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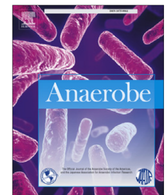

Clinical microbiology

Clostridium difficile and *Clostridium perfringens* from wild carnivore species in Brazil

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ABSTRACT

Despite some case reports, the importance of *Clostridium perfringens* and *Clostridium difficile* for wild carnivores remains unclear. Thus, the objective of this study was to identify *C. perfringens* and *C. difficile* strains in stool samples from wild carnivore species in Brazil. A total of 34 stool samples were collected and subjected to *C. perfringens* and *C. difficile* isolation. Suggestive colonies of *C. perfringens* were then analyzed for genes encoding the major *C. perfringens* toxins (alpha, beta, epsilon and iota) and the beta-2 toxin (*cpb2*), enterotoxin (*cpe*) and NetB (*netb*) genes. *C. difficile* strains were analyzed by multiplex-PCR for toxins A (*tcdA*) and B (*tcdB*) and a binary toxin gene (*cdtB*) and also submitted to a PCR ribotyping. Unthawed aliquots of samples positive for *C. difficile* isolation were subjected to the detection of A/B toxins by a cytotoxicity assay (CTA). *C. perfringens* was isolated from 26 samples (76.5%), all of which were genotyped as type A. The *netb* gene was not detected, whereas the *cpb2* and *cpe* genes were found in nine and three *C. perfringens* strains, respectively. *C. difficile* was isolated from two (5.9%) samples. A non-toxigenic strain was recovered from a non-diarrheic maned wolf (*Chrysocyon brachyurus*). Conversely, a toxigenic strain was found in the sample of a diarrheic ocelot (*Leopardus pardallis*); an unthawed stool sample was also positive for A/B toxins by CTA, indicating a diagnosis of *C. difficile*-associated diarrhea in this animal. The present work suggests that wild carnivore species could carry *C. difficile* strains and that they could be susceptible to *C. difficile* infection.

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1. Introduction

Clostridium perfringens isolates are conventionally 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) [1]. In addition to the major toxins, *C. perfringens* can produce additional virulence factors, such as beta-2 toxin, which has been associated with diarrhea in horses and piglets [2–4], necrotic enteritis toxin B-like (NetB), a pore-forming toxin described as an important virulence factor for necrotic enteritis in broiler chickens [5] and enterotoxin, which is responsible for human foodborne disease and is also associated with diarrhea in dogs and cats [6–8]. *C. perfringens* is commonly found in the enteric microbiota of healthy animals, thus complicating the laboratory diagnosis of infections caused by this microorganism. The knowledge of *C. perfringens* in wild carnivore species is limited to few case reports [9,10] and the role of the most common additional virulence factors of *C. perfringens*, such as beta-2 toxin, enterotoxin and NetB, are

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unknown, and which genotypes of *C. perfringens* are more prevalent in these species, including in healthy animals, is also uncertain.

Clostridium difficile is a spore-forming, anaerobic, Gram-positive bacillus recognized as the pathogen responsible for most cases of antibiotic-associated diarrhea in humans [11] and is an important cause of enterocolitis in various domestic animals, including horses, piglets and dogs [12,13]. Moreover, recent studies have also shown that isolates from humans suffering from *C. difficile* infection (CDI) are highly genetically related to strains isolated from animals, suggesting a hypothetical zoonotic potential [14].

Despite the importance of *C. difficile* as an enteropathogen for human and domestic animals and even as a possible zoonotic agent, the role of CDI in most wild species remains unclear. Indeed, there are only few studies, limited to case reports, that examine its role [15,16]. In addition, some reports have recently raised the importance of wild animals as a reservoir of *C. difficile* for humans and domestic animals, though there are few works on this subject [17,18].

The isolation and screening for virulence factor genes could contribute to the knowledge of *C. difficile* and *C. perfringens* risk factors and epidemiology [19,20]. In light of this, the objective of this study was to identify *C. perfringens* and *C. difficile* strains in stool samples from wild carnivore species in Brazil.

