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Diversity of free-living ticks and serological evidence of spotted fever group *Rickettsia* and ticks associated to dogs, Porto Velho, Western Amazon, Brazil

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Abstract
Rondônia is the only state in the North Region of Brazil to have registered confirmed cases of Brazilian Spotted Fever (BSF). The present study investigated the epidemiological cycle of *Rickettsia* spp. by surveying free-living ixodofauna and tick parasitism of dogs in the municipality of Porto Velho, Rondônia State. Ticks and dogs were tested for the presence of *Rickettsia* spp. DNA and dog serum was tested for reactivity to anti-*Rickettsia* spp. antibodies. Tick collection and dog blood sampling were performed in peri-urban and rural environments at 11 locations. Eight free-living *Amblyomma* species and one *Haemaphysalis* species were collected: *A. scalpturatum*, *A. naponense*, *A. oblongoguttatum*, *A. coelebs*, *A. latepunctatum*, *A. pacae*, *A. ovale*, *Amblyomma* sp., and *H. juxtakochi*. Three tick species were found parasitizing dogs: *Rhipicephalus sanguineus* sensu lato, *A. oblongoguttatum* and *A. ovale*. Molecular analysis did not identify the presence of the *gltA* gene fragment in any tick specimen. Results from an indirect immunofluorescent assay (IFA) showed that 20.8% of peri-urban and 15.4% of rural dog sera exhibited reactivity to *Rickettsia rhipicephali*, *Rickettsia amblyommatis*, *Rickettsia bellii* and *Rickettsia parkeri* antigens. Antibody prevalence in dogs was 16.4%. This study is the first to describe the prevalence of *Rickettsia* spp. infection in dogs from Porto Velho municipality. Our findings enhance current knowledge of *Rickettsia* spp. circulation in the Western Amazon.

Keywords Rickettsiosis · *Amblyomma* spp. · Amazon rainforest · IFA · Sentinel hosts

Introduction

Ticks are competent vectors of etiological agents that are pathogenic to humans and animals (Estrada-Peña and Jongejan 1999; Anderson and Magnarelli 2008). Two forms of rickettsiosis are of utmost importance in Brazil: Brazilian Spotted Fever (BSF), which

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is caused by *Rickettsia rickettsii* and transmitted by *Amblyomma sculptum* Berlese and *Amblyomma aureolatum* Pallas ticks, and *Rickettsia parkeri* Atlantic rainforest strain Spotted Fever (RPSF), which is caused by *R. parkeri* Atlantic rainforest strain and transmitted by *Amblyomma ovale* Koch ticks (Labruna 2009; Szabó et al. 2013a).

Rondônia State is covered primarily by Amazon rainforest and is home to a great diversity of tick species, the most prevalent being ticks of the *Amblyomma* genus (Martins et al. 2014). There is some evidence that rickettsial DNA is present in ticks of this biome. Tick-associated *Rickettsia* has been documented more often in Rondônia than in any other Amazonian state: *Rickettsia amblyommatis* associated to *Amblyomma cajennense* Fabricius, *Amblyomma coelebs* Neumann and *Amblyomma oblongoguttatum* Koch (Labruna et al. 2004a; Aguirre et al. 2018), *Rickettsia rhipicephali* to *Haemaphysalis juxtakochi* Cooley (Labruna et al. 2005a), and *R. amblyommatis* strain Aranha to *Amblyomma longirostre* Koch (Labruna et al. 2004b), belonging to the spotted fever group (SFG), and *Rickettsia bellii* found infecting *Amblyomma humerale* Koch, *A. oblongoguttatum*, *A. ovale* and *Amblyomma rotundatum* Koch (Labruna et al. 2005a). It is important to point out that none of the *Rickettsia* spp. mentioned above have been confirmed to be pathogenic to humans.

According to Brazil's Information System for Notifiable Diseases (Datasus 2021), five cases of BSF have been confirmed in the North Region of Brazil, all in Rondônia State, but only one has been published in the literature (Oliveira et al. 2016). However, none of the reported cases specified which *Rickettsia* species were the causative agent.

Horses are the most suitable sentinel hosts in BSF-endemic areas. *Amblyomma sculptum* is the predominant tick in these areas because horses are its primary host. Horses suffer more exposure to tick vectors in endemic areas and they exhibit higher serological prevalence of anti-*Rickettsia* spp. antibodies than do other animals (Lemos et al. 1996; Ueno et al. 2016).

Dogs play an important role in the transmission of ectoparasites and associated pathogens by acting as a bridge between natural and anthropic environments (Estrada-Pena and Jongejan 1999). Moreover, dogs are considered sentinel and amplifier hosts for *R. rickettsii* in endemic areas where *A. aureolatum* is the most prevalent tick, and *A. aureolatum* may also be an amplifier host for *R. parkeri* (Demma et al. 2005; Piranda et al. 2011; Grasperge et al. 2012; Lado et al. 2015). Dogs are the most suitable sentinel hosts for *A. aureolatum* because they are its primary host and they exhibit the highest serological prevalence of anti-*Rickettsia* spp. antibodies in endemic areas (Moraes-Filho et al. 2009; Pinter et al. 2008).

Understanding how dogs and ticks are associated to *Rickettsia* spp. is of great importance to public health (Silva et al. 2017; Vieira et al. 2018). *Amblyomma* ticks are known to parasitize dogs and *Rhipicephalus sanguineus* sensu lato (s.l.) Latreille (brown dog tick) has been incriminated as a vector of *R. rickettsii* in other countries (Demma et al. 2005). Serological evidence suggests that *R. amblyommatis*, *R. parkeri* and *R. rhipicephali* may occur in dogs in Rondônia State, especially in rural areas (Labruna et al. 2007). In the present study, dogs were treated as suitable sentinel hosts for the study region because dogs are known to be accidental hosts for ticks commonly found in the Amazon (Labruna et al. 2005b), which is considered a quiet region of Brazil (Oliveira et al. 2016).

