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Short Communication

Molecular detection of Anaplasmataceae agents in *Dasyprocta azarae* in northeastern Brazil

Detecção molecular de agentes Anaplasmataceae em *Dasyprocta azarae* no nordeste do Brasil

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Abstract

Recently, the importance of wild-living rodents for maintenance of pathogens of the family Anaplasmataceae in the environment was investigated. These mammals play a role as reservoirs for these pathogens and act as hosts for the immature stages of tick vectors. The aim of the present study was to investigate the prevalence of *Ehrlichia* sp. and *Anaplasma* sp. in 24 specimens of Azara's agouti (*Dasyprocta azarae*) that had been trapped in the Itapiracó Environmental Reserve, in São Luís, Maranhão, northeastern Brazil, using molecular methods. Four animals (16.7%) were positive for *Ehrlichia* spp. in nested PCR assays based on the 16S rRNA gene. In a phylogenetic analysis based on the 16S rRNA gene, using the maximum likelihood method and the GTRGAMMA+I evolutionary model, *Ehrlichia* sp. genotypes detected in Azara's agoutis were found to be closely related to *E. canis* and to genotypes relating to *E. canis* that had previously been detected in free-living animals in Brazil. The present work showed the first molecular detection of *Ehrlichia* sp. in Azara's agoutis in Brazil.

Keywords: Azara's agouti, *Ehrlichia*, rodents, molecular characterization.

Resumo

Recentemente, a importância de roedores selvagens na manutenção de agentes Anaplasmataceae no ambiente tem sido investigada, haja visto o papel que tais mamíferos podem desempenhar como reservatórios para os patógenos e como hospedeiros para estágios imaturos dos carrapatos vetores. O presente estudo objetivou investigar a ocorrência de *Ehrlichia* sp. e *Anaplasma* sp. em 24 cotias (*Dasyprocta azarae*) capturadas na Reserva Ambiental de Itapiracó, em São Luís, Maranhão, nordeste do Brazil, utilizando métodos moleculares. Quatro animais (16,7%) mostraram-se positivos nos ensaios de nested PCR para *Ehrlichia* spp. baseados no gene 16S rRNA gene. Na análise filogenética baseda no gene 16S rRNA e utilizando o método de Máxima Verossimilhança e modelo evolutivo GTRGAMMA+I, os genótipos de *Ehrlichia* sp. detectados em cotias mostraram-se filogeneticamente relacionados às sequências de *E. canis* e outros genótipos relacionados a *E. canis* detectados previamente em animais selvagens no Brasil. O presente trabalho mostrou a primeira detecção molecular de *Ehrlichia* sp. em cotias no Brasil.

Palavras-chave: Cotias, *Ehrlichia*, roedores, caracterização molecular.

The bacteria belonging to the genera *Anaplasma* and *Ehrlichia* are obligate intracellular parasites that replicate in intracytoplasmic vacuoles of neutrophils, monocytes, macrophages and erythrocytes (DUMLER et al., 2001), thus forming microcolonies called morulae. These agents are associated with infections in humans and several species of domestic and free-living animals. Most cases of ehrlichiosis and anaplasmosis described in humans are tick-borne zoonoses (DOUDIER et al., 2010; ISMAIL et al., 2010; WOLDEHIWET, 2010).

Ixodid ticks are competent vectors for *A. phagocytophilum* in the northern hemisphere (RIZZOLI et al., 2014). In Brazil, *A. phagocytophilum* has been molecularly detected in *Rhipicephalus sanguineus* sensu lato and *Amblyomma sculptum* (cited as *A. cajennense*) in the state of Rio de Janeiro (SANTOS et al., 2013). While *Amblyomma americanum* is considered to be the main vector for *Ehrlichia ewingii* and *E. chaffeensis* in North America (RAR & GOLOVLJOVA, 2011), *Dermacentor variabilis* and *Ixodes pacificus*

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possibly act as vectors in other regions (WALKER et al., 2004). *Ehrlichia canis* is transmitted by *Rhipicephalus sanguineus* and *Dermacentor variabilis* (LITTLE, 2010; RAR & GOLOVLJOVA, 2011). Few studies in Brazil have investigated the vector competence of ticks in the epidemiological cycles of pathogens of the family Anaplasmataceae. Recently, it was demonstrated that only the tropical lineage of *R. sanguineus* (SANCHES et al., 2016) showed vector competence for transmitting *E. canis* among dogs in South America (MORAES-FILHO et al., 2015).

