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Research Paper

Detection of toxins A/B and isolation of *Clostridium difficile* and *Clostridium perfringens* from dogs in Minas Gerais, Brazil

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Abstract

The objective of this study was to detect *C. difficile* A/B toxins and to isolate strains of *C. perfringens* and *C. difficile* from diarrheic and non-diarrheic dogs in Brazil. Stool samples were collected from 57 dogs, 35 of which were apparently healthy, and 22 of which were diarrheic. *C. difficile* A/B toxins were detected by ELISA, and *C. perfringens* and *C. difficile* were identified by multiplex PCR. *C. difficile* A/B toxins were detected in 21 samples (36.8%). Of these, 16 (76.2%) were from diarrheic dogs, and five (23.8%) were from non-diarrheic dogs. Twelve *C. difficile* strains (21.1%) were isolated, of which ten were A^+B^+ and two were A^-B^-. All non-toxigenic strains were isolated from non-diarrheic animals. The binary toxin gene *cdtB* was found in one strain, which was A^+B^+ and was derived from a non-diarrheic dog. *C. perfringens* strains were isolated from 40 samples (70.2%). Of these, 18 (45%) were from the diarrheic group, and 22 (55%) belonged to the non-diarrheic group. All isolates were classified as *C. perfringens* type A and there was an association between the detection of the *cpe* gene and the presence of diarrhea. Interestingly, ten strains (25%) were positive for the presence of the *cpb2* gene. The high rate of detection of the A/B toxins in non-diarrheic dogs suggests the occurrence of subclinical disease in dogs or carriage of its toxins without disease. More studies are needed to elucidate the epidemiology of *C. difficile* and *C. perfringens* in dogs and to better our understanding of *C. difficile* as a zoonotic agent. This is the first study to report the binary toxin gene in *C. difficile* strains isolated from dogs in Brazil.

Key words: nosocomial diarrhea, small animals, colitis, enteritis, canine.

Introduction

Clostridium difficile is a spore-forming, anaerobic, Gram-positive bacillus that has been recognized as an important bacterial pathogen in both humans and animals. It has been implicated as a cause of enteric disease in a variety animal species including adult horses, foals, piglets and rabbits (Silva *et al.*, 2013a). In dogs, the importance of *C. difficile* has not been fully determined. There are reports of a diagnosis of chronic and acute diarrhea caused by *C. difficile*, and also an outbreak of infection in dogs from a veterinary hospital has been described (Weese and Armstrong, 2003; Clooten *et al.*, 2008). However, there remains doubt about the role of *C. difficile* as the primary or secondary agent causing diarrhea in dogs.

Most isolates of *C. difficile* produce two types of toxins: toxin A, an enterotoxin, and toxin B, a cytotoxin (Voth and Ballard, 2005). Laboratory diagnosis of *C. difficile* infection is based on the detection of these toxins by cell culture or by enzyme immunoassays (ELISAs) (Delmeé, 2001). In addition, it has been suggested that a binary toxin, called *C. difficile* transferase (CDT), may be an additional important virulence factor (Stubbs *et al.*, 2000). CDT consists of two independent unlinked protein chains that are encoded by two separate genes, designated *cdtA* and *cdtB*.

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According to Schwan *et al.* (2009), this toxin may increase the adherence and colonization of the bacterium. To date, few studies have evaluated the presence of the binary toxin genes in strains isolated from dogs.

C. perfringens is an anaerobic, spore-forming, Gram-positive bacillus that has been associated with outbreaks of acute and often severe diarrhea in human beings, broiler chicken, pigs, dogs and horses (Rudmann *et al.*, 2003; Songer and Uzal, 2005; Silva *et al.*, 2009; Eriksen *et al.*, 2010; Waggett *et al.*, 2010; Silva *et al.*, 2013b). *C. perfringens* isolates are classified as one of five toxigenic types (A-E) based on the capacity to produce one or more of the four major toxins (alpha, beta, epsilon and iota). In addition to the major toxins, *C. perfringens* can produce other toxins such as enterotoxin, which is associated with canine enteritis and large bowel diarrhea (Kruth *et al.*, 1989; Sasaki *et al.*, 1999; Weese *et al.*, 2001), and beta-2 toxin, which is associated with diarrhea in pigs and horses (Herholz *et al.*, 1999, Songer and Uzal, 2005; Silva *et al.*, 2013c).

