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Infection with *Ehrlichia canis* and *Anaplasma platys* (Rickettsiales: Anaplasmataceae) in two lineages of *Rhipicephalus sanguineus* sensu lato (Acari: Ixodidae) from Argentina

Gabriel L. Cicuttin[a], Evelina L. Tarragona[b], M. Nazarena De Salvo[a], Atilio J. Mangold[b], Santiago Nava[b,*]

[a] Instituto de Zoonosis Luis Pasteur, Ministerio de Salud, Gobierno de la Ciudad Autónoma de Buenos Aires, Av Díaz Vélez 4821, C1405DCD Caba, Argentina
[b] Instituto Nacional de Tecnología Agropecuaria, Estación Experimental Agropecuaria Rafaela and Consejo Nacional de Investigaciones Científicas y Técnicas, CC 22, CP 2300 Rafaela, Santa Fe, Argentina

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ABSTRACT

Natural infection with *Ehrlichia canis* and *Anaplasma platys* in ticks belonging to the tropical and temperate lineages of *Rhipicephalus sanguineus* sensu lato from Argentina was evaluated. Samples were tested for *Ehrlichia canis* infection by PCR assays using 16S rRNA, *dsb* and *p28* gene, while detection of *A. platys* was performed with 16S rRNA and *groESL* gene. The assignment of the ticks to each lineage was corroborated with 16S rDNA sequences. All ticks infected with *E. canis* and *A. platys* belonged to the tropical lineage. These results constitute the first record of *E. canis* infection in *R. sanguineus* s.l ticks from Argentina. No ticks from the temperate lineage were found to be infected with *E. canis*, coinciding with previous studies performed in Argentina and Uruguay where *E. canis* infection was not detected in *R. sanguineus* s.l from the temperate lineage. Because the presence of the tropical lineage of *R. sanguineus* s.l has been documented in tropical areas of northern Argentina between 22° and 24° of south latitude, the findings of this work indicate that transmission of *E. canis* and *A. platys* to dogs by *R. sanguineus* s.l probably occurs along this region.

1. Introduction

The taxon *Rhipicephalus sanguineus* sensu lato (Acari: Ixodidae) includes ticks distributed around the world with sanitary and economic importance. They are involved in the transmission of different diseases agents to both dogs and humans (Otranto et al., 2009; Bowman, 2011; Parola et al., 2013) and have a considerable economic relevance for the antiparasitic market of companion animals (Graf et al., 2004). The veterinary importance of *R. sanguineus* s.l is in part attributable to its role in the transmission of *Ehrlichia canis* and *Anaplasma platys*. They are obligate intracellular bacteria of the family *Anaplasmataceae* which cause infectious diseases in dogs (Dumler et al., 2001). *E. canis* is the etiological agent of canine monocytic ehrlichiosis, and it is principally transmitted by nymphs and adults of *R. sanguineus* s.l (Stich et al., 2008). *A. platys* is the causative agent of cyclic thrombocytopenia in dogs, and *R. sanguineus* s.l is suspected to be involved as its principal vector (Inokuma et al., 2000; Sanogo et al., 2003; Abarca et al., 2007). Infection with *A. platys* in *R. sanguineus* s.l was reported in different countries from Asia, Africa and Europe (Inokuma et al., 2000; Sanogo et al., 2003; Ybañez et al., 2012; Latrofa et al., 2014; Ramos et al., 2014). *E. canis* was found infecting *R. sanguineus* s.l in Asia, Africa (Socolovschi et al., 2012; Ybañez et al., 2012), and in South America in Brazil and Venezuela (Unver et al., 2001; Aguiar et al., 2007). Particularly in Argentina, *A. platys* was detected in *R. sanguineus* s.l but not *E. canis* (Oscherov et al., 2011; Cicuttin et al., 2014a,b).

Comparative studies performed with tick populations from different geographical origins have demonstrated that ticks previously determined as *Rhipicephalus sanguineus* sensu stricto belong in fact to different lineages with reproductive incompatibility and significant genetic divergence (Szabó et al., 2005; Burlini et al., 2010; Moraes-Filho et al., 2011; Levin et al., 2012; Nava et al., 2012; Dantas-Torres et al., 2013; Liu et al., 2013). In South America, at least two lineages of *R. sanguineus* s.l were identified: tropical and temperate. The tropical lineage is distributed in tropical areas of northern Argentina, Brazil, Colombia, Paraguay and Peru, and the temperate lineage is associated to temperate and cold localities from Argentina, Brazil, Chile and Uruguay (Moraes-Filho et al.,

* Corresponding author. Tel.: +54 03492440121; fax: +54 03492440114.
E-mail address: nava.santiago@inta.gob.ar (S. Nava).

