

# Occurrence of *Ehrlichia canis* and *Hepatozoon canis* and probable exposure to *Rickettsia amblyommatis* in dogs and cats in Natal, RN

Ocorrência de *Ehrlichia canis*, *Hepatozoon canis* e provável exposição a *Rickettsia amblyommatis* em cães e gatos de Natal, RN

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## Abstract

Occurrence of infection or exposure to *Ehrlichia canis*, *Hepatozoon canis* and *Rickettsia* spp. was detected in feral cats living in two fragments from Atlantic rainforest, in Natal, RN, Brazil, and in dogs living around the parks. While serum samples were collected from 155 animals (53 cats living in the parks; 29 dogs living in human homes around the parks; and 73 dogs living at an animal control center - ACC), spleen samples were collected from 20 dogs that were euthanized at ACC. Serum samples were analyzed to *Rickettsia* spp. and *E. canis* antibodies using the indirect immunofluorescence assay. Seventeen of the 102 dogs (17%) had *E. canis* antibodies and 13% (20/155) of all dogs and cats (i.e. 3% (3/102) of the dogs and 32% (17/53) of the cats) were seropositive for *Rickettsia* spp. antigens. The animals were therefore been exposed to *R. amblyommatis* or by a very closely related genotype. Among the 20 dog spleen samples analyzed, eight were PCR positive for *E. canis* and two for *H. canis* (GenBank accession number MG772657 and MG772658, respectively). In none of the spleen samples were obtained amplicons for *Babesia* spp. through PCR. This study provided the first evidence that *Rickettsia* of the spotted fever group is circulating among dogs and cats in Natal.

**Keywords:** *R. amblyommatis*, PCR, IFA, Atlantic rainforest, Brazil.

## Resumo

A ocorrência de infecção ou exposição para *Ehrlichia canis*, *Hepatozoon canis* e *Rickettsia* spp. foi determinada em gatos ferais que viviam em dois fragmentos da Mata Atlântica, localizados em Natal, RN, Brasil e em cães que viviam em torno dos parques e em outras regiões da cidade. Enquanto amostras de soro foram coletadas de 155 animais (53 gatos que viviam nos parques, 29 cães com domicílio em torno dos parques e 73 cães do Centro de Controle de Animais -CCA), fragmentos de baço foram coletados de 20 cães eutanasiados no CCA. A detecção de anticorpos nas amostras de soros coletadas contra *Rickettsia* spp. e *E. canis* foi realizada pela Reação de Imunofluorescência Indireta. Dezesete dos 102 cães (17%) apresentaram anticorpos anti *E. canis* e 13% (20/155) de todos os cães e gatos (ou seja, 3% (3/102) dos cães e 32% (17/53) dos gatos) foram soropositivos para antígenos de *Rickettsia* spp. Os animais foram considerados expostos à *R. amblyommatis* ou a um genótipo muito relacionado. Entre as 20 amostras de baço de cães analisadas, oito foram positivas para *E. canis* e duas para *Hepatozoon canis* (números de acesso ao Genbank MG772657 e MG772658, respectivamente). Nenhuma das amostras de baço produziram amplicons de *Babesia* spp. na PCR. Observou-se, pela primeira vez, a circulação de *Rickettsia* do grupo da febre maculosa em cães e gatos em Natal, RN.

**Palavras-chave:** *R. amblyommatis*, PCR, RIFI, Mata Atlântica, Brasil.

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## Introduction

Dogs and cats are very popular as pets around the world and can be reservoirs for many microorganisms. *Ehrlichia canis*, *Hepatozoon canis* and *Rickettsia* of the spotted fever group (SFG) may cause diseases in dogs that develop either subclinical infection or severe clinical signs (HARRUS & WANER, 2011, in review; GONDIM et al., 1998; PIRANDA et al., 2008; STILES, 2000). However, the information on clinical signs showing by cats infected by tick-borne agents remains contradictory and requires further characterization (FRITZ & KJEMTRUP, 2003; SHAW et al., 2001). These agents have been reported infecting dogs in many parts of Brazil at wide ranges of occurrence and prevalence rates (COSTA et al., 2015; MIRANDA et al., 2014; KRAWCZAK et al., 2012; RAMOS et al., 2010; ROTONDANO et al., 2015, 2017; SAITO et al., 2008; SILVA et al., 2010; VIEIRA et al., 2011).

