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Molecular detection of *Ehrlichia canis* in dogs from the Pantanal of Mato Grosso State, Brazil

Detecção molecular de *Ehrlichia canis* em cães do Pantanal do Mato Grosso, Brasil

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Abstract

The present study evaluated the presence of *Ehrlichia* DNA in the blood samples of 320 dogs from the urban and rural areas of the municipality of Poconé, Pantanal region, Mato Grosso state, by Polymerase Chain Reaction (PCR), targeting the ehrlichial *dsb* gene. Risk factors for infection in dogs were also evaluated. Forty-eight (15%, 95% CI: 11.4-19.5%) dogs were positive: 25 (15.6%, 95% CI: 10.4-22.2%) from the urban area and 23 (14.4%, 95% CI: 9.3-20.8%) from the rural area (P > 0.05). Partial DNA sequence obtained from PCR products of 18 samples from the urban area and 16 samples from the rural area were 100% identical to *E. canis* from Brazil and the USA. This study reports the first *E. canis* molecular detection in dogs from the northern Pantanal region.

Keywords: Dogs, tick borne disease, *Ehrlichia canis*, Anaplasmataceae, Poconé, Brazil.

Resumo

O presente estudo avaliou a presença de DNA de *Ehrlichia* spp. em 320 cães das áreas urbana e rural do município de Poconé, região do Pantanal de Mato Grosso, pela PCR visando o gene *dsb*. Os fatores de risco para a infecção em cães também foram avaliados. Quarenta e oito (15%, IC 95%: 11,4-19,5%) cães foram positivos, 25 (15,6%, IC 95%: 10,4-22,2%) da área urbana e 23 (14,37%, 95% CI: 9,3-20,8%) da área rural (P > 0,05). Sequências parciais de DNAs obtidos a partir de produtos da PCR de 18 amostras da área urbana e 16 da área rural foram 100% idênticas a *E. canis* do Brasil e EUA. Este estudo relata a primeira detecção molecular de *E. canis* em cães da região norte do Pantanal.

Palavras-chave: Cães, ectoparasitos, *Ehrlichia canis*, Anaplasmataceae, Poconé, Brasil.

Introduction

Bacteria of the genus *Ehrlichia* are tick-borne agents that cause significant diseases in domestic animals (McBRIDE; WALKER, 2010; DUMLER et al., 2001). In Brazil, *Ehrlichia canis* is the agent of canine monocytic ehrlichiosis (CME), a canine disease of nearly worldwide distribution, transmitted by the tick *Rhipicephalus sanguineus* (HARRUS; WANER, 2011; VIEIRA et al., 2011). In North America, dogs can also be infected by *Ehrlichia chaffeensis* and *Ehrlichia ewingii*, which are important agents of zoonoses vectored by *Amblyomma* ticks in that continent (McBRIDE; WALKER, 2010).

Some studies emphasized the possibility that *Ehrlichia* species other than *E. canis* are circulating in Brazil, especially in the Pantanal area, a large periodically wetland area of Brazil that harbors high diversity of mammals and ticks (ALHO et al., 1987; BECHARA et al., 2000). Melo et al. (2011) reported high prevalence of anti-*Ehrlichia* antibodies in domestic dogs from the municipality of Poconé, a northern sub-region of Pantanal. Because some of these seropositive dogs were not infested by *R. sanguineus* ticks (they were rather infested by *Amblyomma* ticks), the authors speculated on the possible occurrence of different *Ehrlichia* species infecting dogs in the region. In the southern area of Pantanal, Widmer et al. (2011) reported a novel ehrlichial agent (genetically related to *Ehrlichia ruminantium*) infecting free-living jaguars (*Panthera onca*) and ticks. Additionally, *E. chaffeensis* was molecularly detected in Brazilian marsh deers

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(*Blastocerus dichotomus*) in a wetland area borderline of Mato Grosso do Sul and São Paulo states (MACHADO et al., 2006). Other reports include molecular detection of *Ehrlichia* sp. in Brazilian wild captive carnivore species maintained in several zoos in Brazil (ANDRÉ et al., 2010, 2012).

