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The incidence of *Clostridioides difficile* and *Clostridium perfringens netF*-positive strains in diarrheic dogs

Amanda Nadia Diniz [a], Fernanda Morcatti Coura [a], Maja Rupnik [b, c], Vicki Adams [d], Thomas L. Stent [d], Julian I. Rood [d], Carlos Augusto de Oliveira Jr. [a], Francisco Carlos Faria Lobato [a], Rodrigo Otávio Silveira Silva [a, *]

[a] *Veterinary School, Universidade Federal de Minas Gerais (UFMG), Antônio Carlos Avenue, 6627, Belo Horizonte, MG, 31.270-901, Brazil*
[b] *Faculty of Medicine, University of Maribor, Slovenia*
[c] *National Laboratory for Health, Environment and Food, Maribor, Slovenia*
[d] *Monash Biomedicine Discovery Institute and Department of Microbiology, Monash University, Clayton, Victoria, Australia*

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ABSTRACT

The aim of this study was to examine the incidence of *Clostridioides* (previously *Clostridium*) *difficile* and *Clostridium perfringens* in the feces of diarrheic and non-diarrheic dogs. Also, the presence of other common canine enteropathogens was examined. Toxigenic *C. difficile* and *C. perfringens* positive for the NetF-encoding gene (*netF*) were detected in 11 (11.9%) and seven (7.6%) diarrheic dogs, respectively. Three dogs were diagnosed simultaneously with toxigenic *C. difficile* and *netF*-positive *C. perfringens*. Among other enteropathogens, *Giardia* sp. was the most common agent detected in dogs positive for toxigenic *C. difficile* or *netF*-positive *C. perfringens*. The results suggest that *C. difficile* and *C. perfringens* occur more frequently as a primary cause of diarrhea.

There are several reports of enteric disorders caused by *Clostridioides* (previously *Clostridium*) *difficile* in dogs. However, its role in canine diarrhea is still uncertain [1,2]. Similarly, several authors have shown a high prevalence of the enterotoxin (CPE)-encoding gene (*cpe*) in *Clostridium perfringens* isolates obtained from diarrheic dogs, which led to the speculation that this toxin was responsible for the pathogenesis of canine *C. perfringens*-associated diarrhea (CPAD) [3]. Recently, two putative pore-forming toxins (NetE and NetF) were described in strains from cases of fatal canine hemorrhagic gastroenteritis and evidence that NetF was the virulence factor in this syndrome was obtained [4,5].

Although these two agents are commonly described as enteropathogens in dogs [6], their role and the involvement of other enteropathogens remain largely unknown. Thus, the aim of this study was to detect and characterize *C. difficile* and *C. perfringens* strains from diarrheic and non-diarrheic dogs. Also, the presence of other common non-clostridial enteropathogens was evaluated to provide a better understanding of the role of these two agents in canine diarrhea.

Stool samples were collected from 154 dogs, of which 92 were diarrheic and 62 were apparently healthy. The samples from diarrheic dogs were obtained directly from the Veterinary Hospital of Universidade Federal de Minas Gerais at the time of the consultation. The clinical history of all animals was recorded. Samples from apparently healthy animals were obtained in city squares in Belo Horizonte (Minas Gerais, Brazil), with prior permission of the owner and only fecal material that did not have contact with the environment was collected. The animals were categorized into four groups based on their age: younger than 6 months (n = 45, 29.2%), from 7 to 12 months (n = 28, 18.1%), from 1 to 5 years (n = 36, 23.3%) and older than 5 years (n = 44, 28.5%). In each age group, at least one apparently healthy dog sample (control) was included for

* Corresponding author.
E-mail address: rodrigo.otaviosilva@gmail.com (R.O.S. Silva).

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each two diarrheic dog samples.

Isolation of *C. difficile* was based on previously described protocols [7,8]. All isolates were subjected to a multiplex-PCR for a housekeeping gene (*tpi*), the toxin A gene (*tcdA*), the toxin B gene (*tcdB*) and a binary toxin gene (*cdtB*) [9] and were PCR ribotyped as previously described [10]. PCR ribotypes for which the reference strains were available are designated by international Cardiff nomenclature, while others are designated by internal nomenclature (BR and number). Stool samples positive for *C. difficile* isolation were also subjected to toxin A/B detection (*C. difficile* Tox A/B II - Techlab Inc., USA).

