Communication

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Occurrence of *Clostridium perfringens* in Wild Mammals in the Amazon Biome

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Simple Summary: *Clostridium perfringens* is a commensal bacterium of humans and animals that can act as a pathogen that causes myonecrosis and enteric diseases. Previous studies have reported *C. perfringens* infection in several wild animals as well as its presence as a commensal, but its role in animals of the Amazon region is largely unknown. Thus, this study investigated the occurrence of *C. perfringens* in samples from wild mammals treated at the Wild Animals Sector of the Veterinary Hospital of the Federal University of Pará in the Amazon biome. *C. perfringens* type A was found in 61.5% of the animals sampled. No other toxinotypes were isolated. The results suggest that *C. perfringens* type A is a common commensal bacterium of animals in the Amazon biome.

Abstract: The objective of this study was to evaluate the occurrence of *Clostridium perfringens* in stool samples and swabs collected from wild mammals in the Amazon biome. Sixty-five faecal and swab samples were collected *in situ* and *ex situ* from 16 species and three genera of wild mammals, some of which were in good health and some of which had diarrhoea. After pre-enrichment, the samples were plated on selective agar for *C. perfringens*. Characteristic colonies were subjected to *multiplex* PCR for the detection of genes encoding the main *C. perfringens* toxins (alpha, beta, epsilon, and iota toxin and enterotoxin). Among the 65 samples, 40 (61.5%) were positive for the gene encoding the alpha toxin and were classified as type A. Faecal samples were more than four times more likely to be positive for *C. perfringens* than were swab samples. No other toxinotypes were found. The findings of this study suggest that *C. perfringens* type A is commonly found as a commensal in mammal species of the Amazon biome. This seems to be the first study to identify *C. perfringens* type A in species such as *B. variegatus* (common ground sloth), *C. didactylus* (two-toed sloth), *P. flavus* (Jupará), *T. tetradactyla* (anteater), *S. collinsi* (squirrel monkey), *S. niger* (black marmoset), and *S. apella* (Guyana capuchin) and in the genus *Didelphis* sp. (opossum).

Keywords: alpha toxin; toxinotype A; wild mammals; diarrhoea

1. Introduction

Clostridium perfringens is a gram-positive, spore-forming bacillus that is classified into seven toxinotypes (A – G) according to the production of six major toxins: alpha (*cpa*), beta (*cpb*), epsilon (*etx*), iota toxin (*itx*), enterotoxin (*cpe*) and necrotic enteritis type B (*netB*) [1–3]. *C. perfringens* is found mainly as a commensal of the gastrointestinal tract of humans and animals and is ubiquitous in the environment [3].

Despite being a common commensal, *C. perfringens* can cause a number of diseases in animals and humans, including gas gangrene and various enteric conditions [1,4]. In humans, it is the second

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most common cause of foodborne illness in the United States and the sixth most common in Brazil [5]. Some studies have demonstrated the presence of *C. perfringens* as a commensal in some species of wild animal in addition to confirming the participation of this agent as a cause of enteritis and enterotoxaemia [6–10]. However, the description of this bacterium and its toxinotypes in wild animals of the Amazon Biome, either as a commensal or as a pathogen, is lacking.

It remains largely unknown which species are colonized by this agent, and among those that have *C. perfringens* as a commensal species, it is not known which toxinotypes are present or which toxins are potentially produced by those strains. Thus, the objective of the present study was to evaluate the isolation frequency and types of *C. perfringens* in wild animals in the Amazon biome.

2. Materials and Methods

2.1. Data Collection

This study was conducted in accordance with the Ethical Principles of Animal Research adopted by the National Council for the Control of Animal Experimentation (CONCEA) and was approved by the Committee on Ethics in the Use of Animals of the Federal University of Pará (CEUA/UFPA) under protocol n° 8888280618. The study was also authorized by the Chico Mendes Institute for Biodiversity Conservation (ICMBio) under n° 67300-1.

