UNIVERSIDADE ESTADUAL PAULISTA - UNESP
CÂMPUS DE JABOTICABAL

DETECÇÃO SOROLÓGICA E CARACTERIZAÇÃO MOLECULAR DE AGENTES TRANSMITIDOS POR ARTRÓPODES EM ANIMAIS SELVAGENS E DOMÉSTICOS NA REGIÃO DO PANTANAL SUL MATOGROSSENSE

Keyla Carstens Marques de Sousa
Médica Veterinária

2017

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Orientador: Prof. Dr. Marcos Rogério André

Tese apresentada a Faculdade de Ciências Agrarias e Veterinárias – UNESP, campus de Jaboticabal, como parte das exigências para a obtenção do titulo de doutor em Medicina Veterinária, área: Medicina Veterinária Preventiva.

2017

Sousa, Keyla Carstens Marques de Sousa
M499a Detecção sorológica e caracterização molecular de agentes transmitidos por artrópodes em animais selvagens e domésticos na região do Pantanal Sul Matogrossense / Keyla Carstens Marques de Sousa. – – Jaboticabal, 2017
vi, 213 p. : il. ; 29 cm

Tese (doutorado) - Universidade Estadual Paulista, Faculdade de Ciências Agrárias e Veterinárias, 2017
Orientador: Marcos Rogério André
Banca examinadora: Estevam Guilherme Lux Hoppe, Heitor Miraglia Herrera, Lúcia Helena O'Dwyer, Rosangela Zacarias Machado
Bibliografia

1. Agentes Anaplasmataceae. 2. *Bartonella* spp.. 3. Micoplasmas hemotróficos. 4. Rickettsiales. 5. *Hepatozoon* sp. 6. Piroplasmídeos. I. Título. II. Jaboticabal-Faculdade de Ciências Agrárias e Veterinárias.

CDU 619:616.728.3:636.92

Ficha catalográfica elaborada pela Seção Técnica de Aquisição e Tratamento da Informação – Serviço Técnico de Biblioteca e Documentação - UNESP, Câmpus de Jaboticabal.

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CERTIFICADO DE APROVAÇÃO

TÍTULO DA TESE: DETECÇÃO SOROLÓGICA E CARACTERIZAÇÃO MOLECULAR DE AGENTES TRANSMITIDOS POR ARTRÓPODES EM ANIMAIS SELVAGENS E DOMÉSTICOS NA REGIÃO DO PANTANAL SUL MATOGROSSENSE

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Aprovada como parte das exigências para obtenção do Título de Doutora em MEDICINA VETERINÁRIA, área: MEDICINA VETERINARIA PREVENTIVA pela Comissão Examinadora:

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Jaboticabal, 03 de fevereiro de 2017

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KEYLA CARSTENS MARQUES DE SOUSA – Filha de João José Marques de Sousa e Rosania Carstens Marques de Sousa, nascida em 18 de Janeiro de 1986 na cidade de Campo Grande, Mato Grosso Sul. Foi graduada em Medicina Veterinária pela Universidade Federal de Mato Grosso do Sul, em dezembro de 2009. Obteve o título de mestre em Medina Veterinária, Área de Concentracão: Patologia Veterinária, na Faculdade de Ciencias Agrarias e Veterinarias, Unesp, Jaboticabal, sob a orientação do Prof. Dr. Gilson Pereira de Oliveira, com a dissertação intitulada: Co-infecção por *Ehrlichia canis, Leishmania infantum chagasi* e *Babesia canis* em cães naturalmente infectados na cidade de Campo Grande, Mato Grosso do Sul. Em março de 2013, ingressou no curso de doutorado em Medicina Veterinária, Área de Concentracão: Medicina Veterinária Preventiva, na Faculdade de Ciencias Agrarias e Veterinarias, Unesp, Jaboticabal– SP, é bolsista de doutorado da Fundação de Amparo à Pesquisa do Estado de São Paulo sob orientação do Prof. Dr. Marcos Rogério André.

Epígrafe

"A vida"

"Depois de muitas quedas, eu descobri que, às vezes, quando tudo dá errado, acontecem coisas tão maravilhosas que jamais teriam acontecido se tudo tivesse dado certo. Eu percebi que quando me amei de verdade pude compreender que, em qualquer circunstância eu estava no lugar certo,na hora certa.
Então pude relaxar... pude perceber que o sofrimento emocional é um sinal de que estou indo contra a minha verdade.
Parei de desejar que a minha vida fosse diferente e comecei a ver que tudo o que acontece contribui para o meu crescimento.
Desisti de querer ter sempre razão e com isso errei muito menos vezes.
Desisti de ficar revivendo o passado e de me preocupar com o futuro. Isso me mantém no presente, que é onde a vida acontece.
Descobri que na vida a gente tem mais é que se jogar, porque os tombos são inevitáveis.
Percebi que a minha mente pode me atormentar e me decepcionar. Mas quando eu a coloco a serviço do meu coração, ela se torna uma grande e valiosa aliada. Também percebi que sem amor, sem carinho e sem verdadeiros amigos a vida é vazia e se torna amarga.
Ser feliz é reconhecer que vale a pena viver, apesar de todos os desafios, incompreensões e períodos de crise. É agradecer a Deus a cada manhã pelo milagre da vida.
Pedras no caminho? Guardo todas, um dia vou construir um castelo.. "

Mario Quintana

Dedicatória

Dedico e ofereço minha tese de doutorado aos meus pais João e Rosania e ao meu irmão Wagner, que sempre estiveram ao meu lado e me apoiaram em tudo. Muito obrigada!

AGRADECIMENTOS

Agradeço...

A Deus, por sempre me dar força na vida.

Á minha mãe, meu pai e meu irmão, por me darem o apoio, estímulo e perseverança sempre que precisei

Ao meu orientador, Prof. Dr. Marcos Rogério André que abriu as portas da pesquisa acadêmica e, com muita paciência, acompanhou o desenvolvimento dos meus trabalhos. Somente com sua ajuda e seu apoio tive a oportunidade de ingressar nesta carreira. Eternamente grata.

Ao Professor Heitor Miraglia Herrera, pela amizade, valiosos ensinamentos, e pela demonstração de companheirismo e solidariedade durante todo o curso, que resultaram na conclusão desse trabalho. Eternamente grata.

A Professora Rosangela Zacarias Machado, pelos conselhos e ensinamentos de uma vida.

A Professora Mirela Tinucci Costa, por aceitar me orientar no começo do curso e por todos os conselhos e ensinamentos.

Ao meu namorado Haroldo Neves, pelos conselhos, por ter me acompanhado durante todo o meu doutorado, pela pasciência e por sempre me apoiar.

Aos meus colegas de laboratório Priscila Ikeda, Priscila Preve, Jyan, Renan, Otávio, Paulo, Kayo, Simone, Inalda, Carlos, Rafaela e Luís, pela companhia prazerosa e pela amizade.

Às minhas amigas Carla Freschi e Marcia Jusi pelas alegrias, risadas e pelos valiosos ensinamentos.

Aos meus colegas Filipe Martins Santos, Wanessa Teixeira Gomes Barreto, Gabriel Carvalho Macedo e João Bosco Campos, por me ajudaram nas colheitas de material no Pantanal, sempre com muita boa vontade.

À Fabiana Lopes Rocha, por me ajudar nas colheitas e pelos valiosos ensinamentos.

Ao professor Pedro Cordeiro Estrela de Andrade Pinto, pelo auxílio na identificação dos pequenos mamíferos.

Aos meus queridos amigos Tâmara e Matheus, pelas alegrias e risadas de sempre que fazem as pequenas coisas importantes na vida.

A todos meus amigos e colegas de Jaboticabal da faculdade ou não que são do convívio e nos dão forças para sempre continuar.

Aos pesquisadores Francisco Borges Costa e Thiago Fernandes Martins, pela amizade, conselhos e por toda a colaboraração dada a esse trabalho.

Ao professor Marcelo Bahia Labruna e a pesquisadora Darci Barros-Battesti pelos valiosos conselhos, ensinamentos, atenção e carinho dispensados para comigo.

Aos professores Karin Werther, Angelo Berchieri Júnior e Rosemeire de Oliveira pelo convívio no Departamento de Patologia Veterinária.

À banca examinadora pelas valiosas críticas e contribuições.

Aos funcionários da FCAV-Unesp/Jaboticabal pela ajuda principalmente do Departamento de Patologia Veterinária, Seção de Pós-Graduação e Biblioteca.

A Universidade Estadual Paulista pela formação formal e informal.

À Fundação de Amparo à Pesquisa do Estado de São Paulo (Fapesp) pela bolsa de estudos concedida, processo FAPESP 2013/13186-5.

Aos meus professores que contribuíram para a minha formação. Obrigada pelo exemplo e dedicação.

Sumário

DETECÇÃO SOROLÓGICA E CARACTERIZAÇÃO MOLECULAR DE AGENTES TRANSMITIDOS POR ARTRÓPODES EM ANIMAIS SELVAGENS E DOMÉSTICOS NA REGIÃO DO PANTANAL SUL MATOGROSSENSE

RESUMO - As enfermidades transmitidas por artrópodes vêm sendo recentemente estudadas em animais selvagens brasileiros, os quais podem atuar como hospedeiros tanto para os vetores quanto para os patógenos, muitos dos quais apresentam potencial zoonótico. O presente estudo tem como objetivo investigar a ocorrência de agentes transmitidos por artrópodes (agentes Anaplasmataceae, *Bartonella* spp., mycoplasmas hemotróficos, *Rickettsia* spp., *Hepatozoon* spp. e piroplasmideos) em animais selvagens, cães domésticos e seus respectivos ectoparasitos, amostrados na região do Pantanal sul matogrossense, por meio de métodos sorológicos e moleculares. Para tal, 31 *Nasua nasua*, 78 *Cerdocyon thous*, sete *L. pardalis*, 42 cães, 110 roedores e 30 marsupiais foram capturados. Os carrapatos recolhidos (1582) dos animais pertenciam às espécies *Amblyomma sculptum*, *Amblyomma parvum*, *Amblyomma ovale*, *Amblyomma tigrinum*, *Rhipicephalus microplus*, *Rhipicephalus sanguineus* sensu lato e *Amblyomma auricularium*. Adicionalmente, 80 pulgas *Polygenis (Polygenis) bohlsi bohlsi* foram recolhidas. Quatorze (17,9%) *C. thous*, sete (16,6%) cães e um (3,2%) *N. nasua* mostraram-se soropositivos (títulos≥80) para *Ehrlichia canis*, com títulos de anticorpos variando de 80 a 1280. Nenhum animal mostrou-se soropositivo *Anaplasma phagocytophilum*. Nove cães, dois *C. thous*, um *N. nasua*, oito roedores, cinco marsupiais e um pool de pulgas *P. (P.) b. bohlsi* mostram-se positivos para *Ehrlichia* spp. Todos os cães e o pool de pulgas *P. (P.) b. bohlsi* positivos para *Ehrlichia* spp. foram também positivos em para o ensaio em tempo real quantitativo (qPCR) para *E. canis*, baseado no gene *dsb*. Sete *N. nasua*, dois *C. thous*, um *L. pardalis*, quatro roedores, três marsupiais, 15 carrapatos *A. sculptum*, dois *A. ovale*, dois *A. parvum* e um pool de larvas de *Amblyomma* spp. foram positivos para *Anaplasma* spp. Co-positividade ou co-soropositividade para *Ehrlichia* spp. e *Anaplasma* spp. foi observada em dois cães, um *N. nasua*, um *C. thous* e dois marsupiais. Trinta e cinco roedores selvagens e três pools de pulgas *P. (P.) b. bohlsi,* mostraram-se positivos nos ensaios moleculares para *Bartonella* spp. A análise filogética revelou que pelo menos dois genótipos diferentes estão circulando entre os roedores amostrados no bioma Pantanal. Os resultados parciais sugerem que a pulga *P. (P.) b. bohlsi* possa estar atuando como um possível vetor de *Bartonella* nesta região. Vinte e quatro *N. nasua*, três *C. thous*, dois cães e um roedor mostraram-se positivos para *Mycoplasma* spp. A análise filogenética apontou que os cães, *C. thous* e *N. nasua* aparentam estar sendo parasitados pela mesma espécie de *Mycoplasma* spp., filogenéticamente relacionada a *M. haemocanis/M. haemofelis* e que provavelmente um novo genótipo de *Mycoplasma* spp. está circulando entre os *N. nasua* e *C. thous* amostrados no bioma Pantanal. Vinte e sete (64,2%) cães, 59 (7,6%) *C. thous* e seis (85,7%) *L. pardalis* mostraram-se soropositivas para pelo menos uma espécie de *Rickettsia*. Para 17 (40,4%) cães, 33 (42,3%) *C. thous* e dois (33,3%) *L. pardalis*, foram observadas reações homólogas para *Rickettsia amblyommatis*. Cento e dezesseis carrapatos (93 *A. parvum*, 14 *A. sculptum,* três *A. auricularim* e seis pools de larvas de *Amblyomma*) e uma amostra

de sangue obtida de *C. thous*, mostraram-se postivas nos ensaios para as espécies de *Rickettsia* do grupo da febre maculosa. As amostras seqüenciadas obtidas dos carrapatos *A. parvum,* mostraram-se filogenéticamente relacionadas com '*Candidatus* Rickettsia andeanae'. Uma alta ocorrência de *Hepatozoon* foi encontrada nos carnívoros amostrados (*C. thous* [91,02%], cães [45,23%], *N. nasua* [41,9%] e *L. pardalis* [71,4%]), porém nenhum artrópode mostrou-se positivo. Adicionalmente, 24 roedores e um marsupial também mostraram-se positivos para *Hepatozoon* spp. Com base na análise filogenética, os *C. thous*, *L. parda*lis, *N. nasua*, cães, roedores e marsupiais aparentam não estar sendo parasitados pela mesma espécie de *Hepatozoon* spp. Sete cães, um *C. thous*, cinco *L. pardalis*, três *N. nasua*, seis roedores, oito carrapatos *A. parvum*, dois *A. sculptum* e um *A. ovale* mostraram-se positivos para os ensaios PCR de piroplasmídeos. Genótipos filogeneticamente relacionados com *Babesia canis vogeli* foram detectados em seis cães e cinco roedores. Genótipos filogeneticamente relacionados com *Babesia caballi* foram detectados em um *C. thous*, um cão, um carrapato *A. ovale* e um *A. sculptum* e genótipos relacionados com *Babesia bigemina* e *Babesia bovis* foram detectados em quatro carrapatos *A. parvum*. Quatro sequências obtidas de *A. parvum*, três *N. nasua* e um roedor mostraram-se filogeneticamente relacionadas com *Theileria equi*. Por fim, *Cytauxzoon* spp. foi detectado em quatro *L. pardalis* .O presente trabalho mostra, portanto, a ocorrência de agentes transmitidos por artrópodes vetores em animais selvagens e domésticos no Panatanal sul matogrossense.

Palavras-chave: Agentes Anaplasmataceae., *Bartonella* spp.*,* micoplasmas hemotróficos*,* Rickettsiales*, Hepatozoon* sp. e piroplasmídeos

SEROLOGICAL DETECTION AND MOLECULAR CHARACTERIZATION OF AGENTS TRANSMITTED BY ARTHROPOD VECTORS IN WILD AND DOMESTIC ANIMALS IN THE REGION OF THE SOUTHERN MATO GROSSO PANTANAL

ABSTRACT - The diseases transmitted by arthropods have been recently studied in Brazilian wildlife, which can act as hosts for vectors and pathogens, many of which have zoonotic potential. The present work aimed to investigate the occurrence of tick-borne agents (Anaplasmataceae agents, *Bartonella* spp., hemotropic mycoplasmas, *Rickettsia* spp., *Hepatozoon* spp. and piroplasms) in wild animals, domestic dogs and their respective ectoparasites, in southern Pantanal region, central-western Brazil, by serological and molecular assays. For this reason, 31 *Nasua nasua*, 78 *Cerdocyon thous*, seven *L. pardalis*, 42 dogs, 110 rodents and 30 marsupials were captured. The ticks collected (1582) from animals belonged to the species *Amblyomma sculptum*, *Amblyomma parvum*, *Amblyomma ovale*, *Amblyomma tigrinum*, *Rhipicephalus microplus, Rhipicephalus sanguineus* sensu lato and *Amblyomma auricularium*. Additionally, 80 *Polygenis* (*Polygenis*) *bohlsi bohlsi* fleas were collected. Overall, 14 (17.9%) *C. thous*, seven (16.6%) dogs and one (3.2%) *N. nasua* were seroreactive (titer≥80) to *Ehrlichia canis*, with titers ranging from 80 to 1280. No animal showed to be seroreactive for *A. phagocytophilum* antigen. Nine dogs, two *C. thous,* one *N. nasua*, eight rodents*,* five marsupials and one *P. (P.) b. bohlsi* pool were positive for *Ehrlichia* spp. All positive dogs and the only *P. (P.) b. bohlsi* pool positive for *Ehrlichia* spp. were also positive in specific *E. canis*-qPCR based on *dsb* gene. Seven *N. nasua*, two dogs, one *C. thous,* one *L. pardalis*, four rodents*,* three marsupials, 15 *A. sculptum*, two *A. ovale,* two *A. parvum* and one *Amblyomma* larvae pool were positive for *Anaplasma* spp. Co-positivity or co-seropositivity for *Ehrlichia* spp. and *Anaplasma* spp. was observed in two dogs, one *N. nasua*, one *C. thous* and two marsupials. Thirty-five rodents and three *P. (P.) b. bohlsi* flea pools, showed to be positive to *Bartonella* spp. in the molecular assays. The phylogenetic analysis revealed that at least two different genotypes are circulating among the rodents sampled in the Pantanal biome. Partial results suggest that the flea *P. (P.) b. bohlsi* may be acting as a possible *Bartonella* vector in this region. Twenty-four *N. nasua*, three *C. thous*, two dogs and one rodent, were positive for *Mycoplasma* spp. Phylogenetic analysis indicated that dogs, *C. thous* and *N. nasua* appear to share the same *Mycoplasma* spp. species, closely related to *M. haemocanis* / *M. haemofelis* and probably a new genotype of *Mycoplasma* spp. is circulating among the *N. nasua* and *C. thous* sampled in the Pantanal biome. Overall, 27 (64.2%) dogs, 59 (75.6%) *C. thous* and six (85.7%) *L. pardalis* were seroreactive to at least one *Rickettsia* species. For 17 (40.4%) dogs, 33 (42.3%) *C. thous*, and two (33.3%) *L. pardalis*, homologous reactions to *Rickettsia amblyommatis* were suggested. One hundred and sixteen ticks (93 *A. parvum*, 14 *A. sculptum*, three *A. auricularim* and six *Amblyomma* larvae pools) and one blood sample obtained from *C. thous* showed positive results for *Rickettsia* spotted fever group species. The sequenced samples obtained from *A. parvum* ticks showed to be closely related to '*Candidatus* Rickettsia andeanae'. A high occurrence of *Hepatozoon* was found in carnivores (*C. thous* [91.02%], dogs [45.23%], *N. nasua* [41.9%] and *L. pardalis* [71.4%]), however no arthropod showed positive results. Additionally, twenty-four rodents and a marsupial also showed positivity to *Hepatozoon* spp. Based on phylogenetic analysis, *C. thous*, *L. pardalis*, *N . nasua*,

dogs, rodents and marsupials appear not to share the same of *Hepatozoon* species. Seven dogs, one *C. thous,* five *L. pardalis*, three *N. nasua*, six rodents, eight *A. parvum*, two *A. sculptum* and one *A. ovale* were positive for piroplasmids-PCR assays. Genotypes closely related to *Babesia vogeli* were detected in six dogs and five rodents. While genotypes closely related to *Babesia caballi* were detected in one *C. thous*, one dog, one *A. ovale* and one *A. sculptum,* genotypes closely related to *Babesia bigemina* and *Babesia bovis* were detected in four *A. parvum* ticks. Four sequences obtained from *A. parvum*, three *N. nasua* and one wild rodent were closely related to *Theileria equi.* Lastly, *Cytauxzoon* spp. was detected in four *L. pardalis.* The present work shows the occurrence of vector-borne agents in wild and domestic animals in southern mato grosso Pantanal.

Keywords: Anaplasmataceae agents, *Bartonella* spp., micoplasmas hemotróficos, Rickettsiales, *Hepatozoon* sp. and piroplasms.

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Certificamos que o Protocolo nº 006772/13 do trabalho de pesquisa intitulado **"Detecção sorológica e caracterização molecular de agentes transmitidos por artrópodes em animais selvagens e domésticos na região do Pantanal Sul-Mato-Grossense"**, sob a responsabilidade da Profª Drª Mirela Tinucci Costa, pelo Colégio Brasileiro de Experimentação (COBEA) e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), em reunião ordinária de 11 de Abril de 2013.

Jaboticabal, 11 de Abril de 2013.

Andrigo Barboza de Nardi

Prof. Dr. Andrigo Barboza de Nardi
Coordenador - CEUA

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Autorização para atividades com finalidade científica

Número: 38787-1	Data da Emissão: 17/05/2013 18:37	Data para Revalidação*: 16/06/2014

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Título do Projeto: DETECÇÃO SOROLÓGICA E CARACTERIZAÇÃO MOLECULAR DE AGENTES TRANSMITIDOS POR ARTRÓPODES EM ANIMAIS SELVAGENS E DOMÉSTICOS NA REGIÃO DO PANTANAL SULMATOGROSSENSE	
Nome da Instituição : UNESP JABOTICABAL	CNPJ: 48.031.918/0012-87

SISBIO

Cronograma de atividades

#	Descrição da atividade	Início (mês/ano)	Fim (mês/ano)
1	Revisão de literatura	04/2013	06/2016
2	Colheita das amostras	06/2013	03/2016
3	PCR para os agentes sob estudo	08/2013	08/2016
4	Clonagem e seqüenciamento dos amplicons positivos	08/2013	10/2016
5	Sorologia	01/2014	06/2016
6	Análise das seqüências - Filogenia molecular	01/2014	11/2016
7	Discussão dos resultados e redação final	01/2017	03/2017

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Outras ressalvas

1	Todos os procedimentos deverão ser supervisionado por médico veterinário

Equipe

#	Nome	Função	CPF	Doc. Identidade	Nacionalidade
1	Heitor Miraglia Herrera	Pesquisador Colaborador	444.869.871-87	049724503 IFP-RJ	Brasileira
2	Rosangela Zacarias Machado	Pesquisadora Colaboradora	125.043.436-04	M4034143 SSP-MG	Brasileira

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Número: 38787-1	Data da Emissão: 17/05/2013 18:37	Data para Revalidação*: 16/06/2014

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Dados do titular

Nome: Marcos Rogério André	CPF: 302.435.148-59
Título do Projeto: DETECÇÃO SOROLÓGICA E CARACTERIZAÇÃO MOLECULAR DE AGENTES TRANSMITIDOS POR ARTRÓPODES EM ANIMAIS SELVAGENS E DOMÉSTICOS NA REGIÃO DO PANTANAL SULMATOGROSSENSE	
Nome da Instituição : UNESP JABOTICABAL	CNPJ: 48.031.918/0012-87

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3	GUILHERME DE MIRANDA MOURÃO	Pesquisador Colaborador- Captura dos animais	488.045.506-78	418653 SSP-MS	Brasileira
4	Keyla Carstens Marques de Sousa	Doutoranda- colheita e processamento das amostras	017.801.431-17	001261455 ssp-MS	Brasileira
5	Fabiana Lopes Rocha	Pesquisadora Colaboradora	696.485.571-87	1751282 SSP-DF	Brasileira
6	Mirela Tinucci Costa	Pesquisadora Colaboradora	022.973.488-00	7758598 ssp-SP	Brasileira

Locais onde as atividades de campo serão executadas

#	Município	UF	Descrição do local	Tipo
1	CORUMBA	MS	Fazenda Nhumirim da Embrapa Pantanal	Fora de UC Federal

Atividades X Táxons

#	Atividade	Táxons
1	Captura de animais silvestres in situ	Clyomys laticeps, Monodelphis domestica, Thylamys macrurus, Leopardus pardalis, Gracilinanus agilis, Nasua nasua, Oecomys mamorae, Cerdocyon thous
2	Coleta/transporte de amostras biológicas in situ	Nasua nasua, Gracilinanus agilis, Thylamys macrurus, Clyomys laticeps, Oecomys mamorae, Leopardus pardalis, Monodelphis domestica, Cerdocyon thous

Material e métodos

1	Amostras biológicas (Carnívoros)	Sangue, Ectoparasita
2	Amostras biológicas (Outros mamíferos)	Ectoparasita, Sangue
3	Método de captura/coleta (Carnívoros)	Armadilha tipo gaiola com atração por iscas (“Box Trap/Tomahawk/Sherman”)
4	Método de captura/coleta (Outros mamíferos)	Armadilha tipo gaiola com atração por iscas (“Box Trap/Tomahawk/Sherman”)

Destino do material biológico coletado

#	Nome local destino	Tipo Destino
1	UNESP JABOTICABAL	

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Táxon*	Qtde.	Tipo de amostra	Qtde.	Data

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* Identificar o espécime no nível taxonômico possível.

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CHAPTER 1 - General considerations

The arthropod-borne diseases have great importance to human and animal health, involving several infectious agents, hosts and vectors (HARRUS AND BANETH, 2006). The geographic distribution of arthropods and vector-borne agents is expanding, due to climatic, ecological and environmental changes. The presence of domestic animals in wild environments and, vice versa, resulted in a closer association between wild reservoirs, arthropod vectors, domestic animals and humans (SHAW et al., 2001; ANDRÉ et al., 2012).

Several studies have been investigated the role of wildlife in the epidemiology of diseases caused by Anaplasmataceae agents. The recent molecular detection of *Anaplasma phagocytophilum* (SANTOS et al., 2011; SILVEIRA et al., 2015) in domestic dogs in Brazil, associated to the detection of new genotypes of *Ehrlichia* spp. and *Anaplasma* spp. in deer (MACHADO et al., 2006; SACCHI et al., 2012; SILVEIRA et al., 2012), carnivorous birds (MACHADO et al., 2012), wild canids (ANDRÉ et al., 2012), domestic cats and neotropical wild felines species (ANDRÉ et al., 2010b; 2012; 2014; 2015) and antibodies anti-*E. chaffeensis* in apparently healthy humans or with clinical signs compatible with tick-borne diseases (COSTA et al., 2005), demonstrate the need for further studies on the molecular characterization of the agents above mentioned. It is also worth mentioning the molecular detection of a closely related to *Ehrlichia ruminantium* agent in (*Panthera onca*) in southern Pantanal (WIDMER et al., 2011) and a new *Ehrlichia* genotype in *Rhipicephalus (Boophilus) microplus* ticks (CABEZAS-CRUZ et al., 2012), which is able to cause clinical symptoms in bovines (AGUIAR et al., 2014), highlighting the importance of future stidies, due to the livestock take part of several economy segments in Brazil (IBGE 2008).

Bartonella species and hemotrophic mycoplasmas (hemoplasmas) are important pathogens that circulate between mammals and invertebrate hosts. Studies regarding the prevalence and pathogenicity of *Bartonella* and hemoplasmas among wild mammals are scarce, especially in Brazil. Until now, *Bartonella* have been detected only in captive felids (GUIMARÃES et al., 2010) and wild and synanthropic rodents (COSTA et al., 2014; FAVACHO et al., 2015; GONÇALVES et

al., 2016). In addition to this, serological evidence of exposure to *Bartonella* spp. has been reported in wild canids maintained in captivity in zoos of São Paulo state (FLEISCHMAN et al., 2015). In relation to the occurrence of hemotrophic mycoplasmas in Brazil, recent studies have detected hemoplasm DNA in wild rodents and non-human neotropical primates (BONATO et al., 2015; GONÇALVES et al., 2015), suggesting the possibility of these animals acting as possible reservoirs for these agents. Besides, hemoplasmas have also been detected in wild carnivores maintained in captivity in zoos (WILLI et al., 2007; GUIMARÃES et al., 2007; ANDRÉ et al., 2011a) and deer (GRAZZIOTIN et al., 2011) in Brazil. Considering the fact that some *Bartonella* and hemotrophic mycoplasmas species are considered emergent zoonotic agents (BREITSCHWERD et al., 2010; MAGGI et al., 2013), studies aiming at assessing the epidemiology of these agents and their respective vectors are much needed.

Rickettsia species genetically classified into the spotted fever group (SFG) are tick-borne agents showing great zoonotic importante in the world (PAROLA et al., 2005). In Brazil, the most important SFG agent is *Rickettsia rickettsii*, the causative agent of Brazilian spotted fever, which is the most deadly rickettsiosis globally (LABRUNA, 2009). Besides, the Atlantic rainforest strain of *Rickettsia parkeri*-like agent has also shown to cause spotted fever in humans (SPOLIDORIO et al., 2010; SILVA et al., 2011). Rickettsial agents have been previously detected in ticks (Acari: Ixodidae) and domestic animals (dogs and horses) in Pantanal biome (WIDMER et al., 2011; ALVES et al., 2014; NIERI-BASTOS et al., 2014; MELO et al., 2011, 2015, 2016; WITTER et al., 2016). Among wild animals sampled in Pantanal, only a serological evidence of exposure to *Rickettsia* spp. has been reported in wild jaguars (*Panthera onca*) in southern Pantanal (WIDMER et al., 2011).

Several studies have been conducted in order to investigate the role of wildlife in the epidemiology of arthropod-borne diseases. For instance, wild animals are considered reservoirs for *Hepatozoon* spp., due to the fact that the infections present usually a subclinical course (METZGER et al, 2008; ANDRÉ et al., 2010a). However, some studies revealed that infections with *Hepatozoon* spp. in young wild canids has been occasionally associated with clinical disease in coyotes (KOCAN et al., 2000; GARRET et al., 2005) and mortality in hyenas (EAST et al., 2008). *Hepatozoon* spp.

may be considered a potential opportunistics pathogens in immunocompromised animals or with concomitant infections (DAVIS et al., 1978; BANETH et al., 1998; KUBO et al., 2006). Therefore, further studies are recommended in order to estimate the impact of these parasites among the Brazilian wildlife.

Piroplasmoses are one of the most prevalent arthropodborne diseases of animals. Piroplasmoses are caused by hemoprotozoan parasites of the phylum Apicomplexa belonging to four related genera: *Babesia*, *Theileria*, *Cytauxzoon* and *Rangelia* (YABSLEY and SHOCK, 2012). These parasites have a great economic and veterinary impact worldwide and are considered to be the second most commonly found parasites in the blood of mammals after trypanosomes (SCHNITTGER et al., 2012). There are few reports concerning the seroprevalence and molecular detection of piroplasmids in wild carnivores. In Brazil, André et al. (2011b) found a seroprevalence of 31.74% and 10.31% against *B. vogeli* antigen in wild felines and canids maintained in captivity, respectively. Among wild felines, André et al. (2011b) detected a closely related genotype to *Babesia leo* in neotropical wild cat (*Oncifelis colocolo*) and cape genet (*Genetta tigrina*) also maintained in captivity in zoos in the state of São Paulo, Brazil. In relation to *Cytauxzoon* spp., fatal cases of cytauxzoonosis were reported in two lions maintained in capitivity in a zoo in Rio de Janeiro state (PEIXOTO et al., 2007). Besides, André et al. (2009) detected *Cytauxzoon* DNA closely related to *C. felis* in asymptomatic neotropical felines also maintained in captivity in zoos in the state of São Paulo. Furthermore, *Cytauxzoon* spp. was detected in domestic cats from Rio de Janeiro (MAIA et al., 2013) and Mato Grosso do Sul (ANDRÉ et al., 2015) states.

Due to the lack of information about the epidemiology of arthropod-borne agents among wildlife in southern Pantanal, the present study aimed to investigate the occurrence of *Hepatozoon* spp., *Bartonella* spp., hemotrophic mycoplasmas*,* Anaplasmataceae and Rickettsiales agentes, and piroplasmas in wild mammals and domestic dogs and their respective ectoparasites, in the region of Pantanal, state of Mato Grosso do Sul, central-western Brazil, using molecular and serological assays,

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CHAPTER 2 - Anaplasmataceae agents among wild mammals and ectoparasites in a Brazilian wetland. Ticks and Tick-Borne Diseases.

Abstract

Anaplasmataceae agents comprise obligate intracellular bacteria that can cause disease in humans and animals. The present work aimed to investigate the occurrence of Anaplasmataceae agents (*Ehrlichia* and *Anaplasma*) in wild mammals, domestic dogs and 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. Canids, felids, rodents and coatis' serum samples were individually tested by Indirect Fluorescent Antibody Test (IFAT) in order to detect IgG antibodies to *Ehrlichia canis* and *Anaplasma phagocytophilum*. Mammals and ectoparasites DNA samples were submitted to a generic quantitative real-time PCR (qPCR) multiplex assay based on *groE* gene and to generic conventional PCR (cPCR) assays based on 16SrRNA gene, in order to detect and quantify *Ehrlichia* spp. and *Anaplasma* spp. DNA. Positive samples in above mentioned cPCR protocols were submitted to specific qPCR and cPCR assays targeting *dsb*, *vlpt, msp-2, groESL, omp-1, trp-36*and *msp-5* genes. Overall, 14 (17.9%) crab-eating foxes, seven (16.6%) dogs and one (3.2%) coati were seroreactive (titer≥80) to *E. canis*, with titers ranging from 80 to 1280. No animal showed to be seroreactive for *A. phagocytophilum* antigen. Nine dogs, two crab-eating fox*,* one coati, eight wild rodents*,* five marsupials and one *Polygenis (Polygenis) bohlsi bohlsi* pool were positive for *Ehrlichia* spp. All positive dogs and the only *Polygenis (P.) bohlsi bohlsi* pool positive for *Ehrlichia* spp. were also positive in specific *E. canis*-qPCR based on *dsb* gene. Seven coatis, two dogs, one crab-eating fox*,* one ocelot, four wild rodents*,* three marsupials, 15 *Amblyomma sculptum*, two *Amblyomma ovale,* two *Amblyomma parvum*and one *Amblyomma* larvae pool were positive for *Anaplasma* spp. Co-positivity or co-seropositivity for *Ehrlichia* spp. and *Anaplasma* spp. was observed in two dogs, one coati, one crab-eating fox and

two marsupials. The present study provided evidences that wild animals from Brazilian southern Pantanal are exposed to Anaplasmataceae agents.

KeyWords: *Ehrlichia, Anaplasma,* dogs, tick, fleas, qPCR, wild mammals

1. Introduction

Anaplasmataceae agents are obligate intracellular bacteria, resident in phagosomes and belonging to the order Rickettsiales, Family Anaplasmataceae, α sub-division of Proteobacteria, whose cycles in the environment involve complex interactions between invertebrate vectors and vertebrate hosts (Dumler et al., 2001). Some Anaplasmataceae agents such as *Ehrlichia* spp. and *Anaplasma* spp. has great importance in veterinary and human medicine, due to their ability to cause severe diseases and even death (Dumler et al., 2001, Tate et al. 2013).

Several studies have investigated the role of wild animals in the epidemiology of diseases caused by Anaplasmataceae agents. The recent molecular detection of *A. phagocytophilum* (Santos et al., 2011, Silveira et al., 2015) in domestic dogs in Brazil, associated to the detection of new genotypes of *Ehrlichia* spp. and *Anaplasma* spp. in deer (Machado et al., 2006, Sacchi et al., 2012, Silveira et al., 2012), carnivorous birds (Machado et al., 2012), wild carnivores (André et al., 2010; 2012; Widmer et al., 2011), domestic cats (André et al., 2014; 2015) and antibodies to *Ehrlichia chaffeensis* in apparently healthy or symptomatic humans (Costa et al., 2005), highlight the need for further studies regarding the molecular characterization of the agents abovementioned.

The aim of the present study was to investigate the occurrence of Anaplasmataceae agents (*Ehrlichia* and *Anaplasma*) in wild mammals and domestic dogs and their respective ectoparasites in the region of Pantanal, state of Mato Grosso do Sul, central-western Brazil.

2. Material and methods

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 **(Figure 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 knownfor 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) (Alves et al., 2016).

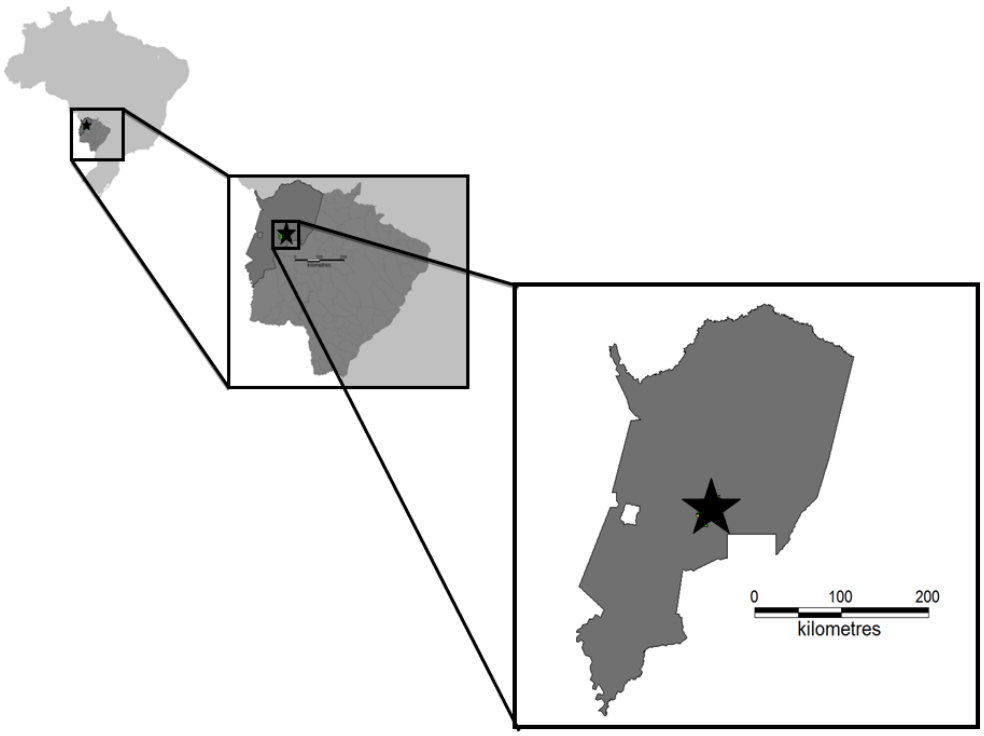

Figure 1. Capture sites. Map of Mato Grosso do Sul State, central-western Brazil, showing the Pantanal region, where mammals' blood and spleensamples and ticks were collected in the present study.

Between August 2013 and March 2015, four field expeditions of approximately 10 days each (August 2013, October 2013, August 2014 and March 2015) were performed. Wild carnivores 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 24h and checked twice a day. The animals were immobilized with an intramuscular injection of a combination of Zolazepan and Tiletamine (Zoletil®) at dosages of 8mg/kg for ocelots and 10mg/kg for crab-eating foxes and coatis. Blood samples were collected

by puncture of the cephalic vein and stored in Vacutainer® tubes with and without EDTA, in order to obtain total blood and serum samples for molecular and serological assays, respectively. All animal captures were in accordance with the licenses obtained from the Brazilian Government Institute for Wildlife and Natural Resources Care (IBAMA) (license number 38787-2) and endorsed by the Ethics Committee of FCAV/UNESP University (Universidade Estadual Paulista [Unesp], Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal, SP, Brasil) nº 006772/13. Additionally, blood samples were collected from domestic dogs (*Canis lupus familiaris*), 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 10m 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 between the two expeditions (August 2014 and March 2015). The identification of specimens was based on external and cranial morphological characters and karyological analyses, as previously described (Bonvicino et al., 2005). The animals were firstly anesthetized with an intramuscular injection of Ketamine (10–30mg/kg) associated with Acepromazine (5–10mg/kg) for rodents (9:1proportion), or Xylazine (2mg/kg) for marsupials (1:1). After anesthesia, blood samples were collected by intracardiac puncture and stored in Vacutainer® tubes without EDTA, in order to obtain serum samples for serological assays. After the blood collection, the animals were euthanized with potassium chloride, which doses ranging from75 to 150mg/kg (Leary et al., 2013). Spleen samples were collected and stored in absolute alcohol (Merck®, Kenilworth, New Jersey, USA). Animal handling procedures followed the Guidelines of the American Society of Mammalogists for the use of wild mammals in research (Sikes and Gannon, 2011). The project had permission from the Brazilian Government Environmental Agency (Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) (SISBIO license number 38145) and was also endorsed by the Ethics Committee of

the FCAV/UNESP University (CEUA -nº 006772/13), in accordance to Brazilian regulations.

Ticks and fleas found parasitizing the sampled animals were detected by inspection of the skin and carefully removed by forceps or manually. The specimens were stored in 100% alcohol (Merck®, Kenilworth, New Jersey, USA) until identification, which was performed using a stereomicroscope (Leica® MZ 16A, Wetzlar, Germany) and following taxonomic literature for adult tick genera (Onofrio et al., 2006; 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).

Canids, felids, coatis and rodents' serum samples were individually tested by IFAT in order to detect IgG antibodies to *E.canis* and *A. phagocytophilum*. For that purpose, *E. canis* crude antigen was obtained from DH82 cells infected with Jaboticabal *E. canis* strain (Aguiar et al., 2007). *Anaplasma phagocytophilum* crude antigen was obtained from HL-60 cells infected with Webster *A. phagocytophilum* strain, kindly provided by Dr. John Stephen Dumler (Uniformed Services University of the Health Sciences, Bethesda, Maryland, USA) (Scorpio et al., 2004). Antigen slides were removed from storage and allowed to thaw at room temperature for 30 min. Ten mililiters of two fold dilutions of sera (starting at 1:80, the cutoff 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 (André et al., 2010, Sousa et al., 2013). *Anaplasma phagocytophilum* seropositive serum sample (titer 1:2560) was obtained from a horse experimentally infected with Webster *A. phagocytophilum* strain at Department of Veterinary Pathology, UNESP, Jaboticabal, SP, Brazil. Slides were incubated at 37º 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 goat anti-mouse IgG (Sigma®, St. Louis, USA) for wild rodents, goat anti-dog IgG (Sigma®, St. Louis, USA) for *Cerdocyon thous* and domestic dogs, goat anti-cat IgG (Sigma®, St. Louis, USA) for *Leopardus pardalis*, goat anti-raccoon IgG (Sigma®, St. Louis, USA) for *Nasua nasua* and goat anti-horse

IgG (Sigma®, St. Louis, USA) for *A. phagocytophilum* positive and negative control serum samples. 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) and anti-horse (dilution of 1:64) were diluted according to the manufacturer's instructions and then added to each well. Slides were incubated again at 37º 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 cover slips, and examined with a fluorescence microscope (Olympus®, Tokyo, Japan). Unfortunately, it was not possible to test the marsupials' serum samples, due to the unavailability of conjugate for this animal group.

