

## Molecular detection of *Ehrlichia canis* and *Babesia canis vogeli* in *Rhipicephalus sanguineus sensu lato* ticks from Cuba\*

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**ABSTRACT.** Navarrete M.G., Cordeiro M.D., Silva C.B., Pires, M.S., Ribeiro C.C.D.U., Cabezas-Cruz A., Massard C.L., López E.R & Foseca A.H. **Molecular detection of *Ehrlichia canis* and *Babesia canis vogeli* in *Rhipicephalus sanguineus sensu lato* ticks from Cuba.** [Detecção molecular de *Ehrlichia canis* e *Babesia canis vogeli* em *Rhipicephalus sanguineus sensu lato* de carrapatos em Cuba.] *Revista Brasileira de Medicina Veterinária*, 38(supl. 3):63-67, 2016. Programa de Pós-Graduação em Ciências Veterinárias, Universidade Federal Rural do Rio de Janeiro, Seropédica, RJ 23897-970, Brasil. E-mail: adivaldofonseca@yahoo.com

Ticks (Acari: Ixodidae) are of relevant medical and veterinary importance worldwide because of the range of pathogens they can transmit. A survey was carried out to identify *Babesia* spp. and *Ehrlichia* spp. in ticks obtained from dogs of Cuba. A total of 431 ticks were collected from 378 dogs and the specimens were properly identified according to a taxonomic key for the genera. All ticks were identified as belong to the *R. sanguineus sensu lato* (s. l.) species. Genomic DNA was extracted from ticks with protocol using phenol/chloroform. Ticks were organized in pools and the DNA extracted were tested by a nested polymerase chain reaction (nPCR) to amplify 398 base pairs (bp) from the 16S ribosomal DNA (*rDNA*) of *E. canis*, and to a PCR to amplify approximately 560 bp from 18S ribosomal DNA (*rDNA*). Of the 49 pools tested, 8.16% (n=4/49) were positive for the *E. canis* by nPCR targeting the 16S *rDNA* gene and only one pool (n=1/49; 2.04%) was positive for the gene 18S *rDNA* for *Babesia canis*. The four sequences obtained for the 16S *rDNA* fragment were identical to each other and resulted in 100% identity with *E. canis* from different countries. The obtained sequence of the 18S *rDNA* gene for *Babesia* spp. presented similarity of 100% with *Babesia canis vogeli* when compared to sequences deposited in Genbank. This is the first molecular detection of these agents in the tick *R. sanguineus* s. l. in Cuba.

**KEY WORDS.** Tick-borne diseases, brown tick, ehrlichiosis, babesiosis, Cuba.

**RESUMO.** Os carrapatos (Acari: Ixodidae) são de importância médica e veterinária relevantes em todo o mundo por causa da variedade de agentes patogênicos que podem transmitir. No presente trabalho, foi realizada uma pesquisa para identificar *Babesia* spp. e *Ehrlichia* spp. em carrapatos co-

letados de cães de Cuba. Foram coletados 431 carrapatos de 378 cães, tendo sido identificados como pertencentes às espécies de *Rhipicephalus sanguineus sensu lato* (s. l.). O DNA genômico foi extraído com protocolo usando fenol/clorofórmio. Os carrapatos foram organizados em "pools" e o DNA extraí-

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do foi testado pela reação em cadeia da polimerase (nPCR) para amplificar 398 pares de bases (pb) do DNA ribossômico 16S (*rDNA*) de *Ehrlichia canis* e PCR para amplificar aproximadamente 560 pb do DNA ribossômico 18S (*rDNA*). Dos 49 pools testados, 8,16% (n = 4/49) foram positivos para o *E. canis* por nPCR visando o gene do 16S *rDNA* e apenas um pool (n = 1/49; 2,04%) foi positivo para o gene 18S *rDNA* para *Babesia canis*. As quatro sequências obtidas para o fragmento de 16S *rDNA* foram idênticas entre si e resultaram em 100% de identidade com *E. canis* de diferentes países. A sequência obtida do gene do 18S *rDNA* para *Babesia* spp. apresentou semelhança de 100% com *Babesia canis vogeli* quando comparada às sequências depositadas no Genbank. Esta foi a primeira detecção molecular desses agentes no carrapato *R. sanguineus* s. l. em Cuba.