Isolation followed by screening for toxin genes leads to a better understanding of transmission patterns and risk factors. The evaluation of the distributions of these strains and the potential association with occurrence of diarrhea are important factors for elucidating the epidemiology of *C. difficile* and *C. perfringens* (Arroyo *et al.*, 2007; Barbut *et al.*, 2005). The objective of this study was to detect *C. difficile* A/B toxins and to isolate strains of *C. perfringens* and *C. difficile* in stool samples from diarrheic and non-diarrheic dogs.

Materials and Methods

Stool samples were collected from 57 dogs, of which 35 were apparently healthy, and 22 were diarrheic. The samples from diarrheic dogs were obtained direct from the rectum, in the Veterinary Hospital of Universidade Federal de Minas Gerais at the time of the consultation and were only collected from dogs for which the main motivation for the consultation was the occurrence of diarrhea. Samples from apparently healthy animals were collected in three city squares in Belo Horizonte city (Minas Gerais, Brazil), with the prior permission of the owner and when the animal was defecating. All samples were stored at -20 °C and were processed within 72 hours after collection.

C. difficile A/B toxins were detected using an ELISA kit (Ridascreen *Clostridium difficile* toxins A/B, R-Biopharm, Germany). The reaction was carried out in accordance the manufacturer's instructions.

To select *C. difficile* spores, equal volumes of stool samples and ethanol 96% (v/v) were mixed, and after incubation for 3 minat room temperature (Silva *et al.*, 2013c), aliquots of 5 μLwere inoculated on plates containing cycloserine-cefoxitin-fructose agar (CFFA, Hi-media, India) supplemented with 7% horse blood. These plates were incubated anaerobically at 37 °C for 72 hours. All colonies with suggestive morphology, Gram stain appearance and typical horse-manure odor (Fedorko and Williams *et al.*, 1997) were collected and suspended in 40 μL of sterile Milli-Q water. DNA extraction was performed according to the method of Baums *et al.* (2004), and samples were stored at 4 °C until use in the PCR assay. Genes encoding toxin A (*tcdA*), toxin B (*tcdB*) and the binary toxin (*cdtB*) were detected by multiplex PCR as previously described by Silva *et al.* (2011).

For isolation of *C. perfringens*, 0.08 to 0.12 g of feces was serially diluted by factors of 10, ranging from 10^{-1} to 10^{-6}. Aliquots of approximately 5 μLof each dilution were plated on sulfite polymyxin sulfadiazine agar (SPS, Difco Laboratories, Detroit, USA) and were incubated anaerobically at 37 °C for 24 hours. After incubation, approximately three to five characteristic colonies were collected and suspended in 40 μLof sterile Milli-Q water. The DNA extraction was performed according to the method of Baums *et al.* (2004), and samples were stored at 4 °C until use in the PCR assay. Genes encoding the beta-2 toxin (*cpb2*), enterotoxin (*cpe*) and major *C. perfringens* toxins (alpha, beta, epsilon and iota) were detected by multiplex PCR (Vieira *et al.*, 2008). For all PCR reactions, amplifications were carried out in a thermocycler (Thermal Cycler Px2 – Thermo Electron Corporation, Milford, USA), and the products were visualized under UV light in a 2% agarose gel stained with ethidium bromide (Sigma-Aldrich, Saint Louis, USA). Chi-square tests were used to evaluate possible association between dependent variables and clinical groups. P values of <0.05 were considered significant.

Results and Discussion

In the detection of *C. difficile* A/B toxins, 21 samples (36.8%) were positive (Table 1). Of these, 16 (76.2%) were from diarrheic dogs, and five (23.8%) were from non-diarrheic dogs. There was a significant association ($p < 0.05$) between the detection of toxin A/B and the presence of diarrhea.

The results corroborate those of previous studies, which have reported an association between the A/B toxins and diarrhea in dogs (Weese *et al.*, 2001; Marks *et al.*, 2002; Clooten *et al.*, 2008). However, the rate of positive animals found in the present study was much greater than that reported previously. Marks *et al.* (2002) found only

Table 1 - Detection of toxins A and B from *C. difficile* by ELISA in stool samples from dogs in Brazil.

Dogs	ELISA	
	Positives	Negatives
Diarrheic	16 (28%)	6 (10.5%)
Non-diarrheic	5 (8.8%)	30 (52.7%)
Total	21 (36.8%)	36 (63.2%)

3.8% (5/130) positives samples, of which four (80%) belonged to the group of diarrheic dogs. In another study, Weese *et al.* (2001) found 15.5% (22/142) positives samples, a proportion greater than that reported by Marks *et al.* (2002) but lower than that found in this study.

