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Original article

# *Rickettsia* spp. among wild mammals and their respective ectoparasites in Pantanal wetland, Brazil

Keyla Carstens Marques de Sousa[a], Heitor Miraglia Herrera[b], Fabiana Lopes Rocha[c], Francisco Borges Costa[d], Thiago Fernandes Martins[d], Marcelo Bahia Labruna[d], Rosangela Zacarias Machado[a], Marcos Rogério André[a,*]

[a] Universidade Estadual Paulista (Unesp), Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal, SP, Brazil
[b] Universidade Católica Dom Bosco, Campo Grande, MS, Brazil
[c] Universidade Federal da Paraíba, Laboratório de Ecologia Animal, Rio Tinto, PB, Brazil
[d] Universidade de São Paulo, Faculdade de Medicina Veterinária e Zootecnia, São Paulo, SP Brazil

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ABSTRACT

The genus *Rickettsia* comprises obligatory intracellular bacteria, well known to cause zoonotic diseases around the world. The present work aimed to investigate the occurrence of *Rickettsia* spp. in wild animals, domestic dogs and their respective ectoparasites in southern Pantanal region, central-western Brazil, by molecular and serological techniques. Between August 2013 and March 2015, serum, whole blood and/or spleen samples were collected from 31 coatis, 78 crab-eating foxes, seven ocelots, 42 dogs, 110 wild rodents, and 30 marsupials. Serum samples from canids, felids, rodents and marsupials were individually tested by indirect fluorescent antibody test (IFAT) in order to detect IgG antibodies to *Rickettsia rickettsii, Rickettsia parkeri* and *Rickettsia amblyommatis*. DNA samples from mammals and ectoparasites were submitted to a multiplex qPCR assay in order to detect and quantify spotted fever group (SFG) and typhus group (TG) rickettsiae and *Orientia tsutsugamushi*. Positive samples in qPCR assays were submitted to conventional PCR assays targeting *gltA*, *ompA, ompB* and *htrA* genes, followed by sequencing and phylogenetic analyses. The ticks collected (1582) from animals belonged to the species *Amblyomma sculptum*, *Amblyomma parvum*, *Amblyomma ovale*, *Amblyomma tigrinum*, *Rhipicephalus (Boophilus) microplus, Rhipicephalus sanguineus* sensu lato and *Amblyomma auricularium*. Overall, 27 (64.2%) dogs, 59 (75.6%) crab-eating foxes and six (85.7%) ocelots were seroreactive (titer ≥ 64) to at least one *Rickettsia* species. For 17 (40.4%) dogs, 33 (42.3%) crab-eating foxes, and two (33.3%) ocelots, homologous reactions to *R. amblyommatis* or a closely related organism were suggested. One hundred and sixteen (23.5%) tick samples and one (1.2%) crab-eating fox blood sample showed positivity in qPCR assays for SFG *Rickettsia* spp. Among SFG *Rickettsia*-positive ticks samples, 93 (80.2%) belonged to *A. parvum*, 14 (12%) belonged to *A. sculptum* species, three (2.5%) belonged to *A. auricularim*, and six (5.2%) were *Amblyomma* larval pools. Thirty samples out of 117 qPCR positive samples for SFG *Rickettsia* spp. also showed positivity in cPCR assays based on *gltA, htrA* and/or *ompB* genes. The Blast analyses showed 100% identity with '*Candidatus* Rickettsia andeanae' in all 30 sequences obtained from *gltA, htrA* and/or *ompB* genes. The concatenated phylogenetic analysis based on *gltA* and 17-kDa *htrA* genes grouped the *Rickettsia* sequences obtained from tick samples in the same clade of '*Candidatus* Rickettsia andeanae'. The present study revealed that wild and domestic animals in southern Pantanal region, Brazil, are exposed to SFG rickettsiae agents. Future studies regarding the pathogenicity of these agents are necessary in order to prevent human cases of rickettsiosis in Brazilian southern Pantanal.

## 1. Introduction

The genus *Rickettsia* includes Gram-negative coccobacilli bacteria that belong to the order Rickettsiales, family Rickettsiaceae, and alphasubdivision of the class Proteobacteria (Dumler et al., 2001). Some *Rickettsia* species are known to be zoonotic pathogens and are transmitted by bloodsucking arthropods (Parola et al., 2005). *Rickettsia* species are classified in phylogenetic groups, namely spotted fever

* Corresponding author at: Laboratório de Imunoparasitologia, Departamento de Patologia Veterinária, Faculdade de Ciências Agrárias e Veterinárias Júlio de Mesquita Filho (UNESP), Campus de Jaboticabal, Via de Acesso Prof. Paulo Donato Castellane, s/n, Zona Rural, CEP: 14884-900, Jaboticabal, São Paulo, Brazil.
*E-mail address:* marcos_andre@fcav.unesp.br (M.R. André).

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group (SFG), typhus group, *Rickettsia bellii* group, and *Rickettsia canadensis* group (Parola et al., 2013).

*Rickettsia rickettsii* is the etiological agent of Rocky Mountain spotted fever, or Brazilian spotted fever, the deadliest rickettsiosis in the world (Guedes et al., 2005; Labruna, 2009). In Brazil, *R. rickettsii* is transmitted to humans primarily by the ticks *Amblyomma sculptum* Berlese [published as *Amblyomma* inclusion of *Rickettsia parkeri*, *Rickettsia rhipicephali*, *Rickettsia amblyommatis* (formerly '*Candidatus* Rickettsia amblyommii'), *Rickettsia monteiroi,* and '*Candidatus* Rickettsia andeanae' (Labruna et al., 2011; Nieri-Bastos et al., 2014).

*'Candidatus* Rickettsia andeanae', *R. parkeri* and *R. amblyommatis* have been detected in ticks (Acari: Ixodidae) sampled in Pantanal biome (Widmer et al., 2011; Alves et al., 2014; Nieri-Bastos et al., 2014; Melo et al., 2015, 2016; Witter et al., 2016). While anti-*Rickettsia* spp. antibodies were detected in dogs Due to the lack of information about the epidemiology of *Rickettsia* spp. among wildlife in southern Pantanal, the present study aimed to investigate the occurrence of *Rickettsia* in wild mammals and domestic dogs and their respective ectoparasites, using molecular and serological assays, in Pantanal wetland, Brazil.

