Isolation and genotyping of *Clostridium perfringens and Clostridium difficile* in Capuchin Monkeys (*Sapajus* spp.)

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ABSTRACT: *The importance of Clostridium perfringens and C. difficile for most wild animal species remains unclear. This study aimed to isolate and genotype C. perfringens and C. difficile in stool samples from free-living and captive capuchin monkeys (Sapajus flavius and Sapajus libidinosus) in Brazil. Ten free-living S. flavius and 14 captive S. libidinosus were sampled for this study. To isolate C. difficile, stool samples were inoculated on plates containing cycloserine-cefoxitin fructose agar supplemented with horse blood and sodium taurocholate. Two different protocols for C. perfringens isolation were tested: direct plating onto selective agar and enrichment in brain heart infusion (BHI) broth followed by plating onto selective agar. C. difficile was not detected in the present study. The results were identical for both protocols tested for isolation of C. perfringens. Four samples (16.7%) were positive for C. perfringens type A, including one sample from a free-living animal (4.2%) and three from captive animals (12.5%), meaning there was no significant difference between these two groups. C. perfringens isolates were negative for all additional virulence factors evaluated, including enterotoxin encoding-gene (cpe) and beta-2 encoding-gene (cpb2). These results suggested that C. perfringens type A is found in the microbiota of capuchin monkeys, although it is less frequent than previously reported in domestic animals.*

Key words: *New World Primates (NWP), enterocolitis, Sapajus libidinosus, Sapajus flavius.*

Isolamento e genotipagem de *Clostridium perfringens* and *Clostridium difficile* em macacos-prego (*Sapajus* spp.)

RESUMO: *A importância de Clostridium perfringens e C. difficile para a maioria das espécies silvestres ainda não está clara. O objetivo do presente estudo foi isolar e genotipar C. perfringens e C. difficile em amostras de fezes de macacos-prego (Sapajus flavius e Sapajus libidinosus) de vida livre e criados em cativeiros no Brasil. Dez S. flavius de vida livre e 14 S. libidinosus de cativeiro foram incluídos no presente estudo. Para isolamento de C. difficile, as amostras de fezes foram inoculadas em agar cicloserina-cefoxitina frutose, suplementado com sangue e taurocolato de sódico. Para isolamento de C. perfringens, foram testados dois protocolos: plaqueamento direto em ágar seletivo e enriquecimento em caldo seguido de plaqueamento em ágar seletivo. C difficile não foi detectado no presente estudo. Os resultados foram idênticos para ambos os protocolos testados para isolamento de C. perfringens, resultando em quatro animais (16,7%) positivos para C. perfringens tipo A. Destes, uma amostra era de um animal de vida livre (4,2%) e três de animais de cativeiro (12,5%), não havendo diferença entre esses dois grupos. Os isolados de C. perfringens foram negativos para todos os fatores de virulência adicionais avaliados, incluindo o gene codificador de enterotoxina (cpe) e o gene codificador beta-2 (cpb2). O presente estudo sugere C. perfringens tipo A como parte da microbiota de macacos-prego, embora esse agente seja menos frequente como comensal, do que relatado anteriormente, em animais domésticos.*

Palavras-chave: *New World Primates (NWP), enterocolite, Sapajus libidinosus, Sapajus flavius.*

Non-human primates (NHP) are known as natural reservoirs of several human pathogens and can serve as natural sentinels for investigation of epizootics and endemic diseases of public health importance (FERREIRA et al., 2015; SVOBODA et al., 2016). Among NHP, capuchin monkeys (*Sapajus* sp.) are widely distributed throughout tropical and subtropical South America, including Brazil. Recently, a new species called *Sapajus flavius* was rediscovered in Brazil (BACALHAO et al., 2016), but it is already listed as an endangered species (BRAZIL, 2014). Despite the known importance of NHP as reservoirs of human pathogens, there are few studies focusing specifically on capuchin monkeys (BATISTA et al., 2013; ROCHA et al., 2015).

