Comparative Immunology, Microbiology and Infectious Diseases 62 (2019) 31–39

Contents lists available at ScienceDirect

Comparative Immunology, Microbiology and Infectious Diseases

journal homepage: www.elsevier.com/locate/cimid

Prevalence, molecular characterization and risk factor analysis of *Ehrlichia canis* and *Anaplasma platys* in domestic dogs from Paraguay

S. Pérez-Macchi[a], R. Pedrozo[b], P. Bittencourt[c], A. Müller[a,*]

[a] *Facultad de Ciencias Veterinarias, Universidad Austral de Chile, Valdivia, Chile*
[b] *Facultad de Ciencias Veterinarias, Universidad Nacional de Asunción, Paraguay*
[c] *Escuela de Medicina Veterinaria, Facultad de Ciencias, Universidad Mayor, Chile*

ARTICLE INFO

Keywords:
PCR
Phylogenetic analysis
Anaplasmataceae
South America

ABSTRACT

This is the first study to investigate the prevalence and risk factors associated with *Ehrlichia canis* and *Anaplasma platys* positivity in dogs from Paraguay. Conventional PCR assays for the *E. canis* 16SrRNA gene and *A. platys* p44 gene were carried out in blood samples from 384 dogs from Asunción city, Paraguay. Sequencing and phylogenetic analysis were performed in selected positive *E. canis* and (16SrRNA gene) and *A. platys* (16S and p44 genes) samples. The overall prevalence of *E. canis* and *A. platys* in dogs in Paraguay was 10.41% (40/384) and 10.67% (41/384), respectively. Older dogs without veterinary care had higher odds for *E. canis* positivity and a higher number of dogs in the same household, as well as absence of anti-tick treatment were considered risk factors for *A. platys*. *Ehrlichia canis* and *A. platys* circulate in the dog population from Asunción, and are described for the first time in Paraguay.

1. Introduction

Ehrlichiosis and anaplasmosis are emerging infectious diseases described in animals and humans, caused by organisms belonging to the subgroup Proteobacteria, order Rickettsiales, Anaplasmataceae family, genus *Ehrlichia* and *Anaplasma*. *Ehrlichia* spp. and *Anaplasma* spp. are small, Gram-negative obligate intracellular bacteria [1,2]. *Ehrlichia canis* and *A. platys* are the most prevalent species in dogs, causing diseases known as monocytic ehrlichiosis and canine cyclic thrombocytopenia, respectively [3–5].

Ehrlichia canis is the most common pathogen detected in domestic dogs, foxes, coyotes and jackals are considered natural reservoirs of the agent [6,7]. *Anaplasma platys* is a platelet-specific microorganism whose morular form in platelets resembles those described in the leukocytes of dogs infected with *E. canis* [5], and has also been identified in cats [8], humans [9,10] and foxes [11].

Ehrlichia canis and *A. platys* are cosmopolitan, predominating in tropical and subtropical areas, related to its vector distribution, R*hipicephalus sanguineus* sensu latto [12–16] [17]. R*hipicephalus sanguineus* s.l. has a worldwide distribution. It has been reported in several countries of the American continent such as Brazil [8,18,19], Uruguay [20], Argentina [21–23], Chile [24], Colombia [25], Venezuela [26], as well as in Europe, Oceania, Asia, and Africa [17,27–34].

Anaplasma platys infections are frequently found in the same geographic regions as *E. canis*, and evidence of exposure to both organisms is often detected in the same dog [35]. The molecular prevalence of *E. canis* and *A. platys* in South America ranges from 5.2%–47.7%, and 1.4%–86.62%, respectively [18,21,23,36–44].

Risk factors associated with *A. platys* and *E. canis* infections in dogs include climatic conditions, the prevalence of ticks, exposure to ticks, as well as the average of tick infestation [45–47]. Agglomeration of dogs, as observed in animal pounds [48], adulthood [49,50], and feeding type [48], are also described as risk factors.

Rhipicephalus sanguineus s.l. ticks were reported in Paraguay for the first time in 1982 [51]. However, in this country *A. platys* and *E. canis* have not yet been molecularly detected in domestic dogs. The aim of this study was to determine the prevalence, perform a molecular characterization, and a risk factor analysis of *E. canis* and *A. platys* in domestic dogs from Asunción city, Paraguay.

2. Materials and methods

2.1. Animals

In order to accurately determine the prevalence of *E. canis* and *A.* platys in Asunción, Paraguay, the sample size required was estimated according to Thrusfield [52]. For this, we considered a prevalence of 50%, which fits the criteria when the prevalence is unknown, with a

---

[*] Corresponding author at: Veterinary Hospital UACh, Fundo Teja Norte S/N, PO Box 567, Valdivia, Chile.
E-mail address: ananda.muller@uach.cl (A. Müller).

https://doi.org/10.1016/j.cimid.2018.11.015
Received 2 May 2018; Received in revised form 21 November 2018; Accepted 27 November 2018

Table 1

Dog population from six locations in Asunción, Paraguay, correlative percentage (%) per sector, number of sampled dogs and PCR prevalence for *Ehrlichia canis* and *Anaplasma platys* in domestic dogs.

Locations				Prevalence	
	Total Number of dogs	%	Number of Sampled dogs	*E. canis*	*A. platys*
Santísima Trinidad	43,240	24.1	93	5.98% (23/384)	3.12% (12/384)
Recoleta	39,015	21.7	83	0.26% (1/384)	1.04% (4/384)
San Roque	39.070	21.8	84	1.30% (5/384)	2.86% (11/384)
La Encarnación	24,086	13.5	52	0.78% (3/384)	1.30% (5/384)
La Catedral	21,078	11.8	45	1.56% (6/384)	0.78% (3/384)
Lambaré	12,747	7.1	27	0.52% (2/384)	1.56% (6/384)
TOTAL	**179,236**	**100**	**384**	**10.41% (40/384)**	**10.67% (41/384)**

Table 2

Summary information of the conventional PCR primer sets and their product sizes used in the study.

Primer	Target	Sequence (5´-3´)	Product size (bp)	Reference
ECC	*16sRNAr*	AGAACGAACGCTGGCGGCAAGC	**477 (primary)**	[53]
ECB	*Ehrlichia* spp.	CGTATTACCGCGGCTGCTGGCA		
ECAN5	*16sRNAr*	CAATTATTTATAGCCTCTGGCTATAGGA	**396 (Nested)**	[53]
HE3	*Ehrlichia canis*	TATAGGTACCGTCATTATCTTCCCTAT		
16S**CNMF**	*16sRNAr*	GTGGCAGACGGGTGAGTAAT	**866**	[54]
817R	Anaplasmataceae	GAGTTTTAGTCTTGCGA		
Apl_p44F3	*P44*	GCT AAG TGG AGC GGT GGC GAT GA CAG	**520**	[10]
Apl_p44R3	*Anaplasma platys*	CGA TCT CCG CCG CTT TCG TAT TCT TC		
RSP19 F	*RPS19*	CCTTCCTCAAAAA/GTCTGGG	**100**	[3]
RSP19 R		GTTCTCATCGTAGGGAGCAAG		

sample of 384 dogs. A precision of 5% was used, with a 95% confidence interval. The study was approved by the bioethics committee of Universidad Austral de Chile (UACh); under the protocol number UACh 238/2015. Over an 8-month period (August 2015 to March 2016), 384 client-owned dogs had their blood sampled by a veterinary team. The dogs came from six locations throughout Asunción city (La Encarnacion, La Catedral, San Roque, Lambaré, Recoleta and Santísima Trinidad) in order to acquire a balanced and representative cohort (Table 1).

Samples were taken from home visits to the pet-owner's household by a veterinary team. Dogs were sampled regardless of age, sex, health, and reproductive status. Data obtained at the time of presentation included age, sex, breed, veterinary care, health status, number of dogs in the same house, tick infestation, and anti-tick treatment; being recorded for potential risk factors. Each owner signed a consent form before sampling. Blood samples were collected aseptically by cephalic or jugular venipuncture, aliquoted in an EDTA collecting plastic tube (Vacutainer®), and submitted to the Laboratory of Animal Infectious Diseases of the Universidad Nacional de Asunción, Paraguay. EDTA anticoagulated blood samples were stored at -20 °C until PCR testing.

2.2. DNA extraction/purification

Frozen EDTA blood samples were thawed at room temperature and vortexed in the Laboratory of Animal Infectious Diseases of the Universidad Nacional de Asunción, Paraguay. DNA extraction and purification from 200 μL of blood was performed using E.Z.N.A. Tissue DNA Kit (Omega®, Georgia, USA), according to the manufacturer's instructions, to obtain 100 μL of purified DNA. DNA concentration and purity were determined (NanoDrop ND-1000 spectrophotometer; Thermo Scientific©, USA). The 260/280 nm absorbance ratio (OD260/OD280) provided an estimate of sample purity, accepting a ratio of 1.8 ± 0.2 as "pure". DNA was stored at −20 °C prior to performing PCR assays.

