MARCOS GOMES LOPES

**Epidemiological aspects of tick-borne diseases in wild and
domestic animals of two environmental protection areas in the city
of Natal, Rio Grande do Norte State, Brazil**

São Paulo

2016

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of two environmental protection areas in the city of Natal, Rio Grande do Norte
State, Brazil**

Tese apresentada ao Programa de Pós-Graduação em Epidemiologia Experimental Aplicada às Zoonoses da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo para a obtenção do título de Doutor em Ciências

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Orientador:

Profa. Dra. Solange Maria Gennari

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BIOÉTICA

CERTIFIED

We certify that the Research "ZOO NOTIC AGENTS IN DOMESTIC AND WILD ANIMALS IN TWO PROTECTED AREAS IN THE NATAL CITY - RIO GRANDE DO NORTE.", protocol number CEUAX 606207, under the responsibility Marcos Gomes Lopes, agree with Ethical Principles in Animal Research adopted by Ethic Committee in the Use of Animals of the School of Veterinary Medicine and Animal Science of University of São Paulo, and was approved in the meeting of day April 14, 2016.

Certificamos que o protocolo do Projeto de Pesquisa intitulado "ESTUDO DE AGENTES ZOONÓTICOS EM ANIMAIS DOMÉSTICOS E SILVESTRES EM DUAS UNIDADES DE CONSERVAÇÃO NO MUNICÍPIO DE NATAL, RIO GRANDE DO NORTE ", protocolado sob o CEUAX nº 606207, sob a responsabilidade de Marcos Gomes Lopes, está de acordo com os princípios éticos de experimentação animal da Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo, e foi aprovado na reunião de 14 de abril de 2016.

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FOLHA DE AVALIAÇÃO

Autor: LOPES, Marcos Gomes

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Dedico a minha mãe Vitória Cedomia Ribeiro Gomes Lopes em cumprimento a nossa promessa. Há 23 anos, numa manhã de inverno, em uma rua calçada de pedras e cercada por casas simples à esquerda e terrenos de capim verde à direita, ela segurava minha mão me levando a escola quando eu olhei em seus olhos e pedi que ela me ajudasse a chegar até aqui. Nós sequer sabíamos onde era, mas ela prometeu. Ela nunca fraquejou em manter sua promessa apesar de eu tê-lo feito em vários momentos.

Obrigado Vitória!

“Sou como a haste fina: qualquer brisa verga, mas nenhuma espada corta!

Pensou que eu ando só? ”

Maria Bethânia: Carta de amor

ABSTRACT

LOPES, M. G. **Epidemiological aspects of tick-borne diseases in wild and domestic animals of two environmental protection areas in the city of Natal, Rio Grande do Norte State, Brazil.** [Aspectos epidemiológicos de agentes transmitidos por carrapatos em animais silvestres e domésticos de duas Unidades de Conservação, na Cidade de Natal, RN]. 2016. 74 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2016.

The aim of this study was to determine the serologic and molecular occurrence of *Rickettsia* spp., *Ehrlichia* spp., *Babesia* spp. and *Hepatozoon* spp. in ticks and domestic and wild animals from two conservation units in the city of Natal, Rio Grande do Norte State, Brazil. The collection period was between October 2012 and August 2013. Serum samples were tested against *Rickettsia* spp. antigens and *Ehrlichia canis* by Indirect fluorescent antibody test. Tissue samples and ticks were processed for molecular detection of the pathogens. Twenty-seven marsupials and four rodents were captured, and up to three animals of each species were euthanized. In addition, serum samples from 155 domestic animals: 53 cats living inside the units, 29 dogs domiciled around the areas and 73 dogs of the Zoonosis Control Center of the City (ZCC). Twenty dogs from ZCC were also euthanized and samples of spleen were obtained. Antibodies to at least one of the *Rickettsia* species tested were detected in six *Didelphis albiventris* and in one *Rattus rattus*; 17% (17/102) of the dogs presented antibodies to *E. canis* and 13% (20/155) of all tested domestic animals (dogs and cats) were seropositive for *Rickettsia* spp. antigens. Three species of ticks (*Amblyomma auricularium*, *Ixodes loricatus* and *Ornithodoros mimon*) were collected and one *A. auricularium* was positive for *Rickettsia amblyommii* by PCR. Two *D. albiventris* spleen samples amplified PCR products for *Ehrlichia* spp. Spleen samples from three *D. albiventris* and spleen and lung sample from one *Necromys lasiurus* were positive for *Babesia* spp. by PCR test. Among the 20 spleen samples from dogs subjected to molecular analysis, eight were positive by PCR for *E. canis* and two for *H. canis*.

Keywords: *Rickettsia*. *Ehrlichia*. *Babesia*. *Hepatozoon*. Brazil.

RESUMO

LOPES, M. G. **Aspectos epidemiológicos de agentes transmitidos por carrapatos em animais silvestres e domésticos de duas Unidades de Conservação, na Cidade de Natal, RN.** [Epidemiological aspects of tick-borne diseases in wild and domestic animals of two environmental protection areas in the city of Natal, Rio Grande do Norte State, Brazil]. 2016. 74 f. Tese (Doutorado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2016.

O objetivo deste estudo foi determinar a ocorrência sorológica e molecular de *Rickettsia*, *Ehrlichia*, *Hepatozoon* e *Babesia* em carrapatos e mamíferos silvestres e domésticos, provenientes de duas unidades de conservação ambiental (UC) na cidade de Natal, Rio Grande do Norte, Brasil. O período de coleta foi de outubro de 2012 a agosto de 2013. Os soros foram testados contra antígenos de *Rickettsia* spp. e *Ehrlichia canis* através da reação de imunofluorescência indireta. Amostras de tecido e carrapatos foram processadas para a detecção molecular dos patógenos. Foram capturados 27 marsupiais e quatro roedores para coleta de sangue, destes foram eutanasiados até três animais de cada espécie e coletadas amostras de baço e pulmão. Paralelo, amostras de soro de 155 animais domésticos: 53 gatos que viviam nas UCs, 29 cães domiciliados no entorno das áreas e 73 cães do Centro de Controle de Zoonoses do município, dos quais 20 tiveram amostras de baço coletadas. Foram detectados anticorpos para, pelo menos, uma das espécies de *Rickettsia* testadas em seis *Didelphis albiventris* e em um *Rattus rattus*, e 17 % (17/102) dos cães apresentaram anticorpos anti-*E. canis* e 13% (20/155) de todos os animais domésticos (cães e gatos) foram soropositivos para antígenos de *Rickettsia* spp. Três espécies de carrapatos (*Amblyomma auricularium*, *Ixodes loricatus* e *Ornithodoros mimon*) foram coletadas e um *A. auricularium* foi positivo para *R. amblyommii* pela PCR. Duas amostras de baço de *D. albiventris* amplificaram produtos de PCR para *Ehrlichia* spp. e amostras de baço de três *D. albiventris* e baço e pulmão de um *Necromys lasiurus* foram positivas para *Babesia* spp. pela PCR. Entre as 20 amostras de baço de cão submetidas a análises moleculares, oito foram positivas na PCR para *E. canis* e duas para *H. canis*.

Palavras-chave: *Rickettsia*. *Ehrlichia*. *Babesia*. *Hepatozoon*. Natal.

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1 INTRODUCTION

1.1 STUDY AREA

The city of Natal, in the state of Rio Grande do Norte, has ten Environmental Protection Areas as defined by the Master Plan of the city. This study was performed at the Environmental Protection Zone 1 (ZPA 1) and 2 (ZPA2) as shown in Figure 1.

Figure 1 - Limits of ZPA 1 and ZPA2

Source: modified from Natal, RN (2008)

The Environmental Protection Sun Valle Zone (ZPA 1) is located in the southern area of Natal and covers the Sun Valle set in Pitimbu neighborhood. It is

seen as the last source of clean water to the city of Natal, supplying 16 districts of the capital.

The Parque da Cidade "Dom Nivaldo Monte", located in the ZPA-1, (5.851067W/35.228306N) (NATAL, RN, 2008), has approximately 64 hectares. This park mainly covers three districts, named: Pitimbú, Candelaria and Cidade Nova (Figure 2).

Figure 2 - Delimitation of Parque Dom Nivaldo Monte within the ZPA 1

Source: Natal, RN (2008).

The original biome is the Atlantic Forest and consists of dune formations covered mostly with salt marsh vegetation (RAMALHO; PIMENTA, 2010). The climate is tropical humid with average annual temperature of 26°C and annual rainfall of 2,500 mm, with most intense rainy season between March and July (RAMALHO; PIMENTA, 2010).

ZPA 2 is located in the east area of Natal and refers to "Parque Estadual das Dunas de Natal". It is covered by the called Coastal Board area and the surrounding areas are field soft grass and dune areas of the coast village (IDEMA 2011).

The Parque das Dunas de Natal (83.818589W/12.034402N) is characterized by a predominance of species peculiar to the Coastal Tablelands, the Atlantic Forest and even the Caatinga vegetation (FREIRE, 1990). The main anthropic influences in

Parque das Dunas are due to the presence of a ring of road that virtually encircles its entire length and in the southwest portion, due to the presence of the campus Center of the Federal University of Rio Grande do Norte (UFRN), covering 123 hectares. Despite this, the park suffers less anthropic pressure than the Parque da Cidade (Figure 3).

Figure 3 - Aerial view of part of the “Parque das Dunas de Natal - Journalist Luiz Maria Alves”

Source: IDEMA (2011).

1.2 JUSTIFICATION

Disturbances are major environmental factors that affect species diversity in natural areas (SOUSA, 1984). These disturbances may be considered events that promote changes in the ecosystems structures and reduce the variety of species by competition in the availability of resources, resulting in a biologic imbalance (CONNELL, 1978; SHER et al., 2000). This is especially true among populations of small mammals, for whom the habitat is the most important dimension by which species can segregate (SCHOENER, 1974).

Changes by human interference in nature have caused serious damage to the equilibrium of ecosystems. Urban sprawl, which can be observed in a direct way in the areas addressed in this study, causes not only loss of habitat for wild species, but also obliges this species occupying modified areas to adapt to the presence of domestic animals and humans. This effect has led to decreased diversity of species

in disturbed areas (VERA Y COUNT; ROCK, 2006) and thus has a direct influence on the increase of diseases caused by zoonotic agents in the world (PATZ et al., 2000). Different mechanisms operate in this process (PATZ; CONFALONIERI, 2005; CONFALONIERI; APARICIO, 2011) and an important step is the expansion of human populations in forest areas, resulting in the exposure of domestic animals and human populations that are immunologically susceptible to pathogens naturally found in the wild (MANDAL, 2011).

