCAATCCAAACAAAATGTA	252[c] 301[d]	(ALVAREZ-PEREZ et al., 2009)
CanineCV	Rep	For_genomic:ATGGCTCAAGCTCAGGTTG Rev 533: CCGCACAGAACCTCCACTTC	533	(KOTSIAS et al., 2019)
CPV-2	VP2	555_for:CAGGAAGATATCCAGAAGGA 555_rev: GGTGCTAGTTGATATGTAATAAACA	583	(BUONAVOGLIA, et al., 2001)

a: primary reaction; b: secondary reaction; c: toxin A; d: toxin B.

infection, followed by *Cryptosporidium* spp. and CPV-2 (10.5%). Among triple infections, *Giardia* spp., *Cryptosporidium* spp. and CPV-2 (6.6% of total cases) was the most frequent (Table 2).

CPV-2 was the main agent involved in single and mixed infections (60/76 cases, 78.94%). In most cases, histopathological lesions suggestive of CPV-2 were reported by the pathologist, including necrotizing enteritis with squamous metaplasia, necrosis and fusion of crypts, villous atrophy (Figure 1A), epithelial syncytia in crypts and Peyer's patch necrosis. DNA sequencing and amino acid analysis of the VP2 protein was performed on 46 CPV-2 positive samples (46/60), indicating that 27 were classified as CPV-2c, 17 as CVP-2a and 2 as CPV-2b. Fourteen samples were not sequenced and classified due to poor DNA quality.

Single infections by CPV-2, *Giardia* spp., *Clostridium perfringens* type A or *Crysptosporidium* spp. were identified in most analyzed dogs (52.6%), with predominance of CPV-2 (36.8%), followed by *Giardia* sp. (11.8%). The age of CPV-2 affected animals ranged from 1 month to 7 years-old. The clinical signs reported were mainly diarrhea, vomiting, prostration, anorexia and convulsion. In cases where only *Giardia* sp. was detected, two dogs developed intestinal lesions characteristic of protozoa infection, as cryptitis (Figure 1B), suggesting giardiasis as primary cause of death. However, five (5/9) dogs positive to *Giardia* sp. presented intestinal lesions highly associated with CPV-2 infection, but viral DNA was not detected by PCR.

Double infections included mostly *Giardia* sp. and CPV-2 (15/28) or *Cryptosporidium* sp. and CPV-2 (8/28). In two cases of co-infection by *Cryptosporidium* sp. and CPV-2, the dogs presented systemic manifestations of other infections, as canine herpesvirus and adenovirus. Young dogs (2 months-

Table 2 - Infections and co-infections detected in dogs presenting severe enteritis in southern Brazil.

Type of infection	Number of cases	%
Single infection	*40*	*52.6*
CPV-2	28	36.8
Giardia sp.	9	11.8
Crysptosporidium sp.	2	2.6
Clostridium perfringens type A	1	1.3
Double infection	*28*	*36.8*
Giardia sp. and CPV-2	15	19.7
Cryptosporidium sp. and CPV-2	8	10.5
Giardia sp. and *Cryptosporidium* sp.	2	2.6
Giardia sp. and *Clostridium perfringens* type A	2	2.6
Clostridium perfringens type A and CPV-2	1	1.3
Triple infection	*8*	*10.5*
Giardia sp., *Cryptosporidium* sp. and CPV-2	5	6.6
Giardia sp., *Sarcocystis* sp. and CPV-2	1	1.3
Giardia sp., *Clostridium difficile* and CPV-2	1	1.3
Giardia sp., *Clostridium perfringens* type A and CPV-2	1	1.3
Total	*76*	*100*

old) co-infected by *Giardia* sp. and *Cryptosporidium* sp. presented clinical and pathological signs suggestive of protozoa (1/2) or CPV-2 infection (1/2). Crypt abscess (or cryptitis) that is a classical lesion of intestinal protozoa infection, was observed in a dog. Co-infection by *Giardia* sp. and *C. perfringens* type A was observed in a single case and the animal presented intestinal lesions characteristic of protozoan infection.