Wild carnivores inhabiting natural areas in periurban locations may be infected by the same pathogens that infect dogs and cats at higher infection rates (MILLÁN et al., 2016). Definition of the reservoirs and vectors involved in the transmission routes is an important step towards prevention of zoonoses.

In this study, we attempted to detect the occurrence of *Babesia* spp., *Ehrlichia canis*, *Hepatozoon canis* and *Rickettsia* spp. in feral cats living in two forest fragments located in the Atlantic Rainforest biome, in the municipality of Natal, state of Rio Grande do Norte (RN), Brazil, and in dogs living around the parks and in other regions of the city.

## Materials and Methods

### Study area and sample collection

For this study, feral cats living in two forest fragments within the Atlantic Rainforest Biome, in Natal, RN, Brazil, were selected: Parque Estadual das Dunas de Natal (Natal Dunes State Park; 5° 49' 41" S and 35° 11' 25" W) and Parque da Cidade (City Park; 5° 50' 45" S and 35° 14' 0" W). In addition, we sampled the population of dogs living in homes located around the parks and dogs at the Animal Control Center (ACC) of the city of Natal, which held dogs from several city regions.

Feral short hair cats were caught manually. These cats were found living in the parks without any care provided and in a state of uncontrolled reproduction, abandoned there by citizens. Apparently healthy dogs, living in their owners' homes around the parks, were sampled by convenience in these homes, according to the accessibility of the place. The dogs at the ACC were euthanized for a variety of reasons, and samples were collected on these occasions.

All the blood samples were collected from the cephalic or jugular vein. The blood serum from each animal was stored separately in microtubes at -20 °C until the laboratory analyses were performed. Some ticks that were found parasitizing the animals were collected and taken to the laboratory for taxonomic identification. The ticks were taxonomically identified following dichotomous keys: Martins et al. (2010) for nymphs; Barros-Battesti et al. (2006) for adults.

### Serological analyses

Canine serum samples were tested individually by means of the indirect immunofluorescence assay (IFA) using *E. canis*-infected DH82 cells as the antigen. The São Paulo strain of *E. canis* was used for this, as previously described (AGUIAR et al., 2007, 2008). Reactions were performed using fluorescein-conjugated rabbit anti-dog IgG diluted 1:1000 (Sigma-Aldrich, St. Louis, MO, USA). Serum was considered to contain antibodies reactive to *E. canis* if it displayed a reaction at the dilution 1:80. Samples that reacted at the screening dilution (1:80) were then titrated using serial twofold dilutions to determine endpoint titers. The positive and negative control serum samples were derived from the study by Aguiar et al. (2007).

Feline and canine antibodies reactive to *Rickettsia* spp. were assayed by simultaneously using six *Rickettsia* isolates from Brazil: *R. bellii* strain Mogi, *R. amblyommatis* strain Ac37, *R. rhipicephali* strain HJ5, *R. rickettsii* strain Taiaçu, *R. parkeri* strain At24, and *Rickettsia felis* strain Pedreira, as previously described (LABRUNA et al., 2007a). Samples that reacted at the screening dilution (1:64) were then titrated using serial twofold dilutions to determine endpoint titers. Fluorescein isothiocyanate-labeled conjugate goat anti-cat IgG, or rabbit anti-dog IgG diluted 1:1000 (Sigma, St Louis, USA) were used. To determine the probable homologous antigens (PHA), serum showing a *Rickettsia* species reactive titer at least four-fold higher than those observed for the other *Rickettsia* species was taken to be homologous to the first *Rickettsia* species or to a very closely related genotype (LABRUNA et al., 2007b; PIRANDA et al., 2008). As control, a dog and cat serum previously shown to be non-reactive (negative controls) and known reactive serum samples (positive control) were used (HORTA et al., 2007). These samples were obtained from the serum bank of the Laboratory of Parasitic Diseases, Department of Preventive Veterinary Medicine and Animal Health (VPS), School of Veterinary Medicine and Animal Science (FMVZ), University of São Paulo (USP).