The presence of known etiologic agents of rickettsiosis (*R. rickettsii* and *R. parkeri*) has not been confirmed in the North Region, and evidence of tick vectors is lacking in some municipalities where confirmed cases of BSF have occurred (Alvorada d'Oeste, Porto Velho and Rolim de Moura). It is possible that ticks considered to be non-vector species may in fact be acting as vectors in this region (Labruna et al. 2005b). The present study aimed to describe free-living tick fauna and its association to dogs by testing ticks and dogs

for the presence of rickettsial DNA and by assessing the prevalence of anti-*Rickettsia* spp. antibodies in dog sera collected in Porto Velho, Rondônia, Brazil.

Materials and methods

Study area and tick collection

This study was conducted in Porto Velho municipality (8°45′0.43″S, 63°54′0.14″W), Rondônia State, North Region, Brazil. The tick collections and dog blood samples were obtained between July 2017 and January 2018. Free-living tick collections were performed once monthly, for a total of six collections at each site. Tick collections and blood samples were taken at the same time, once from each dog, at the location described. According to the Köppen and Geiger (1928) climate classification, Porto Velho municipality has a tropical monsoon climate (Am) and is covered primarily by open ombrophilous forest (IBGE 2012).

Tick collection and dog blood sampling was performed in five peri-urban areas and five rural areas, and at a non-governmental organization (NGO) dog shelter (Associação Protetora dos Animais Desamparados—Amigos de Patas) which houses stray and abandoned dogs from all over Porto Velho (Fig. 1). Rural zones were defined as areas more than 10 km distant from the urban perimeter, as proposed by Costa et al. (2017). The points of tick collections in both peri-urban and rural areas were chosen based on the following criteria: presence of forest fragments where there was evidence of animal trails, water sources (streams and lakes) and close proximity to human and animal shelters (anthropic activity).

Free-living ticks were collected using drag flagging and visual inspection techniques (Oliveira et al. 2000) in forest fragments where wild animal trails were in evidence and water sources (streams and lakes) were close at hand. The ticks were stored alive in plastic flasks and taken to an incubator with controlled environmental conditions:

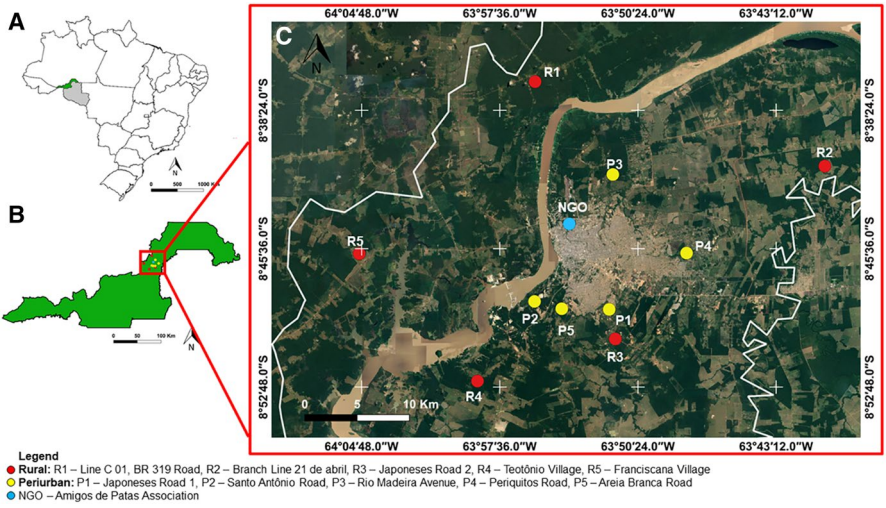

Fig. 1 Distribution of the peri-urban and rural sites, and location of the NGO 'Amigos de Patas' in Porto Velho municipality, Rondônia State, Brazil

23–28 °C, > 90% humidity and L12:D12 photocycle. The tick collections were performed monthly for 6 months to verify whether or not tick species diversity changes during the transition between dry–wet seasons. Tick collection from dogs were performed only once because the number of dogs at each collection site was limited, and because we decided to not restrain the dogs more than once. Consequently, dogs were examined for the presence of tick fauna and for serological evidence of infection by *Rickettsia* spp., without regard for the impact of seasonal changes.

All collections were made in the vicinity of human dwellings and domestic animal enclosures (anthropized areas). Tick collection from dogs was performed by visual inspection and manual detachment using traction and torsion. Adult and nymph *Amblyomma* specimens were identified down to the species level, based on the taxonomic keys of Onofrio et al. (2006) and Martins et al. (2010), respectively; the *Haemaphysalis* ticks were identified down to genus level for nymphs and species level for adults (Dantas-Torres et al. 2019). Nymphs were stored in microtubes containing up to 10 specimens per tube, and adults were stored individually. Samples were preserved in isopropyl alcohol at −20 °C until DNA extraction and polymerase chain reaction (PCR) was performed. No larva was analyzed in this study because no larvae were found in the environment or on dogs.

The tick collections were authorized under SISBIO license protocol number 63827-1.

Dog blood and tick sampling

The number of dogs studied was determined based on the estimated population of dogs in Porto Velho municipality—which is 10% of the human population (Porto Velho: 511,219 inhabitants) (IBGE 2018)—in conjunction with the estimated prevalence of seropositivity to *Rickettsia* spp. (Labruna et al. 2007; Costa et al. 2017). The number was calculated with a confidence level of 95% and a margin of error of 5% using 'Epitools epidemiological calculators—Sample size to estimate a proportion or apparent prevalence with specified precision' (http://epitools.ausvet.com.au/). Thus, the minimal number of dogs necessary to estimate the prevalence of seropositivity to *Rickettsia* spp. was calculated to be 139 individuals. Only healthy and non-pregnant dogs were chosen for collections.