Isolation of *C. perfringens* was also based on previously described protocols [7,8] and isolates were subjected to a PCR protocol [11] for the detection of genes for typing *C. perfringens* (alpha, beta, epsilon and iota toxins), and beta-2 toxin (*cpb2*) and enterotoxin (*cpe*). PCR protocols described by Refs. [12] and [5] were used for the detection of the NetB-, NetE- and NetF-encoding genes (*netB*, *netE* and *netF*, respectively). Stool samples positive for *C. perfringens cpe*$^+$ strains were also subjected to CPE detection (Ridascreen® *Clostridium perfringens* Enterotoxin, R-Biopharm, Germany).

Two *C. perfringens netF*$^+$ isolates (from diarrheic dogs 7 and 8) were selected for whole genome sequencing. Both developed bloody diarrhea without an identifiable predisposing factor and recovered after regular treatment. Library preparations (Nextera kit, Illumina, USA) from genomic DNA [13], were sequenced using a Miseq (Illumina, USA). Reads were trimmed for quality (Nesoni, Paul Harrison (http://www.vicbioinformatics.com/software.nesoni.shtml),), assembled using A5-miseq [14] and auto-annotated using Prokka [15]. Pseudomolecules were generated by scaffolding against the plasmid sequences pJFP838C and pJFP838D [4] for the *netE*-, *netF*- and *cpe*-encoding plasmids, respectively, and manually inspected using Artemis [16]. Plasmid comparisons were generated using the Blastn tool of EasyFig [17]. Plasmid sequences were lodged with NCBI under the following accession numbers (MG456813, MG456814, MG456815 and MG456816).

The presence of parvovirus (CPV), rotavirus, coronavirus and *Giardia* spp. was evaluated by commercial lateral flow tests (Ecodiagnostica, Brazil). For the isolation of *Salmonella* spp., stool samples were inoculated into tetrathionate broth followed by plating on Hektoen enteric agar (Oxoid, USA). Sulfide-producing colonies were subjected to a previously described PCR assay to detect the *Sulmonella ompC* gene [18]. For *Escherichia coli* detection, stool samples were plated on MacConkey agar (Difco, USA) and characteristic lactose-fermenting colonies were analyzed by PCR to detect several genes associated with diarrheagenic *E. coli* [19].

In this survey, diarrheic dogs were more than five times more likely to be positive for *C. difficile* than apparently healthy animals (Table 1). Toxins A and B were detected in eight fecal samples (8.7% of the diarrheic animals) that were PCR positive for toxigenic *C. difficile*, confirming the diagnosis of *C. difficile* infection (CDI) in these animals. Although antibiotic therapy is a known risk factor for CDI in humans and dogs [2,20,21], only one animal that was positive for toxigenic *C. difficile* had been treated with antibiotics (Trimethoprim/Sulfamethoxazole) before the onset of diarrhea. The isolation rate of toxigenic *C. difficile* was slightly higher in adult dogs (older than 1 year) than in young dogs ($p = .043$).

Among toxigenic *C. difficile* isolates, ribotypes 014/020 and 106 were the most common. Ribotype 014/020 seems to be the most frequent in several canine studies and is commonly implicated in human CDI worldwide [32,22,23]. In Brazil, ribotype 106 is common in humans and has also been described in some animals, including dogs [32]. Other authors have suggested pets as a potential source of community acquired CDI in humans due to high genetic similarity between dog and human disease isolates [2,24].

