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Serology, molecular detection and risk factors of *Ehrlichia canis* infection in dogs in Costa Rica

Alexander V. Barrantes-González[a,*], Ana E. Jiménez-Rocha[b], Juan José Romero-Zuñiga[a], Gaby Dolz[a]

[a] *Laboratorio de Entomología, Programa Investigación en Medicina Poblacional, Escuela de Medicina Veterinaria, Universidad Nacional, P.O. Box 86-3000 Heredia, Costa Rica*
[b] *Laboratorio de Parasitología, Escuela de Medicina Veterinaria, Universidad Nacional, P.O. Box 86-3000 Heredia, Costa Rica*

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ABSTRACT

A cross-sectional study combining different serological and molecular techniques for the detection of *Ehrlichia* species in dogs and their ticks was carried out with data from all regions of Costa Rica. A seroprevalence of 32.1% (131/408), and infection with *E. canis* of 3.2% (13/407) was found, whereas 6.9% (9/130) of ticks attached to the dogs were PCR positive to *E. canis*. Higher prevalences were found outside the Greater Metropolitan Area (GMA). Risk factors associated with *E. canis* seropositivity were age, between 2 and 7 years (RR: 1.6, 95% CI: 1.2–2.2) and 8–15 years (RR: 1.8, 95% CI: 1.2–3.0), number of dogs/total of households [Dogs per Household Ratio (DHR) ≥3.1 (RR: 2.0; 95% CI: 1.4–3.0)], number of dogs infested with at least one tick/total of dogs sampled [Tick Infestation Prevalence (TIP) ≥ 31% (RR: 2.1; 95% CI:1.3–3.3)] and living outside the GMA (RR: 1.7; 95% CI: 1.2–2.4) and being a mixed-breed dog (RR: 1.5; 95% CI: 1.1–2.1). Risk factors for *E. canis* PCR positive dogs were a depressive attitude (OR: 11.2; 95% CI: 1.1–115.9), fever (OR:4.8; 95% CI:1.2–19.3), DHR ≥3.1 (OR: 5.7; 95% CI:1.7–19.2)], number of ticks/total of dogs sampled [Tick Distribution Ratio (TDR) ≥2.1 (OR: 6.5; 95% CI: 1.3–31.8)], and TIP ≥40% (OR: 5.7; 95% CI: 1.7–19.2). This paper describes *E. canis* seroprevalence, PCR prevalence and tick analysis in dogs from Costa Rica, with associated clinical signs and owner perceptions. In summary, most of the *E. canis* infections in dogs in our country seemed to pass unnoticed by owners. Since most of the seropositive dogs (97.7%, 131/134) were negative for *E. canis* DNA in their blood, it is important to determine in future studies if these dogs recovered from the *E. canis* infection without any medication, or are persistently infected, and will develop chronic disease.

1. Introduction

Ehrlichiosis is caused mainly by bacteria classified within the group of the alpha-proteobacteria, order Rickettsiales, family Anaplasmataceae, genus *Ehrlichia* (Dumler et al., 2001). This genus consists of obligate intracellular gram-negative bacteria that mainly infect leukocytes such as monocytes, macrophages and granulocytes, and have been found in ticks and mammals (Walker, 1996).

Monocytotropic canine ehrlichiosis is caused mainly by *Ehrlichia canis,* which is found in morulae in the cytoplasm of lymphocytes, monocytes and macrophages (Mylonakis et al., 2003). Other *Ehrlichia* species that cause disease include *E. ewingii*, the causative agent of granulocytic ehrlichiosis, and *E. chaffeensis*, the causative agent of human monocytic ehrlichiosis. Both *E. ewingii* and *E. chaffeensis* infect canines and have been detected in several species of ticks and other vertebrate animals in numerous countries (Harrus et al., 2012). Ticks involved in the transmission of *E. canis* are *Rhipicephalus sanguineus* sensu lato, present in Costa Rica, as well as *Dermacentor variabilis,* which are not reported in the country (Álvarez et al., 2005).

In Costa Rica, *E. canis* was reported for the first time in dogs by Meneses (1995); between 2007 and 2011, four cases of monocytotropic (2) and granulocytotropic (2) ehrlichiosis have been reported in humans in the country (Solano and Villalobos, 2007; Brenes-Valverde et al., 2011).

E. canis was isolated and characterized from blood of sick dogs (Romero et al., 2011), and from *R. sanguineus* s.l. ticks collected from dogs of the country's Central Valley (Ábrego-Sánchez et al.,

* Corresponding author at: Campus Pbro. Benjamín Nuñez, Barreal de Heredia, Heredia, Costa Rica.
E-mail address: avbarrantesgonzalez@gmail.com (A.V. Barrantes-González).

