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Molecular detection of *Ehrlichia canis* in *Rhipicephalus sanguineus* (s.l.) ticks in dogs and their domestic environment in Cuiaba, MT, Brazil

Detecção molecular de Ehrlichia canis em carrapatos Rhipicephalus sanguineus (s.l.), em cães e em seus ambientes domésticos em Cuiabá, MT, Brasil

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ABSTRACT

The central region of Brazil is known to be an endemic area for canine ehrlichiosis. Therefore, this study aims to determine the prevalence rates of *E. canis* infection in dogs and in *Rhipicephalus sanguineus* ticks collected from the dogs and their home environments. Serum samples and genomic DNA from the blood of 20 dogs and 299 ticks were analyzed by IFA and PCR assays in order to detect *Ehrlichia canis* antibodies and DNA. Nine (45%) of the 20 dogs were seropositive for *E. canis*, with titers ranging from 80 to 10240, and 6 dogs (30%) were positive for *Ehrlichia* spp. by PCR. Five free-living ticks were positive (2.89%, 95% confidence interval: 0.94-6.62%), as were six ticks attached to dogs (4.76%; 95% CI: 1.77-10.0%). The two groups showed a similar infection rate (P=0.395). Partial *dsb* DNA sequences of two samples from ticks were identical to each other and 100% (350/350 nucleotides) were identical to *E. canis*. Despite the high serological and molecular rates of canine ehrlichiosis in Cuiabá, the prevalence among infected ticks was lower than that found among dogs. However, adult ticks may remain infective much longer to ensure their infestation and infection of susceptible dogs.

Keywords: *Anaplasmataceae*. Vector. Prevalence. PCR. Antibody.

RESUMO

A região central do Brasil é caracterizada como uma área endêmica para erliquiose canina. Devido a isso, o presente estudo objetivou determinar a prevalência de infecção em cães e seus carrapatos *R. sanguineus*, coletados a partir do mesmo ambiente. As amostras de soro e DNA genômico de sangue de 20 cães e 299 carrapatos foram testadas por RIFI e PCR a fim de detectar anticorpos e DNA de *Ehrlichia canis*. Do total, 9 (45%) eram soropositivos para *E. canis* com títulos variando de 80 a 10240 e 6 cães (30%) positivos para *Ehrlichia* spp. por PCR. Cinco carrapatos de vida livre (2,89%; intervalo de confiança 95%: 0,94-6,62%), e 6 carrapatos fixados em cães (4,76%; IC 95%: 1,77-10,0%) foram positivos. A taxa de infecção foi semelhante entre ambos os grupos (P=0.395). As sequências parciais do gene *dsb* de 2 amostras de carrapatos foram idênticas entre si e 100% (350/350) idênticas à *E. canis*. Apesar das altas taxas sorológicas e moleculares de erliquiose canina em Cuiabá, a prevalência de carrapatos infectados foi menor que o encontrado em cães. Por outro lado, os carrapatos adultos podem permanecer infectados por tempo suficiente para garantir a infestação e infecção aos cães susceptíveis.

Palavras-chave: *Anaplasmataceae*. Vetor. Prevalência. PCR. Anticorpo.

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Introduction

The genus *Ehrlichia* belongs to the order *Rickettsiales*, family *Anaplasmataceae* and consists of six recognized species, namely, *E. canis*, *E. chaffeensis*, *E. ewingii*, *E. muris*, *E. ruminantium* and *E. minasensis* sp. nov. (Cabezas-Cruz et al., 2016; Dumler et al., 2001), which naturally infect a wide variety of vertebrate hosts. All currently known *Ehrlichia* species are transmitted by ticks of the family *Ixodidae* (Bremer et al., 2005). The brown dog tick *Rhipicephalus sanguineus* is the most widespread tick in the world and belongs to the so-called 'Complex R. sanguineus' or *R. sanguineus* group, which includes at least five species morphologically related in the Old World (Pegram et al., 1987; Walker et al., 2000). In addition to dogs, this tick can occasionally parasitize other hosts, including humans; it is highly adapted to live in human dwellings and is active throughout the year, not only in tropical and subtropical regions, but also in some temperate zones (Dantas-Torres, 2010). *Rhipicephalus sanguineus* not only weakens dogs through high infestations, but is also the main vector of *Ehrlichia canis*, the agent of canine monocytic ehrlichiosis (Moraes-Filho et al., 2011).