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Fig. 1. Collection sites of *Rhipicephalus sanguineus* sensu lato in Argentina.

2011; Nava et al., 2012). Furthermore, Moraes-Filho et al. (2013) have found that *R. sanguineus* s.l ticks from tropical America (Sao Paulo, Brazil) were highly competent vectors of *E. canis*, but not *R. sanguineus* s.l ticks from the Southern Cone of South America (Argentina, Uruguay and south of Brazil (Rio Grande do Sul)). In coincide with these results, *R. sanguineus* s.l ticks naturally infected with *E. canis* were detected in tropical areas of Brazil (Aguiar et al., 2007; Da Silva Souza et al., 2010), but no ehrlichial infection was found among *R. sanguineus* s.l ticks collected on dogs in temperate localities from Uruguay (Venzal et al., 2007) and Argentina (Cicuttin et al., 2014a,b).

The possible differences in vectorial competence among different lineages of *R. sanguineus* s.l highlight the need to perform studies to determine the potential role of ticks of each lineage as vector of pathogens causing diseases in companion animals. Therefore, the purpose of this study is to determine the natural infection with *E. canis* and *A. platys* in ticks belonging to the two lineages (tropical and temperate) of *R. sanguineus* s.l in Argentina

2. Materials and methods

Ticks from different populations were collected on dogs in five localities from northern Argentina (Fig. 1): (I) Ingeniero Juarez (IJ), Formosa Province (23°54′S, 61°51′W), collection date: January 2014, number of dogs examined: 4; II) Coronel Juan Solá (JS), Salta Province (23°28′S, 62°52′W), collection date: January 2014, number of dogs examined: 2; III) Machagai (MA), Chaco Province (26°56′S 60°03′W), collection date: November 2013, number of dogs examined: 3; IV) Juan José Castelli (CA), Chaco Province (25°56′S, 60°37′W), collection date: November 2013, number of dogs examined: 13; V) Oberá (OB), Misiones Province (27°29′S, 55°07′W), collection date: September 2013, number of dogs examined: 79. All ticks were collected on clinically healthy adult dogs selected randomly from these localities, and ticks were collected on dogs with the consent of the owners. Ticks were morphologically determined as *R. sanguineus* s.l according to Walker et al. (2000).[1] The assignment of each population to the temperate and tropical lineages was based on Nava et al. (2012), who determined that the geographical boundary separating the two lineages is situated between 24° and 25° of south latitude. According with this scheme, populations of *R. sanguineus* s.l from IJ and JS were considered as belonging to the tropical lineage, while populations from MA, CA and OB were included within the temperate lineage. This a priori assignment was corroborated by means of the analysis of the sequences of a 410 bp fragment of the mitochondrial 16S rRNA gene. DNA was extracted from representative specimens of each population and processed through polymerase chain reaction (PCR) using the methodology and primers described by Mangold et al. (1998). Sequences were compared among each other and with those obtained by Moraes-Filho et al. (2011) and Nava et al. (2012).

For molecular detection of *Ehrlichia* and *Anaplasma*, DNA was extracted from adult ticks (adults individually; nymphs in pools of 5–10 specimens) by using the High Pure PCR Template Preparation Kit (Roche, Mannheim, Germany) following the manufacturer's instructions. Initial screening for Anaplasmataceae was made with a PCR-amplified fragment of the 16S rRNA gene with the primers EHR16SD (GGTACCYACAGAAGAAGTCC) and EHR16SR (TAGCACTCATCGTTTACAGC) (Parola et al., 2000). Samples showed to be positive to *Ehrlichia* were used to amplify a ca. 400-bp fragment of the *dsb* gene with primers dsb-330 (5′-GATGATGTCTGAAGATATGAAACAAAT-3′) and dsb- 728 (5′-CTGCTCGTCTATTTTACTTCTTAAAGT-3′) (Aguiar et al., 2007) and a ca. 500-bp fragment of the *p28* gene with primers 793′ (5′-GCAGGAGCTGTTGGTTACTC-3′) and 1330 (5′-CCTTCCTCCAAGTTCTATGCC-3′) (McBride et al., 1999). Positive samples to *Anaplasma* were subjected to amplification of a ca. 750-bp fragment of the *groESL* gene by using the primers PLA-HS475F (AAGGCGAAAGAAGCAGTCTTA) and PLA-HS1198R (CATAGTCTGAAGTGGAGGAC) (Inokuma et al., 2002).

