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Exposure of dogs to *Rickettsia* spp. in Costa Rica: Risk factors for PCR-positive ectoparasites and seropositivity

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ABSTRACT

Infection of dogs with *Rickettsia* spp. can result in inapparent, mild, or severe disease. Moreover, common dog ticks and fleas are able to transmit rickettsiae to nearby humans. In this study, the seroprevalence of spotted fever group (SFG) rickettsiae was determined in dogs of Costa Rica, as well as possible risk factors associated with exposure. An interview of owners and clinical examinations were performed in a country-wide sample of 441 dogs. IgG antibodies were determined in 399 dogs by indirect immunofluorescence assay (IFA) using antigens of *Rickettsia rickettsii*, *R. amblyommatis*, and *R. felis*. The presence of *Rickettsia* spp. *gltA* gene was evaluated by PCR in ticks and fleas. Poisson regression was performed to assess possible risk factors associated with seropositivity, as well as with having PCR-positive ticks and fleas. The overall seroprevalence to SFG rickettsiae was 10.0% (end titers 64 to 256). *Rhipicephalus sanguineus* s.l. (116/441; 26.3%) and *Ctenocephalides felis* (153/441; 34.7%) were the most common ectoparasites. *Rickettsia* DNA was detected in 30% (39/130) and 32.3% (56/173) of tick and flea pools, respectively. Seropositivity was significantly associated with mean age of 2 to 7 years, scrotal edema, walking problems, large size, and tick and flea infestation. Being a purebred dog was a possible protective factor. The presence of *Rickettsia* PCR-positive ticks was associated with being a purebred dog, while flea treatment was protective. Having PCR-positive fleas was associated with being purebred and the number of people in the dog's environment; protective factors were free roaming and being an outdoor dog. Results confirm that dogs in Costa Rica are exposed to different species of SFG rickettsiae. This may represent a risk to human health and underscores the need for accurate diagnosis in dogs and humans. Surveillance of rickettsial infection in canines may provide useful indicators to understand the epidemiology of these zoonoses.

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1. Introduction

The genus *Rickettsia* includes intracellular bacteria that infect vertebrates and invertebrates, some of which cause disease in humans and domestic animals, including dogs. The spotted fever group (SFG) includes many pathogenic species that are mostly transmitted by ticks. Of these, *Rickettsia rickettsii* is considered the most virulent, with high mortality rates in humans (Parola et al., 2013). In dogs, infection with *R. rickettsii* can cause severe disease and clinical manifestations that are similar to those seen in humans (Keenan et al., 1977; Gasser et al., 2001; Piranda et al., 2008). Disease signs in dogs include fever, lethargy, anemia, ocular lesions, hemorrhage, edema, and neurologic involvement (Shaw et al., 2001; Piranda et al., 2008). However, mortality rates depend on the quantity of bacteria inoculated (Keenan et al., 1977). Given the closeness of dogs and humans, evidence of rickettsial exposure of dogs supports their role as sentinels of human infection (Demma et al., 2005; Paddock et al., 2002; Elchos and Goddard, 2003).

Fleas and ticks are common ectoparasites of dogs in the Neotropical region, especially *Ctenocephalides felis* and *Rhipicephalus sanguineus* s.l. The brown dog tick, *R. sanguineus* s.l. is a competent vector of *R. rickettsii*, and it has been implicated as the principal vector in several cases and outbreaks (Demma et al., 2005; Piranda et al., 2011; Martínez-Caballero et al., 2018). In addition, other rickettsiae have been detected in *R. sanguineus* s.l. in the region, including *R. amblyommatis*, *R. massiliae*, *R. felis*, and *R. akari* (Labruna et al., 2011). Fleas that are common on dogs, such as *C. felis* and *Pulex* spp., can also be frequently infected with rickettsiae like *Rickettsia felis* and *R. asembonensis*, although it is unknown if these species can cause disease in dogs (Hun and Troyo, 2012).

