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Occurrence of *Babesia vogeli*, *Mycoplasma* spp., *Ehrlichia canis* and *Anaplasma* spp. in a hospital dog population of western Paraná

Ocorrência de *Babesia vogeli*, *Mycoplasma* spp., *Ehrlichia canis* e *Anaplasma* spp. em uma população hospitalar de cães do Oeste do Paraná

Carla Rodrigues Bahiense[1]*; Aldair Calistro de Matos[2]; Hugo Luca Abate[3]; Nelson Jesse Rodrigues dos Santos[3]; Odilon Vidotto[4]

Highlights:
Identifies the frequency of *B. vogeli*, *Mycoplasma* spp., *E. canis,* and *Anaplasma* spp.
Lists *Babesia*, *Ehrlichia*, *Mycoplasma* and *Anaplasma* diagnostic tools.
Discusses the vector's influence on the low occurrence of *E. canis*.

Abstract

Ehrlichia canis, *Anaplasma platys*, and *Babesia vogeli* are frequently detected in the veterinary routine with varied pathogenesis that can lead to death, mainly in co-infections. Although canine hemotropic mycoplasmas are considered of low clinical importance, they have recently gained prominence in molecular research. The present study aimed to analyze two hospital populations of dogs, randomly selected from the year 2013 and 2015, from the municipalities of Toledo (n=68) and Cascavel (n=107). Direct examination of blood smears, serology, and PCR were used to detect the presence of *E. canis*, *A. platys, B. vogeli* and *Mycoplasma sp*. Direct blood smear examination was negative for the investigated agents in all samples. Serum immunochromatography (SensPERT ™, VetAll Laboratories, Korea) in 175 animals showed that only 4% (n = 7) had anti-*E. canis* antibodies, while 60% (n = 105) were positive for *Anaplasma sp*. All PCR samples were negative for *E. canis*, while 18.28% (n = 32) were positive for *A. platys*, 3.42% (n = 6) positive for *Babesia vogeli*, and 7.41% (n = 13) positive for *Mycoplasma sp*. The study revealed the presence of *A. platys* in Toledo and Cascavel by PCR, which accentuates the need to monitor vector populations and usual hosts and to evaluate the potential risk of infection in humans.
Key words: Anaplasmosis. Ehrlichiosis. Hemoparasites. *Rhipicephalus sanguineus*. PCR. Immunochromatography.

Resumo

Ehrlichia canis, Anaplasma platys e *Babesia vogeli* são frequentemente detectados na rotina veterinária com patogênese variada que pode levar à morte, principalmente em coinfecções. Embora os micoplasmas

[1] Dr[a]., Universidade Estadual de Londrina, UEL, Londrina, PR, Brasil. E-mail: cbahiense.vet@gmail.com
[2] Técnico Responsável, Laboratório de Moléstias Parasitárias, Departamento de Medicina Veterinária Preventiva, UEL, Londrina, PR, Brasil. E-mail: aldair@uel.br
[3] Discentes do Curso Doutorado do Programa de Pós-Graduação em Ciência Animal, UEL, Londrina, PR, Brasil. E-mail: hugoabate@gmail.com; nelson.jrsantos@uel.br
[4] Prof., Dr., Departamento de Medicina Veterinária, UEL, Londrina, PR, Brasil. E-mail: vidotto@uel.br
* Author for correspondence

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hemotrópicos caninos sejam considerados de baixa importância clínica, recentemente ganharam destaque na pesquisa molecular. O presente estudo teve como objetivo analisar duas populações hospitalares de cães, selecionadas aleatoriamente no ano de 2013 e 2015, dos municípios de Toledo (n = 68) e Cascavel (n = 107). O exame direto de esfregaços de sangue, sorologia e PCR foram usados para detectar a presença de *E. canis*, *A. platys, B. vogeli* e *Mycoplasma* sp. O esfregaço sanguíneo direto foi negativo para os agentes investigados em todas as amostras. A imunocromatografia sérica (SensPERT ™, VetAll Laboratories, Coréia) em 175 animais mostrou que apenas 4% (n = 7) tinham anticorpos contra *E.canis*, enquanto 60% (n = 105) foram positivos para *Anaplasma* sp. Todas as amostras de PCR foram negativas para E. canis, enquanto 18,28% (n = 32) foram positivas para *A. platys*, 3,42% (n = 6) positivas para *Babesia vogeli* e 7,41% (n = 13) positivas para *Mycoplasma* sp. O estudo revelou a presença de *A. platys* em Toledo e Cascavel por PCR, o que acentua a necessidade de monitorar populações de vetores e hospedeiros usuais e avaliar o risco potencial de infecção em humanos.

