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Short Communication

Sarcocystidae in wild birds of southeastern Brazil

Sarcocystidae em aves silvestres do sudeste do Brasil

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Abstract

This study aimed to identify members of the Sarcocystidae family in naturally infected wild birds at a rescue center in the state of Minas Gerais, southeastern Brazil. The heart and brain of 44 wild birds were evaluated by bioassay in mice to detect *T. gondii*, and extracted DNA was used for nested PCR of the 18S ribosomal DNA gene to detect members of the Sarcocystidae family. The positive samples were sequenced, assembled, edited and compared with sequences deposited in GenBank. *Toxoplasma gondii* was isolated from six (13.6%) out of 44 birds. *Toxoplasma gondii* DNA was identified in 10/44 (22.7%) of the birds. The amplified sequences exhibited 100% similarity with the DNA of the ME49 strain of *T. gondii*. *Sarcocystis* DNA (99% similarity) was identified in 5/44 (11.4%) of the birds. *T. gondii* and *Sarcocystis* spp. are common in wild birds in Minas Gerais, Brazil.

Keywords: *Toxoplasma gondii*, *Sarcocystis* spp., nested PCR, wild birds, Brazil.

Resumo

O objetivo deste estudo foi identificar membros da família Sarcocystidae em aves silvestres de vida livre naturalmente infectadas e resgatadas no estado de Minas Gerais, Brasil. Coração e cérebro de 44 aves silvestres foram avaliados por bioensaio em camundongos para detecção de *T. gondii* e extração de DNA para Nested-PCR do gene 18S do DNA ribossomal de membros da família Sarcocystidae. As amostras positivas foram sequenciadas, analisadas, editadas e comparadas com sequências depositadas no GenBank. *Toxoplasma gondii* foi isolado de seis (13,6%) das 44 aves. DNA de *T. gondii* foi identificado em 10/44 (22,7%) das 44 aves. As sequências amplificadas exibiram 100% de similaridade com o DNA da cepa ME49 de *T. gondii*. DNA de *Sarcocystis* (99% de similaridade) foi identificado em 5/44 (11,4%) das 44 aves. *T. gondii* e *Sarcocystis* spp. são encontrados, comumente, em aves silvestres no estado de Minas Gerais, Brasil.

Palavras-chave: *Toxoplasma gondii*, *Sarcocystis* spp., nested PCR, aves silvestres, Brasil.

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Introduction

Parasites of the family Sarcocystidae (Apicomplexa) are associated with a variety of diseases in humans and other animals (Yang et al., 2001). The member genera of the family Sarcocystidae infecting birds are *Sarcocystis*, *Cystoisospora*, *Toxoplasma*, and *Frenkelia* (Ogedengbe et al., 2016). In addition to these genera, *Atoxoplasma* spp., causing a disease known as systemic isosporosis, is a coccidian that can infect several species of birds (Mohr et al., 2017).

The genus *Sarcocystis* comprises approximately 200 species, which vary in their life cycles, pathogenicity and capacities to infect humans, other mammals, birds, reptiles and fish (Odening, 1998). Wild birds can act as definitive and/or intermediate hosts of several species of *Sarcocystis* (Kutkiene & Sruoga, 2004). Among the species affecting birds are *Sarcocystis horvathi*, *S. wenzeli*, *S. rileyi*, *S. lindsayi*, and *S. falcatula* (Olias et al., 2010). The main factors that hinder the identification and characterization of *Sarcocystis* species are molecular and morphological similarity (Yang et al., 2001).

Toxoplasma gondii has been identified in wild birds by both bioassay and PCR (Lukášová et al., 2018; Karakavuk et al., 2018). Raptorial birds can become infected by feeding on other intermediate hosts, such as small rodents (Karakavuk et al., 2018), completing the life cycle of *T. gondii*. Non-raptorial birds can become infected by ingesting oocysts via contaminated water or soil (Lindsay et al., 2003).

This study aimed to identify members of the Sarcocystidae family in the heart and brain of naturally infected wild birds rescued in the state of Minas Gerais, southeastern Brazil.

Material and Methods

The study protocol was approved by the Ethics Committee in Animal Experimentation (CETEA) of the Universidade Federal de Minas Gerais, Brazil (Protocol CEUA 67/2016). Procedures for the collection, transport, and manipulation of free-living wild birds were authorized by the Biodiversity Authorization and Information System of the Federal Government, Brazil (SISBIO no. 52653-1).