2. Material and methods

2.1. Samples

A total of 34 samples from wild carnivores were collected, 11 from *Cerdocyon thous* (crab-eating fox), nine from *Puma concolor* (cougar), four from *Leopardus tigrinus* (oncilla), three from *Leopardus pardalis* (ocelot), four from *Chrysocyon brachyurus* (maned wolf) and one each from *Puma yagouaroundi* (jaguarundi), *Leopardus wiedii*, (margay or tree ocelot) and *Lycalopex vetulus* (hoary fox). The animals were sampled while in captivity and the time of captivation varied between 24 h and 3 years, as shown in Table 1. These species were trapped in farms or around urban areas by the Brazilian group for wildlife. After submitted to laboratory tests, some animals were released in protected areas or kept in captivity when they were unhealthy or unable to readapt into the wild. All stool specimens were collected in sterile containers, and three aliquots were stored at −20 °C until testing was performed.

2.2. Isolation and genotyping of C. perfringens

For the isolation of *C. perfringens*, 0.08–0.12 g of feces was serially diluted by factors of 10, ranging from 10^{-1} to 10^{-3}. Aliquots of 10 μl of each dilution were plated on sulfite polymyxin sulfadiazine agar (SPS, Difco Laboratories, Detroit, MI, 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 [21] for the detection of genes encoding the major *C. perfringens* toxins (alpha, beta, epsilon and iota), beta-2 toxin (*cpb2*) and enterotoxin (*cpe*). In addition, a previously described PCR protocol was applied for the detection of the NetB-encoding gene *netb* [22]. For all of the PCR reactions, the amplifications were performed in a thermocycler (Thermal Cycler Px2 – Thermo Electron Corporation, Milford, MA, USA), and the products were visualized under UV light in a 2% agarose gel stained with ethidium bromide (Sigma–Aldrich Co., St. Louis, MO, USA).

2.3. 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 (Hi-media, Mumbai, India) 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*) [20]. Unthawed aliquots of stool samples positive for *C. difficile* isolation were subjected to A/B toxin detection by CTA using Vero cells [23] and by a commercial ELISA kit (*C. difficile* Tox A/B II - Techlab Inc., Blacksburg, VA, USA). In addition, all *C. difficile* isolates were tested by CTA for *in vitro* toxin production, as previously described [24]. C. *difficile* ATCC 9689 was used as a control for PCR and *in vitro* toxin production.

2.4. PCR ribotyping of C. difficile

All *C. difficile* strains were submitted to PCR ribotyping. Intergenic spacer regions were amplified using Bidet primers as previously described [25]. Amplification products were separated by electrophoresis in 3% agarose gel (Bio-Rad, California, USA) for 5 h at 2.5 V/cm and the gel was analyzed with BioNumerics 7.00 (Applied Maths, Belgium). PCR ribotypes for which the reference strains were available are designated by international Cardiff nomenclature.

2.5. Antimicrobial susceptibility

The minimum inhibitory concentration (MIC) of 16 *C. perfringens* and two *C. difficile* strains were determined using the serial agar

Table 1
Isolation of *Clostridium perfringens* and *C. difficile* from stool samples of wild carnivore species in Brazil.

Species	Common name	Time in captivity			Samples (%)	Isolation					
						C. perfringens			*C. difficile*		
		<1 week	1-to-4 weeks	>4 weeks		Positives	*cpb2*+	*cpe*+	Positives	A+B+	A−B−
C. thous	Crab-eating fox	1	2	9	12 (35.3)	9	4	–	–	–	–
P. concolor	Cougar			9	9 (26.5)	8	1	2	–	–	–
L. tigrinus	Oncilla			4	4 (11.8)	2	2	1	–	–	–
L. pardalis	Ocelot	1	2		3 (8.8)	1	–	–	1	1[a]	–
C. brachyurus	Maned wolf	2		1	3 (8.8)	3	1	–	1	–	1[b]
P. yagouaroundi	Jaguarundi			1	1 (2.9)	1	1	–	–	–	–
L. wiedii	Tree ocelot			1	1 (2.9)	1	–	–	–	–	–
Lycalopex vetulus	hoary fox	1			1 (2.9)	1	–	–	–	–	–
Total		5 (14.7)	4 (11.8)	25 (73.5)	34 (100)	26 (76.5%)	9 (34.6%)	3 (11.5%)	2 (5.9%)	1 (50%)	1 (50%)

[a] PCR Ribotype 046.
[b] PCR Ribotype 053.

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