According to the review of Vieira et al. (2011) prevalence rates of anti-*Ehrlichia* spp. antibodies on dogs in the state of Mato Grosso showed higher prevalence rates of anti-*Ehrlichia* spp. antibodies than any other region in the country (Vieira et al., 2011). Studies carried out in various neighborhoods of the city of Cuiabá have also found high prevalence rates of *E. canis* infection in dogs (Makino et al., 2015; Silva et al., 2010; Witter et al., 2013). Based on this information and in view of the importance of ehrlichiosis in the region, the purpose of this study was to detect the presence of *E. canis* and anti-*E. canis* antibodies in pet dogs in Cuiabá and to evaluate the prevalence rates of *E. canis* infection in the *R. sanguineus* ticks collected from the dogs and their home environments in 17 neighborhoods in the city of Cuiabá, MT.

Materials and Methods

Study area and sample collection

Cuiabá is located in the state of Mato Grosso, Brazil, under the coordinates 15° 35'46" South and 56° 05' 48" West, at 176 meters above sea level. The city, with a population of 607.153 (Instituto Brasileiro de Geografia e Estatística, 2018), has a warm tropical and sub-humid climate. Samples from dogs and ticks were collected from February 2012 to October 2014 in 17 neighborhoods (representing the eastern, western, northern and southern areas of the city of Cuiabá).

This study involved 20 male or female dogs of various ages and breeds treated at the Veterinary Hospital of the Federal University of Mato Grosso, which presented infestation by *R. sanguineus* (s.l.) ticks. The dogs were treated in the veterinary hospital and then the researchers went to their homes and the criteria to be included was the absence of fumigation for several months prior to the study in each house.

Blood samples were collected via jugular or cephalic venipuncture, and the samples were placed in tubes with and without EDTA (ethylenediaminetetraacetic acid). In the laboratory, the serum was separated by centrifugation and the whole blood and serum were stored at -20 °C until analysis.

At each home, ticks were collected directly from the dogs and from the environment (house walls and/or dogs houses). The collected ticks were sent to the Laboratory of Virology and Rickettsial Infections (LVR) of the Veterinary Hospital at the Federal University of Mato Grosso (HOVET-UFMT). The adult ticks were kept in absolute isopropyl alcohol until deoxyribonucleic acid (DNA) was extracted. The engorged nymphs collected from each animal or walls were preserved in a BOD (biochemical oxygen demand) incubator at a temperature of 27 °C and 80% relative humidity in order to undergo ecdysis. All nymphs underwent ecdysis, were identified and stored in isopropyl alcohol until DNA extraction. The ticks were identified according to a specific taxonomic key for adults (Barros-Battesti et al., 2006).

Serological testing

The serum samples were tested by indirect immunofluorescence assay (IFA) using DH82 cells infected with the isolated Cuiaba #16 *E. canis* (Aguiar et al., 2013). Each slide contained nonreactive (negative control) and reactive (positive control; endpoint titer 1280) sera. The IFAT was performed according to Aguiar et al. (2007), and samples that reacted to the screening dilution (1:40) were then titrated using serial two-fold dilutions to determine the final titers.

DNA extraction and PCR amplification

Blood and ticks were subjected to DNA extraction using the guanidine thiocyanate-phenol protocol described by Sangioni et al. (2005). Extractions were performed individually per dog and tick. The extracted DNA was then identified and stored at -20 °C until completion of the polymerase chain reaction (PCR). The DNA of ticks was tested in pools of 3 adults according to the categories: engorged males, engorged females, post-ecdysis males and post-ecdysis females. In order to confirm the presence of genomic DNA in samples, the canine endogenous gene, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Braga et al., 2016) and tick mitochondrial 16S rRNA gene were evaluated in a subset of randomized samples by PCR (Mangold et al., 1998).

The genomic DNA of blood samples and ticks was analyzed by real-time polymerase chain reaction (qPCR), aiming to amplify a fragment of 176-bp of the *dsb* gene of *Ehrlichia* spp., using Dsb-330 sense (5'-GATGATGTCTGAAGATATGAAACAAAT-3') (Labruna et al., 2007) and Dsb-481 antisense primers (5'-TGCTTGTAATGTAGTGCTGCAT-3') (this paper), following the protocol involving 1.25 U GoTaq® Hot Start Polymerase (Promega®), 5.0 µL Flexi Buffer, 2.0mM $MgCl_2$, dNTP mixture (0.2mM each), 0.5 µL of each primer (10 pmol/µL), 0.2 µL ROX™, 2.0 µL SYBR Green, 0.5 µL DNA and 10.05 µL Nuclease Free Water (Promega®), with a final volume of 25 µL, and performed in a StepOnePlus™ Real-Time PCR System. The amplification protocol consisted of an initial denaturation at 95 °C for 15 sec, 40 cycles of denaturation (95 °C, 15 sec), annealing (60 °C 15 sec), immediately followed by a dissociation program consisting of 95 °C for 15 sec, 60 °C for 1 min and 95 °C for 15 sec. The dissociation curves with melting temperature (Tm) ranged from 76 °C to 77 °C.