The hearts and brains of 44 wild birds rescued in the state of Minas Gerais that had been collected on the day of death between August 2016 and June 2017 were used in a bioassay to detect *T. gondii* and subjected to DNA extraction. The organs were collected from the rescued birds after natural death at the *Centro de Triagem de Animais Silvestres* (CETAS; Belo Horizonte, Minas Gerais state), which is associated with the *Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis* (IBAMA). Birds evaluated in this study were: *Amazona aestiva** (1 individual), *Ardea alba* (1), *Aramides saracura* (1), *Asio clamator* (4), *Asio stygius* (1), *Athene cunicularia* (3), *Caracara plancus* (7), *Cariana cristata* (1), *Colaptes campestris** (1), *Coragyps atratus* (2), *Falco sparverius* (2), *Geranoaetus albicaudatus* (1), *Glaucidium brasilianum* (2), *Megascops choliba* (3), *Milvago chimachima* (2), *Patagioenas speciosa** (1), *Pionus maximiliani** (1), *Ramphastos toco* (3), *Rupornis magnirostris* (7). Of the birds, 40 were raptorial, and four (marked by an asterisk) were non-raptorial species.

Half of the heart and half of the brain from each of the 44 birds were cut into small pieces and digested according to Dubey et al. (2004). Digested tissue was inoculated into four Swiss female mice obtained from a *Toxoplasma gondii* free colony at the Federal University of Minas Gerais (CEBIO, UFMG). Two mice were inoculated with digested heart tissue, and the other two were inoculated with digested brain tissue by the subcutaneous via. Mice were provided water and food ad libitum and maintained under a 12 h light/12 h dark light cycle. A technician with specialized training monitored animal health and behavior twice a day for 45 days post infection (d.p.i.). All efforts were made to minimize animal suffering during the course of this study. Moribund and surviving mice (after 45 d.p.i.) were euthanized by an intraperitoneal (i.p.) overdose of ketamine (300 mg/kg) and xylazine (45 mg/kg). Mice were examined for the presence of tachyzoites in the lung and brain cysts. At 45 d.p.i., each surviving mouse was bled by lancing the tail, and blood was collected for an in-house ELISA according to Brandão et al. (2009). Only the brains of seropositive mice were removed for analysis of brain cysts. A bioassay result was considered positive when tachyzoites and/or cysts were detected in the inoculated mouse.

The remaining halves of the hearts and brains from the 44 birds were macerated individually and DNA was extracted using the Wizard Genomic Purification Kit (Promega, Cat. A1120) with modifications proposed by Cunha et al. (2016). When half of the organ weighed more than 20mg (maximum weight recommended by the manufacturer in each extraction), two extractions were performed simultaneously. In these cases, the DNA resulting were homogenized in a single sample. The 18S gene of ribosomal DNA of members of the Sarcocystidae family was

amplified by nested PCR (Vitaliano et al., 2014) using external primers Tg18s48F (5'CCATGCATGTCTAAGTATAAGC3') and Tg18s359R (5'GTTACCCGTCACTGCCAC3'), and internal primers Tg18s58F (5'CTAAGTATAAGCTTTTATACGGC3') and Tg18s348R (5'TGCCACGGTAGTCCAATAC3') (Invitrogen). The DNA extracted from tachyzoites of the RH strain was used as control.

PCR amplification was carried out in a Mastercycler Nexus thermal cycler (Eppendorf) using Invitrogen reagents. The cycling conditions were as follows: initial denaturation at 94 °C for 2 min; followed by 30 cycles of 94 °C for 30 s, annealing at 57 °C and extension at 68 °C for 30 s; and a final extension step at 68 °C for 1 min. For nested PCR, the same conditions used for the initial PCR were used except for the use of 35 amplification cycles. To verify the absence of contaminants, a negative control was included.

