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Molecular epidemiology of *Clostridioides difficile* obtained from fecal samples of wild animals in Brazil[1]

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ABSTRACT.- Lima M.C., Basso R.M., Cerri F.M., Lima H.C., Rahal S.C., Zanon I.P., Carvalho G.M., Silva R.O.S., Arroyo L.G., Oliveira-Filho J.P. & Borges A.S. 2024. **Molecular epidemiology of *Clostridioides difficile* obtained from fecal samples of wild animals in Brazil.** *Pesquisa Veterinária Brasileira, 44:e07385, 2024*. Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista "Júlio de Mesquita Filho", Rua Prof. Doutor Walter Mauricio Correa s/n, Cx. Postal 560, Botucatu, SP 18618-681, Brazil. E-mail: alexandre.s.borges@unesp.br

Clostridioides difficile is a strictly anaerobic, spore-forming Gram-positive bacterium associated with diarrhea, known as *C. difficile* infection (CDI). In domestic animals, *C. difficile* is considered an important pathogen mostly in pigs and horses, but there are also reports in other domestic species. In wild animals, the epidemiology of *C. difficile* is largely unknown, and the role of the bacterium as a cause of diarrhea is unclear. The aim of this study was to determine the prevalence of *C. difficile* in the feces of wild animals referred to the Center of Medicine and Research in Wild Animals (CEMPAS). Fecal samples obtained from 100 animals of 34 different species were subjected to qPCR for the detection of the *C. difficile 16S rRNA* gene and two major toxin genes (*tcdA* and *tcdB*) and to anaerobic bacterial isolation. A total of 63 animals (63%) were positive for *C. difficile* by qPCR, and 16 isolates were recovered. The opossum (*Didelphis* spp.) had the highest number of positive animals in both tests (from 21 samples, 19 were qPCR positive, and four isolates were recovered). Three toxigenic strains (RT 002, 004, and 014), all previously described as infecting humans and animals, were isolated in the following species: bearded dragon (*Pogona vitticeps*), pampas fox (*Lycalopex vetulus*), and marmoset (*Callithrix* sp.). The presence of *C. difficile* in the feces of wild animals highlights the importance of wildlife as potential carriers of infection for production animals or humans.

INDEX TERMS: *Didelphis* spp., qPCR, *tcdB*, *tcdA*, wild animals, *Clostridioides difficile*.

RESUMO.- [**Epidemiologia molecular do *Clostridioides difficile* obtido de amostras de fezes de animais selvagens no Brasil.**]. O *Clostridioides difficile* é uma bactéria Gram-positiva estritamente anaeróbica e formadora de esporos, associada à diarreia e conhecida como infecção por *C. difficile* (CID). Em animais domésticos, o *C. difficile* é considerado um patógeno importante principalmente em porcos e cavalos, mas também há relatos em outras espécies domésticas. Em animais selvagens, a epidemiologia do *C. difficile* é amplamente desconhecida, e o papel da bactéria como causa de diarreia não está claro. O objetivo deste estudo foi determinar a prevalência do *C. difficile* nas fezes de animais selvagens encaminhados ao Centro de Medicina e Pesquisa em Animais Selvagens (CEMPAS). Amostras de fezes obtidas de 100 animais de 34 espécies diferentes foram submetidas à qPCR para a detecção do gene *16S rRNA* do *C. difficile* e dois principais genes de toxina (*tcdA e tcdB*), além de isolamento bacteriano anaeróbico. Um total de 63 animais (63%) foram positivos para *C. difficile* por qPCR, e 16 isolados foram recuperados. O gambá (*Didelphis* spp.) apresentou o maior número de animais positivos em ambos os testes (de 21 amostras, 19 foram positivas na qPCR, e quatro isolados foram recuperados). Três cepas toxigênicas

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(RT 002, 004 e 014), todas previamente descritas como infectando humanos e animais, foram isoladas nas seguintes espécies: dragão barbado (*Pogona vitticeps*), raposa-pampas (*Lycalopex vetulus*) e sagui (*Callithrix* sp.). A presença de *C. difficile* nas fezes de animais selvagens destaca a importância da vida selvagem como potencial portadora de infecção para animais de produção ou seres humanos.

TERMOS DE INDEXAÇÃO: *Didelphis* spp., qPCR, *tcdB*, *tcdA*, animais selvagens, *Clostridioides difficile*.