A non-probabilistic sampling method (for convenience) was used [11] due to all the particular characteristics of the species of the animals treated and hospitalized during the period from August 2017 to October 2022. The collection procedures were monitored by veterinarian residents to monitor animal welfare, including the physical, mental and behavioural states of each species [12].

Stool samples were collected during feeding, which occurred twice a day (morning and afternoon), to reduce contact and handling stress. Pieces of PVC film paper were placed under the animals' enclosure, with subsequent collection of fresh, individual faeces produced without much soiling of the substrate. The collection of samples also occurred during medication applications, physical examinations and weighing [13]. Cloacal and anal swabs were collected under the same conditions, and proper physical and chemical protocols for each species were applied to reduces the risk of accidents and stress [14].

The stool samples were placed in microtubes (2 ml), and the swab samples were transferred to a saline solution, after which the samples were labelled with the animal identification, species and collection date. After labelling, the samples were stored in a refrigerator at -20 °C [15].

2.2. Processing

All samples were sent to the Laboratory of Bacteriosis and Research of the Federal University of Minas Gerais (UFMG) for research and typing of *C. perfringens* [15]. For the isolation of *C. perfringens*, between 0.08 and 0.12 g of faeces were immersed in 1 ml of brain heart infusion broth (BHI, Difco Laboratories, USA) for enrichment and incubated in an anaerobic chamber (Thermo Scientific, BR) for 24 hours at 37 °C. Cloacal and anal swabs were inoculated directly into tubes with BHI and subjected to the same culture conditions. After incubation, 10 µL aliquots of each sample were plated on Shahadi-Ferguson perfringens agar (SFP, Oxoid, UK) and incubated again in an anaerobic chamber at 37 °C for 48 hours [15].

For each plate, up to three characteristic colonies containing sulfite-reducing microorganisms were suspended in 400 µL of ultrapure water (Milli-Q®) in Eppendorf tubes (2 ml) and subjected to thermal DNA extraction at 98 °C for 20 min in a thermoblock (HDV, BR) [15,16]. Subsequently, the microtubes were centrifuged at 3000 x g for 10 min, and the resulting supernatant was used as template DNA in a *multiplex* PCR to identify the genes encoding the alpha, beta, epsilon and iota toxins and enterotoxin [16]. For all the PCRs, the amplifications were performed in a thermocycler (Thermal Cycler Px2, Thermo Electron Corporation, USA), and the bands were visualized with ultraviolet light on a 2% agarose gel stained with ethidium bromide (Sigma–Aldrich Corporation, USA) [15,16].

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2.3. Statistical Analyses

The data were summarized using frequency tables. To measure the association between the categorical variables (presence of diarrhoea, host, sample type [swabs or faeces]) and isolation of *C. perfringens*, a univariate analysis was performed using Fisher's exact test with a significance level of $P \leq 0.05$. The odds ratios with 95% confidence intervals were also calculated. All analyses were performed using R Software 4.0.9 (R Development Core Team, NZ).

3. Results

Sixty-five stool samples and cloacal or anal swabs were obtained from 16 species and three genera of mammals in good health or with diarrhoea (Table 1). Among the species included in the study, five were the most common: *Nasua nasua* (South American coati), with 14 samples (21.4%); *Bradypus variegatus* (common ground sloth), with eight samples (12.3%); *Didelphis* sp. (opossum), with seven samples (10.8%); and *Choloepus didactylus* (sloth), *Saimiri collinsi* (squirrel monkey), and *Sapajus apella* (Guyana capuchin), each with five samples (7.7%). In the present study, 59 (90.8%) of the samples were from healthy individuals, and six (9.2%) were from individuals with diarrhoea. Regarding the type of sample, there was a balance between stool samples (32/65=49.2%) and anal and cloacal swabs (33/65=50.8%). The number of animals sampled *in situ* (19, 29.2%) was lower than the number of animals sampled *ex situ* (46, 70.8%).