The PCR products were purified by the addition of an equal volume of polyethylene glycol (PEG) (20% PEG 8,000 in 2.5 M NaCl) (Promega). After vortex homogenization for 60 s and incubation in a 37 °C water bath for 15 min, the products were centrifuged for 15 min at 15,700 g, and the supernatant was discarded. Each pellet was washed twice with 125 µL of chilled 80% ethanol and then centrifuged for 5 min at 15,700 g. The final pellet was maintained in an oven at 37 °C until the ethanol had fully evaporated. Then, the DNA was rehydrated with 12 µL of ultrapure water for PCR (Phoneutria), quantified with a NanoDrop ND-1000 (Thermo Fisher Scientific) and electrophoresed to confirm the presence of 300 bp product. DNA amplified from each sample was sequenced by Myleus Biotechnology (2020) using the 96-capillary 3730 DNA Analyzer (Thermo Fisher Scientific).

The obtained sequences were assembled and edited with the help of ChromasPro version 2.0.1 (Technelysium Pty Ltd, Australia) to obtain a single consensus sequence. The similarity with sequences deposited in GenBank was analyzed using the Basic Local Alignment Search Tool (BLAST) of the National Center for Biotechnology Information (NCBI, 2020) of the National Library of Medicine, USA.

Results

During bioassay follow-up, *T. gondii* was found in 10 moribund mice (signs of disease occurred between 8 and 15 d.p.i.), and in four surviving seropositive mice, in all cases after euthanasia. Overall, viable *T. gondii* parasites were isolated by mouse bioassay in six of the 44 (13.6%) wild birds assessed in this study. In the six birds yielding positive bioassay results, *T. gondii* was isolated from all of the heart samples but only in 3/6 brain samples (Table 1). *Toxoplasma gondii* DNA was identified in 10/44 (22.7%) of the birds. The amplified sequences in all of these samples exhibited 100% similarity with the DNA of the ME49 strain of *T. gondii*. Only one (a *Colaptes campestris* individual) of the 44 wild birds found positive for *T. gondii* via PCR and/or bioassay belonged to a species not considered raptorial (Table 1).

PCR identified *Sarcocystis* DNA in 5/44 (11.4%) of the birds. The amplified sequences in all of these samples exhibited 99% similarity with the DNA of *Sarcocystis* spp. (Table 1). In 3/44 (6.8%) of the birds, the amount of amplified DNA was insufficient for sequencing. Overall, Sarcocystidae members (*T. gondii* and *Sarcocystis* spp.) were identified in 19 (47.5%) of the 40 raptorial birds and in 25% (1/4) of the non-raptorial birds (Table 1).

Discussion

The molecular methods (amplification and sequencing of *T. gondii* DNA) proved to be more sensitive in detecting *T. gondii* than isolation by bioassay in mice. *Toxoplasma gondii* was isolated from six of the 44 birds. Rêgo et al. (2018) genotyped these isolates and identified five different genotypes by PCR-RFLP. A unique genotype, not previously described was common to two isolates. Genetic sequencing revealed *T. gondii* DNA in heart and/or brain in 10 wild birds, among which *T. gondii* was isolated from only four (*Ramphastos toco*, *Colaptes campestris*, *Megascops choliba* and *Caracara plancus*), all considered raptors, except *C. campestris*. On the other hand, *T. gondii* DNA was not amplified in two birds (*Rupornis magnirostris* and *C. plancus*) from which the parasite was isolated. These results were probably due to an uneven distribution of *T. gondii* in tissue samples of the birds, as previously observed in muscles of naturally infected domestic animals (Rani et al., 2020). The grinding of the whole organ, followed by the separation of two portions for the experiments (bioassay and PCR) could result in greater agreement between the two tests used in the present study. Our results are consistent with those of Vitaliano et al. (2014), who found a higher sensitivity of molecular methods than bioassay when analyzing 226 heart and brain samples from wild animals (with 28 samples found positive by PCR and only 15 samples found positive by bioassay).

