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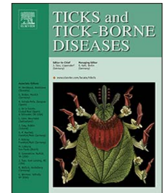

Original article

Assessment of a quantitative 5′ nuclease real-time polymerase chain reaction using *groEL* gene for *Ehrlichia* and *Anaplasma* species in rodents in Brazil

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ABSTRACT

New genotypes of Anaplasmataceae agents have been detected in wild carnivores, birds and deer in Brazil. The present work aimed to investigate the presence of *Ehrlichia* and *Anaplasma* species in rodents sampled in Brazil. Additionally, a newly designed quantitative 5′ nuclease real-time multiplex PCR for *Ehrlichia* and *Anaplasma* spp. detection based on *groEL* gene amplification was designed, showing high specificity and sensitivity (10 *groEL* fragment copy/μL). Between 2000 and 2011, different rodent species [n = 60] were trapped in 5 Brazilian biomes. Among 458 rodent spleen samples, 0.4% (2/458) and 2.4% (11/458) were positive for *Ehrlichia* and *Anaplasma* spp., respectively. Of 458 samples, 2.0% (9/458) and 1.1% (5/458) were positive for *Anaplasma* sp. and *Ehrlichia* sp., respectively, using conventional 16S rRNA PCR assays. Maximum Likelihood phylogenetic analyse based on a small region of 16S rRNA genes positioned the *Anaplasma* genotypes in rodents near *Anaplasma phagocytophilum* or *Anaplasma marginale* and *Anaplasma odocoilei* isolates. *Ehrlichia* genotypes were closely related to *E. canis*. There was a low occurrence of *Anaplasma* and *Ehrlichia* in wild and synanthropic rodents in Brazil, suggesting the circulation of new genotypes of these agents in rodents in the studied areas.

1. Introduction

The Anaplasmataceae family included within the order Rickettsiales is composed of Gram-negative Alphaproteobacteria, comprised of the genera *Anaplasma, Ehrlichia, Neorickettsia, Neoehrlichia,* and *Wolbachia* (Dumler et al., 2001). In vertebrate hosts, these obligate intracellular bacteria replicate within neutrophils, monocytes, macrophages and erythrocytes' intracytoplasmic vacuoles (Dumler et al., 2001; Rar and Golovljova, 2011) forming microcolonies known as morulae and causing infections in humans and several species of domestic and wild animals (Doudier et al., 2010; Ismail et al., 2010; Woldehiwet, 2010).

Ehrlichiosis and anaplasmosis are tick-borne zoonoses (Doudier et al., 2010; Ismail et al., 2010; Woldehiwet, 2010). Identifying the temporal prevalence of infectious agents and their vectors is key to improve the understanding of these diseases (Rejmanek et al., 2011). Recent studies demonstrate the importance of small mammals in the epidemiological cycles of tick-borne pathogens (Szekeres et al., 2014), especially the interaction between tick vectors and mammalian hosts, which play a key role in the maintenance dynamics of these bacteria in the environment (Rizzoli et al., 2014). Rodents can act as hosts for both pathogens and immature stages of tick vectors, consequently posing risks to animal and human health (Foley et al., 2002; Obiegala et al., 2014).

Previous studies report a molecular prevalence for *An. phagocytophilum* ranging from 3.8% (33/652) to 14.5% (47/325) in rodents (*Neotoma fuscipes* and *Tamias ochrogenys*) and ticks (*Ixodes pacificus*, *I. angustus, I. ochotonae* and *I. spinipalpis* species) in California, United States (Foley et al., 2008, 2011; Foley and Nieto, 2011). Using infection

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experiments with *An. phagocytophilum* MRK strain, Nieto and Foley (2009) reported that squirrels belonging to the species *Tamias ochrogenys* could act as potential reservoirs for *An. phagocytophilum*. Besides, these animals show demographic characteristics that contribute to the transmission of the studied agent. When *Neotoma fuscipes* rodents were experimentally infected with *An. phagocytophilum* strains DU-1 and HZ, Rejmanek et al. (2012) found that the agent remains detectable by real-time PCR for at least eight months, confirming their competence as reservoirs for *An. phagocytophilum*, as previously proposed by Nicholson et al. (1999).

On the other hand, the molecular prevalence of *An. phagocytophilum* in rodents in Europe seems to be lower when compared to that found in the United States. For instance, a low prevalence of *An. phagocytophilum* (1.1% [7/631]) is found in *Apodemus sylvaticus*, *Myodes glareolus and Apodemus flavicollis* species rodents in Germany (Obiegala et al., 2014). Similarly, Blanarová et al. (2014) detected *An. phagocytophilum* in 1.6% (11/669) of ear punch samples and 2.2% (9/407) of spleen samples of *Ap. agrarius*, *Ap. flavicollis* and *M. glareolus* species rodents trapped in central Europe.

Additionally, '*Candidatus* Neoehrlichia mikurensis' and *Ehrlichia muris* are also Anaplasmataceae species related to rodents mainly in Europe an USA, respectively (Jahfari et al., 2012; Blanarová et al., 2016; Svitálková et al., 2016). These agents are considered as emerging tick-borne pathogens and may represent risk for human health (Kawahara et al., 2004; Pritt et al., 2009). The *Ixodes scapularis* tick has been incriminated as the primary vector of *Ehrlichia muris*-like in the USA (Pritt et al., 2011; Lynn et al., 2015; Karpathy et al., 2016). Moreover, *Ehrlichia muris*-like DNA has been detected in *Peromyscus leucopus* rodents in the USA (Castillo et al., 2015).

In Europe, '*Candidatus* Neoehrlichia mikurensis' has been detected in eight rodent species, namely *Ap. agrarius, Ap. flavicollis, Ap. sylvaticus, M. glareolus, Microtus agrestis, Mi. arvalis, Mi. arvalis* and *Micromys minutus,* with the highest prevalence in *M. arvalis* (Silaghi et al., 2015, Svitálková et al., 2016). '*Candidatus* Neoehrlichia mikurensis' has also been detected in rodents from China, Japan and Russia and in *I. persulcatus* ticks in Mongolia (Naitou et al., 2006; Tabara et al., 2007; Rar et al., 2010). *Ixodes ricinus* are incriminated as the main vectors of *Candidatus* Neoehrlichia mikurensis in Europe (Jahfari et al., 2012; Svitálková et al., 2016).

There are few reports on the prevalence of Anaplasmataceae agents in wild animals in Brazil. New genotypes of Anaplasmataceae agents were detected in wild carnivores (André et al., 2010; 2012; Widmer et al., 2011), birds (Machado et al., 2012) and deer (Sacchi et al., 2012; Silveira et al., 2012, 2015) in Brazil. Moreover, reports demonstrate serological evidence of exposure to Anaplasmataceae agents in humans in Brazil (Calic et al., 2004; Costa et al., 2005; Vieira et al., 2013). Recently, an *Anaplasma* sp. was detected in a *Hylaeamys megacephalus* rodent sampled in Brazilian Pantanal, in the state of Mato Grosso, central-western Brazil (Wolf et al., 2016).

