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Ehrlichia sp. infection in carthorses of low-income owners, Southern Brazil

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ABSTRACT

Although well established in dogs, *Ehrlichia* sp. infection has been scarcely reported in horses. The aim was to perform a comprehensive serological and molecular survey for the detection of *Ehrlichia* spp. in carthorses from Southern Brazil. Blood samples from 190 carthorses from Paraná State were sampled. Horses were also tested for *Borrelia burgdorferi* and *Anaplasma phagocytophilum*. Anti-*Ehrlichia* sp. antibodies were detected by a commercial rapid ELISA, and immunofluorescence antibody assays (IFA) with *E. chaffeensis* and *E. canis* as crude antigens. The molecular and phylogenetic analysis of *Ehrlichia* sp. was based on 16 S rRNA and *dsb* genes. A total of 52 (27.4%), 4 (2.1%), and 3 (1.6%) horses were positive for *Ehrlichia* spp., *Anaplasma* spp. and *Borrelia burgdorferi*, respectively, by the commercial rapid ELISA. Thirty-eight (20.0%) and 37 (19.5%) horses showed anti-*E. chaffeensis* and anti-*E. canis* antibodies by IFA, respectively. One blood sample that also showed anti-*E. chaffeensis* antibodies was PCR positive for the 16 S rRNA and *dsb* genes of *Ehrlichia* spp., showing an identity of >98.0% to the uncultured *Ehrlichia* sp. previously detected in Brazilian jaguars (*Panthera onca*). Anti-*Ehrlichia* sp. antibodies and *Ehrlichia* DNA were detected in carthorses from Southern Brazil, which may post public health concerns due to intimate contact with low-income owners. This is the first report of a natural infection of this bacteria in horses from South America. Clinical signs and the tick vector remain unknown.

1. Introduction

Ehrlichioses are tick-borne diseases affecting animals and human beings worldwide and caused by at least six bacterial species of the genus *Ehrlichia*: *E. canis*, *E. chaffeensis*, *E. ewingii*, *E. muris*, *E. ruminantium* and *E. mineirensis* [1,2]. Among domestic animals, the disease has been extensively studied in dogs, but mostly neglected in other animal species. In horses, few studies have described the presence of anti-*Ehrlichia* spp. antibodies in serum samples [3–6], and one potentially new *Ehrlichia* species has been described infecting horses from Nicaragua [5].

In Brazil, *E. canis* is the main *Ehrlichia* species found, widely spread in dogs throughout the country [7]. Other identified species include *E. chaffeensis* in marsh deer [8], a possible infection by *E. ewingii* in dogs [9], *Ehrlichia* sp. in a jaguar (*Panthera onca*) [10], and *E. chaffeensis*-like [11] and *Ehrlichia* sp. fox-ES1 [12] in crab-eating foxes (*Cerdocyon thous*). Despite the fact that horses in Brazil are frequently exposed to ticks [13] and are infected by other tick-borne agents [6,14,15], no molecular survey of *Ehrlichia* species infection has been reported to date in such species, particularly in horses with intimate contact with owners. Accordingly, the aim of this study was to perform a comprehensive serological and molecular survey for the detection of *Ehrlichia* spp. in carthorses from low-income owners of Southern Brazil.

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2. Material and methods

2.1. Samples

Blood samples from 190 carthorses previously surveyed for other pathogens [15,16] from low-income owners of Alvorada do Sul (22°54′34.4″ S 51°13′49.1″ W), Colombo (25°25′47″ S 49°16′19″ W), Pinhais (25°26′41″ S 49°11′33″ W) and Curitiba (25°25′47″ S 49°16′19″ W) counties, Paraná state, Southern Brazil was included in this study. All serum and EDTA-blood samples were stored at −80 °C until serological and molecular procedures were performed. This study was approved by the Ethics Committee in Animal Experimentation and Animal Welfare at the Universidade Estadual de Londrina (protocol number 34/2011) and the Universidade Federal do Paraná (protocol number 027/2010), Paraná State, Brazil.