Recently, the importance of free-living rodents for maintenance of pathogens of the family Anaplasmataceae in the environment was investigated. These mammals play a role as reservoirs for these pathogens and act as hosts for the immature stages of tick vectors (FOLEY et al., 2002; OBIEGALA et al., 2014; RIZZOLI et al., 2014).

In the United States, certain rodent species like *Tamias ochrogenys* exhibit demographic characteristics, such as high population density and ability to remain infected for long periods of time, that contribute towards transmission of *Anaplasma phagocytophilum*, the etiological agent of human granulocytic anaplasmosis (NIETO & FOLEY, 2009). Moreover, dispersal of this rodent species may facilitate the spread of infected ticks to other vertebrate hosts (NIETO & FOLEY, 2009). The rodent species *Neotoma fuscipes* has been shown to be an important source of infection for the DU-1, HZ, MRK and Dog_CA strains of *A. phagocytophilum*. It acts as a reservoir for this agent and as hosts for the immature stages of ixodid ticks, which have been recognized as vectors for granulocytic agents of anaplasmosis in the northern hemisphere (NIETO et al., 2010; REJMANEK et al., 2012).

On the other hand, in Europe, prevalence of *A. phagocytophilum* (22.8%) was reported among rodents belonging to the species *Apodemus sylvaticus, Apodemus flavicollis* and *Myodes glareolus*, which were sampled in different regions of France (CHASTAGNER et al., 2016). In Germany, Obiegala et al. (2014) found low prevalence of *A. phagocytophilum* (1.1%) among the rodent species *A. sylvaticus, Myodes glareolus* and *A. flavicollis*. Similarly, low prevalence of *A. phagocytophilum* (0.2%-2.2%) was also reported among rodents in Slovakia (SVITÁLKOVÁ et al., 2015; BLAŇAROVÁ et al., 2014).

Only a few studies have been conducted in Brazil regarding the prevalence of Anaplasmataceae agents in rodents. For instance, Wolf et al. (2016) detected *Anaplasma* sp. in a specimen of *Hylaeamys megacephalus* that had been trapped in the Pantanal of the state of Mato Grosso. More recently, among 458 rodents sampled in different regions of Brazil, Benevenute et al. (2017) reported prevalences of 1.09% and 1.96% for *Ehrlichia* sp. and *Anaplasma* sp., respectively, using previously described conventional PCR (cPCR) assays based on the 16S rRNA gene. Additionally, prevalences of 0.44% and 2.40% for *Ehrlichia* sp. and *Anaplasma* sp., respectively, were reported in the same study, using a newly designed multiplex quantitative real-time PCR based on the *groEL* gene (BENEVENUTE et al., 2017).

Azara's agoutis (*Dasyprocta azarae*) are rodents of terrestrial habit that are distributed in rainforests (Amazon Forest and Atlantic Forest), semi-deciduous forests, savanna and caatinga, and are usually found close to water courses (BONVICINO et al., 2008). Considering the scarcity of studies relating to detection of pathogens of the family Anaplasmataceae in rodents in Brazil, the aim of the present study was to investigate the prevalence of *Ehrlichia* sp. and *Anaplasma* sp. in Azara's agoutis that had been trapped in the Itapiracó Environmental Reserve, in São Luís, Maranhão, northeastern Brazil.

Between January and May 2016, blood samples were collected from 24 Azara's agoutis (16 females and 8 males) that had been trapped in the Itapiracó Environmental Reserve (322 hectares), in the municipality of São Luís, state of Maranhão, northeastern Brazil (Figure 1). The rodents were caught using live traps (Sherman®; H. B. Sherman Traps, Tallahassee, FL, USA; and Tomahawk®; Tomahawk Live Traps, Tomahawk, WI, USA) baited with a mixture of banana, peanut butter, oats and sardine. The traps were set up on seven consecutive nights along linear transects, placed on the ground at ten-meter intervals. The total capture effort was 50 trap-nights. After blood sampling, the animals were ringed and weighed (mean values of 2.65 kg and 2.45 kg for males and females, respectively). All the animal trapping was done in accordance with the licenses obtained from the Brazilian government's Institute for Wildlife and Natural Resource Care (IBAMA) and was endorsed by the Ethics Committee of the State University of Maranhão, São Luis, Maranhão, Brazil), under no. 004/2016.