In Costa Rica, human cases of *R. rickettsii* infection are diagnosed sporadically; unfortunately, some of the diagnoses are made post-mortem (Hun-Opfer, 2008; Hun, 2013; Argüello et al., 2012). No cases of spotted fever rickettsioses in dogs have been confirmed in the country, although there is serological evidence of exposure to *R. rickettsii*, *R. amblyommatis*, *R. rhipicephali*, and *R. felis* (Moreira-Soto et al., 2016; Bermúdez and Troyo, 2018). Moreover, *R. sanguineus* and *C. felis* are the most common ectoparasites of dogs in Costa Rica, and they have been found to be infected with *R. amblyommatis*, *R. felis*, and *R. asembonensis* (Álvarez et al., 2006; Troyo et al., 2012a; Troyo et al., 2012b; Bermúdez and Troyo, 2018). Therefore, the aims of this study were to determine the seroprevalence of SFG rickettsiae in dogs of Costa Rica and to evaluate possible risk factors associated with seropositivity and the presence of *Rickettsia* spp. in ticks and fleas.

2. Material and methods

2.1. Study design, sample size and population analyzed

A cross-sectional, observational, descriptive study was conducted in Costa Rica to determine the exposure of dogs to *Rickettsia* spp. in blood samples, using a serological assay. To obtain country-wide representativeness, the total sample size was estimated to be 385 individuals (50% prevalence, 95% confidence, 5% accepted error) for a calculated population of >40,000 dogs, using Win Episcope 2.0[JRZ1] (Thrusfield et al., 2001). The number of dogs was proportionally allocated by province, according to the estimated population in each. Data and samples from dogs were obtained from June 2011 to September 2012 at 15 highly visited recreational parks throughout the entire country, with the intent to obtain representatives of a mostly healthy population. More details of the population studied and sampling methodology are described in Barrantes-González et al. (2016).

2.2. Clinical examination and sampling

Owners were interviewed to obtain past or current information about the dog, as detailed in Barrantes-González et al. (2016); this included tick/flea infestation and treatment, as well a suspected diagnosis of rickettsiosis. Clinical examination was performed to determine behavior (weak, depressed, docile, alert, nervous, aggressive), capillary refill time (>2 s was considered as delayed), color of mucous membranes (very pale, pale, pink, icteric), rectal temperature (≥39.5 °C was considered as feverish), and other clinical signs that may be compatible with rickettsiosis (weight loss, epistaxis, petechiae, ecchymosis, hematuria, dyspnea, cough, lymphadenomegaly, ataxia, lameness, diarrhea, and scrotal edema) (Shaw et al., 2001; Piranda et al., 2008; Maxie, 2007; Gyles et al., 2010). Treatment with doxycycline was recorded. Blood samples were collected from each dog, stored at 4 °C until serum separation, and frozen at −20 °C prior to serological analyses. If ticks and/or fleas were present, specimens were collected and stored in 70% ethanol at room temperature. In the case of stray dogs living in recreational parks, consent from the administration was obtained to perform the clinical exam, blood sampling and extraction of ectoparasites.

2.3. Identification of ticks and fleas

Taxonomic identification of ticks was performed as described by Fairchild et al. (1966), Barros-Battesti et al. (2006), Nava et al. (2012), and Nava et al. (2014); and fleas were identified following Tipton and Mendez (1966) and Acosta and Morrone (2003). Ticks from each dog were separated and placed in microfuge tubes by species, sex, and developmental stage, whereas fleas were separated by species; all material were preserved in 70% ethanol at −20 °C until DNA extraction as described by Mtambo et al. (2006).

2.4. Serological analyses

Dog sera were analyzed by indirect immunofluorescence assay (IFA) to detect IgG antibodies to spotted fever group (SFG) rickettsiae using antigen from local strains of *Rickettsia rickettsii* (CR-2010), *Rickettsia amblyommatis* (9-CC-1), and *Rickettsia felis* (OP-16-1), and following standard methods that have been described (Labruna et al., 2007; Hun et al., 2011; Argüello et al., 2012; Moreira-Soto et al., 2016). All sera were analyzed initially at a 1:64 dilution in in phosphate-buffered saline (PBS). To determine the end point titer of sera that showed reactivity to one or more antigens, serial two-fold dilutions were prepared and evaluated in the same manner until no reactivity was observed. In each slide, a non-reactive dog serum (negative control) and a reactive to SFG rickettsiae dog serum (positive control) were included at a 1:64 dilution. FITC-labeled anti-dog IgG produced in rabbit (Sigma-Aldrich) was used in all IFAs.