2.3. PCR assays

2.3.1. Conventional (c) PCR controls

The *RPS19* gene was used as an endogenous control assay for canine genomic DNA using the primers RPS19-F y RPS19-R [3]. The reaction mixture for *RPS19* was composed of 12.5 μL Gotaq® Green Master Mix (Promega®, Madisson, USA), 400 nM of each primer (RPS19-F and RPS19-R), 0.5 mM of $MgCL_2$ and 5 μL of template DNA, bringing the total volume to 25 μL with nuclease free water (Thermo Scientific©, USA). The thermic protocol was: 95 °C for 2 min followed by 40 cycles of 95 °C for 20 s, 61 °C for 30 s, 72 °C for 30 s and a final extension of 72 °C for 5 min.

All cPCR runs were performed with nuclease-free water as a negative control. As a positive PCR control, genomic DNA samples from naturally infected dogs were used: *E. canis* genomic DNA, kindly supplied by Dr. Rosangela Zacarias Machado from Universidade Estadual Paulista UNESP, Jaboticabal, Brazil and (2) *A. platys* genomic DNA, supplied by Dr. Nadia Almosny, from the Unversidade Federal Fluminense, Niterói, Brazil; both confirmed by PCR and sequencing. All reactions were performed in a T100™ Thermal Cycler (Bio-Rad, USA). The primer sequences used in cPCR are shown in Table 2.

2.3.2. Conventional (c) nested PCR for Ehrlichia canis screening

All 384 samples were submitted to a nested cPCR to amplify a 396 bp region of the 16SrRNA (16S) *E. canis* gene, as previously described [53].

First amplification targeted a 477 bp region of the *Ehrlichia* spp. 16S gene. The reaction mixture was composed of 12.5 μL Gotaq® Green Master Mix (Promega®, Madisson, USA), 300 nM of each external primer (ECC F and ECB R) and 5 μL of template DNA brought to a total volume of 25 μL with nuclease free water (Thermo Scientific©, USA). The thermal cycling protocol was as follows: 95 °C for 3 min followed by 30 cycles of 94 °C for 1 min, 65 °C for 1 min. and 72 °C for 40 s. Amplification was completed with a final extension of 72 °C for 3 min.

All 384 products from the first cPCR were tested by species-specific cPCR [53] targeting *E. canis* 16S. The reaction mixture was composed of 12.5 μL Gotaq® Green Master Mix (Promega®, Madisson, USA), 300 nM of each primer (ECAN5 F and HE3 R) and 5 μL of template DNA brought to a total volume of 25 μL with nuclease free water (Thermo Scientific©, USA). The thermal cycling protocol was as follows: 95 °C for 3 min followed by 30 cycles of 94 °C for 1 min, 55 °C for 1 min and 72 °C for 40 s. Amplification was completed with a final extension of 72 °C for 3 min.

2.3.3. Conventional (c) PCR for A. platys screening

All 384 samples were submitted to a previously described cPCR protocol [10] to amplify a 520bp region of the *A. platys* p44 gene. The reaction mixture was composed of 12.5 μL Gotaq® Green Master Mix (Promega®, Madisson, USA), 600 nM of each primer (Apl_p44F3 and M Apl_p44R3) and 5 μL of template DNA brought to a total volume of 25 μL with nuclease free water (Thermo Scientific©, USA). The thermal cycling protocol was as follows: 95 °C for 3 min followed by 55 cycles of 94 °C for 15 s, 70 °C for 15 s and 72 °C for 30 s. Amplification was completed with a final extension of 72 °C for 1 min.

2.3.4. Conventional (c) PCR for E. canis and A. platys molecular characterization

Randomly selected positive *E. canis* and *A. platys* samples were further tested by targeting a longer 16S fragment (866 bp) [54] for sequencing and molecular characterization. The 16S cPCR reaction mixture was composed of 12.5 μL Gotaq® Green Master Mix (Promega®, Madisson, USA), 300 nM of each primer (16SCNMF and 817R) and 5 μL of template DNA brought to a total volume of 25 μL with nuclease free water (Thermo Scientific©, USA). The thermal cycling protocol was as follows: 95 °C for 2 min followed by 35 cycles of 95 °C for 1 min, 54 °C for 1 min and 72 °C for 1 min. Amplification was completed with a final extension of 72 °C for 5 min.

Positive *A. platys* p44 cPCR samples were also selected for sequencing and molecular characterization of the *A. platys* p44 (520pb) gene, as previously described [10].

2.3.5. Electrophoresis

Conventional PCR products were separated by 2% agarose gel electrophoresis (LE Agarose Seakem®, Lonza) stained with SYBR© Safe DNA gel stain (Thermo Scientific©, USA). DNA extraction/purification, cPCR amplification and electrophoresis were performed in 3 separate rooms to avoid cross contamination.

2.4. Sequencing

Ehrlichia canis products from the larger 16S protocol and *Anaplasma platys* p44 and 16S products were purified with the commercial Silica Bead DNA Gel Extraction Kit (Thermo Scientific®, USA) following the manufacturer's instructions and sent to Macrogen® (Korea) for sequencing using the Sanger method [56] (BigDye Terminator cycle sequencing; Applied Bio-systems© ABI 3700DNA Analyzer, Carlsbag, CA) in both directions and evaluated by the ABI’s sequence analysis software (ABI, Carlsbad, CA).

2.5. Phylogenetic analysis

The obtained sequences were analyzed initially by BLAST using the NCBI’s Mega-BLAST algorithm [57]. In order to correctly determine the nucleotide composition, the electropherograms were submitted to PhredPhrap analysis [58], with the Phred quality score (peaks around each base call) established as higher than 20 (99% in accuracy of the base call). Final sequences were submitted to GenBank [59] and aligned with published sequences using local alignment search tool (BLASTn). The phylogenetic trees were built with the MEGA version 7 [60] software using the Maximum-likelihood trees model GTR + G + I. The best model of evolution was selected by the MEGA version 7 program, under the Akaike Information Criterion (AIC).

2.6. Data analysis

In order to determine overall *E. canis* and *A. platys* prevalence in dogs from Asunción, Paraguay, the PCR-positive dogs were divided by the total number of tested animals, multiplied by 100, and expressed as a percentage (%).

The relationships between risk factors and cPCR positivity for *E. canis* and *A. platys* were examined using fixed-effect univariable logistic regression. Factors with a likelihood-ratio test p-value < 0.25 were considered for entry into a multivariable logistic regression analysis [61].

The potential risk factors evaluated for *E. canis* and for *A. platys* positivity were: anti-tick treatment (yes *vs.* no), breed (mixed-breed *vs.* breed), sex (male *vs.* female), age ($\leq$ 1 years *vs.* $>$ 1 years), veterinary care (yes *vs.* no) health status (healthy *vs.* sick), number of dogs ($\leq$ 1 *vs.* $>$ 1), tick infestation (yes *vs.* no). Initially, all selected variables were forced into the multivariable logistic regression model. Manual backwards elimination was used for model building, excluding variables with a p-value > 0.05 in the likelihood ratio test. The fit of the fixed-effect in the final model was assessed using Hosmer–Lemeshow goodness-of-fit test [62] and the area under the curve of the receiver-operating characteristic (ROC). Odds ratios (OR) were calculated with a 95% confidence interval (CI). A p value $\leq$ 0.05 was considered statistically significant. Data were analyzed using statistical STATA 13 version.

3. Results

3.1. Ehrlichia canis and Anaplasma platys prevalence in domestic dogs

All 384 DNA samples (Mean [X] and Standard Deviation (SD) of DNA concentration = 25.66 ± 6.55 ng/uL, mean, and SD 260/280 ratio = 1.80 ± 0.15) were positive for the *RPS19* endogenous gene and there was no amplification of negative controls. According to the cPCR protocol, 40 out of 384 samples (10.41%, 95% [CI: 7.4–13.4%]) were positive for *E. canis* and 41 out of 384 samples (10.67%, 95% [CI: 7.5–13.7%]) were positive for *A. platys* (Table 1).

3.2. Phylogenetic analysis

Of 40 *E. canis* 16S-cPCR positive samples, 7 were sequenced for the 16S larger fragment. BLAST and phylogenetic analyses supported the identification of products based on the 16S gene (~800bp) (GenBank accession numbers: MG967461-MG967463, MG967465-MG967468), showing 99%–100 identity to *E. canis* in a dog from Turkey (GenBank accession number KY594915). Those fragments were closely positioned to each other and to *E. canis* isolates from dogs in Venezuela (AF373612), China (AF162860), Spain (AY394465), Peru (DQ915970), Israel (U26740), Japan (AF536827), Italy (EU439944), Brazil (EF195134), and USA (NR_118741), supported by high bootstrap values (100) in Maximum Likelihood phylogenetic analysis (Fig. 1).

Of 41 *A. platys* 16S-cPCR positive samples, 5 were sequenced for the 16S larger fragment. BLAST and phylogenetic analyses supported the identification of products based on the 16S gene (~866bp) (GenBank accession numbers: MH129057, MH129058, MH129060- MH129062), showing 98%–100 identity to *A. platys* in dogs from Cuba (KX792089), Costa Rica (KY389143), and ticks from India (MG050139). In the phylogenetic tree based on the 16S gene, 4 *A. platys* sequences from Paraguay clustered together and with other *A. platys* dog isolates from Uruguay (KX792011) and Japan (AY077619), and one sequence was in another clade, closely related to *A. platys* from Mexico (KT357371) (Fig. 1) (Table 3).

