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Research paper

Tick-borne pathogens in carthorses from Foz do Iguaçu City, Paraná State, southern Brazil: A tri-border area of Brazil, Paraguay and Argentina

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ABSTRACT

Tick-borne diseases (TBD) constitute an important group of illness affecting animals and humans worldwide. In Brazil, carthorses are frequently exposed to ticks and tick-borne pathogens, leading to impairment of horse performance and imposing restrictions by the international veterinary authorities for the importation of horses. Accordingly, this study has aimed to i) determine the prevalence of the TBD agents *Theileria equi*, *Babesia caballi*, *Ehrlichia* spp., and hemotropic mycoplasmas in carthorses, ii) identify the tick species parasitizing the animals, and iii) determine factors associated with exposure/infection in Foz do Iguaçu City, Parana state, southern Brazil. A total of 103 carthorses were screened for anti-*T. equi* and anti-*Ehrlichia* spp. antibodies by indirect fluorescent antibody assays (IFA). Samples were also tested by PCR assays targeting the 18S rRNA gene of *T. equi* and *B. caballi*, and 16S rRNA gene of hemoplasmas. Additionally, PCR assays targeting the 16S rRNA, disulfide bond formation protein (*dsb*) and tandem repeat proteins 36 (*trp36*) genes of *Ehrlichia* spp. were also performed. Antibodies to *T. equi* and *Ehrlichia* spp. were detected in 43/103 (41.75%; 95% CI: 32.10–51.88%) and 5/103 (4.85%; 95% CI: 1.59–10.97%) horses by IFA, respectively. DNA of *T. equi* and *B. caballi* were found in 25/103 (24.27%; 95% CI: 16.36–33.71%) and 10/103 (9.71%; 95% CI: 4.75–17.13%) carthorses, respectively, and all tested negative for *Ehrlichia* spp. and hemoplasmas. All sequences showed ≥99% identity with multiple *T. equi* and *B. caballi* 18S rRNA gene sequences deposited in GenBank. Overall, 191 *Dermacentor nitens* ticks were collected from 25/103 (24.27%) animals. Carthorses older than 5 years were more likely to be positive for *T. equi* ($p < 0.05$). In conclusion, equine piroplasmosis agents are highly prevalent in carthorses from Foz do Iguaçu City. The low prevalence of *Ehrlichia* spp. found may be due to the absence of *Amblyomma* ticks infesting animals, which should be further investigated.

1. Introduction

Tick-borne diseases (TBD) constitute an important group of illnesses affecting animals (Vieira et al., 2013a; Baneth, 2014) and humans in South America (Labruna et al., 2014). TBD of horses are caused by a diverse range of pathogens, including species of piroplasms, *Ehrlichia*, *Anaplasma*, *Rickettsia*, and others (Vieira et al., 2011; Dzięgiel et al., 2013; Souza et al., 2016). Equine piroplasmosis (EP), caused by *Theileria equi* and/or *Babesia caballi*, is the most important group of TBD agents and significantly impacts on horse industry (Friedhoff et al., 1990).

Until the last decade, equine granulocytic anaplasmosis (EGA) caused by *Anaplasma phagocytophilum* (formerly *Ehrlichia equi*) was the only TBD caused by ehrlichial species in horses (Dumler et al., 2001).

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Recently, a potentially novel *Ehrlichia* species infecting horses from Nicaragua and Brazil was detected using molecular assays (O'Nion et al., 2015; Vieira et al., 2016, 2018). However, the clinical impact and the distribution of the *Ehrlichia* sp. infection in this animal species remains unknown.

Molecular detection of hemotropic mycoplasmas (hemoplasmas) has been only described in horses from Germany. The *Mycoplasma* sp. detected was closely related to '*Candidatus* Mycoplasma haemobos' and *Mycoplasma haemofelis* (Dieckmann et al., 2010). A previous study has found that hemoplasma infected horses show unspecific clinical signs such as poor performance, shaggy coat, and apathy, and those < 1 year may develop anemia (Dieckmann et al., 2012). However, the pathogenic potential of hemoplasmas in horses has not been determined. Although previous studies have implicated ticks in the transmission of hemoplasmas (Seneviratna et al., 1973; Neimark et al., 2004), to date there is not enough evidence to support that hemoplasmas are truly a vector-borne pathogen.

Epidemiology of TBD may be influenced by several factors such as urbanization, deforestation, demographic change in developing countries, increased global movement of people and animals, and climate change (Colwell et al., 2011; Dantas-Torres, 2015). Foz do Iguaçu City, Parana State, southern Brazil, is located in the tri-point border of Brazil, Paraguay and Argentina. The city is the third most important tourist destination in this country, due to its natural resources, flora and fauna diversity and ecotourism activities (Souza, 2017; Brasil, 2018). Thus, studies on vector-borne disease (VBD) pathogens are important for monitoring animal health status and public health risks, as horses might act as sentinels. Previous studies on VBD pathogens have been performed in Foz do Iguaçu and reported *Leishmania infantum* in dogs (Thomaz-Soccol et al., 2017) and filarial nematodes in coatis (*Nasua nasua*) and domestic dogs (Moraes et al., 2017). Additionally, hemoplasmas have been detected in coatis (Cubilla et al., 2017a) and non-human primates (Cubilla et al., 2017b), while cervids (*Mazama nana, Mazama Americana, Blastocerus dichotomous*) have tested negative for *Plasmodium* spp. (Santos et al., 2018).