2.2. Detection of antibodies against Anaplasma spp., Borrelia burgdorferi sensu stricto and Ehrlichia spp. by ELISA

All carthorse serum samples were initially tested for antibodies against *Anaplasma* spp. (*A. phagocytophilum* and *A. platys*), *B. burgdorferi* sensu stricto (s.s.), and *Ehrlichia* spp. (*E. canis* and *E. chaffeensis*) using a commercial rapid ELISA test (SNAP® 4Dx®, IDEXX Laboratories Inc., Westbrook, ME, USA), according to the manufacturer's instructions. Although this rapid screening ELISA test has been developed for canine samples [17], the antigen-specific conjugate has been previously validated [18,19] and used for horse samples [3,5,6].

2.3. Detection of anti-Ehrlichia spp. antibodies by indirect immunofluorescent assay

Anti-*Ehrlichia* spp. antibodies in carthorse serum samples were tested by indirect immunofluorescent assay (IFA) using *E. canis* (São Paulo strain) and *E. chaffeensis* (Arkansas strain) as antigens, as previously described [6]. Samples were considered positive when reacting with dilution ≥1:64. Endpoint titers were determined to the largest dilution in which fluorescence was visualized around the bacteria.

2.4. DNA extraction

DNA was extracted from 200 μL of whole blood samples using a commercial kit (Illustra Blood genomicPrep Mini Spin Kit, GE Healthcare, Chalfont, St. Giles, UK), according to the manufacturer's instructions. Ultra-pure water was used as negative control to monitor for cross-contamination in each batch of 30 samples.

2.5. PCR assays and DNA sequencing

A PCR for the horse housekeeping gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH), was performed to ensure successful DNA extraction, as previously described [20]. Samples were evaluated using conventional PCR with genus-specific previously described primers targeting a portion of the *Ehrlichia* 16 S rRNA (344 bp) [21] and disulfide bond formation protein gene (*dsb*) (349 bp) [12]. PCR products of the expected size for each assay were purified from the gel and amplicons sequenced in both directions using the same PCR primers (forward and reverse) by Sanger sequencing.

2.6. Phylogenetic analysis

The generated partial sequence of the *dsb* gene was aligned with sequences from GenBank database using MUSCLE [22].

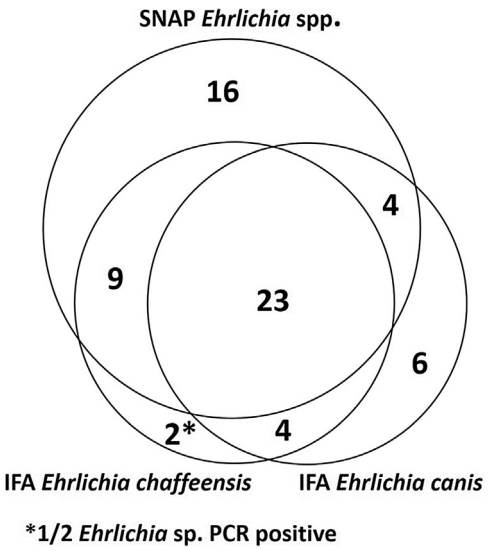

Fig. 1. Proportional Venn diagram showing cross-reactivity by commercial rapid ELISA test (SNAP) and IFA (*E. canis* and *E. chaffeensis* antigens). The diagram was generated by a program available at http://www.cmbi.ru.nl/cdd/biovenn/.

Phylogenetic trees were constructed using the neighbor-joining and maximum-likelihood methods. The Kimura two-parameter method was combined with the neighbor-joining method to correct for nucleotide substitutions. For both methods, data set was resampled 1000 times to generate bootstrap percentages. An available software [23] was used for analyses and the nucleotide sequence of the *Ehrlichia dsb* gene amplified herein was submitted to GenBank database [GenBank: KT148966].