Additionally, the analysis of 9 *A. platys* sequenced products based on the p44 region (GenBank accession numbers MG679902-MG679909, MG679911) showed 99–100% identity to *A. platys* from dogs in Venezuela (GenBank accession number GU357492). Based on the

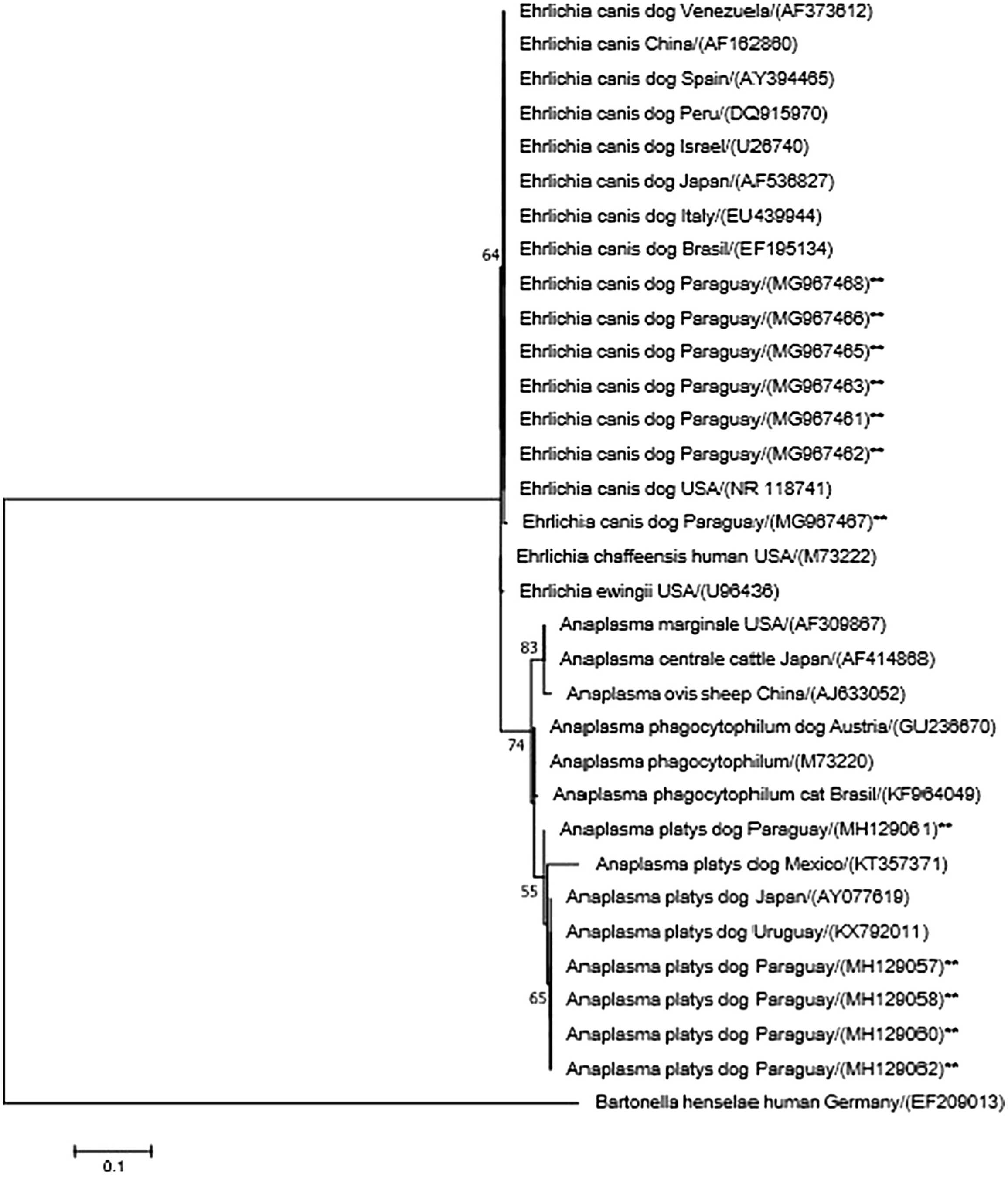

Fig. 1. Phylogenetic relationships within the Anaplasmataceae family based on 800pb fragment of the *16S RNA* gene after alignment. The tree was inferred by using the ML method and evolutive model GTR + G + I. The numbers at the nodes correspond to bootstrap values higher than 50% obtained with 1000 replicates. *Bartonella henselae* was used as and out-group. ** Sequences from the present study.

phylogenetic tree of the p44 gene, all *A. platys* sequences from Paraguay clustered together and were closely related to *A. platys* sequence detected in a domestic dog from Taiwan (KJ155503) (Fig. 2).

3.3. Risk factor analysis

From the eight potential risk factors analyzed in the univariate fixed logistic model for *E. canis*, two passed the multivariate test: age and veterinary care. Concerning *A. platys*, anti-tick treatment and number of dogs in the same household were factors that passed the multivariate test. The number of animals per category, their respective Odds Ratio (OR) and p value are shown for *E. canis* (Table 4) and *A. platys* (Table 5).

When including the two *E. canis* variables in a multivariate logistic analysis, both were considered risk factors in dogs (Table 6). The same pattern was observed when including the two *A. platys* variables in a multivariate logistic regression (Table 7).

4. Discussion

Asunción has a tropical savanna climate characterized by hot humid summers and warm winters [63]. Relative humidity is high in the summer; therefore the heat index is higher than the true air temperature. The country's climate is suitable for *R. sanguineus* s.l. proliferation, being an environment conducive to the appearance of Ehrlichiosis and Anaplasmosis in dogs [64–66]. In South America, while *E. canis* has

Table 3
Sequenced *Ehrlichia canis* and *Anaplasma platys* products from domestic dogs in Asunción, Paraguay, with their closely BLAST identity.

PCR positive samples	16S cPCR	*P44 gene* cPCR	Closest BLAST identity (%)			Query Coverage	E- value
			Organism	Identity	Accession Number		
4C	Positive		*Ehrlichia canis*	(99%)	KY594915	100%	0.0
36SR	Positive		*Ehrlichia canis*	(99%)	KY594915	100%	0.0
47SR	Positive		*Ehrlichia canis*	(99%)	KY594915	98%	0.0
72SR	Positive		*Ehrlichia canis*	(99%)	KY594915	100%	0.0
73SR	Positive		*Ehrlichia canis*	(99%)	KY594915	99%	0.0
75SR	Positive		*Ehrlichia canis*	(99%)	KY594915	100%	0.0
89ST	Positive		*Ehrlichia canis*	(100%)	KY594915	100%	0.0
10E		Positive	*Anaplasma platys*	(99%)	GU357492	100%	0.0
2SR		Positive	*Anaplasma platys*	(99%)	GU357492	99%	0.0
5SR		Positive	*Anaplasma platys*	(99%)	GU357492	100%	0.0
10SR		Positive	*Anaplasma platys*	(99%)	GU357492	99%	0.0
11L		Positive	*Anaplasma platys*	(99%)	GU357492	100%	0.0
13C		Positive	*Anaplasma platys*	(99%)	GU357492	100%	0.0
14		Positive	*Anaplasma platys*	(99%)	GU357492	99%	0.0
14R		Positive	*Anaplasma platys*	(99%)	GU357492	100%	0.0
10E	Positive		*Anaplasma platys*	(98%)	KX792089	98%	0.0
10SR	Positive		*Anaplasma platys*	(98%)	KX792089	98%	0.0
16L	Positive		*Anaplasma platys*	(99%)	MG050139	100%	0.0
17SR	Positive		*Anaplasma platys*	(100%)	KX389143	98%	0.0
52SR	Positive		*Anaplasma platys*	(99%)	KX792089	98%	0.0

