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First description of natural *Ehrlichia canis* and *Anaplasma platys* infections in dogs from Argentina

Diego Fernando Eiras[a,b,*], María Belén Craviotto[a], Darío Vezzani[c], Osnat Eyal[d], Gad Baneth[d]

[a] *Laboratorio DIAP (Diagnóstico en Animales Pequeños), Pueyrredón 1098 (B1828ADD), Banfield, Buenos Aires, Argentina*
[b] *Departamento de Epizootiología y Salud Pública, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, CC 296 (B1900AVW) La Plata, Argentina*
[c] *Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET); and Unidad de Ecología de Reservorios y Vectores de Parásitos, Dto. Ecología, Genética y Evolución, FCEyN, UBA, (C1428EHA) Buenos Aires, Argentina*
[d] *School of Veterinary Medicine, Hebrew University of Jerusalem, P.O. Box 12, Rehovot 76100, Israel*

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ABSTRACT

Bacteria belonging to the Anaplasmataceae family are vector transmitted agents that affect a variety of vertebrate hosts including the tick-borne pathogens *Ehrlichia canis* and *Anaplasma platys*, which cause canine monocytic ehrlichiosis and cyclic thrombocytopenia, respectively. These two infections, typically reported from tropical and sub-tropical regions, have not been previously reported in dogs from Argentina. A total of 86 blood samples from dogs with suspected rickettsial disease and 28 non-suspected dogs were studied. Analysis included evaluation of hematological findings, PCR for *Ehrlichia* and *Anaplasma* species and sequencing of the positive PCR products. *E. canis* was detected in the blood of six dogs and *A. platys* in eighteen. All the dogs categorized as non-suspected were negative by PCR. Co-infection with *Hepatozoon canis* and *Babesia vogeli* was documented. This first report of *E. canis* and *A. platys* infections in dogs from Argentina indicates that these tick-borne infections have a considerably broader range than previously recognized in South America.

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1. Introduction

Canine ehrlichiosis and anaplasmosis are tick-borne diseases caused by bacteria of the Anaplasmataceae family and of the genus *Ehrlichia* and *Anaplasma*, respectively. Agents from both genera are implicated in a variety of tick transmitted diseases in animals and humans.

Ehrlichia canis infection has a worldwide distribution and is the agent of canine monocytic ehrlichiosis. Clinical manifestations of this disease include fever, weight loss, anorexia, bleeding disorders and lymphadenomegaly. The clinicopathological findings include anemia, thrombocytopenia, leucopenia, hyperglobulinemia and hypoalbuminemia [1].

Anaplasma platys infection, also described throughout the world, causes cyclic thrombocytopenia in dogs. Infection is usually mild or asymptomatic [2–4] but may be severe or fatal in some cases particularly when co-infections are involved [5].

Both *E. canis* and *A. platys* are obligatory intracellular bacterium of monocytes and platelets, respectively. The brown dog tick *Rhipicephalus sanguineus* is the proven vector of *E. canis* and the suspected vector of *A. platys* [4]. Dog infection with these agents can be diagnosed by microscopical, serological or molecular methods [6].

* Corresponding author at: Departamento de Epizootiología y Salud Pública, Facultad de Ciencias Veterinarias, Universidad Nacional de La Plata, CC 296 (B1900AVW) La Plata, Argentina. Tel.: +54 11 4242 5489; fax: +54 11 4242 5489.
E-mail addresses: diegoeiras@diap.com.ar, bpleiras@gmail.com (D.F. Eiras).

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Canine ehrlichiosis and anaplasmosis were poorly documented in South America until the last two decades. Canine monocytic ehrlichiosis is currently highly endemic in several regions of Brazil [7] and was also documented in dogs from Venezuela [8,9] and Peru [10]. *A. platys* has been identified in dogs from Chile [11], Venezuela [3], and Brazil [12]. In Argentina, *A. platys* was only reported in *R. sanguineus* ticks from Northeast Argentina [13]. Until now, *E. canis* has not been reported in Argentina and *A. platys* infection has not been reported in dogs from this country. The purpose of this study was to investigate the presence of both bacteria in dogs from temperate Argentina and to characterize them molecularly.

