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Anaplasmataceae agents among wild mammals and ectoparasites in Brazil

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SUMMARY

Anaplasmataceae agents comprise obligate intracellular bacteria that can cause disease in humans and animals. Between August 2013 and March 2015, 31 *Nasua nasua* (coati), 78 *Cerdocyon thous* (crab-eating fox), seven *Leopardus pardalis* (ocelot), 110 wild rodents, 30 marsupials, and 42 dogs were sampled in the Pantanal wetland, Brazil. In addition, ectoparasites found parasitizing the animals were collected and identified. The present work aimed to investigate the occurrence of Anaplasmataceae agents in wild mammals, domestic dogs and ectoparasites, by molecular and serological techniques. Overall, 14 (17·9%) *C. thous*, seven (16·6%) dogs and one (3·2%) *N. nasua* were seroreactive to *Ehrlichia canis*. Nine dogs, two *C. thous,* one *N. nasua*, eight wild rodents, five marsupials, eight *Amblyomma sculptum,* four *Amblyomma parvum,* 13 *A. sculptum* nymphal pools, two *Amblyomma* larvae pools and one *Polygenis (Polygenis) bohlsi bohlsi* flea pool were positive for *Ehrlichia* spp. closely related to *E. canis*. Seven *N. nasua*, two dogs, one *C. thous,* one *L. pardalis*, four wild rodents, three marsupials, 15 *A. sculptum*, two *Amblyomma ovale,* two *A. parvum* and one *Amblyomma* spp. larval pools were positive for *Anaplasma* spp. closely related to *A. phagocytophilum* or *A. bovis*. The present study provided evidence that wild animals from Brazilian Pantanal are exposed to Anaplasmataceae agents.

Key words: *Anaplasma*, dogs, *Ehrlichia*, fleas, qPCR, ticks, wildlife.

INTRODUCTION

Anaplasmataceae agents are obligate intracellular bacteria, resident in phagosomes and belonging to the order Rickettsiales, family Anaplasmataceae, α sub-division of Proteobacteria, whose cycle in the environment involves complex interactions between invertebrate vectors and vertebrate hosts. Some Anaplasmataceae agents, such as *Ehrlichia* spp. and *Anaplasma* spp., have great importance in veterinary and human medicine. For instance, while *Ehrlichia canis* is the etiological agent of canine monocytic ehrlichiosis (CME), a widespread tick-borne disease among dogs around the world, *A. phagocytophilum* and *Ehrlichia chaffeensis* are the main agents responsible for human granulocytic anaplasmosis and human monocytic ehrlichiosis (HME), respectively,

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in humans in North Hemisphere. The clinical presentation caused by Anaplasmataceae agents includes fever, headache, petechiae, myalgia, althralgia, rash, cough, nausea, vomiting, abdominal pain, diarrhea, leukopenia, thrombocythopenia and elevated liver enzymes [1].

In Brazil, the recent molecular detection of *A. phagocytophilum* [2, 3] in domestic dogs, associated with the detection of new genotypes of *Ehrlichia* spp. and *Anaplasma* spp. in deer [4, 5], birds [6], wild canids [7], domestic and wild felids [8, 9], and rodents [10] has shown a diversity of Anaplasmataceae agents circulating in domestic and wild mammals.

The aim of the present study was to investigate the occurence of Anaplasmataceae agents (*Ehrlichia* and *Anaplasma*) in wild mammals, domestic dogs and their respective ectoparasites in the Pantanal wetland, in order to shed some light on the role of wild animals in the epidemiology of Anaplasmataceae agents in wild environments. The Pantanal is a 160 000 km^2 floodplain located in the centre of the South American continent. It is a mosaic of seasonally inundated native grasslands, savannas and scrub savannas, river corridors, lakes, gallery forests and patches of scrub and semi-deciduous forests. In this highly diverse environment, in which humans, wild and domestic animals and arthropods species share the same habitat, the landscape dynamics and resource availability change according to a multi-year variation of flooding intensity [11]. Although the number of studies investigating the role of wild animals in the epidemiology of diseases caused by Anaplasmataceae agents has increased around the world, few studies [12] have focused on the occurrence of these agents in the Pantanal wetland.

MATERIAL AND METHODS

Ethical aspects

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 and 38787-2) and were endorsed by the Ethics Committee of FCAV/UNESP University (Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista 'Júlio de Mesquita Filho', Câmpus Jaboticabal) no. 006772/13.

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–March) and a dry winter (April–September) [13, 14].

Animals sampled

Between the years of 2013 and 2015, four field expeditions were performed in August 2013, October 2013, August 2014 and March 2015. Free-ranging *C. thous* (crab-eating fox), *N. nasua* (coati) and *L. pardalis* (ocelot) were caught using a Zootech® (Curitiba, PR, Brazil) model wire box live trap (1 × 0·40 × 0·50 m^3), which was made with galvanized wire mesh and baited with a piece of bacon every afternoon. Twenty traps were placed on the ground at 2 km intervals, left open during 24 h and checked twice a day for 12 days. The animals were immobilized with an intramuscular injection of zolazepan and tiletamine at dosages of 8 mg/kg for ocelots and 10 mg/kg crab-eating foxes and coatis. Blood samples were collected by puncture of the cephalic vein stored in Vacutainer® containing EDTA and stored at −20 °C until DNA extraction.

