

First study of *Cryptosporidium* spp. occurrence in eared doves (*Zenaida auriculata*)

Primeiro estudo de ocorrência de *Cryptosporidium* spp. em pomba-de-bando (*Zenaida auriculata*)

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Abstract

Cryptosporidium is a protozoan parasite with a wide range of hosts, including humans. However, only a few *Cryptosporidium* species have been described in birds (*C. meleagridis*, *C. baileyi*, *C. galli* and *C. avium*). The aim of this study was to investigate the occurrence of *Cryptosporidium* spp. in feces of eared doves (*Zenaida auriculata*), followed by molecular characterization of the parasite. A total of 196 animals of both sexes were trap-captured; the animals were culled and the intestinal contents were collected for DNA extraction. After extraction, a nested-PCR (nPCR), which amplifies a fragment of the 18S rRNA gene of *Cryptosporidium* spp., was performed. The amplicons obtained were purified and sequenced. PCR analysis revealed that 30 animals (15.3%) were positive for *Cryptosporidium* spp. There was no significant sex-dependent enrichment of *Cryptosporidium* occurrence ($p > 0.05$). Only 15 out of the 30 positive samples were successfully sequenced and their species determined, of which, 13 (86.7%) and 2 (13.3%) were *C. meleagridis* and *C. galli*, respectively. Herein, we present for the first time a molecular characterization of *Cryptosporidium* from feces of eared doves (*Z. auriculata*) and propose that these birds are a potential source of *C. meleagridis* infection in humans.

Keywords: Pigeons, epidemiology, cryptosporidiosis, PCR.

Resumo

Cryptosporidium é um protozoário com uma grande variedade de hospedeiros, incluindo os seres humanos. No entanto, poucas espécies têm sido descritas em aves (*Cryptosporidium meleagridis*, *C. baileyi*, *C. galli* e *C. avium*). O objetivo do presente estudo foi investigar a ocorrência de *Cryptosporidium* spp. em fezes de pombas-de-bando (*Zenaida auriculata*), e realizar a caracterização molecular dos isolados. Um total de 196 animais de ambos os sexos foram capturados, eutanasiados e o conteúdo intestinal recolhido para extração de DNA. Após a extração, realizou-se uma nested-PCR (nPCR), que amplifica um fragmento do gene 18S rRNA do *Cryptosporidium* spp.. Os fragmentos obtidos foram purificados e encaminhados para sequenciamento. Os resultados da n-PCR revelaram 30 animais (15.3%) positivos para *Cryptosporidium* spp.. Quanto ao sexo dos animais não foram observadas diferenças estatísticas significativas ($p > 0.05$). Somente 15 de 30 amostras positivas foram sequenciadas com sucesso e as espécies determinadas, das quais, 13 (86.7%) e 2 (13.3%) foram *C. meleagridis* e *C. galli*, respectivamente. Esse é o primeiro estudo com caracterização molecular de *Cryptosporidium* de fezes de pombas-de-bando (*Z. auriculata*), e propõe serem esses animais potenciais fonte de infecção de *C. meleagridis* para humanos.

Palavras-chave: Pombos, epidemiologia, criptosporidiose, PCR.

Introduction

Cryptosporidium is a protozoa parasite able to infect mammals, reptiles, birds, and fishes (MONIS & THOMPSON, 2003). The first report of *Cryptosporidium* in birds was made by Tyzzer (1929),

who found the parasite in the intestinal epithelium of young hens. To date, 38 species of *Cryptosporidium* have been described worldwide (FENG et al., 2018), however only four species are able to infect birds: *C. baileyi*, *C. galli*, *C. meleagridis* and *C. avium* (NAKAMURA & MEIRELES, 2015; HOLUBOVÁ et al., 2016). *C. meleagridis* has zoonotic potential and causes diarrhea in children and immunodeficient individuals (MBAE et al., 2015). *C. meleagridis* is responsible for approximately 10% of the

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human cryptosporidiosis cases reported in Peru and Thailand (WANG et al., 2014).

Zenaida auriculata (Columbiforme) is endemic from Antilles to Tierra del Fuego, including Brazil. This dove is popularly known as the eared dove and is found in fields, farms, and urban areas (CÂNDIDO et al., 2008), where it can cause damage and potentially transmit pathogens (SHIBATTA et al., 2009).