Palavras-chave: Anaplasmose. Erliquiose. Hemoparasitas. *Rhipicephalus sanguineus*. PCR. Imunocromatografia.

Introduction

Hemoparasitosis is frequent in the veterinary clinic and has a wide geographical distribution, with a more pronounced presence in regions of tropical and subtropical climates, due to the inherent characteristics of the vectors (Belozerov, 1982). It can affect several species, including humans, which endangers public health (Dantas-Torres, Figueredo, & Brandão, 2006).

Canine monocytic ehrlichiosis (EMC) is caused by the mandatory intracellular bacterium *Ehrlichia canis* (Kuehn & Gaunt, 1985), which normally infects members of the *Canidae* family (Shaw et al., 2005), but has been isolated in humans as well (Perez, Rikihisa, & Wen, 1996). In Brazil, *E. canis* has been identified in several states (R. F. C. Vieira et al., 2011) with variable prevalence, which may exceed 70% of seroprevalence (Melo et al., 2011). The severity of EMC varies according to the strain involved, the host's immune status, and the coexistence of other hemoparasitosis (Sousa et al., 2009). Thus, it may result in the death of the infected animal, which justifies the scientific appeal to preventive methods (Trapp, Messick, Vidotto, Jojima, & Morais, 2006).

Anaplasma platys, a bacterium that triggers canine cyclic infectious thrombocytopenia (Nair et al., 2016), commonly affects canids, but has already been identified in other mammals such as humans (Correa et al., 2011; Rufino et al., 2013; Arraga-Alvarado et al., 2014). Correa et al. (2011) found *A. platys* DNA in 13.18% of domestic cats and suggested it acts as a reservoir, given the absence of clinical signs in positive animals. In Brazil, molecular studies have indicated variable prevalence in dogs, reaching 55% in Paraná (Ramos et al., 2009). It is assumed that transmission occurs through the tick *Rhipicephalus sanguineus* (Machado, Dagnone, & Silva, 2010), despite the experimental failure to reproduce this phenomenon (Simpson et al., 1991).

Canine babesiosis can be caused by *Babesia canis, Babesia vogeli, Babesia Rossi, Babesia caballi, Babesia gibsoni, Babesia conradae* and *Babesia microti*-like sp. (Bilic, Kules, Bariac, & Mrljak, 2018). In Brazil, molecular identification of both *B. vogeli* (Passos, Geiger, Ribeiro, Pfister, & Zahler-Rinder, 2005) and *B. gibsoni* (Trapp et al., 2006) has already been reported. The prevalence of the agent in Brazil can reach 66.9%, as described in Minas Gerais (Dantas-Torres & Figueredo, 2006). A. P. Costa et al. (2015) demonstrated that among dogs from Maranhão, those from urban areas were twice as likely to be positive and that this fact was associated with a higher prevalence of *R. sanguineus* in the declared urban population. In Paraná, blood samples from 282 dogs with clinical signs of babesiosis showed that 105 had DNA *from B. vogeli* (Jojima et al., 2008), whose vector

is *R. sanguineus* (Schnittger, Rodriguez, Florin-Christensen, & Morrison, 2012). Moreover, the *B. gibsoni* vector has already been reported in Brazil, namely in Londrina (Trapp et al., 2006; Jojima et al., 2008). The pathogenesis of babesiosis depends on the species involved (Bourdoiseau, 2006), with *B. vogeli* generally being asymptomatic in adult dogs (Schnittger et al., 2012).