**PALAVRAS-CHAVE.** Doenças transmitidas por carrapatos, carrapato marrom, ehrlichiose, babesiose, Cuba.

## INTRODUCTION

Ticks (Acari: Ixodidae) are of relevant medical and veterinary importance worldwide because of the range of pathogens they can transmit. *Rhipicephalus sanguineus sensu lato* is a typical dog tick and a natural transmitter of several hemoparasites (Dantas-Torres et al. 2013). In Cuba, the specie has been found as endemic in the western region (Barros-Battesti et al. 2009).

Tick-transmitted canine disease has drawn both public and scientific attention to this arthropod. In the caribbean region, cases of autochthonous *Ehrlichia* and large *Babesia* infections of dogs have been reported from many caribbean countries (Georges et al. 2008, Yabsley et al. 2008).

Ehrlichiosis and Babesiosis are diseases of great incidence in the veterinary clinics and responsible for variable clinical manifestations, that can determine death. *Ehrlichia canis* is the primary etiologic agent of canine monocytic ehrlichiose (CME), a well know cosmopolitan tick-borne disease of dogs (Harrus et al. 2011). Canine babesiosis is a disease caused by a protozoan parasite, belonging to the Apicomplexa phylum, genus *Babesia* that infect erythrocytes (Solano-Gallego & Baneth 2011).

In Cuba, these two genera have been previously reported in dogs, but their diagnosis has been based on the observation of clinical signs, hematological findings, direct observation by means of blood smears and the detection of antibodies through serological methods (León Goñi & Gómez Rosales 2008, Pérez et al. 2002). These techniques

used in the diagnosis do not allow determining the true situation, due to similarity of the clinical symptoms and the low sensitivity of these methods (Mylonakis et al. 2003, Ramos et al. 2009).

The objective of the present study was to report the first molecular detection of *E. canis* and *Babesia* spp. in *R. sanguineus* s. l. tick collected from dogs in Cuba, since ticks included in this study may harbor pathogens and can also contain infected blood of the host.

## MATERIAL AND METHODS

The fieldwork was conducted between October and December of 2013 in the western region of Cuba. Ticks were collected from 378 dogs' ears, neck, shoulder and toes from four municipalities in western Cuba: three from the Havana province (Habana del Este, Boyeros and Cotorro) and one from the Mayabeque province (San José de las Lajas) (Figure 1).

A convenience sampling was carried out and the inspected animals presented different ages, gender and apparently healthy. The ticks were then collected, and the specimens were properly identified according to a taxonomic key for the genera described by Barros-Battesti et al. (2006) and Barros-Battesti et al. (2009).

The 431 tick specimens collected were distributed in pools with a maximum of ten ticks separated according to species, gender, maturation stage and collected site. The tick pools were stored in polypropylene tubes in freezer -20°C until the extraction of the genetic material.

DNA extraction from ticks was performed following

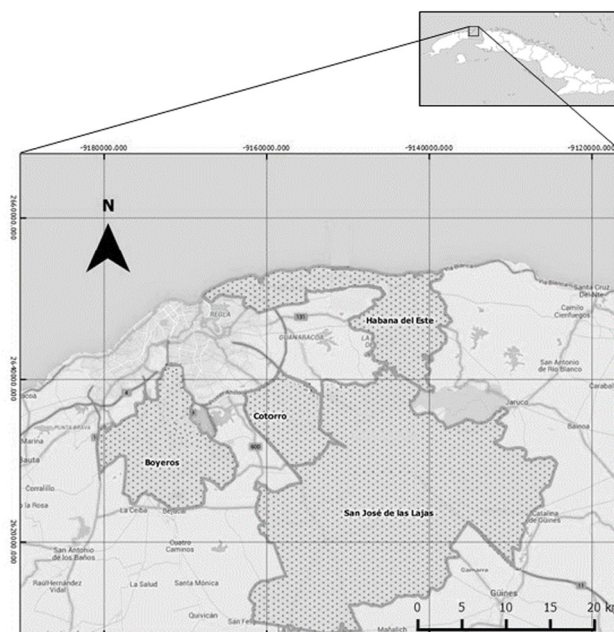


Figura 1. Island of Cuba located in the smaller map. Larger map: location of the municipalities in which they were carried out the collections in Habana (Cotorro, Boyeros, Habana del Este) and Mayabeque (San José de las Lajas) provinces. Scale bar = 20 Km.

