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An exploratory survey of *Trypanosoma cruzi* infection in carnivores (*Urocyon cinereoargenteus* and *Spilogale angustifrons*) of Yucatan, Mexico

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ABSTRACT

OBJECTIVE

We carried out an exploratory study to determine the infection with *Trypanosoma cruzi* in the gray fox (*U. cinereoargenteus*) and the southern spotted skunk (*S. angustifrons*) in Yucatan, Mexico.

METHODS

We used samples from various organs (heart, skeletal muscle, kidney, spleen, liver, esophagus, and stomach) corresponding to individuals of gray fox and spotted skunk, captured in the peridomicile of eight rural localities of Yucatan between the period 1990–2008. The presence of *T. cruzi* DNA in tissue samples was determined by PCR technique and histopathology study.

RESULTS

All the individuals studied had at least one tissue sample with *T. cruzi* DNA. The esophagus (9/13), heart (7/13), and skeletal muscle (6/13) were the organs with the highest frequency of *T. cruzi* DNA. No nests of amastigotes were found, however, microscopic observation revealed lesions characteristic of *T. cruzi* infection, such as inflammatory infiltrate by lymphocytes and histiocytes with or without necrosis of cardiomyocytes cells, and proliferation of fibrocytes, fibroblasts and collagen fibers (fibrosis).

CONCLUSIONS

Our results confirm that these two wild carnivores are natural hosts for *T. cruzi* in Yucatan, Mexico. The synanthropic behavior of these two mammals in Yucatan makes it necessary to focus future studies on their role within the rural peridomiciliary transmission cycle of *T. cruzi* in the Yucatan Peninsula.

DESCRIPTORS

Urocyon, *Spilogale*, Carnivores, *Trypanosoma cruzi*, PCR, Histopathology, Yucatan.

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INTRODUCTION

Trypanosoma cruzi, is a flagellated protozoan that naturally infects approximately 180 species of mammals[1]. In domestic and peridomestic environments, the main hosts of the parasite are dogs and a wide variety of animals, especially marsupials and rodents, which play a very important epidemiological role in the transmission of the parasite[1]. In wild environments, armadillos, marsupials, rodents, and bats are the natural hosts for *T. cruzi*. However, recent studies show a high prevalence of natural infection by *T. cruzi* in carnivores, such as raccoons (*Procyon lotor*), coyotes (*Canis latrans*), gray foxes (*Urocyon cinereoargenteus*), lynxes (*Lynx rufus*) and coaties (*Nasua nasua*), suggesting the importance of carnivores in maintaining the wild cycle of *T. cruzi*[2].

The state of Yucatan, Mexico is considered an endemic area for Chagas disease[3], where several species of wild mammals have been found to be naturally infected with *T. cruzi*, among which opossums *Didelphis virginiana* is considered an important reservoir in the peridomiciliary transmission cycle of the parasite[4-7]. In Yucatan, the gray fox (*Urocyon cinereoargenteus*), the skunk (*Spilogale angustifrons*), and, to a lesser extent, the coati (*Nasua narica*) and the raccoon (*Procyon lotor*) are carnivores that are frequently observed in the backyards of rural houses, mainly where crops are present[8]. Sightings of wildlife in the Yucatecan human environment are increasingly frequent, both in rural and urban locations, probably due to the growth of urban and commercial developments. The presence of gray foxes and skunks is a phenomenon that must be investigated in terms of both ecological impact and the risk of transmission of zoonotic pathogens. The present study aims to explore the presence of infection with *T. cruzi* using preserved biological samples from a bank of tissue samples from synanthropic carnivores, to obtain novel information on natural hosts of *T. cruzi* that contributes to the knowledge of role of wild mammals in the transmission cycle of *T. cruzi* in areas tropical trees of Mexico.

METHODS

Study area

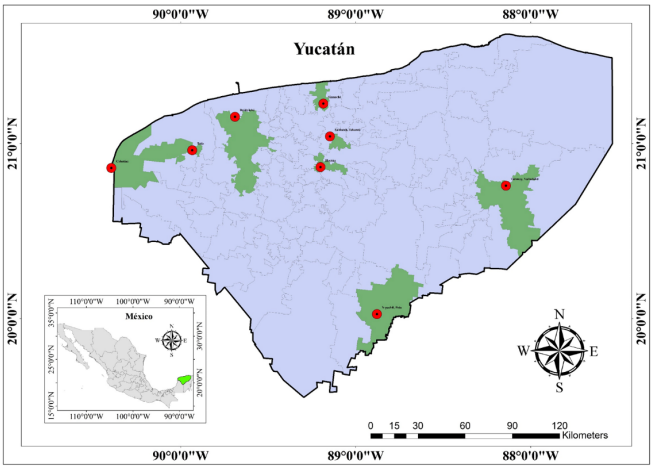
The state of Yucatan is located in the extreme north of the Yucatan Peninsula in southeastern Mexico (19°29' and 21°37' north latitude and 87°32 ' and 90°25' west longitude). It is bordered to the north by the Gulf of Mexico, to the east and southeast by Quintana Roo, and to the west and southwest by Campeche.

