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Absence of infection by *Trichinella* spp. (Nematoda: Trichinellidae) in free-living wild carnivores in Brazil

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Background: Nematodes of the genus *Trichinella* are foodborne zoonotic pathogens that are widespread globally. These parasites have two epidemiological cycles, domestic and sylvatic, with the latter having wild carnivores as the main reservoirs of the parasite. *Trichinella* spp. have been increasingly detected in wild carnivores in Argentina and Chile. Although the disease is absent in domestic animals in Brazil, there is serological evidence that the agent is circulating in wild boars in some areas. This study aimed to diagnose *Trichinella* spp. infection through artificial tissue digestion and histopathology of selected tissues of wild carnivores from São Paulo state, southeastern Brazil.

Methods: Tissue samples (forearm muscles, diaphragm, and tongue) from 53 wild carnivores (21 Canidae, 25 Felidae, 04 Mustelidae, 03 Procyonidae) were used, along with a retrospective study of the slide bank, considering samples from the period 2010 to 2021, totaling 89 free-living carnivores (42 Canidae, 42 Felidae, 03 Mustelidae, 02 Procyonidae).

Results: Either artificial digestion or histopathological analyses did not reveal any larvae suggestive of *Trichinella* spp., indicating that the nematode was not circulating within the target population.

Conclusion: To date, there is no direct evidence of nematode circulation in wild carnivores in the study area.

KEYWORDS

wild animals, zoonoses, parasites, neotropics, one health

Introduction

Nematodes of the genus *Trichinella* Railliet, 1895 (Nematoda: Trichinellidae) are widespread zoonotic pathogens that affect a wide range of hosts, from domestic and wild animals to humans (1). Currently, ten species and three genotypes of *Trichinella* are known, separated into two clades, one which includes species capable of modifying myocytes into nurse cells and encapsulating themselves in the muscle tissue of the hosts, and the second which includes species incapable of this tissue modification (1, 2). Although the genus *Trichinella* has been known and studied since its description in 1835, knowledge about the genus is still dynamic, with a new species described in Canada in 2020 from samples of a wolverine (*Gulo gulo*), a wild mustelid common in North America (1, 3).

Several hosts, such as wild carnivores, rodents, wild boars, birds and reptiles, have already been related to the epidemiological chain of trichinellosis (4, 5). The cycle is divided into two categories: a domestic cycle, where domestic pigs are the main reservoirs, and a sylvatic cycle, which has wild boars and wild carnivores as the main hosts of the nematode (6). Noteworthy are the feeding habits of carnivores, which includes rodents species, which are also an important host for the parasite (7). Transmission occurs through ingestion of muscle tissue infected with larvae, and behaviors such as predation, cannibalism and necrophagy are closely related to infection in animals. In humans, the infection is mainly associated with the consumption of raw or undercooked pork and game meat, also without veterinary inspection (1, 8, 9).

In South America, the parasite has been detected in wild animals such as pumas (*Puma concolor*), opossums (*Didelphis albiventris*), wild boars (*Sus scrofa*), armadillos (*Chaetophractus villosus*) and sea lions (*Otaria flavescens*) in Bolivia, Argentina, Ecuador and Chile (7, 10). In Brazil there is only serological evidence that the agent is circulating in wild boars (11). In contrast, Argentina reports a high number of human infections, with more than 6,000 cases reported between 2012 and 2018 (12). Also, Chile e Bolivia reported cases in humans by the detection of anti-*Trichinella* antibodies (12, 13). However, studies in other South American countries are scarce or absent, hindering the proper comprehension of the epidemiology of human trichinellosis in this region.

Considering the importance of wild carnivores in the epidemiology of trichinellosis, this study aimed to investigate *Trichinella* spp. infection in free-living wild carnivores in the state of São Paulo, Brazil.