the protocol based on the utilization phenol/chloroform as described previously by Costa Santolin et al. (2013). The extracted DNA was stored in a freezer at  $-20^{\circ}\text{C}$ . The purity and concentration of the DNA from all tick's samples were determined by spectrophotometer NanodropND-2000<sup>®</sup> (Thermo Fisher Scientific, Wilmington, DE, USA).

A nested-PCR for amplification of a portion of the 16S *rDNA* gene was performed for detection of *E. canis* in the samples. In this way, the primers ECC (5'-AGAA-CGAACGCTGGCGGCAAGC-3') and ECB (5'-CGTAT-TACCGCGGCTGCTGGCA-3') were used for the first reaction, while the primers ECAN5 (5'-CAATTATTTA-TAGCCTCTGGCTATAGGA-3') and HE3 (5'-TATAGG-TACCGTCATTATCTTCCCTAT-3') for the second reaction (Murphy et al. 1998), amplifying a final fragment of 398 bp from the 16S *rDNA* of *E. canis*.

For the detection of the presence of *Babesia* spp., the primers BJ1 (5'-GTCTTG TAATTGGAATGATGG-3') and BN2 (5'-TAGTTTATGGTTAGGACTACG-3') that amplify a fragment of the 18S *rDNA* gene was used according Casati et al. (2006). All reactions were performed under the same conditions described by the cited authors.

The both positive DNA control used in the reaction were obtained from blood sample of animals from Rio de Janeiro state, Brazil. These animals presented clinic symptomatology, high parasitemia, in acute phase, with infection detected by microscopy and confirmed by the relevant specific molecular assay (Földvári et al. 2005, Murphy et al. 1998).

The amplification products from the reactions were submitted to electrophoresis on a 1.5% agarose gel. The gel was stained with ethidium bromide (0.4 mg/mL) and visualized under ultraviolet light in the Transilluminator UV 302 NM (KASVI - Curitiba, Paraná, Brazil).

The positive samples to *E. canis* and *Babesia* spp. were used for sequencing of 16S *rDNA* and 18S *rDNA* genes, respectively. The products were purified using the Wizard<sup>®</sup>SV Geland PCR Clean-Up System (Promega<sup>®</sup>, Madison, WI, USA), and sequenced by Sanger method (SANGER et al., 1977), using a ABI Prism 310 Genetic Analyzer (Applied Biosystems<sup>®</sup>, Perkin Elmer, CA, USA). The Basic Local Alignment Search Tool (BLAST) was used to evaluate the similarity of the 16S *rDNA* (*E. canis*) and 18S *rDNA* (*B. canis*) genes with nucleotide sequences available in GenBank. For *Ehrlichia* and *Babesia* phylogenetic trees, fragments of 16S *rDNA* and 18S *rDNA* nucleotide sequences were collected from GenBank, respectively. Several species of *Rickettsia* or Apicomplexa were included in the analysis as shown in Figures 2 and 3. The sequences obtained in this study were aligned with the sequences from GenBank for the 16S *rDNA* gene (GU810149, JX629805, U96436, AF147752, AB013008, AF069758, M60313, M73224, M82801, U12457, M73225 and AF036649) and for the 18S *rDNA* gene (KT323936, DQ111760, LC012807, JX962779, AY294206, KU377437, FJ944828, AY534883, KX375828, LC125457, AY260179, FJ213580, KM206780, KJ000484, AY596279, KF928959, AF499604, HQ895985, FJ603460,