Samples with an endpoint titer ≥1:64 to one or more of the three species analyzed were considered as positive, indicating exposure of the corresponding dogs to SFG rickettsiae. When an endpoint titer was at least four-fold higher for one *Rickettsia* sp. than others, it was considered as probably exposure to the species with the highest titer, or to a very closely related species (unknown or not tested), what is known as homologous reaction (Horta et al., 2004).

2.5. Molecular analyses and identification of Rickettsia spp.

DNA was extracted from ticks and fleas using the NucleoSpin® Tissue kit (Macherey-Nagel), as recommended by manufacturer. Adult female ticks from each dog were processed individually, and adult male ticks or nymphs were analyzed in pools (2 to 10 specimens) of the same species. Fleas from each dog were analyzed in pools (2 to 20 individuals) of the same species. For each dog, only one DNA sample (individual or pool) was analyzed for each ectoparasite species identified. All DNA samples from ticks and fleas were stored at −20 °C until PCR analyses.

DNA samples of ticks and fleas were analyzed by conventional one-step PCR to detect a fragment of the *Rickettsia* specific citrate synthase gene (*gltA*). Primers used were CS-78 and CS-323, which amplify a 401 bp product (Labruna et al., 2004). In each assay, a DNA sample of *Rickettsia rickettsii* or *Rickettsia amblyommatis* was included as a positive control, as well as PCR reagents and water as a negative control.

To confirm the presence of *Rickettsia* spp. and identify the most frequent species present in ticks and fleas, technical convenience criteria (amount of material, band resolution at agarose gel) were applied to selected samples of the *gltA* fragments for Sequencing. PCR products were purified using BigDye® XTerminator™ Purification Kit, sequenced with BigDye™ Terminator V 3.1, and visualized in the Genetic Analyzer 3130 (Applied Biosystems/HITACHI). Sequences were edited with BioEdit 7.1.3.0 (Hall, 1999) and aligned with ClustalX 2.1 (Thompson et al., 1997). The resulting sequences were compared against the NCBI database using the Basic Local Aligment Search Tool (BLAST).

2.6. Statistical analysis

Methods for the organization and statistical analysis of data from dogs in this study have been described previously (Barrantes-González et al., 2016). Briefly, the following indicators were determined: tick infestation prevalence (TIP), tick distribution ratio (TDR), *R. sanguineus* s.l. occurrence percentage (R.sOP), flea infestation prevalence (FIP), flea distribution ratio (FDR) and dogs per household ratio (DHR). The frequency distributions of *Rickettsia* seropositive and negative results were determined, with their respective 95% confidence intervals. To assess the risk factors associated with seropositivity, as well as with having *Rickettsia* spp. PCR-positive ticks and fleas, Poisson regression was performed following the same method described in previous studies (Barrantes-González et al., 2016). The data was collected using EpiData Software version 2.0 (Odense, Denmark); and analyzed using STATA version 12 (Stata Corp., USA).

3. Results

A total of 441 dogs were included in this study. A detailed description of this dog population and results of the interviews has been provided elsewhere (Barrantes-González et al., 2016). Clinical examination abnormalities were found in only a minority of the dogs, such as abnormal behavior (weak 0.2%, depressed 0.7%, docile 16.1%), capillary refill time > 2 s (5.2%), pale mucous membranes (very pale 1.1%, pale 11.1%), and rectal temperatures ≥ 39.5 °C (19.5%). Clinical abnormalities also included weight loss (4.5%), lymphadenomegaly (2.0%), petechiae (1.6%), cough (0.9%), scrotal edema (0.9%), ataxia (0.5%) and hematuria (0.2%).

Serum samples of 399 dogs were analyzed by IFA. The frequency of IgG antibodies to SFG *Rickettsia* spp. (end titer ≥ 64) in the dog population was 10.0% (40/399) (Table 1). Exposure to rickettsiae was more frequent in recreational parks located in Alajuela (19.5%) and Guanacaste (22.0%), while no dogs from Heredia nor Puntarenas showed evidence of exposure (Table 1).

Overall, dogs had low end titers ranging from 64 to 256. Some dogs were seroreactive to more than one species of *Rickettsia* tested, showing IgG antibodies to separate of the antigens used, without clear differences in end titers. However, an end titer that was higher (4-fold or more) to one of the antigens was present in 12 dogs: 3 were probably seroreactive to *R. rickettsii* and 9 to *R. amblyommatis*, or closely related species (Table 2).