Table 1. Sequencing of the 18S rRNA gene for detecting members of the Sarcocystidae family in free-living wild birds rescued in the state of Minas Gerais, Brazil

Bird species	Municipality of origin	*T. gondii* isolation rate by bioassay		PCR identification after sequencing		
		Heart	Brain	Heart	Brain	*Pool***
Asio clamator	Belo Horizonte	-	-	100% *T. gondii*	Negative	
Asio clamator	Sabará	-	-	100% *T. gondii*	Negative	
Asio clamator	Sarzedo	-	-	Negative	100% *T. gondii*	
Athene cunicularia	Belo Horizonte	-	-	100% *T. gondii*	Negative	
Athene cunicularia	Betim	-	-	99% *Sarcocystis* spp.	Negative	
Athene cunicularia	Belo Horizonte	-	-	Positive	Positive	
Caracara plancus	Betim	-	-	Positive	Positive	
Caracara plancus	Belo Horizonte	+	+	Negative	Negative	
Caracara plancus	Santa Luzia	+	-	100% *T. gondii*	Negative	
Cariana cristata	Itabirito	-	-	ND	ND	100% *T. gondii*
*Colaptes campestris**	Belo Horizonte	+	+	100% *T. gondii*	100% *T. gondii*	
Coragyps atratus	Betim	-	-	99% *Sarcocystis* spp.	Negative	
Geranoaetus albicaudatus	Belo Horizonte	-	-	ND	ND	99% *Sarcocystis* spp.
Megascops choliba	Belo Horizonte	-	-	Negative	100% *T. gondii*	
Megascops choliba	Belo Horizonte	+	-	100% *T. gondii*	Positive	
Milvago chimachima	Betim	-	-	Negative	Positive	
Ramphastos toco	Cristiano Otoni	+	+	100% *T. gondii*	100% *T. gondii*	
Ramphastos toco	Ribeirão das Neves	-	-	99% *Sarcocystis* spp.	Positive	
Rupornis magnirostris	Belo Horizonte	+	-	ND	ND	Negative
Rupornis magnirostris	Juatuba	-	-	99% *Sarcocystis* spp.	99% *Sarcocystis* spp.	

+ positive bioassay; - negative bioassay; ND: Not done; Negative: There was no amplification; Positive: did not amplify the minimum necessary for sequencing. *non-raptorial bird; **DNA extraction performed in a pool of heart and brain of the same bird.

Based on the organ distribution of *T. gondii* in wild birds in this study, the heart seems to be more frequently parasitized than the brain, by bioassay or by PCR. Our results corroborate observations in South Africa, where a low occurrence (2.7%; 3/110) of *T. gondii* in the brain of wild birds was identified (Lukášová et al., 2018). However, in naturally infected free-range chickens in Brazil, no trend of greater parasitism in heart or brain was observed (Ferreira et al., 2018).

Few studies have been conducted to identify birds as potential hosts of *Sarcocystis*. Twelve species of this genus use birds as definitive hosts, and another 22 use them as intermediate hosts. Two additional species can use birds as final and intermediate hosts (Odening, 1998). In this work, we verified that wild birds rescued in the state of Minas Gerais were infected with members of the genus *Sarcocystis*. However, it was not possible to identify these parasites to the species level in all cases. Recent studies have identified species of *Sarcocystis* in wild birds and have characterized clinical aspects of infection. Gjerde et al. (2017) identified the simultaneous presence of three species of *Sarcocystis* (*S. halieti, S. lari* and *S. truncata)* in a *Haliaeetus albicilla* individual rescued in Western Norway. The DNA of these three species was identified in segments of intestinal mucosa containing sporulated oocysts of *Sarcocystis* via PCR amplification and sequencing of four regions: the 18S and 28S rRNA genes, the ITS1 region and the *cox1* gene. Konradt et al. (2017) described pathological and molecular findings of *Sarcocystis* infection in *Phimosus infuscatus*, which caused necrotizing meningoencephalitis, in southern Brazil. They found that the gene encoding ITS1 in schizonts of the studied *Sarcocystis* sp. had 100% similarity with homologous sequence in *Sarcocystis* sp. which infects *Didelphis albiventris* in Brazil. Further studies using ITS1, 28S, 18S and *cox1* genes (Hoeve-Bakker et al., 2019) should be performed for *Sarcocystis* species identification in wild birds of southeastern Brazil.

An intrinsic limitation of studies designed to molecular diagnosis based on Nested-PCR is the susceptibility to contaminations. To minimize contaminations, different parts of the method were done in physically separate rooms from one another. Other limitation of the present study was the bird size and consequently the organs size, that restrict sample amount analyzed.

Conclusion

Compared with isolation by bioassay, the amplification of the 18S rDNA gene identified *T. gondii* in a larger number of samples. Members of the family Sarcocystidae are common in wild birds in Minas Gerais, Brazil.

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