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 seven consecutive nights along linear transects, placed on the ground at 10 m intervals and alternating between trap type in two field expeditions (August 2014 and March 2015). The total capture effort was 200 traps-nights, equally distributed in two expeditions (August 2014 and March 2015). The captured rodents and marsupials were euthanized in order to perform the identification of the animal's species, based on external and cranial morphological characters and karyological analyses. The animals were firstly anesthetized with an intramuscular injection of ketamine (10–30 mg/kg) with acepromazine (5–10 mg/kg) for rodents (proportion 9 : 1), or xylazine (2 mg/kg) for marsupials (1 : 1). After anesthesia, the animals were euthanized with

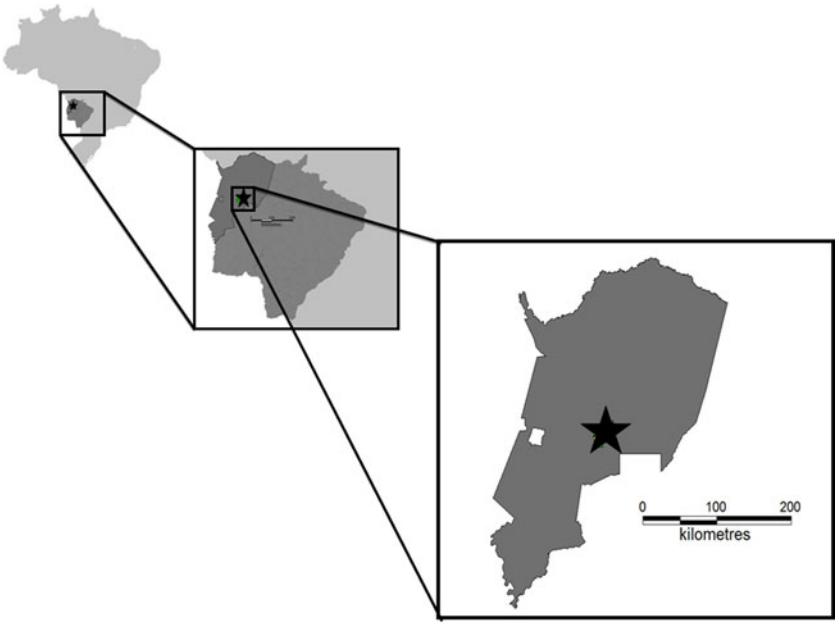

Fig. 1. Capture sites. Map of Mato Grosso do Sul State, central-western Brazil, showing the Pantanal region, where animals samples were sampled in the present study.

potassium chloride, which doses ranged from 75 to 150 mg/kg. Spleen samples were collected and stored in absolute alcohol (Merck®, Kenilworth, Nova Jersey, USA).

A total of 256 animals were captured, including 158 carnivores, including 78 *C. thous*, 31 *N. nasua*, seven *L. pardalis*, 110 wild rodents (77 *Thrichomys fosteri*, 25 *Oecomys mamorae* and eight *Clyomys laticeps*) and 30 wild marsupials (14 *Thylamys macrurus*, 11 *Gracilinanus agilis*, four *Monodelphis domestica* and one *Didelphis albiventris*). Additionally, 42 blood samples from domestic dogs cohabiting the same studied area were collected [13, 14].

Ectoparasites identification

Ticks and fleas found parasitizing the sampled animals were detected by inspection of the skin and carefully removed by forceps or manually. The identification was performed using a stereomicroscope (Leica® MZ 16A, Wetzlar, Germany) and following taxonomic literature for adult tick genera [15, 16], and *Amblyomma* spp. nymphs [17]. *Amblyomma* spp. larvae could not be identified to the species level considering the insufficient literature available until now. The identification of fleas was performed following previously described taxonomic keys [18].

Serological assays

Canids, felids, coatis and rodents' serum samples were individually tested by Indirect Fluorescent Antibody Test (IFAT) in order to detect IgG antibodies to *E. canis* and *A. phagocytophilum*. For that purpose, *E. canis* and *A. phagocytophilum* crude antigens were cultivated in DH82 cells and HL-60 cells infected with Jaboticabal strain of *E. canis* strain [19] and Webster strain of *A. phagocytophilum,* respectively. Briefly, antigen slides were removed from storage and allowed to thaw at room temperature for 30 min. Ten microliters of twofold dilutions of sera (starting at 1 : 80, the cut-off for *E. canis* and *A. phagocytophilum*) were placed in wells on antigen slides. On each slide, previously determined non-reactive and reactive serum samples to *E. canis* were used as negative and positive controls, respectively [9, 20]. A known positive *A. phagocytophilum* serum (titer 1 : 2560) was obtained from a horse experimentally infected with the Webster strain of *A. phagocytophilum* at Department of Veterinary Pathology, UNESP, Jaboticabal, São Paulo, Brazil. A horse serum sample negative for *A. phagocytophilum* was used as negative control. Slides were incubated at 37 °C in a moist chamber for 30 min, washed three times in PBS (pH 7·2) for 5 min, and air dried at room temperature. Then, the slides were incubated with fluorescein isothiocyanate labeled anti-mouse IgG (Sigma®, St. Louis,

USA) for wild rodents, goat anti-dog IgG (Sigma®, St. Louis, USA) for *C. thous* and domestic dogs, goat anti-cat IgG (Sigma®, St. Louis, USA) for *L. pardalis*, goat anti-raccoon IgG (Sigma®, St. Louis, USA) for *N. nasua* and goat anti-horse IgG (Sigma®, St. Louis, USA) for *A. phagocytophilum* control serum sample. Anti-cat conjugate (dilution of 1 : 32) for feline samples, anti-dog conjugate for the canine samples (dilution of 1 : 32), anti-raccoon (dilution of 1 : 10), anti-mouse conjugate for the wild rodents samples and anti-horse (dilution of 1 : 64) were diluted according to the manufacturer's instructions and then added to each well. These slides were incubated again at 37 °C, washed three times in PBS, once more in distilled water, and air dried at room temperature. Finally, slides were overlaid with buffered glycerin (pH 8·7), covered with glass coverslips, and examined with a fluorescence microscope (Olympus®, Tokyo, Japan). Unfortunately, it was not possible to test the marsupial's serum samples, due to the unavailability of conjugate for this animal species in the laboratory.