INTRODUCTION

Clostridioides difficile is a major cause of diarrhea in human patients undergoing antibiotic therapy. *C. difficile* infection (CDI) rates have been increasing in patients even without a previous history of hospitalization (Anjewierden et al. 2020, Maslennikov et al. 2022). In domestic animals, it is currently considered an etiologic agent of neonatal diarrhea in piglets and enteritis in foals and adult horses (Gohari et al. 2014). In cats (Weese et al. 2001, Schneeberg et al. 2012) and dogs (Weese et al. 2001, Busch et al. 2015), its importance as a cause of diarrhea is still questionable.

In Brazil, the agent has already been described in dogs (Rainha et al. 2019), calves (Silva et al. 2015), horses (Silva et al. 2012), wildlife (Silva et al. 2014a, 2014b), and humans (Ferreira et al. 2017, Rizek et al. 2022, Carvalho et al. 2023). In Latin America, there are several reports of CDI in humans, mainly describing the occurrence of ribotypes (RT) 001, 014, 015, 017, 027, 106, 133, and 135 (Acuña-Amador et al. 2022).

In Brazil, cases of CDI in humans have been steadily increasing, especially due to the epidemic spread of RT BI/NAP1/027 (Trindade et al. 2019). Whole-genome analysis of strains isolated from hospitalized patients revealed the presence of a high number of virulence genes (Rizek et al. 2022) and vancomycin resistance genes (Saldanha et al. 2020).

The observation of the same strains of *C. difficile* in animals and rural workers effectively demonstrates the importance of this agent in the concept of One Health (O'Shaughnessey et al. 2019, Redding et al. 2021). The presence of *C. difficile* in animals (Weese et al. 2020), water treatment plants (Chisholm et al. 2022), food (Borji et al. 2023), soil (Marcos et al. 2023), and humans (Monaghan et al. 2022) demonstrates its ability to colonize various species and, consequently, facilitates its dissemination and the exchange of virulence genes (Mitchell et al. 2022).

Evidence of the presence of strains from the ST11 lineage (Ribotype 078) with clonal groups in various animal species and humans reinforces the concept of mutual exchange, both zoonotic and anthropozoonotic, of *C. difficile* (Knight et al. 2019). The identification of identical strains, using next-generation genetic sequencing techniques, in pigs and farm workers highlights the possibility of mutual and common environmental dissemination between humans and animals (Knetsch et al. 2014).

There is limited information on the infection and/or colonization by *C. difficile* in wild animals (Silva et al. 2014a, Bandelj et al. 2018, Weese et al. 2020). Some authors suggest that wild animals may act as a reservoir for *C. difficile* strains relevant to domestic animals and humans (Himsworth et al. 2014, Williams et al. 2018, Krijger et al. 2019, Weese et al. 2019). Thus, the aim of this study was to determine the presence of *C. difficile* in the feces of wild animals using qPCR and classical isolation techniques.

MATERIALS AND METHODS

Animal Ethics. All procedures were approved by the Ethics Committee for the Use of Animals (CEUA) of the FMVZ-Unesp, Botucatu/SP (Protocol CEUA 0088/2022).

Study local and contextualization. Fecal samples were collected (directly from the rectal ampulla or immediately from the environment) during routine daily activities at "Centro de Medicina e Pesquisa em Animais Selvagens" (Center of Medicine and Research in Wild Animals - CEMPAS) in Botucatu, São Paulo (Southeast region), Brazil (22º53'25'' South, 48º27"19"" West), and frozen at -80ºC. The samples were harvested between October and December 2020 from live animals located in Cempas, originating from regions within a 100km radius.

DNA extraction and qPCR. DNA extraction was performed from 200mg of feces using the E.Z.N. A™ Stool DNA Kit (PROMEGA® Madison/WI, USA) and homogenized continuously (Precellys®, Bertin Technologies, Montigny-le-Bretonneux, França). qPCRs (GoTaq® Probe Master Mix – PROMEGA® Madison/WI, USA) were used to detect the *16S rRNA* gene, and in the positive samples, the *tcdA* and *tcdB* genes were analyzed as previously described (Mutters et al. 2009, Kilic et al. 2015).

Classical isolation techniques. Samples positive for *Clostridioides difficile* by qPCR were submitted for bacterial culture as described previously (Silva et al. 2013a). Briefly, approximately 1g of each fecal sample was inoculated in brain heart infusion broth (BHI) (San Luis/MO, USA). After incubation under anaerobic conditions at 37°C for five days, the culture was subjected to alcohol shock and plated on selective agar (Silva et al. 2013b). Suspected *C. difficile* colonies based on characteristic colony appearance and smell were harvested and subjected to DNA extraction and multiplex PCR for detection of the constitutive gene (*tpi*) and *tcdA*, *tcdB,* and *cdtB* genes (Silva et al. 2011). Toxigenic strains (isolates positive for *tcdA* and/or *tcdB*) were also subjected to PCR ribotyping (Bidet et al. 1999). The *C. difficile* library from the "Escola de Veterinária" of the "Universidade Federal de Minas Gerais" (UFMG) was used and additionally, the band patterns were compared using the WEBRIBO[4].