Materials and methods

Animals and study area

This study was conducted only with free-living carnivores, using samples of animals that had been road killed and histopathology slides deposited in a biological collection. The samples came from 51 municipalities in the state of São Paulo, covering an area of approximately 24,236.83 km². The estimated population of the area is 4,257,120 inhabitants, with an HDI (Human Development Index) ranging from 0.681 (Boa Esperança do Sul) to 0.829 (Bebedouro). The study region comprises the Cerrado and Atlantic Forest biomes and ecotones between them. The regional economy is based on agricultural activity, with sugar cane, oranges, soybeans, peanuts, corn and cattle farming predominating (14). Twenty-seven species of wild carnivores, representing the families Felidae, Canidae, Mustelidae, Procyonidae and Mephitidae are registered in these biomes (15). Invasive species are also reported in these area, such as wild boars (*Sus scrofa*), synanthropic rodents, european brown hare (*Lepus europaeus*) and feral domestic dogs (*Canis familiaris*) and cats (*Felis catus*) (16).

Between April 2022 and July 2023, 53 carcasses (21 Canidae, 25 Felidae, 04 Mustelidae, 03 Procyonidae) were collected from animals hit by cars on highways in 17 municipalities of São Paulo state. These carcasses were sent by the Environmental Police, Fire Brigade and highway concessionaires to the Wild Animal Pathology Service (SEPAS) at FCAV/Unesp Jaboticabal/SP, the Bauru/SP Municipal Zoological Park, the São Simão, Santa Rita do Passa Quatro and Luis Antônio experimental stations of the São Paulo Forest Foundation, the "Quinzinho de Barros" Municipal Zoological Park and the Center for Wild Animal Medicine and Research (CEMPAS) - UNESP/FMVZ and kept in freezers at -20°C until processing. After thawing, muscle samples from the arm, diaphragm, and tongue were taken for histopathological analysis and artificial digestion.

In addition, histological slides were selected from 89 free-living wild carnivores (42 Canidae, 42 Felidae, 03 Mustelidae, 02 Procyonidae) deposited in the collection of the Wild Animal Pathology Service (SEPAS) at FCAV/UNESP. Animals with histological sections of at least one of the abovementioned tissues were included in the study. These animals selected came from 39 municipalities in São Paulo and were necropsied between November 2010 and December 2021.

The municipalities of origin of the animals studied are in Figure 1 showing the road killed animals' origins and Figure 2 showing the slide bank samples' origins. Table 1 compiles all the animals used in the study.

Sample collection

Fifty grams of forearm, tongue and diaphragm muscles were collected (17, 18). The samples were stored at -20°C in plastic bags labeled with the species, sex, date and collection place until processing. In addition, a 1 cm^3 fragment of these tissues was fixed in a 10% buffered formalin solution for histological processing.

Artificial digestion (AD) technique

The tissue samples were subjected to the Artificial Digestion Technique (AD) based on the magnetic stirrer method according to European regulation EC 2075/2005, which is used for the surveillance of *Trichinella* spp. infections in wild animals. The samples (20 g) were crushed and digested using 400 ml of artificial digestive fluid (20 ml for each gram of muscle) consisting of 1% pepsin (1:10,000 *US National Formulary*) and 1% hydrochloric acid (HCl). The digested tissue was stirred for 60

