

Molecular epidemiology of *Anaplasma platys*, *Ehrlichia canis* and *Babesia vogeli* in stray dogs in Paraná, Brazil¹

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ABSTRACT.- Ribeiro C.M., Matos A.C., Azzolini T., Bones E.R., Wasnieski E.A., Richini-Pereira V.B., Lucheis S.B. & Vidotto O. 2017. **Molecular epidemiology of *Anaplasma platys*, *Ehrlichia canis* and *Babesia vogeli* in stray dogs in Paraná, Brazil.** *Pesquisa Veterinária Brasileira* 37(2):129-136. Faculdade Educacional de Dois Vizinhos, União de Ensino do Sudoeste do Paraná, Av. Presidente Kennedy 2601, Dois Vizinhos, PR 85660-000, Brazil. E-mail: clau.mribeiro@hotmail.com

Hemoparasitic infections are tick-borne diseases, which affect animals and humans. Considering the importance of canine hemoparasitic infections in veterinary clinics, this study aimed to determine the occurrence of *Anaplasma platys*, *Ehrlichia canis* and *Babesia vogeli* in blood samples from 182 dogs not domiciled in the city of Pato Branco, southwestern region of Paraná State, Brazil, using polymerase chain reaction (PCR). The prevalence of *A. platys* and *B. vogeli* was 32.9% and 10.9% respectively, and *A. platys* infection prevailed ($p < 0.001$). The number of dogs positive for *A. platys* was larger in Winter ($p < 0.05$). All blood samples were negative for *E. canis*. In the dogs, infestation by *Amblyomma cajennense* predominated over that by *Rhipicephalus sanguineus* ($p < 0.001$); but there was no significant association between PCR and the variables presence of ticks, sex and age. Dogs infected by *A. platys* and *B. vogeli* showed thrombocytopenia, lymphopenia and leukocytosis; but there was no correlation between such hematological changes and infection by hemoparasites. This appears to be the first molecular study that demonstrates the existence of *A. platys* and *B. vogeli* in dogs from the southwestern region of Paraná.

INDEX TERMS: *Anaplasma platys*, *Ehrlichia canis*, *Babesia vogeli*, dogs, Paraná, canine thrombocytic anaplasmosis, canine monocytic ehrlichiosis, canine babesiosis, PCR.

RESUMO.- [Epidemiologia molecular de *Anaplasma platys*, *Ehrlichia canis* e *Babesia vogeli* em cães não domiciliados do Paraná.] As hemoparasitoses são enfermidades transmitidas por carrapatos que afetam os animais e os humanos. Considerando a importância das hemoparasitoses caninas na clínica médica veterinária, este estudo ob-

jetivou determinar a ocorrência de *Anaplasma platys*, *Ehrlichia canis* e *Babesia vogeli* em amostras de sangue de 182 cães não domiciliados do município de Pato Branco, região sudoeste paranaense, Brasil, utilizando a reação em cadeia da polimerase (PCR). A prevalência de *A. platys* e *B. vogeli* foi de 32,9% e 10,9%, respectivamente, predominando a infecção por *A. platys* ($p < 0,001$). Constatou-se um maior número de cães positivos para *A. platys* no período do inverno ($p < 0,05$). Todas as amostras de sangue foram negativas para *E. canis*. Nos cães, a infestação por *Amblyomma cajennense* prevaleceu sobre a infestação por *Rhipicephalus sanguineus* ($p < 0,001$), mas não foi observada associação significativa entre a PCR e as variáveis presença de carrapatos, sexo e idade. Cães infectados por *A. platys* e *B. vogeli* apresentaram trombocitopenia, linfopenia e leucocitose, porém não houve correlação destas alterações hematológicas com a infecção pelos hemoparasitas. Este é o primeiro estudo molecular que demonstra a existência de *A. platys* e de *B. vogeli* em cães da região sudoeste paranaense.

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TERMOS DE INDEXAÇÃO: *Anaplasma platys*, *Ehrlichia canis*, *Babesia vogeli*, cães, Paraná, anaplasmose trombocítica canina, erliquiose monocítica canina, babesiose canina, PCR.

INTRODUCTION

Canine tick-borne diseases are caused by bacteria or protozoa that parasitize blood cells and are transmitted by ticks. Among the etiologic agents of hemoparasitic infections, *Ehrlichia canis*, *Anaplasma platys* and *Babesia vogeli* are worthy of note due to their wide geographic distribution and high prevalence (Ramos et al. 2009, Araújo et al. 2015).