Dogs were physically restrained and muzzled during blood sampling. The blood was collected by puncturing the cephalic vein (22G needle coupled to a 5 mL syringe). The gender of the dogs was not recorded because it was not relevant to the objectives of this study. Immediately following blood draw, samples from each dog were transferred to two vacuum tubes, one containing no reagent and one containing the anticoagulant EDTA. To separate serum from the blood cells, blood samples were stored in tubes with no reagent for 12 h at 4 ºC and then centrifuged at 2000×*g* for 5 min. Samples were properly identified and stored at − 20 °C prior to DNA extraction from whole blood and serological testing with serum. Dogs were inspected for presence of ticks at the same time as the blood collections. Only partial and fully engorged ticks were collected. The collected ticks were stored in plastic tubes containing absolute isopropanol, and stored at − 16 °C until identification and DNA extraction. Tick collections from dogs were performed only once because the main objective was to assess the prevalence of seropositivity to *Rickettsia* spp. among dogs. The impact of seasonality among tick-associated dogs was not a concern, therefore the number of dogs examined was based on the estimated prevalence of seropositivity to *Rickettsia* spp. Ticks were collected from dogs solely to evaluate the incidence of ticks parasitizing dogs at the time of blood collection. All procedures involving animals were

approved by the Ethical Committee of Animal Use of Fiocruz Rondônia (CEUA/Fiocruz-RO) under protocol number 2016/06.

Molecular detection of Rickettsia spp. DNA

Tick DNA extraction was based on the guanidine isothiocyanate-phenol technique, as previously described by Sangioni et al. (2005). DNA was extracted from dog blood using the GenomicPrep Mini Spin Kit (GE Healthcare). DNA samples were subjected to PCR using primers CS-78 (5′ GCAAGTATCGGTGAGGATGTAAT 3′) and CS-323 (5′ GCTTCC TTAAAATTCAATAAATCAGGAT 3′) to amplify the 401-base pair (bp) fragment of the citrate synthase (*gltA*) gene, which is present in all species of the *Rickettsia* genus (Labruna et al. 2004c). In addition, we used primers to amplify the tick ITS-2 gene fragment (ITS-2F-5′-CGGATCACATATCAAGAGAG-3′ and ITS-2R-5′-CCCAACTGGAGTGGCCCA GTTT-3′) to validate the assays and ensure the DNA quality.

Indirect immunofluorescence assay (IFA)

Dog serum was subjected to indirect immunofluorescence assay (IFA) to test reactivity against five *Rickettsia* species found in Brazil: *R. rickettsii* strain Taiaçu, *R. parkeri* strain At24, *R. amblyommatis* strain Ac37, *R. rhipicephali* strain HJ5, and *R. bellii* strain Mogi. Bacteria from *Rickettsia* spp. cell cultures was fixed on IFA glass blades, according to Labruna et al. (2007). Dog serum was diluted to 1:64 in phosphate saline buffer pH 7.4 (PBS-0.0084 M Na_2HPO_4, 0.0018 M NaH_2PO_4 and 0.147 M NaCl), applied to the *Rickettsia*-fixed blades, and incubated at 37 °C for 30 min in a humid chamber. Samples were considered positive after successive dilution using a dilution ratio of two to obtain the final titer. Reactions were considered homologous to a specific *Rickettsia* sp. when the titer was at least four times higher for that species than for any other species (Piranda et al. 2008). Serum from a confirmed seropositive dog, infected with *R. parkeri*, was used as positive control, and serum from a healthy dog was used as negative control; both sera were tested in a previous study (Szabó et al. 2013b).

Data analysis

Interpolation and extrapolation curves based on Hill's effective numbers were used to compare species richness and assess sample completeness for each environment. The Hill numbers express the relationship between species richness and relative abundance as a set of diversity numbers of different orders, in this case: order 0 (number of different species), 1 (Shannon index) and 2 (Simpson index) (Chao et al. 2014; Hsieh et al. 2016). Species composition in distinct environments was compared via a permutational multivariate analysis of variance (PERMANOVA) applied on a Bray–Curtis distance matrix calculated on double square root transformed data (Anderson 2001; Anderson and Walsh 2013). All data were analyzed in the statistical environment R (R Development Core Team 2018) using functions from the tidyverse package (Wickham 2017) and iNEXT (Chao et al. 2014; Hsieh et al. 2016).

The estimated minimal infection rate for ticks with *Rickettsia* spp. was calculated as the total number of positive nymph pools plus positive individual adults, divided by the total number of examined ticks, multiplied by 100 (Burket et al. 1998).

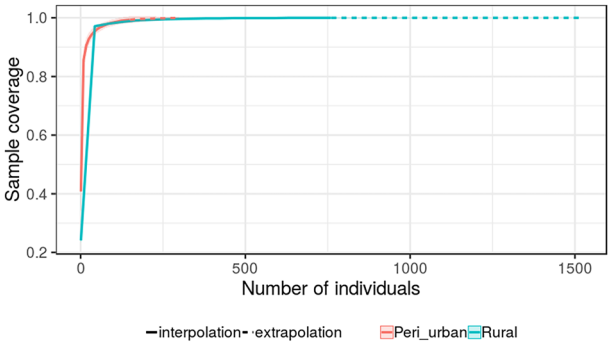

Fig. 2 Sample completeness in peri-urban and rural areas of Porto Velho municipality, Rondônia State, Brazil

The serological prevalence of anti-*Rickettsia* spp. antibodies was calculated as the number of seropositive individuals divided by the total number of examined individuals, multiplied by 100 (Margolis et al. 1982).

