http://dx.doi.org/10.1590/1678-4162-9895

Arq. Bras. Med. Vet. Zootec., v.70, n.6, p.1709-1713, 2018

Isolation of *Clostridium perfringens* and *C. difficile* in crab-eating fox (*Cerdocyon thous* – Linnaeus 1776) from Northeastern Brazil

[*Isolamento de* Clostridium perfringens *e* C. difficile *em cachorro-do-mato* (Cerdocyon thous – *Linnaeus 1776) da região Nordeste do Brasil*]

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ABSTRACT

The aim of the present study was to isolate *Clostridium perfringens* and *C. difficile* in crab-eating fox (*Cerdocyon thous*) from Northeastern Brazil. Stool samples of 18 captive crab-eating foxes from four states of Northeastern Brazil (Alagoas, Bahia, Paraíba e Pernambuco) 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), beta-2 toxin (*cpb2*), enterotoxin (*cpe*), and NetB- (*netB*) and NetF- (*netF*) encoding genes. *C. difficile* strains were analyzed by multiplex-PCR for a housekeeping gene (*tpi*), toxins A (*tcdA*) and B (*tcdB*) and a binary toxin gene (*cdtB*). Unthawed aliquots of stool samples positive for toxigenic *C. difficile* were subjected to a commercial ELISA to evaluate the presence of A/B toxins. *Clostridium perfringens* (type A) was isolated from five (27%) samples, and only one sample was positive for beta-2 enconding gene (*cpb2*). Two (11%) stool samples were positive for *C. difficile*, but negative for A/B toxins. These two wild canids were also positive for *C. perfringens* type A. This is the first report of *C. difficile* in crab-eating fox.

Keywords: diarrhea, carnivores, wild canids

RESUMO

O objetivo deste estudo foi isolar Clostridium perfringens *e* C. difficile *em cachorro-do-mato* (Cerdocyon thous) *da região Nordeste do Brasil. Amostras de fezes de 18 cachorros-do-mato mantidos em cativeiro e oriundos de quatro estados da região Nordeste do Brasil (Alagoas, Bahia, Paraíba e Pernambuco) foram coletadas e submetidas a isolamento de* C. perfringens *e* C. difficile. *As colônias sugestivas de* C. perfringens *foram analisadas para os genes que codificam as principais toxinas de* C. perfringens (*alfa, beta, épsilon e iota), toxina beta-2* (cpb2), *enterotoxina* (cpe) *e NetB-* (netB) *e NetF-* (netF). *As cepas de* C. difficile *foram analisadas por PCR-multiplex para o gene tpi, toxinas A* (tcdA) *e B* (tcdB) *e um gene de toxina binária* (cdtB). *Alíquotas de amostras de fezes positivas para* C. difficile *toxigênico foram submetidas a um ELISA comercial para avaliar a presença de toxinas A/B*. Clostridium perfringens *(tipo A) foi isolado de cinco (27%) amostras, e apenas uma amostra foi positiva para o gene da toxina beta-2 (cpb2). Duas (11%) amostras de fezes foram positivas para* C. difficile, *mas negativas para toxinas A/B. Estes dois canídeos silvestres também foram positivos para* C. perfringens *tipo A. Este é o primeiro relato de* C. difficile *em cachorro-do-mato.*

Palavras-chave: diarreia, carnívoros, canídeos silvestres

INTRODUCTION

The crab-eating fox (*Cerdocyon thous*) is a member of the Canidae family and is widely distributed in South America countries, including Colombia, Venezuela, Paraguay, Uruguay, Argentina and Brazil. Recent studies have suggested that *C. thous* could act as a reservoir of some pathogens, including some responsible for re-emerging diseases like visceral leishmaniais, brucellosis, canine rangeliosis and rabies

Recebido em 15 de março de 2017
Aceito em 17 de janeiro de 2018
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(Carnieli Jr. *et al*., 2009, Oliveira-Filho *et al*., 2012, Soares *et al*., 2014, Tunon *et al*., 2015). The concern about the transmission of diseases from this wild specie to humans and also to domestic animals is increasing in light of the expansion of urban areas at the expense of areas with native vegetation (Souza *et al*., 2016).

