Genotyping of Toxoplasma gondii and Sarcocystis spp. in road-killed wild mammals from the Central Western Region of the State of São Paulo, Brazil

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Abstract

Introduction: Road-killed wild animals host zoonotic pathogens such as Toxoplasma gondii, offering a new opportunity for the epidemiological study of these infectious organisms. Methods: This investigation aimed to determine the presence of T. gondii and other apicomplexan parasites in tissue samples of 64 road-killed wild animals, using polymerase chain reaction (PCR). Positive samples were then typed by PCR-restriction fragment length polymorphism (RFLP) using 7 markers: SAG1, 5′-3′SAG2, SAG3, BTUB, c29-6, PK1, and Apico. PCR-RFLP targeting 18S ribosomal RNA (rRNA) genes was also performed on all samples to detect other apicomplexan parasites. Results: T. gondii DNA was detected in 16 tissue samples from 8 individual animals, as follows: 1 Cerdocyon thous (crab-eating fox), 1 Didelphis albiventris (white-eared opossum), 1 Lutreolina crassicaudata (lutrine opossum), 2 Myrmecophaga tridactyla (giant anteater), 1 Procyon cancrivorus (crab-eating raccoon), and 2 Sphiggurus spinosus (Paraguay hairy dwarf porcupine). Seven different T. gondii genotypes were identified, 6 of which were novel. Typing by 18S rRNA verified these 16 T. gondii-infected samples, and identified 1 Sarcocystis spp.-infected animal [Dasypus novemcinctus (nine-banded armadillo)]. The amplified T. gondii (GenBank accession No. L37415.1) and Sarcocystis spp. 18S rRNA products were confirmed by sequencing. Conclusions: Our results indicate that T. gondii is commonly present in wild mammals, which act as sources of infection for humans and animals, including other wild species. The approach employed herein proved useful for detecting T. gondii and Sarcocystis spp. in the environment and identifying their natural reservoirs, contributing to our understanding of host-parasite interactions.

Keywords: Road-killed animal. Toxoplasma gondii. Sarcocystis spp. Genotyping. Molecular techniques.

INTRODUCTION

Several pathogens derive from wild animals, the study of which is becoming increasingly restrictive, especially when euthanasia is required. Thus, road-killed wild mammals offer an alternative source of such animals for research involving molecular detection of parasites. Although microbiological culture and histopathological analysis using tissue samples from road-killed wild animals are challenging, the identification and typing of pathogens can be achieved through molecular methods¹.

Apicomplexan parasites, principally Toxoplasma gondii, are very common among domestic and wild animals. T. gondii is an obligate intracellular protozoan parasite, prevalent in animals worldwide, and commonly infecting humans. Infection can occur by transplacental transmission, oral ingestion of contaminated soil, raw vegetables, fruits, or water containing sporulated oocysts shed by definitive hosts in their feces, or by ingestion of tissue cysts in raw or undercooked meat or viscera of intermediate hosts²⁻³. This parasite exhibits a highly complex clonal genetic population structure that has been extensively studied in recent years⁴. Several regions of the T. gondii genome have been used for the identification of this organism. The 529- base pair (bp) repetitive sequence, which repeats 200-300 times per genome, provides high sensitivity and specificity, representing an important target for identification⁵⁻⁶. Toxoplasma gondii comprises several clonal lineages whose pathogeneses in humans and animals may differ in progression and severity⁷.

Thus, research concerning the identification of T. gondii by molecular techniques in novel hosts is crucial to clarify its interactions with hosts and molecular epidemiology, and may also provide a good indicator of environmental contamination. Wild animals act as reservoirs of T. gondii infection affecting humans and food animals, necessitating the adoption of
epidemiological and sanitary control measures. With this in mind, the present study aimed to identify new hosts of *T. gondii* and other apicomplexan parasites in tissue samples of road-killed wild mammals using molecular techniques. In addition, parasite genotypes in circulation were determined.

