

**UNIVERSIDADE ESTADUAL PAULISTA – UNESP  
FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS  
CAMPUS DE JABOTICABAL**

**DIVERSIDADE DE PIROPLASMÍDEOS EM MAMÍFEROS E  
AVES SELVAGENS NO BRASIL**

**Ana Cláudia Calchi**

Médica Veterinária

**UNIVERSIDADE ESTADUAL PAULISTA – UNESP**  
**FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS**  
**CAMPUS DE JABOTICABAL**

**DIVERSIDADE DE PIROPLASMÍDEOS EM MAMÍFEROS E**  
**AVES SELVAGENS NO BRASIL**

**Ana Cláudia Calchi**

**Orientador: Prof. Dr. Marcos Rogério André**

Tese apresentada à Faculdade de Ciências Agrárias e Veterinárias – Unesp, Câmpus de Jaboticabal, como parte das exigências para a obtenção do título de Doutora em Medicina Veterinária, área de concentração Patologia Animal.

**2025**

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|-------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|

### **Impacto potencial dessa pesquisa**

A pesquisa revelou uma grande diversidade de espécies e genótipos de piroplasmídeos em animais selvagens no Brasil, contribuindo para um melhor entendimento acerca da distribuição desses patógenos no país. O estudo validou e desenvolveu ensaios moleculares sensíveis e específicos para a detecção em amostras biológicas de animais selvagens e humanos.

### **Potential impact from this research**

The research revealed a high diversity of piroplasmid species and genotypes in wild animals in Brazil, contributing to a better understanding of the distribution of these pathogens in the country. The study also developed and validated sensitive and specific molecular assays for the detection of piroplasmids in biological samples from wild animals and humans.



**CERTIFICADO DE APROVAÇÃO**

TÍTULO DA TESE: DIVERSIDADE DE PIROPLASMÍDEOS EM MAMÍFEROS E AVES SELVAGENS NO BRASIL

**AUTORA: ANA CLÁUDIA CALCHI**

**ORIENTADOR: MARCOS ROGÉRIO ANDRÉ**

Aprovada como parte das exigências para obtenção do Título de Doutora em Medicina Veterinária, área: Patologia Animal pela Comissão Examinadora:



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Jaboticabal, 02 de junho de 2025

## **DADOS CURRICULARES DA AUTORA**

**Ana Cláudia Calchi** – nascida na cidade de Pirassununga, São Paulo, em 13 de março de 1994. Filha de Ana Lúcia de Mattos Calchi e Aldemir Aparecido Calchi. Graduada em Medicina Veterinária pela Universidade Estadual Paulista “Júlio de Mesquita Filho” – Faculdade de Ciências Agrárias e Veterinárias – FCAV/UNESP, Jaboticabal, São Paulo, no ano de 2017. Durante a graduação, foi bolsista de Iniciação Científica da Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) de novembro de 2014 a agosto de 2015 (#2014/15505-3), atuando na área de Produção Animal, voltada para frangos de corte, sob orientação da Profa. Dra. Silvana Martinez Baraldi Artoni. Foi bolsista pela mesma agência de fomento de dezembro de 2015 a julho de 2017 (#2015/22387-0), atuando na área de Parasitologia Veterinária, com ênfase na detecção molecular de agentes da família Anaplasmataceae em canídeos selvagens, sob orientação do Prof. Dr. Marcos Rogério André. Foi monitora voluntária da disciplina de Parasitologia Veterinária II, ministrada pelo Prof. Dr. Marcos Rogério André, de agosto a dezembro de 2016. Em agosto de 2018, ingressou no curso de Mestrado do Programa de Pós-Graduação em Medicina Veterinária (Medicina Veterinária Preventiva) na FCAV/UNESP, sob orientação do Prof. Dr. Marcos Rogério André e coorientação da Prof. Dra. Rosângela Zacarias Machado, com bolsa financiada pela FAPESP (#2018/13838-6), defendendo sua dissertação em julho de 2020. Em agosto de 2020 ingressou no Doutorado no mesmo programa de Pós-Graduação, instituição e sob mesma orientação, porém na área de concentração de Patologia Veterinária, inicialmente com bolsa CAPES e posteriormente com bolsa FAPESP (#2020/07826-5). Em abril de 2023, recebeu o prêmio “Mulheres que fazem a UNESP na Modalidade: Doutorado e Área do Conhecimento Ciências Agrárias”. Entre julho de 2023 a maio de 2024, realizou período de pesquisa no exterior, com bolsa BEPE FAPESP (2022/16555-0), no Intracellular Pathogens Research Laboratory do College of Veterinary Medicine da North Carolina State University em Raleigh, Carolina do Norte, EUA, sob orientação de Prof. Dr. Edward B. Breitschwerdt e Dr. Ricardo G. Maggi.

## EPÍGRAFE

“Foi o tempo que dedicaste à tua rosa que a fez  
tão importante”

(O Pequeno Príncipe)

## DEDICATÓRIA

Dedico a meus pais, à minha tia Nilza e  
àquela criança que tinha o sonho de ser  
professora e cuidar dos animais

## **AGRADECIMENTOS**

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## CEUA – COMISSÃO DE ÉTICA NO USO DE ANIMAIS

### CERTIFICADO

Certificamos que o projeto de pesquisa intitulado **"Diversidade de Piroplasmídeos em mamíferos e aves selvagens no Brasil"**, protocolo nº 9412/2022, sob a responsabilidade do Prof. Dr. Marcos Rogério André, que envolve a produção, manutenção e/ou utilização de animais pertencentes ao Filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica (ou ensino) - encontra-se de acordo com os preceitos da lei nº 11.794, de 08 de outubro de 2008, no decreto 6.899, de 15 de julho de 2009, e com as normas editadas pelo Conselho Nacional de Controle de Experimentação Animal (CONCEA), e foi aprovado pela COMISSÃO DE ÉTICA NO USO DE ANIMAIS (CEUA), da FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS, UNESP - CÂMPUS DE JABOTICABAL-SP, em reunião ordinária de 14 de dezembro de 2022.

Vigência do Projeto: 01/01/2023 a 01/01/2026

|                    |                            |
|--------------------|----------------------------|
| Espécie / Linhagem | Ordem Pilosa               |
| Nº de animais      | 397                        |
| Peso / Idade       | Variável                   |
| Sexo               | Machos e fêmeas            |
| Origem             | Estados de SP, MS, PA e RO |

|                    |                    |
|--------------------|--------------------|
| Espécie / Linhagem | Ordem Cingulata    |
| Nº de animais      | 58                 |
| Peso / Idade       | Variável           |
| Sexo               | Machos e fêmeas    |
| Origem             | Estados de SP e MS |

|                    |                                                    |
|--------------------|----------------------------------------------------|
| Espécie / Linhagem | Ordem Chiroptera                                   |
| Nº de animais      | 273                                                |
| Peso / Idade       | Variável                                           |
| Sexo               | Machos e fêmeas (exceção gestantes e/ou lactantes) |
| Origem             | Estado do Acre                                     |

(continua)

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Câmpus de Jaboticabal



|                    |                            |
|--------------------|----------------------------|
| Espécie / Linhagem | Ordem Artiodactyla         |
| Nº de animais      | 187                        |
| Peso / Idade       | Variável                   |
| Sexo               | Machos e fêmeas            |
| Origem             | Estados do SP, PR, MG e GO |

|                    |                              |
|--------------------|------------------------------|
| Espécie / Linhagem | Ordem carnívora              |
| Nº de animais      | 59                           |
| Peso / Idade       | Variável                     |
| Sexo               | Machos e fêmeas              |
| Origem             | Estado do Mato Grosso do Sul |

|                    |                                                   |
|--------------------|---------------------------------------------------|
| Espécie / Linhagem | Ordem Rodentia                                    |
| Nº de animais      | 113                                               |
| Peso / Idade       | Variável                                          |
| Sexo               | Machos e fêmeas (exceto gestantes e/ou lactantes) |
| Origem             | Estado do Mato Grosso do Sul                      |

|                    |                     |
|--------------------|---------------------|
| Espécie / Linhagem | Ordem Passeriformes |
| Nº de animais      | 501                 |
| Peso / Idade       | Variável            |
| Sexo               | Machos e fêmeas     |
| Origem             | Estados do MG e MS  |

Jaboticabal, 14 de dezembro de 2022.

*Fabiana Pilarski*  
**Profª Drª Fabiana Pilarski**  
Coordenadora – CEUA

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## DIVERSIDADE DE PIROPLASMÍDEOS EM MAMÍFEROS E AVES SELVAGENS NO BRASIL

**RESUMO** - Piroplasmídeos são protozoários apicomplexos transmitidos por carrapatos que compreendem os gêneros *Babesia*, *Rangelia*, *Cytauxzoon* e *Theileria*. Há uma ampla diversidade de espécies que acometem diferentes táxons de mamíferos e aves, as quais podem causar enfermidades em animais e humanos. O presente estudo teve como objetivos: (i) investigar a ocorrência e determinar a diversidade genética de piroplasmídeos em diferentes grupos taxonômicos de vertebrados no Brasil; (ii) validar um protocolo de PCR digital (dPCR) para detecção desses agentes nas amostras de animais selvagens; (iii) desenvolver um protocolo de qPCR/dPCR multiplex para a detecção de espécies zoonóticas de *Babesia*. Para isto, foram analisadas 1543 amostras de DNA extraídas de a partir de sangue ou tecidos de animais selvagens pertencentes a sete grupos taxonômicos (398 Pilosa, 57 Cingulata, 208 Chiroptera, 181 Artiodactyla, 78 Carnivora, 113 Rodentia, sete Didelphimorphia e 501 aves de 14 ordens) amostrados em diferentes estados do país. A triagem foi realizada por meio de nested PCR (nPCR) baseada no gene 18S rRNA (~800pb). Ensaio adicionais de PCR baseados nos genes 18S rRNA (~1500pb), *hsp-70*, *cox-1*, *cox-3*, *cytB*, e das regiões intergênicas ITS-1 e ITS-2 foram utilizados para caracterização molecular das amostras positivas. No total, 217 (14,1%) amostras foram positivas para piroplasmídeos, das quais: 17 tamanduás (10,1%), oito tatus (14%), 136 cervídeos neotropicais (75,1%), três canídeos selvagens (25%) e 53 felinos selvagens (80,3%). Como resultado das análises filogenéticas realizadas, as sequências curtas do gene 18S rRNA obtidas em tatus-Canastra foram alocadas no clado “South American Marsupialia group”, enquanto a obtida em tatu-galinha alocou-se juntamente com uma sequência de *Theileria* sp. previamente detectada em indivíduo pertencente à mesma espécie, formando um subclado dentro do clado *Theileria* sensu stricto. Já na análise filogenética baseada na região ITS-1, as sequências obtidas de tamanduás-bandeira e tamanduás-mirins foram posicionadas em um único clado, separado dos outros clados de piroplasmídeos. Com base em inferências filogenéticas com alvo em diferentes marcadores moleculares (18S rRNA, *hsp70*, *cox-1*, *cox-3*, *cytB* e regiões intergênicas ITS-1, ITS-2, a depender do grupo de animal analisado) e análises de diversidade e de genótipos, o presente estudo mostrou a ocorrência de: (i.) uma nova espécie de *Babesia*, nomeada *Babesia pantanalensis* nov. sp. em cachorros-do-mato (*Cerdocyon thous*) do Pantanal sul-matogrossense; (ii.) *Cytauxzoon brasiliensis* em jaguatiricas e *Cytauxzoon felis* em onças-pintadas amostradas nos biomas do Pantanal, cerrado, Amazonia, Mata Atlântica e caatinga (iii.) diferentes genótipos de *Theileria* sp. filogeneticamente relacionados à *Theileria cervi* ou *Theileria capreoli* em cervídeos. Os resultados aqui obtidos mostram grande diversidade genética de piroplasmídeos em animais selvagens no país. Um protocolo multiplex de dPCR para piroplasmídeos foi padronizado e mostrou-se eficaz para a detecção desses agentes em amostras de animais selvagens, que normalmente apresentam baixa parasitemia, mostrando maior sensibilidade do que técnicas de PCR em tempo real e nPCR. Adicionalmente, foi desenvolvido um protocolo multiplex de qPCR/dPCR para *Babesia divergens*, *Babesia odocoilei*, *Babesia duncani* e *Babesia microti* com base na região ITS-1, tornando possível, detectar pela primeira vez, a ocorrência de coinfeção entre essas espécies em humanos dos EUA, Canadá e México.

**Palavras-chave:** Animais selvagens, Diversidade genética, Piroplasmorida, Técnicas moleculares, *Babesia* spp. zoonóticas

## DIVERSITY OF PIROPLASMIDS IN WILD MAMMALS AND BIRDS FROM BRAZIL

**ABSTRACT** – Piroplasmids are apicomplexan protozoa transmitted by ticks, comprising the genera *Babesia*, *Rangelia*, *Cytauxzoon*, and *Theileria*. A wide diversity of species affects different taxa of mammals and birds, potentially causing diseases in both animals and humans. This study aimed to: (i) investigate the occurrence and genetic diversity of piroplasmids in various vertebrate taxonomic groups in Brazil; (ii) validate a digital PCR (dPCR) protocol for the detection of these agents in wild animal samples.; and (iii) develop a multiplex qPCR/dPCR protocol for detecting zoonotic *Babesia* species. To achieve this, we analyzed 1,543 DNA samples extracted from the blood or tissues of wild animals across seven taxonomic groups: 398 Pilosa, 57 Cingulata, 208 Chiroptera, 181 Artiodactyla, 78 Carnivora, 113 Rodentia, 7 Didelphimorphia, and 501 birds from 14 orders, all sampled from different states in Brazil. Screening was performed using nested PCR (nPCR) based on the 18S rRNA gene (~800 bp). Additional PCR assays targeting the 18S rRNA gene (~1500 bp), *hsp-70*, *cox-1*, *cox-3*, *cytB*, and the ITS-1 and ITS-2 intergenic regions were used for the molecular characterization of the positive samples. In total, 217 (14.1%) samples tested positive for piroplasmids, including 17 anteaters (10.1%), eight armadillos (14%), 136 neotropical deer (75.1%), 3 wild canids (25%), and 53 wild felids (80.3%). Phylogenetic analyses of the small 18S rRNA sequences revealed that sequences from giant armadillos clustered within the “South American Marsupialia group” clade, while sequences from the nine-banded armadillo formed a subclade within the *Theileria* sensu stricto clade, alongside a *Theileria* sp. sequence detected in the same host. On the other hand, in the phylogenetic analysis based on the ITS-1 region, sequences obtained from giant anteaters clustered together in a single clade, distinct from other piroplasmid clades. Phylogenies based on various gene markers (18S rRNA, *hsp70*, *cox-1*, *cox-3*, *cytB*, ITS-1, ITS-2, depending on the animal group) and genotype diversity analyses indicated the occurrence of: (i) a possible new *Babesia* species, named *Babesia pantanalensis* in crab-eating foxes from the Pantanal in Mato Grosso do Sul; (ii) *Cytauxzoon brasiliensis* in ocelots, and *Cytauxzoon felis* in jaguars from the Pantanal, Cerrado, Amazon, Atlantic Forest and Caatinga biomes; (iii) different genotypes of *Theileria* sp., phylogenetically related to *Theileria cervi* or *Theileria capreoli* in deer. The results highlight the significant genetic diversity of piroplasmids in wild animals in Brazil. A multiplex dPCR protocol for detecting piroplasmids were standardized, demonstrating its effectiveness in detecting these agents in wild animal samples, which often exhibit low parasitemia. This protocol showed greater sensitivity than real-time PCR and nPCR techniques. In addition, a multiplex qPCR/dPCR protocol was developed for *Babesia divergens*, *Babesia odocoilei*, *Babesia duncani*, and *Babesia microti* based on the ITS-1 region, enabling, for the first time, the detection of coinfection between these species in humans from the USA, Canada, and Mexico.

**Keywords:** Wild animals, Genetic diversity, Piroplasmorida, Molecular techniques, Zoonotic *Babesia* sp.

## CAPÍTULO 1 – CONSIDERAÇÕES GERAIS

### 1. Introdução

Os piroplasmídeos representam o segundo grupo de hemoparasitos de maior ocorrência em mamíferos, ficando atrás apenas de *Trypanosoma* spp. (Alvarado e Rybak, 2016; Schnittger et al., 2022). Esses protozoários apicomplexos pertencem à Eukarya, domínio Diaphoretickes, Supergrupo TSAR, grupo SAR, clado Alveolata, Filo Apicomplexa, Classe Aconoidasida, Ordem Piroplasmorida (Adl et al., 2018; Burki et al., 2020), que engloba as famílias Babesiidae (gêneros *Babesia* e *Rangelia*) e Theileriidae (gêneros *Theileria* e *Cytauxzoon*) (Solano-Gallego et al., 2011; Schnittger et al., 2012; Alvarado and Rybak, 2016). Parasitam células sanguíneas de vertebrados, incluindo eritrócitos, linfócitos, monócitos ou células endoteliais, a depender do agente. São responsáveis por causar doenças em animais domésticos e selvagens e humanos (Alvarado e Rybak, 2016; Krause et al., 2019; Schnittger et al., 2022).