DNA was extracted from 200 µL of whole blood from each sample, using the QIAamp DNA blood mini kit (QIAGEN®, Valencia, CA, USA), in accordance with the manufacturer's instructions. DNA concentration and quality were measured using the 260/280 nm absorbance ratio (Nanodrop®, Thermo Fisher Scientific, Waltham, MA, USA). In order to verify the presence of amplifiable DNA in the samples, an internal control PCR assay targeting a fragment of mammalian glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (BIRKENHEUER et al., 2003) was performed.

Conventional PCR assays targeting the 16S rRNA gene of *Ehrlichia* spp. (MURPHY et al., 1998) and *Anaplasma* spp. (MASSUNG et al., 1998) were performed, following previously described protocols. In order to achieve further molecular characterization, positive samples were subjected to cPCR assays targeting *groESL/Ehrlichia* and *Anaplasma* spp. (SUMNER et al., 1997, NICHOLSON et al., 1999, LOTRIC-FURLAN et al., 1998) *omp-1/Ehrlichia* spp. (INAYOSHI et al., 2004), and *dsb*/*E. canis* (DOYLE et al., 2005). *Ehrlichia canis* (DE SOUSA et al., 2013), *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* DNA (kindly supplied by Prof. John Stephen Dumler, Uniformed Services University of the Health Sciences, Bethesda, MD, USA) were used as positive controls in cPCR assays. The cPCR assays contained 10X PCR buffer (Life Technologies®, Carlsbad, CA, USA), 1 mM of $MgCl_2$ (Life Technologies®, Carlsbad, CA, USA), 0.2 mM of deoxynucleotide triphosphate (dNTPs) mixture (Life Technologies®, Carlsbad, CA, USA), 1.5 U of Taq DNA polymerase (Life Technologies®, Carlsbad, CA, USA) and 0.5 µM of each primer (Integrated DNA Technologies®, Coralville, IA, USA) (Table 1). Ultra-pure water (Promega®) was used as a negative control in all PCR assays. In order to prevent PCR contamination, the DNA extraction, reaction setup, PCR amplification and electrophoresis were performed in separate rooms. The gels were imaged under ultraviolet light using the Image Lab software version 4.1 (Bio-Rad®). The reaction products were purified using the Silica Bead DNA gel extraction kit (Thermo Fisher Scientific®, Waltham, MA, USA).