2. Materials and methods

Canine blood samples included in the study were submitted by veterinary practitioners for routine testing to the DIAP laboratory in Buenos Aires between November 2011 and February 2012. All the samples included in the study came from eleven municipalities in the Buenos Aires Province, namely: Esteban Echeverría, Almirante Brown, Lomas de Zamora, Florencio Varela, Berazategui, Berisso, La Plata, Lanús, Ezeiza, Avellaneda and Quilmes. Blood was taken by puncture of the cephalic vein, collected in EDTA tubes and stored at 4 °C until it was processed 12–24 h later for hematological tests and then kept at −18 °C until DNA was extracted. Suspicion of *Ehrlichia* or *Anaplasma* infection was based on the following criteria: (a) evidence of possible infection detected upon microscopic evaluation of a May Grünwald-Giemsa stained blood smear, e.g. presence of cytoplasmatic structures that resemble morulae of *Ehrlichia* or *Anaplasma* and/or giant-activated monocytes; (b) dogs with severe thrombocytopenia (<50,000 platelets/μl); and (c) dogs with moderate thrombocytopenia (50,000–149,000 platelets/μl). Samples from dogs suspected for *Ehrlichia* or *Anaplasma* infection were sub-divided into 4 groups: (1) those with suspected blood smear evidence of infection and no thrombocytopenia (>150,000 platelets/μl); (2) those with suspected blood smear evidence of infection and severe thrombocytopenia (<50,000 platelets/μl); (3) those with only severe thrombocytopenia; and, (4) those with only moderate thrombocytopenia (50,000–149,000 platelets/μl). A group of samples was also evaluated from dogs which were not suspected of infection and included samples without cytological evidence suspected of infection by blood smear microscopy and a platelet count ≥150,000/μl.

Platelets count was performed by an automated counter (Abacus, Diatron) and confirmed under light microscope. Thin blood smears were stained by May Grünwald-Giemsa and examined by light microscopy. The detection of inclusions in the blood cells cytoplasm that could resemble morulae of *Ehrlichia* or *Anaplasma* was performed by trained laboratory personnel. The microscopic presence or absence of other hemoparasites was also documented.

DNA was extracted from EDTA blood samples in the DIAP laboratory using the commercial Wizard® Genomic DNA Purification Kit (Promega) according with the manufacturer instructions. The extracted DNA was sent to the Hebrew University of Jerusalem in Israel for molecular testing.

Primers 16S-D (5′-GGTACCYACAGAAGAAGTCC) and 16S-R (5′-TAGCACTCATCGTTTACAGC) (400 nM each) [14] were used to amplify a region of approximately 345 bp from the 16SrRNA gene of *Ehrlichia* and *Anaplasma* species. PCR was performed using a 25 μl reaction of Syntezza PCR-Ready High Specificity (Syntezza Bioscience, Israel). The reaction conditions were as follows: 95 °C for 5 min, 35 cycles of [94 °C for 30 s, 55 °C for 30 s, 72 °C for 90 s] and a final elongation step of 72 °C for 5 min. Positive and negative control samples for *E. canis* were run with each PCR reaction.

Samples were analyzed on a 1.5% agarose gel. Positive samples were at the Center for Genomic Technologies, Institute of Life Sciences, Hebrew University of Jerusalem. Sequencing data were evaluated with the Chromas Lite version 2.01 software and compared to data available from GenBank using the BLASTN 2.2.26 program (http://www.ncbi.nlm.nih.gov/BLAST/).

Co-infecting pathogens observed by microscopy (i.e. *Hepatozoon* gamonts and *Babesia* merozoites) were analyzed by PCR as previously described [15,16] and by sequencing in order to characterize the infecting species.

The percentage of positive dogs according to the PCR assays was compared among the different dog groups with the χ^2 test for multiple independent proportions [17]. Then, to identify groups contributing to significant differences, new tests for multiple proportions were performed using Tukey's procedure [18].

3. Results

A total of 4310 blood samples from dogs living in the greater Buenos Aires area were received for hematological tests at the DIAP laboratory during November 2011 to February 2012. Of these, 86 samples that fitted the study's inclusion criteria were selected for participation in the study and PCR analysis. An additional 28 samples from dogs with normal hematologic findings were included in the study as a control group.