Mycoplasma sp., also known as hemotropic mycoplasmas or hemoplasms, have a higher prevalence when other tick-transmitted pathogens are present (Roura et al., 2010). A study in Italy showed that the prevalence of the agent increased from the north to the south of the country, the warmest region, suggesting climatic influence (Novacco et al., 2010). In Brazil, a study carried out in Ribeirão Preto, SP demonstrated a low prevalence of the agent with only four positives out of 154 dogs tested (one for ‘Candidatus *M. haematoparvum*’ and three for *M. haemocanis*) (Alves et al., 2014). In the Londrina region, 44.7% of dogs from rural areas were positive for hemoplasms, of which 35% were positive for *M. haemocanis,* 20% were positive for Ca. *M. haematoparvum* ‘and the rest for both agents (R. F. C. Vieira et al., 2015).

Both *Mycoplasma* sp. and *Babesia* sp. can be identified by direct microscopy; however, this method has low sensitivity (Bourdoiseau, 2006; Aquino et al., 2016). The different species of *Babesia* sp. and *Mycoplasma* sp. can also be identified by PCR (Jojima et al., 2008; T. S. W. J. Vieira et al., 2013) or by real-time PCR (L. M. Costa et al., 2012; Barker et al., 2004).

Even though A. platys and *Anaplasma* phagocytophilum are routinely diagnosed by light microscopy, this has been replaced by more sensitive and specific techniques, with emphasis on molecular diagnosis (Little, 2010). Traditionally, indirect immunofluorescence (IFAT) has been used to determine antibodies against *E. canis* and, although it can result in false positives (Wen et al., 1997), it is mostly employed to establish prevalence in epidemiological studies. Nevertheless, to nPCR (16S rRNA) and PCR of the disulfide oxidoreductase gene are more indicated to determine the presence of *E. canis* (Aguiar, Saito, Hagiwara, Machado & Labruna, 2007), whereas, for *A. platys*, PCR (16S rRNA) (Eddlestone et al., 2007) or nested PCR (16S rRNA) and nPCR (23SrRNA) (Dahmani, Marié, Mediannikov, Raoult, & Davoust, 2015) are the best options.

Considering the lack of data on *Babesia*, *Mycoplasma*, *E. canis*, and *A. platys* in western Paraná, this study aimed to evaluate the occurrence of these hemoparasites in the hospital populations of dogs in the cities of Toledo and Cascavel.

Material and Methods

Description of the area under study

A total of 175 dogs from the cities of Cascavel (24º ° 57 ‘21″ S; 53º 27’ 19″ W) and Toledo (24º ° 42 ‘49″ S; 53º ° 44’ 35″ W) were evaluated, located in Western Paraná (Figure 1) and characterized by a humid subtropical climate (Instituto Agronômico do Paraná [IAPAR], 2016). Both municipalities have well-defined seasons, Cascavel with a minimum of − 4.2 °C and a maximum of 37.6 °C (IAPAR, 2016) and Toledo with a minimum of − 2.7 ° C and a maximum of 38.0 °C (Instituto Nacional de Meteorologia, 2016).