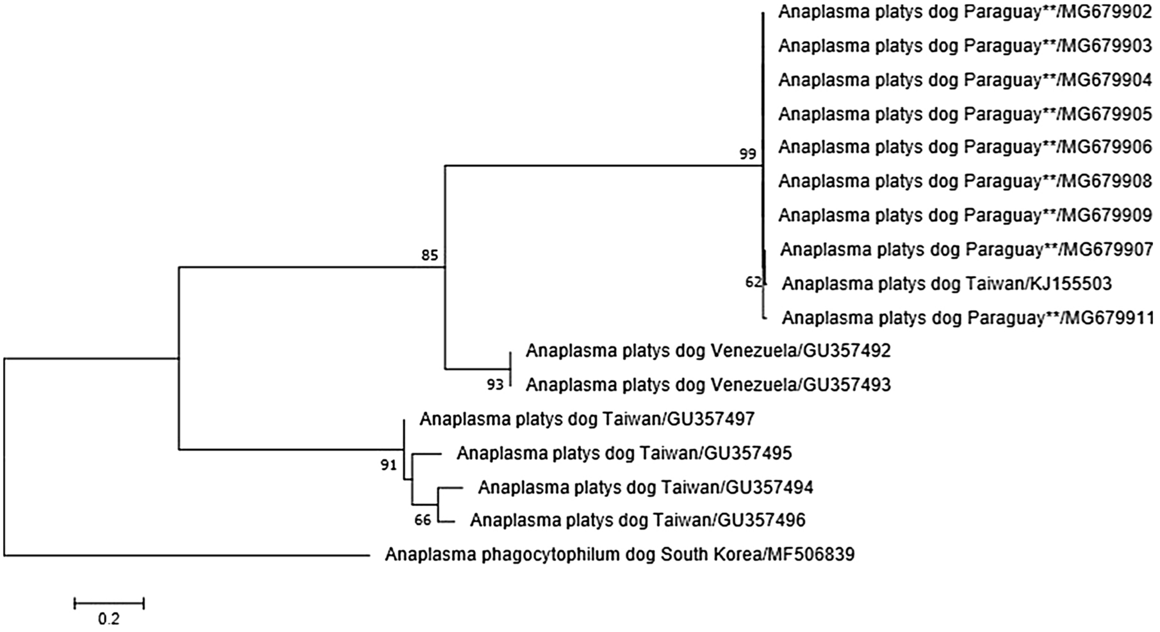

Fig. 2. Phylogenetic relationships within *Anaplasma platys* based on 520pb fragment of the *p44* gene after alignment. The tree was inferred by using the ML method and evolutive model GTR + G + I. The numbers at the nodes correspond to bootstrap values higher than 50% obtained with 1000 replicates. *Anaplasma phagocytophilum* was used as an out-group. ** Sequences from the present study.

been described in Peru [7], Colombia [40], Brazil [18], Chile [67] and Argentina [23], *A. platys* was detected in Chile [68], Argentina [21,23], Uruguay [20], Brazil [18,38,69], Colombia [25], Mexico [36] and Peru [43]. To the best of our knowledge, this is the first molecular detection of *E. canis* and *A. platys* in domestic dogs from Paraguay. Both *E. canis* and *A. platys* were found in all sampled locations, therefore, it could be inferred that they are widely distributed in Asunción city.

The overall prevalence of *E. canis* (10.41%) and *A. platys* (10.67%) in Paraguay was similar to the prevalence described for *A. platys* in Brazil (9.1%–14.09%) [18,70], slightly higher when compared to *E. canis* (6.7%) and *A platys* (7.2%) frequency in Buenos Aires, Argentina [23], and lower than that described for *E. canis* (23.3%–38.9%) in other regions of Brazil [38]. A high *E. canis* prevalence was reported in dogs in Colombia (40.6%) [40], Costa Rica (32%) [71] and Peru (51.3%) [48]. In contrast, low occurrences for *E. canis* (3%) and *A. platys* (4%) were observed in Mexico [36].

A low prevalence of *E. canis* (1.7–6%), and *A. platys* (3.7%), were described in Europe [32,72,73]. In Asia and Africa, studies performed in Iran [74], Malaysia [75], Turkey [76], Algeria [34], Nigeria [77] and South Africa [78], reported frequencies that ranged between 2%–22.5% for *E. canis* and 4%–7.7% for *A. platys*. As for co-infections, a low percentage was observed in Paraguay (1.3%), compared to those detected in Brazil (5.4%) and Panamá (7.5%) [79,80]. The variations in worldwide prevalence can be associated with the infection status, differences in climate and ecology, distribution of vectors, socioeconomic factors, and the tests used to evaluate infection (nested PCR, Real time PCR) [22,81–83].

Phylogenetic analysis of *E. canis* and *A. platys* obtained in this work showed high similarity to worldwide isolates. Phylogenetic analysis based on the secondary structure of 16S rRNA suggests that the gene

Table 4
Univariable logistic regression model of risk factors associated with *E. canis* positivity in domestic dogs (n = 384) from the Asunción city, Paraguay.

Variable	Total	*E. canis* PCR		OR		*p* value
		Positive	Negative		95% CI	
Number of dogs	384	22	24	1.00	0.69-2.60	0.377
≤ 1	236	18	130	1.34		
> 1	148					
Sex	384	19	172	1.00	0.57-2.12	0.765
Male	191	21	172	1.10		
Female	193					
Age	384	9	32	1.00	1.23-6.46	**0.014***
≤ 1	41	31	312	2.83		
> 1	343					
Breed	384	32	236	1.00	0.81-4.10	0.142
Mixed breed	268	8	108	1.83		
Pure Breed	116					
Veterinary care	384	30	307	1.00	1.25-6.11	**0.012***
Yes	337	10	37	2.76		
No	47					
Tick infestation	384	38	318	1.00	0.35-6.80	0.559
Yes	356	2	26	1.55		
No	28					
Anti-tick treatment	384	28	253	1.00	0.54-2.39	0.724
Yes	281	11	87	1.14		
No	98					
Health status	384	37	323	1.00	0.35-4.38	0.731
Healthy	360	3	21	1.24		
Sick	24					

* Statistically significant.

Table 5
Univariable logistic regression model of risk factors associated with *Anaplasma platys* positivity in domestic dogs (n = 384) from the Asunción city, Paraguay.

Variable	Total	*A. platys* PCR		OR	95%CI	*p value*
		Positive	Negative			
Number of dogs	384	18	218	1.00	1.15-4.28	**0.016***
≤ 1	236	23	125	2.22		
> 1	148					
Sex	384	24	167	1.00	0.34-1.29	0.235
Male	191	17	176	0.67		
Female	193					
Age	384	4	37	1.00	0.30-2.65	0.840
≤ 1	41	37	306	0.89		
> 1	343					
Breed	384	27	241	1.00	0.41-1.62	0.562
Mixed Breed	268	14	102	0.81		
Pure Breed	116					
Veterinary care	384	33	304	1.00	0.81-4.38	0.138
Yes	337	8	39	1.88		
No	47					
Tick infestation	384	40	316	1.00	0.45-25.83	0.234
Yes	356	1	27	3.41		
No	28					
Anti-tick treatment	384	24	257	1.00	1.05-4.12	**0.034***
Yes	281	16	82	2.08		
No	98					
Health status	384	40	320	1.00	0.45-2.64	0.308
Healthy	360	1	23	0.34		
Sick	24					

*Statistically significant.

Table 6
Multivariable logistic regression model for risk factors associated with *Ehrlichia canis* positivity in domestic dogs (n = 384) from the Asunción city, Paraguay.

Risk Factor	OR	95% CI	p value
Age	1.00	1.21-6.47	0.016*
≤ 1	2.80		
> 1			
Veterinary care	1.00	1.22-6.11	0.014*
Yes	2.73		
No			

* Statistically significant. AUC: 0.73 Homer Lemeshow x2: 0.13 p = 0.7164.

Table 7
Multivariable logistic regression model for risk factors associated with *Anaplasma platys* positivity in domestic dogs (n = 384) from the Asunción city, Paraguay.

Risk Factor	OR	95% CI	p value
Number of dogs	1.00	1.04-3.96	0.036*
≤ 1	2.03		
> 1			
Anti-tick treatment	1.00	1.02-4.02	0.043*
Yes	2.02		
No			

* Statistically significant. AUC: 0.70 Homer Lemeshow x2 2.41 p = 0.7209.

appeared to be highly conserved in most strains [84]. Studies with less conserved genes such as p30 for *E. canis* [85] and GroEL for *A. platys* [86] are necessary to confirm this high identity between sequences.

Positivity for *E. canis* was more frequently observed in older dogs as opposed to juvenile ones, which was also described in another molecular and serological study in domestic dogs from Brazil [42]. This result is in accordance with several reports [18,49,71,87,88] and may be due to the increased exposure to infection throughout life [48,89,90]. The lower immune system competence of older dogs, which often develop concurrent diseases such as cancer, could also be related to higher odds of infection [91].

The absence of veterinary care was a risk factor for *E. canis* positivity, reflecting that a lack of dogs' heath care may relate to a higher risk of infection. A higher tick infestation is reported in dogs without appropriate protection, and parasitic loads can reach hundreds of ticks per animal, with ticks in all developmental stages [92]. Furthermore, lack of veterinary care leaves the dogs exposed to the acquisition of other diseases; viral, bacterial, fungal or parasitic. A good measure of sanitary protection is a periodic control with a veterinarian, at least once a year [93].

A higher number of dogs from Paraguay in the same household was significantly associated with *A. platys* positivity. A high density was previously described as a risk factor related to *E. canis* seropositivity [90]. It is well known that the population density of dogs is a predisposing factor for a high prevalence and intensity of canine infestation with *R. sanguineus* s.l. [94], which may led to a higher transmission of tick borne pathogens.

Dogs that were not treated with ectoparasiticides had higher odds of being positive for *A. platys*. One study showed that the presence of ticks is a very important factor for the transmission of *A. platys*, demonstrating that the greater the number of ticks, the more effective transmission will be [95]. The lack of treatment with ectoparasiticides or tick repellents results in higher R. *sanguineus* s.l. infestation in dogs [94,96]. It should be mentioned that 3 h of tick attachment to dogs is sufficient to produce efficient transmission of *E. canis* to the vertebrate host [97].