The remaining park areas in cities become remnant lands with great public and ecological importance. However, these forest remnants can dramatically suffer the effects of unplanned urbanization. Among the many negative effects due to urban pressure in the Conservation Units (UCs), stand out pollution in all its forms (noise, visual, air and water), extinction of flora and fauna components and introduction of domestic species in the ecosystem (SALGADO et al., 2007). The main negative consequences of the interaction of domestic animals versus UCs are the loss of the original local biodiversity and the risk of transmitting diseases to animals (wild and domestic) and also to people living near these protected areas (SALGADO et al., 2007).

Tick-borne diseases are commonly identified in animals, whether domestic or wild. Some of these diseases are zoonotic and their etiologic agents can infect humans accidentally when they are exposed to the bite of infected ticks. Ticks can transmit a wide variety of pathogenic microorganisms, such as protozoa, rickettsia, spirochetes and viruses. For this reason, these arthropods are among the most important vectors of diseases that affect animals and humans. Moreover, ticks may cause severe toxic conditions such as paralysis, intoxication, irritation and allergy (JONGEJAN; UILENBERG, 2004).

The knowledge of which agents and tick species are present in conservation areas is of great importance to the prevention and control of disease in wild and domestic animals as well as in humans who frequent these areas.

1.3 TICKS

Ticks are ectoparasites that belong to the phylum Arthropoda, Order Acari order. Worldwide, there are approximately 870 species of ticks, all grouped in the suborder Ixodida, which is divided into three families: Ixodidae (hard ticks), Argasidae (soft ticks) and Nuttalliellidae. In the Brazilian fauna, Ixodida is currently represented by 66 species of ticks, 45 Ixodidae, and 21 Argasidae (MARTINS et al., 2014; NAVA et al., 2014). Some species are important to public and veterinary health by causing direct damage by the bite or transmitting infectious agents (fungi, bacteria, viruses and protozoa) to human and animals (PAROLA; RAOULT, 2001; BARROS-BATTESTI et al., 2006). Among all known invertebrate vectors, ticks are placed first in number of pathogens transmitted to domestic animals followed by ectoparasitic arthropods and mosquitoes that transmit greater variety of pathogens to humans (JONGEJAN; UILENBERG, 2004). Most species of ticks need three hosts to complete their life cycle. The immature stages usually have low host specificity, infecting a wide variety of reptiles, birds and small mammals. Specificity of adults varies depending on the species of tick, as the same species can be found parasitizing both domestic and wild animals (ESTRADA-PEÑA et al., 2004).

1.4 *RICKETTSIA*

The Rickettsiaceae family consists of Gram-negative, aerobic and intracellular obligatory organisms (FORLANO, 2005; SAHNI; RYDKINA, 2009), which are multiplied by binary fission and are associated with invertebrate vectors (BIBERSTEIN; HIRSH, 2003; RAOULT et al., 2005). These bacteria can cause diseases in humans, such as endemic typhus, epidemic typhus and spotted fever (BIBERSTEIN; HIRSH, 2003; GREENE, 2006). Rickettsias are distributed worldwide and are maintained in nature by arthropod vectors (ticks, lice, fleas and mites). They are also able to infect vertebrates, which serve as a source of infection for new vectors (PAROLA et al., 2005). Humans can acquire these microorganisms through

vector parasitism or contact with the feces of infected arthropods (YU; WALKER, 2003). Some records of the symptoms of diseases caused by these pathogens date back centuries, such as epidemic typhus, caused by *Rickettsia prowazekii* and murine typhus caused by *Rickettsia typhi* (RAOULT; ROUX, 1997).

The classic division of *rickettsia* was based on antigenic, molecular and ecological standards and consists of three groups: 1) Typhus group (TG), comprises *R. prowazekii* and *R. typhi*, whose main vectors are insects (lice and fleas, respectively); 2) spotted fever group (SFG), has more than 23 valid species, and transmission is mostly associated with ticks, except for *Rickettsia felis* and *Rickettsia akari*, transmitted by fleas and mites, respectively; 3) ancestor group (AG), which comprises other *Rickettsia* species, such as *Rickettsia bellii* and *Rickettsia canadensis* (YU; WALKER, 2003) .

Recently, other classification based on a multigene approach was proposed, with the division of five groups: 1) (TG) formed by *R. prowazekii* and *R. typhi*; 2) (SFG), the largest group, represented by more than twenty species; 3) (Transition group) in which are inserted *Rickettsia akari*, *Rickettsia felis* and *Rickettsia australis*; 4) (Canadensis group), represented by the species *R. canadensis*; and 5) (Bellii group), represented by the species *R. bellii* and several other genotypes found in insects (WEINERT et al., 2009).

Among the species of rickettsia, *R. rickettsii* is the best known and most pathogenic, especially in Brazil. It has restricted distribution in the Americas, with records of confirmed cases in Canada, USA, Mexico, Costa Rica, Panama, Colombia, Brazil and Argentina (LABRUNA et al., 2011). It causes a severe rickettsial disease in humans, called in Brazil Brazilian Spotted Fever (BSF), "Fiebre Manchaca in Colombia, Panama, Mexico and Costa Rica "and "Rocky Mountain spotted fever" in the United States. *R. rickettsii* multiplies exclusively on endothelial cells of humans, and animals. In the vector of the disease, some species of ticks, it multiplies in cells of different tissues (WEISS; MOULDER, 1984). Wild animals are reservoirs (hosts amplifiers) of these bacteria, such as wild rodents in the United States, and the capybaras and opossums in Brazil (LABRUNA, 2009).

The diagnostic of spotted fever can be done by serological (HORTA et al., 2007), and molecular methods such as Polymerase Chain Reaction (PCR) (KIDD et al., 2008) and by the isolation of bacteria in cell cultures (LABRUNA et al., 2007b).

1.5 EHRLICHIA

Ehrlichiosis is a disease caused by obligate intracellular Gram-negative bacteria with tropism for hematopoietic cells that infects animals and humans in many parts of the world. Bacteria of the genus *Ehrlichia* has been divided into three groups according to their genetic similarities: genogroup "I", constituted by the species *E. canis*, *E. chaffeensis* and *E. ewingii*; Genogroup "II" which includes *E. phagocytophila* (*Anaplasma phagocytophilum* as reclassified), *E. equi* and Human granulocytic ehrlichiosis agent (HGE); and genogroup "III", formed by *E. risticii* and *E. sennetsu* (DUMLER et al., 2001; SKOTARCZAK, 2003).

Ehrlichial infections are widely distributed around the world, particularly frequent in tropical and subtropical areas (HARRUS et al., 1998; PRETORIUS et al., 1998; BATMAZ et al., 2001; WANER et al., 2001; MYLONAKIS et al., 2003; PEREZ et al., 2006). Until now, only five species of the genus *Ehrlichia* have been reported in Brazil: *E. canis*, *E. ewingii*, *E. chaffeensis*, *E. mineirensis* (UFMG –EV) and *E. sp.* UFMT -BV (MACHADO et al., 2006; DACOSTA et al., 2011; CRUZ et al., 2012; AGUIAR et al., 2014). *E. chaffeensis* and *E. ewingii* are recognized as human zoonotic pathogens (ANDERSON et al., 1991; BULLER et al., 1999), and *E. canis* is more prevalent in dogs and often found in the tick *R. sanguineus* (TRAPP et al., 2006; AGUIAR et al., 2007; SOUZA et al., 2010).

The monocytic ehrlichiosis (ME) is caused by *E. canis* (in dogs) and *E. chaffeensis* (human). It is transmitted by the ticks *R. sanguineus* and *Amblyomma americanum*, respectively (SKOTARCZAK, 2003). *E. canis* was first described in Brazil in 1973 (COSTA et al., 1973), and isolation was obtained only in 2002 (TORRES et al., 2002). In Brazil it is a widespread disease in dogs with occurrence varying between different studies (0.7% to 92.3%) and with various diagnostic

methods (SPOLIDORIO et al., 2010; VIEIRA et al., 2011). In South America, human ehrlichiosis caused by the species *E. canis* and *E. chaffeensis*, has been detected by molecular diagnosis in Venezuela (PEREZ et al., 2006; MARTÍNEZ et al., 2008) and there is serologic evidence of the occurrence in Argentina and Chile (RIPOLL et al., 1999; LÓPEZ et al., 2003). In Brazil, there are reports of HIV positive people in the states of Minas Gerais (CALIC et al., 2004) and Espírito Santo (SPOLIDORIO et al., 2010) soropositive for *E. chaffeensis* and *E. canis* antigens.

According to serological studies, *E. canis* can infect wild canids (red fox, *Vulpes vulpes*) (FISHMAN et al., 2004), that are considered potential reservoirs of these bacteria in the United States (GREENE, 2006). *Ehrlichia ruminantium* is another species with wild animals as reservoirs, that infects ruminants in Africa (WALKER; OLWAGE, 1987), and is also considered a zoonotic agent, based on molecular diagnosis in cases of death by ehrlichiosis (LOUW et al., 2005).

In Brazil, studies detected through molecular diagnosis, possible new species of *Ehrlichia*, in tissues of crab-eating fox (*Cerdocyon thous*) (ALMEIDA et al., 2013) and blood and ticks from jaguar (*Panthera onca*) (WIDMER et al., 2011).

The diagnosis of ehrlichiosis can be carried out by blood smear, cytology by Giemsa stain, serology, cell culture and molecular detection (GREENE, 2006; DANTAS -TORRES, 2008 in review).

