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Original article

Prevalence and molecular characterization of piroplasmids in domestic dogs from Paraguay

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ABSTRACT

Canine piroplasmoses, caused by *Babesia* spp., *Theileria* spp. and *Rangelia vitalii*, are emerging vector-borne diseases with a worldwide distribution, transmitted by ticks. The aim of this study was to determine the prevalence and perform molecular characterization of piroplasmids in domestic dogs from Asunción city, Paraguay. Blood samples were taken from 384 domestic dogs from Asunción city, Paraguay. DNA was purified from dog blood samples and submitted to nested PCR assays for piroplasmids (18S rRNA) and sequenced for identification and phylogenetic analysis. Overall piroplasmid prevalence in dogs from Paraguay was 6% (23/384 [CI 95% = 3.6–8.4%]). Phylogenetic studies showed that *Babesia vogeli* was the most prevalent species (91% [21/23]), followed by *Theileria equi* (4% [1/23]) and *Rangelia* sp. closely related to *R. vitalii* (4% [1/23]). *Babesia vogeli*, *T. equi* and *Rangelia* sp. circulate among domestic dogs from Asunción city, and are described for the first time in Paraguay.

1. Introduction

Canine piroplasmosis is an emerging disease worldwide caused by various species of piroplasms and is transmitted by ticks (Jefferies et al., 2007). Piroplasmoses are produced by protozoa belonging to the class Piroplasmea and order Piroplasmorida, including the Babesiidae family, genus *Babesia*, the Theileriidae family, genus *Theileria,* and the species *Rangelia vitalii*, whose taxonomic classification is still under study (Homer et al., 2000; Loretti and Barros, 2005; Soares et al., 2011). *Rangelia vitalii* belongs to the phylum Apicomplexa, order Piroplasmorida (Loretti and Barros, 2005) and is genetically related to protozoans from the Babesiidae family (Soares et al., 2011).

Parasites from the order Piroplasmorida have a cosmopolitan distribution (Criado-Fornelio et al., 2003). The molecular prevalence in dogs varies from 0.1% to 75% for the genus *Babesia,* and 0.1% to 15% for *Theileria*, as previously shown in studies from Europe, America, Africa and Asia (Beck et al., 2017; Cassini et al., 2009; Eiras et al., 2008; Gottlieb et al., 2016; Hamel et al., 2012; Kamani et al., 2013; Matjila et al., 2008; Miró et al., 2015; René-Martellet et al., 2015; Rjeibi et al., 2016; Singh et al., 2014; Soares et al., 2011; Solano-Gallego et al., 2008; Sousa et al., 2013; Suh et al., 2017; Wei et al., 2014; Xu et al., 2015). *Rangelia vitalii* prevalence in Brazil ranges from 5.8% to 30% (Lemos et al., 2012; Soares et al., 2014), and although no prevalence studies were performed in other countries, the presence of *R. vitalii* was molecularly confirmed in Argentina (Eiras et al., 2014) and Uruguay (Soares et al., 2015).

Canine piroplasmosis can vary from subclinical to severe and fatal disease. Lethargy, anorexia, pale mucous membranes, hyperthermia, hemoglobinuria, splenomegaly, hemolytic anemia and thrombocytopenia are clinical and laboratory manifestations regularly described in dogs suffering from piroplasmosis (Cardoso et al., 2016).

Babesia spp. and *Theileria* spp. infections are frequently described in dogs (Baneth et al., 2015) and are reportedly caused by *B. canis*, *B. rossi*, *B. vogeli*, *Babesia* sp. coco, *B. vulpes* (also referred to as *B. microti*-like and *T. annae*), *B. gibsoni*, *B. conradae, T. equi* and *T. annulata* (Baneth et al., 2015; Birkenheuer et al., 2004; Criado-Fornelio and Martinez-Marcos, 2003; Kjemtrup et al., 2006; Matjila et al., 2008; Uilenberg, 2006). *Rangelia vitalii* has only been described in South America and causes severe hemorrhagic syndrome in dogs (Rivero et al., 2017; Soares et al., 2015).

Although cytological evaluation of blood smears has been widely used for detecting piroplasmid inclusions in erythrocytes, this method lacks sensitivity and does not allow the species to be identified (Solano-Gallego et al., 2016). For diagnosis and research purposes, polymerase

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chain reaction (PCR) and its variants are proven to be the most sensitive and specific methods on detecting agents and new strains, as well as being an important tool for evaluating treatment (Birkenheuer et al., 2003; Duarte et al., 2008; Nakaghi et al., 2008; Rubini et al., 2009).