2.7. Statistical analysis

A database was generated and analyzed in a freely available software (Epi Info™ 7.5.1.0, CDC, Atlanta, USA). The Chi-square or Fisher's exact test was used to determine if individual factors (place, presence of ticks, age, or gender) were associated with seropositivity to *Ehrlichia* spp. Odds ratio (OR), 95% confidence interval, and *p* values were calculated separately for each variable. Results were considered significantly different when $p < 0.05$.

The kappa coefficient (κ) of agreement among the serological tests were calculated using a freely available software [24]. The magnitude of κ coefficients was interpreted as follows: ≤0 poor, 0.01–0.2 slight, 0.21–0.4 fair, 0.41–0.6 moderate, 0.61–0.8 substantial, and 0.81–1 almost perfect agreement [25].

3. Results

Seroprevalence values for *Ehrlichia* spp., *Anaplasma* spp., and *B. burgdorferi* by the commercial rapid ELISA and *E. canis* and *E. chaffeensis* by IFA are shown in Table 1. A total of 64 (33.7%) carthorses showed antibodies against at least one of the *Ehrlichia* spp. antigens (*Ehrlichia canis* Outer Membrane Protein, and *E. canis* and *E. chaffeensis* crude antigens), the majority (52/64; 81.2%) being positive by the commercial rapid ELISA. Overlapping to different *Ehrlichia* spp. antigens were also comprehensively analyzed (Fig. 1). Antibody titers by IFA ranged from 1:64 to 1:8192 using *E. canis* antigen and from 1:128 to 1:4096 using *E. chaffeensis* antigen. The seroprevalence of *Ehrlichia* spp. in carthorses based on *E. chaffeensis* antigen within each variable evaluated is summarized in Table 2.

Degree of agreement was moderate ($k = 0.491$, 95% CI: 0.352–0.630) between the commercial rapid ELISA test and IFA with *E. canis* antigen, substantial ($k = 0.624$, 95% CI: 0.485–0.763)

Table 1
Anti-*Ehrlichia* spp. antibodies by commercial rapid ELISA test and IFA in horses from Southern Brazil.

Place	Rapid ELISA						IFA			
	Ehrlichia spp.		*Anaplasma* spp.		*Borrelia burgdorferi*		*E. canis*		*E. chaffeensis*	
	+/N (%)	95%CI	+/N (%)	95% CI	+/N (%)	95%CI	+/N (%)	95%CI	+/N (%)	95%CI
Alvorada do Sul	25/34 (73.5)	55.6–87.1	0/34 (0)	0–10.3	3/34 (8.8)	1.9–23.7	19/34 (55.9)	37.9–72.9	18/34 (52.9)	35.1–70.2
Colombo	11/49 (22.4)	11.8–36.6	4/49 (8.2)	2.3–19.6	0/49 (0)	0–7.3	8/49 (16.3)	7.3–29.7	11/49 (22.4)	11.8–36.6
Pinhais	14/87 (16.1)	9.1–25.5	0/87(0)	0–4.2	0/87(0)	0–4.2	7/87 (8.0)	3.3–15.9	6/87 (6.9)	2.6–14.4
Curitiba	2/20 (10.0)	1.2–31.7	0/20 (0)	0–16.8	0/20 (0)	0–16.8	3/20 (15.0)	3.2–37.9	3/20 (15.0)	3.2–37.9
Total	52/190 (27.4)	21.5–34.7	4/191 (2.1)	0.6–5.3	3/191 (1.6)	0.3–4.5	37/190 (19.5)	14.1–25.8	38/190 (20.0)	14.6–26.4

+, Number of positive animals; N, number of samples per place; 95% CI, 95% confidence interval.

Table 2
Anti-*Ehrlichia* spp. antibodies by IFA (*E. chaffeensis*) in horses within each variable studied.

