

Epizootic amebiasis outbreak in wild black howler monkeys (*Alouatta caraya*) in a wildlife facility during captivity prior to translocation - Recommendations to wildlife management programs

Surto epizootico de amebíase em Bugio preto (*Alouatta caraya*) provenientes de vida livre durante o cativeiro prévio a translocação - Recomendações a programas de manejo de fauna

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Abstract

The capture and quarantine of non-human primates could be necessary in some circumstances including those required under environmental permit. Mortality is undesirable for wildlife management programs and could be related to opportunistic pathogens, for example, deaths due to intestinal protozoa infection outbreaks as described here. Parasitological, necroscopic, microbiological, and molecular tests were used in the diagnosis of severe necrotic enteritis leading to death of three female and two male black howler monkeys (*Alouatta caraya*) captured and held in quarantine prior to translocation. Parasitological tests showed the presence of cysts of *Entamoeba histolytica/dispar* (5/5), *Entamoeba coli* (5/5) and *Giardia duodenalis* (1/5). Necroscopic assessment revealed areas of severe multifocal necrosis in the intestinal mucosa and submucosa. Histopathological examination revealed the presence of structures morphologically compatible to *Entamoeba* spp. in all individuals examined. Furthermore, *G. duodenalis* (1/5) was demonstrated by the nested PCR technique. During temporary captivity of non-human primates in management programs, proper handling protocols, including fast or immediate destination, are suggested in order to mitigate the negative effects of stress and decrease the risk of infections.

Keywords: Neotropical primate. *Entamoeba* spp.. Necrotic enteritis. *Giardia* spp..

Resumo

Óbitos por infecções causadas por protozoários intestinais são indesejáveis para programas de manejo de fauna, exigidos no âmbito do licenciamento ambiental e que demandam a captura e quarentena de primatas não humanos. Exames coproparasitológicos, necroscópicos, microbiológicos e moleculares foram utilizados para a confirmação do diagnóstico de uma severa enterite necrótica que levou a óbito três fêmeas e dois machos de *Alouatta* (*A.*) *caraya* capturados e mantidos em quarentena prévia à translocação. Exames coproparasitológicos revelaram a presença de cistos de *Entamoeba* (*E.*) *histolytica/dispar* (5/5), *Entamoeba* (*E.*) *coli* (5/5) e *Giardia* (*G.*) *duodenalis* (1/5). A avaliação necroscópica revelou áreas de necrose multifocal severa na mucosa e submucosa intestinal. A análise microscópica revelou a presença de estruturas morfológicamente compatíveis com trofozoítos do gênero *Entamoeba* spp. em todos os indivíduos examinados. Além disso, *G. duodenalis* (1/5) foi demonstrada pela técnica de Nested PCR. Sugere-se que quando for necessário o cativeiro temporário de primatas, deverão ser adotados protocolos de manejo adequados buscando a destinação imediata dos animais, de moto a mitigar os efeitos negativos do estresse e reduzir o risco da ocorrência de infecções.

Palavras-chave: Primatas neotropicais. *Entamoeba* spp.. Enterite necrótica. *Giardia* spp..

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Received: 11/12/2015

Approved: 01/06/2016

Introduction

Black howler monkey (*A. caraya*) is a neotropical non-human primate with the broadest geographical distribution in Brazil. It is at risk of extinction in São Paulo due to devastation or fragmentation of its habitat, since there is currently only 1% of the original vegetation of this area (KIERULFF; CARVALHO, 2009). This occurred during the construction of hydroelectric plants. Management and wildlife conservation programs have been developed in order to mitigate these factors in wildlife. These programs conduct the prior monitoring of populations and subsequent capture and quarantine of monkeys, so that they are finally sent to scientific institutions and/or zoos or translocated to other similar areas near those directly affected by the construction.