Rhipicephalus sanguineus sensu lato is widely distributed in Paraguay (Nava et al., 2007; Pallarés and Usher, 1982), *Haemaphysalis* spp. was described in two from the three provinces of Paraguay and *Amblyomma aureolatum* was detected in San Pedro city (Nava et al., 2007). Those ticks are involved in the transmission of piroplasmids (Chauvin et al., 2009; Dixit et al., 2010; Soares et al., 2018; Sousa et al., 2018). However, to date piroplasmids have not been molecularly detected in domestic dogs from Paraguay. The present study aimed to determine the prevalence and perform a molecular characterization of piroplasmids in domestic dogs from Asunción city, Paraguay.

2. Material and methods

2.1. Animals and Area of study

The study was approved by the Universidad Austral de Chile (UACh) bioethics committee under the protocol number UACh 238/2015. In order to determine the prevalence of piroplasmids in Asunción, Paraguay, the sample size required was estimated according to the method described by Thrusfield (2007). The procedure used to calculate sample size is below. According to Thrusfield (2007) the mathematical formula to calculate the sample size for estimate a disease prevalence considering a 95% confidence interval is:

$$n = 1.96^2\ P_{exp}(1\text{-}P_{exp})/D^2$$

Where: $\boldsymbol{n}$ = required sample size (number of dogs required)
$\boldsymbol{P_{exp}}$ = expected prevalence
$\boldsymbol{D}$ = desired absolute precision

The **Pexp** (expected prevalence) of piroplasmids in dogs from Paraguay is not known. There are no previous o pilot studies in the country. The suitable procedure in this cases is to consider a prevalence of 50%, which gives the maximum sample size (Sampaio, 2002; Thrusfield, 2007).

Then, $\boldsymbol{P_{exp}}$ should be = 50%
With a $\boldsymbol{D}$ (desired absolute precision) of 5%,
The mathematical formula should be:

$$n = 1.96^2\ 0.5(1\text{-}0.5)/0.05^2$$

n = 384

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 Asunción locations (Santísima Trinidad, Recoleta, Lambaré, San Roque, La Catedral and La Encarnación) or Neighborhood Units (NU) (NU: i.e., geographically defined areas with relatively homogeneous socioeconomic characteristics), using data provided by the Asunción municipality in order to acquire a balanced cohort. In order to acquire a representative sampling from all Asunción NU, a stratified sampling was performed accordingly to the total number dogs living in each NU and its proportion to the overall dog population in the city, which is detailed in results section of the manuscript (Table 2).

The samples were taken from: (1) dogs during home visits; and (2) dogs admitted to the Veterinary Clinics of Asunción. The dogs were sampled regardless of age, sex, health or reproductive status. Each owner signed a consent form before the samples were taken. Blood samples were collected aseptically by cephalic or jugular venipuncture, collected in EDTA collecting plastic tubes (Vacutainer®), and stored at -20 °C until PCR testing.

2.2. DNA extraction/purification

Frozen EDTA-blood samples were thawed at room temperature and vortexed. DNA extraction and purification from 200 µL of blood was performed using an "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). For every 20 extractions, nuclease-free water (Thermo Scientific©, USA) was used as template to verify possible contamination. DNA was stored at −20 °C prior to performing PCR assays.

2.3. Endogenous conventional PCR

The *RPS19* gene was used to detect canine genomic DNA in order to check the integrity of the DNA template by using the primers RPS19-F y RPS19-R (Brinkhof et al., 2006). The reaction mixture for *RPS19* was composed of 12.5 µL Gotaq® Green Master Mix (Promega®, Madison, USA), 400 nM of each primer (RPS19-F and RPS19-R), 0.5 mM of $MgCl_2$ and 5 µL of template DNA brought to a total volume of 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.