There are no studies of *Cryptosporidium* spp. in eared doves; therefore, we aimed to study the occurrence of *Cryptosporidium* spp. from feces of eared doves and to determine the involved species.

Material and Methods

Sampling and local of capture

A total of 196 eared doves from both sexes were captured in gauze-traps in the urban region of Londrina city, northern of Paraná state, located between 23°08'47" and 23°55'46" south and between 50°52'23" and 51°19'11" west. Sampling was done by area and convenience. The present study was approved by the Brazilian Institute of Environment (IBAMA - SISBIO n. 16428-1) and by the Ethics Committee of Animal Experimentation from the State University of Londrina (n. 70/2008).

Sample collection

After being trap-captured, the birds were euthanized and the gastrointestinal tracts were removed. Intestinal contents were collected in a petri plate and divided into aliquots in microtubes to be frozen at -20 °C and subsequently used for DNA extraction.

DNA extraction

For DNA extraction, fecal samples were frozen and thawed at -80 °C and 30 °C, respectively, three times. After this, 1 mL Tris-EDTA (TE) was added for each 50 µL of fecal samples, then this solution was mixed and centrifuged at 4,000 ×g for 15 minutes. The supernatant was poured off, and the pellet was used for the extraction with the Nucleospin Tissue® kit (Macherey-Nagel, Germany), following the manufacturer's instructions. Negative (ultrapure water) and positive (*C. parvum* oocysts) controls were included in all DNA extraction procedures.

Nested PCR

DNA amplification was performed in triplicate by nPCR targeting the 18S rRNA region using primers previously described for *Cryptosporidium* (XIAO et al., 1999, 2000). In the first reaction, the primers used were Fw1 (5'-TTCTAGAGCTAATACATGCG-3') and Rv2 (5'-CCCATTTCCTTCGAAACAGGA-3'). The second reaction was performed using the primers Fw3 (5'-GGAAGGGTTGTATTTAT'TAGATAAAG-3') and Rv4 (5'-AAGGAGTAAGGAACAACCTCCA-3'). Reactions were performed using 1 × PCR buffer, 2.5 mM of MgCl₂, 0.2 µM of dNTP, 0.2 µM of each primer, 1.2 U of Platinum Taq (Invitrogen),

2.5 µL of DNA and sterilized ultrapure water (total volume of 25 µL). In both reactions, the same cycling conditions were used: 95°C for 5 minutes, followed by 34 cycles of 94°C for 45 seconds, 55°C for 45 seconds and 72°C for 1 minute, with a final extension of 72°C for 5 minutes. DNA from *C. parvum* and ultrapure water were included in all PCR reactions as positive and negative controls respectively. Products from the second amplification were visualized under ultraviolet light after electrophoresis on a 1.5% agarose gel stained with SYBR® Safe (Invitrogen, USA) and photodocumented by LPix Imagem ST Software (Loccus Biotecnologia).

Sequencing and phylogenetic tree

Amplicons from the nPCR were purified using the Purelink Quick Gel Extraction Kit (Invitrogen, USA). After purification, they were submitted for sequencing with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA), at the ABI3730xl Genetic Analyzer (Applied Biosystems, USA). The data obtained from sequencing allowed the comparison between complementary amplicons from the same samples, generating a consensus, which was compared by Blast to the sequences deposited at GenBank for determination of species. Bioedit Sequence Alignment Editor v7.2.5 Software was used to align the sequences, including a reference sample of *Eimeria tenella* (DQ640011.1) as the outgroup.

The consensus sequence was predicted by MEGA6 Software (TAMURA et al., 2013), where the neighbor-joining tree was built with the sequences obtained herein and others deposited at GenBank, using Kimura 2-parameter distance matrix (KIMURA, 1980). Statistical analysis was performed using bootstrapping with 1,000 repetitions.

Statistical analysis

Sex dependence of *Cryptosporidium* occurrence by Chi-square (X²) test with a confidence interval of 95%, using the OpenEpi 3.03a software; p ≤ 0.05 was considered as significant.