Molecular assays

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 DNeasy Blood & Tissue Kit (QIAGEN®, Valencia, CA, USA), according to the manufacturer's instructions. DNA was extracted from 523 tick samples, including 228 (43·5%) adults, 256 (48·9%) pooled nymphs, and 39 (7·4%) pooled larvae. DNA extraction from ticks was processed in pools for nymphs (up to five individuals) and larvae (up to 10 individuals). The adults were processed individually. A total of 39 pooled fleas samples (each one consisting of up to five individuals) were used for DNA extraction. Ticks and fleas were macerated and DNA extracted using the same kit above mentioned [13, 14].

In order to verify the presence of amplifiable DNA in the samples, internal control polymerase chain reaction (PCR) assays targeting fragments of mammalian glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) [21], tick mitochondrial 16S rRNA genes [22] and flea cytochrome-c oxidase subunit I (*COX1*) [23] genes were performed (Table S1).

First, a previously described broad range multiplex qPCR protocol based on *groEL* gene (Table S2) to detect and quantify *Ehrlichia* spp. and *Anaplasma* spp. [10] and cPCR assays targeting 16SrRNA genes [24, 25] (Table S1) of *Ehrlichia* spp. and *Anaplasma* spp. were performed. The limit of detection of *groE* gene fragment quantification for *Ehrlichia* spp. and *Anaplasma* was 10 copies/µl [10]. All positive samples in *groEL* qPCR and 16SrRNA gene cPCR reactions were tested using previously described specific qPCR assays to detect and quantify *A. phagocytophilum* (*msp2*) [26], *E. canis* (*dsb*) [27] and *E. chaffeensis* (*vlpt*) [28] (Table S2). Additionally, cPCR assays targeting four other protein-coding genes, namely *groESL* [29, 30] for *Ehrlichia* and *Anaplasma* spp., *omp1* [31] for *Ehrlichia* spp., *dsb* [27] for *E. canis*, *trp36* [32] for *E. canis* and *Ehrlichia minasensis* sp. nov., and *msp5* [33] for *Anaplasma* spp. (Table S1).

The TaqMan qPCR reactions based on *groEL*, *msp2, dsb* and *vlpt* genes were performed with a final volume of 10 µl containing 5 µl GoTaq® Probe qPCR Master Mix (Promega Corporation, Madison USA), 1·2 µM of each primer and hydrolysis probe (Table S2) 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 plasmid DNA concentrations (Integrated DNA Technologies®, Coralville, Iowa, USA) ($2{\cdot}0 \times 10^{7}$–$2{\cdot}0 \times 10^{0}$ copies/µl). The number of plasmid copies was determined in accordance with the formula (ng/µl DNA/plasmid size (bp) × 660]) × $6{\cdot}022 \times 10^{23}$ × plasmid 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 ($E = 10^{-1/\text{slope}}$). 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% of sensitivity [34].

The cPCR reactions contained 10X PCR buffer (Life Technologies®, Carlsbad, CA, USA), 1 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) (Table S1). *Ehrlichia canis* and *Anaplasma* spp. DNA positive controls were obtained from naturally infected cats [9]. Ultra-pure sterile water (Life Technologies®, Carlsbad, CA, USA) was used as negative control in all PCR

assays. 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 for all positive samples in cPCRs assays, using the BigDye® Terminator v3.1Cycle Sequencing Kit (Thermo Fisher Scientific®, Waltham, MA, USA) and ABI PRISM 310DNA Analyzer (Applied Biosystems®, Foster City, CA, EUA).

Bioinformatics/phylogenetic analysis

Sequences obtained from positive samples in cPCR assays were first submitted to a screening test using Phred-Phrap software version 23 [35] in order to evaluate the electropherogram quality and to obtain consensus sequences from the alignment of sense and antisense sequences. The BLAST program [36] was used to analyze the sequences of nucleotides (BLASTn), aiming to browse and compare with sequences previously deposited in an international database (GenBank) [37]. All sequences that showed appropriate quality standards and identity with *Ehrlichia* spp. or *Anaplasma* spp. were deposited in Genbank. The sequences were aligned with sequences published in GenBank using MAFFT software, version 7 [38].

Phylogenetic inference was based on Bayesian Inference (BI) method. The BI analysis was performed with MrBayes 3.1.2 [39]. 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 [40], under the Akaike Information Criterion (AIC) [41]. All phylogenetic analyses were performed using CIPRES Science Gateway [41]. The trees were examined in Treegraph 2.0.56-381 beta [42].

RESULTS

A total of 1582 ticks parasitizing the sampled mammals were collected, of which 1033 (65·2% [115 adults and 918 nymphs]) belonging to *Amblyomma sculptum* Barlese, 1888 species; 241 (15·2% [78 adults and 163 nymphs]) belonging to *Amblyomma parvum* Aragão, 1908 species; 32 (2%) *Amblyomma ovale* (Koch, 1844) adults; one (0·06%) *Amblyomma tigrinum* Koch, 1844 adult; one (0·06%) *Rhipicephalus (Boophilus) microplus* (Canestrini, 1887) adult; one (0·06%) *Rhipicephalus sanguineus* sensu lato adult; four (0·2%) *Amblyomma auricularium* (Conil, 1878) nymphs; and 269 (17%) *Amblyomma* larvae. Besides, a total of 80 *Polygenis (Polygenis) bohlsi bohlsi* fleas were also collected (Table 1) [13, 14].