E. canis is a Gram-negative bacterium that shows tropism for monocytes and macrophages of dogs. It is the etiologic agent of canine monocytic ehrlichiosis (CME) (Rikihisa 2000), a disease that leads to varied morbidity and mortality rates since it can be acute, subclinical or chronic and manifest as diverse clinical signs such as lethargy, anorexia, pale mucous membranes, fever, bilateral uveitis and thrombocytopenia; in the most serious cases, there is severe anemia (hematocrit < 25%), petechiae, epistaxis and pancytopenia (Shipov et al. 2008, Das & Konar 2013). Transmission of *E. canis* occurs by means of nymphs and adults of the tick *Rhipicephalus sanguineus* (Bremer et al. 2005). *E. canis* is also pathogenic for humans since some strains that cause human ehrlichiosis are genetically and antigenically identical to the strains that cause CME (Perez et al. 2006, Vieira et al. 2013a).

Canine thrombocytic anaplasmosis (CTA) has as etiologic agent the Gram-negative bacterium *A. platys*, which shows tropism for blood platelets (Rikihisa 2000). The epidemiology of CTA has not been fully elucidated yet, and little is known about the vectors involved in the transmission of this bacterium. So far, the probable vector of *A. platys* is *R. sanguineus* since molecular studies have detected the DNA of this bacterium in this tick (Simpson et al. 1991, Sanogo et al. 2003). CTA is characterized by alternate periods of thrombocytopenia which cyclically appear and disappear at every 1-2 weeks (Gaunt et al. 1990, Dyachenko et al. 2012). Most dogs are asymptomatic, but clinical signs such as fever, lethargy, anorexia, pale mucous membranes, petechiae, bilateral uveitis and epistaxis have already been described (Glaze & Gaunt 1986, Caprariis et al. 2011). *A. platys* also has a zoonotic potential since its genetic material was recently detected in a female veterinarian from the USA (Maggi et al. 2013) and in women from Venezuela (Arraga-Alvarado et al. 2014).

In Brazil, *B. vogeli* is the major etiologic agent of canine babesiosis and its transmission occurs by means of larvae, nymphs and adults of *R. sanguineus* (Shortt 1973). In the subclinical form of the disease, animals are asymptomatic. Dogs that develop the clinical form show especially fever, anemia, hemoglobinuria, jaundice and neurological disorders (Salem & Farag 2014, Giroto-Soares & Soares 2015). However, the most severe cases of canine babesiosis are related to co-infection with other hemoparasites (Caprariis et al. 2011).

These pathogens are generally detected based on blood smears or serological tests, which have low sensitivity and specificity, impairing the determination of the prevalence of

such hemoparasites (Silva et al. 2012a, Araujo et al. 2015). Nevertheless, molecular techniques are highly sensitive and specific and can accurately determine the infection status in dogs (Ramos et al. 2009). In Brazil, the prevalence of these hemoparasites, determined by means of molecular methods, can vary from 1.6 to 55% for *A. platys* (Ramos et al. 2009, Lasta et al. 2013, Sousa et al. 2013), from 16.4 to 57% for *E. canis* (Ramos et al. 2009, Silva et al. 2012a, Sousa et al. 2013) and from 3.3 to 57.9% for *B. vogeli* (Jojima et al. 2008, Sousa et al. 2013, Araújo et al. 2015), depending on the geographic region and the studied population.

Considering that hemoparasitic infections are important in the routine of dog medical clinics due to the high casuistry and that there is scarce information about the prevalence of hemoparasites in the southwestern region of Paraná, the present study aimed to determine the prevalence of infection by *A. platys*, *E. canis* and *B. vogeli* among dogs not domiciled in the city of Pato Branco, Paraná State, as well as to assess the epidemiological variables associated with hemoparasitic infections in this region.

MATERIALS AND METHODS

Description of the study area. The dogs evaluated in this study were from the city of Pato Branco, which is in the southwestern region of Paraná (26° 13' 44" S, 52° 40' 15" W) and shows a total area of 539,415 Km²; its population is estimated at 78,136 inhabitants and density at 144,85 hab./Km² (Fig.1). The climate in the region is humid subtropical (Cfa) and the average temperature in the coldest month is inferior to 18°C, while in the hottest month it is above 22°C, showing relatively hot summers, frequent frosts and rainfall well distributed over the year. In the city of Pato Branco, the agricultural sector predominates with corn and soybean planting, poultry and dairy cattle rearing (IBGE 2014, IAPAR 2015).