Clostridium difficile is a major nosocomial pathogen in humans and causes diarrhea and enterocolitis in domestic animals (RODRIGUEZ-PALACIOS et al., 2013). There are few studies about this enteropathogen in wild animals, but confirmed cases of *C. difficile* infection (CDI) have been reported in some species (BOJESEN et al., 2006; SILVA et al., 2013). In addition, recent studies have shown that wild animals can harbor *C. difficile* strains genetically similar to those responsible for CDI in humans (JARDINE et al., 2013; HIMSWORTH et al., 2014; SILVA et al., 2014; ANDRÉS-LASHERAS et al., 2017). Thus, because some studies have hypothesized *C. difficile* to be a zoonotic pathogen (HENSGENS et al., 2012), there is a need to clarify its epidemiology and to study the role of this bacterium in captive and free-living wild animals. So far, there have been no studies evaluating the fecal shedding of *C. difficile* by NHP.

Strains of *C. perfringens* are conventionally classified into five toxigenic types (A-E) based on their capacity to produce one or more of the four major toxins (*alpha*, *beta*, *epsilon* and *iota*) (UZAL et al., 2014). Furthermore, *C. perfringens* can produce additional virulence factors such as enterotoxin, which is responsible for diarrhea in humans (LINDSTRÖM et al., 2011), necrotic enteritis toxin B-like (NetB), which is responsible for necrotic enteritis in broiler chickens (KEYBURN et al., 2008), and NetF, which is associated with acute diarrhea in dogs and foals (GOHARI et al., 2015; DINIZ et al., 2016). Despite its importance as an enteropathogen, *C. perfringens* is commonly reported in the enteric microbiota of healthy animals, thus complicating the laboratory diagnosis of infections caused by this microorganism (SILVA et al., 2015). In wild animals, despite some case reports, the importance of *C. perfringens* remains unclear (SILVA et al., 2015). Specifically, in NHP species, the most common genotypes and the importance of additional virulence factors of *C. perfringens* are largely unknown.

Isolating and screening for virulence factor genes could contribute to the understanding of *C. difficile* and *C. perfringens* transmission patterns, risk factors and epidemiology (SILVA et al., 2014; SILVA et al., 2016). Thus, this study aimed to isolate and genotype *C. difficile* and *C. perfringens* strains from free-living and captive capuchin monkeys (*Sapajus* sp.) in Brazil.

From May 2015 to January 2016, *Sapajus flavius* (ten free-living) and *Sapajus libidinosus* (14 in captivity) were sampled for this study. Free-living animals were trapped with a tomahawk-like trap in a small conservation area called "Mata do Corrego do Inferno" in Pernambuco State. (07°30'47.7"S,

34°58'33.2"W). Stool samples were collected after natural defecation and stored at -20°C until testing. Each individual received a microchip to prevent multiple samples from being collected from the same animal. Captive animals were sampled at a rescue agency for wildlife animals (Centro de Triagem de Animais Silvestres from Pernambuco State - CETAS/PE). Stool samples were collected directly from cages just after defecation, avoiding parts that had contacted the floor, and were stored at -20°C until testing. The present research was authorized by ICMBio/SISBIO (47672-1/2015) and the Ethical Committee from the Universidade Federal de Campina Grande (Protocol number 0048/18032015).

The isolation of *C. difficile* was based on a previously reported protocol (SILVA et al., 2014a). Briefly, stool samples were inoculated on plates containing cycloserine-cefoxitin fructose agar supplemented with 7% horse blood and 0.1% sodium taurocholate (Sigma-Aldrich Co., USA). After anaerobic incubation at 37°C for 72 hours, all colonies were subjected to thermal DNA extraction (BAUMS et al., 2014), and then, multiplex PCR was used to detect a housekeeping gene (*tpi*), toxins A (*tcdA*) and B (*tcdB*) and a binary toxin gene (*cdtB*) as previously described (SILVA et al., 2011).

The isolation of *C. perfringens* was also based on a previously reported protocol (SILVA et al., 2016). Approximately 0.08-0.12g of feces was serially diluted by factors of 10, ranging from 10^{-1} to 10^{-3}. Aliquots of 10μl of each dilution were plated on sulfite polymyxin sulfadiazine agar (SPS, Difco Laboratories, USA) and were anaerobically incubated at 37°C for 24 hours. After incubation, at least three characteristic colonies from each dilution were subjected to thermal DNA extraction (BAUMS et al., 2014), and then, a previously described PCR protocol (VIEIRA et al., 2008) was used to detect genes encoding the major *C. perfringens* toxins (*alpha*, *beta*, *epsilon* and *iota*), *beta*-2 toxin (*cpb2*) and enterotoxin (*cpe*). For detecting the NetB-, NetE-, NetF and NetG-encoding genes (*netB*, *netE*, *netF* and *netG*, respectively), PCR protocols described by KEYBURN et al. (2008) and GOHARI et al. (2015) were applied. Thawed aliquots of stool samples positive for *C. perfringens* cpe^+ strains were subjected to enterotoxin detection with a commercial EIA kit (RIDASCREEN® *Clostridium perfringens* Enterotoxin - R-Biopharm, Germany). Fisher's exact test was used to evaluate associations between variables. Significance was set at a P value of <0.05 (STATA, College Station, Texas, EUA).

