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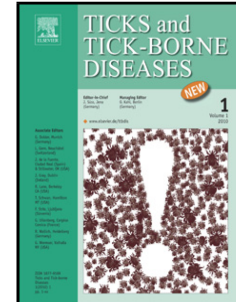

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Molecular detection and identification of Rickettsiales pathogens in dog ticks from Costa Rica

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Abstract

Although vector-borne diseases are globally widespread with considerable impact on animal production and on public health, few reports document their presence in Central America. This study focuses on the detection and molecular identification of species belonging to selected bacterial genera (*Ehrlichia, Anaplasma* and *Rickettsia*) in ticks sampled from dogs in Costa Rica by targeting several genes: 16S rRNA/*dsb* genes for *Ehrlichia*; 16S rRNA/*groEL* genes for *Anaplasma*, and *ompA*/*gltA*/*groEL* genes for *Rickettsia*. PCR and sequence analyses provides evidences of *Ehrlichia canis, Anaplasma platys,* and *Anaplasma phagocytophilum* infection in *Rhipicephalus sanguineus* s.l ticks, and allow establishing the presence of *Rickettsia monacensis* in *Ixodes boliviensis*. Furthermore, the presence of recently discovered Mediterranean *A. platys*-like strains is reported for the first time in Central America. Results provide new background on geographical distribution of selected tick-transmitted bacterial pathogens in Costa Rica and on their molecular epidemiology, and are pivotal to the development of effective and reliable diagnostic tools in Central America.

Keywords

Ehrlichia; *Anaplasma; Rickettsia*; *Rhipicephalus sanguineus* s.l.*; Ixodes boliviensis.*

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Introduction

In tropical regions, tick-transmitted infections are an emerging problem in dogs, causing serious disease or sub clinical infections making companion animals a reservoir for human vector-transmitted infectious agents, since most of tick-borne bacterial agents that infect dogs are zoonotic (Nicholson et al., 2010). The location of Costa Rica in the neotropic ecozone provides the ideal conditions for the establishment and development of a variety of ticks. Most bacterial tick-borne pathogens have been described to cause disease in dogs (Day, 2011): *Ehrlichia canis, Anaplasma phagocytophilum,* and *Rickettsia rickettsii* (Carrade, 2009; Little et al., 2010; Nicholson et al., 2010). The brown dog tick *Rhipicephalus sanguineus sensu lato* (s.l.) (Moares-Filho et al., 2011; Nava et al., 2015) is the most common tick reported in dog infestations in Costa Rica. *Amblyomma cajennense* (reported as *Amblyomma mixtum*)*, Amblyomma maculatum, Amblyomma ovale, Amblyomma pecarium, Ixodes boliviensis* and *Rhipicephalus microplus* have been found sporadically on the same host species (Álvarez et al., 2005; Alvarez et al., 2006; Troyo et al., 2012). Innovative molecular methods have been combined to develop useful, sensitive, and rapid tools for the detection and identification of vector-borne pathogens in arthropods, including ticks (Nicholson et al., 2010). Although there are several reports about prevalence and molecular detection of Rickettsiales agents in dogs (Ábrego et al., 2009; Romero et al., 2010; 2011; Rojas et al., 2014; Troyo et al., 2014), to date, findings in ticks from Costa Rica are still lacking. This study reports the molecular detection and characterization of selected species of the order Rickettsiales (*Ehrlichia, Anaplasma,* and *Rickettsia*) in ticks collected from dogs of Costa Rica.

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Material and methods

Collection and identification of ticks

A prospective observational convenience study was carried out in order to enroll dogs attended by veterinarians. After open invitation, 14 veterinary clinics gave their consent to participate on voluntary basis in the investigation. Clinics were located mainly in the Great Metropolitan Area (GMA) of the country (San José, Alajuela, Cartago and Heredia provinces), and some in the Guanacaste province in the north Pacific coast of Costa Rica. During October 2006 to July 2007, all ticks observed in dogs presented at the clinics or attended by veterinarians in residences were collected and deposited in plastic tubes containing 70% alcohol. Identification of ticks at species level was performed morphologically using a stereoscope and the taxonomic keys proposed by Fairchild et al. (1966) and Barros-Battesti et al. (2006). Identification of nymphs was carried out according to Cooley (1946).