Results

Thirty fecal samples (15.3%) out of 196 were identified as positives using nPCR and submitted for sequencing. Male and female animals had 10.8% (10/92) and 19.2% (20/104) of positive samples (p>0.05), respectively. Because of low DNA quantity, just 15 out of 30 samples had a good sequencing, thirteen (87%) of them matched *C. meleagridis* and two (13%) *C. galli*. The phylogenetic tree (Figure 1) showed two branches: one grouping the 13 samples with genetic similarity of 99% with the standard *C. meleagridis* (AF329186.1, AF180339.1, EU284595.1, KJ851537.1 and JX141294.1), and the second one grouping the two samples with 99% of similarity with *C. galli* (GU734647.1). The nucleotide sequences generated from the positive samples were deposited in GenBank under accession numbers MF405448 to MF405462.

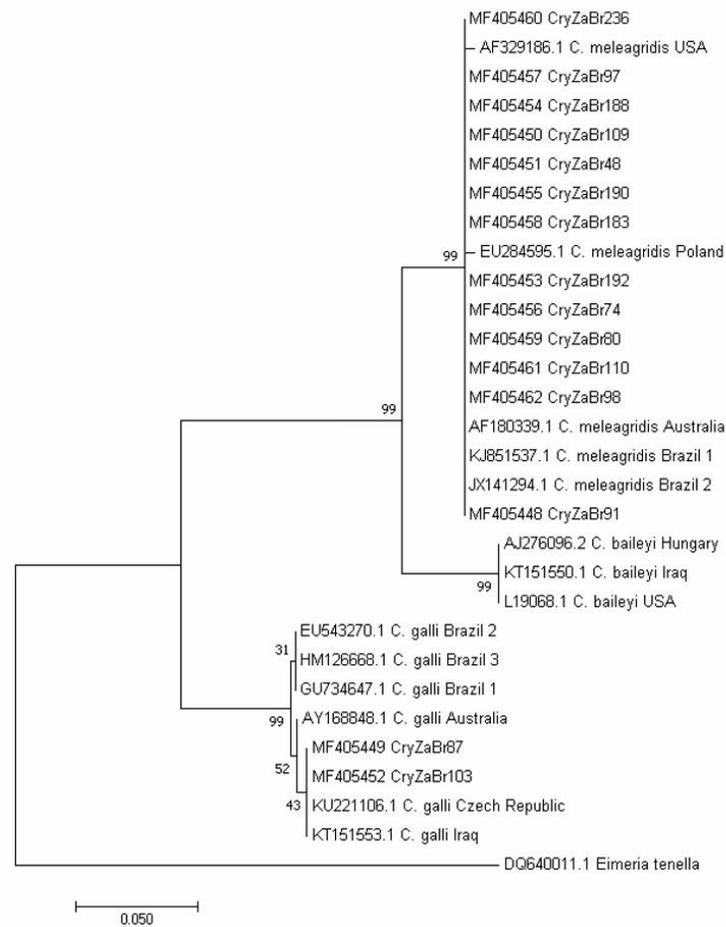


Figure 1. Phylogenetic tree of the 18S rRNA gene sequences of *Cryptosporidium* detected in feces from eared doves *Zenaida auriculata*. CryZaBr# are isolates from our study.

Discussion

To the best of our knowledge, this study shows, for the first time, the molecular prevalence and characterization of *Cryptosporidium* spp. in fecal samples from eared doves. We observed 15.3% positive samples, of which, 87% were identified as *C. meleagridis* and 13% as *C. galli*. The first molecular characterization of *Cryptosporidium* in *Columba livia* (Columbiformes) was reported by Oliveira et al. (2017). Contrary to our results, they observed a lower prevalence (7%) of *Cryptosporidium* and reported *C. parvum* as the predominant parasite species.

Cryptosporidium meleagridis infection affects mainly children and immunodeficient individuals (CHAPPELL et al., 2011; CAMA et al., 2007). The data obtained show the potential of eared doves to act as a source of *C. meleagridis* infection in humans. This protozoan parasite has also been reported in pigeons, chickens, and ducks from China (WANG et al., 2010; LI et al., 2015). The eared doves feed on the ground, mainly in farms, and can fly long distances, from where they sleep to the place they spend the day, therefore, we speculate that eared doves might be acting as vectors of cryptosporidiosis and involved in transmitting the infection from one region to another (BUCHER & BOCCO, 2009).

Conclusions

Our study is the first to report *Cryptosporidium* infection in the eared dove *Z. auriculata*, along with genetic characterization of *C. meleagridis* and *C. galli*. These results suggest that this bird species could be a source of *C. meleagridis* infection in humans.

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