The aim of the present work was to investigate, using molecular methods, the presence of Anaplasmataceae agents in wild and synanthropic rodents sampled in five different biomes in Brazil. Additionally, a newly designed quantitative 5′ nuclease real-time multiplex PCR for *Ehrlichia* and *Anaplasma* species based on *groEL* gene detection is presented.

2. Material and methods

2.1. Distribution and rodent species sampled

The Brazilian territory is composed by six biomes, namely Amazon Forest, Cerrado, Atlantic Forest, Caatinga, Pampa and Pantanal. The Amazon Forest is conditioned by the humid equatorial climate and has a wide variety of plant formations. The Cerrado presents diverse regions, ranging from clean fields devoid of woody vegetation to dense tree formations, and presents two well-defined seasons. The Atlantic Forest is composed of ecosystems with very different structures and composition of flowers, having as a common element the exposure to the humid winds that blow from the ocean. The Caatinga has dry soils and vegetation formed by palm trees and has only two distinguishable seasons: a very hot and dry winter and hot and rainy summer. The Pampa is characterized by herbaceous species and several typologies of the country, composing in some regions by environments integrated with the Araucaria forest. The Pantanal, an alluvial plain influenced by rivers that drain the basin of the Upper Paraguay, is formed by largely sandy terrains, covered by different physiognomies due to the variety of micro regions and flood regimes, by their seasonal inundation and desiccation. They shift between phases of standing water and phases of dry soil, when the water table can be well below the root region (Coutinho, 2006).

Between 2000 and 2011, different rodent genera [n = 32] (Table 1) were trapped in five Brazilian biomes (Fig. 1), namely Amazon Forest, Cerrado, Atlantic Forest, Caatinga, and Pantanal (ftp://ftp.ibge.gov.br/Cartas_e_Mapas/Mapas_Murais). Animals were caught using *Tomahawk* and *Sherman* "live-traps" during previous studies performed by the Laboratories of Trypanosomatid Biology and Biology and Parasitology of Wild Mammals Reservoirs Laboratories, Oswaldo Cruz Institute, Rio de Janeiro, Brazil (Herrera et al., 2005; Oliveira et al., 2009). Euthanasia of animals was performed for taxonomic identification and/or diagnosis of parasites. Rodents were exsanguinated after anesthesia using intramuscular ketamine hydrochloride (100 mg/mL) and acepromazine (10 mg/mL). Euthanasia was assured by intracardiac injection of potassium chloride 19.1% (2 mL/kg).

Spleen tissues from 468 rodents were collected and stored in DNase- and RNase-free microtubes, containing ethanol and maintained at −20 °C until DNA extraction. Sampling procedures were approved by the Brazilian Institute for Environment and Natural Renewable Resources (IBAMA) (IBAMA/CGFAU/LIC 3665-1) and by the Ethics Committee of Oswaldo Cruz Foundation (FIOCRUZ) (P0007-99; P0179-03; P0292/06; L0015-07).

2.2. DNA extraction

DNA was extracted from 10 mg of each rodent spleen tissue using the DNeasy® Blood & Tissue Kit (Qiagen®, Valencia, California, USA), according to manufacturer's instructions. DNA concentration and absorbance ratio (260/280 nm) were measured using a spectrophotometer (Nanodrop, Thermo Scientific, USA). Microtubes containing ultra-pure sterile water were intercalated between each series of twenty rodent spleen samples and used to control for contamination during DNA extraction.

2.3. Evaluation of DNA extraction quality

To evaluate the quality of DNA samples, each spleen DNA was tested by PCR for the mammal *irbp* ("interphotoreceptor retinoid binding protein") gene as an internal control (Ferreira et al., 2010). Samples negative for the abovementioned PCR protocol were tested for another internal control by PCR targeting the *gapdh* (Birkenheuer et al., 2003).

2.4. Quantitative 5′ nuclease real-time multiplex PCR for Ehrlichia spp. and Anaplasma spp.

2.4.1. Primers and hydrolysis probe design

The software Allele ID6 (Premier Biosoft, Palo Alto, CA, USA) was used to design TaqMan primers and probe. The gene target included *Ehrlichia* (alignment of *E. canis, E. chaffeensis, E. muris, and Ehrlichia* sp. (Anan strain, IOE agent) and *Anaplasma* (*An. phagocytophilum, An. platys, An. marginale, An. centrale, An. ovis, and An. bovis*) consensus *groEL* sequences.

Real-time detection was enabled using SYBR® green fluorescent dye and optimized with integration of labeled probe. The result was a

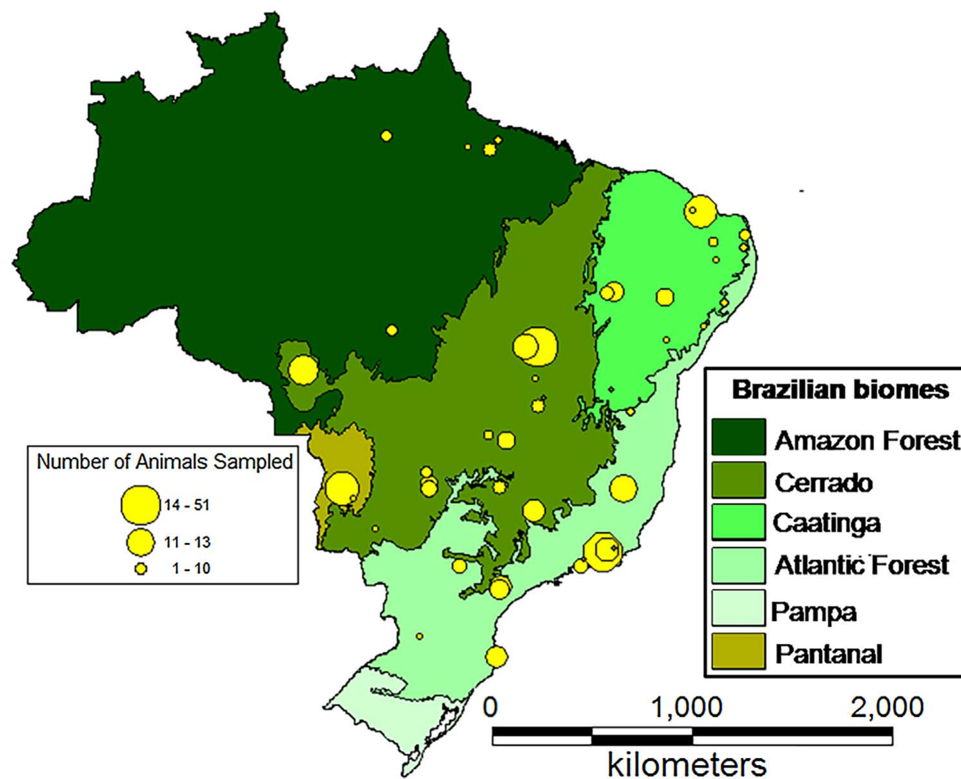

Fig. 1. Distribution of rodents (n = 468) captured in five Brazilian biomes.