Results

Free-living ticks

In total, 910 tick specimens were collected (625 nymphs and 285 adults, i.e., 149 males and 136 females), of which nine species and two genera (*Amblyomma* and *Haemaphysalis*) were identified, with a sample completeness of 99% in both areas (Fig. 2). No free-living larvae were collected in this study. The tick species found here were *Amblyomma scalpturatum* Neuman (n = 301, 33%), followed by *Amblyomma naponense* Packard (n = 244, 27%), *A. coelebs* (n = 210, 23%), and *A. oblongoguttatum* (n = 117, 13%), *Amblyomma* sp. (n = 12, 1.3%), *Amblyomma latepunctatum* Neumann (n = 10, 1.1%), *Haemaphysalis* sp. (n = 9, 1%), *Amblyomma pacae* Aragão (n = 5, 0.5%) and *A. ovale* (n = 2, 0.2%) (Fig. 3a). The proportion of nymphs to the total of collected ticks (nymphs/total ticks × 100) for each species was: 100% for *A. ovale* and *A. pacae* (only nymphs), 99% for *A. coelebs*, 92% for *Amblyomma* sp., 89% for *Haemaphysalis* sp., 78% for *A. oblongoguttatum*, 70% for *A. latepunctatum*, 57% for *A. naponense* and 54% for *A. scalpturatum* (Fig. 3b).

Rural areas exhibited a higher index of species richness and a higher abundance of ticks than peri-urban areas for both common and rare species (85% of all free-living ticks were collected in rural areas) (Table 2). The effective index of species based on Hill's numbers stated that the rural areas had a Shannon index (H′) of 4.65, corresponding to the number of rare species, and a Simpson index (1/D) of 4.15, which is the number of common species. The urban areas had an index of 3.5 rare species (H′) and 2.45 common species (1/D) (Table 1). A total of 762 ticks were collected in rural areas: 212 *A. scalpturatum*, 208 *A. naponense*, 199 *A. coelebs*, 111 *A. oblongoguttatum*, nine *A. latepunctatum*, nine *Haemaphysalis* sp., eight *Amblyomma* sp., four *A. pacae* and two *A. ovale*. Only four ticks could not be identified at the species level (Table 2). Species distribution was similar in both environments. In total, 148 specimens were collected in peri-urban areas: 89 *A. scalpturatum*, 36 *A. naponense*, 11 *A. coelebs*, six *A. oblongoguttatum*, four *Amblyomma* sp., one *A. latepunctatum* and one *A. pacae* (Table 3).

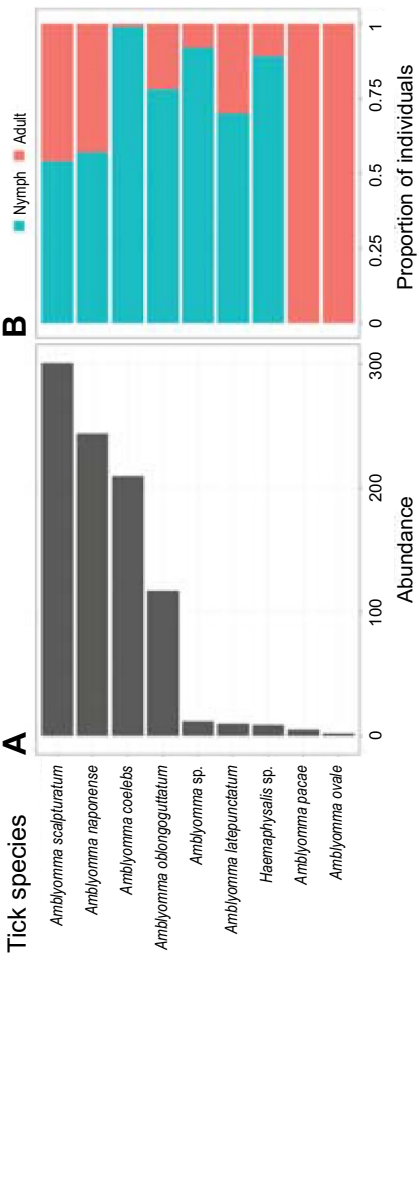

Fig. 3 **a** Abundance and **b** proportion of free-living nymphs and adult ticks collected from peri-urban and rural areas of Porto Velho municipality, Rondônia State, Brazil

Table 1 Diversity estimates for tick fauna collected from peri-urban and rural areas of Porto Velho municipality, Rondônia State, Brazil

Index	Rural			Peri-urban		
	Observed	Estimated	95% CI	Observed	Estimated	95% CI
Richness	9.0	9.0	9.0–9.90	8.0	8.5	8.05–16.10
Shannon	4.65	4.65	4.65–4.90	3.40	3.50	3.40–4.10
Simpson	4.15	4.15	4.15–4.30	2.45	2.45	2.45–2.90

95% CI 95% confidence interval

There was no statistical difference in species composition between rural and peri-urban environments (Permanova pseudo-F = 1.32, p = 0.32). Results were similar when comparing only adults (Permanova pseudo-F = 1.18, p = 0.45) and only nymphs (Permanova pseudo-F = 1.34, p = 0.33).