Table 1. Number of samples (%) of the species and genera of mammals collected and toxinotype identified.

Species	Common Name	Samples (%)	F (*)	Y (*)	A (*)	D (*)	*AND* (*)	*I* (*)	T
B. variegatus	Common ground sloth	8 (12.3%)	3 (3)	5 (3)	8 (6)	0	5 (4)	3 (2)	A
C. didactylus	Two-toed sloth	5 (7.7%)	3 (3)	2 (0)	5 (3)	0	4 (3)	1 (0)	A
P. flavus	Jupará	1 (1.5%)	0	1 (1)	1 (1)	0	1 (1)	0	A
S. collinsi	Squirrel monkey	5 (7.7%)	1 (1)	4 (2)	4 (3)	1 (0)	3 (2)	2 (1)	A
S. niger	Black marmoset	1 (1.5%)	1 (1)	0	1 (1)	0	1 (1)	0	A
S. appeal	Guiana capuchin monkey	5 (7.7%)	4 (3)	1 (0)	4 (2)	1 (1)	2 (2)	3 (1)	A
A. caraya	Howler monkey	1 (1.5%)	1 (0)	0	1 (0)	0	1 (0)	0	-
S. ursulus	Marmoset	1 (1.5%)	0	1 (0)	1 (0)	0	1 (0)	0	-
C. thous	Crab-eating fox	2 (3.1%)	1 (1)	1 (0)	2 (1)	0	2 (1)	0	A
P. yagouaroundi	Black-tailed cat	3 (4.6%)	2 (2)	1 (1)	2 (2)	1 (1)	2 (2)	1 (1)	A
L. pardalis	Ocelot	3 (4.6%)	1 (1)	2 (0)	3 (1)	0	2 (1)	1 (0)	A
T. tetradactyla	Lesser anteater	3 (4.6%)	2 (1)	1 (1)	3 (2)	0	3 (2)	0	A
N. nasua	South American coati	14 (21.5%)	6 (4)	8 (4)	12 (7)	2 (1)	8 (5)	6 (3)	A
American M.	Bush deer	1 (1.5%)	1 (0)	0	1 (0)	0	1 (0)	0	-
M. nemorivaga	Brown brocket	1 (1.5%)	1 (0)	0	1(0)	0	1 (0)	0	-

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D. novemcinctus	Nine-banded armadillo	1 (1.5%)	0	1 (0)	1 (0)	0	1 (0)	0	-
Sapajus sp.	Capuchin monkey	1 (1.5%)	0	1 (1)	1 (1)	0	1 (1)	0	A
Saguinus sp.	Marmoset	2 (3.1%)	0	2 (1)	2 (1)	0	2 (1)	0	A
Didelphis sp.	Opossum	7 (10.8%)	5 (5)	2 (1)	6 (5)	1 (1)	5 (5)	2 (1)	A
	Total	65	32 (25)	33 (15)	59 (36)	6 (4)	46 (31)	19 (9)	

Legend: F – stool; **S –** swab; **A –** asymptomatic patient; **D –** diarrhoeal patient; ***E*** – ex situ; **I –** i*n situ*; **(*)** positive samples.

C. perfringens was isolated from 40 (61.5%) samples. All the isolates were positive only for the gene encoding the alpha toxin and were classified as type A (Table 1). Approximately half of the healthy animals (36/59 =55.4%) were positive for *C. perfringens* type A, while four of the six animals with diarrhoea (4/6 =66.7%) were positive for this toxinotype, with no difference between groups (p=1.0). The animals that were positive for *C. perfringens* Type A and that presented diarrhoea were of the genus *Didelphis* sp. (opossum) and of the species *Sapajus apella* (Guyana capuchin), *Puma yagouaroundi* (buckwheat) and *Nasua nasua* (South American coati).