DNA extraction and polymerase chain reaction (PCR)

DNA was extracted from ticks using DNeasy® Blood & Tissue Kit (QIAGEN, Chatsworth, CA, USA), by following the manufacturer's instructions. All ticks (adults and nymphs) were individually treated. A set of already published primers was selected and used to identify the Rickettsiales bacterial species most frequently reported in dogs (Table 1).

Briefly, the presence of bacterial species belonging to the family Anaplasmataceae and Rickettsiaceae was investigated with primers targeting the 16s rRNA and *ompA*/*gltA*/*groEL* genes, respectively. In order to mine deeper into Anaplasmataceae diversity specific PCR assays were conducted

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to detect *E. canis*, *Ehrlichia chaffeensis*, and *Ehrlichia ewingii* (nested 16S rRNA and *dsb*); *A. phagocytophilum* (nested 16S rRNA and *groEL*), and *A. platys/A. platys-like* (nested 16S rRNA and *groEL*). DNA from *A. phagocytophilum* (strain Trestom) HL-60 infected cells was used as positive control for *A. phagocytophilum* PCR detection; similarly, DNA extracted from blood of a dog positive by PCR to *A. platys* (and confirmed by sequencing) was used as positive control during detection of *A. platys* (Abrego et al., 2009). Plasmids containing segments of DNA from *E. canis*, *E. chaffeensis* and *E. ewingii* were used as positive controls in *Ehrlichia* PCR experiments, whereas *Rickettsia felis* DNA was used as positive control for *Rickettsia* spp. All PCR experiments included water (Fermentas®) used as negative control. PCR products were visualized by agarose gel electrophoresis (1.4%) in TBE (Tris Base, boric acid, EDTA, pH8, 0.5M), and ethidium bromide staining (0.5 µg/ml). GeneRuler 100bp DNA Ladder Plus (Sm0321, Fermentas®) was used for DNA sizing.

Sequencing and sequence analysis

In order to confirm PCR results, amplicons obtained with primers specifically targeting *A. phagocytophilum* (*16S rRNA* and *groEL*)*, A. platys* (*16S rRNA* and *groEL*)*, Rickettsia* spp. (*gltA* and *groEL*), or the ehrlichial *dsb* gene, were sequenced and comparative sequences analyses were performed. Shortly, PCR products were purified using the QIAquick® (QIAGEN) kit, by following the manufacturer's instructions. One *E. canis* sample *(dsb),* two *A. phagocytophilum* samples (16S rRNA and *groEL*), one *A. platys* sample (16S rRNA and *groEL*), one A. platys-like sample (*groEL*), one sample positive to

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Rickettsia spp. (*gltA* and *groEL*), were automatically sequenced by commercial companies. Sequences were aligned with the ClustalW option of BioEdit (Hall, 1999) and compared with sequences deposited in the NCBI database (National Center for Biotechnology Information) using the BLASTn algorithm.

Results

A total of 165 ticks were collected from 165 dogs that were either attended at veterinary clinics (157) or in residences (8), of which 79 came from the province of Heredia, 53 from Alajuela, 21 from San Jose, 8 from Cartago, 2 from Guanacaste (province at the Pacific coast, outside the GMA), and 2 from undefined locations. One hundred fifty-six were adult ticks (104 females and 52 males) whereas 9 were nymphs. The 165 ticks were identified as *R. sanguineus* s.l. (n=160), *A. cajennense* complex (n=4), and *I. boliviensis* (n=1).

Upon an initial screening conducted with primers specific for Anaplasmataceae, 14 out of 165 DNA samples (8.5%) tested positive (Table 2). Subsequently, more specific and sensitive PCR methods were applied in order to mine deeper into the presence of *Anaplasma* and *Ehrlichia* species. *E. canis* was detected in 43 (26%) *R. sanguineus* s.l. ticks (Table 2), of these, 5 were nymphs and 38 were adults (22 females and 16 males). The highest proportion of *E. canis* positive ticks was found in the provinces of Heredia and Alajuela (Table 3) although positivity was detected in all the provinces of the Costa Rican Central Valley. On Blast search the sequence of the *E. canis dsb* gene obtained in this study (KU534872) resulted 100% (288/288 bp) similar to

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several *E. canis* sequences deposited in the GenBank detected in ticks of Argentina and Brazil (KR909452, KP167596), and in human blood donors of Costa Rica (KR732921).