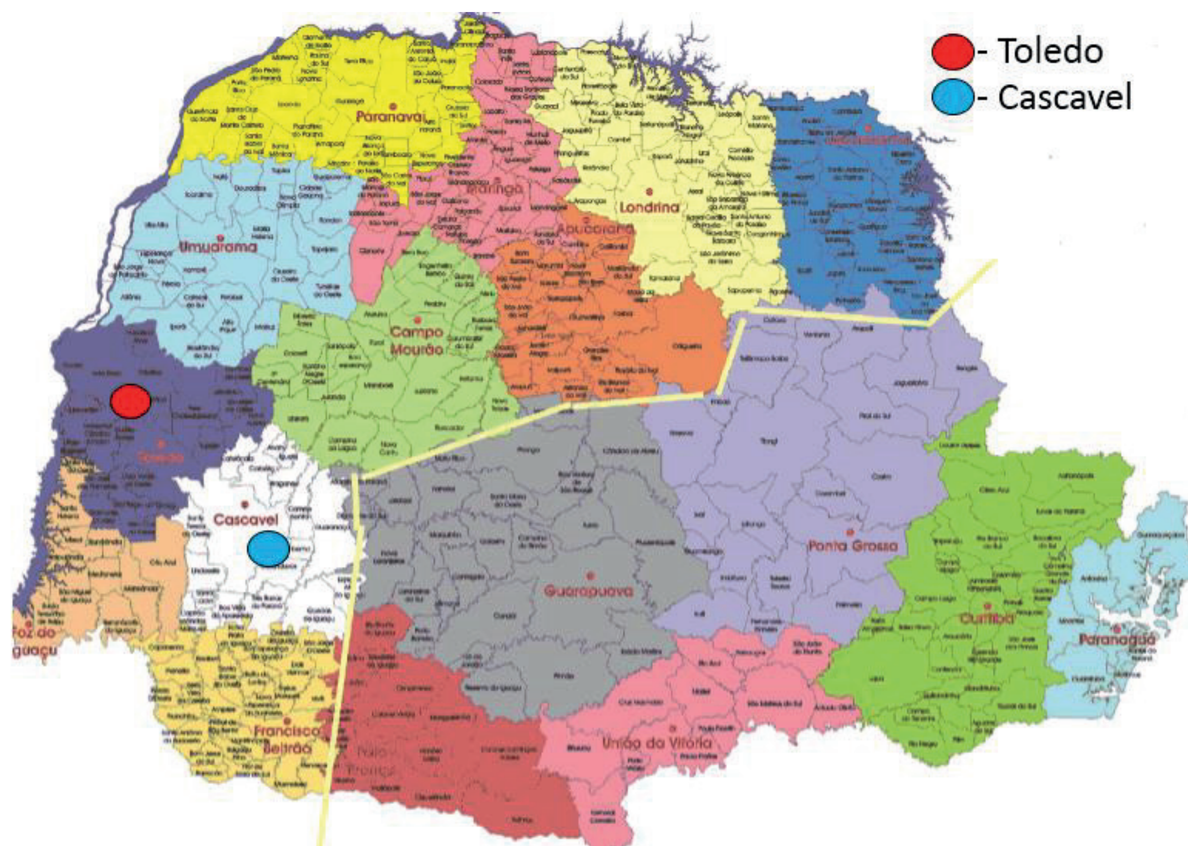

Figure 1. Location of the cities of Toledo and Cascavel located in the western region of the state of Paraná, Brazil, where blood samples were collected from dogs in 2013 and 2015.

Sampling of animals

The animals were randomly selected from two hospital populations, from the Pontifical Catholic University of Paraná (PUCPR) and from the Assis Gurgacz Faculty (FAG), in the Toledo and Cascavel regions, respectively. The sample number was calculated using the Working in Epidemiology 2.0 software, based on the number of annual consultations (n = 1500), a 95% confidence interval, a minimum prevalence of 2.5%, and an error of 5%. With 5% significance, the software revealed 116 was the minimum sample number to represent the population. However, we analyzed samples from 175 dogs, totaling 68 dogs from Toledo and 107 from Cascavel, submitted to descriptive statistical analysis and inference through the chi-square test or Fisher's exact test, with α = 5% and 95% confidence interval. The samples were collected at random - on average, we selected one in five consultations with an indication of venipuncture during the years 2013 and 2015. The project followed the guidelines of the National Council for Animal Control and Experimentation, with approval by the research ethics committee in the use of animals under protocol No. 1005 / 15- PUCPR.

Blood collection

Venous blood samples were fractionated so that 2 mL were transferred to tubes containing EDTA and 3 mL packed in tubes without anticoagulant. A 1 mL aliquot of red blood cells and leukocytes was frozen at −20 ° C for molecular analysis.

DNA extraction and PCR

The sample DNA was extracted from 200 µL of blood cell concentrate using the PureLink Genomic DNA Mini Kit® (Invitrogen) according to the manufacturer's guidelines, which resulted in an eluate of 50 µL that was frozen at -20 °C until its amplification.

Among the samples, 50% were tested using PCR for the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene to ensure that the extraction occurred correctly, as previously described (Birkenheuer, Levy, & Breitschwerdt, 2003).

The *E. canis* virB9 gene (Q6XXL7_EHRCA) was amplified with the primers ECAVB9-F and ECAVB9-R (Felek, Huang & Rikihisa, 2003) and the 16S rRNA gene from *A. platys* with the primer PLATYS-F and PLATYS R (Silva et al., 2012).