It is interesting to note the presence of non-diarrheic animals positive for A/B toxins. This result corroborates those of previous studies (Weese *et al.*, 2001; Marks *et al.*, 2002; Clooten *et al.*, 2008) and suggests the occurrence of subclinical disease in dogs or carriage of A/B toxins without disease. There have been no studies histologically examining the intestine of non-diarrheic dogs positive for toxins A and B. However, in piglets, the occurrence of subclinical animals positive for A/B toxins is common, and when their intestines are evaluated microscopically, these animals show characteristics lesions caused by *C. difficile* (Yaeger *et al.*, 2007). Further studies are needed to determine the importance of the detection of toxins A and B in dogs without diarrhea. These results suggest the possibility that *C. difficile* is a primary or secondary diarrhea-causing agent in dogs.

Twelve (21%) strains of *C. difficile* were isolated, two from diarrheic dogs and ten from non-diarrheic dogs (Table 2). The isolation rate may have been influenced by the high proportion of samples from young dogs (under one year of age): 18 (31.6%) samples were from young dogs, and 39 (68.4%) were from adult animals. In addition, the large number of young dogs in the group of diarrheic animals is noteworthy; they accounted for 60% of samples from this group but accounted for only 17% of the non-diarrheic group. According to Struble *et al.* (1994), the risk of colonization by *C. difficile* increases proportionately with age in dogs. The present study corroborates this statement, as all strains were isolated from adult animals, the youngest of which was aged two years.

In the present study, ten isolates were A^+B^+ and two were A^-B^-. No variant strains were found. Weese *et al.* (2001) obtained *C. difficile* from 1.4% (2/142) of dog stool samples, a lower rate than that observed in the present study. On the other hand, Marks *et al.* (2002) and Struble *et al.* (1994) recovered *C. difficile* from 13.1% (17/130) and 18.4% (28/152) dogs, respectively, and 70.6% and 50% of these strains were toxigenic (A^+B^+), respectively. The absence of variant strains (A^-B^+) corroborates the results of previous reports (Struble *et al.*, 1994; Weese *et al.*, 2001; Marks *et al.*, 2002; Clooten *et al.*, 2008). To date, these strains have been isolated only from dogs that had visited human hospitals (Lefebvre *et al.*, 2006).

Table 2 - Presence of *tcdA* and *tcdB* genes in *C. difficile* strains isolated from diarrheic and non-diarrheic dogs in Brazil.

Dogs	*Clostridium difficile* strains		
	A^+B^+	A^-B^-	Total
Diarrheic	2 (16.7%)	(0%)	2 (16.7%)
Non-diarrheic	8 (66.6%)	2 (16.7%)	(83.3%)
Total	10 (83.3%)	2 (16.7%)	12 (100%)
Dogs	ELISA		
	Positives	Negatives	
Diarrheic	16 (28%)	6 (10.5%)	
Non-diarrheic	5 (8.8%)	30 (52.7%)	
Total	21 (36.8%)	36 (63.2%)	

In this study, all non-toxigenic strains were isolated from healthy animals, corroborating the findings of Alvarez-Perez *et al.* (2009). According to Clooten *et al.* (2008), previous colonization by a non-toxigenic strain reduces the risk of developing diarrhea associated with *C. difficile* in dogs. The same trend has been observed in piglets and in humans (Buggy *et al.*, 1983; Kyne *et al.*, 2000; Silva *et al.*, 2011). As a result, a non-toxigenic strain for competitive exclusion is under development for use in at-risk humans (Songer, 2010).

The binary toxin gene was found in one strain, which was A^+B^+ and was derived from a non-diarrheic animal. This was the first study to report the presence of the binary toxin gene in stool samples from dogs in Brazil. In humans, Persson *et al.* (2008), reported CDT^+ in approximately 26% of human strains, 97.3% of which were A^+B^+. In domestic animals, the presence of the binary toxin was found in approximately 4% of samples from horses (Arroyo *et al.*, 2007) and in more than 50% of samples from piglets (Norman *et al.*, 2009; Silva *et al.*, 2011). Little is known about the clinical relevance and pathogenic role of CDT in *C. difficile* infections, and most studies have focused on human patients. As CDT is a potent cytotoxin, Gonçalves *et al.* (2004) suggested that it might prepare the way for toxins A and B. Alternatively, CDT can also act in synergy with other toxins, depolymerizing the cytoskeleton by a complementary mechanism. It is also important to note that, until now, there is no description of $cdtB^+$ strains in humans in Brazil.