All 298 DNA animal samples amplified the predicted product for *GAPDH* gene. Out of 523 DNA sampled ticks, 31 [5·9% (23 *A. parvum* adults, four *A. sculptum* adults, one *A. ovale* adult, one *A. parvum* nymph and two pooled *Amblyomma* spp. Larvae)] showed negative results for the tick mitochondrial 16S rRNA gene and were excluded from subsequent analyses. Only one flea DNA sample did not amplify the predicted product for *COX1* gene and was also excluded from subsequent analyses [13, 14].

Overall, 14 (17·9%) *C. thous*, seven (16·6%) *C. familiaris* and one(3·2%) *N. nasua* were seroreactive (titre $\geqslant$ 80) to *E. canis*. The seroreactive animals showed titres for *E. canis* ranging from 80 to 1280. No wild rodent showed to be seroreactive to *E. canis* antigen. Ticks were collected from 12 (54·5%) of 22 seroreactive animals. None of the animals was seroreactive for *A. phagocytophilum* (Table 2).

Twenty-seven animals (9%) were positive in cPCR assays for *Ehrlichia* spp. based on 16SrRNA gene, including nine (21·4%) *C. familiaris*, two (7·6%) *C. thous*, one (3·8%) *N. nasua*, four (15·3%) *T. fosteri*, four (15·3%) *O. mamorae*, three (11·5%) *T. macrurus*, two *M. domestica* (50%) and two (7·6%) *G. agilis*. Additionally, eight *A. sculptum* adults, four *A. parvum* adults, 13 *A. sculptum* nymphal pools, two *Amblyomma* larvae pools and one (2·5%) *Polygenis* (*P.*) *bohlsi bohlsi* flea pool showed positive results in cPCR assays for *Ehrlichia* spp. based on 16SrRNA gene. Two *A. sculptum* adults positive in cPCR assays for *Ehrlichia* spp. based on 16SrRNA were collected from a *C. thous* that was also positive in *Ehrlichia*-cPCR assay based on 16SrRNA gene. Four (9·5%) dogs were positive in qPCR assays for *Ehrlichia* spp. based on *groEL* gene. Nine dogs (21·4%) and one *Polygenis* (*P.*) *bohlsi bohlsi* flea pool also showed positive results in a specific qPCR for *E. canis* based on *dsb* gene (Table 3). All 16S rRNA gene-*Ehrlichia* sequences were deposited in Genbank international database under the accession

Table 1. *Tick species collected from wild mammals captured between August 2013 and March 2015 in Pantanal wetland, Brazil*

			Ticks[a]								FLEAS
Animal species	No. 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 (crab-eating fox)	78	35 (44·8)	34M; 55F; 643N	21M; 34F; 3N	1F	4M; 1F				204L	
Nasua nasua (coati)	31	22 (70·9)	10M; 13F; 275N	11M; 6F; 12N		20M; 7F	3N	1F		21L	
Leopardus pardalis (ocelot)	7	2 (28·5)		3M;. 3F							
Canis lupus familiaris (domestic dog)	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	
Thylamys macrurus	14	1 (7·1)		18N						1L	1
Monodelphis domestica	4	0 (0)									4
Gracilinamus 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*.

Table 2. *Number of domestic dogs and wild mammals seroreactive to* Ehrlichia canis *in Pantanal wetland, Brazil*

	Titers for *Ehrlichia canis*				
Animals (no. tested)	80	160	320	640	1280
Canis familiaris (domestic dog, 42)	7	5	2	1	1
Cerdocyon thous (crab-eating fox, 78)	14	7	3	1	–
Leopardus pardalis (ocelot, 7)	0	–	–	–	–
Nasua nasua (coati, 31)	1	1	1	1	–
Wild rodents (110)	0	–	–	–	–
Total (158)	22	13	6	3	1

numbers KY499155-KY499181 and KY930380-KY930407.

Seven (22·5%) *N. nasua*, one (1·2%) *C. thous*, one (14·2%) *L. pardalis*, two (2·5%) *T. fosteri*, one (12·5%) *C. laticeps*, one (9%) *G. agilis*, one (7·1%) *T. macrurus*, two (4·7%) *C. familiaris*, (0·9%), two *A. sculptum* nymphal pools and one (3·1%) *A. ovale* adult showed positivity in cPCR assays for *Anaplasma* spp. based on 16SrRNA gene. Two (3·5%) *T. fosteri,* one (4%) *O. mamorae*, two (18·1%) *G. agilis,* one (7·1%) *T. macrurus,* 10 (3·1%) *A. sculptum* adults, five (2·4%) *A. sculptum* nymphal pools, two (1·5%) *A. parvum* adults, two (6·2%) *A. ovale* adults, and one (2·5%) *Amblyomma* larvae pool, were positive in qPCR for *Anaplasma* spp. based on *groEL* gene (Table 3). Positive samples in cPCR (16SrRNA gene) and qPCR (*groEL*) assays for *Anaplasma* spp. did not show positive results in cPCR assays based on *groESL* and *msp5* genes neither in the qPCR assay for *A. phagocytophilum* based on *msp2* gene. All 16SrRNA gene-*Anaplasma* sequences were deposited in Genbank international database under the accession numbers KY499182 -KY499201.

