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Ehrlichia canis in dogs of Mexico: Prevalence, incidence, co–infection and factors associated

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ABSTRACT

Rickettsial infections in dogs of Mexico were investigated. A total of 246 dogs were blood sampled and initially screened to detect *Ehrlichia canis, E. chaffeensis, E. ewingii*, *Anaplasma phagocytophilum* and *Rickettsia rickettsii* by a quantitative real–time PCR (qPCR) assay. Sixty–five dogs were monitored and sampled twice 7–8 months apart. Using the qPCR, 72 positive dogs to *E. canis* were detected (prevalence of 29.26%). These dogs were also tested by nested PCR to detect the same pathogens. None of the studied dogs were positive to *E. chaffeensis, E. ewingii, R. rickettsii* nor *A. phagocytophilum* by both PCR assays. The cumulative incidence of *E. canis* infection was 38.46%. Sequencing analysis of the nested PCR products revealed 100% and 98.1% identity of *E. canis* and *R. parkeri,* respectively. We found a dog co–infected with *E. canis* and *R. parkeri*.

1. Introduction

Rickettsial infections are caused by various bacterial species that belong to the order Rickettsiales, and the genera *Orientia, Neorickettsia, Neoehrlichia*, *Anaplasma, Rickettsia,* and *Ehrlichia* [1].

The genus *Rickettsia* is distributed worldwide, with some species maintained in nature and transmitted to vertebrate hosts by arthropod vectors [2]. Ticks and mites are the vectors of several species of spotted fever group (SFG) Rickettsiae, while fleas and lice are the vectors transmitting the typhus group Rickettsiae [1]. In Mexico, molecular evidence supports the existence of rickettsemia caused by *R. akari* [3], and *R. typhi* [4] in dogs. However, *R. parkeri* has not been identified in dogs from Mexico. Furthermore, *R. parkeri* has been incriminated as the major causative agent of SFG rickettsioses in South America and in the United States [1,2].

The genus *Ehrlichia* belongs to the family Anaplasmataceae and contains a number of pathogenic species such as *E. muris*–like, *Ehrlichia canis, E. chaffeensis* and *E. ewingii*, all transmitted by Ixodidae ticks. *Ehrlichia* canis is the causative agent of Canine Monocytic Ehrlichiosis (CME), and has been studied to a certain extent in Mexico. In Mexico the national seroprevalence of *E. canis* varies from 33.1% to 74.3% [5,6]. In spite of serological and molecular evidence of *E. canis* infection present in diverse populations of dogs and ticks in Mexico [7,8], there is a lack of molecular surveys to investigate the incidence of *E. canis* and *Rickettsia* spp. infections in dogs in this geographic region.

Previous studies found that *Rhipicephalus sanguineus* sensu lato (s.l.) is the most abundant and widespread tick on dogs in Mexico [9], in addition, several regions of the country have suitable environments for the transmission of rickettsial agents mainly in dogs residing in close contact with people in rural areas [10]. Therefore, the objective of this study was to investigate the prevalence, incidence and factors associated with *Ehrlichia canis* infection, as well as other Rickettsial infections in dogs of rural communities of Yucatan, Mexico.

2. Materials and methods

2.1. Study area and sampling

The study was carried out from June 2016 to March 2017 in three rural communities of Yucatan, Mexico. The studied rural communities were, Sucopo (21°9'0" N, 88°4'0" W), Yaxcheku (21°12'26" N, 87°55'59" W), and Chan San Antonio (21°21'66"N, 88°25'00) [11].

In each community, at least 40 homes reporting tenure of at least one dog were selected for convenience. The communities were divided into four quadrants, using the main streets that cross the towns north to south and east to west as reference. In each quadrant, at least 10 homes

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were selected for dog sampling.

Each of the selected households were visited in June–August 2016 (first sampling) and February–March 2017 (second sampling). A total of 246 (165 at first sampling and 81 at second sampling) dogs were studied and from these 65 dogs samples were gathered and monitored twice. Blood samples were obtained from the dogs' cephalic vein into Vacutainer tubes containing EDTA, individually labeled, and kept cool during transport to the laboratory where they were frozen at −20 °C until tested. According to the rules of the Bioethics Committee of the Campus of Biological and Agricultural Sciences at the Autonomous University of Yucatan (CB–CCBA–D–2016–003) a written consent of each of the dog's owner was obtained.

