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Association of *Bartonella* Species with Wild and Synanthropic Rodents in Different Brazilian Biomes

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ABSTRACT

***Bartonella* spp. comprise an ecologically successful group of microorganisms that infect erythrocytes and have adapted to different hosts, which include a wide range of mammals, besides humans. Rodents are reservoirs of about two-thirds of *Bartonella* spp. described to date; and some of them have been implicated as causative agents of human diseases. In our study, we performed molecular and phylogenetic analyses of *Bartonella* spp. infecting wild rodents from five different Brazilian biomes. In order to characterize the genetic diversity of *Bartonella* spp., we performed a robust analysis based on three target genes, followed by sequencing, Bayesian inference, and maximum likelihood analysis. *Bartonella* spp. were detected in 25.6% (117/457) of rodent spleen samples analyzed, and this occurrence varied among different biomes. The diversity analysis of *gltA* sequences showed the presence of 15 different haplotypes. Analysis of the phylogenetic relationship of *gltA* sequences performed by Bayesian inference and maximum likelihood showed that the *Bartonella* species detected in rodents from Brazil was closely related to the phylogenetic group A detected in other cricetid rodents from North America, probably constituting only one species. Last, the *Bartonella* species genogroup identified in the present study formed a monophyletic group that included *Bartonella* samples from seven different rodent species distributed in three distinct biomes. In conclusion, our study showed that the occurrence of *Bartonella* bacteria in rodents is much more frequent and widespread than previously recognized.**

IMPORTANCE

In the present study, we reported the occurrence of *Bartonella* spp. in some sites in Brazil. The identification and understanding of the distribution of this important group of bacteria may allow the Brazilian authorities to recognize potential regions with the risk of transmission of these pathogens among wild and domestic animals and humans. In addition, our study accessed important gaps in the biology of this group of bacteria in Brazil, such as its low host specificity, high genetic diversity, and relationship with other *Bartonella* spp. detected in rodents trapped in America. Considering the diversity of newly discovered *Bartonella* species and the great ecological plasticity of these bacteria, new studies with the aim of revealing the biological aspects unknown until now are needed and must be performed around the world. In this context, the impact of *Bartonella* spp. associated with rodents in human health should be assessed in future studies.

Bartonella spp. are emerging and reemerging Gram-negative facultative intracellular *Alphaproteobacteria* belonging to the order *Rhizobiales*, family *Bartonellaceae*, that infect erythrocyte and endothelial cells from a wide range of animal species, including humans (1, 2). In the early 1990s, no more than five *Bartonella* spp. were known; however, in the last few decades, the number of species in this genus has been significantly expanded. Considering the increasing number of *Bartonella* spp. described, the criteria used for separation of new genotypes of previously described species remain a big issue (3, 4). Because of the high diversity and ecological plasticity displayed by this bacterial group, new approaches have been proposed to help in solving some conflicts between the definitions of new species (3, 4).

Currently, the *Bartonella* genus contains 33 species, including 20 that have been detected in several rodent species (5). Among these, *B. elizabethae* (6), *B. grahamii* (7), *B. tribocorum* (8), *Bartonella vinsonii* subsp. *arupensis* (9), *B. washoensis* (10), *B. rochalimae* (11), and *B. doshiae* (12) have been implicated in human illnesses.

Rodents represent an important group of potential reservoirs of many *Bartonella* spp. that have been reported worldwide. *Bartonella* infection in rodents usually results in a persistent and subclinical bacteremia lasting for months, characterizing the infected

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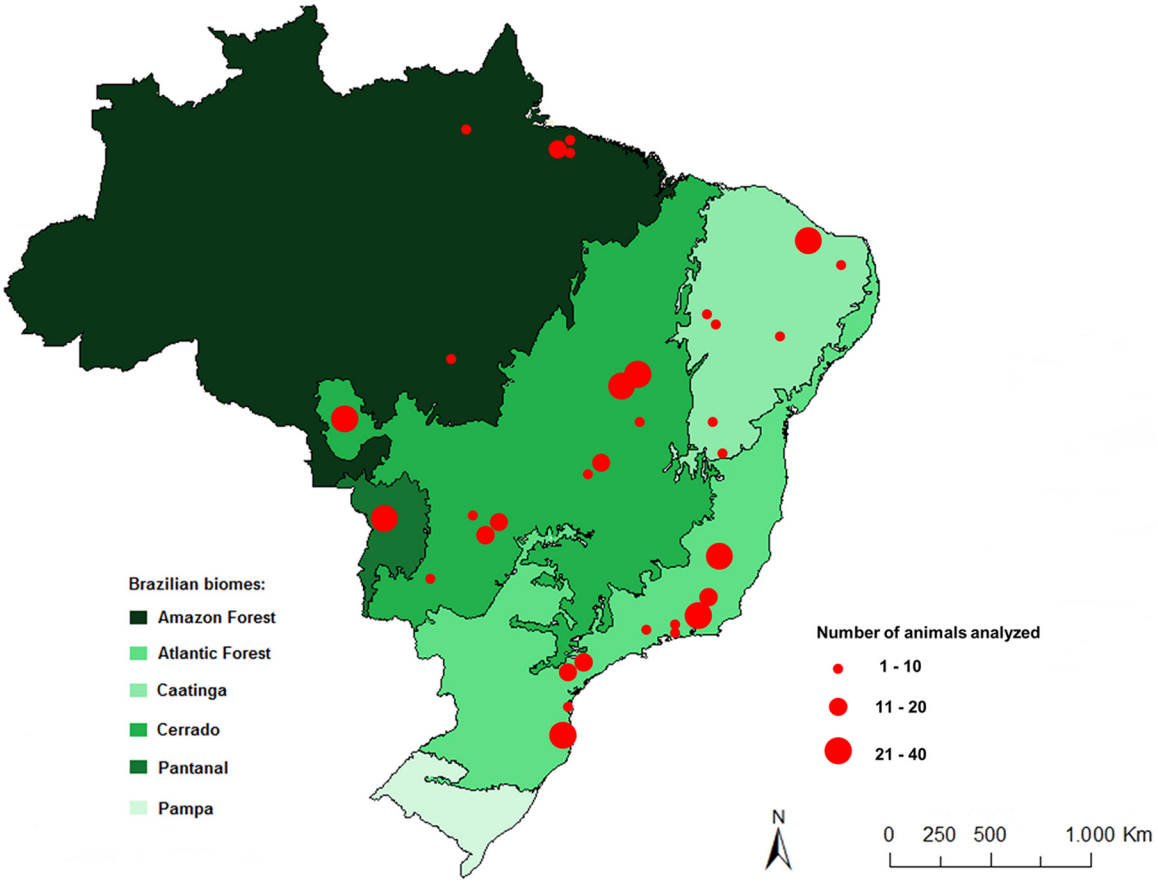

FIG 1 Distribution of rodents sampled in different Brazilian biomes.

rodents as important sources of infection to other susceptible hosts, including humans (13, 14). Some studies have reported a high prevalence of infection in rodent communities, which can reach up to 82% (15). Additionally, factors such the rodent's habitat, behavior, age, specificity, flea abundance, and seasonality represent crucial variables that may influence the establishment of *Bartonella* infections (13).