Dog population. Dogs not domiciled in Pato Branco were captured by the NGO "Associação Lima Protetora dos Animais de Pato Branco" and sent to the Veterinary Hospital of "União do Ensino do Sudoeste do Paraná - UNISEP" for surgical castration. The software Win Episcopo 2.0 was used to calculate the number of dogs necessary to estimate the prevalence of *Anaplasma platys*, *Ehrlichia canis* and *Babesia vogeli* (Thrusfield 2007). The number of samples from animals that showed 5% significance was 127 dogs; however, 182 dogs, male and female, of no defined breed and varied ages, were randomly selected. All dogs were subjected to clinical examination.

Blood samples. From August to November 2014, blood samples were collected from dogs through jugular vein puncture and stored in "vacutainer" tubes containing 10% EDTA. From each sample, 2 mL aliquots were transferred to sterile "ependorf" tubes and kept at -20°C until subsequent use in DNA extraction and PCR.

Complete blood count. The blood samples collected with EDTA were homogenized and subjected to blood cell count in a veterinary hematology counter PochH-100iV Diff (Sysmex®).

Collection and identification of ticks. During blood collection, dogs were examined for the presence of ticks. The found ticks were collected for taxonomical identification according to Aragão & Fonseca (1961).

Extraction and amplification of DNA from *Anaplasma platys*, *Ehrlichia canis* and *Babesia vogeli*. Genomic DNA was extracted from 200 uL blood by using the commercial kit DNeasyBlood & Tissue (Qiagen™), according to the manufacturer's instructions, eluted in 50 µL TE buffer and stored at -20°C until use in PCR.

To detect the DNA of *B. vogeli*, the primers CAN 626R (5'-GAA CTC GAA AAA GCC AAA CGA-3') and CAN 172F (5'-GTT TAT TAG TTT GAA ACC CGC-3') were employed to amplify a fragment of the 18S ribosomal RNA gene (450bp) (Inokuma et al. 2004). PCR was carried out with a final volume of 25 μ L, containing 5 μ L genomic DNA, 0.8pmol of each primer, 200 μ M dNTP, 10X PCR Reaction buffer, 2.0mM MgCl₂ and 1.25U Platinum™ Taq DNA Polymerase (Invitrogen®). The amplification conditions consisted of an initial cycle at 94°C for 5 min, followed by 36 cycles at 94°C for 1 min, annealing at 55°C for 1 min, 72°C for 1 min and a final extension step at 72°C for 7 min.

The primers EcavB9-F (5'-CAT TAT CAT TTC AAT ACG TAA CTC-3') and EcavB9-R (5'-TTT TGA TTT TCT TCT GAC ATA GTG-3') were used to amplify a fragment of the gene virB9 (959bp) from *E. canis*; for *A. platys*, the primers Platys-F (5'-AAG TCG AAC GGA TTT TTG TC-3') and Platys-R (5'-CTC TCC CGG ACT CTA GTC-3') were used to amplify a sequence of the 16S ribosomal RNA gene (504bp) (Silva et al. 2012a). The amplification reactions for *E. canis* and *A. platys* were mounted in a total volume of 25 μ L containing 3 μ L genomic DNA, 0.8pmol of each primer, 200 μ M dNTP, 1X PCR Platinum buffer, 1.5mM MgCl₂ and 1.25U Platinum™ Taq DNA Polymerase (Invitrogen®). The used thermal sequences were: initial cycle at 94°C for 5 min, followed by 35 cycles at 94°C for 1 min, annealing at 58°C for *E. canis* and at 60°C for *A. platys* for 1 min, and final extension at 72°C for 7 min.

As positive control of the reactions, DNA from the target pathogen was employed, while ultrapure water was used as negative control. PCR products underwent electrophoresis on 1.5% agarose gel stained with SYBR® Safe DNA Gel Stain (Invitrogen®) and visualized under UV light.

Sequencing and phylogenetic analysis. Four samples positive for *A. platys* and one sample positive for *B. vogeli* were sequenced with BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems®) and the automated sequencer ABI 3500 Genetic Analyzer (Applied Biosystems®). Multiple sequence alignment was carried out using ClustalW, while phylogenetic trees were built by adopting the Neighbor-Joining method and Mega 7 software (Kumar et al. 2016).