Ticks were found on 28.8% (127/441) of the dogs sampled. The most common tick species was *Rhipicephalus sanguineus* s.l. (116/441; 26.3%); in addition, four dogs were infested with *Amblyomma ovale*, two with *Amblyomma mixtum*, one with

Table 1
Seroprevalence of IgG antibodies to *Rickettsia* spp. in dogs of Costa Rica.

Province	Total serum samples	Number of positive samples	Seroprevalence (%)
San José	171	18	10.5
Alajuela	41	8	19.5
Limón	54	2	3.7
Guanacaste	41	9	22.0
Cartago	35	3	8.6
Heredia	27	0	0.0
Puntarenas	30	0	0.0
Total	399	40	10.0

Amblyomma maculatum, and one with *Ixodes boliviensis*. Mixed infestations were observed in three dogs: two had *R. sanguineus* s.l. and *A. ovale*, while one had *R. sanguineus* s.l. and *A. mixtum*.

A total 130 tick pools were analyzed. Of these, 39 (30.0%) pools (collected from 38 dogs) were positive for *Rickettsia* spp. by PCR (Table 3). The highest positivity of *Rickettsia* spp. in tick pools was observed in the provinces of Puntarenas (66.7%) and Heredia (100%), although only 2 tick pools were evaluated in Heredia (Table 3). Of the PCR-positive ticks, 36 had been identified as *R. sanguineus* s.l. (25 females, 7 males, and 4 nymphs), one as a male *A. mixtum*, one as a female *A. ovale*, and one as a female *I. boliviensis* (Table 4). Eight (22.2%) of these (7 *R. sanguineus* s.l. and 1 *A. mixtum*) were collected from dogs that were seropositive to *Rickettsia* spp.

Fleas were found on 37.2% (164/441) of the dogs sampled. A total of 153 (34.7%) were infested with *Ctenocephalides felis*, nine with *Pulex simulans*, one with *Pulex* sp., and one with *Echidnophaga gallinacea*. Of them, six dogs had mixed infestation with *C. felis*/*P. simulans*, one with *C. felis*/*Pulex* sp., and one with *C. felis*/*E. gallinacea*. A total of 93.7% (509/543) of the fleas collected were *C. felis* and 5.7% (31/543) *P. simulans*.

Of 173 pools of fleas analyzed by PCR, 56 (32.3%) (collected from 56 dogs) were positive for *Rickettsia* spp.; the positivity was higher in Heredia (60.0%) and Cartago (100.0%) (Table 3). Although *Rickettsia* spp. was detected in all flea pools of Cartago, only 4 pools were analyzed (Table 3). Of positive samples, 53 were pools of *C. felis* and 3 were pools of *P. simulans* (Table 4). Only 4 (7.1%) positive pools of *C. felis* were collected from dogs that were also seropositive to *Rickettsia* sp.

To confirm the PCR results and identify the most frequent species of *Rickettsia* in common ticks and fleas of dogs in Costa Rica, 8 sequences of the *gltA* fragments were obtained: 4 from samples of *R. sanguineus* s.l., 3 from *C. felis*, and 1 from *A. mixtum*. *Rickettsia felis* strain URRWXCal2 was identified in *R. sanguineus* s.l. and *C. felis*, *R. asembonensis* in *C. felis*, and *R. amblyommatis* in *A. mixtum* (Table 4). All sequences were identical to reference sequences and sequences of rickettsiae from Costa Rica.

The analysis of risk factors of dogs seropositive to *Rickettsia* spp. (end titers ≥ 1:64 for one or more of the antigens used) detected a significant association with mean age of the dogs (2–7 years, $p = 0.003$), presence of scrotal edema ($p = 0.023$), walking problems in the past ($p = 0.009$), large size ($p = 0.070$), tick infestation indices (1.1–2, $p = 0.008$), and flea infestation indices (>2.1, $p = 0.005$). Moreover, being a purebred dog was determined to be a protective factor ($p = 0.012$) (Table 5). It was not possible to determine risk or protective factors for specific exposure to *R. rickettsii* or *R. amblyommatis* due to the low number of dogs (3 and 9 dogs, respectively) that were specifically seropositive to these two species.