DNA samples from all 114 dogs were tested by PCR for the presence of *Ehrlichia or Anaplasma* species DNA using the 16S-D/R primers. Gel analysis of the PCR products resulted in 24 positive samples, which were sequenced and their identities determined. Sequences obtained were compared by BLAST analysis to GenBank sequences and *Ehrlichia* or *Anaplasma* species were identified based on being the closest match to an existing GenBank accession and being 97–100% identical to it.

A total of 24 out of 86 (27.9%) suspected dogs were positive to *E. canis* or *A. platys* by PCR. All the dogs included in the non-suspected control group ($n = 28$) were negative by PCR. Infection with *E. canis* was recorded in 6 dogs and with *A. platys* in 18 other dogs. Two Argentinean *E. canis* sequences were deposited in GenBank as accession numbers JX261980 and JX261981 and an *A. platys* sequence was deposited as accession no. JX261979. Blood smear evaluation of the positive dogs revealed co-infection with *Hepatozoon* sp. gamonts in three *E. canis* (50%) and four *A. platys* (22.2%) infected dogs. One of the latter was also

Table 1
Results of PCR for *Ehrlichia canis* or *Anaplasma platys* in dogs suspected of infection and in non-suspected control dogs.

	Groups	*N*	PCR positive	Positive for *E. canis* (E) or *A. platys* (A)
Suspected	1. Suspected blood smear evidence[a] without thrombocytopenia	5	3 (60%) a	3 (A)
	2. Suspected blood smear evidence + severe thrombocytopenia	16	9 (56.2%) a	5 (A); 4 (E)
	3. Severe thrombocytopenia without blood smear evidence	50	10 (20%) a	8 (A); 2 (E)
	4. Moderate thrombocytopenia without blood smear evidence	15	2 (13.3%) ab	2 (A)
Non-suspected controls	5. Normal hematology and ≥150,000 platelets/μl	28	0 (0%) b	

Same letters indicate no significant differences ($p > 0.05$) between categories according to the multiple pairwise comparison (Tukey's procedure). No dogs had moderate thrombocytopenia and suspected blood smears.

[a] Presence of cytoplasmatic structures that resemble morulae of *Ehrlichia* or *Anaplasma* and/or giant-activated monocytes.

infected with merozoites of a large *Babesia* sp. Genetic characterization of these co-infecting pathogens was confirmed by PCR and sequencing as *Hepatozoon canis* and *Babesia vogeli*.

According to the results of PCR assays, there were significant differences ($\chi^2_{(4)} = 24.53$, $p < 0.0001$) in the percentage of infected dogs among the different groups compared, with dogs in groups 1–3 differing significantly from group 5, the non-suspected controls (Table 1). Furthermore, dogs infected with *E. canis* were found only in groups 2 and 3 which had severe thrombocytopenia (Table 1), whereas infection with *A. playts* was present also in dogs with moderate thrombocytopenia (group 4) and without thrombocytopenia but with suspected blood smear evidence (group 1).

4. Discussion

This is the first description of *E. canis* and *A. platys* infections in dogs from Argentina. These two pathogens should be included in the differential diagnosis of dogs with compatible clinical signs and hematological abnormalities in Argentina, particularly in regions with suitable environmental conditions supporting the presence of tick vectors, including *R. sanguineus*.

Reports on *E. canis* and *A. platys* infections in dogs from warmer and more tropical South American countries date from the 1970s. Canine mococytic ehrlichiosis was reported in Brazil in 1973 [19], in Venezuela in 1982 [8] and in Peru where a distinct strain of *E. canis* was involved in canine infection in 2007 [10]. *A. platys* infection has been described in several South American countries [11,12] since cyclic thrombocytopenia was first described in a dog from the USA in 1978 [20]. It is not possible to know if these infections, previously described in other regions, have been present but undiagnosed in Argentina or if they have recently emerged in the region.

Parasitism by *R. sanguineus* was described as a risk factor for ehrlichiosis [21]. This tick has been reported in Argentina since the 1940s and its presence is widespread in dogs in the Buenos Aires Province [22]. In this temperate region, the presence of *R. sanguineus* is more abundant in the warmer months. In addition to preferring warm climate regions, *R. sanguineus* can complete its life cycle also in indoor environments in colder regions [23] and it was reported in the last years in different regions in Argentina parasitizing humans, domestic and wild animals [24].