Although anti-tick treatment was a protective factor against *A. platys* infection, the presence of ticks was not considered a risk factor for *A. platys* or *E. canis* positivity. This indicates that not every blood meal that a *R. sanguineus* s.l. consumes on an infected dog results in tick infection (rate of infection in questing ticks). Moreover, a low infection rate of ticks from Paraguay with *E. canis* and *A. platys* might explain this finding [98]. Future studies should be performed to determine the rate of infection on questing ticks from Paraguay.

This result is different to many other studies that correlate tick infestation with molecular and serological positivity for *A. platys* and *E. canis* [95,99]. High exposure to ticks (93%) was observed in sampled dogs from Paraguay and this could be related to the absence of statistically significant results for tick infestation [45].

Sex, breed and health status were not considered risk factors for *E. canis* or *A. platys*, which correlate with the results of other authors [45,83,100–102]. This suggests that there are more environmental than immunological factors, as well as ecological and social factors [71,90] that could influence the risk of infection, and should be considered for future studies.

5. Conclusions

The overall prevalence of *E. canis* and *A. platys* in domestic dogs from Asuncion, Paraguay correlate with that described worldwide. Older dogs without veterinary care had higher odds for *E. canis* positivity and a higher number of dogs in the same household, as well as the absence of anti-tick treatment were considered risk factors for *A. platys*. *Ehrlichia canis* and *A. platys* circulate in the dog population of Asunción and are molecularly described for the first time in Paraguay.

Funding

We acknowledge the financial support of the Postgraduate program, Magister en Ciencias Mención Salud Animal, Universidad Austral de Chile, which enabled this work.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgements

The authors would like to thank the team of Veterinary Doctors from Asunción, Paraguay who helped in the collection of samples and logistical support; the Faculty of Veterinary Sciences of the National University of Asuncion and the team of the Veterinary Clinical Pathology Laboratory at the Austral University of Chile.

References

[1] J.S. Dumler, A.F. Barbet, C. Bekker, G.A. Dasch, G.H. Palmer, S.C. Ray, Y. Rikihisa, F.R. Rurangirwa, Reorganization of genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of Ehrlichia with Anaplasma, Cowdria with Ehrlichia and Ehrlichia with Neorickettsia, descriptions of six new species combi, Int. J. Syst. Evol. Microbiol. 51 (2001) 2145–2165, https://doi.org/10.1099/00207713-51-6-2145.

[2] N. Ismail, K.C. Bloch, J.W. McBride, Human Ehrlichiosis and Anaplasmosis, Clin. Lab. Med. 30 (2010) 261–292, https://doi.org/10.1016/j.cll.2009.10.004.

[3] B. Brinkhof, B. Spee, J. Rothuizen, L.C. Penning, Development and evaluation of canine reference genes for accurate quantification of gene expression, Anal. Biochem. 356 (2006) 36–43, https://doi.org/10.1016/j.ab.2006.06.001.

[4] Z. Woldehiwet, The natural history of Anaplasma phagocytophilum, Vet. Parasitol. 167 (2010) 108–122, https://doi.org/10.1016/j.vetpar.2009.09.013.

[5] S. Gaunt, M. Beall, B. Stillman, L. Lorentzen, P. Diniz, R. Chandrashekar, E. Breitschwerdt, Experimental infection and co-infection of dogs with Anaplasma platys and Ehrlichia canis: hematologic, serologic and molecular findings, Parasit. Vectors 3 (33) (2010), https://doi.org/10.1186/1756-3305-3-33.

[6] D. Sonenshine, M. Roe, Biology of Ticks, 2nd editio, (2014).

[7] J. Vinasco, O. Li, A. Alvarado, D. Diaz, L. Hoyos, L. Tabachi, K. Sirigireddy, C. Ferguson, M.H. Moro, Molecular evidence of a new strain of Ehrlichia canis from South America, J. Clin. Microbiol. 45 (2007) 2716–2719, https://doi.org/10.1128/JCM.01102-07.

[8] D. Mihaljica, Z. Radulovic, S. Tomanovic, S. Cakic, A. Penezic, M. Milutinovic, Molecular detection of Babesia spp. in ticks in northern Serbia, Arch. Biol. Sci. 64 (2012) 1591–1598, https://doi.org/10.2298/ABS1204591M.

[9] R.G. Maggi, P.E. Mascarelli, L.N. Havenga, V. Naidoo, E.B. Breitschwerdt, Co-infection with Anaplasma platys, Bartonella henselae and Candidatus Mycoplasma haematoparvum in a veterinarian, Parasit. Vectors 6 (103) (2013), https://doi.org/10.1186/1756-3305-6-103.

[10] E.B. Breitschwerdt, B.C. Hegarty, B. a Qurollo, T.B. Saito, R.G. Maggi, L.S. Blanton, D.H. Bouyer, Intravascular persistence of Anaplasma platys, Ehrlichia chaffeensis, and Ehrlichia ewingii DNA in the blood of a dog and two family members, Parasit. Vectors 7 (298) (2014), https://doi.org/10.1186/1756-3305-7-298.

[11] L. Cardoso, M. Gilad, H. Cortes, Y. Nachum-Biala, A. Lopes, M. Vila-Viçosa, M. Simões, P.A. Rodrigues, G. Baneth, First report of Anaplasma platys infection in red foxes (Vulpes vulpes) and molecular detection of Ehrlichia canis and Leishmania infantum in foxes from Portugal, Parasit. Vectors 8 (2015) 144, https://doi.org/10.1186/s13071-015-0756-y.

[12] T. Waner, S. Harrus, Canine Monocytic Ehrlichiosis (CME): Recent Advances in Canine Infectious Diseases, International Veterinary Information Service, 2000.

[13] E. Aguirre, A. Sainz, S. Dunner, I. Amusategui, L. López, F. Rodríguez-Franco, I. Luaces, O. Cortés, M.A. Tesouro, First isolation and molecular characterization of Ehrlichia canis in Spain, Vet. Parasitol. 125 (2004) 365–372, https://doi.org/10.1016/j.vetpar.2004.08.007.

[14] M.B. De Castro, R.Z. Machado, L.P.C.T. De Aquino, A.C. Alessi, M.T. Costa, Experimental acute canine monocytic ehrlichiosis: clinicopathological and immunopathological findings, Vet. Parasitol. 119 (2004) 73–86, https://doi.org/10.1016/j.vetpar.2003.10.012.

[15] F. Dantas-Torres, Canine vector-borne diseases in Brazil, Parasit. Vectors 1 (2008) 25, https://doi.org/10.1186/1756-3305-1-25.

[16] J. Moraes-Filho, F.S. Krawczak, F.B. Costa, J.F. Soares, M.B. Labruna, Comparative evaluation of the vector competence of four south american populations of the rhipicephalus sanguineus group for the bacterium Ehrlichia canis, the agent of canine monocytic ehrlichiosis, PLoS One 10 (2015) e0139386, , https://doi.org/10.1371/journal.pone.0139386.

[17] J.P. Baufils, H. Inokuma, J. Martin-Granel, P. Jumelle, M. Barbault-Jumelle, P. Brouqui, Anaplasma platys (Ehrlichia platys) infection in a dog in France: description of the case, and characterization of the agent, Rev. Med. Vet. (Toulouse) 153 (2002) 85–90.

[18] C.S. Lasta, A.P. dos Santos, J.B. Messick, S.T. Oliveira, A.W. Biondo, R.Fda C. Vieira, M.L. Dalmolin, F.H.D. González, Molecular detection of Ehrlichia canis and Anaplasma platys in dogs in Southern Brazil, Rev. Bras. Parasitol. Veterinária. 22 (2013) 360–366, https://doi.org/10.1590/S1984-29612013000300007.

[19] A.S. Dagnone, A.I. de Souza, M.R. André, R.Z. Machado, Molecular diagnosis of Anaplasmataceae organisms in dogs with clinical and microscopical signs of ehrlichiosis, Rev. Bras. Parasitol. Veterinária. 18 (2009) 20–25, https://doi.org/10.4322/rbpv.01804004.

[20] L. Carvalho, M.T. Armua-Fernandez, N. Sosa, M.L. Félix, J.M. Venzal, Anaplasma platys in dogs from Uruguay, ticks tick, Borne. Dis. 8 (2017) 241–245, https://doi.org/10.1016/j.ttbdis.2016.11.005.

[21] D.F. Eiras, M.B. Craviotto, D. Vezzani, O. Eyal, G. Baneth, First description of natural Ehrlichia canis and Anaplasma platys infections in dogs from Argentina, Comp. Immunol. Microbiol. Infect. Dis. 36 (2013) 169–173, https://doi.org/10.1016/j.cimid.2012.11.008.

[22] G.L. Cicuttin, E.L. Tarragona, M.N. De Salvo, A.J. Mangold, S. Nava, Infection with Ehrlichia canis and Anaplasma platys (Rickettsiales: Anaplasmataceae) in two lineages of Rhipicephalus sanguineus sensu lato (Acari: Ixodidae) from Argentina, Ticks Tick, Borne. Dis. 6 (2015) 724–729, https://doi.org/10.1016/j.ttbdis.2015.06.006.