EU277003, KU363043, EU083804) using the MAFFT (Kato & Standley 2013). Non-aligned regions were removed with Gblocks (v 0.91b) (Castresana 2000) implemented in Phylogeny.fr (Dereeper et al. 2008). The final cured alignments contained in all cases more than 250 gap-free nucleotide positions. The best-fit model of the sequence evolution was selected based on Corrected Akaike Information Criterion (cAIC) and Bayesian Information Criterion (BIC) implemented in Molecular Evolutionary Genetics Analysis (MEGA) 6 (Tamura & Nei 1993). The Kimura 2-parameter (Kimura 1980) and TN93 (Tamu-

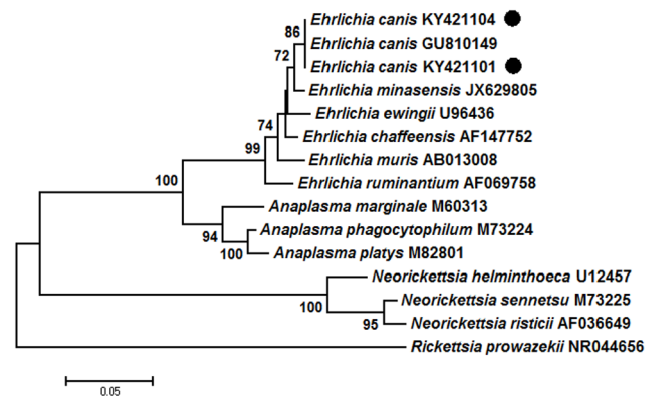


Figura 2. Phylogenetic analysis of *Rickettsia*. A ML phylogenetic tree was built using 16S *rDNA* nucleotide sequences from *Rickettsia* spp. with emphasis in members of the family Anaplasmataceae. The strains of *Ehrlichia canis* identified in ticks from Cuba are shown with a black circle. Numbers on branches are bootstrap support values. Only bootstrap values  $>60\%$  are shown.

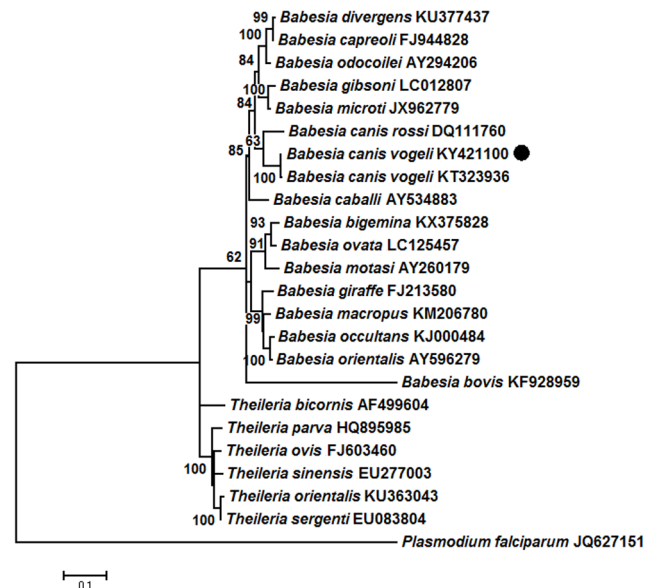


Figura 3. Phylogenetic analysis of Apicomplexa. A ML phylogenetic tree was built using 18S *rDNA* nucleotide sequences from Apicomplexan with emphasis in members of the genera *Babesia* and *Theileria*. The strains of *Babesia canis vogeli* identified in ticks from Cuba are shown with a black circle. Numbers on branches are bootstrap support values. Only bootstrap values  $>60\%$  are shown.

ra & Nei 1993) models, which had the lowest values of cAIC and BIC, were chosen in subsequent phylogenetic analyses for *Rickettsia* or Apicomplexa, respectively. Maximum likelihood (ML) method, implemented in MEGA, was used to obtain the best tree topology. Initial trees for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. *Rickettsia prowazekii* (NR044656) and *Plasmodium falciparum* (JQ627151) were used as outgroups in the phylogenetic trees for *Rickettsia* or Apicomplexa, respectively. A proportion of invariable (+I) and gamma (+G) distributed sites was estimated in MEGA. Reliability of internal branches was assessed using the bootstrapping method (1000 replicates). Graphical representation and editing of the phylogenetic tree was performed with MEGA.

