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Molecular diagnostic for a screening investigation method of tick-borne pathogens in *Didelphis albiventris* road-killed in north of Paraná, Brazil

Investigação de patógenos transmitidos por carrapatos por detecção molecular em *Didelphis albiventris* atropelados no norte do Paraná, Brasil

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Highlights

Possibility of *Didelphis albiventris* acting as reservoirs of *Ehrlichia* sp.
Possibility of circulation of a new species of *Ehrlichia* in *Didelphis* sp.
Feasibility of studies involving run-over animals for the surveillance of zoonoses.

Abstract

The synanthropization of wild animals puts public health at risk by promoting the circulation of zoonotic agents, found naturally in the wild, in the anthropic environment. The objective of this work was to carry out screening by molecular detection of pathogens of the Anaplasmatacea family in *Didelphis albiventris,* a specie characterized as having a synanthropic habit. Opossums that were dead (n = 25) after being road-killed were collected in the North of Paraná state, southern Brazil during the 2016 and 2018 years, through active search. A questionnaire was filled out with information about the animal and collected place. Biological samples of spleen and liver were collected. The genetic material extracted from the spleen and liver was submitted to molecular diagnosis through PCR for amplification of *dsb* of *Ehrlichia* and 16S

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genes for the other agents of the Anaplasmataceae family. One animal was positive for the genus *Ehrlichia* in semi-nested PCR for amplification of the 349 bp fragment of the *dsb* gene in extracted from the liver samples. In PCR for the 16S target no animal was positive. These are preliminary results that reinforce the circulation of *Ehrlichia* in opossums. To improve the knowledge of these agents in opossums more studies are necessary.
Key words: Ehrlichiosis. Opossum synanthropization.

Resumo

A sinantropização de animais silvestres coloca em risco a saúde pública por propiciar a circulação de agentes zoonóticos, encontrados naturalmente em ambiente silvestre, no ambiente antrópico. O trabalho teve como objetivo realizar a triagem por detecção molecular de patógenos da família Anaplasmataceae em *Didelphis albiventris*, espécie caracterizada como de hábito sinantrópico. Gambás mortos (n=25) por atropelamento durante os anos de 2016 e 2018 foram coletados na região norte do Paraná, sul do Brasil, por meio de busca ativa. Realizou-se o preenchimento de formulário com informações sobre a espécie do animal e o local do atropelamento. Foi realizada a necrópsia e coleta de amostras biológicas, de baço e fígado. O material genético extraído de baço e fígado foi submetido a diagnóstico molecular, por meio de PCR, para amplificação dos genes *dsb* de *Ehrlichia* sp. e 16S para os demais agentes da família Anaplasmataceae. Um animal foi positivo para o gênero *Ehrlichia* em semi-nested PCR para a amplificação do fragmento de 349 pb do gene *dsb*, extraído de fígado. Na PCR para detecção do gene 16S nenhum dos animais foi positivo. Esses resultados preliminares reforçam a circulação de *Ehrlichias* em gambás. Para melhorar o conhecimento desses agentes em gambás mais estudos são necessários.
Palavras-chave: Ehrlichiose. Gambá. Sinantropização.

Didelphis albiventris are synanthropic animals. Their omnivorous diet provides access to modified environments. Their proximity to a wild and anthropic environment can increase human exposure to zoonotic agents (Lledó et al., 2010).

Ticks are worldwide distribution vectors. It is the most important specie involved in vector-borne diseases. Some pathogens like genera *Ehrlichia* and *Anaplasma* include zoonotic species of importance in public health that may be transmitted by ticks. Their enzootic cycle involves ticks and vertebrate hosts.

In Brazil molecular and serological studies with Didelphis spp. found positivity for agent *Ehrlichia* spp. in the state of São Paulo and Rio de Janeiro. These animals may be important in the cycle of agents transmitted by ticks (Guimarães et al., 2018; Melo et al., 2016). Serological tests on human biological samples detected seroreactivity for *Ehrlichia* in Brazil (Bezerra et al., 2017).

This study aimed to perform screening by molecular detection of Anaplasmataceae agents in *D. albiventris,* in North of Paraná, Brazil.

Ethics and Animal Experimentation Committee approved the study under number 30/2017, and by the Biodiversity Authorization and Information System (SISBIO) under number 55384-1.