A questionnaire was submitted to each owner to obtain information about age group, gender, body condition, cohabitation with other dog (s), presence of ticks, communities, season of the year, physical activity, and general ectoparasite control practices. The body condition score was assessed using a slightly modified version (we use only three categories) of the method developed by Laflamme [12].

2.2. DNA extraction and polymerase chain reaction (PCR) procedures

DNA was extracted by using DNeasy® Blood & Tissue (Qiagen) following the manufacturer's instructions. DNA extractions were submitted for rickettsiales agents detection to the Texas A&M Veterinary Medical Diagnostic Laboratory (TVMDL) in collaboration with the Department of Veterinary Pathobiology at the College of Veterinary Medicine and Biomedical Science, Texas A&M University (TAMU).

The samples were initially screened for Rickettsiales species by the proprietary TickPath Layerplex (qPCR) at TAMU (https://tvmdl.tamu.edu/tests/tickpath–layerplex–qpcr/) (patent application number US 2019/0093149 A1) [13] to amplify a fragment of the *16S rRNA* gene of *E. canis, E. chaffeensis* and *E. ewingii*, a fragment of the *msp2* gene of *A. phagocytophilum*, and a fragment of *rhhyp* gene of *R. rickettsii* (hypothetical protein A1G_04230).

Positive samples detected by the TickPath Layerplex qPCR were then analyzed by nested PCR at the molecular biology laboratory at the CCBA–UADY. Thirty randomly selected negative dogs by the qPCR were also tested by the nested PCR. The fragments of the *16S rRNA* and *ompB* genes were amplified to detect *Ehrlichia/Anaplasma* and *Rickettsia*, respectively. External primers to distinguish genus and internal to distinguish species were used (primers, target gene and conditions of the nested PCR used are mentioned in Table 1). *Ehrlichia canis*, *E. chaffeensis*, *A. phagocytophilum,* and *R. coronii* plasmids were used as positive controls and nuclease–free water as a negative control.

All studies conducted at TAMU were performed under biosafety level 2 under the Institutional Biosafety Permit (IBC–2013–039 and IBC–2016–051). In addition, samples from Mexico were imported in the US under approved import permits (CDC permit #2015–05–071, USDA APHIS VS permit #128,538).

2.3. Sequencing

Five positive samples to a fragment of the *16S rRNA* of *E. canis* were randomly selected for sequencing. Also one positive sample to a fragment of the *ompB* amplicon of *Rickettsia* spp. was sequenced to determine the species. Products were purified using E.Z.N.A.® gel Extraction Kit (Omega Bio–tek, Inc, Norcross, Georgia, USA) and sequenced in the laboratory DIMYGEN® (Mérida, Yucatán, México; http://www.dimygen.com/). The resulting sequences were compared to sequences of *E. canis* and *R. parkeri* deposited in GenBank® by using BLAST (http://www.ncbi.nlm.nih.gov/blast). All sequences generated in this study were deposited in GenBank®.

Phylogenetic trees were inferred using the Maximum Likelihood method as implemented by the MEGA software version 7 [19]. The evolutionary distances were computed using the Kimura 2–parameter and Tamura–Nei model methods to *E. canis* and *R. parkeri*, respectively and are in the units of the number of base substitutions per site. A bootstrap resampling technique of 10,000 replications was conducted to statistically support the reliabilities of the nodes on the trees.

2.4. Statistical analysis

Prevalence (number of dogs with *E. canis* infection/number of evaluated dogs × 100) and cumulative incidence (number of dogs that become infected with *E. canis* during the study period/number of negative dogs at the beginning of the study period x 100 were calculated [20].

For associated factors analysis, *E. canis* PCR-positive dogs were considered as dependent variable. Age group (< 1 years old, 2–3 years old, > 3 years old), gender (male, female), body condition (poor, medium, good), cohabitation with other dogs (yes, no), presence of ticks (yes, no), community (Sucopo, Chan San Antonio, Yaxcheku), season of the year (rainy, dry), physical activity (hunting dogs with access to forested areas, household dogs with no access to forested areas), and use of ectoparasiticides to control ticks in the last year (yes, no), were considered as independent variables.