**METHODS**

Animals

Sixty-four road-killed wild animals were studied: 1 *Callithrix penicillata* (black-pencilled marmoset) (A1); 4 *Cavia aperea* (Brazilian guinea pig) (A2–A5); 1 *Cebus apella* (tufted capuchin) (A6); 12 *Cerdocyon thous* (crab-eating fox) (A7–A18); 3 *Dasypus novemcinctus* (nine-banded armadillo) (A19–A21); 1 *Dasypus septemcinctus* (Brazilian lesser long-nosed armadillo) (A22); 8 *Didelphis albiventris* (white-eared opossum) (A23–A30); 1 *Eira barbara* (tyara) (A31); 1 *Euphractus sexcinctus* (yellow armadillo) (A32); 2 *Galictis vittata* (greater grison) (A33, A34); 2 *Hydrochoerus hydrochaeris* (capybara) (A35, A36); 3 *Leopardus tigrinus* (oncilla) (A37–A39); 3 *Lepus europaeus* (European hare) (A40–A42); 2 *Lutreolina crassicaudata* (lurine opossum) (A43, A44); 2 *Mazama gouazoubira* (gray brocket) (A45, A46); 1 *Myocastor coypus* (coypu) (A47); 5 *Myrmecophaga tridactyla* (giant anteater) (A48–A52); 3 *Procyon cancrivorus* (crab-eating raccoon) (A53–A55); 1 *Puma concolor* (puma) (A56); 2 *Rattus rattus* (house rat) (A57, A58); 4 *Sphiggurus spinosus* (Paraguayan hairy dwarf porcupine) (A59–A62); and 2 *Tamandua tetradactyla* (southern tamandua) (A63, A64). Only wild animals with no exposed viscera killed 1–7 hours prior to being processed in the laboratory were studied. This study is in accordance with *Instituto Brasileiro do Meio Ambiente e dos Recursos Naturais Renováveis* (IBAMA) normative statement n. 119 of October 11, 2006, Chapter VI, Art. 26, which authorizes the sampling and transport of road-killed wild animals.

Ethical considerations

This work was also approved by the Animal Experimentation Ethics Committee, Faculdade de Medicina Veterinária e Zootecnia, Universidade Estadual Paulista (CEEA/FMVZ n. 052/05).

Sampling

All animals were transported at 4°C to the FMVZ. Lung, spleen, liver, kidney, heart, and mesenteric lymph node samples were collected from each animal, finely chopped, and stored at −80°C in 1.5-mL centrifuge tubes containing sterilized ultrapure water (Life Technologies, Carlsbad, CA, USA), until needed for Deoxyribonucleic acid (DNA) extraction.

Molecular detection

DNA extraction was carried out using an illustra tissue & cells genomicPrep Mini Spin Kit (GE Healthcare, Chicago, IL, USA) according to the manufacturer’s instructions. Polymerase chain reactions (PCRs) were run using the following primers targeting a 529-bp repetitive sequence in the *T. gondii* genome: TOX4 (5′-CGCTGCAGGAGGAAGACGAAAGTTG-3′) and TOX5 (5′-CGCTGCAGACACAGTGCATCTGGATT-3′). Each reaction contained 10mM Tris-HCl (pH 8.0), 50mM KCl, 1.5mM MgCl₂, 0.2mM deoxynucleotides (Life Technologies), 10pmol each primer (Integrated DNA Technologies, Coralville, IA, USA), 0.2 units Taq DNA polymerase (Life Technologies), and 10ng DNA template. All reactions were run on a MasterCycler ep Gradient instrument (Eppendorf, Hauppauge, NY, USA), using the following cycling protocol: initial denaturation for 7 minutes at 94°C, followed by 35 cycles of 1 minute at 94°C, 1 minute at 60°C, and 1 minute at 72°C, before a final extension for 10 minutes at 72°C. Amplicons were analyzed by electrophoresis in 1.5% agarose with SYBR Safe DNA gel stain (Life Technologies), and recorded using a digital gel documentation system, GelDoc-IT™ Imaging System (UVP, Upland, CA, USA).

**RESULTS**

Table 1 contains the taxonomy, sex, and geographic location of the road-killed wild animals for which positive PCR results for *T. gondii* and *Sarcocystis* spp. were obtained, and Table 2 details the corresponding tissue samples and parasite identities. Genotyping results are presented in Table 3.

*Cerdocyon thous* was the most frequently observed species [12/64, 18.6%; 95% confidence interval (CI): 11.1-30.0%]. Of the 8 *T. gondii*-positive samples, 2 were from members of Carnivora (1 *C. thous* and 1 *P. cancrivorus*), 2 from Rodentia
TABLE 1
Taxonomy, sex, and geographic location of PCR-positive road-killed wild animals.