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2013). *E. chaffeensis*and *E. ewingii* were not found in dogs (Romero et al., 2011; Dolz et al., 2013). Seroprevalences of *E. canis* reported in the country ranged from 3.5% in the Southern Pacific region (Scorza et al., 2011) up to 70.0% in the Central Valley (Rímolo, 2008). Reported prevalences of *E. canis* PCR positive dogs ranged between 34.0% and 47.7% (Romero et al., 2011; Rojas et al., 2014).

Diagnosis of *E. canis* in Costa Rican veterinary clinics are performed using commercial rapid assays that detect antibodies. Generally, clinicians initiate a 4-week antibiotic therapy based on a positive serological result. The present study aimed to determine the seroprevalence of *E. canis* in healthy dogs nationwide, and detect DNA of *Ehrlichia* spp. in blood samples from these dogs and their ticks, to establish risk factors and to guide veterinarians in making treatment decisions.

2. Materials and methods

2.1. Study design and sample size

A cross-sectional, observational, descriptive study was conducted to determine the presence of, or exposure to, *Ehrlichia* spp. in blood samples from dogs and their ticks, using molecular and serological assays, respectively. The total sample size was estimated to be 385 individuals (50% prevalence, 95% confidence) for a population of more than 40,000 dogs, calculated using Win Episcope 2.0 (Thrusfield et al., 2001).

2.2. Analyzed population

A survey carried out in 2011 determined that there were 1,211,964 occupied households in the country, distributed by provinces as follows: 33.1% San Jose, 19.5% Alajuela, 10.8% Cartago, 10.1% Heredia, 9.8% Puntarenas, 9.0% Limón, and 7.6% Guanacaste (INEC, 2012; FUPROVI, 2012). Based on an average of 1.6 dogs per household established by List (2009), a population of approximately 1,939,142 dogs was estimated. The canine population was analyzed in accordance with its provincial distribution, and provinces were classified as being located in the Greater Metropolitan Area (GMA: San José, Alajuela, Cartago and Heredia); or outside the GMA, on the Pacific (Puntarenas, Guanacaste) or Atlantic (Limón) coasts. Alajuela was the only province in which samples were taken inside and outside the GMA. Samples were taken in 15 recreational parks, with high levels of concurrency, throughout the entire country, in the period from June 2011 to September 2012, intended to take samples in these settings from a healthy dog population representative of the country as a whole. Each park was visited once during weekends. Interviews, clinical records and samplings were performed for a total of 441 dogs (399 dogs with owners and 42 stray dogs living in the recreational parks), distributed as follows: 198 San José (44.9%), 45 Alajuela (10.2%), 39 Cartago (8.8%), 30 Heredia (6.8%), 32 Puntarenas (7.3%), 54 Limón (12.2%), and 43 Guanacaste (9.8%) (Fig. 1).

2.3. Interview, clinical examination and sampling

A mobile veterinary facility for clinical examination was set up in the middle of each recreational park. Veterinarians approached dog owners walking their dogs in the park, and dog owners also approached the mobile facility. Upon obtaining consent, each owner was interviewed to obtain information about the place of origin, age, breed, household variables (one-dog or multi-dog household), tick infestation, treatment of ticks, and signs suggestive of ehrlichiosis (fever, weight loss, depression, epistaxis, petechiae, ecchymosis, hematuria, dyspnea, cough, lymphadenomegaly, ataxia and scrotal edema), observed by the owners at some point in the lives of their pets, if their veterinarian had suspected ehrlichiosis in the past, if the dog was treated because of this suspicion, and the medications used to treat those dogs. Veterinarians made sure to ask the owners in an appropriate fashion, adapting their vocabulary to the educational level of the respondent, to ensure that the questions were understood correctly. In addition, ticks were collected manually from the dogs from all anatomical sites during 10 min, and a clinical examination was performed to determine attitude (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 ($\geq$39.5°C was considered as feverish) and clinical signs suggestive of ehrlichiosis (weight loss, epistaxis, petechiae, ecchymosis, hematuria, dyspnea, cough, lymphadenomegaly, ataxia, lameness, diarrhea and scrotal edema). Dogs that were treated with Doxycycline were recorded. No exclusion criteria were applied. From stray dogs living in recreational parks, consent from the administration was obtained. Only clinical exam and sampling was performed.

Blood samples were collected from each dog, stored at 4 °C until serum separation, and frozen at −20° prior to the serological and molecular tests. Ticks were stored in 70% alcohol.

2.4. Classification of ticks

Taxonomic classification 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). Ticks from each dog were separated into microfuge tubes by species, sex and stage, and stored at −20°C until DNA extraction.

2.5. Serological analysis

Two commercial techniques were used to detect antibodies against *E. canis*, "Speed Ehrli" Virbac, an Immunochromatography Membrane Assay (IMA) (Bio Veto Test, Rome, Italy; sensitivity 87%, specificity 95%) and "*E. canis* and *A. phagocytophilum* Canine IgG Antibody Kit", an Indirect Inmunofluorescence Assay (IFA) (Fuller Laboratories, California, USA; sensitivity and specificity 100%). The methodologies recommended by the manufacturers were used. Sera were analyzed in IFA only in one dilution (1:80). Sera that exhibited fluorescence in 1:80 dilution were considered positive in IFA. Results of the two tests were compared. To determine seroprevalence, a "parallel testing" methodology was used. A sample was considered seropositive if positive results were obtained from one or both of the testing methodologies (Thrusfield, 2007).