Statistical analysis. Association among epidemiological variables, laboratory tests and hemoparasite prevalence was analyzed according to Chi-square test (χ^2) and Fisher's Exact test, both at 95% confidence interval, using the statistical package

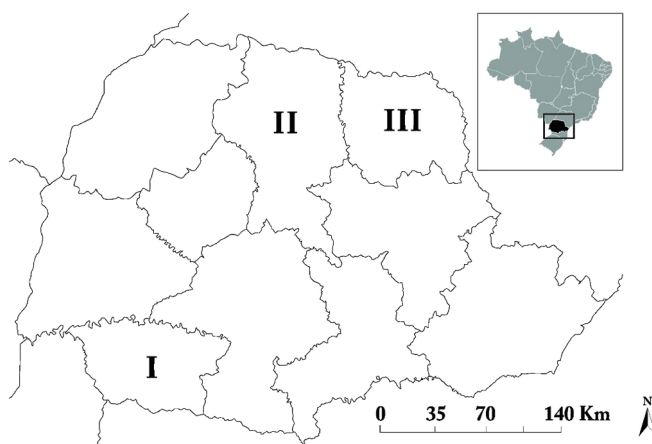


Fig.1. Map of Paraná State, Brazil, showing the southwestern region (I), the site of blood collection from not domiciled dogs, and the central north (II) and pioneer north (III) regions of the state.

GraphPad Prism 6® (GraphPad Prism software, inc. San Diego, CA, USA). All comparisons were made at 5% significance level.

Ethics Committee.- All procedures adopted in the present study were approved by the Ethics Committee on Animal Use of "União de Ensino do Sudoeste do Paraná", Paraná State, Brazil (CEUA-001/2015).

RESULTS

Molecular detection of hemoparasites

Of the studied dogs, 40.1% (73/182) were infected with at least one of the three studied species, while 3.8% (07/182) were co-infected with *Anaplasma platys* and *Babesia vogeli*. Considering the examined blood samples, *A. platys* was detected in 32.9% (60/182) and *B. vogeli* in 10.9% samples (20/182), and infection by *A. platys* predominated over that by *B. vogeli* ($p < 0.001$). No blood sample was positive for *Ehrlichia canis*.

Seasonal variability was not noted for *A. platys* or *B. vogeli* infection ($p > 0.05$); however, in August, there was a

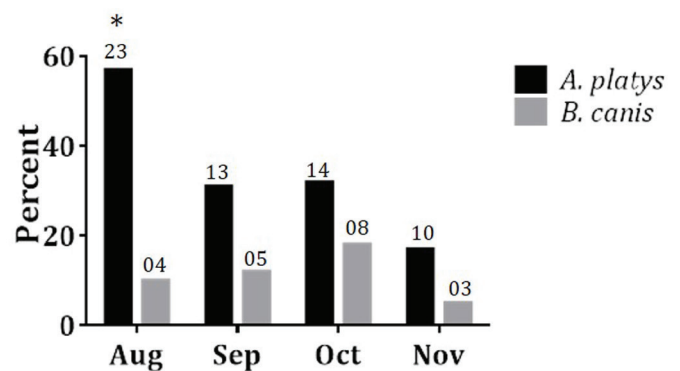


Fig.2. Percentage of dogs infected by *Anaplasma platys* and *Babesia vogeli* per month. Values above each bar indicate the number of animals infected per month. (*) $p < 0.05$.

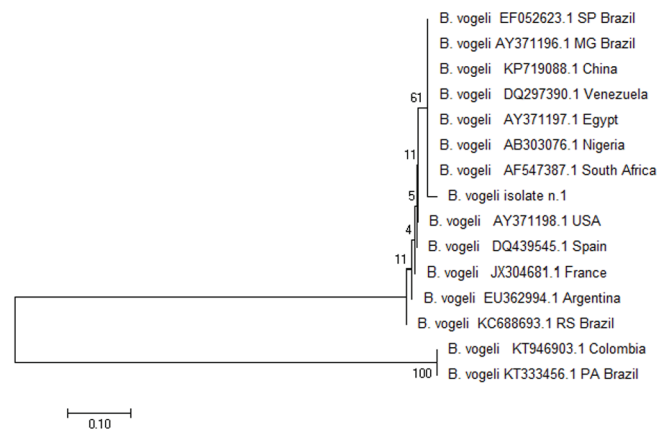


Fig.3. Phylogenetic analysis of partial 18S rRNA gene sequences of *Babesia vogeli* isolated from dogs from the states of Paraná (isolate 1), São Paulo (SP), Minas Gerais (MG), Rio Grande do Sul (RS) and Pará (PA), Brazil, and from dogs from different countries. The optimal tree in which the sum of branch length is equal to 1.32235987 is presented. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The analysis involved 15 nucleotide sequences, totaling 59 positions in the final dataset. The scale bar represents a difference of 0.10 nucleotides between sequences.

larger number of dogs infected by *A. platys* than by *B. canis* ($p < 0.05$) (Fig.2).

