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Captive-bred neotropical birds diagnosed with *Cryptosporidium* Avian genotype III

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ABSTRACT

Currently, there are only three valid species of *Cryptosporidium* infecting avian hosts, namely, *Cryptosporidium meleagridis*, *Cryptosporidium baileyi*, *Cryptosporidium galli* and *Cryptosporidium avium* in addition to 12 genotypes of unknown species status. The objectives of this study were to microscopically diagnose the presence of *Cryptosporidium* in birds from a commercial aviary located in Rio de Janeiro, Brazil; genotypically characterize species and/or genotypes of genus *Cryptosporidum*; and conduct sequencing and phylogenetic analyses to compare the obtained DNA sequences with those deposited in GenBank. A total of 85 fecal samples were collected from wild captive-bred birds: 48 of family Psittacidae and 37 of family Ramphastidae. Initially, a search for the presence of *Cryptosporidium* sp. oocysts was conducted using the centrifugal-flotation in saturated sugar solution technique, after that, the collected samples were analyzed microscopically. *Cryptosporidium* infections were only detected in 24.32% of samples belonging to the family Ramphastidae. DNA was extracted from positive samples and molecular diagnostics was applied targeting the 18S rRNA gene, followed by sequencing and phylogenetic analysis. The *Cryptosporidium* Avian genotype III was diagnosed in this study more closely related to the gastric species. This is the first record of *Cryptosporidium* Avian genotype III in order Piciformes and family Ramphastidae, where three host species (*Ramphastus toco*, *Ramphastus tucanus*, and *Pteroglossus bailloni*) were positive for the etiologic agent. Based on the molecular data obtained, these wild birds raised in captivity do not represent a source of human cryptosporidiosis, considering that *Cryptosporidium* Avian genotype III does not constitute a zoonosis.

1. Introduction

Many species of the class of birds are being bred and raised as pets in cages, nurseries, and even at large. Birds of family Ramphastidae, order Piciformes - commonly called toucans and toucanets - are Neotropical species that live in the forests of Central and South America, whereas birds of family Psittacidae, order Psittaciformes present wide geographic distribution, occupying warm and temperate regions in all continents. Birds of these two families are highly prized as pets, in addition to being easily marketed when bred in captivity.

Currently, there are only four valid species of *Cryptosporidium* infecting avian hosts, namely, *Cryptosporidium meleagridis*, *Cryptosporidium baileyi*, *Cryptosporidium galli* and *Cryptosporidium avium* in addition to 12 genotypes of unknown species status (Nakamura and Meireles, 2015; Holubová et al., 2016).

These species and genotypes of *Cryptosporidum* have been reported infecting more than 30 avian species worldwide distributed in the following orders: Anseriformes, Bucerotiformes, Cathartiformes, Charadriiformes, Cohimbiformes, Columbiformes, Falconiformes, Galliformes, Gruiformes, Paseriformes, Piciformes, Phoenicopteriformes, Psittaformes, Strigiformes, and Struthioniformes (Zahedi et al., 2016). Cryptosporidiosis occurs in three main forms in birds: respiratory, intestinal, and renal, with clinical or subclinical manifestations (Santín, 2013).

There is little knowledge on the distribution of species and genotypes of *Cryptosporidium* in wild animal populations, especially in the class of birds (Appelbee et al., 2005; Ziegler et al., 2007; Ryan et al., 2014).

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However, several studies have shown that pet birds play an important role in the epidemiology of cryptosporidiosis, particularly with respect to proximity to the human host and increased susceptibility to stress owing to captivity. Therefore, it is necessary to know the adaptation between parasites and hosts so that possible implications on public health can be understood (NG et al., 2006; Nakamura et al., 2009; Silva et al., 2010; QI et al., 2011).

Some authors have discussed the importance of waterfowl as disseminators of *Cryptosporidium* oocysts. Cano et al., 2016 analyzed feces of waterbirds in the north of Spain in search of *Giardia* and *Cryptosporidium* oocysts. The authors diagnosed six isolates of *Cryptosporidium* which were characterized as *Cryptosporidium* Avian genotype III (n = 4), duck genotype b (n = 1), and goose genotype Id (n = 1), these findings were considered specific of birds, therefore not presenting risk for human infection. Gorham and Lee, 2016 conducted a literature review in which they emphasized issues regarding bacteria, viruses, and protozoa identified in fecal samples of Canadian geese, and related them to risks for human health through exposure to contaminated recreational waters. Those authors mentioned some classic studies on the prevalence of *Cryptosporidium* in feces of geese (Graczyk et al., 1998; Zhou et al., 2004). However, because prevalence was low in these hosts, it led them to believe that Canadian geese are simply passive transporters of *Cryptosporidium* and play a minor role in zoonotic transmission (Zhou et al., 2004; Graczyk et al., 2007). Nevertheless, the importance of geese in the propagation of human pathogenic *Cryptosporidium* species is unclear to date (Gorham and lee, 2016).

In a previous study also conducted with exotic birds commercialized in pet shops in the state of Rio de Janeiro, Brazil, Gomes et al. (2012) identified the presence of *Cryptosporidium parvum* in passerines from China (*Lonchura striata domestica*) and of Avian genotype III in java sparrow (*Padda oryzivora*) and cockatiel (*Nymphicus hollandicus*).