In Brazil, horses are frequently exposed to *Dermacentor nitens, Amblyomma cajennense* sensu lato (s.l.) and *Rhipicephalus microplus* ticks (Labruna et al., 2001), and these species have also been reported infesting horses in Paraguay and Argentina (Nava et al., 2007, 2017). In urban areas of Brazil, carthorses are used daily by low-income households to collect recycling materials throughout the cities. They therefore pose as sentinels for public health and play an important epidemiological role in the spread of ticks and the pathogens they may transmit. Accordingly, the aims of the present study were i) to determine the prevalence of the TBD agents *T. equi*, *B. caballi* and *Ehrlichia* spp., and hemoplasmas in carthorses, ii) to identify the tick species parasitizing the animals, and iii) to determine factors associated with exposure/infection in Foz do Iguaçu city, Parana state, southern Brazil.

2. Materials and methods

This study was approved by the Ethics Committee in Animal Experimentation and Animal Welfare at the Universidade Federal do Parana (UFPR) (protocol number 046/2016) and conducted according to the ethical principles of animal experimentation, adopted by the Brazilian College of Animal Experimentation.

2.1. Study area

Foz do Iguaçu City (25° 32′ 45″ S 54° 35′ 07″ W), with approximately 265,000 inhabitants distributed in 617.70 km^2, is located in the extreme west of Paraná State, on the border of Brazil, Argentina and Paraguay. The region presents a subtropical climate with rainfall throughout the year and an average temperature of 22.1 °C (INMET, 2018). Foz do Iguaçu city is located in the Atlantic rainforest biome and is internationally recognized by protected areas with diverse fauna, with populations of jaguar (*Panthera onca*), monkeys (*Sapajus nigritus*), capybaras (*Hydrochoerus hydrochaeris*), opossums (*Didelphis* spp.), coatis (*Nasua nasua*), as well as a wide variety of fish, reptiles and birds. This region provides the maintenance of various tick species, to which horses are continually exposed.

2.2. Sampling

A total of 103 clinically healthy carthorses (48 males and 55 females) were voluntarily brought by their owners. Animals were physically restrained, and blood samples (5 mL) were collected by venipuncture of the jugular vein using vacuum tubes containing EDTA (BD Vacutainer®, Franklin Lakes, NJ, EUA) for PCR analysis and kept at −80 °C until testing. In addition, blood samples (5 mL) were collected using vacuum tubes containing a serum separator gel (BD Vacutainer® Franklin Lakes, NJ, EUA) and kept at room temperature (25 °C) until visible clot formation; the samples were then centrifuged at 1500 × *g* for 5 min, serum separated and kept at −20 °C for serological testing.

Packed cell volume (PCV) and total plasmatic protein (TPP) were measured by routine centrifugation and refractometry techniques (Weiss and Wardrop, 2011); a PCV of 0.32 L/L or less, and a TPP of 87.0 g/L or more were used as indicators of anemia and hyperproteinemia, respectively, in the carthorses (Weiss and Wardrop, 2011). Tick specimens infesting horses were removed using a commercial hook (O'TOM®/Tick Twister®, Lavancia, FRA), and kept in absolute ethanol-labeled tubes for identification according to morphological taxonomic keys (Barros-Battesti et al., 2006). Tick examination was conducted by close visual inspection, as described elsewhere (Duell et al., 2013). Briefly, the inspection beginning at the head and neck and proceeding systematically down the mane until the tail was reached. Thereafter, tail, perianal region, foreleg, axillary region, ventrum, hindlegs, inguinal region, and external ears were also examined.

An epidemiological questionnaire was given to each owner addressing age, sex, history of previous tick exposure and presence of ticks at the time of sampling were evaluated. The age of the horses was stratified into groups < 5 years, 5–10 years and > 10 years.

2.3. Detection of anti-Theileria equi and anti-Ehrlichia spp. antibodies by indirect immunofluorescent antibody assays (IFA)

Anti-*T. equi* antibodies in carthorse serum samples were evaluated by IFA, as previously described (Vieira et al., 2015a). Samples were considered positive when reacting with dilution ≥1:80 (Baldani et al., 2010). Titers were determined to the highest dilution in which fluorescence was visualized around the parasite (endpoint titers).