Phylogenetic analysis.

The partial 18S rRNA gene sequence of *B. vogeli* was analyzed in comparison with the sequences deposited in GenBank. The sample *B. vogeli* isolate 1 showed > 99% similarity to the sequences EF052623.1, AY371196.1, KP719088.1, DQ297390.1, AY371197.1, AB303076.1, AF547387.1., AY371198.1, DQ439545.1, JX304681.1, EU362994.1, KC688693.1, KT946903.1 and KT333456.1 (Fig.3).

Alignment of partial 16S rRNA gene sequences isolated from *A. platys* revealed high similarity (> 99%) to the sequences deposited in GenBank under the accession numbers FJ755157.1, JF418996.1, KC989957.1, KJ832067.1, KC109446.1, KF576218.1, KT357373.1, DQ401045.1, KF360842.1, KC525894.1, HE856819.1, JX976181.1, JF683610.1, EU439943.1, LC018183.1, AY821826.1 and AF536828.1. Isolates 1, 2, 3 and 4 of *A. platys* clustered in the same ramification (Fig.4).

Infestation by ticks

Adult ticks were collected from 14.8% (27/182) dogs and identified as *Amblyomma cajennense* and *Rhipicephalus sanguineus*. Considering dogs with ticks, 85.2% (23/27) were parasitized by *A. cajennense* and 14.8% (4/27) by *R. sanguineus*, indicating that *A. cajennense* incidence was greater among the studied animals ($p < 0.001$). Ticks were not found in dogs infected by *A. platys*. Only one dog that was parasitized by *B. vogeli* was also infested by *R. sanguineus* at the moment of visual inspection and tick collection; thus, there was no correlation between presence of ticks and hemoparasite infection ($p > 0.05$).

Hematological changes

After clinical examination, no animal had clinical signs such as fever, pale mucous membranes or lethargy; infections were subclinical. Hematological changes like thrombocytopenia, lymphopenia and leukocytosis were found in dogs in-

fectured by *A. platys* or *B. vogeli*; nevertheless, such alterations were not associated with infection by these pathogens (Table 1) ($p > 0.05$). There were no changes in the complete blood count of dogs co-infected with *A. platys* and *B. vogeli*.

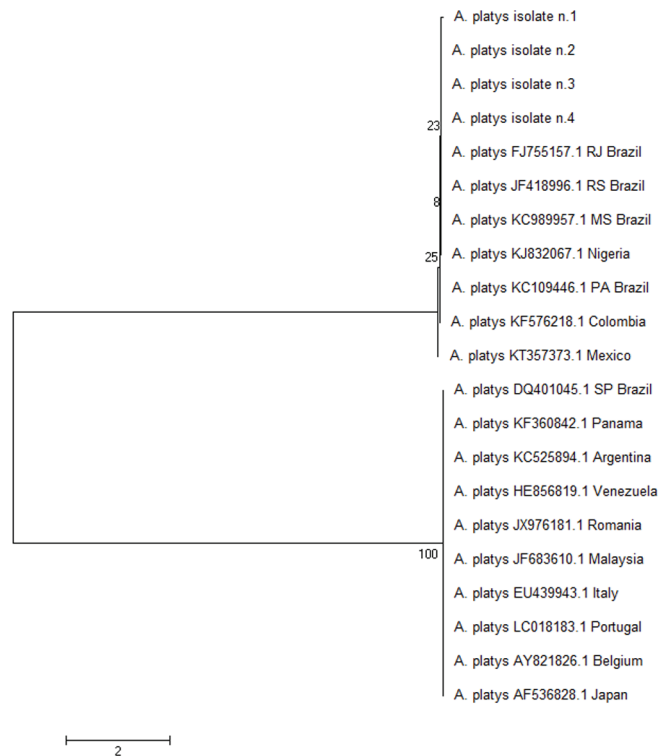


Fig.4. Neighbor-Joining Tree of partial 16S rRNA gene sequences of *Anaplasma platys* isolated from Paraná (isolate 1, 2, 3 and 4), Rio de Janeiro (RJ), Rio Grande do Sul (RS), Minas Gerais (MG), Pará (PA), São Paulo (SP), Brazil, and from countries in different continents. The optimal tree in which the sum of branch length is equal to 16.58608397 is presented. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The analysis involved 21 nucleotide sequences, totaling 235 positions in the final dataset. The scale bar represents a difference of 2 nucleotides between sequences.