The analysis of risk factors for dogs with *Rickettsia* spp. PCR-positive ticks evidenced a significant association with being purebred ($p = 0.035$), while flea treatment was determined to be a protective factor ($p = 0.016$). In dogs with PCR-positive fleas, a significant association was detected with being a purebred dog ($p < 0.001$), as well as a significant association with the number of people in the dog's environment (1 to 3 people $p = 0.002$, >4 people $p = 0.008$); protective factors were free roaming ($p < 0.001$), and being an outdoor dog ($p < 0.006$) (Table 6).

Table 2
Species of *Rickettsia* identified by IFA as possibly responsible for seroreactivity in 12 dogs.

Province	Sample ID	End-point titer			Species identification
		R. rickettsii	*R. amblyommatis*	*R. felis*	
San José	C7	–	128	–	*R. amblyommatis*
	C25	128	–	–	*R. rickettsii*
	C26	–	128	–	*R. amblyommatis*
	C28	–	128	–	*R. amblyommatis*
Alajuela	C62	–	256	–	*R. amblyommatis*
	C67	–	128	–	*R. amblyommatis*
	C68	–	128	–	*R. amblyommatis*
	C78	–	128	–	*R. amblyommatis*
Guanacaste	C121	256	–	–	*R. rickettsii*
	C137	–	128	–	*R. amblyommatis*
Cartago	C243	–	128	–	*R. amblyommatis*
	C246	128	–	–	*R. rickettsii*

(−): no reaction observed at 1:64 serum dilution.

Table 3
Distribution of tick and flea pools that were PCR positive for *Rickettsia* spp. by province in Costa Rica.

Province	PCR-positive tick pools/total (%)	PCR-positive flea pools/total (%)
San José	3/33 (9.1)	18/67(26.9)
Alajuela	3/20 (15.0)	3/15 (20.0)
Cartago	1/15 (6.7)	4/4 (100.0)
Heredia	2/2 (100.0)	15/25 (60.0)
Limón	10/25 (40.0)	12/27 (44.4)
Guanacaste	8/27 (29.6)	0/26 (0)
Puntarenas	12/18 (66.7)	4/9 (44.4)
Total	39/130 (30.0)	56/173 (32.4)

4. Discussion

This is the first cross-sectional study to analyze exposure to SFG rickettsiae in dogs from all provinces of Costa Rica, as well as the risk factors associated with seroprevalence and the presence of *Rickettsia* spp. in their ectoparasites. Since the sampling of dogs was carried out in 2011–2012, it is important to consider that the epidemiological situation may have changed over the years. However, the overall seroprevalence to *Rickettsia* spp. of 10%, agrees with similar studies in Brazil and Panama, where seroprevalence ranged from 4 to 65% (Dantas-Torres, 2008; Silva et al., 2010; Bermúdez et al., 2011). Moreover, IgG end titers in the dogs evaluated were generally low (64 to 256), which is also a common finding in the region (Moreira-Soto et al., 2016; Bermúdez et al., 2011). It is possible that low end titers are due to a past infection that may have been mild or asymptomatic, and perhaps caused by a SFG species of low virulence that cross react with other SFG rickettsiae, such as *R. felis* or *R. amblyommatis* (Rivas et al., 2015; Silva et al., 2010). Interestingly, animals infested by ectoparasites that carry these rickettsiae do not show signs of disease, and seroreactivity may be very low, especially for *R. felis* (Horta et al., 2007; Bermúdez et al., 2011).

Results confirm that dogs in Costa Rica have been exposed to several species of *Rickettsia*, which probably include *R. amblyommatis* and *R. rickettsii* (Moreira-Soto et al., 2016). This is also similar to what has been reported in Panama, where dogs were found to be exposed to *R. amblyommatis*, which seems to be the most common infection (Bermúdez et al., 2011). However, IFA results from this and other studies should be taken with caution, as it is highly unspecific and cannot be used to differentiate between infections by different SFG rickettsiae, especially in areas where several species are present (Dantas-Torres, 2008). Other methods such as neutralization or Western Blot are necessary to determine the specific rickettsiae present (La Scola and Raoult, 1997).

The two most common species of ectoparasites that were identified on dogs were *R. sanguineus* s.l. and *C. felis*, and they may represent the highest risk of rickettsial exposure from dogs to humans explained by their eventual antropophilic behavior (Beall et al., 2012; Piranda et al., 2008). *Rhipicephalus sanguineus* s.l. is also known as the “brown dog tick”. It is an endophilic feeder that is common in peridomestic areas, especially when there are dogs at backyards of houses (Dantas-Torres, 2010). Human infestation is infrequent, but *R. sanguineus* s.l. on humans and other animals (such as rabbits) has been observed in areas of higher temperatures and humidity. Moreover, parasitism of humans is also associated with the presence of highly infested dogs, as well as areas with very high densities of this tick (Dantas-Torres, 2010).