2.6. Molecular analysis

Extraction of DNA from blood samples was performed with the "Wizard Genomic" (Promega®, Wisconsin, USA) while extraction of DNA from ticks was done with the "DNeasy Blood and Tissue Kit" (QIAGEN®, California, USA) according to the manufacturer's instructions. Ticks from each dog were analyzed in pools of the same species. In cases where individual ticks of the same species but different sexes or stages were found, groups of ticks were analyzed according to the following priority: females > nymphs > male > larvae.

The conventional nested PCR, as described by Romero et al. (2011), was used to amplify a portion of the 16S rRNA of *Ehrlichia* spp. PCR products were purified using the QIAquick kit (QIAGEN®), proceeding according to the manufacturer's instructions. Positive samples were sent to Macrogen (Seoul, Korea) for sequencing. Partial sequences were aligned with BioEdit Sequence Aligment Editor® (Hall, 1999) and compared using the BLASTn algorithm

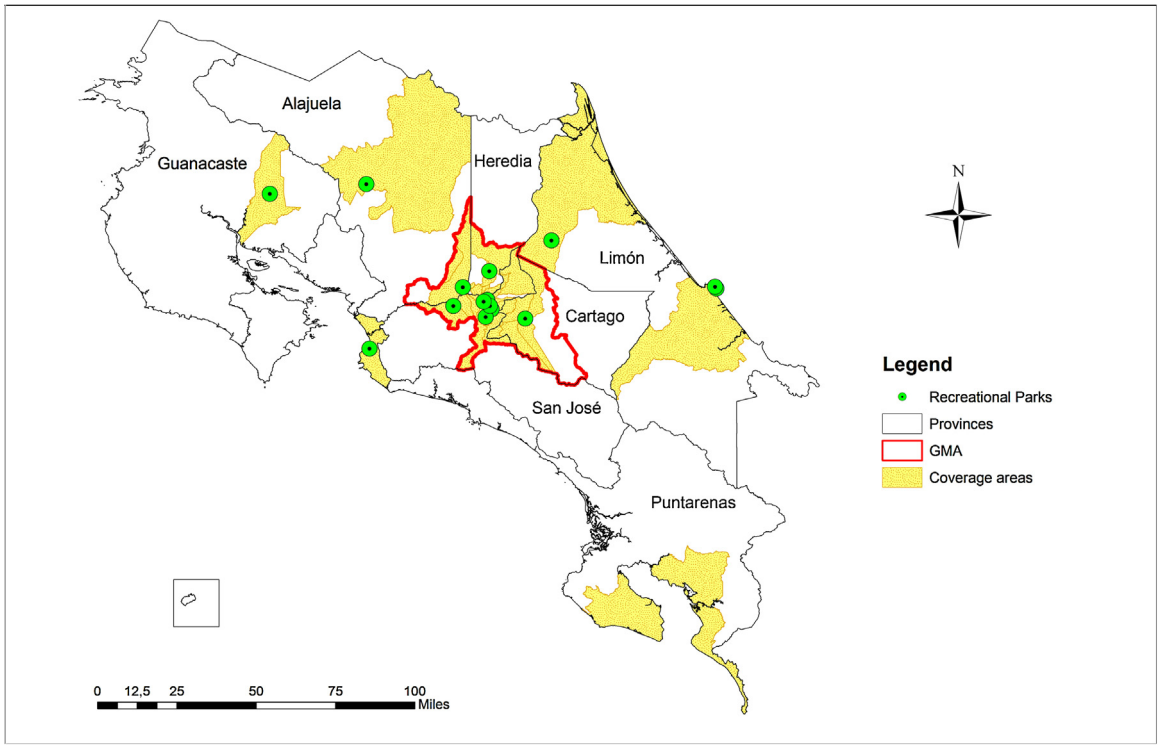

Fig. 1. Sampling sites (circles) and coverage area where the dogs lived (yellow areas). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

with the database of NCBI (National Center for Biotechnology Information).