A transmissão ocorre principalmente através da picada de carrapatos ixodídeos, os quais transmitem o agente pela saliva (Jalovecka et al., 2019). Porém, outras rotas menos comuns já foram relatadas, tais como: transfusão sanguínea, já descrita em humanos (*Babesia microti*, *Babesia duncani*, *Babesia divergens*-like) e em cães, sendo nestes últimos devido à infecção por *Babesia gibsoni* (Stegeman et al., 2003; Tang and Tran, 2020; Drews et al., 2023) ; transplacentária em cães (*B. gibsoni* e *Babesia vogeli*), equinos (*Theileria equi*), bovinos (*Babesia bovis* e *Babesia bigemina*) e humanos (*B. microti*) (Corrêa, 1974; Fukumoto et al., 2005; Krause and Vannier, 2012; Costa et al., 2016; Sant et al., 2016; Agrawal et al., 2020; Rojas-Pirela et al., 2021; Bonato et al., 2023); e vias iatrogênicas (materiais e agulhas contaminados) (Scoles and Ueti, 2015; Karasová et al., 2022; Schnittger et al., 2022). Além disso, suspeita-se da transmissão direta de *B. gibsoni* entre cães via brigas, provavelmente por meio de mordidas (Birkenheuer et al., 1999; Matsuu et al., 2004; Jefferies et al., 2007). Corduneanu et al. (2020) demonstraram a presença de DNA de *Babesia canis* em órgãos de camundongos que ingeriram carrapatos (*Dermacentor reticulatus*) infectados com o agente, gerando uma nova hipótese acerca da possibilidade de infecção por via oral.

O diagnóstico poder ser confirmado por esfregaços sanguíneos de sangue periférico, principalmente de orelha ou da cauda, no qual inclusões (merozoítos e/ou



trofozoítos) podem ser observadas no interior das células sanguíneas (Dantas-Torres et al., 2016). Porém, a sensibilidade do teste depende do nível de parasitemia e da fase da doença na qual ele se encontra, por estar diretamente relacionadas com o número de células infectadas presentes na circulação sanguínea. Um segundo método para a detecção é a realização de testes sorológicos, porém eles não confirmam infecção vigente e podem ocorrer reações cruzadas entre diferentes espécies (Todorovic, 1975; Herwaldt et al., 2003; Kukina et al., 2018). Por fim, métodos moleculares, principalmente por meio de ensaios de PCR estão sendo amplamente utilizados, devido a maior sensibilidade e especificidade (Dantas-Torres et al., 2016). Novas técnicas mais sensíveis vêm sendo desenvolvidas e exploradas. Atualmente, destacam-se a PCR digital (dPCR), a digital droplet PCR (ddPCR) (Feng et al., 2017; Mirabile et al., 2024). As técnicas de primeira e segunda geração são PCR convencional (cPCR), PCR em tempo real (qPCR) e qPCR microfluídica (MF qPCR), respectivamente, que são as mais difundidas até o momento (Zhang et al., 2022).

Diversas novas espécies de piroplasmídeos vêm sendo descritas nos últimos anos. Muitas vezes, as linhagens descritas são associadas de acordo com o táxon do hospedeiro vertebrado, sugerindo co-especiação. Entretanto, algumas espécies de piroplasmídeos podem infectar diferentes espécies de hospedeiros, fato observado principalmente para o grupo de *Babesia* sensu stricto, uma vez que a capacidade de transmissão transovariana nos carrapatos vetores favorece a troca para um novo hospedeiro da mesma espécie ou de espécie diferente. O mesmo pode ser observado, porém em menor escala, para as demais linhagens de piroplasmídeos. Uma vez que para as demais linhagens filogenéticas de piroplasmídeos verifica-se somente transmissão transestadial, a capacidade de atingir diferentes hospedeiros é menor, a não ser que as espécies de carrapatos vetores sejam di ou trioxenos (Jalovecka et al., 2019).

No Brasil, vários genótipos e novas espécies de piroplasmídeos vêm sendo descritos em diferentes espécies de animais selvagens. *Babesia* spp. foi detectada em gato-palheiro-do-Pantanal (*Leopardus braccatus*) (André et al., 2011), capivaras (*Hydrochoerus hydrochaeris*) (Criado-Fornelio et al., 2009; Krawczak et al., 2023; Neves et al., 2023), cachorros-do-mato (*Cerdocyon thous*) (de Sousa et al., 2017; Calchi et al., 2024b), roedores selvagens (Wolf et al., 2016; de Sousa et al., 2017;

Pacheco et al., 2025), gambás (Serra-Freire et al., 1979; Soares et al., 2017; Gonçalves et al., 2021; de Oliveira et al., 2023; Braga et al., 2023), cervídeos (Silveira et al., 2011; Silveira et al., 2013; Soares et al., 2017), morcegos não-hematófagos (Ikeda et al., 2021) e hematófagos (de Mello et al., 2023a) e aves (Quillfeldt et al., 2014; Werther et al., 2017; Sgarioni et al., 2023). *Theileria* spp. foi detectada em cervídeos (Silveira et al., 2011; Silveira et al., 2013; Soares et al., 2017; Fava et al., 2022; Calchi et al., 2024a), tatus-galinha (*Dasypus novemcinctus*), cutias (*Dasyprocta* sp.), pacas (*Cuniculus paca*), queixadas (*Tayassu pecari*) (Soares et al., 2017), antas (*Tapirus terrestris*) (da Silveira et al., 2017; Gonçalves et al., 2020; Silva et al., 2021; Mongruel et al., 2023), roedores (de Sousa et al., 2017), quatis (*Nasua nasua*) (de Sousa et al., 2017) e morcegos hematófagos (de Mello et al., 2023a). *Cytauxzoon* sp., por sua vez, foi detectado em diferentes espécies de felídeos selvagens (Peixoto et al., 2007; André et al., 2009; Filoni et al., 2011; de Sousa et al., 2017; Furtado et al., 2017; Soares et al., 2017; Silva et al., 2021; de Paula et al., 2022; Fagundes-Moreira et al., 2022; Calchi et al., 2025). Por fim, *Rangelia vitalli* vem sendo detectada em canídeos selvagens sintomáticos e assintomáticos (Soares et al., 2014a; de Quadros et al., 2015; Fredo et al., 2015; Silveira et al., 2016; de Souza et al., 2019; Copat et al., 2019; de Lorenzo et al., 2021).

A pesquisa de hemoparasitos em animais selvagens é importante tanto para a promoção da conservação e saúde desses animais quanto para a saúde humana, uma vez que eles são considerados fontes de infecção, atuando como reservatórios, de diferentes patógenos de importância para a saúde animal e humana. De acordo com dados publicados pela Organização Mundial da Saúde, 60% dos agentes patogênicos existentes possuem potencial zoonótico, sendo responsáveis por 75% das doenças emergentes atuais (Daszak et al., 2000; Zanella, 2016). A identificação de agentes transmitidos por vetores em animais selvagens pode contribuir para o conhecimento do papel desses animais na epidemiologia das enfermidades e sua relação com patógenos zoonóticos, estabelecimento de áreas de risco para humanos e animais domésticos e criação de medidas de caráter preventivo para as enfermidades.

## 2. Objetivos

### 2.1. Objetivo Geral

O presente estudo teve como objetivo investigar a ocorrência e diversidade genética de piroplasmídeos em diferentes grupos taxonômicos de animais selvagens no Brasil. Adicionalmente, este trabalho objetivou validar um ensaio de dPCR para piroplasmídeos para amostras biológicas de animais selvagens, e desenvolver ensaios de qPCR específicos para a detecção de espécies zoonóticas de *Babesia*.

## 2.2. Objetivos Específicos

- Investigar, por meio de métodos moleculares, a ocorrência de agentes da ordem Piroplasmorida em amostras biológicas (sangue ou baço) de animais das Ordens Pilosa, Cingulata, Chiroptera, Artiodactyla, Carnivora, Rodentia, Didelphimorphia e Passeriformes de vida-livre no Brasil;
- Traçar inferências filogenéticas acerca do DNA de piroplasmídeos detectados em mamíferos amostrados com base nos genes *cox-1*, *cox-3*, 18S rRNA, *hsp70*, *cytB* e regiões intergênicas ITS-1 e ITS-2;
- Investigar a diversidade de haplótipos de piroplasmídeos circulantes nos animais amostrados;
- Padronizar um ensaio de dPCR para a detecção de piroplasmídeos em amostras biológicas coletadas de animais selvagens;
- Desenvolver e padronizar um protocolo de qPCR e dPCR multiplex específicas para a detecção de espécies zoonóticas de *Babesia* spp. (*Babesia odocoilei*, *Babesia microti*, *Babesia divergens* e *Babesia duncani*).

## 3. Revisão de Literatura

### 3.1. Histórico e classificação dos Piroplasmídeos

A primeira descrição de piroplasmídeos foi realizada por Victor Babes em 1888, que descobriu um microrganismo infectando eritrócitos de bovinos apresentando “Redwater fever”, caracterizada por anemia, hemoglobinúria e febre na Romênia. O referido pesquisador nomeou o agente como *Haematococcus bovis*, por pensar tratar-se de uma bactéria (Babes, 1888). Em 1892, Victor Babes encontrou parasitos semelhantes àqueles anteriormente relatados infectando hemácias de ovinos (Babes, 1892; Uilenberg, 2006). Smith & Kilbourne (1893) descreveram *Pyrosoma bigeminum*, agente causador da “Febre do Texas” em bovinos nos Estados Unidos. Os mesmos

autores demonstraram que o referido agente era transmitido por carrapatos (*Boophilus annulatus*) (Smith and Kilborne, 1893). No mesmo ano, Starcovici, aluno de Victor Babes, verificou que, de fato, *Haematococcus bovis* era um protozoário e não uma bactéria, criando então o gênero *Babesia* para homenagear Victor Babes e nomeando as espécies até então descobertas como *B. bovis*, *Babesia ovis* e *B. bigemina* (Starcovici, 1893). Uma vez que o nome *Pyrosoma* já existia antes dessa nomenclatura ser adotada, Patton (1895) renomeou *Pyrosoma bigeminum* como *Piroplasma bigeminum*, devido ao formato de pera dos parasitos após multiplicação (Penzhorn, 2020). Naquele mesmo ano, foi descoberta a espécie *Pyrosoma bigeminum* var. *canis* ou *Piroplasma canis* circulando em cães na Itália (Pianna and Galli-Valerio, 1895). du Toit (1918) reclassificou as espécies e adotou a nomenclatura dada por Starcovici, a qual foi descrita primeiramente (du Toit, 1918; Penzhorn, 2020).

Duas diferentes espécies de piroplasmídeos foram descritas em bovinos e nomeadas como *Piroplasma kochi* Stephens and Christopher, 1903 (sinônimo de *Piroplasma parvum* Theiler, 1904) e *Piroplasma annulatum* Dschunkowsky and Luhs, 1904. Porém, como foram observadas a presença de esquizontes no ciclo evolutivo de ambas as espécies, elas foram removidas do gênero *Piroplasma* e realocadas em um novo gênero nomeado de *Theileria*/ Bettencourt, Franca and Borges, 1907, passando a ser denominadas *Theileria parva* e *Theileria annulata* (Bettencourt et al., 1907; Neitz and Jansen, 1956). O mesmo ocorreu com um parasito encontrado em cães e primeiramente denominado *Piroplasma vitalii* Pestana, 1910 e que, por apresentar esquizogonia extra-eritrocitária e por infectar leucócitos e células endoteliais, passou a ser inserido em novo gênero e denominado *Rangelia vitalii* Carini e Manciel, 1914 (Soares et al., 2014b). du Toit (1918) considerou apenas duas famílias: Theileriidae, composta pelos gêneros *Theileria* e *Rangelia*, e família Babesidae, composta pelos gêneros *Babesia*, *Piroplasma*, *Nicolliia*, *Nuttallia*, *Smithia*, *Rossiella* e *Gonderia*. A divisão levou em consideração que membros da primeira família realizam esquizogonia nos linfócitos e divisão binária nas hemácias, enquanto membros da segunda família se multiplicavam apenas em hemácias e davam origem a duas ou quatro células-filhas (Neitz and Jansen, 1955).

Wenyon (1926), ao revisar a classificação outrora realizada, manteve a separação em apenas duas famílias, mas elencou as espécies em apenas dois

gêneros: *Theileria* e *Babesia*. O referido autor acreditava que formas evolutivas encontradas em canídeos infectados com *Rangelia vitalii* representavam co-infecção por *B. canis* e *Toxoplasma gondii* (Thomson, 1933; Neitz and Jansen, 1956).

*Cytauxzoon sylvicaprae* Neitz and Thomson, 1948 foi descrita em duiker comum (*Sylvicapra grimmia*) na África do Sul. Um novo gênero (*Cytauxzoon*) foi criado, uma vez que, apesar das semelhanças morfológicas com *Theileria*, a esquizogonia dessa espécie foi observada em células histiocíticas e não em células do sistema linfático como ocorre em *Theileria*. Além disso, a espécie descrita também apresentou divisão binária em hemáceas (Neitz and Thomson, 1948).

Novas reclassificações foram sugeridas no decorrer dos anos (Donatien and Lestoquardi, 1930; Thomson, 1933; Neitz and Jansen, 1956). Atualmente, estão alocados em Eukarya, domínio Diaphoretickes, Supergrupo TSAR, grupo SAR, clado Alveolata, Filo Apicomplexa, Classe Aconoidasida, Ordem Piroplasmorida (Adl et al., 2018; Burki et al., 2020). É um grupo de protozoários composto por duas famílias, Babesiidae (gêneros *Babesia* e *Rangelia*) e Theileriidae (gêneros *Theileria* e *Cytauxzoon*), que estão distribuídos em todo o mundo (Alvarado-Rybak et al., 2016; Schnittger et al., 2022). As espécies normalmente são classificadas de acordo com três critérios: (I) tamanho e forma dos merozoítos nos eritrócitos, sendo essa classificação mais utilizada para o gênero *Babesia*, as quais podem ser consideradas pequenas (1,0-2,5 µm) ou grandes (2,5-5,0 µm); (II) de acordo com o número de merozoítos; (III) com base no hospedeiro, sendo esta última não mais utilizada com tanta frequência, uma vez que várias espécies de piroplasmídeos são encontrados em diferentes hospedeiros, não sendo hospedeiro-específicas; e (IV) posicionamento filogenético (Alvarado-Rybak et al., 2016; Dantas-Torres et al., 2016; Jalovecka et al., 2019).

### 3.2. Filogenia e linhagens de Piroplasmídeos

Os piroplasmídeos constituem um grupo polifilético. Jalovecka et al. (2019), a partir de estudos filogenéticos realizados previamente com base no gene 18S rRNA, estabeleceram dez clados diferentes, a saber: (I) *B. microti*-like composta por *Babesia rhodaini* (infecta roedores), *Babesia felis* (infecta felinos), *Babesia vulpes* (canídeos e mustelídeos), *B. microti* (roedores e primatas); (II) Monotremata (*Theileria*

*ornythorhynchi*); (III) Western Clade (*Babesia duncani*, *Babesia lengau*, *Babesia conradae* e *Babesia negevi*) que podem acometer as famílias Bovidae, Cervidae, Canidae, Felidae, Herpestidae e humanos; (IV) Peircei (*Babesia peircei*, *Babesia poelea* e *Babesia ugwiensis*) que infectam aves; (V) Marsupialia, composta por *Theileria* spp. que acometem marsupiais da Austrália; (VI) Rhinocerotidae (*Theileria bicornis*); (VII) *Cytauxzoon* spp. (*Cytauxzoon felis*, *Cytauxzoon manul*, *Cytauxzoon otrantorum*, *Cytauxzoon banethi*, *Cytauxzoon europaeus* e *Cytauxzoon brasiliensis*) que ocorre em animais das famílias Felidae, Ursidae, Herpestidae; (VIII) Equus (*T. equi* e *Theileria haneyi*) que acomete equídeos; (IX) *Theileria* sensu stricto (*T. annulata*, *T. parva*, *Theileria lestoquardi* e *Theileria orientalis*) que acometem as famílias Bovidae e Cervidae; e grupo (X) *Babesia* sensu stricto (*B. bovis*, *B. bigemina*, *B. canis* e *B. ovis*) descrita em várias espécies de animais (Marsupialia, Bovidae, Cervidae, Giraffidae, Rodentia, Canidae, Mustelidae, Ursidae, Felidae, aves e humanos). *Rangelia vitalli*, piroplasmídeo encontrado em canídeos domésticos e selvagens na América do Sul (Brasil, Argentina e Uruguai), é inserido no clado de *Babesia* sensu stricto (Soares et al., 2011; Lemos et al., 2012; Eiras et al., 2014; Soares et al., 2014b; 2015) (**Figura 1**).

Novos clados vêm sendo inseridos na filogenia conforme novas espécies são descobertas. Até o momento, cinco novos clados foram descritos: (I) Phyllostomidae group, composto por piroplasmídeos detectados em morcegos não-hematófagos da família Phyllostomidae (Ikeda et al., 2021); (II) South American Marsupialia group, composto por *Babesia* spp. detectadas em gambás (*Didelphis* spp.) e *Amblyomma dubitatum* (Gonçalves et al., 2021; de Oliveira et al., 2023; Braga et al., 2023), (III) Capybara group, composto por *Babesia goianiaensis*, descrita em capivaras (*Hydrochoerus hydrocharis*) (Krawczak et al., 2023); (IV) *T. terrestris* group formado por *Theileira terrestris* descrita em antas (Mongruel et al., 2023) e (V) South American Rodentia group, que compreende Piroplasmida spp. detectada em roedores da espécie *Thrichomys pachyuris* (Pacheco et al., 2025) (**Figura 1**).

