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SEROLOGICAL SURVEY OF *Ehrlichia* SPECIES IN DOGS, HORSES AND HUMANS: ZOONOTIC SCENERY IN A RURAL SETTLEMENT FROM SOUTHERN BRAZIL

Rafael Felipe da Costa VIEIRA(1,2), Thállitha Samih Wischral Jayme VIEIRA(2), Denise do Amaral Gomes NASCIMENTO(2), Thiago F. MARTINS(3), Felipe S. KRAWCZAK(3), Marcelo B. LABRUNA(3), Ramaswamy CHANDRASHEKAR(4), Mary MARCONDES(5), Alexander Welker BIONDO(6) & Odilon VIDOTTO(2)

SUMMARY

The aims of this study were to determine the seroprevalence of *Ehrlichia* spp. and risk factors for exposure in a restricted population of dogs, horses, and humans highly exposed to tick bites in a Brazilian rural settlement using a commercial ELISA rapid test and two indirect immunofluorescent assays (IFA) with *E. canis* and *E. chaffeensis* crude antigens. Serum samples from 132 dogs, 16 horses and 100 humans were used. Fifty-six out of 132 (42.4%) dogs were seropositive for *E. canis*. Dogs > one year were more likely to be seropositive for *E. canis* than dogs ≤ one year (p = 0.0051). Ten/16 (62.5%) and 8/16 (50%) horses were seropositive by the commercial ELISA and IFA, respectively. Five out of 100 (5%) humans were seropositive for *E. canis* and *E. chaffeensis*. *Rhipicephalus sanguineus* (n = 291, 97.98%) on dogs and *Amblyomma cajennense* (n = 25, 96.15%) on horses were the most common ticks found. In conclusion, anti-*Ehrlichia* spp. antibodies were found in horses; however, the lack of a molecular characterization precludes any conclusion regarding the agent involved. Additionally, the higher seroprevalence of *E. canis* in dogs and the evidence of anti-*Ehrlichia* spp. antibodies in humans suggest that human cases of ehrlichiosis in Brazil might be caused by *E. canis*, or other closely related species.

KEYWORDS: *Ehrlichia canis; Ehrlichia chaffeensis;* IFA; ELISA.

INTRODUCTION

Ehrlichiosis is a tick-borne disease caused by *Ehrlichia* spp. that affects animals and humans worldwide[9,27,39,54]. The disease is historically endemic in tropical and subtropical regions and has increasingly been recognized, not only in traditionally endemic areas, but also in temperate regions[25]. This may be attributed to several factors, including the improved diagnostic tools, and both environmental and climate changes which directly influences the distribution of ticks[24].

In some regions of Brazil, dogs and horses are frequently exposed to ticks[29,30]. Dogs and humans are exposed and susceptible to infection by many of the very same tick-borne bacterial pathogens in the order Rickettsiales, including *Ehrlichia* spp.[24]. *Ehrlichia canis* is the causative agent of canine monocytic ehrlichiosis and is the main *Ehrlichia* species present in dogs in Brazil[54]. Additionally, *E. canis* DNA was also amplified from the blood of six human patients with clinical signs of human monocytic ehrlichiosis in Venezuela, suggesting that *E. canis* can also be associated with clinical manifestation in humans[44].

In humans there are two recognized diseases to date caused by *Ehrlichia* species; human monocytic ehrlichiosis (HME), caused by *Ehrlichia chaffeensis*; and human granulocytic ehrlichiosis (HGE) due to *Ehrlichia ewingii*[41]. Human ehrlichiosis cases have been serologically identified in Brazil since 1980[6,11,12]; however, the *Ehrlichia* species associated with these cases were not identified. Additional cases of human ehrlichiosis have been serologically diagnosed in other South American countries, including Argentina[45], Chile[31], Peru[38] and Venezuela[43,44].

Equine monocytic ehrlichiosis (EME), caused by *Neorickettsia risticii* (formerly *E. risticii*), and equine granulocytic anaplasmosis (EGA) caused by *Anaplasma phagocytophilum* (formerly *E. equi*) are the two recognized diseases caused by ehrlichial species[16]. Ticks have never been implicated in the transmission of *N. risticii*[3,16], whereas ticks belonging to the *Ixodes* genus are the vectors of *A. phagocytophilum*[56]. While clinical cases of EME have been reported in southern and southeastern regions of Brazil[10,17,18], horses serologically reactive to *A. phagocytophilum* were reported with clinical alterations in the central-west region of the country[48].