Co-positivity was observed between the molecular assays to *Ehrlichia* spp. and *Anaplasma* spp. or between the molecular and serological assays to *Ehrlichia* spp. and *Ehrlichia canis* in two dogs, one *N. nasua*, one *C. thous,* one *T. macrurus* and one *G. agilis* (Table 4).

The BLAST analysis of the 16SrRNA gene of *Ehrlichia* spp. showed that sequences obtained from *C. thous*, *N. nasua*, *M. domestica*, *T. macrurus,* *G. agilis*, *T. fosteri*, *O. mamorae,* dogs, a pool of *Polygenis* (*P.*) *bohlsi bohlsi*, *A. sculptum* nymphal pools, *A. sculptum* adults, *Amblyomma* spp. larvae pools and *A. parvum* adults samples, showed 99%–100% identity with *Ehrlichia* spp. sequences obtained from free-living wild animals from Brazil (KX898136, JQ260861, JQ260855), an *E. canis* sequence from Brazil (JX118827), an *E. canis* sequence obtained from *R. sanguineus* ticks collected in Philippines (JN121379), and with an *E. canis* sequence from Malaysia (KR920044). The 16SrRNA gene sequences of *Anaplasma* spp. obtained from *N. nasua*, *L. pardalis*, *C. thous*, dogs, *T. fosteri*, *C. laticeps*, *T. macrurus*, *G. agilis*, *A. sculptum* nymphal pool and *A. ovale* samples, showed 98–100% of identity with an *Anaplasma* spp. sequence obtained from *Amblyomma cajennense sensu lato* ticks (KJ831219) collected in the state of Mato Grosso, Brazil, *Anaplasma platys* (KU500914, KU534873) and *A. phagocytophilum* (CP006618, GU064900) by BLAST analysis (Table S3).

The phylogenetic analysis based on 16SrRNA gene fragments of *Ehrlichia* spp. grouped the sequences obtained from mammals, ticks and a flea pool (KY499155-KY499181 and KY930380-KY930407) together and in the same branch of *E. canis* sequences from Brazil (EF195135) and other countries (EF011111, EU106856, U26740) and with *Ehrlichia* spp. sequences obtained from free-living *N. jubata* and wild felids from Brazil (KX898136, EU376114), based on Bayesian analysis, with clade support of 61 (Fig. 2). Regarding the phylogenetic analysis of *Anaplasma* based on 16SrRNA gene fragments, *Anaplasma* spp. sequences obtained from one *N. nasua* (KY499193), one *C. familiaris* (KY499188) and one *A. sculptum* nymphal pool (KY499182) were grouped in the same clade and together with one sequence obtained from *Carcara plancus* from Brazil (JN217096), one *A. phagocytophilum* sequence (GU236670) obtained from an Austrian dog and one *Anaplasma* sp. sequence (KF964051) obtained from a domestic cat from Brazil, based on BI analysis. Four *Anaplasma* spp. 16SrRNA gene sequences obtained from *N. nasua* (KY499184, KY499187, KY499194, KY499195), one sequence obtained from *L. pardalis* (KY499183), one sequence obtained from *C. thous* (KY499185), two sequences obtained from *T. fosteri* (KY499197, KY499198), one sequence obtained from *C. laticeps* (KY499196), one sequence obtained from *T. macrurus* (KY499200), one sequence obtained from *G. agilis* (KY499199), one sequence obtained from one *A. sculptum* nymphal pool (KY499201) and one sequence obtained from one *A. ovale* adult (KY499191) were positioned in

Table 3. *Number of domestic dogs and wild mammals positive in qPCR and cPCR assays for* Ehrlichia *and* Anaplasma

	Agents									
	Ehrlichia							*Anaplasma*		
HOST	qPCR *groEL*	qPCR *dsb*	cPCR 16SrRNA	qPCR *groEL*+ qPCR *dsb*	qPCR *groEL*+ cPCR 16SrRNA	qPCR *dsb* + cPCR 16SrRNA	qPCR *groEL* + qPCR *dsb* + cPCR 16SrRNA	qPCR *groEL*	cPCR 16SrRNA	qPCR *groEL*+ cPCR 16SrRNA
Canis familiaris (domestic dog)	4	9	9	1	2	4	3	–	2	–
Cerdocyon thous (crab-eating fox)	–	–	2	–	–	–	–	–	1	–
Nasua nasua (coati)	–	–	1	–	–	–	–	–	7	–
Leopardus pardalis (ocelot)	–	–	–	–	–	–	–	–	1	–
Clyomys laticeps	–	–	–	–	–	–	–	–	1	–
Thrichomys fosteri	–	–	4	–	–	–	–	2	2	2
Oecomys mamorae	–	–	4	–	–	–	–	1	–	–
Gracilinamus agilis	–	–	2	–	–	–	–	2	1	1
Thylamys macrurus	–	–	3	–	–	–	–	1	1	1
Monodelphis domestica	–	–	2	–	–	–	–	–	–	
Amblyomma ovale adult	–	–	–	–	–	–	–	2	1	1
Amblyomma sculptum adult	–	–	8	–	–	–	–	10	–	–
Amblyomma sculptum nymphal pool	–	–	13	–	–	–	–	5	2	2
Amblyomma parvum adult	–	–	4	–	–	–	–	2	–	–
Amblyomma larvae pool	–	–	2	–	–	–	–	1	–	–
Polygenis (Polygenis) bohlsi bohlsi	–	–	1	–	–	1	–	–	–	–
Total	4	9	55	1	2	5	3	26	19	7