Biological samples

Fifty tissue samples from heart, muscle, spleen, liver, kidney, esophagus, and stomach of 10 adult individuals of *U. cinereoargenteus* (41 samples) and 2 adult individuals and a juvenile individual of *S. angustifrons* (nine samples) were used. All samples were obtained from the bank of biological samples of Laboratorio de Zoonosis y Otras ETV's of the Centro de Investigaciones Regionales "Dr. Hideyo Noguchi" of the Universidad Autónoma de Yucatán. This bank stores different kind of biological samples obtained from mammals captured from different research projects carried out between the period 1990–2008 in household backyards of rural localities in the state of Yucatan.

The selected samples for the study, come from wild carnivores collected in different rural localities (Figure 1), and all of them were preserved in paraffin cubes and preparations of histological sections stained with Hematoxylin-Eosin (H&E).

Figure 1. Geographical location of the rural localities of the state of Yucatan where the animals whose samples analyzed in this study were captured.



Diagnosis of Trypanosoma cruzi infection by PCR

DNA extraction from paraffin-embedded samples: Five µm-thick sections were obtained with the aid of a microtome and placed in 1.5 mL microcentrifuge tubes. The paraffin was removed by consecutive washes of xylene, 100% ethanol, 70% ethanol, and distilled water (20 min each). Subsequently, 200 µl of lysis buffer (200 mM Tris-HCl pH 7.5, 250 mM NaCl, 25 mM EDTA and 0.5% SDS) and Proteinase K (10 µg/ml) were added to each sample and incubated at 60 °C for at least two and a half hours[9]. Extraction and purification of DNA, was completed with the Maxwell® 16 semi-automated extraction system and using the FFPE Tissue LEV DNA Purification Kit (Promega, Madison, WI, USA), following the manufacturer's instructions.

DNA extraction from stained histological preparations: For tissues obtained from H&E-stained histological preparations, tissue fragments were manually removed from the slides using a scalpel edge for each sample. Subsequently, the removed samples were deparaffinized by consecutive washes with xylene, 100% ethanol, 70% ethanol and distilled water for 10 minutes for each solution. Finally, the samples were allowed to dry for 20 minutes at room temperature and were placed in 200 µl PCR tubes and then for their conservation in freezing at -20 °C until their analysis[10,11] (see below).

Polymerase Chain Reaction: For the paraffin-embedded samples 3 µL of DNA were mixed with Go Taq Green Master Mix (Promega, Madison, WI, USA) 1X and 10 pM of each synthetic oligonucleotide: 121 (sense) 5'- AAATAATGTACGG (T/G) GAGATGCATGA-3 'and 122 (antisense) 5'- GGTTCGATTGGGGTTGGTGTAATATA-3', which amplify 330 base pairs of the kDNA of the parasite and a sensitivity of 95 %[12-14]. For the samples from stained histological preparations, a direct PCR was done using the Phusion Blood Direct PCR kit (Thermo Scientific, Carlsbad, CA, USA), which does not require DNA extraction. 1X Phusion Blood PCR Buffer, water, and 10 pM of each synthetic oligonucleotide (primers 121 and 122), were added to these samples[12]. After several tests and in order to improve the quality of the aforementioned PCR product[15], reamplification was performed using the Go Taq Green Master Mix (Promega) 1X. Genomic DNA of the *T. cruzi* strain H4 was used as a positive control and the reaction mixture without DNA was used as a negative control.

The PCR product of both types of samples, was run by submarine electrophoresis in 1.5% agarose stained with ethidium bromide (5 µg/mL) for 30 min at 100V in 1X TBE buffer (tris-boric acid-EDTA).

Histopathological study

All paraffin-embedded samples were processed for the

preparation of 5 µm thick histological sections, stained with H&E, and mounted with synthetic resin on slides. Before being processed for DNA extraction, all the samples preserved in histological preparations stained with H&E, were also examined microscopically at different magnifications of 4×, 10×and 40×. The positive analysis consisted of observing microscopic lesions compatible with *T. cruzi* infection[16].