Anti-*Ehrlichia* spp. antibodies in carthorse serum samples were tested by IFA using *E. canis* (São Paulo strain) as antigens, with samples considered positive when reacting at a dilution ≥1:40 (Aguiar et al., 2007). Crude antigens were produced by culturing ehrlichiae in DH82 cells, as previously described (Aguiar et al., 2007; Souza et al., 2010). Additionally, considering that *Rhipicephalus microplus* ticks may parasitize horses in Brazil, carthorse serum samples that seroreacted to *E. canis* were also tested by IFA using *E. minasensis* (Cuiabá strain) crude antigens, as previously described (Aguiar et al., 2007). Endpoint titers were determined to the highest dilution in which fluorescence was visualized around the bacteria.

2.4. DNA extraction

DNA was extracted from all horse blood samples using a commercially available kit (Illustra™ blood genomicPrep Mini Spin Kit, GE Healthcare, Chalfont, St. Giles, UK), according to the manufacturer's instructions. Ultra-pure water was used in parallel to monitor cross-contamination in each batch of 20 samples.

2.5. Detection of *Theileria equi*, *Babesia caballi*, *Ehrlichia* spp. and hemoplasmas by conventional polymerase chain reaction

Samples were evaluated using PCR with previously described species-specific primers targeting a portion of the 18S rRNA gene of *T. equi* (392 bp) and *B. caballi* (540 bp) (Alhassan et al., 2005). The PCR mixture contained 2.5 μL of 10x PCR buffer (Invitrogen, Carlsbad, CA, USA), 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP (dATP, dGTP, dCTP, dTTP), 0.2 μM of each primer, 1.0 U of Platinum® *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA), 5 μL of DNA template made up to 25 μL with water. Cycling conditions consisted of a 1 min denaturation at 94 °C followed by 40 cycles of 94 °C for 30 s, 60 °C (*T. equi*) and 55 °C (*B. caballi*) for 30 s and 72 °C for 30 s, with a final extension of 72 °C for 1 min and cooling at 4 °C (SimpliAmp® Thermal Cycler, Applied Biosystems®, Foster City, CA, USA). Horse samples known to be infected with *T. equi* and *B. caballi*, and nuclease-free water were used as positive and negative control, respectively. A conventional PCR for the horse housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) was performed to monitor DNA extraction, as described elsewhere (Birkenheuer et al., 2003).

Samples were also evaluated using PCR with previously described genus-specific primers and protocols targeting portions of the 16S rRNA gene (344 bp) (O'Nion et al., 2015), disulfide bond formation protein gene (*dsb*) (349 bp) (Almeida et al., 2013), and tandem repeat proteins 36 (*trp*36) gene (Aguiar et al., 2014) of *Ehrlichia* spp. Dog samples known to be infected with *E. canis* and nuclease-free water were used as positive and negative control, respectively. Additionally, samples were screened for hemoplasmas using previously described pan-hemoplasma primers targeting the 16S rRNA gene (~900 bp) (Hoelzle et al., 2011). The PCR-hemoplasma mixture contained 2.5 μL of 10x PCR buffer (Invitrogen), 1.5 mM of $MgCl_2$, 0.2 mM of each dNTP, 0.4 mM of each primer, 1.0 U of Platinum® *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA), 5 μL of DNA template made up to 25 μL with water. Cycling conditions consisted of a 3 min denaturation at 94 °C followed by 35 cycles of 94 °C for 45 s, 59 °C for 45 s and 72 °C for 90 s, with a final extension of 72 °C for 10 min and cooling at 4 °C (SimpliAmp® Thermal Cycler). Cat samples known to be infected with *M. haemofelis* and nuclease-free water were used as positive and negative control, respectively. The amplified PCR products were subjected to gel electrophoresis in 1.5% agarose gels for 1 h at 100 V, followed by ethidium bromide staining (5 μg/ml), and were viewed under a 312 nm UV light transilluminator (LTB HE, Loccus do Brasil, São Paulo, Brazil).

2.6. Sequencing and phylogenetic analysis

A fragment of the 18S rRNA gene from three *T. equi* (~392 bp) and two *B. caballi* (~540 bp) isolates were sequenced. PCR products were purified by enzymatic purification (ExoSAP-IT™ PCR Product Cleanup Reagent, Thermo Scientific, Waltham, USA), evaluated by spectrophotometry for concentration and purity (NanoDrop™ Spectrophotometers, Thermo Scientific, Waltham, USA), and sequenced in both directions by Sanger method using 3500 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA). The raw data were trimmed and assembled using Geneious Prime 2019.1.1 (Biomatters Limited, Auckland, NZ), and the consensus sequences were subjected to BLASTn analysis (Altschul et al., 1990) for determining the identity with the sequences deposited in GenBank® database. The nucleotide sequences of *T. equi* and *B. caballi* amplified were submitted to GenBank database under accession nos.: MH053402, MH059519 to MH059522.