Since serological tests cannot distinguish between infection by *E. canis* and other *Ehrlichia* species (KELLY et al., 1994; ALLSOPP; ALLSOPP, 2001), the present study aimed to evaluate the presence of *Ehrlichia* species by molecular tools in blood samples of dogs from the municipality of Poconé, Pantanal region, Brazil, where higher canine seroprevalence of *Ehrlichia* spp. has been recently reported (MELO et al., 2011).

Materials and Methods

1. Study site and blood collection

The study was conducted in the municipality of Poconé (16° 15' W and 56° 37' S), 100 km southwest of Cuiabá, the capital of Mato Grosso state. Samples were obtained between July and September 2009 by Melo et al. (2011), who evaluated the prevalence of antibodies against *Rickettsia* spp. and *E. canis*, as well as the presence of ectoparasites in dogs from the related area. In the urban areas, samples were collected from 160 dogs of all (12) neighborhoods (~13 dogs per neighborhood), whereas in the rural areas, samples were obtained from 160 dogs of 25 farms (~4.7 dogs per farm) and three rural communities (~14 dogs per community; Figure 1). The blood samples were collected from the jugular vein in vacuum tubes with ethylenediamine tetraacetic acid (EDTA) and stored in 1.5 mL microtubes at –20 °C until processing. A questionnaire was given to each dog owner in order to obtain information related to age, sex, ectoparasites infestation, habitat, and hunting practice. The sample protocol was approved by the Ethics Committee for Animal Research of the Federal University of Mato Grosso (Protocol number 23108.019742/09-9).

2. Nucleic acid extraction and polymerase chain reaction

DNA was extracted from blood samples in EDTA solution using an UltraClean Blood DNA Isolation kit (MO BIO Laboratories Inc., Carlsbad, CA) according to the manufacturer's instructions. The DNA samples were initially processed by a PCR protocol designed to amplify a 409-bp fragment of the *dsb* gene of *Ehrlichia* genus (LABRUNA et al., 2007) using primers 330 forward (5' GATGATGTCTGAAGATATGAAACAAAT 3') and 728 reverse