A primary screening was performed using 2 × 2 contingency tables (univariate ($\chi 2$ analysis) of exposure variables (Statistix software, version 9). All variables with $p < 0.20$ were analyzed by a logistic–binomial regression model of fixed effects (multivariate analysis) using the SPSS program version 18.0 for Windows, which provides exact regression estimates, 95% confidence intervals, odds ratio (OR), and *P*–values. A *P*–value < 0.05 was considered statistically significant.

3. Results

From June 2016 to March 2017 a total of 246 dogs from three rural communities of Yucatan were submitted for diagnoses of Rickettsial agents. Using qPCR, 72 positive dogs to *E. canis* was detected (prevalence of 29.26%, 72/246). None of the studied dogs were positive to *E. chaffeensis*, *E. ewingii*, *R. rickettsii* nor *A. phagocytophilum.*

All 72 positive dogs to *E. canis* in the qPCR were positive in the nested PCR (100% of concordance). Thirty randomly selected negative dogs in the qPCR were negative in the nested PCR (100% of concordance).

From the 52 negative dogs in the first sampling, 20 dogs became positive at the second sampling time point (211–248 days between the first and second sampling). The cumulative incidence of *E. canis* infection was 38.46% (20 new positive dogs /52 negative dogs at risk/).

From the 13 infected dogs with *E. canis* at the first sampling, 11 dogs (84.61%) remained positive at the second sampling (211–248 days between the first and second sampling).

Sequencing analysis of the nested PCR products amplifying the *16S rRNA* gene fragment of *E. canis* in five dogs confirmed the results, and showed 100% identity with CP025749.1, EU106856.1, and CP000107.1. Sequencing the nested PCR product of the *ompB* gene fragment of *Rickettsia* spp. of one dog of the five sequenced (coinfected with *E. canis*) showed 98.1% of identity to *R. parkeri* partial gene KY113111.1, CP003341.1, KY113111.1, and AF123717.1. The phylogenetic tree of *E. canis* was inferred based on the *16S rRNA* sequences obtained in this study and compared with *E. chaffeensis* (NR_074500.2); *Cowdria* (*Ehrlichia*) *ruminantium* (X61659.1) and *Anaplasma phagocytophilum* (CP006617.1) (Fig. 1). The phylogenetic tree of *R. parkeri* was inferred based on the *ompB* sequence obtained in this study and KJ663756.1, KJ675443.1, AE006914.1, KC847318.1, AP017601.1, CP012420.1, CP013133.1, AF123705.1, KX034059.1, and CP000053.1 (Fig. 2). In the nested PCR a dog co–infected with *E. canis* and *R. parkeri* was detected.

Representative sequences obtained in this study have been deposited in the GenBank® database under the following accession numbers: MH374119 (*16S rRNA* gene fragment of *E. canis*) and MH401420 (*ompB* gene fragment of *R. parkeri*).

Table 1
Pathogens tested, primers, and target gene of nested PCR used in this study.

Primer	Pathogen	Primer sequency	Name of primer (Reference)	Target gene	PCR conditions Reference
External	*Ehrlichia* spp.	5´-agaacgaacgctggcggcaagcc-3´ 5´-cgtattaccgcggctgctggc-3´	ECC ECB [14]	*16 s rRNA*	[14]
	Anaplasma spp.	5´-cacatgcaagtcgaacggattattc-3´ 5´-ttccgttaagaaggatctaatctcc-3´	Ge3a Ge10r [15]	*16 s rRNA*	[15]
	Rickettsia spp.	5´-gtaaccggaagtaatcgtttcgtaa-3´ 5´-gctttataaccagctaaaccacc-3´	rompB-OF rompB-OR [16]	*ompB*	[16]
Internal	*Ehrlichia canis*	5´-caattatttatagcctctggctataggaa-3´ 5'- tataggtaccgtcattatcttccctat-3'	ECAN5 HE-3 [14]	*16 s rRNA*	[14]
	Ehrlichia chaffeensis	5´-caattgcttataacctttttggttataaat-3´ 5'- tataggtaccgtcattatcttccctat-3'	HE1 HE3 [14]	*16 s rRNA*	[17]
	Ehrlichia ewingii	5´-cgaacaattcctaaatagtctctgac-3´ 5'-tataggtaccgtcattatcttccctat-3'	EE52 HE3 [18]	*16 s rRNA*	[18]
	Anaplasma phagocytophilum	5´-aacggattattctttatagcttgct-3´ 5´-ggcagtattaaaagcagctccagg-3´	Ge9f Ge2 [15]	*16 s rRNA*	[15]
	Rickettsia spp.	5´-gtttaatacgtgctgctaaccaa-3´ 5´- ggtttggcccatataccataag-3´	*ompB* SFG *opmB* SFG/TG [16]	*ompB*	[16]