The 18S rRNA sequences of *T. equi* and *B. caballi* were aligned using MAFFT 7.110 (Katoh and Standley, 2013) on the Guidance 2 server (Sela et al., 2015) for each gene. The phylogenetic analysis was based on Bayesian Inference (BI) performed in the MrBayes 3.1.2 software (Ronquist and Huelsenbeck, 2003). We run 1,000,000 generations of Monte Carlo Markov chain (MCMC) with one sampling per 100 generations and a 10% burn-in. The substitution model was estimated as F81 + G using jModeltest 2.1.10 (Darriba et al., 2012). Reconstruction was visualized with FigTree 1.4.4 software (Rambaut, 2014).

2.7. Statistical analysis

A non-parametric Mann–Whitney test or a parametric unpaired Student's *t*-test were used to compare the PCV and TPP concentrations between *T. equi* and/or *B. caballi*-infected and non-infected animals. The Chi-square or Fisher's exact test was used to determine the difference between whether individual factors were associated with seropositivity to *T. equi* and *Ehrlichia* spp., or with infection by *T. equi* and *B. caballi*. Odds ratio (OR), 95% confidence interval and p values were calculated for each variable. Results considered significantly different when $p < 0.05$. Data were compiled and analyzed in Epi Info™ software (version 7.1.5, CDC).

3. Results

A total of 54/103 (52.43%; 95% CI: 42.35–62.36%) carthorses were anemic. The mean PCV concentration for carthorses was 0.31 L/L (SD ± 0.04). All carthorses presented normal TPP values, with a mean TPP concentration of 74.6 g/L (SD ± 5.50).

A total of 191 tick specimens (67 males, 74 females, 44 nymphs and six larvae) were collected from 25/103 (24.27%; 95% CI:16.36–33.71%) carthorses, ranging from one to 42 ticks per animal (mean: 7.64 ticks per animal). All adult and nymphal ticks were identified as *D. nitens*.

Anti-*T. equi* antibodies were detected in 43/103 (41.75%; 95% CI: 32.10–51.88%) carthorses by IFA, with reciprocal antibody titers ranging from 80 to 1280. Five out of 103 (4.85%; 95% CI: 1.59–10.97%) carthorse serum samples tested were positive by IFA for *E. canis* with reciprocal antibody titers ranging from 80 to 320. From the total of seropositive animals, 3/5 (60.00%) also reacted to *E. minasensis* antigens by IFA, with reciprocal antibody titers ranging from 320 to 640. Two carthorses were seropositive for *T. equi* and *Ehrlichia* spp. (*E. canis* and *E. minasensis* crude antigens).

The *gapdh* gene was consistently amplified from all DNA samples. When samples were tested for EP agents, a total of 25/103 (24.27%; 95% CI: 16.36–33.71%) and 10/103 (9.71%; 95% CI: 4.75–17.13%) carthorses were positive for *T. equi* and *B. caballi* by PCR, respectively. Two out of 103 (1.94%; 95% CI: 0.24–6.84%) carthorses tested PCR-positive for both *T. equi* and *B. caballi*. The prevalence of *T. equi* by IFA and PCR within each variable evaluated are summarized in Table 1. All 103 carthorses tested negative for *Ehrlichia* spp. and hemoplasmas by PCR. The prevalence of *B. caballi* and *Ehrlichia* spp. within each variable evaluated are summarized in Table 2.

Carthorses 5–10 yrs old were 4.29 times more likely to be PCR-positive to *T. equi* ($p = 0.018$) (Table 1). Additionally, carthorses 5–10 and > 10 yrs old were 3.59 ($p = 0.010$) and 3.75 ($p = 0.022$) times more likely to be seropositive to *T. equi*, respectively. No significant association was found between sex ($p = 0.688$), anemia ($p = 0.774$) or history of previous tick exposure ($p = 0.459$) and positivity to *T. equi* (Table 1).

Phylogenetic analysis based on the *T. equi* 18S rRNA gene sequences detected herein showed two major groups (A and C) (Fig. 2). One *T. equi* sequence was grouped in group A and showed ≥99% identity with multiple *T. equi* 18S rRNA gene sequences deposited in GenBank database, including sequences from Brazil and other countries (Fig. 2). Two *T. equi* sequences were grouped in group C and showed ≥99% identity with multiple *T. equi* 18S rRNA gene sequences deposited in GenBank database, including sequences from Brazil and other countries (Fig. 2).

B. caballi sequences were grouped in group A and showed ≥99% identity with isolates from Brazil and other regions of the world (Fig. 3).

Table 1
Prevalence of *Theileria equi* in carthorses within each variable studied, Paraná state, southern Brazil.