TaqMan (5′-nuclease) assay for the CFX PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Cy5 and TET were the fluorophores used to detect the *groEL* of *Anaplasma* and *Ehrlichia* species, respectively. Primers and probes were synthesized by Integrated DNA Technologies (IDT, Coralville, IA, USA).

Primers and hydrolysis probe were identified that met the following conditions (default parameters according AlleleID): primers and hydrolysis probes length of 16–18 nucleotides, primers melting temperatures of 50.0 ± 2 °C, hydrolysis probe melting temperatures of 7.0 ± 2 °C, amplicon size of 75–150 bp. Primers and hydrolysis probe specificity was tested *in silico* using BLAST algorithms. Aiming for the best amplification conditions, both the annealing temperature and concentration of primers and hydrolysis probe in the reaction were determined experimentally. The annealing temperature tested ranged from 50 to 57 °C and the concentration of primers and hydrolysis probe ranged from 0.2 to 1.2 μM. The sequences of primers and hydrolysis probes obtained using AlleleID6 were F-Ehr (5′-GCGAGCATAATTACTCAGAG-3′), R-Ehr (5′-AGTATGGAGCATGTAGTAG-3′) and F-TET-5′-CATTGGCT CTTGCTATTGCTAAT-3′[BHQ2a-Q] and F-Anap (5′-TTATCGTTACATT GAGAAGC-3′)R-Anap (5′-GATATAAAGTTATTAAAAGTATAAAGC-3′) and Cy5- 5′-CCACCTTATCATTACACTGAGACG-3′[BHQ2a-Q] for *Ehrlichia* and *Anaplasma* species, respectively. The standardization of qPCR followed the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) (Bustin et al., 2009).

2.4.2. Analytical specificity

The specificity of the assay was assessed using DNA from several genomic bacterial (*E. canis, E. chaffeensis, E. muris, Ehrlichia* sp. (Anan strain, IOE agent), *An. phagocytophilum, An. platys, An. marginale, An. centrale, An. ovis, An. bovis, B. henselae, B. quintana, B. elizabethae, B. vinsonii berkhofii, B. phoceensis, B. schoenbuchii, B. chomelli, B. bovis, B. alsaticae B. birtlesii, Bartonella* sp. Th239, *R. rickettsii, R. slovaca, R. typhi, R. prowazekii, N. risticii, N. helminthoeca, N. sennetsu* and *Orientia tsutsugamushi*) and protozoa (*Plasmodium falciparum, P. malariae, Trypanosoma brucei rhodesiense*). *Anaplasma, Ehrlichia* and *Rickettsia* samples were provided courtesy of Anthony Barbet (University of Florida, Gainesville, FL), Guy Palmer (Washington State University, Pullman, WA), David Walker (University of Texas Medical Branch, Galveston), and Nahed Ismail (University of Pittsburgh, PA); *Bartonella* spp. DNA samples were kindly supplied by Dr. Bruno Chomel (University of California, Davis, CA, USA) and Dr. Edward Breitschwerdt (North Carolina State University, Raleigh, NC, USA). *Trypanosoma brucei* DNA sample was kindly supplied by Dr. Dennis J. Grab (The Johns Hopkins University School of Medicine, Baltimore, MD, USA).

2.4.3. Analytical sensitivity

The analytical sensitivity of the real-time PCR was determined using 10-fold serial dilutions of pIDTSMART plasmids (Integrated DNA Technologies, Coralville, IA, USA) encoding *groEL Ehrlichia* and *Anaplasma* consensus sequences (insert containing 83 bp). The number of plasmid copies was determined according to the formula (Xg/μL DNA/[plasmid length in bp x 660]) x 6.022×10^{23} x plasmid copies/μL. Plasmid copy number was adjusted and serially diluted in both TE (10 mmol/L, Tris–HCl, 0.1 mmol/L), EDTA, pH 8.0) and TE buffer combined with 30 μg/mL of herring sperm DNA (Sigma-Aldrich, St Louis, MO, USA) aiming to mock the presence of host DNA in the sample.

2.4.4. Amplification efficiency and limit of detection

Amplification efficiency (E) was calculated from the slope of the standard curve in each run using the following formula ($E = 10^{-1/\text{slope}}$). To determine the limit of detection of the *TaqMan* assay, the standard curves generated by 10-fold dilutions were used to determine the amount of DNA that could be detected with 95% sensitivity (Bustin et al., 2009).

2.4.5. SYBR green and TaqMan PCR reactions

For SYBR Green PCR reactions, 10 μL PCR mixtures contained 2 μL of IQ™ SYBR® Green Supermix (BioRad) with a final concentration of 2 μM of each primer and 1 μL of DNA sample. For TaqMan qPCR reactions, 10 μL PCR mixtures contained 5 μL of Go Taq® Probe qPCR

Table 1
Species, number of animals and rodent capture sites analyzed in the present study.

Rodent Species	Biomes				
	Amazon Forest	Caatinga	Cerrado	Atlantic Forest	Pantanal
Akodon cursor	–	–	–	1	–
Akodon montensis	–	–	9	5	–
Akodon sp.	–	–	4	39	–
Bolomys lasiurus	–	1	4	–	–
Bolomys sp.	–	–	–	4	–
Brucepattersonius sp.	–	–	3	1	–
Calomys cerqueirai	–	–	–	3	–
Calomys tener	–	–	3	4	–
Calomys sp.	12	–	28	–	–
Cavia sp.	–	1	–	–	–
Cerradomys marinhus	–	–	1	–	–
Cerradomys sp.	–	–	6	–	–
Clyomys laticeps	–	–	–	–	8
Clyomys sp.	–	–	–	–	11
Delomys dorsalis	–	–	–	8	–
Euryoryzomys russatus	–	–	–	10	–
Euryoryzomys sp.	–	–	1	–	–
Galea spixii	–	18	–	–	–
Gracilinanus agilis	–	–	–	–	–
Holochilus sp.	–	–	–	–	3
Hylaeamys sp.	–	–	11	–	–
Hylaeamys megacephalus	–	–	3	–	–
Juliomys sp.	–	–	1	–	–
Mus musculus	1	5	2	2	–
Necromys lasiurus	13	–	25	–	–
Necromys sp.	3	–	1	–	–
Nectomys squamipes	–	–	1	6	–
Nectomys sp.	–	–	3	–	–
Nectomys rattus	–	–	6	–	–
Oecomys sp.	–	–	7	–	2
Oecomys gr. bicolor	–	–	2	–	–
Oecomys gr. concolor	–	–	2	–	–
Oligoryzomys nigripes	–	–	6	17	–
Oligoryzomys sp.	–	–	10	5	–
Oligoryzomys flavescens	–	–	–	3	–
Oryzomys scotti	–	–	1	–	–
Oryzomys sp.	–	–	3	–	–
Oryzomys megacephalus	–	–	1	–	–
Oxymycterus sp.	3	–	–	4	–
Oxymycterus dasythricus	–	–	–	3	–
Proechimys goeldii	7	–	–	–	–
Proechimys sp.	3	–	2	–	–
Proechimys roberti	2	–	1	–	–
Rattus rattus	9	14	–	–	–
Rhipidomys macrurus	–	5	–	–	–
Rhipidomys sp.	–	1	14	–	–
Sciurus sp.	–	–	–	1	–
Sooretamys angouya	–	–	–	1	–
Sphiggurus sp.	–	–	–	1	–
Sphiggurus villosus	–	–	–	1	–
Thrichomys pachyurus	–	–	–	–	–
Thrichomys laurentius	–	24	–	–	–
Thrichomys apereoides	–	–	–	10	–
Thrichomys sp.	–	4	1	–	1
Thrichomys inermis	–	7	–	–	–
Trinomys dimidiatus	–	–	–	4	–
Trinomys iheringi	–	–	–	1	–
Wiedomys sp.	–	–	1	–	–
Wiedomys pyrrhorinus	–	1	–	–	–
Zygodontomys sp.	–	2	–	–	–
TOTAL	53	83	163	134	25