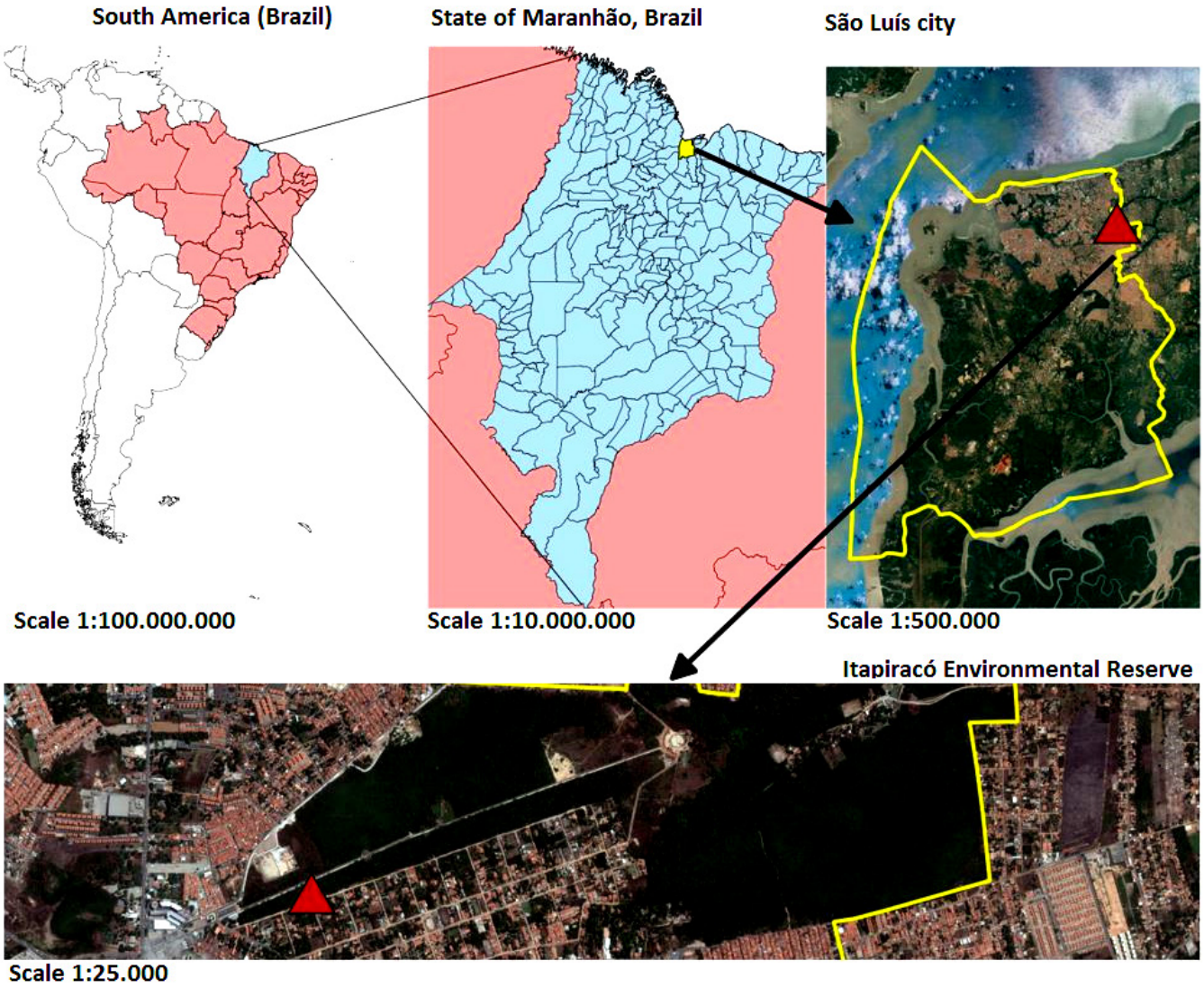

Figure 1. Map of Reserva Ambiental do Itapiracó, São Luís city, state of Maranhão, northeastern Brazil, where Azara's agouti blood samples were collected between January and May 2016.

Sanger sequencing was performed using the BigDye® Terminator v3.1 cycle sequencing kit (Thermo Fisher Scientific®, Waltham, MA, USA) and the ABI PRISM 310 DNA analyzer (Applied Biosystems®, Foster City, CA, USA) (SANGER et al., 1977).

Sequences obtained from positive samples were first subjected to a screening test using the Phred-Phrap software, version 23 (EWING & GREEN, 1998; EWING et al., 1998), in order to evaluate the electropherogram quality and obtain consensus sequences through alignment of sense and antisense sequences. The BLAST software (ALTSCHUL et al., 1990) was used to analyze the nucleotide sequences (BLASTn), with the aim of browsing through them and comparing them with sequences that had previously been deposited in an international database (GenBank) (BENSON et al., 2017). All sequences that showed appropriate quality standards and similarity with *Ehrlichia* spp. or *Anaplasma* spp. were deposited in GenBank. The sequences were aligned with sequences that had previously been published in GenBank, using the MAFFT software, version 7 (KATOH & STANDLEY, 2013).

Phylogenetic inferences were made based on the maximum likelihood (ML) method. The ML analysis was done using the RAxML-HPC BlackBox software 7.6.3 (STAMATAKIS et al., 2008) (which includes estimation of bootstrap node support), using 1000 bootstrapping replicates. The best evolutionary model (GTRGAMMA+I) was selected through the jModelTest2 software (version 2.1.6) on XSEDE (DARRIBA et al., 2012), under the Akaike Information Criterion (AIC) (POSADA et al., 2004). All phylogenetic analyses were performed using the CIPRES Science Gateway (MILLER et al., 2010). The trees were examined using Treegraph 2.0.56-381 beta (STOVER & MULLER, 2010).