Variable		*T. equi* – PCR					*T. equi* – IFA				
		+/n	(%)	OR	95% CI	p-value	+/n	(%)	OR	95% CI	p-value
Age[a]	> 10	6/22	27.27	2.719	0.67–11.07	0.175	12/22	54.55	3.750	1.18–11.92	0.022
	5–10	16/43	37.21	4.296	1.27–14.47	0.018	23/43	53.49	3.594	1.33–9.73	0.010
	< 5	4/33	12.12				8/33	24.24			
Sex	Male	13/48	27.08	1.200	0.49–2.92	0.688	21/48	43.75	1.167	0.53–2.55	0.700
	Female	13/55	23.64				22/55	40.00			
Anemia	Yes	13/54	24.07	0.878	0.36–2.13	0.774	20/54	37.04	0.665	0.30–1.46	0.309
	No	13/49	26.53				23/49	46.94			
History of tick bite	Yes	12/54	22.22	0.714	0.29–1.74	0.459	22/54	40.74	0.917	0.42–2.00	0.828
	No	14/49	28.57				21/49	42.86			
Presence of ticks	Yes	10/25	40.00	2.583	0.98–6.82	0.051	10/25	40.00	0.909	0.36–2.27	0.839
	No	16/78	20.51				33/78	42.31			

+, Number of positive animals; n, number of samples; 95% CI, 95% confidence interval.

[a] No age data of five animals.

4. Discussion

To the author's knowledge, this is the first study to assess serological, molecular and epidemiological data for TBD agents (*T. equi*, *B. caballi* and *Ehrlichia* spp.) in carthorses from Foz do Iguaçu City, a tri-border area of Brazil, Paraguay and Argentina. Herein, 54.42% carthorses were positive for at least one TBD agent, which are widespread in the city (Fig. 1).

In horses, EP caused by *T. equi* and/or *B. caballi*, is the most important group of TBD agents and significantly impacts on horse industry (Friedhoff et al., 1990). In the present study, 49.5% carthorses were positive for at least one EP agent when combining IFA and PCR. A previous study in carthorses from different regions and biomes (Atlantic Rainforest and Cerrado) of Parana State have found 95% carthorses positive for EP agents (Vieira et al., 2013b). In Brazil, *T. equi* and *B. caballi* are mainly transmitted by *R. microplus* and *D. nitens*, respectively (Scoles and Ueti, 2015), although some evidence suggests that *A. sculptum* (published as *A. cajennense*) ticks are associated with *T. equi* infection (Kerber et al., 2009). Herein, 24.2% carthorses were solely infested by *D. nitens* ticks at the time of sampling. Although Foz do Iguaçu City is located in the Atlantic Rainforest biome, *A. cajennense* s.l. ticks have not yet been found infesting horses in the region, which may be due to the high humidity and cooler temperatures during the winter which might be the limiting factor for the establishment of this tick species (Martins et al., 2016). Additionally, the differences in the prevalence of EP agents between studies may also be due to the diagnostic test used (IFA vs cELISA) (Vieira et al., 2013b).

In the present study, 24.27% and 9.71% carthorses were PCR-positive to *T. equi* and *B. caballi*, respectively, in agreement with previous studies, which have found that *T. equi* infections are more common than *B. caballi* (Rothschild, 2013). EP is typically responsible for a decrease of 20% in PCV, but may fall to 10% or lower (Ambawat et al., 1999; Rothschild, 2013). Although 52.43% horses were anemic, association between anemia and positivity for EP agents was not found ($p = 0.445$). Other important causes of anemia in horses may include equine infectious anemia, clostridiosis, toxicosis, immune-mediated hemolytic anemia, and intestinal parasites (Satué et al., 2014), which unfortunately were not evaluated in the present study.

Theileria equi 18S rRNA gene has been widely used for species identification, phylogenetic, and genotype studies, and three major groups have been reported (Bhoora et al., 2009; Hall et al., 2013; Liu et al., 2016; Manna et al., 2018; Sant et al., 2019; Wang et al., 2019). Herein, two *T. equi* genotypes clustered to groups A and C, as previously described for Brazilian horse samples (Vitari et al., 2019). The genotypes of *T. equi* do not seem to be geographically restricted, since the sequences from Brazil and other countries have clustered together (Fig. 2). In the present study, different *T. equi* genotypes were detected in carthorse samples from the same locality. This may be attributed to the movement of these animals and a subsequent genetic divergence or the genetic recombination of the parasites during the sexual stage development in their tick vectors (Bhoora et al., 2009; Salim et al., 2010).

Babesia caballi 18S rRNA gene sequences clustered into A and B groups (Bhoora et al., 2009; Wang et al., 2019; Manna et al., 2018; Sant et al., 2019). The *B. caballi* 18S rRNA gene sequences found have clustered in group A with sequences from Brazil (Braga et al., 2017; Nogueira et al., 2017), Trinidad (Sant et al., 2019), Montenegro (Davitkov et al., 2016) and China (Wang et al., 2019) (Fig. 3). Although the phylogenetic analysis has showed two *B. caballi* genotypes, *B. caballi*

Table 2
Prevalence of *Babesia caballi* and *Ehrlichia* spp. in carthorses within each variable studied, Paraná state, southern Brazil.