Master Mix, dTTP (Promega) with a final concentration of 1.2 μM of each primer and hydrolysis probe and 1 μL of DNA sample. PCR amplifications were conducted in Low-Profile Multiplate™ Unskirted PCR Plates (BioRad) using CFX96 Thermal Cycler (BioRad). The amplification conditions were 95 °C for 3 min followed by 40 cycles of 95 °C for 10 min and 52.7 °C for 30 s.

2.4.6. Repeatability and reproducibility

The intra-assay variance (repeatability or short-term precision) was determined by running five replicates of the plasmid dilutions (10^7 to 1 copy of plasmid /reaction) in the same run. The standard curve was then generated using the CFX Manager Software version 2.1 (BioRad). The inter-assay variance (reproducibility or long-term precision) was determined by running triplicates of the same plasmid dilution in five different runs, on separate days. These replicates were used to determine the mean, standard deviation and coefficient of variation in Cq values for each plasmid dilution.

2.5. Conventional(c) PCR assays for Ehrlichia spp. and Anaplasma spp.

Previously described cPCR assays for *Ehrlichia* spp. *and Anaplasma* spp., based on 16S rRNA gene, were performed for rodents' DNA samples. The primers sequences and cycling conditions used in cPCR assays were based on protocols described by Murphy et al. (1998) for *Ehrlichia* sp. (478 bp) and *Ehrlichia canis* (358 bp), Kocan et al. (2000) for *Ehrlichia chaffeensis* (410 bp) and Massung et al. (1998) for *Anaplasma* spp. (456 bp). Positive samples in qPCR and cPCR for *Ehrlichia* spp. and *Anaplasma* spp. based on *groEL* and 16S rRNA gene, respectively, were submitted to cPCR assays for amplification of partial fragments of *dsb* (Doyle et al., 2005), *groESL* (Sumner et al., 1997; Nicholson et al., 1999; Lotric-Furlan et al., 1998), *msp-4* (De La Fuente et al., 2005; Bown et al., 2007) and *omp-1* (Inayoshi et al., 2004) genes, using previously described protocols.

PCR products were separated by electrophoresis on a 1% agarose gel stained with ethidium bromide. In order to prevent PCR contamination, DNA extraction, reaction setup, PCR amplification and electrophoresis were performed in separated 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 (Fermentas, São Paulo, SP, Brazil). Purified amplified DNA fragments from positive samples were subjected to sequencing for confirmation in an automatic sequencer (ABI Prism 310 genetic analyzer; Applied Biosystems/Perkin-Elmer) and were used for subsequent phylogenetic analyses.

2.6. qPCR assays for E. canis (dsb gene), E. chaffeensis (vlpt gene) and An. phagocytophilum (msp-2 gene)

Positive samples in qPCR and cPCR assays for *Ehrlichia* spp. and *Anaplasma* spp., based on *groEL* and 16S rRNA genes, respectively, were submitted to specific qPCR assays designed for *E. canis* based on *dsb* gene (Doyle et al., 2005), *E. chaffeensis* based on *vlpt* gene (Reller et al., 2009), and *An. phagocytophilum* based on *msp-2* gene (Drazenovich et al., 2006). The amplification reactions were performed using a final volume reaction of 10 μL containing a mixture of 1 μL of the DNA sample, 0.2 μM of each oligonucleotide and hydrolysis probe, 5 μL of PCR buffer (Go Taq® Probe qPCR Master Mix, dTTP, Promega, Madison, Wisconsin, USA) and sterilized ultra-pure water (Nuclease-Free Water, Promega®, Madison, Wisconsin, USA) q.s.p 10 μL. Amplification reactions were conducted in a CFX96 Thermal Cycler thermal cycler (BioRad®, Hercules, California, USA). The sensitivity of real-time reactions was determined using 10-fold serial dilutions of pIDTSMART plasmids (Integrated DNA Technologies, Coralville, IA, USA) encoding inserts containing 378 bp of *E. canis dsb* gene, 79 bp of *E. chaffeensis vlpt* gene and 289 bp of *An. phagocytophilum msp-2* gene. The number of plasmid copies was determined according to the formula (Xg/μL DNA/

[plasmid length in bp x 660]) x 6.022 × 10^{23} x plasmid copies/μL.

2.6.1. Phylogenetic analyses

The analysis of electropherograms generated by sequencing was performed observing the quality of peaks corresponding to each base sequenced, using Bioedit v. 7.0.5.3 (Hall, 1999) and *Phred/Phrap/Consed* softwares (Gordon et al., 1998; Ewing et al., 1998; Ewing and Green, 1998). The BLAST software (Altschul et al., 1990) was used in order to compare the sequences obtained with those previously deposited in GenBank (http://www.ncbi.nlm.nih.gov/genbank).

The sequences obtained in 16S rRNA-cPCR assays were identified using BLASTn (nucleotide BLAST 2.4.0 – Megablast with default parameters). Subsequently, a multiple alignment with the new sequences and those available in GenBank was performed using Clustal/W v. 7.0.5.3 (using default parameters) (Thompson et al., 1994) and, posteriorly, MAFFT (**M**ultiple **A**lignment using **F**ast **F**ourier **T**ransform) (also using default parameters) (Katoh et al., 2002). Sequences where manually adjusted in Bioedit (v. 7.0.5.3) (Hall, 1999). The alignments saved in “FASTA” mode were transformed into Nexus, Phylip and Mega by the Alignment Transformation Environment (Glez-Peña et al., 2010) site. The Maximum Likelihood (ML) phylogenetic analysis was inferred with RAxML-HPC BlackBox (v. 7.6.3; the bootstrap values higher than 50% were accessed with 1000 replicates) (Stamatakis et al., 2008), both analyses were performed through the CIPRES Science Gateway (Miller et al., 2010). Akaike information criterion available on *jModelTest 2* (Darriba et al., 2012; Guindon and Gascuel, 2003) was applied to identify the most appropriate model of nucleotide substitution. The most appropriate model of nucleotide substitution (lower AIC value) was TIM2 + I + R.