DNA was extracted from 200µL of each whole blood (wild carnivores and domestic dogs) and 10mg of spleen (small mammals) samples using the QIAamp DNA Blood Mini kit (QIAGEN®, Valencia, CA, USA), according to the manufacturer's instructions. While DNA extraction from ticks was processed in pools for nymphs (up to 5 individuals) and larvae (up to 10 individuals), the adults were processed individually. 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. DNA concentration and quality were measured using 260/280nm absorbance ratio (Nanodrop®, Thermo Fisher Scientific, Waltham, MA, USA). In order to verify the presence of amplifiable DNA in the samples, internal control PCR assays targeting fragments of mammalian glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Birkenheuer et al., 2003), mitochondrial 16S rRNA ticks gene (Black and Piesman,1994) and fleas cytochrome-c oxidase subunit I(Folmer et al., 1994) genes were performed **(Table 1).**

Table 1. Oligonucleotides sequences, target genes and cycling conditions used in cPCR assays performed in biological samples from wild mammals, domestic dogs and their respective ectoparasites, sampled in southern Pantanal, state of Mato Grosso do Sul, central-western Brazil.

Oligonucleotides sequences (5'-3')	Target gene	Cycling conditions	PCR Product Size	References
GAPDH-F (CCTTCATTGACCTCAACTACAT) GAPDH-R (CCAAAGTTGTCATGGATGACC)	GAPDH/ Mammals	95°C for 5 min; 35 cycles of 95°C for 15 sec, 50°C for 30 sec and 72°C for 30 sec; and final extension of 72°C for 5min.	400pb	Birkenheuer et al. (2003)
16S+1(CTGCTCAATGATTTTTTAAATTGCTGTGG) 16S-1(CCGGTCTGAACTCAGATCAAGT)	16SrRNA /Ticks	10 cycles of 92°C for 1 min, 48°C for 1min and 72°C for 1min, followed by 32 cycles of 92°C for 1 min, 54°C for 35 sec and 72°C for 1,35 min, and final extension of 72°C for 7min.	460pb	Black andPiesman (1994)
HC02198 (TAAACTTCAGGGTGACCAAAAAATCA) LCO1490 (GGTCAACAAATCATAAAGATATTGG)	COX1/ Fleas	95°C for 1min, 35 cycles of 95°C for 15 sec, 55°C for 15 sec and 72°C for 10 sec, and final extension of 72°C for 5min.	710pb	Folmer et al (1994)
ECC (GAACGAACGCTGGCGGCAAGC) ECB (CGTATTACCGCGGCTGCTGGCA) ECAN-5 (CAATTATTTATAGCCTCTGGCTATAGGA) HE3 (TATAGGTACCGTCATTATCTTCCCTAT)	*16SrRNA /Ehrlichia* spp.	1ºRound: 94°C for 5 min, 40 cycles of 94°C for 1 min, 60°C for 1 min and 72°C for 1min; and final extension of 72°C for 5min 2ºRound: 94°C for 5 min, 40 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 1min; and final extension of 72°C for 5min	358pb	Murphy et al. (1998)
gE3a (CACATGCAAGTCGAACGGATTATTC) gE10R (TTCCGTTAAGAAGGATCTAATCTCC) gE2r (GGCAGTATTAAAAGCAGCTCCAGG) gE9f (AACGGATTATTCTTTATAGCTTGCT)	*16SrRNA /Anaplasma* spp.	94°C for 5 min, 40 cycles of 94°C for 30sec, 55°C for 30sec and 72°C for 1min; and final extension of 72°C for 5min (1º and 2ºRounds)	546pb	Massung et al., 1998
HS1a (AITGGGCTGGTAITGAAAT) EHR-CS778R (CCICCIGGIACIAIACCTTC) HS43 (AT(A/T)GC(A/T)AA(G/A)GAAGCATAGTC) HSVR (CTCAACAGCAGCTCTAGTAGC)	*groESL/ Anaplasma* spp.and *Ehrlichia spp.*	1ºRound: 3 cycles of 94°C for 1 min, 48°C for 2min and 72°C for 1,5min followed by 37 cycles of 94°C for 1 min, 48°C for 2 min and 72°C for 1,5min; and final extension of 72°C for 5min, 2ºRound: the annealing temperature rises to 55°C	600pb	Sumner et al. (1997), Nicholson et al. (1999)
conP28-F1 (AT(C/T)AGT(G/C)AAA(A/G)TA(T/C)(A/G)T(G/A)CCAA) conP28-R1 (TTA(G/A)AA(A/G)G(C/T)AAA(C/T)CT(T/G)CCTCC) conP28-F2 (CAATGG(A/G)(T/A)GG(T/C)CC(A/C)AGA(AG)TAG) conP28-R2 (TTCC(T/C)TG(A/G)TA(A/G)G(A/C)AA(T/G)TTTAGG)	*omp-1/ Ehrlichia* spp.	94°C for 3 min, 35 cycles of 94°C for 1min, 50°C for 1min and 72°C for 2min; and final extension of 72°C for 5min (1º and 2ºRounds)	300pb	Inayoshi et al. (2004)
dsb-330 (GATGATGTCTGAAGATATGAAACAAAT) dsb-728 (CTGCTCGTCTATTTTACTTCTTAAAGT)	*dsb/E. canis*	95°C for 2min, 50 cycles of 95°C for 15 sec, 58°C for 15 sec and 72°C for 30 sec, and final extension of 72°C for 5min.	409 pb	Doyle et al. (2005)
TRP36-F1 (TTTAAAACAAAATTAACACACTA) TRP36-R1 (AAGATTAACTTAATACTCAATATTACT)	*TRP36/E. canis and E. minassensis*	95 °C for 30 s, 45 °C for 30 s, and 72°C for 1 min for 30 cycles.	800-1000pb	Aguiar et al. (2014)
Amar msp5 eF (GCATAGCCTCCGCGTCTTTC) Amar msp5 eR: (TCCTCGCCTTGGCCCTCAGA) Amar msp5 iF: (TACACGTGCCCTACCGAGTTA) Amar msp5 eR: (TCCTCGCCTTGGCCCTCAGA)	*msp-5/Anaplasma* spp.	94 °C for 5 min, 34 cycles at 94 °C for 1 min, 58 °C for 1 min and 72 °C for 1 min, and final extension at 72 °C for 10 min (1º and 2ºRounds)	351 bp	Singh et al. (2012)

Firstly, a previously described broad range multiplex qPCR protocol based on *groE* gene (**Table 2**) was used aiming to detect and quantify *Ehrlichia* spp. and *Anaplasma* spp. DNA (Benevenute et al., 2017). Additionally, cPCR assays targeting 16SrRNA gene of *Ehrlichia* spp. (Murphy et al., 1998) and *Anaplasma* spp. (Massung et al., 1998) were also performed (**Table 1**). All positive samples in *groE* qPCR and 16SrRNA cPCR reactions were submitted to previously described species-specific qPCR assays (**Table 2**) aiming at detecting and quantify *A. phagocytophilum* (*msp-2*gene) (Drazenovich et al., 2006), *E. canis* (*dsb* gene) (Doyle et al., 2005) and *E. chaffeensis* (*vlpt* gene) (Reller et al., 2010) DNA. Conventional PCR assays (**Table 1**) targeting four other protein-coding genes, namely *groESL/Ehrlichia* and *Anaplasma* spp. (Sumner et al., 1997, Nicholson et al., 1999), *omp-1/Ehrlichia* spp. (Inayoshi et al., 2004), *dsb*/*E. canis* (Doyle et al., 2005), *TRP36*/*E. canis* and *Ehrlichia minassensis* (Aguiar et a., 2014) and *msp-5/Anaplasma* spp. (Singh et al., 2012) were also performed.

The Taq Man qPCR reactions were performed with a final volume of 10μL contained 5μL GoTaq® Probe qPCR Master Mix (Promega Corporation, Madison USA), 1.2μM of each primer and hydrolysis probe (**Table 2**) and 1μL 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) ($2.0x10^7$ to $2.0x10^0$ copies/μL). The number of plasmid copies was determined in accordance with the formula (Xg/μL DNA/[plasmid size (bp)x660])x$6.022x10^{23}$xplasmid 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).

Table 2. Oligonucleotides and hydrolysis probes sequences, target genes and cycling conditions used in qPCR assays that were performed in biological samples from wild mammals, domestic dogs and their respective ectoparasites, sampled in southern Pantanal, state of Mato Grosso do Sul, central-western Brazil.

Oligonucleotides and hydrolysis probes sequences (5'-3')	Target gene	Cycling conditions	References
EHRF (GCGAGCATAATTACTCAGAG) EHRR (CAGTATGGAGCATGTAGTAG) EHR [TET]CATTGGCTCTTGCTATTGCTAAT [BHQ2a-Q]3'	*groE/ Ehrlichia* spp. and *Anaplasma* spp.	95°C for 3 minutes followed by 50 cycles at 95°C for 10 seconds and 52.7°C for 30 seconds	Benevenute et al. (2017)
ANAF (TTATCGTTACATTGAGAAGC) ANAR (GATATAAAGTTATTAAAAGTATAAAGC) ANA [Cy-5] CCACCTTATCATTACACTGAGACG [BHQ2a-Q]3'			
903f (AGTTTGACTGGAACACACCTGATC) 1024r (CTCGTAACCAATCTCAAGCTCAAC) [FAM]-TTAAGGACAACATGCTTGTAGCTATGGAAGGCA [TAMRA] 3'	*msp-2/ A. phagocytophilum*	50°C for 2 min, 95°C for 3 minutes followed by 40 cycles at 95°C for 15 seconds and 60°C for 1min.	Drazenovich et al. (2006)
F (CTAATTCTGATTTACACGAGTCTTC) R (GCATCATCTTCGAATTGAACTTC) 5'[TAMRA] TTGAGTGTCC[BHQ2a-Q]3'	*vlpt/ E. chaffeensis*	95°C for 3 minutes followed by 40 cycles at 95°C for 10 seconds and 55°C for 30 seconds	Reller et al. (2010)
dsb-330 (GATGATGTCTGAAGATATGAAACAAAT) dsb-728 (CTGCTCGTCTATTTTACTTCTTAAAGT) 5'[FAM] AGCTAGTGCTGCTTGGGCAACTTTGAGTGAA-3 [BHQ-1]3'	*dsb/ E. canis*	95°C for 5 minutes followed by 40 cycles at 95°C for 15 seconds and 60°C for 1min.	Doyle et al. (2005)

The mixture of cPCR assays contained 10X PCR buffer (Life Technologies®, Carlsbad, CA, USA), 1.0mM $MgCl_2$ (Life Technologies®, Carlsbad, CA, USA), 0.2mM deoxynucleotide triphosphate (dNTPs) mixture (Life Technologies®, Carlsbad, CA, USA), 1.5U Taq DNA Polymerase (Life Technologies®, Carlsbad, CA, USA), and 0.5µM of each primer (Integrated DNA Technologies®, Coralville, IA, USA) (**Table 1**). *Ehrlichia canis* and *Anaplasma* spp. DNA positive controls were obtained from naturally infected dogs (Sousa et al., 2013). Ultra-pure sterile water (Life Technologies®, Carlsbad, CA, USA) was used as negative control. 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 v3.1Cycle Sequencing Kit (Thermo Fisher Scientific®, Waltham, MA, USA).and ABI PRISM 310DNA Analyzer (Applied Biosystems®, Foster City, CA, EUA) (Sanger et al., 1977).

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 program (Altschul et al., 1990) was used to analyze the sequences of nucleotides (BLASTn), aiming to browse and compare with sequences previously deposited in an international database (GenBank) (Benson et al., 2002). 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 (Katoh and Standley, 2013).

Phylogenetic inference was based on Bayesian Inference (BI) and Maximum Likelihood (ML) methods. The Bayesian inference (BI) 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 Maximum-likelihood (ML) analysis was inferred with RAxML-HPC BlackBox 7.6.3 (Stamatakis et al., 2008) (which includes an estimation of bootstrap node support), using 1000 bootstrapping replicates. 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 et al., 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

A total of 256 animals were captured in the central region of Pantanal, municipality of Corumbá, state of Mato Grosso do Sul: 158 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 cohabiting the same studied area were collected.

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 *A. sculptum* Berlese species, 241 (15.2% [78 adults and 163 nymphs]) belonging to *A. parvum* Aragão species, 32(2%) *A. ovale* Koch adults, one (0.06%) *Amblyomma tigrinum* Koch adult, one (0.06%) *Rhipicephalus (Boophilus) microplus* (Canestrini) adult, one (0.06%) *Rhipicephalus sanguineus* sensu lato (Latreille) adult, four (0.2%) *Amblyomma auricularium* (Conil) nymphs, and 269 (17%) *Amblyomma* larvae (**Table 3**). Besides, a total of 75 *Polygenis* (*P.*) *bohlsi bohlsi* (Wagner) fleas were collected from *T. fosteri,* four from *M. domestica* and one flea was collected from *T. macrurus.*

Table 3. Ticks species collected from wild mammals captured between August 2013 and March 2015 in southern Pantanal, state of Mato Grosso do Sul, central-western Brazil.

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

L – larvae, N – nymph, M –male adult, F – female adult, 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.

All 298 DNA animal samples amplified the predicted product for GAPDH gene, the concentration mean and absorbance ratio (260/280) were 145.3ng/µL (SD ± 95.3) and 2.13(SD ± 2.18), respectively. The amount of tick DNA extracted was 523 (314 *A. sculptum*; 132 *A. parvum*; 32 *A. ovale;* 3 *A. auricularium;* 1 *A. tigrinum;* 1 *R. sanguineus;* 1 *R.* (*B.*) *microplus;* 39 *Amblyomma* larvae pools), of which 228 (43.5%) were from adults, 256 (48.9%) pooled nymphs, and 39 (7.4%) from pooled larvae, the concentration mean and absorbance ratio (260/280) were 45.9ng/µL (SD ± 84.3) and 1.81(SD ± 1.96), respectively. Out of 523 sampled ticks, 31 (5.9% [23 *A. parvum*adults, 4 *A. sculptum* adults, 1 *A. ovale* adult, 1 *A. parvum* nymph and 2 pooled *Amblyomma* larvae]) showed negative results for the tick mitochondrial 16S rRNA gene and were excluded from subsequent analyses. A total of 39 pooled fleas samples were submitted to DNA extraction, the concentration mean and absorbance ratio (260/280) were 7ng/µL (SD ± 8.43) and 1.12(SD ± 1.03), respectively. Only one flea DNA sample did not amplify the predicted product for cox-1 and was also excluded from subsequent analyses.

Overall, 14 (17.9%) crab-eating foxes, seven (16.6%) dogs and one (3.2%) coati were seroreactive (titer≥80) to *E. canis*. The seroreactive animals showed titers for *E. canis* ranging from 80 to 1280. While dogs showed titers to *E. canis* ranging from 80 to 1280, crab-eating foxes showed titers ranging from 80–640. The seropositive coati showed a titer of 640. No wild rodent showed to be seroreactive to *E. canis* antigen. Ticks were collected from 12 (54.5%) out of 22 *E. canis*-seroreactive animals. No sampled mammal showed to be seroreactive for *A. phagocytophilum* antigen.

Twenty-five animals (8.3%) were positive in cPCR assays for *Ehrlichia* spp.based on 16SrRNA gene. Among the positive animals, seven (28%) were dogs, two (8%) *C. thous*, one (4%) *N. nasua*, four (16%) *T. fosteri*, four (16%) *O. mamorae*, three (12%) *T. macrurus*, and two (8%) *G. agilis*. Additionally, one (2.5%) *Polygenis (P.) bohlsi bohlsi* flea pool was also positive in *Ehrlichia* spp. cPCR based on 16SrRNA gene. Four (9.5%) dogs were positive for *Ehrlichia* spp. *groE* qPCR. The number of copies of *Ehrlichia groE* fragment/µL ranged from 1.990×10^{1} to 2.478×10^{3}. The efficiency, correlation coefficient and slope of qPCR reactions ranged from

90.2% to 97.9% (mean=94%), 0.917 to 0.995 (mean=0.956) and -3.373 to -3.582 (mean=-3.477), respectively (**Table 4**).

Table 4. Positive samples for *Ehrlichia* spp. based on cPCR (16SrRNA) and qPCR (*groE*) assays, with their respective quantification values and qPCR reaction parameters.

Host	N° of *groE* copies/µL	Efficiency (%)	R^2	Slope	Y-int	cPCR 16SrRNA
Canis familiaris	2.478×10^3	90.2	0.995	-3.582	43	-
Canis familiaris	3.59×10^2	90.2	0.995	-3.582	43	-
Canis familiaris	2.14×10^2	90.2	0.995	-3.582	43	+
Canis familiaris	1.99×10^1	97.9	0.917	-3.373	36.9	+
Canis familiaris	-	97.9	0.917	-3.373	36.9	+
Canis familiaris	-	97.9	0.917	-3.373	36.9	+
Canis familiaris	-	97.9	0.917	-3.373	36.9	+
Canis familiaris	-	90.2	0.995	-3.582	43	+
Canis familiaris	-	90.2	0.995	-3.582	43	+
Cerdocyon thous	-	93.3	0.999	-3.494	40.147	+
Cerdocyon thous	-	93.3	0.999	-3.494	40.147	+
Nasua nasua	-	93.3	0.999	-3.494	40.147	+
Thrichomys fosteri	-	91.3	0.999	-3.550	41.689	+
Thrichomys fosteri	-	91.3	0.999	-3.550	41.689	+
Thrichomys fosteri	-	90.5	0.997	-3.572	41.510	+
Thrichomys fosteri	-	90.5	0.997	-3.572	41.510	+
Oecomys mamorae	-	90.6	0.994	-3.571	39.896	+
Oecomys mamorae	-	90.5	0.997	-3.572	41.510	+
Oecomys mamorae	-	95	0.996	-3.448	37.642	+
Oecomys mamorae	-	95	0.996	-3.448	37.642	+
Gracilinamus agilis	-	91.3	0.999	-3.550	41.689	+
Gracilinamus agilis	-	91.3	0.999	-3.550	41.689	+
Thylamys macrurus	-	91.3	0.999	-3.550	41.689	+
Thylamys macrurus	-	95	0.996	-3.448	37.642	+
Thylamys macrurus	-	90.5	0.997	-3.572	41.510	+
Monodelphis domestica	-	90.5	0.997	-3.572	41.510	+
Monodelphis domestica	-	95	0.996	-3.448	37.642	+
Polygenis (P.) bohlsi bohlsi	-	90.6	0.994	-3.571	39.896	+

N° of copies - Number of copies/µL, R^2 - Correlation coefficient, Y-int - intercepton the axis Y

Two out of seven positive dogs in *Ehrlichia*spp.16SrRNA-cPCR were also positive in *Ehrlichia* spp. *groE* qPCR. Nine dogs (21.4%) and one *Polygenis (P.) bohlsi bohlsi* flea pool also showed positive results in qPCR for *E. canis* based on *dsb* gene. Two dogs showed positive results in both *Ehrlichia*-qPCR (based on *groE* and *dsb* genes) and cPCR (16SrRNA) assays. The number of copies of *E. canis dsb* fragment/µL ranged from $4.00X10^{1}$ to $1.652x10^{4}$. The efficiency, correlation coefficient, and slope of qPCR assays ranged from 90.3% to 101.4% (mean=95.7%), 0.989 to 0.998(mean=0.994), and -3.579 to -3.288(mean=-3.432), respectively (**Table 5**). No samples positive for *Ehrlichia* spp. based on cPCR (16SrRNA) and qPCR (*groE*) assays showed positive results in previously described cPCR protocols based on *omp-1*, *dsb, TRP36* and *groESL* genes. Besides, all *Ehrlichia* spp.-positive samples were also negative in qPCR assay for *E. chaffeensis* based on *vlpt* gene. All 16S rRNA *Ehrlichia* sequences obtained in the present study were deposited in Genbank international database under the accession numbers KY499155-KY499181.

Table 5. Positive samples for *Ehrlichia canis* based on cPCR (16SrRNA) and qPCR (*dsb*) assays, with their respective quantification values and qPCR reaction parameters.

Host	N° of copies/µL	Efficiency (%)	R^2	Slope	Y-int	cPCR 16SrRNA
Canis familiaris	1.652×10^{4}	95.6	0.996	-3.431	42.6	+
Canis familiaris	2.395×10^{3}	95.6	0.996	-3.431	42.6	+
Canis familiaris	1.958×10^{3}	95.6	0.996	-3.431	42.6	+
Canis familiaris	1.043×10^{3}	90.3	0.998	-3.579	44.5	+
Canis familiaris	4.920×10^{2}	95.6	0.996	-3.431	42.6	+
Canis familiaris	2.778×10^{2}	90.3	0.998	-3.579	44.5	+
Canis familiaris	1.136×10^{2}	90.3	0.998	-3.579	44.5	+
Canis familiaris	9.722×10^{1}	90.3	0.998	-3.579	44.5	-
Canis familiaris	5.171×10^{1}	90.3	0.998	-3.579	44.5	-
Polygenis (P.) bohlsi bohlsi	4.00×10^{1}	101.4	0.989	-3.288	42.4	+

N° of copies - Number of copies/µL, R^2 - Correlation coefficient, Y-int - intercepton the axis Y

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*, three (7.1%) dogs, (0.9%) two *A. sculptum* nymphs pools and one (3.1%) *A. ovale* adult showed positivity in cPCR for *Anaplasma* spp. on 16SrRNA gene. Two (3.5%) *T. fosteri,* one (4%) *O. mamorae*, two (18.1%) *G. agilis,* one (7.1%) *T. macrurus,* ten (3.1%) *A. sculptum* adults, five (2.4%) *A. sculptum* nymph pools, two (1.5%) *A. parvum* adults, two (6.2%) *A. ovale* adults, and one (2.5%) *Amblyomma* larvae pools were positive in qPCR for *Anaplasma* spp. based on *groE* gene (**Table 6**). The number of copies of *Anaplasma groE* fragment/µL ranged from 4.496 x 10^0 to 2.304 x 10^3. The efficiency, correlation coefficient, and slope of qPCR reactions ranged from 90% to 100.8% (mean=94%), 0.987 to 0.998 (mean=0.993), and -3.362 to -3.391 (mean=-3.588), respectively (**Table 6**). Two *T. fosteri,* one *T. macrurus,* one *G. agilis,* two *A. sculptum* nymph pools, and one *A. ovale* adult were positive in both 16SrRNA-cPCR and *groE*-qPCR assays for *Anaplasma* spp. (**Table 6**). A *A. sculptum* nymph pool that was positive for *Anaplasma* in both 16SrRNA-cPCR and *groE*-qPCR assays was collected from a coati which was also positive in *Anaplasma* cPCR based on 16SrRNA. No positive sample for *Anaplasma* spp. based on 16SrRNA-cPCR or *groE*-qPCR assays showed positive results in qPCR for *A. phagocytophilum* based on *msp-2* gene, and cPCR reactions based on *groESL* and *msp-5* genes. *Anaplasma* sequences obtained in the present study were deposited in Genbank international database under the accession numbers KY499182 - KY499201.

Table 6. Positive samples for *Anaplasma* spp. based on cPCR (16SrRNA) and qPCR (*groE*) assays, with their respective quantification values and qPCR reaction parameters.

Host	N° of copies/µL	Efficiency (%)	R^2	Slope	Y-int	cPCR 16SrRNA
Thrichomys fosteri	2.304×10^3	98.4	0.987	-3.362	39.3	+
Thylamys macrurus	2.246×10^3	93	0.994	-3.501	43.8	+
Gracilinamus agilis	1.997×10^3	93	0.994	-3.501	43.8	+
Gracilinamus agilis	7.242×10^1	93	0.994	-3.501	43.8	-
Amblyomma sculptum nymph	4.00×10^1	90.9	0.997	-3.563	39.293	+
Amblyomma parvum	4.00×10^1	93.5	0.996	-3.489	38.643	-
Amblyomma sculptum	3.97×10^1	93.5	0.996	-3.489	38.643	-
Amblyomma ovale	3.96×10^1	91.1	0.987	-3.554	40.6	+
Amblyomma sculptum	3.95×10^1	90.4	0.99	-3.576	40.608	-
Amblyomma sculptum nymph	3.94×10^1	91.1	0.987	-3.554	40.6	+
Amblyomma sculptum	3.91×10^1	95.4	0.994	-3.436	39.359	-
Amblyomma sculptum	3.91×10^1	93.6	0.99	-3.484	39.2	-
Amblyomma parvum	3.90×10^1	97.2	0.998	-3.391	37.5	-
Amblyomma sculptum	3.90×10^1	95.4	0.994	-3.436	39.359	-
Amblyomma sculptum nymph	3.90×10^1	90.2	0.994	-3.581	41.2	-
Amblyomma sculptum	3.89×10^1	95.4	0.994	-3.436	39.359	-
Amblyomma sculptum	3.86×10^1	93.5	0.996	-3.489	38.643	-
Amblyomma sculptum nymph	3.85×10^1	91.9	0.992	-3.532	39.856	-
Amblyomma sculptum nymph	3.85×10^1	90	0.998	-3.588	39.368	-
Amblyomma sculptum	3.83×10^1	97.2	0.998	-3.391	37.5	-
Amblyomma sculptum	3.82×10^1	97.2	0.998	-3.391	37.5	-
Amblyomma sculptum	3.81×10^1	93.5	0.996	-3.489	38.643	-
Amblyomma ovale	3.78×10^1	95.4	0.994	-3.436	39.359	-
Amblyomma larvae	3.75×10^1	97.2	0.998	-3.391	37.5	-
Thrichomys fosteri	3.693×10^1	93	0.994	-3.501	43.8	+
Oecomys mamorae	4.496×10^0	100.8	0.992	-3.304	42.9	-
Leopardus pardalis	-	97.1	0.984	-3.394	38.608	+
Nasua nasua	-	97.1	0.984	-3.394	38.608	+
Nasua nasua	-	98.7	0.968	-3.355	39.836	+
Nasua nasua	-	97.1	0.984	-3.394	38.608	+
Nasua nasua	-	97.1	0.984	-3.394	38.608	+
Nasua nasua	-	97.1	0.984	-3.394	38.608	+
Nasua nasua	-	97.1	0.984	-3.394	38.608	+
Nasua nasua	-	98.7	0.968	-3.355	39.836	+
Cerdocyon thous	-	98.7	0.968	-3.355	39.836	+
Canis familiaris	-	98.7	0.968	-3.355	39.836	+
Canis familiaris	-	98.7	0.968	-3.355	39.836	+
Canis familiaris	-	98.7	0.968	-3.355	39.836	+
Clyomys laticeps	-	93	0.994	-3.501	43.8	+

N° of copies - Number of copies/µL, R^2 - Correlation coefficient, Y-int - intercepton the axis Y

Two seropositive dogs to *E. canis* also showed positivity in qPCR for *E. canis* based on *dsb* gene. One coati showed antibodies against *E. canis* and was positive in cPCR assays for *Ehrlichia* spp.and *Anaplasma* spp. based on 16SrRNA gene. A seroreactive crab-eating fox to *E. canis* also showed positivity in cPCR for *Ehrlichia* spp. based on 16SrRNA gene. One *T. macrurus* was positive for both *Ehrlichia* spp. and *Anaplasma* spp. based on cPCR assays based on 16S rRNA. One *G. agilis* showed positivity in cPCR for *Ehrlichia* spp. based on 16SrRNA gene and qPCR for *Anaplasma* based on *groE* gene (**Table 7**).

Table 7. Co-positivity between molecular assays for *Ehrlichia* spp. and *Anaplasma* spp. and between molecular and serological assays for *Ehrlichia* spp. and *Ehrlichia canis*.

Host/ID	**Serology**	**cPCR**		**qPCR**		
	E. canis	*Ehrlichia* spp.	*Anaplasma* spp.	*Ehrlichia* spp.	*Anaplasma* spp.	*E.canis*
Canis familiaris	+	+	-	+	-	+
Canis familiaris	+	-	-	-	-	+
Nasua nasua	+	+	+	-	-	-
Cerdocyon thous	+	+	-	-	-	-
Thylamys macrurus	-	+	+	-	-	-
Gracilinamus agilis	-	+	-	-	+	-

The BLAST analysis of the 16SrRNA *Ehrlichia* spp. DNA fragments (358pb) showed that two sequences obtained from *C. thous*, two sequences obtained from *M. domestica,* and three sequences obtained from *T. macrurus* showed 99-100% of identity with *Ehrlichia* sp. from free-living Orenoco goose (*Neochen jubata)* from Brazil (KX898136). Seven 16SrRNA *Ehrlichia* spp. sequences obtained from dogs blood samples showed 100% identity with the Brazilian isolate of *E. canis* (JX118827).Two *Ehrlichia* spp. sequences obtained from *G. agilis* spleen samples showed 99% of identity with an *E. canis* isolate obtained from *R. sanguineus* ticks from the Philippines (JN121379). The only 16SrRNA *Ehrlichia* spp. sequence obtained from a *N. nasua* blood sample showed 100% of identity with an *E. canis* isolate from Malaysia (KR920044). Eight 16SrRNA *Ehrlichia* spp. sequences

obtained from wild rodents spleen samples (four from *T. fosteri* and four from *O. mamorae*) showed 100% of identity with *Ehrlichia* sp. detected in *C. thous* from Brazil (JQ260861). The 16SrRNA *Ehrlichia* spp. sequence obtained from one *Polygenis (P.) bohlsi bohlsi* fleas pool showed 99% of identity with an *Ehrlichia* spp. sequence detected in a *Puma yagouaroundi* specimen from Brazil (JQ260855) (**Table 8**).

The 16S rRNA sequences of *Anaplasma* spp. obtained from seven coatis, one ocelot, one crab-eating fox, two *T. fosteri,* one *C. laticeps,* one *T. macrurus* and one *A. sculptum* nymph pool showed 99% of identity with an *Anaplasma* spp. sequence obtained from an *Amblyomma cajennense* tick (KJ831219) collected in the state of Mato Grosso, Brazil, by BLAST analysis (**Table 8**). Two 16SrRNA *Anaplasma* spp.sequences obtained from dogs' blood samples and one 16SrRNA *Anaplasma* spp. sequence obtained from a *G. agilis* spleen sample showed 100% and 99% of identity to *A. platys* (KU500914, KU534873), respectively (**Table 8**). The 16SrRNA *Anaplasma* spp.sequences obtained from one *A. sculptum* nymph pool and one *A. ovale* adult showed 99% and 98% of identity with *A.phagocytophilum*, respectively (CP006618, GU064900) (**Table 8**).

Table 8. Maximum identity of 16S rRNA *Anaplasma* and *Ehrlichia* sequences detected in mammals and ticks in southern Pantanal, Brazil, by BLAST analysis.

Host	Numberofsequencesanalized	Target Gene	% of identity by BLAST analysis
Leopardus pardalis	1	16SrRNA	99%-Uncultured *Anaplasma* sp. clone Pocone Ac48 (KJ831219)
Nasua nasua	7	16SrRNA	99%-Uncultured *Anaplasma* sp. clone Pocone Ac48 (KJ831219)
Cerdocyon thous	1	16SrRNA	99%-Uncultured *Anaplasma* sp. clone Pocone Ac48 (KJ831219)
Canis familiaris	2	16SrRNA	100%-*A. platys* (KU500914)
Thrichomys fosteri	2	16SrRNA	99%-Uncultured *Anaplasma* sp. clone Pocone Ac48 (KJ831219)
Clyomys laticeps	1	16SrRNA	99%-Uncultured *Anaplasma* sp. clone Pocone Ac48 (KJ831219)
Thylamys macrurus	1	16SrRNA	99%-Uncultured *Anaplasma* sp. clone Pocone Ac48 (KJ831219)
Gracilinamus agilis	1	16SrRNA	99%- *A.platys*(KU534873)
Amblyomma sculptum nymph	1	16SrRNA	99%- Uncultured *Anaplasma* sp. clone Pocone Ac48 (KJ831219)
Amblyomma sculptum nymph	1	16SrRNA	99%- *A. phagocytophilum* str. Dog2 genome (CP006618)
Amblyomma ovale	1	16SrRNA	98%- *A. phagocytophilum* clone HLAP260 (GU064900)
Cerdocyon thous	2	16SrRNA	99%- *Ehrlichia* spp. *Neochen jubata*, Brazil (KX898136)
Nasua nasua	1	16SrRNA	100%- *E. canis*, Malásia (KR920044)
Canis familiaris	7	16SrRNA	100%- *E. canis*, Brazil (KR920044)
Thrichomys fosteri	4	16SrRNA	100% - Uncultured *Ehrlichia* sp., *Cerdocyon thous*, Brazil (JQ260861)
Oecomys mamorae	4	16SrRNA	100% - Uncultured *Ehrlichia* sp., *Cerdocyon thous*, Brazil (JQ260861)
Gracilinamus agilis	2	16SrRNA	99% - Uncultured *Ehrlichia* sp., *Rhipicephalus sanguineus* (JN121379)
Monodelphis domestica	2	16SrRNA	99%- *Ehrlichia* sp. *Neochen jubata*, Brazil (KX898136)
Thylamys macrurus	3	16SrRNA	99%- *Ehrlichia* sp. *Neochen jubata*, Brazil (KX898136)
Polygenis (Polygenis) bohlsi bohlsi	1	16SrRNA	99%- Uncultured *Ehrlichia* sp. clone jaguarundi, Brasil (JQ260855)

The phylogenetic analysis based on 358pb 16S rRNA gene fragment positioned the *Ehrlichia* spp. sequences obtained from sampled mammals and one flea pool (KY499155-KY499181) in the same branch of *E. canis* sequences detected in Brazil (EF195135) and other countries (EF011111, EU106856, U26740), and with *Ehrlichia* spp. sequences obtained from free-living *N. jubata* and wild felines from Brazil (KX898136, EU376114), based on ML and BI analyses, with relatively significant clade support (61) only in Bayesian analysis (**Figures 2 and 3**).

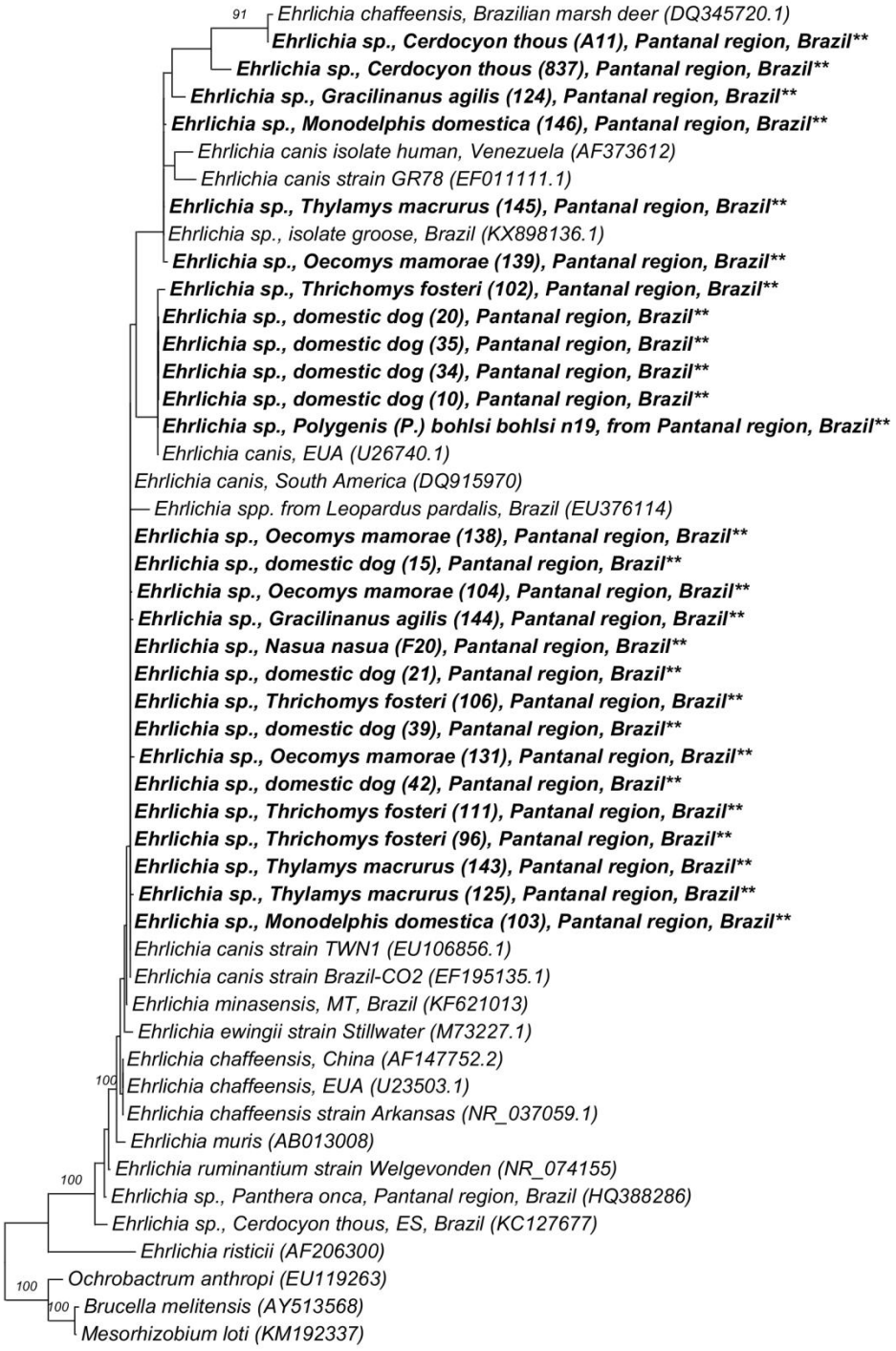

Figure 2. Phylogenetic tree constructed with 860 pb *Ehrlichia* spp.16SrRNA sequences, using Maximum Likelihood (ML) method and GTR+G+I evolutionary model. Numbers at nodes correspond to ML bootstrap values over 50, using *Mesorhizobium loti* (KM192337), *Brucella melitensis* (AY513568), *Ochrobactrum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

Figure 3. 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 phylogenetic analysis based on a 546pb 16S rRNA fragment positioned *Anaplasma* spp. sequences obtained from one coati (KY499193), one dog (KY499188) and one *A. sculptum* nymph pool (KY499182) in the same clade of an *Anaplasma* sequence detected in *Caracara 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, with clade support of 84 and 88, based on ML and BI analyses, respectively(**Figure 4 and 5**). Four 16SrRNA *Anaplasma* spp. sequences obtained from coatis (KY499184, KY499187, KY499194, KY499195), one sequence obtained from *L. pardalis* (KY499183), one sequence obtained from *C. thous* (KY499185), two sequences obtained from of *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* nymph pool (KY499201), and one sequence obtained from one *A. ovale* adult (KY499191) were positioned in the same clade of *A. bovis* (LC012812), with significant clade support (84) based only on BI analysis (**Figure 5**).

Figure 4. Phylogenetic tree constructed with 600 pb *Anaplasma* spp.16SrRNA sequences, using Maximum Likelihood (ML) method and GTR+G+I evolutionary model. Numbers at nodes correspond to ML bootstrap values over 50, using *Mesorhizobium loti* (KM192337), *Brucella melitensis* (AY513568), *Ochrobactrum anthropi* (EU119263) and as outgroups.

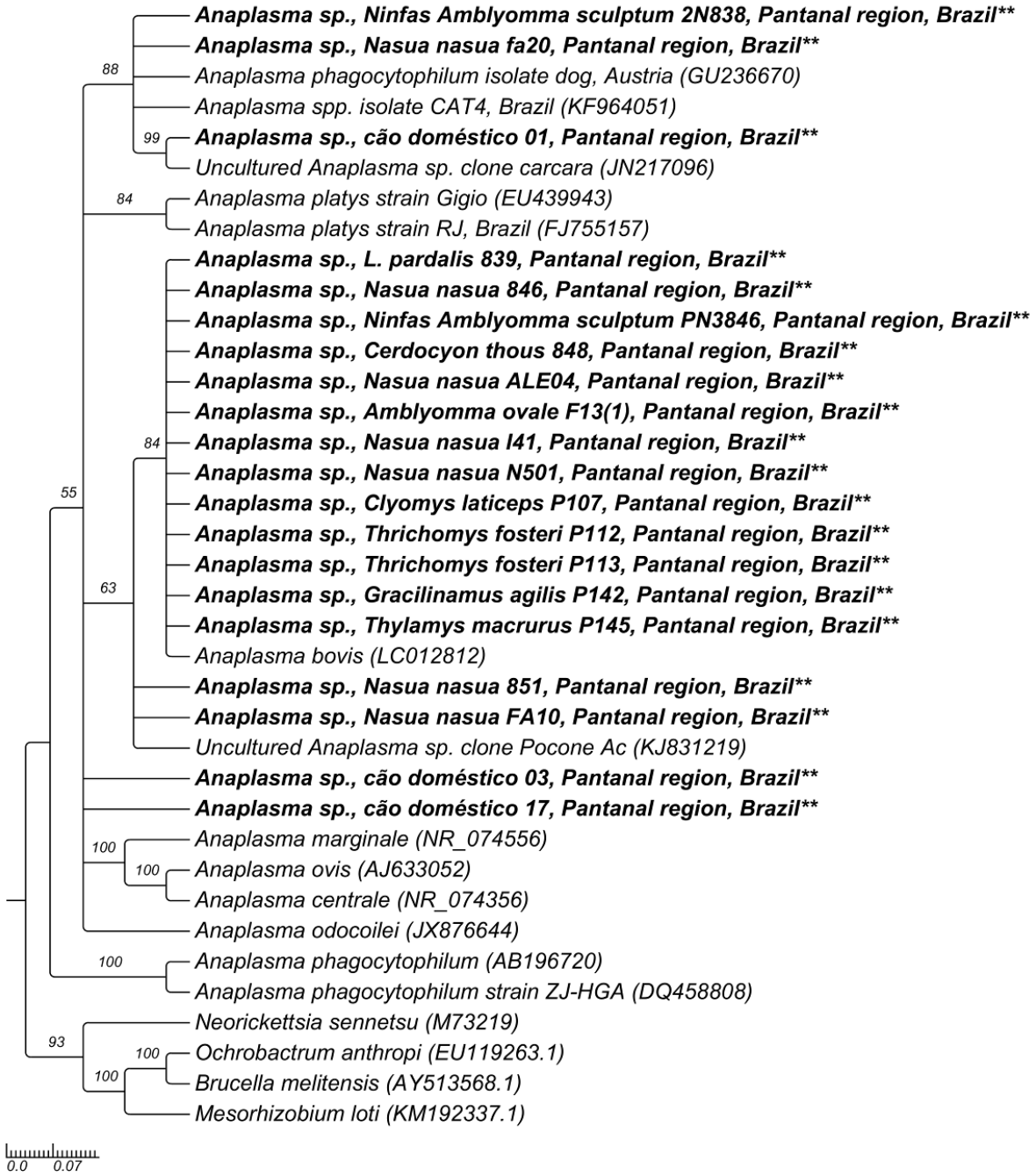

Figure 5. Phylogenetic tree constructed with 600 pb *Anaplasma* spp 16SrRNA sequences, using Bayesian method and GTR+G+I evolutionary model. Numbers at nodes correspond to Bayesian posterior probabilities over 50, using *Mesorhizobium loti* (KM192337), *Brucella melitensis* (AY513568) and *Ochrobactrum anthropi* (EU119263) as outgroups.