### Molecular analyses

DNA was extracted from dog spleen fragments of approximately 20mg each, using the Wizard® genomic DNA purification kit (Promega Corporation, Madison, Wisconsin, USA), in accordance with the manufacturer's instructions. DNA samples were tested by Polymerase Chain Reaction (PCR) using the sets of primers described in Table 1. Reactions were performed in a final volume of 25 µl containing 10 mM of Tris-HCl (pH 8.3), 50 µM of KCl, 1.5 mM of MgCl<sub>2</sub>, 0.2 mM of each desoxynucleoside triphosphate, 1.5 U of Taq DNA polymerase (Invitrogen), 11 pmol of each primer and approximately 100 ng of canine genomic DNA. The amplified products were viewed under ultraviolet light after electrophoresis on agarose gel (1.5%) stained with SyBr gold (Invitrogen). The PCR products were purified using ExoSap (USB) and were sequenced in an automatic sequencer (model ABI Prism 310 Genetic; Applied Biosystems/Perkin Elmer, California, USA), in accordance with the manufacturer's protocol, and with the same primers as used in the PCR. The partial sequences obtained were

subjected to BLAST analyses (ALTSCHUL et al., 1990) to infer the closest similarities to sequences in GenBank.

## Results

Between October 2012 and August 2013, four campaigns were conducted, and serum samples were collected from 155 animals: 53 feral cats (*Felis catus*) living in the parks; 29 dogs (*Canis familiaris*) living in human homes around the parks; and 73 dogs at the ACC. Furthermore, spleen fragments from 20 dogs that were euthanized at the ACC were also collected.

The serological results showed that 17% (17/102) of the dogs showed anti-*E. canis* antibodies and 13% (20/155) of all the animals tested, i.e. 3% (3/102) of the dogs and 32% (17/53) of the cats, were seropositive for *Rickettsia* spp. antigens (Table 2), with endpoint titers ranging from 64 to 2048 (Table 3). Except for one dog serum that only showed *R. bellii* as the PHA, and one cat serum that showed *R. parkeri* as the PHA, all the other positive animals showed seroreactivity with the highest titers for *R. amblyommatis*. At least 13 animals presented *R. amblyommatis* as the PHA, for which endpoint titers were four-fold higher than the endpoint titers shown for the other five *Rickettsia* species. These 13 animals might have been exposed to *R. amblyommatis* or a very closely related genotype (Table 3).

Parasitism by ticks of the species *Rhipicephalus sanguineus* sensu lato (s.l.) was observed in 38% (11/29) of the dogs, Park<sup>1</sup> 23,5% (4/17) and Park<sup>2</sup> 58,3% (7/12). One cat (2% [1/49]) from

Parque da Cidade was found to be parasitized by one nymph stage of *Amblyomma auricularium*.

Among the 20 dog spleen samples, subjected to molecular analyses, eight were found to be positive in the PCR targeting a fragment of the 16S rRNA gene of Anaplasmataceae, and in the PCR targeting a fragment of the *dsb* gene of *Ehrlichia* spp. PCR products of 16S rRNA and *dsb* gene generated sequences 100% similarity to *E. canis*, which is found in several parts of the world, including Brazil (16S rRNA: KP642752, KP182941, KP182942; *dsb*: MG967467, KY594915, CP025749, KR920044). Furthermore, two samples were positive for *Hepatozoon canis*, which were confirmed by DNA sequences and BLAST analysis, showing to be 100% identity to sequences already reported from dogs around the world (KJ513193, KJ513198, KF621083). None of the canine spleen samples yielded amplicons in the PCR for *Babesia* spp.