The *B. vogeli* 18S rRNA gene was amplified with the primers CAN 626 R and CAN 172 F (Ribeiro et al., 2017), whereas the 16S rRNA gene for the group of hemotropic mycoplasmas with the aid of the primers 16S_HAEMOforw and 16S_HAEMOrev (Hoelzle et al., 2011).

The amplifications were performed with a volume of 25µL, containing 3 µL of DNA, 1.0 µL of each primer, 0.25 µL of Taq polymerase, and 12.5 µL of MIX 1.5. For *E. canis* the thermocycler was programmed to perform 35 cycles (91 ° C for 1 ‘; 94 ° C for 1’; 58 ° C for 1 ‘; 72 ° C for 1’) followed by an extension cycle 72 ° C by 7 ‘; thermal cycles for *A. platys* consisted of 35 cycles (91 ° C by 1 ‘; 94 ° C by 1’; 60 ° C by 1 ‘; 72 ° C by 1’) followed by an extension cycle 72 ° C by 7 ‘; for *B. canis* the thermocycler was programmed to perform 35 cycles (94 ° C for 5 ‘; 94 ° C for 1’; 60 ° C for 1 ‘; 72 ° C for 1’) followed by an extension cycle 72 ° C by 7 ‘; the thermal cycles for hemoplasma consisted of 32 cycles (94 ° C by 2 ‘; 95 ° C by 30 ”; 50 ° C by 30”; 68 ° C by 1’30 ”) followed by an extension cycle72 ° C for 7 ‘.

We used ultrapure water as a negative control and DNA samples from agents previously stored in the laboratory as a positive one, as used in previous experiments (Silva et al., 2012). The products of the PCR amplifications were subjected to electrophoresis on an agarose gel with 6 µg/mL of SYBR® Safe DNA Gel Stain (Invitrogen, CA, USA) and 1.5% bromophenol blue dye for later observation under UV light.

Hemoparasite Serology and Research

Serological tests for *E. canis* and *Anaplasma* sp. were performed using a commercial rapid immunochromatography test (SensPERTTM, VetAll Laboratories, South Korea) on the 175 serum samples, according to the manufacturer's specifications.

The hemoparasite test was carried out in 10 fields on the fringe of each blood smear stained with Romannowsky.

Results and Discussion

Molecular detection

None of the 175 samples from Cascavel and Toledo, Paraná, were positive for *E. canis* (Table 1), despite its high occurrence in most countries (R. F. C. Vieira et al., 2011) and some medical records that report vector infestation. *E. canis* DNA studies, in random samples of canines in the State of Paraná, showed a 16.4% of positive results that in Jataizinho, a city located 400 km to the north (Silva et al., 2012). In Maringá, located 280 km to the north, and Londrina, located 380 km to the north, animals suspected of EMC were sampled and analyzed by PCR, which identified the DNA of *E. canis* in 51.8% and 21.7% of the dogs, respectively (Souza et al., 2011; Dagnone, Morais, Vidotto, Jojima & Vidotto, 2003).

Table 1
Occurrence of *Ehrlichia canis* and *Anaplasma platys* using PCR and serology in dogs from a hospital population in the municipalities of Cascavel and Toledo, Paraná, 2015.

Resultados	PCR *E. canis*	Immunochromatography *E. canis*	PCR *A. platys*	Immunochromatography *Anaplasma spp.*
Canine Positive	0 (0%)	7 (4%)	32 (18.28%)	105 (60%)
Canine Negative	175 (100%)	168 (96%)	143 (81.71%)	70 (40%)
Total	175	175	175	175