Recent studies have revealed a complex interaction between *Bartonella* spp. and rodent species with a high degree of genetic diversity, coinfection, horizontal gene acquisitions, recombination events, and interactions with other vector-borne microbes within each microenvironment (arthropod vectors and/or blood from vertebrate hosts) (16–19). However, limited information about the epidemiological, genetic diversity, distribution, and ecological aspects of *Bartonella* infections in Brazil is available as yet.

To the best of our knowledge, until now only two studies evaluating the occurrence of *Bartonella* spp. have been conducted in Brazil: one in synanthropic rodents (*Rattus norvegicus*) in the state of Bahia (northeastern Brazil) and one in wild rodents in the state of Mato Grosso do Sul (midwestern Brazil). These studies reported the first isolation of *B. queenslandensis* (5/26) and *B. tribocorum* (1/26) in synanthropic rodents (20) and the first detection of *Bartonella vinsonii* subsp. *arupensis* in wild rodents (21). Therefore, more studies are needed to assess the genetic diversity of *Bartonella* spp. within rodent communities in Brazil.

The majority of human infections by *Bartonella* spp. in Brazil is caused by *B. henselae* and *B. quintana* (22, 23). Although *Bartonella* spp. associated with rodents have never been detected in humans from Brazil until now, the high number of *Bartonella* spp. associated with rodents coupled with the fact that Rodentia are widely distributed in different habitats and represent the largest order of mammals (24) emphasizes the need for further studies to assess the pathogenicity of rodent-associated *Bartonella* spp. in humans.

The present study aimed to (i) investigate the occurrence of *Bartonella* infection in wild rodents from five different Brazilian biomes by quantitative PCR (qPCR), (ii) quantify the number of DNA copies of *Bartonella* spp. detected in the infected rodents, (iii) identify and characterize the *Bartonella* spp. infecting wild and synanthropic rodents using *gltA*, *ftsZ*, and *groEL* genes, and (iv) analyze the haplotype diversity of *Bartonella* sequences detected.

MATERIALS AND METHODS

Distribution and rodent species sampled. Between 2000 and 2011, different rodent species ($n = 52$) were trapped in five Brazilian biomes (Fig. 1) (http://www.mma.gov.br/biomas). Animals were caught using Tomahawk and Sherman live traps during previous studies performed by the Laboratory of Trypanosomatid Biology, Laboratory of Biology and Parasitology of Wild Reservoirs Mammals, and the Laboratory of Hantaviruses and Rickettsioses, Oswaldo Cruz Institute, Rio de Janeiro, Brazil (25–27). Euthanasia of animals was performed for taxonomic identification and/or diagnosis of parasites. Rodents were chemically immobilized using a combination of ketamine hydrochloride (100 mg/ml) and

acepromazine (10 mg/ml) intramuscularly. When the death of anesthetized animal did not occur after exsanguination, then euthanasia was performed through intracardiac injection of 19.1% potassium chloride (2 ml/kg).

Spleen tissues from 500 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 the Environment and the Natural Renewable Resources (IBAMA) (IBAMA/CGFAU/LIC 3665-1) and the Oswaldo Cruz Foundation (FIOCRUZ) ethics committee (P0007-99, P0179-03, P0292/06, L0015-07).

DNA extraction. DNA was extracted from 10 mg of each rodent spleen tissue using the DNeasy blood and tissue kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. The DNA concentration and absorbance ratio (260/280 nm) were measured using a NanoDrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). Microtubes containing ultrapure sterile water were intercalated between each series of 20 rodent spleen samples and underwent DNA extraction.

Evaluation of DNA extraction quality. In order to evaluate the quality of DNA samples, each sample of spleen-extracted DNA was used as a template in an internal control PCR targeting the mammalian IRBP (interphotoreceptor retinoid binding protein) gene (28). Three microliters of DNA was used as a template in 25-μl reaction mixtures containing 10× PCR buffer, 1.0 mM $MgCl_2$, 0.6 mM deoxynucleotide triphosphate (dNTPs) mixture, 1.5 U of *Taq* DNA polymerase (Life Technologies), 1.25 μl of dimethyl sulfoxide (DMSO) (Sigma-Aldrich), and 0.5 μM IRBPfwd (5′-TCCAACACCACCACTGAGATCTGGAC-3′) and IRBPrev (5′-GTG AGGAAGAAATCGGACTGGCC-3′) primers. PCR amplifications were performed at 94°C for 4 min followed by 35 repetitive cycles of 94°C for 30 s, 57°C for 1 min, and 72°C for 1 min, followed by a final extension at 72°C for 5 min. Samples negative for the IRBP gene by PCR were subsequently submitted to another internal control PCR targeting the GAPDH gene (29), using GAPDH-F (5′-CCTTCATTGACCTCAACTACAT-3′) and GAPDH-R (5′-CCAAAGTTGTCATGGATGACC-3′) primers and the same concentration of reagents described for IRBP PCR (except DMSO). PCR amplifications were performed at 94°C for 5 min followed by 35 repetitive cycles of 94°C for 30 s, 50°C for 30 s, and 72°C for 1 min, followed by a final extension at 72°C for 5 min. Samples positive for the above-described internal control PCR protocols were subsequently submitted to qPCR assays targeting a fragment of the *nuoG* gene of *Bartonella* spp.