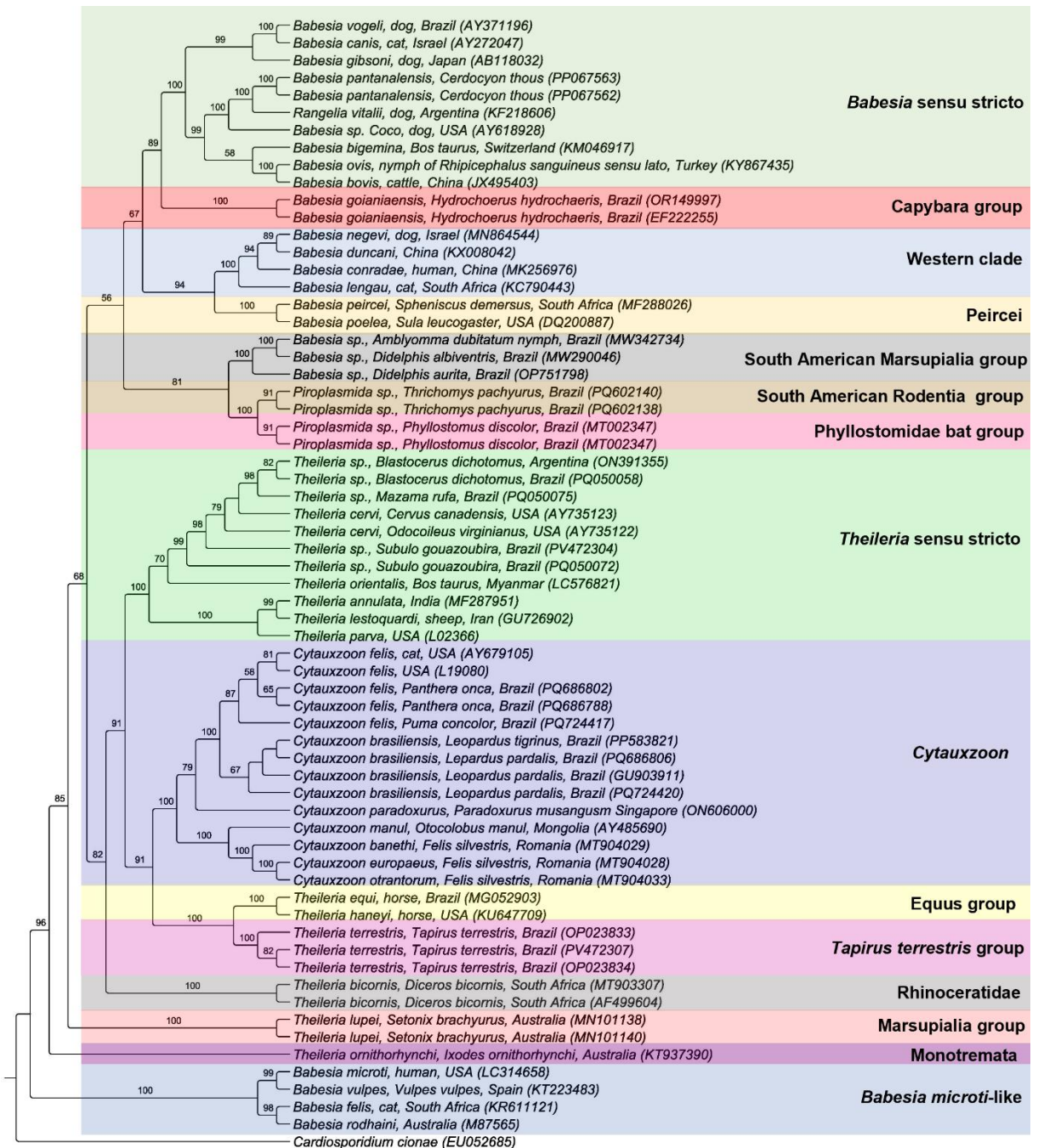


**Figura 1.** Análise filogenética por Máxima Verossimilhança baseada em um alinhamento de 1726 caracteres de sequências do gene 18S rRNA de piroplasmídeos e modelo evolutivo TIM3+I+G. *Cardiosporidium cionae* foi utilizado como grupo externo (Fonte: arquivo pessoal).

### 3.3. Ciclo Biológico

Os estágios biológicos do desenvolvimento de vida dos piroplasmídeos compreende as fases de merogonia e esquizogonia, as quais representam formas de

reprodução assexuada no hospedeiro vertebrado (hospedeiro intermediário), de gametogonia, fecundação e esporogonia, que compreendem reprodução sexuada e assexuada. A gametogonia tem início no hospedeiro vertebrado e término nos carrapatos vetores (hospedeiros definitivos) e a esporogonia ocorre exclusivamente no vetor. Uma diferença observada entre os gêneros é que a fase de esquizogonia ocorre apenas em *Theileria*, *Cytauxzoon* e *Rangelia*, não sendo confirmada em *Babesia* sensu stricto (Uilenberg et al., 2006; Jalovecka et al., 2019). Além disso, a multiplicação no hospedeiro vertebrado ocorre de forma assincrônica, uma vez que diferentes fases evolutivas podem ser observadas nas células ao mesmo tempo (Chauvin et al., 2009).

O carrapato vetor inocula esporozoítos na circulação sanguínea do hospedeiro vertebrado durante o repasto sanguíneo. Em *Babesia* sensu stricto, esses esporozoítos chocam-se com eritrócitos de forma aleatória e, por meio de proteínas do complexo apical e enzimas, penetram nessas células. Já no meio intra-eritrocítico, desenvolvem-se em trofozoítos, os quais realizam merogonia formando merozoítos; estes, por sua vez, rompem os eritrócitos, caem na circulação, infectam novas células e realizam o ciclo novamente. Após algum tempo, alguns merozoítos darão origem a gametócitos. Diferentemente de outros protozoários apicomplexas, piroplasmídeos não se localizam dentro de vacúolos parasitóforos nas células, ficando em contato direto com o citoplasma (Jalovecka et al., 2018, 2019; Schnittger et al., 2022).

Já para os gêneros *Theileria*, *Cytauxzoon* e *Rangelia*, esporozoítos, ao caírem na circulação sanguínea invadem primeiramente leucócitos, linfócitos, macrófagos e/ou células endoteliais, a depender da espécie, e tornam-se esquizontes multinucleados (corpúsculos de Koch), por meio da esquizogonia; tais formas evolutivas posteriormente desenvolvem-se em merozoítos, os quais são liberados na circulação sanguínea, infectam eritrócitos, tornam-se trofozoítos e posteriormente merozoítos, dando continuidade ao ciclo. A merogonia é realizada apenas em eritrócitos em todas as espécies, com exceção de *R. vitalii*, a qual pode ocorrer também em leucócitos (Silva et al., 2011; França et al., 2014; Jalovecka et al., 2019). Adicionalmente, em algumas espécies de *Theileria* sensu stricto, conhecidas como transformadoras (*T. annulata*, *T. parva* e *T. lestoquardi*), a invasão de leucócitos juntamente com a modulação da resposta imune (inibição de apoptose) causa uma



transformação neoplásica da célula, de forma a haver uma proliferação descontrolada de esquizontes (Sivakumar et al., 2014; Mans et al., 2015).

Os carrapatos vetores, ao realizarem repasto sanguíneo em um animal infectado, ingerem eritrócitos contendo gametócitos. Uma vez no lúmen do intestino médio do vetor, estes são diferenciados em gametas, os quais em *Babesia* spp. são denominados corpos raiados e não se diferem morfológicamente, enquanto em *Theileria* spp. são diferenciados morfológicamente em micro e macrogametas. Após a fertilização, há formação do zigoto, que penetra na matriz peritrófica e invade as células epiteliais do intestino do vetor, onde sofrem meiose e dão origem a cinetos primários. Em *Theileria* spp., os cinetos primários migram diretamente para as glândulas salivares, diferentemente de *Babesia* spp. em que há uma invasão prévia de tecidos (hemócitos, fibras musculares, túbulos de Malpighi, células peritraqueais e ovários para espécies sensu stricto; e nefrócitos e glândulas salivares para espécies sensu lato), onde ocorre reprodução assexuada e formação de cinetos secundários, os quais vão para as glândulas salivares. Uma vez nessas glândulas, os cinetos transformam-se em esporontes, os quais irão se diferenciar em esporoblastos; estes, por sua vez, ficam em estado dormente durante a ecdise do carrapato até a próxima alimentação do artrópode, quando tornar-se-ão ativos e darão origem aos esporozoítos, os quais serão liberados de forma contínua para a corrente sanguínea do hospedeiro. No vetor, verifica-se transmissão transestadial para todos os piroplasmídeos e transmissão transovariana para espécies de *Babesia* sensu stricto (Jalovecka et al., 2018, 2019).

### **3.4. Patogenia e Sinais Clínicos**

Uma vez que esses parasitos invadem as células sanguíneas, a patogenia e sinais clínicos estão associados à destruição dessas células. A hemólise intra- e extravascular culmina em anemia hemolítica e em alterações que incluem vasodilatação, estase vascular, baixa pressão sanguínea, edema e alterações na coagulação (Krause et al., 2007; Fanelli, 2021). Trombocitopenia também pode ser observada nas infecções por esses hemoparasitos e pode ocorrer devido à destruição das plaquetas mediada por anticorpos (como ocorre na babesiose) e/ou devido ao consumo por sequestro esplênico ou pelas lesões provocadas no endotélio dos vasos,

como ocorre na rangelirose (Brandão and Hagiwara, 2002; Figuera et al., 2010; Paim et al., 2012; Fernandes et al., 2013; França et al., 2013).

As principais alterações e sinais clínicos observados na fase aguda das piroplasmoses são febre, apatia, letargia, anorexia, anemia, fraqueza, vômitos, hemoglobinúria, bilirrubinúria, icterícia, petéquias e equimoses, espleno e linfadenomegalia. Na fase crônica os animais são normalmente assintomáticos (Irwin, 2010; Schnittger et al., 2012). Sinais neurológicos como incoordenação, convulsão, movimentos de pedalagem e paralisia podem ser observados em bovinos e cães infectados por *B. bovis* e *Babesia rossi*, respectivamente, uma vez que há sequestro e maior tropismo de eritrócitos infectados em capilares cerebrais (Jacobson, 2006; Sondgeroth et al., 2013; Dantas-Torres et al., 2016). Sinais graves como diáteses hemorrágicas, edema pulmonar, hipotensão refratária e insuficiência renal aguda também podem ser observados (Irwin, 2010).

As espécies de *Theileria* descritas como transformadoras são responsáveis por induzir blastogênese, a qual causa uma proliferação descontrolada dos esquizontes e uma transformação incompleta dos leucócitos, culminando em leucocitólise, febre, letargia, linfadenomegalia, icterícia, anemia, insuficiência respiratória e morte, se não tratado (Hayashida et al., 2013; Mans et al., 2015; Tretina et al., 2015; Agina et al., 2020; Clift et al., 2020). Já as espécies não-transformadoras são consideradas benignas e não-patogênicas para os animais (Mans et al., 2015; Clift et al., 2020).

*Rangelia vitalii*, por parasitar e lesionar o endotélio de vasos sanguíneos, levando a ocorrência de hemorragias persistentes que são vistas principalmente nas cavidades (orelhas, narinas, ânus, boca, olhos), diarreia sanguinolenta e os sinais já mencionados na fase aguda de piroplasmoses (Figuera et al., 2010; Soares et al., 2014b).

A severidade da doença está associada às características do hospedeiro, como idade, resposta imune, presença de outras doenças ou infecções associadas, e fatores genéticos (Schnittger et al., 2012). Além disso, as infecções em animais selvagens são geralmente subclínicas e sem sinais clínicos, diferentemente do que ocorre em animais domésticos (Penzhorn, 2006; Alvarado-Rybak et al., 2016).

### **3.5. Principais espécies que acometem animais domésticos e de produção**

Cães são mais acometidos por espécies de *Babesia* que podem causar desde casos subclínicos e assintomáticos a doenças severas. Nove espécies são descritas atualmente que acometem esses animais, as quais se distribuem em três diferentes clados filogenéticos, a saber: (I) clado de *Babesia* sensu stricto (*B. canis*, *B. vogeli*, *B. rossi*, *B. gibsoni*, *Babesia* sp. 'Coco' e *R. vitalii*); (II) Western clade (composto por *B. conradae* e *B. negevi*) e (III) clado de *B. microti*-like (*B. vulpes*) (Baneth et al., 2020; Schnittger et al., 2022; Zygner et al., 2023). Além disso, há registros da ocorrência de *Babesia caballi*, *B. bigemina*, *Babesia* spp. detectada primeiramente em gambás e *T. equi* em cães da América do Sul e Turquia (de Sousa et al., 2018; Inácio et al., 2018; de Oliveira et al., 2023; Oskan et al., 2024). Três espécies ocorrem no Brasil: *R. vitalii*, *B. vogeli* e *B. gibsoni* (esta última em menor proporção), cuja ocorrência está relacionada à distribuição dos carrapatos vetores: *Amblyomma aureolatum* para *R. vitalii*, *Rhipicephalus sanguineus* sensu stricto e *Rhipicephalus linnaei* para *B. vogeli* e *B. gibsoni* (Trapp et al., 2006; André et al., 2014; 2015; Malheiros et al., 2016; Soares et al., 2018; de Souza et al., 2019; de Lorenzo et al., 2021; de Oliveira et al., 2023; Schnitiger et al., 2022).

Gatos domésticos são acometidos mais frequentemente por espécimes do gênero *Cytauxzoon*. Apesar de seis espécies terem sido descritas até o momento circulando em felinos selvagens (*C. felis* – nos EUA, *C. manul* – na Ásia, *C. europaeus*, *C. banethi*, *C. otrantorum* – na Europa e *Cytauxzoon brasiliensis* – no Brasil), apenas duas, *C. felis* e *C. europaeus*, foram descritas em gatos domésticos dos EUA e Suíça, respectivamente (Wagner et al., 1975; Reichard et al., 2005; Wang et al., 2017; Panait et al., 2021; Willi et al., 2022; Reichard et al., 2021; Duarte et al., 2024). Adicionalmente, genótipos de *Cytauxzoon* sp. têm sido detectados na Europa, porém nenhuma espécie foi associada ou os autores erroneamente chamaram de *C. felis*, que comprovadamente só ocorre nas Américas (Carli et al., 2012; Alho et al., 2016; Díaz-Reganon et al., 2017; Wang et al., 2017; Panait et al., 2020; Grillini et al., 2021; Schnittiger et al., 2022).

*Cytauxzoon felis* é considerada a espécie mais patogênica que acomete felinos domésticos, cuja mortalidade já chegou a atingir 100% dos animais acometidos. Atualmente com a melhoria nos métodos de diagnóstico e tratamento, essa taxa vem diminuindo (Lloret et al., 2015; Reichard et al., 2021). No Brasil, genótipos

filogeneticamente associados a *C. felis* vêm sendo descritos, porém devido ao pequeno tamanho do fragmento do gene 18S rRNA amplificado, não é possível dizer se são genovariantes de *C. felis*, *C. brasiliensis* ou até mesmo uma possível nova espécie de *Cytauxzoon* (Miceli et al., 2013; André et al., 2015; Raimundo et al., 2021; André et al., 2022; de Oliveira et al., 2022; Palmer et al., 2022; Carvalho et al., 2024). Até o momento, não há registros de óbitos de felinos domésticos por *Cytauxzoon* spp. no país. Além disso, não há a presença das espécies de carrapatos (*Dermacentor variabilis* e *Amblyomma americanum*) conhecidas como vetores de *C. felis* nos Estados Unidos em território brasileiro (André et al., 2017).

Felinos domésticos também podem ser acometidos por espécies de *Babesia*, tais como: *Babesia felis*, *Babesia leo*, *Babesia lengau*, *Babesia panickeri*, *Babesia* sp. Western Cape, *Babesia hongkongensis*, *Babesia canis presentii*, *B. vogeli*, *B. canis*, *B. gibsoni*, *B. vulpes*, *B. microti* e *B. lohiae* (Panicker et al., 2020; Penzhorn and Oosthuizen, 2020; Schnittiger et al., 2022). No Brasil, *B. vogeli* tem sido a principal espécie desse gênero encontrada em gatos (André et al., 2014; André et al., 2015; Malheiros et al., 2015; André et al., 2022). Adicionalmente, *Theileria* sp. filogeneticamente relacionada àquelas detectadas em equídeos, ruminantes e animais selvagens já foram detectadas em gatos no país (André et al., 2014; 2015).

Equídeos podem ser acometidos por três espécies de piroplasmídeos: *Babesia caballi*, *T. equi* e *T. haneyi*. Pelo menos 33 espécies de carrapatos pertencentes aos gêneros *Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Hyalomma*, *Ixodes*, and *Rhipicephalus* são consideradas vetores comprovados ou suspeitos desses agentes. Adicionalmente, transmissão por via iatrogênica foi comprovada para *T. equi* e *B. caballi*, e transmissão transplacentária foi comprovada para *T. equi* (Scoles and Ueti, 2015; Sant et al., 2016; Schnittger et al., 2022). No Brasil, ainda não houve detecção de *T. haneyi*, porém *B. caballi* e *T. equi* são espécies endêmicas no país (Heim et al., 2007; Santos et al., 2011; Guimarães et al., 2016; Peckle et al., 2018; Bonadimann et al., 2021; Peckle et al., 2022; de Albuquerque et al., 2024; Fernandes et al., 2025).

Bovinos e bubalinos são acometidos por uma ampla gama de espécies de piroplasmídeos, tais como: *B. bovis*, *B. bigemina*, *Babesia divergens*, *Babesia ovata*, *Babesia major*, *Babesia occultans*, *Babesia orientalis*, *Babesia motasi*, *Babesia* sp., *T. parva*, *Theileria taurotragi*, *Theileria orientalis*, *Theileria mutans* e *Theileria velifera*.

Por outro lado, pequenos ruminantes podem ser parasitados por *B. ovis*, *Babesia crassa*, *Babesia motasi*, *Babesia motasi-like*, *Babesia venatorum*, *Babesia* sp., *Theileria uilenberg*, *Theileria luwenshuni*, *T. lestoquardi*, *Theileria ovis*, *Theileria separata* e *Theileria* sp. (Schnittger et al., 2022). No Brasil, *B. bovis* e *B. bigemina* são as espécies de maior importância para os bovinos e as mais frequentemente detectadas devido à presença do vetor no país (*Rhipicephalus microplus*). Pode causar sinais severos em bovinos que vivem em áreas de instabilidade enzoótica (Grisi et al., 2014; Mendes et al., 2019). Adicionalmente, genótipos de *Theileria* spp. associados às espécies que infectam ruminantes foram identificados em um rebanho de búfalos (*Bubalus bubalis*) no Pará (Silveira et al., 2016).