In most cases of triple infection (6/8), the pathological lesions and clinical signs were also suggestive of CPV-2 infection. The only one case in which the animal presented triple infection by *Giardia* sp., *Sarcocystis* spp. and CPV-2, the clinical signs and pathological changes (necrotizing colitis with intralesional protozoa) were suggestive of enteritis by protozoa, even though CPV-2 had been detected by PCR.

Enterotoxemia by *Clostridium perfringens* type A was the probable cause of death of one dog (Fig 1C and D), in which DNA of toxin A was detected by PCR. *Cryptosporidium* spp. was also suggested as the cause of death of one animal in which it was the only agent detected. In a dog with mixed CPV-2 and *Crysptosporidium* sp. infection, CPV-2 was considered as the primary agent. DNA of *Toxoplasma gondii*, *Neospora caninum* and Canine CV were not detected in any analyzed samples.

DISCUSSION

Acute gastroenteritis is a very common disease in dogs around the world, frequently evolving to dehydration, apathy, prostration and death, mainly in puppies (HUBBARD et al., 2007). Several pathogens have been associated with acute gastroenteritis of fatal course in dogs, but CPV-2 is the most frequent agent detected (DOWGIER et al., 2017; DUIJVESTIJN et al., 2016; GIZZI et al., 2014).

In the present study, CPV-2 was the main agent involved, either in single or mixed infections and affecting mainly young, but also adult animals. Among the CPV subtypes, CPV-2c has been more frequently associated with severe enteritis, affecting both adult and vaccinated dogs (DECARO & BUONAVOGLIA, 2012; DE OLIVEIRA et al., 2018). Although, CPV-2c was the most prevalent subtype (45%, 27/60) we did not observe differences in the histological lesions caused by any of the subtypes. CPV-2 is highly transmissible and associated with severe enteritis mainly in young dogs (DECARO et al., 2006), but CPV-2-associated disease has also been reported in adult and dogs with a complete vaccination protocol (MIRA et al., 2018). Interestingly, even in a few cases in which CPV-2 was not detected, histopathological changes suggested it as the probable cause of the disease and death. The lesions observed in cases of parvovirosis are well defined and characterized by necrotizing enteritis with squamous metaplasia, necrosis and fusion of crypts, villous atrophy, epithelial syncytia in crypts and Peyer's patch necrosis (DE OLIVEIRA et al., 2018; GODDARD & LEISEWITZ, 2010; MOON et al., 2008; NOVOSEL et al., 2019), similar to those