Table 4. *Co-positivity observed between the molecular assays to* Ehrlichia *spp. and* Anaplasma *spp. and between molecular and serological assays to* Ehrlichia *spp. and* Ehrlichia canis

Host	Serology	cPCR		qPCR		
	Ehrlichia canis	*Ehrlichia* spp.	*Anaplasma* spp.	*Ehrlichia* spp.	*Anaplasma* spp.	*Ehrlichia canis*
Canis familiaris (domestic dog)	+	+	–	+	–	+
Canis familiaris (domestic dog)	+	–	–	–	–	+
Nasua nasua (coati)	+	+	+	–	–	–
Cerdocyon thous (crabeating fox)	+	+	–	–	–	–
Thylamys macrurus	–	+	+	–	–	–
Gracilinamus agilis	–	+	–	–	+	–

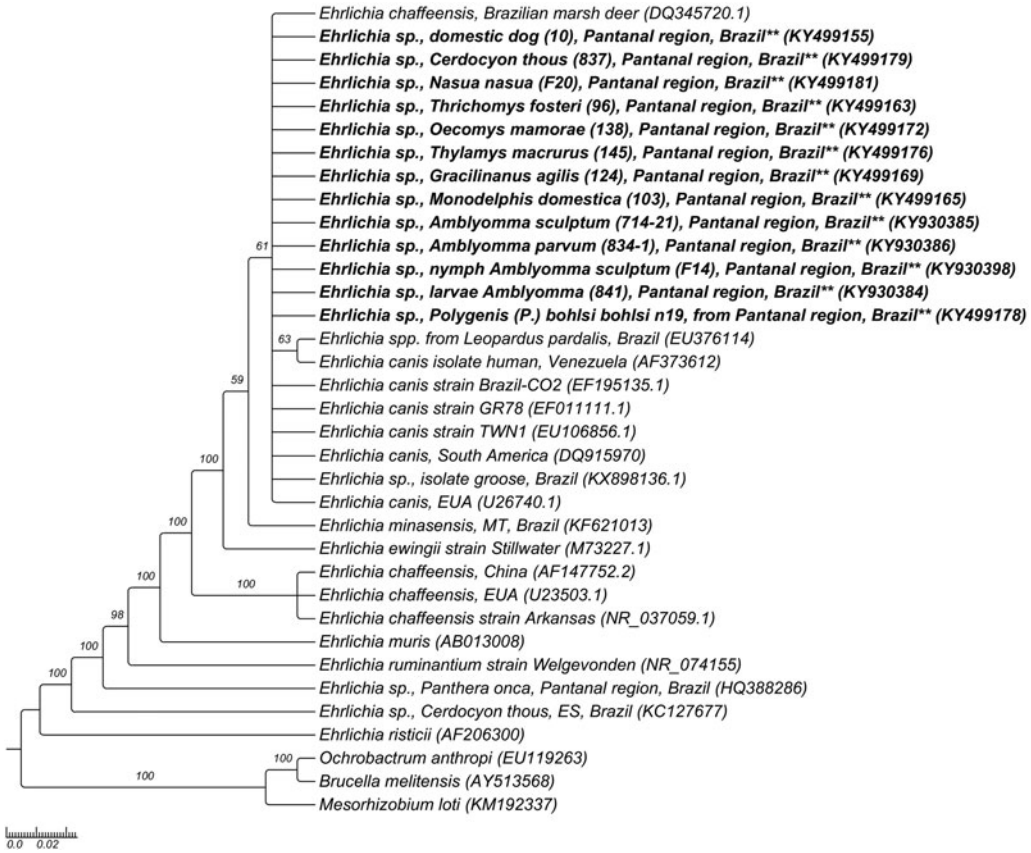

Fig. 2. Phylogenetic tree constructed with 860 pb *Ehrlichia* spp.-16SrRNA sequences, using Bayesian method and GTR + G + I evolutionary model. Numbers at nodes correspond to Bayesian posterior probabilities over 50, using *Mesorhizobium loti* (KM192337), *Brucella melitensis* (AY513568) and *Ochrobactrum anthropi* (EU119263) as outgroups.

the same clade of *A. bovis* (LC012812), with clade support of 84 (Fig. 3).

DISCUSSION

In the present study, the circulation of *E. canis* among dogs in Brazilian Pantanal wetland was confirmed by both molecular and serological methods. Considering the pathogenic potential of *E. canis* in domestic dogs [1], the role of these animals as a source of *E. canis* infection to wild carnivores cohabiting the same area and the impact of the infection on wildlife health requires further studies.

Reports on seropositivity to *E. canis* among wild carnivores are scarce in Brazil. In fact, there is only