### **3.6. Piropasmídeos em animais selvagens no Brasil**

#### **3.6.1. Ordem Artiodactyla**

##### **3.6.1.1. Família Cervidae**

No Brasil, a maioria dos genótipos (fragmentos de 400 a 500 pb) do gene 18S rRNA de piropasmídeos detectados em cervídeos correspondem à *Theileria cervi* e foram descritos em veados-catingueiros (*Subulo gouazoubira*) (52,9% [9/17]) e cervos-do-Pantanal (*Blastocerus dichotomus*) (25% [1/4]) amostrados no estado de Minas Gerais (Silveira., 2011), veados-campeiros (*Ozotocerus bezoarticus*) (20% [12/60]) amostrados no Pantanal sul-matogrossense (Silveira et al., 2013) e em veados-mateiros (*Mazama americana*) (100% [2/2]) no Pará (Soares et al., 2017).

*Babesia bovis* foi detectada em cervos-do-Pantanal (1/4) e em veados-campeiro (3/60) amostrados nos estados de Minas Gerais e Mato Grosso do Sul, respectivamente. *Babesia bigemina* foi detectada em veado-catingueiro (1/12) e veados-campeiros (2/60) amostrados nos estados acima mencionados (Silveira et al., 2011; Silveira et al., 2013).

##### **3.6.1.2. Família Tayassuidae**

Uma (11,1% [1/9]) queixada amostrada no estado do Pará mostrou-se positiva na PCR para piropasmídeos. A sequência de 514 pb do gene 18S rRNA foi posicionada em um clado próximo à uma sequência de *Babesia* sp. WA1 detectada em uma criança na Califórnia e ao clado de *B. lengau*, *B. gibsoni* e *B. conradae*

(Soares et al., 2017). Adicionalmente, inclusões intracitoplasmáticas sugestivas de piroplasmídeos foram encontradas em esfregaço sanguíneo de um caititu (*Pecari tacaju*) que mostrou-se negativo na PCR para os agentes em questão (Fava et al., 2022).

### 3.6.2. Ordem Carnivora

#### 3.6.2.1. Família Canidae

A positividade molecular para piroplasmídeos obtida em canídeos selvagens nos estudos realizados até o momento variou de 1,2 a 38,1% (Soares et al., 2014a; de Sousa et al., 2018; de Souza et al., 2019; de Lorenzo et al., 2021; Fava et al., 2021). As espécies descritas circulando nesses animais no país são: *Rangelia vitalii* e *Babesia* sp. filogeneticamente relacionada com *B. caballi* (Soares et al., 2014a; de Sousa et al., 2018). Além disso, Cansi et al. (2012) relataram um caso clínico de um lobo-guará (*Chrysocyon brachyurus*) apresentando feridas por punções com miíase, prostração, anemia normocítica normocrômica e anisocitose no zoológico de Brasília. Inclusões em eritrócitos foram observadas e diagnosticadas morfolologicamente como *B. canis*. O animal veio a óbito enquanto estava sob tratamento. À necropsia foram observadas hepato e esplenomegalia, equimoses no corpo peniano, e destruição das camadas medular e cortical dos rins devido ao parasitismo por *Dioctophyme renale*. Como não foram realizadas outras análises para confirmar a espécie de piroplasmídeo encontrada, não foi possível realmente afirmar tratar-se de *B. canis*, uma vez que esta espécie é morfolologicamente semelhante a outras espécies de *Babesia* spp. e *R. vitalii* e nunca foi diagnosticada previamente por técnicas moleculares no país.

*Rangelia vitalii* foi descrita circulando em canídeos selvagens das espécies *C. thous* (Soares et al., 2014a; Fredo et al., 2015; Copat et al., 2019; de Souza et al., 2019; de Lorenzo et al., 2021; de Souza Júnior et al., 2023), *C. brachyurus* (Silveira et al., 2016) e *Lycalopex gymnocercus* (graxaim-do-campo) (de Quadros et al., 2015; Fredo et al., 2015; da Silva et al., 2018; de Souza et al., 2019). Ocorre nas regiões sul e sudeste do país, associada à presença do carrapato-vetor *Amblyomma aureolatum* (Soares et al., 2018). *Cerdocyon thous* é considerado hospedeiro natural e reservatório desse agente, por ser a espécie de animal com maior relato de positividade na literatura, por não desenvolver sinais clínicos (suspeita-se que a fase de esquizogonia

seja curta e auto-limitante), com raras exceções, por manter a infecção por longos períodos e por ser considerado o principal hospedeiro para *A. aureolatum* (Labruna et al., 2005; Soares et al., 2014a; de Lorenzo et al., 2021; de Souza Júnior et al., 2023).

De Quadros et al. (2015) descreveram o caso de uma fêmea de *L. gymnocercus* de vida-livre em Santa Catarina que apresentava mucosas pálidas, apatia, hipotermia, desidratação e incoordenação motora. Exames laboratoriais evidenciaram a presença de linfocitose, neutropenia, policromasia, eritroblastose e corpúsculos de Howell-Jolly. O animal veio a óbito e o parasitismo por *R. vitalii* foi confirmado por PCR baseada no gene 18S rRNA e pela presença de estruturas morfológicamente compatíveis ao hemoparasito em tecidos renais e cardíacos. Além de *R. vitalii*, o animal estava co-infectado por *Hepatozoon canis* e *Calodium hepaticum*. Já Copat et al. (2019) relataram um caso clínico de um cachorro-do-mato resgatado no Rio Grande do Sul que apresentava icterícia, incoordenação motora, fezes de coloração enegrecida e infestação por *A. aureolatum*. Foram detectadas inclusões piriformes em hemácias no esfregaço sanguíneo; o hemograma mostrou anemia normocítica normocrômica, anisocitose, policromasia, leucocitose, neutrofilia, monocitose e trombocitopenia. Os exames bioquímicos mostraram elevação dos níveis de uréia e creatinina, hipoalbuminemia e hipoproteinemia. À necropsia foram encontrados icterícia generalizada, esplenomegalia, sangramento no pâncreas, petéquias na mucosa intestinal e melena. Em cortes histológicos foi observada a presença do parasito no endotélio vascular de vários órgãos. DNA do parasito foi detectado pela PCR (Copat et al., 2019). Outros trabalhos também relataram a presença do parasito no citoplasma de células endoteliais de diferentes órgãos em um lobo-guará de Minas Gerais (Silveira et al., 2016) e graxaim-do-campo e cachorro-do-mato no Rio Grande do Sul (Fredo et al., 2015). Em ambos os estudos os animais apresentavam diferentes sinais clínicos inespecíficos e vieram a óbito, porém não foi possível determinar se os sinais clínicos e causa da morte tenham sido decorrentes da infecção por *R. vitalii*, pois os animais apresentavam co-infecção com outros agentes que poderiam causar os mesmos sinais clínicos. O lobo-guará estava parasitado por vários helmintos, além de *Hepatozoon* sp., *Leishmania* sp. e *Entamoeba* sp.; o graxaim-do-campo havia sido atacado por um cão; e o cachorro-do-mato estava infectado pelo vírus da cinomose (Fredo et al., 2015; Silveira et al., 2016).

Por fim, de Sousa et al. (2018) detectaram a presença de *Babesia* sp. filogeneticamente relacionada à *B. caballi* em um *C. thous* (1,2% [1/78]) amostrado no Pantanal Sul-matogrossense. Infelizmente, o fragmento obtido do gene 18S rRNA era pequeno (739 pb), o que dificultou uma melhor interpretação acerca de seu posicionamento filogenético.

### 3.6.2.2. Família Felidae

Até o momento, o gênero de piroplasmídeos descrito com maior frequência em felinos selvagens no Brasil é *Cytauxzoon* spp. Há relatos da detecção desse agente em animais de cativeiro e de vida-livre das seguintes espécies: *Leopardus pardalis* (jaguar), *Leopardus tigrinus* (gato-macambira), *Leopardus guttulus* (gato-do-mato-do-sul), *Panthera onca* (onça-pintada), *Puma concolor* (onça-parda) e *Panthera leo* (leão) (Juliano et al., 2004; Amaral, 2006; Peixoto et al., 2007; André et al., 2009; Filoni et al., 2012; Furtado et al., 2017; Soares et al., 2017; Antunes et al., 2018; de Sousa et al., 2018; Silva et al., 2020; Guizellini et al., 2021; Silva et al., 2021; de Paula et al., 2022; Fagundes-Moreira et al., 2022; Fava et al., 2022; Castillo et al., 2024; Duarte et al., 2024; Alves et al., 2025). A positividade molecular variou de 0,92 a 98,1% nos estudos (André et al., 2009; Filoni et al., 2012; Furtado et al., 2017; de Sousa et al., 2018; Silva et al., 2020; Silva et al., 2021; Fagundes-Moreira et al., 2022; Alves et al., 2025).

Embora a maioria dos trabalhos realizados relatam a ocorrência de *C. felis* em felídeos selvagens no Brasil, todos esses estudos foram baseados em um fragmento curto do gene 18S rRNA, o qual é altamente conservado, dificultando a identificação precisa da espécie. Duarte et al. (2024), a partir da obtenção de um fragmento quase completo do gene 18S rRNA (1459 pb) e de um fragmento longo do gene *cytB* (1097pb), descreveram uma nova espécie no país, denominada *C. brasiliensis* e detectada primeiramente em *L. tigrinus*. Essa espécie se posicionou em um clado distinto, porém irmão ao clado de *C. felis*. A divergência encontrada entre *C. felis* e *C. brasiliensis* foi de 0,4% para o gene 18S rRNA e aproximadamente 6,8% para o gene *cytB*.

Casos fatais de citauxzoonose no país foram registrados em felídeos selvagens mantidos em cativeiro: uma leoa e seu filhote no Rio de Janeiro (Peixoto et al., 2007)



e em uma onça-pintada no Mato Grosso do Sul (Guizelini et al., 2021). No primeiro caso, o filhote de leão veio a óbito e, após 40 dias, a leoa começou a apresentar perda de peso, apatia, anemia, mucosas pálidas, perda de pelo, urina amarronzada, taquipneia, nistagmo, incoordenação motora, surdez, descargas nasais e oculares e acabou vindo a óbito também. Os exames laboratoriais mostraram anemia, neutrofilia, monocitose, linfopenia, eosinopenia, trombocitopenia e níveis elevados de uréia no sangue, além de piúria, hematúria e proteinúria na urinálise. À necropsia de ambos os animais foram encontrados esquizontes intra-histiocitários obstruindo vasos sanguíneos de diferentes órgãos, achado patognomônico associado à citauxzoonose (Peixoto et al., 2007). No segundo caso, um filhote de onça-pintada (5 meses) nascido em cativeiro, apresentou hiporexia e veio a óbito mesmo após administração de antibioticoterapia. À necropsia foram observados a presença de icterícia em tecido subcutâneo, mucosa oral e ocular, esplenomegalia e fígado com padrão lobular. Além disso, foi registrada a presença de macrófagos contendo esquizontes obstruindo vasos sanguíneos do cérebro, medula espinhal, leptomeninges, pulmões, coração, musculatura esquelética, adrenais, rins, baço, intestino delgado e pâncreas no exame histopatológico. O diagnóstico foi confirmado por PCR e sequenciamento (Guizelini et al., 2021). Casos fatais de citauxzoonose são raros em felinos selvagens. Há registros de óbito devido à infecção por *Cytauxzoon* spp. em tigres (*Panthera tigris*) mantidos em cativeiro na Alemanha (Jakob and Wesemeier, 1996) e nos EUA (Garner et al., 1996), e em um lince-pardo (*Lynx rufus*) de vida-livre nos EUA (Nietfield e Pollock, 2002).

Nos EUA, *L. rufus* são considerados os principais hospedeiros naturais e reservatórios de *C. felis*. A fase da esquizogonia nesses animais é curta e autolimitante (Glenn et al., 1983; 1987). No Brasil, suspeita-se que onças-pintadas exerçam essa função para as espécies circulantes no país, devido à alta positividade encontrada nesta espécie animal. Vários estudos mostraram uma ocorrência acima de 96,5% de *Cytauxzoon* spp. nesses animais (Furtado et al., 2017; Fagundes-Moreira et al., 2022; Alves et al., 2025). Além disso, há registros de animais que foram recapturados em diferentes períodos (com intervalo de 60 dias a até quatro anos entre as coletas) e continuaram positivos para o agente, demonstrando que tais animais

mantêm a infecção ou são constantemente reinfectados (Furtado et al., 2017; Fagundes-Moreira et al., 2022).

Ainda não há comprovação experimental de qual espécie de carrapato possa estar envolvida na transmissão de *Cytauxzoon* spp. no Brasil. Porém, Fagundes-Moreira et al. (2022) sugeriram *Amblyomma sculptum* como um possível vetor, uma vez que foi a espécie mais frequentemente encontrada nos felinos selvagens amostrados e positivos para *Cytauxzoon* spp. nos estados de Mato Grosso e Mato Grosso do Sul. Todas as amostras de sangue de felinos selvagens amostrados no estado do Rio Grande do Sul mostraram-se negativas para o referido agente, enquanto a positividade encontrada nos animais amostrados nos estados do centro-oeste do país foi de 98,11%. *Amblyomma sculptum* não ocorre no estado do Rio Grande do Sul, reforçando a hipótese que ele possa atuar no ciclo biológico do parasito nas demais localidades estudadas. Adicionalmente, DNA de *Cytauxzoon* sp. (gene 18S rRNA) foi detectado em ninfas de *A. sculptum* coletadas de javalis no estado de São Paulo. Os autores sugeriram, hipoteticamente, que larvas desta espécie de carrapato tenham se infectado ao realizarem hematofagismo em felinos domésticos ou selvagens e carregaram a infecção para o estágio biológico do desenvolvimento de ninfa (Santana et al., 2022).

No que diz respeito a outros gêneros de piroplasmídeos detectados em felinos selvagens no Brasil, André et al. (2011) detectaram DNA de *Babesia* sp. a partir da amplificação de um fragmento do gene 18S rRNA em um gato-dos-pampas mantido em cativeiro em zoológico no estado de São Paulo. A sequência obtida (790 pb) mostrou identidade de 97 a 99% com *B. leo* detectada em gatos e leões da África do Sul. Ademais, 31% (54/169) dos felinos selvagens amostrados foram soropositivos para *B. vogeli* pela Reação de Imunofluorescência Indireta, incluindo o espécime de *L. braccatus* que havia sido positivo na PCR.

### 3.6.2.3. Família Procyonidae

Escassos são os relatos de piroplasmídeos em procionídeos no Brasil. Até o momento, *Theileria* sp. foi detectada, morfológica e molecularmente, em três quatis (9,6% [3/31]) amostrados no Pantanal sul-matogrossense. As três sequências 18S rRNA obtidas (fragmentos variando de 450 a 564 pb) foram alocadas no mesmo clado

de *Theileria* sp. previamente detectada em gatos no Brasil, formando um clado irmão ao de *T. equi* (de Sousa et al., 2018). Em contrapartida, Estevam et al. (2020) detectaram *Babesia* sp. (380 pb) filogeneticamente associada a *B. bigemina* em três quatis (1,4% [3/209]) amostrados em um parque de Belo Horizonte, estado de Minas Gerais. Adicionalmente, dois quatis e dois mão-peladas (*Procyon cancrivorous*) amostrados em centros de reabilitação de Goiás e Minas Gerais mostraram-se positivos na PCR baseada no gene 18S rRNA de piroplasmídeos, porém nenhuma amostra foi sequenciada (Fava et al., 2022).

Perles et al. (2023) não encontrou quatis positivos para piroplasmídeos em animais amostrados em um parque e em uma área residencial na cidade de Campo Grande, Mato Grosso do Sul. Por outro lado, DNA de piroplasmídeos foi detectado em ectoparasitos coletados desses animais. Um *pool* de larvas de *Amblyomma* sp. e dois *pools* de ninfas de *Amblyomma dubitatum* foram positivos na nested PCR baseada no gene 18S rRNA de piroplasmídeos e as sequências foram posicionadas no clado “South American Marsupialia Group”. Adicionalmente, sequências detectadas em dois *pools* de ninfas de *A. sculptum* alocaram-se em um clado contendo sequências de *Babesia* spp. previamente detectadas em capivaras amostradas em Campo Grande (MS) e Pelotas (RS), e em espécimes de *A. dubitatum* coletados de *Rattus rattus* em Campo Grande. Em estudo realizado com ectoparasitos coletados de *N. nasua* no Parque Nacional do Iguaçu, Paraná, ninfas de *Amblyomma coelebs* mostraram-se positivas para *Theileria* sp. As duas sequências obtidas (fragmentos de 458 e 588 pb) foram alocadas juntas em um clado filogeneticamente relacionado ao clado de *T. cervi* (Araújo et al., 2023).

### 3.6.3. Ordem Chiroptera

#### 3.6.3.1. Família Phyllostomidae

No Brasil, dois estudos relataram taxas de positividade molecular para piroplasmídeos de 18,77% (43/229) e 12,6% (17/135) em morcegos não-hematófagos e hematófagos, respectivamente (Ikeda et al., 2021; de Mello et al., 2023a).

