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HELMINTHOLOGY - SHORT COMMUNICATION

High prevalence of *Ancylostoma caninum* infection in black-eared opossums (*Didelphis aurita*) in an urban environment

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Abstract

Marsupials of the genus *Didelphis*, such as black-eared opossums (*Didelphis aurita*), are common synanthropic animals in urban areas of Brazil. These marsupials are frequently parasitized by numerous helminth species, including ancylostomatid nematodes. This study aimed to report the occurrence of *Ancylostoma caninum* in black-eared opossums captured in an urban environment of Southeastern Brazil and discuss the potential impact of these findings for public health. From January to June 2019, we collected fecal samples from 49 restrained opossums and evaluated by a simple flotation method; Helminth eggs were observed at different magnifications and identified according to morphological and morphometric features. Genomic DNA was extracted from Ancylostomatidae eggs and screened by duplex PCR for *Ancylostoma* spp. and *Necator americanus* using primers that amplify a region of internal transcribed spacer 2 and the 28S ribosomal RNA (ITS2-28S rRNA). *Ancylostoma* spp. eggs were detected in 65.3% (32/49) of the animals. Sequence analysis revealed 100% homology with *A. caninum* sequences from GenBank. Our results demonstrate a new host-parasite interaction for *A. caninum*, suggesting that black-eared opossums may participate in the zoonotic cycle of this parasite in urban areas of Brazil.

Keywords Ancylostomatidae · Marsupials · Public health · Wildlife · Nematodes

Introduction

Nematodes account for an important part of gastrointestinal infections in humans and animals. Within the phylum Nematoda, worms of the Ancylostomatidae family are widely prevalent in mammals, and the heavy infection by these parasites causes deleterious effects (i.e., anemia, diarrhea, and hemorrhagic enteritis) on the host (Seguel and Gottdenker 2017). Among the many species belonging to this family, hookworms (*Ancylostoma* spp.) present substantial importance to public health. For example, the species *Ancylostoma braziliense*, *Ancylostoma caninum*, and *Uncinaria stenocephala* cause the so-called cutaneous *larva migrans*, a neglected tropical zoonosis that affects people in developing countries (Heukelbach and Feldmeier 2008; Oliveira-Arbex et al. 2016).

From a "One Health" perspective, zoonotic nematode infections in synanthropic animals are particularly important due to their potential as a source of infection for humans and domestic animals (Cunningham et al. 2017). Marsupials of the genus *Didelphis* are perfect examples of synanthropic animals, as they are well adapted to urban environments, and their opportunistic dietary behavior facilitates infection by gastrointestinal parasites such as *Ancylostoma* spp. (Teodoro et al. 2019).

This study aimed to report the occurrence of *A. caninum* in black-eared opossums (*Didelphis aurita*) captured in an urban environment of Southeastern Brazil and discuss the potential impact of these findings for public health.

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Material and methods

The study area included urban sites of the city of Viçosa (latitude 20° 45′ 14″ south and longitude 42° 52′ 54″ west), Minas Gerais, Brazil. The region is classified as Cwa (Köppen climate classification) and is 650 m above sea level, presenting an annual average temperature varying from 20 to 22 °C. Opossums were captured from January to June 2019 through Tomahawk live traps (0.45 × 0.21 × 0.21 m) baited with a mix of corn flour, canned fish, and banana. Animals were classified according to their sex and age group (Supplementary material), being subsequently marked with a small V cut at the right ear to avoid collecting samples from recaptured individuals. After sampling procedures, marsupials were released at the same site of capture.

Fresh fecal samples (5 to 10 g per animal) were collected from the traps as soon as the animals defecated or directly from the cloaca. Subsequently, each sample was evaluated by the simple flotation technique of Willis-Mollay (Willis 1921). Ancylostomatidae eggs were analyzed at different magnifications (× 400 and × 1000) with an Olympus CX31 microscope and identified according to morphological and morphometric features previously described (Taylor et al. 2007; Bowman 2010).

Eggs were isolated by filtration and centrifugation with sulfuric ether and hypochlorite 1%. The material was observed under optic microscopy, and individual eggs were recovered with a micropipette (Zuccherato et al. 2018). Genomic DNA was extracted from 30 eggs recovered from three animals (10 eggs per animal), following a protocol previously described (Lake et al. 2009). Thereafter, DNA samples were screened by duplex PCR for *Ancylostoma* spp. and *Necator americanus* using primers that amplify a region of internal transcribed spacer 2 and the 28S ribosomal RNA (ITS2-28S rRNA). Forward primers AD1 (5′CGACTTTA GAACGTTTCGGC3′) and NA (5′ATGTGCACGTTATT CACT3′) and the reverse primer NC2 (5′TTAGTTTC TTTTCCTCCGCT3′) were used for DNA amplification, resulting in 250 bp if positive for *N. americanus* and in 130 bp if positive for *Ancylostoma* spp. (Sahimin et al. 2017). Reactions were performed using 0.2 μM of each primer, 1 U of Taq DNA polymerase (Phoneutria, Brazil), 200 μM of deoxynucleoside triphosphate (dNTPs), 1× reaction buffer, 5 μL of DNA sample, and ultrapure water to complete a 10-μL final volume. Genomic DNA of hookworms (*N. americanus* and *Ancylostoma* spp.) and nuclease-free water were used as the positive and negative controls, respectively. Electrophoresis was performed in 1% agarose gel with 0.5× TAE buffer and GelRed ™ (Biotium, EUA) to observe the amplified products. The amplicons were further processed using a PCR Purification Kit (Illustra GFX™ PCR DNA and Gel Band Purification Kit, GE Healthcare, UK) according to manufacturer's recommendations. Purified PCR products were sequenced in both directions using the Sanger's method (Sanger et al. 1977) in an automated sequencer AB 3500 Genetic Analyzer. DNA sequences were aligned using Mega7 software (Kumar et al. 2016) and compared with sequences from the GenBank database via the basic local alignment search tool (BLAST).