With the intention to determinate the sensibility of qPCR assay, DNA from the Cuiabá#1 strain of *E. canis* cultured in DH82 cells were submitted to a conventional PCR using Dsb-330 and Dsb-481 primers. The amplicon was purified by the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare®) and then quantified in QubitR 2.0 Fluorometer (Life Invitrogen®). The DNA sample was concentrated on 1ng/µL followed by serial 10-fold dilution and then submitted to the qPCR above described. The specificity of this new *dsb* protocol for *E. canis* was determined by testing genomic DNA of *E. chaffeensis* and *E. minasensis*, which are the closest species related to *E. canis*. This species was obtained from cell culture of canine monocytes (DH82) in our laboratory.

The DNA of the tick pools that were positive for *Ehrlichia* spp. were then tested individually to determine the exact number of positive individuals. Subsequently, in order to obtain an amplicon of 409-bp of *dsb* gene for sequencing analysis, positive samples were subjected to a PCR assay, according to the protocol described by Labruna et al. (2007), using DSB-330 sense and Dsb-728 antisense primers (5'-CTGCTCGTCTATTTTACTTCTTAAAGT-3'). The products of amplification were purified using Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare®) commercial kits, according to the manufacturers' recommendations, and their sequences were determined on an automated DNA sequencer, Applied Biosystems 3500/3500xL Genetic Analyzer, following the manufacturer's instructions. The sequences thus obtained were compared by Blast (Basic Local Alignment Search Tool) Analysis with other *Ehrlichia* spp. sequences available in GenBank - NCBI (National Center for Biotechnology Information).

Statistical analysis

Confidence intervals (95%) were calculated for prevalence rates in free-living ticks and ticks collected from dogs. The PCR results of each group were evaluated by the chi-square test. Values were considered significant at $P<0.05$.

Results

To ensure DNA extraction from dogs and ticks samples, PCR targeting GAPDH and tick mitochondrial genes respectively were applied. All tested samples resulted positive.

Sensibility and specificity. The sensibility of qPCR assay using the primer Dsb-481 of *Ehrlichia*. The positivity was observed until the 10^{th} dilution (1/2 copy of DNA) which means 2 copies of *E. canis dsb* gene in 4 µL of DNA template. PCR product was not amplified from *E. chaffeensis* but was positive for *E. minasensis* DNA extracted from cell culture.

Dogs. Nine (45%) of the 20 dogs were seropositive for *E. canis*. Antibody titers ranged from 80 to 10240 (Table 1). Six dogs (30%) were positive for *Ehrlichia* spp. by PCR and

Table 1 - Number of homes, dogs and collected ticks from 17 neighborhoods of Cuiabá evaluated for the presence of *Ehrlichia canis*

Home	Dogs				Ticks	
	Number	Evaluated	PCR	IFA Titer	Collected	Positive
1	2	1	Neg	NR	19	0
2	1	1	Neg	NR	16	1 post-ecdysis female from wall
3	1	1	Neg	NR	15	0
4	1	1	Neg	NR	8	0
5	2	1	Pos	2560	15	1 female collected from wall
6	1	1	Neg	NR	21	0
7	1	1	Pos	5120	24	0
8	1	1	Neg	NR	12	0
9	1	1	Neg	640	21	0
10	1	1	Neg	NR	12	1 female collected from a dog
11	2	1	Neg	2560	8	1 female collected from a dog
12	1	1	Neg	80	18	1 female collected from a dog
13	1	1	Pos	NR	15	0
14	3	1	Neg	1280	10	1 male collected from a dog
15	1	1	Pos	10240	15	0
16	1	1	Pos	10240	14	2 males collected from a dog
17	1	1	Neg	NR	16	1 male and 2 females from wall
18	1	1	Pos	NR	12	0
19	1	1	Neg	NR	16	0
20	1	1	Neg	160	12	0

NR = Not reactive.

their average titer (n=6, average titer=4693.3) was higher than that of the PCR negative dogs (n=5, average titer=944). Only two dogs testing positive by PCR had no antibodies. There was no association between PCR positive dogs and seroreactivity by IFAT.