The use of a 16S rRNA *A. phagocytophilum* specific PCR allowed identifying this pathogen in 2 (1.3%) adult female *R. sanguineus* s.l. ticks from Heredia and San Jose (Table 3). After sequencing, one of the sequences, designated as 16S_AphagoCR1 and deposited in GenBank under KU534874, was found 100% (343/343 pb) similar to *A. phagocytophilum* strains isolated in different countries and different hosts. The second sequence, named 16S_AphagoCR2 and deposited in GenBank under KU534875, shared 99.7% homology (310/311pb) with the same strains. When these two ticks were analyzed by *A. phagocytophilum* specific *groEL* PCR, only one sample, from which sequence 16S_AphagoCR1 was obtained, generated a positive reaction. *GroEL* sequencing resulted in a sequence designated as GroEL_AphagoCR1 and deposited in GenBank under KU534870, that had 100% (530/530pb) homology with *A. phagocytophilum* pathogenic strains isolated in the USA and in the Mediterranean area (AF172163 and AY848750, respectively).

Using a 16S rRNA *A. platys* specific PCR this pathogen was identified in 5 (3.0%) *R. sanguineus* s.l. tick samples, of which four were adults (3 females and 1 male) and one was a nymph (Table 2). Positive samples came from the provinces of Heredia and Alajuela (Table 3). Sequencing and BlastN analyses showed that the 16S rRNA sequence generated in this study (KU534873) was 98.5% similar to sequences representative of the 16S rRNA gene of *A. platys* (AF_156784), and of several related strains, such as that found in

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dromedaries in Tunisia (KC800963) and that described as *Candidatus* Anaplasma camelii in Saudi Arabia (KF843823). The presence of *A. platys* was further investigated by targeting the *groEL* gene from samples that yielded positive results in the 16S RNA-PCR for A*. phagocytophilum* (n=2) and 16S RNA-PCR for *A. platys* (n=4). Only DNA extracted from one *R. sanguineus* s.l. tick was found positive to *A. platys groEL* PCR, sequencing and homology search revealed that this sequence (KU534871) was 100% (476/476 pb) similar to sequence KC335256, isolated from a calf in the Mediterranean area, belonging to a cluster of strains similar to but different from *A. platys*, and infecting neutrophil granulocytes. Interestingly this tick positive to *A. plat*ys–like by *groEL* PCR was also found positive to *A. phagocytophilum* (sequence 16S_AphagoCR1).

A total of 26 (16.2%) ticks, 25 *R. sanguineus* s.l. and 1 *I. boliviensis,* tested positive to *ompA* and *gltA Rickettisia* spp. specific PCR (Table 2). Sequencing and homology revealed the presence of an invariable *gltA* (KU529481) and *groEL* (KX447668) sequence in the female *I. boliviensis* tick from Heredia, that on BlastN comparison was 99.7% (378/379 bp) and 99.8% (582/583 bp) similar to *Rickettsia monacensis* sp. nov. (type strain, IrR/MunichT) from an *Ixodes ricinus* tick collected in Germany (AF141906.1), respectively. The *groEL* sequence was 99.2% (376/379bp) similar to *R. monacensis* strain WB9/Ir Pavullo (HM210739.1) isolated from *I. ricinus* from Italy.

Discussion

R. sanguineus s.l. is the most common tick infesting dogs of Costa Rica (Álvarez et al., 2005; Álvarez et al., 2006; Jiménez-Rocha et al., 2013). Dogs

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are the closest animals to humans and although reports in the literature about human bites by ticks are few and interaction of *R. sanguineus* s.l. with humans must be further investigated, parasitism by these ticks have been documented, indicating a potential risk for transmission of pathogens to humans (Dantas-Torres, 2008). Thus, the detection of *E. canis, A. phagocytophilum, A. platys*, and *A. platys*-like in this tick species is of particular importance. However, since ticks were collected directly from dogs, our results for Anaplasmataceae could have originated from infected blood ingested by these ticks, and not necessarily from infected ticks.

Only 14 out of 165 DNA samples tested positive for Anaplasmataceae, what can be explained by the lower sensitivity of this PCR compared to more specific and nested PCRs. However, it is important to notice that only 8 samples were confirmed as *E. canis* positive, or *E. canis* and *A. platys* positive (2 samples), whereas the remaining 4 samples yielded negative results. This may suggest the presence of a novel bacterial strains belonging to this family, that can not be detected with species-specific PCRs, or the presence of other bacterial groups, not related to ticks.