## 2. Material and methods

### 2.1. Study area

The fieldwork was conducted at the Nhumirim ranch (56°39′W, 18°59′S), located in the central region of the Pantanal, municipality of Corumbá, state of Mato Grosso do Sul, central-western Brazil (Fig. 1). This region is characterized by a mosaic of semi-deciduous forest, arboreal savannas, seasonally flooded fields covered by grasslands with dispersed shrubs and several temporary and permanent ponds. The Pantanal is the largest Neotropical floodplain, being well known for its rich biodiversity. Two well-defined seasons are recognized in that region: a rainy summer (October to March) and a dry winter (April to September) (Sousa et al., 2017a,b,c).

### 2.2. Biological sampling

Between August 2013 and March 2015, four field expeditions (August 2013, October 2013, August 2014 and March 2015) were performed. The free-ranging carnivores (*Cerdocyon thous*, *Nasua nasua* and *Leopardus pardalis*) were caught used a Zootech® (Curitiba, PR, Brazil) model wire box live trap (1 × 0.40 × 0.50 m), which was made with galvanized wire mesh and baited with a piece of bacon every afternoon. Traps were armed during 24 h and checked twice a day. The animals were immobilized with an intramuscular injection of a combination of zolazepan and tiletamine (Zoletil®) at dosages of 8•mg/kg for ocelots and 10 mg/kg crab-eating foxes and coatis. Additionally, blood samples were collected from domestic dogs, which were cohabiting the same studied area.

Small mammals (rodents and marsupials) were captured 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, oat and sardines. Traps were set up for 7 consecutive nights along linear transects, placed on the ground at 10 m intervals and alternating between trap type in 2 field expeditions (August 2014 and March 2015). The total capture effort was 200 traps-nights, equally distributed between the 2 expeditions (August 2014 and March 2015). The identification of specimens was based on external and cranial morphological characters and karyological analyses.

Blood samples were collected from carnivores and domestic dogs by puncture of the cephalic vein and stored in Vacutainer® tubes with EDTA and without EDTA, in order to obtain total blood and serum samples for molecular and serological assays, respectively. Spleen samples were collected from small mammals and stored in absolute ethanol (Merck®, Kenilworth, Nova Jersey, USA) for molecular assays. Blood samples were collected by small mammals' intracardiac puncture and stored in Vacutainer® tubes without EDTA, in order to obtain serum samples for serological assays. All blood and serum samples were stored at −20°C. The DNA extraction and serological assays were performed one week after the captures. All animal captures were in accordance with the licenses obtained from the Brazilian Government Institute for Wildlife and Natural Resources Care (IBAMA) (license numbers 38145, 38787-2) and endorsed by the Ethics Committee of FCAV/UNESP University (Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus Jaboticabal) n° 006772/13 (Sousa et al., 2017a,b,c).

Ticks and fleas found parasitizing the sampled animals were detected by inspection of the skin and carefully removed by forceps or

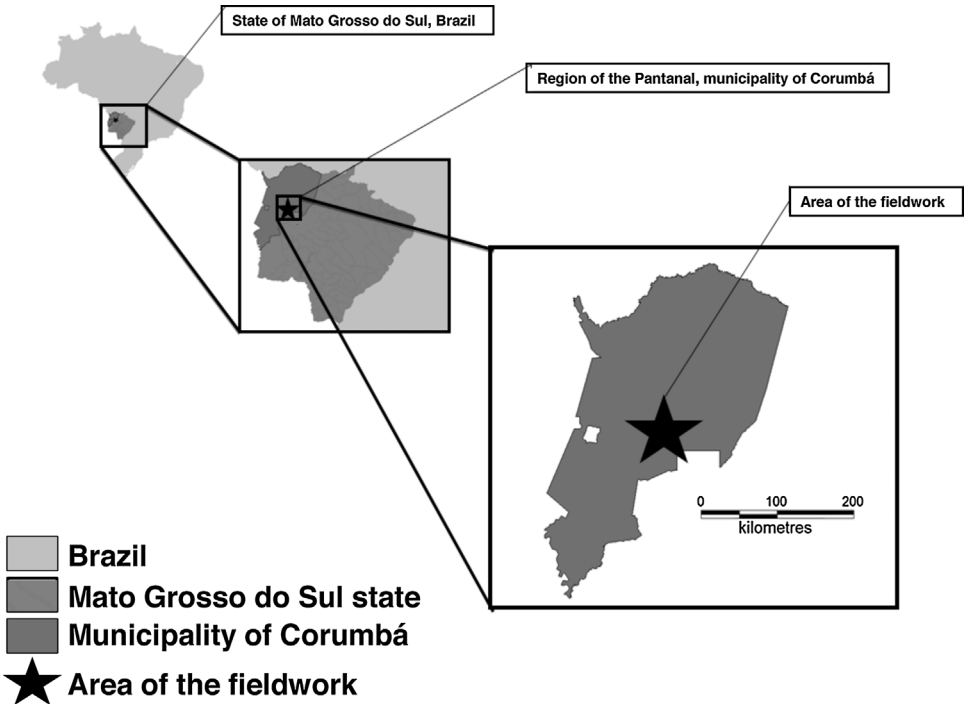


**Fig. 1.** Capture sites. Map of Mato Grosso do Sul State, central-western Brazil, showing the Pantanal region, where animals samples were collected in the present study.

**Table 1**
Oligonucleotides and hydrolysis probes sequences, target genes and cycling conditions used in qPCR and cPCR assays that were performed in biological samples from wild mammals, domestic dogs and their respective ectoparasites, sampled in Pantanal wetland, Brazil.