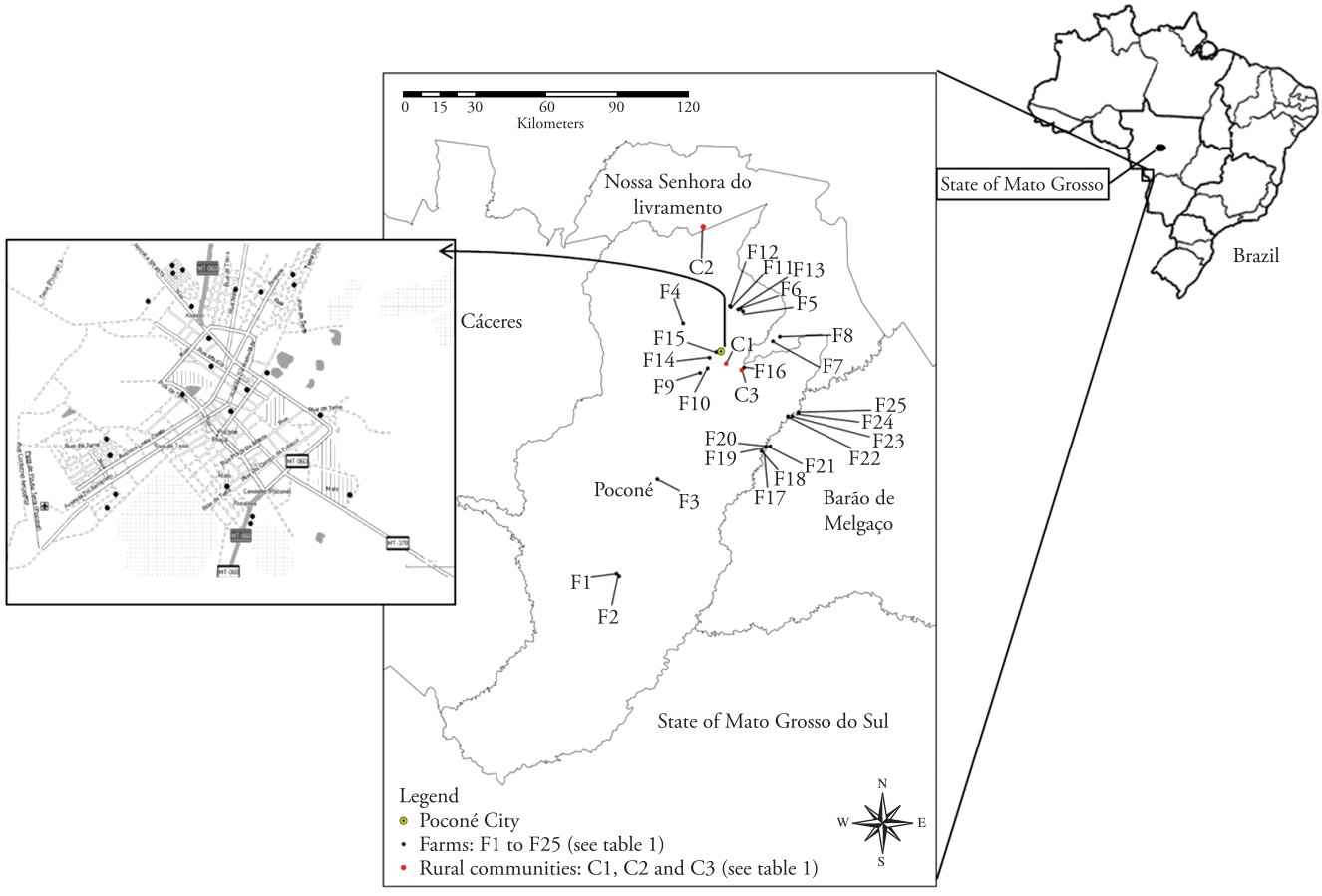

Figure 1. Poconé and neighboring municipalities of Mato Grosso State, Brazil, indicating the urban area (city of Poconé), 25 farms, and 3 rural communities where dogs were sampled in the present study. In the urban area, places where positive dogs were sampled are indicated (●).

(5' CTGCTCGTCTATTTTACTTCTTAAAGT 3)'. In addition, samples were also tested by a heminested-PCR targeting portions of 401-bp (1st reaction) and 349-bp (2nd reaction) of the *dsb* gene of all *Ehrlichia* species (ALMEIDA, 2011, Table 1). PCR products were analyzed by 1.5% agarose gel electrophoresis stained with ethidium bromide and examined by UV transillumination. Samples that yielded amplicon of the expected size by the above PCR protocols were subjected to a *dsb* real-time PCR in order to confirm previous findings through an *E. canis*-specific probe assay, as previously described (DOYLE et al., 2005). In all reactions, a positive control (DNA of *E. canis*-infected DH82 cells) as well as three negative controls (water) were included.

3. Purification and genetic sequencing

Conventional PCR products were purified using illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Bio-Sciences) according to manufacturer's instructions and sequenced directly using the primers Dsb–380 and Dsb-720 and Big Dye Kit (Applied Biosystems), according to manufacturer's instructions in ABI-PRISM 3100 Genetic Analyzer. The sequences were edited using SeqMan software (Lasergene, DNAstar, Madison, WI USA.) and similarity was analyzed using the program Basic Local Alignment Search Tool (BLAST two sequences analysis) (ALTSCHUL et al., 1990) to check for homology with corresponding sequences available in GenBank.