Concerning *C. felis*, its geographical distribution is cosmopolitan, mostly due to the movement of humans and their infested pets (Soto, 2009). Consequently, pathogens transmitted by fleas also follow this distribution and can be disseminated quickly in human populations due to their frequent feeding patron and extraordinary capacity for dispersal (Azad et al., 1997).

In the present study, *R. felis* sequences were detected in both *C. felis* fleas and *R. sanguineus* s.l. ticks, whereas *R. amblyommatis* was detected in a tick identified as *A. mixtum*. The presence of rickettsial DNA in ectoparasites from dogs is in accordance with

Table 4
Identification of *Rickettsia* spp. in samples of tick and flea pools that were PCR positive.

Species	No. pools analyzed	*Rickettsia* spp. positive pools (%)	No. sequences obtained	Province (pools sequenced)	Sequence similarity (%)	Sequence length (bp)	*Rickettsia* sp. [GenBank accession number]
R. sanguineus s.l.	119	36 (30.2)	4	Limón, San José, Guanacaste	100	349	*R. felis* [KX544809; CP00053]
A. mixtum	3	1 (33.3)	1	Guanacaste	100	349	*R. amblyommatis* [KX544812; JF694089]
A. ovale	6	1 (16.6)	0	–	–	–	–
I. boliviensis	1	1 (100.0)	0	–	–	–	–
C. felis	161	53 (34.6)	2	San Jose, Puntarenas	100	349	*R. felis* [KX544809; CP00053]
			1	Limón	100	349	*R. asembonensis* [KX544807; AF5116333]
P. simulans	9	3 (33.3)	0	–	–	–	–

Table 5
Poisson regression analysis of risk factors of dogs seropositive for *Rickettsia* spp.

Variable	Class	n	Seropositive	%	95% $CI_{\%}$		RR	95% CI_{RR}		*p*	p-Wald
					LL	UL		LL	UL		
Age (years)	0–1	198	11	5.6	3.9	7.2					0.007
	2–7	166	27	16.3	13.4	19.1	2.9	1.5	5.9	0.003	
	8–15	35	2	5.7	1.8	9.6	1.0	0.2	4.6	0.971	
Scrotal edema	No	395	38	9.6	1.0	99.0					
	Yes	4	2	50.0	6.7	12.5	5.2	1.3	21.5	0.023	
Gait abnormalities	No	337	28	8.3	7.8	51.1					
	Yes	17	5	29.4	5.4	11.3	3.5	1.4	9.2	0.009	
Size	Small	91	5	5.5	3.1	7.9					0.159
	Medium	300	33	11.0	9.2	12.8	2.0	0.8	5.1	0.148	
	Large	8	2	25.0	9.7	40.3	4.6	0.9	23.5	0.070	
Tick infestation indices	≤1	188	11	5.9	4.1	7.6					0.003
	1.1–2.0	97	16	16.5	12.7	20.3	2.8	1.3	6.1	0.008	
	≥2.1	114	13	11.4	8.4	14.4	1.9	0.9	4.4	0.103	
Flea infestation indices	≤1	267	20	7.5	5.9	9.1					
	1.1–2.0	75	8	10.7	7.1	14.2	1.4	0.6	3.2	0.398	0.019
	≥2.1	57	12	21.1	15.7	26.5	2.8	1.4	5.7	0.005	
Breed	Mixed	188	27	14.4	2.9	9.4					
	Purebred	211	13	6.2	9.3	19.4	0.4	0.2	0.8	0.012	

n: number of individuals +: number of seropositives %: seropositive percentage CI: confidence interval RR: rate ratio LL: lower limit UL: upper limit p: rate ratio probability p-Wald: Wald test probability.