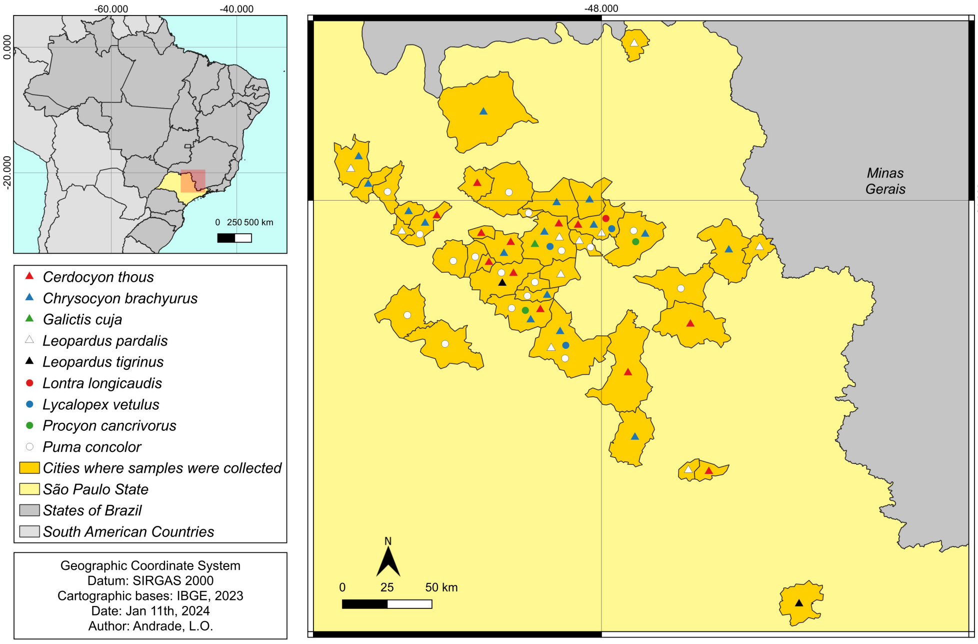

FIGURE 1
Cities and species from São Paulo State where the road killed carcasses were obtained (Assis, Bauru, Bebedouro, Boa Esperança do Sul, Itápolis, Jaú, Jundiaí, Martinópolis, Monte Azul Paulista, Palmital, Presidente Bernardes, Presidente Prudente, Piratininga, Santa Cruz do Rio Pardo, Santa Rita do Passa Quatro, São Simão, Tambaú) between April 2022 and July 2023.