Espécimes de quatro espécies de morcegos não-hematófagos amostrados na região periurbana de Campo Grande, Mato Grosso do Sul, mostraram-se positivas na nPCR baseada no gene 18S rRNA de piroplasmídeos: *Artibeus planirostris*, *Artibeus*

*lituratus*, *Phyllostomus discolor* e *Platyrrhinus lineatus*. Sequências com mais de 1450 pb do referido gene foram obtidas a partir de amostras de DNA de sangue de *P. discolor* e foram posicionadas na filogenia em um novo clado, indicando tratar-se de uma possível nova espécie. O clado foi nomeado como “Phyllostomidae bat group”. Em contrapartida, só foi possível obter sequências curtas (de até 740 pb) de piroplasmídeos a partir de amostras biológicas de *A. plasnirostris* e *A. lituratus*, as quais foram alocadas no clado de “*Babesia* sensu stricto”, juntamente com *B. vogeli* (Ikeda et al., 2021).

Taxas de positividade para piroplasmídeos de 18,4% (42/228) e 100% (1/1) foram relatadas em espécimes de *Desmodus rotundus* e *Diaemus youngi* amostrados em diferentes estados da região norte do país. Sequências curtas (de 474 a 828 pb) e longas (> 1400 pb) do gene 18S rRNA foram obtidas para as amostras amplificadas a partir do DNA extraído de baço de *D. rotundus*. Na análise filogenética, as sequências amplificadas foram distribuídas em três diferentes clados: “South American Marsupialia Group”, “*Tapirus terrestris* group” e “*Theileria* sensu stricto”, evidenciando uma alta diversidade de piroplasmídeos nesse grupo de animais (de Mello et al., 2023a). Adicionalmente, estudo realizado por de Mello et al. (2023b) com amostras de DNA extraídas a partir de fragmentos de fígado de *D. rotundus* e *Diphylla ecaudata*, amostrados em diversos estados do Brasil, mostraram-se negativas para os agentes em questão, resultado provavelmente influenciado pelo tipo de amostra utilizada.

### **3.6.4. Ordem Didelphimorphia**

#### **3.6.4.1. Família Didelphidae**

O primeiro registro de piroplasmídeos em marsupiais no Brasil foi realizado em 1961 a partir de esfregaços sanguíneos de *Didelphis marsupialis* (gambá-comum) amostrados no estado do Pará (Denae and Deane, 1961). Posteriormente, Serra-Freire (1979) encontrou formas evolutivas de *Babesia brasiliensis*, primeiramente descrita como *Babesia ernestoi*, em esfregaços sanguíneos de *D. marsupialis* e *Didelphis albiventris* (gambá-de-orelha-branca) amostrados no Rio de Janeiro.

A detecção por meio de PCR já foi descrita em espécimes de *Monodelphis domestica* (cuíca-de-rabo-curto), *D. albiventris*, *D. marsupialis* e *Didelphis aurita* (gambá-da-orelha-preta) amostrados nos estados do Distrito Federal, Goiás,

Maranhão, Mato Grosso, Mato Grosso do Sul, Minas Gerais, Pará e Rio de Janeiro. A positividade obtida variou de 2,22 a 50% (sendo esta última positividade baseada em apenas dois animais amostrados) (Wolf et al., 2016; Soares et al., 2017; Colle et al., 2019; Fava et al., 2021; Gonçalves et al., 2021; Braga et al., 2023; de Oliveira et al., 2023).

Um espécime de *D. aurita*, amostrado por de Oliveira et al. (2023) e positivo na PCR para piroplasmídeos, mostrou sinais clínicos como letargia, perda de peso, icterícia, mucosas pálidas e anemia confirmada por exames laboratoriais, sinais clínicos e hematológicos previamente descritos em *D. marsupialis* com alta parasitemia por *B.ernestoi* (Serra-Freire, 1979).

A partir da análise de sequências quase completas do gene 18S rRNA e sequências parciais de outros marcadores genéticos (*cox-1* e *cox-3*) amplificadas a partir de amostras de sangue de *D. albiventris* oriundos do Distrito Federal e Mato Grosso do Sul, e de *D. aurita* amostrados no Rio de Janeiro, um novo clado filogenético foi descrito e denominado “South American Marsupialia group”. A princípio, nenhuma espécie foi nomeada, mas acredita-se que seja pertencente ao gênero *Babesia* (Gonçalves et al., 2021; de Oliveira et al., 2023).

Em estudo realizado por Braga et al. (2023) foi obtida apenas uma sequência curta do gene 18S rRNA amplificada a partir de amostra de sangue de espécime de *D. marsupialis* (2,2% 1/45) capturado no estado do Maranhão. A sequência em questão foi posicionada no clado “South American Marsupialia group”, levantando evidências da existência de uma nova espécie de *Babesia* spp. em animais do gênero *Didelphis*.

### **3.6.5. Ordem Perissodactyla**

#### **3.6.5.1. Família Tapiridae**

Uma anta resgatada em Campo Grande (MS) apresentava fratura no membro pélvico esquerdo, apatia, hipertermia e anorexia. Exames laboratoriais indicaram anemia normocítica normocrômica, leucocitose, neutrofilia, linfocitose, anisocitose e inclusões em eritrócitos sugestivas de piroplasmídeos no esfregaço sanguíneo. Parasitismo por piroplasmídeos foi confirmada por PCR. A sequência obtida (414 pb) mostrou 98% de identidade com *T. equi* em análise pelo BLASTn. Como o animal

possuía uma fratura e ostemioelite, não foi possível concluir que os achados clínicos e laboratoriais estavam relacionados à infecção pelo agente (da Silveira et al., 2017).

Posteriormente, antas mantidas em cativeiro e amostradas nos estados do Amazonas e Pará, mostraram-se positivas (57,9% [11/19]) na PCR para piroplasmídeos baseada no gene 18S rRNA a partir da amplificação e sequenciamento de fragmentos de 392 a 475 pb (Gonçalves et al., 2020). Silva et al. (2021) relataram positividade de 35,3% (6/17) para piroplasmídeos em amostras de baço e sangue de *T. terrestris* atropeladas no Mato Grosso. Duas sequências de 776 pb alocaram-se no mesmo clado que sequências de *Theileria* sp. previamente detectadas em gatos no Brasil, clado este filogeneticamente próximo ao clado de *T. equi*. Adicionalmente, Fava et al. (2021) detectaram DNA de piroplasmídeos em uma anta (1/2 [50%]). A sequência de 18S rRNA (553 pb) obtida alocou-se em um clado separado, porém filogeneticamente relacionado a *T. equi*.

Em estudo realizado com amostras de sangue de antas de vida-livre amostradas no estado do Mato Grosso do Sul, 56,7% (56/99) foram positivas para piroplasmídeos. Análises filogenéticas baseadas em sequências quase completas do gene 18S rRNA e sequências dos genes *cox-1* e *hsp70*, associadas à presença de merozoítos dispostos em “Cruz-de-Malta” em eritrócitos, embasaram a descrição de uma nova espécie, denominada de *Theileria terrestris*. Na filogenia, as sequências obtidas foram posicionadas em um clado novo (“*Tapirus terrestris* group”) filogeneticamente relacionado com o clado “Equus group” (Mongruel et al., 2023).

### 3.6.6. Ordem Primates

Não há estudos específicos voltados para a detecção de piroplasmídeos em primatas não-humanos no Brasil. Apenas dois trabalhos que objetivaram detectar hemoparasitos em diferentes grupos de animais cativos amostrados em Goiás e Minas Gerais detectaram a ocorrência de piroplasmídeos nesses animais, porém nenhuma sequência foi obtida (Fava et al., 2021; Castillo et al., 2024).

No estudo realizado por Fava et al. (2021) em animais mantidos em centros de reabilitação nos estados de Goiás e Minas Gerais, dos 28 primatas não-humanos das espécies *Alouatta caraya* (bugiu-preto) e *Callithrix penicillata* (sagui-de-tufos-pretos) amostrados, cinco (17,5%) animais foram positivos na PCR para piroplasmídeos e em

13 (46,4%) foram observadas inclusões intracitoplasmáticas sugestivas dos referidos agentes em esfregaço sanguíneo. Já Castillo et al. (2024) relataram 22,2% (4/18) de positividade para piroplasmídeos em primatas não-humanos amostrados no estado de Minas Gerais. Os animais positivos eram representantes de *Ateles* sp. (macaco-aranha), *Sapajus apella* (macaco-prego) e *Gorilla gorila* (gorila).

### 3.6.7. Ordem Rodentia

#### 3.6.7.1. Família Caviidae

Criado-Fornelio et al. (2009) registraram ocorrência molecular de 14,3% (2/14) para piroplasmídeos em capivaras amostradas em Pelotas, no Rio Grande do Sul. A sequência de 1714 pb do gene 18S rRNA obtida foi posicionada em um clado irmão ao clado de “*Babesia sensu stricto*” e foi denominada *Babesia* sp. capybara. Posteriormente, positividade de 39,3% (11/28) para o referido agente foi relatada em um estudo realizado no estado de Goiás. No referido trabalho, foram amplificadas sequências longas do gene 18S rRNA (1519 - 1561 pb) que se alocaram em um clado único, juntamente com a sequência citada previamente. Além disso, foram amplificadas sequências dos genes *hsp70* e *cox-3*, as quais também foram posicionadas em um clado à parte dos demais nas filogenias. Devido a isso, uma nova espécie foi descrita (*Babesia goaniaensis*) e mais um clado (“Capybara group”) foi adicionado à filogenia de piroplasmídeos (Krawczak et al., 2023; Neves et al., 2023).

Adicionalmente, fragmentos curtos do gene 18S rRNA foram amplificados a partir de amostras de DNA extraídas de ninfas e adultos de *A. dubitatum* coletados de capivaras (Gonçalves et al., 2021; Neves et al., 2023), *Rattus rattus* (Gonçalves et al., 2021) e quatis (Perles et al., 2023) amostrados em Mato Grosso do Sul e Goiás, bem como em espécimes de *A. sculptum* coletados de capivaras (Neves et al., 2023; Krawczak et al., 2023) e quatis (Perles et al., 2023) nos mesmos estados. Tais sequências foram filogeneticamente associadas a *Babesia* sp. detectada em capivaras, sugerindo que *A. dubitatum* e *A. sculptum* possam atuar como vetores desse agente. Entretanto, experimentos de competência vetorial devem ser realizados para tal confirmação (Neves et al., 2023; Krawczak et al., 2023).

### 3.6.7.2. Família Cuniculidae

Quatro pacas (12,1% [4/33]) amostradas no estado de Mato Grosso foram positivas para piroplasmídeos com base em PCR baseada no gene 18S rRNA. A sequência de 537 pb foi posicionada em um clado filogeneticamente associado com *Babesia* sp. capybara (Soares et al., 2017).

### 3.6.7.3. Família Dasyproctidae

Uma única cutia amostrada no estado de Mato Grosso mostrou-se positiva na PCR para piroplasmídeos. A sequência obtida (314 pb) do gene 18S rRNA foi alocada em um clado próximo a sequências de *Theileria* sp. detectadas em tatu-galinha amostrados no mesmo estudo. Este clado foi posicionado próximo a *Theileria* sensu stricto (Soares et al., al 2017).

### 3.6.7.4. Família Echimyidae

O primeiro estudo que detectou molecularmente piroplasmídeos em roedores selvagens no Brasil foi realizado por Wolf et al. (2016) em animais amostrados no Pantanal de Poconé, estado de Mato Grosso. De 11 *Trichomys pachyurus* capturados, três (27,3%) foram positivos para os agentes sob estudo. A sequência de 532 pb obtida na amplificação do gene 18S rRNA foi posicionada em um clado contendo uma sequência de *Babesia* sp. detectada em *M. domestica* obtida no mesmo estudo.

Posteriormente, de Sousa et al. (2018) detectaram DNA de piroplasmídeos em seis *Trichomys fosteri* (7,8% [6/110]) no Pantanal do estado do Mato Grosso do Sul. Cinco das sequências obtidas (aproximadamente 700 pb) foram posicionadas em um clado juntamente com *B. vogeli*. Em contrapartida, uma sequência alocou-se juntamente com sequências de *T. equi*.

Um estudo recente conduzido no estado do Mato Grosso relatou positividade de 11,7% (17/145) para piroplasmídeos nos roedores capturados. Todas as amostras positivas eram oriundas de *T. pachyurus*. Foram obtidas sequências longas do gene 18S rRNA (1200 a 1230 pb) e sequências entre 700 e 800 pb do gene *cytB*. Em ambas as filogenias as sequências se alocaram em um clado único. Na filogenia do gene 18S rRNA, as sequências se posicionaram em um clado irmão àquele "Phyllostomidae bat



goup”. O novo clado foi denominado “South American Rodentia group” (Pacheco et al., 2025).

Adicionalmente, outros trabalhos relataram a presença de formas evolutivas de piroplasmídeos em esfregaços sanguíneos de roedores da família Cricetidae (*Oligoryzomys nigripes*, *Akodon montanensis*, *Delomys sublineatus* e *Nectomys squamipes*) e Muridae (*Rattus norvegicus*) amostrados no Rio de Janeiro (Gazeta et al., 2004; Silva et al., 2007).

### 3.6.8. Superordem Xenarthra (Ordens Cingulata e Pilosa)

A Superordem Xenarthra compreende duas ordens: Cingulata (composta pelos tatus) e Pilosa (composta por tamanduás e preguiças). No Brasil, a primeira descrição de piroplasmídeos nesses animais foi realizada por Laison et al. (1979), que encontraram formas evolutivas de *Theileria* sp. em esfregaços sanguíneos de tatu-galinha. Posteriormente, Soares et al. (2017) detectaram um novo genótipo do gene 18S rRNA (473 pb) em tatus-galinha (9,37% [3/32]) amostrados no estado do Mato Grosso (Soares et al., 2017). Esse novo genótipo alocou-se em um clado separado dos demais, próximo a *Theileria* sp. detectada em cutia amostrada no mesmo trabalho (Soares et al., 2017).

Adicionalmente, positividade de 12,5% (4/32) para piroplasmídeos foi obtida por meio de PCR para o gene 18S rRNA em amostras biológicas de tamanduás-bandeira (*Myrmecophaga tridactyla*) e tamanduás-mirim (*Tamandua tetradactyla*) coletadas de animais em centros de reabilitação de Goiás e Minas Gerais. Infelizmente, nenhuma sequência foi obtida (Fava et al., 2021).

### 3.6.9. Aves

Poucos estudos foram conduzidos no que diz respeito à ocorrência de piroplasmídeos em aves selvagens no Brasil. Neste sentido, Quillfeldt et al. (2014) relataram a presença de DNA de *Babesia* spp. filogeneticamente relacionada a *B. poelea* (clado “Percei group”- gene 18S rRNA) em atobá-pardo (*Sula leucogaster*) (7% [12/172]) e atobá-grande (*S. dactylatra*) (13,1% [22/168]) amostrados em ilhas do litoral brasileiro (Arquipélago de Abrolhos, Fernando de Noronha, Atol das Rocas e Arquipélago de São Pedro e São Paulo).

Seis (9,68% [6/62]) gansos-do-Orinoco (*Neochen jubata*) amostrados no estado de Goiás mostraram-se positivos para piroplasmídeos. Uma das amostras foi sequenciada (fragmento de 693 pb do gene 18S rRNA) e alocou-se no clado de *B. vogeli* (Werther et al., 2017).

Por fim, em estudo realizado em uma praia de Florianópolis, Santa Catarina, de 144 aves marinhas amostradas, apenas um albatroz-de-bico-amarelo-do-Atlântico (*Thalassarche chlororhynchos*) mostrou-se positivo para piroplasmídeos (0,69%). A sequência obtida (672 pb) do gene 18S rRNA foi alocada no clado de *Babesia* sensu stricto, juntamente com sequências de *Babesia kiwiensis* previamente detectadas em *Apteryx australis mantellii* (Kiwi-marrom-da-ilha-Norte) e de *Babesia* sp. detectadas em *Turdus falcklandii* (tordo-austral) e *Morus serrator* (atobá-australiano) (Sgarioni et al., 2023).

A **Tabela Suplementar 1** mostra um resumo de todos os estudos acerca da ocorrência e identidade molecular (quando presente) de piroplasmídeos em animais selvagens realizados no país.

### 3.7. Piroplasmídeos que acometem humanos

Babesiose vem sendo considerada uma zoonose emergente e humanos são considerados hospedeiros acidentais para tais agentes (Kumar et al., 2021; Karshima et al., 2022). O primeiro caso de babesiose humana foi descrito na antiga Iugoslávia em 1957 (Skrabalo and Deanovis, 1957). As espécies que acometem humanos e foram identificadas até o momento são: *B. microti* (incluindo *B. microti*-like), *B. duncani*, *B. divergens* (incluindo *B. divergens*-like MO1), *B. venatorum*, *B. motasi*-like, *Babesia crassa* (incluindo *B. crassa*-like), *B. bigemina* e *B. odocoilei* (Herwaldt et al., 2004; Vannier et al., 2012; Yasbley et al., 2013; Arsuaga et al., 2016; Chen et al., 2019; Gray et al., 2019; Hong et al., 2019; Kumar et al., 2021; Scott et al., 2021; Doderer-Lang et al., 2022; Karshima et al., 2022; Calvopina et al., 2023; Maggi et al., 2024).

Casos já foram relatados na América do Norte, América do Sul, Europa, Ásia e Austrália (Krause, 2019; Kumar et al., 2022), sendo que 95% deles ocorreram nos EUA e Canadá (Yang et al., 2021). No Brasil, foram descritos, a partir de esfregaços sanguíneos, casos de babesiose humana sintomática (febre, inapetência e dores no

corpo) em Pernambuco, cuja espécie envolvida não foi identificada, e assintomático no Rio Grande do Sul (para o qual levantou-se a hipótese de que a provável espécie envolvida era *B. microti*) (Alecrim et al., 1983; Rech et al., 2004). Adicionalmente, anticorpos contra *B. bovis* foram detectados em pacientes que apresentavam Síndrome de Baggio-Yoshinari (*Lyme-like disease*) no estado de São Paulo, embora reações sorológicas cruzadas não puderam ser descartadas (Yoshinari et al., 2003).