Bacterial infections associated with NHPs constitute the more common zoonoses and include

gastrointestinal infections caused by several enteric pathogens (ADAMS et al., 1995). Despite the known importance of *C. perfringens* and *C. difficile* for animals and their possible roles as zoonotic pathogens, there are few studies evaluating these agents in NHP species. In the present study, *C. difficile* was not detected. So far, there is only one reported outbreak of *C. difficile* infection in NHP. In that report, five individuals from a colony of cotton-top tamarins (*Saguinus oedipus*) died spontaneously after antibiotic therapy for infectious diarrhea associated with *Campylobacter* spp. (ROLLAND et al., 1997). *C. difficile* has been reported in few other wild species, and it seems to be more common in synanthropic rodents and in captive animals under antibiotic therapy (SILVA et al., 2013; JARDINE et al., 2013; RODRIGUEZ-PALACIOS et al., 2013; HIMSWORTH et al., 2014; SILVA et al., 2014a; SILVA et al., 2014b). As this was not the case in the present study, the absence of *C. difficile* in the sampled animals was not a surprise.

Four capuchin monkeys (16.7%) were positive for *C. perfringens* type A in the present research. Although, *C. perfringens* is commonly isolated from up to 90% of healthy individuals in domestic species, its prevalence seems to vary widely among wild animals (SILVA et al., 2015; MILTON et al., 2017). In addition, there have been few studies about *C. perfringens* in monkeys and none specifically with *Sapajus* sp., making comparisons difficult. The reported frequencies of *C. perfringens* in these previous studies with NHP vary from 0% in black-eared marmosets (*Callithrix penicillata*) to 80% in captive chimpanzees (FUJITA & KAGEYAMA, 2006; MAFRUZA et al., 2012; MTSHALI et al., 2012). It is also interesting to note that among the four isolates obtained, one sample was from a free-living animal (4.2%), and three were from captive monkeys (12.5%), meaning there was no significant difference between these two groups (p=0.6146). These results are in contrast with a previous study with chimpanzees, which reported that captive individuals were more likely to be positive for *C. perfringens* (FUJITA & KAGEYAMA et al., 2006). These previous reports and the present study suggested differences in the frequency of *C. perfringens* among different NHP species, implying that broad studies with each species, including both free-living and captive individuals, are necessary to clarify the roles of this agent as a commensal in NHP.

All four isolates obtained in the present study were negative for all additional virulence factors tested, including *beta*-2 toxin encoding-gene (*cpb2*), already reported in other wild animals, and enterotoxin-encoding gene (*cpe*), an important virulence factor for *C. perfringens* associated with disease in humans (GORMLEY et al., 2011; SILVA et al., 2015; MILTON et al., 2017). This is the first study to genotype and evaluate these common additional virulence factors in *C. perfringens* isolated from NHPs, so it is impossible to compare these findings to those from other primate species. Regardless, our results suggested that these additional virulence factors are less common in capuchin monkeys than in other wild animals including ruminants, canids, felids and birds (SILVA et al., 2014; SILVA et al., 2015; MILTON et al., 2017).

It is also important to note that the results were identical for both isolation protocols tested. This is similar to results previously reported in dogs (GOLDSTEIN et al., 2012) but different from a study with South American coatis (*Nasua nasua*), which reported that the colonization rate of *C. perfringens* in this species would be underestimated if only direct plating onto selective agar was used (SILVA et al., 2016). Thus, the present study suggested that simple direct plating on selective agar can be applied for further studies with *Sapajus* spp**.** Finally, the present study suggests that *C. perfringens* type A is part of the microbiota of capuchin monkeys, although it is less frequent than previously reported in domestic animals.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest.

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