4. Discussion

The present study showed the presence of antibodies to *E. canis* in serum samples from crab-eating foxes, domestic dogs and one coati, and *Ehrlichia* spp. and *Anaplasma* spp. DNA in blood or spleen samples from wild carnivores, domestic dogs, rodents and ticks sampled in the southern region of Pantanal, state of Mato Grosso do Sul, central-western Brazil.

Ehrlichia canis is a wide spread tick-borne bacterium in dogs in Brazil (Aguiar et al., 2007, Dagnone 2009, Sousa et al., 2013; Santos et al., 2013, Melo et al., 2016a), mainly in tropical and subtropical regions (Andereg and Passos, 1999), in accordance with the distribution of the tropical lineage of *R. sanguineus* s.l (Moraes-Filho et al., 2015). In the present study, the molecular occurrence of *E. canis* (21.4%) among sampled dogs was higher than that found (15%) in a previous study involving dogs in northern Pantanal (Melo et al., 2016a), but lower than that found (45%) in a previous study in an urban area from the same state of Mato Grosso do Sul (Sousa et al., 2013). On the other hand, the seroprevalence (16.6%) to *E. canis* found among dogs was lower than that found (67.5%) in a previous study in northern Pantanal (Melo et al., 2011) and dogs (65%) sampled in an urban area from the same state of Mato Grosso do Sul state (Sousa et al., 2013). The differences found on seroprevalence rates may be due to the higher cut-off (≥80) used in the present study than that ones used in previous studies (≥64) in northern Pantanal and in the urban area of Mato Grosso do Sul state (Melo et al., 2011; Sousa et al., 2013). The elevation of cut-off to 80 was performed in order to minimize cross-reactions between Anaplasmataceae agents. Since most of the urban and rural dogs are mainly parasitized by *R. sanguineus* s.l. and *Amblyomma* ticks, respectively (Labruna and Pereira, 2001), a higher occurrence of *E. canis* is expected in dogs from urban areas (Sousa et al., 2013). Furthermore, considering the pathogenic potential of *E. canis* in domestic dogs (Dumler et al., 2001), 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 should be better investigated.

Reports of seropositivity to *E. canis* among wild carnivores are scarce in Brazil. In fact, there is only one report concerning the serological detection of antibodies to *E. canis* among wild felids maintained in captivity in zoos of São Paulo

state and Brazil, Brazil (André et al., 2010). Herein, a seroprevalence of 17.9% and 3.2% were found among free-living crab-eating foxes and coatis, respectively. To the best authors' knowledge, this was the first serological evidence of exposure to Anaplasmataceae agents among free-living wild crab-eating foxes and coatis in Brazil. Although antibodies to *E. canis* were detected among crab-eating foxes and coatis, no wild carnivore showed positive results in specific qPCR assays for *E. canis* based on *dsb* gene. Due to the unavailability of others *Ehrlichia* species antigens, cross-reactions between *E. canis* with closely related agents could not be discarded. Only two (2.5%) crab-eating foxes and one (3.2%) coati were positive for *Ehrlichia* spp. based only on 16SrRNA cPCR assays. A previous study detected a higher molecular occurrence (10%) of *Ehrlichia* spp. among wild canids (*C. thous*, *Speothos venaticus* and *Canis lupus*) maintained in captivity in Brazilian zoos (André et al., 2014). André et al. (2010) also detected *Ehrlichia* spp. DNA in 15% (11/72) of wild felids (*L. pardalis, Leopardus tigrinus, Puma concolor, Puma yagouaroundi* and *P. onca*) sampled in zoos from São Paulo state and Brasilia, Brazil. A previous study involving road-killed crab-eating foxes in the state of Espírito Santo, Brazil, also detected a higher molecular occurrence of *E. canis*. (10.3%) when compared to the present study (Almeida et al., 2013). However, it is worth noticing that while spleen fragments were used as biological samples in the previous study (Almeida et al., 2013), whole blood was used in this present study. A higher sensitivity of *Ehrlichia* detection is achieved when spleen aspirates instead blood samples from subclinically experimentally infected dogs is used in molecular assays (Harrus et al., 1998, 2004).

The present study also detected *Ehrlichia* DNA in 7.2% of wild rodents (4 *T. fosteri* and 4 *O. mamorae*) and in 23.3% of marsupials (3 *T. macrurus,* 2 *M. domestica* and 2 *G. agilis*) spleen samples. Previously, Melo et al. (2016b) detected antibodies to *E. canis* in 14.6% of opossums (*Didelphis aurita* and *Didelphis albiventris*) trapped in São Paulo state, Brazil. Although serological assays were not perform in marsupials' serum samples collected in the present study, *Ehrlichia* DNA was detected in 23.3% of sampled marsupials. Our study and the previous one involving Brazilian opossums (Melo et al., 2016b) highlight that marsupials participates in some way in the biological cycle of *Ehrlichia* in the environment. Thus, the role of these animals in *Ehrlichia* epidemiology should be better investigated.

Recently, Benevenute et al. (2017) detected *Ehrlichia* DNA in 24% of wild rodents (*O. mamorae, T. fosteri, C. laticeps* and *Calomys cerqueirai*) sampled in the same biome Pantanal. Although rodents are considered hosts for *E. chaffeensis* in China and Korea (Gao et al., 2000, Chae et al., 2008, Dong et al., 2013), the role of wild rodents in *Ehrlichia* epidemiology remains unknown in Brazil, as well as the arthropod vector involved in the transmission cycles.

Ehrlichia canis DNA was detected in one *Polygenis (P.) bohlsi bohlsi* flea pool collected from a specimen *T. fosteri* rodent that was negative in PCR assays for *Ehrlichia* spp. In a previous study, Torina et al. (2013) collected fleas *(Xenopsylla cheopis*, *Ctenocephalides canis*, *Ctenocephalides felis* and *Cediopsylla inaequalis*) from red foxes (*Vulpes vulpes*) in Italy, and reported the molecular detection of *E. canis* in one (1/1) *C. inaequalis* and 3% (2/75) of the *X. cheopis* fleas. According to the author, since two out of three positive fleas were collected from *E. canis*-positive foxes, an association between *E. canis* and fleas should be further investigated (Torina et al., 2013). On the other hand, others studies have failed to detect *Ehrlichia* species in fleas collected from cats in Australia (Barrs et al., 2010), dogs in Thailand (Foongladda et al., 2011), rodents (*Rattus norvegicus, Rattus rattus, Mus musculus),* canids *(Vulpes rueppelli),* mustelids *(Mustela nivalis,)* and wild ruminants(*Capra hircus*) sampled in Egypt (Loftis et al., 2006). Although *E. canis* was detected in a *Polygenis (P.) bohlsi bohlsi* flea pool by qPCR and cPCR assays, no association between positivity in fleas and hosts was observed, so further studies are needed in order to clarify the vectorial capacity of fleas in transmitting Anaplasmataceae agents. Alternatively, residual ehrlichial DNA from host blood in the siphonapteran digestive tract may be responsible for the positivity of fleas for the agent in the present study.

Herein, *Anaplasma* DNA was detected in three sampled dogs (7.1%). A similar *Anaplasma* occurrence was found in a previous study (7.19%) among dogs sampled in northern Brazilian Pantanal (Melo et al., 2016a). Besides, *Anaplasma* DNA was also detected among carnivores (one crab-eating fox [1.2%], one ocelot [14.2%] and seven coatis [22.5%]). Previously, André et al. (2012) detected *Anaplasma* DNA in three wild felids (*L. tigrinus*) and one wild canid (*S. venaticus*) maintained in captivity in zoos in the state of São Paulo. To the best authors' knowledge, this was the first

molecular evidence of exposure to *Anaplasma* agents among free-living crab-eating foxes, ocelots, coatis and marsupials in Brazil. Additionally, four wild rodents (3.6%) were also positive for *Anaplasma* spp. Recently, Benevenute et al. (2017) detected *Anaplasma* DNA in nine rodents (*Rattus rattus*, *Akodon* sp., *Sphiggurus villosus* and *C. cerqueirae*) sampled in Atlantic forest and Caatinga biomes. In the northern region of the Brazilian Pantanal, *Anaplasma* DNA was detected in only one *Hylaeamys megacephalus* wild rodent (1/4) (Wolf et al., 2016). Additional studies should be done in order to access the role of rodents and marsupials in the *Anaplasma* epidemiology in South America.

Thus, the present study reported the molecular detection of *Anaplasma* spp. among *A. sculptum, A. ovale* and *A. parvum* ticks. While *Ixodes* and *Haemaphysalis ticks* are considered vectors for *A. phagocytophilum* in the USA (Rizzoli et al., 2014) and Korea (Kang et al., 2016), respectively, the arthropod vectors involved in *Anaplasma* and *Ehrlichia* species transmission cycles among wild mammals are still unknown in Brazil. Recently, an unclassified *Anaplasma* sp. was detected in one *A. sculptum* tick collected from a dog in northern Pantanal (Melo et al., 2016a). Herein, *Anaplasma* DNA was detected in an *A. sculptum* nymph pool collected from an *Anaplasma* positive coati, suggesting some association between *A. sculptum* ticks and *Anaplasma* spp. Alternatively, residual *Anaplasma* DNA from host blood in the arthropods digestive tract may be responsible for the positivity of tick species for the agent in the present study.

The phylogenetic inferences based on a small fragment of 16S rRNA gene fragment positioned the *Ehrlichia* genotypes detected in dogs, *C. thous*, *N. nasua*, *T. fosteri*, *O. mamorae*, *T. macrurus*, *G. agilis* and *Polygenis (P.) bohlsi bohlsi* flea pool in the same clade of *E. canis*. *Ehrlichia* genotypes closely related to *E. canis* have already been detected in wild carnivores (André et al., 2012), wild birds (Machado et al., 2012), domestic cats (André et al., 2015) and wild rodents (Benevenute et al., 2017) in Brazil, and rodents in Korea (Kim et al., 2006). The wild animals positive samples at cPCR and qPCR assays for *Ehrlichia* spp. based on *groE* and 16S rRNA genes, respectively, were negative at specific qPCR assays for *E. canis* and *E. chaffeensis* based on *dsb* and *vlpt* genes, respectively, suggesting the possible circulation of *Ehrlichia* genotypes in wild mammals not yet isolated. On the other

hand, the presence of *E. canis* DNA in nine (21.4%) dogs' blood samples and one *Polygenis* flea pool was confirmed by a qPCR assay based on *dsb* gene.

The phylogenetic analyses based on a small fragment of 16S rRNA gene positioned the *Anaplasma* genotypes detected in four coatis, one ocelot, one crab-eating fox, two *T. fosteri*, one *C. laticeps*, one *T. macrurus*, one *G. agilis*, one *A. sculptum* nymph pool and one *A. ovale* adult in the same clade of *A. bovis* sequences. Although *A. bovis* is known to cause anaplasmosis in domestic and wild ruminants (Jilintai et al., 2009), *A. bovis* DNA has already been detected in dogs from Japan (Sakamoto et al., 2010), rodents from the USA (Goethert and Telford, 2003), cats from Japan (Sasaki et al., 2012) and small mammals from Taiwan (Masuzawa et al., 2014). Among wild animals, *A. bovis* has already been detected in wild felids (*Prionailurus iriomotensis*, *Prionailurus bengalensiseuptilura*) from Japan (Tateno et al., 2013) and Korea (Hwang et al., 2015), and in wild swines (*Sus scrofa*) and Pangolim-Malay (*Manis javanica*) from Malaysia (Koh et al., 2016). These findings indicate a possible circulation of new *Anaplasma* genotypes in wild animals in Brazil, whose zoonotic and pathogenic potential is still unknown.

Despite the relatedness of the 16S rRNA gene sequences detected in wild mammals apparently infected with a closely genotype to *E. canis* and *A. bovis*, the positive samples in 16S rRNA cPCR and *groE* qPCR assays were all negative in cPCR assays targeting four other genes (*groESL, omp-1, dsb, msp-5*), precluding additional phylogenetic inferences. The variable amplification of different target genes could be explained by the low level of bacteremia in animals' blood or spleen samples and ectoparasites. Moreover, PCR protocols used for amplification of different target genes may have proved unsuitable for amplification of variants of *Anaplasma* and *Ehrlichia* species infecting wild mammals in Brazil. The phylogenetic analysis based on short 16S rRNA gene fragments (358bp and 546pb) did not provide sufficient genetic discrimination to identify *Ehrlichia* and *Anaplasma* species. Based on phylogenetic analyses of 16S rRNA gene and *dsb* partial sequences, an *Ehrlichia* sp. found in 2 jaguars from the same region of this study, and in six crab-eating foxes sampled in Espirito Santo state, Brazil, were grouped into a cluster, albeit distantly, with different genotypes of *Ehrlichia ruminantium*, (Widmer et al., 2011, Almeida et al., 2013). While genotypes closely related to *E. chaffeensis*has

been detected detected in deer (Machado et al., 2006; Sacchi et al., 2012; Silveira et al., 2012), genotypes closely related to *A. phagocytophilum* have already been detected in wild carnivores (André et al., 2012), deer (Silveira et al., 2012) and birds (Machado et al., 2012), as well as in domestic cats (André et al., 2014) in Brazil. However, *E. chaffeensis* and *A. phagocytophilum* infection among wild mammals from Pantanal region was ruled out by specific qPCR assays targeting the *E. chaffeensis vlpt* gene and *A. phagocytophilum msp-2* gene.

In conclusion, the present study revealed that wild animals and ticks in southern Pantanal region, Brazil, are exposed to Anaplasmataceae agents. Antibodies against *E. canis* were detected for the first time in crab-eating foxes and coatis in Brazil. The role of domestic dogs as a source of *E. canis* infection to wild animals should be better investigated. Wild animals and *Amblyomma* ticks seemed to be eventually infected by genotypes closely related to *A. bovis*. Wild animals and *Polygenis* fleas are exposed to a genotype closely related to *E. canis* in Brazilian Pantanal. The present work showed the first molecular detection of *Ehrlichia* spp. and *Anaplasma* spp. in marsupials and coatis in Brazil. Previously described qPCR and cPCRs protocols aimed at amplifying different target genes may have proved unsuitable for amplification of variants of *Anaplasma* and *Ehrlichia* species that infect wild carnivores in Brazil. Therefore, future studies are much needed in order to isolate tick-borne bacteria that are circulating among wildlife from Brazil, for a better characterization of the Brazilian Anaplasmataceae strains.

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CHAPTER 3 – Molecular detection of *Bartonella* spp. in wild mammals and ectoparasites, with insights of a possible vector among rodents in Brazilian Pantanal. Microbes & Infection

Abstract.

Bartonella are fastidious bacteria that infect mammalian erythrocytes and endothelial cells. The present work aimed to investigate the occurrence of *Bartonella* in mammals and ectoparasites in Pantanal wetlands, Brazil. Between August 2013 and March 2015, 31 *Nasua nasua*, 78 *Cerdocyon thous*, seven *Leopardus pardalis*, 110 wild rodents, 30 marsupials, and 42 dogs were sampled. DNA samples were submitted to a qPCR assay targeting *Bartonella-nuoG* gene. Positive samples in qPCR were submitted to conventional PCR assays targeting *gltA*, *rpoB, ribC, nuoG* and *ftsZ* genes, followed by sequencing and phylogenetic analyses. Thirty-five wild rodents and three *Polygenis (P.) bohlsi bohlsi* flea pools showed positive results in *nuoG*-qPCR for *Bartonella* spp. Thirty**-**seven out of 38 positive samples in qPCR were also positive in cPCR assays based on *ftsZ* gene, nine in *nuoG*-cPCR, and six in *gltA*-cPCR**.** Concatenated phylogenetic analyses showed that *Bartonella* sequences detected in *Oecomys mamorae* rodents and *fleas* pooled in the same branch of genotypes previously detected in wild rodents in Brazil. *Bartonella* sequences detected in *Thrichomys fosteri* rodents were positioned in the same clade as *Bartonella alsatica*. These results showed that *Bartonella* spp. circulate among wild rodents and suggest that fleas could act as possible vectors in Pantanal wetlands, Brazil.

KeyWords: Bartonellaceae, wild carnivores, fleas, qPCR, wild rodents, Brazilian wetland

Running title: *Bartonella* in wild mammals in Pantanal

1. Introduction

Bartonella species comprise facultative, fastidious, gram-negative intracellular bacteria belonging to the alpha-2 class of Proteobacteria [1]. The transmission of

these agents occurs mainly through blood-sucking arthropod vectors. Seventeen *Bartonella* species have been associated with diseases in animals and humans [2].

Rodents are considered natural reservoirs for several *Bartonella* species. The association between rodents and *Bartonella* shows great importance, since these animals present persistent and subclinical bacteremia for long periods of time. Infections by a wide variety of *Bartonella* species have been reported in many different rodent species worldwide [3].

In Brazil, *Bartonella* spp.have been detected in synanthropic rodents in Salvador city, state of Bahia (Atlantic forest biome) [4] and in wild rodents from peri-urban areas from Mato Grosso do Sul state (Cerrado biome) [5]. Recently, different *Bartonella* genotypes have been detected in wild and synanthropic rodents from different Brazilian biomes [6]. In addition to this, *Bartonella* spp.have been detected among cats [7], dogs [8] and wild carnivores [9,10] in Brazil.

Due to the lack of information about the epidemiology and transmission routes of *Bartonella* spp. among wildlife in Brazil, the present study aimed to investigate the occurrence of *Bartonella* in wild mammals, domestic dogs and ectoparasites in Brazilian Pantanal wetland.

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 semideciduous 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 and it is wellknownfor a rich biodiversity. Two well-defined seasons are recognized: a rainy summer (October to March) and a dry winter (April to September).

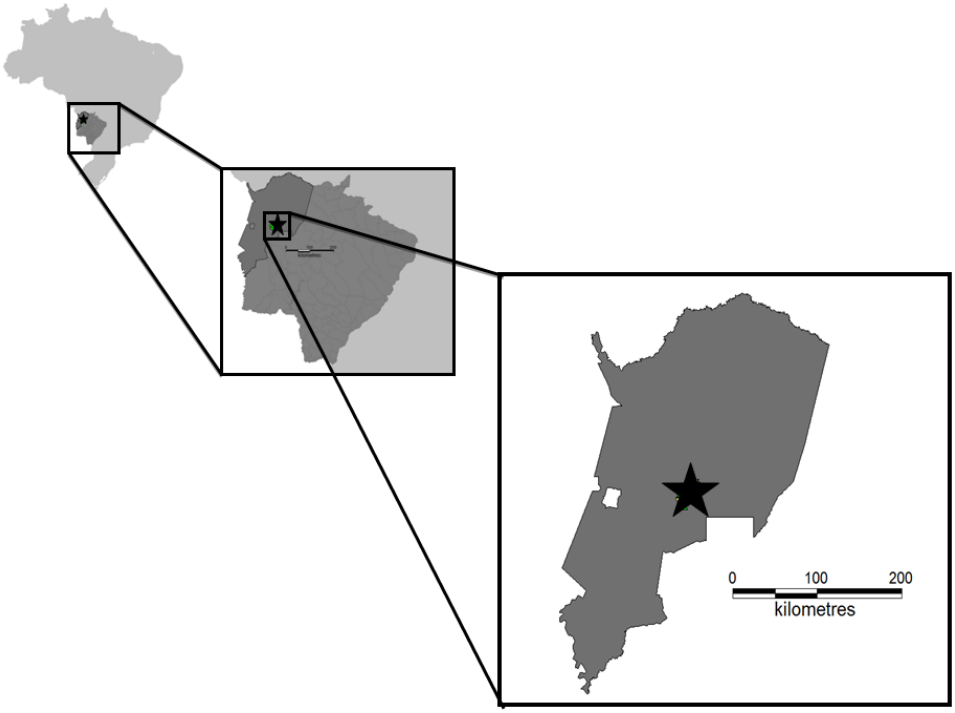

Figure 1. Capture sites. Map of Mato Grosso do Sul State, central-western Brazil, showing the Pantanal region, where animals samples were collected in the present study.

2.2. Capture methods and biological sampling

Between August 2013 and March 2015, four field expeditions (August 2013, October 2013, August 2014 and March 2015) were performed. Free-ranging carnivores 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 set up during 24h 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 *L. pardalis* and 10 mg/kg for *C. thous* and *N. nasua*. Blood samples were collected by puncture of the cephalic vein stored in Vacutainer® EDTA-containing tubes and stored at -20°C until DNA extraction and ectoparasites found parasitizing the animals were carefully removed by forceps or manually and stored in 100% alcohol (Merck®, Kenilworth, Nova Jersey, USA) until identification. After complete recovery of the animals, they

were returned to the nature. Additionally, blood samples and ectoparasites were collected from domestic dogs (*Canis lupus familiaris*), 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 seven consecutive nights along linear transects, placed on the ground at 10m intervals and alternating between trap type in 2 field expeditions (August 2014 and March 2015). The total capture effort was 200 traps per night, equally distributed between the two expeditions (August 2014 and March 2015). The identification of specimens was based on external and cranial morphological characters and karyological analyses, as previously described [11]. The animals were firstly anesthetized with an intramuscular injection of Ketamine (10–30 mg/kg) associated with Acepromazine (5–10 mg/kg) for rodents (proportion 9:1), or Xylazine (2 mg/kg) for marsupials (1:1) and ectoparasites were carefully removed by forceps or manually and stored in 100% alcohol (Merck®, Kenilworth, Nova Jersey, USA) until identification. After anesthesia, the animals were euthanized with potassium chloride, which doses ranging from 75 to 150 mg/kg. Spleen samples were collected and stored in absolute alcohol (Merck®, Kenilworth, Nova Jersey, USA). Animal handling procedures followed the Guidelines of the American Society of Mammalogists for the use of wild mammals in research [12].

2.3. 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 [13,14], and *Amblyomma* nymphs [15]. *Amblyomma* larvae could not be identified to the species level because there is insufficient literature available until now. The identification of fleas also was performed following taxonomic literature [16].

2.4. Molecular analysis

DNA was extracted from 200µL of each whole blood (wild carnivores and domestic dogs) and 10 mg of spleen tissues (small mammals) samples using the QIAamp DNA Blood Mini kit (QIAGEN®, Valencia, CA, USA), according to the manufacturer's instructions. While ticks DNA extraction was processed in pools for nymphs (up to 5 individuals) and larvae (up to 10 individuals), the adults were processed individually. The fleas DNA extraction also was processed in pools consisting of up to 5 individuals. Ticks and fleas were macerated and submitted to DNA extraction, using the same kit abovementioned. DNA concentration and quality was measured using absorbance ratio between 260/280 nm (Nanodrop®, Thermo Fisher Scientific, Waltham, MA, USA). In order to verify the existence of amplifiable DNA in the samples, internal control PCR assays targeting glyceraldehyde-3-phosphatedehydrogenase (GAPDH) mammals gene [7], mitochondrial 16S rRNA ticks gene [18] and a fragment of the cytochrome c oxidase subunit I (cox1) coding for COX1 from fleas [19] were performed **(Table 1).**

Table 1. Oligonucleotides sequences, target genes and thermal conditions used in conventional PCR assays targeting GAPDH endogenous genes and *gltA, rpoB, nuoG, ftsZ* and *ribC*gene fragments of *Bartonella* spp. in biological samples from wild mammals and domestic dogs, trapped and sampled, respectively, in Pantanal wetland, Brazil.

Oligonucleotide sequences (5'-3')	Gene	Cycling conditions	References
GAPDH-F (CCTTCATTGACCTCAACTACAT) GAPDH-R (CCAAAGTTGTCATGGATGACC)	GAPDH/ Mammals	95°C for 5 min; 35 cycles of 95°C for 15 sec, 50°C for 30 sec and 72°C for 30 sec; and final extension of 72°C for 5min.	[17]
16S+1 (CTGCTCAATGATTTTTTAAATTGCTGTGG) 16S-1 (CCGGTCTGAACTCAGATCAAGT)	16SrRNA /Ticks	10 cycles of 92°C for 1 min, 48°C for 1min and 72°C for 1min, followed by 32 cycles of 92°C for 1 min, 54°C for 35 sec and 72°C for 1,35 min, and final extension of 72°C for 7min.	[18]
HC02198 (TAAACTTCAGGGTGACCAAAAAATCA) LCO1490 (GGTCAACAAATCATAAAGATATTGG)	COX1/ Fleas	95°C for 1min, 35 cycles of 95°C for 15 sec, 55°C for 15 sec and 72°C for 10 sec, and final extension of 72°C for 5min.	[19]
443f (GCTATGTCTGCATTCTATCA) 1210R (GATCYTCAATCATTTCTTTCCA)	*gltA/ Bartonella* spp.	94°C for 5 min, 35 cycles of 94°C for 30 sec, 54°C for 30 sec and 72°C for 1 min, and final extension of 72°C for 5min.	[21]
1615s (ATYACYCATAARCGYCGTCTTTCTGCTCTGG) 2267as (GGATCTAAATCTTCYGTYGCACGRATACG)	*rpoB/ Bartonella* spp.	95°C for 2 min, 55 cycles of 94°C for 15 sec, 62°C for 15 sec and 72°C for 15 sec, and final extension of 72°C for 5min.	[8]
nuoGF (GGCGTGATTGTTCTCGTTA) nuoGR (CACGACCACGGCTATCAAT)	*nuoG /Bartonella* spp.	94°C for 5 min, 35 cycles of 94°C for 30 sec, 53°C for 30 sec and 72°C for 1 min, and final extension of 72°C for 5min.	[23]
ftszF (CATATGGTTTTCATTACTGCYGGTATGG) ftszR (TTCTTCGCGAATACGATTAGCAGCTTC)	*ftsZ /Bartonella* spp.	94°C for 5 min, 35 cycles of 94°C for 30 sec, 61°C for 30 sec and 72°C for 1 min, and final extension of 72°C for 5min.	[22]
ribcF (TYGGTTGTGTKGAAGATGT) ribcR (AATAATMAGAACATCAAAAA)	*ribC/ Bartonella* spp.	94°C for 5 min, 35 cycles of 94°C for 30 sec, 61°C for 30 sec and 72°C for 1 min, and final extension of 72°C for 5min.	[22]

Firstly, a previously described broad range quantitative real-time PCR (qPCR) protocol based on *nuoG* gene was used aiming to detect and quantify *Bartonella* spp. DNA copies (number of copies/µL) [7]. The Taq Man qPCR reactions were performed with a final volume of 10µL contained 5µL GoTaq® Probe qPCR Master Mix (Promega Corporation, Madison USA), 1.2µM of each primer F-Bart (5'-CAATCTTCTTTTGCTTCACC-3'), R-Bart (5'- TCAGGGCTTTATGTGAATAC-3') and hydrolysis probe TexasRed-5'-TTYGTCATTTGAACACG-3'[BHQ2a-Q]-3', and 1µL of each DNA sample. The amplification conditions were 95°C for 3 minutes followed by 40 cycles at 95°C for 10 seconds and 52.8°C for 30 seconds. PCR amplifications were conducted 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 concentrations of plasmid DNA (pIDTSMART - Integrated DNA Technologies) ($2.0x10^7$ to $2.0x10^0$ copies/µL), which encoded an 83bp *Bartonella henselae-nuoG* gene fragment. The number of plasmid copies was determined in accordance with the formula (Xg/µL DNA/[plasmid size (bp)x660])x$6.022x10^{23}$xplasmid copies/µL. Each qPCR assay was performed including duplicates of each DNA sample. All the duplicates showingCq 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 [20].

All positive samples in qPCR reactions were submitted to previously described conventional PCR (cPCR) assays to five other protein-coding genes, namely *gltA* (750 bp) [21], *ftsZ* (600 bp) [22], *nuoG* (400 bp) [23], *ribC* (420bp) [22] and *rpoB* (585 bp) [8] (**Table 1**). Each sample of extracted DNA was used as a template in 25µL PCR assay reactions. The mixture containing 10X PCR buffer (Life Technologies®, Carlsbad, CA, USA), 1.0mM $MgCl_2$ (Life Technologies®, Carlsbad, CA, USA), 0.2mM deoxynucleotide triphosphate (dNTPs) mixture(Life Technologies®, Carlsbad,CA,USA), 1.5U Taq DNA Polymerase(Life Technologies®, Carlsbad, CA, USA), and 0.5µM of each primer (Integrated DNA Technologies®, Coralville, IA,

USA). *Bartonella henselae* DNA obtained from a naturally infected cat [7] and ultra-pure sterile water (Life Technologies®, Carlsbad, CA, USA) were used as positive and negative controls. 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). The sequencing was carried out 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).

2.5. Bioinformatics/Phylogenetic analysis

The sequences obtained from positive samples were first submitted to a screening test using Phred-Phrap software version 23 [24] to evaluate the electropherogram quality and to obtain consensus sequences from the alignment of the sense and antisense sequences. The BLAST program was used to analyze the sequences of nucleotides (BLASTn), aiming to browse and compare with sequences previously deposited in GenBank international database. All sequences that showed appropriate quality standards and identity with *Bartonella* spp. were deposited in the Genbank international database. Samples showing positive results for more than one protocol had their sequences concatenated, using the fragment merger software, version 1 [25]. The sequences were aligned with sequences retrieved from GenBank using MAFFT software, version 7 [26].

Phylogenetic inference was based on Bayesian Inference (BI). The Bayesian Inference analysis was performed with MrBayes 3.1.2 [27]. 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%.

Additionally, analysis of nucleotide polymorphisms of sequences obtained in the present study was performed. The sequences were aligned using Clustal/W [28]. The number of haplotypes, haplotype diversity (Hd), nucleotide diversity (Pi),

andnumber of variable sites were determined using the program DnaSP 5, version 5.10.01 [29].

2.6. 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 was 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.

3. Results

A total of 298 animals were captured in Brazilian Pantanal: 158carnivores, including 78 crab-eating foxes (*C. thous*), 31 coatis (*N. nasua*), seven ocelots (*L. pardalis*) and 42 domestic dogs; 140 small mammals, including 110 wild rodents (77 *T. fosteri*, 25 *O. mamorae* and 8 *Clyomys laticeps*) and 30 wild marsupials (14 *Thylamys macrurus,* 11 *Gracilinanus agilis*, 4 *Monodelphis domestica* and 1 *Didelphis albiventris*).

One thousand five hundred and eighty-two ticks parasitizing the sampled wild mammals were collected, among 1033 belonging to *Amblyomma sculptum* Berlese species, 241 belonging to *Amblyomma parvum* Aragão species, 32 *Amblyomma ovale* Koch adults, one *Amblyomma tigrinum* Koch adult, one *Rhipicephalus (Boophilus) microplus* (Canestrini) adult, one *Rhipicephalus sanguineus* s.l. (Latreille) adult, four *Amblyomma auricularium* (Conil) nymphs, and 269 *Amblyomma* larvae (**Table 2**). Besides, a total of 75 *Polygenis* (*Polygenis*) *bohlsi bohlsi* (Wagner) fleas were collected from *T. fosteri,* four from *M. domestica* and one flea was collected from *T. macrurus.*

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

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

L – larvae. N – nymph. M –male adult.F – female.adult
N°anim. – number of sampled animals
No 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.

All 298 DNA animal samples amplified the predicted product for GAPDH gene. The DNA concentration mean and absorbance ratio (260/280) were 145.3 ng/µL (SD ± 95.3) and 2.13(SD ± 2.18), respectively. The number of tick DNA samples extracted was 523, of which 228 (43.5%) were from adults, 256 (48.9%) pooled nymphs, and 39 (7.4%) from pooled larvae. The concentration mean and absorbance ratio (260/280) were 45.9ng/µL (SD ± 84.3) and 1.81(SD ± 1.96), respectively. Out of523 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* larvae]) showed negative results for the tick mitochondrial 16S rRNA gene cPCR and were excluded from subsequent analyses. A total of 39 pooled fleas samples were submitted to DNA extraction, whose concentration mean and absorbance ratio (260/280) were 7 ng/µL (SD ± 8.43) and 1.12(SD ± 1.03), respectively. Only one flea DNA sample did not amplify the predicted product for cox-1 and was also excluded from subsequent analyses.

Thirty-five (31.8%) wild rodents and three (7.8%) *Polygenis (P.) bohlsi bohlsi* flea pools (collected from three *T. fosteri* wild rodents) showed positivity in qPCR for *Bartonella* spp. based on the *nuoG* gene. None of sampled *C. thous, N. nasua, L. pardalis*, marsupials, domestic dogs and ticks showedpositivity in qPCR for *Bartonella* spp. based on the *nuoG* gene. Among *Bartonella*-positive rodents, 30 (85.7%) belonged to *T. fosteri* species and five (14.2%) belonged to *O. mamorae* species. The number of copies of *Bartonella-nuoG* fragment/µL ranged from 1.33 x 10^0 to 2.61 x 10^6 (mean= 6.913 x 10^4). The efficiency and correlation coefficient of qPCR reactions ranged from 91.1% to 101.3% and 0.948 to 0.998, respectively. The quantification mean between the groups of hosts were: *T. fosteri* = 3.73 x 10^2copies/µL (1.33 x 10^0 - 3.58 x 10^3), *O. mamorae* = 5.23 x 10^5copies/µL (1.57 x 10^1 - 2.61 x 10^6) and *Polygenis* (*P.*) *bohlsi bohlsi* = 3.11 x 10^1copies/µL (2.77 x 10^1 - 3.51 x 10^1). The *Bartonella*-positive fleas in qPCR assays were not collected from any positive wild rodent.

Thirty-seven samples (97.3%) out of 38 qPCR positive samples for *Bartonella* also showed positivity in cPCR based on the *ftsZ* gene, of which 29 belonged to *T. fosteri*species*,* five belonged to *O. mamorae* speciesand threebelonged to *Polygenis (P.) bohlsi bohlsi* flea species. Four *O. mamorae* and two flea pools positive samples

in *ftsZ* cPCR also showed positivity in cPCR based on *gltA* gene. In addition to this, nine *T. fosteri* positive samples in *ftsZ* cPCR also showed positivity in cPCR based on *nuoG* gene. No animal or arthropod was positive in cPCR assays based on *rpoB* and *ribC* genes. All *gltA, ftsZ* and *nuoG* sequences obtained from positive animals and fleas were deposited in the international database Genbank under the following accession numbers: KX578719, KX827420, KY273622-KY273657, KY304482-KY304486.

The BLAST analysis of a 750bp fragment of *Bartonella gltA* gene obtained from four *O. mamorae* spleen samples showed 96% identity with the American isolated R-phy1 *Bartonella* spp. (Z70010). The sequences of *gltA* gene of *Bartonella* spp. obtained from flea samples showed 95% identity with a *Bartonella* spp. sequence obtained from a wild Brazilian rodent (KX086733). On the other hand, The BLAST analysis of a 600bp fragment of *Bartonella ftsZ* gene, obtained from the same four *O. mamorae* spleen samples, showed 91% identity with *B. vinsonii* subsp. berkhoffii (CP003124). Thirty *Bartonella ftsZ* sequences obtained from *T. fosteri* spleen samples showed 94-96% identity with *B. alsatica* (AF467763).Three *Bartonella ftsZ* sequences obtained from flea pools showed 92% identity with a *Bartonella* spp. sequence obtained from a wild Brazilian rodent (KX036239). The BLAST analysis of a 400bp fragment of *Bartonella nuoG* gene obtained from nine *T. fosteri* spleen samples showed 94-95% identity with *B. alsatica* (EF659935).

The concatenated phylogenetic analysis of *Bartonella* based on *gltA* and *ftsZ* genes showed that eight *Bartonella gltA+ ftsZ* sequences obtained from *O. mamorae* wild rodents and four *Bartonella gltA+ftsZ* sequences obtained from *Polygenis (P.) bohlsi bohlsi* pools in the present study were pooled in the same branch of *Bartonella* sequences obtained from Brazilian wild rodents previously deposited in Genbank, with clade support of 100, based on BI analyses, (**Fig 2**). Besides, the concatenated phylogenetic analysis of *Bartonella* based on *gltA* and *ftsZ* genes showed that *Bartonella* spp. sequences obtained from *O. mamorae* wild rodents and *Polygenis (P.) bohlsi bohlsi* pools from the present study were closely related to *Bartonella* genotypes detected in *Sigmodon hispidus* in the USA and *Bartonella* genotypes detected in Brazilian wild rodents previously deposited in the Genbank (**Fig 2**). Additionally, the concatenated phylogenetic analysis of *Bartonella* based on *nuoG*

and *ftsZ* genes showed that 18 *nuoG +ftsZ Bartonella* sequences obtained from *T. fosteri* wild rodents were grouped in the same clade of *B. alsatica* with high clade support of 100, based on BI analyses (**Fig 3**).

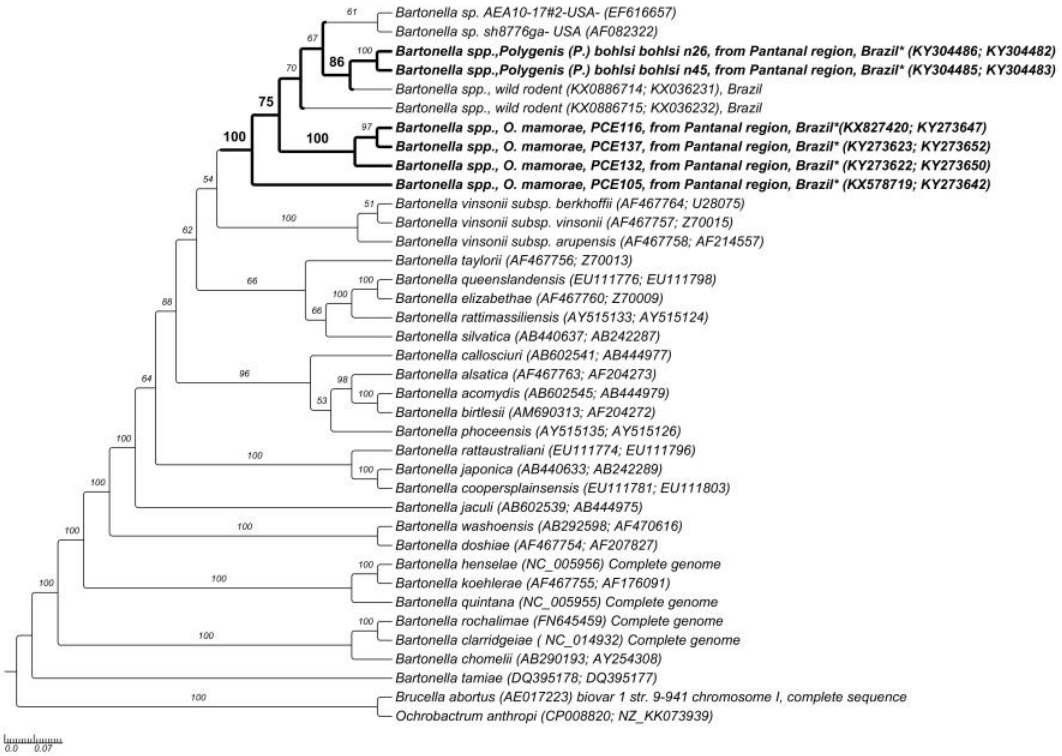

Figure 2. Phylogenetic tree constructed with 1245bp *Bartonella gltA+ftsZ* sequences, using Bayesian method and GTR+G+I evolutionary model. Numbers at nodes correspond to Bayesian posterior probabilities over 50, using *Ochrobactrum anthropi* (CP008820, NZ_KK073939) and *Brucella abortus* (AE017223) as outgroups.

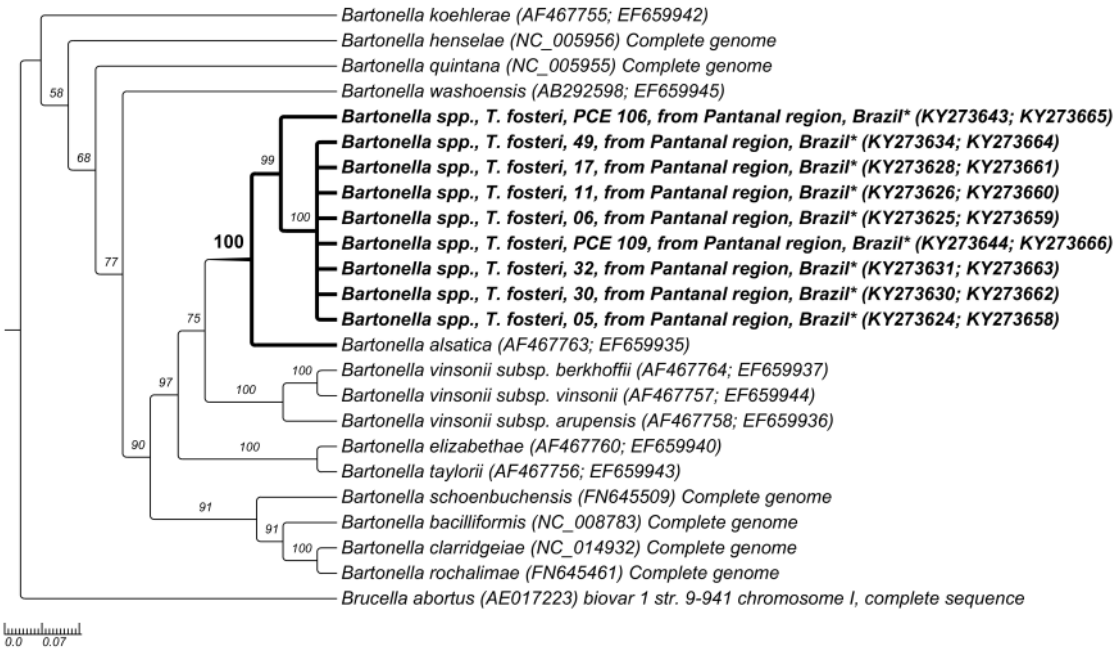

Figure 3. Phylogenetic tree constructed with 820bp *Bartonella nuoG+ftsZ* sequences, using Bayesian method and GTR+G+I evolutionary model. Numbers at nodes correspond to Bayesian posterior probabilities over 50, using *Brucella abortus* (AE017223) as outgroup.

