Prevalence of *Ehrlichia canis* (Rickettsiales: Ehrlichiae) DNA in Tissues From *Rhipicephalus sanguineus* (Acari: Ixodidae) Ticks in Areas Endemic for Canine Monocytic Ehrlichiosis in Brazil

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Abstract

Canine monocytic ehrlichiosis (CME) is a disease caused by the obligate intracellular bacterium *Ehrlichia canis*. Tropical lineages of *Rhipicephalus sanguineus* ticks play an essential role in the transmission of this pathogen. The aim of the present study was to evaluate the prevalence of *E. canis* DNA in tissue from *R. sanguineus* ticks in areas endemic for CME in Brazil and quantify levels of *E. canis* DNA in dissected tissues from these samples. A total of 720 ticks were collected from 72 dogs (36 dogs from the city Araçatuba in São Paulo state and 36 from Campo Grande in the state of Mato Grosso do Sul). Ticks were dissected to collect the guts, ovaries and salivary gland. A quantitative polymerase chain reaction (qPCR) targeting the disulphide bond formation (*dsb*) gene was performed to quantify the level of *E. canis* infection. The *E. canis dsb*-qPCR assay was positive for 31.9, 10, and 15.2% of the gut, ovary, and salivary glands, respectively. The average gut, ovary, and salivary gland bacterial load estimated by qPCR was 1.21 × 10^3, 2.60 × 10^3, and 4.92 × 10^3 gene copies/µl, respectively. This is the first report of *E. canis* DNA in ovaries of *R. sanguineus* ticks parasitizing dogs in these CME-endemic areas. These observations raise the possibility of *E. canis* trans-ovarial transmission.

Key words: tick, tissue, ehrlichiosis

Ehrlichiosis, caused by obligate intracellular bacteria belonging to the family Anaplasmataceae, genus *Ehrlichia*, is a highly pathogenic disease that can occur with high incidence in affected areas (Nakaghi et al. 2008). Canine monocytic ehrlichiosis (CME) can assume a multisystemic pattern, and is described as having three phases, designated acute, subclinical (asymptomatic) and chronic (Harrus et al. 1997). The diagnosis can be performed by examining blood smears. The presence of the morula stage in leukocytes is indicative of the acute phase of the infection (Moreira et al. 2003, Nakaghi et al. 2008). Serology and molecular assays can also be used for diagnosis (Sousa et al. 2013).

In Brazil, two species of *Ehrlichia* have been recognized in dogs, *Ehrlichia canis* (Costa 1973) and *Ehrlichia ewingii* (Oliveira et al. 2009). *Ehrlichia canis* is transmitted by the tropical lineage of *Rhipicephalus sanguineus* (Moraes-Filho et al. 2015). CME is prevalent in tropical and subtropical areas because of the wide geographical distribution of the tick vector (Moraes-Filho et al. 2015, Sanches et al. 2016). *Rhipicephalus sanguineus* is present throughout Brazil, especially in urban areas, where large concentrations of stray and domestic dogs occur (Labruna 2004). Populations of *R. sanguineus* ticks from South America differ in their capacity to transmit *E. canis*. It has been demonstrated that tick lineages from São Paulo, located in southeast Brazil where tropical climatic conditions prevail, are able to transmit *E. canis*. However, *R. sanguineus* lineages from temperate climatic conditions, as found in Argentina,
Uruguay, and Southern Brazil, were not capable of transmitting *E. canis* (Moraes-Filho et al. 2015).

*E. canis* multiplies in the gut, hemocytes and in epithelial cells of the salivary glands of *R. sanguineus*. Trans-stadial transmission occurs but trans-ovarial transmission has not been reported. Dogs are considered the major reservoir for this pathogen (Stich et al. 2008). Previous studies showed that infected engorged female ticks were not able to transmit *E. canis* to their progeny, since the bacterium was not detected in the ovary of experimentally infected ticks by electron microscopy (Woody 1982, Hoskins 1991, Woody 1991).