1.6 HEPATOZOON

The *Hepatozoon* genus comprises more than 300 species of protozoa belonging to the phylum Apicomplexa (ALMOSNY, 2002; EWING; PANCIERA, 2003, in review), that affect a wide variety of domestic and wild animals (VICENT JOHNSON et al., 2003). Canine hepatozoonosis is caused by two different species, *Hepatozoon canis* and *Hepatozoon americanum* (VICENT JOHNSON et al., 2003 in review). In Brazil, the main species is *H. canis* (RUBINI et al., 2005), transmitted by *Rhipicephalus sanguineus*, which is a known vector of *H. canis* in the Old World, and may also play a role in the transmission of this pathogen in Brazil. Infection occurs by

ingestion of tick containing mature oocytes (ALMOSNY, 2002; DANTAS-TORRES, 2008 in review). Other tick species likely associated with *Hepatozoon* transmission in Brazil are: *Amblyomma aureolatum*, *Amblyomma ovale* and *A.cajennense* (O'DWYER et al., 2001; FORLANO et al., 2005).

In the United States, canine hepatozoonosis is caused by *H. americanum* transmitted by *Amblyomma maculatum* (EWING; PANCIERA, 2003; GREENE, 2006). Besides the genetic difference between *H. canis* and *H. americanum*, there are also differences in pathophysiology of each agent. These two protozoa gamontes forms are observed in infected leukocytes however, the merogony and gamogony phases occur in monocytes in infections by *H. americanum* and neutrophils in infections by *H. canis*. Another difference is regarding the form of meronts, which have different morphology and topology among these species (EWING; PANCIERA, 2003).

In Brazil, this disease was first reported in the states of Rio de Janeiro and Rio Grande do Sul (MASSARD, 1979) through direct diagnosis in blood smears and serological techniques. The infection has been described in dogs in Sao Paulo (GONDIM et al., 1998; RUBINI et al., 2008), Espírito Santo, Minas Gerais, Mato Grosso do SUL (MUNDIM et al., 1992; MUNDIM et al., 2008; SPOLIDORIO et al., 2009; SPOLIDORIO et al., 2010; RAMOS et al., 2015), Rio de Janeiro (FORLANO et al., 2007) and Rio Grande do Norte (GONÇALVEZ et al., 2013). Different genotypes of *Hepatozoon* spp. have been described in dogs and wild canids from Brazil (PALUDO et al., 2005; ANDRÉ et al., 2010).

The diagnosis of hepatozoonosis is based on clinical symptoms, cytological analyses through blood smear by visualization of the gamontes parasitizing leukocytes, biopsy, immunohistochemistry, serology and PCR (EWING; PANCIERA, 2003; KARAGENC et al., 2006; LI et al., 2008)

1.7 BABESIA

Babesiosis is a cosmopolitan disease caused by haemoprotozoans of the genus *Babesia*, family Babesiidae, order Piroplasmida and Filo Apicomplexa (IRWIN,

2009). These parasites infect red blood cells of the host, leading to haemolysis (ALMOSNY, 2002; TABOADA; LOBETTI, 2006). In the last decade, based on molecular analysis, the species *B. canis* was divided into three genetically distinct subspecies: *Babesia canis canis*, *Babesia canis rossi* and *Babesia canis vogeli* (ALMOSNY, 2002; IRWIN, 2009).

The most prevalent specie in dogs in Brazil is *B. canis vogeli* (PASSOS et al., 2005; TRAPP et al., 2006; COSTA JR et al., 2009; SPOLIDORIO et al., 2010). Babesiosis is transmitted by ticks of the family Ixodidae, and *B. canis canis* is mainly transmitted by *Dermacentor reticulatus*; *Babesia canis rossi* by *Haemaphysalis leachi* and *Babesia canis vogeli* by *R. sanguineus* (TABOADA; LOBETTI, 2006 in review; IRWIN, 2009).

Some *Babesia* species infecting cats have already been reported: *Babesia felis*, *Babesia cati*, *Babesia canis presentii* and *Babesia herpailuri* (ALMOSNY, 2002; TABOADA; LOBETTI, 2006, in review).

In humans it causes an acute febrile disease that can be confused with malaria and it is possibly fatal (ALMOSNY, 2002). Babesiosis in humans can be caused by several species such as *Babesia microti*, *Babesia divergens*, "*Babesia divergens*-like" among others (TABOADA; LOBETTI, 2006, in review). In Brazil, there is only one account of direct diagnosis of human babesiosis, but without molecular confirmation; and another study shows existence of coinfection with etiological agents of Lyme borreliosis and babesiosis in human (ROSEMARY et al., 1983; YOSHINARI et al., 2003).

Studies in wild animals have shown infection with *Babesia* spp. through molecular detection, in several species of canids, such as the red fox (*Vulpes vulpes*) and the gray fox (*Urocyon inereogenteus*) in North America (BIRKENHEUER et al., 2010). *B. canis rossi* has been found in the African wild dog (*Lycaon pictus*) in South Africa (MATJILA et al., 2008). There are reports of *Babesia* spp. in Brazil infecting the hoary fox (*Pseudalopex vetulus*) (MARTINS et al., 2006), the raccoon (*Lycalopex gymnocercus*) (RUAS et al., 2003), lobo-guará (*Chrysocyon brachyurus*) (SERRA-FREIRE et al., 1993) and cachorro-do-mato (*Cerdocyon thous*) (PARAENSE; VIANNA, 1948), with cytological examination through blood smear used as a diagnostic method.

Diagnosis can be accomplished by microscopic visualization of the parasite agent in erythrocytes by peripheral blood smears and serological and molecular detection techniques (TABOADA; LOBETTI, 2006, in review).

1.8 OBJECTIVES

1.8.1 General Purpose

To investigate the occurrence of tick-borne disease agents in ticks, tissue and serum of wild and domestic animals from Parque das Dunas de Natal "Jornalista Luiz Maria Alves" and Parque da Cidade "Dom Nivaldo Monte" in the city of Natal, Rio Grande do Norte State, Brazil.

1.8.2 Specific objectives

- To examine tissues of small mammals and their ticks, serologically and molecularly to evaluate tick-borne diseases agents (*Rickettsia* spp. and *Ehrlichia* spp.).
- To detect the occurrence of infection by *Ehrlichia canis*, *Hepatozoon canis* and *Rickettsia* spp. in feral cats living in the parks, and in dogs that live around the units as well in other regions of the city.
- To evaluate the molecular occurrence of hemoparasites of the piroplasmida order in small mammals captured in the parks.

2 RICKETTSIAL AND ERLICHIAL INFECTION IN SMALL MAMMALS FROM NORTHEAST BRAZIL

ABSTRACT

The aim of this study was to determine the serological and molecular occurrence of *Rickettsia* spp. and *Ehrlichia* spp. in small mammals and their ticks in two wildlife conservation units at Natal, Rio Grande do Norte State, Brazil. Samples were collected during October 2012 and February 2013. Sera were tested against antigens of *Rickettsia amblyommii*, *Rickettsia rhipicephali*, *Rickettsia parkeri*, *Rickettsia rickettsii*, *Rickettsia felis* and *Rickettsia bellii* by indirect fluorescence antibody assay (IFAT). Samples of spleen, lungs and the ticks were processed for molecular detection of organisms of the genus *Rickettsia* and *Ehrlichia*. Twenty seven marsupials (23 *Didelphis albiventris* and 4 *Monodelphis domestica*) and 4 rodents (2 *Necromys lasiurus*, 1 *Thrichomys apereoides*, 1 *Rattus rattus*) were captured and antibodies were detected in 6 *D. albiventris* and in 1 *R. rattus* (IFAT > 64) to at least one of the tested rickettsiae, with final titers four times greater for *R. amblyommii*, *R. bellii* or *R. parkeri* compared with other rickettsia species tested. Three tick species (*Ornithodoros mimon*, *Amblyomma auricularium* and *Ixodes loricatus*) were collected from marsupials, and one *A. auricularium* was infected by *R. amblyommii* by Polymerase Chain Reaction (PCR) and DNA sequencing (rickettsial *gltA* and *ompA* gene). One *D. albiventris* spleen yielded PCR amplicons for 2 Ehrlichial genes (16S rRNA and *dsb*). DNA sequencing of the 16S rRNA amplicon generated a sequence that was closest (99% identity) to several uncultured *Ehrlichia* spp. from different parts of the world. The *dsb* sequence was closest (80-81%) to *E. chaffeensis* and *E. mineirensis*. This is the first detection of *Rickettsia* spp and *Ehrlichia* spp. in wild animals from the State of Rio Grande do Norte, Brazil.

Keywords: *Rickettsia*. *Ehrlichia*. *Didelphis*. *Amblyomma*. Brazil.

RESUMO

O objetivo deste estudo foi determinar a ocorrência sorológica e molecular de *Rickettsia* spp. e *Ehrlichia* spp. em pequenos mamíferos e carrapatos presentes nestes animais, provenientes de duas unidades de conservação ambiental na cidade de Natal, Rio Grande do Norte, Brasil. As coletas foram realizadas em outubro de 2012 e fevereiro de 2013. Os soros foram testados contra antígenos de *Rickettsia amblyommii*, *Rickettsia rhipicephali*, *Rickettsia parkeri*, *Rickettsia rickettsii*, *Rickettsia felis* e *Rickettsia bellii* através do teste de imunofluorescência indireta (IFI). Amostras de tecido e carrapatos foram processadas para a detecção molecular de organismos do gênero *Rickettsia* e *Ehrlichia*. Vinte e sete marsupiais (23 *Didelphis albiventris* e quatro *Monodelphis domestica*) e quatro roedores (dois *Necromys lasiurus*, um *Thrichomys apereoides* e um *Rattus rattus*) foram capturados. Anticorpos, para pelo menos uma das rickettsias testadas, foram detectados em seis *D. albiventris* no *R. rattus* (IFI > 64), com títulos finais quatro vezes maior para *R. amblyommii*, *R. bellii* ou *R. parkeri* em comparação com as outras espécies de *Rickettsia* testadas. Três espécies de carrapatos (*Ornithodoros mimon*, *Amblyomma auricularium* e *Ixodes loricatus*) foram coletadas de marsupiais e um *A. auricularium* foi positivo para *R. amblyommii* por Reação de polimerase em cadeia (PCR) e sequenciamento de DNA (gene *gltA* rickettsial). Uma amostra de baço de *D. albiventris* rendeu amplificadores de PCR para dois genes de *Ehrlichia* (16S rRNA e *dsb*). O sequenciamento do fragmento amplificado de 16S rRNA gerou uma sequência com 99% de identidade com *Ehrlichia* spp. ainda não isoladas. As sequências de *dsb* mostraram de 80-81% de identidade para *E. chaffeensis* e *E. mineirensis*. Este é o primeiro relato de ocorrência de *Rickettsia* spp e *Ehrlichia* spp. em animais selvagens do Estado do Rio Grande do Norte, Brasil.