Among the six *Bartonella gltA* sequences analyzed, four different haplotypes were found, showing Pi = 0.02889, hd = 0.8667 and K = 16.6667. The haplotypes #2 and #4 were formed by two sequences each, while the haplotypes #1 and #3 were formed by only one sequence each. The haplotypes #1, #2 and #3 were formed only by *gltA* sequences obtained from *O. mamorae* and the haplotype #4 was formed by sequences obtained from fleas samples. Analyzing the alignment of 37 *Bartonella fstZ* sequences, four different haplotypes were also found, with Pi = 0.03523, hd = 0.413 and K = 9.3018. The haplotype#1 was formed by only one *Bartonella fstZ* sequence obtained from *T. fosteri*, the haplotype #2 was formed by 28 sequences also obtained from *T. fosteri,* the haplotype #3 was formed by five sequences obtained from *O. mamorae* and the haplotype #4 was formed by three sequences obtained from fleas samples. The *Bartonella nuoG* alignment did not present variable sites, so it was not possible to observe any polymorphism or nucleotide diversity between the sequences of this gene.

4. Discussion

The present study showed the presence of *Bartonella* DNA in spleen samples of wild rodents and in *Polygenis (P.) bohlsi bohlsi* fleas collected from rodents in Brazilian Pantanal wetlands. To the best authors' knowledge, this was the first molecular detection of *Bartonella* spp. among blood sucking arthropods from Brazil.

The found occurrence of *Bartonella* spp. among wild rodents sampled in the present study was higher (35/110) than that found in synanthropic rodents (5/26) in the city of Salvador, state of Bahia, northeastern Brazil [4], but similar to that found in a previous recent study involving wild rodents (11/32) sampled in southern Pantanal [6]. However, the occurrence of *Bartonella* infection among wild rodents in Pantanal biome in the present study was lower than that found in wild rodents (18/42) in peri-urban areas from the same state where the present study was performed [5]. A closer contact among wild rodents from peri-urban areas with different hosts species (including domestic animals) and arthropods could explain the differences observed in prevalence rates.

A genotype closely related to *Bartonella* spp. (phylogenetic group A and strain R-phy1) detected in Cricetidae rodents from North America [1, 30] and Brazil [6] was detected in *O. mamorae* rodents, also belonging to Cricetidae family. On the other hand, a different *Bartonella* genotype was detected in *T. fosteri* rodents belong to Echimyidae family sampled in the same region. A higher *Bartonella* host specificity for Cricetidae rodents (*Sigmodon hispidus* and *Peromyscus leucopus*) has been experimentally demonstrated when compared to BALB/c mice and Wistar rats [31]. These partial results suggest the presence of a Cricetidae–specific strain of *Bartonella* circulating among wild rodents from North and South America.

To the best of authors' knowledge, a genotype closely related to *B. alsatica* was detected, for the first time, among *T. fosteri* rodents in Brazil. Although it is known that *Bartonella* belonging to phylogenetic group A does not cause illness in experimentally infected *S. hispidus* [32] and its zoonotic potential is still unknown [6], *B. alsatica* is known to cause lymphadenitis [33] and endocarditis in humans [34], highlighting the importance of future studies to elucidate the ecological pathways involving this *Bartonella* genotype among rodents and vectors in Pantanal region.

Among the ectoparasites that infest wild rodents, fleas are considered the main vectors for *Bartonella* species [35]. Fleas are also considered important reservoirs for *Bartonella* [36]. In the present study, a *Bartonella* closely related to R-phy1 genotype was detected in both *O. mamorae* rodents and *Polygenis (P.) bohlsi bohlsi* fleas in Pantanal. Although fleas that were positive for *Bartonella* were not collected from rodents positive for this agent, the phylogenetic analysis showed that the genotypes were closely related, suggesting that *Polygenis (P.) bohlsi bohlsi* fleas may act as possible *Bartonella* vectors to Cricetidae rodents from Brazil. Similar evidence was reported in *Polygenis gwyni* fleas parasitizing Cricetidae rodents (*Sigmodon hispidus*) in the United States [30].

Although a previous study demonstrated the vectorial competence of *Ixodes ricinus* ticks in transmitting *B. birtlesii* to rodents [37], the biological role of ticks in the transmission of *Bartonella* in the environment has not yet been confirmed, and its epidemiological role is considered secondary [38]. Due to the fact that no tick of the present study showed positivity for *Bartonella* spp., the importance of ticks in the bartonellosis epidemiology in the studied region in Brazil remains unclear.

The *Bartonella* diversity based on *gltA* sequences (Pi = 0.028,hd = 0.8667) found in the present study was similar to that found in previous studies among rodents sampled in different biomes in Brazil (Pi = 0.024) [6], Asia (Pi= 0.02154) and North America (Pi = 0.01427) [39], but higher than that found among *B. grahamii*genotypes detected in *Myodes rutilus*, *Microtus fortis* and *Apodemus agrarius*rodentsin China(Pi = 0.012) [39]. On the other hand, the found*gltA* diversity was lower than that found among *Bartonella* genotypes detected in *Myodes glareolus* rodents sampled in Paris, France (Pi= 0.077) [40]. These findings reinforce the hypotheses that within the same gene (*gltA*), recombination and mutation events may happen, promoting distinct haplotypes diversity in a certain population. In addition to this, the *ftsZ* nucleotide diversity observed (Pi = 0.035) was also similar to that found among *Bartonella* genotypes detected in rodents in a previous study in Brazil (Pi = 0.037) [6], but higher than those previously reported among *B. grahamii* genotypes detected in rodents in France (Pi = 0.016) [40] and China (Pi = 0.010) [39]. These results reveal some degree of *Bartonella* genetic diversity among the populations of wild rodents in Brazil.

Previously,*Bartonella* spp. weremolecularly detected in *six different species of wild felids, namely Leopardus wiedii, L.pardalis, Leopardus tigrinus, Leopardus geoffroyi, Puma yagouaroundi* and *Panthera onca*, maintained in captivity in Paraná state, southern Brazil [9]. Additionally, antibodies to *Bartonella clarridgeiae* and *Bartonella vinsonii* subsp. *berkhoffii* were *detected in four different species of wild canids, namely C. thous, Speothos venaticus, Canis lupus* and *Lycalopex vetulus*, *maintained in captivity in the states of São Paulo and Mato Grosso, Brazil [10]*. In the present study, none of the sampled wild carnivores showed presence of *Bartonella* DNA in blood samples. In a previous study involving free-roaming domestic cats in a zoo environment in Brazil [41], *Bartonella* DNA was detected in 30% of the sampled cats. Considering that domestic cats are the main reservoirs for *B. henselae* and *B. clarridgeiae* [2], the presence of infected cats in a zoo environment may have facilitated the *Bartonella* transmission to the wild carnivores maintained in captivity, which probably did not happen among wild canids and felids sampled in the present study, since they live free and the contact with domestic cats is scarce.

None of the domestic dogs sampled in the present study showed to be positive for *Bartonella* in qPCR assays, corroborating the hypothesis that dogs are considered only accidental hosts for *Bartonella* [2]. In Brazil, there is only one report showing a low occurence (1% for *B. henselae* and 0.5% for *B. vinsonii* subsp. *berkoffii*) in dogs from São Paulo state [8]. Besides, the diagnosis of *Bartonella* spp. based only on PCR amplification shows lack of sensitivity, due to the fact the bacteremia found in dogs may be usually low. A previous study showed that a combined approach using isolation culture-based growth medium, such as BAPGM, followed by PCR amplification, can provide a substantial improvement in chances of detection of *B. henselae* and *B. vinsonii* (*berkhoffii*) in the blood of naturally infected dogs [42].

In conclusion, the present study revealed that wild rodents in southern Pantanal region, Brazil, are exposed, at least, to two different genotypes of *Bartonella* spp. Considering the fact that some *Bartonella* genotypes found in this present study showed to be closely related to *B. alsatica,* a well known zoonotic *Bartonella* species, studies aiming at assessing the transmission routes among rodents, involved vectors and the zoonotic potential of the isolates are much needed

in order to prevent human cases of bartonellosis. Lastly, this study provided an evidence of the possible participation of *Polygenis (P.) bohlsi bohlsi* in *Bartonella* transmission cycles among wild rodents in Brazil. However, in order to confirm the competence of *P.bohlsi* fleas to transmit *Bartonella,* experimental studies must be performed.

Declaration of conflict of interest

None.

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CHAPTER 4 - Occurrence and molecular characterization of hemoplasmas in domestic dogs and wild mammals in a Brazilian wetland. Acta Tropica

Abstract

Hemotropic mycoplasmas are known to cause anemia in several mammalian species. The present work aimed to investigate the occurrence of *Mycoplasma* spp. in wild animals, domestic dogs and their respective ectoparasites, in southern Pantanal region, central-western Brazil. Between August2013 and March2015, 31 coatis, 78 crab-eating foxes, seven ocelots, 42 dogs, 110 wild rodents, and 30 marsupials were trapped and ectoparasites (ticks and fleas) found parasitizing the animals were collected. Mammals and ectoparasites DNA samples were submitted to conventional PCR assays for *Mycoplasma* spp. targeting 16S rRNA and *RnaseP* genes. Twenty-four coatis, three crab-eating foxes, two domestic dogs, one ocelot and one wild rodent were positive for both 16S rRNA PCR protocols. Fourteen coatis samples were also positive in *RnaseP* PCR. No marsupial and arthropod showed positivity for *Mycoplasma* spp. The phylogenetic analyses based on 16SrRNA gene showed that all sequences obtained from dogs, two sequences obtained from crab-eating foxes and ten sequences obtained from coatis showed to be closely related to *M. haemocanis*/*M. haemofelis* species. Genotypes closely related to '*Candidatus* Mycoplasma haemominutum' and *M. haemomuris* were detected in the ocelot and in the wild rodent, respectively. Probably a novel *Mycoplasma* genotype, closely related to a sequence obtained from a Brazilian capybara was detected in one crab-eating fox and 14 coati, based on a concatenated phylogenetic analysis of 16S rRNA and *RnaseP* genes. The present study revealed that wild animals in southern Pantanal region, Brazil, are exposed to different species of hemoplasmas.

KeyWords: *Mycoplasma* spp., wild carnivores, coatis, wild rodents, Pantanal.

1. Introduction

Hemotropic mycoplasmas (hemoplasmas) are epicellular erythrocytic bacteria lacking cell wall. In contrast to several mucosal mycoplasmas, hemoplasmas have

never been grown successfully in culture so far. These pathogens are known to be the causative agents of infectious anemia in several mammalian species and may induce acute hemolysis in some cases (Tasker, 2010). The disease is characterized by anorexia, lethargy, dehydration, weight loss and in some cases, canlead to death (Willi et al., 2007). Furthermore, hemotropic mycoplasmas are considered emergent zoonotic agents, mainly in immunocompromised individuals or those highly exposed to arthropod vectors (dos Santos et al., 2008; Maggi et al., 2013a).

The transmission of hemoplasmas between domestic cats and dogs seems to occur mainly by bloodsucking arthropods, such as ticks and fleas, blood transfusion, contaminated fomites and transplacentally (Seneviratna et al., 1973; Messick, 2003; Lappin et al., 2006). In addition to this, direct transmission through biting and fighting is another possible route of transmission.

Although hemoplasmas have been detected in domestic cats (Braga et al., 2012; Miceli et al., 2013; André et al., 2014; Santis et al., 2014) and dogs (Ramos et al., 2010; Alves et al., 2014; Valle et al., 2014; Soares et al., 2016) from several localities in Brazil, few reports have documented the occurrence of hemoplasma species in wild animals. For instance, hemoplasmas have been detected in wild carnivores maintained in captivity in zoos (Willi et al., 2007; Guimarães et al., 2007; André et al., 2011), wild rodents (Vieira et al., 2009; Conrado et al., 2015; Gonçalves et al., 2015), monkeys (Bonato et al., 2015) and deer (Grazziotin et al., 2011) in Brazil.

Due to the lack of information about the epidemiology and routes of transmission of hemoplasmas among the wildlife from Brazil, the present study aimed to investigate the occurrence of hemotropic mycoplasmas in wild mammals and domestic dogs and their respective ectoparasites in the region of Pantanal, state of MatoGrosso do Sul, central-western Brazil.

2. Material and Methods

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 **(Figure 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) (Alves et al., 2016).

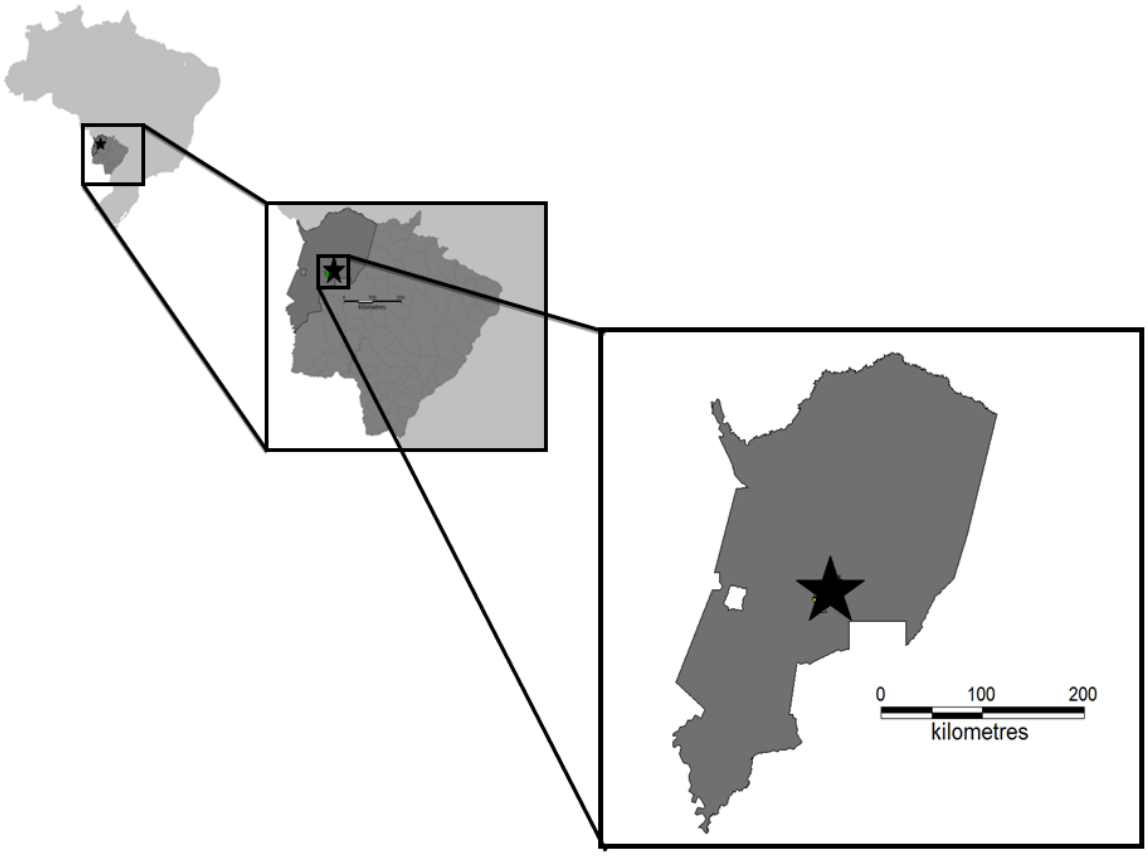

Figure 1. Capture sites. Map of Mato Grosso do Sul State, central-western Brazil, showing the Pantanal region, where animals samples were collected in the present study.

Between August 2013 and March 2015, four field expeditions of approximately 10 days each (August 2013, October 2013, August 2014 and March 2015) were performed. Wild 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 24h and checked twice a

day. The animals were immobilized with an intramuscular injection of a combination of Zolazepan and Tiletamine (Zoletil®) at dosages of 8mg/kg for ocelots and 10mg/kg for crab-eating foxes and coatis. Blood samples were collected by puncture of the cephalic vein and stored in Vacutainer® containing EDTA tubes at -20°C until DNA extraction. All animal captures were in accordance with the licenses obtained from the Brazilian Government Institute for Wildlife and Natural Resources Care (IBAMA) (license number 38787-2) and endorsed by the Ethics Committee of FCAV/UNESP University (Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista "Júlio de MesquitaFilho", Câmpus Jaboticabal) nº 006772/13. Additionally, blood samples were collected from domestic dogs (*Canis lupus familiaris*), 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 10m 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 between the two expeditions (August 2014 and March 2015). The identification of specimens was based on external and cranial morphological characters and karyological analyses, as previously described (Bonvicino et al., 2005). The animals were firstly anesthetized with an intramuscular injection of Ketamine (10–30mg/kg) associated with Acepromazine (5–10mg/kg) for rodents (9:1proportion), or Xylazine (2mg/kg) for marsupials (1:1). After anesthesia, the animals were euthanized with potassium chloride, which doses ranging from75 to 150mg/kg (Leary et al., 2013). Spleen samples were collected and stored in absolute alcohol (Merck®,Kenilworth, Nova Jersey,USA). Animal handling procedures followed the Guidelines of the American Society of Mammalogists for the use of wild mammals in research (Sikes and Gannon, 2011). The project had permission from the Brazilian Government Environmental Agency (Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) (SISBIO license number 38145) and was also endorsed by the Ethics Committee of the FCAV/UNESP University (CEUA -nº 006772/13), in accordance to Brazilian regulations.

Ticks and fleas found parasitizing the sampled animals were detected by inspection of the skin and carefully removed by forceps or manually. The specimens were stored in 100% alcohol (Merck®, Kenilworth, NewJersey, USA) until identification, which was performed using a stereomicroscope (Leica® MZ 16A, Wetzlar, Germany) and following taxonomic literature for adult tick genera (Onofrio et al., 2006; 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).

DNA was extracted from 200µL of each whole blood (wild carnivores and domestic dogs) and 10mg of spleen (small mammals) samples using the QIAamp DNA Blood Mini kit (QIAGEN®, Valencia, CA, USA), according to the manufacturer's instructions. While DNA extraction from ticks was processed in pools for nymphs (up to 5 individuals) and larvae (up to 10 individuals), the adults were processed individually. 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. DNA concentration and quality were measured using 260/280nm absorbance ratio (Nanodrop®, Thermo Fisher Scientific, Waltham, MA, USA). In order to verify the presence of amplifiable DNA in the samples, internal control PCR assays targeting fragments of mammalian glyceraldehyde-3-phosphatedehydrogenase (GAPDH) (Birkenheuer et al., 2003), ticks mitochondrial 16S rRNA (Black and Piesman, 1994) and fleas cytochrome-c oxidase subunit I (COX1) (Folmer et., 1994) genes were performed (**Table1**)**.**

Table 1. Oligonucleotides sequences, target genes and thermal conditions used in conventional PCR assays targeting endogenous genes and 16S rRNA and *RNaseP* fragments of hemoplamas in biological samples from wild mammals and domestic dogs, trapped and sampled, respectively, in southern Pantanal, state of Mato Grosso do Sul, central-western Brazil.

Primers sequences (5'-3')	Gene	Thermal conditions	References
GAPDH-F (CCTTCATTGACCTCAACTACAT) GAPDH-R (CCAAAGTTGTCATGGATGACC)	GAPDH/ Mammals	95°C for 5 min; 35 cycles of 95°C for 15 sec, 50°C for 30 sec and 72°C for 30 sec; and final extension of 72°C for 5min.	Birkenheuer et al. (2003)
16S+1 (CTGCTCAATGATTTTTTAAATTGCTGTGG) 16S-1 (CCGGTCTGAACTCAGATCAAGT)	16SrRNA /Ticks	10 cycles of 92°C for 1 min, 48°C for 1min and 72°C for 1min, followed by 32 cycles of 92°C for 1 min, 54°C for 35 sec and 72°C for 1,35 min, and final extension of 72°C for 7min.	Black and Piesman (1994)
HC02198 (TAAACTTCAGGGTGACCAAAAAATCA) LCO1490 (GGTCAACAAATCATAAAGATATTGG)	COX1/ Fleas	95°C for 1min, 35 cycles of 95°C for 15 sec, 55°C for 15 sec and 72°C for 10 sec, and final extension of 72°C for 5min.	Folmer et al. (1994)
HemMycop16S-41s (GYATGCMTAAYACATGCAAGTCGARCG) HemMyco16S-938as (CTCCACCACTTGTTCAGGTCCCCGTC)	16S rRNA 1° set / Hemoplasmas	94°C for 2 min, 55 cycles of 94°C for 15 sec, 68°C for 15 sec and 72°C for 18 sec, and final extension of 72°C for 1min.	Maggi et al. (2013b)
HemMycop16S- 322s (GCCCATATTCCTACGGGAAGCAGCAGT) HemMycop16S-1420as (GTTTGACGGGCGGTGTGTACAAGACC)	16S rRNA 2° set / Hemoplasmas	94°C for 2 min, 55 cycles of 94°C for 15 sec, 68°C for 15 sec and 72°C for 18 sec, and final extension of 72°C for 1min.	Maggi et al. (2013b)
HemoMyco RNaseP30s (GATKGTGYGAGYATATAAAAAATAAARCTCRAC) HemoMyco RNaseP200as (GMGGRGTTTACCGCGTTTCAC)	*RNaseP*/ Hemoplasmas	94°C for 2 min, 55 cycles of 94°C for 15 sec, 59°C for 15 sec and 72°C for 18 sec, and final extension of 72°C for 1min.	Maggi et al. (2013b)

Previously described PCR protocols based on 16SrRNA gene were performed in order to amplify *Mycoplasma* spp. DNA, using two sets of oligonucleotides (Maggi et al., 2013b) (**Table 1**). Five microliters of DNA were used as a template in 25µL reaction mixtures containing 10X PCR buffer, 1.0mM $MgCl_2$, 0.8mM deoxynucleotide triphosphate (dNTPs) mixture, 1.5U Taq DNA Polymerase (Life Technologies®) and 0.3µM of each oligonucleotide. *Mycoplasma haemofelis* DNA obtained from a naturally infected cat (Miceli et al., 2013) and ultra-pure sterile water were used as positive and negative controls, respectively.16SrRNA-*Mycoplasma* spp.-positive samples were additionally submitted to a previously described *RNaseP* gene-*Mycoplasma* spp. (165bp) PCR assay (Maggi et al., 2013b) (**Table1**). The sequencing was performed using the BigDye® Terminator v3.1Cycle Sequencing Kit (Thermo Fisher Scientific®, Waltham, MA, USA) and ABI PRISM 310DNA Analyzer (Applied Biosystems®, Foster City, CA, USA) (Sanger et al., 1977).

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 program (Altschul et al., 1990) was used to analyze the sequences of nucleotides (BLASTn), aiming to browse and compare with sequences previously deposited in an international database (GenBank) (Benson et al., 2002). All sequences that showed appropriate quality standards and identity with hemoplasmas were deposited in Genbank, except *RNaseP* sequences, whose small fragment size (165pb) precluded being deposited in the international database. Samples showing positive results for both16SrRNA and *RNaseP* protocols had their sequences concatenated, using the fragment merger software, version 1 (Bell and Kramvis, 2013). The sequences were aligned using Clustal/W (Thompson et al., 1994) in Bioedit v. 7.0.5.3 (Hall, 1999) and MAFFT software, version 7 (Katoh and Standley, 2013).

Phylogenetic inferences were based on Bayesian and Maximum Likelihood (ML) methods. 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 Maximum-likelihood (ML) analysis was inferred with RAxML-

HPC BlackBox 7.6.3 (Stamatakis et al., 2008) (which includes an estimation of bootstrap node support), using 1000 bootstrapping replicates. 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 et al., 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).

Logistic regression models were employed to assess the effect of the putative predictor variables (i.e. gender and animal species) on the logit of the probability of infection by hemoplasmas. All analyses were performed using R software 3.0.2 (R Core Team. 2013).

Additionaly, ananalysis of nucleotide polymorphisms of 16SrRNA sequences obtained in the present study was performed. The sequences were aligned using Clustal/W (Thompson et al., 1994) in Bioedit v. 7.0.5.3 (Hall, 1999). The number of haplotypes, haplotype diversity (Hd) and nucleotide diversity (Pi) were estimated to explore the levels of genetic differentiation among the species of hosts with higher number of positive samples for hemoplasmas, using the program DnaSP 5, version 5.10.01 (Librado and Rozas, 2009).

3. Results

A total of 256 animals were captured in the central region of the Pantanal, municipality of Corumbá, state of Mato Grosso do Sul: 158 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 cohabiting the same studied area were collected.

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* Berlese species, 241 (15.2% [78 adults and 163 nymphs]) belonging to *Amblyomma parvum* Aragão species, 32 (2%) *Amblyomma*

ovale Koch adults, one (0.06%) *Amblyomma tigrinum* Koch adult, one (0.06%) *Rhipicephalus (Boophilus) microplus* (Canestrini) adult, one (0.06%) *Rhipicephalus sanguineus* s.l. (Latreille) adult, four (0.2%) *Amblyomma auricularium* (Conil) nymphs, and 269 (17%) *Amblyomma* larvae. Besides, a total of 80 *Polygenis* (*Polygenis*) *bohlsi bohlsi* (Wagner) fleas were collected.

All 298 DNA animal samples amplified the predicted product for GAPDH gen, the concentration mean and absorbance ratio (260/280) were 145.3 ng/µL (SD ± 95.3) and 2.13(SD ± 2.18), respectively. The amount of tick DNA extracted was 523, of which 228 (43.5%) were from adults, 256 (48.9%) pooled nymphs, and 39 (7.4%) from pooled larvae, the concentration mean and absorbance ratio (260/280) were 45.9 ng/µL (SD ± 84.3) and 1.81(SD ± 1.96), respectively. Out of523 sampled ticks, 31 (5.9% [23 *A. parvum* adults, 4 *A. sculptum* adults, 1 *A. ovale* adult, 1 *A. parvum* nymph and 2 pooled *Amblyomma* larvae]) showed negative results for the tick mitochondrial 16S rRNA gene and were excluded from subsequent analyses. A total of 39 pooled fleas samples were submitted to DNA extraction, the concentration mean and absorbance ratio (260/280) were 7 ng/µL (SD ± 8.43) and 1.12(SD ± 1.03), respectively. Only one flea DNA sample did not amplify the predicted product for cox-1 and was also excluded from subsequent analyses.

Twenty-four (77.4%) *N. nasua,* three (3.8%) *C. thous,* two (4.7%) domestic dogs, one (14.2%) *L. pardalis* and one (0.9%) wild rodent (*O. mamorae*) were positive for both hemoplasmas-PCR protocols based on16SrRNA gene. Fourteen (4.6%) *N. nasua* were also positive in PCR for hemoplasmas based on *RnaseP* gene. None of sampled marsupials and arthropods (tick or flea) showed positivity for *Mycoplasma* spp. All 16S rRNA sequences obtained from positive animals were deposited in the international database Genbank under the following accession numbers: KY002649-KY002679. Logistic regression results evidenced no significant difference (P> 0.05) between sexes for positivity in PCR assays for *Mycoplasma* spp. However, compared to other animal species (ocelots, crab-eating foxes, domestic dogs and wild rodents), coatis showed a significantly (p-value < 0.01) higher chance to acquire *Mycoplasma* spp. infection. Odds ratios for *Mycoplasma* spp. infection between coatis and each of other animal species were all above 20.57.

The BLAST analysis was performed with sequences obtained from an overlapping of approximately 600bp of two hemoplasmas-16SrRNA fragments (800bp and 1000bp), and from 165bp fragment of hemoplasmas-*RnaseP* gene. Twenty-four hemoplasmas-16SrRNA sequences obtained from *N. nasua* blood samples showed 99% identity with a sequence of *Mycoplasma* sp. detected in a raccon (*Procyon lotor*) sampled in the United States (KF743735). Three hemoplasmas-16SrRNA sequences obtained from *C. thous* blood samples and two hemoplasmas-16SrRNA sequences obtained from domestic dogs blood samples showed 100% identity with *M. haemocanis* (KP715859). Hemoplasmas-16SrRNA sequences obtained from one *L. pardalis* blood sample and one *O. mamorae* spleen sample showed 100% identity with '*Candidatus* Mycoplasma haemominutum' (EU839985) and with *Mycoplasma* sp. sequence obtained from a Brazilian wild rodent (KT215634), respectively, based on BLAST analysis. Additionally, all 14 hemoplasmas-*RNAseP* sequences obtained from *N. nasua* blood samples presented 91% identity with *M. haemofelis* (JN368074) **(Table 2).**

Table 2. Maximum identity of 16S rRNA and *RnaseP Mycoplasma* spp. sequences detected in wild and domestic animals sampled in southern Pantanal, Brazil, by BLAST analysis.

Animal species	Number of sequences analized	Target Gene	% of identify by BLAST®- analysis
O. mamorae	1		Uncultured *Mycoplasma* sp. from a wild rodent, Brazil (KT215634) 100%
L. pardalis	1		*'Candidatus* Mycoplasma haemominutum' (EU839985) 99%
Domestic dogs	2	16SrRNA	*Mycoplasma haemocanis* (KP715859) 100%
C. thous	3		*Mycoplasma haemocanis* (KP715859) 100%
N. nasua	24		*Mycoplasma* sp. (Raccon USA) (KF743735) 99%
N. nasua	12	*RNaseP*	*Mycoplasma haemofelis* (JN368074) 91%

All hemoplasmas-16SrRNA sequences obtained from dogs' blood samples (KY002678, KY002679), two hemoplasmas-16SrRNA sequences obtained from *C. thous* (KY002677, KY002675) and ten hemoplasmas-16SrRNA sequences obtained from *N. nasua* (KY002652, KY002653, KY002657, KY002661, KY002662, KY002665, KY002667, KY002672, KY002673, KY002674) were grouped in the same large branch comprising 16SrRNA sequences of *M. haemocanis* (HQ918287, AY150973) and *M. haemofelis* (EU930823, KJ135316) previously deposited in Genbank, with clade support of 98 and 100, based on ML and Bayesian analyses, respectively (**Figures 2 and 3**). A hemoplasma-16SrRNA sequence obtained from one *C. thous* blood sample (KY002676) and 14 hemoplasmas-16SrRNA sequences obtained from *N. nasua* blood samples (KY002651, KY002654, KY002655, KY002656, KY002658, KY002659, KY002660, KY002663, KY002664, KY002666, KY002668, KY002669, KY002670, KY002671) were pooled in a separate clade from the others *Mycoplasma* species previously detected, with clade support of 87 and 100,based on ML and Bayesian analyses, respectively (**Figures 2 and 3**), but closely related with a new hemoplasma genotype obtained from a Brazilian capybara (FJ667773) and in the same large branch of *M. coccoides* (AY171918), '*Candidatus* Mycoplasma turicensis'(KJ095696) and a new genotype detected in a synanthropic rodent from Brazil (KM203857), with clade support of 53 and 69, based on ML and Bayesian analyses, respectively (**Figures 2 and 3**).The hemoplasma-16SrRNA sequence obtained from an *O. mamorae* wild rodent (KY002650) was positioned in the same clade of one hemoplasma-16SrRNA sequence obtained from *T. fosteri* wild rodent sampled in the same region of Pantanal, Brazil (KT215621), with clade support of 100 and 100, based on ML and Bayesian analyses, respectively (**Figures 2 and 3**). All hemoplasmas-16SrRNA sequences obtained from *N. nasua, C. thous, O. mamorae* and dogs were grouped into the same large branch of "haemofelis group", based on ML and Bayesian analyses (**Figures 2 and 3**). The hemoplasma-16SrRNA sequence obtained from one *L. pardalis* (KY002649) blood sample was placed in the same clade of '*Candidatus* Mycoplasma haemominutum' (KJ135327), with clade support of 93 and 100, based on ML and Bayesian analyses, respectively, and in the same large branch of "suis group" (**Figures 2 and 3**). Phylogenetic inferences based on ML and Bayesian methods were performed using the

evolutionary model GTR + G + I, using *Mesorhizobium loti* (KM192337), *Brucella melitensis* (AY513568) and *Ochrobactrum anthropi* (EU119263) as outgroups.

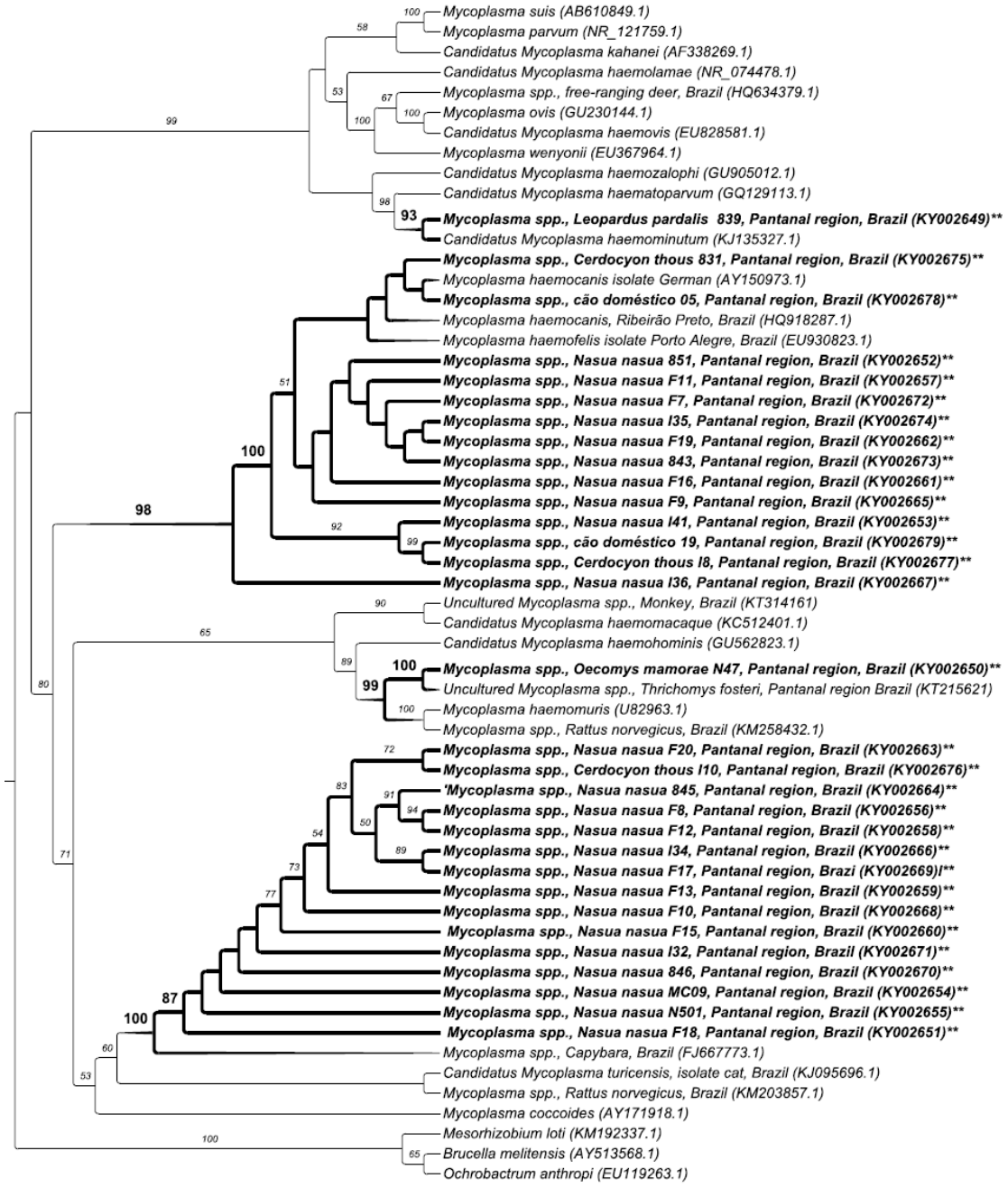

Figure 2. Phylogenetic tree constructed with 1535pb *Mycoplasma* spp.16SrRNA sequences, using Maximum Likelihood (ML) method and GTR+G+I evolutionary model. Numbers at nodes correspond to ML bootstrap values over 50, using *Mesorhizobium loti* (KM192337), *Brucella melitensis* (AY513568) and *Ochrobactrum anthropi* (EU119263) as outgroups.

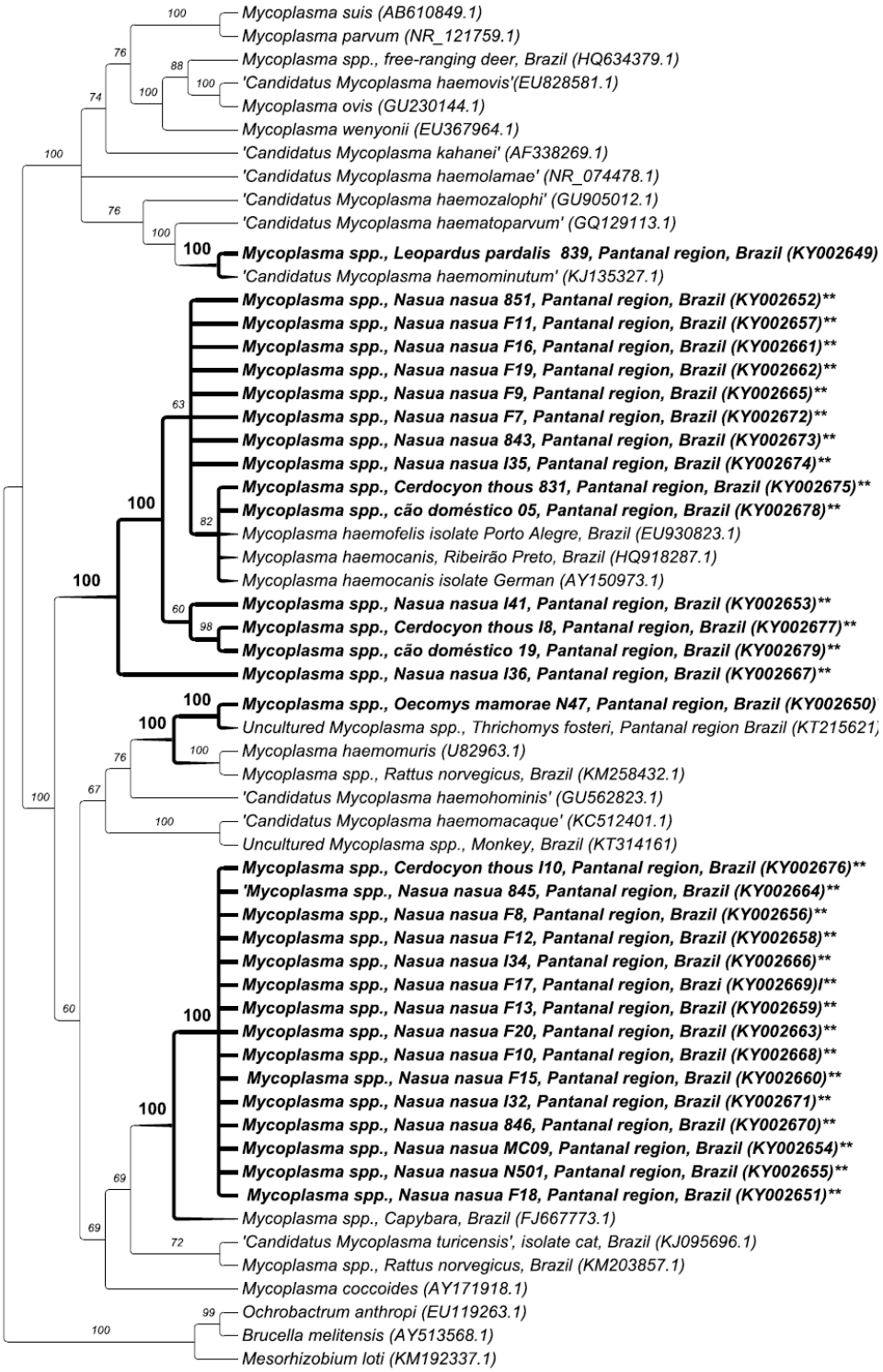

Figure 3. Phylogenetic tree constructed with 1535 pb *Mycoplasma* spp.16SrRNA sequences, using Bayesian method and GTR+G+I evolutionary model. Numbers at nodes correspond to Bayesian posterior probabilities over 50, using *Mesorhizobium loti* (KM192337), *Brucella melitensis* (AY513568) and *Ochrobactrum anthropi* (EU119263) as outgroups.

Regarding the concatenated phylogenetic analysis of hemoplasmas based on *RnaseP* and 16SrRNA genes, six *Mycoplasma* spp. sequences obtained from *N. nasua* blood samples (KY002654, KY002651, KY002655, KY002666, KY002668, KY002671) was pooled in a separate clade from the others *Mycoplasma* species previously detected, with clade support of 100 and 100, based on ML and Bayesian analyses, respectively (**Figures 4 and 5**), but in the same large branch of *M. coccoides* (AY171918; EU078619) and '*Candidatus* Mycoplasma turicensis'(KJ095696; EF212002), with clade support of 91 and 100, based on ML and Bayesian analyses, respectively (**Figures 4 and 5**). The remaining eight hemoplasmas *RnaseP*+16SrRNA concatenated sequences obtained from *N. nasua* blood samples (KY002652, KY002653, KY002657, KY002661, KY002662, KY002665, KY002672, KY002674) were grouped in the same large branch of *M.haemofelis* (KJ135316; EU078617) and *M. haemocanis* (AY150973; EU078618) with clade support of 100 and 100, based on ML and Bayesian analyses, respectively (**Figures 4 and 5**). Phylogenetic inferences based on ML and Bayesian methods were performed using the GTR + G + I evolution model and *Clostridium innocuum* (GQ456215; U64878) as outgroup.