3. Results

3.1. Viability of rodent DNA samples

Among the 468 rodent spleen samples analyzed, 458 were positive on amplification of a fragment of irbp gene (endogenous control of reaction). The ten samples negative for *irbp* PCR assay were also negative on the cPCR based on *gapdh* gene and were excluded from subsequent analysis. The average concentration of DNA and 260/280 ratio were 154.62 ng/μL (0.1–812.0 ng/μL) and 1.95 (0.7–6.18), respectively.

3.2. Specificity analysis

The designed primers and probe amplified all genomic DNA from six *Anaplasma* species and four *Ehrlichia* species. No amplification signal was obtained from no template controls and from *Bartonella*, *Neorickettsia*, *Orientia*, *Rickettsia*, *Trypanosoma* and *Plasmodium* species.

3.3. Repeatability and reproducibility of qPCR assays for Anaplasma spp. and Ehrlichia spp.

The efficiency mean of qPCR assays for *Anaplasma* spp. was E = 90.9% (slope = −3.466; r^2 = 0.992). As few as 10 copies of plasmid diluted in TE/reaction were detected in this assay. When the plasmid dilutions contained 30 μg/mL of herring sperm, the efficiency was E = 93.9% (slope = −3.476; r^2 = 0.985) and the assay also detected as few as 10 copies of plasmid/reaction. For *Ehrlichia* spp., the efficiency mean of qPCR assays was E = 91.7% (slope = −3.551; r^2 = 0.961), when using the plasmid standards diluted in TE. When the plasmid dilutions contained 30 μg/mL of herring sperm, the efficiency was E = 90.5% (slope = −3.594; r^2 = 0.951). The intra- and inter-assays repeatability and reproducibility of the herein designed qPCR assay for *Ehrlichia* spp. and *Anaplasma* spp. based on *groEL* gene are shown in Tables 2 and 3.

Table 2
Intra- and inter-assay repeatability and reproducibility for the qPCR *groEL* assay for *Anaplasma* species.

Plasmid copies	Intra-assay repeatability				Inter-assay reproducibility			
	GBlock diluted in 1 x TE		GBlock diluted in DNA		GBlock diluted in 1 x TE		GBlock diluted in DNA	
	Mean Crossing point (Cq ± SD)	CV (%)	Mean Crossing point (Cq ± SD)	CV (%)	Mean Crossing point (Cq ± SD)	CV (%)	Mean Crossing point (Cq ± SD)	CV (%)
10^7	13.36	4.1	13.28	13.6	13.99	1.86	13.85	2.85
10^6	17.10	2.2	15.89	3.6	17.70	4.22	17.13	3.01
10^5	20.81	3.3	20.41	0.9	20.87	1.87	20.71	1.29
10^4	24.02	0.6	24.36	1.2	24.50	0.79	24.46	0.83
10^3	27.87	0.9	27.70	0.9	27.69	0.97	27.97	1.35
10^2	31.07	1.5	31.68	0.8	31.44	1.09	31.61	1.36
10	35.89	3.7	35.14	4.3	34.76	3.29	35.31	4.37

Table 3
Intra- and inter-assay repeatability and reproducibility for the qPCR *groEL* assay for *Ehrlichia* species.

Plasmid copies	Intra-assay repeatability				Inter-assay reproducibility			
	GBlock diluted in 1 x TE		GBlock diluted in DNA		GBlock diluted in 1 x TE		GBlock diluted in DNA	
	Mean Crossing point (Cq ± SD)	CV (%)	Mean Crossing point (Cq ± SD)	CV (%)	Mean Crossing point (Cq ± SD)	CV (%)	Mean Crossing point (Cq ± SD)	CV (%)
10^7	12.86	15.4%	12.48	5.6%	13.44	3.85	13.32	4.47
10^6	15.94	7.0%	16.24	1.8%	17.06	2.92	16.63	4.40
10^5	20.16	1.4%	19.44	3.8%	20.84	2.01	20.57	1.76
10^4	23.88	1.3%	23.96	1.3%	24.40	2.32	24.43	1.32
10^3	27.25	1.4%	27.15	1.1%	27.78	1.57	27.88	1.71
10^2	30.63	1.6%	31.58	2.5%	31.80	1.59	31.88	2.27
10	36.60	4.3%	34.35	0.9%	35.70	4.11	35.32	8.82

3.4. Molecular detection of *Anaplasma* spp. and *Ehrlichia* spp. by qPCR based on *groEL* gene

Among the 458 rodent spleen samples tested, 0.44% (2/458) and 2.40% (11/458) were positive for *Ehrlichia* spp. and *Anaplasma* spp., respectively. The cycles of quantification (Cqs) mean of positive samples for *Ehrlichia* spp. was 31.95. The mean of *groEL* gene fragment quantification for *Ehrlichia* spp. varied from 1.071×10^2 to 2.808×10^2 copies/μL. The positive samples for *Anaplasma* spp. showed a Cq mean value of 33.70, with number of copies of *groEL* gene fragment ranging from 1.123×10^0 to 1.310×10^2 copies/μL (Table 4).

Overall, 15 multiplex qPCR assays for *Ehrlichia* and *Anaplasma* plates were performed. The mean value of efficiency (E) of qPCR assays for *Ehrlichia* spp. was: E = 92.4% [(90.1–99.7%, SD ± 3.08); *slope* = −3.044; r^2 = 0.989]. For *Anaplasma* spp., the mean value of efficiency was: E = 95.9% [(90.4% –102.4%, SD ± 3.55); *slope* = −3.447; r2 = 0.993]. The limit of detection was ten copies of plasmid/microliter. However, five samples show a number of copies of DNA lower than 10 copies/μL of *Anaplasma* sp.

3.5. Molecular detection of *Anaplasma* spp. and *Ehrlichia* spp. by cPCR assays

Out of 458 samples tested, 1.97% (9/458) were positive for *Anaplasma* sp.-cPCR assay based on 16S rRNA gene: 5 *Rattus rattus* from Jaguaruana city (Ceará state); 2 *Akodon montensis* captured from Teresópolis city (Rio de Janeiro state); 1 *Sphiggurus villosus* from Piraí city (Rio de Janeiro state), and 1 *Calomys cerqueirae* from Capitão Andrade (Minas Gerais state). Five other rodents (1.09%) were positive for *Ehrlichia* sp. cPCR assay based on 16S rRNA gene: 1 *Thrichomys laurentius* from Coronel José Dias city (Piauí state, Caatinga biome); 1 *Calomys cerqueirai* from Capitão Andrade city; 2 *Clyomys laticeps* and 1 *Holochilus fosteri* from Corumbá city (Mato Grosso do Sul state, Pantanal biome) (Table 5).

None of the rodent DNA samples positive in multiplex qPCR assays based on *groEL* gene and cPCR assays based on 16S rRNA were positive in cPCR assays based on *dsb*, *omp-1*, *groEL, msp-4* and *msp-5* genes. None of the samples were positive for both *groEL*-multiplex qPCR and 16S rRNA cPCR assays.