Table 2 Free-living and dog-related ticks species collected between August 2017 and January 2018 at 10 sites (five per environment) in peri-urban (PU) and rural (R) areas of Porto Velho municipality, Rondônia State, Brazil, and number of pools/samples processed for molecular detection of *Rickettsia* spp

Origin	Species	Stage (number of specimens)			
		Nymph (pools/samples)	Female	Male	Total
Peri-urban (PU) environment	*Amblyomma scalpturatum*	58 (6)	11	20	89
	A. naponense	13 (2)	10	13	36
	A. coelebs	11 (1)	–	–	11
	A. oblongoguttatum	5 (1)	1	–	6
	A. latepunctatum	1 (1)	-	–	1
	A. pacae	–	1	–	1
	Amblyomma spp.	4 (1)	–	–	4
Total PU environment		**92 (12)**	**23**	**33**	**148**
Rural (R) environment	*A. scalpturatum*	104 (11)	45	63	212
	A. naponense	126 (13)	49	33	208
	A. coelebs	196 (20)	2	1	199
	A. oblongoguttatum	86 (9)	14	11	111
	A. latepunctatum	6 (1)	1	2	9
	A. pacae	–	–	4	4
	A. ovale	–	1	1	2
	Amblyomma spp.	7 (1)	1	–	8
	Haemaphysalis spp.	8 (1)	–	1	9
Total R environment		**533 (56)**	**113**	**116**	**762**
PU dogs	*Rhipicephalus sanguineus* s.l	2 (1)	53	27	82
	A. ovale	–	2	1	3
R dogs	*R. sanguineus* s.l	6 (1)	17	26	49
	A. oblongoguttatum	–	2	6	8
Total		**633 (70)**	**210**	**209**	**1052**

Table 3 Total number of dogs parasitized by ticks and total number of ticks collected from dogs between August 2017 and January 2018 in peri-urban (PU1-5) and rural (R1-5) areas of Porto Velho municipality, Rondônia State, Brazil

Species			*Rhipicephalus sanguineus*	*Amblyomma oblongogut-tatum*	*Amblyomma ovale*	Total
Peri-urban	PU1	Dogs (%)	9 (25.7)	–	–	9 (25.7)
		N	–	–	–	–
		A (F/M)	34 (24/10)	–	–	34 (24/10)
	PU2	Dogs (%)	4 (11.4)	–	–	4 (11.4)
		N	–	–	–	–
		A (F/M)	4 (1/3)	–	–	4 (1/3)
	PU3	Dogs (%)	4 (11.4)	–	2 (5.7)	6 (17.1)
		N	–	–	–	–
		A (F/M)	10 (7/3)	–	3 (2/1)	13 (9/4)
	PU4	Dogs (%)	12 (34.3)	–	–	12 (34.3)
		N	2	–	–	2
		A (F/M)	17 (10/7)	–	–	17 (10/7)
	PU5	Dogs (%)	4 (11.4)	–	–	4 (11.4)
		N	–	–	–	–
		A (F/M)	15 (11/4)	–	–	15 (11/4)
	Total PU	Dogs (%)	33 (94.3)	–	2 (5.7)	35 (100)
		N	2	–	–	2
		A (F/M)	80 (53/27)	–	3 (2/1)	83 (55/28)
Rural	R1	Dogs (%)	6 (31.6)	–	–	6 (31.6)
		N	–	–	–	–
		A (F/M)	9 (4/5)	–	–	9 (4/5)
	R2	Dogs (%)	–	–	–	–
		N	–	–	–	–
		A (F/M)	–	–	–	–
	R3	Dogs (%)	2 (10.5)	1 (5.3)	–	3 (15.8)
		N	–	–	–	–
		A (F/M)	2 (1/1)	1 (0/1)	–	3 (1/2)
	R4	Dogs (%)	8 (42.1)	4 (21.1)	–	9 (47.4)
		N	1	–	–	1
		A (F/M)	30 (11/19)	6 (2/4)	–	36 (13/23)
	R5	Dogs (%)	1 (5.3)	1 (5.3)	–	1 (5.3)
		N	5	–	–	5
		A (F/M)	2 (1/1)	1 (0/1)	–	3 (1/2)
	Total R	Dogs (%)	17 (89.5)	6 (31.6)	–	19 (100)
		N	6	–	–	6
		A (F/M)	43 (17/26)	8 (2/6)	–	51 (19/32)
Total number of ticks (%)			131 (92.3)	8 (5.6)	3 (2.1)	142 (100)

N nymphs, *A* (*F*/*M*) Adults (female/male)

Ticks parasitizing dogs

A total of 190 dogs were examined for ticks (97 from peri-urban areas, 77 from rural areas, and 16 from the 'Amigos de Patas' dog shelter). In total, 54 dogs (28.4%) were found with attached ticks: 50 with *R. sanguineus* s.l. (92.6%), six with *A. oblongoguttatum* (11.1%) and two with *A. ovale* (3.7%). Four dogs had mixed infestations of *R. sanguineus* s.l. and *A. oblongoguttatum* (Table 3). Ticks were found on 36.1% of peri-urban dogs, 24.7% of rural dogs and no dogs from NGO presented ticks. *Rhipicephalus sanguineus* s.l. (brown dog tick) was the most abundant species; it was found on 26.3% of examined dogs (22.1% of rural dogs and 34% of peri-urban dogs). The other tick species found on dogs were *A. oblongoguttatum* (3.2% of dogs) and *A. ovale* (1.1% of dogs). *Amblyomma ovale* was found on 2.1% of peri-urban dogs, and *A. oblongoguttatum* was found on 7.8% of rural dogs.

Tick collection data is detailed in Table 3. Percentage values for dogs were calculated as the total number of dogs with ticks from each collection site divided by the total number of dogs found with ticks from peri-urban or rural areas. Percentage values for ticks were calculated as the total number of ticks from each tick species divided by the total number of ticks collected from all examined dogs. Tick collection data from the 'Amigos de Patas' dog shelter has been excluded because no attached ticks were found at this site.

Rickettsia spp. DNA detection

DNA was extracted from all collected ticks and from 171 samples of dog blood and PCR targeting the *gltA* gene fragment was performed: a total of 1052 specimens (both free-living and parasitic) comprised of 633 nymphs (70 pools of up to 10 individuals per pool), 419 adults (individuals). Notwithstanding, no amplification was detected.