| Oligonucleotides and hydrolysis probes sequences (5′-3′) | Target gene | Cycling conditions | References |
|---|---|---|---|
| GAPDH-F (CCTTCATTGACCTCAACTACAT)<br>GAPDH-R (CCAAAGTTGTCATGGATGACC) | GAPDH/Mammals | 95 °C for 5 min; 35 cycles of 95 °C for 15 s, 50 °C for 30 s and 72 °C for 30 s; and final extension of 72 °C for 5 min. | Birkenheuer et al. (2003) |
| 16S + 1(CTGCTCAATGATTTTTTAAATTGCTGTGG)<br>16S-1(CCGGTCTGAACTCAGATCAAGT) | 16SrRNA/Ticks | 10 cycles of 92 °C for 1 min, 48 °C for 1 min and 72 °C for 1 min, followed by 32 cycles of 92 °C for 1 min, 54 °C for 35 s and 72 °C for 1,35 min, and final extension of 72 °C for 7 min. | Black and Piesman (1994) |
| HC02198 (TAAACTTCAGGGTGACCAAAAAATCA)<br>LCO1490 (GGTCAACAAATCATAAAGATATTGG) | COX1/Fleas | 95 °C for 1 min, 35 cycles of 95 °C for 15 s, 55 °C for 15 s and 72 °C for 10 s, and final extension of 72 °C for 5 min. | Folmer et al. (1994) |
| F (TGTCAGGCTCTGAAGCTAAA)<br>R (AGCACCTGCCGTTGTGATATC)<br>[FAM] TAGCCGCAGTCCCTACAACAC[BHQ2a-Q] | *ompA*/Spotted fever group *Rickettsia* | 95 °C for 3 min followed by 40 cycles at 95 °C for 10 s and 55 °C for 30 s | Prakash et al. (2009) |
| F (ACTTGGTTCTCAATTCGGTCAC)<br>R (GACACTTGCACCGATTTGTCC)<br>[TXRED] TGCCCCAAGTAATGCGCC[BHQ2a-Q] | 17-kDa lipoprotein/ Typhus group *Rickettsia* | | |
| F (GGTGGTAATGCTTTCGCTAAT)<br>R (TGCTGCTTCTTGCGCTGTAG)<br>[Cy-5] TGCTGCTGTTGCTGCCCTTGCC [BHQ2a-Q] | 56-kDa antigen/*O. tsutsugamushi* | | |
| CS-78 (GCAAGTATCGGTGAGGATGTAAT)<br>CS-323 (GCTTCCTTAAAATTCAATAAATCAGGAT) | *gltA/Rickettsia* spp. | 94 °C for 5 min, 40 cycles of 94 °C for 30 s, 48 °C for 30 s and 72 °C for 1 min, and final extension of 72 °C for 5 min. | Labruna et al. (2004a,b) |
| Rr190.70p (ATGGCGAATATTTCTCCAAAA)<br>Rr190.602n (AGTGCAGCATTCGCTCCCCCT) | *ompA/Rickettsia* spp. | 94 °C for 5 min, 40 cycles of 94 °C for 30 s, 48 °C for 30 s and 72 °C for 1 min, and final extension of 72 °C for 5 min. | Regnery et al. (1991) |
| 120-M59 (CCGCAGGGTTGGTAACTGC)<br>120–807 (CCTTTTAGATTACCGCCTAA) | *ompB/Rickettsia* spp. | 94 °C for 5 min, 40 cycles of 94 °C for 30 s, 48 °C for 30 s and 72 °C for 1 min, and final extension of 72 °C for 5 min. | Roux and Raoult (2000) |
| 17kD1 (GCTCTTGCAACTTCTATGTT)<br>17kD2 (CATTGTTCGTCAGGTTGGCG) | *htr*A17KDa/*Rickettsia* spp. | 94 °C for 5 min, 40 cycles of 94 °C for 30 s, 48 °C for 30 s and 72 °C for 1 min, and final extension of 72 °C for 5 min. | Labruna et al. (2004a,b) |

manually. The specimens were stored in 100% alcohol (Merck®, Kenilworth, Nova Jersey, EUA) until identification using a stereomicroscope (Leica® MZ 16A, Wetzlar, Germany) and following taxonomic literature for adult tick genera (Guimarães et al., 2001; Martins et al., 2016), and *Amblyomma* nymphs (Martins et al., 2010). *Amblyomma* larvae could not be identified to the species level because there is insufficient literature available until now. The identification of fleas was performed following the dichotomous keys elaborated by Linardi and Guimarães (2000). All specimens were stored in 100% ethanol (Merck®, Kenilworth, New Jersey, USA) until DNA extraction

### 2.3. Immunofluorescence assays

Canids, felids, rodents and marsupials' serum samples were individually tested by IFAT in order to verify the reactivity to SFG rickettsiae, using *R. rickettsii, R. parkeri* or *R. amblyommatis* antigens. For this purpose, *Rickettsia* crude antigens were cultivated in Vero cells as previously described (Horta et al., 2007; Labruna et al., 2007). Each serum sample was initially diluted (1:64) in phosphate-buffered saline (PBS), pH 7.2. On each slide, previously determined non-reactive and reactive serum samples to SFG rickettsiae were used as negative and positive controls, respectively (Labruna et al., 2007; Widmer et al., 2011; Coelho et al., 2016). The slides were incubated at 37 °C for 30 min in a humidity box, rinsed once, and were held twice for 15 min per wash in PBS. Then, the slides were incubated with fluorescein isothiocyanate labeled goat anti-mouse IgG (Sigma®, St. Louis, USA) for *O. mamorae*, goat anti-guinea pig IgG (Sigma®, St. Louis, USA) for *T. fosteri* and *C. laticeps*, sheep anti-opossum IgG (CCZ, São Paulo, Brazil) for *T. macrurus, G. agilis*, *M. domestica* and *D. albiventris,* goat anti-dog IgG (Sigma®, St. Louis, USA) for *C. thous* and domestic dogs, and goat anti-cat IgG (Sigma®, St. Louis, USA) for *L. pardalis*. IgG fluorescein-labeled conjugate was diluted at 1:1000, except for opossum that was diluted at 1:500. After drying, each slide was examined under a fluorescence microscope (Olympus®, Tokyo, Japan). For each serum sample, the endpoint titer reacting with each of the three *Rickettsia* antigens was determined. Sera showing antibodies titers at least four fold higher than that observed for any other *Rickettsia* species were considered to be homologous to the first *Rickettsia* species or to a closely related species (possible antigen involved in a homologous reaction) (Horta et al., 2007; Labruna et al., 2007). Unfortunately, it was not possible to test the coatis serum samples, due to the unavailability of a feasible conjugate.

### 2.4. DNA extraction

DNA was extracted from 200 µL of each whole blood (158 wild carnivores and 42 domestic dogs) and 10 mg of spleen (140 small mammals) samples using the QIAamp DNA Blood Mini kit (QIAGEN®, Valencia, CA, USA), according to the manufacturer's instructions and stored at −20 °C. The total number of individual tick samples or pools was 523, of which 228 (43.5%) were from adults, 256 (48.9%) nymphal pools, and 39 (7.4%) from pooled larvae. DNA extraction from ticks was processed in pools for nymphs (up to 5 individuals) and larvae (up to 10 individuals), while the adults were processed individually. A total of 39 pooled fleas samples were submitted to DNA extraction. The fleas DNA extraction was also performed in pools consisting of up to five individuals. Ticks and fleas were macerated and submitted to DNA extraction, using the same kit before mentioned. Immediately after the DNA extraction of all biological samples, the molecular analyses were performed (Sousa et al., 2017a,b,c).