Screening and quantification of *Bartonella* species DNA (qPCR assay). A previously described broad-range qPCR protocol based on the *nuoG* gene was used with the aim of detecting and quantifying *Bartonella* species DNA copies (number of copies per microliter) (30). The TaqMan qPCRs were performed with a final volume of 10 μl containing 5 μl of GoTaq probe qPCR master mix (Promega Corporation, Madison, WI, USA), 1.2 μM each primer, F-Bart (5′-CAATCTTCTTTTGCTTC ACC-3′) and R-Bart (5′-TCAGGGCTTTATGTGAATAC-3′), and hydrolysis probe TexasRed-5′-TTYGTCATTTGAACACG-3′[BHQ2a-Q]-3′, and 1 μl of each DNA sample. The amplification conditions were 95°C for 3 min followed by 40 cycles at 95°C for 10 s and 52.8°C for 30 s (30). PCR amplifications were conducted in low-profile multiplate unskirted PCR plates (Bio-Rad, CA, USA) using a CFX96 thermal cycler (Bio-Rad, CA, USA).

Serial dilutions were performed with the aim of constructing standard curves with different concentrations of plasmid DNA (pIDTSmart; Integrated DNA Technologies) (2.0×10^7 to 2.0×10^0 copies/μl), which encoded a 83-bp *Bartonella henselae nuoG* gene fragment (30). The number of plasmid copies was determined in accordance with the formula (x grams per microliter of DNA/[plasmid size (base pairs) × 660]) × 6.022×10^{23} × plasmid copies per microliter. Each qPCR assay was performed, including duplicates of each rodent DNA sample. All of the duplicate samples with quantification cycle (C_q) values higher than 0.5 of difference were tested again. Amplification efficiency (E) was calculated from the slope of the standard curve in each run using the formula $E = 10^{-1/\text{slope}}$. To determine the limit of detection from the qPCR assay, the standard curves generated by 10-fold dilutions were used to determine the amount of DNA that could be detected with 95% sensitivity (31).

Molecular characterization of *Bartonella* spp. All positive qPCR samples from rodents were submitted to the previously described conventional PCR (cPCR) assays for three other protein-coding genes, namely, *gltA* (350 bp), *ftsZ* (515 bp), and *groEL* (752 bp) genes (1, 17, 32, 33). *Bartonella henselae* DNA obtained from a naturally infected cat was used as a positive control (30). All PCR products that showed high intensity of bands with expected sizes were purified using a silica bead DNA gel extraction kit (Fermentas, São Paulo, SP, Brazil). Purified amplified DNA fragments from positive samples were submitted to sequence confirmation in an automatic sequencer (ABI Prism 310 genetic analyzer; Applied Biosystems/PerkinElmer) in both directions. The electropherogram quality was initially analyzed in the FinchTV 1.4.0. program (http://www.geospiza.com/ftvdlinfo.html). Only sequences with expected sizes and without background in the electropherograms were submitted to BLASTn, phylogenetic, and diversity analyses. The consensus sequences were obtained through the analysis of the sense and antisense sequences using the CAP3 program (http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py) (34). Last, in order to correctly determine the nucleotide composition, the electropherograms were also submitted to the Phred-Phrap program (35) and compared through alignment in BioEdit. The Phred quality score (peaks around each base call) was established at >20 (99% in accuracy of the base call). Subsequently, the sequences were submitted to phylogenetic analyses.

Phylogenetic analyses. The sequences obtained from *gltA*, *ftsZ*, and *groEL* cPCR assays were identified by BLASTn (nucleotide BLAST 2.4.0 between 2 April 2016 to 15 April 2016) using megaBLAST (with default parameters) and, *a posteriori*, a multiple sequence alignment with sequences available in GenBank was performed using Clustal/W v. 7.0.5.3 (using default parameters) (36) and manually adjusted in BioEdit (v. 7.0.5.3) (37). Phylogenetic analysis based on Bayesian inference (BI) was done using MrBayes on XSEDE (v. 3.2.6) (the *a posteriori* probability values higher than 50% were accessed with 10^7 replicates; the first 25% trees were discarded as burn-in) (38). 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 1,000 replicates) (39); both analyses were performed through the CIPRES Science Gateway (40). Additionally, we performed a concatenated phylogenetic analysis with the sequences which were simultaneously positive in PCR assays based on *gltA*, *ftsZ*, and *groEL* genes. The Akaike information criterion (AIC) available in MEGA 5.05 (41) was applied to identify the most appropriate model of nucleotide substitution. The most appropriate models of nucleotide substitution (lower AIC value) were T92+G+I, GTR+G+I, TN93+G+I, and GTR+G+I for the *gltA*, *ftsZ*, *groEL*, and concatenated phylogenetic analyses, respectively.

***Bartonella* genetic diversity and distribution analysis.** The alignment sequences of the *gltA*, *ftsZ*, and *groEL* genes amplified in the present study were utilized to calculate the nucleotide diversity (π), the polymorphic level (haplotype diversity [Hd]), the number of variable sites (v), and the average number of nucleotide differences (K) using DnaSP (v. 5.10) (using default parameters) (42). The sequences of the *gltA* gene were submitted to a median-joining network (43) inferred using the Population Analysis with Reticulate Trees (popART) (v. 1.7) (using default parameters) (44).

Accession number(s). The sequences of *Bartonella* generated in this study were deposited in the GenBank database (https://www.ncbi.nlm.nih.gov/genbank/) under accession numbers KX086714 to KX086733 for *gltA*, KX036231 to KX036245 for *ftsZ*, and KX086734 to KX086737 for *groEL*.

RESULTS

Quality of DNA samples and qPCR assay. Out of the 500 rodent spleen samples analyzed, 457 were positive for the internal control (IRBP gene) PCR (Table 1). All samples negative for the IRBP gene

TABLE 1 Numbers and distribution of rodent species positive for *Bartonella* spp. in qPCR