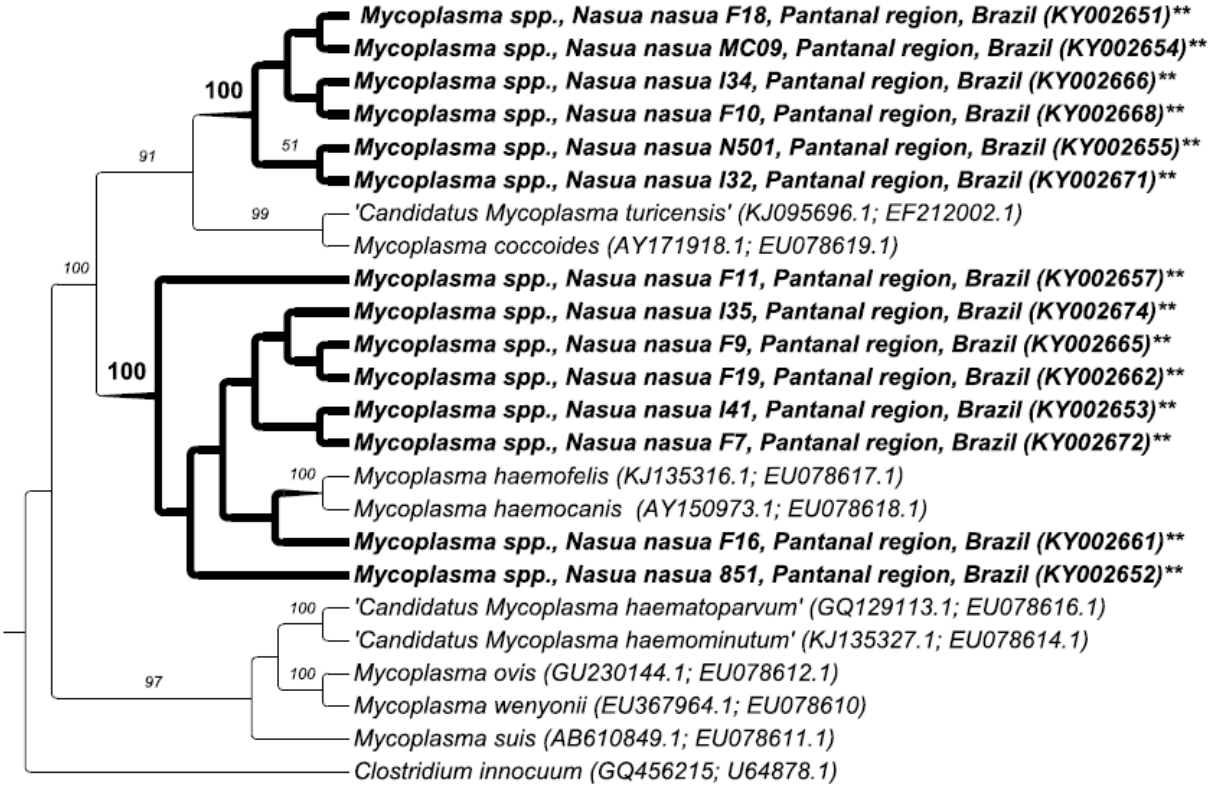

Figure 4. Phylogenetic tree constructed with 1750pb *Mycoplasma* spp.16SrRNA + *RnaseP* sequences, using Maximum Likelihood (ML) method and GTR+G+I evolutionary model. Numbers at nodes correspond to ML bootstrap values over 50 and *Clostridium innocuum* (GQ456215; U64878) as outgroups.

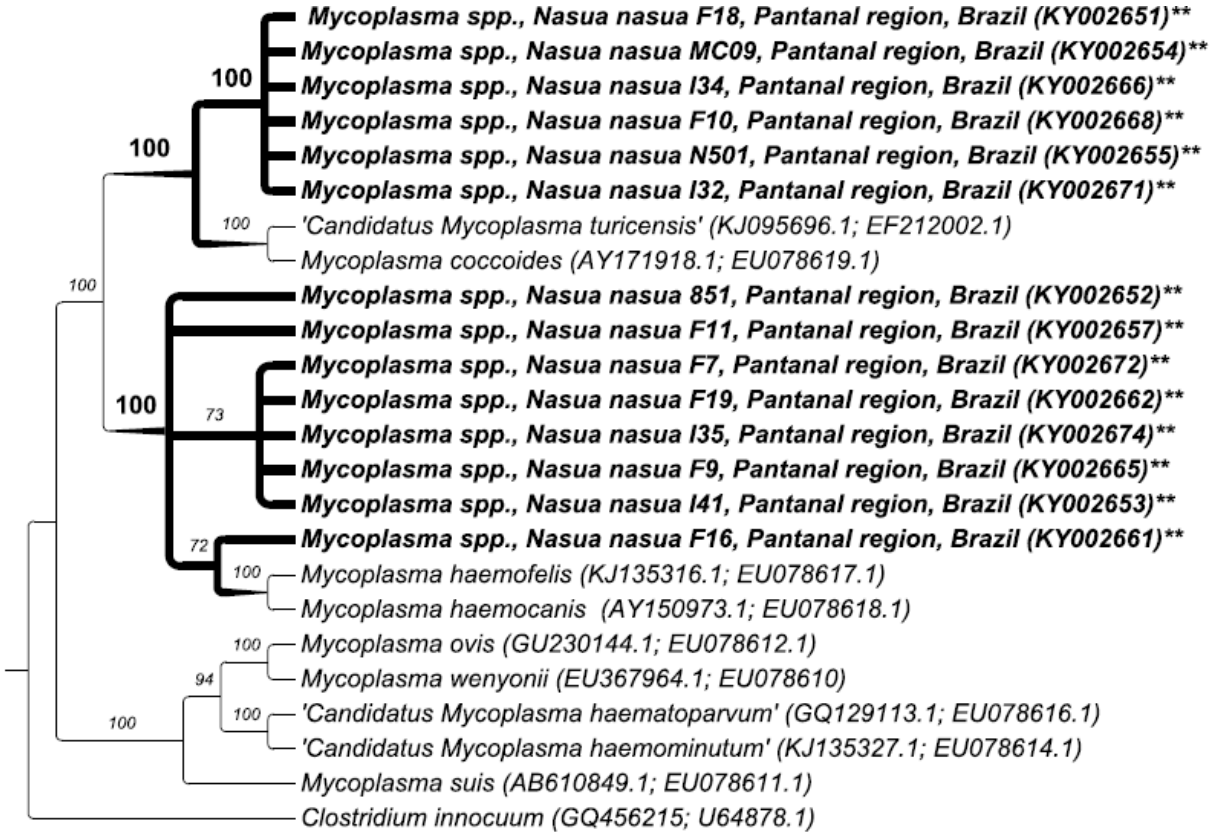

Figure 5. Phylogenetic tree constructed with 1750pb *Mycoplasma* spp.16SrRNA + *RnaseP* sequences, using Bayesian method and GTR+G+I evolutionary model. Numbers at nodes correspond to Bayesian posterior probabilities over 50 and *Clostridium innocuum* (GQ456215; U64878) as outgroup.

One sequence obtained from *C. thous* blood sample (KY002676) and 14 sequences obtained from *N. nasua* blood samples (KY002651, KY002654, KY002655, KY002656, KY002658, KY002659, KY002660, KY002663, KY002664, KY002666, KY002668, KY002669, KY002670, KY002671), were pooled in a separate clade from others *Mycoplasma* species previously detected, based on the 16SrRNA and *RnaseP* phylogenetic analysis **(Figures 2-5).**

Among the animals captured, coatis showed higher positivity in *Mycoplasma* PCR assays when compared to other mammals groups (dogs, ocelots, crab-eating foxes and wild rodents). The 16SrRNA alignment (1247pb) of 24 sequences obtained from coatis blood samples showed the presence of four haplotypes (haplotype diversity (hd): 0.569; Standard Deviation (SD): =0.074), nucleotide diversity (Pi) of 0.05270 (SD=0,00563) and average number of nucleotide differences, k: 19.44565

(**Table 3**). Nucleotide polymorphisms were not found in 120bp *RnaseP* gene fragment alignment of 14 sequences obtained from coatis blood samples. Fourteen sequences shared the same haplotype (#hap 1), while the sequences (10) closed related to *M. haemocanis/M. haemofelis* showed tree different haplotypes (#hap2, #hap3, #hap4) (**Figure 6**).

Table 3. Polymorphisms of *Mycoplasma* spp. 16SrRNADNA sequences obtained from *Nasua nasua* blood samples.

Population (Hosts)	Nº of 16SrRNA sequences	AS	NVS	H	Hd	Pl	k
N. nasua	24	1247	48	4	0.569	Pi= 0.05270 (SD=0.00563)	19.44565

Nºof 16S rRNA sequences, Number of 16S rRNA hemoplasma sequences obtained from *N. nasua* blood samples;AS, Alignment Size; NVS, Number of Variable Sites; H, Number oh haprotypes; Hd, Haplotype diversity,Pi, Nucleotide Diversity; SD, Standard Deviation; k, Average number of nucleotide differences.

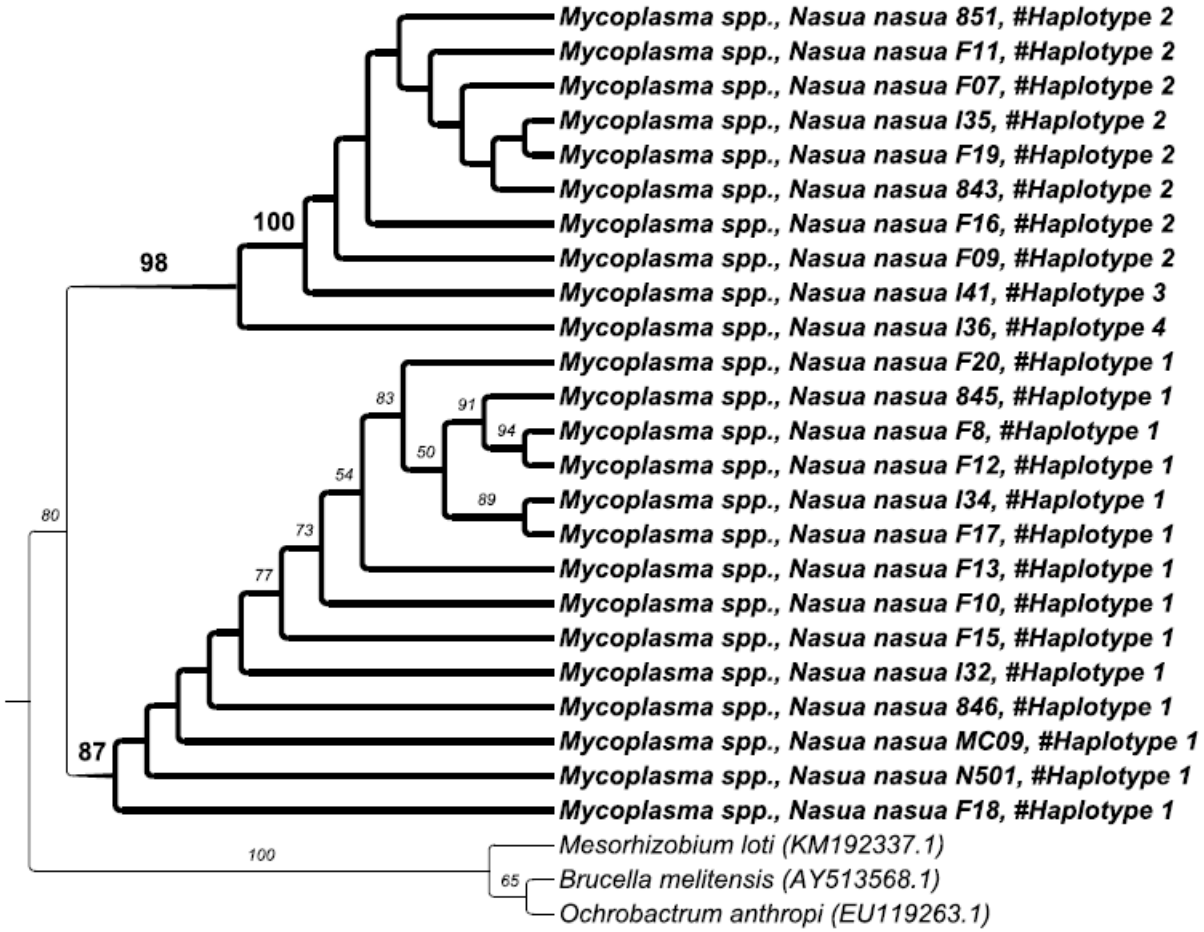

Figure 6. Tree distribution of four haplotypes found among the hemoplasmas sequences obtained from coatis blood samples, based on the polymorphisms analysis of 16S rRNA alignment.

4. Discussion

The present study showed the presence of *Mycoplasma* spp. DNA in blood or spleen samples of wild carnivores, domestic dogs and wild rodents in the region of Pantanal, state of Mato Grosso do Sul, central-western Brazil. To the best authors' knowledge, this was the first molecular detection of *Mycoplasma* spp. among wild coatis and crab-eating foxes from Brazil.

The molecular analyses of hemoplasmas-16SrRNA sequences revealed a low (2/42) occurrence of *M. haemocanis* among sampled dogs. The occurrence of *M. haemocanis* (4.7%) found in the present study was similar to that reported in dogs sampled in an urban area (4.2%) located in the same state of Mato Grosso do Sul (Soares et al., 2016). On the other hand, the found occurrence of *M. haemocanis*

was higher than that found in dogs sampled in the states of Pernambuco (Ramos et al., 2010), northeastern Brazil (1/205), and São Paulo (Alves et al., 2014) (3/154), but lower than that found in dogs sampled in the state of Rio Grande do Sul (17/331) (Valle et al., 2014). Only two studies reported the molecular detection of '*Candidatus* M. haematoparvum' in dogs from Brazil, in the states of São Paulo (Alves et al., 2014) and Rio Grande do Sul (Valle et al., 2014). The results of the present study reinforces that the mostly common species of *Mycoplasma* parasitizing dogs in Brazil is *M. haemocanis.*

A genotype closely related to *M. haemocanis/haemofelis* was detected in blood samples from two crab-eating foxes in the present study, based on the molecular analysis of 16SrRNA sequences. Unfortunately, the two positive *C. thous* samples did not show positivity to *RnaseP* PCR protocol, precluding the differentiation of these *Mycoplasma* species. In previous studies, a genotype closely related to *M. haemocanis* was detected in raccoon dogs (*Nyctereutes procyonoides viverrinus*) from Japan (Harasawa et al., 2014) and arctic foxes (*Vulpes lagopus*) from Canada (Mascarelli et al., 2015). Besides, genotypes closely related to *M. haemocanis, M. haemofelis* and '*Candidatus* M. turicensis' were detected in Darwin's foxes (*Lycalopex fulvipes*) from Chile (Cabello et al., 2013). In Brazil, among wild canids maintained in captivity in zoos, two bush dogs (*Speothos venaticus*) and two European wolves (*Canis lupus*) were positive for *Mycoplasma* species closely related to '*Candidatus* M. haematoparvum and '*Candidatus* M. haemominutum', respectively (André et al., 2011).

Mycoplasma haemofelis and '*Candidatus* Mycoplasma haemominutum' were detected in the saliva and salivary glands of hemoplasma infected cats, suggesting that these bacteria could be transmitted by social interactions (Dean et al., 2008). Considering that, it could be hypothesized that the gregarious behavior of coatis (Blanco and Hirsch, 2006) may have contributed for a higher incidence of *Mycoplasma* spp. infection among this carnivore species compared to the other animal species sampled in the present study.

Among wild coatis, ten hemoplasmas sequences showed to be closely related to *M. haemocanis/haemofelis* based on 16SrRNA and *RnaseP* phylogenetic analyses. Additionally, a new '*Candidatus* Mycoplasma' species is proposed for the

hemoplasma detected in 14 coatis and one crab-eating fox, which hgrouped in a separate branch with high values of clade support, and was closely related to a new *Mycoplasma* genotype detected in a capybara from Brazil (Vieira et al., 2009), based on ML and Bayesian phylogenetic analyses of 16S rRNA gene. The concatenated phylogenetic analysis of 16SrRNA and *RnaseP Mycoplasma* sequences also positioned six sequences obtained from coatis in a distinct branch with a significant value of clade support. Due to the inability to culture hemoplasmas in vitro, the only method to differentiate these species relies on the phylogenetic analysis of 16SrRNA and *RnaseP* genes (Peters et al., 2008; Neimark et al., 2005). In addition to this, the 16SrRNA sequences obtained from coatis blood samples comprises only one haplotype (#hap 1), which showed to be different from the others three haplotypes found in the 16SrRNA sequences obtained from coatis closely related to *M. haemocanis/M haemofelis*. Although the *Mycoplasma* sequences obtained from coatis and a crab-eating fox showed to be closely related to the new hemoplasma genotype detected in a Brazilian capybara, these genotypes were not positioned in the same clade, highlighting the circulation of a new genotype of *Mycoplasma* among carnivores in Pantanal region, Brazil.

'Candidatus Mycoplasma haemominutum' was the most common feline hemoplasma species found in previous studies involving Iberian lynxes (*Lynx pardinus*) from Spain, Eurasian lynxes (*Lynx lynx*) from Switzerland, lions (*Panthera leo*) from Tanzania, and seven Brazilian neotropic felid species namely Geoffroy's cats (*Oncifelis geoffroyi*), jaguarundis (*Puma yaguaroundi*), margays (*Leopardus wiedii*), ocelots (*Leopardus pardalis*), oncillas (*Leopardus tigrinus*), pumas (*Puma concolor*), and jaguars (*Panthera onca*) (Willi et al., 2007; André et al., 2011). On the other hand, '*Candidatus* M. turicensis' was the most prevalent hemoplasma species among European wildcats (*Felis silvestris silvestris*) (Willi et al., 2007). The only report on hemoplasma infection in a Brazilian wild felid showed a coinfection with *M. haemofelis* and '*Candidatus* M. turicensis' in a ocelot (Willi et al., 2007), whereas *'Candidatus* M. haemominutum' was the only hemoplasma species detected in a wild ocelot in the present study, corroborating to previous reports on the occurrence of hemotrophic mycoplasmas in ocelots maintained in captivity in zoos in Brazil (André et al., 2011). Wild animals may be more susceptible to become infected by

hemoplasmas when compared to those animals maintained in captivity, since they are more likely to be exposed to a great diversity of bloodsucking arthropods, multiple pathogens and aggressive interactions (Filoni et al., 2006). Further studies should be perform in order to sample a higher number of wild felids aiming to know the most common hemoplasma species circulating among wild Brazilian felids and the impact of infection on animal health.

The found occurrence of *Mycoplasma* among wild rodents sampled in the present study was lower (1/110) than that found in a previous recent study involving wild rodents (4/32) in the Pantanal, Brazil (Gonçalves et al., 2015). However, the occurrence of *Mycoplasma* infection found among wild rodents in Pantanal biome in the abovementioned study was significantly lower than that found in the others three Brazilian biomes (Cerrado, Amazon Forest, and Atlantic Forest) (Gonçalves et al., 2015). Additionally, high occurrence of hemoplasmas was found among capybaras (64%), synanthropic and laboratory rodents (*Rattus novergicus*) (63.5%) from urban areas in the state of Paraná, Brazil (Vieira et al., 2009; Conrado et al., 2015). A closer contact among rodents from urban areas with different host species and arthropods could explain the differences observed in prevalence rates. In spite of that, a high diversity of *Mycoplasma* genotypes has been detected in rodents from Japan, Hungary and Brazil (Vieira et al., 2009; Sashida et al., 2013; Conrado et al., 2015; Gonçalves et al., 2015; Hornok et al., 2015). A genotype closely related to *M. haemomuris* was detected in a wild rodent (*O. mamorae*) in the present study. The same genotype has been already detected in wild rodents from the same biome Pantanal (Gonçalves et al., 2015), suggesting that *M. haemomuris,* besides being the most common hemoplasma species found in synantropic rodents (Conrado et al., 2015; Hornok et al., 2015) seems to be the main hemotrophic mycoplasma species parasitizing wild rodents in the Pantanal biome in Brazil.

Although bloodsucking arthropods are suspected to be the vectors of hemoplasmas, the real role of these ectoparasites in the transmission cycles remains inconclusive (Woods et al., 2005). Supporting that hypothesis, '*Candidatus* Mycoplasma haemominutum' and *M. haemofelis* were detected in fleas (*Ctenocephalides felis*) collect from cats in the USA (Lappin et al., 2006), although experimental attempts of hemoplasmas transmission between felines via fleas were

inconclusive (Woods et al., 2005). In addition, *M. haemofelis* and '*Candidatus* M. haemominutum' DNA were detected in dog brown tick *Rhipicephalus sanguineus* collected from lions in Tanzania (Fyumagwa et al., 2008). The tick *R. sanguineus* s.l.is considered the main vector of *M. haemocanis* (Seneviratna et al., 1973). Despite of that, even in the localities where this tick species is well distributed and high rates of infestation are documented in Brazil (Labruna and Campos Pereira, 2001) the occurrence of hemotropic mycoplasmas in dogs was generally low (Ramos et al., 2010; Alves et al., 2014; Valle et al., 2014; Soares et al., 2016). In fact, there is only one study about the ability of *R. sanguineus* to transmit *M. haemocanis* to dogs Seneviratna et al., 1973). The others reports were based only in positive statistical correlations between *M. haemocanis* infection and *R. sanguineus* infestation rates (Soares et al., 2016). Keeping that in mind, the role of the tick *R. sanguineus* in *M. haemocanis* epidemiology remains unclear. The lice *Polypax spinulosa* and *Polyplax serrata* are known to be able to transmit *M. coccoides* to rodents (Eliot, 1936; Berkenkamp and Wescott, 1988). Recently, *Mycoplasma* phylotypes were detected in fleas (*Synosternus cleopatrae*) collected from rodents (*Gerbillus andersoni*) from Israel, suggesting the possible participation of fleas as vectors of *Mycoplasma* spp. to rodents (Cohen et al., 2015). The possible arthropod vector of hemoplasmas among wild animals in Brazil remains unknown. In the present study, none of the ticks or fleas collected from wild animals showed to be positive for *Mycoplasma* spp. The participation of other arthropods or other transmission routes, such as aggressive interactions or predation among Brazilian wildlife should be better investigated.

In conclusion, the present study revealed that wild animals in southern Pantanal region, Brazil, are exposed to different species of hemotropic hemoplasmas; some of them are known to cause clinical diseases in domestic animals. Domestic dogs, some crab-eating foxes and *N. nasua* seemed to share the same *Mycoplasma* spp. species, closely related to *M. haemocanis/M. haemofelis*. The role of domestic dogs as a source of *Mycoplasma* infection to wild animals should be better investigated. On the other hand, wild felines and rodents appear to be infected by specific host species of *Mycoplasma* spp. The real role of arthropods in the hemoplasmas transmission cycles remains unknown. Finally, probably a new genotype of *Mycoplasma* was detected among wild coatis and crab-eating foxes in

the present study. Therefore, future studies are much needed in order to elucidate the main and alternatives routes of *Mycoplasma* spp. transmission and to estimate the impact of hemoplasma infection among the Brazilian wildlife from Pantanal biome.

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CHAPTER 5 - *Rickettsia* spp. among wild mammals and their respective ectoparasites in Pantanal wetland, Brazil. Ticks and Tick-Borne Diseases.

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. Canids, felids, rodents and marsupials' serum samples were individually tested by Indirect Fluorescent Antibody Test (IFAT) in order to detect IgG antibodies to *Rickettsia rickettsii, Rickettsia parkeri* or *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 (1,582) 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%) *Cerdocyon thous* blood sample showed positivity in qPCR assays for SPF *Rickettsia* spp. Among SPF *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. Seventeen samples out of 117 qPCR positive samples for SPF *Rickettsia*spp. also showed positivity in cPCR

based on the *gltA* gene. Ten and three samples also showed positivity on 17-kDa *htrA* and *ompB* cPCR assays, respectively. 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.

KeyWords: *Rickettsia rickettsii, Rickettsia parkeri*, *Rickettsia amblyommatis,* '*Candidatus* Rickettsia andeanae', serology, qPCR, ticks

1. Introduction

The genus *Rickettsia* includes Gram-negative coccobacilli bacteria that belong to the order Rickettsiales, family Rickettsiaceae, and alpha-subdivision 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 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 cajennense* (Fabricius)] and *Amblyomma aureolatum* (Pallas) (Guedes et al. 2005, Labruna 2009). Besides, a *Rickettsia parkeri*-like agent (strain Atlantic rainforest) has also been showed to cause spotted fever rickettsiosis in humans in Brazil (Spolidorio et al. 2010, Silva et al. 2011). Although *R. rickettsii,* and *Ricketsia typhi* were known as the *Rickettsia* species endemic in Brazil seventeen years ago (Labruna, 2009),this number has risen to seven, with the inclusion of *Rickettsia parkeri*, *Rickettsia rhipicephali*, *Rickettsia amblyommatis* (formerly ‘*Candidatus* R. 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, Melo et al., 2016, Witter et al., 2016). While anti-*Rickettsia* spp. antibodies were detected in dogs (Melo et al., 2011) and horses (Alves et al., 2014) in northern Pantanal, among wild animals, antibodies anti-*Rickettsia* spp.have only been detected in wild jaguars (*Panthera onca*) in southern Pantanal (Widmer et al., 2011).

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

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 **(Figure 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) (Alves et al., 2016).

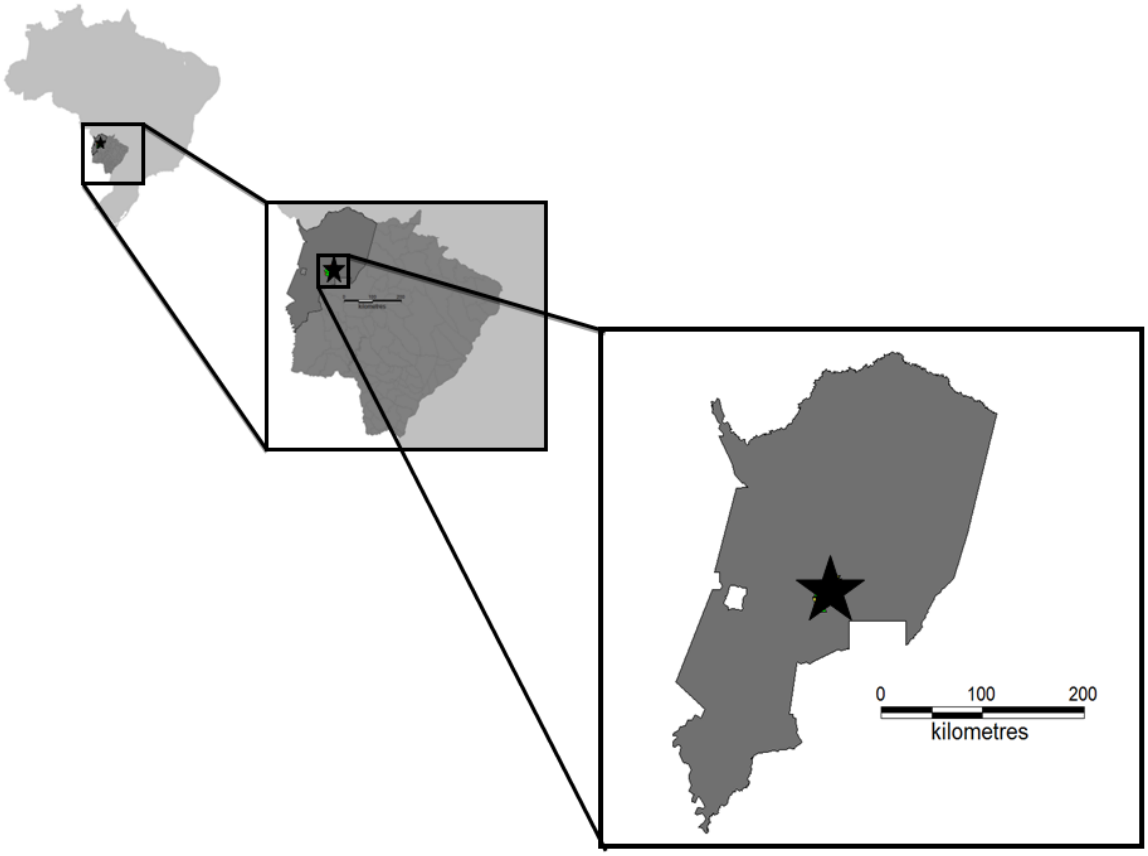

Figure 1. Capture sites. Map of Mato Grosso do Sul State, central-western Brazil, showing the Pantanal region, where animals samples were collected in the present study.

Between August 2013 and March 2015, four field expeditions of approximately 10 days each (August 2013, October 2013, August 2014, and March 2015) were performed. Wild carnivores (*C. 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 24h and checked twice a day. The animals were immobilized with an intramuscular injection of a combination of Zolazepan and Tiletamine (Zoletil®) at dosages of 8mg/kg for ocelots (*L. pardalis*) and 10mg/kg for crab-eating foxes (*C. thous*) and coatis (*N. nasua*). Blood samples were collected 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. Additionally, blood samples were collected from domestic dogs (*Canis lupus familiaris*), 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 10m 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 between the two expeditions (August 2014 and March 2015). The identification of specimens was based on external and cranial morphological characters and karyological analyses, as previously described (Bonvicino et al., 2005). The animals were firstly anesthetized with an intramuscular injection of Ketamine (10–30mg/kg) associated with Acepromazine (5–10mg/kg) for rodents (9:1proportion), or Xylazine (2mg/kg) for marsupials (1:1). After anesthesia, blood samples were collected by intracardiac puncture and stored in Vacutainer® tubes without EDTA, in order to obtain serum samples for serological assays. After the blood collection, the animals were euthanized with potassium chloride, which doses ranging from75 to 150mg/kg (Leary et al., 2013). Spleen samples were collected and stored in absolute alcohol (Merck®,Kenilworth, Nova Jersey,USA). Animal handling procedures followed the Guidelines of the American Society of Mammalogists for the use of wild mammals in research (Sikes and Gannon, 2011). All animal captures were in accordance with the licenses obtained from the Brazilian Government Institute for Wildlife and Natural Resources Care (IBAMA) (license numbers38145, 38787-2) and endorsed by the Ethics Committee of FCAV/UNESP University (Universidade Estadual Paulista [Unesp], Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal, SP, Brasil) nº 006772/13.

Ticks and fleas found parasitizing the sampled animals were detected by inspection of the skin and carefully removed by forceps or manually. The specimens were stored in 100% alcohol (Merck®,Kenilworth, New Jersey, USA) until identification, which was performed using a stereomicroscope (Leica® MZ 16A, Wetzlar, Germany) and following taxonomic literature for adult tick genera (Onofrio et

al., 2006; 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).

Canids, felids, rodents and marsupials' serum samples were individually tested by Indirect Fluorescent Antibody Test (IFAT) in order to detect IgG antibodies reactive to *R. rickettsii, R. parkeri* or *R. amblyommatis*. 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 37ºC 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 *Oecomys mamorae*, goat anti-guinea pig IgG (Sigma®, St. Louis, USA) for *Thrichomys fosteri* and *Clyomys laticeps*, sheep anti-opossum IgG (CCZ, São Paulo, Brazil) for *Thylamys macrurus, Gracilinanus agilis*,*Monodelphis domestica* and *Didelphis 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 fourfold 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.

DNA was extracted from 200µL of each whole blood (wild carnivores and domestic dogs) and 10mg of spleen (small mammals) samples using the QIAamp DNA Blood Mini kit (QIAGEN®, Valencia, CA, USA), according to the manufacturer's

instructions. While DNA extraction from ticks was processed in pools for nymphs (up to 5 individuals) and larvae (up to 10 individuals), the adults were processed individually. Flea DNA extractions were 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. DNA concentration and quality were measured using 260/280nm absorbance ratio (Nanodrop®, Thermo Fisher Scientific, Waltham, MA, USA). In order to verify the presence of amplifiable DNA in the samples, internal control PCR assays targeting fragments of mammalian glyceraldehyde-3-phosphatedehydrogenase (GAPDH) (Birkenheuer et al., 2003), mitochondrial 16S rRNA ticks gene (Black and Piesman, 1994),and fleas cytochrome-c oxidase subunit I(Folmer et al., 1994) genes were performed **(Table 1).**

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

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-kDalipoprotein 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 10µL contained 5µL GoTaq® Probe qPCR Master Mix (Promega Corporation, Madison USA), 1.2µM of each primer and hydrolysis probe (**Table 1**) and 1µL 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 (500bp)concentrations (Integrated DNA Technologies®, Coralville, Iowa, USA) ($2.0x10^7$ to $2.0x10^0$ copies/µL).The number of gBlockcopies was determined in accordance with the formula (Xg/µL DNA/[gBlock size (bp)x660])x$6.022x10^{23}$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).

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* (530bp) (Regnery et al., 1991), *ompB* (862 bp) (Roux and Raoult, 2000) and *htrA* 17-kDa(440bp) (Labruna et al., 2004b) (**Table 1**).The mixture contained10X PCR buffer(Life Technologies®, Carlsbad, CA, USA), 1.0mM $MgCl_2$(Life Technologies®, Carlsbad, CA, USA), 0.2mM deoxynucleotide triphosphate (dNTPs) mixture(Life Technologies®, Carlsbad, CA, USA), 1.5U Taq DNA Polymerase(Life Technologies®, Carlsbad, CA, USA), and 0.5µM of eachprimer (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 v3.1Cycle Sequencing Kit (Thermo Fisher Scientific®, Waltham, MA, USA) and ABI PRISM 310DNA Analyzer (Applied Biosystems®, Foster City, CA, EUA) (Sanger et al., 1977).

Sequences obtained from positive samples were firstly 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 program(Altschul et al., 1990) was used to analyze the sequences of nucleotides (BLASTn), aiming to browse and compare with sequences previously deposited in an international database (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 (BI) method. The Bayesian inference (BI) 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 et al., 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

A total of 256 animals were captured in the central region of Pantanal, municipality of Corumbá, state of Mato Grosso do Sul: 158 carnivores, among78 crab-eating foxes (*C. thous*), 31 coatis (*N. nasua*) and seven ocelots (*L. pardalis*); 140 small mammals, among 110 wild rodents (77 *T. fosteri*, 25 *O. mamorae* and 8 *C. laticeps*) and 30 wild marsupials (14 *T. macrurus,* 11 *G. agilis*, 4 *M. domestica* and 1 *D. albiventris*). Additionally, 42 blood samples from domestic dogs cohabiting the same studied area were collected.

One thousand five hundred and eighty-two ticks parasitizing the sampled mammals were collected, comprising 1033 (65.2% [115 adults and 918 nymphs]) *A. sculptum,* 241 (15.2% [78 adults and 163 nymphs]) *A.parvum* Aragão, 32 (2%) *A. ovale* Koch adults, one (0.06%) *A. tigrinum* Koch adult, one (0.06%) *R.* (*B.*) *microplus* (Canestrini) adult, one (0.06%) *R. sanguineus* sensu lato (Latreille) adult, four (0.2%) *A. auricularium* (Conil) nymphs, and 269 (17%) *Amblyomma* spp. larvae (**Table 2**). Besides, a total of 80 *Polygenis* (*Polygenis*) *bohlsi bohlsi* (Wagner) fleas were collected.

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

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

L – larvae, N – nymph, M –male adult, F – female.adult, 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.

All 298 DNA animal samples amplified the predicted product for GAPDH gene, the concentration mean and absorbance ratio (260/280) were 145.3ng/µL (SD ± 95.3) and 2.13(SD ± 2.18), respectively. The amount of tick DNA extracted was 523, of which 228 (43.5%) were from adults, 256 (48.9%) pooled nymphs, and 39 (7.4%) from pooled larvae, the concentration mean and absorbance ratio (260/280) were 45.9ng/µL (SD ± 84.3) and 1.81(SD ± 1.96), respectively. Out of 523 sampled ticks, 31 (5.9% [23 *A. parvum* adults, 4 *A. sculptum* adults, 1 *A. ovale* adult, 1 *A. parvum* nymph and 2 pooled *Amblyomma* larvae]) showed negative results for the tick mitochondrial 16S rRNA gene and were excluded from subsequent analyses. A total of 39 pooled fleas samples were submitted to DNA extraction, the concentration mean and absorbance ratio (260/280) were 7ng/µL (SD ± 8.43) and 1.12(SD ± 1.03), respectively. Only one flea DNA sample did not amplify the predicted product for cox-1 and was also excluded from subsequent analyses.

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 (**Table 3**).The seroreactive animals showed endpoint titers for *Rickettsia* spp. ranging from 64 to 4096. Among the 27 seroreactive dogs, titers for *Rickettsia* species ranged as following: *R. rickettsii*, 64–1024;*R. parkeri*, 64–2048; and *R. amblyommatis,* 64 – 4096. Among the 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 two (33.3%) ocelots, anti-*R. amblyommatis* endpoint antibodies titers were at least fourfold 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 wild rodent or marsupial showed to be seroreactive for any of the three employed rickettsial antigens.

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

Animals (no. tested)	N°. 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 (52.3)	23 (54.7)	27 (64.2)	19 (*R. amblyommatis*)
Cerdocyon thous (78)	38 (48.7)	40 (51.2)	59 (75.6)	33 (*R. amblyommatis*)
Leopardus pardalis (7)	5 (71.4)	6 (85.7)	5 (71.4)	2 (*R. amblyommatis*)
Total (127)	65 (51.1)	69 (54.3)	91 (71.6)	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

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 nymph-pool, 44 adults, and three egg pools), 14 (12%) belonged to *A. sculptum* species (five-nymph pool and nine adults), three-nymph pool (2.5%) belonged to *A. auricularium* species (all nymph samples) and six (5.2%) larval pool containing ten *Amblyomma* spp. individuals. The number of copies of SFG *Rickettsia-ompA* fragment/µL ranged from 2.53 x 10^1 to 4.52 x 10^1. The efficiency, correlation coefficient, and slope of qPCR reactions ranged from 90.3% to 102.3% (mean=94.3%), 0.952 to 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 x 10^1copies of SFG *Rickettsia-ompA* fragment/µL. The quantification mean between the groups of hosts were: *A. parvum* = 3.54 x 10^1copies/µL (3.53 x 10^1 - 4.52x 10^1), *A. sculptum*= 3.89 x 10^1copies/µL (3.4x 10^1 - 4.41 x 10^1), *A. auricularium*= 3.62 x 10^1copies/µL (3.58x 10^1 - 3.65 x 10^1), *Amblyomma* larvae= 3.68 x 10^1copies/µL (3.54x 10^1 - 3.93 x 10^1).The quantification of *Rickettsia* sp. *ompA* DNA in *C. thous* blood sample was 3.85x 10^1copies/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 (five-nymph pool, eight adults

and two egg pools) and two positive samples belonged to *Amblyomma* spp. larval pools. Nine *gltA* cPCR-positive *A. parvum* samples and one *Amblyomma* spp. larval pool also showed positivity in the cPCR assay based on 17-kDa *htrA* gene. In addition, three *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 the GenBank international database under the following accession numbers:KY402168-KY402196.

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

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	% of identify by BLAST®- analysis
A. parvum nymphs	5	*gltA*	*'Candidatus* Rickettsia andeanae' isolate LIC4328 (KT153033) 100%
Amblyomma larvae	2	*gltA*	*'Candidatus* Rickettsia andeanae' isolate LIC4328 (KT153033) 100%
A. parvum eggs	2	*gltA*	*'Candidatus* Rickettsia andeanae' isolate LIC4328 (KT153033) 100%
A. parvum adult	8	*gltA*	*'Candidatus* Rickettsia andeanae' isolate LIC4328 (KT153033) 100%
A. parvum adult	2	*ompB*	*'Candidatus* Rickettsia andeanae' isolate T163 (GU395297) 99%
A. parvum eggs	1	*ompB*	*'Candidatus* Rickettsia andeanae' isolate T163 (GU395297) 99%
A. parvum nymphs	4	*htrA*17KDA	*'Candidatus* Rickettsia andeanae' isolate T163 (GU395295) 100%
Amblyomma larvae	1	*htrA*17KDA	*'Candidatus* Rickettsia andeanae' isolate T163 (GU395295) 100%
A. parvum adult	5	*htrA*17KDA	*'Candidatus* Rickettsia andeanae' isolate T163 (GU395295) 100%

The concatenated phylogenetic analysis of *Rickettsia* based on *gltA* and 17-kDa *htrA* genes grouped the nine sequences obtained from *A. parvum* (KY402168; KY402184; KY402169; KY402185; KY402178; KY402189; KY402170; KY402186; KY402172; KY402187; KY402179; KY402190; KY402181; KY402192; KY402182; KY402193; KY402180; KY402191) and the sequences obtained from an *Amblyomma* larvae pool (KY402173; KY402188) in the same clade of '*Ca.* R. andeanae', with clade support of 100 based on BI analysis. The concatenated phylogenetic analysis of *Rickettsia* based on *gltA,*17-kDa *htrA* and *ompB* genes corroborated with the phylogenetic analysis of *Rickettsia* based on *gltA* and 17-kDa *htrA* genes. The concatenated *Rickettsia gltA*+17-kDa *htrA*+*ompB* sequences (KY402181; KY402192; KY402196) obtained from *A. parvum* adult was pooled in the same branch of '*Ca.* R. andeanae', with high clade support of 100, based on BI analysis and using *R. tamurae* as outgroup (**Figures 2 and 3**).

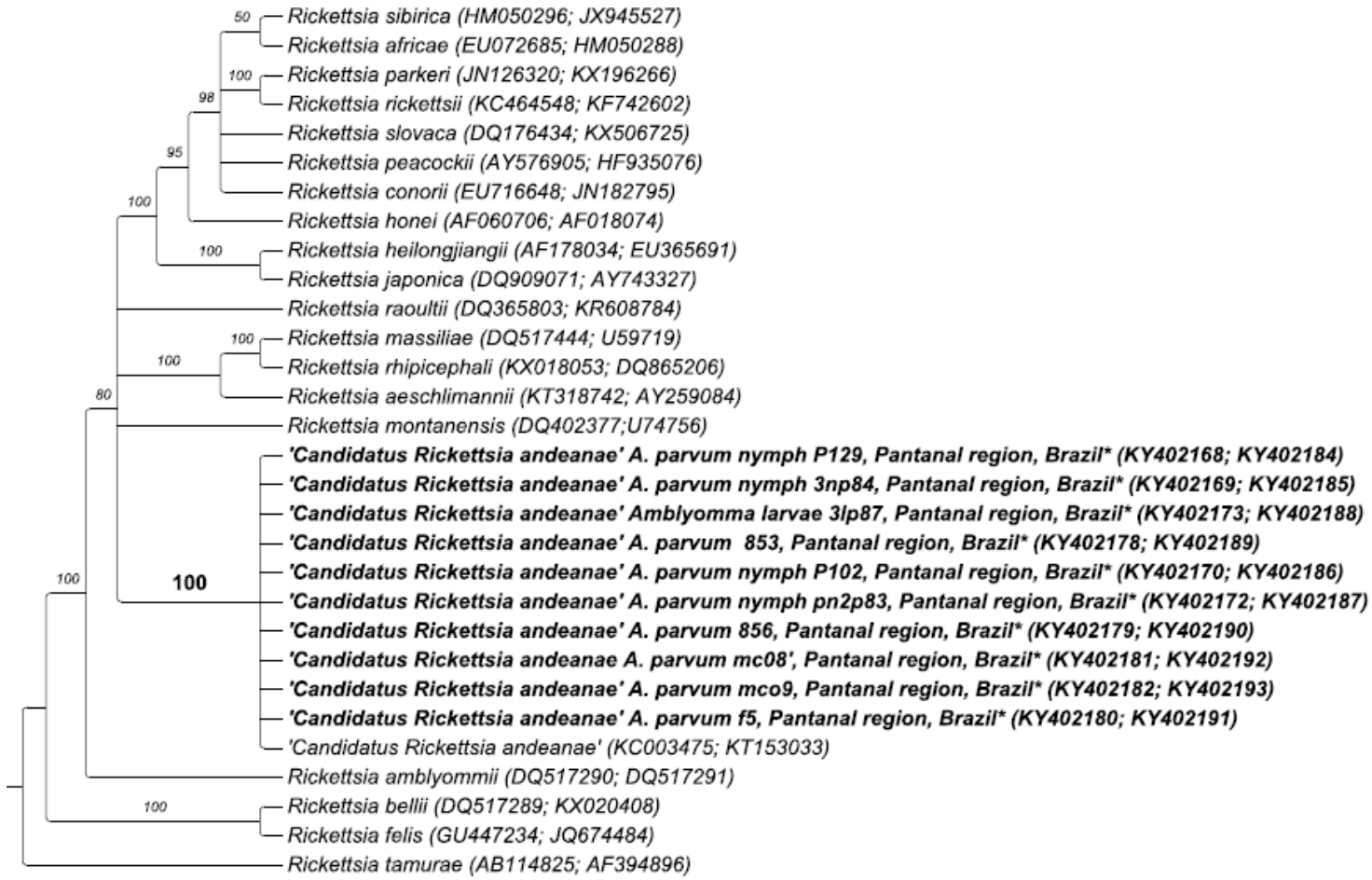

Figure 2. Phylogenetic tree constructed with approximately 740bp *Rickettsia* spp. *gltA* + *htrA* sequences, using Bayesian method and GTR+G+I evolutionary model. Numbers at nodes correspond to Bayesian posterior probabilities over 50, using *Rickettsia tamurae* (AB114825; AF394896) as outgroup.