Anti-*Rickettsia* spp. antibody detection

Within the 190 tick-collected dogs, IFA was performed on 171 samples of dog serum, which have the blood collected, from peri-urban areas (77), rural areas (78), and the 'Amigo de Patas' dog shelter (16). Serum from 16/77 (21%) peri-urban dogs and 12/78 (15.4%) rural dogs exhibited reactivity to *R. rhipicephali*, *R. amblyommatis*, *R. bellii* or *R. parkeri*. Titrations varied from 1:64 to 1:1024. The total prevalence of anti-*Rickettsia* spp. antibodies was 16.4% (28/171). Among the serum-reactive dogs, the prevalence of anti-*Rickettsia* spp. antibodies was 58.6% for *R. bellii*, 27.6% for *R. amblyommatis*, 10.3% for *R. rhipicephali* and 3.4% for *R. parkeri*. No dog from the 'Amigo de Patas' dog shelter was serum-reactive (Table 4).

Discussion

Amblyomma scalpturatum was the most abundant tick species in both peri-urban and rural environments. In Brazil, the geographic distribution of this species extends from the northern states of Acre, Amazonas, Pará, Roraima and Rondônia, through the middle western states of Mato Grosso and Mato Grosso do Sul, to the southern state of Paraná (Pereira

Table 4 Indirect immunofluorescence assay (IFA) titers for dog sera exhibiting reactivity to five *Rickettsia* species from peri-urban (PU1-5) and rural (R1-5) areas of Porto Velho municipality, Rondônia State, Brazil

Local	Total dogs reactive/ tested (%)	Reactive sample ID	IFA titers					PAIHR
			R. rickettsii	*R. parkeri*	*R. amblyommatis*	*R. rhipicephali*	*R. bellii*	
PU1	4/15 (26.7)	2	–	–	–	–	512	*R. bellii*
		3	–	–	–	–	512	*R. bellii*
		5	–	–	512	–		*R. amblyommatis*
		12	–	–	–	–	256	*R. bellii*
PU2	2/16 (12.5)	48	–	–	–	–	128	*R. bellii*
		54	–	–	128	–	–	*R. amblyommatis*
PU3	4/16 (25.0)	16	–	–	256	–	–	*R. amblyommatis*
		20	–	–	128	–	–	*R. amblyommatis*
		21	–	–	64	–	–	–
		27	–	–	–	64	–	–
PU4	2/15 (13.3)	33	–	–	–	–	256	*R. bellii*
		39	–	–	–	–	256	*R. bellii*
PU5	4/16 (25.0)	62	–	–	64	–	–	–
		65	–	64	–	64	–	–
		70	–	–	–	–	128	*R. bellii*
		77	–	–	–	–	1024	*R. bellii*
R1	3/15 (20.0)	100	–	–	–	–	64	–
		101	–	–	256	–	–	*R. amblyommatis*
		107	–	–	–	–	512	*R. bellii*
R2	0/15 (0.0)	–	–	–	–	–	–	–
R3	5/16 (31.3)	78	–	–	–	–	64	–
		83	–	–	–	–	512	*R. bellii*
		85	–	–	64	–	–	–
		86	–	–	–	–	512	*R. bellii*
		94	–	–	–	–	1024	*R. bellii*
R4	2/15 (13.3)	117	–	–	–	–	1024	*R. bellii*
		121	–	–	–	–	1024	*R. bellii*
R5	2/16 (12.5)	138	–	–	–	–	1024	*R. bellii*
		139	–	–	–	128	256	–
NGO	0/16 (0.0)	–	–	–	–	–	–	–

ID identification number of each serum sample, *PAIHR* possible antigen involved in the homologous reaction

et al. 2000; Labruna et al. 2005c; Onofrio et al. 2006, 2010; Lima et al. 2018; Aguirre et al. 2019).

Amblyomma scalpturatum has a preference for tapirs (*Tapirus terrestris*) and wild suid (Suidae), but its parasitism is distributed evenly among other animals, such as anteaters (*Myrmecophaga tridactyla*) and domestic dogs. Immature stages of this species can also parasitize humans (Labruna et al. 2005b; Aguirre et al. 2019). These results may be contingent upon the fact that the study was conducted near regions of human activity and that the wild hosts happened to be present in those regions. *Amblyomma scalpturatum* has been associated to *R. bellii*, which is not pathogenic to humans or domestic animals (Labruna et al. 2004b; Silva et al. 2016). The first record of an *A. scalpturatum* tick infected with a SFG *Rickettsia* was published recently, in an Amazon region of the state of Mato Grosso (Colle et al. 2020). Future studies will need to investigate *A. scalpturatum*'s role as a reservoir for SFG *Rickettsia*, given that *A. scalpturatum* comes in frequent contact with humans, its immature stages exhibit low specificity for hosts, and it occurs in high abundance in the Amazon (Labruna et al. 2005c).

Three other tick species were abundant in rural environments: *A. coelebs, A. naponense* and *A. oblongoguttatum*. Both *A. coelebs* and *A. oblongoguttatum* are considered putative vectors of infectious agents, such as *R. amblyommatis* which belongs to the SFG (Labruna et al. 2004b; Aguirre et al. 2018). Serological evidence from patients diagnosed with Rocky Mountain Spotted Fever (RMSF) in the USA suggests that *R. amblyommatis* may be pathogenic to humans (Apperson et al. 2008).