2.4. Nested PCR (nPCR) for piroplasmids

All 384 samples were tested by a nested PCR (nPCR) protocol, as previously described (Jefferies, R., Ryan, U.M., Irwin, 2007), for amplifying a partial region of the 18S rRNA gene of piroplasmids (*Babesia* spp.; *Theileria* spp. and *Rangelia* sp.). This nPCR has an analytical sensitivity of 2.7×10^{-6} for the primary round of amplification and 2.7×10^{-7} for the second one (Jefferies et al., 2007). A set of external primers was BTF1 and BTR1 (930bp), and internal primers BTF2 and BTR2 (800bp) were used. The reaction mixture was composed of 12.5 µL Gotaq® Green Master Mix (Promega®, Madison, USA), 200 nM of each primer (BTF1 and BTR1) and 5 µL of template DNA brought to a total volume of 25 µL with nuclease-free water (Thermo Scientific©, USA). For the primary round of amplification the thermal cycling protocol was as follows: 94 °C for 3 min followed by 45 cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 30 s. Amplification was completed with a final extension of 72 °C for 3 min.

The same conditions were used for the secondary round of amplification, except the set of primers (BRF2 and BTR2) and the annealing temperature which was increased to 62 °C, using 5 µL of the DNA amplicon from the primary reaction. All PCR runs were performed with nuclease-free water (Thermo Scientific©, USA) as a negative control. *Babesia vogeli* genomic DNA obtained from a naturally infected dog was kindly provided by Dr. Marcos Rogério André from Universidade Estadual Paulista, UNESP Jaboticabal, Brazil, and used as a positive control.

All reactions were performed in a T100 TM Thermal Cycler (Bio-Rad, USA). nPCR 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, nPCR amplification, and electrophoresis were performed in three separate rooms to avoid cross contamination. The summary of primers used in this study is shown in Table 1.

All 18S rRNA nPCR positive products (n = 23) were purified by enzymatic reaction (ExoSAP-IT™ PCR Product Cleanup Reagent) (Thermo Scientific©, USA) following the manufacturer's instructions, and sent to Macrogen® (Korea) for sequencing by the Sanger method (Sanger et al., 1977) in both directions, and evaluated by the ABI's sequence analysis software (ABI, Carlsbad, CA). The obtained sequences were analyzed initially by BLAST through the NCBI's Mega-BLAST algorithm (Altschul et al., 1990). In order to correctly determine nucleotide composition, the electropherograms were submitted to PhredPhrap analysis (Ewing et al., 2005), with the Phred quality score (peaks around each base call) established as higher than 20 (99% in accuracy of the base call). Final consensus sequences were submitted to

Table 1
Summary information on the different primer sets and product sizes used in nested PCR assays performed in this study.

Primers	Target-gene	Sequence (5´- 3´)	Product size pb	Reference
RPS19 R	RPS19	CCTTCCTCAAAAA/GTCTGGG	100	(Brinkhof et al., 2006)
RPS19 F		GTTCTCATCGTAGGGAGCAAG		
BTF1 (external)	18S rRNA	GGCTCATTACAACAGTTATAGCCCAAAGACTTTGATTTCTCTC	930	(Jefferies et al., 2007)
BTR1 (external)		CCGTGCTAATTGTAGGGCTAATACGGACTACGACGGTATCTGATCG		
BTF2 (internal)	18S rRNA	GGCTCATTACAACAGTTATAGCCCAAAGACTTTGATTTCTCTC	800	(Jefferies et al., 2007)
BTR2 (internal)		CCGTGCTAATTGTAGGGCTAATACGGACTACGACGGTATCTGATCG		

GenBank (Benson et al., 2004). Percentages of identities were obtained using BLASTn.

A Neighbor-Joining phylogenetic tree was constructed using MEGA version 7 (Altschul et al., 1990; Benson et al., 2004; Kumar et al., 2016). The best nucleotide substitution model was chosen using the MEGA 7 software (Kumar et al., 2016), under the Akaike information criterion (AIC). The data set was analyzed 1000 times to generate the Bootstrap values.

In order to determine overall piroplasmid prevalence in dogs from Asunción, Paraguay, the PCR-positive dogs were divided by the total number of animals and multiplied by 100. The observed prevalence was expressed in percentages, and the 95% IC was calculated.

3. Results

All 384 DNA samples (Mean and Standard Deviation (SD) of DNA concentration = 26.06 ± 6.54 ng/uL; mean and SD 260/280 ratio = 1.80 ± 0.15) were positive for the *RPS-19* reference gene and there was no amplification of negative controls. Overall the molecular prevalence of piroplasmids in dogs from Asunción using 18 s rRNA nPCR was 6% (23/384 [CI 95% = 3.6–8.4%]). Positive dogs were found in all geographic sampled areas, with the following occurrence by sector: Santísima Trinidad 4% (4/93); Recoleta 4% (3/83); San Roque 7% (6/84); La Encarnación 8% (4/52); Catedral 9% (4/45); Lambaré 7% (2/27) (Table 2).