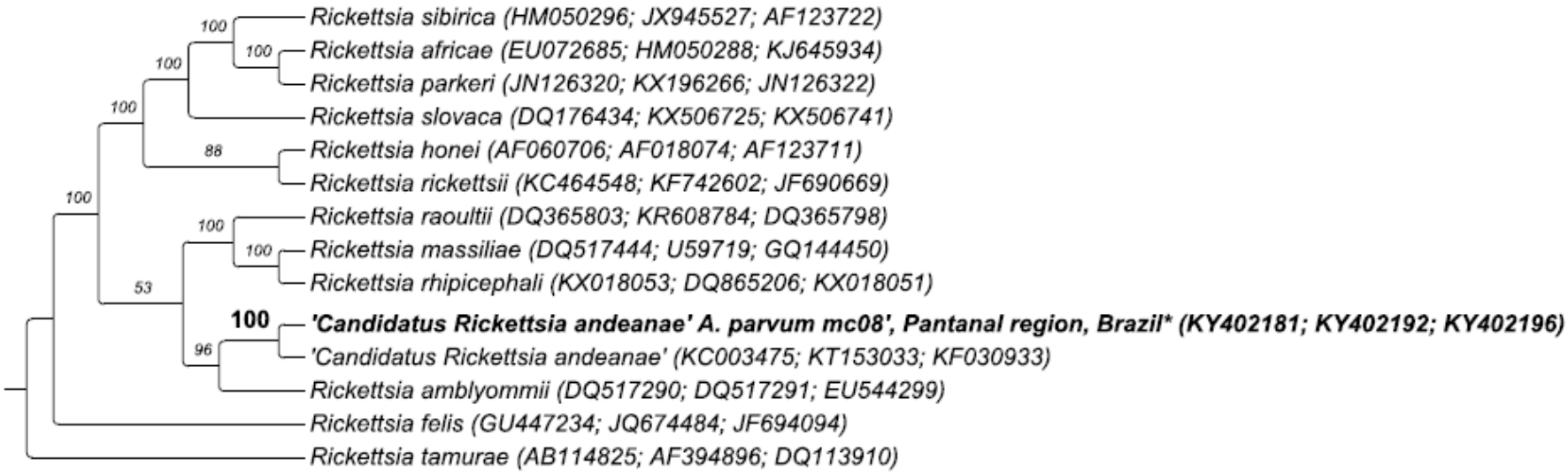

Figure3. Phylogenetic tree constructed with approximately 1490bp *Rickettsia* spp. *gltA* + *htrA*+ *ompB* sequences, using Bayesian method and GTR+G+I evolutionary model. Numbers at nodes correspond to Bayesian posterior probabilities over 50, using *Rickettsia tamurae* (AB114825; AF394896; DQ113910) as outgroup.

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 sampled in the present study in southern Pantanal. Similar results (75.6%) were found in dogs sampled in a previous study in northern Pantanal (Melo et al., 2011). *Rickettsia amblyommatis* represented the only rickettsial homologous reaction detected in the present study, corroborating with the hypothesis that *R. amblyommatis* or a very closely related agent seems to be the most common rickettsial agent infecting dogs in Pantanal biome, Brazil (Melo et al., 2011).

Although only one *A. sculptum* tick had been found parasitizing a dog sampled in the present study, previous studies reported higher numbers (209/930 - Melo et al., 2011; 152/557 - Melo et al., 2016) of *A. sculptum* parasitizing dogs in northern Pantanal, suggesting that infestation by this tick species among dogs from northern Pantanal seems to be frequent. In fact, a positive correlation between seropositive dogs to *Rickettsia* and infestation by *A. sculptum* ticks in northern Pantanal has been reported (Melo et al., 2011). Recently, *R. amblyommatis* DNA has been reported in *A. sculptum* collected from dogs (Melo et al., 2016) and horses (Alves et al., 2014) in northern Pantanal, suggesting that *A. sculptum* may play a role as a vector of *R. amblyommatis* for mammals in this Brazilian biome.

Similarly to that observed among domestic dogs, a high seroprevalence (75.6%) to *Rickettsia* spp. was also found among *C. thous* sampled in the present study, with homologous reactions to *R. amblyommatis.* Unfortunately, the ten positive *A. sculptum* specimens (9 adults and 1 nymph pool) collected from crab-eating foxes and showing positive results in qPCR assays SFG for *Rickettsia* did not show positivity in cPCR assays, which precluded their sequencing and phylogenetic inferences. However, the high anti-*R. amblyommatis* antibodies titers associated with the high number of *A. sculptum* (89 adults and 643 nymphs) ticks (which is supposed to be the main vector for *R. amblyommatis* in Pantanal) collected from *C. thous*,

suggests that this rickettsial agent or a very closely related agent is likely to be infect crab-eating foxes in southern Pantanal. On the other hand, the *Rickettsia* DNA detected in eight *A. parvum* specimens collected from *C. thous* showed to be closely related to '*Ca.* R. andeanae'. Due to the unavailability of '*Ca.* R. andeanae' antigen, cross-reactions between the related species '*Ca.* R. andeanae' and *R. amblyommatis* or a closely related agent could not 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), these findings highlight 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 '*Ca.* R. andeanae'. In Brazil, '*Ca.* R. andeanae' was previously detected only in 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 '*Ca*. R. 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 '*Ca*. R. andeanae' as the rickettsial agent presented in seven 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 '*Ca*. R. 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 *'Ca. R. 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, seroprevalence rates 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., 2012). 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., 2012). 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* ticks 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., 2012, Coelho et al., 2016). Keeping that in mind, the differences in seroprevalence rates 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, six (85.7%) out of seven ocelots trapped were seroreactive to at least one *Rickettsia* species. In a previous study, all ten jaguars (*P. onca*) sampled in Pantanal were seropositive to at least one *Rickettsia* antigen, with homologous reactions to *R. bellii* and *R. parkeri* in two animals (Widmer et al., 2011). In the present study, two 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. parkeri* DNA has been previously detected in an *A. triste* specimen collected from a jaguar (Widmer et al., 2011),'*Ca*. R. 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.

In a study encompassing five 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* or a closely related species. 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 '*Ca.* R. andeanae'. Additionally, studies aiming at investigating the circulation of '*Ca.* R. 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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Supplementary Material

Table 1. Positive samples in qPCR assays for *Rickettsia* spp. (SFG) spp. based on *ompA* gene with their respective quantification values and reaction parameters.

Host	Collected from	N° of copies/µL	Efficiency (%)	R^2	Slope	Y-int
1N *A. parvum*	*N. nasua*	$4.52X10^1$	100.8	0.987	-3.304	39.483
PN *A. sculptum*	*N. nasua*	$4.41X10^1$	90.5	0.997	-3.572	41.510
PN *A. sculptum*	*N. nasua*	$4.35X10^1$	90.5	0.997	-3.572	41.510
A. sculptum	*C. thous*	$4.25X10^1$	90.5	0.997	-3.572	41.510
A. sculptum	*C. thous*	$4.14X10^1$	93.3	0.999	-3.494	40.147
1N *A. parvum*	*O. mamorae*	$3.98X10^1$	102.2	0.960	-3.269	38.110
1N *A. parvum*	*C. thous*	$3.96X10^1$	91.3	0.999	-3.550	41.689
2L*Amblyomma*	*T. fosteri*	$3.93X10^1$	102.2	0.960	-3.269	38.110
A. sculptum	*C. thous*	$3.91X10^1$	90.3	0.994	-3.577	39.628
A. sculptum	*C. thous*	$3.90X10^1$	95	0.996	-3.448	37.642
PN *A. sculptum*	*N. nasua*	$3.89X10^1$	102.3	0.974	-3.269	39.059
C. thous	-	$3.85X10^1$	97.1	0.984	-3.394	38.608
2N *A. parvum*	*T. macrurus*	$3.85X10^1$	102.2	0.960	-3.269	38.110
A. parvum	*C. thous*	$3.84X10^1$	90.6	0.994	-3.571	39.896
3N *A. parvum*	*N. nasua*	$3.83X10^1$	98.7	0.968	-3.355	39.836
A. parvum	*C. thous*	$3.81X10^1$	93.3	0.999	-3.494	40.147
3N *A. parvum*	*T. fosteri*	$3.79X10^1$	102.2	0.960	-3.269	38.110
PN *A. sculptum*	*N. nasua*	$3.78X10^1$	90.3	0.994	-3.577	39.628
A. sculptum	*C. thous*	$3.75X10^1$	90.5	0.997	-3.572	41.510
A. parvum eggs	*C. thous*	$3.75X10^1$	95	0.996	-3.448	37.642
A. parvum	*N. nasua*	$3.73X10^1$	98.7	0.968	-3.355	39.836
1N *A. parvum*	*T. fosteri*	$3.72X10^1$	94	0.952	-3.475	40.365
A. sculptum	*C. thous*	$3.72X10^1$	95	0.996	-3.448	37.642
1N *A. parvum*	*N. nasua*	$3.71X10^1$	100.8	0.987	-3.304	39.483
PN *A. sculptum*	*C. thous*	$3.71X10^1$	95.7	0.976	-3.428	39.094
3N *A. parvum*	*T. fosteri*	$3.70X10^1$	102.2	0.960	-3.269	38.110
PN *A. parvum*	*T. fosteri*	$3.70X10^1$	102.2	0.960	-3.269	38.110
3L*Amblyomma*	*T. fosteri*	$3.70X10^1$	94	0.952	-3.475	40.365
1N *A. parvum*	*T. fosteri*	$3.69X10^1$	102.2	0.960	-3.269	38.110
PL *Amblyomma*	*N. nasua*	$3.68X10^1$	90.6	0.998	-3.571	40.981
A. sculptum	*C. thous*	$3.68X10^1$	95	0.996	-3.448	37.642
9L*Amblyomma*	*T. fosteri*	$3.68X10^1$	94	0.952	-3.475	40.365
A. parvum	*C. thous*	$3.68X10^1$	97.1	0.984	-3.394	38.608
A. parvum	*N. nasua*	$3.68X10^1$	90.6	0.994	-3.571	39.896
2N *A. parvum*	*T. fosteri*	$3.67X10^1$	102.2	0.960	-3.269	38.110
A. parvum	*N. nasua*	$3.66X10^1$	100.8	0.987	-3.304	39.483
1N *A. parvum*	*T. fosteri*	$3.65X10^1$	102.2	0.960	-3.269	38.110
1N*A. auricularium*	*N. nasua*	$3.65X10^1$	100.8	0.987	-3.304	39.483
A. parvum	*L. pardalis*	$3.64X10^1$	94	0.952	-3.475	40.365
1N*A. auricularium*	*C. laticeps*	$3.62X10^1$	94	0.952	-3.475	40.365
A. parvum	*C. thous*	$3.62X10^1$	97.1	0.984	-3.394	38.608

1N A. parvum	*N. nasua*	$3.62X10^{1}$	90.6	0.994	-3.571	39.896
A. parvum	*C. thous*	$3.61X10^{1}$	90.3	0.994	-3.577	39.628
4N *A. parvum*	*T. fosteri*	$3.60X10^{1}$	102.2	0.960	-3.269	38.110
A. parvum	*C. thous*	$3.60X10^{1}$	90.6	0.998	-3.571	40.981
3N *A. parvum*	*T. fosteri*	$3.60X10^{1}$	102.2	0.960	-3.269	38.110
A. parvum	*C. thous*	$3.60X10^{1}$	93.3	0.999	-3.494	40.147
4N *A. parvum*	*T. fosteri*	$3.60X10^{1}$	94	0.952	-3.475	40.365
A. parvum	*C. thous*	$3.60X10^{1}$	92.2	0.997	-3.523	39.873
A. parvum	*C. thous*	$3.59X10^{1}$	92.2	0.997	-3.523	39.873
4N *A. parvum*	*T. fosteri*	$3.59X10^{1}$	102.2	0.960	-3.269	38.110
3N *A. parvum*	*T. fosteri*	$3.58X10^{1}$	94	0.952	-3.475	40.365
2N*A. auricularium*	*N. nasua*	$3.58X10^{1}$	90.6	0.994	-3.571	39.896
A. parvum	*C. thous*	$3.58X10^{1}$	90.3	0.994	-3.577	39.628
3N *A. parvum*	*T. fosteri*	$3.56X10^{1}$	102.2	0.960	-3.269	38.110
A. parvum	*C. thous*	$3.56X10^{1}$	90.3	0.994	-3.577	39.628
PN *A. parvum*	*T. fosteri*	$3.56X10^{1}$	94	0.952	-3.475	40.365
A. sculptum	*C. thous*	$3.56X10^{1}$	95	0.996	-3.448	37.642
3N *A. parvum*	*C. laticeps*	$3.56X10^{1}$	102.2	0.960	-3.269	38.110
7L*Amblyomma*	*C. laticeps*	$3.56X10^{1}$	94	0.952	-3.475	40.365
PN *A. parvum*	*T. fosteri*	$3.56X10^{1}$	94	0.952	-3.475	40.365
A. parvum	*N. nasua*	$3.55X10^{1}$	94	0.952	-3.475	40.365
A. parvum	*N. nasua*	$3.55X10^{1}$	92.2	0.997	-3.523	39.873
A. parvum	*C. thous*	$3.55X10^{1}$	92.2	0.997	-3.523	39.873
A. parvum	*C. thous*	$3.55X10^{1}$	90.6	0.998	-3.571	40.981
A. parvum	*C. thous*	$3.55X10^{1}$	95	0.996	-3.448	37.642
4N *A. parvum*	*T. fosteri*	$3.54X10^{1}$	102.2	0.960	-3.269	38.110
7L*Amblyomma*	*T. fosteri*	$3.54X10^{1}$	102.2	0.960	-3.269	38.110
A. parvum	*C. thous*	$3.54X10^{1}$	92.2	0.997	-3.523	39.873
A. parvum	*C. thous*	$3.54X10^{1}$	92.2	0.997	-3.523	39.873
A. parvum	*C. thous*	$3.54X10^{1}$	92.2	0.997	-3.523	39.873
3N *A. parvum*	*T. fosteri*	$3.53X10^{1}$	94	0.952	-3.475	40.365
PN *A. parvum*	*T. fosteri*	$3.53X10^{1}$	94	0.952	-3.475	40.365
1N *A. parvum*	*T. fosteri*	$3.53X10^{1}$	102.2	0.960	-3.269	38.110
A. parvum	*N. nasua*	$3.53X10^{1}$	94	0.952	-3.475	40.365
4 N *A. parvum*	*N. nasua*	$3.53X10^{1}$	90.6	0.998	-3.571	40.981
A. parvum	*C. thous*	$3.52X10^{1}$	95	0.996	-3.448	37.642
2N *A. parvum*	*T. fosteri*	$3.52X10^{1}$	102.2	0.960	-3.269	38.110
A. parvum	*C. thous*	$3.52X10^{1}$	92.2	0.997	-3.523	39.873
A. parvum	*L. pardalis*	$3.51X10^{1}$	94	0.952	-3.475	40.365
PN *A. parvum*	*T. fosteri*	$3.51X10^{1}$	102.2	0.960	-3.269	38.110
3N *A. parvum*	*T. fosteri*	$3.50X10^{1}$	94	0.952	-3.475	40.365
A. parvum	*C. thous*	$3.50X10^{1}$	95.7	0.976	-3.428	39.094
A. parvum	*N. nasua*	$3.50X10^{1}$	98.7	0.968	-3.355	39.836
A. parvum	*C. thous*	$3.49X10^{1}$	95.7	0.976	-3.428	39.094

A. parvum	*C. thous*	$3.48X10^{1}$	95	0.996	-3.448	37.642
A. parvum	*C. thous*	$3.48X10^{1}$	92.2	0.997	-3.523	39.873
PN *A. parvum*	*T. fosteri*	$3.48X10^{1}$	94	0.952	-3.475	40.365
PN *A. parvum*	*T. fosteri*	$3.48X10^{1}$	94	0.952	-3.475	40.365
A. parvum	*C. thous*	$3.48X10^{1}$	97.1	0.984	-3.394	38.608
PN *A. parvum*	*C. thous*	$3.48X10^{1}$	90.6	0.998	-3.571	40.981
PN *A. parvum*	*T. fosteri*	$3.48X10^{1}$	94	0.952	-3.475	40.365
4N *A. parvum*	*T. fosteri*	$3.47X10^{1}$	102.2	0.960	-3.269	38.110
PN *A. parvum*	*T. fosteri*	$3.47X10^{1}$	94	0.952	-3.475	40.365
A. parvum	*C. thous*	$3.47X10^{1}$	95	0.996	-3.448	37.642
A. parvum	*N. nasua*	$3.47X10^{1}$	94	0.952	-3.475	40.365
PN *A. parvum*	*T. fosteri*	$3.47X10^{1}$	94	0.952	-3.475	40.365
A. parvum eggs	*C. thous*	$3.45X10^{1}$	100.9	0.940	-3.300	39.092
PN *A. parvum*	*C. laticeps*	$3.44X10^{1}$	94	0.952	-3.475	40.365
A. parvum	*C. thous*	$3.44X10^{1}$	92.2	0.997	-3.523	39.873
A. parvum	*N. nasua*	$3.41X10^{1}$	94	0.952	-3.475	40.365
1N *A. parvum*	*T. fosteri*	$3.40X10^{1}$	102.2	0.960	-3.269	38.110
A. sculptum	*C. thous*	$3.40X10^{1}$	95	0.996	-3.448	37.642
PN *A. parvum*	*T. fosteri*	$3.40X10^{1}$	102.2	0.960	-3.269	38.110
A. parvum	*C. thous*	$3.38X10^{1}$	95	0.996	-3.448	37.642
A. parvum eggs	*C. thous*	$3.37X10^{1}$	95	0.996	-3.448	37.642
A. parvum	*C. thous*	$3.37X10^{1}$	95	0.996	-3.448	37.642
PN *A. parvum*	*T. macrurus*	$3.36X10^{1}$	102.2	0.960	-3.269	38.110
PN *A. parvum*	*T. macrurus*	$3.35X10^{1}$	102.2	0.960	-3.269	38.110
PN *A. parvum*	*T. fosteri*	$3.34X10^{1}$	102.2	0.960	-3.269	38.110
PN *A. parvum*	*T. macrurus*	$3.33X10^{1}$	102.2	0.960	-3.269	38.110
A. parvum	*N. nasua*	$3.31X10^{1}$	100.8	0.987	-3.304	39.483
A. parvum	*C. thous*	$3.31X10^{1}$	97.1	0.984	-3.394	38.608
A. parvum	*C. thous*	$3.26X10^{1}$	95	0.996	-3.448	37.642
A. parvum	*C. thous*	$3.23X10^{1}$	95	0.996	-3.448	37.642
A. parvum	*C. thous*	$3.04X10^{1}$	94	0.952	-3.475	40.365
PN *A. parvum*	*C. laticeps*	$2.53X10^{1}$	94	0.952	-3.475	40.365

C. thous – Cerdocyon thous, L. pardalis – Leopardus pardalis, N. nasua – Nasua nasua, L – larvae. N – nymph, PN – nymph pools, L – larvae, PL – larvae pools, *A.s – Amblyomma sculptum. A.p – Amblyomma parvum. A. auricularium – Amblyomma auricularium,* N° of copies - Number of copies/µL, R^2 - Correlation coefficient, Y-int - intercepton the axis Y

CHAPTER 6 - Molecular detection of *Hepatozoon* spp. in domestic dogs and wild mammals in southern Pantanal, Brazil with implications in the route. Veterinary Parasitology.

Abstract

Hepatozoon parasites comprise intracellular apicomplexan parasites transmitted to vertebrate animals by ingestion of arthropods definitive hosts. The present work aimed to investigate the occurrence of *Hepatozoon* spp. in wild animals, domestic dogs and their respective ectoparasites, in southern Pantanal region, central-western Brazil, by molecular techniques. Between August 2013 and March 2015, 31 coatis (*Nasua nasua*), 78 crab-eating foxes (*Cerdocyon thous*), seven ocelots (*Leopardus pardalis*), 42 dogs (*Canis lupus familiaris*), 110 wild rodents (77 *Thichomys fosteri,* 25 *Oecomys mamorae,* and 8 *Clyomys laticeps*), 30 marsupials (14 *Thylamys macrurus,* 11 *Gracilinanus agilis,* 4 *Monodelphis domestica* and 1 *Didelphis albiventris*), and 1582 ticks and 80 fleas collected from the sampled animals were investigated. DNA samples were submitted to PCR assays for *Hepatozoon* spp. targeting 18S rRNA gene. Purified amplicons were directly sequenced and submitted to phylogenetic analysis. A high prevalence of *Hepatozoon* among carnivores (*C. thous* [91.02%], dogs [45.23%], *N. nasua* [41.9%] and *L. pardalis* [71.4%]) was found. However, ticks and fleas were negative to *Hepatozoon* PCR assays. By phylogenetic analysis based on 18S rRNA sequences, Hepatozoon sequences amplified from crab-eating foxes, dogs, coatis and ocelots clustered with sequences of *H. canis, H. americanum* and *H. felis.* The closely related positioning of *Hepatozoon* sequences amplified from wild rodents and *T. macrurus* marsupial to *Hepatozoon* from reptiles and amphibians suggest a possible transmission of those *Hepatozoon* species between hosts by ectoparasites or by predation. *Hepatozoon* haplotypes found circulating in wild rodents seem to present a higher degree of polymorphism when compared to those found in other groups of animals. Although rodents seem not to participate as source of *Hepatozoon* infection to wild carnivores and domestic dogs, they may play an important role in the transmission of *Hepatozoon* to reptiles and amphibians in Pantanal biome.

Keywords: *Hepatozoon* spp., ticks, wild mammals, dogs, phylogenetic analysis, Brazil.

1. Introduction

Hepatozoon spp. are apicomplexan parasites that infect a wide variety of vertebrate hosts, which play a role as intermediate hosts and acquire infection through the ingestion of arthropod definitive host containing oocysts (Smith, 1996). Additionally, other routes of transmission, such as the predation of infected vertebrates containing *Hepatozoon* cysts in their tissues (Johnson et al., 2007) and transplacental transmission (Baneth et al., 2013), have been described.

Regarding the occurrence of *Hepatozoon* spp. in wild and captive animals in Brazil, the protozoa has been so far molecularly detected in crab-eating foxes (*Cerdocyon thous*) (André et al., 2010; Almeida et al., 2013); bush dogs (*Speothos venaticus*) (André et al., 2010); maned wolf (*Cerdocyon brachyurus*); ocelots (*Leopardus pardalis*) (Metzger et al., 2008; André et al., 2010); little-spotted-cats (*Leopardus tigrinus*) (André et al., 2010); yagourandi (*Puma yagouaroundi*), pumas (*Puma concolor*); rodents (*Akodon* sp., *Oligoryzomys nigripes*, *Oligoryzomys flavescens, Calomys callosus*) (Demoner et al., 2016; Wolf et al., 2016); rattlesnake (*Crotalus durissus terrificus*) (O'Dwyer et al., 2013); crocodiles (*Caiman yacare)* (Viana et al., 2010); lizards (*Hemidactylus mabouia, Phyllopezus periosus*, *Phyllopezus pollicaris*) (Harris et al., 2015); and anuran amphibians (*Leptodactylus chaquensis*, *Leptodactylus podicipinus*) (Leal et al., 2015).

Although *Hepatozoon* infections in wild animals are usually subclinical (Kocan et al., 2000; Metzger et al., 2008), some studies associated the *Hepatozoon* infection with the presence of clinical disease in coyotes (Kocan et al., 2000; Garret et al., 2005), mortality in hyenas (East et al., 2008) and necrotizing inflammatory lesion in unnatural reptilian hosts (Wozniak et al., 1995). Moreover, *Hepatozoon* spp. may be a potential pathogen and an opportunistic parasite in immunocompromised animals or if occurring in concomitant infections (Davis et al., 1978; Baneth et al., 1998; Kubo et al., 2006). For that reason, there is a need to assess the distribution of *Hepatozoon* spp. in free-living animals, especially for endangered or elusive, free-living host species that are difficult to sample (Wobeser, 2007). Furthermore, large-

scale parasite screening has the potential for determining the distribution of similar lineages in different hosts, providing information on parasite transmission dynamics, which represents an important issue for endangered species (Fayer et al., 2004).

There is a lack of information on the vectorial competence of arthropods that may act as definitive hosts for *Hepatozoon* spp. in Brazil. Although *Rhipicephalus sanguineus* sensu lato (s. l.) is considered the main biological vector for *Hepatozoon canis* (Smith, 1996), preliminary studies indicated that this tick species has little or no importance in the transmission of *Hepatozoon* sp. in Brazil (Demoner et al., 2013). Additionally, *H. canis* oocysts have been found in the hemocoel of *Amblyomma ovale* (Forlano et al., 2005) and *Rhipicephalus* (*Boophilus*) *microplus* (Miranda et al., 2011). Furthermore, the role of other invertebrate hosts in the transmission cycles of *Hepatozoon* sp. has been investigated. For instance, Watkins et al. (2006) observed oocysts of *Hepatozoon* sp. in *Megabothris abantis* fleas collected from rodents, suggesting the participation of this ectoparasite in the biological cycle of *Hepatozoon* sp.

The predation of paratenic hosts seems to be another important infection route for *Hepatozoon* spp. (Johnson et al., 2009). Sequences of *Hepatozoon* spp. obtained from rodents and wild canids in South Africa showed to be phylogenetically related, suggesting a potential for transmission by predation of rodents by foxes (*Vulpes pallida*) (Maia et al., 2014). Additionally, *Hepatozoon* genotype sp. detected in snakes (*Python regius* and *Boa constrictor imperator*) were closely related to sequences of *Hepatozoon* spp. obtained from rodents in North America (Sloboda et al, 2008; Allen et al, 2011). In Brazil, the prevalence of *H. canis* among domestic dogs in rural areas has showed to be higher than in urban areas. Although dogs from rural areas often roam the forests and probably predate *Hepatozoon* sp.-infected animals (O'Dwyer, 2011), the analysis of sequences obtained from wild rodents from an endemic area for *H. canis* showed that wild rodent species in Brazil were infected with other *Hepatozoon* species, rejecting the hypothesis that rodents act as reservoirs for *H. canis* in the state of São Paulo, southeastern Brazil (Demoner et al., 2016). However, monozoic cysts were found in the sampled rodents' tissues, suggesting that rodents may act as paratenic hosts for *Hepatozoon* spp. in Brazil (Demoner et al., 2016).

The aim of the present study was to investigate the occurence of *Hepatozoon* spp. in wild mammals and domestic dogs and their respective ectoparasites in the region of Pantanal, state of Mato Grosso do Sul, central-western Brazil.

2. Material and methods

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 semideciduous 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 and it is well known for a rich biodiversity. Two well-defined seasons are recognized: a rainy summer (October to March) and a dry winter (April to September) (Alves et al., 2016).

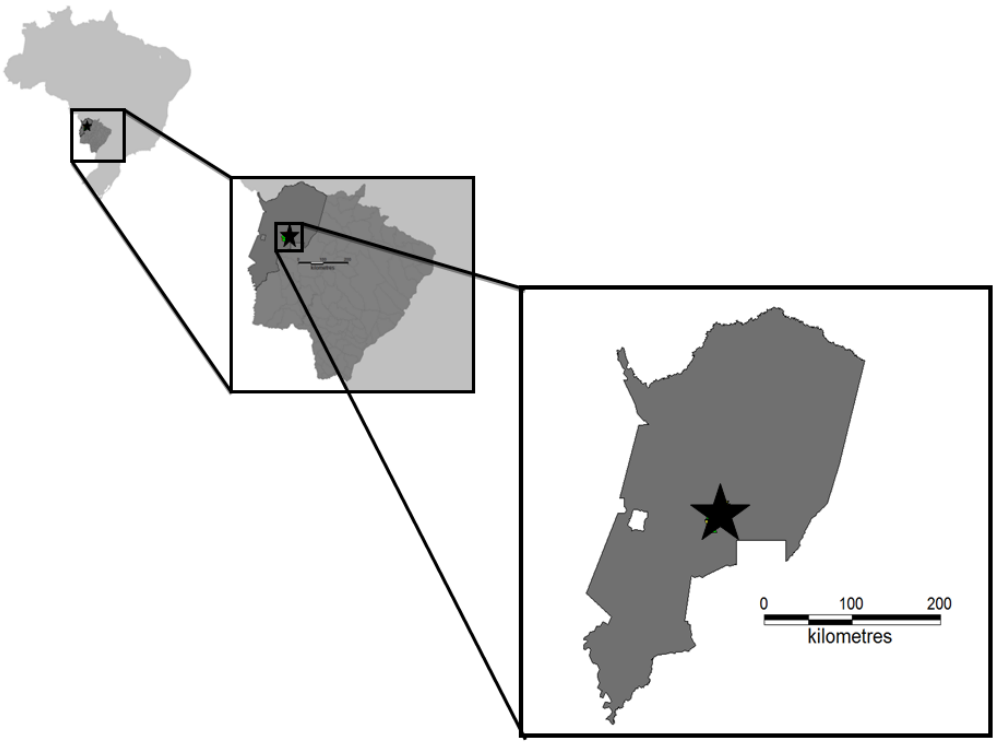

Figure 1. Capture sites. Map of Mato Grosso do Sul State, central-western Brazil, showing the Pantanal region, where animals samples were collected in the present study.

Between August 2013 and March 2015, four field expeditions (August 2013, October 2013, August 2014 and March 2015) were performed. The free-ranging carnivores (*C. thous*, *N. nasua* and *L. 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 24h 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. Blood samples were collected by puncture of the cephalic vein stored in Vacutainer® containing EDTA and stored at -20°C until DNA extraction. Additionally, blood samples were collected from 42 domestic dogs, which were cohabiting the same studied area. In addition to this, blood smears were performed and fixed with methanol and stained with Giemsa® (Giemsa® stain, modified, Sigma-Aldrich, St. Louis, MO,USA).

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 10m 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 among the 2 expeditions (August 2014 and March 2015). The identification of specimens was based on external and cranial morphological characters and karyological analyses, as previously described by Bonvicino et al. (2005). The animals were firstly anesthetized with an intramuscular injection of ketamine (10–30 mg/kg) associated 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 potassium chloride, which dosis ranged from 75 to 150 mg/kg (Leary et al., 2013). Spleen samples were collected and stored in absolute alcohol (Merck®, Kenilworth, Nova Jersey, USA). Animal handling procedures followed the Guidelines of the American Society of Mammalogists for the use of wild mammals in research (Sikes and Gannon, 2011). The project had permission from the Brazilian Government Environmental Agency (Brazilian Institute

of Environment and Renewable Natural Resources (IBAMA) (SISBIO licenses numbers 38145, 38787-2) and was also endorsed by the Ethics Committee of the FCAV/UNESP University (CEUA -nº 006772/13), in accordance to Brazilian regulations.

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

DNA was extracted from 200µL of each whole blood (wild carnivores and domestic dogs) and spleen (small mammals) samples using the QIAamp DNA Blood Mini kit (QIAGEN®, Valencia, CA, USA), according to the manufacturer's instructions. While ticks DNA extraction was processed in pools for nymphs (up to 5 individuals) and larvae (up to 10 individuals), the adults were processed individually. The fleas DNA extraction also was processed in pools consisting of up to five individuals. Ticks and fleas were macerated and prepared for DNA extraction, using the same kit before mentioned. DNA concentration and quality was measured using absorbance ratio between 260/280 nm (Nanodrop®, Thermo Fisher Scientific, Waltham, MA, USA).

In order to verify the existence of amplifiable DNA in the samples, internal control PCR assays targeting the glyceraldehyde-3-phosphatedehydrogenase (GAPDH) mammals gene (Birkenheuer et al., 2003), mitochondrial 16S rRNA ticks gene (Black and Piesman, 1994) and a fragment of the cytochrome c oxidase subunit I (cox1) coding for COX1 from fleas (Folmer et al., 1994) were perfomed**.** Two different PCR protocols were used aiming amplify different regions of 18SrRNA of *Hepatozoon*, based on Ujvari et al. (2004) and Perkins and Keller (2001) protocols **(Table 1)**. Thus, the two *Hepatozoon* 18S rRNA sequences obtained by the two PCR protocols were concatenated in order to obtain a large 18S rRNA fragment (1300pb)

to be used in phylogenetic analyses. DNA positive control was obtained from a naturally infected dog (Malheiros et al., 2016) and ultra-pure sterile water (Life Technologies®, Carlsbad, CA, USA) was used as negative control.

Table 1. Oligonucleotides sequences, target genes and thermal conditions used in conventional PCR assays targeting endogenous genes and *Hepatozoon* 18S rRNA gene fragments in biological samples from wild mammals and domestic dogs, trapped and sampled, respectively, in southern Pantanal, state of Mato Grosso do Sul, central-western Brazil.

Oligonucleotides sequences (5'-3')	Target gene	Thermal conditions	References
GAPDH-F (CCTTCATTGACCTCAACTACAT) GAPDH-R (CCAAAGTTGTCATGGATGACC)	GAPDH/ Mammals	95°C for 5 min; 35 cycles of 95°C for 15 sec, 50°C for 30 sec and 72°C for 30 sec; and final extension of 72°C for 5min.	Birkenheuer et al. (2003)
16S+1 (CTGCTCAATGATTTTTTAAATTGCTGTGG) 16S-1 (CCGGTCTGAACTCAGATCAAGT)	16SrRNA /Ticks	10 cycles of 92°C for 1 min, 48°C for 1min and 72°C for 1min, followed by 32 cycles of 92°C for 1 min, 54°C for 35 sec and 72°C for 1,35 min, and final extension of 72°C for 7min.	Black and Piesman (1994)
HC02198 (TAAACTTCAGGGTGACCAAAAAATCA) LCO1490 (GGTCAACAAATCATAAAGATATTGG)	COX1/ Fleas	95°C for 1min, 35 cycles of 95°C for 15 sec, 55°C for 15 sec and 72°C for 10 sec, and final extension of 72°C for 5min.	Folmer et al (1994)
HepF300 (GTTTCTGACCTATCAGCTTTCGACG) HepR900 (CAAATCTAAGAATTTCACCTCTGAC)	18SrRNA / *Hepatozoon*	94°C for 3 min, 35 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 1 min, and final extension of 72°C for 7min.	Ujvari et al. (2004)
HEMO1 (TATTGGTTTTAAGAACTAATTTTATGATTG) HEMO2 (CTTCTCCTTCCTTTAAGTGATAAGGTTCAC)	18SrRNA / *Hepatozoon*	94°C for 3 min, 35 cycles of 94°C for 45 sec, 48°C for 60 sec and 72°C for 1 min, and final extension of 72°C for 7min.	Perkins and Keller (2001)

The reaction products were purified using the Silica Bead DNA gel extraction kit (Thermo Fisher Scientific®, Waltham, MA, USA). The sequencing of the two different regions of 18S rRNA *Hepatozoon* spp. gene fragments of was carried out 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) (Sanger et al., 1977).

The 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) to evaluate the electropherogram quality and to obtain consensus sequences from the alignment of the sense and antisense sequences. The BLAST program (Altschul et al., 1990) was used to analyze the sequences of nucleotides (BLASTn), aiming to browse and compare with sequences from international database (GenBank) (Benson et al., 2002). All sequences that showed appropriate quality standards and identity with *Hepatozoon* spp. were deposited in the international database Genbank. Samples showing positive results for both PCR protocols 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 (BI) and Maximum Likelihood (ML) methods. The Bayesian inference (BI) 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 Maximum-likelihood (ML) analysis was inferred with RAxML-HPC BlackBox 7.6.3 (Stamatakis et al., 2008) (which includes an estimation of bootstrap node support), using 1000 bootstrapping replicates. 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 et al., 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).

Additionally, an analysis of nucleotide polymorphisms of the 18S rRNA sequences obtained in the present study was performed. The sequences were

aligned using Clustal/W (Thompson et al., 1994) in Bioedit v. 7.0.5.3 (Hall, 1999). The number of haplotypes, haplotype diversity (Hd), nucleotide diversity (Pi) and DNA divergence between populations (group of hosts different species) that were estimated to explore the levels of genetic differentiation among the populations were determined using the program DnaSP 5, version 5.10.01 (Librado and Rozas, 2009).

3. Results

A total of 256 animals were captured. One hundred fifty-eight carnivores: Seventy-eight crab-eating foxes, 31 coatis and seven ocelots. One hundred and forty small mammals: 110 wild rodents (77 *T. fosteri*, 25 *O. mamorae* and 8 *C. laticeps*) and 30 wild marsupials (14 *T. macrurus,* 11 *G. agilis*, 4 *M. domestica* and 1 *D. albiventris*). We also sampled blood of 42 domestic dogs.

One thousand five hundred and eighty-two ticks found parasitizing the sampled mammals were collected, of which 1033 (65.2% [115 adults and 918 nymphs]) belonging to *Amblyomma sculptum* Berlese species, 241 (15.2% [78 adults and 163 nymphs]) belonging to *Amblyomma parvum* Aragão species, 32 (2%) *Amblyomma ovale* Koch adults, one (0.06% *Amblyomma tigrinum* Koch adult, one (0.06%) *Rhipicephalus (Boophilus) microplus* (Canestrini) adult, one (0.06%) *Rhipicephalus sanguineus* s.l. (Latreille) adult, four (0.2%) *Amblyomma auricularium* (Conil) nymphs, and 269 (17%) *Amblyomma* larvae (**Table 2**). Besides, a total of 80 *Polygenis* (*Polygenis*) *bohlsi bohlsi* (Wagner) fleas were collected. Seventy-five fleas (93.7%) were collected from 16 (5.4%) *T. fosteri* rodents*,* four (5%) from two (0.6%) *M. domestica,* and one (1.25%) fleas from one (0.3%) specimen of *T. macrurus.*

Table 2. Ticks species collected from wild mammals captured between August 2013 and March 2015 in southern Pantanal, state of Mato Grosso do Sul, central-western Brazil.

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

N^{o} anim. – number of sampled animals

No 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.

All 298 DNA animal samples amplified the predicted product for GAPDH gene with an average concentration of 145.3 (SD=95,3) ηG/µL, which indicated a successful DNA extraction. The number of tick DNA samples extracted was 523, of which 228 (43.5%) were from adults, 256 (48.9%) pooled nymphs and 39 (7.4%) pooled larvae with an average concentration of 45.9 (SD=84,3) ηG/µL. Out of 523 sampled ticks, 31 (5.9% [23 *A. parvum* adults, 4 *A. sculptum* adults, 1 *A. ovale* adult, 1 *A. parvum* nymph and 2 pooled *Amblyomma* larvae]) showed negative results for the mitochondrial 16S rRNA tick gene and were excluded from analysis. A total of 39 pooled fleas samples were extracted with an average concentration of 7 (SD=8,43) ηG/µL and only one sample didn't amplified the predicted product for cox-1 from fleas and was also was excluded from further analysis.

No gamont suggestive of *Hepatozoon* was found in the blood smears from sampled animals. Out of 298 sampled animals, 61 (78.2%) crab-eating foxes, 16 (38%) domestic dogs, five (71.4%) ocelots, two (8%) *O. mamorae* and one (1.2%) *T. fosteri* were positive for 18SrRNA *Hepatozoon* spp.-PCR based on Perkins and Keller (2001) protocol. Thirty crab-eating foxes samples (38.4%), 13 (30.9%) domestic dogs, 13 (41.9%) coatis, 12 (15.5%) *T. fosteri,* nine (36%) *O. mamorae* and one (7.1%) *T. macrurus* showed positive results in 18SrRNA *Hepatozoon* spp.-PCR based on Ujvari et al. (2004) protocol. Twenty (25.6%) crab-eating foxes and ten (23.8%) domestic dogs samples showed positive results for both protocols, which allowed the concatenation of obtained sequences. All arthropod (fleas and ticks) DNA samples were negative for both PCR protocols. All sequences obtained from the positive animals were deposited in the international database Genbank under the following accession numbers: KT881500 -KT881535 and KX776286 - KX776408.

All the 18S rRNA *Hepatozoon* sequences obtained from domestic dogs (n=29), showed 99-100% identity (100% of coverage) with *H. canis* previously deposited in GenBank (AY150067) by BLAST analysis. The 18S rRNA *Hepatozoon* sequences obtained from *C. thous* (n=91) showed 98-99% identity (97-100% of coverage) with *Hepatozoon* spp. sequences obtained from lizards from Portugal (JX531925), *H. felis* isolate Cuiaba (KM435071) and other *H. felis* genotype from Japan (AB771501). The sequences amplified from *T. fosteri* spleen samples (n=13) showed 97-99% identity (97-100% of coverage) with *Hepatozoon* sp. sequence obtained from *Crotalus durissus terrificus* from Brazil (KC342523) and *Hepatozoon fitzsimonsi* (KR069084). Additionally, *Hepatozoon* sequences obtained from *O.*

mamorae (n=11) showed 99% identity (100% of coverage) with *Hepatozoon* sp. sequence amplified from a Brazilian lizard (KM234617) and *Hepatozoon* spp. sequences amplified from rodents in Brazil (KU667309). The unique 18S rRNA *Hepatozoon* sequence obtained from *T. macrurus* also showed 99% identity (88% of coverage) with *Hepatozoon* spp. sequences amplified from rodents in Brazil (KU667309). The 18S rRNA *Hepatozoon* sequences obtained from *L. pardalis* sequences (n=5) and from *N. nasua* (n=12) showed 98-99% identity (99-100% of coverage) with *H. felis* isolate Cuiaba (KM435071) and *H. felis* isolate Japan (AB771501), respectively **(Table 3)**.

Table 3. Maximum identity of 18S rRNA *Hepatozoon* spp. sequences detected in wild and domestic animals by BLAST analysis.