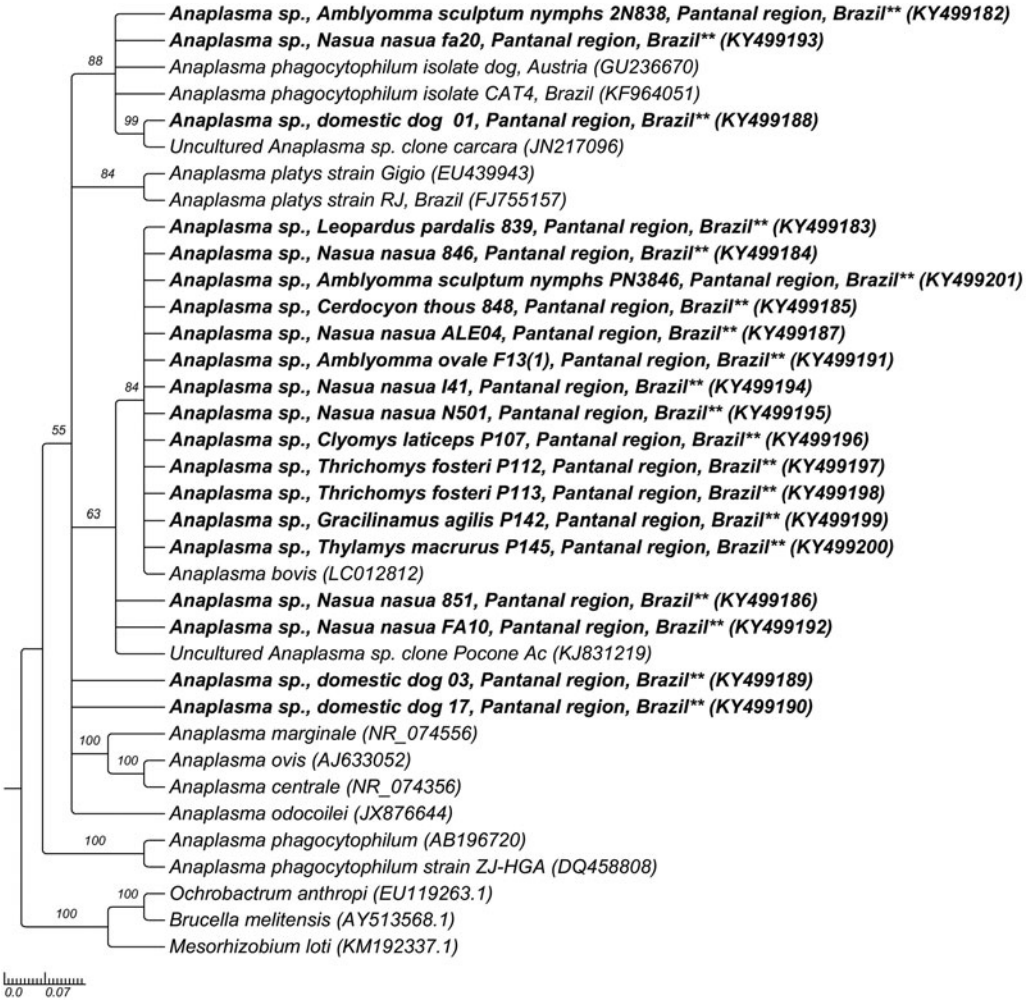

Fig. 3. Phylogenetic tree constructed with 600 pb *Anaplasma* spp.-16SrRNA sequences, using Bayesian method and GTR + G + I evolutionary model. Numbers at nodes correspond to Bayesian posterior probabilities over 50, using *Mesorhizobium loti* (KM192337), *Brucella melitensis* (AY513568) and *Ochrobactrum anthropi* (EU119263) as outgroups.

one report concerning the serological detection of antibodies to *E. canis* among wild felids maintained in captivity in zoos in the states of São Paulo and Mato Grosso state, Brazil [8]. Herein, seroprevalence rates of 17·9% and 3·2% were found among free-living *C. thous* and *N. nasua*, respectively. However, seropositive animals showed negative results in the specific qPCR assay for *E. canis* based on *dsb* gene. Due to the unavailability of other *Ehrlichia* species antigens, serological cross-reactions between *E. canis* and closely related agents could not be excluded

Ehrlichia spp. DNA was detected in two *C. thous* from Pantanal wetland. Previous studies had already detected *Ehrlichia* spp. DNA among wild canids (*C. thous*, *Speothos venaticus* and *Canis lupus*) maintained in captivity in Brazilian zoos [8] and among road-killed *C. thous* in the state of Espírito Santo, southeastern Brazil [43]. However, it is worth noticing that while *Ehrlichia* spp. DNA has been detected among Brazilian canids, no clinical signs suggestive of ehrlichiosis have been reported until now in this group of wild mammals. Future studies are needed in order to clarify the role of these animals in the epidemiology of *Ehrlichia* spp. in nature.

Among the wild felids, all seven *L. pardalis* trapped in this present study showed negative results in molecular and serological assays for *Ehrlichia* spp. In a previous study, an *Ehrlichia dsb* sequence obtained in one out of 10 jaguars also sampled in the Pantanal biome were closely related to *Ehrlichia ruminantium* [44]. While *A. sculptum* (formerly named as *A. cajennense*), *Amblyomma triste* and *R.* (*B.*) *microplus* were previously found parasitizing jaguars in Pantanal [44], only *A. parvum* was found parasitizing *L. pardalis* in

the present study at the time of sample collection. Further studies are needed to determine the real role of different tick species in the transmission of ehrlichial agents among wild felids in Brazil.

Few studies have assessed the occurrence of Anaplasmataceae agents in marsupials. Previously, antibodies to *E. canis* were detected in 14·6% of opossums (*Didelphis aurita* and *Didelphis albiventris*) trapped in São Paulo state, southeastern Brazil [45]. Although serological assays were not performed in marsupials' serum samples collected in the present study, *Ehrlichia* DNA was detected in 23·3% of sampled marsupials. The present study highlights that marsupials may participate in the biological cycles of *Ehrlichia* in the environment. To the best authors' knowledge, this was the first molecular evidence of exposure to *Ehrlichia* spp. among free-living marsupials in the world until now.