The sequences were edited using BioEdit Sequence Alignment Editor (Hall, 1999) with manual edition whenever it was necessary and aligned with the program Clustal W (Larkin et al., 2007). They were compared with those sequences of *Ehrlichia* and *Anaplasma* deposited in GenBank. Sequences were used to perform phylogenetic analyses with Maximum-likelihood (ML) methods by using the program Mega 5.0 (Tamura et al., 2011). Best fitting substitution models were determined with the Akaike Information Criterion using the ML model test implemented in MEGA 5.0. Support for the topologies was tested by bootstrapping over 1000 replications and gaps were excluded from the comparisons.

3. Results

The analysis of the 16S rDNA sequences of *R. sanguineus* s.l ticks from northern Argentina validated the a priori assignment of each geographic population to the tropical or temperate lineages. 16S rDNA sequences from IJ and JS clustered with those sequences belonging to the tropical lineage of *R. sanguineus* s.l, and sequences of ticks from MA, CA and OB were grouped with the sequences of the temperate lineage of *R. sanguineus* s.l (Fig. 2). Pairwise differences among the sequences within each lineage ranged from 0 to 0.8%.

[1] In this work the name *R. sanguineus* s.l applies to the two lineages present in the Neotropical Region, see Moraes-Filho et al. (2011) and Nava et al. (2012).

