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Questing ticks in Atlantic rainforest (Mata Atlantica) fragments in the municipality of Divino, Minas Gerais state, Brazil

Carrapatos na fase de vida livre em fragmentos de Mata Atlântica do município de Divino, Minas Gerais, Brasil

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Highlights

Adult, nymphal, and larval stages of ticks were frequent in Atlantic Forest fragments.
The flannel drag technique was efficient for collecting ticks.
No hemoparasites were detected in the 1,122 collected tick.

Abstract

The tick species and tick-borne pathogens present in a group of questing ticks collected from forest fragments in a rural area in the municipality of Divino, Minas Gerais state, Brazil were evaluated. The collected ticks were divided into two groups those collected from around the edges of the fragments and those collected from the interior of the forest. In all the fragments, the ticks were collected using a dragging and flagging technique and by harvesting them from white fabric gaiters. The larvae, nymphs, and adults were all morphologically identified using specific taxonomic keys. The larvae were identified to the genus level. DNA was extracted from the ticks and tested for the presence of *Rickettsia* spp., *Borrelia* spp., *Ehrlichia* spp., *Anaplasma* spp., *Babesia* spp., and *Theileria* spp. using a conventional polymerase chain reaction (PCR). In total, 1,122 questing ticks (750 larvae, 367 nymphs, and five adults) and 18 larval clusters were evaluated. The main species found in the collected tick population were *Amblyomma sculptum*, *A. auricularium*, *A. aureolatum*, and *A. pseudoconcolor*, along with the larvae of *Amblyomma* spp. and *Dermacentor* spp. None of the tick samples gave a positive result when tested by PCR for the presence of DNA from *Rickettsia* spp., *Borrelia* spp., *Ehrlichia* spp., *Anaplasma* spp., *Babesia* spp., or *Theileria* spp.

Key words: Arthropods. *Amblyomma*. hemoparasites. Atlantic rainforest. Tick-borne pathogens.

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Resumo

Neste estudo avaliou-se a presença de espécies de carrapatos e a detecção de agentes patogênicos a eles associados. Os carrapatos de vida livre foram coletados em fragmentos florestais da área rural do Município de Divino, Minas Gerais, Brasil. Os carrapatos coletados foram divididos em dois grupos, aqueles que foram coletados na área da margem do fragmento (Borda) e os coletados no interior da floresta (Mata). Em todos os fragmentos, os carrapatos foram coletados de acordo com a técnica de arraste aéreo ou no chão e com o uso de perneiras e de flanela. Tanto, as larvas, quanto as ninfas e os adultos foram morfologicamente identificados usando chaves taxonômicas específicas. No caso das larvas, estas foram identificadas até o nível de gênero. Foi realizada extração de DNA dos carrapatos e o DNA extraído foi testado para a presença de *Rickettsia* spp., *Borrelia* spp., *Ehrlichia* spp., *Anaplasma* spp., *Babesia* spp., e *Theileria* spp. por meio de uma estratégia de reação em cadeia da polimerase convencional. No total, 1.122 carrapatos em fase de vida livre (750 larvas, 367 ninfas e 5 adultos) e 18 clusters de larvas foram usados no estudo. As principais espécies identificadas na população de carrapatos coletada foram: *Amblyomma sculptum, Amblyomma auricularium, Amblyomma aureolatum* e *Amblyomma pseudoconcolor* e larvas de *Amblyomma* spp. e *Dermacentor* spp. Como resultado da detecção de patógenos não foi possível achar DNA de nenhum dos agentes analisados, assim todas as amostras de DNA dos carrapatos testados foram negativas tanto para *Rickettsia* spp. quanto para *Borrelia* spp., *Ehrlichia* spp., *Anaplasma* spp., *Babesia* spp. e *Theileria* spp.

Palavras-chave: Artrópodes. *Amblyomma.* Hemoparasitos. Mata Atlântica. Patógenos associados a carrapatos.

Ticks and the diseases they transmit are relevant to both commercial livestock and small animal producers. Many vector-borne pathogens constitute a major challenge to animal production in tropical and subtropical regions of the world.