The high infection rate of *E. canis* in Costa Rica in *R. sanguineus* s.l. ticks are in accordance with the high seroprevalence observed previously in dogs (Barrantes-González et al., 2013). Populations of *R. sanguineus* s.l. ticks from Central America are included within a group called tropical or northern lineage (Dolz et al., 2015), which is known to be associated with the transmission of *E. canis* to dogs in the tropical areas of South America (Moraes-Filho et al., 2016).

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DNA of *A. phagocytophilum* has been reported in *R. sanguineus* s.l. from Brazil (Santos et al., 2013), but to date not in Central America. In Costa Rica two possible human cases were diagnosed previously but based on clinical signs and detection of granulocytic morulae in blood smears (Rojas-Solano and Villalobos-Vindas, 2007; Hernández-de Mezerville and Padilla-Cuadra, 2007). This is in accordance with our results that identified DNA of *A. phagocytophilum*. However, it remains to establish which other genetic *A. phagocytophilum* variants are present in Costa Rica (Dugat et al., 2015), and if *R. sanguineus* s.l. plays a role in the transmission of this pathogen to humans and other animals.

Infections with *A. platys* in *R. sanguineus* s.l. have been reported in different countries from Africa, Asia, and Europe (Latrofa et al. 2014; Ramos et al. 2014; Sanogo et al. 2003; Ybañez et al. 2012;). The potential role of this tick as biological vector was suggested (Harvey et al., 1978; Simpson et al., 1991; Ramos et al., 2014), our results obtained in this study show additional evidence.

Finding *A. platys*-like strains in ticks in Costa Rica are in agreement with recent detection of this agent in Mediterranean and Chinese ruminants (Zobba et al, 2014; Yang et al 2015), and suggest that species diversity of Anaplasmataceae has yet to be fully investigated, and that further studies are needed to identify more species and variants in the future.

The only rickettsias reported to date in *I. boliviensis* were *Candidatus* Rickettsia andaneae, detected in a tick from a horse in Peru (Blair et al., 2004), and an undescribed *Rickettsia* sp. found in a dog tick in Costa Rica (Troyo et al., 2014). This rickettsia was named strain IbR/CRC, and placed in

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the group of *R. monacensis*, but was also found close to an endosymbiont of *Ixodes scapularis* and other undescribed rickettsiae. Our results using *gltA* and *groEL* protocols confirmed the presence of *R. monacensis* in *I. boliviensis* tick from Costa Rica.

Most ticks were infected with a single pathogen, however, four *R. sanguineus* s.l. ticks infected with *E. canis* displayed also infections with *A. platys* (2), *A. phagocytophilum*CR2 (1) and *R. amblyommii* (1), whereas one *R. sanguineus* s.l. tick showed mixed infection of *A. phagocytophilum*CR1 and *A. platys*-like, and what is extensively reported in the literature (Latrofa et al., 2014).

E. chaffeensis and *E. ewingii* were not detected in the present study. They have been reported in dog ticks in the United States using molecular techniques (Breitschwerdt et al. 1998; Varde et al., 1998), and are primarily transmitted by *Amblyomma americanum*. This tick is widely distributed in the southeastern United States and northern of New York (Means and White, 1997), but has not been reported in Costa Rica (Alvarez et al., 2005). Mainly *R. sanguineus* s.l. ticks were analyzed in the present study, what could explain why *E. chaffeensis* and *E. ewingii* were not detected. The presence of these agents in ticks of other regions should not be ruled out, and it is recommended to continue investigations to detect these agents in Costa Rica.

Conclusions

This study reports the identification of tick-borne pathogens in dog ticks of Costa Rica, some of them of zoonotic interest, pointing out the increasing awareness of diseases associated to these ectoparasites, and suggesting a risk for the emergence of tick-borne diseases in dogs and humans of Costa

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Rica. Further studies are needed to investigate the presence of Rickettsiales in the country, to establish the epidemiological and clinical importance of these pathogens, and to set up surveillance plans for zoonotic diseases transmitted by ticks.