### 2.5. cPCR for endogenous genes

In order to verify the presence of amplifiable DNA in the samples, internal control PCR assays targeting fragments of mammalian glyceraldehyde-3-phosphatedehydrogenase (GAPDH), ticks mitochondrial 16S rRNA and fleas cytochrome-c oxidase subunit I (COX1) genes were performed (Table 1).

### 2.6. qPCR screening test

A previously described broad range multiplex quantitative real-time PCR (qPCR) protocol was used in order to detect and quantify spotted fever group (SFG) rickettsiae (*ompA* gene), typhus group (TG) rickettsiae (17-kDa lipoprotein precursor gene) and *O. tsutsugamushi* (56-kD antigen gene) DNA copies (number of copies/µL) (Prakash et al., 2009). The Taq Man qPCR reactions were performed with a final volume of 10 µL contained 5 µL GoTaq® Probe qPCR Master Mix (Promega

Corporation, Madison USA), 1.2 µM of each primer and hydrolysis probe (Table 1) and 1 µL of each DNA sample. PCR amplifications were performed in low-profile multiplate unskirted PCR plates (BioRad®, CA USA) using a CFX96 Thermal Cycler (BioRad®, CA USA).

Serial dilutions were performed aiming to construct standard curves with different gBlock (500 bp) concentrations (Integrated DNA Technologies®, Coralville, Iowa, USA) ($2.0 \times 10^7$ to $2.0 \times 10^0$ copies/µL). The number of gBlock copies was determined in accordance with the formula (Xg/µL DNA/[gBlock size (bp)x660]) x$6.022 \times 10^{23}$xgBlock copies/µL. Each qPCR assay was performed including duplicates of each DNA sample. All the duplicates showing Cq difference values higher than 0.5 were re-tested. Amplification efficiency (E) was calculated from the slope of the standard curve in each run using the following formula ($E = 10^{-1/slope}$). 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% of sensitivity (Bustin et al., 2009).

### 2.7. Conventional PCRs assays

All positive samples in qPCR reactions were submitted to previously described conventional PCR (cPCR) assays targeting five other protein-coding genes, namely *gltA* (401 bp) (Labruna et al., 2004a), *ompA* (530 bp) (Regnery et al., 1991), *ompB* (862 bp) (Roux and Raoult, 2000) and *htrA* 17-kDa (440 bp) (Labruna et al., 2004b) (Table 1). The mixture contained 10X PCR buffer (Life Technologies®, Carlsbad, CA, USA), 1.0 mM $MgCl_2$ (Life Technologies®, Carlsbad, CA, USA), 0.2 mM deoxynucleotide triphosphate (dNTPs) mixture (Life Technologies®, Carlsbad, CA, USA), 1.5 U Taq DNA Polymerase (Life Technologies®, Carlsbad, CA, USA), and 0.5 µM of each primer (Integrated DNA Technologies®, Coralville, IA, USA). *Rickettsia rickettsii* DNA, kindly provided by Fundação Oswaldo Cruz (Fiocruz, Rio de Janeiro, Brazil), and ultra-pure sterile water (Life Technologies®, Carlsbad, CA, USA) were used as positive and negative controls, respectively. PCR products were separated by electrophoresis on a 1% agarose gel stained with ethidium bromide (Life Technologies®, Carlsbad, CA, USA). 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 (Thermo Fisher Scientific®, Waltham, MA, USA). Sanger sequencing was performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific®, Waltham, MA, USA) and ABI PRISM 310DNA Analyzer (Applied Biosystems®, Foster City, CA, EUA) (Sanger et al., 1977).

### 2.8. Bioinformatics/Phylogenetic analysis

Sequences obtained from positive samples were first submitted to a screening test using Phred-Phrap software version 23 (Ewing and Green, 1998; Ewing et al., 1998) in order to evaluate the electropherogram quality and to obtain consensus sequences from the alignment of sense and antisense sequences. The BLAST tool available in Genbank (Altschul et al., 1990) was used to analyze the sequences of nucleotides (BLASTn), aiming to browse and compare with sequences previously deposited in Genbank (Benson et al., 2002). All sequences that showed appropriate quality standards and identity with *Rickettsia* spp. were deposited in Genbank. Samples showing positive results for two or more target genes had their sequences concatenated, using the Fragment Merger software version 1 (Bell and Kramvis, 2013). The sequences were aligned with sequences published in GenBank using MAFFT software, version 7 (Katoh and Standley, 2013).

Phylogenetic inference was based on Bayesian method (BI). The Bayesian inference analysis was performed with MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003). Markov chain Monte Carlo (MCMC) simulations were run for $10^9$ generations with a sampling frequency of every 100 generations and a burn-in of 25%. The best model of evolution was selected by the program jModelTest2 (version 2.1.6) on XSEDE (Darriba et al., 2012), under the Akaike Information Criterion (AIC) (Posada and Buckley, 2004). All phylogenetic analyses were performed using CIPRES Science Gateway (Miller et al., 2010). The trees were examined in Treegraph 2.0.56-381 beta (Stover and Muller, 2010).

## 3. Results

Between August 2013 and March 2015, a total of 256 animals were captured in the central region of the Pantanal, municipality of Corumbá, state of Mato Grosso do Sul: 116 carnivores, among 78 crab-eating foxes (*C. thous*), 31 coatis (*N. nasua*) and seven ocelots (*L. pardalis*); 140 small mammals, among 110 wild rodents (77 *Thrichomys fosteri*, 25 *Oecomys mamorae* and 8 *Clyomys laticeps*) and 30 wild marsupials (14 *Thylamys macrurus*, 11 *Gracilinanus agilis*, 4 *Monodelphis domestica* and 1 *Didelphis albiventris*). Additionally, 42 blood samples from domestic dogs were collected (Sousa et al., 2017a,b,c).