Tick-borne diseases are common in human and veterinary medicine. Examples of tick-borne zoonoses with an increased incidence include Lyme borreliosis, human anaplasmosis, babesiosis, human monocytic ehrlichiosis, spotted fever group rickettsiosis, and Powassan virus encephalitis (POWV) (Rosenberg et al., 2018).

The present study generated information on the acarological fauna of a forest fragment biome in the Atlantic Forest, which is characterized by livestock farming and a high degree of anthropization in rural areas in the municipality of Divino, Minas Gerais state, Brazil (Figure 1). These forest fragments had variable extents and were contiguous with areas exploited for agriculture, particularly the production of milk and coffee. The level of conservation of native forest in this area varies greatly in relation to human intervention. The study aimed to describe the species of ixodid ticks that occur in this biome and search for the agents potentially transmissible by the ticks.

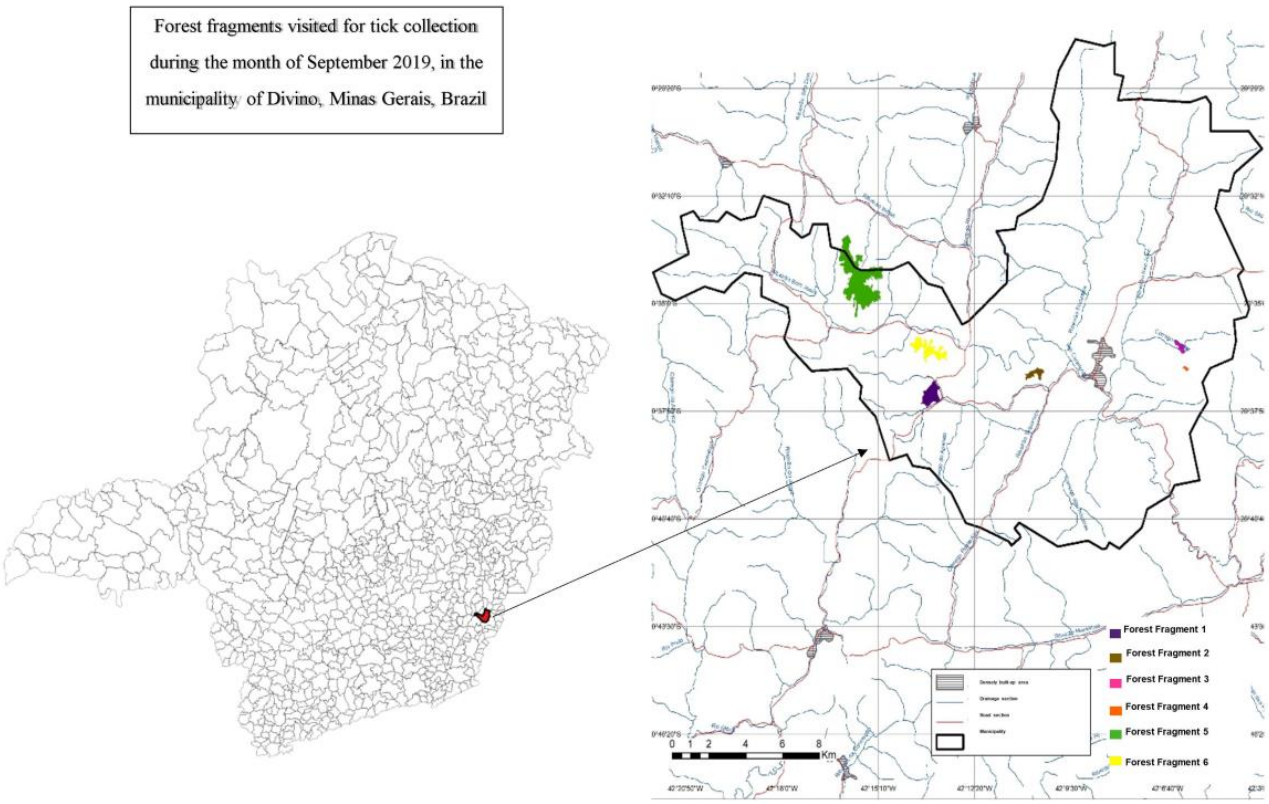

Figure 1. Forest fragments visited for tick collection during the month of September 2019, in the municipality of Divino, Minas Gerais.
(a) Fragment 1 - *Amblyomma* spp, larvae; *A. auriculatum nynphs* and *A. sculptum* adults; (b) Fragment 2- *Amblyomma* spp larvae; *Dermacentor* spp; *A. auricularium* and *A. sculptum nynphs*, *A. sculptum* adults. (c) Fragment 3 - *Amblyomma* spp larvae and *A. sculptum nynphs.* (d) Fragment 4 - Larvas de *Amblyomma* spp e *Dermacentor* spp e Ninfas de *A. sculptum*; (e) Fragment 5 - *Amblyomma* spp larvae; *A. sculptum* and *A. pseudoconcolor nynph*; *A. sculptum* adults. (f) Fragment 6 – *Amblyomma* spp. larvae.

The relief of this region is predominated by mountains and narrow valleys. The Atlantic Forest biome originally formed the vegetative cover but, due to intense deforestation over the years, this continuous vegetation cover has been transformed into small forest fragments restricted mainly to the highest elevations. The main economic activity of the municipality is coffee, grown on small properties, with small forest fragments commonly found among these coffee plantations (Figure 1). Livestock are also present in this municipality, occupying large areas of pasture.

For the capture of free-living ticks, forest fragments located in areas containing coffee plantations or cattle were chosen, these fragments lying between the coordinates: 20°33'41.13"S, 42°16'33.80"W; 20°37'57.89"S, 42°14'02.03"W; 20°34'55.14"S, 42°04'28.25"W; and 20°37'16.98"S, 42°04'23.24"W.

Six forest fragments were chosen, with areas varying between 4.16 and 355 km^2, as well as a property where the circulation of cattle, horses, and capybaras was frequent. Table 1 provides the most relevant characteristics of the vegetation and physical features of the fragments.