## RESULTS AND DISCUSSION

From the inspected animals, a total of 431 ticks were collected. All identified ticks belonged to the *R. sanguineus* s. l. species. A total of 158 ticks (84 males, 48 females, 24 nymphs, 2 larvae) were collected from Habana del Este, followed by Boyeros with 153 (95 males, 44 females, 14 nymphs) specimens, San José de las Lajas with 73 (48 males, 16 females, 9 nymphs) and Cotorro with 47 (23 males, 8 females, 16 nymphs).

The total number of ticks was distributed in 15 female pools, 27 male pools, six nymph pools and one larval pool, totaling 49 samples. These data were partially presented by da Silva et al. (2016) when reporting the first molecular detection of *Anaplasma platys* in dogs and their ticks in Cuba. Of the 49 pools tested, four were positive for the *E. canis* 16S *rDNA* gene, of which one pool of female, two pools of males and one pool of nymphs, and only one pool of female ticks was positive for the gene 18S *rDNA* for *Babesia* spp. The four sequences obtained for the 16S *rDNA* fragment were identical to each other and resulted in 100% identity with *E. canis* from different countries. For this reason of similarity, two samples of Cuba ticks positive to *E. canis* were inserted in the phylogenetic analysis as representative of the others. The obtained sequence of the 18S *rDNA* gene for *Babesia* spp. presented similarity of 100% with *B. canis vogeli* when compared to sequences deposited in Genbank.

The partial sequences of 16S *rDNA* gene for *E. canis* were submitted to GenBank under the accession numbers: KY421101, KY421102, KY421103, and KY421104, and from and 18S *rDNA* gene for *B. canis vogeli* the accession number KY421100.

As shown in figure 2, the analyses of 16S *rDNA* sequences showed that the Cuban isolates clustered together with other *E. canis* isolate deposited in GenBank, segregating away from any reference sequences of the others genus species used. In the same way, the analyses of 18S *rDNA* sequence (Figure 3) for *Babesia* spp., allowed to observe that the tick sample from Cuba formed a cluster together with *B. canis vogeli*.

The identification of ticks infesting dogs corresponded to *R. sanguineus* s. l. as being the only species present. This arthropod is widely distributed throughout the country, especially in urban areas as already reported by Barros-Battesti et al. (2009).

Up to present moment, there are still no reports of molecular diagnosis of *E. canis* in dogs resident in Cuba, but the CME is an infectious disease of dogs found in the clinical routine in Cuba mainly by the symptomatology aspects (León Goñi & Gómez Rosales 2008).

The results demonstrate the presence of *E. canis* and *B. canis* in *R. sanguineus* s. l. as its vector. To our knowledge, this is the first report these hemoparasites in this tick from Cuba; although previously, the presence of the CME agent in Cuban territory was performed through clinical and pathological reports by through hematological data and serology using ImmunoComb® kit (León Goñi & Gómez Rosales 2008).

The nPCR may improve the sensitivity of detection of target nucleotide sequence (Haff 1994). This technique has been shown to be sensitive for direct identification of ehrlichiae in ticks (Kramer et al. 1999).

Among tick-borne diseases in dogs, babesiosis stands out for its worldwide distribution (de Sa et al. 2006), presenting the common clinical aspects including fever, anorexia, depression, oliguria, hemoglobinuria, vomiting, lethargy, dehydration, icterus, pale mucous membranes, splenomegaly and dyspnea (Irwin 2009). However, sometimes the animals were asymptomatic for babesiosis, and thus, the group of positive dogs may probably be in the subclinical stage of the infection (Vilela et al. 2013). On the other hand, no method evidenced the presence of *Babesia* spp. in *R. sanguineus* s. l. ticks or dogs in Cuban territory up to the present time. A first approach to the detection of *R. sanguineus* s. l. positive for *Babesia* spp. was performed by Rodriguez et al. (2014), but none of the ticks tested were positive.

This is the first molecular detection of both *E. canis* and *B. canis vogeli* infecting *R. sanguineus* s. l.

ticks in Cuba. The identification of these agents in dogs is necessary to prove the association of this vector in the transmission of these hemoparasites in companion animals in Cuba.

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