RESULTS

Trypanosoma cruzi DNA amplification in tissues

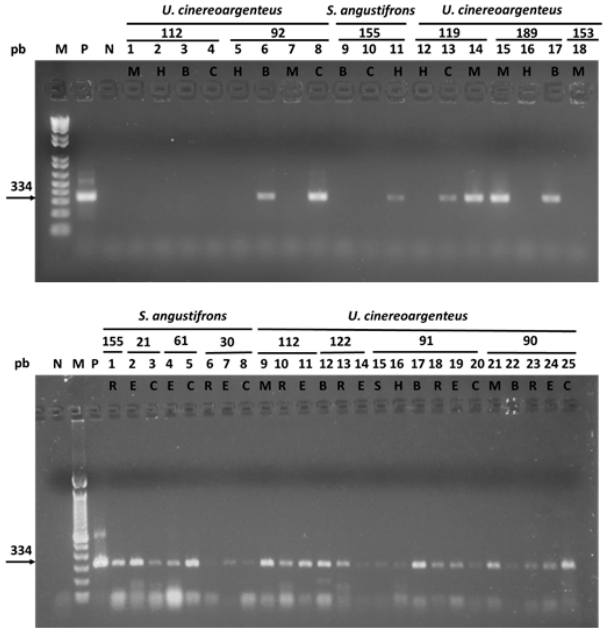
At least one tissue sample from all animals studied, and 76% of the total analyzed samples (37/50), revealed the presence of *T. cruzi* DNA (31 gray fox samples, and 6 from skunk samples) (Figure 2). According to the results of the PCR, the esophagus (90%), skeletal muscle (85%), and heart (70%), were the tissues with the highest frequency of *T. cruzi* DNA (Table 1).

Table 1. List of tissue samples positive and negative for *Trypanosoma cruzi* DNA by organs and host species.

Total	*U. cinereoargenteus*		*S. angustifrons*	
Organs	Positive	Negative	Positive	Negative
Heart	5	2	2	1
Spleen	5	1	NE	1
Liver	2	4	1	NE
Kidney	5	1	1	1
Skeletal muscle	6	1	NE	NE
Esophagus	7	1	2	NE
Stomach	1	NE	NE	NE

NE = Non-Existent

Figure 2. 1.5% agarose gel electrophoresis of paraffin-embedded and histological preparation samples of *U. cinereoargenteus* and *S. angustifrons*. M = weight molecular marker (100 bp), P = positive control (*T. cruzi* DNA), N = negative control (mixture without DNA). The numbers below the scientific names correspond to the code of each individual and the numbers below these correspond to the lanes. The letters correspond to the type of tissue analyzed: C = heart; E = esophagus; R = kidney; B = spleen; M = muscle; H = liver; S = stomach.



Histopathological study

Lesions compatible with infection with *T. cruzi* were observed in tissue samples of both species, however, amastigote nests were not found (See Table 2; Figure 3). Overall, all injuries observed in the tissue samples were mild. In *U. cinereoargenteus*, lesions compatible with *T. cruzi* were found in cardiac muscle in one of five PCR-positive individuals (20%), in one of six (17%) in skeletal muscle and in one of seven (14%) in the esophagus; in the case of parenchymal organs, the kidney was the most affected in three out of five individuals (60%), and in one of two (50%) the liver was the most affected. In *S. angustifrons*, in one of two individuals (50%) positive for PCR compatible with *T. cruzi*, were found only in cardiac muscle; in parenchymal organs, only the liver of one individual had compatible lesions. In both *U. cinereoargenteus* and *S. angustifrons*, the PCR negative samples showed lesions, however, these were not compatible with the histopathology associated with *T. cruzi* (Table 2).

Figure 3. Histological lesions observed in tissue samples of *U. cinereoargenteus* (**A-C**) and *S. angustifrons* (**D**) PCR-positive to ADN of *T. cruzi*. A) Lymphohistiocytic infiltrate in heart (10×), B) cardiomyocytes necrosis in heart (10×), C) esophagus with inflammatory infiltrate (10×), D) inflammatory cell in cardiac tisue (40×).