Animal species	Number of sequences analized	Protocol	% coverage by BLAST®	% identity by BLAST®
Canis familiaris	29	Perkins and Keller (2001) and Ujvari et al. (2004)	100%	99-100% *H. canis* (AY150067).
Cerdocyon thous	14	Perkins and Keller (2001)	97%	98-99% of identity with *Hepatozoon* spp. from lizards from Portugal (JX531925)
Cerdocyon thous	47	Perkins and Keller (2001)	99-100%	98-99% *H. felis* isolate Cuiaba (KM435071)
Cerdocyon thous	30	Ujvari et al. (2004)	100%	98% *H. felis* from Japan (AB771501)
Thrichomys fosteri	1	Perkins and Keller (2001)	100%	99% *Hepatozoon* spp. from *Crotalus durissus terrificus* from Brazil (KC342523)
Thrichomys fosteri	12	Ujvari et al. (2004)	97-99%	97% *H. fitzsimonsi* (KR069084).
Oecomys mamorae	2	Perkins and Keller (2001)	100%	99% *Hepatozoon* spp. from a lizard from Brazil (KM234617)
Oecomys mamorae	9	Ujvari et al. (2004)	100%	99% *Hepatozoon* spp. from a rodent from Brazil (KU667309)
Thylamys macrurus	1	Ujvari et al. (2004)	88%	99% *Hepatozoon* spp. from a rodent from Brazil (KU667309)
Leopardus. pardalis	5	Perkins and Keller (2001)	98%	98-99% *H. felis* isolate Cuiaba (KM435071)
Nasua nasua	12	Ujvari et al. (2004)	99%	98-99% *H. felis* isolate Japan (AB771501)

The phylogenetic tree of *Hepatozoon* spp. 18S rRNA sequences clustered basically in two large branches: one of them composed by *Hepatozoon* sequences amplified from *C. thous, C. L. familiaris, N. nasua* and *L. pardalis* from the present study and sequences of *H. canis, H. americanum* and *H. felis* retrieved from Genbank. The other large branch grouped: i. *Hepatozoon* sequences amplified in wild rodents species (*T. fosteri* and *O. mamorae*) and *T. macrurus* marsupial species from the present study; ii. *Hepatozoon* sequences amplified from rodents sampled in from Brazil (Mato Grosso and São Paulo, states) and other countries (Chile, Spain, Thailand, Ghana); iii. *Hepatozoon* sequences amplified from reptiles (snakes, tortoise and lizards) and amphibians previously deposited in Genbank, supported by significant clade support. *Adelina* sp., *Theileria* sp., *Isospora* sp., and *Sarcocystis* sp. were used as outgroups (**Fig 2).**

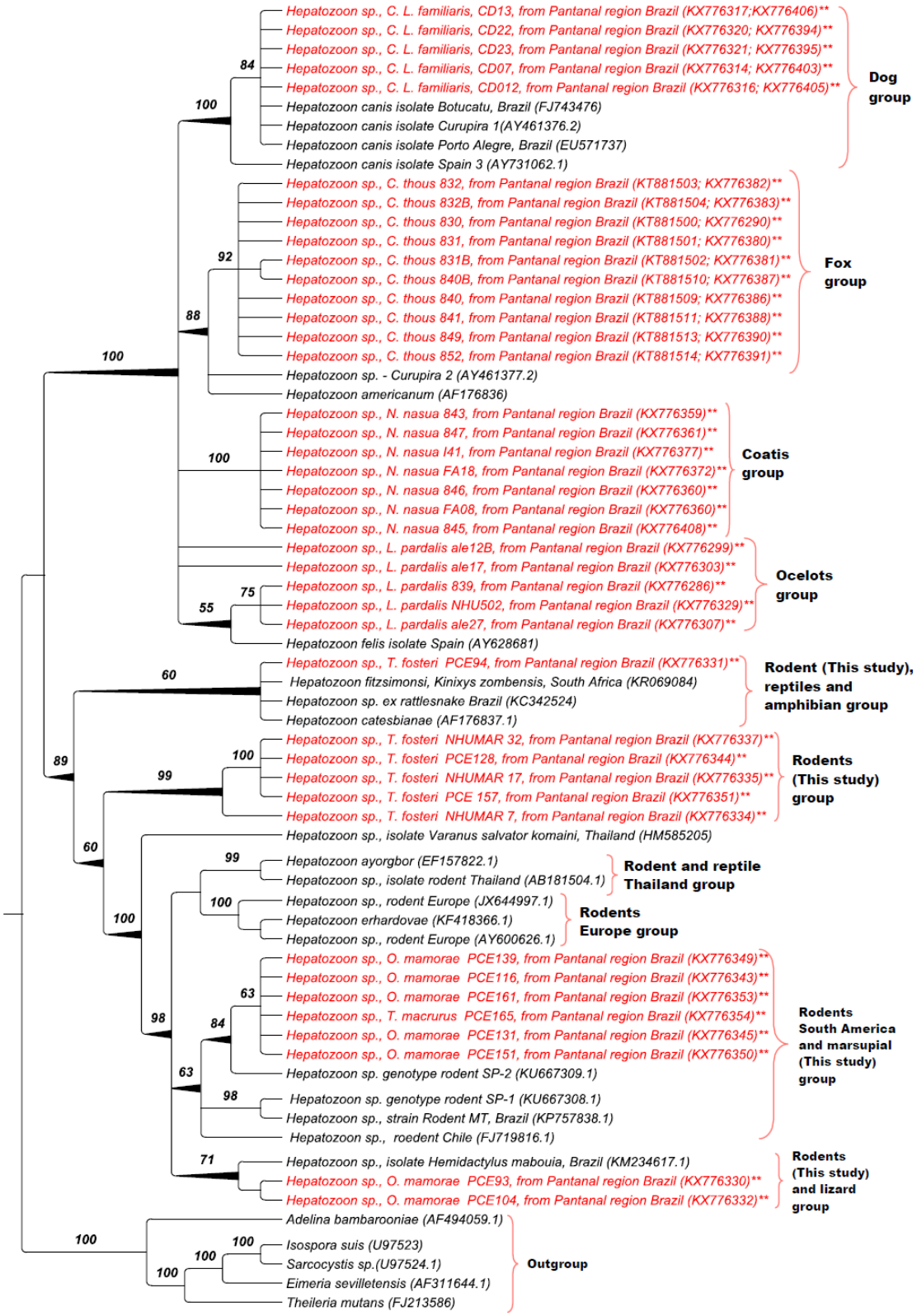

Figure 2. Phylogenetic tree based on an alignment of 1900bp fragment of *Hepatozoon* spp. 18SrRNA sequences, using Bayesian inference (BI) method and GTR+G+I evolutionary model. Numbers at nodes correspond to Bayesian posterior probabilities over 50.

Nucleotide polymorphisms and DNA divergence between populations (groups of different host species) were also analyzed among the sequences obtained in the present study. The alignments were analyzed in separate, because Perkins and Keller (2001) and Ujvari et al. (2004) PCR protocols amplify different regions of 18SrRNA gene. Both DNA fragments obtained from two different regions from 18SrRNA gene showed to be quite conserved. The analysis of nucleotide polymorphisms of 18S rRNA sequences obtained from both protocols showed a small number of haplotypes (4 [Perkins and Keller (2001) PCR protocol] and 7 [Ujvari et al. (2004) protocol]) among the population of different hosts sampled. The alignment of sequences obtained from Ujvari et al. (2004) protocol showed seven haplotypes (haplotype diversity (hd): 0.771; Standard Deviation (SD): =0.032) and nucleotide diversity (Pi) of 0.02084 (SD= 0.00142). Within the *T. fosteri* group, three different *Hepatozoon* haplotypes and a noteworthy Pi (0.00813) were observed. Except for the *Hepatozoon* sequences of rodents, divergence values between sampled host populations, based on the alignment of sequences obtained from Perkins and Keller (2001) PCR protocol, was very low, probably because the alignment generated only four haplotypes (hd: 0.4392; SD: =0.048) with low number of variable sites (3) to analyze. Although the number of haplotypes found had been small, some differences were observed analyzing the DNA divergence between populations (group of different host species). Analyzing the alignment of 18S rRNA *Hepatozoon* sequences from both protocols, the Pi value among the rodent populations of *T. fosteri* and *O. mamorae* was high compared to other host species. The alignment of 18S rRNA *Hepatozoon* sequences obtained from Ujvari et al. (2004) protocol showed a high level of divergence between the haplotypes found in domestic dogs and *T. fosteri* rodents groups (Pi=0.04293). Additionally, the Pi value (0.0074) found between the 18S rRNA *Hepatozoon* sequences obtained from *N. nasua* and *C. thous* populations was lower in relation to other host species.

4. Discussion

The present study showed the presence of *Hepatozoon* spp. in blood or spleen samples of wild carnivorous, domestic dogs, rodents and marsupials in the region of Pantanal, state of Mato Grosso do Sul, central-western Brazil. Molecular

analyses based on 18S rRNA gene revealed a high occurrence of *Hepatozoon* spp. among sampled animals.

Five out seven free-living ocelots captured showed to be positive for *Hepatozoon* spp. The occurrence of *Hepatozoon* was higher (83.3%) than that reported by Metzger et al. (2008) in wild *L. pardalis* (17.2%) sampled in the states of Maranhão and Ceará, northeastern Brazil. Based on the phylogenetic analysis, three 18S rRNA *Hepatozoon* sequences detected in sampled *L. pardalis* and sequences previously detected in wild felids from northeastern Brazil (Metzger et al., 2008) were closely related to *Hepatozoon* sp. genotype from Spanish cats. A genotype closely related to *Hepatozoon* sp. isolated from Spanish cats has also been found in a pampas gray fox (*Lycalopex gymnocercus*) that was co-infected with canine distemper virus in Argentina (Giannitti et al., 2012). The gray fox was euthanized after had showed severe incoordination; on necropsy, *Hepatozoon* cysts were observed in skeletal and myocardium muscles. Keeping in mind that this genotype closely related to *H. felis* is circulating in Brazil, more studies are much needed in order to monitor the impact of this parasite among the Brazilian wildlife.

The occurrence of *Hepatozoon* spp. among sampled crab-eating foxes found in the present study was very high (91%) when compared to previous studies, which had found percentages of positivity of 43.1% in wild *C. thous* sampled in the state of Espírito Santo, southeastern Brazil (Almeida et al., 2013), and 55% in wild *C. thous* sampled in the state of Rio Grande do Sul, southern Brazil (Criado-Fornelio et al., 2006). The phylogenetic analysis demonstrated that the *Hepatozoon* genotype infecting crab-eating foxes in the region of Pantanal was closely related to *Hepatozoon* spp. Isolate Curupira 2, an *H. americanum*-related organism, and *H. americanum*. The *H. americanum*-related haplotypes have been reported in crab-eating foxes from the Brazilian states of Rio Grande do Sul (Criado-Fornelio et al., 2006), São Paulo (André et al., 2010), and Espírito Santo (Almeida et al., 2013). Thus, this haplotype appears to be common among foxes in Brazil. Although *H. americanum* is considered a high pathogenic protozoa species for domestic dogs in the United States (Mathew et al. 1998), the *H. americanum*-related haplotype-infected crab-eating foxes in Brazil were apparently healthy (Criado-Fornelio et al., 2006, André et al., 2010, Almeida et al., 2013).

Hepatozoon canis infection was more prevalent (43.2%) among domestic dogs sampled in the present study than in dogs (3.63%) from urban areas from the

same state of Mato Grosso do Sul, Brazil (Ramos et al., 2015). The higher prevalence of *H. canis* among dogs from rural areas when compared to dogs from urban areas has been already reported in several states from Brazil (Rubini et al., 2008, Gomes et al., 2010, Ramos et al., 2010, Miranda et al., 2014, Ramos et al., 2015). The phylogenetic positioning reinforced the hypothesis that the domestic dogs from rural areas in Brazil are commonly infected by *H. canis* (Miranda et al., 2014).

To the best of authors' knowledge, the present study reported the first molecular detection of *Hepatozoon* spp. among coatis. Although Rodrigues et al. (2007) had previously detected *Hepatozoon* in two coatis from the state of Minas Gerais, southeastern Brazil, the diagnosis relied only on morphological and morphometric features of gametocytes in blood-stained smears. The *Hepatozoon* sequences detected in coatis were grouped within the large branch composed by the closely related *H. canis, H. americanum* and *H. felis* sequences. Similarly, *Hepatozoon* detected in raccoons (*Procyon lotor),* another member of Procyonidae family from the United States, showed to be closely related to *H. canis* (Allen et al., 2011). Considering that coatis can represent preys for wild felids (Novack et al., 2005), future studies should be performed in order to investigate the role of coatis as source of *Hepatozoon* infection for wild carnivores in Pantanal biome.

Although *Hepatozoon* spp. oocysts have been found in *A. ovale* (Rubini et al., 2009) and *R.* (*B.*) *microplus* (Miranda et al., 2011) ticks hemocoel, only *A. ovale* has been showed to be a competent vector for *Hepatozoon* in Brazil (Rubini et al., 2009, Demoner et al., 2013). In the present study, no tick, even *A. ovale,* was positive for *Hepatozoon* spp. Similar results have been found in northern Pantanal (Melo et al., 2016), where only one (1/930) *A. sculptum* was positive for *Hepatozoon* spp. Similarly, *H. canis* oocysts were found in only one (1/31) *R.* (*B.*) *microplus* ticks' hemocoel in an endemic area for canine hepatozoonosis in southeastern Brazil (Demoner et al., 2016). In fact, both *A. sculptum* and *R. microplus* species seem to show little or no importance in the hepatozoonosis epidemiology (Demoner et al., 2013, Demoner et al., 2016). Although the role of fleas as invertebrate hosts for *Hepatozoon* species infecting rodents in the United States have been proposed by Watkins et al. (2006), the *P.* (*P.*) *b. bohlsi* fleas collected from rodents in the present study did not show positivity for *Hepatozoon* in the molecular assays. Also, the role of other transmission routes, such as transplacental and predation, and the participation

of different arthropods species as source of *Hepatozoon* infection should be better investigated in endemic areas.

The prevalence of *Hepatozoon* among wild rodents (*T. fosteri* and *O. mamorae*) was 21.8% (n=24/110), lower than that reported (55.2%) in rodents (*O. nigripes, O. flavescens, Akodon sp., Necromys lasiurus* and *Sooretamys angouya*) sampled in an endemic area for canine hepatozoonosis in the state of São Paulo state, southeastern Brazil (Demoner et al., 2016). However, the found prevalence was higher than that reported (7.1%) in *C. callosus* rodents from northern Pantanal, state of Mato Grosso (Wolf et al., 2016). Although *Hepatozoon* DNA has been detected in wild rodents from Brazil (Demoner et al., 2016, Wolf et al., 2016), this is the first molecular detection of this parasite in *T. fosteri* and *O. mamorae* rodents and in *T. macrurus* marsupial in Brazil.

Although the transmission of *H. americanum* has been experimentally confirmed by the ingestion of cysts containing - rodent tissues by dogs (Johnson et al., 2009), previous studies showed evidence that the *Hepatozoon* species found in free-living rodents in Brazil differ from those detected in domestic and wild canids (Maia et al., 2014, Demoner et al., 2016). Herein, the phylogenetic positioning and DNA divergence analysis reinforce the hypothesis that the transmission of *Hepatozoon* from preys (rodents) to canids is a rare event and may not contribute to the spread of the parasite among canids in Brazil (Maia et al., 2014, Demoner et al., 2016).

Although the transmission of *Hepatozoon* spp. by carnivorism has been well documented in systems involving snakes as intermediate hosts, and frogs or lizards as paratenic hosts (Smith, 1996; Smith et al., 1999), Sloboda et al. (2008) showed that snakes could get experimentally infected by feeding tissues from *Hepatozoon*-infected rodents. Furthermore, Allen et al. (2011) and Demoner et al. (2016) detected *Hepatozoon*-rodent sequences closely related to *Hepatozoon*-reptile sequences. In addition to this, monozoic cysts containing cystozoites have been recently found in the lung of a free-living wild rodent sampled in the state of São Paulo State, Brazil (Demoner et al., 2016). Herein, the phylogenetic positioning of *Hepatozoon* detected in wild rodents suggested a possible transmission of *Hepatozoon* species between rodents and reptiles and amphibians by predation. Further studies focusing on the detection of monozoic cysts in rodents' tissues should be performed in order to support this hypothesis.

Although the *Hepatozoon* fragments obtained from two 18SrRNA different regions were quite conserved, nucleotide polymorphisms and DNA divergence between populations (groups of different host species) were observed in the present study. The present study provided some novel data concerning the *Hepatozoon* spp. diversity in Brazil. Within the *T. fosteri* group, three different *Hepatozoon* haplotypes and a noteworthy Pi value (0.00813) were observed. In addition to this, the Pi value found among the rodent populations of *T. fosteri* and *O. mamorae* was higher than that found among other host species, suggesting some degree of *Hepatozoon* genetic diversity among the population of wild rodents from Brazil. This fact may be due to the diversity of hosts (rodents, reptiles and amphibians) sharing the same closely related *Hepatozoon* species, revealed by the phylogenetic analysis.

In conclusion, the present study showed a high occurence of *Hepatozoon* spp. among wild animals in southern Pantanal region, Brazil. Carnivores, rodents and domestic dogs seemed not to share the same *Hepatozoon* species in the studied region. Rodents may play a role in the routes of *Hepatozoon* transmission to reptiles and amphibians. An evidence of some degree of *Hepatozoon* genetic diversity among the population of wild rodents from Brazil was reported for the first time.

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SUPPLEMENTARY MATERIAL

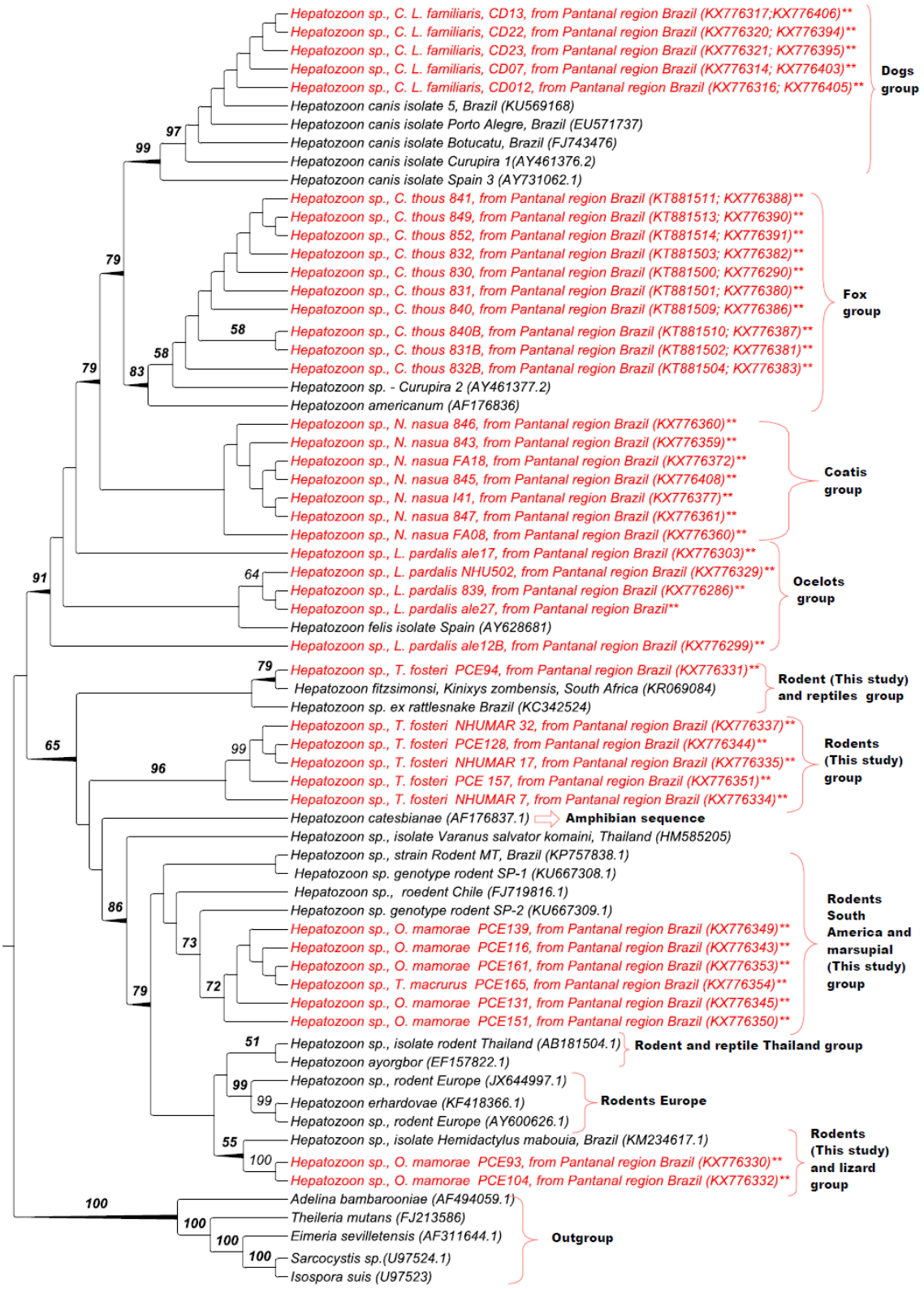

Figure 1. Phylogenetic tree based on an alignment of 1900bp fragment of *Hepatozoon* spp. 18SrRNA sequences, using Maximum Likelihood (ML) method and GTR+G+I evolutionary model. Numbers at nodes correspond to ML bootstrap values over 50.

Table 1. Polymorphisms of *Hepatozoon* DNA sequences based on two different regions of 18SrRNA gene.

PCR protocol for *Hepatozoon* spp. (18S rRNA gene)	SS	AS	NVS	PI	k
Ujvari et al. (2004)	75	760	12	Pi= 0.02084 (SD= 0.00142)	3.50126
Perkins and Keller (2001)	85	903	3	Pi= 0.00394 (SD= 0. 00051)	0.48011

SS, Sample Size; AS, Alignment Size; NVS, Number of Variable Sites; Pi, Nucleotide Diversity; SD, Standard Deviation; k, Average number of nucleotide differences.

Table 2. *Hepatozoon* haplotypes based on 760pb alignment size of 18SrRNA fragment obtained from Ujvari et al. (2004) PCR protocol.

Population (Hosts)	Sample Size	Haplotype
Cerdocyon thous	30	Haplotype 1
Nasua nasua	12	Haplotype 2
Dogs	13	Haplotype 3
Thylamys macrurus	1	Haplotype 4
Oecomys mamorae	8	Haplotype 4
Thrichomys fosteri	11	Haplotype 5 (n=6). Haplotype 6 (n=1). Haplotype 7 (n=4)
Total	75	7 (hd:0.771; SD: =0.032)

Hd, Haplotype (gene) diversity; SD, Standard Deviation

Table 3. *Hepatozoon* DNA divergence between wild and domestic mammals populations in southern Brazilian Pantanal, based on a 760pb alignment size of 18SrRNA fragment obtained from Ujvari et al. (2004) PCR protocol.

	Cerdocyon thous	***Nasua nasua***	***Thylamys macrurus***	**Dogs**	***Oecomys mamorae***	***Thrichomys fosteri***
Cerdocyon thous	Pi= 0.00 (k=0.0)	Pi= 0.0074 (k=1.254)	Pi=0.00154 (k=0.258)	Pi= 0.01542 (k=2.591)	Pi= 0.00813 (k=1.366)	Pi= 0.00761 (k=1.278)
Nasua nasua	-	Pi= 0.00 (k=0.0)	Pi= 0.0073 (k=2.308)	Pi=0.02461 (k=7.800)	Pi= 0.02448 (k=7.074)	Pi=0.02930 (k=9.289)
Thylamys macrurus	-	-	Pi= 0.00 (k=0.0)	Pi=0.00867 (k=3.857)	Pi= 0.00 (k=0.0)	Pi=0.01286 (k=4.848)
Dogs	-	-	-	Pi= 0.00 (k=0.0)	Pi= 0.02448 (k=7.074)	Pi=0.04293 (k=16.141)
Oecomys mamorae	-	-	-	-	Pi= 0.00 (k=0.0)	Pi=0.02153 (k=7.170)
Thrichomys fosteri	-	-	-	-	-	Pi=0.00813 (k=3.082)

Pi, Nucleotide Diversity; k, Average number of nucleotide differences.

Table 4. *Hepatozoon* haplotypes based on 903pb alignment size of 18SrRNA fragment obtained from Perkins and Keller (2001) PCR protocol.

Population (Hosts)	Sample Size	Haplotype
Cerdocyon thous	61	Haplotype 1 (n=60), Haplotype 3 (n=1)
Dogs	16	Haplotype 2
Leopardus pardalis	5	Haplotype 2
Oecomys mamorae	2	Haplotype 4
Thrichomys fosteri	1	Haplotype 2
Total:	85	4 (hd:0.4392; SD: =0.048)

Hd, Haplotype (gene) diversity; SD, Standard Deviation.

Table 5. *Hepatozoon* DNA divergence between wild and domestic mammals populations in southern Brazilian Pantanal, based on 903pb alignment size of 18SrRNA fragment obtained from Perkins and Keller (2001) PCR protocol.

	Cerdocyon thous	***Leopardus pardalis***	**Dogs**	***Oecomys mamorae***	***Thrichomys fosteri***
Cerdocyon thous	Pi= 0.00027 (k=0.033)	Pi= 0.00141 (k=0.172)	Pi= 0.00295 (k=0.360)	Pi= 0.00128 (k=0.157)	Pi= 0.00053 (k=0.065)
Leopardus pardalis	-	Pi= 0.00132 (k=0.889)	Pi=0.00284 (k=1.876)	Pi= 0.00976 (k=6.571)	Pi=0.00723 (k=4.867)
Dogs	-	-	Pi= 0.00 (k=0.0)	Pi= 0.00486 (k=3.346)	Pi=0.00295 (k=0.360)
Oecomys mamorae	-	-	-	Pi= 0.00 (k=0.0)	Pi=0.01577 (k=12.000)
Thrichomys fosteri	-	-	-	-	Pi= 0.00 (k=0.0)

Pi, Nucleotide Diversity; k, Average number of nucleotide difference

CHAPTER 7 - Diversity of piroplasmids among wild and domestic mammals and ectoparasites in southern Pantanal, Brazil. Parasitology Journal

SUMMARY

Piroplasmoses are one of the most prevalent arthropod-borne diseases of animals. The present work aimed to investigate the occurrence of piroplasmids in wild mammals, domestic dogs and ectoparasites in southern Pantanal region, central-western Brazil. For that purpose,, 31 coatis, 78 crab-eating foxes, seven ocelots, 42 dogs, 110 wild rodents, and 30 marsupials blood or tissue samples and 1582 ticks were submitted to PCR assays for piroplasmids targeting 18SrRNA and hps70 genes. Seven dogs, one crab-eating fox*,* five ocelots, three coatis, six wild rodents*,* eight *A. parvum*, two *A. sculptum* and one *A. ovale* were positive for piroplasmids-PCR assays. Genotypes closely related to *Babesia vogeli* were detected in six dogs and five wild rodents. While genotypes closely related to *Babesia caballi* were detected in one crab-eating fox, one dog, one *A. ovale* and one *A. sculptum,* genotypes closely related to *Babesia bigemina* and *Babesia bovis* were detected in four *A. parvum* ticks. Four sequences obtained from *A. parvum*, three coatis and one wild rodent were closely related to *Theileria equi. Cytauxzoon* spp*.* was detected in four ocelots*.* The present study revealed that wild and domestic animals in Brazilian southern Pantanal are exposed to different piroplasmids species.

KeyWords: *Babesia, Cytauxzoon,* dogs, phylogenetic analysis, *Theileria*, *Rangelia,* wildlife

INTRODUCTION

Piroplasmids (Piroplasmida) are apicomplexan protozoa comprising the genera *Babesia*, *Theileria*, *Cytauxzoon* and *Rangelia* (Yabsley and Shock, 2013). These agents are tick-borne protozoan that parasitizes blood cells of numerous wild and domestic vertebrates worldwide (Alvarado-Rybak *et al*, 2016). These parasites have a great economic and veterinary impact, being considered the second most commonly parasites found in the blood of mammals after trypanosomes (Schnittger *et al*., 2012). In the vertebrate hosts, the infection is usually characterized by fever,

anemia and haemoglubinuria, and in severe cases, can lead to death (Kuttler, 1988). Although some of these parasites can cause diseases in animals and humans, the vectors are still unknown for many piroplasms species of (Kjemtrup *et al*., 2000; Hersh *et al*., 2012).

Previously, the classification of piroplasmids relied only on host of origin, size and shape of trophozoites (small or large) and the number of merozoites within erythrocytes. However, the identification based on host origin has been invalidated, since many of these parasites are not host-specific (Penzhorn, 2006; Criado-Fornelio *et al*., 2003; Yabsley and Shock, 2013). Besides, the diagnosis based only on direct observations of blood smears does not allow species identification and usually molecular assays are necessary in order to perform the etiological agent involved (Criado-Fornelio *et al*., 2003). In the last few years, the advent of molecular techniques has contributed to a expressive increase in the number of studies reporting infection with piroplasmids in wild animals worldwide (Alvarado-Rybak *et al*, 2016).

In Brazil, there are few reports concerning the seroprevalence and molecular detection of piroplasmids in wild carnivores. For instance, André *et al*. (2011) found a seroprevalence of 31.7% and 10.3% against *B. vogeli* antigen among wild felines and canids maintained in captivity, respectively. Additionally, André *et al*. (2011) detected a genotype closely related to *B. leo* in a neotropical wild cat (*Oncifelis colocolo*) and Cape genet (*Genetta tigrina*) also maintained in captivity in zoos in the state of São Paulo, Brazil. In addition to this, fatal cases of cytauxzoonosis were reported in two lions maintained in capitivity in a zoo in the state of Rio de Janeiro (Peixoto *et al*., 2007). Besides, André *et al.* (2009) detected *Cytauxzoon* DNA closely related to *Cytauxzoon felis* in asymptomatic neotropical felines also maintained in captivity in zoos in the state of São Paulo and Brasília. Furthermore, *Cytauxzoon* spp. has also been molecular detected in domestic cats from the states of Rio de Janeiro (Maia *et al*., 2013) and Mato Grosso do Sul (André *et al*., 2015).

Due to the lack of information about the epidemiology and transmission routes of piroplasms among wild animals in Brazil, the present study aimed to investigate the occurrence of piroplasmids in wild mammals and domestic dogs and their

respective ectoparasites in the region of Pantanal, state of Mato Grosso do Sul, central-western Brazil.

MATERIALS AND METHODS

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 **(Figure 1).** This region is characterized by a mosaic of semideciduous 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 and it is well known for its rich biodiversity. Two well-defined seasons are recognized: a rainy summer (October to March) and a dry winter (April to September) (Alves *et al*., 2016).

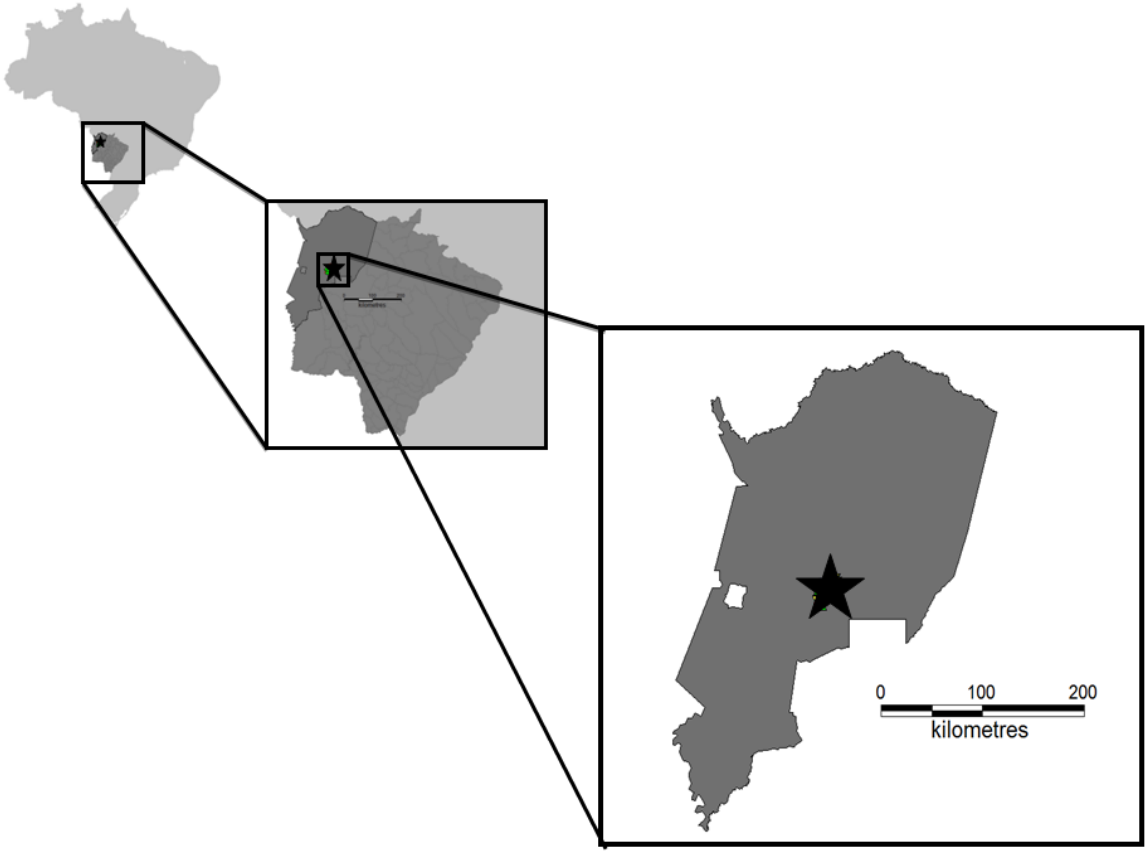

Figure 1. Capture sites. Map of Mato Grosso do Sul State, central-western Brazil, showing the Pantanal region, where animals samples were collected in the present study.

Between August 2013 and March 2015, four field expeditions (August 2013, October 2013, August 2014 and March 2015) were performed. The free-ranging carnivores 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 24h and checked twice a day. The animals were immobilized with an intramuscular injection of a combination of zolazepan and tiletamine (Zoletil®) at dosages of 8mg/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. Canids' blood samples were also collected in Vacutainer® tubes without EDTA, in order to obtain serum samples for serological assays. All animal captures were in accordance with the licenses obtained from the Brazilian Government Institute for Wildlife and Natural Resources Care (IBAMA) (license number 38787-2) and was 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. Additionally, blood samples were collected from 42 domestic dogs, which were cohabiting the same studied area. In addition to this, blood smears were performed and fixed with methanol and stained with Giemsa® (Sigma-Aldrich, St. Louis, MO,USA). In order to detect IgG antibodies to *B. vogeli*, canids serum samples were individually tested by an enzyme-linked immunosorbent assay (ELISA) kit (IMUNODOT, Diagnósticos Ltda®, Jaboticabal, SP, Brazil), according to the manufacturer's instructions.

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 10m 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 between the two expeditions (August 2014 and March 2015). The identification of specimens was based on external and cranial morphological characters and karyological analyses, as previously described by Bonvicino *et al*. (2005). The animals were firstly anesthetized with an intramuscular

injection of ketamine (10–30mg/kg) associated with acepromazine (5–10mg/kg) for rodents (proportion 9:1), or xylazine (2 mg/kg) for marsupials (1:1). After anesthesia, the animals were euthanized with potassium chloride, which doses ranged from 75 to 150mg/kg (Leary *et al*., 2013). Spleen samples were collected and stored in absolute alcohol (Merck®, Kenilworth, Nova Jersey, USA). Animal handling procedures followed the Guidelines of the American Society of Mammalogists for the use of wild mammals in research (Sikes and Gannon, 2011). The project had permission from the Brazilian Government Environmental Agency (Brazilian Institute of Environment and Renewable Natural Resources (IBAMA) (SISBIO license number 38145) and was also endorsed by the Ethics Committee of the FCAV/UNESP University (CEUA - nº 006772/13), in accordance to Brazilian regulations.

Ticks and fleas found parasitizing the sampled animals were detected by inspection of the skin and carefully removed by forceps or manually. The specimens were stored in 100% alcohol (Merck®, Kenilworth, NewJersey, USA) until identification using a stereomicroscope (Leica® MZ 16A, Wetzlar, Germany) and following taxonomic literature for adult tick genera (Onofrio *et al*., 2006; 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).

DNA was extracted from 200µL of each whole blood (wild carnivores and domestic dogs) and spleen (small mammals) samples using the QIAamp DNA Blood Mini kit (QIAGEN®, Valencia, CA, USA), according to the manufacturer's instructions. While ticks DNA extraction was processed in pools for nymphs (up to 5 individuals) and larvae (up to 10 individuals), the adults were processed individually. The fleas DNA extraction also was processed in pools consisting of up to five individuals. Ticks and fleas were macerated and prepared for DNA extraction, using the same kit aforementioned. DNA concentration and quality was measured using absorbance ratio between 260/280 nm (Nanodrop®, Thermo Fisher Scientific, Waltham, MA, USA). In order to verify the existence of amplifiable DNA in the samples, internal control PCR assays targeting the glyceraldehyde-3-phosphatedehydrogenase (GAPDH) mammals gene (Birkenheuer *et al*., 2003), mitochondrial 16S rRNA ticks

gene (Black and Piesman, 1994) and a fragment of the cytochrome c oxidase subunit I (cox1) coding for COX1 from fleas (Folmer *et al*., 1994) were performed **(Table 1).**

Table 1. Oligonucleotides sequences, target genes, cycling conditions and PCR products sizes used in conventional PCR assays targeting mammals and ticks endogenous genes, and 18S rRNA and heat shock protein 70 (*hsp70*) piroplasmids genes in biological samples from wild mammals domestic dogs and and their respective ectoparasites, sampled in southern Pantanal, state of Mato Grosso do Sul, central-western Brazil.

Oligonucleotides sequences (5'-3')	Target gene	Cycling conditions	PCR Product Size	References
GAPDH-F (CCTTCATTGACCTCAACTACAT) GAPDH-R (CCAAAGTTGTCATGGATGACC)	GAPDH/ Mammals	95°C for 5 min; 35 cycles of 95°C for 15 sec, 50°C for 30 sec and 72°C for 30 sec; and final extension of 72°C for 5min.	400pb	Birkenheuer *et al*., 2003
16S+1 (CTGCTCAATGATTTTTTAAATTGCTGTGG) 16S-1 (CCGGTCTGAACTCAGATCAAGT)	16SrRNA /Ticks	10 cycles of 92°C for 1 min, 48°C for 1min and 72°C for 1min, followed by 32 cycles of 92°C for 1 min, 54°C for 35 sec and 72°C for 1,35 min, and final extension of 72°C for 7min.	460 pb	Black and Piesman, 1994
HC02198 (TAAACTTCAGGGTGACCAAAAAATCA) LCO1490 (GGTCAACAAATCATAAAGATATTGG)	COX1/Fleas	95°C for 1min, 35 cycles of 95°C for 15 sec, 55°C for 15 sec and 72°C for 10 sec, and final extension of 72°C for 5min.	710 pb	Folmer *et al*.,1994
HepF300 (GTTTCTGACCTATCAGCTTTCGACG) HepR900 (CAAATCTAAGAATTTCACCTCTGAC)	18SrRNA / *Hepatozoon*	94°C for 3 min, 35 cycles of 94°C for 30 sec, 60°C for 30 sec and 72°C for 1 min, and final extension of 72°C for 7min.	600pb	Ujvari *et al*., 2004
BTF1 (GGCTCATTACAACAGTTATAG) BTR1 (CCCAAAGACTTTGATTTCTCTC) BTF2 (CCGTGCTAATTGTAGGGCTAATAC) BTR2 (GGACTACGACGGTATCTGATCG)	18SrRNA / *Babesia* spp., *Theileria* spp., *Rangelia vitalii* and *Cytauxzoon* sp.	1ºRound: 94°C for 3 min, 1 min for 58°C and 2 min for 72°C; 45 cycles of 94°C for 30 sec, 58°C for 20 sec and 72°C for 30 sec; and final extension of 72°C for 5min. 2º Round: 94°C for 3 min, 1 min for 62°C and 2 min for 72°C; 45 cycles of 94°C for 30 sec, 64°C for 20 sec and 72°C for 30 sec; and final extension of 72°C for 5min.	800pb	Jefferies *et al*., 2007
Cy-F (GCGAATCGCATTGCTTTATGCT) Cy-R (CCAAATGATACTCCGGAAAGAG)	18SrRNA/ *Cytauxzoon* sp.	94°C for 5 min; 40 cycles of 95°C for 45 sec, 59°C for 45 sec and 72°C for 1min; and final extension of 72°C for 5min.	300pb	Birkenheuer *et al*., 2006
hsp70F1 (CATGAAGCACTGGCCHTTCAA) hsp70 R1 (GCNCKGCTGATGGTGGTGTTGTA)	*hsp70*/ *Babesia* spp., *Rangelia vitalii* and *Theileria* spp.	95°C for 5 min; 35 cycles of 95°C for 15 sec, 60°C for 30 sec and 72°C for 30 sec; and final extension of 72°C for 5min.	740pb	Soares *et al*., 2011

Previously described PCR protocols based on 18SrRNA gene and heat shock protein 70 (*hsp70*) were performed in order to amplify *Babesia* spp., *Cytauxzoon* sp. and *Theileria* spp. DNA (Ujvari *et al*., 2004; Birkenheuer *et al*. 2006; Jefferies *et al*., 2007; Soares *et al*., 2011) (**Table 1**). Each sample of extracted DNA was used as a template in 25µL PCR reactions. The mixture containing 10X PCR buffer (Life Technologies®, Carlsbad, CA, USA), 1.0mM $MgCl_2$ (Life Technologies®, Carlsbad, CA, USA), 0.2mM deoxynucleotide triphosphate (dNTPs) mixture (Life Technologies®, Carlsbad, CA, USA), 1.5U Taq DNA Polymerase (Life Technologies®, Carlsbad, CA, USA), and 0.5µM of each primer (Integrated DNA Technologies®, Coralville, IA, USA). *Babesia vogeli, Cytauxzoon* sp. and *Theileria* sp. DNA positive controls were obtained from naturally infected cats (André *et al*., 2015). Ultra-pure sterile water (Life Technologies®, Carlsbad, CA, USA) was used as negative control. PCR products were separated by electrophoresis on 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). The sequencing was carried out 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) (Sanger *et al*., 1977).