Table 1
Description of the physical and vegetative characteristics of the fragments selected for tick collection in the municipality of Divino, Minas Gerais, Brazil

Fragment	Characteristics
1	Highly conserved forest. Humid environment. Dense vegetation. Trails and wild animal burrows. Mixture of activities nearby (livestock and coffee plantations).
2	Partially conserved forest. Humid environment. Dense vegetation with eucalyptus plantations. Agricultural activities nearby (livestock and coffee plantations). Dry soil.
3	Highly conserved forest. Humid environment. Dense vegetation. Trails and wild animal burrows. Steep terrain. Agricultural activities nearby (coffee plantations).
4	Highly conserved forest. Humid environment. Dense vegetation. Trails and wild animal burrows. Steep terrain. Livestock-based activities nearby (cattle).
5	Semi-conserved forest. Moderately dense forest. Trails and wild animal burrows. High humidity. Steep terrain.
6	Semi-conserved forest. Moderately dense forest. High humidity. High anthropogenic activity. Human trails. Some sectors with a predominance of bamboo.

The sample collections took place in September 2019, the time of the year with the highest number of Brazilian spotted fever cases, according to the Information System For Notifiable Diseases, [SINAN], (2021). The collections were carried out over the 10 consecutive days of the expedition, between 8 am and 1 pm. Each work area covered approximately 150 m^2, with the ticks being sampled using flannel-drag and walking-trap techniques. In the drag technique, a piece of white flannel (1.50 × 0.80 m) was attached to a wooden pole (0.85 m long) by a rope and dragged over the vegetation. In the walking-trap technique, the collector walked through the vegetation wearing white clothing and white flannel leggings to capture ticks on the vegetation, waiting for a host. The team consisted of four to six members per campaign. Periodically, the flannel and the bodies of the collectors were inspected with the naked eye to establish the presence of ticks (Figure 2). Ticks were collected separately in two groups, the first collected from around the edge of the forest (i.e., around the periphery and in the first 30-m strip) and labeled the marginal group, the second collected from the interior of the forest, beyond 30 m, and designated the Mata group. The tick specimens were placed in 2-ml microtubes containing isopropyl alcohol. A count of the individuals in the different life stages was performed. The larvae were identified to the genus level, with adults and nymphs identified