[23] G.L. Cicuttin, M.N. De Salvo, F.E. Gury Dohmen, Molecular characterization of Ehrlichia canis infecting dogs, Buenos Aires, Ticks Tick. Dis. 7 (2016) 954–957, https://doi.org/10.1016/j.ttbdis.2016.04.017.

[24] K. Abarca V, J.L.ópez Del P, P. González A, J. Dabanch P, M. Torres H, V. Solari G, C. Perret P, Evidencia seroepidemiológica de exposición humana a Anaplasma sp en Santiago, Chile, Rev. Chil. Infectología. 25 (2008) 358–361, https://doi.org/10.4067/S0716-10182008000500008.

[25] G. Vargas-Hernandez, M.R. André, D.M. Cendales, K.C.M. de Sousa, L.R. Gonçalves, M.C.H. Rondelli, R.Z. Machado, M. Tinucci-Costa, Molecular detection of Anaplasma species in dogs in Colombia, Rev. Bras. Parasitol. Veterinária. 25 (2016) 459–464, https://doi.org/10.1590/s1984-29612016066.

[26] C. Arraga-Alvarado, M. Palmar, O. Parra, P. Salas, Ehrlichia platys (Anaplasma platys) in dogs from Maracaibo, Venezuela: an ultrastructural study of experimental and natural infections, Vet. Pathol. 40 (2003) 149–156, https://doi.org/10.1354/vp.40-2-149.

[27] a Sainz, I. Amusategui, M. a Tesouro, Ehrlichia platys infection and disease in dogs in Spain, J. Vet. Diagn. Invest. 11 (1999) 382–384, https://doi.org/10.1177/104063879901100419.

[28] Y. Motoi, H. Satoh, H. Inokuma, T. Kiyuuna, Y. Muramatsu, H. Ueno, C. Morita, First detection of Ehrlichia platys in dogs and ticks in Okinawa, Japan, Microbiol. Immunol. 45 (2001) 89–91.

[29] A.R. Martin, G.K. Brown, R. Hugh Dunstan, T.K. Roberts, Anaplasma platys: an improved PCR for its detection in dogs, Exp. Parasitol. 109 (2005) 176–180, https://doi.org/10.1016/j.exppara.2004.11.007.

[30] J. De La Fuente, A. Torina, V. Naranjo, S. Nicosia, A. Alongi, F. La Mantia, K.M. Kocan, Molecular characterization of Anaplasma platys strains from dogs in Sicily, Italy, BMC Vet. Res. 2 (2006) 1–5, https://doi.org/10.1186/1746-6148-2-24.

[31] D. Pinyoowong, S. Jittapalapong, F. Suksawat, R.W. Stich, A. Thamchaipenet, Molecular characterization of Thai Ehrlichia canis and Anaplasma platys strains detected in dogs, Infect. Genet. Evol. 8 (2008) 433–438, https://doi.org/10.1016/

j.meegid.2007.06.002.

[32] M. Trotta, A. Fogliazza, T. Furlanello, L. Solano-Gallego, A molecular and serological study of exposure to tick-borne pathogens in sick dogs from Italy, Clin. Microbiol. Infect. 15 (2009) 62–63, https://doi.org/10.1111/j.1469-0691.2008.02279.x.

[33] S. Harrus, T. Waner, Diagnosis of canine monocytotropic ehrlichiosis (Ehrlichia canis): an overview, Vet. J. 187 (2011) 292–296, https://doi.org/10.1016/j.tvjl.2010.02.001.

[34] M. Dahmani, A. Loudahi, O. Mediannikov, F. Fenollar, D. Raoult, B. Davoust, Molecular detection of Anaplasma platys and Ehrlichia canis in dogs from Kabylie, Algeria, Ticks Tick, Borne. Dis. 6 (2015) 198–203, https://doi.org/10.1016/j.ttbdis.2014.12.007.

[35] R. Soares, C.A. Ramos, T. Pedroso, V. Babo-Terra, H. Cleveland, F. De Araújo, Molecular survey of Anaplasma platys and Ehrlichia canis in dogs from Campo Grande, Mato Grosso do sul, Brazil, An. Acad. Bras. Cienc. 89 (2017) 301–306, https://doi.org/10.1590/0001-3765201720150556.

[36] C. Almazán, V.H. González-Álvarez, I.G. Fernández de Mera, A. Cabezas-Cruz, R. Rodríguez-Martínez, J. de la Fuente, Molecular identification and characterization of Anaplasma platys and Ehrlichia canis in dogs in Mexico, ticks Tick, Borne. Dis. 7 (2016) 276–283, https://doi.org/10.1016/j.ttbdis.2015.11.002.

[37] A.S. Dagnone, H.S.A. de Morais, M.C. Vidotto, F.S. Jojima, O. Vidotto, Ehrlichiosis in anemic, thrombocytopenic, or tick-infested dogs from a hospital population in South Brazil, Vet. Parasitol. 117 (2003) 285–290, https://doi.org/10.1016/j.vetpar.2003.10.001.

[38] F. Santos, J.S. Coppede, A.L.A. Pereira, L.P. Oliveira, P.G. Roberto, R.B.R. Benedetti, L.B. Zucoloto, F. Lucas, L. Sobreira, M. Marins, Molecular evaluation of the incidence of Ehrlichia canis, Anaplasma platys and Babesia spp. In dogs from Ribeirão Preto, Brazil, Vet. J. 179 (2009) 145–148, https://doi.org/10.1016/j.tvjl.2007.08.017.

[39] L.E. Romero, A.I. Meneses, L. Salazar, M. Jiménez, J.J. Romero, D.M. Aguiar, M.B. Labruna, G. Dolz, First isolation and molecular characterization of Ehrlichia canis in Costa rica, Central America, Res. Vet. Sci. 91 (2011) 95–97, https://doi.org/10.1016/j.rvsc.2010.07.021.

[40] G. Vargas-Hernández, M.R. André, J.L.M. Faria, T.D. Munhoz, M. Hernandez-Rodriguez, R.Z. Machado, M. Tinucci-Costa, Molecular and serological detection of Ehrlichia canis and Babesia vogeli in dogs in Colombia, Vet. Parasitol. 186 (2012) 254–260, https://doi.org/10.1016/j.vetpar.2011.11.011.

[41] G. Dolz, L. Ábrego, L. Romero, L. Campos, L. Bouza, A. Jiménez-Rocha, Ehrlichiosis y anaplasmosis en Costa Rica, Acta Med. Costarric. 55 (2013) 34–40.

[42] P.E.B. Guedes, T.N. de, A. Oliveira, F.S. Carvalho, R.S.A. Carlos, G.R. Albuquerque, A.D. Munhoz, A.A. Wenceslau, F.L. Silva, Canine ehrlichiosis: prevalence and epidemiology in northeast Brazil, Rev. Bras. Parasitol. Veterinária. 24 (2015) 115–121, https://doi.org/10.1590/S1984-29612015030.

[43] T. Viviana Tateishi, E. Olga Lí, S. Luis Hoyos, G. Hermelinda Rivera, S. Alberto Manchego, A. Luis Barrios, B. Juan More, Identificación Hematológica y Molecular de Anaplasma platys en Caninos Domésticos de Lima Metropolitana con Signos Clínicos Compatibles con Anaplasmosis, Rev. Investig. Vet. Peru 26 (2015) 111, https://doi.org/10.15381/rivep.v26i1.10920.

[44] D.C.P. Evangelista Pedro, Martinez Ana Pula, Pereira Marques Thiago, Curitiba, ParanáFrequência de hemoparasitoses em cães na Região Sul Fluminense – Rj., in: 42° Congresso Bras. de Medicina Veterinária e 1° congresso sul-brasileiro da anclivepa2018, Frequência de hemoparasitoses em cães na Região Sul Fluminense – Rj., in: 42° Congresso Bras. de Medicina Veterinária e 1° congresso sul-brasileiro da anclivepa (2018) n.d..

[45] L.M. Costa-Júnior, K. Rembeck, L.M.F. Passos, M.F.B. Ribeiro, Factors associated with epidemiology of Anaplasma platys in dogs in rural and urban areas of Minas Gerais State, Brazil, Prev. Vet. Med. 109 (2013) 321–326, https://doi.org/10.1016/j.prevetmed.2012.10.011.

[46] P.E. Parham, J. Waldock, G.K. Christophides, D. Hemming, F. Agusto, K.J. Evans, N. Fefferman, H. Gaff, A. Gumel, S. LaDeau, S. Lenhart, R.E. Mickens, E.N. Naumova, R.S. Ostfeld, P.D. Ready, M.B. Thomas, J. Velasco-Hernandez, E. Michael, Climate, environmental and socio-economic change: weighing up the balance in vector-borne disease transmission, Philos. Trans. R. Soc. B Biol. Sci. 370 (2015), https://doi.org/10.1098/rstb.2013.0551 20130551–20130551.

[47] C.S. McMahan, D. Wang, M.J. Beall, D.D. Bowman, S.E. Little, P.O. Pithua, J.L. Sharp, R.W. Stich, M.J. Yabsley, R.B. Lund, Factors associated with Anaplasma spp. Seroprevalence among dogs in the United States, Parasit. Vectors 9 (2016) 169, https://doi.org/10.1186/s13071-016-1431-7.