There are few reports on the occurrence of *E. canis* in *R. sanguineus* populations in Brazil. For instance, Aguiar et al. (2007) detected *E. canis* in 2.5–6% of *R. sanguineus* s.l. sampled in northern and northeastern regions of Brazil. The aim of this study was to evaluate the prevalence and quantify levels of *E. canis* in isolated gut, salivary gland, and ovaries of *R. sanguineus* from two areas endemic for CME in Brazil.

**Materials and Methods**

This study was approved by the Animal Use Ethics Committee (Comitê de Ética no Uso de Animais - CEUA) of the São Paulo State University (UNESP), School of Veterinary Medicine, Araçatuba, process FOA 2014-01458.

**Tick Sample Collection**

Ticks were sampled in the Zoonosis Control Centers in the municipalities of Araçatuba, state of São Paulo (Latitude: 21°12’41”S, Longitude: 49°25’34”W), and Campo Grande, state of Mato Grosso do Sul (Latitude: 20°26’37”S, Longitude: 54°38’52”W), Brazil. Dogs were selected randomly from individuals that were infested with at least 10 engorged adult *R. sanguineus* females. A total of 720 ticks, 10 per dog, were collected from 72 dogs (36 from Araçatuba and 36 from Campo Grande).

Ticks species were identified using the dichotomous keys of Walker et al. (2000). Immediately after identification, ticks were rinsed in phosphate buffered saline pH 7.4 (PBS) followed by ethanol, dried on a paper towel, and attached onto Petri dishes using paraffin. Gut, ovary and salivary glands were carefully dissected from each tick according to Edwards et al. (2009) and Dantas-Torres et al. (2010). Extensive precautions were taken to avoid rupturing the gut and cross-contaminating other tissues. Each dissected organ was washed three times in PBS and approximately half of the material placed into a microtube containing PBS, one organ per tube. The other half of each tissue sample was placed in a microtube containing 10% buffered formalin for preparing hematoyxin–eosin stained histological sections. Sections were examined to confirm the morphology of each organ. Tissue samples from the same organ and the same dog were pooled and stored at −80°C for subsequent DNA extraction. A total of 72 pools of gut, ovary and salivary glands were obtained in this manner. DNA was extracted according to Sangioni et al. (2005) and stored at −80°C.

**Polymerase Chain Reaction**

To quantify *E. canis* DNA concentration in each tissue pool, DNA samples were analyzed by quantitative polymerase chain reaction (qPCR) using a protocol based on the disulfide bond protein (*dsb*) gene. The qPCR reactions had a final reaction volume of 10 µl, containing 1 µl DNA, 0.2 µM of each primer (5’ −TTGCAAAAATGTGCTGAAAAATATGAAACA − 3’ and 5’ −GCTGCTCACCACAAATGTATCCYCTT− 3’), 10 µl of hydrolysis probe (10 pmol) (5’ FAM AGCTAGTGCTGGCTGGAAC TTTGAGTGAA-[BHQ1−3’]) (Doyle et al. 2005), 5 µl of MasterMix (GoTaq Probe qPCR MasterMix, Promega, Madison, WI) and ultrapure water (Nuclease-Free Water, Promega). These reactions were run in 96 wells plates (BioRad, Hercules, CA) using a CFX96 Thermal Cycler (BioRad), with the following thermal program: 95°C for 5 min, followed by 39 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was tested in duplicate. No-template negative controls were run using water to substitute for DNA in the qPCR reaction. Quantification of the number of target DNA copies/µl was performed using plasmid DNA (IDT pSMART, Integrated DNA Technologies, Coralville, IA) containing the target sequence serially diluted from 2.0 × 10³ copies/µl to 2.0 copies/µl. These serial dilutions were also used to estimate the efficiency and correlation coefficient of reactions. The plasmid copy number was determined according to the formula (Xg/µl DNA [size of plasmid in bp × 660]) × 6.022 × 10¹⁴ copies of plasmid/µl. The standardization of Quantitative Real-Time PCR (qPCR) followed the Minimum Information for Publication of Quantitative Real-Time PCR Experiments according to MIQE Guidelines (Bustin et al. 2009). Data analysis consisted of descriptive statistics.