DNA sequences generated in this study were deposited in GenBank under the accession numbers MH118746 for *E. canis* 16S rRNA partial sequence, MG772657 for *E. canis dsb* partial sequence, and MG772658 for *H. canis* 18S rRNA partial sequence.

## Discussion

The results from the serological analyses on *Rickettsia* spp. showed that the number of seropositive dogs was low (3%), while it was relatively high (32%) among the sampled cats (Table 2). This can possibly be explained by the fact that the cats were living inside the parks and were exposed to parasitism by *A. auricularium*,

**Table 1.** Primer pairs used in the present study for detecting tick-borne agents.

| Target agents (gene)                     | Primers              | Primer sequences (5'-3')                                      | (Bp) | Reference             |
|------------------------------------------|----------------------|---------------------------------------------------------------|------|-----------------------|
| <i>Babesia</i> spp. (18S rRNA)           | BAB1<br>BAB4         | GTGAACCTTATCACTTAAAGG<br>CAACTCCTCCACGCAATCG                  | 590  | Duarte et al. (2008)  |
| Anaplasmataceae (16S rRNA)               | GE2'F2'<br>HE3       | GTTAGTGGCAGACGGGTGAGT<br>TATAGGTACCGTCATTATCTTCCCTAT          | 360  | Aguiar et al. (2008)  |
| <i>Ehrlichia</i> spp. ( <i>dsb</i> gene) | DSB-330<br>DSB-728   | GATGATGTCTGAAGATATGAAACAAAT<br>CTGCTCGTCTATTTTACTTCTTAAAGT    | 409  | Doyle et al. (2005)   |
| <i>Hepatozoon</i> sp. (18S rRNA)         | HEP2-169<br>HEP2-718 | F- GGTAATTCTAGAGCTAATACATGAGC<br>R- ACAATAAAGTAAAAACAYTTCAAAG | 574  | Almeida et al. (2012) |

Bp = Base Pairs.

**Table 2.** Distribution according to area of the numbers of dogs and cats in the state of Rio Grande do Norte, Brazil, that were analyzed for *Rickettsia* spp. and *E. canis* using the indirect immunofluorescence assay (IFA), showing the numbers of positive cases and percentages.

| Animals                                      | No. seropositive animals / No. tested (% seropositivity) |             |                         |
|----------------------------------------------|----------------------------------------------------------|-------------|-------------------------|
|                                              | IFA for <i>Rickettsia</i> spp.                           |             | IFA for <i>E. canis</i> |
| <i>Felis catus</i> (Park <sup>1</sup> )      | 1/4 (25)                                                 | 17/53 (32%) | Not tested              |
| <i>Felis catus</i> (Park <sup>2</sup> )      | 16/49 (33)                                               |             |                         |
| <i>Canis familiaris</i> (Park <sup>1</sup> ) | 1/17 (6)                                                 | 3/102 (3%)  | 5/17 (25)               |
| <i>Canis familiaris</i> (Park <sup>2</sup> ) | 2/12 (16)                                                |             | 3/12 (25)               |
| <i>Canis familiaris</i> (ACC)                | 0/73 (0)                                                 |             | 9/73 (12)               |
| <b>Total</b>                                 | 20/155 (13)                                              |             | 17/102 (17)             |

<sup>1</sup>Parque Estadual das Dunas de Natal; <sup>2</sup>Parque da Cidade; ACC = animal control center.

**Table 3.** Results from indirect immunofluorescence assays (IFA) against six *Rickettsia* species among serum samples from dogs and cats in Natal, Rio Grande do Norte, Brazil.