The road-killer animals were collected from municipalities in the North of Paraná (Figure 1) with the help of an active team in specific transects (Figure 1) or upon notification by the second Company of Military Environmental Police and Military Highway Police. The animals collected were in rigor mortis and with no evisceration. Fragments of the organs such as the liver, spleen, and skin were collected from all animals and stored at -20 °C. The animals were assessed for the presence of ectoparasites. The dichotomous key proposed by Onofrio (2009, 2020) identified the tick.

DNA extraction from the liver and spleen, and the ectoparasite found was performed using PureLink Genomic DNA Mini Kit (Thermo Fisher Scientific, Madison, WI, USA). Ultrapure water (Milli-Q, Millipore, Merck, Darmstadt, Germany) was used as a negative control for each extraction. The extracted DNA was quantified using L-QUANT (Loccus Biotechnology, Cotia, Brazil). The samples had a minimum DNA concentration of 25 ng/µL. The DNA samples were stored in a 1.5 mL microtube at -20 °C for subsequent PCR.

DNA extracted from spleen and liver tissues was used for *Ehrlichia* and Anaplasmataceae reactions. Details regarding the primers are shown in Table 1. The negative control used ultrapure water. For positive controls, DNA extracted from the whole blood samples of dogs positive for *E. canis* and cattle for *A. marginale,* at the Veterinary Hospital of the State University of Londrina.

The positive sample to *Ehrlichia* spp. it was submitted to a new PCR for the specie *Ehrlichia canis* detection according to Ribeiro et al. (2017). The tick underwent molecular diagnosis for Anaplasmataceae.

The PCR products were purified using PureLink Gel Extraction Kit (Invitrogen, Molecular Probes, Eugene, OR, USA) and quantified using L-QUANT. For the sequencing, a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Carlsbad, CA, USA) was used, with the manufacturer's instructions. The sequences were analyzed using the PHRED software (http://asparagin.cenargen.embrapa.br/phph). Consensus sequences were determined using the CAP3 software (http://asparagin.cenargen.embrapa.br/cgi-bin/phph/cap3.pl). The obtained sequence identity was determined by comparing our results with those available in GenBank, using the BLAST software (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