2.7. Statistical analysis

Data obtained from the interview, clinical examination and results of diagnostic tests (serology and PCR) were entered in a digital database. The following canine demographic parameters were determined: Tick infestation prevalence (TIP = number of dogs infested with at least 1 tick/total of dogs sampled), tick distribution ratio (TDR = number of ticks/total of dogs sampled), *R. sanguineus* s.l. occurrence percentage (R.sOP = number of *R. sanguineus* s.l. ticks/total of ticks sampled), and dogs per household ratio (DHR = number of dogs/total of households). Statistical analysis was performed using STATA IC 13 (Stata Corp., USA). A descriptive analysis of the results through measures of central tendency (mean), measures of dispersion (standard deviation and confidence interval 95%) and frequency (%) by factors was performed. The presence and absence of *E. canis* was determined and the frequency distributions of positive and negative results with their respective percentages were determined. Furthermore, to assess the risk factors associated with seropositivity and PCR-positivity to *E. canis,* Poisson and logistic regressions were performed, respectively. The regression procedures consisted of 2 steps: 1) univariable analysis, 2) multivariable analysis. In the second step, all variables with $p < 0.25$ in the univariable analysis were included. All variables with $p < 0.25$ in the univariable analysis were taken to bivariate analysis to test potential confounders (living place according to GMA, sex, breed, age and ownership status, that is, stray dogs or dogs with an owner) and look for modifiers and then they were taken to the multivariate analysis. A backward elimination model was used, based on the likelihood ratio test. The process of exclusion-inclusion of each variable into the multivariable model tested for confounding and interaction by comparison of the estimated coefficients in the new model with the estimated coefficients and likelihood ratio of the old model. Confounding was considered to be present if at least one coefficient changed more than 10% (if the rate ratio had a value between 0.7 and 1.5) or if at least one coefficient changed more than 25% (if the rate ratio had a value <0.7 or >1.5). Finally, variables that were excluded in the univariable step were evaluated for collinearity with the variables in the final model to check for potential confounding by calculation of simple correlations. The data was collected using EpiData Software version 2.0 (Odense, Denmark); and analyzed using STATA version 12 (Stata Corp., USA)

3. Results

Of the total of dogs (n = 441) that participated in the study, 399 had owners, and it was possible to conduct an owner interview, while 42 (9.5%) dogs were residents of the parks and had no known owner. Higher values of DHR were found in provinces outside the GMA (2.7), including Limón (3.2) and Guanacaste (3.1), than in provinces in the GMA ($p < 0.001$) (Table 1). A total of 55.9% of the owners had observed ticks on their pets, only 2.0% of the dogs were diagnosed previously by a veterinarian with ehrlichiosis; 77.8% of them had been treated (28.6% with doxycycline and 71.4% did not remember the name of the medication), and in 3.6% of cases the veterinarian suspected that the pets had ehrlichiosis. Clinical signs suggestive of ehrlichiosis observed by the owners at some point in the lives of their pets were: bleeding (17.0%), petechiae (9.5%), weight loss (4.5%), and hematuria (1.8%).

Clinical examination encountered abnormalities in only a minority of the dogs, such as attitude (weak 0.2%, depressed 0.7%, docile 16.1%), capillary refill time >2 s (5.2%), mucous membranes (very pale 1.1%, pale 11.1%), and rectal temperatures ≥39.5°C (19.5%). Clinical abnormalities 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%).

Table 1
Canine demographic parameters as tick infestation percentage (TIP), tick distribution ratio (TDR), *R. sanguineus* s.l. occurrence percentage (RsOP), and dogs per household ratio (DHR) determined in different provinces of Costa Rica.

Province	Tick Infestation Prevalence (TIP)[1]			*R. sanguineus* s.l. Occurrence Percentage (RsOP)[1]			Tick Distribution Ratio (TDR) TT/TDS[2]		Dogs per Household Ratio (DHR) TD/TH[2]	
	DIT/TDS (%)		(%) Globaln = 441*	TRs/TT (%)		(%) Globaln = 608°				
San José	40/198 (20.2)	a,c,f	9.0	123/129 (95.3)	a	20.3	129/198 (0.7)	a,b	343/174 (2.0)	a,b
Alajuela	17/44 (38.6)	a,b,c,d,e	3.8	78/82 (95.1)	a	12.8	82/44 (1.9)	a,b,c	94/42 (2.2)	a,b,c
Cartago	0/35 (0)	f	0	51/51 (100)	a,b	8.4	51/35 (1.5)	a	53/33 (1.6)	a
Heredia	4/35 (6.6)	c,f	0.9	3/6 (50.0)	c	0.3	5/35 (0.1)	a	75/34 (2.2)	b,c
Limón	26/54 (48.1)	b,e	5.9	198/198 (100)	b	32.6	198/54 (3.6)	c	159/49 (3.2)	c
Guanacaste	26/43 (60.5)	d,e	5.9	133/135 (98.5)	a,b	21.9	135/43 (3.1)	b,c	111/36 (3.1)	b,c
Puntarenas	14/32 (56.3)	a,b,c,d,e	3.2	23/24 (95.8)	a,b	3.8	24/32 (0.7)	a,b	70/31 (2.3)	a,b,c
Total	127/441 (28.8)		28.8	608/624 (97.4)		100	623/441 (1.4)		905/399 (2.3)	
GMA	53/294 (18.0)	a	12.0	206/215 (95.8)	a	33.9	215/294 (0.7)	a	523/266 (2.0)	a
NO GMA	74/147 (50.3)	b	16.8	402/409 (98.3)	a	66.1	408/147 (2.7)	b	364/133 (2.7)	b