In the present study, 37.28% (44/118) of the animals with anemia and/or thrombocytopenia were negative for PCR. This result is in line with that obtained by Gottlieb et al. (2016), who reported that among 58 animals suspected of hemoparasitosis, none of them were positive for *E. canis* in Rio Grande do Sul. Furthermore, Malheiros, Costa, Amaral, Sousa, & André (2016) did not obtain any positive results in 110 dogs from Passo Fundo, RS. Our study disagrees with the estimate that one in five thrombocytopenic animals is infected with the agent (Dagnone et al., 2003), which may result from the low sample number of thrombocytopenic dogs (n = 32 / 27.11%), laboratory characteristics most striking of the infection found in 100% of the positive dogs in the PCR in Botucatu (Ueno et al., 2009), and in 66.7% of the dogs in a survey in Brasília (Cesar, 2008). This discrepancy may be explained by classifying the study areas according to their climates, which suggests that this there are different species of the *R. sanguineus* vector (one temperate and the other tropical), as described by (Moraes et al., 2011)

Of the 175 animals analyzed by PCR for *A. platys*, 18.28% (n = 32) were positive (Table 1). Similar studies have shown lower prevalence, as observed in the Pantanal, where 7.19% of the dogs were positive (Melo et al., 2016) and in Cuiabá, with 9.1% of positive results (Witter et al., 2013). There was a significant difference (p = 0.000028) between the two populations, demonstrating that the dogs of Cascavel (28.03%) were more likely to present the DNA of the pathogen when compared to animals from Toledo (2.94%).

Some populations of *R. sanguineus* collected in Montevideo (Uruguay), Santa Fe (Argentina), and Cachoeira do Sul (RS; Brazil) are not competent as vectors of *E. canis* (Moraes et al., 2015). Based on the fact that Cachoeira do Sul is 750 km south of Cascavel and that a significantly higher prevalence of this pathogen has been reported in the canine population located approximately that same distance to the north (Silva et al., 2010), we suggest that the area under study belongs to a transition band between different R. sanguineus populations that can be competent or not to act as *E. canis* vectors. This may be caused by the subtropical climate of Cascavel and Toledo, a classification in which cities where the existence of these non-competent populations was characterized (Moraes et al., 2015).

Even in the absence of ectoparasites studies in the region and the possibility of other ixodid ticks acting as vectors (Johnson et al., 1998), *R. sanguineus* is probably the most frequent because 83% of the population was declared urban and 74% of these inhabited the interior of their homes. This fact strengthens the theory of a regional vector populations unable to transmit the agent, since a portion of the individuals had vector infestation. A previous study in Argentina showed that *A. platys* prevalence in dogs was three times higher than

that of *E. canis* (Eiras, Craviotto, Vezzani, Eyal & Baneth, 2013). This result is consistent with our work, in which the prevalence of *A. platys* was higher since no sample was positive for *E. canis,* yet another evidence that may be associated with vector characteristics.

We found evidence of *B. canis* DNA in 3.42% of the dogs, and their blood samples were analyzed using PCR with specific primers for the 18S rRNA gene. When comparing to studies with similar conditions, such as random subject collection and PCR as detection method, *B. canis* prevalence in the present study was lower than that found in Rio de Janeiro (11.9%) (Vilela et al., 2013), and similar to that observed in Mato Grosso (3.13%) (Melo et al., 2016). The lower prevalence rates observed in southern regions of Brazil suggest a climatic influence on the vector population, *R. sanguineus*. Although it was isolated in cold climates (Schnittger et al., 2012), this ixodid tick can reproduce up to four generations per year in tropical climatic conditions, which contributes to the population increase (Dantas-Torres & Figueredo, 2006).

Research based on the IFI for *B. vogeli* reveals more discrepant data, as is the case of Minas Gerais (18.8%) (Maia, Costa, Haddad, Passos, & Ribeiro, 2007) and Londrina (36%) (Trapp et al., 2009), which is expected due to the presence of antibodies that remain in the bloodstream after the acute phase of the disease persisting in healthy animals. The sample selection of animals suspected of babesiosis demonstrated that only 37.4% were positive for PCR in Londrina (Jojima et al., 2008), which suggests that even in the face of clinical and laboratory suspicion, differential diagnoses should always be considered.

Only 13 (7.42%) of the 175 samples subjected to PCR for hemoplasma were positive. The prevalence found in this study was higher than that in Ribeirão Preto (2.59%) (Alves et al., 2014), and lower than that observed in the rural area of Londrina (44.7%) (R. F. C. Vieira et al., 2015). In regions where the presence of the *R. sanguineus* vector is uncommon, hemoplasma infection is rare (Kenny, Shaw, Beugnet, & Tasker, 2004); however, no studies have attempted to investigate this in the region under study.