The increasing number of people living in rural settlements in Brazil, with poor-resources and precarious living conditions, inadequate sanitary

(1) Departmento de Ciências Veterinárias, Centro de Ciências Agrárias, Universidade Federal da Paraíba, Bairro Universitário, 58397-000 Areia, Paraíba, Brazil.
(2) Departmento de Medicina Veterinária Preventiva, Universidade Estadual de Londrina, Rodovia Celso Garcia Cid, PR445 Km 380, 86051-990 Londrina, Paraná, Brazil.
(3) Departamento de Medicina Veterinária Preventiva e Saúde Animal, FMVZ, Universidade de São Paulo, Av. Prof. Orlando Marques de Paiva 87, 05508-270 São Paulo, SP, Brazil.
(4) IDEXX Laboratories Inc., Westbrook, ME 04092, USA.
(5) Departmento de Clínica, Cirurgia e Reprodução Animal, Universidade Estadual Paulista, 16050-680 Araçatuba, São Paulo, Brazil.
(6) Departmento de Medicina Veterinária, Universidade Federal do Paraná, Rua dos Funcionários 1540, 80035-050 Curitiba, Paraná, Brazil and Department of Veterinary Pathobiology, University of Illinois, IL, 61802, USA.
Correspondence to: Odilon Vidotto, Departamento de Medicina Veterinária Preventiva, Universidade Estadual de Londrina, Rodovia Celso Garcia Cid, PR 445, Km 380. Campus Universitário, 86051-990 Londrina, Paraná, Brasil. Phone: +55 43 3371 5958, Fax: +55 43 3371 4485. E-mail: vidotto@uel.br

care, and sanitary education, associated to the presence of pets, production animals, wild animals, and ticks sharing the same environment, may represent an important source of several zoonotic pathogens. Thus, the aims of the present study were to i) determine the seroprevalence of *Ehrlichia* species in a restricted population of dogs, horses, and humans highly exposed to tick bites, ii) identify the tick species parasitizing dogs and horses, and iii) determine risk factors for exposure in a rural settlement from Paraná State, southern Brazil.

MATERIALS AND METHODS

Ethical principles: The study was approved by the Ethics Committee in Animal Experimentation and Animal Welfare at Universidade Estadual de Londrina (UEL) (protocol number 34/2011), and was conducted according to the ethical principles of animal experimentation, adopted by the Brazilian College of Animal Experimentation. Collection of human blood samples was approved by the Ethics Committee on Human Research at UEL (protocol number 53/2011).

Area: The rural settlement is located in Alvorada do Sul county (22° 54' 34.4" S 51° 13' 49.1" W), Paraná State, southern Brazil. The area is located within the rural perimeter of Alvorada do Sul, 16 km from downtown, 380 m above sea level. The region presents a subtropical climate with rainfall throughout the year, with a higher concentration in summer months (average temperature of 25 °C)[26].

The region is subdivided in 60 homesteads, each with an area of approximately 12 hectares, amounting to 786 hectares. The main activity of subsistence is the cultivation of grains and vegetables. The area also comprises 20% of a native forest having a diverse fauna, with populations of capuchin monkeys, capybaras, opossums, coatis, and wild canids, as well as a wide variety of birds and fishes. There is a barrage close to the area, where the habitants often fish and bath. This region provides the maintenance of various ticks, to which dogs, horses, and humans are continually exposed.

Study design: According to the seasonal dynamics of adult ticks[50,52], samples were collected in March 2011, which represents the end of the summer in the South hemisphere. Sampling was performed house-to-house, comprising all 60 homesteads of the area.

A questionnaire focused on epidemiological aspects was given to each owner. Breed, age, gender of their dogs and horses, and presence or previous contact with ticks were evaluated. The age of the dogs was classified into groups of ≤ one year and > one year. Age, gender and history of previous contact with ticks were also addressed for humans.

Collection of ticks: A total of 297 and 26 tick specimens were collected from dogs and horses, respectively. Ticks were removed and kept in 70% ethanol-labeled tubes in order to identify each host. Ticks were classified according to taxonomic keys[2,20,34,42].

Sampling: Dog (n = 132) and horse (n = 16) blood samples (up to 10 mL *per individual*) were collected by venipuncture of the jugular vein. Blood specimens from human (n = 100) were collected by nurses by venipuncture of the brachial vein. All samples were collected in tubes without anti-coagulant and kept at room temperature (25 °C) until visible clot retraction, centrifuged at 1500 *g* for five min, and the serum was separated and kept at -20 °C until processing.