The isolation of *cpe*-positive *C. perfringens* was associated with diarrhea to at least some extent (Table 1), corroborating previous studies [7,8,25]. In addition, *C. perfringens* strains positive for NetE- and NetF-encoding genes were detected in seven (7.6%) samples from diarrheic dogs ($p = .042$). The *netE* and *netF* genes were identified only in *C. perfringens cpe*$^+$ strains and were exclusively isolated from adult dogs with bloody diarrhea, corroborating the results of [5]. Genomic sequencing of two *netE*$^+$*netF*$^+$*cpe*$^+$ strains from different diarrheic dogs revealed that plasmids in both of these Brazilian strains had significant identity (Fig. 1) to plasmids previously identified from dogs in Canada [5]. These results are consistent with recent findings that showed that the *netEF* plasmids, and to a lesser extent the *cpe* plasmids, are highly conserved in canine and equine isolates of *C. perfringens* from diarrheic animals [4] and support the hypothesis that *C. perfringens netF*$^+$ strains are enteropathogenic in adult dogs.

Table 1
Frequency of enteric pathogens and detection of selected virulence factors and virulence genes in diarrheic (n = 92) and apparently healthy (n = 62) dogs.

Enteropathogens		Dogs (n = 154)		*p* value
		Diarrheic (n = 96) (%)	Non diarrheic (n = 62) (%)	
Clostridioides difficile	A$^+$B$^+$CDT$^-$	11 (11.9)*	0 (0)	0.032
	A$^-$B$^-$ CDT$^-$	8 (8.7)	3 (4.8)	0.526
	A/B toxins[b]	8/11 (72.3)	–	–
Clostridium perfringens	Type A	46 (50)	22 (34.5)	0.099
	cpe$^+$	10 (10.8)*	0 (0)	**0.006**
	cpe$^+$ *netF*$^+$	7 (7.6)*	0 (0)	0.042
	CPE[a]	5/10 (50)	–	–
Escherichia coli	Enteropathogenic (EPEC)	11 (11.9)	11 (17.7)	0.352
	Shiga Toxin-Producing (STEC)	1 (0.9)	2 (3.2)	0.565
	Enterotoxigenic (ETEC)	3 (3.2)	1 (1.6)	0.648
	Atypical	1 (0.9)	0 (0)	1.000
Salmonella sp.		0 (0)	0 (0)	–
Parvovirus (CPV)		10 (10.8)*	0 (0)	**0.006**
Canine Coronavirus		2 (2.1)	2 (3.2)	1.000
Rotavirus		1 (0.9)	1 (1.6)	1.000
Giardia sp.		10 (10.8)	2 (3.2)	0.124

*Chi-square test or Fisher's exact test were used to evaluate possible association between enteropathogens in diarrheic and healthy dogs or age groups. P values of <0.05 were considered significant (in bold).

[a] Enterotoxin (CPE) detection by commercial EIA on unthawed aliquots of stool samples positive for *C. perfringens cpe*$^+$isolation.

[b] A/B toxin detection by commercial EIA on unthawed aliquots of stool samples positive for toxigenic *C. difficile* isolation.