| Animals IDs                       | Endpoint titers for rickettsial antigens |                              |                             |                              |                              |                              | PHA <sup>7</sup> |
|-----------------------------------|------------------------------------------|------------------------------|-----------------------------|------------------------------|------------------------------|------------------------------|------------------|
|                                   | <i>R. ambly</i> <sup>1</sup>             | <i>R. rhipi</i> <sup>2</sup> | <i>R. rick</i> <sup>3</sup> | <i>R. parke</i> <sup>4</sup> | <i>R. belli</i> <sup>5</sup> | <i>R. felis</i> <sup>6</sup> |                  |
| Dog 10 (park <sup>1</sup> )       | 0                                        | 0                            | 0                           | 0                            | 256                          | 0                            | <i>R. belli</i>  |
| Dog 23 (park <sup>2</sup> )       | 1024                                     | 0                            | 0                           | 0                            | 0                            | 0                            | <i>R. ambly</i>  |
| Dog 25 (park <sup>2</sup> )       | 512                                      | 0                            | 0                           | 0                            | 64                           | 0                            | <i>R. ambly</i>  |
| Cat 33 (park <sup>2</sup> )       | 256                                      | 0                            | 0                           | 0                            | 0                            | 0                            | <i>R. ambly</i>  |
| Cat 5 (park <sup>2</sup> )        | 64                                       | 0                            | 0                           | 64                           | 0                            | 0                            | -                |
| Cat 8 (park <sup>2</sup> )        | 64                                       | 0                            | 0                           | 0                            | 0                            | 0                            | -                |
| Cat 19 (park <sup>2</sup> )       | 2048                                     | 512                          | 256                         | 256                          | 128                          | 64                           | <i>R. ambly</i>  |
| Cat 20 (park <sup>2</sup> )       | 1024                                     | 0                            | 0                           | 0                            | 0                            | 0                            | <i>R. ambly</i>  |
| Cat 9 (28.2) (park <sup>2</sup> ) | 512                                      | 0                            | 0                           | 0                            | 0                            | 0                            | <i>R. ambly</i>  |
| Cat 6 (1.3) (park <sup>2</sup> )  | 2048                                     | 256                          | 256                         | 0                            | 256                          | 0                            | <i>R. ambly</i>  |
| Cat 35 (park <sup>2</sup> )       | 256                                      | 0                            | 0                           | 0                            | 0                            | 0                            | <i>R. ambly</i>  |
| Cat 24 (park <sup>2</sup> )       | 1024                                     | 0                            | 0                           | 0                            | 0                            | 0                            | <i>R. ambly</i>  |
| Cat 23 (park <sup>2</sup> )       | 512                                      | 0                            | 0                           | 0                            | 0                            | 0                            | <i>R. ambly</i>  |
| Cat 32 (park <sup>2</sup> )       | 64                                       | 0                            | 0                           | 0                            | 0                            | 0                            | -                |
| Cat 7 (park <sup>2</sup> )        | 0                                        | 0                            | 0                           | 64                           | 0                            | 0                            | -                |
| Cat 9 (park <sup>2</sup> )        | 512                                      | 256                          | 0                           | 1024                         | 0                            | 0                            | -                |
| Cat 11 (park <sup>2</sup> )       | 256                                      | 0                            | 0                           | 0                            | 0                            | 0                            | <i>R. ambly</i>  |
| Cat 1 (1.3) (park <sup>1</sup> )  | 1024                                     | 0                            | 0                           | 0                            | 0                            | 0                            | <i>R. ambly</i>  |
| Cat 31 (park <sup>2</sup> )       | 512                                      | 0                            | 0                           | 0                            | 0                            | 0                            | <i>R. ambly</i>  |
| Cat 9 (18.2) (park <sup>2</sup> ) | 0                                        | 0                            | 0                           | 256                          | 0                            | 0                            | <i>R. parke</i>  |

<sup>1</sup>*Rickettsia amblyommatis*; <sup>2</sup>*R. rhipicephali*; <sup>3</sup>*R. rickettsii*; <sup>4</sup>*R. parkeri*; <sup>5</sup>*R. belli*; <sup>6</sup>*R. felis*; <sup>7</sup>PHA = probable homologous antigens; park<sup>1</sup> = Parque Estadual das Dunas de Natal; park<sup>2</sup> = Parque da Cidade.

as observed on this work. It is known that this tick is a competent vector for *R. amblyommatis* (SARAIVA et al., 2013). Moreover, two dogs living in the area around the parks showed antibodies against *R. amblyommatis* while the dogs at the ACC were all negative. In a previous study in the state of São Paulo, Horta et al. (2007) showed that cats could be better rickettsial sentinels than dogs. Cats may be more exposed to ticks than other domestic animals because of their habits. In the present study, the cats abandoned in the parks had become feral, living in direct contact with the wild environment.

