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Clostridium perfringens and *C. difficile* in parvovirus-positive dogs

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ABSTRACT

The aim of this study was to investigate *Clostridium difficile* and *Clostridium perfringens* in 82 diarrheic dogs positive for canine parvovirus type 2 (CPV). Enterotoxigenic *C. perfringens* type A was isolated from three (3.6%) dogs. One (1.2%) strain was also positive for NetE- and NetF-encoding genes, which are commonly associated with diarrhea in dogs. Toxigenic *C. difficile* was isolated from one animal (1.2%), which was also positive for A/B toxins. The present study identified *C. difficile* and *C. perfringens* infection in CPV-positive dogs. Further studies are necessary to clarify if clostridial infections may predispose or potentiate CPV-infection in dogs or vice versa.

1. Introduction

Canine parvovirus type 2 (CPV) is recognized as the most common viral enteric pathogen in dogs worldwide, and it is responsible for a life-threatening illness that occurs predominantly in puppies. CPV type 2 emerged in 1970s, but few years later this type was replaced by a new genetic variants, called CPV-2a, -2b and -2c [22]. The predisposing factors and clinical relevance of CPV are well described [4,7,11]. It is also known that CPV induces suppression of the immune system and thus may facilitate infection with other agents [3], but the role of bacterial enteropathogens in CPV-infected dogs is still poorly understood.

Some authors have hypothesized that CPV infection may predispose dogs to *Clostridium perfringens*-associated diarrhea (CPAD) [32,34,27]. At this time, this synergism has not been confirmed, as there were no virulence markers to differentiate when *C. perfringens* is present as part of the microbiota or as an enteropathogen. Previous studies have shown an association between isolation of *C. perfringens cpe*$^{+}$ (also referred as enterotoxigenic *C. perfringens*) and diarrhea, suggesting that enterotoxin (CPE) might be involved in CPAD in dogs. However, enterotoxigenic *C. perfringens* or CPE can be found in healthy dogs. Thus, although these methods could suggest that *C. perfringens* is involved, they are not confirmatory [30]. Recently, studies have shown that two pore-forming toxins (NetE, NetF) present in *C. perfringens* type A strains are strongly associated with canine hemorrhagic gastroenteritis [8,14], suggesting these toxin-encoding genes could be used as virulence markers to diagnose CPAD in dogs.

Clostridium difficile is also commonly described as an enteropathogen in dogs, but the role of this agent and the main predisposing factors involved are largely unknown [26]. In humans, hospitalization and antibiotic therapy are known risk factors for the development of *C. difficile* infection (CDI) [15], whereas, in dogs, most cases of CDI seem to be community-acquired [26]. It is still controversial whether *C. difficile* is a primary or secondary agent in canine diarrhea [19], as other enteropathogens, including CPV, have been isolated in dogs diagnosed with CDI [9]. In this scenario, the purpose of the present study was to detect and characterize

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C. difficile and *C. perfringens* from CPV-positive dogs.

2. Material and methods

2.1. Animals, selected epidemiological data and blood analysis

A convenience sample of dogs was used in the current study. The following selected epidemiological data were obtained for further analysis: age, vaccination status, and clinical outcome. Vaccination status of animals were classified according to the Vaccination Guidelines of the World Small Animal Veterinary Association [41]. Data on whole blood count from some animals were analyzed, as well. Stool samples were obtained directly from the rectum at the moment the animals were admitted in the Veterinary Hospital. Each sample was divided in three microtubes of about 300 μL, and stored at −80 °C.