A doença em humanos é considerada moderada, podendo ser assintomática ou com sintomas como fadiga, letargia, anorexia, febre intermitente, sudorese, náuseas, mialgia, dores abdominais, distúrbios do sono, hematúria e hepatoesplenomegalia. Pacientes imunossuprimidos (esplenectomizados, ou que possuam outras doenças associadas como câncer, HIV/AIDS, doenças cardíacas, pulmonares, renais ou hepáticas, ou que estão sendo submetidos a tratamentos com drogas imunossupressoras) podem apresentar sinais mais severos, como anemia hemolítica e falência de órgãos, podendo levar a óbito (Setty et al., 2003; Krause et al., 2008; Martinot et al., 2011; Vannier et al., 2012; Yasbley et al., 2013; Akel and Mobarakai, 2017; Kukina et al., 2018; Locke et al., 2023). Além disso, a babesiose humana vem sendo relacionada com complicações neurológicas, como dores de cabeça, síncope, confusão mental, delírio, alterações do nível de consciência e neuropatias (Usmani-Brown et al., 2013; Bradshaw et al., 2021; Venigalla et al., 2022; Locke et al., 2023).

Os principais vetores associados com a transmissão de *Babesia* spp. para humanos são *Ixodes scapularis* nos EUA e Canadá, *Ixodes ricinus* na Europa, e *Ixodes ovatus* no Japão (Yabsley et al., 2013; Krause et al., 2019). Além da transmissão por picada de carrapatos, transfusão sanguínea, transmissão transplacentária e por transplante já foram relatadas em menores proporções (Herwaldt et al., 2011; Yabsley et al., 2013; Brennan et al., 2016; Krause et al., 2019).

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## **CAPÍTULO 2\* - Molecular detection of piroplasmids in mammals from the Superorder Xenarthra in Brazil\***

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### **Abstract**

Xenarthra mammals can be found from southern North America to southern South America, including all Brazilian biomes. Although it's been shown that Xenarthra mammals can play a role as reservoirs for several zoonotic agents, few studies investigate the diversity of piroplasmids (Apicomplexa: Piroplasmida) in this group of mammals. Taking into account that piroplasmids can cause disease in animals and

humans, understanding the prevalence and diversity of piroplasmids in Xenarthra mammals would contribute to conservation efforts for this group of animals as well as to infer risk areas for transmission of emergent zoonosis. The present study aimed to investigate the occurrence and molecular identity of piroplasmids in free-living mammals of the Superorder Xenarthra from four Brazilian states (Mato Grosso do Sul, São Paulo, Rondônia and Pará). For this, DNA was extracted from blood or spleen samples from 455 animals. A nested PCR based on the 18S rRNA gene was used as screening for piroplasmids. Of the 455 samples analyzed, 25 (5.5%) were positive. Additionally, PCR assays based on 18S rRNA near-complete, *cox-1*, *cox-3*, *hsp70*, *cytB*, *β-tubulin* genes and the ITS-1 intergenic region were performed. Five out of 25 positive samples also tested positive for ITS-1-based PCR. The phylogenetic analysis positioned three 18S rRNA sequences detected in *Priodontes maximus* into the same clade of *Babesia* sp. detected in marsupials (*Didelphis albiventris*, *Didelphis marsupialis* and *Monodelphis domestica*) and *Amblyomma dubitatum* collected from opossums and coatis in Brazil. On the other hand, the 18S rRNA sequence obtained from *Dasypus novemcinctus* was closely related to a *Theileria* sp. sequence previously detected in armadillos from Mato Grosso State, grouping in a subclade within the *Theileria sensu stricto* clade. In the phylogenetic analysis based on the ITS-1 region, the sequences obtained from *Myrmecophaga tridactyla* and *Tamandua tetradactyla* were placed into a single clade, apart from the other piroplasmid clades. The present study demonstrated the molecular occurrence of Piroplasmida in anteaters and *Babesia* sp. and *Theileria* sp. in armadillos from Brazil.

**Keywords:** piroplasmids, armadillos, anteaters, sloths

## Introduction

Diseases transmitted by arthropod vectors represent 22.8% of the current emerging diseases (Zanella, 2016). Climate, environmental and ecological changes favor the disruption of cycles of naturally established microorganisms and the consequent emergence of diseases, as well as the expansion of the distribution of vectors and greater proximity of wildlife with domestic animals and humans. Therefore, there is a greater interaction between reservoirs, vectors and susceptible hosts, leading to serious problems for public and animal health (Shaw et al., 2001; André, 2018).

The Superorder Xenarthra comprises the orders Cingulata and Pilosa. The first includes different species of armadillos, which are divided into the families Dasipodidae and Chlammyphoridae. The second order is composed of four families: Bradypodidae and Megalonychidae, which include sloths, and Cyclopedidae and Myrmecophagidae, which include the silk anteaters and anteaters (Gardner, 2005). Currently, 38 species are registered, of which six species of sloths, seven species of silk anteater, three

species of anteater and 22 species of armadillo (ASASG, 2023). They are distributed from southern North America to southern South America (Gardner, 2005). In Brazil, these mammals are distributed throughout all of the country biomes. According to the IUCN (International Union for Conservation of Nature) red list of threatened species, the pygmy three-toed sloth (*Bradypus pygmaeus*) is classified as critically endangered. Maned three-toed sloth (*Bradypus torquatus*), giant anteater (*Myrmecophaga tridactyla*), giant armadillo (*Priodontes maximus*) and Brazilian three-banded armadillo (*Tolypeutes tricinctus*) are species classified as vulnerable to extinction (IUCN, 2023). Regarding the occurrence of vector-borne agents in this group of mammals, *Anaplasma marginale* (Guillemi et al., 2016), a new genotype of *Ehrlichia* (Soares et al., 2017), *Ehrlichia canis*, *Ehrlichia minasensis*, two putative novel *Anaplasma* ('*Candidatus Anaplasma brasiliensis*' and '*Candidatus Anaplasma amazonensis*') (Calchi et al, 2020a), a putative novel *Bartonella* ('*Candidatus Bartonella washoensis* subsp. brasiliensis') (Calchi et al., 2020b) and two new putative hemotropic *Mycoplasma* ('*Candidatus Mycoplasma haematotetradactyla*' and '*Candidatus Mycoplasma haematomaximus*') (de Oliveira et al., 2022) have been recently detected in Xenarthra mammals from South America.

Piroplasmids are tick-borne apicomplexan protozoa belonging to the order Piroplasmida, which is composed of the families Babesiidae (genera *Babesia* and *Rangelia*) and Theileriidae (genera *Theileria* and *Cytauxzoon*) (Solano-Gallego and Baneth, 2011; Shock et al., 2012; França et al., 2014). These agents parasitize blood cells of vertebrate animals, such as erythrocytes, lymphocytes, monocytes, or endothelial cells, depending on the involved agent (Alvarado-Rybak et al., 2016). These protozoa can cause illnesses in domestic and wild animals (Hunfeld et al. 2008; Alavarado-Rybak et al., 2016) as well as humans (Homer et al., 2000; Kjemtrup and Conrad, 2000; Zintl et al., 2003; Hunfeld et al., 2008; Leiby, 2011).

Despite the worldwide occurrence of piroplasmids in wild animals, few are the reports about the occurrence and molecular identity of piroplasmids in mammals of the Superorder Xenarthra. In the past, two Piroplasmida species have been described, namely *Theileria brimont*, which was detected in pale-throated sloth (*Bradypus tridactylus*) from French Guiana (Leger and Mouzels, 1917), and *Babesia choloepi*, detected in Linnaeus's two-toed sloth (*Choloepus didactylus*), in the same country

(Dedet et al., 1988). In addition, *Theileria* sp. has been morphologically detected in nine-banded armadillo (*Dasypus novemcinctus*) from Pará state, Brazil (Laisson et al., 1979). *Babesia* sp. has been detected in *Bradypus didactylus*, *Dasypus novemcinctus*, greater long-nosed armadillos (*Dasypus kappleri*) and silky anteaters (*Cyclops didactylus*) from French Guiana (de Thoisy et al., 2000). All four descriptions were based only on the finding of intraerythrocytic parasites in blood smears. More recently, Soares et al. (2017) detected a new genotype of *Theileria* sp. in *D. novemcinctus* in the states of Pará and Mato Grosso, Brazil. In the phylogeny based on the partial 18S rRNA gene, the armadillo-associated sequences were grouped into a new clade along with a genotype detected in agouti (*Dasyprocta* sp.), closely related to the *Theileria sensu stricto* clade. Finally, piroplasmids were detected by blood smears and PCR assays based on the 18S rRNA gene in giant anteater (*Myrmecophaga tridactyla*) and southern tamandua (*Tamandua tetradactyla*) kept in rehabilitation centers in the states of Goiás and Minas Gerais, Brazil. However, no amplified product was sequenced (Fava et al., 2022).

Recently, the description of putative novel piroplasmids in Brazil (Ikeda et al., 2021; Gonçalves et al., 2021; Mongrue et al., 2022; Oliveira et al., 2023) has contributed to the expansion of the Piroplasmida clades previously proposed by Jalovecka et al. (2019), emphasizing the need for additional studies aiming at untangling the complex diversity of this group of apicomplexan protozoa. The present study aimed to investigate the prevalence and diversity of piroplasmids in Xenarthra mammals sampled in four different Brazilian states.

## **Material and methods**

### **Studied area and sampled mammal species**

Between 2011 and 2022, a total of 455 biological samples (blood or spleen) were collected from: 230 sloths (191 brown-throated three-toed sloths [*Bradypus variegatus*], 3 *Bradypus* sp., 5 Linnaeus's two-toed sloths [*C. didactylus*], 31 *Choloepus* sp.), 168 anteaters (37 southern tamandua [*T. tetradactyla*], 131 giant anteaters [*M. tridactyla*]) and 57 armadillos (4 southern naked-tailed armadillos [*Cabassous unicinctus unicinctus*], 10 nine-banded armadillos [*D. novemcinctus*], 11



six-banded armadillos [*Euphractus sexcinctus*], 32 giant armadillos [*Priodontes maximus*]]. The samples were collected in four Brazilian states: São Paulo (SP), Mato Grosso do Sul (MS), Pará (PA) and Rondônia (RO) and covered three different biomes, namely Cerrado (SP and MS), Pantanal (MS), and Amazon (PA and RO) (**Figure 1**). A small fragment of spleen was cut during necropsy of the animals and stored in DNase/RNase-free microtubes. Blood from live animals was collected in EDTA tubes. The collected samples were stored in a freezer at -20°C until use.

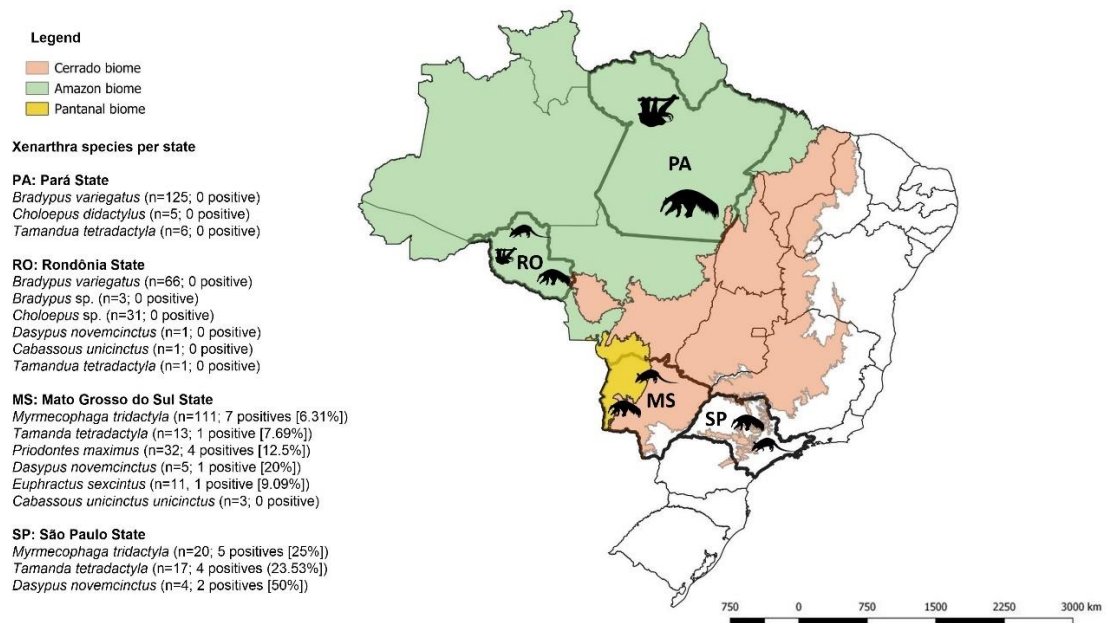


**Figure 1.** Map of Brazil with the number of Xenarthra species sampled in the present study per state and the number and percentage of positive animals. The figures within the states represent which animals were collected in each state.

The biological samples from Mato Grosso do Sul state were represented by spleen samples (n=81) collected during necropsies of road-killed animals and blood samples (n=93) collected from free-living animals captured on site. All the samples were collected by the Anteaters & Highways Project and by the Giant Armadillo Conservation Program. In São Paulo state, only spleen samples (n=39) were collected from animals necropsied by the Wildlife Pathology Service of the School of Agricultural and Veterinarian Sciences. Finally, the blood samples obtained from the states of Pará (n=137) and Rondônia (n=105) are part of the DNA/Tissues collection (TCC-USP) of the Laboratory of Phylogeny and Molecular Taxonomy of Trypanosomatids, Department of Parasitology, Institute of Biomedical Sciences (ICB).

## DNA extraction and quality assessment

DNA was extracted from 10 mg of each spleen tissue and 200  $\mu$ L of each blood sample using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. In order to verify the presence of amplifiable DNA and to discard the presence of PCR inhibitors, DNA samples were subjected to a conventional PCR (cPCR) assay targeting the mammalian endogenous glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) gene (Birkenheuer et al., 2003). The positive samples were subjected to specific PCR assays for piroplasmids.

## Molecular detection and characterization of piroplasmids

Firstly, DNA samples were screened for piroplasmids using a nested PCR (nPCR) assay targeting a fragment (~800 bp) of the 18S rRNA gene (Jefferies et al. 2007). Positive samples were subjected to further molecular characterization using conventional PCR assays targeting different molecular markers: near-complete 18S rRNA gene (~1,500 bp) (Kawabuchi et al., 2005; Merino et al., 2006; Quillfeldt et al., 2014; Yabsley et al., 2017; Greay et al. 2018), *cox-1* (~800 bp; Corduneanu et al. 2017), *cox-3* (~600 bp; Schreeg et al., 2016; Barbosa et al. 2019), *hsp70* (~700 bp; Soares et al. 2011), *cytB* (~1 kb; Barbosa et al. 2019),  *$\beta$ -tubulin* (~1,214 bp; Zamoto et al., 2004) and ITS-1 (~450 bp; Shock et al., 2011). All primer sequences and thermal cycles used in the PCR assays are described in **Table 1**.

**Table 1.** Description of primers, amplicon sizes and thermal sequences used in conventional and nested PCR assays

|                    | Gene/ name<br>primer | Primers sequences             | Size (bp) | Thermal sequences                       | Reference                    |
|--------------------|----------------------|-------------------------------|-----------|-----------------------------------------|------------------------------|
| Endogenous<br>gene | <i>gapdh</i>         | 5'TCCAACACCACCACTGAGATCGGAC - | 400       | 95°C for 5 min;                         | Birkenheuer<br>et al. (2003) |
|                    | -GAPDHF              | 3'                            |           | 35 cycles: 95°C for 15s, 50°C for       |                              |
|                    | -GAPDHR              | 5'- GTGAGAAGAAATCGGACTGGCC3'  |           | 30s and 72°C for 30s;<br>72°C for 5 min |                              |