Proportions and ratios bearing different superscript letters differ significantly ($p < 0.05$) [1]: Pearson's Chi Square Test $p < 0.001$ [2]: ANOVA Test $p < 0.001$ *: Total of dogs sampled °: Total of *R. sanguineus* s.l. ticks collected DIT: Dogs infested with ticks TT: Total ticks collected TDS: Total dogs sampled TRs: Total of *R. sanguineus* s.l. ticks collected TD: Total of dogs living in households (dogs living in the same household with the sampled dog were included) TH: Total of households GMA: Greater Metropolitan Area NO GMA: Outside the Greater Metropolitan Area.

Ticks were found on 28.8% (127/441) of the dogs sampled. A total of 116 dogs were infested with *R. sanguineus* s.l., four with *Amblyomma ovale*, two with *Amblyomma mixtum*, one with *Amblyomma maculatum* and one with *Ixodes boliviensis*. In addition, one dog presented a mixed infestation of *R. sanguineus* s.l. and *A. mixtum*, and two dogs presented mixed infestations of *R. sanguineus* s.l. and *A. ovale*. A total of 99.2% (608/623) of ticks were *R. sanguineus* s.l.; the remaining ticks included nine *A. ovale*, three *I. boliviensis,* two *A. mixtum,* and one *A. maculatum,* which were found in Heredia, Guanacaste, and Alajuela. Tick infestation and distribution was determined to be higher outside the GMA (TIP 50.3%; TDR 2.7) than in the GMA (18.0% and 0.7%, respectively) ($p < 0.05$), with the highest values occurring in the province of Guanacaste (Table 1). Furthemore, *R. sanguineus* s.l. infestations of dogs were more frequent outside the GMA (47.6%) than in the GMA (16.7%) ($p < 0.05$), and the highest global RsOPs were found in Limón (32.6%) and Guanacaste (21.9%) ($p < 0.001$) (Table 1).

A total of 23.2% (95/408) of the samples analyzed by IFA showed antibodies against *E. canis*, while 30.0% (121/403) of the samples analyzed by IMA yielded positive results, and 32.1% (131) dogs were determined to be seropositive to *E. canis*. A total of 398 dogs were tested with both tests. The concordance between the two serological assays was 89.7%. The distribution by provinces of dogs that were seropositive to *E. canis* is shown in Table 2. Seroprevalences were lower in the GMA than outside the GMA ($p < 0.05$), with most of the seropositive dogs found in the provinces of Guanacaste (70.7%), and Puntarenas (63.3%) ($p < 0.05$).

In 3.2% (13/407) of the blood samples analyzed by nested PCR, DNA of *E. canis* was detected, and in 10 (77.0%) of these samples, antibodies against *E. canis* were also detected. Of the total of 130 tick groups analyzed by PCR, 9 (6.9%) were positive for *E. canis*. All PCR-positive ticks were identified as *R. sanguineus* s.l. (five groups of females, two groups of nymphs, and two groups of males), which were collected from 9 dogs: 7 seropositive but PCR-negative dogs, and 2 seropositive and PCR-positive dogs. Sequencing of the PCR products of three blood samples from dogs and one *R. sanguineus* s.l. tick confirmed the results and showed 100% identity between them, and 99.3% (424/427) identity with *E. canis* strain Jake (074283.1). These samples were deposited in GenBank. *E. chaffeensis* and *E. ewingii* were not detected in any of the blood samples or ticks analyzed.

Most of the PCR-positive dogs were detected in the provinces of Guanacaste (9.8%), Limón (5.5%), and Alajuela (4.9%). While most of the PCR-positive ticks were found in Guanacaste (18.5%), Puntarenas (11.1%), Limón (9.1%) and Alajuela (5.0%). The PCR prevalences were always higher outside the GMA (4.9%) than in the GMA (2.0%), and samples outside the GMA of Alajuela province had higher seroprevalences than samples collected from this province in the GMA, although there were no significant differences. The PCR prevalences of *E. canis* in dogs and their ticks by provinces is shown in Table 2. A total of 25% of PCR positive ticks were attached to PCR positive dogs, and 13% of PCR positive ticks were attached to seropositive dogs. In contrast, 6.3% of PCR-positive ticks were attached to PCR negative dogs, and 3.0% of PCR positive ticks attached to serological negative dogs.

An analysis of risk factors using Poisson regression found a significant association ($p < 0.05$) of seropositive dogs with age, between 2 and 7 years (RR: 1.6, 95% CI: 1.2–2.2) and 8–15 years (RR: 1.8, 95% CI: 1.2–3.0), DHR ≥3.1 (RR: 2.0, 95% CI: 1.4–3.0), TIP ≥31% (RR: 2.1, 95% CI: 1.2–3.4), living outside the GMA (RR: 1.7, 95% CI: 1.2–2.4) and being a mixed-breed (RR: 1.5, 95% CI: 1.1–2.1) (Table 3).