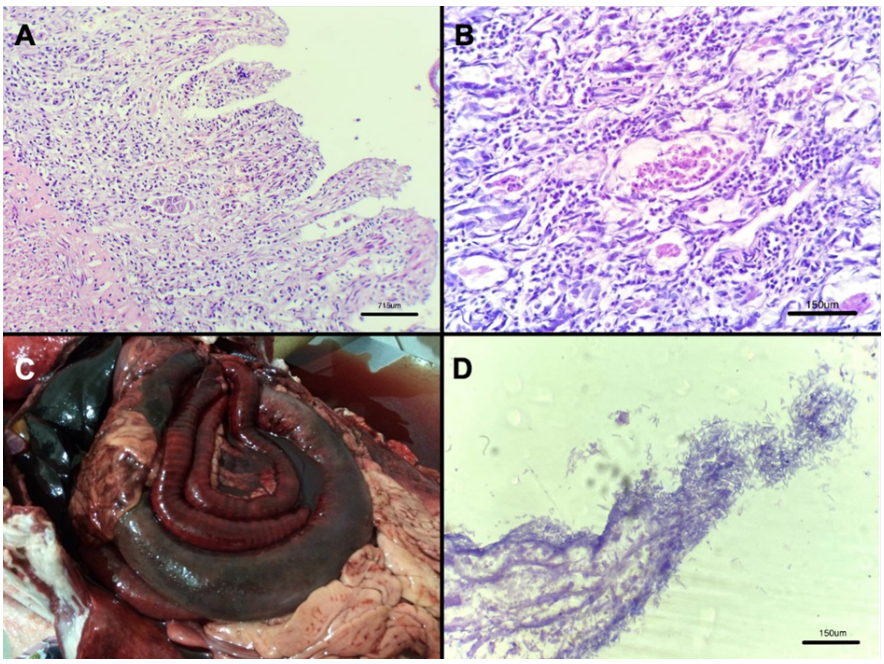

Figure 1 - Pathological lesions observed in the intestine of dogs with severe enteritis caused by viruses, protozoa or bacteria. A) Necrosis and collapse of crypts, with villous atrophy and squamous metaplasia of intestinal cells in the small intestine of a dog infected with canine parvovirus type 2. Hematoxylin and eosin (HE). Bar: 150µm. B) Multifocal crypt abscesses in the small intestine of a dog infected with *Giardia* sp. Multiple dilated crypts are filled with cellular debris and degenerated neutrophils. HE, bar: 150 µm. C) Intestine of a dog infected with *Clostridium perfringens* A presenting massive necro-hemorrhagic enteritis. D) Myriad of basophilic bacilli lining a necrotic intestinal villus of a dog infected with *Clostridium perfringens* A. HE, bar: 150 µm.

observed in most cases of enteritis associated with CPV-2 reported here (Figure 1A). Other viral agents commonly related to enteric disease in dogs promote different histopathology lesions. Coronavirus causes atrophy and fusion of intestinal villi and deepening of crypts, increase in cellularity of the lamina propria, and flattening of epithelial cells (KEENAN et al., 1976; PRATELLI et al., 2001); and distemper virus produce eosinophilic inclusion bodies in the epithelial cells of the lung, intestine, renal pelvis and urinary bladder, and gastroenteritis with degeneration of epithelia, lymphocyte necrosis and neutrophil infiltration of the lamina propria (OKITA et al., 1997).

The association between CPV-2 and *Giardia* spp., *Cryptosporidium* spp. or *Clostridium* sp. were the most frequent cases of double infections. A similar finding was observed in diarrheic dogs from southern Brazil (Curitiba, PR), in which co-infections were detected only in diarrheic dogs (GIZZI et al., 2014). These findings indicated that multiple agents should be investigated in cases of diarrhea or severe enteritis in dogs.

Association between CPV-2 and *Giardia* sp. was the most frequent co-infection detected in our study. A significant association of these enteropathogens was already reported in feces of domestic (KUZI et al., 2020) and sheltered dogs (DUIJVESTIJN et al., 2016). In most of the mixed infections examined here, the histopathological lesions were predominantly related to CPV-2 infection, as previously described. These findings suggested that CPV-2 was the main primary agent involved in most cases of gastroenteritis and death of the animals, since *Giardia* sp. is frequently detected in healthy, asymptomatic dogs (DUIJVESTIJN et al., 2016). However, in a single case, a crypt abscess/cryptitis was observed, that is a characteristic lesion induced by protozoa (COTTON et al., 2011). Then, the protozoa co-infection was the suggestive cause of the pathology and death of this dog.

The combination of CPV-2 and *Crypstoporidium* spp. was also frequently observed in our study. However, the animals had only intestinal

lesions characteristic of CPV-2 infection, suggesting a circumstantial presence of the protozoa in the feces. No cases of mixed infection presented pathological changes suggestive of protozoa enteritis as cause of the death. Co-infection by CPV-2 and *Cryptosporidium* spp. has been frequently reported in diarrheic dogs and has been suggested that the protozoa may aggravate the CPV-2 infection (DENHOLM et al., 2001).

Two cases of multiple infections involving *Giardia* sp. and *Cryptosporidium* spp., and *Giardia* sp. and *C. perfringens* type A, resulted in lesions characteristic of protozoa infection, which was considerate the cause of death. Enteric protozoa, as *Giardia* sp., *Cryptosporidium* spp. and *Cystoisospora* spp., are frequently detected in asymptomatic dogs (TUPLER et al., 2012) or in dogs with transient diarrhea and vomiting (GIZZI et al., 2014). Hence, they were related as cause of some cases of fatal gastroenteritis in dogs (DUIJVESTIJN et al., 2016). In these cases of co-infections, cryptitis/crypt abscess and villous necrosis, which are histopathological lesions related to infection by protozoa (SCORZA & TANGTRONGSUP, 2010) were observed. Single infection by *Giardia* sp. was also associated with intestinal lesions and death in two cases of puppies with history of gastroenteropathy, hypovolemia and hypothermia, that presented mild to accentuated multifocal necrosupurative cryptitis (Figure 1B). Thus, it was suggested that *Giardia* sp. and/or *Crypstoporidium* sp. were the primary cause of the disease and death in these cases. These findings reinforce the importance of protozoa as agents of severe enteritis in dogs, and they should be included in the list of differential diagnosis of enteric agents.