*Detection of anti-*Ehrlichia spp *antibodies:* Serum samples of 138 dogs and 16 horses were tested for *E. canis* using a commercial ELISA rapid test (SNAP® 4Dx®, IDEXX Laboratories Inc., Westbrook, ME, USA), according to the manufacturer's instructions. The kit also detects antibodies anti-*A. phagocytophilum,* anti-*B. burgdorferi* (s.l.)*,* and anti-*Dirofilaria immitis* antigen.

Anti-*Ehrlichia* spp. antibodies in horses and human serum samples were evaluated by indirect immunofluorescent assay (IFA) using *E. canis* (São Paulo strain) and *E. chaffeensis* (Arkansas strain) as antigens. Crude antigens were produced by culturing ehrlichiae in DH82 cells, as previously described[1,51]. IFA was performed with 10 μL of serum samples incubated at 37 °C for 30 min in slides previously seeded with *E. canis* or *E. chaffeensis,* washed three times for five min in phosphate buffered saline (PBS, pH 7.2), additionally washed by distilled water, then dried at room temperature. Twenty microliters of fluorescein isothiocyanate-conjugated rabbit anti-human IgG (Sigma-Aldrich, St. Louis, MO) at 1:800 dilution in PBS with 1% of bovine serum albumin and 1% Evans blue were applied onto the slide. Horse samples were titred with a dilution of 1:1200 of fluorescein isothiocyanate-conjugated rabbit anti-horse IgG (Sigma-Aldrich, St. Louis, MO). Slides were then incubated at 37 °C for 30 min, three times washed for five min, additionally washed by distilled water, allowed to air dry and subsequently examined using a microscope with a fluorescent light source. Samples were considered positive when reacting with dilution ≥ 1:64[5,11,19,39]. Titers were determined to the largest dilution in which fluorescence was visualized around the bacteria (endpoint titers).

Statistical analysis: The Chi-square or Fisher's exact test were used to determine the difference between whether individual factors 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 $p < 0.05$. Data were compiled and analyzed in Epi Info™ Software (version 3.5.3).

RESULTS

A total of 132 dogs were sampled, 83 (62.8%) of which were males and 49 (37.2%) females. All were mixed breed, with ages varying from six months to 12 years. By the commercial ELISA rapid test, 56/132 (42.4%; 95%CI: 33.9 - 51.3%) dogs were seropositive for *E. canis*. Dogs > one year were more likely to be seropositive for *E. canis* than dogs ≤ one year (OR = 3.13, 95% CI, 1.45 - 6.75%). No significant association was found between gender or presence of ticks, and seropositivity to *E. canis*. Data on *E. canis* seroprevalence are shown in Table 1. Additionally, anti-*A. phagocytophilum* antibodies were found in 3/132 (2.3%; 95% CI: 0.5 - 6.5%) dogs. Anti-*B. burgdorferi* antibodies and *D. immitis* antigens were not found in dogs.

Horses included nine (56.2%) males and seven (43.8%) females, all mixed breed, with ages ranging from three to 15 years. When serum samples were evaluated by each test, 10/16 (62.5%; 95% CI: 35.4 - 84.8%) and 8/16 (50%; 95% CI: 24.7 - 75.3%) were positive for *E. canis* antigen by the commercial ELISA rapid test and IFA, respectively. Ten out of 16 (62.5%; 95% CI: 35.4 - 84.8%) horses were positive for *E. chaffeensis* antigen. Antibodies titers ranged from 256 to 2048 for *E. canis* and from 256 to 1024 for *E. chaffeensis* by IFA. Additionally, antibodies anti-*B. burgdorferi* were found in 2/16 (12.5%; 95% CI: 1.6 - 38.3%)

Table 1
Seroprevalence of *E. canis* in dogs within each variable studied from a rural settlement, Paraná State, southern Brazil

Variable	+/N (%)	OR	95% CI	*p*-value
Presence of ticks				
Yes	35/73 (47.9%)	1.66	0.82-3.36	0.1534
No	21/59 (35.6%)			
Age (years)				
> 1	43/82 (52.4%)	3.13	1.45-6.75	0.0051
≤1	13/50 (26%)			
Gender				
Male	33/83 (39.8%)	0.74	0.36-1.52	0.4200
Female	23/49 (46.9%)			

+, Number of positive animals; N, number of samples per variable; OR, odds ratio; 95% CI, 95% confidence interval.

horses. Antibodies anti-*A. phagocytophilum* were not found.

Humans included 48 (48%) males and 52 (52%) females, with ages varying from two to 79 years. Five out of 100 (5%; 95% CI: 1.6 - 11.3%) humans were seropositive for *E. canis* and *E. chaffeensis* antibodies, respectively. From the total of seropositive samples, two reacted for both agents. Antibodies titers ranged from 64 to 512 for *E. canis* and from 64 to 256 for *E. chaffeensis* by IFA. Seventy-five out of 100 (75%) humans recollected having been bitten by ticks. No significant association was found between age, gender or previous exposure to tick bites, and seropositivity to *Ehrlichia* spp.