to the species level, by applying taxonomic keys for ticks in Brazil (Dantas-Torres, Martins, Muñoz-Leal, Onofrio, & Barros-Battesti, 2019; Martins, Onofrio, Barros-Battesti, & Labruna, 2010). Once identified, all the specimens were used for molecular analysis and tested for hemoparasites. Each specimen was dried on paper towel for approximately 30 min until the alcohol had completely evaporated and was then transferred individually to a 1.5-ml tube and three times with 1 ml of phosphate-buffered saline solution (pH 7). Once the final aliquot of buffer had been discarded, the tubes were frozen at −20°C until the DNA extraction was performed.

Figure 2. (A) Researcher manipulating flannel fixed to a wooden pole in a forest area. (B) Procedure for collecting the instars present on the flannel. (C) Flannel deposited in an area containing ticks, as observed with the naked eye. (D) Presence of ticks on a researcher's clothing.

DNA was extracted from the individual ticks using a HotSHOT method adapted for ticks. Briefly, the ticks were cut longitudinally and transferred to a fresh tube, to which 50 µl of alkaline (pH 12) sodium hydroxide + sodium ethylenediaminetetraacetic acid buffer solution (25 mM NaOH + 0.2 mM Na2EDTA) was added. The tubes were placed in an oven at 90°C for 30 min, and then transferred to ice for 5 min and stabilized with 50 µl of tris(hydroxymethyl)aminomethane hydrochloride neutralizing solution (40 mM Tris HCl, pH 5). A final volume of 100 µl was obtained from the solution containing DNA from each tick.

Approximately 10% of the obtained DNA samples were subjected to polymerase chain reaction (PCR) for the amplification of a fragment of the mitochondrial gene (16S rDNA) of the ticks from the family Ixodidae. Extrapolation of the results of the mitochondrial 16S rDNA amplification to all tested samples served as the baseline criterion for considering all the extracted DNA to be amplifiable DNA via conventional PCR to continue with pathogen DNA detection.

The extracted DNA was tested using a battery of PCR assays targeting microorganisms of the *Rickettsia, Borrelia, Anaplasma, Ehrlichia,* and *Babesia/Theileria* genera. Specific oligonucleotide primers, previously reported by several authors, were used. The primer sequences are listed in Table 2. The amplification protocol followed for each pathogen was as described in the original work cited in Table 2. The PCR conditions for the family Ixodidae were as described by Mangold, Bargues, and Mas-Coma (1998). Rickettsia's following M. Labruna et al. (2004), for *Ehrlichia,* described by Almeida, Souza, Marcili, and Labruna (2013) and for *Borrelia* spp. according Blanco et al. (2017). The amplification conditions for the two reactions of the nested PCR for *Anaplasma* spp. were described by Barlough, Madigan, DeRock, and Bigornia (1996).

Regarding all the collection points, a total of 1,122 ticks were collected, of which 750 were larvae, 367 were nymphs, and five were adults. In addition to these specimens, 18 larval clusters were collected from four of the fragments, and five larvae were used from each cluster in the study. Of the 750 larvae, 709 were assigned to the genus *Amblyomma* and the remaining 41 to the genus *Dermacentor.* Regarding the nymphs, 365 were assigned to the species *Amblyomma sculptum,* one specimen was identified as *A. pseudoconcolor,* and one as *A. auricularium.* Of the five adult ticks, two came from Fragment 1, two from Fragment 2, and one from Fragment 5. The adult specimens in Fragments 1 and 2 were females of the species *A. sculptum,* while the specimen from Fragment 5 was a female of the species *A. aureolatum.* The name and number of each species and their collection points are shown in Table 2.