Risk factors for *E. canis* PCR-positive dogs analyzed by logistic regression established a significant association ($p < 0.05$) with depressive attitude (OR: 11.2, 95% IC: 1.1–115.9), fever (OR: 4.8, 95% IC: 1.2–19.3), and DHR ≥ 3.1 (OR: 5.7, 95% IC: 1.7–19.2), TDR ≥ 2.1 (OR: 6.5, 95% IC: 1.3–31.8) and TIP ≥ 40% (OR: 5.7, 1.7–19.2) (Table 4).

4. Discussion

This work represents the first cross-sectional study in dogs from Costa Rica combining different diagnostic techniques to determine the seroprevalence of and infection with *E. canis* with analysis of attached ticks, and the risk factors associated with these criteria. The seroprevalence and prevalence established in this study show the presence and wide distribution of *E. canis* in the country.

A national seroprevalence of 32.1% (131/408) was determined, whereas seroprevalences in the different provinces ranged between 18.5% and 70.7%. This wide range of seroprevalence is in agreement with previous reports in the country ranging from 3.5% (3/84) in a Southern Pacific region (Scorza et al., 2011) up to 70% (21/30) in the GMA (Rímolo, 2008). The high seroprevalence determined by Rímolo (2008) was potentially due to the fact that only sick dogs with a suspicion of ehrlichiosis were analyzed. The reason that IFA did not detect 35 positive sera could be due to the sensitivity of the test and that only one dilution (1:80) was used.

DNA from *E. canis* was found in only 3.2% (13/407) of dogs analyzed, which does not agree with results reported by Romero et al. (2011) of 47.7% (148/310). This is most likely due to the fact that only sick dogs with signs of ehrlichiosis were evaluated in the previous study, whereas this study looked at primarily healthy dogs. Our

Table 2
Geographical distribution of seropositive dogs, PCR positive dogs and PCR positive ticks to *E. canis* by provinces of Costa Rica.

Provincce	Serology[1]			Blood PCR[2]		Tick PCR[1]		
	+/Total (%)		(%) Globaln = 408*	+/Total (%)	(%) Globaln = 407*	+/Total (%)		(%) Globaln = 130*
San José	43/180 (23.9)	b	10.5	3/179 (1.7)	0.7	0/33 (0)	b	0
Alajuela	13/41 (31.7)	a,b	3.2	2/41 (4.9)	0.5	1/20 (5.0)	a,b,c	0.8
Cartago	11/35 (29.0)	a,b	2.7	1/35 (2.9)	0.2	0/5 (0)	a,b,c	0
Heredia	6/27 (22.2)	b	1.5	0/27 (0)	0	0/2 (0)	a,b,c	0
Limón	10/54 (18.5)	b	2.5	3/54 (5.5)	0.7	1/25 (4.0)	a,b,c	0.8
Guanacaste	29/41 (70.7)	c	7.1	4/41 (9.8)	1.0	5/27 (18.5)	c	3.8
Puntarenas	19/30 (63.3)	a,c	4.7	0/30 (0)	0	2/18 (11.1)	a,b,c	1.5
Total	131/408 (32.2)		32.2	13/407 (3.2)	3.2	9/130 (6.9)		6.9
GMA	68/265 (25.7)	a	16.7	6/264 (2.0)	1.5	1/55 (1.8)	a	0.8
NO GMA	63/143 (44.1)	b	15.4	7/143 (4.9)	1.7	8/75 (10.6)	b	6.2

Proportions bearing different superscript letters differ significantly ($p < 0.05$) [1]: Pearson's Chi Square Test $p < 0.001$ [2]: Pearson's Chi Square Test P = 0.088 *: Total blood samples or tick pools analyzed GMA: Greater Metropolitan Area NO GMA: Outside the Greater Metropolitan Area.

Table 3
Risk factors associated with dogs seropositive to *E. canis* determined by Poisson regression.

Variable	Class	n	+	%	95% $CI_{\%}$		RR	95% CI_{RR}		*p*	*p*-Wald
					LL	UL		LL	UL		
Age (years)	0–1	204	47	23.0	20.1	26.0					0.002
	2–7	169	65	38.5	34.7	42.2	1.6	1.2	2.2	0.001	
	8–15	36	19	52.8	44.5	61.1	1.8	1.2	3.0	0.011	
DHR	≤ 2.5	288	79	27.4	24.8	30.1					0.001
	2.6–3.0	51	14	27.5	21.2	33.7	1.0	0.6	1.8	0.998	
	≥3.1	69	38	55.1	49.1	61.1	2.0	1.4	3.0	< 0.001	
TIP	≤30%	221	48	21.7	18.9	24.5	0.001				
	31–40%	47	21	44.7	37.4	51.9	2.1	1.2	3.4	0.006	
	41–50%	75	33	44.0	38.3	49.7	2.0	1.3	3.2	0.002	
	> 50%	65	29	44.6	38.4	50.8	2.1	1.3	3.3	0.002	
Area	GMA	264	68	25.8	35.6	51.9					
	NO GMA	144	63	43.8	20.5	31.0	1.7	1.2	2.4	0.024	
Breed	Pure	218	57	26.1	32.0	45.9					
	Mixed	190	74	38.9	20.3	32.0	1.5	1.1	2.10	0.024	