All 23 18S rRNA-nPCR positive samples were sequenced. BLAST and phylogenetic analyses supported the identification of 91% (21/23) products (~715 - 765bp and two sequences with ~368 - 420bp) as *B. vogeli,* (GenBank accession numbers MH100702-MH100704, MH100706, MH100708-MH100724), showing 98–100% identity with *B. vogeli* sequences from dogs (GenBank accession numbers KT333456 and DQ297390); 4% (1/23) products (~785bp) as *T. equi* (GenBank accession number MH100725) being its closest identity (100%) *T. equi* sequences from horses (GenBank accession numbers JX177672 and JX177670); and 4% (1/23) as *Rangelia* sp. (GenBank accession number MH100726) (~740pb) with a 95% identity to other *R. vitalii* sequences from dogs in Argentina and Brazil (GenBank accession numbers KT288200 and KT288203), with a high query coverage (100%). The highest prevalence was observed for *B. vogeli* (5.5% [21/384]), followed by *T. equi* (0.3% [1/384]) and *Rangelia* sp. (0.3% [1/384]) (Table 2).

Table 2
Dog population, number of sampled dogs and prevalence of piroplasmid infection in domestic dogs (n = 384) from Asunción sectors, Paraguay.

Locality	Dog Population Number/ percentage	Sampled dogs (n)	Piroplasmid's prevalence
La Encarnación	24,086 (13.5%)	52	8% (4/52)
La Catedral[1]	21,078 (11.8%)	45	9% (4/45)
San Roque[2]	39,070 (21.8%)	84	7% (6/84)
Lambaré	12,747 (7.1%)	27	7% (2/27)
Recoleta	39,015 (21.7%)	83	4% (3/83)
Santísima Trinidad	43,240 (24.1%)	93	4% (4/93)
TOTAL	179,236 (100%)	384	6% (23/384)

1.*Theileria equi* positive dog was from La Catedral; 2. *Rangelia* sp. positive dog was from San Roque.

Babesia vogeli from Paraguay clustered with *B. vogeli* sequences detected in dogs, cats and ticks from Brazil (AY371195, KT333456 and KT323935), Australia (MG758132), and China (MG586234, MG586235). *Theileria equi* was closely positioned to *T. equi* sequences from horses in Brazil (KU240068 and JX177672) and *Theileria* sp. in domestic cat from Brazil (KP410273). Finally, *Rangelia* sp. detected in this study (MH100726) was closely positioned to *R. vitalii* sequences from dogs in Argentina (KF218605 and KF218606) and Brazil (KT323931 and KT288200) (Fig. 1).

4. Discussion

Canine piroplasmosis is an emerging disease that is widely distributed, especially in tropical and subtropical countries (Jefferies et al., 2007). Its geographical distribution is largely determined by the ecological ranges of its vector arthropods (Cardoso et al., 2010; Laha et al., 2015). Studies on piroplasmosis in dogs have been performed throughout the world (Irwin, 2009; Solano-Gallego and Baneth, 2011).

In South America, there are reports of piroplasmids in dogs from Brazil (França et al., 2014; Gottlieb et al., 2016; Lemos et al., 2012; Passos et al., 2005; Soares et al., 2011; Sousa et al., 2013, 2018), Argentina (Eiras et al., 2008, 2014), Uruguay (Soares et al., 2015), Venezuela (Criado-Fornelio et al., 2007), Ecuador (Zoubi et al., 2016) and Colombia (Vargas-Hernández et al., 2012). To the best of our knowledge, *B. vogeli*, *T. equi* and *Rangelia* sp. are reported for the first time in domestic dogs from Paraguay in this study.

Babesia vogeli from Paraguay was very similar to other *B. vogeli* sequences detected in dogs, ticks and cats worldwide, confirming that the *B. vogeli* from Paraguay presented an affiliation with other isolates from different geographical regions. This high degree of similarity has been described before (Duarte et al., 2011; Gülanber et al., 2006).