Intestinal protozoa are a common finding in neotropical monkeys and the most frequent species of parasites detected, being usually opportunistic and non-pathogenic. However, these agents can lead individuals in immune-suppression situations to death (AMATO et al., 2002; CRISTÓBAL-AZKARATE et al., 2010; MONTENEGRO et al., 2010). Immune-suppression occurs during periods of chronic stress, which may be due to factors such as environmental disturbance, inadequate maintenance in captivity, transportation, release, adaptation to a new group, among others (MOBERG, 2000; TEIXEIRA et al., 2007). Influence of such factors in stress in *A. caraya* have been documented during wildlife management programs (SÁNCHEZ-SARMIENTO et al., 2015).

Entamoeba spp. and *Giardia* spp. may be considered the main species of protozoa with zoonotic potential with high clinical significance

(THOMPSON; SMITH, 2011). *Giardia* spp. presents seven different genotypes, among them A and B, which are known to infect humans, non-human primates and a wide variety of mammals (VOLOTÃO et al., 2008). Its life cycle has two stages; cysts are responsible for fecal-oral transmission and trophozoites are responsible for the onset of infection in the duodenum of the host (THOMPSON, 1994). In the life cycle of *Entamoeba*, the cyst (also its transmission state) occasionally germinates in the duodenum and originates trophozoites that will settle in the colon and cecum (SCHUSTER; VISVESVARA, 2004).

The management of populations of non-human primates implies a zoonotic hazard, since there is a risk of transmitting protozoan agents to humans. It must also be considered that monkeys have a greater risk of obtaining bacterial and viral infections from humans. In this context, increased personal protection and a thorough etiological approach are suggested (ULRICH et al., 2010). In addition, research on parasitic infections in animals from wildlife and those kept in captivity provides new tools for disease prevention and optimization of wildlife management plans as proposed in this study.

Material and Methods**Captivity**

A small hydropower plant (SHP), built in Sapucaí-Mirim River, state of São Paulo, Brazil (20°28'S and 47°52'W), has a wildlife screening center (WSC) in order to support rescue and monitoring activities of wildlife directly affected by vegetation suppression and flooding of the area for reservoir construction. Between May and November 2009, 22 *A. caraya* were captured before vegetation suppression. The quarantine period referred to non-human primates was 60 days prior to translocation. Early in this period, animals were evaluated by physical and biometrical tests and biological samples were collected. All howler monkeys were considered healthy in physical tests; with good body condition, without any lesions and

evidence of changes on palpation or auscultation. Prophylactically, animals were dewormed with ivermectin (Ivomec® 200 µg/kg, Merial, Campinas, SP, Brazil). The cages were in the external area of WSC, distant 200 meters from each other. Each cage (2.50 m x 2.50 m x 2.50 m cages) was equipped with a bed, trunks and a platform for food and water, and had no artificial ground cover. Animals were gathered in family groups to facilitate adaptation and further translocation.

Water was provided *ad libitum*, which was supplied by the Sapucaí-Mirim River. In addition, animals were fed with vegetables, greens and fruits twice daily. Food was cleaned with 10% sodium hypochlorite solution for 30 minutes, followed by rinsing in water for ten minutes to minimize contamination.

Case study

Five *A. caraya*, two males (adult and juvenile) and three females (two adults, A and B, and one sub-adult), died between August and October 2009. The period in captivity was 94 days for the young male and 142 for the adult male. For females, this period was 97 and 112 days for adults and 144 days for the sub-adult female.

The clinical history was similar for the five individuals despite some minor differences in the state and length of clinical signs. The animals began to show apathy, appetite loss, severe dehydration, hypothermia, hypoglycemia, apparent weight of loss, abdominal distension with gases on palpation. Feces showed a liquid aspect, brownish, melena and foul odor. Infective forms of protozoa were detected (cysts) on parasitological examinations. Treatment included antiparasitic, Nitazoxanide (Annita® 0.375 mL/kg, FQM, Rio de Janeiro, RJ, Brazil); antibiotic, Ceftiofur (Excenel® RTU 0.6 mL, IV, Zoetis, São Paulo, SP, Brazil); intravenous fluid, Ringer solution with sodium lactate (15 mg/kg, SC, Baxter Hospital, São Paulo, SP, Brazil); hyperglycemic medication, monohydrate glucose 5 g (glycol-saline solution, 15

mg/kg, IV, Baxter Hospital, São Paulo, SP, Brazil); Vitamin B12 (Catosal®, 1.0m L, IM, Bayer, São Paulo, SP, Brazil) and warming.