DHR: Dogs per Household Ratio TIP: Tick Infestation Prevalence GMA: Greater Metropolitan Area NO GMA: Outside the Greater Metropolitan Area n: Number of individuals+: Number of seropositives %: Seropositive percentage RR: Rate Ratio CI: Confidence Interval LL: Lower Limit UL: Upper Limit *p*: Rate Ratio probability *p*-Wald: Wald test probability.

Table 4
Risk factors associated with dogs PCR-positive to E. canis determined by logistic regression.

Variable	Class	n	+	%	95% $CI_{\%}$		OR	95% CI_{OR}		*p*	*p*-Wald
					LL	UL		LL	UL		
Attitude	Alert	416	12	2.9	−17.4	67.4					
	Depressed	4	1	25.0	1.3	4.5	11.2	1.1	115.9	0.042	
Fever	No	343	8	2.3	0.7	3.9					
	Yes	29	3	10.3	−0.7	21.4	4.8	1.2	19.3	0.026	
DHR	≤ 2.5	299	5	1.7	0.9	2.4					0.019
	2.6–3.0	53	2	3.8	1.2	6.4	2.3	0.4	12.2	0.326	
	≥3.1	68	6	8.8	5.4	12.3	5.7	1.7	19.2	0.005	
TDR	≤1	204	2	1.0	0.3	1.7					0.069
	1.1–2.0	100	4	4.0	2.0	6.0	4.2	0.8	23.4	0.101	
	≥2.1	116	7	6.0	3.8	8.2	6.5	1.3	31.8	0.021	
TIP	≤ 20%	299	5	1.7	0.9	2.4					0.019
	21–39%	53	2	3.8	1.2	6.4	2.3	0.4	12.2	0.326	
	≥40%	68	6	8.8	5.4	12.3	5.7	1.7	19.2	0.005	

DHR: Dogs per Household Ratio TDR: Tick Distribution Ratio TIP: Tick Infestation Prevalence n: Number of individuals +: Number of seropositives %: Seropositive percentage OR: Odds Ratio CI: Confidence Interval LL: Lower Limit UL: Upper Limit *p*: Rate Ratio probability *p*-Wald: Wald test probability.

results also contrast with those of Rojas et al. (2014), who determined 34.2% (50/146) of the dogs to be PCR-positive to *E. canis*, ranging between 4.8% to 57.9% among different regions. However, this discrepancy could also be due to higher sensitivity of Real Time PCR used in latter, however, we strongly state that it was due to differences in dog populations outside the GMA.

Sequencing confirmed the presence of *E. canis*. *E. chaffeensis* and *E. ewingii* were not found. The absence of other *Ehrlichia* species in dogs from Costa Rica agrees with the results of Romero et al. (2011), and is probably due to the absence of competent vectors (*Amblyomma americanum* and *D. variabilis*) in the country (Romero et al., 2011). The tick flora found in the present study is consistent

with previous reports, pointing out that *R. sanguineus* s.l. is the most common dog tick in Costa Rica. DNA from *E. canis* was detected in 6.9% (9/130) of ticks analyzed. In a previous study, Ábrego-Sánchez et al. (2013) found 26.0% (43/65) of *R. sanguineus* s.l. ticks positive, while Souza et al. (2010) reported 21.9% (7/32) of ticks infected with *E. canis* in Brazil.

Differences between seroprevalence (32.2%) and PCR prevalence (3.2%) obtained in the present study are consistent with studies carried out in Brazil that reported *E. canis* seroprevalence of 69.4% (75/108) and PCR positivity of 3.7% (4/108) (Tanikawa et al., 2013). The great discrepancy between PCR and serology results could be related to the infection state of the dogs. The three seronegative but PCR positive dogs were probably in an early stage of the acute phase of *E. canis* infection, which can be easily detected by PCR in blood (Tanikawa et al., 2013), because antibodies are not detected before 2–3 weeks post-infection when seroconversion takes place (Neer et al., 2002). The other ten dogs with positive results in both assays (serology and PCR) could be related either to acute *E. canis* infections, persistent chronic infections or even subclinically infected dogs, where bacteria can also circulate intermittently in peripheral blood. Most of the dogs (121, 29.6%) were determined to be seropositive and PCR negative, what could be related to past infections, or to subclinical or chronic infections without an ongoing disease condition. To confirm the presence of the latter two types of infections, spleen and bone marrow are the most appropriate tissues to analyze (Harrus et al., 2004; Mylonakis et al., 2003). Dogs in these phases are known to be carriers of *E. canis* and remain clinically healthy for months or years, until they recover from the infection spontaneously or develop severe illness (Harrus et al., 1998). Since the owners in the present study did not remember having observed clinical signs of ehrlichiosis in their pets, and the dogs were never treated with doxycycline, we hypothesize that the infection may occur silently in our country, and probably is resolved by the animals on their own. However, this must be confirmed in future studies.