One thousand five hundred and eighty-two ticks parasitizing the sampled mammals were collected, of which 1033 (65.2% [115 adults and 918 nymphs]) belonging to *Amblyomma sculptum* species; 241 (15.2% [78 adults and 163 nymphs]) belonging to *Amblyomma parvum* species; 32 (2%) *Amblyomma ovale* adults; one (0.06%) *Amblyomma tigrinum* adult; one (0.06%) *Rhipicephalus (Boophilus) microplus* adult; one (0.06%) *Rhipicephalus sanguineus* s.l. adult; four (0.2%) *Amblyomma auricularium* nymphs; and 269 (17%) *Amblyomma* larvae (Table 2). Besides, a total of 80 *Polygenis (Polygenis) bohlsi bohlsi* fleas were collected (Sousa et al., 2017a,b,c).

All 298 DNA animal samples amplified the predicted product for GAPDH gene. Out of 523 tick samples, 31 (5.9%) showed negative results for the tick mitochondrial 16S rRNA gene and were excluded from subsequent analyses (23 *A. parvum* adults, 4 *A. sculptum* adults, 1 *A. ovale* adult, 1 *A. parvum* nymph and 2 pooled *Amblyomma* larvae). Only one flea DNA sample did not amplify the predicted product for cox-1 and was also excluded from subsequent analyses (Sousa et al., 2017a,b,c).

Overall, 27 (64.2%) dogs, 59 (75.6%) crab-eating foxes and 6 (85.7%) ocelots were seroreactive (titer ≥ 64) to at least one *Rickettsia* species (Table 3). The seroreactive animals showed endpoint titers for *Rickettsia* spp. ranging from 64 to 4096. Among the 27 seroreactive dogs and 59 seroreactive crab-eating foxes, titers for *Rickettsia* species ranged as following: *R. rickettsii*, 64–2048; *R. parkeri*, 64–2048; and *R. amblyommatis,* 64–4096. Lastly, among the six seroreactive ocelots, titers for *Rickettsia* species ranged as following: *R. rickettsii*, 64–256; *R. parkeri*, 64–512; and *R. amblyommatis,* 64–1024. For 19 (70.3%) dogs, 33 (55.9%) crab-eating foxes and 2 (33.3%) ocelots, anti-*R. amblyommatis* endpoint antibodies titers were at least four fold higher than the endpoint titers to the remaining rickettsial antigens, suggesting homologous reactions to *R. amblyommatis* or a closely related organism (Table 3). Ticks were collected from 31 (33.6%) out of 92 seroreactive animals. None of the wild rodents or marsupials showed seroreactivity for any of the three employed rickettsial antigens.

One hundred and sixteen (23.5%) tick samples and one (1.2%) *C. thous* blood sample showed positivity in qPCR for SFG *Rickettsia* spp. based on *ompA* gene. None of the samples showed positivity in qPCR for TG *Rickettsia* spp. or *O. tsutsugamushi,* based on 17-kDa lipoprotein precursor and 56-kD antigen genes, respectively. Among SFG *Rickettsia*-positive ticks, 93 (80.2%) belonged to *A. parvum* species (46 nymphal pools, 44 adults, and 3 egg pools), 14 (12%) belonged to *A. sculptum* species (5 nymphal pools and 9 adults), 3 nymphal pools (2.5%) belonged to *A. auricularium* species (all nymphal samples), and 6 (5.2%) larval pools contained 10 *Amblyomma* spp. individuals. The number of copies of SFG *Rickettsia-ompA* fragment/µL ranged from $2.53 \times 10^1$ to $4.52 \times 10^1$. The efficiency, correlation coefficient and slope of qPCR reactions ranged from 90.3% to 102.3% (mean = 94.3%), 0.952 to

**Table 2**
Tick species collected from wild mammals captured between August 2013 and March 2015 in Pantanal wetland, Brazil.

| Animal species | | | TICKS[a] | | | | | | | FLEAS | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | N° of anim. | Infest (%) | *A. sculptum* | *A. parvum* | *A. tigrinum* | *A. ovale* | *A. auricularium* | *R. (B.) microplus* | *R. sanguineus* s. l. | *Amblyomma* spp. | *Polygenis (Polygenis) bohlsi bohlsi* |
| *Cerdocyon thous* | 78 | 35 (44.8) | 34 M; 55F; 643N | 21 M; 34F; 3N | 1F | 4 M. 1F | | | | 204L | |
| *Nasua nasua* | 31 | 22 (70.9) | 10 M; 13F; 275N | 11 M;. 6F;.12N | | 20 M;7F | 3N | 1F | | 21L | |
| *Leopardus pardalis* | 7 | 2 (28.5) | | 3 M;. 3F | | | | | | | |
| *Canis Lupus familiaris* | 42 | 1 (2.3) | 1F | | | | | | 1M | | |
| *Thrichomys fosteri* | 77 | 23 (29.8) | 2N | 116N | | | | | | 36L | 75 |
| *Oecomys mamorae* | 25 | 1 (4) | | 1N | | | | | | | |
| *Clyomys laticeps* | 8 | 3 (37.5) | | 13N | | | 1N | | | 7L | |
| *Tylamys macrurus* | 14 | 1 (7.1) | | 18N | | | | | | 1L | 1 |
| *Monodelphis domestica* | 4 | 0 (0) | | | | | | | | | 4 |
| *Gracicinamus agilis* | 11 | 0 (0) | | | | | | | | | |
| *Didelphis albiventris* | 1 | 0 (0) | | | | | | | | | |
| Total | 298 | 88 (29.6) | 1033 | 241 | 1 | 32 | 4 | 1 | 1 | 269 | 80 |

L – larvae, N – nymphs, M – male adults, F – female adults, N°anim. – number of sampled animals, N° infest. – number of infested animals according to host species.
[a] *A. sculptum* – *Amblyomma sculptum*, *A. parvum* – *Amblyomma parvum*, *A. tigrinum* – *Amblyomma tigrinum*, *A. ovale* – *Amblyomma ovale*, *A. auricularium* – *Amblyomma auricularium*, *R.(B.) microplus* – *Rhipicephalus (Boophilus) microplus*, *R. sanguineus* s.l.– *Rhipicephalus sanguineus* sensu lato.