Biome/state	Species	No. of rodents positive for the internal control (IRBP gene)	No. of rodents positive for *Bartonella* spp. (qPCR *nuoG* gene)
Atlantic forest			
Rio de Janeiro	*Akodon* sp.	27	16
	Necromys lasiurus	4	3
	Brucepattersonius sp.	1	
	Delomys dorsalis	9	4
	Euryoryzomys russatus	2	
	Oligoryzomys nigripes	4	
	Oligoryzomys sp.	1	
	Oxymycterus dasythricus	3	3
	Oxymycterus sp.	3	3
	Trinomys dimidiatus	1	
	Trinomys iheringi	3	
	Sciurus sp.	1	
	Sphiggurus sp.	1	
	Sphiggurus villosus	1	
Minas Gerais	*Nectomys squamipes*	4	2
	Thrichomys apereoides	10	1
	Oligoryzomys nigripes	7	
	Calomys cerqueirai	3	1
São Paulo	*Oligoryzomys* sp.	4	
	Oligoryzomys nigripes	11	4
	Oligoryzomys flavescens	3	2
	Akodon montensis	11	4
	Calomys tener	10	
	Mus musculus	2	
Santa Catarina	*Akodon montensis*	5	1
	Mus musculus	1	
	Euryoryzomys russatus	10	6
	Sooretamys angouya	2	1
	Mus musculus	1	
Amazon forest			
Pará	*Rattus rattus*	8	
	Mus musculus	1	
	Proechimys gouldii	7	
	Proechimys roberti	2	
	Proechimys gr. cuvieri	2	
	Proechimys sp.	3	
	Zygodontomys sp.	2	
Mato Grosso	*Rattus rattus*	2	
	Proechimys sp.	3	
	Necromys lasiurus	1	1
Caatinga			
Bahia	*Necromys lasiurus*	1	
	Mus musculus	1	
	Thrichomys inermis	7	
	Thrichomys laurentius	2	
	Thrichomys sp.	3	
	Galea spixii	8	
Ceará	*Galea spixii*	12	
	Mus musculus	7	
	Rattus rattus	12	
	Thrichomys laurentius	8	1
Piauí	*Rhipidomys macrurus*	3	2
	Thrichomys laurentius	7	1
Rio Grande do Norte	*Cavia* sp.	1	
	Thrichomys laurentius	3	1
Cerrado			
Mato Grosso	*Rattus rattus*	3	1
	Necromys lasiurus	15	5

(Continued on following page)

TABLE 1 (Continued)

Biome/state	Species	No. of rodents positive for the internal control (IRBP gene)	No. of rodents positive for *Bartonella* spp. (qPCR *nuoG* gene)
	Calomys sp.	16	
Mato Grosso do Sul	*Calomys expulsus*	1	
	Necromys lasiurus	6	3
	Nectomys rattus	3	1
	Nectomys sp.	3	2
	Nectomys squamipes	2	2
Goiás	*Necromys lasiurus*	15	5
	Cerradomys sp.	3	
	Hylaeamys megacephalus	7	
	Nectomys rattus	5	5
	Nectomys sp.	2	2
	Oecomys gr. bicolor	4	
	Oryzomys megacephalus	2	1
	Rattus rattus	4	1
	Rhipidomys sp.	10	5
	Oligoryzomys nigripes	2	
	Oligoryzomys fornesi	2	
Tocantins	*Calomys* sp.	27	
	Hylaeamys sp.	9	6
	Necromys lasiurus	9	5
	Rhipidomys sp.	5	3
	Mus musculus	1	
	Juliomys sp.	1	1
	Oligoryzomys sp.	6	
	Cerradomys sp.	3	
	Oecomys sp.	6	1
	Oryzomys sp.	2	
Pantanal			
Mato Grosso do Sul	*Clyomys laticeps*	8	
	Holochilus sp.	6	4
	Thrichomys fosteri	18	7
Total (no. [%])		457	117 (25.6)

PCR were also negative for the GAPDH gene PCR and were excluded from subsequent analyses. The concentration mean and absorbance ratio (260/280 nm) from the DNA spleen samples extracted were 221.6 ng/μl (ranging from 29.4 to 511.7 ng/μl; standard deviation [SD], ±130.6) and 2.1 (ranging from 1.78 to 2.25; SD, ±0.15), respectively. The efficiency mean of qPCR assays was $E = 96.6\%$ (90.8% to 101%; SD, ±2.78), slope = −3.407, and $r^2 = 0.994$. The limit of detection was 10 copies of plasmid/μl with 95% sensitivity. However, quantitative data of 11 DNA rodent samples showed numbers of copies lower than 10 copies/μl. Fifteen DNA samples positive for *Bartonella* spp. showed inconsistency in quantitative results (replicates with C_q values of >0.5), probably due to a low bacterial load in the tissue. Therefore, quantitative data from these samples were not used in the mean of quantification.

Occurrence of the *Bartonella* spp. in wild rodents. Among all DNA rodent spleen samples analyzed, 25.6% (117/457) were positive for *Bartonella* spp. by qPCR. Eight-one (69.2%), 56 (47.8%), and 35 (29.9%) samples were positive in *ftsZ*, *gltA*, and *groEL* cPCR assays, respectively. Among them, only 25 samples were simultaneously positive in *gltA*, *ftsZ*, and *groEL* cPCR assays. Additionally, among the 52 different rodent species analyzed in the present study, *Bartonella* DNA was detected in 25 rodent species distributed in 17 different genera trapped in five Brazilian biomes (Table 1). Although it may not reflect the natural abundance, a higher occurrence of *Bartonella* was observed in rodents sampled in Atlantic forest (35.9% [52/145]). On the other hand, a lower occurrence of *Bartonella* was observed in rodents caught in Amazon forest (3.2% [1/31]). The occurrence of *Bartonella* spp. among rodents trapped in different biomes and the mean of absolute quantification of *nuoG*-positive *Bartonella* DNA in rodents are shown in Table 2.

Molecular identification and phylogenetic analysis. To identify the *Bartonella* genotypes circulating in rodents from different Brazilian biomes, partial sequences of *gltA* (20), *ftsZ* (15), and *groEL* (4) genes were compared with other sequences previously deposited in GenBank by BLASTn and submitted to BI and ML analysis.