C. perfringens type A is a Gram-positive rod naturally found in intestinal flora animals (MCCLANE et al., 2014; SILVA et al., 2017), and produces a wide variety of exotoxins and enzymes that could cause enteritis in dogs, associated with severe hemorrhagic diarrhea (SCHLEGEL et al., 2012). A similar finding was observed in an 8 years-old dog in this study, which presented hemorrhagic diarrhea, vomit with blood, and died after developed hypothermia. At necropsy, necrotizing enteritis, hemorrhagic, suppurative and diffuse, with aggregates of rod bacteria were observed, lesions compatible with enterotoxaemia/clostridiosis (Figure 1C, D). However, since *C. perfringens* type A is a natural habitant of the intestinal flora, DNA detection would not suffice to confirm the diagnosis (SCHLEGEL et al., 2012). Rather, this finding needs to be associated with bacteria quantitation, toxin detection and/or pathological lesions to reach the final diagnosis of clostridia enterotoxemia (MARKS & KATHER, 2003). Since no characteristic lesions of clostridiosis were observed in other four cases where DNA of *C. perfringens* type A was detected associated with other agents, it is unlikely that *C. perfringens* type A was the primary cause of the disease and death.

In few cases, the pathological findings and molecular diagnosis were divergent. In one of them, the intestinal lesions were compatible with *Clostridium perfringens* type A infection, but only DNA of *Giardia* spp. was detected. In another five cases, parvovirus was the diagnosis suggested by the pathologist, but only *Giardia* sp. was detected. Our hypothesis is that the long storage time at -20 ºC of the samples and the freezing and thawing process may have contributed to the DNA degradation that affected mainly the CPV-2 PCR sensibility, since we used a conventional PCR to CPV-2 and nested-PCR to *Giardia* spp. (high sensitivity test).

Canine enteric coronavirus and canine distemper virus, as well as other low-prevalence agents (such as bocavirus, astrovirus, and kobuvirus), have been detected in dogs with enteric diseases worldwide (BARROS et al., 2019; BODEWES et al., 2014; CHOI et al., 2014; DOWGIER et al., 2017; LI et al., 2011). Although, we have not observed characteristic/pathognomonic intestinal lesions associated with these agents, we cannot completely rule out their involvement in some cases. Our study was conducted using long-term storage samples at -20 °C, which may not be ideal for detecting RNA viruses or other agents that are prone to degradation over time. Future studies, using fresh and well-preserved samples, may provide more accurate results and allow the investigation of these agents.

CONCLUSION

Taken together, our results showed that single and mixed infections are associated with severe enteritis in dogs from Southern Brazil. Although CPV-2 was the main pathogen involved in these cases, *Giardia* sp., *Crypstoporidium* sp. and *C. perfringens* type A were also detected and may contribute for the disease and, thus, should be included in differential diagnosis.

ACKNOWLEDGEMENTS

PSBO, EFF and FSFV are recipientes of Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) fellowships. APGM and MGS are recipientes of Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) fellowships, Brasil - Finance code 001.

DECLARATION OF CONFLICT OF INTEREST

The authors declare no conflict of interest.

BIOETHICS AND BIOSSECURITY COMMITTEE APPROVAL

No approval of research ethics committees was required to accomplish the goals of this study because experimental research was conducted with samples received for diagnosis.

AUTHORS' CONTRIBUTIONS

All authors contributed to perform research, interpreting the results, writing and critically revising the manuscript. All authors approved the final version of the manuscript.

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