RESULTS

qPCR and classical isolation techniques

A total of 100 fecal samples from 34 different species were analyzed from June to November 2020. qPCR detected the presence of *Clostridioides difficile* DNA in 63 (63%) fecal samples, of which 19 were positive for toxins A and B (*tcdA/tcdB*) (Table 1). *C. difficile* was isolated from 16 samples, of which three isolates (Bearded dragon – *P. vitticeps*, Pampas fox – *Lycalopex vetulus,* and Marmoset – *Callithrix* sp.) were toxigenic (all positive for the *tcdA* and *tcdB* genes) and classified as ribotypes 004, 014 and 002, respectively.

DISCUSSION

The frequency of *Clostridioides difficile* in opossum (*Didelphis* spp.) deserves to be highlighted: 19 out of the 21 sampled opossum (90.48%) were positive for *C. difficile* by qPCR, nine of which were classified as toxigenic by qPCR. Additionally, four strains (21.05%) were isolated. One hypothesis for this high frequency is the cohabitation of these animals in areas with domestic animals and humans, including in urban centers (Silva et al. 2014a, Zlender et al. 2022).

Samples were collected from seven carnivorous species (*Cerdocyon thous, Leopardus tigrinus, Puma yagouaroundi,*

[4] Available at <https://webribo.ages.at/> Accessed on May 11, 2022.

Leopardus pardalis, Chrysocyon brachyurus, Puma concolor, and Lycalopex vetulus) (n=22). *C. difficile* DNA was detected by qPCR in 20 (91%) of the samples, of which five were culture-positive. This frequency suggests that wild canids and felids can harbor and disseminate *C. difficile* strains, similar to those previously reported by Silva et al. (2014b). In addition to its possible role as an asymptomatic carrier, *C. difficile* can also cause disease in these animals, which was previously observed in Brazil, where this pathogen has already been confirmed to cause fatal diarrhea in an ocelot (*L. pardalis*) (Silva et al. 2014b).

C. difficile DNA was detected in primates (*Sapajus apella* and *Callithrix* sp.) and, for the first time, in giant anteaters (*Myrmecophaga tridactyla*). There are very few studies on the prevalence of *C. difficile* in primates, and a previous study in Brazil (Carvalho et al. 2022) failed to isolate *C. difficile* from 24 capuchin monkeys (*Sapajus* spp.). On the other hand, CDI has already been described in several primate species, including marmoset (*Callithrix* sp.) (Armstrong et al. 2019). Among reptiles, seven were positive by qPCR, and two isolates were recovered. *C. difficile* was previously reported to colonize apparently healthy reptiles (Andrés-Lasheras et al. 2018, Ramos et al. 2019). Interestingly, the red-faced tortoise (*Chelonoidis carbonaria*), which is commonly kept as a pet, stands out with four positive samples (4/7, 57%).

Synanthropic animals and reptiles had the highest number of positive fecal samples, and therefore, they may be a reservoir for *C. difficile* strains (Andrés-Lasheras et al. 2018, Williams et

Table 1. Molecular detection of *Clostridioides difficile*, toxin genes *tcdA* and *tcdB*, and selective bacterial isolation in fecal samples of wild animals