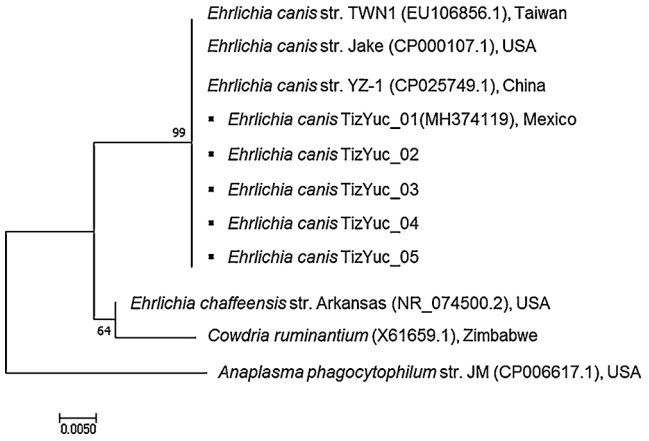

Fig. 1. Phylogenetic trees based on the partial 16S rRNA sequences of *Ehrlichia canis* isolates. Accession numbers for *E. canis* isolates and other sequences of *E. chaffeensis*, *Cowdria* (*Ehrlichia*) *ruminantium*, and *Anaplasma phagocytophilum* are given in parentheses. The scale bar indicates the number of substitutions per nucleotide position.

The frequency of *E. canis* infection in dogs with the following variables were: age group (≤1 years old: 27.1%, 2–3 years old: 19.0%, > 3 years old: 37.2%), gender (male: 28.8%, female: 30.2%), body condition (poor: 33.3%, medium: 31.5%, good: 28.3%), cohabitation with other dogs (yes: 32.2%, no: 26.0%), presence of ticks (yes: 31.4%, no: 26.8%), community (Sucopo: 22.3%, Yaxcheku: 34.2%, Chan San Antonio: 34.2%), season of the year (rainy: 28.4%, dry: 30.8%), physical activity (hunting dogs with access to forested areas: 35.9%, household dogs with no access to forested areas: 32.6%), and use of ectoparasites to control ticks in the last year (yes: 28.4%, no: 30.0%).

In the statistical analysis of χ^2 of the variables, only age, community and co–habitation with other dogs had *P*–value < 0.2 and were included in the logistic regression. Table 2 shows the logistic regression analysis wherein dogs > 3 years old were 2.41 (OR value, $p = 0.022$) times more likely to be infected with *E. canis* compared with dogs 2–3 years old.

4. Discussion

Anaplasma phagocytophilum, Ehrlichia canis, E. ewingii, E. chaffeensis and *Rickettsia* spp. are tick–borne pathogens of a wide range of vertebrate hosts including humans [21]. Their ecology involves hard ticks (Ixodidae) as vectors, wildlife and domestic animals, and human as hosts [21]. In Mexico, only two cases of human ehrlichiosis by *E. chaffeensis* have been diagnosed by using an immunofluorescence antibody test [22] and nested PCR [23]. Also, *E. chaffeensis* has been identified in *Peromyscus* spp. rodents (5.5%) collected from 31 sites in Mexico, and in *R. sanguineus* s.l. and *Amblyomma mixtum* ticks [24], which are found throughout Mexico. *Anaplasma phagocytophilum* is another pathogen that has been detected in opossums and dogs from Mexico with a prevalence of 3 and 27%, respectively [25]. In this study we did not detect positive dogs to *E. chaffeensis*, *E. ewingii* nor A. *phagocytophilum*, despite the fact that these studies were conducted in dogs from rural communities and might be exposed to several tick species [9], including ticks from the forest (i.e. *Ixodes affinis*).