2.2. CPV detection and sequencing

All stool samples were submitted to total DNA extraction with Invisorb Spin Tissue Mini Kit (Invitek, EUA). Amplification was performed as previously described by Ref. [1] with primers 555F-5′CAG GAA GAT ATC CAG AAG GA3′ and 555R-5′GGT GCT AGT TGA TAT GTA ATA AACA3′, resulting in a fragment of 583 bp from VP2. Aliquots of stool samples positive for CPV by PCR (n = 82) were further submitted to *C. difficile* and *C. perfringens* detection, as described next. In addition, 20 random CPV positive samples were selected for VP2 sequencing, as described previously [1]. Briefly, PCR products were purified using Agencourt AMPure XP (Beckman Coulter Company, Beverly, Massachusetts, USA) according to the manufacturer's instructions, and each product was sequenced in both directions using primers the 554F and 555R, and the Big Dye V3.1 Terminator Kit (Applied Biosystems, USA) using an ABI 3500 DNA analyzer (Applied Biosystems, California, USA). In order to prevent detection of canine CPV post-vaccination shedding, dogs without vaccination status record, or vaccinated less than 3 weeks before the analysis were excluded from the study. Also, all dogs simultaneously positive for CPV and *C. perfringens* or *C. difficile* infection were selected for VP2 sequencing.

2.3. Clostridium perfringens genotyping and CPE detection

To perform the isolation of *C. perfringens*, 0.08–0.12 g of feces were serially diluted tenfold, ranging from 10^{-1} to 10^{-3}. Aliquots of 10 μl of each dilution were plated on sulfite polymyxin sulfadiazine agar (SPS, Difco Laboratories, USA), and were anaerobically incubated at 37 °C for 24 h. After incubation, at least three characteristic colonies from each dilution were subjected to a previously described PCR protocol for the detection of genes encoding the major *C. perfringens* toxins (alpha, beta, epsilon and iota), beta-2 toxin (*cpb2*) and enterotoxin (*cpe*) [20]. For the detection of the NetB-, NetE-, NetF and NetG-encoding genes (*netB*, *netE*, *netF*, and *netG*, respectively) PCR protocols described by Refs. [17] and [14] were applied. One aliquot of each stool sample positive for *C. perfringens cpe*$^{+}$ strains were subjected to CPE detection in the commercial EIA kit (RIDASCREEN® *Clostridium perfringens* Enterotoxin - R-Biopharm, Germany).

2.4. Clostridium difficile isolation, PCR and A/B toxin detection

To isolate *C. difficile* spores, equal volumes of stool samples and 96% ethanol (v/v) were mixed; after incubation for 30 min at room temperature, 50-μl aliquots were inoculated on plates containing cycloserine-cefoxitin fructose agar supplemented with 7% horse blood and 0.1% sodium taurocholate (Sigma-Aldrich Co., St. Louis, MO, USA). After anaerobic incubation at 37 °C for 72 h, all colonies with suggestive morphology were subjected to a previously described multiplex-PCR for a housekeeping gene (*tpi*), toxins A (*tcdA*) and B (*tcdB*), and a binary toxin gene (*cdtB*) [28]. One aliquot of each stool sample positive for toxigenic *C. difficile* were subjected to A/B toxin detection by an enzyme-linked immunosorbent assay (EIA) kit (*C. difficile* Tox A/B II - Techlab Inc., Blacksburg, VA, USA).

3. Results

Details on age, outcome, vaccination status, and leukogram of the dogs included in the present study are summarized in Table 1. The majority of animals included were puppies, unvaccinated or intermittently vaccinated against CPV, and showed leukopenia in their whole blood count. All samples submitted to CPV sequencing (n = 24) were typed as CPV-2b. Forty dogs (48.8%) were positive for *C. perfringens* type A, but only three strains were also positive for the enterotoxin-encoding gene (*cpe*) (Table 2). One of these strains was also positive for *netE* and *netF*. The stool sample of this dog was positive for CPE in the commercial EIA kit. Two out of the three dogs positive for enterotoxigenic *C. perfringens*, including the one positive for *C. perfringens netF*$^{+}$, survived after treatment. *C. difficile* was isolated from ten (12.2%) dogs, but only one (1.2%) strain was toxigenic (A + B + CDT-) (Table 2). This stool sample was also positive for A/B toxins in EIA. This dog also recovered after treatment. No dog was simultaneously positive for CDI and enterotoxigenic *C. perfringens* in the present study.