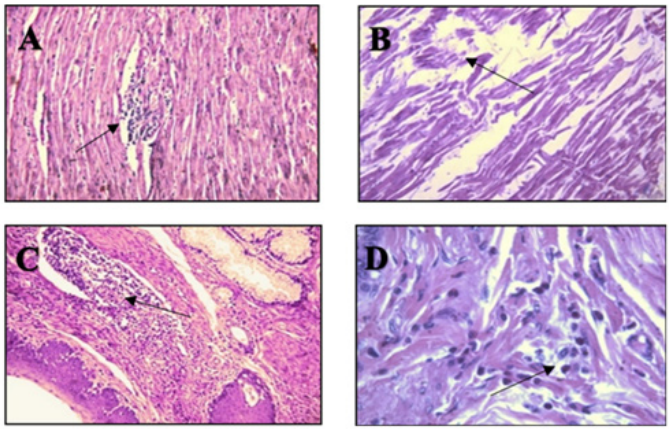


Table 2. Histological lesions and their frequency observed in tissue samples of *Urocyon cinereoargenteus* and *Spilogale putorius* positive and negative to DNA of *Trypanosoma cruzi*.

Species	Tissue	*T. cruzi* DNA	Histological lesion	n (%) tissue samples
U. cinereoargenteus	Heart	Positive	Moderate multifocal cardiomyocyte necrosis	1/5 (20%)
			Mild multifocal lymphohistiocytic inflammation	1/5 (20%)
		Negative	Moderate multifocal lymphocytic, histiocytic, eosinophilic infiltrate	1/2 (50%)
			Cardiomyocyte necrosis	1/2 (50%)
			Hydropic degeneration	1/2 (50%)
	Skeletal muscle	Positive	Mild multifocal necrosis	1/6 (16.6%)
	Esophagus	Positive	Moderate multifocal acanthotic hyperplasia	4/7 (57.1%)
			Mild focal lymphocytic infiltrate	1/7 (14.2%)
		Negative	Moderate acanthotic hyperplasia	1/1 (100%)
	Kidney	Positive	Mild multifocal lymphocytic interstitial infiltrate	3/5 (60%)
			Hydropic degeneration	1/5 (20%)
			Tubular epithelium necrosis	1/5 (20%)
		Negative	Hydropic degeneration	1/1 (100%)
	Liver	Positive	Lymphohistiocytic interstitial infiltrate	1/2 (50%)
		Negative	Multifocal hydropic degeneration	2/4 (50%)
	Spleen	Positive	Lymphoreticular tissue atrophy	1/5 (20%)
			Multifocal hyperplasia of lymphoid follicles	2/5 (40%)
		Negative	Moderate lymphoreticular hyperplasia	1/1 (100%)
S. angustifrons	Heart	Positive	Mild multifocal hydropic degeneration	1/2 (50%)
		Negative	Muscle necrosis	1/1 (100%)
			Mild multifocal infohistiocytic infiltrate	1/1 (100%)
	Esophagus	Positive	Multifocal moderate acanthotic hyperplasia	2/2 (100%)
			Mild multifocal eosinophilic infiltrate	2/2 (100%)
	Kidney	Negative	Multifocal hydropic degeneration of the tubular epithelium	1/1 (100%)
			Tubular epithelium necrosis	1/1 (100%)
	Liver	Positive	Moderate multifocal hydropic degeneration	1/1 (100%)
			Periportal lymphocytic infiltrate	1/1 (100%)
	Spleen	Negative	Moderate lymphoreticular hyperplasia	1/1 (100%)

DISCUSSION

The presence of *Trypanosoma cruzi* DNA in tissues samples of all individuals from *Urocyon cinereoargenteus* and *Spilogale angustifrons* captured in different localities of the state of Yucatan, reveals that these carnivores are part of the natural hosts of this protozoan in the region. Although [17]Zavala-Velázquez *et al*. (1996) in Yucatan, and [18]Zamora-Ledesma *et al*. (2016) in Queretaro, had already reported serological reactivity for *T. cruzi* in gray fox, our findings confirm its natural infection with *T. cruzi*, and the first infection record for *S. angustifrons* in Mexico.

Although vectorial *T. cruzi* transmission is the epidemiologically most important pathway for humans, this does not appear to be the case for natural hosts of *T. cruzi*. Various studies have indicated that habits of mammals such as grooming, feeding, and the predator-prey relationship, are common direct transmission routes of the parasite between its different natural hosts[19,20]. The acquisition of infection with *T. cruzi* via the oral route is probably the most important among species with carnivorous habits. This has been demonstrated experimentally in skunks (*Mephitis mephitis*) and naturally in raccoons (*Procyon lotor*), through the ingestion of infected triatomines, and meat contaminated with trypomastigotes, respectively[21,22]. Furthermore, it has been shown that *T. cruzi* can invade and replicate in the epithelium of the gastric mucosa[23,24]. It is very likely that, due to the feeding type of foxes (omnivorous opportunistic) and skunks (omnivorous), the predation of carrion and small prey such as rodents, and insects in dry seasons[25,26], confer them a natural susceptibility to acquiring infection with *T. cruzi* in their sylvatic habitat. In Yucatan, a high prevalence of *T. cruzi* has been reported in commensal rodents and a high rate of capture and infection of *Triatoma dimidiata* in the dry season in peridomiciles of the state[6,27], which constitutes a suitable epidemiological scenario to explain that the high number of positive samples in digestive tract (esophagus, and stomach) of the mammals found in this study, were acquired through natural predation of gray foxes and skunks in their habitats.