Variable		*B. caballi* – PCR					*Ehrlichia* spp. – IFA				
		+/n	(%)	OR	95% CI	p-value	+/n	(%)	OR	95% CI	p-value
Age[a]	> 10	1/22	4.55	0.345	0.03–3.31	0.638	3/22	13.64	–	–	0.059
	5–10	3/43	6.98	0.544	0.11–2.62	0.460	2/43	4.65	–	–	0.502
	< 5	4/33	12.12				0/33	00.00			
Sex	Male	4/48	8.33	0.742	0.20–2.80	0.747	2/48	4.17	0.754	0.12–4.71	1.000
	Female	6/55	10.91				3/55	5.45			
Anemia	Yes	4/54	7.41	0.573	0.15–2.17	0.512	1/54	1.85	0.212	0.02–1.97	0.189
	No	6/49	12.24				4/49	8.16			
History of tick bite	Yes	3/54	5.56	0.353	0.08–1.45	0.187	1/54	1.85	0.212	0.02–1.97	0.189
	No	7/49	14.29				4/49	8.16			
Presence of ticks	Yes	1/25	4.00	0.319	0.04–2.65	0.444	0/25	0.00	–	–	0.332
	No	9/78	11.54				5/78	6.41			

+, Number of positive animals; n, number of samples; 95% CI, 95% confidence interval.

[a] No age data of five animals.

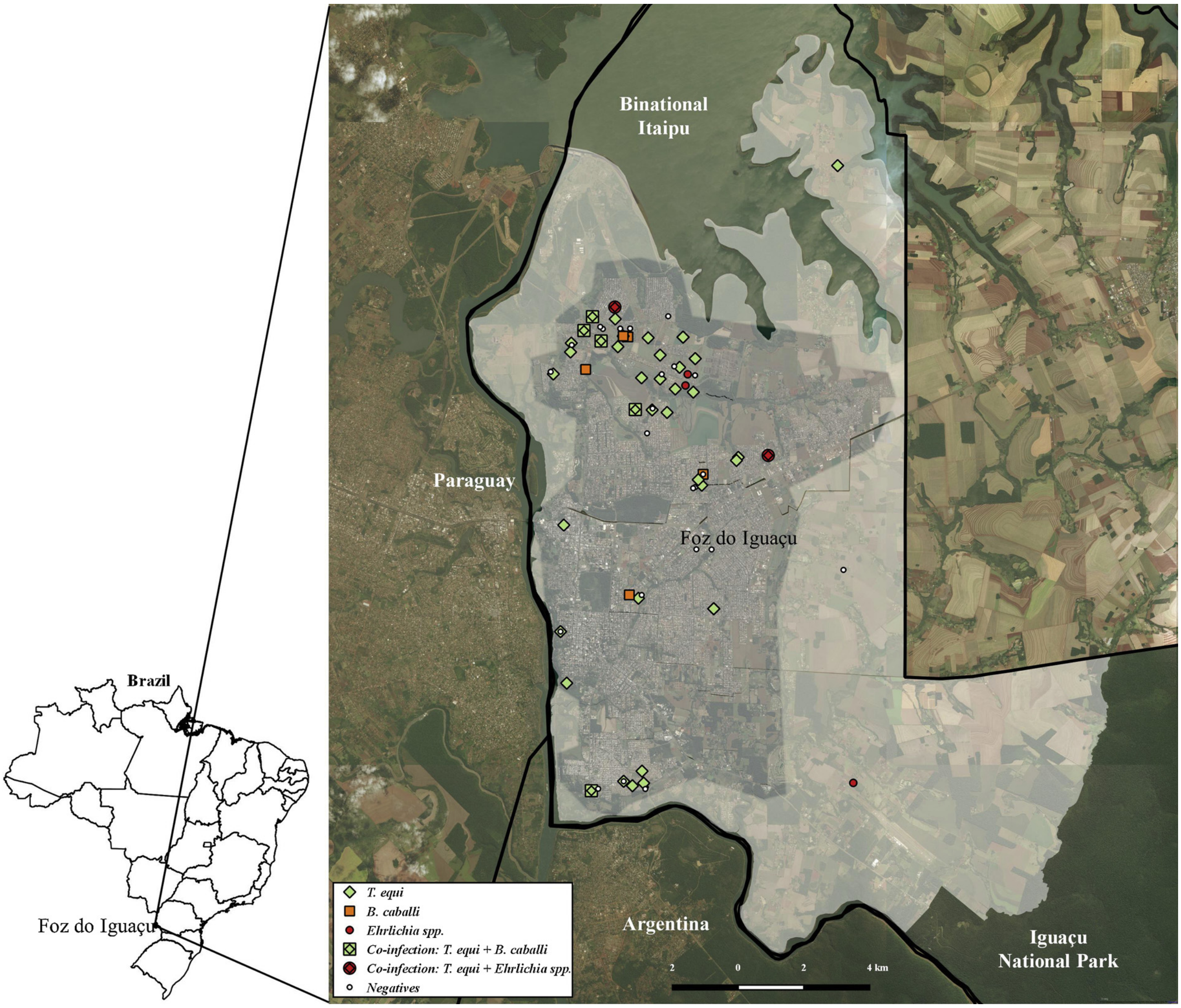

Fig. 1. Map of Foz do Iguaçu City (Paraná state, southern Brazil) showing the location of carthorses sampled and tick-borne disease pathogens occurrence.

sequences have showed less genetic variation when compared to the *T. equi* sequences, as previously described (Bhoora et al., 2009; Braga et al., 2017).