Palavras-chave: *Rickettsia*. *Ehrlichia*. *Didelphis*. *Amblyomma*. Brasil.

2.1 INTRODUCTION

Rickettsia spp. (Rickettsiaceae) and *Ehrlichia* spp. (Anaplasmataceae) are tick-borne pathogens classified within the order Rickettsiales. They are obligate intracellular Gram-negative bacteria, occupying respectively intracytoplasmic and an intravacuolar compartments within infected host cells (DUMLER et al., 2001).

The Atlantic Forest of Northeastern Brazil is currently reduced to less than 6% of its original pre-Colombian extent, exhibiting high levels of forest fragmentation (GALINDO-LEAL; CÂMARA, 2003). Considering this fragmented landscape, ecological studies of interactions between tick-borne bacteria and their mammal hosts, suggest that areas of disturbed Atlantic Forest provide an environment for small mammals and ticks and that small mammals living in these areas are highly exposed to tick-borne pathogens (DANTAS-TORRES et al., 2012). It has been proposed that these mammals are good natural indicators of the circulation of rickettsial agents in a particular area, since they have limited dispersion and short lifespan and thus can serve as a natural environmental dispersion (MILAGRES et al., 2013). Besides, disorders in that kind of natural ecosystem can eventually bring humans into contact with wildlife-associated pathogens (BRADLEY; ALTIZER, 2006).

Until now, only five species of the genus *Ehrlichia* have been reported in Brazil: *Ehrlichia canis*, *Ehrlichia ewingii*, *Ehrlichia chaffeensis*, *Ehrlichia mineirensis* (UFMG –EV) and *Ehrlichia* sp. UFMT -BV (MACHADO et al., 2006; DACOSTA et al., 2011; CRUZ et al., 2012, AGUIAR et al., 2014). Both, *E. chaffeensis* and *E. ewingii* represent human zoonotic pathogens (BULLER et al., 1999; ANDERSON et al., 1991) and *E. canis* is more prevalent in dogs and often found in the tick *Rhipicephalus sanguineus* (TRAPP et al., 2006; AGUIAR et al., 2007; SOUZA et al., 2010). Reports around the world suggest that some other *Ehrlichia* species (i. e. *E. canis*, *E. muris* and *E. ruminantium*-like organisms and Panama Mountain *Ehrlichia*) might be also human pathogens (LOUW et al., 2005; PEREZ et al., 2006; NEFEDOVA et al., 2008; REEVES et al., 2008; PRITT et al., 2011).

In order to assess the presence of tick-borne pathogens in urban region surrounding Atlantic Forest areas, in the Northeast of Brazil, in this study serological and molecular technique were used to access *Rickettsia* and *Ehrlichia* organisms, in small mammals and their associated ticks.

2.2 METHODS

2.2.1 Ethical permission

Permits for capture and euthanasia of three specimens per species in each of the study areas and transportation of individuals were granted by the System Authorization and Information on Biodiversity (SISBio – nº 32104 -2. The study was approved by - Instituto de Defesa do Meio Ambiente de Natal (IDEMA–RN) and by the Ethics Committee on Animal Use of the Institute of Biomedical Sciences, USP, and protocol number 204.

2.2.2 Study Site

Two field campaigns to capture wild mammals were conducted, one during the dry season (October 2012) and the other during the rainy season (February 2013), in two peri-urban wildlife conservation units: Parque Estadual das Dunas de Natal (5.851067W/35.228306N) and Parque da Cidade (83.818589W/12.034402N) composed by Atlantic Forest vegetation, in the city of Natal, Rio Grande do Norte State, Brazil. Noteworthy to mention, people usually frequenting both sites for recreational or working purposes.

2.2.3 Capture of small mammals and ticks

Animals were trapped with Sherman and Tomahawk-like traps with different baits (a mixture of cornmeal, sardines and bananas) distributed in specific sites with animal activity signs along path. For the collection of ticks, the animals were anesthetized with xylazine (mean dose 5mg/kg) and ketamine (mean dose 50mg/kg) by cardiac, tail vein or cephalic vein puncture. When possible, three animals of each species were euthanized, and fragments of lungs and spleen were collected for molecular analyses. After species determination, the skins of the euthanized animals were deposited in the Museum of Natural History at the Catholic University of Minas Gerais, Brazil.

Collected ticks were separated by host and stored in 100% ethanol until taxonomic identification and molecular analyses. Species identification for ticks of the Ixodidae family was performed following Onofrio et al. (2006, 2009) and ticks of the Argasidae family according Kohls et al. (1969) and Barros-Battesti et al. (2013) taxonomic keys.

2.2.4 Investigation of IgG to *Rickettsia* spp. antibodies

Antibodies to *Rickettsia* spp. were detected by indirect fluorescence antibody assay (IFAT) using simultaneously five *Rickettsia* isolates, all from Brazil: *R. bellii* strain Mogi, *R. amblyommii* strain Ac37, *R. rhipicephali* strain HJ5, *R. rickettsii* strain Taiacu and *R. parkeri* strain At24, as previously described (HORTA et al., 2004). Samples that reacted at the screening dilution (1:64) were then titrated using serial two-fold dilutions to determine endpoint titers. Serum presenting a *Rickettsia* species titer at least four times higher than those observed for the other *Rickettsia* species was considered to be homologous to the first *Rickettsia* species or to a very closely related genotype (HORTA et al., 2004). In all reactions, previously known positive and negative controls and antigen controls were used.

2.2.5 Molecular analyses

DNA extraction using the Wizard® genomic DNA purification kit (Promega corporation, Madison / USA), in accordance with the manufacturer's instructions, was individually performed to tissue samples obtained from the euthanized animals and to adults, nymphs and larvae of collected ticks. For nymphs and larvae, pools of three specimens were used. Subsequently, extracted-DNA was submitted to different PCR protocols. To confirm morphological identifications, a PCR using primers 5'-CCGGTCTGAACTCAGATCAAGT-3' and 5'-GCTCAATGATTTTTTAAATTGCTGT-3' targeting a ≈460-bp fragment of the tick mitochondrial 16S rRNA gene we performed, as described elsewhere (MANGOLD et al., 1998). The final volume of 25 µl, containing 10 mM of Tris-HCl (pH 8.3), 50 µM of KCl, and 1.5 mM of MgCl₂, 0.2 mM of each deoxynucleoside triphosphate, 1.5 U of Taq DNA polymerase (Invitrogen; Waltham, MA, USA), 11 pmol of each primer and approximately 100 ng of genomic DNA.

To test rickettsial infection in ticks, an initial PCR screening using the primers CS-78 5'-GCAAGTATCGGTGAGGATGTAAT-3' and CS-323 5'-GCTTCCTTAAAATTCAATAAATCAGGAT-3', which amplify a ≈398-bp fragment of the citrate synthase gene (*glta*) was performed to all known *Rickettsia* species (LABRUNA et al. 2004), in all the tick-extracted DNA samples. Positive samples were subsequently tested with the primers Rr190.70F 5'-ATGGCGAATATTTCTCCAAAA-3' and Rr190.701R 5'-GTTCCGTTAATGGCAGCATCT-3', which amplify an outer membrane protein (*ompA*) 532-bp fragment of the 190-kDa present in most of the Spotted Fever group *Rickettsia* species (ROUX et al., 1996).

The DNA extracted from spleen and lung of the small mammals was tested by conventional PCR using a first reaction with the primers GE2'F2' 5'-GTTAGTGGCAGACGGGTGAGT-3' and HE3 5'-TATAGGTACCGTCATTATCTTCCCTAT-3' that amplify a ≈360-bp fragment of the 16S rRNA gene of the Anaplasmataceae bacteria (AGUIAR et al., 2008), and then positive samples were tested with primers DSB-330 5'-GATGATGTCTGAAGATATGAAACAAAT-3' and DSB-728 5'-CTGCTCGTCTATTTTACTTCTTAAAGT-3', which amplify a ≈409-bp fragment of the

Ehrlichia genus-specific disulfide bond formation protein gene (*dsb*) (DOYLE et al., 2005).

All PCR products were visualized under ultraviolet light after electrophoresis through SyBr gold (Invitrogen; Waltham, MA, USA) stained agarose gel, expected size amplicons were purified using ExoSap-IT (USB) and posteriorly sequenced in an automatic sequencer (Applied Biosystems/Perkin Elmer, model ABI Prism 310 Genetic, California, USA), with the same primers used in PCR. Obtained partial sequences were assembled and subjected to BLAST analyses (ALTSCHUL et al., 1990) to infer the closest similarities with other organisms available in GenBank database.

2.2.6 Phylogenetic analyses

An alignment of the mitochondrial 16S rRNA and *dsb* partial sequences of the Ehrlichial agents amplified in this study with corresponding sequences of *Ehrlichia* spp. available in GenBank, was performed using the T-COFFEE 8.93 program (MCWILLIAM et al., 2013) (Figure 4 and 5). Both phylogenetic trees were inferred by Bayesian method using Mrbayes_v3.2.5 software based on The Jukes–Cantor model combined with the models of gamma distribution (G). It was used 1,000,000 generations for the 16S tree and 300,000 for the *dsb* tree. Both trees were sampled every 1,000 generations, ran four times beginning with random starting trees, and the first 25% of the trees represented burn-in, being the remaining trees used to calculate Bayesian posterior probability (BPP). The sequence of *Rickettsia prowazekii* was used as outgroup for the rooted 16S analysis and we constructed an unrooted tree was constructed for the *dsb* gene sequences (HUELSENBECK; RONQUIST, 2001).

2.3 RESULTS

Twenty-seven marsupials (23 *Didelphis albiventris* and 4 *Monodelphis domestica*), 4 rodents (2 *Necomys lasiurus*, 1 *Thrichomys apereoides*, and 1 *Rattus rattus*) and 2 Xenarthral (*Euphractus sexcinctus*) were captured. Serum samples were collected and tick search was realized in all of them. The lung and spleen tissue samples were collected from the euthanized animals and from 3 opossums found dead.