Due to their biology and ecology, carnivores can have an important effect on multi-host parasitic transmission networks, as reflected in the high prevalence of infection with *T. cruzi* reported in carnivores[19,22,28-31]. In Brazil, the coati (*Nasua nasua*) seems to be playing a role in the maintenance and dispersal of *T. cruzi*, due to its high parasitic load and long duration[28]. In some areas of the United States, the high prevalence of infection with *T. cruzi* in wild carnivores, particularly for *U. cinereoargenteus*, varies from 7.6 to 13%[31,32]. Similarly, there are few studies that report infection with *T. cruzi* in skunks, mainly focused on *Mephitis mephitis*, in which prevalence of infection in a range of 3 to 32% have been reported[29,33]. In the state of Yucatan, only one study has reported seropositivity for *T. cruzi* in gray foxes, but not for skunks[17].

In the histopathological analysis of the tissue samples studied for both species, no parasite nests were observed, and the lesions found, most frequently in *U. cinereoargenteus*, were both cardiac and skeletal muscle. For *S. angustifrons*, compatible lesions due to *T. cruzi* were observed only in cardiac tissue since skeletal muscle samples, were not available for this species. The lesions found in this study, were similar to those reported in other natural hosts of *T. cruzi*, including carnivores[22,31,34-36]. Regarding the parenchymal organs, the kidney and the liver were the most affected for *U. cinereoargenteus* and *S. angustifrons*, respectively, which are similar to those lesions reported by different authors in these same organs[34-36]. The "absence" of *T. cruzi* nests in the tissue sections analyzed could be showing a mild parasite burden in these hosts, therefore supporting their role as natural reservoirs. Furthermore, observing parasite nests in a single 5 micron-thick slice of an entire organ may indicate high luck or evidence of an extensive cellular invasion by the parasite. The latter does not appear to be the case in this study.

Despite that in this study some lesions, such as infiltrates (multifocal lymphocytic, histiocytic, and eosinophilic), cardiomyocyte necrosis, and hydropic degeneration of the heart, acanthotic hyperplasia of esophagus, hydropic degeneration of hepatocytes, and lymphoreticular hyperplasia of the spleen, were observed in PCR negative samples, these were not compatible with infection with *T. cruzi*.

Although the findings obtained in this study are of preliminary and exploratory type, and the samples used were obtained and preserved for 10 and 30 years ago, we consider that they are relevant because they provide information on natural circulation of *T. cruzi* in wild synanthropic carnivores in the state of Yucatan. However, a recent study that synthesizes the cases of zoonotic diseases reported between 1995 and 2019 in the Yucatan Peninsula, shows that *T. cruzi* continues to circulate among synanthropic animals mainly rodents, opossums and dogs, as well as wild animals such as bats and rodents[37]. The capture of these carnivores in human ecotopes (such as all individuals analyzed in this study), suggests that these mammals could be involved in the peridomestic cycle and the flow of strains of *T. cruzi* of wild origin towards the rural human peridomiciliary area of Yucatan. Therefore, in future studies, it is necessary to identify the DTU (*Discrete typing units*) of the *T. cruzi* strains that circulate in these species. Understanding the population genetics of *T. cruzi* is critical to discerning parasite flow within landscapes, particularly in relation to fragmentation and change in land use, where there are native and non-native community structures[38]. A recent study in the Yucatan Peninsula provides us with an idea about the ecological connectivity of the host communities of mammals, vectors, and *T. cruzi*[39].

Finally, due to the scarce existing information on the ecology and biology of the natural populations of *S. angustifrons* and *U. cinereoargenteus* in the Yucatan Peninsula, it is necessary to carry out studies on the subject to elucidate the extent to which these carnivores could be participating in the transmission cycles of *T. cruzi*.

CONCLUSION

This study reveals that gray fox and southern spotted skunk are natural hosts of *T. cruzi* in Yucatan and was reported the first infection record for *S. angustifrons* in Mexico.

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