[48] E. Huerto-Medina, B. Dámaso-Mata, [Factors associated with Ehrlichia canis infection in dogs infested with ticks from Huanuco, Peru], Rev. Peru. Med. Exp. Salud Publica. 32 (2015) 756–760 http://www.ncbi.nlm.nih.gov/pubmed/26732926.

[49] S.M. Trapp, A.S. Dagnone, O. Vidotto, R.L. Freire, A.M. Amude, H.S.A. de Morais, Seroepidemiology of canine babesiosis and ehrlichiosis in a hospital population, Vet. Parasitol. 140 (2006) 223–230, https://doi.org/10.1016/j.vetpar.2006.03.030.

[50] C.J.A. Orjuela, A.G.F. García, J.G. Imbachi, Análisis epidemiológico de la presentación de Ehrlichia sp. en caninos de Florencia Caquetá, Colombia, Rev. Electron. Vet. 16 (2015) 1–10.

[51] C.A. 1982 Pallarés, R., Benítez Usher, La distribucion de Ixodina (Vander Hammen, 1968) en el Paraguay., Rev. Paraguaya Microbiol. (n.d.) 17, 49–52.

[52] M. Thrusfield, Veterinary Epidemiology, 4th editio, Wiley (Ed.), 2018 p. 888.

[53] G.L. Murphy, S. Ewing, L.C. Whitworth, J.C. Fox, A.A. Kocan, A molecular and serologic survey of Ehrlichia canis, E. chaffeensis, and E. ewingii in dogs and ticks from Oklahoma, Vet. Parasitol. 79 (1998) 325–339, https://doi.org/10.1016/S0304-4017(98)00179-4.

[54] A. Hodžić, R. Cézanne, G.G. Duscher, J. Harl, W. Glawischnig, H.-P. Fuehrer, Candidatus Neoehrlichia sp. In an Austrian fox is distinct from Candidatus Neoehrlichia mikurensis, but closer related to Candidatus Neoehrlichia lotoris, Parasit. Vectors 8 (2015) 539, https://doi.org/10.1186/s13071-015-1163-0.

[56] F. Sanger, S. Nicklen, A.R. Coulson, DNA sequencing with chain-terminating inhibitors, Proc. Natl. Acad. Sci. 74 (1977) 5463–5467, https://doi.org/10.1073/pnas.74.12.5463.

[57] S.F. Altschul, W. Gish, W. Miller, E.W. Myers, D.J. Lipman, Basic local alignment search tool, J. Mol. Biol. 215 (1990) 403–410, https://doi.org/10.1016/S0022-2836(05)80360-2.

[58] B. Ewing, L. Hillier, M.C. Wendl, P. Green, Base-calling of automated sequencer traces using phred. I. Accuracy assessment, Genome Res. 8 (1998) 175–185, https://doi.org/10.1101/gr.8.3.175.

[59] D.A. Benson, GenBank, Nucleic Acids Res. 33 (2004) D34–D38, https://doi.org/10.1093/nar/gki063.

[60] S. Kumar, G. Stecher, K. Tamura, MEGA7: molecular evolutionary genetics analysis version 7.0 for bigger datasets, Mol. Biol. Evol. 33 (2016) 1870–1874, https://doi.org/10.1093/molbev/msw054.

[61] I. Dohoo, W. Martin, H. Stryhn, Veterinary Epidemiology Research, 2nd editio, (2009).

[62] S.L. Gortmaker, D.W. Hosmer, S. Lemeshow, Applied logistic regression, Contemp. Sociol. 23 (159) (1994), https://doi.org/10.2307/2074954.

[63] M.C. Peel, B.L. Finlayson, T.A. McMahon, Updated world map of the Köppen-Geiger climate classification, Hydrol. Earth Syst. Sci. 11 (2007) 1633–1644, https://doi.org/10.5194/hess-11-1633-2007.

[64] Y. Suto, A. Suto, H. Inokuma, H. Obayashi, T. Hayashi, First confirmed canine case of Ehrlichia canis infection in Japan, Vet. Rec. 148 (2001) 809–811 http://www.ncbi.nlm.nih.gov/pubmed/11467609.

[65] A.L.T. Melo, T.F. Martins, M.C. Horta, J. Moraes-Filho, R.C. Pacheco, M.B. Labruna, D.M. Aguiar, Seroprevalence and risk factors to Ehrlichia spp. and Rickettsia spp. in dogs from the Pantanal Region of Mato Grosso State, Brazil, Ticks Tick, Borne. Dis. 2 (2011) 213–218, https://doi.org/10.1016/j.ttbdis.2011.09.007.

[66] S. Nava, M. Mastropaolo, J.M. Venzal, A.J. Mangold, A.A. Guglielmone, Mitochondrial DNA analysis of Rhipicephalus sanguineus sensu lato (Acari: Ixodidae) in the Southern Cone of South America, Vet. Parasitol. 190 (2012) 547–555, https://doi.org/10.1016/j.vetpar.2012.06.032.

[67] J. López, K. Abarca, M.I. Mundaca, C. Caballero, F. Valiente-Echeverría, Identificación molecular de Ehrlichia canis en un canino de la ciudad de Arica, Chile, Rev. Chil. Infectología. 29 (2012) 527–530, https://doi.org/10.4067/S0716-10182012000600008.

[68] K. Abarca, J. López, C. Perret, J. Guerrero, P. Godoy, A. Veloz, F. Valiente-Echeverría, U. León, C. Gutjahr, T. Azócar, Anaplasma platys in dogs, Chile, Emerg. Infect. Dis. 13 (2007) 1392–1395, https://doi.org/10.3201/eid1309.070021.

[69] R.F. Ferreira, A. De Mello, F. Cerqueira, A.G. De Sá, S. Abreu, C.L. Massard, Anaplasma platys diagnosis in dogs : comparison between morphological and molecular tests, Intern J Appl Res Vet Med. 5 (2007) 113–119.

[70] R. Witter, S.N. Vecchi, T.D.A. Pacheco, A.L.T. Melo, A. Borsa, A.L. Sinkoc, A.J. Mendonça, D.M. de Aguiar, Prevalência da erliquiose monocítica canina e anaplasmose trombocítica em cães suspeitos de hemoparasitose em Cuiabá, Mato Grosso, Semin. Ciências Agrárias. 34 (2013) 3811, https://doi.org/10.5433/1679-0359.2013v34n6Supl2p3811.

[71] A.V. Barrantes-González, A.E. Jiménez-Rocha, J.J. Romero-Zuñiga, G. Dolz, Serology, molecular detection and risk factors of Ehrlichia canis infection in dogs in Costa rica, Ticks Tick, Borne. Dis. 7 (2016) 1245–1251, https://doi.org/10.1016/j.ttbdis.2016.07.006.

[72] U. Ravnik, B.P. Bajuk, L. Lusa, N. Tozon, Serum protein profiles, circulating immune complexes and proteinuria in dogs naturally infected with Anaplasma phagocytophilum, Vet. Microbiol. 173 (2014) 160–165, https://doi.org/10.1016/j.vetmic.2014.07.007.

[73] V.V. Ebani, S. Nardoni, G. Fognani, L. Mugnaini, F. Bertelloni, G. Rocchigiani, R.A. Papini, F. Stefani, F. Mancianti, Molecular detection of vector-borne bacteria and protozoa in healthy hunting dogs from Central Italy, Asian Pac, J. Trop. Biomed. 5 (2015) 108–112, https://doi.org/10.1016/S2221-1691(15)30153-2.

[74] N. Maazi, A. Malmasi, P. Shayan, S.M. Nassiri, T.Z. Salehi, M.S. Fard, Molecular and serological detection of Ehrlichia canis in naturally exposed dogs in Iran: an analysis on associated risk factors, Rev. Bras. Parasitol. Vet. 23 (2014) 16–22.

[75] M. Nazari, S.Y. Lim, M. Watanabe, R.S.K. Sharma, N.A.B.Y. Cheng, M. Watanabe, Molecular detection of Ehrlichia canis in dogs in Malaysia, PLoS Negl. Trop. Dis. 7 (2013) e1982, https://doi.org/10.1371/journal.pntd.0001982.

[76] H. Çetinkaya, E. Matur, İ. Akyazi, E.E. Ekiz, L. Aydin, M. Toparlak, Serological and molecular investigation of Ehrlichia spp. And Anaplasma spp. In ticks and blood of dogs, in the Thrace Region of Turkey, Ticks Tick, Borne. Dis. 7 (2016) 706–714, https://doi.org/10.1016/j.ttbdis.2016.02.021.

[77] J. Kamani, C.-C. Lee, A.M. Haruna, P.-J. Chung, P.R. Weka, Y.-T. Chung, First detection and molecular characterization of Ehrlichia canis from dogs in Nigeria, Res. Vet. Sci. 94 (2013) 27–32, https://doi.org/10.1016/j.rvsc.2012.07.031.

[78] P.T. Matjila, A.L. Leisewitz, F. Jongejan, B.L. Penzhorn, Molecular detection of tick-borne protozoal and ehrlichial infections in domestic dogs in South Africa, Vet. Parasitol. 155 (2008) 152–157, https://doi.org/10.1016/j.vetpar.2008.04.012.