Clostridium perfringens and *C. difficile* are Gram-positive sporogenic anaerobic bacterium and are recognized as pathogens responsible for intestinal disease in human and animals (Silva and Lobato *et al*., 2015, Rodriguez *et al*., 2016). *C. perfringens* is commonly found in the enteric microbiota of healthy animals but also responsible for a several diseases in humans and animals. *C. perfringens* strains are divided into five types (from A to E) according to four major toxins (alpha, beta, epsilon and iota), while some additional toxins have been associated with intestinal infections (Uzal *et al*., 2014). The screening for virulence factors genes could contribute to the knowledge of *C. perfringens* epidemiology in wild animals but, despite few studies, the main genotypes and the most common additional virulence factors of *C. perfringens* strains isolated from Canidae are largely unknown (Silva *et al*., 2016). *C. difficile* produces toxin A and toxin B, known as the main virulence factors of this microorganism. Some strains may also produce a binary toxin (CDT) associated with enhanced virulence (Schwan *et al*., 2009). *C. difficile* infection (CDI) commonly occurs in elderly hospitalized patients submitted to antibiotic therapy, so CDI is recognized as a primarily nosocomial disease (Loo *et al*., 2011). On the other hand, this concept is now being challenged due cases of CDI reported in populations without any previous antibiotic therapy or hospitalization (Hensgens *et al*., 2012). Some studies have also shown a genetic overlap from *C. difficile* strains from humans and animals, suggesting it might be a zoonotic pathogen (Knetsch *et al*., 2014).

In light of this, the purpose of this study was to isolate *C. perfringens* and *C. difficile* in crab-eating fox (*Cerdocyon thous*) from Northeastern Brazil.

MATERIAL AND METHODS

This study was carried out in accordance with the Ethical Principles in Animal Research adopted by the Brazilian College of Animal Experimentation and was approved by the Animal Use Ethics Committee from Universidade Federal Rural de Pernambuco (license number 27/2015) and by the Brazilian Institute for the Environment and Renewable Natural Resources (IBAMA) under number 47677.

Captive crab-eating foxes (*C. thous*) from different states of Northeastern Brazil were sampled by this study as follows: eight animals from Pernambuco, three animals from Alagoas, four from Bahia and ten from Paraíba. Chemical restraint (1% xylazine hydrochloride and 10% cetamine hydrochloride) was required. In Paraíba state was not possible to collect fecal samples individually due to local management conditions; thereby the crab-eating foxes were grouped according to their relationship to each other, resulting in three pools of stool samples. In this specific case, fresh fecal samples were collected manually from the ground. Thus, eighteen fecal specimens (15 individually samples and three pools) were collected in sterile containers and were kept cool at -20^{o}C until further processing. None of the sampled animals had diarrhea or any other clinical signs previously or at the sampling moment.

To perform the isolation of *C. perfringens*, 0.08–0.12g of feces were 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 (Difco Laboratories, Detroit, MI, USA) and were anaerobically incubated at 37°C for 24 hours. After incubation, all sulfate-reducing colonies from each dilution were subjected to a previously described PCR protocol (Vieira *et al*., 2008) for the detection of genes encoding the major *C. perfringens* toxins (alpha, beta, epsilon and iota), beta-2 toxin (*cpb2*) and enterotoxin (*cpe*). The PCR protocol described by Keyburn *et al*. (2008) and Gohari *et al*. (2015) was applied for the detection of the NetB- and NetF-encoding genes (*netB* and *netF*, respectively). The PCR product was subjected to electrophoresis in 2% agarose gel stained with ethidium bromide (Sigma–Aldrich Co., St. Louis, MO, USA). The bands were visualized under ultraviolet light in a photodocumentation system.

The isolation of *C. difficile* spores was carried out as follows: equal volumes of fecal samples and 96% ethanol (v/v) were mixed. After incubation at room temperature for 30 minutes, 50µl aliquots were inoculated on plates contained with 7% horse blood and 0.1% sodium taurocholate (Sigma–Aldrich Co., St. Louis, MO, USA). After anaerobic incubation at 37°C for at least 72 hours, all colonies with suggestive morphology were subjected to a multiplex-PCR for a housekeeping gene (*tpi*), toxins A (*tcdA*) and B (*tcdB*) and a binary toxin gene (*cdtB*). Unthawed aliquots of stool positive samples for *C. difficile* were subjected to a commercial enzyme-linked immunosorbent assay (ELISA) kit to evaluate the presence of A/B toxins (*C. difficile*Tox A/B II - Techlab Inc., Blacksburg, VA, USA).

RESULTS AND DISCUSSION

C. perfringens was isolated from 27% (5/18) samples, all genotyped as type A. Four positive samples were detected in animals from Pernambuco. The other positive sample was a pool of fecal sample of three adults crab-eating foxes from Paraíba. The isolation of *C. perfringens* type A and the absence of other genotypes corroborate previous studies with some Canidae species (Silva *et al*., 2014a). It is also interesting to note that only one sample, obtained from Pernambuco state, was positive for beta-2 enconding gene (*cpb2*), while Silva *et al*. (2014a) reported 34.6% rate in a study with several carnivore species including *C. thous*, *Puma concolor* (cougar), *Leopardus tigrinus* (oncilla), *Leopardus pardalis* (ocelot), *Chrysocyon brachyurus* (maned wolf) and others. Other additional toxin genes, including enterotoxin (*cpe*), NetB (*netB*) and the recently described NetF (*netF*) were not detected in the present study.