**Results and Discussion**

The *dsb*-qPCR assay of *R. sanguineus* tissues was positive in 31.9, 10, and 15.2% of gut, ovary, and salivary gland samples, respectively (Supp. Table S1). The qPCR found average gut, ovary, and salivary gland bacterial loads of 1.21 × 10⁴, 2.60 × 10⁴ and 4.92 × 10⁴ copies/µl, respectively. The qPCR efficiency ranged from 90.2 to 99% (average: 95.66%). The correlation coefficient of the qPCR ranged from 0.767 to 0.995 (average: 0.949) and the slope ranging from −3.346 to −3.581 (mean = −3.442) (Supp. Table S2).

All tick pools positive for *E. canis* DNA in the salivary glands or ovary were also positive in the gut sample. Two ticks were positive for *E. canis* DNA in all tissue pools (2.78%) and seven ticks were positive only in the gut pool (9.72%) (Table 1). Although *E. canis* had never been reported in the ovaries, our results can be explained by the fact that *E. canis* is present in different tissues depending on its multiplication stage in *R. sanguineus* (Woody 1991). *Ehrlichia* is known to be present in the hemolymph (Cruz et al. 2012), and thus, it is possible that the detection of *E. canis* in the ovaries of ticks is due to *E. canis* being attached to the surface of the ovaries and not due to the invasion of reproductive tissues.

Although the molecular phylogeny of *R. sanguineus* was not examined in the present study, the high occurrence of *E. canis* DNA in the examined ticks (32%) is likely because our sampled populations were from regions where the tropical lineage of this tick occurs. These lineages are important in the epidemiology of CME because, recently, the tropical lineage’s vectorial competence for *E. canis* (Sanches et al. 2016) was demonstrated (Moraes-Filho et al. 2015). The high percentage of *E. canis* positive ticks we found may explain the high occurrence of *E. canis* in dogs from Campo Grande reported by Sousa et al. (2013).

<table>
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<th>Table 1. Qualitative results of <em>dsb</em>-qPCR assay targeting <em>Ehrlichia canis</em> in three <em>Rhipicephalus sanguineus</em> tissue pools</th>
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<tr>
<td><strong>dsb-qPCR</strong></td>
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It is important to note that *E. canis* DNA was detected in the gut and salivary glands of *R. sanguineus* tick pools collected from dogs. This observation is consistent with this agent’s multiplication in these organs, as well as in the hemocoele, favoring trans-stadial transmission (Stich et al. 2008). Early studies showed that infected engorged females were unable to transmit *E. canis* to their progeny, and bacteria could not be detected in the ovaries of experimentally infected ticks using electron microscopy (Wooody 1982, Hoskins 1991, Woody 1991). Contrary to these reports, we detected *E. canis* DNA in 10% of *R. sanguineus* ovary pools, based on the dsb-qPCR assay. This finding justifies future studies to re-evaluate the competence of various *R. sanguineus* lineages for their competence to transmit *E. canis* trans-ovarially.

A significant percentage of *R. sanguineus* tick pools in our study were positive for *E. canis* DNA. This contrasts with the report of Aguiar et al. (2007) finding 2.5 to 6% *E. canis* positive *R. sanguineus* ticks from populations in the North and Southeast regions of Brazil and Souza et al. (2010) finding 21.9% in a study carried out in the northeastern region of Brazil. The prevalence of *E. canis* DNA in our samples of *R. sanguineus* was higher than previously described in other countries, such as Portugal (Sanches et al. 2018), Mexico (Pat-Nah et al. 2015, Ojeda-chi et al. 2018) and Ivory Coast (Socolovschi et al. 2012).

The methodology described in our work to detect *E. canis* was previously used to detect several other pathogens transmitted by several species of ticks such as Babesia spp., Theileria spp., and Anaplasma spp. (Georges et al. 2001, Borrelia spp. (Xu et al. 2013) and Leishmania spp. in *R. sanguineus* (Viol et al. 2016).

In conclusion, we detected *E. canis* DNA in gut, salivary gland and ovary of *R. sanguineus* ticks parasitizing dogs in two cities of Brazil located in CME-endemic areas. The presence of this pathogen in some of the tick ovary samples raises the possibility that trans-ovarian transmission is occurring.

Supplementary Data
Supplementary data are available at *Journal of Medical Entomology* online.

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References Cited