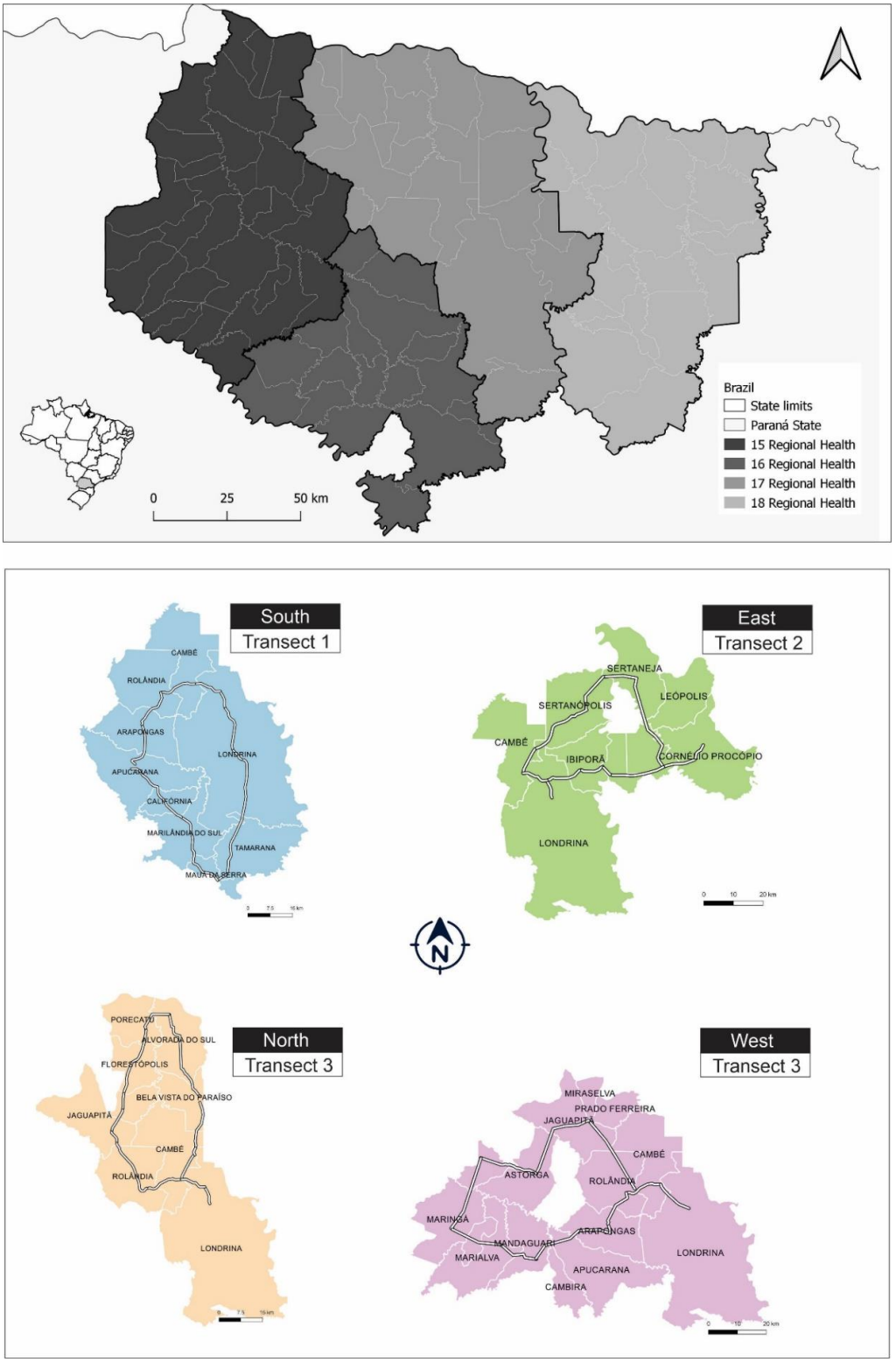

Figure 1. Schematic map of the 15th, 16th, 17th and 18th Health Regional of the State of Paraná and its municipalities and maps with the transects covered by the team to actively search for run over animals.

Table 1
Primers and targets used in PCR assays for detection of *Ehrlicha* sp, *Ehrlichia canis*, and Anaplasmataceae family

Specificity	Primers	Gene fragment	References
Ehrlichia sp	DSB 330	401 bp – gene dsb 349 bp – gene dsb	Doyle et al., 2005 Almeida et al., 2013
	DSB 720		
	DSB 380		
Ehrlichia canis	EcavB9-F	959 bp – gene virB9	Ribeiro et al., 2017
	EcavB9-R		
Anaplasmataceae family	EHR 16SD	345 bp – gene 16S	Inokuma et al., 2000
	EHR 16SR		

Twenty-five specimens of *D. albiventris* were collected between November 2016 and October 2018, of which 68.0% were male (Table 2). The season with the highest collection of animals was autumn with 44% (11/25) of the total samples. Ectoparasites were found in only 4.0% (1/25) of the animals, and the parasite was identified as female *Ixodes loricatus* after morphological analysis. The tick was negative for all agents in molecular diagnosis. Of the animals analyzed, 4.0% (1/25) were positive for *Ehrlichia* spp. (liver sample). The same sample of the positive animal was submitted to a new PCR for the specie *E. canis* and did not present amplification. The positive animal was an adult male collected in the winter of 2017 in the city of Ibiporã, Paraná, Brazil. None of the analyzed samples were positive in PCR for the Anaplasmatacea family.

D. albiventris can be parasitized by a variety of tick species based on their age, host behavior, and environmental factors. Although the present study found ectoparasites of the species *I. loricatus* in only 4.0% of the analyzed animals, this data should not be considered representative of the parasitism reality in Didelphis spp. It is important to note that the analyzed animals were dead, and the time between collection and inspection may have favored the disappearance of these ectoparasites from the specimens. Among the currently reported species of ticks on marsupials: *I.loricatus, Amblyomma sp., Amblyomma parvum, Amblyomma auricularium,* and *Ornithodoros mimon* (Oliveira et al., 2020; Lopes et al., 2018). Through molecular diagnosis, Lopes et al. (2018) detected positivity for *Rickettsia amblyommatis* in ticks of the species *Amblyomma auricularium* and suggested the possibility of the same species or even of *I. loricatus* acting as vectors of *Ehrlichia* sp. Natal strain.