	Species	Animals	qPCR		Culture	
		N	16	*tcdA*⁺ *tcdB*⁺	Isolation	*tcdA*⁺ *tcdB*⁺
Synanthropic mammals	Opossum (*Didelphis* spp.)	21	19	8	4	0
	Rabbit (*Oryctolagus cuniculus*)	3	0	0	0	0
	Rat mouse (*Mus musculus*)	1	1	1	0	0
	Hare (*Lepus europaeus*)	1	0	0	0	0
	TOTAL	26	20	9	4	0
Carnivorous mammals	Crab-eating fox (*Cerdocyon thous*)	9	7	1*	1	0
	Pampas fox (*Lycalopex vetulus*)	3	3	1	2	1
	Maned wolf (*Chrysocyon brachyurus*)	3	3	0	0	0
	Puma (*Puma concolor*)	3	3	1	1	0
	Wild cat (*Leopardus tigrinus*)	2	2	0	0	0
	Jaguarundi (*Puma yagouaroundi*)	1	1	0	1	0
	Ocelot (*Leopardus pardalis*)	1	1	1**	0	0
	TOTAL	22	20	4	5	1
Other mammals	Howler monkey *(Alouatta guariba*)	2	0	0	0	0
	Capybara (*Hydrochoerus hydrochaeris*)	1	0	0	0	0
	Water opossum (*Chironectes minimus*)	1	1	1	1	0
	Capuchin monkey (*Sapajus apella*)	6	4	0	1	0
	Crab-eating raccoon (*Procyon cancrivorus*)	1	1	1	0	0
	Hairy dwarf porcupine (*Erinaceus roumanicus*)	3	2	0	0	0
	Hairy dwarf porcupine (*Coendou prehensilis*)	2	0	0	0	0
	Marmoset (*Callithrix sp.*)	7	1	1	1	1
	Giant anteater (*Myrmecophaga tridactyla*)	7	5	1**	2	0
	Nine-banded armadillo (*Dasypus novemcinctus*)	1	1	0	0	0
	Armadillo (*Euphractus sexcinctus*)	1	0	0	0	0
	Brown deer (*Mazama gouazoubira*)	2	1	0	0	0
	TOTAL	34	16	4	5	1
Reptiles	Leopard gecko (*Eublepharis macularius*)	1	0	0	0	0
	Red-fronted tortoise (*Chelonoidis carbonaria*)	7	4	0	1	0
	Boa constrictor (*Boa constrictor constrictor*)	7	2	2	0	0
	Bearded dragon (*Pogona vitticeps*)	1	1	1**	1	1
	Python (*Python molurus*)	1	0	0	0	0
	Teiu (*Tupinambis merianae*)	1	0	0	0	0
	TOTAL	18	7	3	2	1
	TOTAL %	100	63	19	16	3

* Only *tcdA* positive, ** only *tcdB* positive.

al. 2018, Ramos et al. 2019). In this context, the identification of 13 non-toxigenic strains (*Didelphis* spp.*, Cerdocyon thous, Lycalopex vetulus, Puma concolor, Puma yagouaroundi, Chironectes minimus, Sapajus apella, Myrmecophaga tridactyla, Chelonoidis carbonaria*), even though they are not associated with the development of CDI cases, can hold epidemiological significance. This group of strains, despite lacking the pathogenicity locus (*PaLoc*), has the potential to disseminate resistance genes through mobile genetic elements such as plasmids and transposons, as documented by Kartalidis et al. (2021). Non-toxigenic strains can also be capable of encoding virulence factors and acquiring the *PaLoc*, becoming virulent (Chowdhury et al. 2016).

Interestingly, the detection of ribotypes 002, 006, and 014, which are commonly associated with CDI in humans (Dauby et al. 2017), suggests that these toxigenic strains could infect humans or vice versa (Himsworth et al. 2014, Krijger et al. 2019). Ribotypes 002 and 014 have already been described in captive and wild animals (Zlender et al. 2022). Ribotype 014 is the most frequently observed, being the primary driver of the global spread of this agent (Berger et al. 2020, Wen et al. 2022). This ribotype exhibits significant genetic diversity, facilitating the transmission of antimicrobial resistance genes, such as tetracycline, erythromycin, and aminoglycosides (Knight et al. 2017, Andino-Molina et al. 2019). Recently, the presence of RT014/20 strains in metronidazole-resistant dogs was demonstrated (Leite et al. 2023).

The difference between the qPCR results and isolation can be explained by the high sensitivity of the technique (Maestri et al. 2022). We believe that the use of BHI without a previous alcohol shock would have reduced the sensitivity of the isolation method. It´s also possible that qPCR was able to detect strains that were not viable or in enough quantity to be cultured (Jia et al. 2023). This technique is highly practical due to its rapid execution and greater feasibility under anaerobic conditions compared to isolation (Okanda et al. 2020). The use of primers for constituent genes (*tpi)* (Kilic et al. 2015) and for the gene encoding the 60kDa chaperonin protein (*cpn60*) (Kohler et al. 2022) can improve the specificity of the qPCR results.

The primary limitation of this study is the inability to gather data regarding the animals' location, habitat, interactions with humans and other animal species, as well as their clinical history.

CONCLUSION

Considering the number of isolates recovered, the ribotypes found and the rate of qPCR positivity in the feces of the species included, it is important to consider wild animals as possible reservoirs or carriers of *Clostridioides difficile* and the possibility of transmission to humans and other domestic animals.

Conflict of interest statement.- The authors declare that there are no conflicts of interest.

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