Seropositive human samples were: female, 12 years-old, and kept five dogs at home, all seropositive for *E. canis*; male, 13 years-old, owned a dog seronegative for *E. canis*; female, 29 years-old, kept two dogs at home, seronegative for *E. canis*; male, 30 years-old, kept three dogs at home and all were seronegative for *E. canis*; female, 72 years-old, owned a dog seropositive for *E. canis*. All humans recalled tick and insect bites, and recollected acute febrile syndromes in the past.

A total of 297 ticks (154 males, 104 females, 34 nymphs and five larvae) were collected from 73/132 (55.3%; 95% CI, 46.4 - 64%) dogs, ranging from one to 26 ticks per animal. Three tick species were identified: *Rhipicephalus sanguineus* (n = 291, 97.98%), *Amblyomma ovale* (n = 5, 1.68%) and *Amblyomma cajennense* (n = 1, 0.34%). From the total of 73 dogs found infested by ticks, 68/73 (93.15%; 95% CI, 84.95 - 97%) were infested by *R. sanguineus*, 3/73 (4.11%; 95% CI, 1.41 - 11.4%) by *A. ovale*, and 1/73 (1.37%; 95% CI, 0.24 - 7.36%) by *A. ovale* and *A. cajennense*.

Twenty-six ticks (18 males and eight females) were collected from 7/16 (43.7%; 95% CI, 23.1 - 66.8%) horses, ranging from one to nine ticks per animal. Two tick species were identified: *A. cajennense* (n = 25, 96.15%) and *Rhipicephalus* (*Boophilus*) *microplus* (n = 1, 3.85%). *A. cajennense* ticks were found infesting 6/7 (85.7%; 95% CI, 48.6 - 97.4%) horses, and *R.* (*B.*) *microplus* in 1/7 (14.3%; 95% CI, 2.5 - 5.1%) horse.

DISCUSSION

Anti-*Ehrlichia* spp. antibodies were found in 62.5% of horses from southern Brazil by using the commercial ELISA rapid test. Despite this test has been developed for screening canine samples[8], the assay uses antigen-specific conjugate and was previously validated for screening *B. burgdorferi* and *A. phagocytophilum* in horses[7,28]. Using this same commercial ELISA in horses from Denmark, France, French Guyana and Africa, anti-*Ehrlichia* spp. antibodies were not found[21,35,36]. Previous studies using this point-of-care ELISA assay in dog serum samples reported cross-reactivity between *E. canis* and *E. chaffeensis* antibodies[40]. In the present study, 50% of the horses were positive by IFA using *E. canis* and *E. chaffeensis* as antigens. Thus, differences in the number of seropositive horses found by each test were somewhat expected, since the commercial ELISA rapid test utilizes synthetic peptides from p30 and p30-1 outer membrane proteins of *E. canis* as antigen[40], while IFA used *E. canis* and *E. chaffeensis* crude antigens.

A weak cross-reactivity between *N. risticii* and *E. canis* by IFA, ELISA and Western blot methods has been reported[4,23,46,49]. Recently developed Western blot tests coupled with serum absorption techniques have been used in order to identify the organisms involved and solve the serological cross-reactivity between these agents[22]. In the present study, horse antibody endpoint titers ranged from 256 to 2048 for *E. canis* and from 256 to 1024 for *E. chaffeensis* by IFA. Moreover, 43.7% of the horses were infested by ticks, in the majority *A. cajennense* (96.1%). *Ehrlichia canis* is transmitted through the bite of the brown dog tick *R. sanguineus*[15], while *E. chaffeensis* is mainly transmitted by *A. americanum* ticks[55]. Neither horse infestations by *R. sanguineus* nor *E. canis* infection in *Amblyomma* ticks are natural host-parasite interactions. In addition, no data are currently available on the vector competence of *A. cajennense* for *E. chaffeensis*[55]. On the other hand, ticks have never been implicated in the transmission of *N. risticii*[16], which is transmitted upon ingestion of this bacterium in the metacercarial stage of trematodes encysting in aquatic insects by horses[47]. Thus, authors did not exclude the possibility on the involvement of a not-yet-described *Ehrlichia* species in the population of horses herein studied, which should be further molecularly identified and characterized.