Three species of two tick families (*Ixodidae* and *Argasidae*) were identified (Table - 1). The nymphs and adult ticks were identified by morphology and the larvae stages by molecular analysis.

A total of 2 adults, 13 nymphs and 16 larvae of *Amblyomma auricularium*; 9 *Ixodes loricatus* and 25 *Ornithodoros mimon* larvae were found parasiting 9 of the 23 captured *D. albiventris*. A number of 29 adults, 125 nymphs and 30 larvae of *A. auricularium*; and 7 *O. mimon* were found parasiting the 2 captured armadillos (*E. sexcinctus*) (Table - 1).

Regarding the rickettsial infection analyses, 2 tick species (*A. auricularium* and *I. loricatus*) were tested by PCR; and DNA sequencing targeting rickettsial *gltA* and *ompA* genes. All the 29 *A. auricularium* adult ticks and the pools of larvae and nymphs, from the armadillos, were found respectively 100% (29\29), 100% (30/30) and 90% (113\125) positive for *R. amblyommii*. One of the two *A. auricularium* specimen, found in *D. albiventris*, was infected by *R. amblyommii*. All the fragments of the gene sequences demonstrated 100 % identity with *R. amblyommii* AAPE isolated strain of *A. auricularim* collected in armadillos in the state of Pernambuco (GenBank accession numbers KJ534310 (*gltA*), KJ534312 (*ompA*). *I. loricatus* and *O. mimon* were not positive for *Rickettsia* spp. (Table – 1).

Table 1 - Quantities and species of ticks collected from small mammals captured in studied areas, and tick score infection by *Rickettsia amblyommii*

Tick Species (stage)	<i>Didelphis albiventris</i>		<i>Euphractus sexcinctus</i>	
	Ticks/Hosts harboring	Infected/ Tested (%)	Ticks/Hosts harboring	Infected/ Tested (%)
<i>A. auricularium</i> – A	(02/01) - 1M, 1F*	01/02 (50)	(29/02) - 23M, 6F*	29/29 (100)
<i>A. auricularium</i> – N	(13/06)	06/13 (46)	(125/02)	113/125 (90)
<i>A. auricularium</i> –L	(16/02)	16/16 (100)	(30/02)	30/30 (100)
<i>I. loricatus</i> – A	(09/06) - 6M, 3F*	00/09 (00)	(00/02)	00/00 (00)
<i>O. mimon</i> – L	(25/04)	00/25 (00)	(07/02)	00/07 (00)

Subtitles: * F: females; M: males ; A: Adult; N: nymphs; L: larvae

Serum samples were tested for the presence of antibodies to *Rickettsia* spp. by IFAT in 31 captured small mammals (23 *D. albiventris*, 4 *M. domestica*, 2 *N. lasiurus*, 1 *T. apereoides* and 1 *R. rattus*) (Table - 2). Antibodies to *Rickettsia* spp. were detected in 6 *D. albiventris* and in 1 *R. rattus* for at least one of the tested *Rickettsia* antigens. The final titers showed that *R. amblyommii*, *R. bellii* and *R. parkeri* were four times greater when compared with other tested rickettsial antigens. Therefore they are, probably, the homologous antigens (PHA) (Table 3).

Table 2 - Number and animals species sampled in two forest fragments areas in the city Natal- RN (Parque das Dunas and Parque da cidade), positive to at least one of the *Rickettsia* species tested by IFAT

Host	IFAT Positive animals/ Tested (%)		Total %
	Parque das Dunas	Parque da Cidade	
<i>Didelphis albiventris</i>	2/8 (25)	4/15 (26,6)	6/23 (26)
<i>Monodelphis domestica</i>	0/0	0/4	0/4
<i>Necromys lasiurus</i>	0/0	0/2	0/2
<i>Thrichomys apereoides</i>	0/1	0/0	0/1
<i>Rattus rattus</i>	1/1 (100)	0/0	1/1 (100)
Total	3/10 (19,35)	4/21 (25,9)	7/31 (24,1)

Table 3 - Antibodies titres (IFAT) for the six tested Rickettsial species in positive serum of small mammals, showing the titers and the probable homologous antigens (PHA)

Host/ID	Antibodies titers for the rickettsial antigens						PHA
	¹ R.A	² R.Rh	³ R.R	⁴ R.P	⁵ R.B	⁶ R.F	
<i>D. albiventris</i> 106	128	0	0	0	1024	0	<i>R. bellii</i>
<i>D. albiventris</i> 67	2048	64	0	0		1024	Indefinite
<i>D. albiventris</i> 68	0	0	0	128	0	0	<i>R. parkeri</i>
<i>D. albiventris</i> 08	256	0	0	0	0	128	Indefinite
<i>D. albiventris</i> 85	1024	0	0	0	0	0	<i>R. amblyommii</i>
<i>R. rattus</i> 01	512	256	0	0	256	0	Indefinite
<i>D. albiventris</i> 11	512	0	0	0	64	0	<i>R. amblyommii</i>

Subtitle: ¹*Rickettsia Amblyommii*; ²*R. rhipicephali*; ³*R. rickettsii*; ⁴*R. parkeri*; ⁵*R. bellii*; ⁶*R. felis*

One *D. albiventris* spleen yielded PCR amplicons for 2 Ehrlichial genes (16S rRNA and *dsb*). DNA sequencing of the 16S rRNA amplicon generated a sequence that was the closest (99% identity) to several uncultured *Ehrlichia* spp. from several parts of the world. The *dsb* sequence was the closest (80-81%) to *E. chaffeensis* and *E. mineirensis*.

Phylogenetic trees based on the 16S rRNA and *dsb* gene sequences are showed in the Figures 4 and 5. A probably novel species, *Ehrlichia* sp. Natalensis, that was found in this study is described as well.

Figure 4 - Phylogenetic tree based on the 16S rRNA (A) gene sequences from members of the family Anaplasmataceae. The tree shows that *Ehrlichia* sp. Natalensis falls in a clade separated from all the previous reported sequences. Posterior probability (BPP) values are shown as % in the internal branch. *Rickettsia prowazekii* 16S rRNA sequence was used to root the 16S rRNA tree. The GenBank accession numbers of the sequences used to build the 16S rRNA tree are: *E. muris*, AB013008; *E. chaffeensis*, AF147752; *E. ruminantium*, AF069758; *E. ewingii*, U96436; *A. marginale*, M60313; *A. phagocytophilum*, M73224; *A. platys*, M82801; *N. helminthoeca*, U12457; *N. sennetsu*, M73225; *N. risticii*, AF036649; *E. canis*, GU810149; *R. prowazekii*, NR044656. *E. mineirensis* JX629805

Fonte: (LOPES, M. G., 2016)

Figure 5 - Phylogenetic unrooted tree based on the *dsb* gene sequences from members of the family Anaplasmataceae. The tree shows that *Ehrlichia* sp. Natalensis falls in a clade separated from all the previous reported sequences and the previously reported. Posterior probability (BPP) values are show as % in the internal branch. The GenBank accession numbers of the *dsb* sequences used to build the tree are: *E. canis*, AF403710; *E. canis* Uberlandia, GU586135; *E. canis* Jaboticabal, DQ460716; *E. canis* Sao Paulo, DQ460715; *E. muris*, AY236484; *E. chaffeensis*, AF403711; *E. ruminantium*, AF308669, clon 18hw; *E. ewingii*, AY428950, *E. mineirensis* JX629808

Fonte: (LOPES, M. G., 2016)

2.4 DISCUSSION

Our results of *A. auricularium*, *I. loricatus* and *O. mimon* are supported by current literature. Adult stages of *A. auricularium* have moderate host specificity, parasite mainly armadillos, although immature stages are also found feeding on small rodents (GUGLIELMONE et al., 2003; HORTA et al., 2011; SARAIVA et al.,

2012). The adults of the species *I. loricatus* are commonly found on marsupials and preferably opossums *Didelphis* spp., and their immature stages rather feed on small wild rodents and small marsupials (BARROS-BATTESTI et al., 2000; SARAIVA et al., 2012); and also *O. mimon*, that for a long time was believed to exclusively parasite species of bats, recently has been reported parasitizing on humans and marsupials (*D. albiventris*) (LABRUNA et al., 2014).

Most of the captured small mammals are *D. albiventris*. This fact could be an environmental degradation indicator in this area (BONVICINO et al., 2002). In this study immature tick stages were found parasitizing chiefly the opossums and armadillos, and not the rodents, as usually expected. Coupled with the ecological aspect, the observed low host specificity of larvae and nymphs of *A. auricularium* is relevant from an epidemiological standpoint.

The high *R. amblyommii* natural infection found for *A. auricularium* is supported by the results reported by Saraiva et al. (2013). They showed that in laboratory conditions, *R. amblyommii* is efficiently maintained by 100% transovarial transmission and transstadial perpetuation in *A. auricularium* ticks. Until now *R. amblyommii* has been reported in the northeast of Brazil infecting immature stages of *A. longirostre* collected from birds and *Amblyomma varium* (OGRZEWALSKA et al., 2011; LUGARINI et al., 2015), and in adult ticks collected from thin-spined porcupine (*Chaetomys subspinosus*) and hairy dwarf porcupine (*Coendou insidiosus*) (MCINTOSH et al., 2015). In immatures and adults of *A. auricularium* collected from the striped hog-nosed skunk (*Conepatus semistriatus*), and armadillos (*Euphractus sexcinctus*), in the state of Pernambuco, were also infected by *R. amblyommii* (SARAIVA et al., 2013).

The current study extends the distribution of *R. amblyommii* in northeastern Brazil. The bacteria was detected in all parasitic stages of the tick *A. auricularium* collected from *D. albiventris* and *E. sexcinctus* from both studied areas.

Previous reports found *Didelphis* sp. and *R. rattus* positive to rickettsial antigens (PENA et al., 2009; MILAGRES et al., 2010; DANTAS-TORRES et al., 2011; MILAGRES et al., 2013). The present study suggests that the disturbed forest fragments could provide an enabling environment for small mammals and ticks; and that small mammal, living in these areas, are potentially exposed to rickettsial organisms.

Currently, *R. amblyommii* is considered to be a potential human pathogen, since there has been serological evidence of human infection by this agent in the United States (APPERSON et al., 2008; VAUGHN et al., 2014). One nymph of *A. auricularum* was collected from a researcher involved in the fieldwork (data not shown). Nonetheless, analyses of that tick found negative for rickettsial infection. People who perform activities (work or recreation) in these areas might be at risk of exposure to ticks and rickettsial organisms.