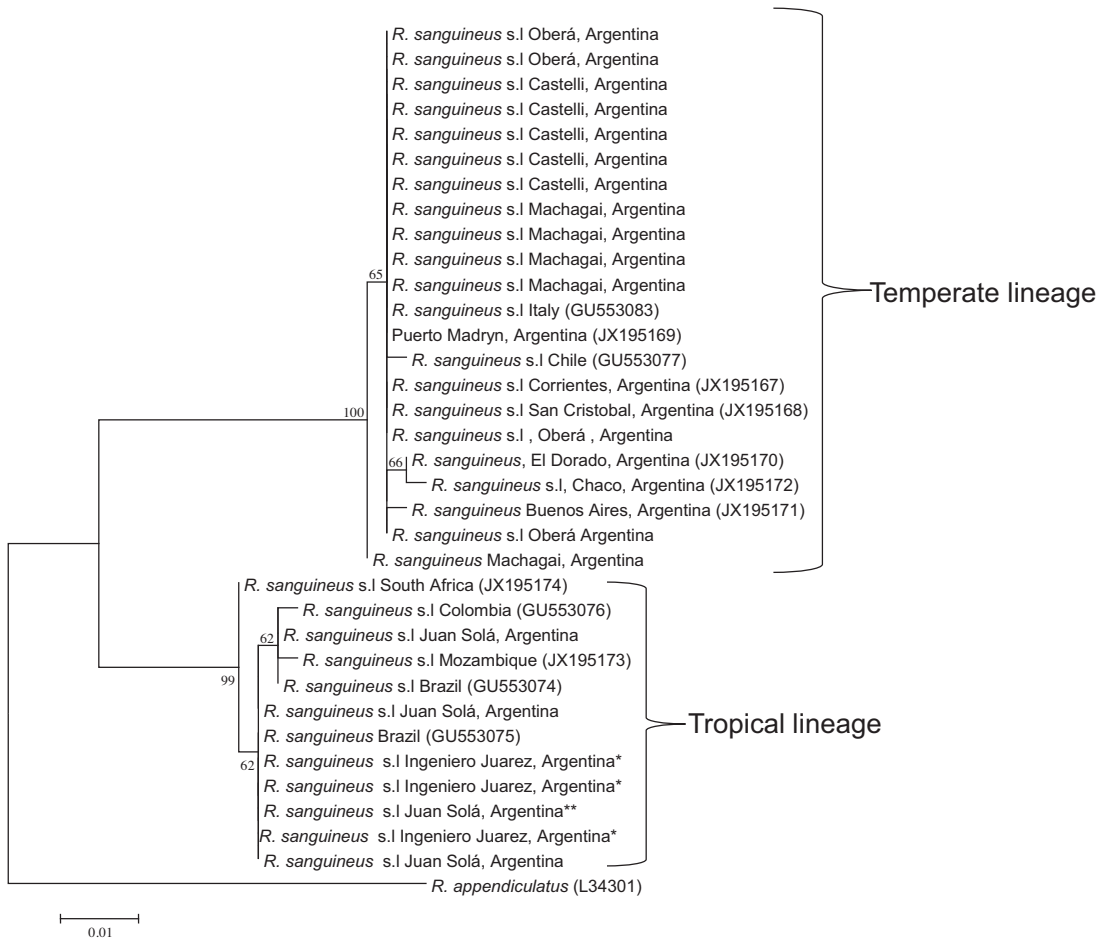

Fig. 2. Maximum-likelihood tree constructed from 16S rDNA sequences of *Rhipicephalus sanguineus* sensu lato (substitution model: GTR + G). Numbers represent bootstrap support generated from 1000 replications. GenBank accession numbers are in brackets. *Sequences of the samples infected with *Ehrlichia canis*. **Sequence of the sample infected with *Anaplasma platys*. Only bootstrap values higher than 60 are shown.

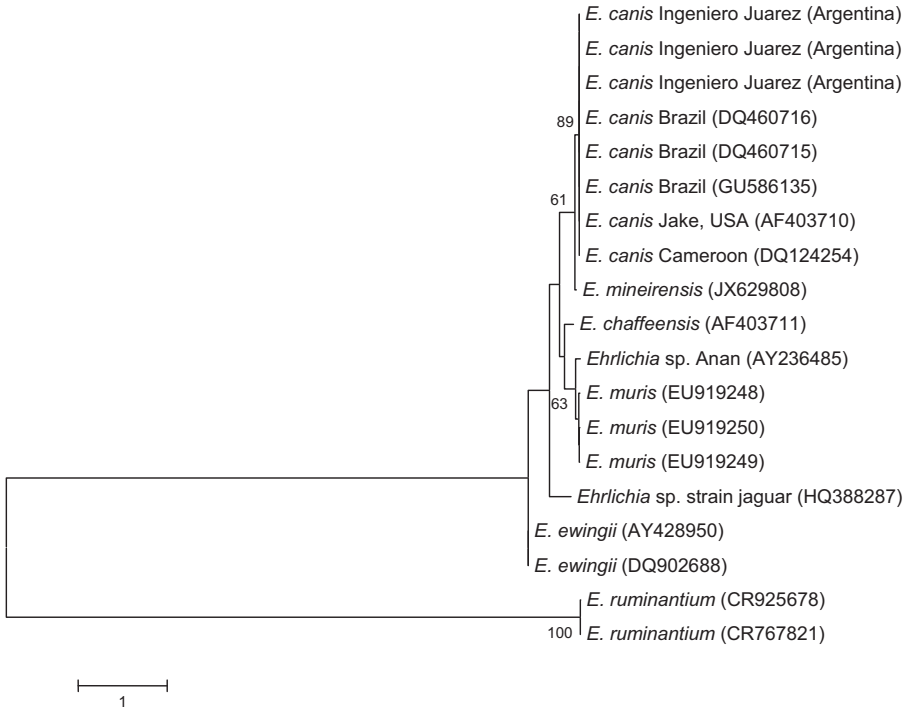

Fig. 3. Maximum-likelihood tree constructed from *dsb* sequences of *Ehrlichia* spp. (substitution model: Tamura 3 parameter + G). Numbers represent bootstrap support generated from 1000 replications. GenBank accession numbers are in brackets. Only bootstrap values higher than 60 are shown.