The samples from all 24 Azara's agoutis amplified a GAPDH gene fragment. Four animals (16.7%) were positive for *Ehrlichia* spp.

Table 1. Description of oligonucleotides, PCR product size and references used in cPCR assays for *Ehrlichia* spp. and *Anaplasma* spp.

Agent (Target gene)	Oligonucleotide sequences (5'-3')	Product size (bp)	References
Ehrlichia spp. *(16S rRNA gene)*	ECC (GAACGAACGCTGGCGGCAAGC) ECB (CGTATTACCGCGGCTGCTGGCA)	478	Murphy et al. (1998)
Nested *E. canis* *(16S rRNA gene)*	ECAN-5 (CAA TTATTTATAGCCTCTGGCTATAGGA) HE3 (TATAGGTACCGTCATTATCTTCCCTAT)	358	Murphy et al. (1998)
Nested *E. chaffeensis* *(16S rRNA gene)*	CHAFF (CAA TTGCTTATAACCTGGTTATAAAT) GAIUR (GACTTTGCCGGGACTTCTTCT)	410	Kocan et al. (2000)
A. phagocytophilum *(16S rRNA gene)*	gE3a (CACATGCAAGTCGAACGGATTATTC) gE10R (TTCCGTTAAGAAGGATCTAATCTCC)	932	Massung et al. (1998)
	gE2 (GGCAGTATTAAAAGCAGCTCCAGG) gE9f (AACGGATTATTCTTTATAGCTTGCT)	546	
Ehrlichia/Anaplasma spp. (*groESL* gene)	AACGGATTATTCTTTATAGCTTGCT HS1a AITGGGCTGGTAITGAAAT	1300 - 1450	Sumner et al. (1997),
	HS6a CCICCIGGIACIAIACCTTC		Nicholson et al. (1999),
	HS43 AT(A/T)GC(A/T)AA(G/A)GAAGCATAGTC HSVR CTCAACAGCAGCTCTAGTAGC	1297	Lotric-Furlan et al. (1998)
Ehrlichia spp. (*omp-1* gene)	conP28-F1 AT(C/T)AGTG(G/C)AAA(A/G)TA(T/C)(A/G)T(G/A)CCAA conP28-R1 TTA(G/A)AA(A/G)G(C/T)AAA(C/T)CT(T/G)CCTCC	713	Inayoshi et al. (2004)
	conP28-F2 CAATGG(A/G)(T/A)GG(T/C)CC(A/C)AGA(AG)TAG conP28-R2 TTCC(T/C)TG(A/G)TA(A/G)G(A/C)AA(T/G)TTTAGG 5'-TTCC(T/C)TG(A/G)TA(A/G)G(A/C)AA(T/G)TTTAGG-3'	300	
Ehrlichia spp. (*dsb* gen*e)*	dsb-330 GATGATGTCTGAAGATATGAAACAAAT dsb-728 CTGCTCGTCTATTTTACTTCTTAAAGT	409	Doyle et al. (2005)

in nested PCR assays based on the 16S rRNA gene. Three out of four positive amplicons were subjected to sequencing based on a high-intensity band that was observed on agarose gel. The sequences obtained (MF435278 - *Dasyprocta* #1; MF435279 - *Dasyprocta* #3; and MF435280 - *Dasyprocta* #18) showed 98-99% similarity to *E. canis* (GenBank access numbers KX766395.1, KJ995838.1 and KC109445.1). All the samples were negative for *Anaplasma* spp. in cPCR assays based on the 16S rRNA. Samples that were positive for *Ehrlichia* spp. based on the 16S rRNA gene were negative in cPCR assays based on *groESL, dsb* and *omp-1* genes, thus precluding further molecular characterization. In the phylogenetic analysis based on the 16S rRNA gene using the maximum likelihood method and GTRGAMMA+I evolutionary model, the *Ehrlichia* sp. genotypes detected in Azara's agoutis were closely related to *E. canis* and to genotypes relating to *E. canis* that had previously been detected in free-living animals in Brazil (Figure 2).