Nevertheless, despite the records described in the avian medicine literature, there has been little documentation on the epidemiology of parasites in ornamental and pet birds, mainly regarding cryptosporidiosis. Therefore, the purpose of this study was to investigate the occurrence of *Cryptosporidium* in birds of the families Ramphastidae and Psittacidae bred in captivity for marketing.

2. Material and methods

2.1. Site and procedures for collection of fecal samples

Fecal samples were collected at a commercial aviary legalized at the Brazilian Institute of the Environment and Renewable Natural Resources (IBAMA) located in the district of Guaratiba, west zone of the municipality of Rio de Janeiro, Rio de Janeiro state, Brazil. A search for *Cryptosporidium* sp. was conducted in two families of birds: Ramphastidae and Psittacidae. This aviary has a license for breeding and marketing birds intended for pets, exportation, and other aviaries. A total of 85 fecal samples from captive-bred birds were collected from March 2015 to February 2016.

In the aviary, the birds were housed in screened cages to avoid contact, promoting comfort and preventing fights. The birds were fed their ration adequate for each species and a daily portion of fruits and vegetables. Potable water from the local distributor was provided in appropriate pots replaced twice a day. Couples were usually placed in each cage for mating in captivity. The facilities presented good-quality sanitary conditions, with periodic cleaning and veterinary assistance. Free-living birds attracted by food, mainly pigeons and turtledoves (*Patagioenas picazuro*, *Columbina*), were often observed in these places.

A total of 37 fecal samples from family Ramphastidae were evaluated: 28 samples from the species *Ramphastus toco* (Toucan toco), four from the species *Ramphastus tucanus* (White-throated toucan), two from the species *Ramphastus dicolorus* (Green-billed toucan), two from *Pteroglossus bailloni* (Saffron toucanet), and one from *Selenidera maculirostris* (Spot-billed Toucanet).

A total of 48 fecal samples from family Psittacidae were analyzed: 14 samples from the species *Psittacus eritachus* (African grey parrot), four from the species *Ara ararauna* (Blew-and-yellow macaw), one from the species *Ara macao* (Scarlet macaw), 19 from *Amazona aestiva* (True parrot), eight from *Eclectus roratus* (Eclectus parrot), and two from *Pionites leucogaster* (Green-thighed parrot).

Fecal samples from the two families investigated in this study were collected individually; even when there was a bird couple inside the premises, separation was possible because there were removable partitions that could be used to separate the animals without harming them.

2.2. Fecal sample processing and microscopic diagnostics

In the laboratory, the fecal samples were cataloged, processed, and diagnosed by optical microscopy (OM) using the centrifugal-flotation in saturated sugar solution technique by (Sheather, 1923; Huber et al., 2007) with some modifications as described ahead. The amount of feces processed per sample varied according to the size of the birds, ranging from 5 to 10 g.

The fecal samples were identified, homogenized with distilled water, and filtered using a disposable plastic sieve lined with cheesecloth for removal of coarse residues. After filtering, the solution was distributed into two 15 mL conical glass tubes and centrifuged for 10 min at 402.4 g. Next, the supernatant was discarded and only the sediment remained. One tube from each sample was randomly selected and homogenized again with saturated sugar solution (specific density of 1.30 g/mL), whereas the other tube was stored under refrigeration at a temperature of approximately 4 °C. After homogenization of the tube containing the sediment with saturated sugar solution, the material was once again centrifuged for 10 min at 402.4 g.

Subsequently, in the second centrifugation, the tubes of each sample were filled with saturated sugar solution to form a meniscus on the surface. The tubes were covered with a glass coverslip for five minutes; the coverslips were then removed from the surface of the tube and placed on a sterile glass slide to perform the parasitological microscopic diagnosis using 400 x magnification. Samples were considered positive for *Cryptosporidium* spp. if oocysts were identified by light field and phase contrast optical microscopy.

For the samples diagnosed as positive for *Cryptosporidium* spp. oocysts, the 15 mL tube corresponding to the same sample that had been preserved under refrigeration was later used for DNA extraction and subsequent analyses.

2.3. DNA extraction

The commercial QIAamp DNA Stool Mini Kit (Qiagen) was used in this phase, according to the manufacturer's recommendations, with minor modifications with respect to the two incubation periods of the material when samples were subjected to a temperature of 95 °C, with a longer incubation of 10 min, using a temperature controlled stirrer at 800 rpm. At the end of extraction, the samples were eluted in 100 uL of buffer AE (supplied by the manufacturer) (Fayer, 2010).

2.4. Primary and nested-PCR targeting subunit 18S gene

Polymerase chain reaction (PCR) was conducted in two phases, corresponding to the 18S ribosomal RNA gene of the parasite. The following primers were used in the primary PCR: 18SF: 5'- TTC TAG AGC TAA TAC ATG CG-3' (forward) and 18SR: 5'- CCC ATT TCC TTC GAA ACA GGA-3' (reverse), with expected amplicon sizes of approximately 1325 pb (Xiao et al., 1999; Fayer, 2010).

The following primers were used in the nested-PCR: 18SNF: 5'- GGA AGG GTT GTA TTT ATT AGA TAA AG-3' (forward) and 18SNR: 5'- AAG GAG TAA GGA ACA ACC TCC A-3' (reverse), with expected amplicon size ranging from 826 to 864 pb depending on the species of