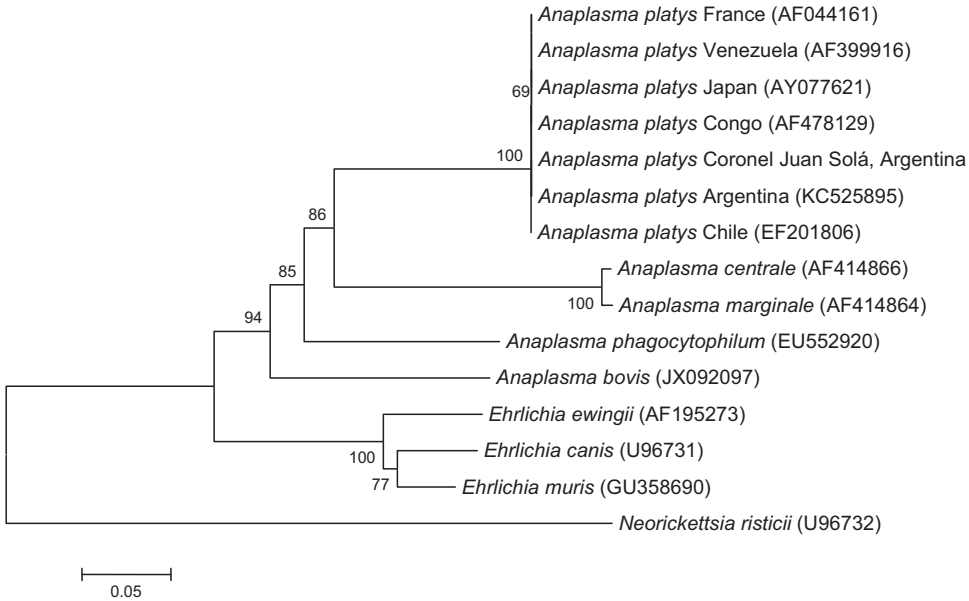

Fig. 4. Maximum-likelihood tree constructed from *groESL* sequences of *Anaplasma* spp. (substitution model: GTR + G). Numbers represent bootstrap support generated from 1000 replications. GenBank accession numbers are in brackets. Only bootstrap values higher than 60 are shown.

A total of 181 ticks (146 adults and 35 nymphs) were tested for *Ehrlichia* and *Anaplasma* infection, 147 belonging to the temperate lineage (n MA: 27; n CA: 51; n OB: 79) and 34 from the tropical lineage (n IJ: 29; n JS: 5). Three adult ticks from IJ were shown to be positive to *Ehrlichia* spp. and one pool of two nymphs from JS was positive to *Anaplasma* spp. by 16S rRNA PCR assay. No PCR products were generated from the remaining ticks. Samples from IJ shown by PCR to be positive to *Ehrlichia* were further used to obtain sequences of *dsb* gene. The three sequences were identical with each other (GenBank accession number: KR909452) and with those *dsb* sequences of *E. canis* from Brazil (Jaboticabal, Uberlândia and São Paulo; GenBank accession numbers: DQ460716, GU586135, DQ460715), USA (strain Jake; GenBank accession number: AF403710) and Cameroon (GenBank accession number: DQ124254) (Fig. 3). Additionally, a partial sequence (ca. 500 bp) of the *p28* gene was obtained from one positive sample to *E. canis* from IJ. This sequence was 99.2% identical to *E. canis* strain Jake (GenBank accession number: CP000107) and *E. canis* strain Oklahoma (GenBank accession number: AF078553).

The sample from JS scored positive for *Anaplasma* by 16S rRNA PCR assay was employed to amplify a partial sequence (ca. 700 bp) of the *groESL* gene. This sequence (GenBank accession number: KR909453) clustered with sequences of *A. platys* from Chile, Congo, France, Japan, Uruguay, Venezuela, and with a sequence of *A. platys* obtained from blood samples of dogs in Argentina (Fig. 4). The degree of nucleotide sequence similarity among the *A. platys* sequences included in Fig. 4 ranged from 99.8 to 100%.

The 16S rDNA sequence of the four specimens of *R. sanguineus* s.l infected with *E. canis* and *A. platys* from IJ and JS were included in the phylogenetic tree (Fig. 2) to confirm they belong to the tropical lineage. Representative 16S rDNA sequences of the different haplotypes have been deposited in GenBank as follow: Haplotype Castelli I (KR909454), haplotype Obera I (KR909455), haplotype Machagai I (KR909456), haplotype Machagai II (KR909457), haplotype Juan Sola I (KR909458) and haplotype Ingeniero Juarez (KR909459).