3.6. qPCR assays for *E. canis* (*dsb* gene), *E. chaffeensis* (*vlpt* gene) and *An. phagocytophilum* (*msp-2* gene)

None of the 27 rodent spleen samples positive in multiplex qPCR and cPCR assays based on *groEL* and 16S rRNA genes, respectively, showed to be positive in specific qPCR assays for *E. canis*, *E. chaffeensis* and *An. phagocytophilum* based on *dsb*, *vlpt* and *msp-2* genes, respectively.

3.7. BLAST and phylogenetic analyses of *Anaplasma* spp. and *Ehrlichia* spp. 16S rRNA sequences

Three out of nine 16S rRNA-*Anaplasma* spp. amplicons were submitted to sequencing, due the high intensity of bands in agarose gels. The other sequences showed insufficient DNA for sequencing. The BLAST analysis on one *Anaplasma* sp. 16S rRNA sequenced product (GenBank accession number KY391802) detected in one *Calomys cerqueirai* captured in the state of Minas Gerais (Atlantic Forest biome) showed 99% sequence identity with *Anaplasma phagocytophilum* (GenBank accession numbers KU705197 and KX646192) and *Anaplasma* sp. detected in an Orinoco goose (*Neochen jubata*) in Brazil (GenBank accession number KX886808). Additionally, one *Anaplasma* sp. 16S rRNA sequenced product (GenBank accession number KY391803) detected in one *Rattus rattus* from the state of Ceará (Caatinga biome) showed 99% sequence identity with *Anaplasma*

Table 4
Rodent species positive for *Ehrlichia* spp. and *Anaplasma* spp. qPCR multiplex assays based on *groEL* gene, their respective biome, and mean of Cq values and quantification.

Rodent species	Biome	Detection (Fluorophore)	Mean of Cq values	Mean of Quantification (copies/μL)
Thrichomys inermis	Caatinga	*Anaplasma* sp. (Cy5)	37,06	2.972×10^0
Thrichomys pachyurus	Pantanal	*Ehrlichia* sp. (TET)	32,50	1.071×10^2
Oecomys laticeps	Pantanal	*Ehrlichia* sp. (TET)	31,40	2.808×10^2
Calomys expulsus	Cerrado	*Anaplasma* sp. (Cy5)	35,70	6.971×10^0
Euryoryzomys russatus	Atlantic Forest	*Anaplasma* sp. (Cy5)	31,28	1.310×10^2
			32,75	2.107×10^1
Akodon montensis	Atlantic Forest	*Anaplasma* sp. (Cy5)	35,18	4.264×10^0
Euryoryzomys russatus	Atlantic Forest	*Anaplasma* sp. (Cy5)	32,43	2.601×10^1
Akodon montensis	Atlantic Forest	*Anaplasma* sp. (Cy5)	32,68	2.207×10^1
Euryoryzomys russatus	Atlantic Forest	*Anaplasma* sp. (Cy5)	33,19	1.573×10^1
Euryoryzomys russatus	Atlantic Forest	*Anaplasma* sp. (Cy5)	33,19	1.573×10^1
Thrichomys laurentius	Caatinga	*Anaplasma* sp. (Cy5)	33,95	9.55×10^0
Cavia spixii	Caatinga	*Anaplasma* sp. (Cy5)	32,87	1.94×10^1
			34,04	8.99×10^0
Oligoryzomys nigripes	Atlantic Forest	*Anaplasma* sp. (Cy5)	37,06	2.972×10^0
Thrichomys inermis	Caatinga	*Anaplasma* sp. (Cy5)	37,06	2.972×10^0

Table 5
Molecular prevalence of *Ehrlichia* spp. and *Anaplasma* spp. based on 16S rRNA cPCR assays in rodents trapped on different Brazilian biomes.

Biomes	Positive samples for *Ehrlichia* spp.	Positive samples for *Anaplasma* spp.	Total of positive samples by Biome
Caatinga	1/1[a] *Trichomys laurentius*	5/9 *Rattus rattus*	6/458 (1.3%)
Atlantic Forest	–	2/9 *Akodon montensis* 1/9 *Sphiggurus villosus* 1/9 *Calomys cerqueirae*	4/458 (0.9%)
Pantanal	1/4 *Holochilus fosteri* 2/4 *Clyomys laticeps* 1/4 *Calomys cerqueirai*	–	4/458 (0.9%)
TOTAL	5/458 (1.1%)	9/458 (2%)	14/458 (3.1%)

[a] no. positive/number tested for indicated species.

marginale detected in cattle from Philippines, Uganda and China (GenBank accession numbers JQ839009, KU686794, FJ389579, respectively). One *Anaplasma* sp. 16S rRNA sequence detected in one *Sphiggurus villosus* (GenBank accession number KY391804) captured in the state of Rio de Janeiro (Atlantic Forest biome) showed 99% identity with *Anaplasma* sp. detected in an *Amblyomma cajennense* tick collected in Poconé, state of Mato Grosso, Brazil (GenBank accession number KJ831219) and 98% identity with sequences of *Anaplasma* sp. and *Anaplasma odocoilei* detected in *Mazama gouazoubira*, in Brazil (GenBank accession number KF020580) and *Odocoileus hemionus columbianus*, in the United States (GenBank accession number KT870134), respectively.

The ML phylogenetic analysis based on 546 bp fragment of 16S rRNA gene positioned the *Anaplasma* spp. genotypes detected in specimens of rodents sampled in Atlantic forest and Caatinga biomes near *An. phagocytophium* (LC012812.1) and *An. odocoilei* (KT870139.1; DQ007352) (Fig. 2), respectively. The *Anaplasma* sp. sequence obtained from *Rattus rattus* trapped in Caatinga biome was positioned with a genotype of *Anaplasma* sp. detected in a specimen of *Hylaeamys megacephalus*, in Pantanal biome, state of Mato Grosso, Brazil, by cPCR based on 16S rRNA gene (KP757841).

The analysis on five sequenced products (358 bp) based on the 16S rRNA region (GenBank accession numbers KY391797, KY391798, KY391799, KY391800 and KY391801) showed 99% sequence identity with *E. canis* sequences (GenBank accession numbers KP844663, KF972450 and AB723708) by BLAST analysis. The ML phylogenetic inference positioned the found *Ehrlichia* spp. in the same clade of *E. canis* sequences detected in Brazil (KC109445; KP642753; EU376114; EU376115; EU376116), India (LC053451), Peru (DQ915970), Taiwan (EU106856) and strain Jake of *E. canis* (NC_007354) (Fig. 2).