Variable	+/N (%)	OR	95% CI	*p*-value
Place				
Alvorada do Sul	18/34 (52.9%)	6.37	1.57–25.85	0.0057
Colombo	11/49 (22.4%)	1.64	0.4–6.6	0.3665
Pinhais	6/87 (6.9%)	0.42	0.09–1.8	0.2213
Curitiba	3/20 (15%)	Ref.		
Presence of Ticks				
Yes	16/41 (39%)	3.69	1.7–8.0	0.0005
No	22/149 (14.8%)	Ref.		
Age (Years)				
>10	12/34 (26.1%)	0.72	0.30–1.6	0.4505
5–10	16/79 (20.3%)	1.13	0.4–2.8	0.7936
<5	9/49 (23.4%)	Ref.		
Gender				
Female	21/88 (23.9%)	1.56	0.7–3.2	0.2162
Male	17/102 (16.7%)	Ref.		

+, Number of positive animals; N, number of samples per variable; OR, odds ratio; 95% CI, 95% confidence interval; Ref. variable used as a reference value.

between the commercial rapid ELISA test and IFA with *E. chaffeensis* antigen, and substantial ($k = 0.6512$, 95% CI: 0.509–0.793) between the IFA with *E. canis* and IFA with *E. chaffeensis* antigens.

All carthorse DNA samples consistently amplified the GAPDH gene, ensuring successful DNA extraction. Only 1/190 (0.5%; 95% CI: 0.0–2.9%) sample has yielded amplicons of the expected size for the *Ehrlichia* 16 S rRNA and *dsb* genes by PCR. This animal was seropositive to *E. chaffensis* by IFA only, with a titer of 1:512. Sequencing of the 16 S rDNA fragment (306 bp) showed that this gene region is 98–99% identical to all five *Ehrlichia* species described to date and up to 100% identical to multiple other *Ehrlichia* spp. genotypes deposited in GenBank. Although results confirmed the detection of *Ehrlichia* sp. in carthorses, proper sequence discrimination for phylogenetic analysis solely based in this gene fragment was not possible.

DNA sequence obtained from the *Ehrlichia* sp. *dsb* gene (307 bp) has shown variable degrees of divergence with gene sequences deposited in GenBank. Sequence was closest related to two Brazilian native species: uncultured *Ehrlichia* sp. clone J6 (98.0%; 301/307; HQ388287) from free-ranging jaguars (*Panthera onca*) of the Central-Western region [10] and uncultured *Ehrlichia* sp. clone F2 (84.0%; 254/303; KC127678) from crab-eating fox (*Cerdocyon thous*) of the Southeastern region [12]. In addition, closest identity was also observed with *Ehrlichia ruminantium* (82.0%; 249/305; CR925678) from South Africa [26].

Phylogenetic *dsb* gene fragment analysis confirmed the close relationship of the horse ehrlichial genotype of the present study with the uncultured *Ehrlichia* sp. clone J6 detected in Brazilian jaguars, under >99% bootstrap support. Both genotypes formed a strongly supported clade of >92% bootstrap with the *Ehrlichia* sp. detected in crab-eating fox and *E. ruminantium* (Fig. 2).

4. Discussion

To the authors' knowledge, this is the first report of a natural *Ehrlichia* sp. infection in horses from South America, a likely infection confirmed by DNA detection and sequencing, associated to an antibody titer of 1:512 by IFA with *E. chaffeensis* crude antigen.

Actually, *Ehrlichia* sp. infecting horses was only reported in Nicaragua, Central America, with a novel species revealed by 16 S rRNA, *sodB*, and *groEL* sequencing and gene analysis [5]. Since studies have sequenced different genes and different 16 S rRNA regions, whether the *Ehrlichia* sp. found herein is the same infecting horses in Nicaragua or a novel species remains to be further determined. Interestingly, although with different sequence alignments, both strains have similarly been placed in the *E. ruminantium* (CR925678) clade in Neighbor-Joining and Maximum Likelihood phylogenetic trees (Fig. 2). When compared to similar studies, partial 16 S rRNA sequence in the present study has shown 100% identity with multiple other *Ehrlichia* spp. found in ticks from Asia (KJ410253, HQ697589, AY30997, KF728356, GU075696, and GU0756981). Partial *dsb* gene sequence has revealed *Ehrlichia* species closely related to previously reported in Brazilian free-ranging jaguars (Fig. 2).