Agents of the *Ehrlichia* genus had already been found by Melo et al. (2016) in a serological study in which 14.67% (16/109) of didelphids (*D. aurita* and *D. albiventris*) were seroreactive, with titers between 40 and 160 for *E. canis,* in indirect immunofluorescence. Guimarães et al. (2018) detected *Ehrlichia* spp. genotype 100% identical to *E. canis* in *D. aurita* using 16S rRNA PCR with species-specific primers for *E. canis* and *E.chaffeensis.*

Table 2
***Didelphis albiventris* run over specimens collected between the years 2016 and 2018 in north of Paraná, Brazil**

ID	Date of road-killed	City	Sex	Age range	Seasons	Ectoparasite	Molecular analysis
1	April 8th, 2017	Londrina	Female	Juvenile	Autumn	Absent	Negative
2	April 17th, 2017	Uraí	Female	Juvenile	Autumn	Absent	Negative
3	May 1st, 2017	Londrina	Male	Adult	Autumn	Absent	Negative
4	May 12th, 2017	Cambé	Male	Adult	Autumn	Absent	Negative
5	May 30th, 2017	Mauá da Serra	Male	Adult	Autumn	Absent	Negative
6	June 8th, 2017	Londrina	Male	Juvenile	Autumn	Absent	Negative
7	June 8th, 2017	Londrina	Male	Adult	Autumn	Absent	Negative
8	June 8th, 2017	Londrina	Male	Juvenile	Autumn	Absent	Negative
9	June 12th, 2017	Londrina	Male	Juvenile	Autumn	Absent	Negative
10	June 19th, 2017	Londrina	Female	Juvenile	Autumn	Absent	Negative
11	June 10th, 2017	Londrina/ Guaravera	Male	Juvenile	Winter	Absent	Negative
12[1]	July 17th, 2017	Ibiporã	Male	Adult	Winter	Absent	Positive for *E.canis*
13	July 23rd, 2017	Londrina	Male	Puppy	Winter	Absent	Negative
14	August 21st, 2017	Londrina	Male	Adult	Winter	Absent	Negative
15	August 23rd, 2017	Londrina	Male	Adult	Winter	Absent	Negative
16[2]	August 31st, 2017	Londrina	Male	Juvenile	Winter	Present	Negative
17	October 1st, 2017	Londrina	-	-	Spring	Absent	Negative
18	November 23rd, 2017	Londrina	Female	Adult	Spring	Absent	Negative
19	November 28th, 2017	Londrina	-	Adult	Spring	Absent	Negative
20	November 29th, 2017	Londrina	Male	Juvenile	Spring	Absent	Negative
21	January 11th, 2018	Londrina	Male	Juvenile	Summer	Absent	Negative
22	May 17th, 2018	Londrina	Male	Juvenile	Autumn	Absent	Negative
23	June 8th, 2018	Londrina	Female	Juvenile	Winter	Absent	Negative
24	October 10th, 2018	Londrina	Male	Juvenile	Spring	Absent	Negative
25	October 3rd, 2018	Londrina	Female	Juvenile	Spring	Absent	Negative

[1]Animal positive for *Erlichia* [2]Animal parasitized by *Ixodes loricatus.*

Recent works corroborate this study and indicate the possibility of *Didelphis* spp. act as reservoirs for hemoparasites such as *Ehrlichia* spp. (Oliveira et al., 2020; Lopes et al., 2018; Guimarães et al., 2018; Melo et al., 2016). Studies have reported the presence of organisms genetically similar to *E. canis* in non-synanthropic wild animals, thereby suggesting the existence of a wild cycle of the parasite and the possibility of the involvement of other vector species (Santoro et al., 2016; André et al., 2010; Ebani et al., 2017). Further studies are needed to understand the role of opossums in the epidemiological cycle of *Ehrlichia* spp. as well as the risks that the circulation of *Didelphis* spp. between the wild and anthropic environments can bring to both wild animals and humans. There are few studies performed on hemoparasites in opossums in Brazil. The negative results found in other Anaplasmatacea agents corroborate those of Colle et al. (2019). In the molecular diagnosis (PCR) in blood, spleen, and liver samples of 230 animals, marsupials, and rodents of the Amazon biome, every presented negative results for the agents of the family Anaplasmataceae. These negative results may be associated with the lack of sensitivity of *D. albiventris* to infections by *Anaplasma* spp. It has been demonstrated that *D. virginiana* is capable of reducing infection by *A. phagocytophilum* via a mechanism that is still unknown (Levin et al., 2002). Further studies are required to clarify the interaction between *D. albiventris* and *Anaplasma* spp.

These are preliminary results that reinforce the circulation of *Ehrlichia* in opossums. A larger sample size is necessary to improve the analysis.

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