Herein, *Ehrlichia* DNA was detected in 7·2% of wild rodents (*T. fosteri* and *O. mamorae*) sampled in southern Pantanal. Recently, *Ehrlichia* DNA was detected in 24% of wild rodents (*O. mamorae, T. fosteri*, *C. laticeps* and *Calomys cerqueirai*) sampled in the biome Pantanal [10]. Although rodents are considered hosts for *E. chaffeensis* in China and Korea [46–48], the role of wild rodents in natural cycles of *Ehrlichia* species and the arthropod vectors involved in the transmission routes remain unknown in Brazil.

Ehrlichia canis DNA was detected in one *Polygenis (P.) bohlsi bohlsi*flea pool collected from a specimen of *T. fosteri* rodent that was negative in PCR assays for *Ehrlichia* spp. In a previous study, *E. canis* DNA was detected in one (1/1) *Cediopsylla inaequalis* and 3% (2/75) of the *Xenopsylla cheopis* fleas collected from red foxes (*Vulpes vulpes*) in Italy [49]. Although *E. canis* DNA was detected in a *Polygenis (P.) bohlsi bohlsi*flea pool by qPCR and cPCR assays, we believe that the positivity observed was due to residual ehrlichial DNA from host blood in the siphonapteran digestive tract.

Anaplasma DNA was detected among wild carnivores (*C. thous*, *L. pardalis* and *N. nasua*) and dogs sampled in the present study. Previously, *Anaplasma* DNA was detected in three wild felids (*L. tigrinus*) and one wild canid (*S. venaticus*) maintained in captivity in zoos in the state of São Paulo [7]. Additionally, wild rodents and marsupials were also positive for *Anaplasma* spp. Recently, *Anaplasma* DNA was detected in nine rodents (*Rattus rattus*, *Akodon* sp., *Sphiggurus villosus* and *Calomys cerqueirae*) sampled in Atlantic Forest and Caatinga biomes in Brazil [10]. In the northern region of the Brazilian Pantanal, *Anaplasma* DNA was also detected in only one *Hylaeamys megacephalus* wild rodent (1/4) [50]. Additional studies should be done in order to access the role of wild carnivores, rodents and marsupials in the *Anaplasma* epidemiology in South America.

The arthropod vectors involved in *Anaplasma* and *Ehrlichia* species transmission cycles among wild mammals in Brazil are still unknown. Previously, unclassified *Anaplasma* spp. and *Ehrlichia* spp. were detected in one *A. sculptum* tick collected from a dog [12] and in *A. sculptum* (formerly named as *A. cajennense*), *A. triste* and *Amblyomma* spp. nymph ticks collected from *P. onca* [44], respectively, in Pantanal biome. The present study reports the molecular detection of *Anaplasma* spp. among *A. sculptum, A. ovale* and *A. parvum* ticks, and *Ehrlichia* spp. in *A. sculptum, A. parvum* and *Amblyomma* spp. larvae. According to some authors, questing tick samples are preferable in order to assess the real role of arthropods as vectors of Anaplasmataceae agents [51]. Considering the fact that we detected *Ehrlichia* and *Anaplasma* DNA in ticks found parasitizing sampled animals, we assumed that tick positivity could be related to the remnant of infected host blood meal.

The phylogenetic inferences based on a small fragment of 16S rRNA gene positioned the *Ehrlichia* and *Anaplasma* genotypes in the same clade of *E. canis* and *A. bovis*, respectively. The variable amplification of different target genes in the present study, which precluded additional phylogenetic inferences, could be explained by low bacteraemia level in mammalian blood or spleen samples and ectoparasites. Moreover, PCR protocols used for amplification of different target genes have been proven unsuitable for amplification of variants of *Anaplasma* and *Ehrlichia* species infecting wild mammals in Brazil, as previously reported [6, 7, 10]. The phylogenetic analysis based on short 16S rRNA gene fragments did not provide sufficient genetic discrimination to allow the identification of *Ehrlichia* and *Anaplasma* species. Although genotypes closely related to *E. chaffeensis, E. canis* and *A. phagocytophilum* were detected in deer [4, 5], wild carnivores [7], birds [6], and domestic cats [9] in Brazil, these new genotypes have not been isolated so far. Alternatively, species-specific qPCR assays might not have sufficient sensitivity to detect the presence of *E. canis* and *E. chaffeensis* in wild animals' biological samples in the present study.

In conclusion, the present study revealed that wild animals and ticks in southern Pantanal region, Brazil, are exposed to Anaplasmataceae agents. The role of wild animals in the epidemiology of Anaplasmataceae agents as reservoirs and the impact of the infection on wildlife health should be further investigated. Although *Anaplasma* and *Ehrlichia* have been molecularly detected among ticks and fleas collected from sampled animals, the vectors involved in the natural cycles of Anaplasmataceae agents remain unknown in wild environments in Brazil. Therefore, future studies aiming at isolating tick-borne bacteria that circulates among wildlife are much needed in order to achieve a deeply molecular and antigenic characterization of new Anaplasmataceae strains in Brazil.

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AUTHORS' CONTRIBUTIONS

KCMS performed the study, carried out sampling and laboratory work, and drafted the manuscript; ACC helped with molecular analyzes; HMH helped with the animals captures in Pantanal; JSD donated Webster strain of *A. phagocytophilum* and prepared antigen slides; DMBB helped with arthropods identification; RZM donated Jaboticabal strain of *E. canis* and prepared antigen slides; MRA conceived the study and reviewed the manuscript critically for important scientific and intellectual content. All authors read, reviewed, and approved the submitted version.

DECLARATION OF INTEREST

None.

SUPPLEMENTARY MATERIAL

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