4. Discussion

In the present study, a quantitative real-time multiplex qPCR assay was designed to detect *Anaplasma* and *Ehrlichia* species DNA in splenic tissues from wild and synanthropic rodents collected in Brazil. This assay showed high sensitivity (up to 1×10^1 copies of a fragment of the *groEL* gene/μL) and specificity. This approach can be used for molecular screening of *Ehrlichia* and *Anaplasma* in animal and human populations as well as arthropod vectors. Moreover, multiplex PCR assays show several advantages when compared to singleplex reactions, such as low cost, shortened time, and optimization of the reagents used on each assay by simultaneous amplification of more than two genera/species in only one reaction tube. The proposed qPCR protocol differs from those previously designed that aimed to detect Anaplasmataceae agents based on the use of species-specific primers and hydrolysis probes. For instance, previously described qPCR protocols were designed for specific detection of *An. phagocytophilum* based on the amplification of partial fragments of *ank* (Stephenson et al., 2015), *groEL* (Jahfari et al., 2012), and *msp-2* genes (Courtney et al., 2004; Drazenovich et al., 2006). Similarly, previously qPCR protocols were designed for specific amplification of *E. canis*, based on *dsb* gene (Doyle et al., 2005), and *E. chaffeensis*, based on *dsb* and *vlpt* (Doyle et al., 2005, Reller et al., 2009). Additionally, a TaqMan singleplex qPCR assay for detection of *Anaplasma* and *Ehrlichia* species based on the 16S rRNA gene, but without the possibility of distinguishing the amplified genetic material for each genus of these agents, has also been described (Kim et al., 2004).

Herein, qPCR multiplex assays showed a low occurrence of *Ehrlichia* (0.44% [2/458] and *Anaplasma* spp. [2.40% [11/458]) among sampled rodents in Brazil. These data were similar to those reported by Chastagner et al. (2017) in France, who found differences in the occurrence of *An. phagocytophilum* in specimens of *Ap. sylvaticus*, *Ap. flavicollis* and *M. glareolus*. In Germany, Obiegala et al. (2014) also found a low occurrence (1.1% [7/631]) for *An. phagocytophilum* using qPCR assays based on *msp-2* gene in *Ap. sylvaticus*, *M. glareolus* and *Ap. flavicollis* rodents. Similarly, Blanarová et al. (2014) also reported a very low occurrence (0.2% [1/606]) for *An. phagocytophilum* in rodents (*Ap. agrarius, Ap. flavicollis* and *M. glareolus*) trapped in the central region of Europe, using qPCR assays based on *msp-2* gene.

On the other hand, a high occurrence for *Anaplasma* sp./*Ehrlichia* spp. (70.5% [299/424]) was reported in rodents sampled in Korea, using qPCR assays based on 16S rRNA gene (Kim et al., 2006). In the United States of America, an occurrence of 10.6% (15/141) for *An.*

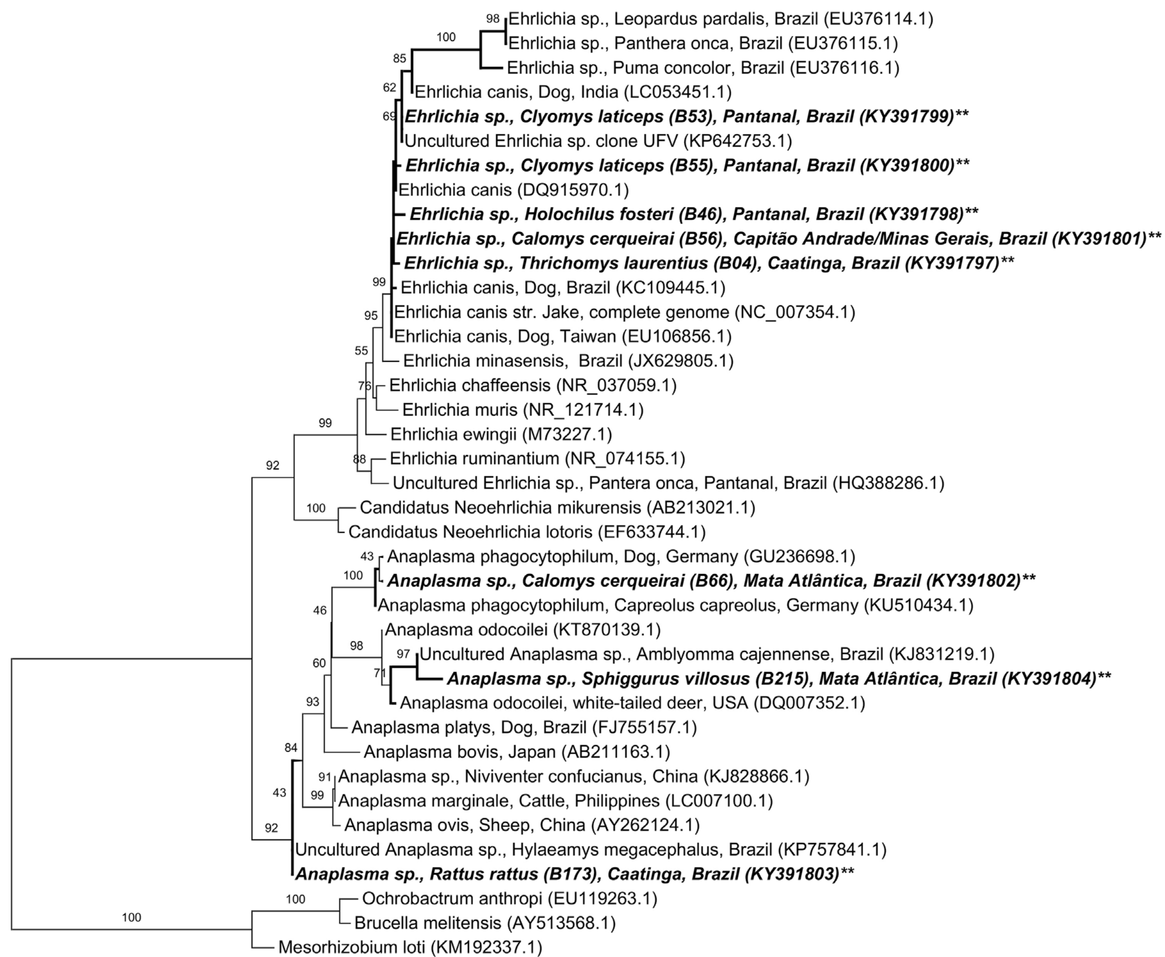

Fig. 2. Phylogenetic positioning of *Anaplasma* spp. and *Ehrlichia* spp. 16S rRNA gene sequences based on Maximum Likelihood (ML).The tree was drawn using the Maximum Likelihood (ML) estimation method and the TIM2 + I + R model. The numbers in the tree indicate the bootstrap values above 50 for the branches. Accession numbers are shown next to the sequences. 16S rRNA partial sequences of *Ochrobactrum anthropi* (EU119263.1), *Brucella melitensis* (AY513568.1) and *Mesorhizobium loti* (KM192337) were used as outgroups.*In bold: sequences of the present study.