<table>
<thead>
<tr>
<th>Order or superorder</th>
<th>Family</th>
<th>Species</th>
<th>Sex</th>
<th>Animal</th>
<th>Geographic location</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carnivora</td>
<td>Canidae</td>
<td><em>Cerdocyon thous</em></td>
<td>Male</td>
<td>A7</td>
<td>22°53'36.49&quot;S 48°28'35.76&quot;W</td>
</tr>
<tr>
<td></td>
<td>Procyonidae</td>
<td><em>Procyon cancrivorus</em></td>
<td>Male</td>
<td>A53</td>
<td>22°50'51.83&quot;S 48°30'09.69&quot;W</td>
</tr>
<tr>
<td>Didelphimorphia</td>
<td>Didelphidae</td>
<td><em>Didelphis albiventris</em></td>
<td>Male</td>
<td>A23</td>
<td>22°53'35.49&quot;S 48°26'48.50&quot;W</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Lutreolina crassicaudata</em></td>
<td>Male</td>
<td>A43</td>
<td>22°53'28.55&quot;S 48°29'29.25&quot;W</td>
</tr>
<tr>
<td>Rodentia</td>
<td>Erethizontidae</td>
<td><em>Sphiggurus spinosus</em></td>
<td>Female</td>
<td>A59</td>
<td>23°01'53.00&quot;S 48°04'53.19&quot;W</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Female</td>
<td>A60</td>
<td>22°49'58.71&quot;S 48°25'24.75&quot;W</td>
</tr>
<tr>
<td></td>
<td>Dasypodidae</td>
<td><em>Dasypus novemcinctus</em></td>
<td>Male</td>
<td>A19</td>
<td>22°53'09.20&quot;S 48°27'35.59&quot;W</td>
</tr>
<tr>
<td>Xenarthra</td>
<td>Myrmecophagidae</td>
<td><em>Myrmecophaga tridactyla</em></td>
<td>Female</td>
<td>A49</td>
<td>23°01'51.18&quot;S 48°30'47.26&quot;W</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Male</td>
<td>A50</td>
<td>22°56'55.51&quot;S 48°15'29.35&quot;W</td>
</tr>
</tbody>
</table>

PCR: polymerase chain reaction.

TABLE 2
Molecular identification by PCR and sequencing of *Toxoplasma gondii* and *Sarcocystis* spp. in tissue samples from road-killed animals.

<table>
<thead>
<tr>
<th>Animal</th>
<th>Tissue (PCR-positive)</th>
<th>Identity %/GenBank accession No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>A7</td>
<td>Heart</td>
<td>100%/L37415.1 <em>Toxoplasma gondii</em></td>
</tr>
<tr>
<td>A53</td>
<td>Kidney</td>
<td>100%/L37415.1 <em>Toxoplasma gondii</em></td>
</tr>
<tr>
<td>A23</td>
<td>Liver</td>
<td>100%/L37415.1 <em>Toxoplasma gondii</em></td>
</tr>
<tr>
<td>A43</td>
<td>Mesenteric lymph node</td>
<td>100%/L37415.1 <em>Toxoplasma gondii</em></td>
</tr>
<tr>
<td>A59</td>
<td>Lung, spleen</td>
<td>100%/L37415.1 <em>Toxoplasma gondii</em></td>
</tr>
<tr>
<td>A60</td>
<td>Mesenteric lymph node</td>
<td>100%/L37415.1 <em>Toxoplasma gondii</em></td>
</tr>
<tr>
<td>A19</td>
<td>Liver</td>
<td>100%/AY656815.1, AY628220.1, AY628219.1, AF252406.1, U33149.1 <em>Sarcocystis</em> spp.</td>
</tr>
<tr>
<td>A49</td>
<td>Lung, liver, kidney</td>
<td>100%/L37415.1 <em>Toxoplasma gondii</em></td>
</tr>
<tr>
<td>A50</td>
<td>Lung, spleen, liver, kidney, heart, mesenteric lymph node</td>
<td>100%/L37415.1 <em>Toxoplasma gondii</em></td>
</tr>
</tbody>
</table>

PCR: polymerase chain reaction; RNA: ribonucleic acid; GenBank accession No.: L37415.1: *Toxoplasma gondii* 18S ribosomal RNA gene, complete sequence; AY656815.1: *Sarcocystis felis* sporocysts small subunit ribosomal RNA gene, partial sequence; AY628220.1: *Sarcocystis falcata* strain Stiles small subunit ribosomal RNA gene, partial sequence; AY628219.1: *Sarcocystis neurona* isolate MIOP17 small subunit ribosomal RNA gene, partial sequence; AF252406.1: *Sarcocystis neurona* 18S ribosomal RNA gene, partial sequence; U33149.1: *Sarcocystis neurona* 18S small subunit ribosomal RNA gene, 5′ partial sequence.
(1 Sphiggurus spinosus), 2 from Didelphimorphia (1 L. crassicaudata and 1 D. albiventris), and 2 from Xenarthra (1 M. tridactyla). One sample from a species in this latter order (1 D. novemcinctus) tested positive for Sarcocystis spp. Seven of the 8 T. gondii isolates in the present study exhibited novel genotypes.