Our findings were partially expected due to the cosmopolitan nature of *R. sanguineus* and the previously reported presence of other *R. sanguineus*-borne pathogens of dogs in Argentina. However, knowledge on the uniformity of the species *R. sanguineus* in South America and the capacity of its variants to transmit *E. canis* and other pathogens is currently incomplete. Several studies have reported morphological, biological and genetic variations among *R. sanguineus* ticks from different locations in South America [25,26,28].

The recent descriptions of *H. canis* and *B. vogeli* infections, two canine protozoal pathogens transmitted by *R. sangineus* in Buenos Aires [29,30], may suggest that *E. canis* and *A. platys* could be newly introduced pathogens to this area.

In one study made in Brazil, of 115 dogs infected naturally by *Hepatozoon* sp., 22.61% were concurrently infected with *Babesia* sp. or *Ehrlichia* sp. [31]. Despite the fact that molecular characterization of *H. canis* and *B. vogeli* was previously reported in dogs from Buenos Aires, no evidence of co-infection was documented before. This study also represents the first report of co-infection with different hemoparasites transmitted by the same tick in Argentina.

Canine ehrlichiosis caused by *E. canis* is a severe disease in which clinical acute or chronic febrile illness can be observed, although the clinical manifestations can be exacerbated when other vector borne pathogens are present [32].

Anaplasmosis caused by *A. platys* has long been considered mostly a non-clinical infection in dogs [5] although clinical illness in dogs in Europe may be more severe than in the Americas [33] and might be related to more virulent *A. platys* strains. In a study performed by de Caprariis et al. [6], *A. platys* infection was associated with thrombocytopenia, lymphadenomegaly, weight loss and hematological abnormalities but mainly in dogs co-infected with *B. vogeli*. Despite this, clinical and clinical-pathological abnormalities (anorexia, lethargy, anemia, thrombocytopenia, lymph node follicular hyperplasia and splenic hemorrhage) can be found in *A. platys* infected dogs without obvious co-infection [34].

Molecular diagnostic techniques were used in this study to confirm infection of dogs with *Ehrlichia* and *Anaplasma* species. Direct observation of morulae on microscopy may be difficult for the diagnosis of canine ehrlichiosis and anaplasmosis and therefore suspected infection must be confirmed by other methods like serology or PCR. Furthermore, suspicion of infection may be missed in

inexperienced veterinary laboratories in areas or countries (e.g. Argentina) where these infections are considered rare or have not been reported before. Interestingly, *E. canis* was only detected in dogs with severe thrombocytopenia while *A. platys* was found also in dogs with moderate thrombocytopenia and in three dogs with no thrombocytopenia. Although thrombocytopenia is a typical finding in *E. canis* and *A. platys* infections, some dogs may be infected without having thrombocytopenia [6–35]. Therefore, despite the fact that the index of suspicion for these infections should be higher in thrombocytopenic dogs, they cannot be ruled out solely on the basis of absence of thrombocytopenia, and additional parameters such as blood smear abnormalities should be considered with ultimate confirmation by PCR.

Several species of *Ehrlichia* and *Anaplasma* are known to be zoonotic disease agents infecting people in many parts of the world [32–36]. Although *A. platys* has not been described to infect humans, a strain of *E. canis* has been reported to cause human disease in Venezuela [37]. The potential for transmission of zoonotic rickettsial infections by ticks in Argentina including Rocky Mountain spotted fever and ehrlichiosis emphasizes the importance of a One Health approach where physicians and veterinarian join forces in the management of these diseases [38].

In conclusion, *E. canis* and *A. platys* have been found to infect dogs in Buenos Aires, in addition to other canine tick borne pathogens including *B. vogeli* and *H. canis* recently reported in this region. The emergence of these pathogens and the expansion of these canine vector borne diseases to Argentina should be brought to the awareness of health workers, veterinarians, diagnosticians and dog owners in an effort to diagnose and treat infected dogs and to prevent new infections.

Conflict of interest

The authors do not have a conflict of interest that could inappropriately influence or bias the content of this paper.

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