The most prevalent piroplasmid species in our study was *B. vogeli*, which is in accordance with results reported in Brazil (Sousa et al., 2013, 2018; Trapp et al., 2006), Tunisia (Rjeibi et al., 2016), Palestine (Azmi et al., 2016), and China (Xu et al., 2015), where *B. vogeli* was the most prevalent piroplasmid, and in Colombia (Vargas-Hernández et al., 2012) where *B. vogeli* was the only piroplasmid detected in dogs. *Babesia vogeli* has the most widespread distribution (Allison et al., 2011; Simões et al., 2011), being present in Europe (Solano-Gallego et al., 2008), as well as in tropical areas or the subtropics of Africa (Inokuma et al., 2004; Matjila et al., 2004), Australia (Jefferies et al., 2003), North and South America (Birkenheuer et al., 2005; Passos et al., 2005; Sousa et al., 2013, 2018).

The prevalence of *B. vogeli* (5.5%) in dogs from Paraguay resembles that observed in Argentina (3–7%) (Mascarelli et al., 2016), Colombia (5.4%) (Vargas-Hernández et al., 2012) and Costa Rica (5.5%) (Rojas et al., 2014). The presence of *R. sanguineus* s.l. has been confirmed in Paraguay, being widely distributed throughout the country (Nava et al., 2007; Pallarés and Usher, 1982), and thus, could be related to piroplasmid transmission. More studies are needed to elucidate the distribution of ticks in dogs and confirm their competence for *B. vogeli* in Paraguay.