0.997 (mean = 0.975) and −3.304 to −3.269 (mean = −3.428), respectively. The quantification mean of all 16 performed reactions was $3.59 \times 10^1$copies of SFG *Rickettsia-ompA* fragment/μL. The quantification mean between the groups of hosts were: *A. parvum* = $3.54 \times 10^1$copies/μL ($3.53 \times 10^1$ – $4.52 \times 10^1$), *A. sculptum* = $3.89 \times 10^1$copies/μL ($3.4 \times 10^1$ – $4.41 \times 10^1$), *A. auricularium* = $3.62 \times 10^1$copies/μL ($3.58 \times 10^1$ – $3.65 \times 10^1$), *Amblyomma* larvae = $3.68 \times 10^1$copies/μL ($3.54 \times 10^1$ – $3.93 \times 10^1$). The quantification of *Rickettsia* sp. *ompA* DNA in *C. thous* blood sample was $3.85 \times 10^1$copies/μL.

Seventeen samples (14.5%) out of 117 qPCR positive samples for SFG *Rickettsia* spp. also showed positivity in cPCR based on *gltA* gene, of which 15 positive samples belonged to *A. parvum* tick species (5 nymphal pools, 8 adults and 2 egg pools) and 2 positive samples belonged to *Amblyomma* spp. larval pools. Nine *gltA* cPCR-positive *A. parvum* samples and 1 *Amblyomma* spp. larval pool also showed positivity in the cPCR assay based on 17-kDa *htrA* gene. In addition, 3 *A. parvum* positive samples in *gltA* and17-kDa *htrA* cPCR assays also showed positivity in cPCR assay based on *ompB* gene. No tick was positive in cPCR assay based on *ompA* gene. The *gltA,* 17-kDa *htrA* and *ompB* sequences obtained from positive ticks were deposited in GenBank under the following accession numbers: KY402168-KY402196.

The BLAST analysis of a 300–350 bp *Rickettsia gltA* gene fragment obtained from 15 *A. parvum* tick species (5 nymphal pools, 8 adults and 2 egg pools) and 2 positive samples belonged to *Amblyomma* spp. larval pools showed 100% identity with '*Candidatus* Rickettsia andeanae' isolate LIC4328 (KT153033). The BLAST analysis of a 350–380 bp *Rickettsia* 17-kDa *htrA* gene fragment obtained from 9 *A. parvum* and one *Amblyomma* spp. larval pool showed 100% identity with '*Candidatus* Rickettsia andeanae' isolateT163 (GU395295). Lastly, the BLAST analysis of an 800 bp *Rickettsia ompB* gene fragment obtained from 3 *A. parvum* specimens (2 adults and 1 egg pool) showed 99% identity also with '*Candidatus* Rickettsia andeanae' isolate T163 (GU395297) (Table 4).

The concatenated phylogenetic analysis of *Rickettsia* based on *gltA,* 17-kDa *htrA* and *ompB* genes grouped sequences obtained from *A. parvum* adult in the same clade of '*Candidatus* Rickettsia andeanae', with clade support of 100 based on BI analysis, and using *R. tamurae* as outgroup (Fig. 2).

## 4. Discussion

The present study showed the presence of antibodies anti-*Rickettsia* spp. in serum samples from crab-eating foxes, ocelots and domestic dogs, and SFG *Rickettsia* DNA in *A. parvum, A. sculptum* and *A. auricularium* ticks collected from mammals sampled in the region of Pantanal, state of Mato Grosso do Sul, central-western Brazil.

A high seroprevalence (64.2%) to *Rickettsia* spp. was found among dogs and *C. thous* sampled in the present study, with homologous reactions to *R. amblyommatis.* On the other hand, the *Rickettsia* DNA detected in eight *A. parvum* specimens collected from *C. thous* showed to be closely related to '*Candidatus* Rickettsia andeanae'. Due to the unavailability of '*Candidatus* Rickettsia andeanae' antigen, cross-reactions between the related species '*Candidatus* Rickettsia andeanae' and *R. amblyommatis* or a closely related agent cannot be discarded. Besides, SFG *Rickettsia* DNA was detected in a *C. thous* blood sample. Considering that the molecular detection of *Rickettsia* from blood samples shows low sensitivity, mainly because the rickettsemia occurs only on the first stage of the disease (Znazen et al., 2015), highlighting the finding that crab-eating foxes from southern Pantanal seems to be frequently exposed to SFG rickettsial agents. To the best authors' knowledge, this was the first serological evidence of exposure to rickettsial agents among wild crab-eating foxes in Brazil.

Herein, all *Rickettsia* sequences amplified from *A. parvum* specimens showed to be closely related to '*Candidatus* Rickettsia andeanae'. In Brazil, '*Candidatus* Rickettsia andeanae' was previously detected only in

**Table 3**
IFAT results found among mammals in Pantanal wetland, Brazil.

| Animals (no. tested) | No. of seroreactive animals to each of *Rickettsia* species (% seroreactivity for each animal species) | | | No. of animals with determined homologous reaction (PAHIR in parentheses) |
|---|---|---|---|---|
| | *R. rickettsii* | *R. parkeri* | *R. amblyommatis* | |
| Dogs (42) | 22 (52) | 23 (55) | 27 (64) | 19 (*R. amblyommatis*) |
| *Cerdocyon thous* (78) | 38 (49) | 40 (51) | 59 (76) | 33 (*R. amblyommatis*) |
| *Leopardus pardalis* (7) | 5 (71) | 6 (86) | 5 (71) | 2 (*R. amblyommatis*) |
| Total (127) | 65 (51) | 69 (54) | 91 (72) | 54 (*R. amblyommatis*) |

*R. rickettsii* – *Rickettsia rickettsii, R.parkeri* – *Rickettsia parkeri, R.amblyommatis* – *Rickettsia amblyommatis*, PAHIR – A homologous reaction was suggested when an endpoint titer to a *Rickettsia* species was at least 4-fold higher than those observed for the other *Rickettsia* species. In this case, the *Rickettsia* species involved in the highest endpoint titer was considered the possible antigen involved in a homologous reaction.

questing *A. parvum* from southern Pantanal biome (Nieri-Bastos et al., 2014), *A. parvum* and *A. auricularium* collected from horses and *Turdus amaurochalinus* in northeastern Brazil (Nieri-Bastos et al., 2014; Lugarini et al., 2015), and *A. sculptum* collected from a wild animal, whose species was not informed, in the state of Mato Grosso, central-western Brazil (Witter et al., 2016). Although '*Candidatus* Rickettsia andeanae' has also been reported infecting ticks in Peru (*Amblyomma maculatum* and *Ixodes bolivensis*) (Blair et al., 2004), Argentina (*A. parvum*) (Pacheco et al., 2007), the United States (*A. maculatum*) (Paddock et al., 2010), Chile (*Amblyomma triste*) (Abarca et al., 2012), and Paraguay (*A. parvum*) (Ogrzewalska et al., 2014), the role of this rickettsia as a human pathogen is still unknown (Ferrari et al., 2013).