previous studies in the country, where the prevalence of infection in pools of ticks and fleas was estimated to be between 30 and 35% (Moreira-Soto et al., 2016). Other similar studies in the region have determined a prevalence of *Rickettsia* spp. DNA of 12.3% in *R. sanguineus* s.l., all of which were *R. amblyommatis* (Bermúdez et al., 2011). Moreover, the *Amblyomma cajennense* species complex, which includes *A. mixtum*, is commonly associated with *R. amblyommatis* in Central America (Bermúdez and Troyo, 2018). In Costa Rica, *R. amblyommatis* has been reported in *A. mixtum*, *A. longirostre*, *A. ovale*, *Dermacentor nitens*, and *R. sanguineus* s.l., while *R. felis* has been reported in *C. felis* (Hun et al., 2011; Ogrzewalska et al., 2015; Troyo et al., 2016; Bermúdez and Troyo, 2018). However, previous studies have shown rickettsiae in 67.7% of *A. mixtum* tick pools from this country, most of which are *R. amblyommatis* (Troyo et al., 2016).

The infection rate of *R. felis* in fleas has been estimated to be approximately 3.8%, and studies have confirmed that it is maintained successfully in flea populations through transovarial transmission (Azad et al., 1997; Wedincamp and Foil, 2002). Considering that *R. felis* was detected in both *R. sanguineus* and *C. felis* in the present study, it is possible that *R. sanguineus* acquire the initial infection horizontally by co-feeding in proximity to fleas that share the same host (Oliveira et al., 2008). This may also be true with other species of rickettsiae like *R. asembonensis*, which is common in fleas but has also been detected in ticks collected from dogs (Troyo et al., 2016; Bermúdez and Troyo, 2018). In addition to *C. felis* and *R. sanguineus* s.l., *R. asembonensis* has been reported in Costa Rica in *Pulex simulans* and *A. ovale* from dogs (Troyo et al., 2016).

Overall, the prevalence of *Rickettsia* spp. in ectoparasites in Costa Rica agrees with other studies in the region. However, it greatly varies according to species and geographical location, from the absence of detection to almost 100% positivity, as has been observed in this and other studies (Hun et al., 2011; Troyo et al., 2012a; Troyo et al., 2016). Another factor to take cautiously

Table 6
Risk factors associated of dogs with ticks and fleas PCR-positive to *Rickettsia* spp. determined by Poisson regression analysis.

Ectoparasite	Variable	Class	n	PCR positive	%	95% $IC_{\%}$		RR	IC_{RR}		*p*	p-Wald
						LL	UL		LI	LS		
Ticks	Breed	Mixed	73	21	28.8	18.7	43.1					
		Purebred	55	17	30.9	18.4	39.2	1.2	1.0	1.4	0.035	
	Flea treatment	No	41	18	43.9	11.5	31.5					
		Yes	65	14	21.5	28.7	59.1	0.4	0.6	0.8	0.016	
Fleas	Breed	Mixed	100	31	31.0	27.1	51.0					
		Purebred	64	25	39.1	21.9	40.1	1.4	1.2	1.6	<0.001	
	Number of people living with the dog	0	28	8	28.6	20.0	37.1					0.001
		1–3	95	35	36.8	31.9	41.8	1.7	1.2	2.4	0.002	
		≥4	41	13	31.7	24.4	39.0	1.6	1.1	2.3	0.008	
	Having an owner	Yes	133	48	36.1	10.4	41.2					
		No	31	8	25.8	27.9	44.3	0.6	0.4	0.8	<0.001	
	Being an indoor or outdoor dog	Indoor	49	21	42.9	35.8	49.9					0.006
		Indoor/outdoor	29	10	34.5	25.7	43.3	1.0	0.8	1.3	0.802	
		Outdoor	86	25	29.1	24.2	34.0	0.8	0.6	0.9	0.006	

n: number of individuals +: number of seropositives %: seropositive percentage CI: confidence interval RR: rate ratio LL: lower limit UL: upper limit p: rate ratio probability p-Wald: Wald test probability.

is what happen in some locations (for instance, Cartago and San José) where rickettsial DNA was detected with low prevalence in ticks, although there was found higher serological evidence of possible exposure to rickettsiae. This may have occurred when dogs got contact previously in other areas of higher prevalence of rickettsiae and then moved. In contrast, in some regions many ticks were positive for *Rickettsia* sp., where no serological evidence of exposure to rickettsiae was demonstrated, this is like in Puntarenas and Heredia. Moreover, *R. felis* seems to be frequent in both ticks and fleas in the areas studied, although a specific immune response to *R. felis* was not clearly observed. This confirms that dogs rarely elicit an immune response upon contact or infection with this organism, or that *R. felis* is not readily transmitted to dogs. The same may be possibly true about *R. asembonensis*. However, studies to specifically investigate infection and pathogenicity of *R. felis* and *R. asembonensis* in dogs are required to confirm this.