The 16S rRNA gene has been considered sufficient to classify different species of bacteria, presenting a high level of conservation with a low evolutionary rate (WOESE et al., 1987; WEN et al., 2002; CRUZ et al., 2012). In addition, a fragment of the gene that encodes for homologous immunoreactive thio-disulfide oxidoreductases, or disulfide bond formation (*dsb*) proteins from *Ehrlichia* sp. was analyzed (DOYLE et al., 2005), and the high posterior probability values in the phylogenetic trees (Figures 4 and 5), support the position of *Ehrlichia* sp. Natalensis as a novel species of the genus *Ehrlichia*. In both phylogenetical analyses the *Ehrlichia* sp. Natalensis takes a position different to the others that already have been reported, falling, possibly, in a different clade.

Two hard tick species (*I. loricatus* and *A. auricularum*) were found in the area where the opossums were captured. Although, both species were found parasiting the opossums, on the animals that DNA of the *Ehrlichia* sp. Natalensis were detected, only *I. loricatus* was found and it was negative for the molecular analyses for ehrlichial agents. Further studies should be conducted to define the role of this new agent in the epidemiology of ehrlichiosis and its interaction with the local fauna and ticks.

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3 *EHRLICHIA CANIS*, *HEPATOZOOM CANIS* AND *RICKETTSIA AMBLYOMMII* OCCURRENCE IN DOGS AND CATS FROM NATAL, RIO GRANDE DO NORTE, BRAZIL.

ABSTRACT

In this study, we attempted to detect the occurrence of infection by *Ehrlichia canis*, *Hepatozoon canis* and *Rickettsia* spp. in feral cats living in two Atlantic Rainforest fragments in the municipality of Natal, Rio Grande do Norte State, Brazil, and in dogs living around the area and in other regions of the city. The animals were sampled between October 2012 to August 2013. Serum samples from 155 animals: 53 feral *Felis catus* from the forest area; 29 domiciled *Canis familiaris* living surrounding the forest area and 73 dogs from Zoonosis Control Center (ZCC) of the municipality. Spleen fragments from 20 dogs euthanized at the ZCC were also collected. Blood serum was analyzed for detection of antibodies to *Rickettsia* spp. and to *Ehrlichia canis* by indirect fluorescent antibody test. The serological results showed that 17% (17/102) of the dogs had antibodies to *E. canis* and 13% (20/155) of all the animals (dogs and cats) were seropositive to *Rickettsia* spp. antigens. The animals were considered to be infected by *R. amblyommii* or a very closely related genotype. The spleen samples were subjected to molecular analyses and 8 of the 20 (40%) were positive for *E. canis* and 2 (10%) for *H. canis*. None of the canine spleen samples were PCR positive to piroplasmid agents.

Keywords: *Ehrlichia*. *Hepatozoon*. *Rickettsia*. Cat. Dog.

RESUMO

O objetivo deste estudo foi determinar a ocorrência de infecção por *Ehrlichia canis*, *Hepatozoon canis* e *Rickettsia* spp. em gatos selvagens que vivem em dois fragmentos florestais, localizadas no Bioma de Mata Atlântica, em Natal, RN, Brasil; e em cães que vivem no entorno dessa área e em outras regiões da cidade de Natal. As amostras foram obtidas entre outubro de 2012 a agosto de 2013, e foram

coletados sangue de 155 animais: 53 *Felis catus*, 29 *Canis familiaris* domiciliados no entorno das áreas florestais e 73 cães do Centro de Controle de zoonoses (CCZ). Fragmentos de baço de 20 dos cães eutanasiados no CCZ foram obtidos para análise molecular. As amostras de soro foram analisadas para detecção de anticorpos anti-*Rickettsia* spp e anti- *E. canis* pela reação de Imunofluorescência indireta. Os resultados serológicos mostraram que 17% (17/102) dos cães tinham anticorpos anti- *E. canis* e 13% (20/155) de todos os animais testados (cães e gatos) eram soropositivos para pelo menos um dos antígenos de *Rickettsia* spp. testados sendo a mais provável *R. amblyommii* ou um genótipo próximo. Entre as amostras de baço dos 20 cães que foram submetidas a análises moleculares, oito (40%) foram positivas para *E. canis* e duas (10%) para *H. canis*. Nenhuma das amostras de baço foi positiva para piroplasmidas pela PCR.

Palavras-chave: *Ehrlichia*. *Hepatozoon*. *Rickettsia*. Gato. Cão.

3.1 INTRODUCTION

Ehrlichia canis, *Hepatozoon canis* and spotted fever group (SFG) *Rickettsia* may cause diseases in dogs that develop either subclinical infection or severe clinical signs (HARRUS; WANER, 2011, in review; GONDIN et al., 1998; STILES, 2000; PIRANDA et al., 2008). Clinical symptoms in cats caused by tick-borne diseases are still contradictory and require more information (SHAW et al., 2001; FRITZ; KJEMTRUP, 2003). However these agents had been reported infecting dogs in many parts of Brazil at a wide range of occurrence (SAITO et al., 2008; RAMOS et al., 2010; SILVA et al., 2010; VIEIRA et al., 2011; DE MIRANDA et al., 2014; COSTA et al., 2015).

Wild carnivores inhabiting natural areas in periurban location can be infected by the same pathogens that infect dogs and cats with a higher frequency of infection (MILLÁN et al., 2016). The definition of the reservoirs and vectors involved on the routes of transmission are important steps to prevention of zoonosis.

In this study, the occurrence of infection by *Ehrlichia canis*, *Hepatozoon canis* and *Rickettsia* spp. were determined in feral cats living in two forest fragments, located in the Atlantic Rainforest Biome, in Natal, RN, Brazil, in dogs that live surrounding the areas and dogs from different regions of the city.

3.2 METHODS

3.2.1 Study area and collection of the samples

From two forest fragments, located in the Atlantic Rainforest Biome, in Natal, RN, Brazil, named Parque Estadual das Dunas de Natal (5.851067W/35.228306N) and Parque da Cidade (83.818589W/12.034402N) samples of the feral cats living inside the parks were obtained. Samples from dogs, living in homes located around the parks and dogs from the Zoonosis Control Center (ZCC) of the city of Natal, which held animals from several city regions, were also collected.

These cats had been living in the parks without care and under uncontrolled reproduction. Owned and apparently healthy dogs from houses around the parks were sampled by convenience directly from their residences, according to accessibility of the place. The dogs from ZCC were euthanized due to different causes, and in these occasions samples of spleen were obtained.

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 analyses. The ticks found parasiting the animals were collected for taxonomic identification (BARROS-BATTESTI et al., 2006; MARTINS et al., 2010).

Permits for capture and euthanasia of three specimens per species in each of the study areas and transportation of individuals were granted by the System Authorization and Information on Biodiversity (SISBio – nº 32104 -2. The study was approved by - Instituto de Defesa do Meio Ambiente de Natal (IDEMA–RN) and by

the Ethics Committee on Animal Use of the Institute of Biomedical Sciences, USP, and protocol number 204.

3.2.2 Serological analyses

Canine serum samples were tested individually by means of the indirect indirect fluorescence antibody test (IFAT) using *E. canis*-infected DH82 cells as antigen. The São Paulo strain of *E. canis* was used (AGUIAR et al., 2007b, 2008). Reactions were performed using fluorescein isothiocyanate-labelled rabbit anti-dog IgG (Sigma, St Louis, MO, USA) and IFAT cut-off to *Ehrlichia* spp. was 1:80 (KRAWCZAK et al., 2012). Samples that reacted at the screening dilution (1:80) were two-fold serial diluted for titration.

Feline and canine antibodies reactive to *Rickettsia* spp. were assayed simultaneously by six *Rickettsia* isolates from Brazil: *R. bellii* strain Mogi, *R. amblyommii* 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., 2007). Reactions were performed using fluorescein isothiocyanate-labelled rabbit anti-dog IgG and anti-cat IgG (Sigma, St Louis, MO, USA). Samples that reacted at the screening dilution (1:64) were then titrated using serial two-fold dilutions to determine endpoint titers. To specify the Probable Homologous Antigens (PHA), serum showing a *Rickettsia* species titer at least four times higher than those observed for the other *Rickettsia* species was considered to be homologous to the first *Rickettsia* species or to a very closely related genotype (LABRUNA et al., 2007; PIRANDA et al., 2008). For all reactions, a nonreactive canine serum specimen (negative control) and a known reactive canine serum specimen (positive control) were included on each slide.

3.2.3 Molecular analyses

DNA was extracted from spleen fragments of approximately 1,5cm³ each, using the Wizard® genomic DNA purification kit (Promega corporation, Madison / USA), in accordance with the manufacturer's instructions. DNA samples were tested by means of the polymerase chain reaction (PCR) using the sets of primers described in Table 4. Reactions were performed in a final volume of 25 µl, containing 10 mM of Tris–HCl (pH 8.3), 50 µM of KCl, and 1.5 mM of MgCl₂, 0.2 mM of each deoxynucleoside triphosphate, 1.5 U of Taq DNA polymerase (Invitrogen; Waltham, MA, USA), 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 stained with SyBr gold (Invitrogen; Waltham, MA, USA). The PCR products were purified using ExoSap (USB) and were sequenced in an automatic sequencer (Applied Biosystems/Perkin Elmer, model ABI Prism 310 Genetic, California, USA), in accordance with the manufacturer's protocol and with the same primers used in PCR. The partial sequences obtained were subjected to BLAST analyses (ALTSCHUL et al., 1990) to infer the closest similarities to samples in GenBank.