Although the role of free-living rodents in the epidemiology of Anaplasmataceae pathogens has been extensively studied in the United States and Europe, there are few reports on the prevalence of *Ehrlichia* and *Anaplasma* in rodents in South America. In fact, it was only recently that *Ehrlichia* spp. and *Anaplasma* spp. DNA was detected in free-living rodents in Brazil (WOLF et al., 2016; BENEVENUTE et al., 2017).

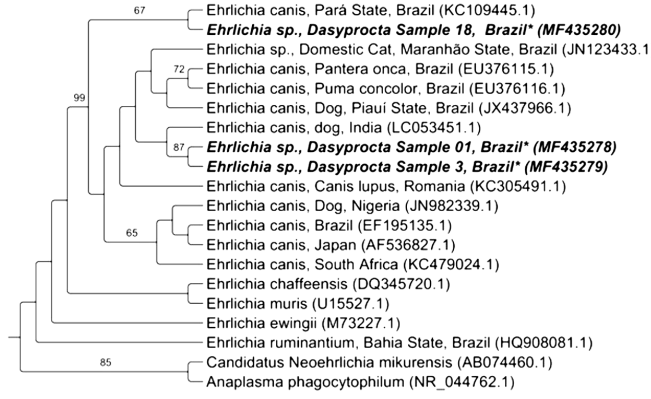

Figure 2. Phylogenetic tree constructed with *Ehrlichia* spp.-16SrRNA sequences, using Maximum Likelihood method and GTRGAMMA+I evolutionary model. The numbers at the nodes correspond to bootstrap values higher than 50% accessed with 1,000 replicates, using '*Candidatus* Neoehrlichia mikurensis' (AB074460.1) and *Anaplasma phagocytophilum* (NR_0447621) as outgroups. *The sequences amplified in the present study are highlighted in bold.

Here, all 24 Azara's agoutis were shown to be negative for *Anaplasma* spp. Previously, one out of 42 rodents sampled in the Pantanal of Mato Grosso was positive for *Anaplasma* sp., but

the phylogenetic positioning of this specimen was not accessed. More recently, Benevenute et al. (2017) detected 16S rRNA DNA fragments of *Anaplasma* sp. in 21 (4.6%) out of 458 spleen samples from rodents that had been sampled in five distinct biomes in Brazil. The *Anaplasma* genotypes were detected in rodents from the Caatinga, Cerrado and Atlantic Forest biomes (BENEVENUTE et al., 2017).

In the present study, four Azara's agoutis that were sampled in the Amazon biome were positive for an *Ehrlichia* genotype closely related to *E. canis*. These findings have been corroborated through the results recently found by Benevenute et al. (2017), who detected a similar genotype in 7 (1.5%) out of 458 rodents belonging to the species *Thrichomys pachyurus, Clyomys laticeps, Holochilus fosteri and Calomys cerqueirai*, which had been trapped in the Pantanal biome, and *Thrichomys laurentius*, which had been caught in the Caatinga biome. Interestingly, the genotypes detected in Azara's agoutis were also closely related to an *Ehrlichia* sp. previously detected in a domestic cat sampled on the island of São Luis (BRAGA et al., 2012). Genotypes closely related to *E. canis* have also been detected in rodents in Korea (KIM et al., 2006), free-living birds (MACHADO et al., 2012), wild-living carnivores (ANDRÉ et al., 2010, 2012) and domestic cats (ANDRÉ et al., 2015) in Brazil. Unfortunately, attempts to make additional phylogenetic inferences based on genes rather than 16S were unsuccessful, thus corroborating the results found by Benevenute et al. (2017). The real identity at species level of these genotypes that circulate in wild-living rodents in Brazil needs to be further investigated in the future, through isolation, with the aim of achieving better molecular and antigenic characterization.

To the best of the authors' knowledge, the present work showed the first molecular detection of *Ehrlichia* sp. in Azara's agoutis in Brazil. The pathogenic and zoonotic potential of these genotypes circulating in wild-living rodents in Brazil, their competent vectors and natural cycles need to be further investigated in the future.

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