4. Statistical analysis

Prevalence values were calculated with a 95% confidence interval. The association between *Ehrlichia* infection in dogs and the independent variables was performed by the Chi-Square test (χ^2), or Fisher's exact test when necessary. In the rural area, the presence of tick species in farms was evaluated as another variable. Variables that presented $P \leq 0.05$ were considered significant. The statistical software EPIINFO 7.0 was used for analysis.

Results and Discussion

Forty-eight dogs (15%; 95% CI: 11.4- 19.5%) tested positive by both PCR and heminested-PCR, targeting portions of the ehrlichial *dsb* gene in order to increase the diagnostic sensitivity (ALMEIDA, 2011). All 48 dogs from the urban and rural areas were shown by *dsb* real-time PCR to contain *E. canis* DNA. Partial DNA sequences (250-bp) of 34 PCR-positive samples were generated; they were identical to each other and 100% identical to the multiple corresponding *E. canis* sequences available in GenBank (AF403710, CP000107, DQ460715, DQ460716). *Dsb* gene sequence from the identified *E. canis* isolate in this study was deposited in GenBank and assigned the nucleotide accession number JQ419757.1.

In the urban area, 25 dogs (15.6%; 95% CI: 10.4-22.2%) were positive, whereas in the rural area 23 dogs (14.4%; CI: 9.3-20.8%) from nine farms (36.0%) and all communities (100%) were positive (Table 2). However, no statistical difference for *E. canis* positivity between urban and rural dogs was observed ($P > 0.05$). Similar results were observed in the previous study by Melo et al. (2011), who detected, by IFAT, positive reaction against *Ehrlichia* spp. in 227 (70.9%) dogs: 119 (74.3%) from urban areas and 108 (67.5%) from rural areas ($P > 0.05$). Additionally, *R. sanguineus* was present in all urban neighborhoods, and was not found in only three farms of the currently PCR positive dogs (Table 2); however, the presence of *R. sanguineus* ticks on the dogs studied was not considered a risk factor for *E. canis* infection in the farms. Nevertheless, Melo et al. (2011) reported that some of these rural dogs were frequently taken to urban or suburban environments, which may explain the positivity for *E. canis* regardless of the presence of *R. sanguineus* in the farm. On the other hand, *Amblyomma* spp. were associated ($P < 0.05$) with the presence of PCR-negative dogs in the farms, emphasizing the relevance of *R. sanguineus* in the transmission of *E. canis* to dogs (HARRUS; WANER, 2011). Breed, sex and age were not associated with PCR positivity. Melo et al. (2011), studying this same dogs, reported greater frequency of seropositive dogs as they become older, probably due to a higher chance of becoming infested with ticks over time.

Table 1. *Ehrlichia* species and primer sequences*.

Ehrlichia **species (Genbank accession number)**	**Primer Dsb-330 (forward) 5'-3'**	**Primer Dsb- 720 (reverse) 5'-3'**	**Primer Dsb – 380 (forward) 5' - 3'**
Primer sequence	GATGATGCTTGAAGATATSAAACAAAT	CTATTTTACTTCTTAAAGTTGATAWATC	ATTTTTAGRGATTTTCCAATACTTGG
E. chaffeensis (AF403711)	A.....C.....	.....A.....	....A.....
E. canis (AF403710)	A.....C.....	.....	....A....C.....
E. muris (AY236484)	A.....C.....	.....C.....T.....A.....	....A....C.....C....T.....C.....
E. ruminantium (AF308669)	A.....C.....	.....	.....
Anan strain (AY236485)	.....	.....A.....	....A.....C....T.....C.....

*Primers used in heminested-PCR as follows: Dsb-330 and Dsb-720, in the first reaction; Dsb–380 and Dsb-720, in the second reaction.