While *A. parvum* adult stage preferably parasitizes medium to large-sized mammals (ruminants, horses and carnivores), larvae and nymphs are mainly found parasitizing small mammals (Aragão, 1936; Nava et al., 2008). Previous studies conducted in Argentina (Nava et al., 2008) and Brazil (Horta et al., 2011) suggested that *Galea* and *Thrichomys* rodents are important hosts for *A. parvum* sub-adults. In the present study, the majority of ticks (148/159) collected from small mammals were *A. parvum* nymphs. Besides, 45 out of the 54 DNA tick samples extracted from *A. parvum* nymphs collected from small mammals showed positivity in *Rickettsia* (SFG)-qPCR, whose sequencing pointed '*Candidatus* Rickettsia andeanae' as the rickettsial agent presented in 7 tick specimens.

Surprisingly, no rodent or marsupial showed seropositivity in IFAT for any rickettsial agent. One explanation for these findings is that *A. parvum* nymphs may not be able to transmit '*Candidatus* Rickettsia andeanae' to their hosts, highlighting the importance of future studies aiming at evaluating the vectorial competence of *A. parvum* nymphs for this *Rickettsia* species. Interestingly, a laboratory study in the United States demonstrated that '*Candidatus* Rickettsia *andeanae*' was not efficiently transmitted to the host skin during tick feeding (Grasperge et al., 2014).

The seroprevalence to *Rickettsia* spp. found among small mammals (rodents and marsupials) from others Brazilian states was higher than that found in the present study. For instance, seroprevalences ranging from 19.6% to 68.1% with homologous reactions to *R. rickettsii*, *R. bellii* and *R. parkeri* were reported among marsupials and *R. rickettsii* and *R. parkeri* were reported among rodents in the state of São Paulo, southeastern Brazil (Horta et al., 2007; Ogrzewalska et al., 2012; Szabó et al., 2013). Besides, the ticks collected from the small mammals sampled in the state of São Paulo belonged to *A. ovale*, *Amblyomma fuscum*, *Amblyomma brasiliense*, *A. sculptum* (formerly named as *A. cajennense*), *Amblyomma dubitatum*, *Ixodes loricatus* and *Haemaphysalis juxtakochi* (Horta et al., 2007; Ogrzewalska et al., 2012; Szabó et al., 2013). In the state of Pernambuco, northeastern Brazil, 68.8% (150/218) of the small mammals were seroreactive to one or more *Rickettsia* antigens; although both marsupials and rodents showed to be seroreactive to *R. rickettsii*, *R. bellii* and *R. amblyommatis* antigens, no homologous reactions were suggested. In addition to this, ticks belonging to *A. fuscum*, *A. dubitatum*, *Haemaphysalis leporispalustris* and *I. loricatus* tick species were found parasitizing wild rodents and marsupials sampled in the state of Pernambuco (Dantas-Torres et al., 2012). Recently, 70 out of 416 small mammals (16.8%) sampled in the state of Minas Gerais, southeastern Brazil, showed to be seroreactive to *Rickettsia* spp., with homologous reactions to *R. rickettsii, R. parkeri, R. rhipicephali* and *R. bellii* (Coelho et al., 2016). Only two tick species (*A. dubitatum* and *I. loricatus*) were found parasitizing the animals sampled in Minas Gerais state (Coelho et al., 2016). In contrast to the present study, all previous studies assessing the rickettsial serological profile of small mammals in Brazil did not report infestations by *A. parvum* ticks among the sampled animals (Horta et al., 2007; Dantas-Torres et al., 2012; Ogrzewalska et al., 2012; Szabó et al., 2013; Coelho et al., 2016). Keeping that in mind, the differences in seroprevalences found in the present study could be due to the absence of competent tick vectors parasitizing the small mammals from southern Pantanal.

Among the wild felids, 6 (85.7%) out of 7 ocelots trapped were seroreactive to at least one *Rickettsia* species. In a previous study, all 10 jaguars (*P. onca*) sampled in Pantanal were seropositive to at least one *Rickettsia* antigen, with homologous reactions to *R. bellii* and *R. parkeri* in 2 animals (Widmer et al., 2011). In the present study, 2 ocelots showed endpoint titers suggesting homologous reaction to *R. amblyommatis* or a very closely related species. While *A. cajennense*, *A. triste* and *R.* (*B.*) *microplus* were found parasitizing jaguars in the above-mentioned study (Widmer et al., 2011), only *A. parvum* was found parasitizing ocelots trapped in the present study. Moreover, while *R.*

**Table 4**
Maximum identity of *Rickettsia* spp. *gltA, ompB* and *htrA* 17KDA sequences detected in ticks collected from wild mammals sampled in Pantanal wetland, Brazil, by BLAST analysis.

| Host | Number of sequences analized | Target Gene | Identity by BLAST® analysis |
|---|---|---|---|
| *A. parvum* nymphs | 5 | *gltA* | '*Candidatus* Rickettsia andeanae' isolate LIC4328 (KT153033) 294/294 (1) |
| *Amblyomma* larvae | 2 | *gltA* | '*Candidatus* Rickettsia andeanae' isolate LIC4328 (KT153033) 317/317 (1) |
| *A. parvum* eggs | 2 | *gltA* | '*Candidatus* Rickettsia andeanae' isolate LIC4328 (KT153033) 345/345 (1) |
| *A. parvum* adult | 8 | *gltA* | '*Candidatus* Rickettsia andeanae' isolate LIC4328 (KT153033) 339/339 (1) |
| *A. parvum* adult | 2 | *ompB* | '*Candidatus* Rickettsia andeanae' isolate T163 (GU395297) 735/736 (0.9) |
| *A. parvum* eggs | 1 | *ompB* | '*Candidatus* Rickettsia andeanae' isolate T163 (GU395297) 499/500 (0.9) |
| *A. parvum* nymphs | 4 | *htrA*17KDA | '*Candidatus* Rickettsia andeanae' isolate T163 (GU395295) 459/459 (1) |
| *Amblyomma* larvae | 1 | *htrA*17KDA | '*Candidatus* Rickettsia andeanae' isolate T163 (GU395295) 496/496 (1) |
| *A. parvum* adult | 5 | *htrA*17KDA | '*Candidatus* Rickettsia andeanae' isolate T163 (GU395295) 535/535 (1) |