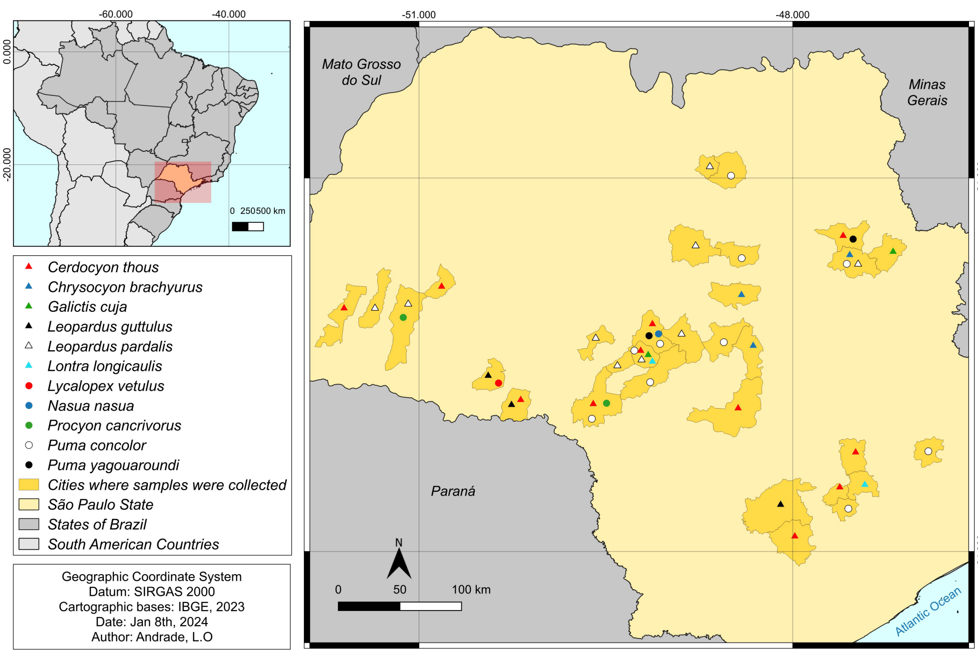

FIGURE 2
Cities and species from São Paulo State where the slide bank carcasses were obtained (Araraquara, Aramina, Barretos, Barrinha, Borborema, Cajuru, Cândido Rodrigues, Catanduva, Catiguá, Cássia dos Coqueiros, Cedral, Cordeirópolis, Dobrada, Elisiário, Fernando Prestes, Guariba, Ibitinga, Itirapina, Jaboticabal, Jundiaí, Matão, Monte Alto, Monte Azul Paulista, Pitangueiras, Pontal, Ribeirão Preto, Santa Adélia, Santa Rita do Passa Quatro, Santa Gertrudes, Santa Ernestina, São José do Rio Preto, São Carlos, São Simão, Sertãozinho, Taquaritinga, Taquaral, Uchôa and Vista Alegre do Alto) between November 2010 to December 2021.

TABLE 1 Species of carcasses and collection of slides of wild carnivorous mammals used in the study.

	Roadkill carcasses	Slide bank	Total
Canidae			
Cerdocyon thous	17	19	36
Chrysocyon brachyurus	03	20	23
Lycalopex vetulus	01	03	04
Felidae			
Puma concolor	11	29	40
Leopardus pardalis	09	11	20
Leopardus guttulus	03	02	05
Herpailurus yagouaroundi	02	N/A	02
Mustelidae			
Lontra longicaudis	02	02	04
Galictis cuja	02	01	03
Procyonidae			
Procyon cancrivorous	02	02	04
Nasua nasua	01	N/A	01
Total	53	89	142

N/A, not available.

min or more at 44-46°C in a 600 ml glass beaker using a heated magnetic stirrer plate. After digestion, the fluid was sieved through a 180 µm mesh sieve into the separatory funnel and left to stand for 30 minutes. After this period, 40 ml of the sediment sample was quickly released from the funnel into a 50 ml beaker and sedimented again for 10 minutes. Next, 30 ml of supernatant was removed from the 50 ml beaker and the 10 ml of sediment was poured into a Petri dish. Finally, the 50 ml beaker was rinsed with 10 ml of water and the liquid was added to the Petri dish. The sample was analyzed under a stereomicroscope (Leica EZ4 HD, Leica Microsystems© Limited) at 15 to 40x magnification.

Histopathology

The tissues collected after 24 to 48 hours in 10% phosphate-buffered formalin (pH 7.4) were processed according to routine, embedded in paraffin, cut at 3µm and mounted on histological slides stained with hematoxylin and eosin. Readings were made using an Olympus BX-51® optical microscope equipped with a Q-color3® camera at 10x and 40x magnification.

Results and discussions

No *Trichinella* spp. larvae were found in this study, either by artificial digestion or histopathological analysis. To date, there has been no record of diagnosis of nematode larvae in any study carried out in Brazil, whether in domestic or wild animals. More than 15,000 domestic pigs had samples analyzed by AD in the state of Paraná between 2002 and 2011, all of which were negative (19–21). Furthermore, 14,852 horses were tested in the states of Minas Gerais, Goiás, and Bahia between 2014 and 2016, with no observation of the nematode (22). In addition, 594 rats were tested in the region of Santos, São Paulo and not a single sample was positive (23). Only a few published studies have tested for trichinellosis in wild animals using artificial digestion and/or other diagnostic methods in Brazil. Between 2018 and 2020, 15 wild carnivores (05 *Puma concolor*, 03 *Leopardus pardalis*, 02 *Chrysocyon brachyurus*, 04 *Cerdocyon thous* e 01 *Leopardus guttulus*) were tested in São Paulo state (11), while 71 other wild animals (29 *Didelphis albiventris*, 11 *Nasua nasua*, 10 *Cerdocyon thous*, 07 *Dasypus novemcinctus*, 06 *Leopardus guttulus*, 06 *Sphiggurus spinosus* and 02 *Puma concolor*) run over in the northern region of Paraná, were analyzed from 2016 to 2021 (24). The data from the present study add further evidence of the absence of circulation of the agent in wild carnivores in Brazil, despite serological evidence of infection by *Trichinella* spp. in wild boar (11).