Theileria equi is usually found in horses (Beck et al., 2009; Homer

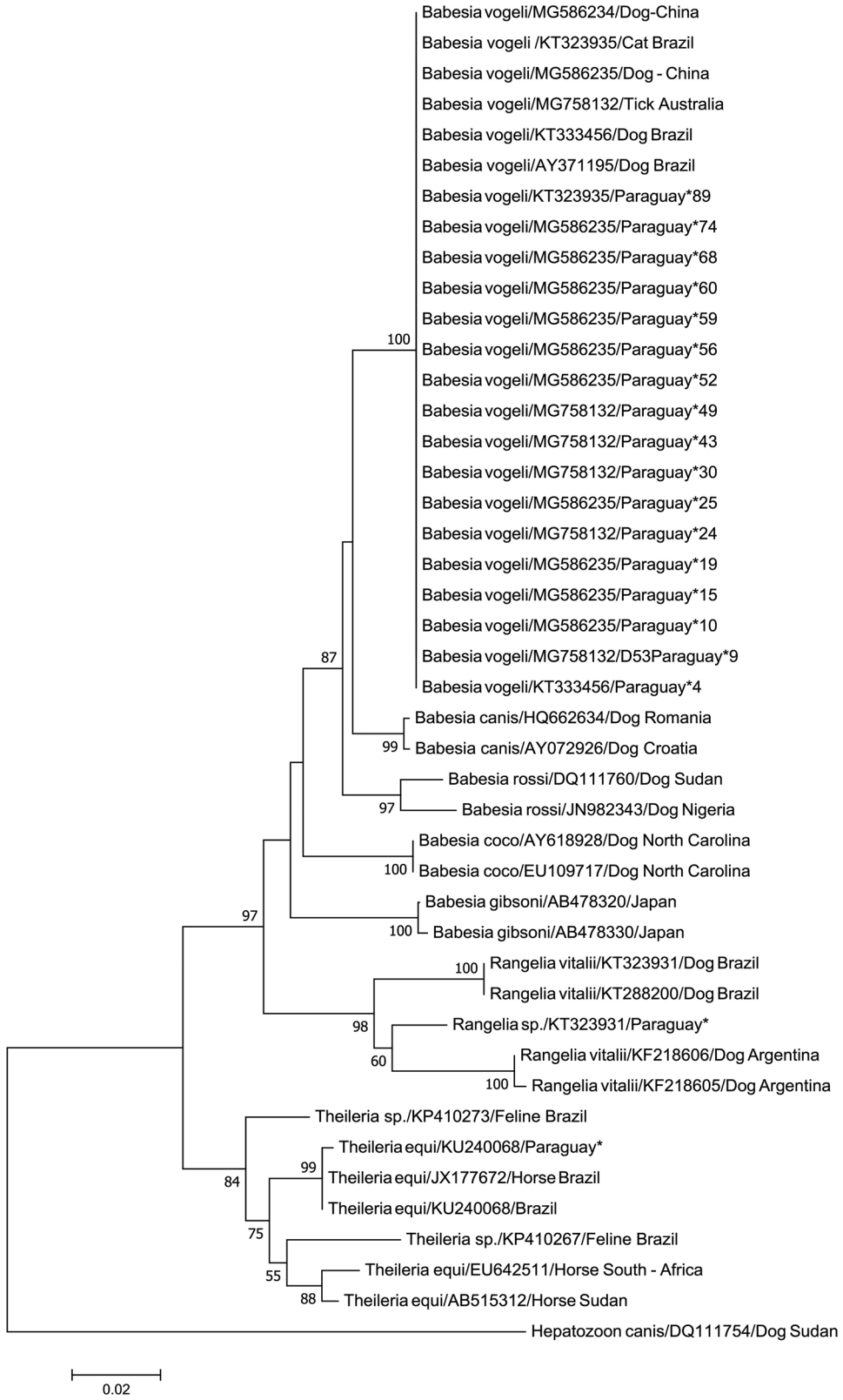

Fig. 1. Neighbor-Joining phylogenetic relationships within species belonging to the Piroplasmorida order based on a ~715-bp fragment of the 18S rRNA. The tree was inferred by using the ML method and evolutive model GTR + G + I. The numbers at the nodes correspond to percentage of supported bootstrap values (1000 replicates). *Hepatozoon canis* was used as an outgroup.

et al., 2000), however, infection with *T. equi* was described in a dog from Croatia (Beck et al., 2009), and previously reported in asymptomatic dogs (n = 3) and in a symptomatic dog (n = 1) from Spain (Criado-Fornelio and Martinez-Marcos, 2003). The clinical signs of theileriosis in dogs are often non-specific, which prevents diagnostic accuracy (Krause, 2003; Quintão-Silva and Ribeiro, 2003; Wise et al., 2013). Low *T. equi* prevalence (4% [3/72]) was also described in dogs from Nigeria (Adamu et al., 2014). Our study presents the first report of

T. equi in a domestic dog from South America.

Theileria equi is transmitted by a series of tick species within the genera *Hyalomma*, *Dermacentor* and *Rhipicephalus*. *Dermacentor nitens* ticks have been found in horses from Paraguay (Pallarés and Usher, 1982; Quinlan et al., 1980) and ticks from the genus *Rhipicephalus* have been confirmed in dogs from Paraguay (Nava et al., 2007).

A genotype of *Theileria* sp. closely related to *T. equi* was found in blood samples from Brazilian cats (André et al., 2014, 2015). *Theileria equi* detected in Paraguay was grouped into the same clade with *T. equi* from horses, which may indicate a genetic relationship between the piroplasmids of these two animal species. This could suggest a spill-over between animal species (Criado-Fornelio et al., 2003). There are no reports of piroplasmids infecting horses from Paraguay, however future studies should aim to identify piroplasmoses and its vectors in horses in the country.

Rangelia sp. detected in Paraguay was grouped with *Rangelia vitalli* from Argentina and Brazil, in a separate branch with 95% identity in 18S rRNA and high query coverage. More studies should be carried out with less conserved genes such as *hsp70* (Soares et al., 2011) and *β-Tubulina* (Zamoto et al., 2004) to elucidate whether it could be a closely related species or a variant of *R. vitalii*.

The prevalence of *Rangelia* sp. in dogs from Paraguay (4%) was lower than that described for *R. vitalii* in dogs from Brazil (6.8%–30%) (Lemos et al., 2012; Soares et al., 2014). *Rangelia vitalii,* has only been detected in dogs from south and southeastern regions of Brazil (Lemos et al., 2012; Lóes et al., 2013; Loretti and Barros, 2005; Soares et al., 2011; Soares, 2014) Argentina (Eiras et al., 2014) and Uruguay (Soares et al., 2015).

Amblyomma aureolatum is the vector of *R. vitalii* in Brazil (Soares et al., 2018). Asunción has a tropical savanna climate with a favorable microclimate for *A. aureolatum* ticks maintenance (Pinter et al., 2004; Rodrigues et al., 2002), being this species of tick described before in San Pedro (Nava et al., 2007), a city located nearby Asunción, 195 miles apart. As the *Rangelia* sp. positive dog had no history of traveling, further studies must check the presence of *A. aureolatum* in Asunción and its vectorial competence for *Rangelia* sp. transmission in the country.

5. Conclusions

The findings of this study revealed the presence of *B. vogeli*, *T. equi* and *Rangelia* sp. for the first time in domestic dogs from Paraguay. Piroplasmids were widely distributed in Asunción city. Those pathogens should be included in the differential diagnosis of dogs exposed to vector-borne pathogens, and thus contribute to the successful implementation of control strategies in the country. Finally, further studies are needed to identify the major vectors of these pathogens in the region.

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Declaration of interest

None.

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