The analyses of possible risk factors associated with *Rickettsia* spp. seropositivity detected an age of 2 to 7 years as a risk factor, which agrees with the reports by Pinter et al. (2008), who have also reported higher seroprevalence in older dogs due to increased time of exposure to infected ticks and rickettsiae. Other risk factors in that study included: tick infestation index (direct association) and flea infestation indices >2.1 (direct association). Concerning ectoparasites, Melo et al. (2011) also reported a higher *Rickettsia* spp. infection rate in dogs infested with *A. mixtum*. Anorexia is another factor found to be associated with seropositivity that has been reported previously (Breitschwerdt et al., 1985; Maxie, 2007; Piranda et al., 2008; Gyles et al., 2010). In addition, difficulty walking and testicular edema were associated with rickettsiosis in a retrospective study by Gasser et al. (2001), as well as other authors (Hirsh et al., 2004; Maxie, 2007; Greene, 2012). Another factor positively associated with seropositivity to *Rickettsia* spp. in the present study was large size, but, to the authors' knowledge, this has not been reported elsewhere.

Being a purebred dog was a protective factor for evidence of exposure to *Rickettsia* spp. in Costa Rica. This contrasts with reports by Weiser and Greene (1989), who found a positive association with being purebred, and reported that they seem more prone to developing the disease than mixed breeds, especially German Shepherds and English Springer Spaniels. The reasons for these apparent contradictions may be that, in the present study, the proportion of dogs infested by ectoparasites was much higher in mixed breed than purebred dogs; also, antiparasitic treatment in mixed breed dogs may be less common than in purebreds, which may affect exposure to rickettsiae.

Interestingly, being a purebred dog was a factor positively associated with *Rickettsia* spp. positive ticks. This does agree with Weiser and Greene (1989) and Greene (2012), but contrasts with the results of the serological portion of this study. A possible protective factor for dogs with *Rickettsia* spp. positive ticks was having had treatment for flea infestations. It may be possible to hypothesize that several of the insecticide treatments available against fleas can also control ticks, and may reduce exposure to rickettsiae. In contrast, risk factors positively associated with dogs that had *Rickettsia* spp. in fleas in this study were a high number of people living with the dog. This finding had not been reported previously in other studies, and more investigations will be necessary to determine the cause of this association. In addition, male dogs and dogs that had free range or were outdoors seem less likely to have *Rickettsia*-positive fleas.

Until now, no studies had reported that free roaming or being outdoor dogs could be a protective factor for the presence of fleas with *Rickettsia* spp. on them.

Although the epidemiological situation may have changed somewhat in the past years, the findings of this study present a first attempt to describe the seroprevalence and possible risk factors of *Rickettsia* spp. in dogs of Costa Rica. Our results suggest that, although dog ticks and fleas harbor different species of rickettsiae, infections of dogs seems to occur in a smaller proportion. Furthermore, there are several possible risk factors that may be influencing the exposure of dogs to rickettsiae. Future studies must focus on accurately identifying the species of *Rickettsia* infecting local dogs, and to determine which of them may cause illness or if they only induce a serological response. For this reason, accurate diagnostic techniques must be implemented.

Relevance of domestic and free roaming dogs in public health have usually been associated with their use as sentinels of Rocky Mountain spotted fever in areas where are at relative high risk of transmission. However, recent experimental studies and outbreak investigations at Brazil, México and U.S.A. have strongly suggested that dog populations and it's most frequent ectoparasite, *R. sanguineus* s.l., may support maintenance of *R. rickettsii*, and possibly other co-circulating rickettsiae, in an urban context where dogs serves as amplifying hosts and *R. sanguineus* s.l. as main vector in the transmission of dogs, and even humans in near contact with them (Burgdorfer et al., 1988; Piranda et al., 2011; Pacheco et al., 2011; Rozental et al., 2008; Álvarez-Hernández et al., 2017). Therefore, surveillance and diagnosis of rickettsial infection in canines may provide useful indicators to understand the epidemiology of these zoonoses.

Declaration of competing interest

None.

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