Differences in isolation rates and toxinotype frequencies may result from differences in management practices, the clustering of cases (Arroyo *et al.*, 2007) and differences in geographical distribution. A recent study has suggested prevalent variation of certain genotypes of *C. difficile* in different geographic regions (Avbersek *et al.*, 2009). In addition, the carrier state of *C. difficile* seems to vary among asymptomatic individuals by species and within the same species, depending on age and other population characteristics (Keel and Songer *et al.*, 2006). The presence of a CDT^+ strain observed in the present study highlights the importance of future work to evaluate the distribution and role of *C. difficile* in dogs.

Recently, *C. difficile* was isolated from ready-to-eat retail meats and salads. Many of these strains were of ribotypes associated with *C. difficile* infection in humans and food animals (Bakri *et al.*, 2005, Rodriguez-Palacios *et al.*, 2007; Songer *et al.*, 2009). It is also important to note

Table 3 - Presence of *cpb2* and *cpe* genes in *C. perfringens* type A strains isolated from diarrheic and non-diarrheic dogs.

Dogs	*C. perfringens* type A strains				Total
	$cpb2^{-}cpe^{-}$	$cpb2^{+}cpe^{-}$	$cpb2^{-}cpe^{+}$	$cpb2^{+}cpe^{+}$	
Diarrheic	9 (22.5%)	0 (0%)	4 (10%)	5 (12.5%)	18 (45%)
Non-diarrheic	15 (37.5%)	1 (2.5%)	2 (5%)	4 (10%)	22 (55%)
Total	24 (60%)	1 (2.5%)	6 (15%)	9 (22.5%)	40 (100%)

that the two most common toxigenic ribotypes in dogs, which account for 90% of isolates, are also recognized as causes of *C. difficile* disease in humans (Arroyo *et al.*, 2007). All of these reports raise the possibility of *C. difficile* transmission from human fecal contamination to dogs or even as a zoonotic disease, but more studies are needed for confirmation.

A low percentage of animals were positive for both the isolation and detection of A/B toxins simultaneously. This result corroborates those found in other studies with dogs (Struble *et al.*, 1994; Marks *et al.*, 2002) and is related to the difficulty of isolating this bacteria species. In many cases, toxins A/B are found in fecal content, but the bacterium itself is not isolated. The vegetative form of *C. difficile* can persist for a short time under aerobic conditions and is dependent on sporulation for it to remain viable in the feces (Buggy *et al.*, 1983). In addition, some strains cannot grow on CCFA due to the sensitivity to one or both antibiotics used in selective media (Songer and Uzal, 2005).

C. perfringens strains were isolated from 40 samples (70.2%). Of these, 18 (45%) belonged to diarrheic dogs, and 22 (55%) belonged to non-diarrheic dogs. This result is similar to those obtained by Marks *et al.* (2002) and Weese *et al.* (2001), who obtained *C. perfringens* in 78% and 80% of dog stool samples, respectively. As in the present study, both Weese *et al.* (2001) and Marks *et al.* (2002) found no significant differences in the isolation rates between the diarrheic group and the non-diarrheic group.

All isolates obtained were classified as *C. perfringens* type A, whereas the *cpe* gene, responsible for encoding the enterotoxin, was found in 15 strains (37.5%), six (17.1%) were from apparently healthy animals, and nine (40.9%) were from diarrheic dogs (Table 3). This result corroborates those of previous studies that have reported that type A is the most frequent *C. perfringens* type in dogs (Sasaki *et al.*, 1999; Marks *et al.*, 2002; Siqueira *et al.*, 2012). Moreover, there was a significant association ($p > 0.05$) between the detection of the *cpe* gene and the presence of diarrhea, result similar to those reported by Marks *et al.* (2002).

Interestingly, ten strains (25%) were positive for the presence of *cpb2* gene. In other domestic animals, such as pigs and horses, *C. perfringens* $cpb2^{+}$ strains have been associated with diarrhea and typhlocolitis, respectively (Herholz *et al.*, 1999; Songer and Uzal, 2005; Silva *et al.*, 2013c). More studies are needed to elucidate the role and the importance of beta-2 toxin in *C. perfringens* diarrhea in dogs.

The next step of this study is to evaluate the minimum inhibitory concentrations of antibiotics commonly used in small animal clinics against *C. difficile* and *C. perfringens* strains. In addition, PCR-ribotyping could be useful to elucidate the epidemiology of *C. difficile* in dogs and would add more information about the role of *C. difficile* as zoonotic agent. This is the first study about *C. perfringens* and *C. difficile* from dogs in Brazil.

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