The 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 obtain consensus sequences from the alignment of sense and antisense sequences. The BLAST program (Altschul *et al*., 1990) was used to analyze the sequences of nucleotides (BLASTn), aiming to browse and compare with sequences from international database (GenBank) (Benson *et al*., 2002). All sequences that showed appropriate quality standards and identity with piroplasmids species were deposited in Genbank. Samples showing positive results for both PCR protocols (18SrRNA and *hsp70*) 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 Inference (BI) and Maximum Likelihood (ML) methods. The Bayesian inference (BI) 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 Maximum-likelihood (ML) analysis was inferred with RAxML-HPC BlackBox 7.6.3 (Stamatakis *et al*., 2008) (which includes an estimation of bootstrap node support), using 1000 bootstrapping replicates. 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 *et al*., 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).

RESULTS

A total of 256 animals were captured: 158 carnivores, including 78 crab-eating foxes (*Cerdocyon thous*), 31 coatis (*Nasua nasua*) and seven ocelots (*Leopardus pardalis*); 140 small mammals, including 110 wild rodents (77 *Thrichomys fosteri*, 25 *Oecomys mamorae* and 8 *Clyomys laticeps*) and 30 wild marsupials (14 *Tylamys macrurus,* 11 *Gracilinanus agilis*, 4 *Monodelphis domestica* and 1 *Didelphis albiventris*). Additionally, blood samples were collected from 42 domestic dogs.

One thousand five hundred and eighty-two ticks found parasitizing the sampled mammals were collected, of which 1033 (65.2% [115 adults and 918 nymphs]) belonging to *Amblyomma sculptum* Berlese species, 241 (15.2% [78 adults and 163 nymphs]) belonging to *Amblyomma parvum* Aragão species, 32 (2%) *Amblyomma ovale* Koch adults, one (0.06%) *Amblyomma tigrinum* Koch adult, one (0.06%) *Rhipicephalus (Boophilus) microplus* (Canestrini) adult, one (0.06%) *Rhipicephalus sanguineus* s.l. (Latreille) adult, four (0.2%) *Amblyomma auricularium* (Conil) nymphs, and 269 (17%) *Amblyomma* larvae (**Table 2**).

Table 2. Ticks species collected from wild mammals captured between August 2013 and March 2015 in southern Pantanal, state of Mato Grosso do Sul, central-western Brazil.

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

L – larvae, N – nymph, M – adult male, F – adult female, N° anim. – number of sampled animals, Infest (%). – percentage 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.

All 298 DNA animal samples amplified the predicted product for GAPDH gene with an average concentration of 145.3 (SD=95,3) ηG/µL, which indicated a successful DNA extraction. The number of tick DNA samples extracted was 523 (314 *A. sculptum*; 132 *A. parvum*; 32 *A. ovale;* 3 *A. auricularium;* 1 *A. tigrinum;* 1 *R. sanguineus;* 1 *R.* (*B.*) *microplus;* 39 *Amblyomma* larvae pools) of which 228 (43.5%) were from adults, 256 (48.9%) pooled nymphs and 39 (7.4%) pooled larvae with an average concentration of 45.9 (SD=84,3) ηG/µL. Out of 523 sampled ticks, 31 specimens (5.9% [23 *A. parvum* adults, 4 *A. sculptum* adults, 1 *A. ovale* adult, 1 *A. parvum* nymph and 2 pooled *Amblyomma* larvae]) showed negative results for the mitochondrial 16S rRNA tick gene and were excluded from analysis. A total of 39 pooled fleas samples were extracted with an average concentration of 7 (SD=8,43) ηG/µL and only one sample didn't amplified the predicted product for cox-1 from fleas and it was also was excluded from further analysis.

The average absorbance of negative sera provided in ELISA kit was 0.157 (0.198 ± 0.129), resulting in a cut-off value of 0.393. Thirty-nine (92.8%) dogs and 42 (53.8%) crab-eating foxes were seroreactive to *B. vogeli* antigen. The mean antibodies absorbance values of seropositive dogs and crab-eating foxes were 0.734 (1.431 ± 0.450) and 0.691 (1.211 ± 0.411), respectively.

Seven (16.6%) dogs, one (1.2%) crab-eating fox, three (42.8%) ocelots, six (7.8%) *T. fosteri* wild rodents, eight (6%) *A. parvum* (5 adults and 3 nymph pools), two (0.6%) *A. sculptum* (1 adults and 1 nymph pool) and one (3.1%) *A. ovale* adult (1.2%) were positive for 18SrRNA-PCR based on Jefferies *et al*. (2007) protocol. Three (9.6%) coatis and one (14.2%) ocelot were positive for 18SrRNA-PCR based on Ujvari *et al*. (2004) and Birkenheuer *et al*. (2006) protocols, respectively. Three out of seven positive dogs in 18SrRNA-PCR and two out of eight positive *A. parvum* in 18SrRNA-PCR were also positive in *hsp70*-PCR based on Soares *et al*. (2011) protocol. All the sampled fleas were negative in PCRs assays for piroplasmids. All sequences obtained from the positive samples were deposited in Genbank international database under the accession numbers KY450716-KY450752.

Regarding the presence of inclusions suggestive of piroplasmids in Giemsa-stained blood smears, piroplasms and Maltese cross forms were observed within one ocelot´s erythrocytes (**Figures 2**). Additionally, piroplasms were found in one coati´s

erythrocytes (**Figure 2**). Both animals were positive in 18SrRNA PCR assays for piroplasmids.

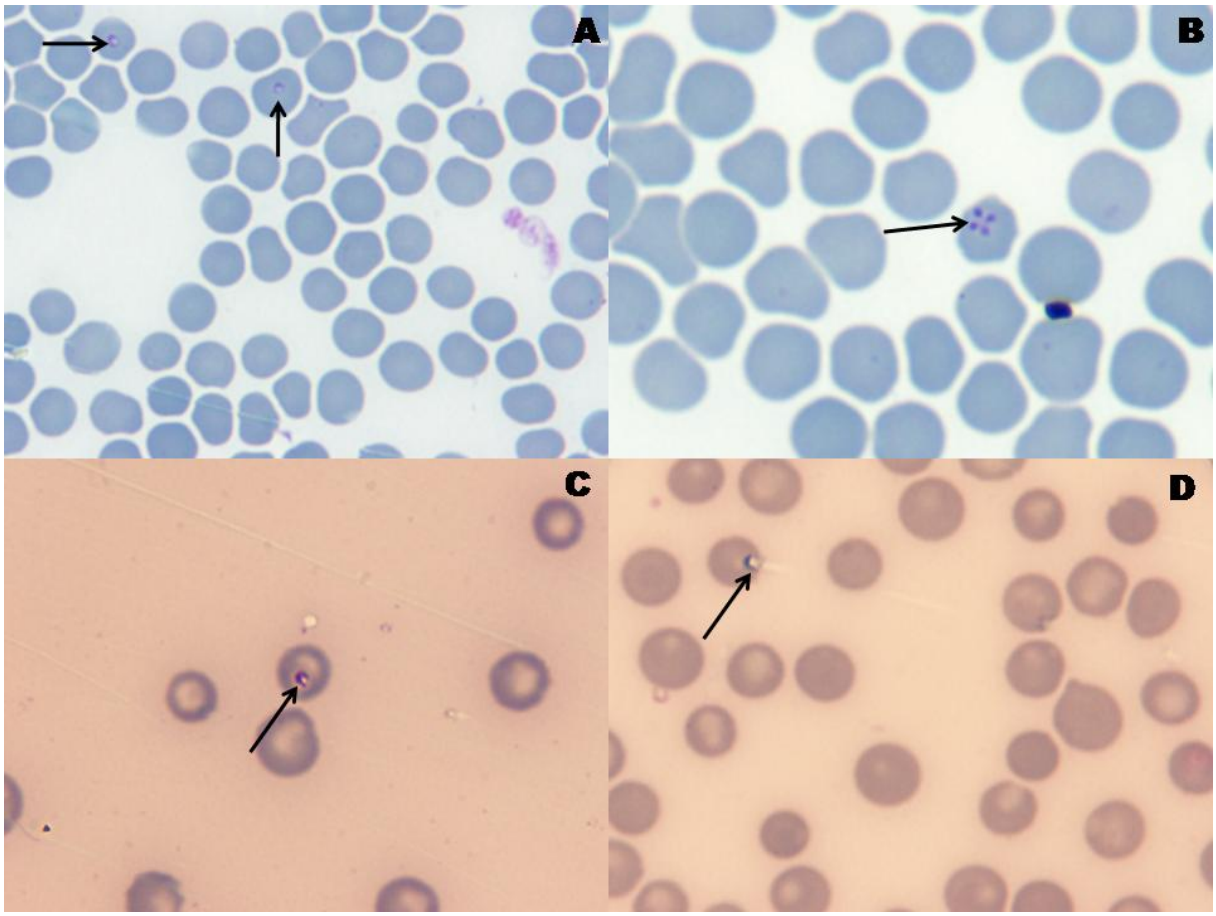

Figure 2. Piroplasmids suggestive forms found inGiemsa-stained blood smears from sampled mammals in Brazilian southern Pantanal. (A)Individual forms found within erythrocytesfrom an ocelot. (B) Maltese-cross inclusion found within erythrocytes from an ocelot. (C; D) Individual forms found within erythrocytes from coatis sampled in southern Pantanal.

Six out of seven 18S rRNA-piroplasmids sequences obtained from domestic dogs showed 100% of identity with *B. vogeli* previously deposited in GenBank (KT323934) by BLAST analysis. One 18S rRNA-piroplasmid sequence obtained from a domestic dog showed 100% of identity with *B. caballi* (EU642512). While five out of six 18S rRNA-piroplasmids sequences obtained from *T. fosteri* showed 99% of identity with *B. vogeli* (KT323934), one of them showed 99% of identity with *T. equi*

(KU672386) by BLAST analysis. One 18S rRNA-piroplasmid sequence obtained from a crab-eating fox, one sequence obtained from an *A. ovale* adult and one sequence obtained from an *A. sculptum* adult showed 100% of identity with *B. caballi* previously deposited in GenBank (EU642512). While four 18S rRNA-piroplasmids sequences obtained from *A. parvum* adults showed 99% of identity with *T. equi* (KU672386), four others 18S rRNA-piroplasmids sequences obtained from *A. parvum* (1 adult and three nymph pools) showed 97% of identity with a sequence of *Babesia* sp. obtained from *Odocoileus virginianus*, from USA (HQ264119). A 18S rRNA-piroplasmid sequence obtained from an *A. sculptum* nymph pool showed 99% of identity with a sequence of *Theileria* spp. obtained from *Rangifer tarandus tarandus*, also from USA (JN086224). Surprisingly, three positive coatis samples amplified using 18SrRNA-*Hepatozoon* protocol (Ujvari *et al*., 2004) showed 100% of identity with a sequence of *Theileria* spp. obtained from a domestic cat sampled in Brazil (KF970930). All 18S rRNA-piroplasmids sequences obtained from ocelots (n=4) showed 99% of identity with *C. felis* previously deposited in GenBank (GU903911) by BLAST analysis (**Tables 3 and 4**). Three *hsp70*-piroplasmids sequences obtained from domestic dogs showed 100% of identity with *B. vogeli* (AB248733). Finally, two *hsp70*-piroplasmids sequences obtained from *A. parvum* nymph pools showed 90% of identity with *B. canis* (AB248735), by BLAST analysis.

Table 3. Maximum identity of 18S rRNA and *hsp70* piroplasmids sequences detected in wild and domestic animals in Brazilian southern Pantanal by BLAST analysis.

Host	Number of sequences analized	Target gene	% identity by BLAST®-analysis
Cerdocyon thous	1	18SrRNA	100% *Babesia caballi* genotype A_CABEQ30 (EU642512)
Thrichomys fosteri	5	18SrRNA	99% *Babesia vogeli* (KT323934)
Thrichomys fosteri	1	18SrRNA	99%*Theileria equi* (KU672386)
Canis familiaris	5	18SrRNA	100% *Babesia vogeli* (KT323934)
Canis familiaris	1	18SrRNA	100% *Babesia caballi* genotype A_CABEQ30 (EU642512)
Nasua nasua	3	18SrRNA	100% *Theileria* sp. (KP410272)
Leopardus pardalis	4	18SrRNA	99% *Cytauxzoon felis* (GU903911)
Canis familiaris	3	*hsp70*	100% *Babesia vogeli* (AB248733)

Table 4. Maximum identity of 18S rRNA and *hsp70* piroplasmids sequences detected in ticks collected from wild animals in Brazilian southern Pantanal by BLAST analysis.

Tick specimen	Host	Target gene	% identity by BLAST® analysis
Amblyoma parvum adult	*Cerdocyon thous*	18SrRNA	99%*Theileria equi* (KU672386)
Amblyoma parvum adult	*Cerdocyon thous*	18SrRNA	99%*Theileria equi* (KU672386)
Amblyoma parvum adult	*Cerdocyon thous*	18SrRNA	99%*Theileria equi* (KU672386)
Amblyoma parvum adult	*Cerdocyon thous*	18SrRNA	99%*Theileria equi* (KU672386)
Amblyoma parvum adult	*Cerdocyon thous*	18SrRNA	97% *Babesia* sp. from *Odocoileus virginianus* (HQ264119)
Amblyoma parvum nymph	*Thrichomys fosteri*	18SrRNA	97% *Babesia* sp. from *Odocoileus virginianus* (HQ264119)
Amblyoma parvum nymph	*Thrichomys fosteri*	18SrRNA	97% *Babesia* sp. from *Odocoileus virginianus* (HQ264119)
Amblyoma parvum nymph	*Clyomys laticeps*	18SrRNA	97% *Babesia* sp. from *Odocoileus virginianus* (HQ264119)
Amblyomma sculptum adult	*Cerdocyon thous*	18SrRNA	100% *Babesia caballi* genotype A_CABEQ30 (EU642512)
Amblyomma sculptum nymph	*Cerdocyon thous*	18SrRNA	99% *Theileria* sp. from *Rangifer tarandus tarandus* (JN086224)
Amblyomma ovale adult	*Cerdocyon thous*	18SrRNA	100% *Babesia caballi* genotype A_CABEQ30 (EU642512)
Amblyoma parvum nymph	*Thrichomys fosteri*	*hsp70*	90% *Babesia canis canis*(AB248735)
Amblyoma parvum nymph	*Clyomys laticeps*	*hsp70*	90% *Babesia canis canis*(AB248735)

The phylogenetic analysis based on 18S rRNA gene fragments clustered six piroplasmids sequences obtained from dogs' blood samples (KY450732, KY450733, KY450734, KY450735, KY450736, KY450737) and five sequences obtained from *T. fosteri* spleen samples (KY450738, KY450739, KY450740, KY450741, KY450742) in a branch comprising *B. vogeli* sequences (AY371196, HM590440) previously deposited in Genbank, with clade support values of 100 and 100, based on ML and

BI analyses, respectively (**Figures 3 and 4**). 18SrRNA-piroplasmids sequences obtained from one *C. thous* blood sample (KY450725), one domestic dog blood sample (KY450731), one *A. ovale* adult (KY450726), and one *A. sculptum* adult (KY450730) were closely related to *B. caballi* (EU888901, EU642512), with clade support values of 100 and 100, based on ML and BI, respectively (**Figures 3 and 4**). Four 18SrRNA-piroplasmids sequences obtained from *A. parvum* (1 adult [KY450743], three sequences obtained from nymph pools [KY450727, KY450728, KY450729]), and a *Babesia* spp. sequence, previously deposited in Genbank and obtained from *Odocoileus virginianus* sampled in the USA (HQ264119), were grouped in the same clade of *B. bigemina* (JQ723014) based on ML analysis, and in the same clade of *B. bigemina* (JQ723014) and *B bovis* (L19077) based on BI analysis (**Figures 3 and 4).** 18SrRNA-piroplasmids sequences obtained from four *A. parvum* adults (KY450716, KY450717, KY450718, KY450716), three coatis blood samples (KY450722, KY450723, KY450724) and one *T. fosteri* spleen sample (KY450720) were grouped into the same large branch of *T. equi* sequences (KU672386, AY150064, AB515315), with clade support values of 50 and 99, based on ML and BI analyses, respectively (**Figures 3 and 4**). One 18SrRNA-piroplasmid sequence obtained from an *A. sculptum* nymph pool (KY450721) was placed in the same clade of *Theileria* spp. sequence obtained from *Rangifer tarandus tarandus* (JN086224) sampled in the USA, with clade support values of 99 and 100, based on ML and BI analyses, respectively (**Figures 3 and 4**). Four 18SrRNA-piroplasmids sequences obtained from ocelots blood samples (KY450744, KY450745, KY450746, KY450747) was pooled in the same clade of *C. felis* sequences previously deposited in Genbank (DQ382277, L19080), with clade support values of 100 and 100, based on ML BI analyses, respectively (**Figures 3 and 4**). 18SrRNA-phylogenetic inferences based on ML and BI methods were performed using the evolutionary model GTR + G + I and *Coccidia* sp. (HM117907), *Isospora suis* (U97523), *Sarcocystis* sp. (U97524), and *Adelina bambarooniae* (AF494059) as outgroups.

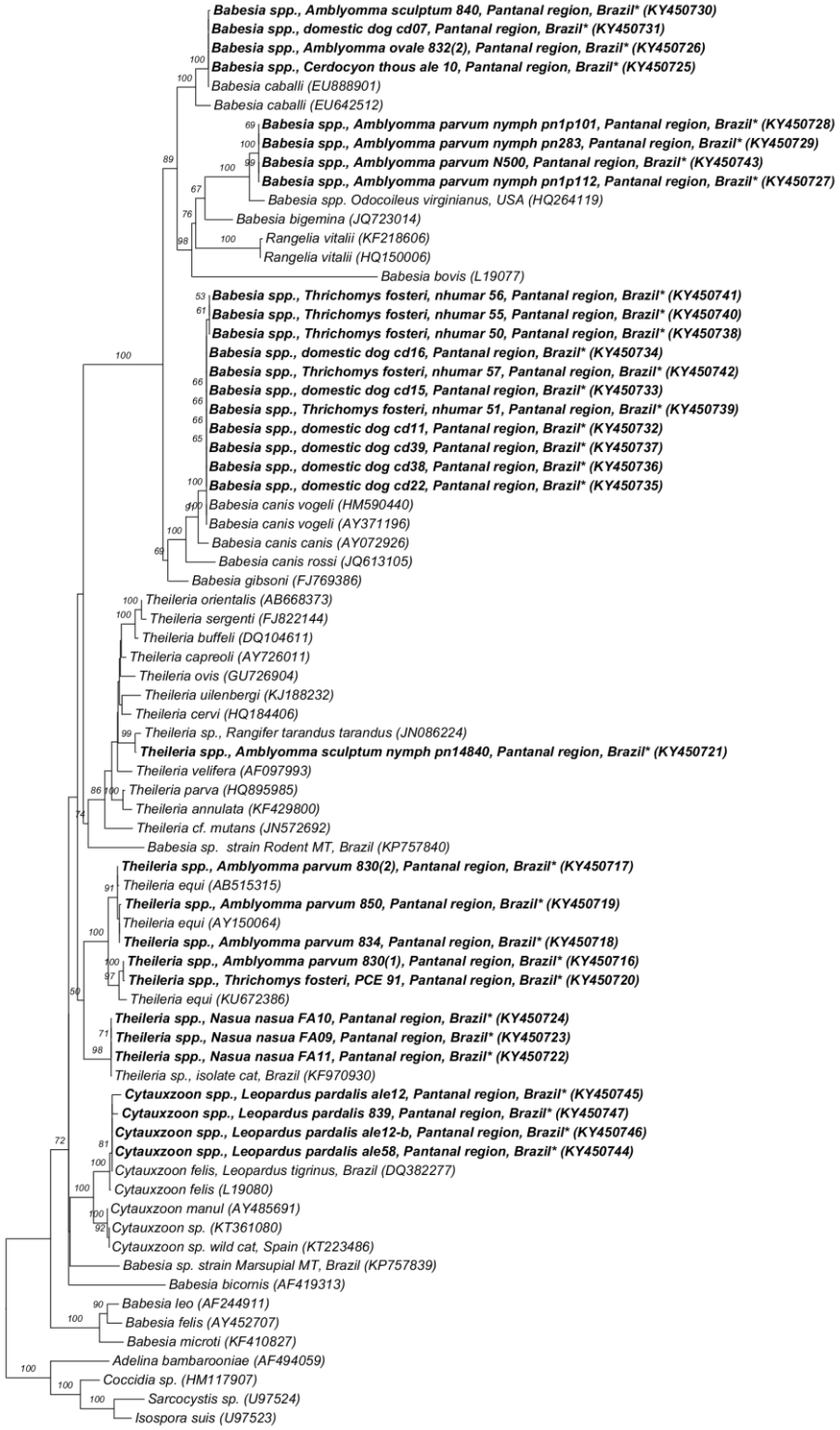

Figure 3. Phylogenetic tree constructed with 2000pb of piroplasmids-18SrRNA sequences, using Maximum Likelihood (ML) method and GTR+G+I evolutionary model. Numbers at nodes correspond to ML bootstrap values over 50, using and *Coccidia* sp. (HM117907), *Isospora suis* (U97523), *Sarcocystis* sp. (U97524),and *Adelina bambarooniae* (AF494059) as outgroups.

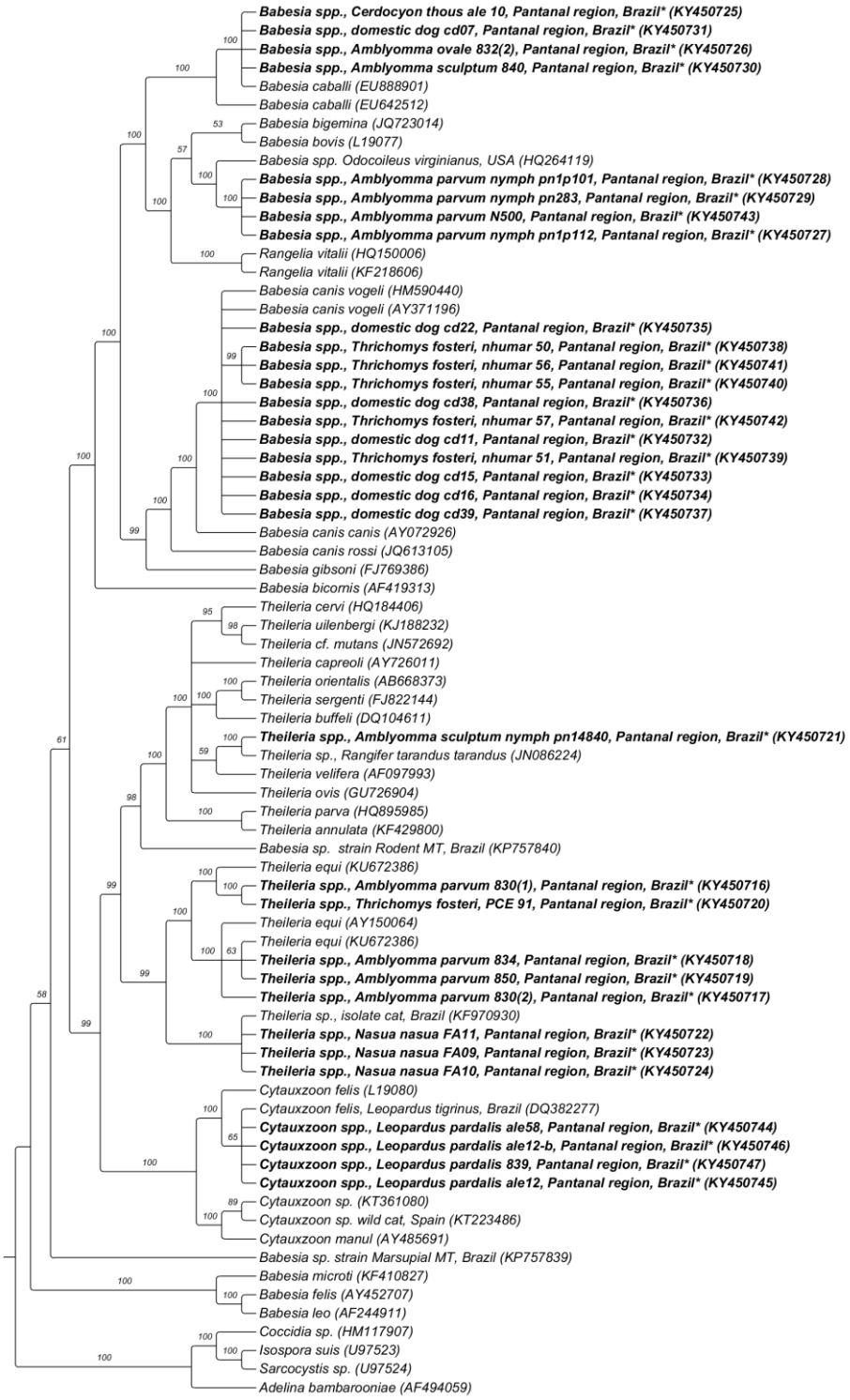

Figure 4. Phylogenetic tree constructed with 2000pb of piroplasmids-18SrRNA sequences, using Bayesian method and GTR+G+I evolutionary model. Numbers at nodes correspond to Bayesian posterior probabilities over 50, using *Coccidia* sp. (HM117907), *Isospora suis* (U97523), *Sarcocystis* sp. (U97524),and *Adelina bambarooniae* (AF494059) as outgroups.

Regarding the concatenated phylogenetic analysis of piroplasmids based on 18SrRNA and *hsp70* genes fragments, two piroplasms sequences obtained from *A. parvum* nymphs pools were pooled in the same branch of *R. vitalii* sequences (JF279603; KF218606), with clade support values of 53 and 94, based on ML and BI analyses, respectively. The remaining three piroplasms *hsp70*+18SrRNA concatenated sequences obtained from dogs blood samples were grouped in the same clade of *B. vogeli* sequences (AB248733; AY371196), with clade support values of 100 in both ML and BI analyses (**Figures 5 and 6**). Concatenated 18SrRNA+*hsp70*-phylogenetic inferences based on ML and BI methods were performed using the GTR + G + I evolution model and *Plasmodium falciparum* (M19753; JQ627152) as outgroup.

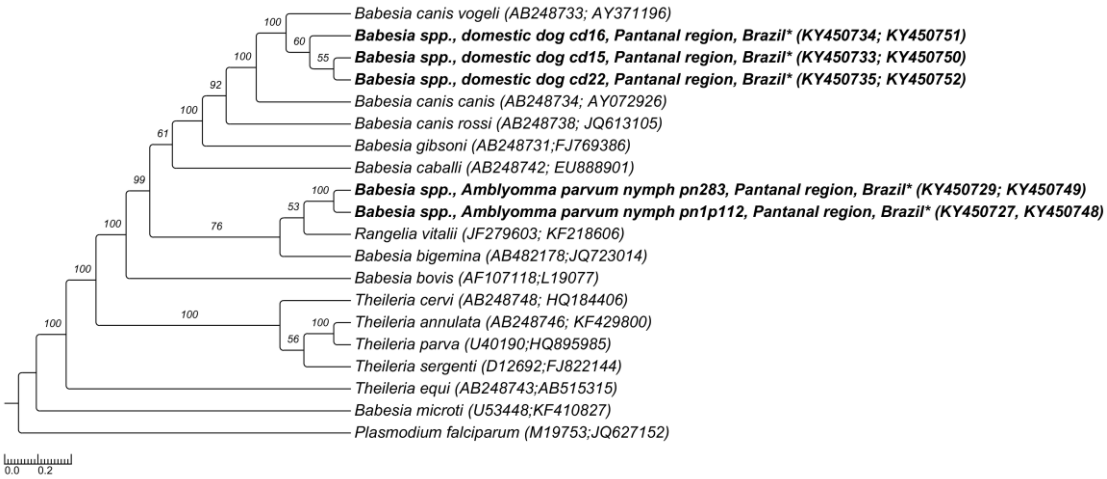

Figure 5. Phylogenetic tree constructed with 4800pb of piroplasmids 18SrRNA + *hsp70* sequences, using Maximum Likelihood (ML) method and GTR+G+I evolutionary model. Numbers at nodes correspond to ML bootstrap values over 50. *Plasmodium falciparum* (M19753;JQ627152) was used as outgroup.

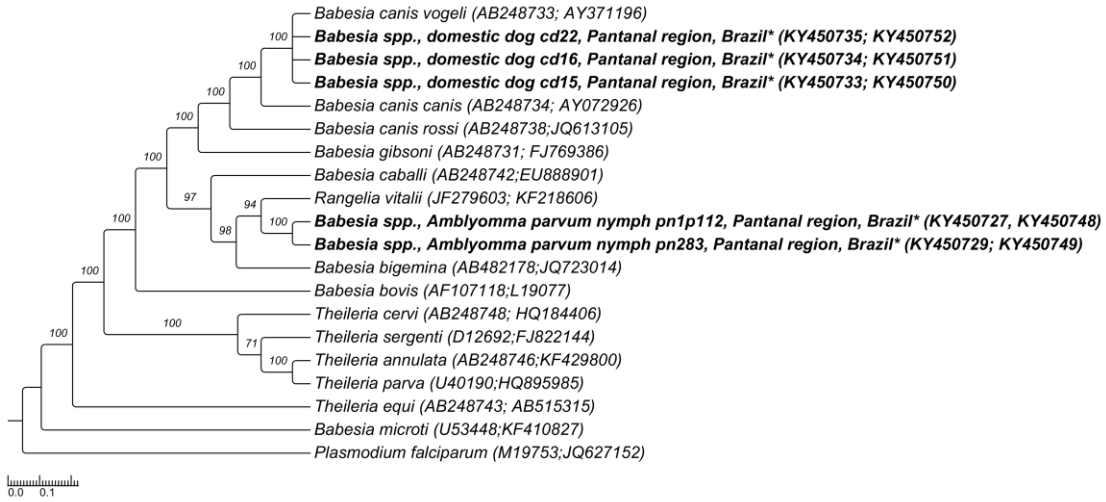

Figure 6. Phylogenetic tree constructed with 4800pb of piroplasmids 18SrRNA + *hsp70* sequences, using Bayesian method and GTR+G+I evolutionary model. Numbers at nodes correspond to Bayesian posterior probabilities over 50.*Plasmodium falciparum* (M19753;JQ627152) was used as outgroup.

DISCUSSION

The present study showed the presence of piroplasmids DNA in blood or spleen samples from wild carnivores, domestic dogs, rodents and ticks sampled in the southern region of Pantanal, state of Mato Grosso do Sul, central-western Brazil. Molecular analyses based on 18S rRNA and *hsp70* genes revealed an occurrence of different piroplasmids species among sampled mammals and arthropods.

Babesia vogeli is a widespread tick-borne protozoan in dogs in Brazil (Passos *et al*., 2005; O'Dwyer *et al*., 2009; Sousa *et al*., 2013; da Costa *et al*., 2015; Moraes *et al*., 2015), in accordance with the wide distribution of its vector, *R. sanguineus* s.l., mainly in urban and peripheral urban areas (Labruna and Pereira, 2001). In the present study, the molecular occurrence of *B. vogeli* (16.6%) among sampled dogs was higher than that found (3.3%) in a previous study involving dogs in an urban area from the same state of Mato Grosso do Sul (Sousa *et al*., 2013) and dogs (3.13%) sampled in northern Pantanal, state of Mato Grosso (Melo *et al*., 2016). Herein, the high seroprevalence (92.8%) to *B. vogeli* found among dogs was similar to that found (81.6%) among dogs sampled in an urban area from the same state of Mato Grosso do Sul (Sousa *et al*., 2013). Infection by *B. vogeli* in dogs seems to be common among dogs from the state of Mato Grosso do Sul.

On the other hand, reports of seropositivity to *B. vogeli* among wild canids are scarce in Brazil (André *et al*., 2011). A higher seroprevalence (53.8%) was found among free-living crab-eating foxes sampled in the present study when compared to that found among with wild canids (10%) maintained in captivity in Brazilian zoos (André *et al*., 2011). Although this work showed the first serological evidence of exposure to *B. vogeli* among free-living wild canids in Brazil, cross-reactions between *Babesia* and *Rangelia* could not be discarded. Furthermore, considering the high occurrence of *B. vogeli* found in domestic dogs sampled in the studied area, the role of these animals as a source of *B. vogeli* infection to wild carnivores cohabiting the same area should be better investigated.

A genotype closely related to *B. vogeli* was also detected in five *T. fosteri* wild rodents. It seems that the host range for *B. vogeli* or a genotype closed related to is wider than previously supposed, since the protozoa has also been detected in cats (André *et al*., 2014; 2015), besides dogs and rodents, in Brazil. Recently, a genotype closely related to *Babesia bicornis* was detected in *T. fosteri* rodents sampled in northern Pantanal (Wolf *et al*., 2016). Changes in land use may facilitate the circulation of piroplasmids-infected ticks between wild rodents and domestic and wild animals, since those animals can share the same ecotypes.

Equine piroplasmoses (EP) are tick-borne diseases of domestic and wild equines caused by the exclusively intraerythrocytic protozoa *B. caballi* or by the intraerythrocytic and intralymphocytic protozoa *T. equi* (Kerber *et al*., 1999). Although Brazil is considered an endemic area for equine piroplasmids (Barbosa *et al*., 1995; Baldani *et al*., 2004; 2007; Costa Pereira *et al*., 2005; Heim *et al*., 2007; Kerber *et al*., 2009; Vieira *et al*., 2013), the molecular diversity of these agents in South America remains scarce (Heim *et al*., 2007). In the present study, while genotypes closely related to *B. caballi* were detected in one dog and one crab-eating fox, genotypes closely related to *T. equi* were detected in three coatis and in one wild *T. fosteri* rodent. In addition to this, *Theileria*-suggestive piroplasms inclusions were found in a coati blood smear. Recently, genotypes closely related to *T. equi* have also been detected in cats in Brazil (André *et al*., 2014; 2015).

Although *Anocentor nitens* (Roby and Anthony, 1963) and *R.* (*B*.) *microplus* (Battsetseg *et al*., 2002) ticks are considered, respectively, the main vectors for *B.*

caballi and *T. equi* in Brazil, some evidences have suggested the participation of *Amblyomma cajennense* ticks in *T. equi* (Kerber *et al*., 2009; Ribeiro *et al*., 2011, Scoles and Ueti, 2013) and *B. caballi* (Vieira *et al*., 2013) transmission cycles. Herein, while *B. caballi* DNA was detected in *A. ovale* and *A. sculptum* ticks, *T. equi* DNA was detected in *A. parvum* ticks. These findings highlight the importance of future studies regarding the role of *Amblyomma* species in the epidemiological cycles of *B. caballi* and *T. equi.*

In South America*, Rangelia vitalii,* a piroplasm species associated to hemolytic disorders (Soares *et al*., 2011) in canids.,has been reported in domestic and wild canids from southeastern and southern regions of Brazil (Soares *et al*., 2011, 2014; Gottlieb *et al*., 2016; Quadros *et al*., 2015; Fredo *et al*., 2015; Silveira *et al*., 2016), and in domestic dogs from Argentina (Eiras *et al*., 2014) and Uruguay (Soares *et al*., 2015). Herein, the concatenated phylogenetic analysis revealed the presence of a genotype closely related to *R. vitalii* in *A. parvum* ticks collected from *T. fosteri* and *C. laticeps* wild rodents. Previous studies suggested that crab-eating foxes are the natural reservoirs for *R. vitalii* in Brazil (Soares *et al*., 2014; Fredo *et al*., 2015). Considering the fact that *C. thous* is the second most frequent wild mammal species found in Pantanal (Mamede and Alho, 2006), future studies should be performed in order to assess the real significance of the circulation of this genotype in this Brazilian wetland.

A genotype closely related to *Theileria* sp. previously detected in a wild deer (*R. t. tarandus*) sampled in the USA was detected in *A. sculptum* nymphs collected from *C. thous* in the present study. Previously, a genotype showing 90–100% identicalness to *Theileria cervi* was detected in 52% of sampled pampas deer (*Ozotoceros bezoarticus*) in the same region of Pantanal (Silveira *et al*., 2013). Genotypes closely related to *Theileria* spp. associated to wild ruminants have also been detected in cats from Campo Grande, an urban area in the state of Mato Grosso do Sul (André *et al*., 2015). Future studies aiming at investigate the host and vectors range of wild ruminants-associated *Theileria* genotypes should be investigated in Brazilian Pantanal.

While the epidemiology of *Cytauxzoon* spp. has been extensively studied in the USA (Blouin *et al*., 1984; Meinkoth and Kocan, 2005; Yabsley *et al*., 2006; Haber

et al., 2007), the occurrence of these parasites in other regions of the world has been scarcely reported. In fact, while clinical and fatal cytauxzoonosis has been well reported in the USA (Garner *et al*., 1996, Nietfield and Pollock, 2002), Germany (Jakob and Wesemeier, 1996) and Italy (Carli *et al.,* 2012), the parasite has been rarely described and not associated with clinical disease in domestic cats in Brazil so far (Maia *et al*., 2013; André *et al*., 2015). Although *Cytauxzoon* spp. have already been reported in neotropical and exotic wild felids maintained in captivity in zoos in Brazil (Peixoto *et al*., 2007; André *et al*., 2009), the present work showed the first molecular detection of *Cytauxzoon* spp. among free-living wild felids in South America. Herein, a genotype closely related to *C. felis* was detected in four apparently asymptomatic ocelots. *Cytauxzoon*-suggestive maltese cross inclusions were found in one ocelot blood smear. The pathogenic potential of *Cytauxzoon* isolates in Brazil is still unknown. In fact, while fatal cytauxzoonosis has been reported in lions maintained in captivity in a zoo in the state of Rio de Janeiro (Peixoto *et al*., 2007), the parasite has been detected in apparently asymptomatic wild felids maintained in captivity in the state of São Paulo and Brasília (André *et al*., 2009). The role of neotropical wild felids as reservoirs for *Cytauxzoon* in Brazil should be further investigated. Although *A. parvum* ticks were found parasitizing the sampled ocelots, *Cytauxzoon* DNA was not detected in any collected tick specimen. While *Dermacentor variabilis* (Blouin *et al*., 1984) and *A. americanum* (Reichard *et al*., 2010) are responsible for transmitting *C. felis* among felids in the USA, the vectors involved in the *Cytauxzoon* epidemiological cycles in Brazil remains unknown.

Recently, phylogenetic analyses based on mitochondrial genome sequences concatenated along with 18S rRNA sequences identified five distinct Piroplasmida lineages with different biological features, namely: i. *Babesia* sensu stricto (*B. caballi*, *B. bigemina*, *B. canis*, *B. rossi*, *B. vogeli*, *B. gibsoni* and *B. bovis*); ii. *Theileria* spp. and *Cytauxzoon* sp.; iii. *T. equi*; iv. Western *Babesia* group (*B. conradae*); v. *B. microti* group (Schreeg *et al*., 2016). Although the mitochondrial genome analysis was not performed in the present study, sequences apparently belonging to three out of five Piroplasmida lineages were identified. Genotypes closely related to *B. caballi* and *B. vogeli,* obtained from *C. thous, T. fosteri, A. ovale* and *A. sculptum* from the present study seems to belong to *Babesia* sensu stricto lineage, which is

characterized by infecting erythrocytes from different vertebrate species and transovarial and transstadial transmission in ticks hosts (Schreeg *et al*., 2016). On the other hand, the sequences closely related to *Theileria* spp. and *C. felis,* obtained from *A. sculptum* nymphs and ocelots seems to belong to *Theileria* spp. and *Cytauxzoon* sp. lineage. Protozoans belonging to this lineage have developed strategies to enhance their propagation, which has been evidenced by enlarged schizont-infected leukocytes, suggesting a blocking of host cell apoptosis (Hagiwara *et al*., 1997; Susta *et al*., 2009). Piroplasmids sequences obtained from three coatis, one *T. fosteri* four *A. parvum* ticks seems to belong to *T. equi* lineage*,* in which host leukocyte manipulation has not been observed, differently from *Theileria* spp. and *Cytauxzoon* sp. lineage (Schreeg *et al*., 2016). In fact, the mitochondrial genome structure of *T. equi* has showed to be radically divergent from any other Piroplasmida species, suggesting that *T. equi* was recovered as a unique distinct lineage (Schreeg *et al.*, 2016). Finally, although a genotype closely related to *R. vitalii* was detected in *A. parvum* ticks in the present study, the mitochondrial genome structure of *R. vitalii* has not been analysed yet, precluding its positioning in piroplasmids phylogroups. Schreeg *et al*. (2016) suggested that new lineages would be reported if a higher number of newly described piroplasmids were included in phylogenetic analyses. From a preliminary point of view, based on 18S rRNA and *hsp70*-concatenated phylogenetic analyses, *R. vitalii* and associated genotype detected in the present study grouped with protozoans allocated in *Babesia* s.s. group.

In conclusion, the present study revealed that wild animals and ticks in southern Pantanal region, Brazil, are exposed to a high diversity of piroplasmids species. The role of domestic dogs as a source of *B. vogeli* infection to wild animals should be better investigated. Wild and domestic animals and *Amblyomma* ticks seemed to be eventually infected by genotypes closely related to equine piroplasmids. Genotypes closely related to *R. vitalii* and ruminants-associated *Theileria* species circulate in Pantanal region. Finally, *Cytauxzoon* spp. was detected among free-living wild felids for the first time in South America. Therefore, future studies are much needed in order to estimate the impact of piroplasmids infections among the Brazilian wildlife in Pantanal biome.

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CHAPTER 8 - Final considerations

Conclusions

- Wild animals from southern Pantanal region, central-western Brazil, are exposed to *Hepatozoon* spp., *Bartonella* spp., hemotropic mycoplasmas, Anaplasmataceae, piroplasmids and *Rickettsia* spp. agents;
- Wild carnivores, rodents and domestic dogs sampled in southern Pantanal seemed not to share the same *Hepatozoon* species;
- Rodents in Pantanal region may play a role in the routes of transmission for *Hepatozoon* species to reptiles and amphibians;
- Wild rodents in southern Pantanal region, Brazil, are exposed, at least, to two different *Bartonella* species;
- *Polygenis (P.) bohlsi bohlsi* fleas may act as a possible vectors for *Bartonella* spp. among rodents in southern Pantanal, Brazil*;*
- Domestic dogs, crab-eating foxes and coatis seemed to share the same *Mycoplasma* spp. species, closely related to *M. haemocanis/M. haemofelis*;
- Wild felines and rodents appear to be infected by specific hemoplasmas species;
- The real role of arthropods in hemoplasmas and *Hepatozoon* transmission cycles in southern Pantanal remains unknown;
- A new *Mycoplasma* genotype was detected among wild coatis and crab-eating foxes in southern Pantanal;
- Domestic dogs, crab-eating foxes and ocelots seemed to be exposed to *R. amblyommatis* sp. nov. or a closely related species;
- A high occurrence of '*Candidatus.* Rickettsia andeanae' was found among *Amblyomma parvum* ticks collected from sampled wild animals in southern Pantanal region.