Horses > 5 years were more likely to be seropositive to *T. equi* ($p = 0.004$), in agreement with previous studies (Vieira et al., 2013b; Afridi et al., 2017; Ayala-Valdovinos et al., 2017; Montes Cortés et al., 2017; Stefania et al., 2017; Zhang et al., 2017). This finding may be explained since adult animals may have been exposed to ticks for a longer period than young animals, and thus, more likely to be infected by EP agents (Montes Cortés et al., 2017). Moreover, seroprevalence of *T. equi* increases with age, since animals infected with *T. equi* may become lifelong carriers (Scoles and Ueti, 2015), while infection with *B. caballi* is self-limiting, lasting up to 4 years after infection (Holbrook, 1969).

A potentially novel *Ehrlichia* species has been molecularly detected infecting horses from Nicaragua and Brazil (O'Nion et al., 2015; Vieira et al., 2016, 2018), and serological evidences has suggested that an ehrlichial agent is circulating in horses from Brazil (Vieira et al., 2013a, 2016) and USA (Duell et al., 2013; Carmichael et al., 2014). Although all carthorses tested negative by PCR assays targeting 16S rRNA, *dsb* and *trp36* genes of *Ehrlichia* spp., 5/103 (4.85%) animal serum samples reacted to *E. canis* (reciprocal antibody titers ranging from 80 to 320), and 3/5 also reacted to *E. minasensis* crude antigens (reciprocal antibody titers ranging from 320 to 640). Previous studies with *Ehrlichia* spp. in Brazilian horses have found seroprevalence rates ranging from 27.4% to 62.5%, with seropositive animals presenting higher antibody titers to *E. canis* (reciprocal antibody titers ranging from 64 to 8192) than to *E. chaffeensis* (titers ranging from 128 to 4096) crude antigens by IFA (Vieira et al., 2013a, 2016). It is important to state that higher seroprevalence rates of *Ehrlichia* spp. in horses have been found in areas where *Amblyomma* ticks are present (Duell et al., 2013; Vieira et al., 2013a; Carmichael et al., 2014; Vieira et al., 2016), which may explain the low prevalence found in the present study, and reinforces that ticks from this genus may be involved in the transmission of this ehrlichial agent. Moreover, phylogenetic analysis of the partial sequences of the 16S rRNA, *sodB* and *groEL* genes revealed that the potentially novel *Ehrlichia* sp. found in horses was closely related to *E. ruminantium* and distant from *E. canis*, *E. chaffeensis* and *E. minasensis* (O'Nion et al., 2015; Vieira et al., 2018). Considering that cross-reaction between *Ehrlichia* spp. is a common finding when using serological assays (Holland et al., 1985; O'Connor et al., 2006; Hegarty et al., 2009; Vieira et al., 2016), a multi-modal approach should always be performed in horses as to increase the odds of finding and further characterizing this emerging ehrlichial agent, as previously suggested (Vieira et al., 2016).

To date, hemoplasmas have only been reported in horses from Germany (Dieckmann et al., 2010). In the present study, all horses were negative for hemoplasmas. Along with previous studies, a total of 436 horses (clinically healthy, anemic and/or infested by ticks) from different biomes of Brazil have been screened for hemoplasmas and all tested negative by PCR (Vieira et al., 2015b, c; Ferreira et al., 2016). Considering that the *Mycoplasma* sp. detected in horses from Germany was closely related to '*ca.* M. haemobos' and *M. haemofelis* (Dieckmann

Fig. 2. Phylogenetic tree of *Theileria equi* inferred from partial sequences of the 18S rRNA gene found in carthorses from Foz do Iguaçu, Paraná state, southern Brazil.

et al., 2010), typically found in cattle and cats respectively, hemoplasma infection in horses may be considered accidental or a uncommon infection.

In conclusion, EP agents is highly prevalent in carthorses from Foz do Iguaçu City. The low prevalence of *Ehrlichia* spp. found may be due to the absence of *Amblyomma* ticks infesting animals, which should be further investigated. Hemoplasma infection in horses may be considered accidental or an uncommon infection.