Canine Monocytic Ehrlichiosis (CME) is endemic to Yucatan, Mexico, largely because regional climate conditions in this subtropical zone provide an adequate environment for its main vector, the tick *Rhipicephalus sanguineus* s.l. [9]. In Yucatan the seroprevalence of *E. canis* varies from 9 to 45% in dogs [5,26] depending on the area of study and the serological test used. The high endemicity of this bacteria in the region is attributed to the high *R. sanguineus* s.l. infestation on dogs [9] and the high *E. canis* infection rate (18.5%) in this tick species [7]. The prevalence of *E. canis* active infection in dogs from three rural communities of Yucatan studied in our survey (29.2%), is similar to molecular diagnosis reported in an urban area (36%) of the same state of Yucatan [7].

Prevalence and incidence have different applications. Prevalence is useful if interest is focused on existing cases. Cumulative incidence is used to predict an individual´s change in health status because indicates the probability of an individual becoming infected over a specified period of time [20]. The incidence of *E. canis* infection depends on certain epidemiological factors, such as the distribution of the vector, infection rate, animal behavior, and the average age of the study population [5]. In our study the cumulative incidence of *E. canis* infection was 38.46%. To our knowledge, this is the first study to report

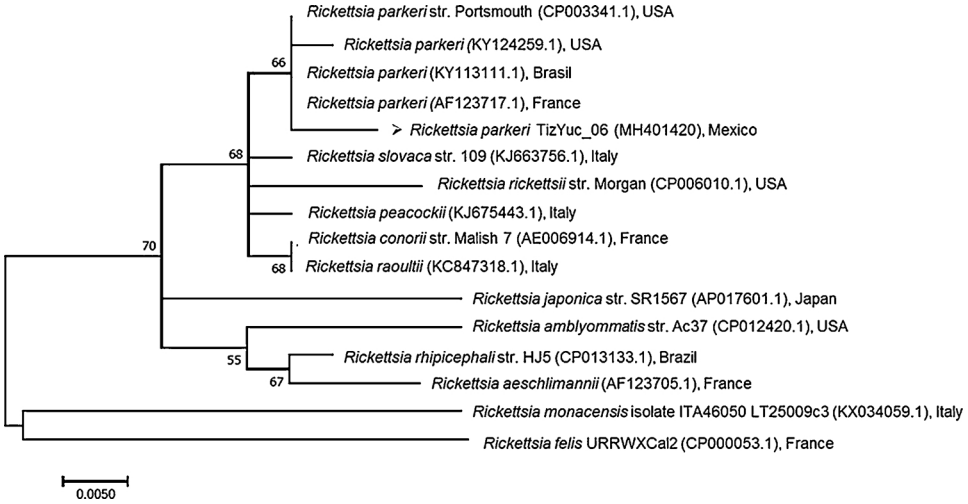

Fig. 2. Phylogenetic trees based on the partial ompB sequences of *Rickettsia parkeri* isolate. Accession numbers for *R. parkeri* isolate and other sequences of *R. slovaca, R. peacockii, R. conorii, R. raoultii, R. japonica, R. amblyommatis, R. rhipicephali, R. aeschlimannii, R. monacensis,* and *R. felis* are given in parentheses. The scale bar indicates the number of substitutions per nucleotide position.

cumulative incidence rate of *E. canis* in dogs.

Due to the lack of information on incidence of *E. canis* infection in dogs elsewhere, it is not possible to compare the cumulative incidence rate found in this study. However, our findings indicate a need to increase veterinary educational efforts about the risks for acquiring *E. canis* and to develop the clinical illness in dogs in the region, and in particular, to expand prevention awareness of medically important *R. sanguineus* s.l. ticks.

In the absence of transovarial transmission in ticks, maintenance of *Ehrlichia* spp. requires persistently infected vertebrate hosts as reservoir hosts [21]. The present study shows that 84.61% of dogs remained PCR-positive for *E. canis* after 211–248 days of evaluation. The PCR-positive during the second sampling period might be due to the *E. canis* re-infection or the long–term infection. The long–term infection has previously been reported in dogs infected with *E. canis* and *E. ewingii* where the bacterial DNA can be detected in blood by PCR up to 1020 [27] and 773 days post–infection [28], respectively.