The importance of *R. amblyommatis* in relation to pathogenicity for humans is not yet fully elucidated. However, some Rocky Mountain spotted fever (RMSF) cases in the United States that were diagnosed as due to *R. rickettsii* may have instead been due to *R. amblyommatis* (APPERSON et al., 2008).

The IFA test showed that antibodies for *E. canis* were found in 17% (17/102) of the dogs analyzed in this study. A similar seropositivity rate was previously observed by Costa et al. (2015), who found antibodies to this agent in 14.6% of the dogs from rural and urban areas of the state of Maranhão, Brazil. Higher seropositivity rates among dogs have been reported in studies conducted in other states in northeastern Brazil: Paraíba 72.5%, 69.4%, 23% and 34% (ARAES-SANTOS et al., 2015; AZEVEDO et al., 2011; ROTONDANO et al., 2015; TANIKAWA et al., 2013); and Bahia 23.0%, 36% and 35.6% (ARAES-SANTOS et al., 2015; CARLOS et al., 2007; SOUZA et al., 2010).

Confirming the serological findings, *E. canis* DNA was detected by PCR in 40% (8/20) of the canine spleen samples.

Furthermore, 75% (6/8) of these PCR-positive dogs were also positive in serological analysis for *E. canis* antibodies. Previous studies in the state of Rio Grande do Norte showed results that corroborate the findings of the present study: fragments of DNA sequences of *E. canis* and *H. canis* were detected in blood samples collected from dogs in the city of Mossoró (GONÇALVES et al., 2014). Furthermore, inclusions suggestive of *Ehrlichia* spp. were detected in 6.5% (13/198) of dogs showing clinical signs suggestive of canine monocytic ehrlichiosis in the same city (MEDEIROS & LIMA, 2004). These results revealed that *E. canis* was the main tick-borne pathogen infecting dogs in northeastern Brazil, a condition corroborated by the present study.

In Brazil, *Hepatozoon canis* infection has been reported in many regions and the occurrence rate may range from 8.6% to 100% in the southeastern region (MIRANDA et al., 2014; MUNDIM et al., 2008; O'DWYER et al., 2001; SPOLIDORIO et al., 2009) and 3.6% to 73% in the central-western region (MELO et al., 2016; MUNDIM ECS. et al., 2008; PALUDO et al., 2003; RAMOS et al., 2015), while two cases with molecular analyses has been reported in the southern region (LASTA et al., 2009; MALHEIROS et al., 2016). Studies in northeastern Brazil have shown lower occurrence rates ranging from 0.49% in Pernambuco to 9.3% in Paraíba (BERNARDINO et al., 2016; RAMOS et al., 2010; ROTONDANO et al., 2015). Gonçalves et al. (2014) reported *H. canis* infection cases in Mossoró, RN. Our results strengthen the data on circulation of this parasite in this study region through showing that 10% (2/20) of the dog spleen samples were positive for *H. canis* by means of PCR.

*Rhipicephalus sanguineus* s.l. was the only tick species found on the dogs evaluated in this study. This Ixodidae is a competent vector for *E. canis* (DANTAS-TORRES, 2008) corroborating with the serological and molecular results of the present study.

In conclusion, *Rickettsia* spp., represented mainly by anti-*R. amblyommatis* antibodies, are circulating among cats and dogs in the anthropized fragments of the Atlantic Rainforest biome in the municipality of Natal, RN, Brazil. Cats were shown to be an important sentinel for *Rickettsia* spp., and *E. canis* and *H. canis* are also infecting dogs in the urban area of Natal. To our knowledge, the present study provided the first evidence suggesting that *Rickettsia* of the spotted fever group is circulating among dogs and cats in the region analyzed here in the state of Rio Grande do Norte, northeastern Brazil.

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