Countries near Brazil, such as Chile and Argentina, have recorded the presence of *Trichinella* spp larvae. (*T. spiralis, T. patagoniensis, T. pseudospiralis* e *T. britovi)* in wild carnivores, such as the guigna (*Leopardus guigna*), lesser grison (*Galictis cuja*) and jaguar (*Puma concolor*), using the diagnostic method of AD (9, 12). Due to its high sensitivity, this technique is considered the gold standard for diagnosing *Trichinella* spp. by the World Organization for Animal Health (WOAH) (25).

The health of wild animals is known to be related to human health given their importance as carriers or reservoirs of zoonotic pathogens (26, 27). Currently, the transmission of trichinellosis is more related to the consumption of undercooked meat from wild animals than from domestic pigs due to technological advances in pork farms (28). However, international trade requires testing for *Trichinella* spp. in pig production in countries where the disease occurs, increasing the cost of importing products (2). Additionally, subsistence-type farms with low confinement as well as free-range or organic pig production increase the risk of exposure to *Trichinella* and many outbreaks in Easterns Europe and Argentina are related to this type of production (11, 29).

Despite the negative results obtained using the artificial digestion method, a Brazilian government agency reported 24 positive cases for *Trichinella* spp. out of 554 wild boars tested using the ELISA technique (IDEXX xChek®) between 2012 and 2016 (12). The samples were obtained from wild boar slaughtered in the states of São Paulo, Mato Grosso, Mato Grosso do Sul, Rio Grande do Sul, and Santa Catarina, resulting in the change of Brazil's health status for *Trichinella* from "never reported" to "restricted in some areas" (30). Additionally, 07 wild boars out of 115 animals with serum tested by indirect ELISA in São Paulo state from 2018 to 2020 were also positive for trichinellosis (11). The use of indirect diagnostic methods such as ELISA can be an essential tool in the surveillance of zoonotic diseases such as trichinellosis in wild animals. However, the validation of serological assays for several wild animals is hindered by the lack of reference sera (31) and due to the numerous parasitic, bacterial, fungal and viral agents that can affect these animals, the chances of cross-reactions and false positives are high (32). Another factor that makes it difficult to carry out serologies on the carcasses of animals that have been road killed is the coagulation and autolysis of the blood. Direct methods are more suitable for diagnosing *Trichinella* spp. in these cases. Furthermore, using the carcasses of road killed animals is a non-invasive way of performing epidemiological surveillance of several pathogens, maximizing the use of biological samples and reducing the risk to both humans and animals.

Microscopy showed in 14% (20/142) of the animals (07 *Puma concolor*, 04 *Cerdocyon thous*, 03 *Leopardus pardalis*, 03 *Lontra longicaulis*, 01 *Nasua nasua*, 01 *Chrysocyon brachyurus*, 01 *Leopardus guttulus*), rounded, basophilic and encapsulated structures in the striated muscle cells, compatible with Sarcocystidae protozoan cysts. The protozoan cysts visualized under microscopy, even if they were accidental findings, show the effectiveness of the technique in visualizing intracellular parasites in muscles when present. As observed in a study in 2011 in Texas, USA, using the diagnostic methods of histology and artificial digestion of the tongues of 77 coyotes (*Canis latrans*) to test for *Trichinella* spp., one animal tested positive only by histology. In addition to being in agreement with AD, offering greater accuracy in the result (33). Therefore, it constitutes an auxiliary technique for the diagnosis of trichinosis. In 2003, researchers in Chile diagnosed trichinosis infection in an Inca human mummy from around 1500 AD, using histological techniques and indirect immunofluorescence (34). Despite not being the gold standard for diagnosing this nematode, histopathology is an alternative for confirming infection and diagnosis.