In this study we identified indirectly one dog coinfected with *R. parkeri* and *E. canis*. This bacterium has previously been reported in dogs from Bolivia, Brazil and the USA [2,29,30]; in Mexico this is the first report in dogs. All documented USA human cases of *R. parkeri* rickettsiosis occurred within the known geographic range of *A. maculatum* ticks, predominantly in coastal states of the Eastern Seaboard and along the Gulf of Mexico. Confirmed cases of *R. parkeri* rickettsiosis have also been reported in Uruguay and Argentina, where *A. triste* and *A. tigrinum* ticks serve as the principal vector species [31]. Additional studies reported *R. parkeri* in *A. ovale* from Colombia and Belize, and *A. maculatum* tick from Belize [32,33]. Recently, a distinct strain of *R. parkeri*, was reported in the tick *Dermacentor parumapertus* in Chihuahua and Sonora [34]. In Yucatan, *A. maculatum* and *A. ovale* have been reported to infest dogs [9]. A laboratory study showed that *A. ovale* is a competent vector of *R. parkeri* [33]. In our study the diagnosis of *R. parkeri* in dogs was based on single *ompB* gen with 420 pb, additional genes analysis to confirm the *R. parkeri* infection and the role in the transmission of this pathogen needs to be further investigated in dogs and ticks.

The logistic regression revealed that dogs > 3–year had higher probability (OR: 2.41, $p = 0.022$) to be infected with *E. canis* in relation to young animals. This finding is in agreement with Rodriguez–Vivas et al. [5] who found 4–6 higher probability to find *E. canis* seropositive in dogs of > 2–year compared with young animals. Possible explanations include the immunologic status of the host or more likely, the greater probability to be exposed to infected vectors [35]. In this study, other variables such as the season of the year, ectoparasite control practices, gender, body condition, cohabitation with other dogs, community and physical activity were not associated with dogs infected with *E. canis*. Despite this, in previous studies dogs with tick infestation, regions with less urbanization and lower socioeconomic status and absence of veterinary care are more likely to be infected with *E. canis* [36–38].

PCR and sequencing are sensitive methods for detecting and characterizing tick–borne infectious agents affecting humans and animals, and in particular in the detection of *E. canis* DNA [21] in both animals and ticks. In this study the novel patent pending TickPath Layerplex qPCR (US 2019/0093149 A1) and conventional nested PCR methodologies had 100% of concordance, demonstrating that these methodologies can complement current diagnostic efforts in the veterinary field. The results obtained in this study, confirmed that both assays had an excellent concordance and are strongly recommended for *E. canis* detection in blood samples of dogs.

Table 2
Logistic regression analysis to detect associated factors to *Ehrlichia canis* infection in dogs from three rural communities of Yucatan, Mexico.

Factor	Total tested dogs	No. Positive	Frequency	Odds ratio	CI 95%	*P* value
Rural community						
*Sucopo	103	23	22.33	1.00		
Yaxcheku	73	25	34.24	1.00	0.49–2.02	0.998
Chan San Antonio	70	24	34.28	1.60	0.86–3.25	0.175
Age						
*2–3	63	12	19.04	1.00		
≤1	81	22	27.16	1.64	0.86–3.11	0.127
> 3	102	38	37.25	2.41	1.13–5.14	0.022
*Co-habitation with other dogs						
No	119	31	26.05			
Yes	127	41	32.28	1.19	0.67–2.13	0.544

* Used as a reference, CI 95%: Confidence interval at 95%.

To understand the public health impact of rickettsial diseases, it is important to know the relationship between pathogens, vector species and human and animal hosts, as well as the impacts of varying environmental conditions on these interactions [39]. Without rigorous epidemiological surveillance programs, health burdens of tick–borne diseases are nearly impossible to track. Moreover, without information about disease prevalence and incidence, field surveillance is required for cautious clinical diagnoses and risk assessment. In the absence of epidemiological and clinical data, field surveillance for infected ticks and reservoir hosts can shed light on human and animal risk to tick-–borne disease exposure [40].

5. Conclusions

It is concluded that high prevalence of *Ehrlichia canis* in the dog population of Yucatan was detected. The age (> 3 years old) was the only factor associated with *E. canis* infection in dogs. Furthermore, to the best of the author's knowledge, this study presents the first report of a *R. parkeri* active infection in a domestic dog in the state of Yucatan, Mexico, presented as a co–infection with *E. canis*.

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Declaration of Competing Interest

The authors declare that there are no conflicts of interest.

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