4. Discussion

The results of this work constitute the first record of *E. canis* infection in *R. sanguineus* s.l ticks from Argentina. In South America, this association has already been reported in Brazil (Aguiar et al., 2007) and Venezuela (Unver et al., 2001) but not in Argentina, where the previous record of this pathogen corresponds to the description of *E. canis* infection in blood samples from dogs in Buenos Aires Province (Eiras et al., 2013). However, the diagnostic presented by Eiras et al. (2013) is subject to confirmation because it was only based on sequences of a short fragment (318 bp) of the 16S RNA gene, which exhibits low levels of polymorphism within the genus *Ehrlichia*. In fact, the comparison of the two sequences obtained by Eiras et al. (2013) (GenBank accession numbers: JX261980 and JX261981) with those sequences available in GenBank by using BLAST program (http://blast.ncbi.nlm.nih.gov/Blast) has showed a similarity from 99 to 100% not only with different isolates of *E. canis* but also with *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, *Ehrlichia muris* and several sequences of *Ehrlichia* sp.

Canine monocytic ehrlichiosis caused by *E. canis* has been described around the world, but it appears to be particularly prevalent in tropical regions where is principally vectored by *R. sanguineus* s.l ticks (Bremer et al., 2005; Stich et al., 2008). In this work, all ticks infected with *E. canis* were determined as belonging to the tropical lineage of *R. sanguineus* s.l. Because the presence of the tropical lineage of *R. sanguineus* s.l has been documented in tropical areas of northern Argentina between 22° and 24° of south latitude (Nava et al., 2012), the findings of this work indicate that transmission of *E. canis* to dogs by *R. sanguineus* s.l probably occurs along this region of Argentina.

Moraes-Filho et al. (2013) have experimentally demonstrated differences in the vectorial competence to transmit *E. canis* between the tropical and temperate lineages of *R. sanguineus* s.l. This author found that *R. sanguineus* s.l ticks from tropical America (Sao Paulo, Brazil) are highly competent vectors of *E. canis* but not *R. sanguineus* s.l ticks from temperate areas of South America (Argentina, Uruguay and south of Brazil). The analyses of the natural infection of *R. sanguineus* s.l with *E. canis* performed in this work are in agreement with the results obtained by Moraes-Filho et al. (2013), because all *E. canis*-infected ticks belonged to the tropical lineage of *R. sanguineus* s.l. Even thought the number of ticks from the temperate lineage tested for *E. canis* infection was higher than the number of tested ticks from the tropical lineage, no tick from the temperate lineage was found to be infected with *E. canis* during

this work. These results coincide with previous studies performed in Argentina (Cicuttin et al., 2014a,b) and Uruguay (Venzal et al., 2007), where *E. canis* infection was not detected in natural populations of *R. sanguineus* s.l from the temperate lineage. A caveat of this work is that whole body of ticks was used for DNA extraction. Although the ticks analyzed were not engorged and collected from clinically healthy adult dogs, there is a possibility of acquiring the infection by blood meal without colonization of salivary glands. Therefore, comparison between the prevalence of the *E. canis* infection obtained in this study and those values reported in previous works where genomic DNA was extracted from the salivary glands (e.g. Aguiar et al., 2007) is not feasible.

One sample of *R. sanguineus* s.l from JS belonging to the tropical lineage (see Fig. 2) was also found to be infected with *A. platys* during this study. This result is not unexpected since *A. platys* infection in *R. sanguineus* s.l ticks was previously described in countries from Africa, Asia and Europe (Inokuma et al., 2000; Sanogo et al., 2003; Ybañez et al., 2012; Latrofa et al., 2014; Ramos et al., 2014). In Argentina, Oscherov et al. (2011) have found *A. platys* infection in *R. sanguineus* s.l ticks from an area of Corrientes Province where populations of *R. sanguineus* s.l of the temperate lineage prevail (Nava et al., 2012). Consequently, *A. platys* infection in Argentina is present in both tropical and temperate lineages of *R. sanguineus* s.l. From these results it can be concluded that *A. platys* infection in *R. sanguineus* s.l is an ubiquitous phenomenon worldwide, independently of the association of the infected *R. sanguineus* s.l ticks with a particular lineage.

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