In addition to molecular findings, carthorses from Southern Brazil may be highly exposed to *Ehrlichia* spp. infection. This fact is supported since anti-*Ehrlichia* spp. antibodies were detected by two independent serological methods (ELISA and IFA) using three different *Ehrlichia* antigens [p30/p30-1 outer membrane proteins (OMPs) of *E. canis*, and crude antigens of *E. canis* and *E. chaffeensis*], as shown on Venn diagram (Fig. 1). However, it is important to note that serological methods may yield false-positive results because these techniques do not differentiate between infection and previous exposure to *Ehrlichia* sp. On the other hand, only one horse was found to be infected by *Ehrlichia* sp. in the present study. This found may be explained by the fact that false-negative results by

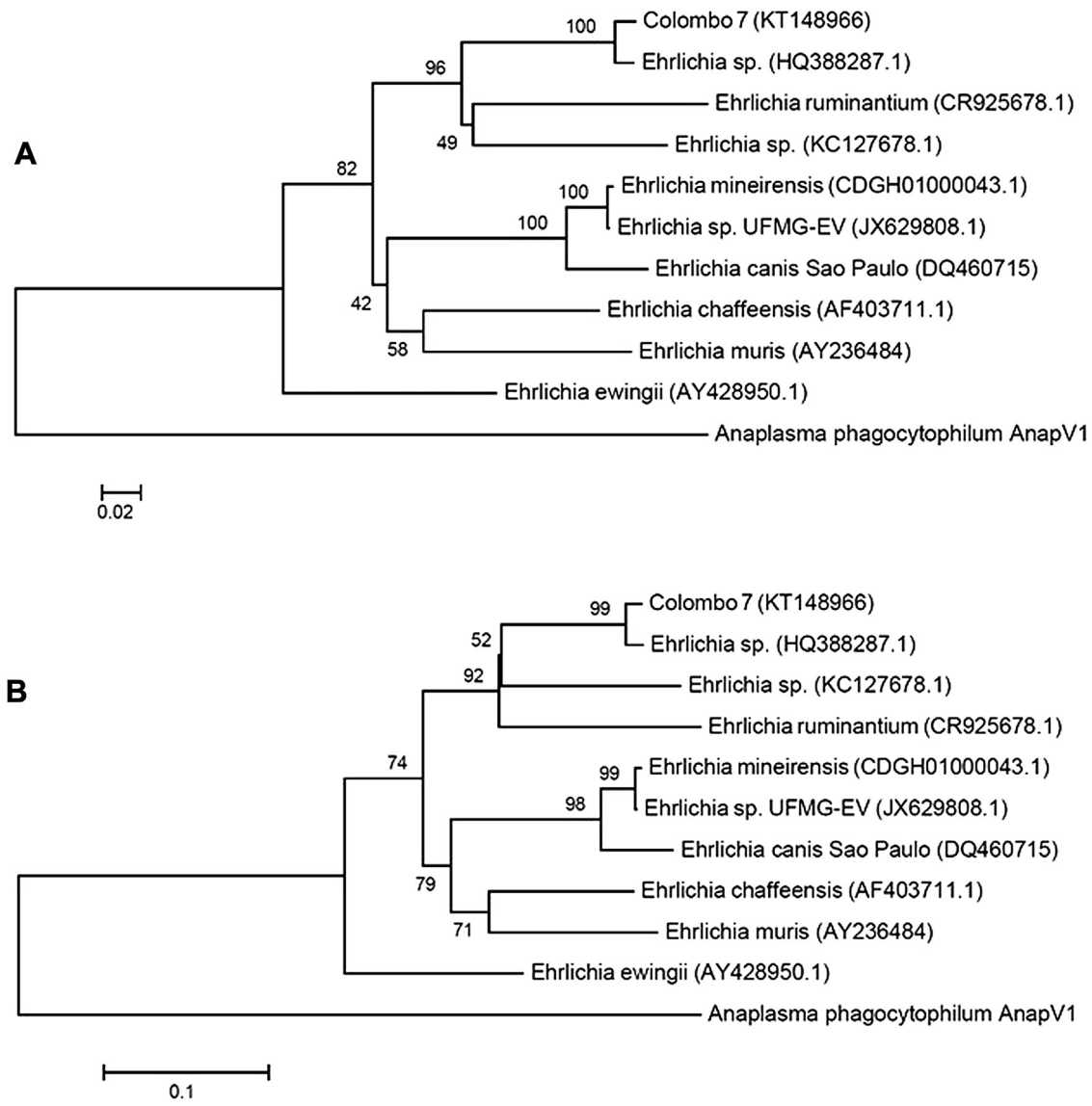

Fig. 2. Phylogenetic trees based on partial sequences of the disulfide bond formation protein (*dsb*) gene, showing the relationship between the *Ehrlichia* sp. detected in the Brazilian horse from this study and other ehrlichial agents. *Anaplasma phagocytophilum* strain AnapV1 was used as an outgroup. The GenBank accession number is in parentheses after the species name of each bacterium. Bootstrap percentage values, obtained from 1000 resamplings, are indicated at each node. A) Phylogenetic tree constructed using the neighbor-joining method with the Kimura two-parameter model. B) Phylogenetic tree constructed using Maximum Likelihood method. Bar, substitution per nucleotide.

PCR in peripheral blood may also occur, as previously described in subclinically or chronically infected dogs [27].

Despite the commercial rapid ELISA kit used in the present study have been developed for screening dog samples [28], this kit has been previously used for screening *Ehrlichia* spp. in horses from Denmark, France, French Guyana, Africa, United States and Brazil [29–31]. In the present study, 27.4% horses were seropositive for *Ehrlichia* spp. by using this commercial kit. However, when the three serological tests were combined the seroprevalence rate increased for 33.7%, in agreement with previous studies which stated that the