Table 2
Sequences of oligonucleotide primers used for the detection of microorganisms of the genera *Rickettsia, Borrelia, Ehrlichia,* and *Anaplasma* and the phylum Apicomplexa (genera *Babesia* and *Theileria*) in the DNA of ticks from the municipality of Divino, Minas Gerais, Brazil

Primers	Gene	Organismo	Sequências de nucleotídeos (5'-3')	Tamanho do Fragmento	Referência
16S+	16S rRNA	Família Ixodidae	CCGGTCTGAACTCAGATCAAGT	460pb	(Mangold et al., 1998)
16S-			GCTCAATGATTTTTTAAATTGCTGT		
CS239 F	gltA	Rickettsia spp.	GCTCTTCTCATCCTATGGCTATTAT	834 bp	(M. Labruna et al., 2004)
CS1069 R			CAGGGTCTTCGTGCATTTCTT		
BorFlaF1	flaB	Borrelia spp.	TACATCAGCTATTAATGCTTCAAGAA	948 bp (1)	(Blanco et al., 2017)
BorFlaR1			GCAATCATWGCCATTGCRGATTG		
BorFlaF2			CTGATGATGCTGCTGGWATGG	790 bp (2)	
BorFlaR2			TCATCTGTCATTRTWGCATCTT		
BT-F3	18S rRNA	Phylum Apicomplexa	TGGGGGGAGTATGGTCGCAAG	650 bp	(Seo et al., 2013)
BT-R3			CTCCTTCCTTTAAGTGATAAG		
BTF1	18S rRNA	Order Piroplasmida	GGCTCATTACAACAGTTATAG	961 bp	(Jefferies, Ryan, & Irwin, 2007)
BTR1			CCCAAAGACTTTGATTTCTCTC		
BTF2			CCGTGCTAATTGTAGGGCTAATAC	825 bp	
BTR2			GGACTACGACGGTATCTGATCG		
DSB-330	dsb	Ehrlichia spp.	GATGATGCTTGAAGATATSAAACAAAT	349 bp	(Almeida et al., 2013)
DSB-380			ATTTTTAGRGATTTTCCAATACTTGG		
DSB-720			TCCTGGCTCAGAACGAACGCTGGCGGC		
EE1	16S rRNA	Anaplasma spp.	TCCTGGCTCAGAACGAACGCTGGCGGC	1433 bp	(Barlough et al., 1996)
EE2			AGTCACTGACCCAACCTTAAATGGCTG		
EE3			GTCGAACGGATTATTCTTTATAGCTTGC	926 bp	
EE4			CCCTTCCGTTAAGAAGGATCTAATCTCC		

pb – pares de base; (1) e (2): Nested-PCR.

There was no DNA amplification for the tested genes in any of the ticks in this study. It can be inferred that the infection rate and parasite load of these arthropods were so low that the molecular methodology used could not detect the low number of copies of the DNA of any of the analyzed parasites likely to be present in them. The constant improvement of certain detection methodologies is a topic of great importance Complementation with other techniques, such as nucleic acid hybridization, particularly Southern blotting, increases the sensitivity and specificity of such methods.

The Atlantic Forest, considered a biodiversity hotspot, has undergone extensive devastation throughout Brazil's history, becoming one of the most threatened biomes in the world. When observing the landscapes surrounding the region of Divino, the significant loss of green space, giving

way to different plantation areas, is notable. The habitat loss and fragmentation taking place in the municipality of Divino reflect the biodiversity loss occurring in many parts of the world, which is increasing the risk of animal species extinctions, and may potentially lead to the emergence of infectious diseases in humans and wild or domestic animal species (Dobson & Foufopoulos, 2001).

The present study evaluated the acarological fauna in an Atlantic Forest biome region in Minas Gerais that is currently highly degraded, with small forest fragments occurring among intensively cultivated coffee plantations and pastures designated for cattle raising, with or without horse raising. Importantly, these fragments have a very high degree of anthropization, showing, for example, remnants of cut trees around their edges and interiors, along with trails made by human or horses activities leading into the interior of the forest. Although the distance covered in the interior varied between the fragments it was not possible to get very far into the interior of the forest in several fragments due to ensuring the safety of the team it was always possible to obtain specimens from the edge and interior of the forest.

The most abundant tick species found in our study was *A. sculptum,* a member of the *A. cajennense* complex (*A. cajennense sensu lato*), together with the species *A. cajennense sensu stricto, A. mixtum, A. interandium, A. tonelliae,* and *A. patinoi* (Nava et al., 2014).