Except for *gltA* sequences, all amplified sequences (*ftsZ* and *groEL* genes) showed a low percentage of identity (ranging from 91 to 93%) with other *Bartonella* spp., mainly with the *B. vinsonii* species complex. Eighteen amplified *gltA* sequences shared percentages of identity ranging from 96 to 98% with *Bartonella* spp. detected in wild rodents (*Sigmodon hispidus* and *Oryzomys palus-*

TABLE 2 Occurrence and absolute quantification of *Bartonella* spp. DNA among rodents trapped in five distinct Brazilian biomes

Biome	Occurrence of *Bartonella* spp. (% [no.])	Absolute quantification (*nuoG* copies/μl)[a]
Amazon forest	3.2 (1/31)	0.1×10^2
Atlantic forest	35.9 (52/145)	38×10^2
Caatinga	6.7 (5/75)	7.5×10^2
Cerrado	27.6 (48/174)	120×10^2
Pantanal	34.4 (11/32)	8.0×10^2
Total	25.6 (117/457)	34.7×10^2

[a] Mean of absolute quantification.

tris) and fleas (*Polygenis gwyni*) from the United States and previously named phylogenetic group A (45) (92.6% identity with *B. vinsonii*), a frequently found but not very well molecularly characterized *Bartonella* genogroup isolated from *S. hispidus* in the southeastern United States. Two other *gltA* sequences (7,554 and 11,790), both detected in *Necromys lasiurus* from the Cerrado biome, were closely related (sharing 98 and 97% of identity, respectively) to *Bartonella* species strain R-phy1 detected in wild rodents (*Phyllotis* sp.) from Peru (1). All sequences amplified in the present study showed query coverages ranging from 92 to 100%.

The phylogenetic analyses performed using different methods (BI and ML) yielded similar tree topologies and the same relationships for all genes analyzed in this study. The Bayesian tree was

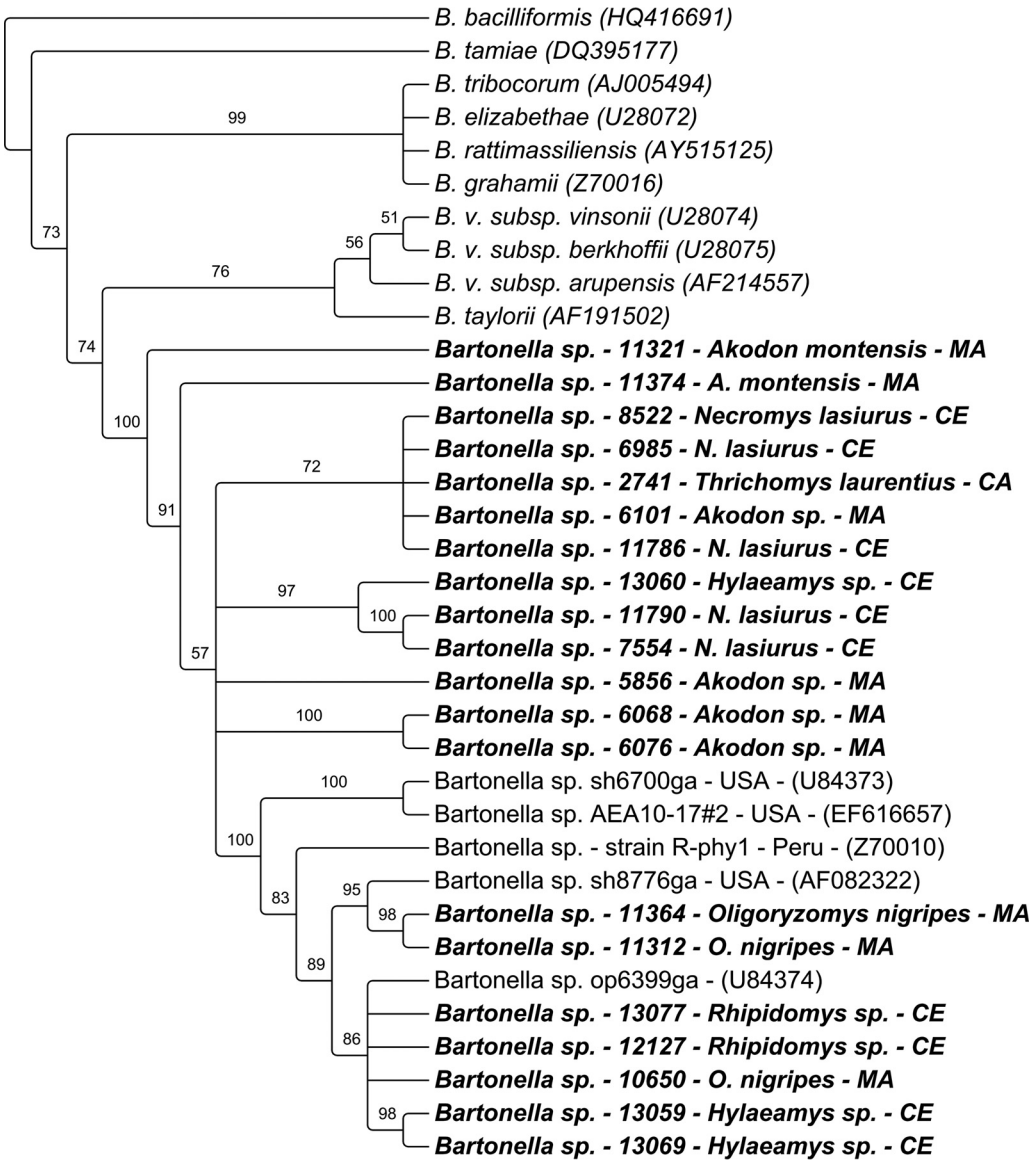

FIG 2 Phylogenetic relationships within the *Bartonella* genus based on the *gltA* gene. The tree was inferred by using the Bayesian inference (BI) with the T92+G+I model. The sequences detected in the present study are highlighted in bold. The numbers at the nodes correspond to *a posteriori* probability values higher than 50% accessed with 10^7 replicates. *Bartonella bacilliformis* was used as an outgroup. MA, Mata Atlântica biome; CE, Cerrado biome; CA, Caatinga biome.