Ticks. At the homes, 127 engorged nymphs (95 from walls and 32 from dogs), 78 adult males (33 from walls and 45 from dogs), 94 adult females (47 from walls and 47 from dogs) were collected. A total of 299 ticks (175 from walls and 124 attached to dogs) were identified as belonging to the *R. sanguineus* complex. The DNA of pools that were PCR positive was tested individually, resulting in 5 (2.89%; 95% confidence interval: 0.94% - 6.62%) positive free-living ticks: 1 male and 4 females (1 non-engorged post-ecdysis and 3 non-engorged). Six positive ticks were attached to dogs (4.76%; 95% CI: 1.77-10.0%), three males and three females. The two groups showed a similar infection rate (P=0.395). Partial *dsb* DNA sequences from 2 samples from ticks were identical to each other and 100% (350/350 nucleotides) identical to *E. canis* (GenBank CP000107).

Discussion

The present study evaluated the *E. canis* infection rates in a population of *R. sanguineus* s.l. ticks in the central region of Brazil. The city of Cuiabá is located in the exact center of South America, and its warm tropical and sub-humid climate is favorable for the maintenance of *R. sanguineus* s.l. tick populations (Jacobs et al., 2001; Louly et al., 2007). Hence, the occurrence of dogs from this city parasitized by ticks is usually high throughout the year (personal information). In this way to avoid analyzing places under effect of treatment, the criterion of inclusion of the tick population was based on the history of parasitism by *R. sanguineus* in dogs living in environments that had not been fumigated for several recent months prior to this study.

For this study, a new protocol to detect DNA from *E. canis* was developed based on a qPCR assay targeting fragment of the *dsb* gene. The sensibility observed for *E. canis* (2 copies) supported its application for diagnostic procedures or epidemiological inquiries in our region, which is considered endemic for canine ehrlichiosis. In the other side, the primers were not specific for *E. canis* since they amplified *dsb* fragments of *E. minasensis*. *Ehrlichia canis* and *E. minasensis* are two related species from the *Ehrlichia genus*. These two species have been evolved from a variable clade within the genus, and this proximity could difficult a better PCR specificity (Cabezas-Cruz et al., 2014).

According to the PCR and IFA analyses, 30% (n=6) and 45% (n=9) of the dogs were positive, respectively. These data corroborate previous results reported for the region (Silva et al., 2010; Witter et al., 2013), which described Cuiabá as an endemic area for ehrlichiosis in Brazil. Two of the dogs in this study were seronegative, but *Ehrlichia* spp. DNA was identified in their blood. Most dogs become seropositive after 28 days of infection (Harrus et al., 1997), although

DNA can be identified 14 days after infection, characterizing the acute phase of ehrlichiosis (Aguiar et al., 2007).

Despite their different origins, the PCR results showed no difference between free-living ticks collected directly from house walls, and ticks attached to dogs. Ticks collected from dogs that tested positive can be associated with rickettsemia (e.g., the blood sample of a dog from house #16 was positive and the ticks attached to it were also positive). On the other hand, although ticks in the parasitic phase were positive, the dogs to which they were attached were PCR negative (house # 10, 11, 12 and 14). Three of these dogs were seropositive, suggesting the chronic phase, when some cycles of bacteremia can occur, at which time ticks are susceptible to infection. The seronegative dogs and the PCR negative dog (house #10) were parasitized by positive *R. sanguineus* ticks, exemplifying the period when natural transmission of *E. canis* by the tick may occur.

In this regard, positive ticks were collected from the walls of homes #2 and #17 where seronegative and PCR negative dogs lived. According to Aguiar et al. (2007), the maintenance of *E. canis* in the environment or in *R. sanguineus* populations requires dogs suffering from rickettsemia, since transplacental transmission does not occur in ticks. Hence, ticks cannot maintain *E. canis* infections for more than one generation. On the other hand, adults of *R. sanguineus* ticks are able to keep *E. canis* viable for up to 155 days after molt (Lewis et al., 1977). Therefore, even without the presence of an infected dog living at a site, in some circumstances, unfed adult *R. sanguineus* ticks can survive for long periods and may be able to infect susceptible dogs.

Conclusion

The development of a new and sensitive diagnostic tool for detection of *Ehrlichia* spp. was reported. Despite the high serological and molecular rates of canine ehrlichiosis in Cuiabá, the prevalence rate of infected ticks was much lower than that of dogs. However, adult ticks may remain infective much longer to ensure their infestation and infection of susceptible dogs.

Conflict of Interest

The authors state they have no conflicts of interest to declare.

Ethics Statement

This study was evaluated by the Animal Research Ethics Committee of the Federal University of Mato Grosso and was approved under Protocol nº. 23108.051170/10-0.

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