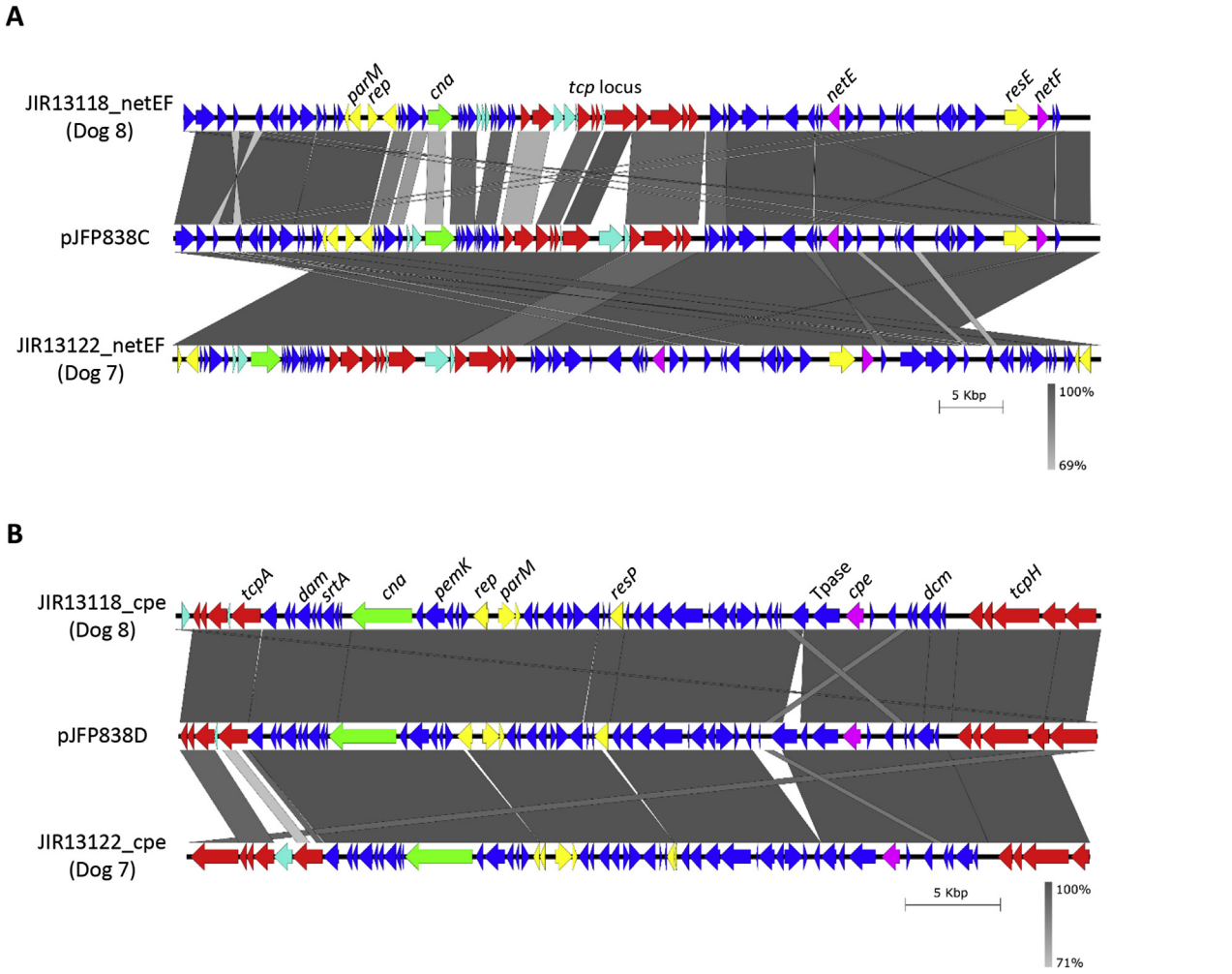

Fig. 1. Comparison of the plasmids from Dog 7 and 8 with plasmids pJFP838C and pJFP838D: A) Blastn analysis using EasyFig to align pJFP838C against the pseudomolecules encoding the *netEF* genes from the genome sequences obtained from dog 7 and 8 isolates. **B)** An EasyFig alignment of pJFP838D compared to pseudomolecules encoding the *cpe* gene from genome data derived from dog 7 and 8. Legend: grey shaded regions indicate nucleotide identity. ORFs are represented as arrows and are coloured as follows: magenta – toxin genes (as indicated), red – conjugation locus, yellow – plasmid replication and maintenance, green - putative collagen adhesion, dark blue – conserved ORF, light blue – poorly or non-conserved ORFs. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

All dogs positive for *C. perfringens netF*$^{+}$ were apparently healthy before the onset of diarrhea. Additional stool samples were collected from four dogs, five to eight months after the diarrheal episode. In two instances, dogs housed together with the previously affected animals were also sampled. The *netF*$^{+}$ gene was not detected in any of these healthy dogs. These results are consistent with the finding that *netF-*$^{+}$ *C. perfringens* strains have only been isolated from dogs during diarrheal episodes [5].

In the present study, toxigenic *C. difficile* and *netF*-positive *C. perfringens* were detected together in three animals (Table 2). These are the first confirmed coinfections of these strains in dogs and two of these cases have been described in more details in a case report [26]. Among other enteropathogens, *Giardia* sp. was the most common agent detected in dogs positive for toxigenic *C. difficile* or *netF*-positive *C. perfringens* (Table 2), with dogs positive for *Giardia* sp. more likely to be positive for *C. perfringens* ($p = .0057$), similar to a previous report [27]. Veterinary practitioners commonly recognize giardiasis as a cause of mild and uncomplicated diarrhea in dogs, but the role of this parasite as a predisposing factor for acute diarrhea associated with other enteropathogens is not known.