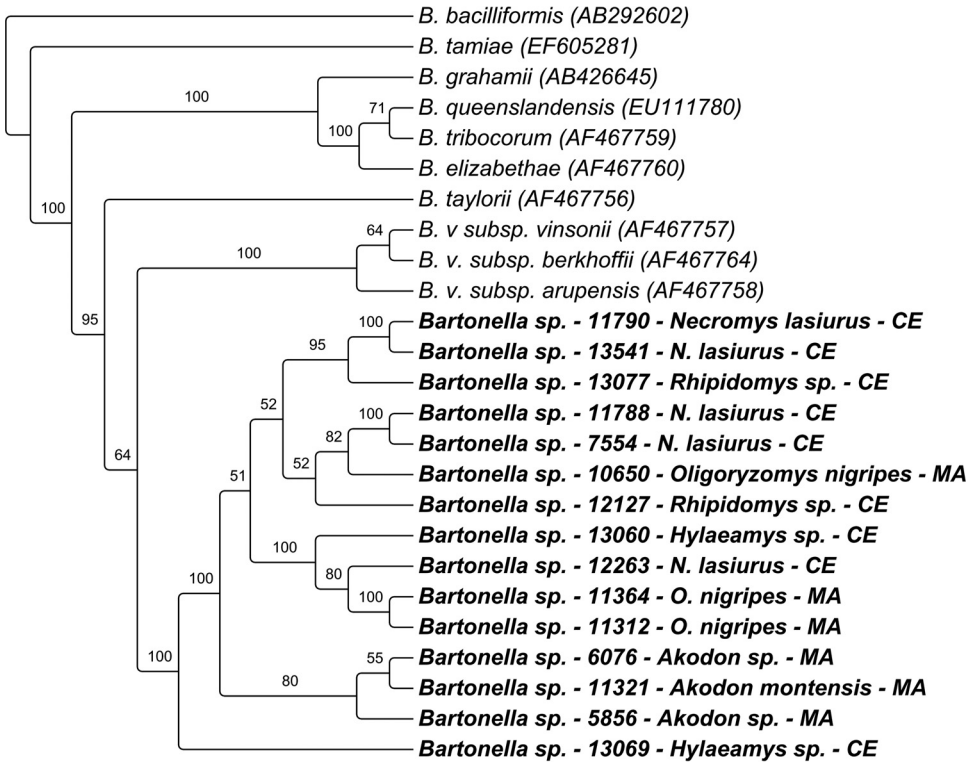

FIG 3 Phylogenetic relationships within the *Bartonella* genus based on the *ftsZ* gene. The tree was inferred by using the Bayesian inference (BI) with the GTR+G+I model. The sequences detected in the present study are highlighted in bold. The numbers at the nodes correspond to *a posteriori* probability values higher than 50% accessed with 10^7 replicates. *Bartonella bacilliformis* was used as an outgroup. MA: Mata Atlântica; CE: Cerrado biomes.

chosen to represent the phylogenetic relationships among the *gltA* sequences (Fig. 2). According to BLASTn analysis, the *gltA* sequences amplified in the present study were positioned nearest to phylogenetic group A and branched more closely to *B. taylorii* and *B. vinsonii* complex. The tree inferred by BI as *ftsZ* sequences (Fig. 3) and by ML as *groEL* sequences (Fig. 4), compared with other previously described *Bartonella* sequences, showed a clear separation in both genes analyzed, constituting a monophyletic group, clustering closely to the *Bartonella vinsonii* complex. Additionally, the concatenated phylogenetic tree (*gltA*, *ftsZ*, and *groEL* genes) generated by the maximum likelihood method using *B. vinsonii* and *B. elizabethae* species complexes, *B. grahamii*, *B. taylorii* and *B.*

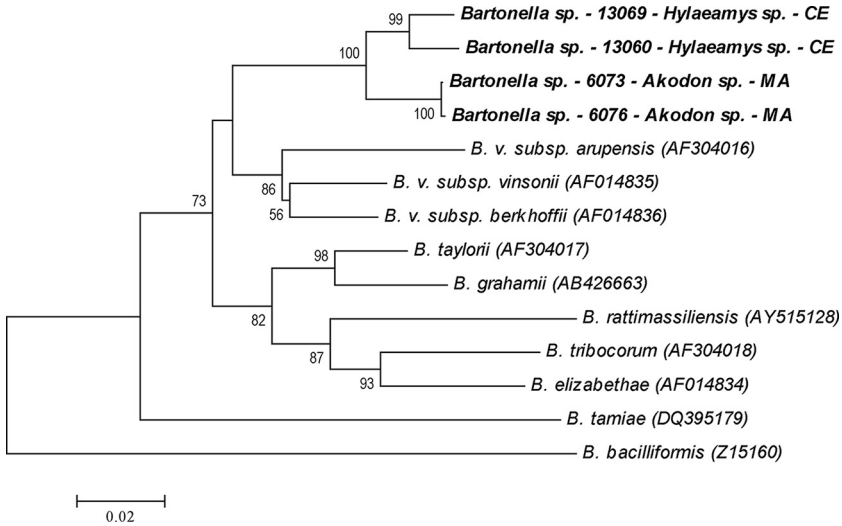

FIG 4 Phylogenetic relationships within the *Bartonella* genus based on the *groEL* gene. The tree was inferred by using the maximum likelihood (ML) method with the TN93+G+I model. The sequences detected in the present study are highlighted in bold. The numbers at the nodes correspond to bootstrap values higher than 50% accessed with 1,000 replicates. *Bartonella bacilliformis* was used as an outgroup. MA, Mata Atlântica biome; CE, Cerrado biome.

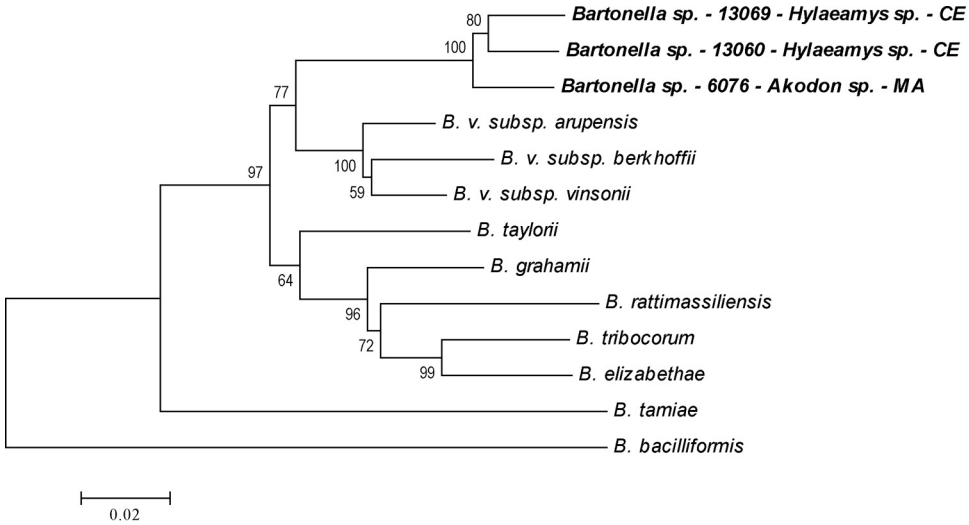

FIG 5 Phylogenetic relationships within the *Bartonella* genus based on a concatenated tree (*gltA*, *ftsZ*, and *groEL* genes). The tree was inferred by using the maximum likelihood (ML) method with the GTR+G+I model. The sequences detected in the present study are highlighted in bold. The numbers at the nodes correspond to bootstrap values higher than 50% accessed with 10^7 replicates. *Bartonella bacilliformis* was used as an outgroup. MA, Mata Atlântica biome; CE, Cerrado biome.

tamiae species, and three sequences amplified in our study showed that the *Bartonella* detected in rodents trapped in distinct Brazilian biomes are more closely related to the *B. vinsonii* species complex (Fig. 5). These findings revealed that *Bartonella* detected in the present study are a monophyletic and widespread group among seven different rodent species trapped in three distinct Brazilian biomes. The other phylogenetic trees are available in Fig. S1 to S4 in the supplemental material.