IFA is considered the gold standard test for diagnosis of HME, although cross-reactivity between *E. canis* and *E. chaffeensis* by serological methods commonly occur[54]. In the present study, 5% of the humans were seropositive by IFA for either *E. canis* or *E. chaffeensis* antigens. Although clinical cases of human ehrlichiosis have been reported in Brazil by using *E. chaffeensis* antigens[6,11,12], the existing cross-reactions between *Ehrlichia* species in serological assays precluded the determination of the etiological agent of human patients. Although molecular detection of *E. chaffeensis* DNA has already been related in wildlife in Brazil[32,33], *E. canis* is indeed the most prevalent *Ehrlichia* species in the country[54]. Since 75% of humans of the present study reported past tick bites, and *R. sanguineus* ticks, the main vector of *E. canis*- were the most common tick species found (90%)- it is possible that anti-*Ehrlichia* spp. antibodies detected in this study were due to exposure to *E. canis*-infected *R. sanguineus* ticks, although this statement needs to be confirmed by direct detection of the agent in vertebrate blood.

Seropositivity to *E. canis* was found in 42.4% of the dogs. Serological surveys of *E. canis* in dogs from rural areas have found prevalence data

ranging from 24.7% to 65.6% by different methods[37,54]. We found that age (> one year-old) is associated with seropositivity to *E. canis* ($p = 0.0051$), corroborating with other studies performed in the veterinary teaching hospital in Londrina City, southern Brazil[53]. Previous studies have reported that male dogs previously exposed to tick bites were at high risk of being seropositive for *E. canis*[13]. In the present study, besides 56.2% of the dogs were males infested by ticks; association between gender or presence of ticks and seropositivity to *E. canis* was not observed. In addition, previous studies have stated that the commercial ELISA rapid test is able to identify dogs with titers > 320[40]. Thus, seroprevalence for *E. canis* in dogs from the studied area might be higher, since dogs with low titers may have not been recognized when a point-of-care ELISA assay is used[14].

CONCLUSION

Antibodies anti-*Ehrlichia* species were found in horses by two different serological methods. However, the lack of a molecular characterization precludes any conclusion regarding the agent involved. The higher *E. canis* seroprevalence in dogs and the detection of anti-*Ehrlichia* spp. antibodies in humans suggest that human cases of ehrlichiosis in Brazil might be caused by *E. canis*, or other related species.

RESUMO

Investigação sorológica de espécies de *Ehrlichia* em cães, equinos e humanos de um assentamento rural do sul do Brasil

Objetivou-se determinar a soroprevalência de *Ehrlichia* spp. e os fatores de risco associados a exposição em uma população restrita de cães, cavalos e humanos altamente expostos a picadas de carrapatos em um assentamento rural brasileiro utilizando um teste comercial de ELISA rápido e dois testes de imunofluorescência indireta (IFI) com antígenos brutos de *E. canis* e *E. chaffeensis*. Amostras de soro de 132 cães, 16 cavalos e 100 humanos foram utilizadas. Cinquenta e seis/132 (42,4%) cães foram soropositivos para *E. canis*. Cães > um ano apresentaram mais chance de serem soropositivos para *E. canis* do que cães ≤ um ano (p = 0,0051). Dez/16 (62,5%) e 8/16 (50%) cavalos foram soropositivos pelo ELISA comercial e IFI, respectivamente. Cinco/100 (5%) humanos foram soropositivos para *E. canis* e *E. chaffeensis*. *Rhipicephalus sanguineus* (n = 291, 97,98%) nos cães e *A. cajennense* (n = 25, 96,15%) nos cavalos foram os carrapatos mais encontrados. Concluindo, anticorpos anti-*Ehrlichia* spp. foram encontrados em cavalos; entretanto, a ausência de uma caracterização molecular impede qualquer conclusão sobre agente envolvido. Além disso, a alta soroprevalência de *E. canis* em cães e a evidência de anticorpos anti-*Ehrlichia* sp. em humanos, sugere que os casos de erliquiose humana no Brasil possam ser causados por *E. canis* ou outra espécie intimamente relacionada.

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CONFLICT OF INTEREST

The authors have declared that there are no conflicting interests.

AUTHOR CONTRIBUTIONS

Conceived and designed the experiments: RFC Vieira, TSWJ Vieira, AW Biondo, O Vidotto. Performed the experiments: RFC Vieira, TSWJ Vieira, DAG Nascimento, TF Martins, FS Krawczak. Analyzed the data: RFC Vieira, TSWJ Vieira, MB Labruna, R Chandrashekar, M Marcondes, AW Biondo, O Vidotto. Contributed reagents/materials/analysis tools: R Chandrashekar, MB Labruna, M Marcondes. Wrote the paper: RFC Vieira, TSWJ Vieira, MB Labruna, O Vidotto.

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