Sick individuals were also isolated. However, the animals' response was not satisfactory, evolving into severe prostration, anorexia and evident weakness. After an average of 118 days, the five animals died.

After death, the other individuals of their group were allocated to research centers and zoos, which allowed reallocating the population. Thus, new cages were assembled and following was performed in cages for a period of at least seven days. This was carried out through disinfection (detergent and water) and sterilization (fire broom) of bars for the arrival of new individuals. Each cage received its own set of drinking and feeding bowls.

Diagnostic procedures

While animals exhibited clinical signs, parasitological tests were performed using the pool of cages. Direct parasitological tests by flotation methods were performed at the Laboratory of Parasitic Diseases of the School of Veterinary Medicine and Animal Science - University of São Paulo (FMVZ/USP), Brazil, with individual samples and group (cages). The techniques used were centrifugal-flotation in supersaturated sucrose solution ($d = 1.205 \text{ g/cm}^3$) and the Willis technique, and sedimentation in water-ether solution (1:1) (FAYER; XIAO, 2008; SANTOS, 2014). Additionally, fecal samples positive for *Giardia* spp. underwent polymerase chain reaction (PCR) with the nested PCR technique for the glutamate dehydrogenase gene (GDH) at the Laboratory of Molecular Biology and Serology, FMVZ/USP (XIAO; FAYER, 2008). In addition, necropsy, histopathology and microbiology were performed in the five dead individuals in order to determine cause of death. During necropsy, fragments of all organs were collected, fixed in 10% buffered formalin (pH 7.4), and processed through the inclusion technique in paraffin wax, sectioned at 5 µm and stained with hematoxylin & eosin (H&E) for histopathological evaluation at the

Laboratory of Wildlife Comparative Pathology (LAPCOM), FMVZ/USP.

Bacteriological-culture and mycological-culture of liver, lung, spleen, small and large intestines and blood of dead animals were performed at the Laboratory of Infectious Diseases, FMVZ/USP.

Results

Coprological: Feces from the cages showed the presence of *Giardia* spp., *Entamoeba* spp. and *Chilomastix* spp. protozoan cysts. Among the dead animals, the presence of *E. histolytica/dispar* and *E. coli*, solely on the sub-adult female, was confirmed. Enzymatic profile or antigenic or genetic tests were not made in order to determine differences between *E. histolytica (dysenteriae)* (pathological specie) and *E. dispar (not pathological species)*, thereby the term *E. histolytica/dispar* was adopted.

Necropsy: In the five animals, the necropsy findings were similar, especially in the intestinal tract, where severe coalescing necrotic enteritis was diagnosed with greater severity in colon and cecum (Figure 1).



Figure 1 – Photography of cecum (juvenile male). Note severe necrosis and with mucosa detachment

Histopathology: In all dead animals,

histopathological exams revealed the presence of structures morphologically similar to trophozoites of *E. histolytica/dispar*, alone or in small multifocal distribution groups in the mucosa, submucosa and lamina propria (Figure 2). In addition, secondary lesions were reported in lungs, liver and kidneys, associated with the chronicity of the infection.