Among the 20 *gltA* sequences analyzed (distributed in 11 different sites), 15 different haplotypes were identified showing $\pi = 0.02459$, Hd = 0.958, and $K = 8.1894$. Only the haplotype numbers 1 (with four sequences), 5 (with two sequences), and 14 (with two sequences) had one or more sequences (Fig. 6). We also noted subtle intergene variation in levels of diversity among the *ftsZ* ($\pi = 0.037$) and *groEL* ($\pi = 0.027$) genes compared to that for *gltA*. The haplotype diversity results of the additional genes are shown in Table 3. The median-joining network of the *gltA* sequences showed a strong geographic separation between the haplotypes detected. Except for the sequence 2741 (belonging to haplotype 1) detected in *Thrichomys laurentius* sampled in the Caatinga biome, all of the haplotypes identified were unique to a particular biome.

DISCUSSION

Several studies suggest that *Bartonella* spp. have adapted to rodent species. As a probable result, rodents develop a long-lasting subclinical bacteremia and a high diversity of *Bartonella* genotypes (13, 46–48). Although the genetic diversity of this group of pathogens infecting rodents has been very well documented (49–52), this high variability has caused a laborious challenge in the taxonomic constitution of this group when the old criteria, such as DNA-DNA hybridization, comparison of 16S rRNA gene sequences, and phenotypical characteristics, are used to describe *Bartonella* spp.

Herein we reported the occurrence of *Bartonella* spp. in wild rodents from Caatinga, Cerrado, Atlantic forest, and Amazon forest biomes in Brazil.

Among all amplified *gltA* sequences, the maximum nucleotide divergence was 3.6%, suggesting that all sequences amplified in the present study belong to the same *Bartonella* spp., according to the species definition proposed by La Scola et al. (3).

Among the 117 (25.6%) samples positive for *Bartonella* spp. by qPCR, only 25 (21.3%) were simultaneously positive by *gltA*, *ftsZ*, and *groEL* cPCR assays. These findings reveal a higher sensitivity of qPCR than of cPCR assays, highlighting the use of multiple approaches in order to increase the sensitivity of *Bartonella* detection. Additionally, the logistic regression analysis performed by André et al. (30) showed that a 10-fold increase in copy numbers assessed by qPCR was associated with the odds of positive results in cPCR, demonstrating the better performance of qPCR over cPCR in detecting low *Bartonella* DNA copy numbers (30).

Interestingly, we found differences in the occurrence of *Bartonella* spp. among different biomes. However, such findings should be analyzed with prudence, since in the present study, the hosts were not deeply sampled to accurately determine *Bartonella* abundance in each biome.

The phylogenetic analysis of partial sequences of *gltA*, *ftsZ*, and *groEL* genes when analyzed separately or by concatenated tree, showed a clear separation between the sequences obtained in the present study and other *Bartonella* spp. previously described in GenBank, corroborating the low percentage of identity obtained by BLASTn analysis. However, *gltA* sequences detected in infected rodents were closely related to *Bartonella* spp. (phylogenetic group A and strain R-phy1) detected in rodents belong to the Cricetidae family and fleas parasitizing some of these rodents in North America (1, 45, 53).

These findings raise an interesting question about the phylogenetic relationship among them: would all *gltA* sequences detected in Cricetidae rodents from North America (phylogenetic group A), Peru (strain R-phy1), and now in Brazil (besides one Echimyidae *Thrichomys laurentius*) represent an unique *Bartonella* species? According to the genetic identity values proposed by La Scola et al. (3), which discriminate *Bartonella* at the species level, the answer is yes.