4. Discussion

4.1. Sampled dogs' details and parvovirus sequencing

The high percentage of puppies and unvaccinated or intermittently vaccinated dogs in the present study is not surprising, and corroborates previous studies that showed age and absence of vaccination as common predisposing factors for CPV infection [9,23,39]. Leukopenia, which is also a typical finding in CPV infected dogs, occurred in around three of every four animals. The survival rate of the present study (89.3%) is also similar to that of previous studies that reported percentages of recovery ranging from 80 to 90% [35].

Table 1
Details of age, outcome, vaccination status and leukogram of CPV-positive dogs (n = 82) submitted to *C. difficile* and *C. perfringens* isolation.

Age (n = 71)			Vaccination status (n = 71)		Leukogram (n = 56)		Outcome (n = 71)	
Puppies		>1 year old[a]	Not vaccinated[b]	Vaccinated	Normal	Leukopenia	Recovered	Death
<6 months	6-12 months							
57 (80.3%) 67 (94.4%)	10 (14.1%)	4 (5.6%)	62 (87.3%)	9 (12.7%)	13 (23.2%)	43 (76.8%)	67 (89.3%)	8 (10.7%)

[a] The four adult dogs positive for CPV in this study had one, two, seven and 14 years-old.
[b] Animals that were not vaccinated or intermittently vaccinated according to WSAVA [41].

Table 2
Results of isolation of *C. difficile* and *C. perfringens* from stool samples of CPV-positive dogs (n = 82).

Agent		Results (%)	Additional information
C. perfringens type A[a]	*cpe- netE-*	37 (45.1)	
	cpe + *netE-*	2 (2.4)	Both CPE negative[b]
	cpe + *netE+*	1 (1.2)	CPE positive[b]
	Total	40 (48.8)	
C. difficile	Non toxigenic	9 (11)	
	Toxigenic	1 (1.2)	A/B toxins positive[c]
	Total	10 (12.2)	

[a] Other genotypes of *C. perfringens* were not detected in the presente study.
[b] Enterotoxin (CPE) detection by an EIA kit (Ridascreen® *Clostridium perfringens* Enterotoxin - R-Biopharm, Germany).
[c] A/B toxin detection by an EIA kit (*C. difficile* Tox A/B II - Techlab Inc., USA).

The detection of only CPV-2b was unexpected, as previous studies in Brazil showed high prevalence of CPV-2c [12,25]. Considering that these studies were carried out in other Brazilian regions, it is believed that this variation might be due to geographical differences and highlight the need for regional studies to better understand CPV epidemiology in Brazil.

Although it is known that CPV induces suppression of the immune system, which facilitates the development of other infections [3], few studies focused on the role of this agent as a predisposing factor for infections by other enteric pathogens. The co-infection of canine coronavirus (CCoV) and CPV is the only synergism confirmed so far [9,40]. The influence of other coinfections by major enteropathogens is still poorly understood.

4.2. C. perfringens in CPV-positive dogs

Once *C. perfringens* belongs to the enteric microbiota, detection of this agent in diarrheic dogs is not confirmatory diagnosis [30,38]. Conversely, previous studies have shown a putative association between isolation of *C. perfringens cpe*$^+$ and diarrhea, suggesting that the CPE might be at least partially responsible for CPAD in dogs [2,18,21,29,33,37]. In the present study, almost half of the dogs were positive for *C. perfringens* type A, but only three of these isolates (3.6%) were positive for the enterotoxin-encoding gene (*cpe*). Among these three enterotoxigenic *C. perfringens* isolates, two were negative for *netE* and *netF*. These two dogs were puppies aged three and six months old, and were negative for CPE in their stool samples. Previous studies have shown an association between isolation of enterotoxigenic *C. perfringens* and canine diarrhea, but it is known that these strains can be isolated from healthy dogs. Thus, although these methods could suggest that *C. perfringens* is involved, they are not confirmatory [30]. More recently, two pore-forming toxins (NetE and NetF) that are always present in *C. perfringens* type A *cpe*$^+$ strains, were recently described in a strong association with fatal canine hemorrhagic gastroenteritis [14]. In the present study, one of the three enterotoxigenic *C. perfringens* isolates was also positive for *netE* and *netF*. This sample was obtained from an adult dog, aged two years old. So far, *C. perfringens netE*$^+$ strains were exclusively recovered from diarrheic adult dogs elsewhere [8,14], which corroborates our findings. In addition, the stool sample of this dog was positive for CPE in EIA. Together, these results strongly suggest that this adult dog was simultaneously infected with CPV and *C. perfringens cpe*$^+$ *netE*$^+$.