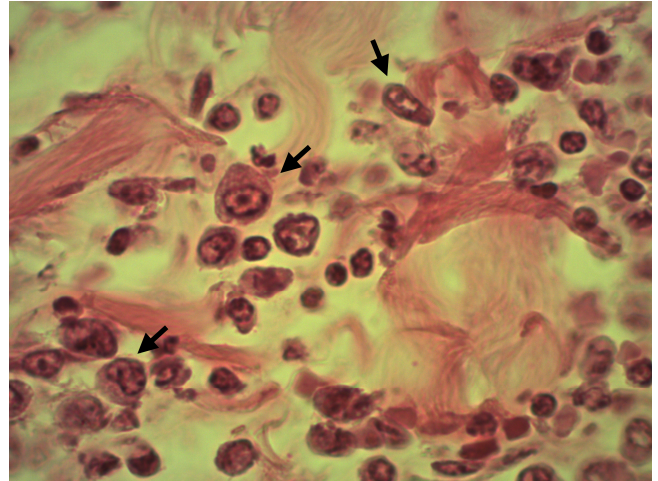


Figure 2 – Photomicrograph of large intestine (adult female). Observe the necrosis and mixed inflammatory cell exudation in lamina propria associated with structures morphologically similar to trophozoites of *Entamoeba histolytica/dispar* (arrows) (H&E 100X)

Microbiology: The results of blood culture reported the growth of *Escherichia coli* was present in the adult females. The culture of samples of small and large intestines showed the presence of *Escherichia coli*, *Enterococcus faecalis* and *Bacillus* spp. in adult female A. Cultures of liver, lung and spleen fragments showed no bacterial growth in any of the individuals. Those results were compatible with histopathological evaluation; no bacterial cluster was detected in tissues collected for microbiology.

Molecular: In coproparasitological examination, and subsequently, in the molecular diagnostics by nested PCR technique, only the sub-adult female was positive for *G. duodenalis*. In Table 1, the relevant results of each diagnostic technique can be observed in detail.

Table 1 – Diagnostic method and relevant results related to parasitosis that resulted in death of *Alouatta caraya* – São Paulo – 2009

DIAGNOSTIC METHOD	RESULTS
Parasitological:	<ul style="list-style-type: none"> • Pools of feces from the cage tested by centrifugal flotation and direct stool examination: Cysts from <i>Giardia</i> spp. and <i>Entamoeba</i> spp. • Pools of feces from the second enclosure (Included dead animals): <ul style="list-style-type: none"> ○ Amoebic cysts (rare); ○ Low quantity of <i>Chilomastix</i> spp. (+), ○ Moderate quantity of <i>Giardia</i> spp. (++) cysts. • For dead animals, only sub-adult female presented: <ul style="list-style-type: none"> ○ <i>E. histolytica/dispar</i> (rare) ○ Moderate quantity of <i>E. coli</i> (++)
Necropsy:	<ul style="list-style-type: none"> • Small intestine: Greenish-brown or goeey white liquid. Mucosa: thickened and swollen with multifocal loss. • Large intestine: Whitish and friable plates (75% of whole mucosa). The remaining 25% of mucosa was thickened, swollen and congested. High quantity of greenish to brownish and goeey liquid with putrid odor. Cecum and colon with erosive appearance and widespread detachment of mucosa (Tiflocolitis). • Mesenteric lymph nodes: lymphadenomegaly. Black color, outside and inside (when cut).
Histopathology:	<ul style="list-style-type: none"> • Severe multifocal necrotic enteritis • Lamina propria: Structures morphologically similar to <i>E. histolytica/dispar</i>, isolated, forming cavities in the tissue or in small multifocal distribution groups. • Sub-mucosa: Multifocal and moderate lymphoplasmacytic cell infiltration. • Amoeboid parasitic forms: Different stages and sizes, spherical, irregular. Trophozoite, spongy and vacuous cytoplasm and a single nucleus, approximate size of 15-20 µm. These structures were also observed in blood vessels • Lymph nodes: Various moderate multifocal areas of lymphoid depletion. • Adrenal glands: Adrenalitis with thrombosis, suggesting disseminated intra vascular coagulopathy. • Lungs: Chronic interstitial pneumonia. • Liver: Chronic hepatitis with confluent necrosis. • Kidneys: Chronic interstitial glomerulonephritis.
Microbiology:	<ul style="list-style-type: none"> • Blood Culture: <i>Escherichia coli</i> in both adult females. • Small and large intestines: <i>Escherichia coli</i>, <i>Enterococcus faecalis</i> and <i>Bacillus</i> spp. in adult female A.
Molecular:	<ul style="list-style-type: none"> • Sub-adult female positive to <i>Giardia duodenalis</i> in parasitological tests: positive in molecular diagnosis (Nested PCR).