Table 2. Detection of *Ehrlichia canis* DNA in dogs from the Pantanal and results of serological and ticks data in the municipality of Poconé

Locality*	Nº. tested dogs	Detection of *Ehrlichia* DNA		Number of seroreactive dogs to *Ehrlichia* spp. (%)[a]	Ticks found in dogs[a]
		Nº. positive (%)	Related species		
Urban area	160	25 (15.6)	*E. canis*	119 (74.3)	*R. sanguineus, A. cajennense*
Farm 1	06	0	-	0	-
Farm 2	17	0	-	13 (76.4)	*R. sanguineus, A. cajennense*
Farm 3	10	0	-	7 (70.0)	*A. cajennense*
Farm 4	09	1 (11.1)	*E. canis*	8 (88.8)	*R. sanguineus, A. cajennense*
Farm 5	07	1 (14.2)	*E. canis*	7 (100.0)	*R. sanguineus, A. cajennense*
Farm 6	04	1 (25.0)	*E. canis*	3 (75.0)	*R. sanguineus, A. cajennense*
Farm 7	08	4 (50.0)	*E. canis*	7 (87.5)	*R. sanguineus, A. cajennense*
Farm 8	04	2 (50.0)	*E. canis*	4 (100.0)	-
Farm 9	04	1 (25.0)	*E. canis*	3 (75.0)	*R. sanguineus, A. cajennense*
Farm 10	06	1 (16.6)	*E. canis*	2 (3.3)	*A. cajennense*
Farm 11	04	1 (25.0)	*E. canis*	2 (50.0)	-
Farm 12	04	0	-	2 (50.0)	*R. sanguineus*
Farm 13	02	0	-	1 (50.0)	*R. sanguineus*
Farm 14	02	0	-	0	*R. sanguineus*
Farm 15	05	2 (40.0)	*E. canis*	5 (100.0)	*R. sanguineus*
Farm 16	05	0	-	4 (80.0)	*A. cajennense*
Farm 17	02	0	-	2 (100.0)	-
Farm 18	01	0	-	0	*A. cajennense*
Farm 19	03	0	-	0	*A. cajennense*
Farm 20	02	0	-	2 (100.0)	*A. cajennense*
Farm 21	03	0	-	1 (33.3)	*A. cajennense*
Farm 22	02	0	-	1 (50.0)	*A. cajennense*
Farm 23	03	0	-	1 (33.3)	*A. cajennense, A. ovale*
Farm 24	03	0	-	2 (66.6)	*A. cajennense, A. ovale*
Farm 25	02	0	-	1 (50.0)	*A. cajennense*
Community 1	22	3 (13.6)	*E. canis*	12 (54.5)	*R. sanguineus, A. cajennense, A. ovale*
Community 2	09	2 (22.2)	*E. canis*	9 (100.0)	*R. sanguineus, A. cajennense*
Community 3	11	4 (36.3)	*E. canis*	9 (81.8)	*R. sanguineus, A. cajennense, A. ovale*

[a]According to Melo et al. (2011); Nº - number; % - percentage.

Despite the recent reports of ehrlichial agents other than *E. canis* in Brazil (MACHADO et al., 2006; SILVEIRA et al., 2012; ANDRÉ et al., 2010, 2012; SACCHI et al., 2012), molecular detection of new genotypic variants in Brazilian wildlife animals has been reported, since those animals play the role of sentinels for vector-borne pathogens because they can act as hosts for both bacteria and arthropod, including wildlife of the Pantanal region (WIDMER et al., 2011). However, the present work failed to detect *Ehrlichia* species different from *E. canis* infecting dogs from northern Pantanal, even though we used a high sensitive PCR capable of detecting all known *Ehrlichia* species (LABRUNA et al., 2007). These data corroborated previous studies that reported *E. canis* as the only *Ehrlichia* species that has been isolated *in vitro* from dogs in Brazil (AGUIAR et al., 2007, 2008; TORRES et al., 2002).

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