Serology

Only 4% (7/175) of the analyzed samples presented anti-E. *canis* antibodies, as assessed by the Sens PERTTM immunochromatography serological test (Table 1). Another serological test based on ELISA (3dx IDEXX) was validated by Harrus et al. (2002), and when comparing it with positive samples in the IFAT, it revealed 100% specificity and 71% sensitivity. This low sensitivity was attributed to the titration, indicating the author to repeat the exam in 1-2 weeks to increase the sensitivity of the test. Belanger et al. (2002) performed a similar comparative study with similar results.

In Brazil, the seroprevalence of *E. canis* in regions close to the Equator line reaches 72.5%, as is the case in Paraíba (Azevedo et al., 2011). In Cuiabá, located in the central-west region, seroprevalence is 42.5% (Silva et al., 2010). Areas even farther from the Equator line showed a seroprevalence of up to 20.4%, as in Guarapuava, a city with subtropical climate located 250 km from the area under study (Dobrowolski, Gheller, , Carrasco & Seki, 2015). Bowman et al. (2008) demonstrated when analyzing different regions of the USA, that seroprevalence increased in southern regions. Therefore, our results corroborate the recurrent correlation of *E. canis* seroprevalence to climatic factors.

We found antibodies to *Anaplasma* sp. in 60% (105) of the 175 samples evaluated, and only 11 of them were also positive for *A. platys,* as determined by PCR (Table 1). Some hypotheses to explain this discrepancy between positive animals detected by PCR and serology could be suggested. The fact that samples are randomly collected decrease the probability of these animals being in the acute

phase, resulting in a PCR false negative, since IgG in dogs can be found as early as seven days after infection (Nair et al., 2016). In addition, platelet parasitemia is cyclical, with platelet inclusions appearing for a short period during the acute phase of the disease. Eddlestone et al. (2007) used real-time PCR to analyze leukocyte samples as well as and splenic and medullary tissue from dogs that had been experimentally infected with *A. platys.* These animals were monitored for 28 days and tested for *A. platys* DNA between the 4th and the 14th day after infection. The authors concluded that the subsequent negative tests could be the result of the sequestration of infected platelets by phagocytic cells of the reticulum endothelial system.

The manufacturer of the commercial ELISA 3 Dx (IDEXX) reports the possibility of cross-reaction between *A. phagocytophilum* and *A. platys*, which could justify the high number of positives. However, Ferreira et al. (2008) tested 16 PCR positives for *A. platys* with ELISA, resulting in a negative result for *A. phagocytophilum*, which may also be due to the absence of antibodies in the initial stage of the disease. Although the kit they utilized was not from IDEXX, it was also based on immunochromatography.

Hematozoa research

Direct examination of all analyzed samples (n = 118) was negative for both *E. canis* and *A. platys*. The negative results for *E. canis* can be supported by the absence of DNA in 100% of these samples and by the low sensitivity of the technique, which is a dependent examiner. Evidence of *A. platys* was not observed in any of the 34 samples that contained the pathogen's DNA, which is explained by the non-specificity of platelet inclusions and the low or absent parasitemia (Ferreira et al., 2008; Machado et al., 2010). Direct examination can thus result in false negatives, as in the case of a 21% occurrence of *A. platys* against 55% positives identified by nPCR in the same sample number (Ramos et al., 2009), and false positives, as pointed out in a study where only 44.4% of the samples with platelet inclusions were also positive when analyzed by PCR for *A. platys* (Correa et al., 2011).

Conclusion

Using molecular techniques, our work demonstrated the presence of *A. platys, Mycoplasma* sp., *and B. vogeli* in domestic animal dogs from the Cascavel and Toledo region of Western Paraná.

No sample was positive in the direct blood smear examination, evidencing the low sensitivity of this technique to diagnose asymptomatic animals.

This study contributes to the epidemiology of dog hemoparasites, since it reports the occurrence of *E. canis*, *A. platys*, *B. vogeli*, and hemoplasma in dogs domiciled in the western region of Paraná.

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