association of different methods for antibody detection of vector-borne pathogens improve sensitivity and specificity [32,33]. Considering that cell culture isolation of the *Ehrlichia* sp. infecting horses has not been performed yet, our findings suggest that a multi-modal approach should always be performed in horses as to increase the sensitivity of the infection detection and consequently the odds of finding and further characterizing this emerging ehrlichial agent.

Among factors associated to exposure, carthorses living in Alvorada do Sul City were 6.37 times more likely to present *Ehrlichia* spp. antibodies than carthorses living in Curitiba City ($p = 0.0057$). Moreover, carthorses infested by ticks were more likely to be *Ehrlichia* spp. seroreactive than carthorses without ticks ($p = 0.0005$). Unfortunately, only ticks from carthorses from Alvorada do Sul City were collected, with the majority identified as *Amblyomma cajennense* sensu lato (69.4%, 127/183) and *Dermacentor nitens* (30%, 55/183) [15]. A previous study reported that horses highly infested by *Amblyomma americanum* ticks were more commonly seropositive for *Ehrlichia* spp. [4]. Future efforts should focus on sampling, identification and detection of *Ehrlichia* DNA in ticks parasitizing horses in order to fully establish the putative tick vector of this *Ehrlichia* sp. infecting horses in Brazil.

Finally, carthorses from low-income owners in the present study were mostly used for carrying recycling material in urban areas of Brazil, as previously described [15]. Therefore, *Ehrlichia* sp. infection in Brazilian carthorses may post public health concerns due to intimate contact with low-income owners.

5. Conclusions

Anti-*Ehrlichia* sp. antibodies and *Ehrlichia* DNA were detected in carthorses from Southern Brazil, which may post public health concerns due to intimate contact with low-income owners. This is the first report of a natural infection of this bacteria in horses from South America. Clinical signs and the tick vector remain unknown.

Competing interests

The authors have declared that no competing interests exist.

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