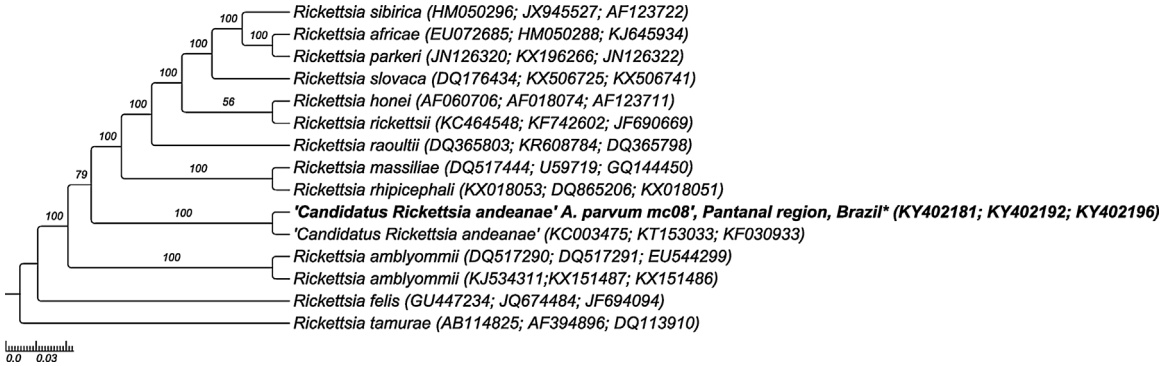


**Fig. 2.** Phylogenetic tree constructed with approximately 1490 bp *Rickettsia* spp. *gltA* + *htrA* + *ompB* sequences, using Bayesian method and GTR + G + I evolutionary model. Numbers at nodes correspond to Bayesian posterior probabilities over 50, using *Rickettsia tamurae* (AB114825; AF394896; DQ113910) as outgroup.

*parkeri* DNA has been previously detected in an *A. triste* specimen collected from a jaguar (Widmer et al., 2011), '*Candidatus* Rickettsia andeanae' was detected in *A. parvum* collected from the ocelots sampled in the present study. Further studies aiming at sampling a higher number of felids should be performed in order to evaluate the occurrence of these agents and the impact among endangered neotropical wild felids.

Surprisingly, only 30 samples out of 117 qPCR positive samples for SFG *Rickettsia* spp. showed positivity in cPCR assays based on *gltA, ompB or htrA* genes. In a previous study, the sensitivity of 4 cPCR protocols based on *ompB, rpoB*, *gltA* and *hrtA* genes and 1 SYBR Green-based qPCR assay were compared for the detection of rickettsial DNA in blood and tissue samples from *Rickettsia*- infected laboratory animals (n = 87). While rickettsial DNA was detected in 39.1% of samples by qPCR, cPCR assays detected *Rickettsia* DNA in 14.9% of samples (Zemtsova et al., 2015). The results of the present study corroborate those found by Zemtsova et al. (2015) that showed that qPCR assays show a higher sensitivity when compared to more traditional assays in detecting rickettsial DNA.

Herein, only one specimen of *A. tigrinum, R. (B) microplus* and *R. sanguineus* s.l. were found parasitizing the sampled animals. Previously, Witter et al. (2016) also collected only one *R. (B) microplus* in a jaguar, one *R. sanguineus* s.l. in one *C. thous* and one *A. trigrinum* in one *Chrysocyon brachyurus* from 27 animals belonging to order Carnivora captured in the state of Mato Grosso. In the same area of the present study, Cançado et al. (2017) found only one *A. tigrinum* and none *R. (B) microplus* and *R. sanguineus* s.l. when analyzed 42 foxes, 37 coatis and 24 dogs, indicating low prevalence of these tick species among the wild carnivores and domestic dogs in this region. Although infestations by *R. (B) microplus* have been observed in cattle (37%) in this same area of Pantanal (Cançado et al., 2017), accidental parasitism could be observed in wild animals in low frequency.

In a study encompassing 5 areas in the state of São Paulo, southeastern Brazil, *R. felis* DNA was detected in the fleas *Polygenis* (*Neopolygenis*) *atopus* (5/99) and *Ctenocephalides felis felis* (66/144), all collected from opossums (*Didelphis albiventris, Didelphis aurita*). At the same time, there was no serologic evidence that these same opossums were exposed to *R. felis* infection (Horta et al., 2007). Herein, *Polygenis* (*Polygenis*) *bohlsi bohlsi* collected from a *T. fosteri* wild rodent and *M. domestica* and *T. macrurus* opossums did not show positive results in qPCR assays. Whereas São Paulo state is known to be an endemic area for *R. felis*-infected fleas (mainly *C. felis felis*) (Horta et al., 2014), little is known about the ecology of fleas and flea-borne rickettsial agents in Pantanal biome. Although Melo et al. (2011) detected antibodies anti-*R. felis* among dogs parasitized by *C. felis felis* in northern Pantanal, homologous reactions were found only to *R. amblyommatis*, *R. parkeri, R. bellii, R. rickettsii* and *R. rhipicephali.*

In conclusion, the present study revealed that wild animals in Pantanal wetland, Brazil, are exposed to SFG rickettsial agents. Domestic dogs and *C. thous* seemed to be exposed to *R. amblyommatis*. However, serological cross-reactions between the related species '*Candidatus* Rickettsia andeanae' and *R. amblyommatis* or a closely related agent cannot be discarded, since all sequences obtained from the collected ticks belonged only to '*Candidatus* Rickettsia andeanae'. Although it was not possible to assess the presence of antibodies to *Rickettsia* spp. in coatis serum samples, the detection of SFG rickettsial agents DNA in ticks collected from these animals suggests that these animals have also been exposed to rickettsial agents. Therefore, future studies are much needed in order to evaluate the capacity of ticks in transmitting '*Candidatus* Rickettsia andeanae'. Additionally, studies aiming at investigating the circulation of '*Candidatus* Rickettsia andeanae' in humans and other mammal hosts should be performed, in order to estimate its zoonotic potential and the impact of rickettsial infection among endangered wild animals in Brazil.

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ttbdis.2017.10.015.

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