[79] G.C.F. da Silva, A.D.N. Benitez, A. Girotto, A. Taroda, M.C. Vidotto, J.L. Garcia, J.C. de Freitas, S.A. Headley, O. Vidotto, Occurrence of Ehrlichia canis and Anaplasma platys in household dogs from northern Parana, Rev. Bras. Parasitol. Veterinária. 21 (2012) 379–385, https://doi.org/10.1590/S1984-

29612012005000009.

[80] A. Santamaria, J.E. Calzada, A. Saldaña, M.J. Yabsley, N.L. Gottdenker, Molecular diagnosis and species identification of Ehrlichia and Anaplasma infections in dogs from Panama, Central America, Vector-Borne Zoonotic Dis. 14 (2014) 368–370, https://doi.org/10.1089/vbz.2013.1488.

[81] C.A.N. Ramos, R.A.N. Ramos, F.R. Araújo, D.S. Guedes Júnior, I.I.F. Souza, T.M. Ono, D.S. Pimentel, E.O. Rosas, M.A.G. Faustino, L.C. Alves, Comparação de nested-PCR com o diagnóstico direto na detecção de Ehrlichia canis e Anaplasma platys em cães, Rev. Bras. Parasitol. Veterinária. 18 (2009) 58–62, https://doi.org/10.4322/rbpv.018e1011.

[82] O. Peleg, G. Baneth, O. Eyal, J. Inbar, S. Harrus, Multiplex real-time qPCR for the detection of Ehrlichia canis and Babesia canis vogeli, Vet. Parasitol. 173 (2010) 292–299, https://doi.org/10.1016/j.vetpar.2010.06.039.

[83] R.I. Rodriguez-Vivas, R.E.F. Albornoz, G.M.E. Bolio, Ehrlichia canis in dogs in Yucatan, Mexico: seroprevalence, prevalence of infection and associated factors, Vet. Parasitol. 127 (2005) 75–79, https://doi.org/10.1016/j.vetpar.2004.08.022.

[84] V.I. Siarkou, M.E. Mylonakis, E. Bourtzi-Hatzopoulou, A.F. Koutinas, Sequence and phylogenetic analysis of the 16S rRNA gene of Ehrlichia canis strains in dogs with clinical monocytic ehrlichiosis, Vet. Microbiol. 125 (2007) 304–312, https://doi.org/10.1016/j.vetmic.2007.05.021.

[85] S. Felek, R. Greene, Y. Rikihisa, Transcriptional analysis of p30 major outer membrane protein genes of Ehrlichia canis in naturally infected ticks and sequence analysis of p30-10 of E. Canis from diverse geographic regions, J. Clin. Microbiol. 41 (2003) 886–888, https://doi.org/10.1128/JCM.41.2.886-888.2003.

[86] T. Bouzouraa, M. René-Martellet, J. Chêne, C. Attipa, I. Lebert, K. Chalvet-Monfray, J.-L. Cadoré, L. Halos, L. Chabanne, Clinical and laboratory features of canine Anaplasma platys infection in 32 naturally infected dogs in the Mediterranean basin, Ticks Tick. Dis. 7 (2016) 1256–1264, https://doi.org/10.1016/j.ttbdis.2016.07.004.

[87] R.F. da, C. Vieira, A.W. Biondo, A.M.S. Guimarães, A.P. dos Santos, R.P. dos Santos, L.H. Dutra, P.P.V. de, P. Diniz, H.A. de Morais, J.B. Messick, M.B. Labruna, O. Vidotto, Ehrlichiosis in Brazil, Rev. Bras. Parasitol. Veterinária. 20 (2011) 1–12, https://doi.org/10.1590/S1984-29612011000100002.

[88] A. Pinter, M.C. Horta, R.C. Pacheco, J. Moraes-Filho, M.B. Labruna, Serosurvey of Rickettsia spp. In dogs and humans from an endemic area for Brazilian spotted fever in the State of São Paulo, Brazil, Cad. Saude Publica 24 (2008) 247–252, https://doi.org/10.1590/S0102-311X2008000200003.

[89] S.S. de Azevedo, D.M. Aguiar, S.F. de Aquino, R.C. Orlandelli, A.Rda F. Fernandes, I.C.P. Uchôa, Soroprevalência e fatores de risco associados à soropositividade para Ehrlichia canis em cães do semiárido da Paraíba, Brazilian J. Vet. Res. Anim. Sci. 48 (2011) 14, https://doi.org/10.11606/S1413-95962011000100002.

[90] R.W. Stich, B.L. Blagburn, D.D. Bowman, C. Carpenter, M. Cortinas, S.A. Ewing, D. Foley, J.E. Foley, H. Gaff, G.J. Hickling, R. Lash, S.E. Little, C. Lund, R. Lund, T.N. Mather, G.R. Needham, W.L. Nicholson, J. Sharp, A. Varela-Stokes, D. Wang, Quantitative factors proposed to influence the prevalence of canine tick-borne disease agents in the United States, Parasit. Vectors 7 (2014) 417, https://doi.org/10.1186/1756-3305-7-417.

[91] E.H. Greeley, R.D. Kealy, J.M. Ballam, D.F. Lawler, M. Segre, The influence of age on the canine immune system, Vet. Immunol. Immunopathol. 55 (1996) 1–10, https://doi.org/10.1016/S0165-2427(96)05563-8.

[92] Á. Sainz, X. Roura, G. Miró, A. Estrada-Peña, B. Kohn, S. Harrus, L. Solano-Gallego, Guideline for veterinary practitioners on canine ehrlichiosis and anaplasmosis in Europe, Parasit. Vectors 8 (2015) 75, https://doi.org/10.1186/s13071-015-0649-0.

[93] M. Torres, L. Javier, V. Solari, J. Leonor, K. Abarca, C. Perret, Recomendaciones para el cuidado y manejo responsable de mascotas y su impacto en salud humana, (2014).

[94] F. Dantas-Torres, Biology and ecology of the brown dog tick, Rhipicephalus sanguineus, Parasit. Vectors 3 (2010) 26, https://doi.org/10.1186/1756-3305-3-26.

[95] Y. Yuasa, Y.-L. Tsai, C.-C. Chang, T.-H. Hsu, C.-C. Chou, The prevalence of *Anaplasma platys* and a potential novel *Anaplasma* species exceed that of *Ehrlichia canis* in asymptomatic dogs and *Rhipicephalus sanguineus* in Taiwan, J. Vet. Med. Sci. 79 (2017) 1494–1502, https://doi.org/10.1292/jvms.17-0224.

[96] V. Loruusso, F. Dantas-Torres, R.P. Lia, V.D. Tarallo, N. Mencke, G. Capelli, D. Otranto, Seasonal dynamics of the brown dog tick, Rhipicephalus sanguineus, on a confined dog population in Italy, Med. Vet. Entomol. 24 (2010), https://doi.org/10.1111/j.1365-2915.2010.00885.x no-no.

[97] J.J. Fourie, D. Stanneck, H.G. Luus, F. Beugnet, M. Wijnveld, F. Jongejan, Transmission of Ehrlichia canis by Rhipicephalus sanguineus ticks feeding on dogs and on artificial membranes, Vet. Parasitol. 197 (2013) 595–603, https://doi.org/10.1016/j.vetpar.2013.07.026.

[98] D.M. Aguiar, G.T. Cavalcante, A. Pinter, S.M. Gennari, L.M. a Camargo, M.B. Labruna, Prevalence of Ehrlichia canis (Rickettsiales: anaplasmataceae) in dogs and Rhipicephalus sanguineus (Acari: ixodidae) ticks from Brazil, J. Med. Entomol. 44 (2007) 126–132, https://doi.org/10.1603/0022-2585(2007)44.

[99] M. Ansari-Mood, J. Khoshnegah, M. Mohri, S.M. Rajaei, Seroprevalence and risk factors of Ehrlichia canis infection among companion dogs of Mashhad, north east of Iran, 2009-2010, J. Arthropod. Dis. 9 (2015) 184–194 http://www.ncbi.nlm.nih.gov/pubmed/26623430.

[100] A. Tanikawa, M.B. Labruna, A. Costa, D.M. Aguiar, S.V. Justiniano, R.S. Mendes, A.L.T. Melo, C.J. Alves, S.S. Azevedo, Ehrlichia canis in dogs in a semiarid region of Northeastern Brazil: serology, molecular detection and associated factors, Res. Vet. Sci. 94 (2013) 474–477, https://doi.org/10.1016/j.rvsc.2012.10.007.

[101] S. Hii, R. Traub, M. Thompson, J. Henning, C. O'Leary, A. Burleigh, S. McMahon, R. Rees, S. Kopp, Canine tick-borne pathogens and associated risk factors in dogs presenting with and without clinical signs consistent with tick-borne diseases in northern Australia, Aust. Vet. J. 93 (2015) 58–66, https://doi.org/10.1111/avj.12293.

[102] M. Ben Said, H. Belkahia, L. Messadi, Anaplasma spp. In North Africa: a review on molecular epidemiology, associated risk factors and genetic characteristics, Ticks Tick, Borne. Dis. 9 (2018) 543–555, https://doi.org/10.1016/j.ttbdis.2018.01.003.