Canine coronavirus, CPV and enteropathogenic *E. coli* were also detected in association with toxigenic *C. difficile* or *netF*-positive *C. perfringens* (Table 2). Despite previous reports that suggested that CPV could be an important predisposing factor for CPAD in dogs [28,29], enterotoxigenic *C. perfringens* was not recovered from any CPV-positive animals. However, the only adult dog positive for CPV in the present study (Dog 12) was also positive for CDI and *Giardia* sp (Table 2). Sequencing of the VP2-encoding gene from this sample revealed the involvement of CPV-2b [30]. These data suggest that, in addition to CPV-2c, CPV-2b can also cause infection in repeatedly vaccinated dogs and highlight the need for broad studies focusing on a possible synergism of *C. difficile* infection and canine CPV in adult dogs [31].

In summary, the present study reinforces the association of *netF*-positive *C. perfringens* and toxigenic *C. dificile* as enteropathogens in dogs, with these isolates commonly associated with bloody diarrhea in adult individuals. A possible coinfection of these two agents and also with other enteropathogens is feasible based on these data, but further studies are required to assess the significance of this association.

Table 2
Details of all dogs positive for *Clostridium perfringens cpe*$^+$ or toxigenic *Clostridium difficile*.

ID	Age (months)	Feacal characteristic	*Clostridium difficile*			*Clostridium perfringens*				Other enteropathogens	Outcome
			A/B toxin	Isolation	Ribotype[a]	*cpe*	*netE/F*	*netG*	CPE		
1	48	Mushy	–	–	–	+	–	–	+	–	Recovered.
2	3	Bloody	–	–	–	+	–	–	+	Coronavirus	Died
3	125	Bloody		–	–	+	–	–	–	–	Recovered.
4	61	Bloody	–	–	–	+	+	–	–	*Giardia* sp.	Recovered.
5	18	Bloody	–	–	–	+	+	+	+	–	Recovered.
6	133	Bloody	–	–	–	+	+	–	+	–	Recovered.
7	18	Bloody	–	–	–	+	+	–	–	–	Recovered
8	19	Bloody	+	$A^+B^+CDT^-$	014/020	+	+	–	–	–	Recovered.
9	145	Bloody	+	$A^+B^+CDT^-$	014/020	+	+	+	–	–	Died.
10	36	Bloody	+	$A^+B^+CDT^-$	106	+	+	+	+	EPEC (*eae*$^+$)	Died.
11	19	Bloody	+	A + B + CDT-	014/020	–	–	–	–	–	Recovered
12	181	Bloody	+	A + B + CDT-	BR1	–	–	–	–	CPV-2b and *Giardia* sp.	Recovered
13	6	Bloody	+	A + B + CDT-	BR1	–	–	–	–	–	Died
14	168	Mushy	+	A + B + CDT-	014/020	–	–	–	–	–	Recovered
15	48	Bloody	+	A + B + CDT-	106	–	–	–	–	–	Died
16	121	Bloody	–	A + B + CDT-	BR2	–	–	–	–	–	Died
17	5	Bloody	–	A + B + CDT-	BR3	–	–	–	–	–	Died
18	23	Mushy	–	A + B + CDT-	BR4	–	–	-	-	*Giardia* sp.	Recovered.
19	120	Mushy	–	A + B + CDT-	602	–	–	–	–	–	Recovered.

Legend: EPEC – Enteropathogenic *E. coli*; CPV-2b – Canine parvovirus type 2b; CPE – *C. perfringens* enterotoxin; A – Toxin A encoding gene (*tcdA*); B – Toxin B encoding gene (*tcdB*); CDT – binary toxin gene (*cdtB*).

[a] PCR ribotypes for which the reference strains were available are designated by international Cardiff nomenclature, while others are designated by internal nomenclature (BRA and number).

Conflicts of interest

The authors declare that they have no conflicts of interest.

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