Table 1. Frequency of infection by *Anaplasma platys* and *Babesia vogeli* in not domiciled dogs, according to sex, age and hematological changes

	<i>Anaplasma platys</i>					<i>Babesia vogeli</i>						
	Negative		Positive		Statistics	Negative		Positive		Statistics		
	N	(%)	N	%		N	%	N	%			
Female	68	(55.7)	30	(50.0)	0.532 (0.52)	1.27 (0.62-2.31)	86	(53.1)	12	(60.0)	0.342 (0.63)	0.74 (0.2-1,9)
Male	54	(44.3)	30	(50.0)			76	(46.9)	8	(40.0)		
< 5 years old	101	(82.8)	47	(78.3)	0.525 (0.54)	1.33 (0.67-2.82)	129	(79.6)	19	(95.0)	2.768 (0.13)	0.27 (0.1-1.5)
> 5 years old	21	(17.2)	13	(21.7)			33	(20.4)	1	(5.0)		
Thrombocytopenia												
Yes	0	(0.0)	4	(6.7)	8.316 (0.53)	0.59 (0.27-0.96)	2	(1.3)	2	(10.0)	6.363 (0.50)	0.13 (0.10-0.84)
No	122	(100.0)	56	(93.3)			160	(98.7)	18	(90.0)		
Lymphopenia												
Yes	5	(4.1)	24	(40.0)	3.780 (0.43)	0.67 (0.65-0.72)	22	(13.6)	7	(35.0)	6.098 (0.42)	0.21 (0.10-0.81)
No	117	(95.9)	36	(60.0)			140	(86.4)	13	(65.0)		
Leukocytosis												
Yes	2	(1.6)	3	(5.0)	0.731 (0.33)	0.35 (0.19-0.51)	3	(1.8)	2	(10.0)	4.424 (0.49)	0.16 (0.02-1.08)
No	120	(98.4)	57	(95.0)			159	(98.2)	18	(90.0)		

N = number.

Age and sex of dogs

There was no association between positive PCR and the variables sex and age of dogs ($p > 0.05$) (Table 1).

DISCUSSION

Tick-borne pathogens are of great importance for small animal clinics and public health since they are increasingly prevalent among dogs and can infect humans (Jojima et al. 2008, Maggi et al. 2013). In Brazil, there is lack of information about the distribution of hemoparasites and their vectors. This is the first study that evaluates the prevalence of *Anaplasma platys*, *Ehrlichia canis* and *Babesia vogeli* among dogs not domiciled in the southwestern region of Paraná, South of Brazil.

All PCR-tested blood samples were negative for *E. canis*. The absence of *E. canis* in this study differs from the results obtained in other regions of Paraná State. In the city of Jataizinho, pioneer north region of Paraná, *E. canis* prevalence was 16.4%, while co-infection with *E. canis* and *A. platys* was verified for 5.4% of the evaluated dogs (Silva et al. 2012a). *E. canis* was also detected in 21.7% of the dogs showing anemia and attended at a veterinary school hospital in the city of Londrina, central north region of Paraná (Dagnone et al. 2003). Serological tests in the city of Londrina demonstrated that dogs from the urban area were more infested by *R. sanguineus* and had greater seroprevalence for *E. canis* (64.7%) than those from the rural area (25.7%) (Vieira et al. 2013b). Seroprevalence for *E. canis* was also high in the city of Alvorada do Sul (42.4%), central north region of Paraná (Vieira et al. 2013a).

CME is endemic to several Brazilian states, and *E. canis* frequency is high in São Paulo (40%) (Ueno et al. 2009), Mato Grosso do Sul (45%) (Sousa et al. 2013), Bahia (25.6%) (Guedes et al. 2015), Pernambuco (57%) (Ramos et al. 2009) and Paraíba (25%) (Rotondano et al. 2015). Nevertheless, in Rio Grande do Sul, the occurrence of this rickettsia is low or null, although *R. sanguineus* is present (Saito et al. 2008, Lasta et al. 2013).