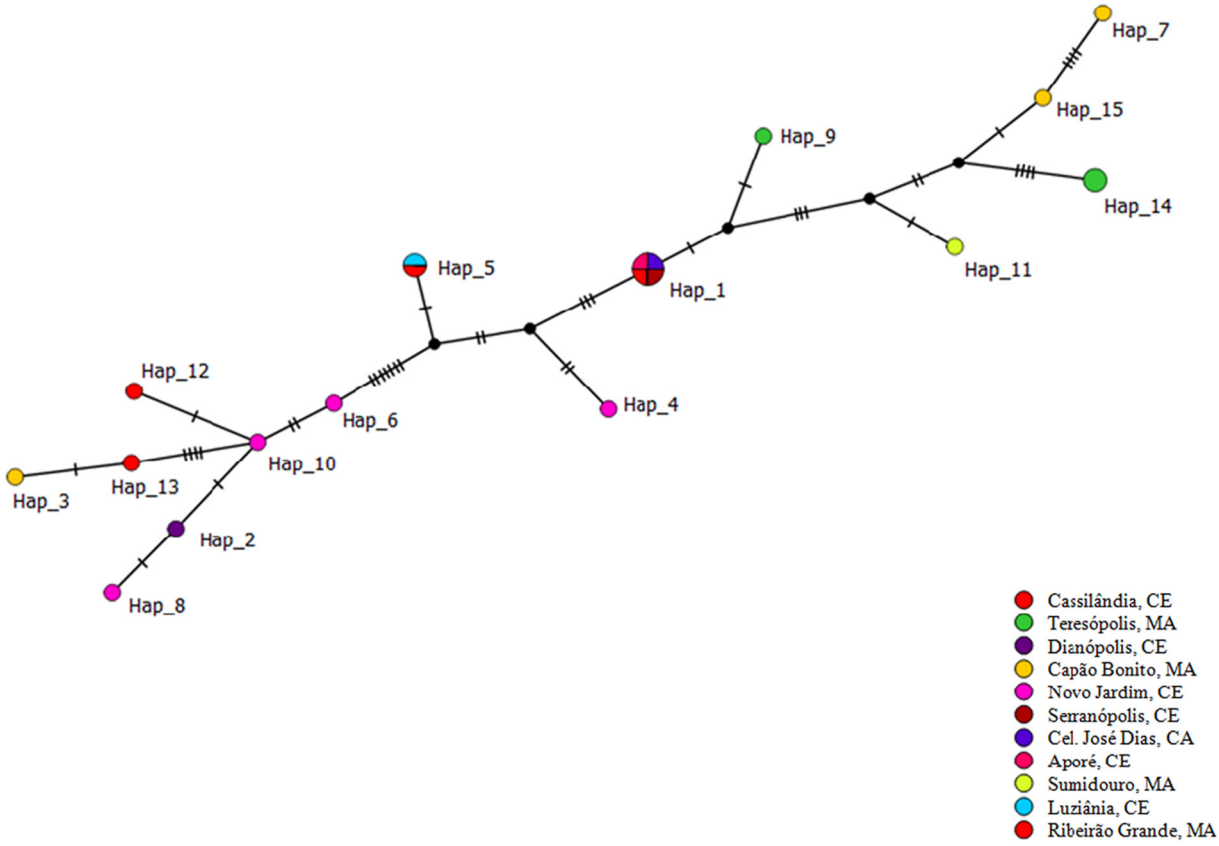

FIG 6 Median-joining network of *gltA* haplotypes detected in wild rodents sampled in 11 different sites in Brazil. MA, Mata Atlântica biome; CE, Cerrado biome; CA, Caatinga biome.

The same *Bartonella* sp. was detected in different rodent species derived from distinct (and distant) Brazilian biomes, showing a probable dominance of this genotype. Furthermore, it is important to emphasize that there is a huge overlap of distribution areas for the majority of rodent specimens found infected by *Bartonella* spp. in the present study (54). Consequently, a given genotype may circulate in different rodent species coexisting in a certain biome, as described for hemoplasmas, another group of pathogens also transmitted between rodents by arthropods (55). Additionally, the number of *Bartonella* spp. infecting wild rodents in Brazil is probably higher, as demonstrated by Favacho et al. when they analyzed rodents from the state of Mato Grosso do Sul, Brazilian Pantanal (21).

Although the Kosoy et al. research group (45, 48) showed host specificity for the phylogenetic group A, our results demonstrate that *Bartonella* spp. detected in wild rodents from Brazil are not host-specific and highlight a probable spillover event in the rodent species analyzed. Our results are similar to those reported in the United Kingdom, where one of the *Bartonella* spp. identified was found in five different rodent species (56). The reason for this distinct scenario may be related to virulence factors that limit or provide the ability to "jump" from one rodent species to another (57).

TABLE 3 Polymorphism and genetic diversity of *Bartonella* spp. detected in wild rodents in Brazil[a]

Gene	bp	No.	VS	h	GC (%)	Hd (mean ± SD)	π (mean ± SD)	*K*
gltA	333	20	26	15	35.0	0.958 ± 0.033	0.024 ± 0.001	8.189
ftsZ	551	15	56	15	47.2	1.000 ± 0.024	0.037 ± 0.003	20.047
groEL	750	4	33	4	41.7	1.000 ± 0.117	0.027 ± 0.006	20.000

[a] No., number of sequences analyzed; VS, number of variable sites; h, number of haplotypes; GC, G+ C content; Hd, haplotype diversity; π, nucleotide diversity (per site = PI); *K*, average number of nucleotide differences.

The haplotype diversity is controlled by different processes, including but not limited to mutation, recombination, and demography. The diversity analysis performed in the present study suggests that the genetic diversity detected in *gltA* sequences of *Bartonella* spp. infecting wild rodents in Brazil is similar to that reported for *B. grahamii* (a *Bartonella* species with high genetic diversity) upon comparison with *gltA* sequences obtained in strains from Asia ($\pi = 0.02154$; Hd = 0.943) and North America and Europe ($\pi = 0.01427$; Hd = 0.889) (58). However, it was lower than that demonstrated in *Myodes glareolus* ($\pi = 0.077$) sampled in Paris, France (49). In addition, the *Bartonella* species nucleotide diversity revealed in our study was higher than that reported in *B. grahamii* detected in rodents belonging to the species *Myodes rutilus*, *Microtus fortis*, and *Apodemus agrarius* ($\pi = 0.012$; Hd = 0.700) sampled in 4 sites from China (58). These findings highlight the fact that even within the same species and gene (*B. grahamii* and *gltA*, respectively), different rates of mutation, recombination, and specific demographic characteristics might promote distinct haplotype diversity. Last, the nucleotide

diversity observed in the *ftsZ* sequences ($\pi = 0.037$; Hd = 1.000) amplified in the present study was higher than those previously reported within *ftsZ* sequences of *B. grahamii* amplified in France ($\pi = 0.016$) (49) and China ($\pi = 0.010$; Hd = 1.000) (58). On the other hand, the nucleotide diversity observed in the *groEL* sequences ($\pi = 0.027$) was similar to that demonstrated in France among *groEL* sequences of *B. grahamii* ($\pi = 0.029$) (49). These findings reveal that the genetic diversity of *Bartonella* detected in wild rodents from Brazil is similar to or higher than that in some strains of *B. grahamii* detected around the world.

Although it is known that *Bartonella* spp. belonging to phylogenetic group A did not cause illness in experimentally infected *S. hispidus* (59), the zoonotic potential of these bacteria is still unknown. Considering the increasing destruction of natural habitats associated with intense human activities in forests and the presence of wild rodents in peridomiciliar areas, wild rodents and humans can also share arthropod vectors and pathogens. Because of this, a One Health approach (60) is required to elucidate the vector ecology and transmission dynamics of these bacteria detected among wild Brazilian rodents and the possible role in human infections.

In a nutshell, our study showed that the occurrence of *Bartonella* spp. in rodents is much more frequent and widespread that previously recognized, and this trait may be also influenced by the characteristics of rodent fauna and environment from different Brazilian biomes. Additionally, the *Bartonella* genotypes detected were closely related to those detected in other Cricetidae rodents from Americas, probably representing an unique species.

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