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Molecular identification of *Toxoplasma gondii* in roadkill wild animals in Yucatan, Mexico

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Abstract

Toxoplasma gondii is an obligate intracellular protozoan parasite, recognized as the etiologic agent of toxoplasmosis, a zoonotic endemic disease in several countries, including Mexico. In the Yucatan State of Mexico, Toxoplasma infection has a high impact in both human and domestic animal health. Wild animals can also host zoonotic pathogens such as *Toxoplasma gondii*. The presence of *Toxoplasma gondii* DNA in roadkill wild animals in Yucatan was detected using a nested Polymerase Chain Reaction. *Toxoplasma gondii* DNA was identified in several organs retrieved from a Yucatan squirrel (*Sciurus yucatanensis*), a coatimundi (*Nasua narica*), and a greater grison (*Galictis vittata*). The amplified fragments of *Toxoplasma gondii* DNA were purified, sequenced, and certified by BLAST analysis. Our results confirm that *Toxoplasma gondii* can infect wild mammals from Yucatan, which could act as intermediate hosts and contribute to the transmission of the disease to humans and domestic animals, as well as other wild animal species. We present the first molecular evidence of *Toxoplasma gondii* in a squirrel and a coatimundi from Yucatan, and quite possibly in a greater grison at a global level.

Keywords: *Toxoplasma gondii*; roadkill; wild animals; Yucatan; Mexico

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Introduction

Toxoplasma gondii is an obligate intracellular protozoan parasite that infects a large variety of warm-blooded animal species, including humans. One-third of the human population has likely been exposed to this parasite. Moreover, Toxoplasma infection has been recognized in some countries as an important concern for animal and public health.[1] Infection occurs mainly by accidental ingestion of oocysts present in the environment, the consumption of bradyzoites found in contaminated tissues or organs of intermediate hosts, or by transplacental transmission of tachyzoites (vertical infection).[2]

The *T. gondii* infection (clinically named toxoplasmosis) affects several organs, especially the lungs, the central nervous system (CNS) and the eyes.[3] The prevalence rate varies between countries (10%-80%) due to diverse climate conditions that impact oocyte viability. Human factors such as personal hygiene, sanitary conditions, feeding habits, drinking water quality and management practices in livestock production systems can also affect infection rates.[4]

Many wild animal species are susceptible to be infected by *T. gondii*. In fact, several studies have identified high infection rates in zoo animals and wild birds.[5] Moreover, *T. gondii* has a high level of genetic variation in these animals[6]. Recognizing the distribution of *T. gondii* in wild animals is essential to understand the transmission cycle of this parasite.[5] Circulation of *T. gondii* in wild ecosystems is a result of environmental contamination with oocysts disseminated by wild or domestic felids (final hosts).[6]

In Mexico, human toxoplasmosis has been detected in different regions of the country, particularly in tropical areas where the parasite remains infectious for long periods of time, due to the prevailing environmental conditions (*i.e.* optimal temperature and humidity). Several studies have also been performed in diverse mexican animal populations (wild and domestic), which have reported different infection rates.[7]

The aim of this study was to identify *T. gondii* DNA in tissue samples from roadkill wild animals in Yucatan, Mexico, to contribute with the epidemiologic understanding of toxoplasmosis in the region. We present the first molecular evidence of *Toxoplasma gondii* in a squirrel and a coatimundi from Yucatan, and quite possibly in a greater grison at a global level.

Material and methods

Animal sampling

Four roadkill wild animals were studied: a coatimundi, a greater grison, a Yucatan squirrel, and a tayra (*Eira barbara*). This last species is also known in the region as *"cabeza de viejo"*. Species identification was performed by veterinarians, according to information found in the book "A Field Guide to the Mammals of Central America and Southeast Mexico".[8]

All animals were collected from October to December 2016, at different points of the "Merida-Cenotillo" highway. Carcasses were inspected before retrieval to ascertain post-mortem changes and were later transported to the laboratory inside a plastic cooler with ice. Upon arrival, specimens were submitted to a second inspection, and a necropsy was performed. All animals seemed to have had a good

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body condition before death. Nematodes inside cardiac cavities and trachea were found only in the coatimundi carcass (data not shown).