Age, in a directly proportional relationship, was determined as a risk factor associated with *E. canis* seropositivity, which is consistent with several studies, stating that the older the dog the greater the probability of exposure to infected vectors (Pinter et al., 2008; Vieira et al., 2013). Furthermore, living outside the GMA was determined as a risk factor associated with seropositivity; this is in agreement with Melo et al. (2011) who found that dogs from rural areas had a higher chance to be seropositive than urban dogs. Furthermore, the more infested the dogs with ticks (TIP in a directly proportional relation) the greater the possibility of being seropositive. Although this finding is quite expectable, the association has not been previously established in any other study. The number of dogs per household (DHR > 3.1) was determined to be a risk factor associated with seropositivity to *E. canis,* which has been considered as a potential contributor to seroprevalence of tick-borne pathogens (Stich et al., 2014).

Seropositive mixed-breed dogs (55.7%) showed a higher frequency of tick infestation (14.3%) and were less medicated (9.7%) against ticks than purebreds. This could explain why being purebred was found to be a protective factor for seropositivity to *E. canis*, suggesting that more environmental factors than immunological factors lie behind this association.

Risk factors associated with dogs that were PCR-positive to *E. canis* included depressed attitude and fever, signs that have been associated with acute or chronic phase of the disease (Buhles et al., 1975; Dantas-Torres, 2008), and also having more than 3 dogs per household (DHR ≥ 3.1), dogs having more than 2 ticks (TDR ≥ 2.1) and dogs living in a region with a tick infestation prevalence higher than 40%.

Coinfections must be considered when determining associations with risk factors and have been described previously (Rojas et al., 2014; Wei et al., 2014).

The highest frequencies of seroprevalence, PCR prevalence, and percentage of dogs with PCR-positive ticks were found in the Guanacaste province, located in the northern Pacific coast of Costa Rica, outside the GMA. This high circulation of *E. canis*, is probably due to high TIP, TDR, and DHR, which were determined to be risk factors, and high RsOP. In contrast, Heredia, a province located in the GMA, showed the lowest seroprevalence; neither PCR-positive dogs nor dogs with PCR-positive ticks were found. This is probably due to the low TIP, TDR, DHR, and RsOP values encountered in this province.

Higher seroprevalence, PCR prevalence and percentage of dogs with PCR-positive ticks found outside the GMA are probably due to the higher values of TIP, TDR, and DHR compared to the GMA and also to the higher presence of mixed-breed dogs and higher Global RsOP values outside the GMA.

Taking into consideration only seropositive dogs, most of the dogs outside the GMA had higher TIP, TDR and DHR values, and the majority were mixed-breeds, compared to seropositives in the GMA, where most of the dogs had lower TIP, TDR and DHR values, and the majority were purebreds.

The DHR values determined in the present study were much higher than those reported by List (2009); 2.0 dogs per household were found in the GMA, whereas 2.7 dogs were found outside the GMA. This, and the fact that 66.1% of collected *R. sanguineus* s.l. ticks were found outside the GMA, could explain the higher circulation of *E. canis* in these peri-urban areas (Álvarez et al., 2005; Labruna and Pereira, 2001). Furthermore, it seems that regions with less urbanization and a lower socioeconomic status had higher seropositivity and circulation of *E. canis*, probably due to the lack of veterinary care or lack of flea and tick prevention.

The differences in seroprevalence and presence of *E. canis* in blood of dogs and their ticks detected in the different provinces are probably influenced by ecological factors such as vector presence, host factors, and social factors (Stich et al., 2014). These factors must be considered in future studies.

5. Conclusions

In conclusion, the findings of this study suggest that although *E. canis* infection is highly endemic in Costa Rica, most of the infections in dogs seem to occur without being noticed by owners. Future studies must determine if these dogs resolved the *E. canis* infection without any medication, or are persistently infected, and will develop chronic disease. Until then, clinicians evaluating dogs suspected of ehrlichiosis are advised to treat only seropositive dogs that show clinical signs with doxycycline, until blood or tissue PCR results are available. This investigation corroborates other studies that have reported *E. canis* as the only ehrlichial agent infecting dogs in the country. A higher level of circulation of the agent was determined to exist outside the Greater Metropolitan Area, and factors that favor conditions for the *R. sanguineus* s.l. tick were found to be important for *E. canis* infection.

Conflict of interest

No conflict of interest is declared.

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