Table 4 - Primer pairs used in the present study for detecting tick-borne agents

Target agents (gene)	Primers	Primer sequences (5'-3')	(bp)	References
<i>Babesia</i> spp (18S rRNA)	BAB1 BAB4	GTGAACCTTATCACTTAAAGG CAACTCCTCCACGCAATCG	590	Duarte et al., (2008)
Anaplasmataceae (16S rRNA)	GE2'F2' HE3	GTTAGTGGCAGACGGGTGAGT TATAGGTACCGTCATTATCTTCCCTAT	360	Aguiar et al., (2008)
<i>Ehrlichia</i> spp	DSB-330 DSB-728	GATGATGTCTGAAGATATGAAACAAAT CTGCTCGTCTATTTTACTTCTTAAAGT	409	Doyle et al., (2005)
<i>Hepatozoon</i> spp. (18S rRNA)	HEP2-169 HEP2-718	F- GGTAATTCTAGAGCTAATACATGAGC R- ACAATAAAGTAAAAACAYTTCAAAG	574	Almeida et al., (2013)

3.3 RESULTS

During October 2012 to August 2013, serum samples were collected from 155 animals (53 feral *Felis catus* that lived in the parks; 29 *Canis familiaris* domiciled around the parks and 73 from ZCC). Spleen fragments from 20 dogs, euthanized at the ZCC, were also collected.

The animals had samples of their blood serum analyzed for detection of antibodies anti-*Rickettsia* spp and anti-*E. canis* by IFAT. The serological results showed that 17% (17/102) of the dogs had antibodies anti-*E. canis* and 13% (20/155) of all the animals tested (dogs and cats) were seropositive to *Rickettsia* spp antigens (Table 5), with titers ranging from 64 to 2048 (Table 6). With the exception of one dog that showed only antibodies anti-*R. bellii* and 2 cats that showed only antibodies against *R. parkeri*, all other positive animals showed seroreactivity and highest titers to *R. amblyommii*. At least 15 animals presented endpoint titers that were four times higher than the endpoint titers showed for the other five *Rickettsia* species. These 15 animals were considered to have been infected by *R. amblyommii* or a very closely related genotype (Table 6).

Parasitism by Ticks of the specie *Rhipicephalus sanguineus* sensu lato (s.l.) was observed in 48% of the dogs, and one *Amblyomma auricularium* was found in a cat.

Table 5 - Number and animal species positive to at least one the *Rickettsia* spp. tested and to *Ehrlichia canis* by IFAT

Animals	Positive animals / Tested (%)	
	IFA- <i>Rickettsia</i> spp	IFA- <i>E. canis</i>
<i>Felis catus</i>	17/53 (32%)	Not tested
<i>Canis familiaris</i> (Parks)	3/29 (10%)	8/29 (28%)
<i>Canis familiaris</i> (ZCC)	00/73 (0%)	9/73 (12%)
Total	20/155 (13%)	17/102 (17%)

Table 6 – Occurrence of antibodies to six *Rickettsia* species in dogs and cats from Natal, RN, Brazil

Animals IDs	Endpoint titers for the following rickettsial antigens:						
	R.A ¹	R. R ²	R. R ³	R.P ⁴	R.B ⁵	R.F ⁶	PHA ⁷
Dog 10	0	0	0	0	256	0	<i>R. bellii</i>
Dog 23	1024	0	0	0	0	0	<i>R. amblyommii</i>
Dog 25	512	0	0	0	64	0	<i>R. amblyommii</i>
Cat 33	256	0	0	0	0	0	<i>R. amblyommii</i>
Cat 5	64	0	0	64	0	0	Undefined
Cat 8	64	0	0	0	0	0	Undefined
Cat 19	2048	512	256	256	128	64	<i>R. amblyommii</i>
Cat 20	1024	0	0	0	0	0	<i>R. amblyommii</i>
Cat 9 (28.2)	512	0	0	0	0	0	<i>R. amblyommii</i>
Cat 06 (1.3)	2048	256	256	0	256	0	<i>R. amblyommii</i>
Cat 35	256	0	0	0	0	0	<i>R. amblyommii</i>
Cat 24	1024	0	0	0	0	0	<i>R. amblyommii</i>
Cat 23	512	0	0	0	0	0	<i>R. amblyommii</i>
Cat 32	64	0	0	0	0		Undefined
Cat 7	0	0	0	64	0	0	Undefined
Cat 9	512	256	0	1024	0	0	Undefined
Cat 11	256	0	0	0	0	0	<i>R. amblyommii</i>
Cat 1 (1.3)	1024	0	0	0	0	0	<i>R. amblyommii</i>
Cat 31	512	0	0	0	0	0	<i>R. amblyommii</i>
Cat 9 (18.2)	0	0	0	256	0	0	<i>R. parkeri</i>

Subtitle: ¹*Rickettsia amblyommii*; ²*R. rhipicephali*; ³*R. rickettsii*; ⁴*R. parkeri*; ⁵*R. bellii*; ⁶*R. felis*; ⁷PHA - Probable Homologous Antigens.

Among the 20 dog spleen samples subjected to molecular analyses, 8 (40%) were positive on the PCR for Anaplasmataceae by the 16S rRNA partial sequences, and for *Ehrlichia* spp. by *dsb* partial sequences. All the fragments of both genes sequences demonstrated 100 % identity with *E. canis*. Two samples were positive for *Hepatozoon canis*, confirmed by DNA sequences and BLAST analysis, showing to be 100% identical to available sequences already related in dogs from Rio Grande do Norte (GONÇALVES et al., 2014). None of the canine spleen samples yielded amplicons in the *Babesia* spp. PCR.

3.4 DISCUSSION

The results of serological analyses of *Rickettsia* spp. showed a low number of positives dogs (10%) and highest occurrence for the cats (32%). The result could be explained by the fact that the cats were living inside the parks and more exposed to *A. auricularium* that was found infesting cats and spread at the environment (Data not shown). It is known that this tick is a competent vector of *R. amblyommii* (SARAIVA et al., 2013). Besides that, two dogs that lived around the parks showed antibodies to *R. amblyommii* while dogs from other region of the city were all negative. Horta et al. (2007) showed that cat could be better rickettsial sentinels than dogs. Cats may be more exposed to ticks than other domestic animals because of their habits, and in this study, the cats abandoned in the parks have become feral, sharing the wildlife environment.

R. sanguineus was the only species of tick found parasitizing dogs in this study. It is known that this species is the vector of *E. canis* in Brazil, and the only vector of *H. canis* in the Old World (DANTAS-TORRES, 2008 in review; VIEIRA et al., 2011 in review). Until now four *Ehrlichia* species have been reported in Brazil: *E. canis*, infecting mainly dogs (VIEIRA et al., 2011); *E. ewingii*, infecting dogs (OLIVEIRA et al., 2009), *E. chaffeensis*, infecting deer (MACHADO et al., 2006), and *E. minasensis*, infecting cattle (CRUZ et al., 2012; AGUIAR et al., 2014). However, only *E. canis* has been reported in the Northeast region (SOUZA et al, 2010; VIEIRA et al, 2011).

IFAT antibody for *E. canis* was found in 17% of the dogs analyzed in this study, a similar seropositivity has been previously observed by Costa et al. (2015) that found 14.6% on dogs from rural and urban areas of Maranhão, also a state located in the northeast Brazil. Other highest values of seropositivity in dogs were reported in Northeastern Brazil: Paraíba 72.5%, 69.4%, 23% and 34% (AZEVEDO et al., 2011; TANIKAWA et al., 2013; ARAES-SANTOS et al., 2015; ROTONDANO et al., 2015), and Bahia 23.0%, 36% and 35.6% (CARLOS et al., 2007; SOUZA et al., 2010; ARAES-SANTOS et al., 2015).

Confirming the serological findings *E. canis* DNA was detected by PCR in 40% (8/20) of the spleen samples of the dogs and 75% (6/8) of these samples are also positive to *E. canis* antibodies. Previous studies on Rio Grande do Norte state show results that corroborate the findings in this study. DNA of *E. canis* and *H. canis* was detected in blood from dogs (GONÇALVES et al., 2014) and inclusions, suggestive of *Ehrlichia* spp., were detected in 6.5% (13/198) of dogs with clinical signs suggestive of canine monocytic ehrlichiosis (MEDEIROS; LIMA, 2004). These results revealed that *E. canis* was the main tick-borne pathogen infecting dogs on the region of the study.

In Brazil, canine infection due to *Hepatozoon canis* has been reported in many regions and the occurrence may range between: 8.6% and 100% on Southeast (O'DWYER et al., 2001; MUNDIM et al., 2008a; SPOLIDORIO et al., 2009; DE MIRANDA et al., 2014); 3.6 % and 73%, on Midwest (PALUDO et al., 2003; MUNDIM et al., 2008b; RAMOS et al., 2015; MELO et al., 2016), and one case with molecular analyses in the South region (LASTA et al., 2009). The studies in northeastern Brazil have showed lower values of occurrence that range from 0.49% on Pernambuco to 9.3% on Paraíba (RAMOS et al., 2010; ROTONDANO et al., 2015; BERNARDINO et al., 2016). Gonçalves et al. (2014) reported a low *H. canis* infection in dogs from Mossoro, RN, reinforcing the presence of the parasite in the region, as reported within the present study (10%).

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4 PIROPLASMID INFECTIONS IN SMALL MAMMALS OF NORTH-EASTERN BRAZIL

ABSTRACT

The aim of this study was to evaluate the molecular occurrence of hemoparasites of the piroplasmida order in small mammals of two environment conservation units in the municipality of Natal, Rio Grande do Norte State, Brazil. Sampling occurred between October 2012 and February 2013. Tissue samples (blood, spleen and lungs) were processed for molecular detection of nucleotide sequences of 18S rRNA gene common in *Babesia*, *Theileria* and *Cythauxzoon* genus. Molecular analyzis was performed in 39 tissue samples of six *Didelphis albiventris*, three *Monodelphis domestica*, two *Necromys lasiurus*, one *Thrichomys apereoides*, and one *Rattus rattus* captured in the areas. Five samples (spleen of three *D. albiventris*, and spleen and lung of one *N. lasiurus*) were positive in PCR. Phylogenetic analysis of near-full lenght 18S rRNA (1607 bp) suggested a new species of *Babesia* closely related to two species of avian *Babesia* found in the pacific region: *Babesia poelea* (DQ200887), parasite of brown boobies (*Sula leucogaster*), and another species of *Babesia* sp. (FJ717705), still unnamed, found in common murres (*Uria aalge*).

Keywords: *Babesia*. *Didelphis*. *Necromys*. Brazil.