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Table 1. Primers used in this study for amplifying selected tick-borne pathogens

Pathogen	Gen	Primer (Reference)	Sequences (5´- 3´)	Fragment length (bp)
Anaplasmataceae	16S rRNA	EHR16SD EHR16SR (Inokuma et al., 2000)	GGTACCYACAGAAGAAGTCC TAGCACTCATCGTTTACAGC	345
Rickettsia spp.	*gltA*	Cs78 Cs323 (Labruna et al., 2004)	GCAAGTATCGGTGAGGATGTAAT GCTTCCTTAAAATTCAATAAATCAGGAT	401
Rickettsia spp.	*ompA*	Rr190.70p Rr190.602n (Regnery et al., 1991)	ATGGCGAATATTTCTCCAAAA AGTGCAGCATTCGCTCCCCCT	532
Rickettsia spp.	*groEL*	RgroMAR2-F RgroMAR2-R RgroMAR1-F RgroMAR1-R (Chisu et al., 2016)	AAAAGCTCGTGAGCAAATGC GTGATAACCGTTGAAGAAG GAGAGATGGAAGCAAGTAC GAAAGATGGATAGTCGCTGA	508
Ehrlichia spp.	16S rRNA	ECC ECB (Romero et al., 2011)	AGAACGAACGCTGGCGGCAAGC CGTATTACCGCGGCTGCTGGCA	478
E. canis	16S rRNA	ECAN5 HE3 (Romero et al., 2011)	CAATTATTTATAGCCTCTGGCTATAGGA TATAGGTACCGTCATTATCTTCCCTAT	389
E. chaffeensis	16S rRNA	HE1 HE3 (Romero et al., 2011)	CAATTGCTTATAACCTTTTGGTTATAAAT TATAGGTACCGTCATTATCTTCCCTAT	390
E. ewingii	16S rRNA	EE5 HE3 (Romero et al., 2011)	CAATTCCTAAATAGTCTCTGACTATTTAG TATAGGTACCGTCATTATCTTCCCTAT	392
Ehrlichia spp.	*dsb*	Dsb-330 Dsb-728 (Romero et al., 2011)	GATGATGTCTGAAGATATGAAACAAAT CTGCTCGTCTATTTTACTTCTTAAAGT	409
A. platys	16S rRNA	8F 1448R EHR16SR PLATYS	AGTTTGATCATGGCTCAG CCATGGCGTGACGGGCAGTGTG TAGCACTCATCGTTTACAGC GATTTTTGTCGTAGCTTGCTATG	678

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		(Ábrego et al., 2009)		
A. phagocytophilum	16S rRNA	Ge3a Ge10r Ge9f Ge2 (Massung et al., 1998)	CACATGCAAGTCGAACGGATTATTC TTCCGTTAAGAAGGATCTAATCTCC AACGGATTATTCTTTATAGCTTGCT GGCAGTATTAAAAGCAGCTCCAGG	546
Anaplasma spp.	*groEL*	EphplgroEL EphplgroEL (Alberti et al., 2005)	ATGGTATGCAGTTTGATCGC TCTACTCTGTCTTTGCGTTC	624
A. platys	*groEL*	EphplgroEL EplgroEL (Zobba et al., 2014)	ATGGTATGCAGTTTGATCGC CATAGTCTGAAGTGGAGGAC	515
A. phagocytophilum	*groEL*	EphplgroEL EphgroEL (Alberti et al., 2005)	ATGGTATGCAGTTTGATCGC TTGAGTACAGCAACACCACCGGAA	573

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Table 2. Number of ticks positive for selected tick-borne pathogens amplifying different genes with PCR

Pathogen	16S rRNA +/total	*dsb* +/total	*groEL* +/total	*gltA* +/total	*ompA* +/total
Anaplasmataceae	14/165	-	-	-	-
E. canis	43/165	1/1	-	-	-
E. chaffeensis	0/165	-	-	-	-
E. ewingii	0/165	-	-	-	-
A. platys	5/165	-	0/4	-	-
A. phagocytophilum	2/163	-	1/2	-	-
A. platys-like	-	-	1/6	-	-
Rickettsiaceae	-	-	1/1	26/160	26/160

Table 3. Geographical origin and infection rates of ticks

Province	Ticks	Infection rates				
	(N)	*E. canis*	*A. platys*	*A. phagocytophilum*	*A. platys-like*	*R. monacensis*
Heredia	79	21 (26.6%)	1 (1.3%)	1 (0.6%)	1 (1.3%)	1 (0.6%)
Alajuela	53	17 (32.0%)	4 (7.5%)	-	-	-
San José	21	2 (9.5%)	-	1 (0.6%)	-	-
Cartago	8	2 (25%)	-	-	-	-
Guanacaste	2	-	-	-	-	-
Not known	2	1 (50%)	-	-	-	-
Total	165	43 (26%)	5 (3%)	2 (1.2%)	1 (0.6%)	1 (0,6%)