**DISCUSSION**

Utilizing road-killed wild animals for molecular detection of T. gondii represents a feasible and efficient alternative to the use of live animals in research, as indicated by animal research ethics committees. Notably, most studies having used road-killed wild animals have identified a large number of mammalian species. In addition, sensitive and specific molecular tools enable pathogen identification without the need for laborious microbiological cultures and histopathological examination.

In this paper, molecular detection of T. gondii in several wild species was attempted using PCR. A number of studies have reported the presence of this parasite in wild rodents and members of Carnivora, Didelphimorphia, and Xenarthra(18)(19)(20). These findings confirm the worldwide distribution of T. gondii, and highlight the wide variety of intermediate hosts that form part of the epidemiological chain responsible for transmission of this infection and the associated disease.

Here, 22 specimens were from members of the order Carnivora, with C. thous predominating. These animals can be found in several environments, from Cerrado savanna to the Atlantic Forest(21). Their abundance may be due to their generalist and, of preference, nocturnal feeding habits, moving through tracks at forest edges and surviving in degraded and anthropic areas(22). They are frequently seen on roadsides searching for food, which may include other road-killed animals, meaning that, as a carnivorous species, C. thous has a high road-kill rate(23). T. gondii DNA was detected in samples from 1 C. thous and 1 P. cancrivorus. In the literature, similar results have been obtained using molecular assays(20)(24).

Toxoplasma gondii DNA was not detected in animals of the orders Artiodactyla (2 M. gouazoubira), Lagomorpha (3 L. europaeus), and Primates (1 C. apella and 1 C. penicillata), but these groups are nevertheless important in the epidemiology of this parasite, since several reports of T. gondii infection in cervids, lagomorphs, and primates have been published(25).

Toxoplasma gondii DNA was detected in 2/13 (15.4%) specimens of the order Rodentia. The positive S. spinosus samples emphasize the importance of this species as a carrier of T. gondii and several other pathogens with zoonotic potential(26). Although the number of infected animals of this order was small, further assessment of this group is needed, since Truppel et al.(27) and Yai et al.(28) successfully isolated this parasite from capybaras (H. hydrochaeris), detecting its presence by serology.

Members of Didelphidae, represented here by D. albiventris and L. crassicaudata (10 specimens), are generalists and inhabit areas close to human dwellings, including farms, backyards, and urban centers(29). Due to the destruction of their habitat, L. crassicaudata seeks shelter and food in urban areas(29). This group is considered a reservoir of several potentially zoonotic organisms(30). In our study, T. gondii DNA was detected in 1 L. crassicaudata and 1 D. albiventris.
Of the 12 animals belonging to the superorder Xenarthra, 3 (25%; 95%CI: 9.1-53.8%) gave positive PCR results, 1 for *Sarcocystis* spp. This reinforces the importance of this taxon in the epidemiology of *T. gondii* infection. The fact that this parasite was not detected in *E. sexcinctus* may reflect the differences between this animal’s feeding habits and habitat and those of the other species examined. *E. sexcinctus* feeds on carrion found on the ground, and constructs its burrows in drier environments and open fields.

*Toxoplasma gondii* samples from 8 animals were genotyped, 7 of which yielded previously unreported marker combinations (Table 3), and 1 of which demonstrated a profile similar to that already reported in RFLP studies performed by Dardé et al. (17), Su et al. (8), Sousa et al. (15), Dubey et al. (10) (16), and Velmurugan et al. (17). Two of these genotypes, TgCatBr38 and TgCatBr44, were identified in cats from Aracatuba and Conchas, both in the State of São Paulo (14). In contrast to Pena et al. (14), in this study, typing data was obtained from only 7 of the markers tested. It is likely that the remainder were negative due to low parasite loads. Having complete typing data for all 11 markers would certainly provide a more accurate picture of the present study sample. However, the 7 unique results obtained emphasize the importance of wild animals and the utility of road-killed specimens to the study of pathogens causing infectious diseases. The distinctiveness of these genotypes demonstrates that *T. gondii* is constantly adapting to its environment, as observed by Su et al. (8), Pena et al. (14), and Da Silva et al. (4), with mutations and adaptive changes in clonal populations. Most of the animals identified in this work become infected through different routes. Therefore, further study of these species may provide valuable epidemiological information, supplying answers to the many questions concerning the adaptation and transmission of *T. gondii* to new hosts, its resistance, and the development of future vaccines.

Thus, road-killed wild animals may serve as an important *T. gondii* reservoir, contributing to its transmission to domestic and wild animals, as well as humans.

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Conflict of interest

The author declares there is no conflict of interest.

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