Different tissues were collected during the necropsy (care was taken to only sample tissues that were inside body cavities and that did not contact the floor at any time, to avoid possible environmental cross-contamination). Samples were kept frozen at -70 °C in 1.5 mL centrifuge tubes containing 96% ethanol, until processed for total DNA extraction.

Total DNA extraction and Toxoplasma gondii molecular identification

All biological samples were washed with bidistilled water for five minutes to eliminate alcohol excess, before the total DNA extraction process.

For total DNA extraction a QIAamp DNA Mini Kit® was used (QIAGEN; Hilden, Germany). DNA purification from tissues followed manufacturer specifications. The DNA extraction process was performed inside a laminar flow hood (LABCONCO®; Kansas City, United States) to prevent contamination. Extracted DNA was quantified by spectrophotometry (NanoDrop 2000^{TM}, Thermo Scientific®, Wilmington, United States) and preserved at -70°C until used for PCR.

A fragment of *T. gondii* B1 gene was identified by nested polymerase chain reaction (nested PCR).

For the first reaction, the primers described by Sroka *et al.* (2009) were used: Pml/S1 (5′-TGTTCTGTCCTATCGCAACG-3 ′) and Pml/AS1 (5′-ACGGATGCAGTTCCTTTCTG-3′). For the second reaction, the following primers were used: Pml/S2 (5′-TCTTCCCAGACGTGGATTTC-3′) and Pml/AS2 (5′-CTCGACAATACGCTGCTTGA-3′). Both reactions amplified a 560 base pair (bp) segment. Reagents used in both reactions had the following concentrations in a final volume of 25μL: 5X PCR Buffer, 25mM MgCl2, 1mM dNTP′s, 10μM of each primer, 5U Taq polymerase (Thermo Scientific® Inc.; Waltham, Massachusetts, United States), 3ml template DNA, and distilled water. The thermocycler conditions were: initial denaturation at 95 °C for three minutes, followed by 35 cycles at 95 °C for 30 seconds, 64.2 °C for 30 seconds, and 45 °C for 45 seconds; the final extension was 72° C for five minutes.

All reactions included positive (total DNA from a brain of a [BALB/c] mouse infected with *T. gondii*) and negative controls (sterile water). Electrophoresis was performed in 8% agarose gels stained with ethidium bromide. To visualize bands, a Bio-Rad® photodocumentation system was used (Bio-Rad®, California, United States).

Sequencing and alignment analyses

PCR positive amplicons were purified with the Zymoclean^{TM} Gel DNA Recovery kit (Zymo Research, The Epigenetics Company^{TM}, California, United States) and sent to a private laboratory for sequencing (DIMYGEN http:// http://www.dimygen.com/).

Obtained sequences were contrasted with data stored in the GenBank®, using the Basic Local Alignment Search Tool (BLAST), implemented by the

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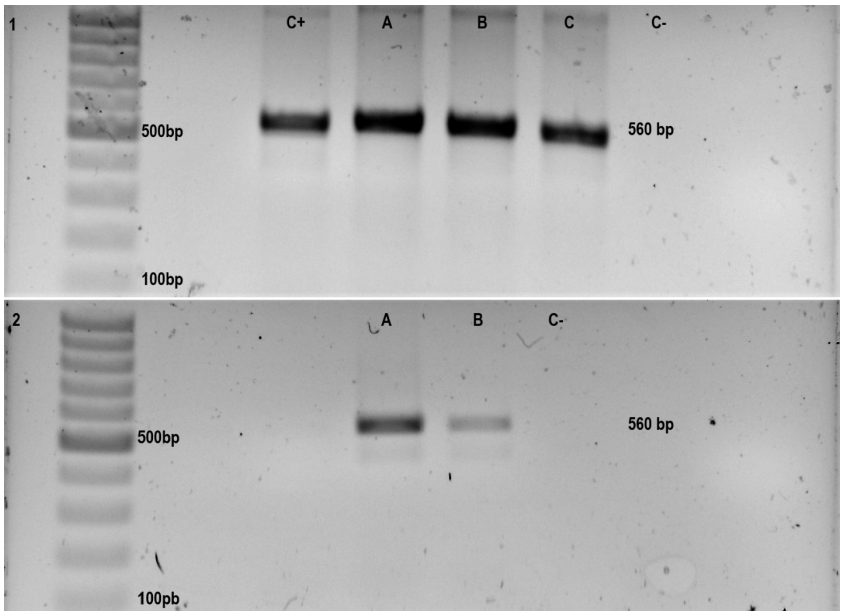