The Isolation of *C. perfringens* was greater in the stool samples than in the swab samples (p = 0.0105): a stool sample was approximately four times more likely to be positive than a swab sample (OR = 4.28; CI: 1.47-12.6). There was no difference in the frequency of isolation between ex *situ* and *in situ* animals (p=0.16), with 31 (47.7%) and nine (13.8%) positive animals, respectively.

Samples from *Alouatta caraya* (black howler monkey, n=1), *Saguinus ursulus* (marmoset, n=1), *Mazama americana* (Mateiro deer, n=1), *Mazama nemorivaga* (brown deer, n=1) and *Dasypus novemcinctus* (nine-banded armadillo, n=1) were negative for *C. perfringens* in the present study.

4. Discussion

Most previous studies that investigated the prevalence of *C. perfringens* in wild animals have focused on a single animal species or on groups belonging to the same family and with similar feeding habits [9,15,17–19]. This appears to be the first study covering this diversity of forest mammals within Brazil, allowing a better understanding of the occurrence of *C. perfringens* in the Amazon biome.

The finding that more than 60% of the samples were positive for *C. perfringens* type A corroborates the findings of previous studies of other species of wild animals, showing that type A is the most prevalent commensal [9,15,17–20]. Some species included in the present study were negative for this anaerobic microorganism. This result may be related to the low sampling rate of some animals, especially for those with only one sample. A previous study also proposed the hypothesis of non-colonization or low colonization of some species [21].

In addition, the present study revealed that swabs had a lower rate of *C. perfringens* isolation than faeces and excreta, which may also have influenced *C. perfringens* detection in some species. Interestingly, a study conducted with toucans in Brazil revealed no difference between the isolation rate of *C. perfringens* from faeces and that from swabs [21]. Furthermore, it is worth noting that most of the stool samples obtained in the present study were collected *ex situ*, representing more than two-thirds of the samples obtained. This pattern could have affected the rate of isolation, since studies have suggested that *C. perfringens* is apparently more likely to be observed in *ex situ* samples than in *in situ* samples [18].

Despite the importance of *C. perfringens* as an enteropathogen in domestic animals and humans, the actual role of type A in diarrhoeal diseases remains uncertain in several wild species due to the absence of a marker that allows for the differentiation of *C. perfringens* when it is present as an enteropathogen or as a commensal [15,17,18]. Nevertheless, there are reports suggesting that type A is the cause of lethal haemorrhagic enteritis in several species, such as *Panthera tigris altaica* (Siberian

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tiger), *Panthera leo* (lion) [10], *Vulpes vulpes* (red fox) [22], *Loxodonta africana* (elephant) [23], *Selenarctos thibetanus* (Asian black bear) [24] and *Papio hamadryas* (hamadryas baboon) [25]. Previous studies have suggested that factors other than the toxinotype may be associated with the occurrence of enteric disease caused by *C. perfringens* type A in animals and humans, such as stress, dysregulation of the intestinal microbiota, and parasitic and viral infections [5,18,22,24–30].

The results of the present study corroborate previous findings suggesting that *C. perfringens* type A is a commensal in *N. nasua* (coati) [15], *C. thous* (crab-eating fox) [17], *P. yagouaroundi* (black-tailed cat), and *L. pardalis* (ocelot) [19]. On the other hand, this seems to be the first report of *C. perfringens* in some species, such as *B. variegatus* (common ground sloth), *C. didactylus* (two-toed sloth), *T. tetradactyla* (anteater), *Didelphis* sp. (opossum), *S. apella* (Guyana capuchin), *S. collinsi* (squirrel monkey), *S. niger* and *P. flavus* (Jupará).

5. Conclusions

The findings of the present study suggest that *C. perfringens* is a commensal bacterium of several species of wild mammals in the Amazon biome. Similar to the vast majority of domestic animals, type A seems to be the most common type among the animals sampled**.**

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