Prevalence was determined by the proportion of positive samples in the study area. The normality of data was checked using the Lilliefors test, and the chi-square test was used to analyze *A. caninum* infections and the association with sex and age of positive animals. A 5% significance level was considered for all parameters tested ($p < 0.05$). The BioEstat 5.3 software was used to perform all statistical analyses.

Results and discussion

Hookworm eggs were detected in 32 (65.3%) out of 49 fecal samples of the black-eared opossums tested (Table 1). No statistically significant difference was observed for parasitism regarding sex ($X_2 = 0.35$; $p = 0.56$) or age of the animals ($X_2 = 0.16$; $p = 0.68$). Sequence analysis of the PCR products revealed *A. caninum* with query cover of 100% and identity of 99.72% (accession numbers: KP844730.1; DQ438075.1; DQ438071.1) as compared with sequences available in the GenBank database. The sequences obtained in this study were deposited in GenBank under the accession numbers MT130904 to MT130933.

The detection of *A. caninum* in *D. aurita* is of great importance from a "One Health" point of view, since this marsupial species circulates among urban, rural, and wild environments (De Castro et al. 2017). Helminths of the family Ancylostomatidae have been previously reported in black-eared opossums (*D. aurita*) in Brazil with a prevalence of 41.1% (Teodoro et al. 2019). Such results have also been observed for *Didelphis albiventris*, *Didelphis virginiana*, and *Didelphis marsupialis*, with 100.0%, 84.5%, and 60.0% prevalence, respectively (Rueda et al. 2014; Aragón-Pech et al. 2018; Teodoro et al. 2019). However, these studies were

Table 1 Prevalence of *Ancylostoma caninum* in *Didelphis aurita* according to sex and age of the animals

Age/sex	Captured animals	Prevalence (%)
Adult male	10	70.0 (7/10)
Adult female	14	57.1 (8/14)
Subadult male	12	75.0 (9/12)
Subadult female	12	66.7 (8/12)
Pup male	1	0 (0/1)
Pup female	0	0 (0/0)
Total	49	65.3 (32/49)

restricted to morphological identification of eggs, not allowing the classification at species level. In our study, a high prevalence was also detected, and as observed in those previous studies, it may suggest that marsupials of the genus *Didelphis* are important hosts for *Ancylostoma* spp., having implications on the health of humans and domestic animals.

Several families of wildlife species are affected by parasites of the genus *Ancylostoma*; however, only individuals of the families Canidae (*Canis latrans*, *Vulpes vulpes*, *Canis lupus*, *Canis lupus dingo*, *Canis aureus*, *Urocyon cinereoargenteus* and *Canis rufus*), Felidae (*Lynx rufus* and *Lynx canadensis*), and Ursidae (*Ursus americanus*) are recorded harboring *A. caninum* (Seguel and Gottdenker 2017). Our study adds *D. aurita* as a new host for this nematode, therefore including the family Didelphidae within the range of host families for *A. caninum*.

Studies on zoonotic helminths in urban environments attributed feces of dogs and cats as the only source of *A. caninum* contamination of soil and sandboxes (Leon et al. 2019; Da Silva et al. 2019). However, black-eared opossums also circulate in these environments looking for a food source (Muller et al. 2005). Thus, they are suggested to have a role in the zoonotic cycle of this nematode in urban settings. Moreover, *D. aurita* feces may contaminate vegetables and water used for human and domestic animal consumption in rural surroundings, therefore enabling the oral route of infection through the ingestion of *A. caninum* larvae (Landmann and Prociv 2003; Jung et al. 2020).

Among domestic animals, dogs are definitive hosts for *A. caninum* (Shepherd et al. 2018); however, phylogenetic analysis suggests that cats are primary hosts for this nematode in some regions and that cross-infection between different host species sharing the same environment are possible (Liu et al. 2013). In this aspect, domestic animals are in close contact with *Didelphis* spp. For example, it is common to observe dogs attacking and killing these marsupials in cities and rural settings (Lessa et al. 2016). Besides this, ingestion of *A. caninum* larvae through contaminated food may be a driver to cross-infection among these animals (Rehman et al. 2017).

Our results showed a new host-parasite interaction for *A. caninum*, suggesting that black-eared opossums may participate in the zoonotic cycle of this nematode species; however, further studies on the involvement of *D. aurita* in the spreading of *A. caninum* in urban environments are needed.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All protocols used in this study were approved by the Ethics Committee for Animal Experimentation (ECAE) of the Universidade Federal de Viçosa (license number: 80/2018), and the capture of the animals was authorized by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) through the Biodiversity Information and Authorization System (SISBIO), license number: 64930-1.

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