Discussion

Outbreak's Modular Cofactors

Stress is an adaptive response to a stressful event. However, during chronic stress, there is a permanent activation of the hypothalamic-pituitary-adrenal and sympathetic adreno medullary systems, resulting in constant physiological dysregulation, brain changes, accelerated aging, illness or death (MCEWEN, 2007). Teixeira et al. (2007) considered that in translocation and reintroduction programs, the conditions: environmental disturbance, captivity, transport, release, adaptation to the new environment and monitoring are sources of chronic stress. Furthermore, in the present case, the quarantine period was longer than 60 days due to waiting for permission for translocation from the environmental agencies. The group evaluated in this study experienced all these factors, and we believe they could promote immunosuppression in all of the five individuals, a decisive event before the high parasite load and subsequent clinical manifestation of the disease and death.

The group of non-human primates experienced loss and fragmentation of their habitat during these events; its members were captured and kept in captivity. At this stage, they were associated with other conditions, such as environmental contamination by urban sewage and management.

Balantidiasis (*Balantidium coli*), giardiasis, and amoebic dysentery, caused by *E. histolytica* are among zoonotic waterborne infections. Both are transmitted by fecal contamination of water or food with cysts, which are discharged in feces. These diseases emerge from the lack of rigorous quality control of water and effluent (SCHUSTER; VISVESVARA, 2004) and inadequate infrastructure receiving fecal waste from humans (GRACZYK et al., 2002). It is known that urban effluents are not often treated before being discharged directly into rivers, as occurred in the city of São Joaquim before the dam was built and quarantine was located. Consequently, sanitary control was difficult, favoring continuous reinfections.

The management of a captive population can be a factor that promotes the maintenance of parasites in the group and transmission among individuals, so the development of a primate management program should consider age, reproductive status, diet, temperature, humidity of cages, and animal population density (MONTENEGRO et al., 2010).

In an attempt to keep social ties, WSC cages had a high population density, with different age levels and reproductive status, which can cause stress due to behavioral changes, such as an increase in aggressiveness, which has been described in marmosets (CAPEROS et al., 2011). It is suggested in other primates (*Cebus capucinos*) as a way to reduce this aggressive behavior to decrease contact with individuals in areas with high population densities (VAN WOLKENTEN et al., 2006).

Data on the natural history of species should be considered while preparing cages. In nature, *Alouatta* primates have very broad areas of use, either by individual or by group (GÓMEZ-POSADA et al., 2007; GAVAZZI et al., 2008; DUQUE; GÓMEZ-POSADA, 2009), but cages failed to meet all the specific requirements for the species, and it may have increased the likelihood of contact of animal feces with feeders or drinkers, which possibly contributed to reinfection. However, as individuals were relocated due to the outbreak, and after allocation to zoos, this population factor may not have the same relevance any longer. Thus, immediate allocation is recommended so that the population load in WSC is not high and appropriate sanitation management is facilitated.

Diagnosis

Different authors have reported a very diverse prevalence of intestinal parasites in non-human primates regarding different species of helminthes and protozoa (ECKERT et al., 2006; VITAZKOVA; WADE, 2006; BRANDÃO et al., 2009). However, their presence is not associated with death in the wild. In this study, the presence of *E. histolytica/dispar* was

associated with mortality in wild *A. caraya*, which were temporarily kept in cages.