An increasingly common diagnosis of CPV infection in repeatedly vaccinated, adult dogs has been reported by some authors. CPV-2c is commonly detected in these cases, and it has been speculated that commercial vaccines might offer limited protection against this CPV type [5,6]. Because of this, our initial suspicion regarding the detection of a CPV-positive in this well-vaccinated adult dog was immunization failure associated with CPV-2c. However, the sequencing of VP2 revealed the involvement of CPV-2b, the same type that is commonly found in Brazilian CPV vaccines. It is hard to hypothesize, then, which of these two enteropathogens (enterotoxigenic *C. perfringens netE+* and CPV) might have initiated the disease. These data strongly reinforce the need for further studies focusing on a possible synergism of enterotoxigenic *C. perfringens* and canine CPV in adult dogs.

Predisposing factors associated with CPAD in dogs are still unknown. It has been speculated that disruption of the normal enteric microbiota, associated with sudden change in the diet may lead to overgrowth of *C. perfringens* and diarrhea [30]. In turn, all three dogs positive for enterotoxigenic C. *perfringens* in the present study were fed only commercial feed. Thus, changes in the diet could be ruled out in these cases. Infection by other enteropathogens, including CPV, has been also suggested as a risk factor for CPAD in dogs [32,34]. So far, this speculation is based only on findings from case reports and the current study is the first that focuses specifically on CPV-infected dogs. Our findings showed, for the first time, that *C. perfringens* type A *cpe*$^+$ and also *netE*$^+$ could be detected in CPV infected dogs. Due to few studies screening healthy and diarrheic dogs for major enteropathogens, including CPV and *C. perfringens*, it is difficult to evaluate this data and to establish comparisons, once previous studies focused only on the isolation of *C. perfringens*, rarely testing the *C. perfringens* isolates for *cpe*. To our knowledge, the current study is the first one that evaluated the presence of *netE* and *netF* genes of *C. perfringens* among CPV-positive dogs.

Similar to previous studies, *C. perfringens* type C was not detected [9,10,18,29,37]. Although some authors refer to *C. perfringens* type C as a cause of hemorrhagic diarrhea in young dogs [19,38], there is no confirmed report in the literature, so far.

4.3. C. difficile in CPV-positive dogs

Only one animal, aged two months old, was positive for toxigenic *C. difficile* (A + B + CDT-) in the present study (Table 2). The detection of A/B toxins in the stool sample of this animal confirmed that this dog was not only shedding this bacterium, but was also suffering from CDI. There are several reports of enteric disorders caused by *C. difficile* in dogs, ranging from chronic cases to an outbreak of acute diarrhea in a Veterinary Hospital [36,38]. In addition, studies screening for multiple canine enteropathogens commonly show an association between diarrhea and isolation of toxigenic *C. difficile* or detection of A/B toxins in dogs, suggesting that this agent is an important enteropathogen in this domestic species [13,18,29]. However, the main predisposing factors involved are still largely unknown [26]. In humans, CDI is commonly a nosocomial infection associated with antibiotic therapy [15], whereas in dogs, although antibiotic therapy is known to increase the risk of colonization by *C. difficile*, most studies reported community-acquired infection in companion animals [9,26,36]. Corroborating these reports, the dog diagnosed with CDI in the

present study was sampled at the time of admission, and was not under antibiotic therapy before stool collection.

It is also important to note that the high genetic similarity of isolates from dogs and humans raises the concern of *C. difficile* as a zoonotic enteropathogen [31]. Some authors have already suggested that pets may be potential sources of community-acquired CDI in humans [24,31], which highlights the need for more studies to clarify the role of this agent in canine diarrhea.

The present study identified *C. difficile* and *C. perfringens* infection in CPV-positive dogs. Further studies are necessary to clarify if clostridial infections may predispose or potentiate CPV-infection in dogs or vice-versa.

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