|                                        |                  |                                |            |                                                               |           |                                                                                                                 |                                               |
|----------------------------------------|------------------|--------------------------------|------------|---------------------------------------------------------------|-----------|-----------------------------------------------------------------------------------------------------------------|-----------------------------------------------|
| <b>Piroplasmids (screening)</b>        | 18S rRNA         |                                |            | 94°C for 3 min, 58°C for 1 min, 72°C for 2 min;               | 800       | 45 cycles: 94°C for 30s, 58°C for 20s and 72°C for 30s; 72°C for 7 min<br>Annealing temperature 2nd round =62°C | Jefferies et al.(2007)                        |
|                                        | -BTF1            | 5'GGCTCATTACAACAGTTATAG-3'     |            |                                                               |           |                                                                                                                 |                                               |
|                                        | -BTR1            | 5'CCCAAAGACTTTGATTCTCTC-3'     |            |                                                               |           |                                                                                                                 |                                               |
|                                        | -BTF2            | 5'CCGTGCTAATTGTAGGGCTAATAC-3'  |            |                                                               |           |                                                                                                                 |                                               |
|                                        | -BTR2            | 5'GGACTACGACGGTATCTGATCG-3'    |            |                                                               |           |                                                                                                                 |                                               |
| <b>Piroplasmids (characterization)</b> |                  | 5'-GCCAGTAGTCATATGCTTGTGTTA-3' |            |                                                               |           |                                                                                                                 |                                               |
|                                        | 18S rRNA         | 5'-                            |            |                                                               |           |                                                                                                                 |                                               |
|                                        | 1st reaction     | CTCCTTCCTYTAAGTGATAAGGTTAC-    |            | 95°C for 3 min;                                               |           |                                                                                                                 |                                               |
|                                        | -Piro 0F         | 3'                             |            | 33 cycles: 95°C for 1min, 59°C for 1 min and 72°C for 2 min;  | 1500      |                                                                                                                 | Kawabuchi et al., 2005                        |
|                                        | -Piro 6R         | 5'-                            |            |                                                               |           |                                                                                                                 |                                               |
|                                        | 2nd reaction     | CCATGCATGTCTWAGTAYAARCTTTTA    |            | 72°C for 10 min                                               |           |                                                                                                                 |                                               |
|                                        | -Piro 1F         | -3'                            |            |                                                               |           |                                                                                                                 |                                               |
|                                        | -Piro 5.5R       | 5'-                            |            |                                                               |           |                                                                                                                 |                                               |
| <b>Piroplasmids (characterization)</b> | 18S rRNA         | 5'-GGATAACCGTGCTAATTGT-3'      |            | 95°C for 10 min;                                              |           |                                                                                                                 | Merino et al. (2006); Quillfeld et al. (2014) |
|                                        | -NBAB1Bab        | 5'-AAAGGGCAGGGACGTAATC-3'      | 1484       | 40 cycles: 95°C for 30s, 55°C for 30s and 72°C for 90s;       |           |                                                                                                                 |                                               |
|                                        | -Hep1615R        |                                |            | 72°C for 10 min                                               |           |                                                                                                                 |                                               |
|                                        |                  |                                |            |                                                               |           |                                                                                                                 |                                               |
| <b>Piroplasmids (characterization)</b> | 18S rRNA         | 5'-CCTGGTTGATCCTGCCAGTAGT-3'   |            | 94°C for 1 min;                                               |           |                                                                                                                 | Yabsley et al. (2017)                         |
|                                        | 1st reaction     | 5'-                            |            | 30 cycles: 94°C for 1 min, 48°C for 1 min and 72°C for 2 min; | ~1700     |                                                                                                                 |                                               |
|                                        | -5.1             | CCC GGATCCAAGCTTGATCCTTCTGC    | (2nd       |                                                               |           |                                                                                                                 |                                               |
|                                        | -B               | AGGTTCACCTAC-3'                | reaction – | 72°C for 5 min                                                |           |                                                                                                                 |                                               |
|                                        | 2nd reaction – 1 | 5'-CATATGCTTGCTTAAA-3'         | 1)         |                                                               |           |                                                                                                                 |                                               |
|                                        | -5.1 V2          | 5'-CTCCTTCCTTTAAGTGATAAG-3'    |            | Annealing temperature 2nd round - 1=52°C                      | ~500 (2nd |                                                                                                                 |                                               |
|                                        | -3.1             | 5'-                            |            |                                                               |           |                                                                                                                 |                                               |
|                                        | 2n reaction -2   | GAGGTAGTGACAAGAAATAACAATA-     | reaction – | Annealing temperature 2nd round - 2=50°C                      |           |                                                                                                                 |                                               |
|                                        | -RLBF            | 3'                             | 2)         |                                                               |           |                                                                                                                 |                                               |
|                                        | -RLBR            | 5'-TCTTCGATCCCCTAACTTTC-3'     |            |                                                               |           |                                                                                                                 |                                               |
| <b>Piroplasmids (characterization)</b> | 18S rRNA         | 5'-                            |            | 95°C for 5 min;                                               |           |                                                                                                                 | Greay et al. (2018)                           |
|                                        | -Nbab_1F         | AAGCCATGCATGTCTAAGTATAAGCTT    | 1500       | 50 cycles: 95°C for 45s, 60°C for 45s and 72°C for 2min;      |           |                                                                                                                 |                                               |
|                                        | -18SApiR         | TT-3'                          |            | 72°C for 5 min                                                |           |                                                                                                                 |                                               |
| <b>Piroplasmids (characterization)</b> | <i>cox-1</i>     | 5'ATWGGATTYTATATGAGTAT3'       |            | 95°C for 1 min;                                               |           |                                                                                                                 | Corduneanu et al. (2017)                      |
|                                        | -Bab_for1        | 5'ATAATCWGGWATYCTCCTTGG3'      |            | 35 cycles; 95°C for 15s, 45°C for 30s and 72°C for 1 min      |           |                                                                                                                 |                                               |
|                                        | -Bab_Rev1        | 5'TCTCTWCATGGWTTAATTATGATAT3'  |            | 72°C for 10 min                                               | 924       |                                                                                                                 |                                               |
|                                        | -Bab_for2        | ,                              |            | Annealing temperature 2nd round =49°C                         |           |                                                                                                                 |                                               |
|                                        | -Bab_rev2        | 5'TAGCTCCAATTGAHARWACAAAGTG    |            |                                                               |           |                                                                                                                 |                                               |
|                                        |                  | 3'                             |            |                                                               |           |                                                                                                                 |                                               |

|                                            |                                                   |                                                                                                                               |      |                                                                                                                                         |                                               |
|--------------------------------------------|---------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------|------|-----------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------|
| <b>Piroplasmids<br/>(characterization)</b> | <i>cox-3</i><br>-Cox3F<br>-Cox3R                  | 5'-ACTGTCAGCTAAAACGTATC-3'<br>5'-ACAGGATTAGATACCCTGG-3'                                                                       | 600  | 94°C for 5 min;<br>50 cycles: 94°C for 20s, 55°C for 30s and 68°C for 45s;<br>72°C for 7 min                                            | Schreeg et al.(2016);<br>Barbosa et al.(2019) |
| <b>Piroplasmids<br/>(characterization)</b> | <i>hsp70</i><br>-Hsp70F1<br>-Hsp70R1              | 5' CATGAAGCACTGGCCHTTCAA 3'<br>5' GCNCKGCTGATGGTGGTGTGTA 3'                                                                   | 740  | 95°C for 5 min;<br>35 cycles: 95°C for 15s, 60°C for 30s and 72°C for 30s;<br>72°C for 5 min                                            | Soares et al. (2011)                          |
| <b>Piroplasmids<br/>(characterization)</b> | <i>CytB</i><br>-F<br>-R                           | 5'- TTAGTGAAGGAACTTGACAGGT 3'<br>5'-CGGTAAATCTTTCCTATTCCTTACG 3'<br>5'-CAAATWGGYGCM AARTTYTGGA-3'                             | 1Kb  | 94°C for 5 min;<br>50 cycles: 94°C for 20s, 56°C for 30s and 68°C for 1 min;<br>72°C for 7 min                                          | Schreeg et al.(2016);<br>Barbosa et al.(2019) |
| <b>Piroplasmids<br/>(characterization)</b> | <i>B-tubulin</i><br>-Tubu-63F<br>-Tubu-3          | 3'<br>5'-<br>TCGTCCATACCTTCWCCSGTRTACCAGTG-3'                                                                                 | 1214 | 95°C for 5 min;<br>50 cycles: 94°C for 40s, 55°C for 1min and 72°C for 90s;<br>72°C for 5 min                                           | Zamoto et al., (2004)                         |
| <b>Piroplasmids<br/>(characterization)</b> | ITS-1<br>-ITS13B<br>-ITS15C<br>-ITS13C<br>-ITS15D | 5'-CGATCGAGTGATCCGGTGAATTA-3'<br>5'-GCTGCGTCCTTCATCGTTGTG-3'<br>5'-AAGGAAGGAGAAGTCGTAACAAGG-3'<br>5'-TTGTGTGAGCCAAGACATCCA-3' | 450  | 94°C for 1 min;<br>34 cycles: 94°C for 30s, 52°C for 30s and 72°C for 1 min.<br>72°C for 5 min<br>Annealing temperature 2nd round =49°C | Shock et al. (2011)                           |

The assays were performed using 5 µL of the DNA samples in a mixture containing 0.75 U Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, California, USA), PCR buffer (PCR buffer 10 X – 100 nM Tris-HCl, pH 9.0, 500 mM KCl), 0.2 mM deoxynucleotides (dATP, dTTP, dCTP, and dGTP) (Invitrogen, Carlsbad, California, United States), 1.5 mM of Magnesium chloride (Invitrogen, Carlsbad, CA, United States), 0.5 µM of each primer (Invitrogen, Carlsbad, California, USA), and sterile ultrapure water (Invitrogen, Carlsbad, California, USA) comprising a total volume of 25 µL. In nPCR assays, 1 µL of the amplified product from the first PCR reaction was used as the target DNA in the second reaction. A DNA sample obtained from a dog experimentally infected with *Babesia vogeli* (Jaboticabal strain) (Furuta et al., 2009) and sterile ultrapure water were used as positive and negative controls, respectively, in all PCR assays for piroplasmids.

The products obtained in PCR assays were separated by horizontal electrophoresis on a 1% agarose gel stained with ethidium bromide (Life Technologies™, Carlsbad, CA, USA) in TEB running buffer pH 8.0 at 100 V/150 mA for 50 min. The gels were

examined under ultraviolet light illumination using ChemiDoc MP Imaging System (Bio Rad, Hercules, CA, USA) and photographed using Image Lab Software v.4.1 (Biorad, Hercules, CA, USA).

### **Sequencing, BLAST and phylogenetic analyses**

The amplified products that showed high band intensity on agarose gels were purified using Wizard SV gel and PCR clean-up system (Promega, Madison, WI, USA) and sequenced by Sanger's method using the BigDye Terminator v3.1 Cycle Sequencing kit (Thermo Fisher Scientific, Waltham, MA, USA) and the ABI PRISM 310 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) at the "Centro de Recursos Biológicos e Biologia Genômica (CREBIO -FCAV - UNESP).

The sequences obtained were submitted to a quality-screening test using Phred-Phrap software (version 23) (Ewing and Green, 1998; Ewing et al., 1998) to evaluate the quality of the electropherograms and to obtain the consensus sequences from the alignment of the sense and antisense sequences. Consensus sequences were trimmed so that only nucleotides with Phred index above 20 were used. The BLASTn program (Altschul et al., 1990) was used to compare the obtained sequences with previously deposited sequences in the GenBank database (Benson et al., 2002). The sequences saved in "FASTA" format were aligned with other homologous sequences of each agent retrieved from the database (Genbank), using the MAFFT software (Kato and Standley, 2013) and edited via Bioedit v. 7.0.5.3 (Hall, 1999). W-IQ-Tree software was used for choosing the evolutionary model following BIC criterion as well as for phylogenetic analysis by the Maximum Likelihood method (available online: <http://iqtree.cibiv.univie.ac.at/>) (Trifinopoulos et al., 2016). Clade support indices were evaluated through bootstrap analyses of 1000 repetitions. The phylogenetic trees were edited using Treegraph 2.0.56-381 beta software (Stover and Muller, 2010). The choice of sequences and outgroups used in the phylogenetic analyses were based on the topology described by Jalovecka et al. (2019), and updated by works performed by Ikeda et al. (2021), Gonçalves et al. (2021), Mongruel et al. (2022), and Oliveira et al. (2023).

## Results

### Molecular assays

All 455 DNA samples analyzed were positive for mammalian endogenous gene (*gapdh*) and subjected to a nPCR assay based on the 18S rRNA gene of piroplasmids. Of the 455 analyzed samples, 25 (5.49% [25/455]) were positive: 12 (9.16% [12/131]) giant anteaters, five (13.51% [5/37]) southern tamanduas, three (30% [3/10]) nine-banded armadillos, four (12.5% [4/32]) giant armadillos and one (9.09% [1/11]) six-banded armadillo (**Table 2**). Due to the low intensity of the bands in agarose gel, only four samples (three giant armadillos and one armadillo) were subjected to sequencing (**Table 3**).

**Table 2.** Positive animals by state and type of sample analyzed.

| Species                        | Positive mammals per States of Brazil |               | Positives per type of sample |                |
|--------------------------------|---------------------------------------|---------------|------------------------------|----------------|
|                                | Mato Grosso do Sul                    | São Paulo     | Blood samples                | Spleen samples |
| <i>Tamandua tetradactyla</i>   | 0.57% (1/175)                         | 9.76% (4/41)  | 0                            | 4.13% (5/121)  |
| <i>Myrmecophaga tridactyla</i> | 4.00% (7/175)                         | 12.19% (5/41) | 0                            | 9.92% (12/121) |
| <i>Dasypus novemcinctus</i>    | 0.57% (1/175)                         | 4.88% (2/41)  | 0                            | 2.48% (3/121)  |
| <i>Euphractus sexcinctus</i>   | 0.57% (1/175)                         | 0             | 0                            | 0.83% (1/121)  |
| <i>Priodontes maximus</i>      | 2.28% (4/175)                         | 0             | 1.20 (4/334)                 | 0              |
| TOTAL                          | 14                                    | 11            | 4                            | 21             |

Out of the 25 samples positive in the nPCR for piroplasmids, 5 (20%; three southern tamanduas and two nine-banded armadillos), and 5 (20%; two southern tamanduas, two giant anteaters and one nine-banded armadillo) were positive for the *cox-1* gene and the intergenic region (ITS-1), respectively (**Table 3**). Due to the low intensity of the obtained bands in agarose gel, only three ITS-1 amplicons were successfully sequenced. All 25 samples positive in the nPCR for piroplasmids were negative in the PCR assays based on the nearly complete 18S rRNA gene, *cox-3*, *hsp70*, *cytB* and  $\beta$ -*tubulin* genes.

**Table 3.** Positive animals in the PCR assays for piroplasmids performed in the present study.

| Xenarthra species<br>(ID) | Location | 18S rRNA<br>gene | ITS-1 |
|---------------------------|----------|------------------|-------|
|---------------------------|----------|------------------|-------|

|                                            |    |    |        |
|--------------------------------------------|----|----|--------|
| <i>Tamandua tetradactyla</i><br>(NEC16)    | MS | NS | -      |
| <i>Myrmecophaga tridactyla</i><br>(NEC 28) | MS | NS | -      |
| <i>Dasypus novemcinctus</i> (NEC<br>20)    | MS | S  | -      |
| <i>Myrmecophaga tridactyla</i><br>(NEC 24) | MS | NS | -      |
| <i>Myrmecophaga tridactyla</i><br>(NEC 32) | MS | NS | -      |
| <i>Myrmecophaga tridactyla</i><br>(NEC 34) | MS | NS | -      |
| <i>Myrmecophaga tridactyla</i><br>(NEC 36) | MS | NS | -      |
| <i>Euphractus sexcinctus</i> (NEC<br>52)   | MS | NS | -      |
| <i>Myrmecophaga tridactyla</i><br>(NEC 69) | MS | NS | -      |
| <i>Myrmecophaga tridactyla</i><br>(NEC 71) | MS | NS | -      |
| <i>Priodontes maximus</i> (DN02)           | MS | NS | -      |
| <i>Priodontes maximus</i> (TC11)           | MS | S  | -      |
| <i>Priodontes maximus</i> (TC12)           | MS | S  | -      |
| <i>Priodontes maximus</i> (TC16)           | MS | S  | -      |
| <i>Tamandua tetradactyla</i><br>(33433)    | SP | NS | + (S)  |
| <i>Tamandua tetradactyla</i><br>(34255)    | SP | NS | -      |
| <i>Dasypus novemcinctus</i><br>(35881)     | SP | NS | + (NS) |
| <i>Dasypus novemcinctus</i><br>(36563)     | SP | NS | -      |
| <i>Myrmecophaga tridactyla</i><br>(40088)  | SP | NS | + (S)  |
| <i>Myrmecophaga tridactyla</i><br>(40766)  | SP | NS | + (S)  |
| <i>Tamandua tetradactyla</i><br>(41060)    | SP | NS | + (NS) |

|                                           |    |    |   |
|-------------------------------------------|----|----|---|
| <i>Myrmecophaga tridactyla</i><br>(41201) | SP | NS | - |
| <i>Myrmecophaga tridactyla</i><br>(41662) | SP | NS | - |
| <i>Myrmecophaga tridactyla</i><br>(42046) | SP | NS | - |
| <i>Tamandua tetradactyla</i><br>(42535)   | SP | NS | - |

MS = Mato Grosso do Sul State, SP= São Paulo State, NS= non-sequenced samples, S= sequenced samples

### BLASTn analysis

In the BLASTn analysis of the 18S rRNA sequences obtained in this study, the three sequences from the giant armadillos were 100% identical to one sequence of *Babesia* sp. previously detected in a white-eared opossum (*Didelphis albiventris*) from Brazil. The sequence detected in a nine-banded armadillo showed 91.4% identity with *Theileria* sp. detected in goats from China. The three ITS-1 sequences obtained from two giant anteaters and one southern anteater showed identity ranging from 94.2 to 94.4% to *Cytauxzoon felis* previously detected in *Dermacentor variabilis* from the USA (Table 4).