phagocytophilum was reported in *Tamias ochrogenys* rodents sampled in California (Foley and Nieto, 2011). Additionally, a similar occurrence of *An. phagocytophilum* was found in spleen samples collected from *Neotoma fuscipes* [11.8% (4/42)] and *Tamias ochrogenys* [8.9% (6/81)] in California by qPCR assays based on *msp-2* gene (Foley et al., 2011*)*. While *Ixodes* and *Haemaphysalis* ticks are considered vectors for *An. phagocytophilum* in the USA *(*Rizzoli et al., 2014*) and Korea* (Kang et al., 2016), respectively, the arthropod vectors involved in Anaplasma and Ehrlichia species transmission cycles among rodents are still unknown in Brazil. Nevertheless, nymphs of *Amblyomma dubitatum* and *Ixodes loricatus* and larvae of *Amblyomma* spp. and *Ixodes* spp. ticks were found parasitizing small mammals in peri-urban areas of Uberlandia, state of Minas Gerais, southeastern Brazil (Coelho et al., 2016). Additionally, *A. cajennense* and *A. triste* nymphs were collected on specimens of *Holochilus sciureus* sampled in Pantanal Biome, state of Mato Grosso (Wolf et al., 2016). The organization of ectoparasite communities found in rodents is host species-dependent, mainly for mites and lice rather than ticks (Sponchiado et al., 2016). The lack of tick specificity to rodents may favor the transmission of *Ehrlichia* and *Anaplasma* among several rodent species. In Brazil, *Anaplasma* 16S rRNA gene fragment was detected in a specimen of *Amblyomma triste* tick collected from a *Holochilus sciureus* rodent in the Pantanal, state of Mato Grosso (Wolf et al., 2016).

The phylogenetic analyses based on a small fragment of 16S *rRNA* gene positioned the *Anaplasma* genotypes detected in *C. cerqueirai* near to *An. phagocytophilum* sequences. On the other hand, *Anaplasma* genotypes detected in *S. villosus* and *R. rattus* were closely related to *An. odocoilei* and *An. bovis*, respectively, pathogens found in wild and domestic ruminants. *Anaplasma* genotypes closely related to *An. phagocytophilum* have already been detected in wild carnivores (André et al., 2012), deer (Silveira et al., 2012) and birds (Machado et al., 2012), as well as in domestic cats (André et al., 2014) in Brazil. However, the positive samples by qPCR e cPCR assays based on *groEL* and 16S *rRNA* genes, respectively, were negative by a qPCR specific to *An. phagocytophilum* based on the *msp-2* gene. These findings indicate a possible circulation of new *Anaplasma* genotypes in wild animals in Brazil, whose zoonotic and pathogenic potential is still unknown. *Anaplasma phagocytophilum* has already been detected in dogs and deer in Brazil, based on PCR assays targeting *msp-2* and *msp-4* genes, respectively (Santos et al., 2013; Silveira et al., 2014a,b, 2015). According to the low occurrence of *Anaplasma* among sampled rodents, it seems that this group of mammals might not play an important role in the epidemiology of *Anaplasma* species in Brazil. On the other hand, *Neotoma fuscipes* and *Peromyscus* spp. rodent species are recognized as important sources of infections for the HZ, MRK and Dog_CA strains of *An. phagocytophilum* in the USA (Nieto et al., 2010).

The phylogenetic inferences based on a small fragment of 16S *rRNA* gene fragment positioned the *Ehrlichia* genotypes detected in *Clyomys laticeps*, *Holochilus fosteri*, *Clyomys laticeps* and *C. cerqueirai* rodents in the same clade of *E. canis*. *Ehrlichia* genotypes closely related to *E. canis* have already been detected in wild carnivores (André et al., 2012), wild birds (Machado et al., 2012) and domestic cats (André et al., 2015) in Brazil, and rodents in Korea (Kim et al., 2006). In addition, *Ehrlichia minasensis,* a species closely related to *Ehrlichia canis* that has been

identified infecting *Rhipicephalus microplus* ticks and cattle in Brazil (Carvalho et al., 2016; Cabezas-Cruz et al., 2016), also showed phylogenetic relatedness with the sequences obtained in the present study, but lower when compared to *Ehrlichia canis*. Following the same pattern observed with *Anaplasma* genotypes detected in the present study, the positive samples at cPCR and qPCR assays for *Ehrlichia* spp. based on *groEL* and 16S rRNA genes, respectively, were negative at specific qPCR assays for *E. canis* and *E. chaffeensis* based on *dsb* and *vlpt* genes, respectively, suggesting once again the possible circulation of *Ehrlichia* genotypes in rodents not yet isolated.

The negative results observed in PCR assays based on 16S rRNA gene for samples that were previously positive at qPCR assays based on *groEL* gene could be explained considering the low bacterial loads in rodents tissue samples, precluding amplification by conventional PCR assays, given the lower sensitivity of cPCR assays when compared to qPCR assays. These findings were also reported by Chastagner et al. (2017), who submitted rodent spleen DNA samples to qPCR and cPCR for *Ehrlichia* and *Anaplasma* species. Since the amplification of other genes (*dsb, groEL, omp-1, msp-4*) fragments was not achieved in the present study, despite several attempts, an accurate phylogenetic positioning of the found *Ehrlichia* and *Anaplasma* genotypes was not possible. Similarly, although Wolf et al. (2016) reported the occurrence of an *Anaplasma* genotype in a specimen of *Hylaeamys megacephalus* by conventional PCR targeting 16S rRNA gene, an accurate phylogenetic positioning of the found sequence was not performed. Furthermore, five samples were positive for the amplification of the 16S rRNA gene of *Ehrlichia* spp. and *Anaplasma* spp., but negative at multiplex qPCR assays, once again indicating the occurrence of different *Anaplasma* and *Ehrlichia* genotypes circulating in rodents in Brazil. Since the primers and the hydrolysis probes used in the qPCR assays were designed based on sequences of the heat shock protein encoding gene of six known *Anaplasma* species and four *Ehrlichia* species, we believe that the used oligonucleotides could not hybridize into new genotypes DNA fragments. On the other hand, since oligonucleotides used in cPCR assays target 16S rRNA, a more conserved gene compared to *groEL*, a successful amplification was achieved. Considering the fact that ehrlichiosis and anaplasmosis in humans and animals are mainly treated with doxycycline and tetracyclines (Bowman, 2011), a sensitive qPCR assay, even generating negative results in conventional PCR protocols, would contributed to a fast and reliable diagnosis of these diseases, aiming at achieving a quick and accurate treatment. On the other hand, from an epidemiological point of view, a sensitive qPCR assay would be useful in screening ticks, human and animal populations for the main already described *Ehrlichia* and *Anaplasma* species, avoiding running amount of samples to several protocols. Unfortunately, '*Candidatus* Neoehrlichia mikurensis' *groEL* sequences were not used in the alignment for primers and probes design in the newly presented PCR protocol, representing a limitation of this technique for screening rodent populations around the world. This agent has been molecularly detected in rodents and ticks from Europe and Asia (Tabara et al., 2007; Naitou et al., 2006; Rar et al., 2010; Jahfari et al., 2012; Silaghi et al., 2015, Svitálková et al., 2016).

In a nutshell, the present work presented the standardization of a newly designed quantitative 5′ nuclease real-time PCR for *Ehrlichia* and *Anaplasma* species detection based on *groEL* gene, which showed high specificity and sensitivity. Additionally, the present study showed a low occurrence of *Anaplasma* and *Ehrlichia* in rodents in Brazil.

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