Eight specimens of *Haemaphysalis* sp. and one male of *H. juxtakochi* were collected. This is the only species of the *Haemaphysalis* genus known to occur in Rondônia State. It has a preference for deer but also parasitizes other mammals, including dogs (Labruna et al. 2005b). *Haemaphysalis juxtakochi* is known to be associated to *R. rhipicephali*, which belongs to the SFG although its pathogenicity to humans and domestic animals is unknown (Labruna et al. 2005a).

The present study is the first to report the presence of *A. pacae* and *A. ovale* in Porto Velho municipality, Rondônia State. *Amblyomma ovale* is known to be a vector of *R. parkeri* Atlantic rainforest strain, which is pathogenic to humans (Faccini-Martínez et al. 2018). Two cases of Spotted Fever in Porto Velho municipality have been reported to Datasus (2021); nevertheless, the *Rickettsia* spp. responsible for these human infections and tick vectors remain unknown. Moreover, no study has detected pathogenic *Rickettsia* in Rondônia State until now.

A study conducted by Szabó et al. (2001) examined 140 dogs from Franca municipality in São Paulo State, and found that 27.5% of urban dogs were parasitized solely by *R. sanguineus* s.l. The same study found ticks on 36.8% of rural dogs; half of these dogs were parasitized by *R. sanguineus* s.l. (18.4%) whereas the other half were parasitized by *A. cajennense* s.l., *Rhipicephalus microplus* and *A. ovale*. More peri-urban dogs than rural dogs were parasitized by *R. sanguineus* s.l. in the present study. In the metropolitan region of Curitiba in Paraná State, a recent study of dogs from urban and rural areas found that 57.7% of dogs were parasitized by *R. sanguineus* s.l., 38.5% were parasitized by *A. aureolatum* and 3.8% were parasitized by *A. ovale* (which was found only on rural dogs) (Silva et al. 2017). In the present study, *A. ovale* was found only on peri-urban dogs. In Espírito Santo State, another study found that 40.7% of rural and urban dogs were parasitized by *R. sanguineus* s.l., whereas 0.53% were parasitized by *A. sculptum* and 0.26% by *A. ovale* (Vieira et al. 2018).

In the present study, *A. ovale* was found on dogs from peri-urban areas. It is important to note that peri-urban environments in this study were similar to rural environments, in

that peri-urban dogs had free access to preserved forest fragments. *Amblyomma ovale* is commonly found on rural dogs because these dogs have accesses to environments that harbor wild animals, which are *A. ovale*'s primary hosts. Labruna et al. (2005b) found that most dogs from urban and rural areas across Rondônia State were parasitized by *A. ovale*, which suggests that *A. ovale* may be the most common dog-parasitizing tick in some areas of Rondônia.

Amblyomma oblongoguttatum was collected only in rural environments (at distinct sites). In Uruará municipality, Pará State, Labruna et al. (2000) found that rural dogs were frequently exposed to *A. oblongoguttatum* and that this was the predominant dog-parasitizing tick in the North Region of Brazil. Dogs have been shown to be the primary hosts for the adult stage of *A. oblongoguttatum* under laboratory conditions (Martins et al. 2017). However, *A. oblongoguttatum*'s role in transmitting pathogenic agents to humans and domestic animals has not been studied, even though this species is known to be associated to *R. amblyommatis* (Aguirre et al. 2018).

Dogs were seropositivity to *Rickettsia* spp. but did not present *Rickettsia* DNA in blood samples. It is not necessary for the same animal to test positive to *Rickettsia* in both the IFA and PCR assays. In order to test positive by both methods, the animal needs to have been infected with *Rickettsia* spp. for some days, its plasma cells (transformed B lymphocytes) need to have started producing IgG, and *Rickettsia* needs to be circulating in the peripherical blood. In the case of this study, it is possible that all dogs that presented IgG anti-*Rickettsia* in the IFA acquired a *Rickettsia* infection some time ago, but at the moment of blood collection all dogs were healthy. Notwithstanding, this apparent absence could be explained by the low incidence of *Rickettsia* in dogs or by PCR's low sensitivity to circulating *Rickettsia* (Stenos et al. 2005). Some studies have shown that nested PCR may be a more effective means of detecting *Rickettsia* DNA because this form of PCR increases analytic sensitivity (Fournier and Raoult 2004; Choi et al. 2005; Sousa et al. 2005, Santibañez et al. 2013). Pathogenic *Rickettsia* grows in endothelial cells which suggests that molecular diagnosis would be more effective if it were applied to tissues or internal organs (Faccini-Martínez et al. 2018).

The prevalence of anti-*Ricketssia* spp. antibodies in dog blood samples was 16.4% (28/171), which indicates that dogs have been exposed to *Rickettsia* spp. in Porto Velho municipality. Although 28 dog serum samples exhibited reactivity, only 20 samples had high enough titers to be considered homologous reactions: 15 for *R. bellii* (75%) and five for *R. amblyommatis* (25%). Two samples exhibited low titers so the *Rickettsia* spp. for these samples could not be determined. The other serum-reactive samples exhibited cross-reaction with two or more *Rickettsia* spp. (Table 4). According to Brezina et al. (1973), when cross-reaction occurs it is not possible to determine which *Rickettsia* sp. was responsible for the reactions.

Nevertheless, Labruna et al. (2007) found that most dog serum-reactive samples tested by IFA exhibit reactivity to more than one *Rickettsia* spp., and that 11.6% (19/164) of rural dogs and 3.9% (6/153) of urban dogs were serum-reactive. In that study, the most prevalent reactions were to *R. parkeri*, *R. rhipicephali* and *R. amblyommatis*. The prevalence of serum-reactive animals was higher in the present study. In an area non-endemic for BSF in Espírito Santo State, 5.13% of examined dogs were found to be serum-reactive to *Rickettsia* spp., and 1.6% of examined dogs exhibited reactivity to *R. rickettsii* antigens (Vieira et al. 2018). In a BSF-endemic area of Rio de Janeiro State, 28% of dogs were found to be serum-reactive to *Rickettsia* spp. (Cunha et al. 2014).