CRediT authorship contribution statement

Jessica D.M. Valente: Data curation, Formal analysis, Methodology, Writing - original draft. **Anna C.B. Mongruel:** Data curation, Methodology, Writing - original draft. **Carolina A.L. Machado:** Data curation, Methodology, Writing - original draft. **Luciana Chiyo:** Data curation, Investigation. **Andre S. Leandro:** Conceptualization, Data curation, Investigation, Methodology, Resources. **André S. Britto:** Data curation, Formal analysis. **Thiago F. Martins:** Formal analysis, Methodology, Writing - review & editing. **Ivan R. Barros-Filho:** Conceptualization, Data curation, Investigation, Methodology, Supervision, Resources, Writing - review & editing. **Alexander W. Biondo:** Investigation, Writing - review & editing. **João H. Perotta:** Conceptualization, Data curation, Investigation, Methodology, Supervision. **Amanda N.S. Campos:** Formal analysis,

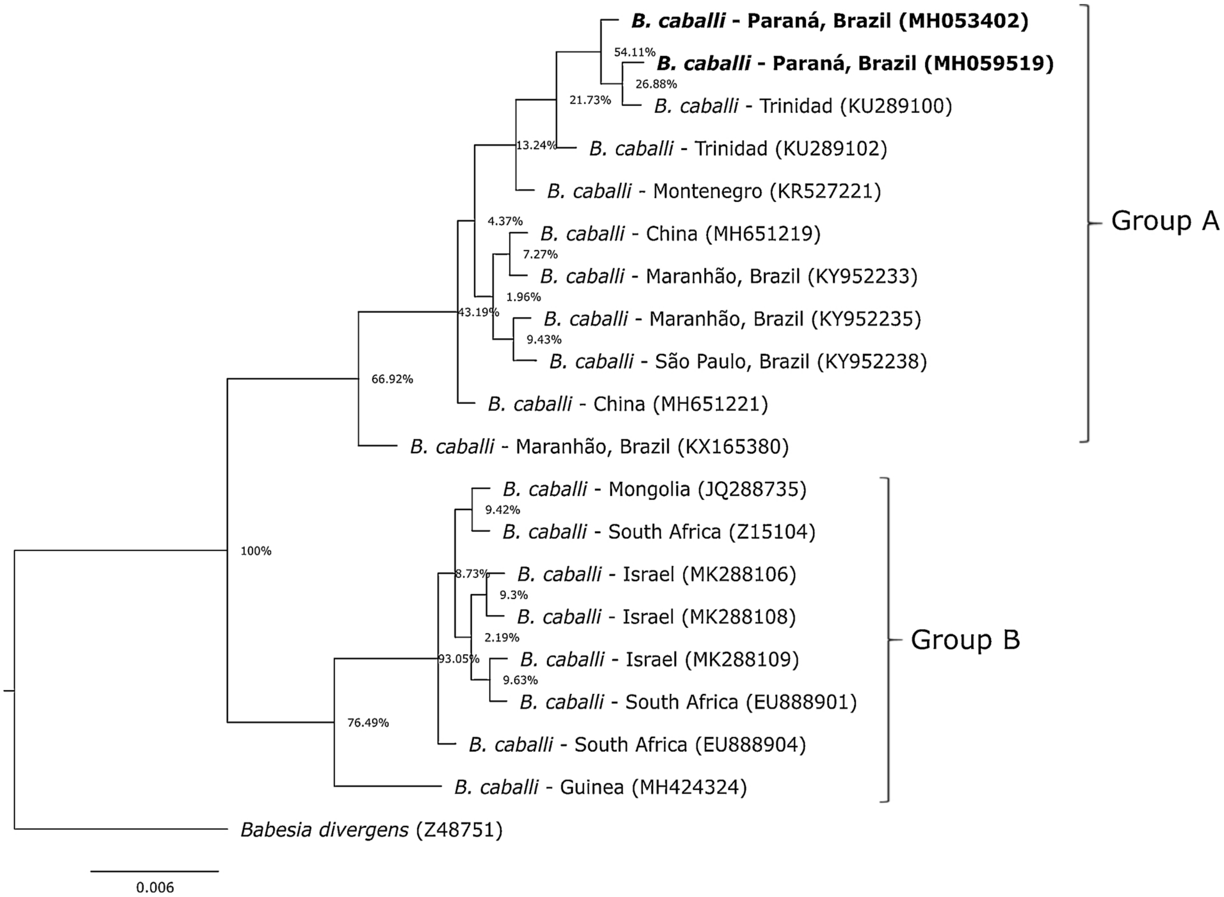

Fig. 3. Phylogenetic tree of *Babesia caballi* inferred from partial sequences of the 18S rRNA gene found in carthorses from Foz do Iguaçu, Paraná state, southern Brazil.

Methodology. **Odilon Vidotto:** Formal analysis, Methodology, Resources. **Marcelo B. Labruna:** Investigation, Writing - review & editing. **Daniel M. Aguiar:** Investigation, Methodology, Resources, Writing - review & editing. **Thállitha S.W.J. Vieira:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing - original draft, Writing - review & editing. **Rafael F.C. Vieira:** Conceptualization, Data curation, Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Resources, Supervision, Writing - original draft, Writing - review & editing.

Declaration of Competing Interest

The authors declared no conflict of interest.

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