This difference in *E. canis* occurrence between the southwestern region of Paraná and other regions of this and other Brazilian states could be explained by the genetic diversity of *R. sanguineus* present in this region. Phylogenetic studies have indicated that there are two genetically distinct populations of *R. sanguineus* in Latin America: a tropical and a temperate one (Moraes-Filho et al. 2011). The tropical *R. sanguineus* strain is of African origin and is distributed over the tropical and subtropical America, i.e., north of Argentina, Colombia, Paraguay, Peru, and the Brazilian states Piauí, Pernambuco, Paraíba, Mato Grosso, Mato Grosso do Sul, Minas Gerais, Espírito Santo, São Paulo and Santa Catarina; the temperate *R. sanguineus* strain is of European origin and is present in temperate Latin America, i.e., Chile, Argentina, Uruguay, and the Brazilian state Rio Grande do Sul (Moraes-filho et al. 2011, Nava et al. 2012). *R. sanguineus* from tropical America has high vector competence and is capable of transmitting *E. canis*. On the other hand, *R. sanguineus* from temperate Latin America has low vector competence, not transmitting *E. canis* to dogs (Moraes-Filho et al. 2013).

Cicuttin et al. (2015) demonstrated that both tropical and temperate *R. sanguineus* strains are found in the north region of Argentina. Those authors found the tropical *R. sanguineus* strain infected by *E. canis* in the cities of Ingeniero Juárez, Formosa Province and Coronel Juan Solá, Salta Province; these provinces are at the frontier with Paraguay and Bolivia. For the cities Machagai and Juan José Castelli, both in Chaco Province, and in the city of Oberá in Misiones Province, only temperate *R. sanguineus* strains were found and all these ticks were negative for *E. canis*. Misiones Province is at the frontier with the southwestern region of Paraná, and Oberá City is located at less than 500 Km apart from Pato Branco City. Therefore, it is possible that the temperate *R. sanguineus* strain predominates in the southwestern region of Paraná and that *E. canis* transmission to dogs does not occur, as observed in this study. However, future studies are needed to determine the *R. sanguineus* strain that prevails in this region and its vector competence for *E. canis*. In addition, more blood samples from urban and rural dogs should be analyzed by using primers that amplify fragments from other target genes of *E. canis* in order to conclude whether this pathogen is absent or its occurrence is rare in the southwestern region of Paraná.

In the present study, *B. vogeli* prevalence detected by means of PCR was lower (10.9%) than that obtained in the central north region of Paraná State (23.4%) (Jojima et al. 2008) but similar to that found in Minas Gerais (10.8%) (Costa-Júnior et al. 2012) and higher than those noted for the states of São Paulo (8%) (O'Dwyer et al. 2009), Mato Grosso do Sul (3.3%) (Sousa et al. 2013) and Maranhão (3.3%) (Silva et al. 2012b).

B. vogeli isolate in this study clustered in the same sub-clade as strains found in dogs from the states of São Paulo and Minas Gerais and from countries belonging to different continents, such as Venezuela, Nigeria, South Africa, Egypt and China (Matjila et al. 2004, Passos et al. 2005, Rey-Valeirón et al. 2007, Sasaki et al. 2007, Oliveira et al. 2009, Xu et al. 2015) (Fig.3).

Even though *B. vogeli* was detected, *R. sanguineus* was only found in a few dogs and the tick *A. cajennense* was prevalent ($p < 0.001$). This occurred because the studied dogs were from a predominantly rural area, where *A. cajennense* is abundant; *R. sanguineus* is found especially in urban areas (Costa-Júnior et al. 2013, Vieira et al. 2013a). As a probable consequence, *B. vogeli* frequency was lower than that verified in the central north region of Paraná, a predominantly urban area. These data agree with those obtained by O'Dwyer et al. (2009), who reported that infection by *Babesia* spp. in dogs from rural areas is not as prevalent as in urban areas.

Considering the hemoparasites investigated in the southwestern region of Paraná, infection by *A. platys* prevailed (32.9%) ($p < 0.001$); however, Silva et al. (2012a) detected lower prevalence of this bacterium in the pioneer north region of Paraná, a predominantly urban area. In fact, the frequency of *A. platys* among dogs has already been reported to be greater in the rural than in the urban zone (Costa-Júnior et al. 2013). This bacterium was also

identified in the states of Pernambuco (55%) (Ramos et al. 2009), Minas Gerais (13.9%) (Costa-Júnior et al. 2013), Rio de Janeiro (14.8%) (Ferreira et al. 2007), São Paulo (3.9%) (Dagnone et al. 2009) and Rio Grande do Sul (14%) (Lasta et al. 2013).