Figure 1. Agarose gel presenting PCR amplicons (560 bp) positive to *Toxoplasma gondii*. 1) C+: positive control; A: Yucatan squirrel liver sample; B: great grison femoral muscle sample; C: coatimundi kidney sample; C-: negative control. 2) A: Yucatan squirrel brain sample; B: great grison lung sample; C-: negative control.

National Institute of Health (NIH, United States; http://blast.ncbi.nlm.nih.gov/Blast.cgi), to determine identity similarity and coverage percentages.[9-12]

Results and discussion

Toxoplasma gondii DNA was detected in several tissues from a Yucatan squirrel, a coatimundi, and a great grison (Fig. 1). Table 1 summarizes wild animals studied, tissues sampled, and nested PCR results.

Identity and coverage percentages obtained in the BLAST analysis are shown in Table 2. All sequences were homologous to a previously sequenced *T. gondii*.

The use of wild animals for the molecular detection of *T. gondii* and other zoonotic pathogens in roadkill samples is an efficient alternative to live wild animal research. Moreover, molecular tools allow *T. gondii* identification with high sensitivity and specificity, without the need of arduous microbiological cultures and histopathological examinations.[13]

In Yucatan, Mexico, *T. gondii* is widespread. There are frequent reports of infection in domestic animals such as cats[14] and pigs,[15,16] as well as in wild animals such as opossums[17] and synanthropic rodents,[18] and also in humans.[19-22] Additionally, *T. gondii* oocysts have been identified in drinking water sources,[23] which states the relevance of the toxoplasmosis in the region. However, *T. gondii* identification in other wildlife populations are very scarce.[7]

Intermediate hosts, such as domestic livestock (pigs, chickens, goats, and sheep) and wild animals (rodents, wild boars, foxes, and wild birds) get infected by ingesting sporulated *T. gondii* oocysts found in food or water, or by consuming infected tissue cysts.[24]

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Table 1. Species identification and food habits, sampled tissues, PCR results and recovery site coordinates of roadkill wild animals from Yucatan, Mexico

Species common name	Scientific name	Feeding habits	Sampled tissue	*T. gondii* PCR results	Recovery site coordinates
Yucatan squirrel	*Sciurus yucatanensis*	Frugivore, insectivorous	Brain Lung Liver Kidney Spleen Femoral muscle Heart Masseter muscle	+ + + + + - - -	21°05'24.5"N, 88°30'56.0"W
Cabeza de viejo	*Eira barbara*	Omnivorous	Brain Tongue Liver Kidney Spleen Femoral muscle Heart Masseter muscle	- - - - - - - -	21°11'04.5"N, 88°33'07.1"W
Coatimundi	*Nasua narica*	Omnivorous	Brain Tongue Liver Kidney Spleen Femoral muscle Heart Masseter muscle	- - - + - - - -	21°11'33.1"N, 88°47'17.9"W
Greater grison	*Galictis vittata*	Omnivorous	Brain Lung Liver Kidney Spleen Femoral muscle Heart Masseter muscle	- + - - - + - -	21°10'44.0"N, 88°29'33.4"W