All positive crab-eating foxes were asymptomatic at the sampling moment and there were no previous episodes of diarrhea, so the isolated *C. perfringens* by the present study might be part of their microbiota. However severe clinical cases of *C. perfringens* infection in wild carnivores have been described, such as the death of a Siberian tiger (*Panthera tigris altaica*) and a lion (*Panthera leo*) by hemorrhagic enterocolitis caused by *C. perfringens* type A (Zhang *et al*., 2012) and a suspected case of neurotoxicity in a tiger (*P. tigris*) due to *C. perfringens* type B (Zeira *et al*., 2012). Despite the known potential of *C. perfringens* as an enteropathogen for wild Canidae and Felidae, virulence factors involved in enteric disease are still clouded. Recent studies described two novel pore-forming toxins (NetF and NetB) that are clearly associated with necrotic enteritis and hemorrhagic diarrhea in dogs and broiler chicken, respectively (Keyburn *et al*., 2008, Gohari *et al*., 2015). Further studies, including diarrheic animals, are essential to clarify if any of the described toxin genes could be used as a virulence marker for C. *perfringens* isolated from wild carnivore species.

Two (11%) stool samples were positive for *C. difficile* (Figure 1), but negative for A/B toxins by ELISA. These strains, one toxigenic (A+B+CDT-) and one non-toxigenic (A-B-CDT-), were isolated from animals from Pernambuco and Alagoas state, respectively.

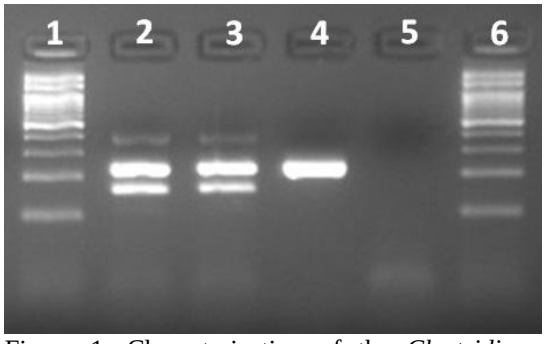

Figure 1**.** Characterization of the *Clostridium difficile* isolates by multiplex PCR. First and sixth lane, 100bp ladder. Second lane, *C. difficile* ATCC 9689 (positive control); third lane, toxigenic (A+B+) *C. difficile* isolate; fourth lane, nontoxigenic (A−B−) *C. difficile* strain; fifth lane, negative control.

These two wild canids were also positive for *C. perfringens* type A. Both animals were apparently healthy during the sampling, but one of them was under antibiotic therapy due to surgery to repair a fractured pelvis. To the best of the author´s knowledge, this is the first report of *C. difficile* in crab-eating fox. A low rate of fecal shedding of *C. difficile* has been described in several wild species, including synanthropic and wild rodents, zebra, procynoids and some wild carnivores (Himsworth *et al*., 2014, Silva *et al*., 2013, Álvarez-Pérez *et al*., 2014, Silva *et al*.,

2014a,b). Similar to the present study, Silva *et al*. (2014a) described the isolation of *C. difficile* from an apparently healthy maned wolf (*Chrysocyon brachyurus*) and from a diarrheic ocelot (*Leopardus pardalis*), both under antibiotic therapy at the time of collection. The influence of antibiotic administration on the incidence of CDI is well-known in humans and also in some domestic animals (Rodriguez *et al*., 2016). *C. difficile* infection (CDI) has been detected also in other non-carnivore species, including elephants and harbor seals (Bojensen *et al*., 2006, Anderson *et al*., 2015). Anyway, both *C. difficile* positive animals in the present study were apparently healthy and, even in the crab-eating foxes positive for a toxigenic strain, the stool samples were negative for A/B toxins, thus both cases should be interpreted as fecal shedding and not to CDI.

CONCLUSION

This study suggests that crab-eating foxes could shed *C. difficile* in their feces. It is important to emphasize that this is the first report of *C. difficile* in crab-eating fox. Concerning to the detection of *C. perfringens*, the microorganism may be considered part of the normal intestinal microbiota of these wild carnivores. Additional studies are needed to clarify the role of *C. perfringens* and its virulence factors in wild carnivore species.

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