The histopathological findings, together with clinical history and the results of fecal examinations, revealed that deaths were due to severe chronic necrotizing enterocolitis caused by protozoa, especially *Entamoeba* spp. In this study, clinical signs and histopathological lesions were consistent with those presented by *Rhesus* monkeys with chronic enterocolitis caused by protozoa (SESTAK et al., 2003) and *A. caraya* diagnosed with entamoebiasis caused by *E. histolytica* (MONTENEGRO et al., 2010). In these studies, the presence of severe hematochezia, loss of appetite, apathy, pale mucous membranes, chronic diarrhea and affection are described, especially in colon, cecum and terminal ileum. Mononuclear infiltrates were also found in mucosa, submucosa, lamina propria, blood vessels, as well as presence of aggregates and isolated forms of these amoeboid stages in these regions.

Micro-abscesses in liver, brain and lungs are also described. After penetrating the intestine, *E. histolytica* may be carried by the bloodstream to other organs, causing micro-abscesses (SCHUSTER; VISVESVARA, 2004; MONTENEGRO et al., 2010), although such lesions were not observed in these organs on histopathological evaluation.

In contrast, other studies have corroborated the great diversity of species of protozoa affecting primates in captivity (GÓMEZ et al., 1996; LEVECKE et al., 2007; MARTÍNEZ-DÍAZ et al., 2011). In Brazil, in breeding *Cebusapella*, *Macaca mulatta*, *Callithrix jacchus* and *Callithrix penicillata* in captivity, the presence of *Cryptosporidium*, *Giardia*, *Cystoisospora* and *Balantidium* has been reported (SILVA et al., 2008). Fecal examinations in *A. caraya* evaluated in this study also revealed the presence of intestinal flagellate protozoa *Chilomastix* and *Giardia*. These and others, such as *Retortomonas* and *Trichomonas*, can be parasitic or commensal microorganisms in humans, and may also affect *Alouatta* spp. (STUART et al., 1998).

In Belize, asymptomatic *Alouatta pigra* individuals were identified with *G. duodenalis*, with the genotypes A and B transmissible from man to non-human primates (VITAZKOVA; WADE, 2006). In Brazil, *G. duodenalis*, genotype A, has been reported in asymptomatic *Alouatta clamitans* and *A. caraya* individuals kept in captivity (VOLOTÃO et al., 2008; SANTOS et al., 2011).

The other protozoa detected in this study, for example, *Chilomastix* spp., was considered as commensal or opportunistic protozoa (SESTAK et al., 2003), and its presence was not necessarily involved with the clinical condition observed in the animals.

Prophylaxis

Despite conventional prophylactic treatments, several factors are associated with the difficulty of controlling protozoa in non-human primates kept in captivity, among them, the direct cycle of the parasite, the situations favored by captivity, life habits, institution infrastructure and challenges posed by human resources (GÓMEZ et al., 1996). All these factors may be involved in disease outbreaks because even though they had an adequate and supportive treatment (KINDLOVITS; KINDLOVITS, 2009; KRAMER et al., 2009), this procedure was not enough to avoid the death of the five animals. However, the wildlife management program adopted effective prophylactic measures, which rapidly decreased the number of sick animals and prevented new deaths.

Interspecies transmission implications

People susceptible to infection by *E. histolytica* and *E. coli* are those who work with monkeys, slaughter animals for food or are pet monkey owners (SCHUSTER; VISVESVARA, 2004). The presence of protozoa could be related to interspecies transmission between WSC staff and howler monkeys, which was reduced with appropriate management practices. Furthermore, it is possible that the displacement of primate populations increased the contact with

human populations, favoring protozoa and other microorganism transmission.

This study showed that *Entamoeba* spp., *Giardia* spp. and *Chilomastix* spp. are intestinal protozoa found in non-human primates captured from wildlife, which may lead to death due to stressful conditions inherent to captivity. Multiple diagnostic techniques can be used to detect protozoa, including histopathological, molecular and serological ones (LANGHOUT et al., 2010). On the other hand, as performed in this study, the implementation of basic laboratory techniques (parasitology) in WSC enables the epidemiological surveillance of captive populations, establishment of an accurate and prompt diagnosis, the provision of effective preventive measures and settlement of adequate treatments.

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