Wild predators are associated with the maintenance of *Trichinella* in the wild cycle of all disease sites in the world (28). The meat consumption from these animals is cultural in some Latin American countries, such as Chile. Although hunting wild carnivores is illegal in Chile, researchers confirmed the presence of *Trichinella spiralis* in a semi-cured carcass of a jaguar (*Puma concolor*) that was slaughtered for consumption (35). Also prohibited in Brazil, this practice is considered cultural in some regions (36, 37). Carnivores such as the ocelot (*L. pardalis*), oncilla (*L. guttulus*), Jaguarundi (*H. yagouaroundi*) and crab-eating fox (*C. thous*) have already been reported in studies as a source of protein for consumption and/or medicinal use (38, 39).

Access to samples of wild carnivores, especially endangered animals protected by law, can be hampered by various factors. Therefore, the necropsy of carcasses of animals that have already died, as well as not interfering with conservation or causing stress to the animals, provides us with a lot of information (40). It is possible to observe places of occurrence, points of greatest trampling, and evaluate stomach contents, among other ways of using them for educational purposes. It is also possible to monitor numerous diseases of human and animal importance.

The sensitivity of the AD diagnostic is theoretically 1 larva per gram of muscle. However, due to the non-uniform larvae distribution in the tissues and limitations of the technique, the current sensitivity is considered 3 to 5 larvae per gram for 1g of muscle (41, 42). However, it is not known whether this method has the same sensitivity for detecting pre-encapsulated larvae (43). With regard to species from the non-encapsulated clade, studies show that the sensitivity of AD is between 7 and 17 larvae per gram (44). Therefore, factors such as the distribution of larvae in the muscles of carnivores and low parasite rates can lead to false negatives. Samples freezing may reduce the sensitivity of the technique, especially in the case of larvae that are not resistant to freezing, and therefore the tissues should ideally be maintained at 2-8°C (45). However, due to the logistics of the distance between the laboratory and the institutions that collected the carcasses, freezing is the best alternative of accessing a greater number and diversity of carnivore species.

The epidemiological surveillance of trichinellosis in wild boars is already being carried out in Brazil, as these animals are important hosts for the nematode (46). Wild animals, especially carnivores, are natural hosts of *Trichinella* spp. and the parasite develops better in these animals than in domestic animals (39). Given the way trichinellosis is transmitted and the fact that these animals are at the top of the food chain, epidemiological surveillance of wild carnivores and wild boars is essential to understanding the epidemiology of the disease in Brazil.

Conclusion

To date, there is no direct evidence of *Trichinella* spp. nematodes circulating in wild carnivores in São Paulo state

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was approved by FCAV/Unesp Ethics Committee for the Use of Animals. The study was conducted in accordance with the local legislation and institutional requirements. All the procedures adopted in this study are in accordance with current international standards. This work was authorized by the Chico Mendes Institute for Biodiversity Conservation (ICMBio/Sisbio #82767-4), by the Ethics Committee for the Use of Animals (CEUA) at UNESP/FCAV (proc. #2729/22) and by the Environmental Research Institute of the Secretariat for the Environment, Infrastructure and Logistics of the São Paulo State Government (#2180/2023)..

Author contributions

LA: Conceptualization, Writing – original draft, Writing – review & editing, Methodology. PP: Methodology, Writing – original draft. CA-P: Methodology, Writing – original draft, Writing – review & editing. CA: Methodology, Writing – original draft. FL: Methodology, Writing – original draft. FP: Methodology, Writing – review & editing. LS-N: Methodology, Writing – review & editing. AB: Methodology, Writing – review & editing. WO: Methodology, Writing – original draft. RI: Methodology, Writing – original draft. AC: Methodology, Writing – original draft. PR: Methodology, Writing – original draft. KW: Methodology, Writing – review & editing. EL: Conceptualization, Funding acquisition, Supervision, Writing – original draft, Writing – review & editing.

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Conflict of interest

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