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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].

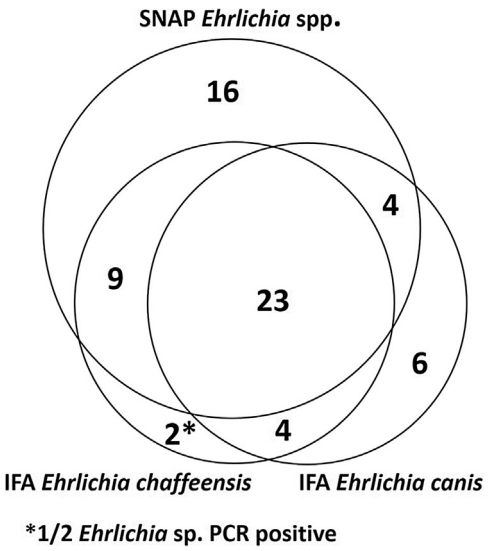

Fig. 1. Proportional Venn diagram showing cross-reactivity by commercial rapid ELISA test (SNAP) and IFA (*E. canis* and *E. chaffeensis* antigens). The diagram was generated by a program available at http://www.cmbi.ru.nl/cdd/biovenn/.

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)