In this study, the isolates 1, 2, 3 and 4 of *A. platys* were clustered in one same subclade and had evolutionary homology to strains from the states of Pará, Mato Grosso do Sul, Rio de Janeiro and Rio Grande do Sul and from the countries: Colombia and Nigeria (Fig.4) (Lasta et al. 2013, Rufino et al. 2013, Sousa et al. 2013, Miranda & Mattar 2015).

Co-infection with *A. platys* and *B. vogeli* was verified for 3.8% of the dogs. These results corroborate those found by Otranto et al. (2010) and Caprariis et al. (2011) in Italy and suggest that *R. sanguineus* is the vector responsible for the transmission of both pathogens. However, it is important to consider that in August, a dry winter month, there was a larger number of dogs infected by *A. platys* than by *B. canis* ($p < 0.05$) and, during this period, very few dogs are exposed to infestation by *R. sanguineus*. This tick was considered the major transmitter of *A. platys*, but epidemiological studies have detected the DNA of this anaplasmataceae in a low percentage of *R. sanguineus*, demonstrating that *A. platys* is more prevalent in dogs than in this tick (Brown et al. 2005, Ybañez et al. 2012, Latrofa et al. 2014).

It is possible that other tick species act as vectors of *A. platys*, since molecular tools have detected the DNA of this anaplasmataceae in nymphs and adults of the ixodidea *Haemaphysalis longicornis* and *H. flava*, as well as in larvae and nymphs of *Ixodes nipponensis* (Chae et al. 2008). Other arthropods are also thought to be capable of transmitting *A. platys*. Brown et al. (2005) detected the DNA of *A. platys* in *Heterodoxus spiniger* and suggested that this louse species can be the vector of this bacterium, especially in winter months, because the prevalence of *A. platys* and *H. spiniger* in this season is high.

There was no correlation between infection by *A. platys* and infestation by *A. cajennense* ($p > 0.05$), since no ticks were found in dogs positive for this bacterium. However, it must be considered that *A. cajennense* is a trioxenic tick, which remains in a certain host for a few days to feed on lymph and blood and then returns to the soil for ecdysis. This was the only tick species found in the dogs investigated from mid August to September. In fact, between May and October, when the average temperatures and rainfall are low, larval and nymph instars of *A. cajennense* predominate on the pastures or parasitizing the animals (Oliveira et al. 2000). Thus, we support the hypothesis that *A. cajennense* can act as a vector of *A. platys* in the winter, which must be proven by future studies.

Regardless of sex and age, dogs had equal opportunity to become infected with *A. platys* and *B. vogeli*, and there was no association of these variables with infection by hemoparasites. Molecular methods have demonstrated similar results for *A. platys* in Minas Gerais (Costa-Júnior et al. 2013) and for *B. vogeli* in Maranhão (Silva et al. 2012b).

Thrombocytopenia has been cited as the most frequent hematological change found in CTA dogs, initially due to

the destruction of blood platelets caused by *A. platys* proliferation and then as a consequence of immune-mediated mechanisms (Bodié et al. 2011). Considering canine babesiosis, thrombocytopenia has been reported to manifest especially when there is co-infection with other pathogens (Caprariis et al. 2011, Silva et al. 2012b). However, in the present study most dogs with thrombocytopenia were negative according to PCR, and there was no association between infection by hemoparasites and thrombocytopenia ($p > 0.05$).

Leukocytosis and lymphopenia were observed in dogs positive for *A. platys* and *B. vogeli*; however, there was no association between infection and hematological changes ($p > 0.05$). Nevertheless, leukocytosis due to neutrophilia has been cited as one of the most frequent changes in canine babesiosis (Giroto-Soares & Soares 2015), while some dogs can present leucopenia (Jojima et al. 2008).

CONCLUSIONS

In the present study, infection by *Anaplasma platys* and *Babesia vogeli* was described for the first time in the southwestern region of Paraná based on the detection of DNA of these pathogens in the blood of dogs not domiciled in the city of Pato Branco.

Ehrlichia canis was not found in this region and the dogs were more infested by *Amblyoma cajennense* than by *Rhipicephalus sanguineus*.

Results demonstrate that hematological changes should not be used separately to establish a diagnosis of CTA or canine babesiosis, but should be evaluated in association with molecular methods.

This study provides epidemiological information about the prevalence of pathogens transmitted by ticks in the state of Paraná, Brazil, contributing to small animal clinics in order to establish control and preventive measures against hemoparasitic infection in dogs.

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