+: Positive
-: Negative
N: North
W: West

Table 2. Identity and coverage percentages obtained in BLAST analyses

Purified product name	Identity (%)	Coverage (%)	GenBank accession of the homologous sequence
T. gondii coatimundi (kidney)	99	98	AF179871.1
T. gondii great grison (femoral muscle)	100	99	AF179871.1
T. gondii great grison (lung)	100	98	AF179871.1
T. gondii Yucatecan squirrel (liver)	99	99	AF179871.1
T. gondii Yucatecan squirrel (brain)	100	100	AF179871.1

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There are several reports of *T. gondii* infection in species of squirrels all around the world, with the infection attaining different organs and tissues. Indeed, Fayyad *et al.,*[25] reported a fatal systemic *T. gondii* infection in a red squirrel (*Sciurus vulgaris*) and in a Swinhoe's striped squirrel (*Tamiops swinhoei*) from Germany. Also, Kik *et al.,*[26] described that 20 of 37 red squirrels died of a disseminated *T. gondii* infection in the Netherlands, and Jokelainen and Nylund,[27] mentioned three cases of fatal toxoplasmosis in red squirrels from Finland. This stresses the importance of this species in the life cycle of toxoplasmosis. Conversely, Suzán and Ceballos[28] did not find serological evidence of *T. gondii* in two rock squirrels (*Spermophilus variegatus*) oror a gray squirrel (*Sciurus aureogaster*) in Mexico. However, this could be due to the small sample size (n = 3).

Mode of transmission of *T. gondii* in squirrels remains unclear, but it is known that the parasite's life cycle depends on infecting felids (domestic or wild).[29] Oocysts shed in cat faeces may contaminate nuts, fungi, plant shoots, or berries, all of which are part of the diet of squirrels.[26] Omnivorous squirrels could also get infected through ingestion of contaminated animal tissues.[27]

The presence of *T. gondii* in Yucatan squirrels may indicate an important issue for human health nationwide since squirrel meat is consumed in various Mexican states;[30] especially in communities where subsistence hunting is common practice. However, further studies are needed to confirm this hypothesis.

Information about *T. gondii* in greater grison is very scarce worldwide. *Khan et al.*,[31] described the isolation of a *T. gondii* strain from one individual in the French Guiana; and Richini-Pereira *et al.*[13] reported not to have found the infection in two animals from Brazil. It is thus possible that the results shown here are the first molecular evidence of *T. gondii* in this species. Evidence of *T. gondii* infection in carnivores other than the greater grison is extensive, so the mode of transmission for this species could be similar, *i.e.* by eating infected animals such as rodents and birds.[13]

Rendón-Franco *et al.* have described a *T. gondii* infection in a coatimundi from Mexico though a serological study.[32] The identification of *T. gondii* infection in a coatimundi could have important zoonotic consequences, since this species is also hunted and consumed by several inhabitants of Mexico.[33] This is the first molecular evidence of the presence of *T. gondii* in a coatimundi from Yucatan.

Our results contribute to ascertain that *T. gondii* has a worldwide distribution, and also underline that a broad diversity of intermediate hosts may take part in the epidemiological chain of toxoplasmosis.[13] Further epidemiological studies are necessary to identify the *T. gondii* genotype(s) present in wild animals from Yucatan, Mexico.[7]

Conclusions

Results of this work confirm that *T. gondii* is present in wild mammals in Yucatan. Also, the studied species could act as intermediate hosts, helping to spread the infection to humans and other animals.[13] Finally, our study presents the first molecular evidence of *Toxoplasma gondii* in a squirrel and a coatimundi from Yucatan, and quite possibly in a greater grison at a global level.

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Conflicts of interest

The authors declare that they have no conflict of interest.

Author contributions

Marco Antonio Torres Castro: Study design, sample collection, manuscript writing and approval.

Rodrigo Adán Medina Pinto: Sample collection, manuscript writing, and approval.

Henry René Noh Pech: Data analyses and laboratory work.

Fernando I. Puerto: Manuscript writing and approval.

Roger Iván Rodríguez Vivas: Study design, manuscript writing, and approval.

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