Amblyomma sculptum is adapted to the dry conditions of the Cerrado biome; however, due to the intense deforestation in the Atlantic Forest biome, it has also advanced into this region, being abundant therein (Nava et al., 2014). According to Szabó et al. (2009), the low incidence of light, lower temperatures, and high humidity in the forest are factors that hinder its maintenance in this biome; however, this species is common in the Atlantic Forest surroundings, deforested regions, and anthropic areas. Of note is the abundance of *A. sculptum* nymphs found around the edges of, and inside, the forest. This is probably due to horses entering the perimeter, as both tracks and equine feces were observed at some sites.

The other *Amblyomma* species found were *A. auricularium, A. pseudoconcolor,* and *A. aureolatum. Both A. auricularium* and *A. pseudoconcolor* are mainly parasites of animals of the family Dasypodidae (armadillos). Botelho, Linardi, and Encarnação (1989) found that armadillos of the genera *Cabassous* and *Dasypus* had a higher level of infestation with *A. pseudoconcolor* than those of the genera *Euphractes* and *Priodontes* in a study carried out in Minas Gerais. In agreement with this finding, some burrows were sighted in the trawled places in this study, although no animals were sighted.

In this study, the presence of domestic animals, particularly dogs, horses, and bovines, were noted over a large part of the collection area. Some dogs showed signs of hemoparasitoses, such as anorexia, epistaxis, and pale mucous membranes, suggesting anemia. It is therefore worth mentioning that there are reports in the literature of *A. aureolatum* occurring in rural and semi-urban areas located close to forest fragments, with the adult form parasitizing mainly domestic dogs (Guglielmone et al., 2006).

Although no pathogens of medical or veterinary importance were detected, the role of ticks of the genus *Amblyomma,*

particularly *A. sculptum* and *A. aureolatum,* which were found in this study as vectors of the Rickettisiae is known. The transmission of rickettisiae occurs through the bite of infected ticks, regardless of their life stage (larva, nymph, or adult) (M. B. Labruna, 2009). Ticks in Brazil are known to be responsible for the transmission of spotted fever, which has affected thousands of Brazilians in recent years, in addition to being involved in the transmission of Lyme simile borreliosis, babesiosis, ehrlichiosis, anaplasmosis, and arboviruses. Furthermore, in addition to acting as a vector of *Rickettsia rickettsii, A. sculptum* is responsible for maintaining rickettsia through successive generations, acting as a reservoir for this bacterium in nature (Soares, Soares, Barbieri, & Labruna, 2012).

Notwithstanding no ticks infected by any of the evaluated agents were detected, it is important to note that the presence of *A. sculptum* and *A. aureolatum* in the sampled sites, and the characteristics of these sites in association with anthropogenic activities, constitute a great risk of the presence or establishment of agents infectious to humans and transmitted by ticks, such as *Rickettsia* spp. *Dermacentor* spp. larvae were also found in the study area, probably related to the passage of horses through some of the collection sites. Popularly known as "horse-ear tick," the species *D. nitens* is widely distributed throughout the Brazilian territory, with horses being its main hosts.

Most samples were collected in the larval form, followed by nymphs and then adults. This is probably related to the time of year during which the study was carried out (September i.e., late winter in Brazil), suggesting that tick development periods are strongly influenced by climatic factors. The coincidence of the tick development period and early winter means that ticks can emerge in a new stage the following spring. All tick stages, not just the larvae, spread their emergence times across different months and years, and the extent of this spread can vary from year to year due to climatic variations (Randolph, 2004).

Although no samples tested positive for the evaluated pathogens, it is necessary to closely monitor both suspected and confirmed cases of tick transmitted diseases in the population in the study area and continue with similar studies that contribute to establishing the pertinent measures required to reduce the risk of this type of zoonosis. The ticks found in this study are of great importance for public health and may yet represent a risk, as the latest data from DATASUS show 41 cases of spotted fever in the state of Minas Gerais alone. (Sistema de Informacão de Agravos de Notificação [SINAN], 2021).

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