Horses are treated as sentinel hosts in BSF-endemic areas where the predominant vector is *A. sculptum*, because horses are this vector's primary host and horses in these areas

usually exhibit seroprevalence higher than 50% (57.1–90%). Dogs exhibit lower seroprevalence (8–66.7%) because they are only accidental hosts for *A. sculptum* (Sangioni et al. 2005). Conversely, dogs are treated as sentinel hosts in BSF-endemic areas where *A. aureolatum* is the predominant vector, because dogs are this vector's primary host and dogs exhibit seroprevalence higher than 60% in these areas (Pinter et al. 2008). An area is considered to be at high-risk for BSF transmission when 50% of the sentinel hosts are serum-reactive (Vieira et al. 2018). In the present study, 16.4% of dogs were serum-reactive to *Rickettsia* spp. and the majority of homologous reactions were homologous to *R. bellii* (Table 4). Dog serum reactive to *R. amblyommatis* (which belongs to the SFG) was collected at four sites in this study: PU1 (6.7%), PU2 (6.3%), PU3 (12.5%) and R1 (6.7%). Dogs in the region have been found parasitized by the following tick species common to the Amazon: *A. cajennense* s.l., *A. naponense*, *A. oblongoguttatum*, *A. ovale*, *A. pacae*, *A. scalpturatum*, *A. tigrinum* and *H. juxtakochi* (Labruna et al. 2005b). This suggests that the seroprevalence of *Rickettsia* spp. has a unique profile in this region relative to BSF-endemic regions, given that there have been two confirmed cases of BSF in the municipality, but no dogs tested positive for reactivity to *R. rickettsii*.

Dog serum samples exhibited reactivity to three SFG *Rickettsia* spp. (at titrations of 1:64 to 1:1024): *R. amblyommatis*, *R. parkeri* and *R. rhipicephali*. Of these, only *R. parkeri* (Atlantic rainforest strain) is known to be pathogenic in Brazil (Szabó et al. 2013a). The pathogenicity of *R. rhipicephali* and *R. amblyommatis* remains unknown. However, there is serological and symptomatological evidence which suggests that humans can be infected by *R. amblyommatis* (Apperson et al. 2008; Delisle, et al. 2016).

In the IFA, serum from 10 dogs exhibited reactivity to *R. amblyommatis*, *R. rhipicephali* and *R. bellii* in dogs parasitized by *R. sanguineus* s.l., *A. oblongoguttatum* and *A. ovale*. This does not prove that these ticks transmitted these rickettsial agents, but it does demonstrate that the dogs experienced simultaneous exposure to both. According to Labruna et al. (2005a), *R. bellii* is commonly found associated to the ticks *A. ovale*, *A. oblongoguttatum* and *A. scalpturatum*, whereas *R. amblyommatis* is associated to the ticks *A. cajennense* s.l. and s.s., *A. coelebs* and *A. oblongoguttatum*; and *R. rhipicephali* is associated to the tick *H. juxtakochi* (Labruna et al. 2004b; Aguirre et al. 2018). This study collected only free-living *H. juxtakochi*. In the present study, the sera of dogs parasitized only by *R. sanguineus* s.l. ticks exhibited reactivity to *R. bellii*, *R. amblyomatis* and *R. rhipicephali*, whereas dogs parasitized by *A. oblongoguttatum* and *A. ovale* exhibited reactivity to *R. bellii* and *R. amblyommatis*, respectively. Once again, this does not prove that these ticks transmitted these rickettsial agents, but it does demonstrate simultaneous exposure.

This study is the first to demonstrate the prevalence of anti-*Rickettsia* antibodies in dog sera from Porto Velho, Rondônia State. The study was conducted in response to recent reports of BSF in the region (Datasus 2021). Dogs were screened for anti-*Rickettsia* antibodies because dogs experience high exposure to several tick species, and dogs live in close proximity to humans in the study region. Furthermore, dogs are considered suitable sentinels for monitoring *Rickettsia* spp. circulation in regions where *A. sculptum* is not the predominant tick (Demma et al. 2005; Pinter et al. 2008).

For the majority of dog sera, antibody titers were low and thus taken to be evidence of mild or earlier infections. Given that rickettsial infection appears to be a longstanding condition among dogs in the region, studies should be conducted in other municipalities where human cases are confirmed or suspected, and these studies should aim to identify which *Rickettsia* spp. are responsible for the human cases. To this end, a retrospective study should be conducted to examine sera from humans with a history of acute

exanthematous febrile illness. Finally, horses and wild animals (such as capybaras, small rodents, marsupials, and wild carnivores) should be examined in order to form a complete picture of the epidemiological cycle of *Rickettsia* spp. in Rondônia State.

The presence of serum-reactive animals in the study region indicates that *Rickettsia* spp. (including SFG vectors) are present in Porto Velho municipality. It is still not possible to confirm which *Rickettsia* spp. were responsible for the human cases in Rondônia State, nor is it possible to confirm which tick species are acting as vectors in the region. Future studies will be needed to clarify the epidemiological cycle of *Rickettsia* spp. and assess the risk factors of BSF (and other possible tick-borne agents) in the Amazon region.

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Data availability The authors declare that the data herein provide an accurate and transparent account of our research.

Declarations

Conflict of interest The authors have no conflict of interest.

Ethical approval This study and its animal handling procedures were analyzed and approved by the Institutional Animal Care and Use Committee under protocol number 2016/06 (CEUA/Fiocruz-RO).

Informed consent All dog owners gave consent for their dogs to participate in scientific research. The dog owners gave consent with the understanding that no personal information would be included in the published data.

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