RESUMO

O objetivo deste estudo foi determinar a ocorrência molecular de hemoparasitas da ordem piroplasmida em pequenos mamíferos de duas unidades de conservação ambiental no município de Natal, Rio Grande do Norte, Brasil. As coletas foram realizadas entre os meses de outubro de 2012 e fevereiro de 2013. As amostras de tecidos (sangue, baço e pulmão) foram processados para detecção molecular de sequências de nucleotídeos do gene 18S rRNA comum aos gêneros *Babesia*, *Theileria* e *Cythauxzoon*. A análise molecular foi realizada em 39 amostras de tecidos de seis *Didelphis albiventris*, três *Monodelphis domestica*, dois *Necromys*

lasiurus, um *Thrichomys apereoides* e um *Rattus rattus*. Cinco amostras (baço dos três *D. albiventris* e baço e pulmão de um *N. lasiurus*) amplificaram produtos na PCR. A análise filogenética da sequência de 18S rRNA (1600 pb) suporta a indicação de uma nova espécie de *Babesia* próxima à duas espécies de *Babesia* de aves marinhas da região do Pacífico: *Babesia poelea* (DQ200887) encontrada em atobás (*Sula leucogaster*) e outra espécie de *Babesia* sp. (FJ717705), encontrada em arau-comum (*Uria aalge*).

Palavras-chave: *Babesia*. *Didelphis*. *Necromys*. Brasil.

4.1 INTRODUCTION

Members of the genera *Babesia*, *Theileria* and *Cytauxzoon* are the most common tick-borne blood parasites of vertebrates and depending on the direct relationship between the vector ticks and their hosts. Belonging to the phylum Apicomplexa, they are eukaryotes and all members live parasitically. They can cause serious illness in their mammalian hosts and are therefore of great medical and veterinary importance (HOMER et al., 2000).

The aim of this study was to evaluate the molecular occurrence of hemoparasites of the piroplasmida order in small mammals of two environment conservation units in the municipality of Natal, Rio Grande do Norte State, Brazil.

4.2 METHODS

For this study two environmental conservation units were selected, located in the Atlantic Rainforest Biome, in the city of Natal, northeast of Brazil (Parque Estadual das Dunas de Natal (5.851067W/35.228306N) and Parque da Cidade (83.818589W/12.034402N).

Two field campaigns (October 2012 and February 2013) were conducted for the capture of wild animals. Permits for capture and euthanasia of three specimens per species in each of the study areas and transportation of individuals were granted by the System Authorization and Information on Biodiversity (SISBio) – nº 32104 -2, by Instituto de Defesa do Meio Ambiente de Natal (IDEMA–RN) and by the Ethics Committee on Animal Use of the Institute of Biomedical Sciences, USP, protocol number 204. Trap type cages (Shermman and Tomahawk) were distributed in spots along the paths inside the parks and a mixture of cornmeal, sardines and bananas was used as bait. Blood, spleen and lungs fragments were collected from some animals for molecular analysis. The caught animals were anesthetized using xylazine, mean dose 5mg/kg, and ketamine, mean dose 50mg/kg. The skins of the euthanized animals were send to the Museum of Natural History at the Catholic University of Minas Gerais after identification of the species. Both studied areas are commonly frequented by people for recreation or work.

DNA from lung, spleen and blood samples was extracted using the Wizard® genomic DNA purification kit (Promega corporation, Madison / USA), in accordance with the manufacturer's instructions. The DNA extracted from spleen and lung of the small mammals was tested by PCR DNA targeting overlapping portions of the 18S rRNA gene of *Babesia* spp: primers BAB-33-57 (5'-GCCAGTAGTCATATGCTTGTCTTAA-3') and BAB-432-409 (5'-TTCCTTAGATGTGGTAGCCGTTTC-3'), designed to amplify a ≈370-bp fragment (SPOLIDORIO et al., 2009); and then, for positive samples, we used the primers Piro0F (5'-GCCAGTAGTCATATGCTTGTGTTA-3') and Piro5.5R (5'-CCTYTAAGTGATAAGGTTACAAAACCTT-3') (KAWABUCHI et al., 2005) to amplify near full-length Babesial 18S rRNA (≈1648-pb) gene sequence.

The polymerase chain reaction (PCR) was performed with a final volume of 25 µl, containing 10 mM of Tris–HCl (pH 8.3), 50 µM of KCl, and 1.5 mM of MgCl₂, 0.2 mM of each deoxynucleoside triphosphate, 1.5 U of Taq DNA polymerase (Invitrogen; Waltham, MA, USA), 11 pmol of each primer and approximately 100 ng of genomic DNA. The amplified products were viewed under ultraviolet light after electrophoresis on agarose gel stained with SyBr gold (Invitrogen; Waltham, MA, USA). The PCR products were purified using ExoSap (USB) and were sequenced in an automatic sequencer (Applied Biosystems/Perkin Elmer, model ABI Prism 310

Genetic, California, USA), in accordance with the manufacturer's protocol and with the same primers used in the PCR. The obtained sequences were subjected to BLAST analyses (ALTSCHUL et al., 1990) to infer the closest similarities to samples in GenBank.

The alignment with other sequences of different piroplasmids species was performed using the Clustal Omega Web Services program (MCWILLIAM et al., 2013). The sequence of *Plasmodium falsiparum* (M19172) was used as outgroup. Phylogenetic trees were inferred by Bayesian method and were performed with Mrbayes_3.2.5 software with 1,000,000 generations. Trees being sampled every 2,000 generations, running 4 times beginning with random starting trees. The Genreal Times reversible (GTR) model (HUELSENBECK; RONQUIST, 2001) was used combined with the models of rate variation among sites: Gamma distribution (G) and extent of static, unchanging sites in a dataset (I). The first 25% of the trees represented burn-in, and the remaining trees were used to calculate Bayesian posterior probability (BPP).

4.3 RESULTS AND DISCUSSION

Blood, lungs and spleen tissue samples were collected from 03 *Didelphis albiventris*, 03 *Monodelphis domestica*, 02 *Necromys lasiurus*, 01 *Thrichomys apereoides* and 01 *Rattus rattus* euthanized and from 03 opossums found dead in the area. The total of samples analyzed was 39 for the 13 animals. Of the molecular analyses 13% (05/39) of the samples had products amplified in the PCR assay (spleen of three *D. albiventris*, and spleen and lung of one *N. lasiurus*).

From these PCR-positive samples, a near full-length Babesial 18S rRNA sequence was generated with 1600-pb and by BLAST analyses demonstrated a maximum of 94% identity with the other sequences found in Genbank with 98% of query cover. In the phylogenetical analyses the obtained sequence, named "Babesia sp. Natal", was positioned separated of the clades that commonly appear dividing piroplasmids phylogenetical trees (ALLSOPP; ALLSOPP, 2006; YABSLEY et al.,

2006; PAPARINIA et al., 2015), forming a monophyletic branch basal to *Cytauxzoon* spp. and *Theileria* spp. clades. This information suggests that the analyzed sequence could belong to a new species of piroplasmid (Figure -6)

Figure 6- Bayesian tree inferred from near full-length Babesial 18S rRNA sequences. Numbers represent the Bayesian Posterior Probability (%). GenBank accession numbers of each sequence are indicated in parenthesis. *Babesia* sp. Natal is indicated in bold

Fonte: (LOPES, M. G., 2016)

A clade formed by avian piroplasm appears near to “*Babesia* sp. Natal” position. That includes a sequence (Genbank - KC754965) found in seabirds from offshore islands of the Atlantic coast of Brazil, of which is known that they could have a migration route through the Brazilian coastal region (DIAZ et al., 2012). Besides that, migrating birds play an important role as distributors of ticks around the world (SÁNDOR et al., 2014) and, although we did not find *Babesia* positive ticks collected from the analyzed hosts (*Ornithodoros mimon*, *Amblyomma auricularium* and *Ixodes loricatus*, data not shown) further studies should be done to evaluate this possible link, since several species of ticks are known to parasite seabirds, i.e., *Ixodes uriae*, *Ixodes signatus*, *Ornithodoros capensis*, and *Ornithodoros maritimus* (MUZAFFAR; JONES, 2004).

Another hypothesis can be raised when comparing the studies by Serra-Freire (1978) that describes characteristics of a new *Babesia* species found in *Didelphis* sp., from Rio de Janeiro coastline, based on morphological analyses. The new parasite was named as “*Babesia ernesto*” and was described as belonging to the group of the large *Babesia* spp., also specificity to the genus *Didelphis*, with low parasitemia and a low frequency of piriform stages on the smears. Considering that both species of parasites were found in animals of the genus *Didelphis*, which were collected in Brazilian coastal areas of Atlantic forest; the findings of Serra Freire (1978) are most likely a reference to the same parasite of which the sequences analyzed in this study originated.

Recently a study about tick-borne diseases of small terrestrial mammals of the Pantanal, Brazil, reported one marsupial (*Monodelphis domestica*) and three rodents (*Thrichomys pachyurus*) positive to novel piroplasmid genotypes based 18S rDNA gene partial sequences (500bp) related to *Theileria bicornis*, *Cytauxzoon manul*, and *Cytauxzoon felis* (WOLF et al., 2016). These sequences show significant differences in relation to what was found in this study with an identity of 85 % (KP757840) and 95 % (KP757839) for the homologous corresponding 500bp fragment in a Identity matrix. The fact that Wolf et al. (2016) used only ≈500bp fragments, it could not be included in our phylogenetic analysis that analysed ≈1600bp fragments.

These results show that there is a great diversity of piroplasmids infecting small mammals in Brazil and therefore encourage further research on tick-borne diseases involving small mammals.

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5 CONSIDERAÇÕES FINAIS

Conclui-se que a presença de patógenos dos gêneros *Rickettsia*, *Ehrlichia*, *Babesia*, e *Hepatozoon* ocorrem nas unidades de conservação estudadas no município de Natal, RN, bem como em cães que vivem no entorno em outras regiões do município.

Amblyomma auricularum, *Ixodes loricatus*, *Ornithodoros mimon* são espécies de carrapatos que parasitam animais silvestres nas unidades de conservação abordadas nesse estudo.

Rhipicephalus sanguineus parasita cães na cidade de Natal, e como vetor competente conhecido pode estar envolvido com a transmissão de *Hepatozoon canis* e *Ehrlichia canis*.

Este trabalho evidencia uma espécie nova de *Babesia* e uma de *Ehrlichia* parasitando pequenos mamíferos, sobretudo *Didelphis albiventris*, em Natal, RN.

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