**Table 4.** BLASTn results of the sequences obtained in the amplification of target genes for piroplasmids in *Xenarthra* blood or spleen samples.

| Specie/<br>Identification                    | Location | Target<br>gene | Size<br>(bp) | Query-<br>cover<br>(%) | E-value | Identity<br>(%) | GenBank Sequence<br>(access number)                                                                                            |
|----------------------------------------------|----------|----------------|--------------|------------------------|---------|-----------------|--------------------------------------------------------------------------------------------------------------------------------|
| <i>Prionomys</i><br><i>maximus</i> /<br>TC11 | MS       | 18S<br>rRNA    | 728          | 100                    | 0       | 100             | <i>Babesia</i> sp., detected<br>in <i>Amblyomma</i><br><i>dubitatum</i> from Mato<br>Grosso do Sul State,<br>Brazil (MW342734) |
| <i>Prionomys</i><br><i>maximus</i> /<br>TC16 | MS       | 18S<br>rRNA    | 638          | 100                    | 0       | 100             | <i>Babesia</i> sp., detected<br>in <i>Amblyomma</i><br><i>dubitatum</i> from Mato<br>Grosso do Sul State,<br>Brazil (MW342734) |



|                                        |    |              |     |     |                     |       |                                                                                                             |
|----------------------------------------|----|--------------|-----|-----|---------------------|-------|-------------------------------------------------------------------------------------------------------------|
| <i>Priodontes maximus</i> / TC12       | MS | 18S rRNA     | 709 | 99  | 0                   | 100   | <i>Babesia</i> sp., detected in <i>Amblyomma dubitatum</i> from Mato Grosso do Sul State, Brazil (MW342734) |
| <i>Dasypus novemcinctus</i> / NEC20    | MS | 18S rRNA     | 731 | 100 | 0                   | 91,39 | <i>Theileria</i> sp. detected in goat from China (MG930118)                                                 |
| <i>Myrmecophaga tridactyla</i> / 40088 | SP | Região ITS-1 | 242 | 27  | 4 X10 <sup>18</sup> | 94,20 | <i>Cytauxzoon felis</i> detected in <i>Dermacentor variabilis</i> from the USA (KC119625)                   |
| <i>Myrmecophaga tridactyla</i> / 40766 | SP | Região ITS-1 | 233 | 29  | 3X10 <sup>19</sup>  | 94,37 | <i>Cytauxzoon felis</i> detected in <i>Dermacentor variabilis</i> from the USA (KC119625)                   |
| <i>Tamandua tetradactyla</i> / 33433   | SP | Região ITS-1 | 138 | 48  | 4 X10 <sup>18</sup> | 94,20 | <i>Cytauxzoon felis</i> detected in <i>Dermacentor variabilis</i> from USA (KC119625)                       |

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\*SP=São Paulo State; MS=Mato Grosso do Sul State

### Phylogenetic analyses

The phylogenetic analysis based on an 805 bp alignment of the 18S rRNA gene and inferred by Maximum Likelihood method and TIM3+I+G evolutionary model positioned the piroplasmid sequences obtained from *P. maximus* into the same clade of *Babesia* sp. previously detected in marsupials (*Monodelphis domestica*, *Didelphis albiventris*, *Didelphis marsupialis* and *Didelphis aurita*) and *Amblyomma dubitatum* ticks collected from opossums and coatis (*Nasua nasua*) from central-western Brazil, with a bootstrap of 100%. The sequence detected in *D. novemcinctus* was closely related to *Theileria*

sp. detected in nine-banded armadillos in the state of Mato Grosso State, Brazil, forming a subclade within the *Theileria sensu stricto* clade with a bootstrap of 94% (Figure 2).

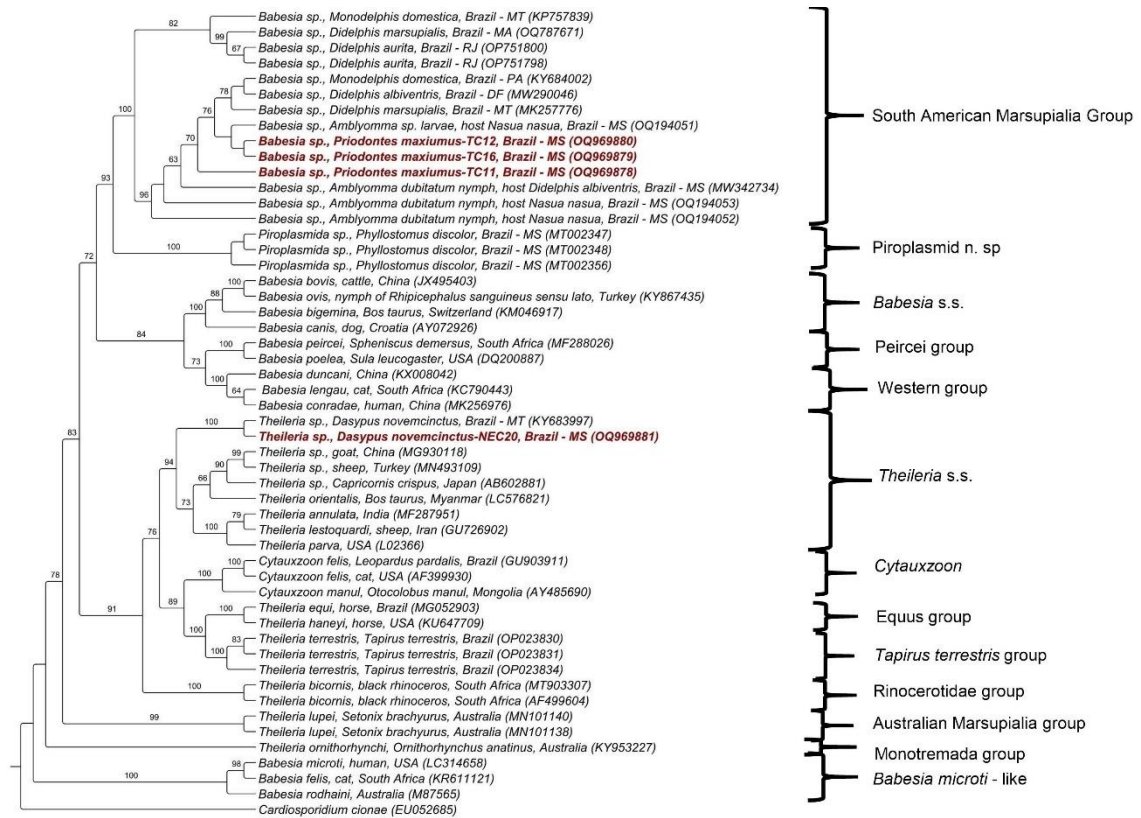


**Figure 2.** Phylogenetic analysis of Piroplasmids 18S rRNA sequences inferred from 805 bp alignment generated by Maximum Likelihood and with TIM3+I+G evolutionary model. The bootstrap values and the branch lengths are represented at the nodes of each clade. *Cardiosporidium cionae* was used as outgroup.

The phylogenetic analysis based on a 937 bp alignment of the ITS-1 region and inferred by Maximum Likelihood method and HKY+G evolutionary model positioned the three sequences obtained from *T. tetradactyla* and *M. tridactyla* into a single clade, apart from the other piroplasmid clades, with a bootstrap of 95% (Figure 3).

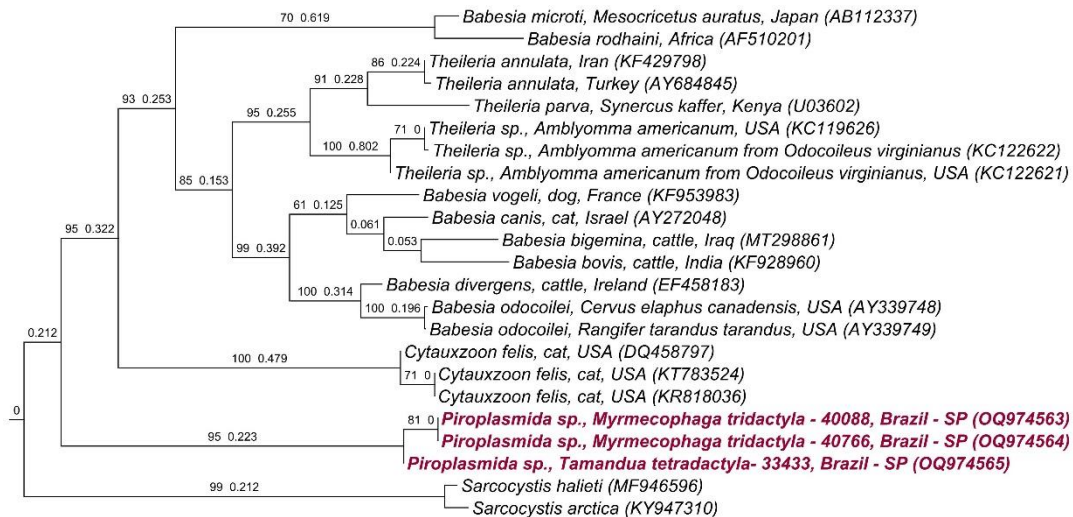


**Figure 3.** Phylogenetic analysis of Piroplasmids ITS-1 sequences inferred from 937 bp alignment generated by Maximum Likelihood and with HKY+G evolutionary model. The bootstrap values and the branch lengths are represented at the nodes of each clade. *Sarcocystis halioti* and *Sarcocystis arctica* were used as outgroup.

## Discussion

The present study reported a positivity of 5.5% (25/455) for piroplasmids in mammals from the Superorder Xenarthra sampled in the states of São Paulo, Mato Grosso do Sul, Rondônia e Pará. All animals from the states of Pará and Rondônia were negative for these agents. This positivity is in agreement with frequencies reported in previous studies conducted with Xenarthra from Brazil, which ranged from 0 to 11.76% (Soares et al., 2017; Silva et al., 2021; Fava et al., 2022).

Even though *Babesia* spp. and *Theileria* spp. have been morphologically described in xenarthrans from South America (Leger and Mouzels, 1917; Dedet et al., 1988; Laissou et al., 1979), piroplasmid 18S rRNA sequences from these mammals were first described in a study conducted in the Amazon biome, in the states of Amazonas and Mato Grosso. A putative novel piroplasmid genotype, which was positioned in a sister clade to the *Theileria sensu stricto* clade, was detected in *D. novemcinctus* (6.25%) (Soares et al., 2017).

The phylogenetic analysis based on 18S rRNA gene demonstrated the occurrence of two different genera of piroplasmids in Xenarthra. The sequences detected in *P. maximus* were positioned into the “South American Marsupialia group”, a recently described clade that contains putative novel species of *Babesia* sp. detected in marsupials and *A. dubitatum* ticks from Brazil (Gonçalves et al., 2021; de Oliveira et

al., 2023; Perles et al., 2023). The sequence detected in *D. novemcinctus* was positioned within the *Theileria sensu stricto* clade along with a *Theileria* sp. previously detected in armadillos (Soares et al., 2017). Despite several attempts, the amplification of the near-complete 18S rRNA was not successful, probably due to the low parasitemia in these animals or the poor quality or integrity of the DNA extracted. Additionally, the primer specificity might have prevented amplification of the DNA of piroplasmid species that occurs in Xenarthra, directly influencing the sensitivity of the test performed and precluding robust phylogenetic inferences about the Xenarthra-associated piroplasmids.

The phylogenetic analysis based on the ITS-1 positioned the sequences obtained from *M. tridactyla* and *T. tetradactyla* into a single clade, apart from the other ones containing known species of piroplasmids. This finding suggests the occurrence of putative novel piroplasmids in this group of mammals. Unfortunately, the existence of few piroplasmids ITS-1 sequences deposited in GenBank hindered further conclusions regarding the phylogenetic positioning of the obtained sequences. According to Gou et al., 2012, the ITS region is easily amplified in PCR assays for piroplasmids since it is present in multiple copies in the genome. Most likely, it was possible to obtain amplicons for this molecular marker despite the lack of amplification for the other genic regions.

The tick vectors involved in the transmission of piroplasmids among xenarthrans are yet to be described. Several studies conducted in Brazil have shown a wide variety of tick species parasitizing these animals (Labruna et al., 2002; Marques et al., 2002; Arzua et al., 2005; Dantas-Torres et al., 2009; Garcia et al., 2013; Sanches et al., 2014; Soares et al., 2014; Acosta et al., 2016; Kluyber et al., 2016; Luz et al., 2018; Szabó et al., 2019; Bernardes et al., 2022; Martins et al., 2023). The great diversity of life habits and behaviors among the different members of this group of mammals together with the wide geographical distribution and the diversified associated biomes may contribute to the tick diversity associated with xenarthrans. For instance, Desbiez and Kluyber (2013) demonstrated that the burrows built by giant armadillos are used by different animals, including other Xenarthra species. In addition, the authors described that giant armadillo burrows maintain a constant temperature (26°C) that can attract arthropod vectors, being considered a very relevant “hotspot” for pathogen

transmission. The authors also reported having seen giant anteaters and other animals taking sand baths in the sand mounds outside the burrows, which may favor contact with ticks. Anthropization and deforestation of biomes can favor the spread of more generalist tick species, while decrease the number of ticks showing high host specificity (Ogrzewalska et al., 2011; Ramos et al., 2020). These facts may favor the exchange of ectoparasites and pathogens between animals and humans and may explain the occurrence of, at least, two different piroplasmids in armadillos. In addition, the behavior described above together with the diversity of ticks and vector-borne agents that these mammals can harbor, highlights the potential role that Xenarthra mammals can play in the epidemiological cycles of piroplasmids. Further studies should be carried out to unravel the real molecular identity of piroplasmids that parasitize Xenarthra mammals, as well as the tick species associated with their transmission. Finally, studies aiming at investigating the role of Xenarthra mammals as hosts for piroplasmids that infect other wildlife species (e.g. opossums) and humans are much needed.

## Conclusion

The present study demonstrated the molecular occurrence of piroplasmids in animals of the superorder Xenarthra from Brazil. This is the first work to report the presence of piroplasmids DNA in xenarthrans in the states of Mato Grosso do Sul and São Paulo. At least two different piroplasmid species parasitize armadillos, since the obtained sequences were positioned into two different clades. While the sequences detected in *P. maximus* were positioned within the South American Marsupialia clade, the sequence detected in *D. novemcinctus* was positioned along with another sequence detected in the same host species, forming a subclade within the *Theileria sensu stricto* clade. A putative novel Piroplasmida was detected in anteaters from Brazil. These results demonstrated the circulation of different species of piroplasmids in Xenarthra and their possible role in the maintenance and transmission of these agents. Further studies should be conducted to clarify the role of these agents in public and wildlife health.

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## **Declarations**

### **Ethical approval**

Animal procedures and management protocols were approved by Institute Chico Mendes for Conservation of Biodiversity (SISBIO number 53798–5) and by the Ethics Committees on Animal use of the ICB—USP (protocol number 98) and the School of Agricultural and Veterinarian Sciences (FCAV/UNESP) (protocol number 9412/2022). The DNA samples were registered under SISGEN #A4C38D5 (“Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado”).

### **Consent to participate and consent to publish**

Not applicable.

### **Conflict of interest**

The authors declare no conflict of interest.

### **Author’s contribution**

Conceptualization: Ana Cláudia Calchi and Marcos Rogério André; Methodology: Ana Cláudia Calchi, Débora Regina Yogui, Mario Henrique Alves, Arnaud Leonard Jean Desbiez, Danilo Kluyber, Juliana Gaboardi Vultão, Paulo Vitor Cadina Arantes, Mariele de Santi, Karin Werther, and Marta Maria Geraldês Teixeira; Writing – original draft preparation: Ana Cláudia Calchi, Débora Regina Yougui, Mario Henrique Alves, Arnaud Leonard Jean Desbiez, Danilo Kluyber, Juliana Gaboardi Vultão, Mariele de Santi, Paulo Vitor Cadina Arantes, Karin Werther, Marta Maria Geraldês Teixeira,

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### **Availability of data**

The datasets generated and analyzed during the current study are available in the NCBI GenBank Nucleotide platform (<https://www.ncbi.nlm.nih.gov/genbank/>) and can be accessed through accession numbers: OQ969878-OQ969881, OQ974563-OQ974565

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**CAPÍTULO 3\* - Phylogenetic inferences based on distinct molecular markers reveals a novel *Babesia* (*Babesia pantanalensis* nov. sp.) and a *Hepatozoon americanum*-related genotype in crab-eating foxes (*Cerdocyon thous*)\***

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**Abstract**

Piroplasmids and *Hepatozoon* spp. are apicomplexan protozoa that may cause disease in several canid species. The present study aimed to expand the knowledge on the diversity of piroplasmids and *Hepatozoon* in crab-eating foxes (*Cerdocyon thous*; n=12) sampled in the Pantanal of Mato Grosso do Sul State, central-western Brazil. PCR assays based on the 18S rRNA were used as screening. Three (25%) and

11 (91.7%) were positive for piroplasmids and *Hepatozoon* spp., respectively. Co-infection was found in three *C. thous*. Phylogenetic analyses based on the near-complete 18S rRNA, *cox-1* and *hsp70* genes evidenced the occurrence of a novel of *Babesia* spp. (namely *Babesia pantanalensis* nov. sp.) closely related to *Rangelia vitalii* and *Babesia* sp. 'Coco'. This finding was supported by the genetic divergence analysis which showed (i) high divergence, ranging from 4.17 to 5.62% for 18S rRNA, 6.16% for *hps70* and 4.91 to 9.25% for *cox-1* and (ii) the genotype network (which displayed sequences separated from the previously described Piroplasmida species by median vectors and several mutational events). Also, phylogenetic analysis based on the 18S rRNA gene of *Hepatozoon* spp. positioned the sequences obtained herein in a clade phylogenetically related to *Hepatozoon* sp. 'Curupira 2', *Hepatozoon* sp. detected in domestic and wild canids from Uruguay and *Hepatozoon americanum*. The present study described *Babesia pantanalensis* nov sp. and *Hepatozoon* closely related to *H. americanum* in crab-eating foxes from Brazil. Moreover, the coinfection by piroplasmids and *Hepatozoon* sp. for the first time in crab-eating foxes strongly suggesting that this wild canid species potentially acts as a bio-accumulate of hemoprotozoan in wild environment.

**Keywords:** molecular diagnosis, apicomplexan protozoa, *Babesia*, *Hepatozoon*, crab-eating foxes, phylogeny

## 1. Introduction

The crab-eating fox, *Cerdocyon thous* (Linnaeus, 1766), (Mammalia: Carnivora) is a medium-sized wild canid with wide geographic distribution from Central (Panama) to the south of South America (Argentina). In Brazil, it is distributed throughout the entire territory except for the Amazon basin [1]. It is considered a species of least concern for extinction by the IUCN (International Union for Conservation of Nature) Red List of threatened species [1]. This canid species has nocturnal and crepuscular habits and usually lives in pairs or small groups [2]. It is considered an omnivore, feeding on small mammals, reptiles, amphibians, birds, insects, crustaceans, fruits, and grasses [3, 4]. Also, this wild canid plays an important role in seed dispersion and rodent control [5]. Furthermore, since crab-eating fox is a synanthropic species and has been recorded concomitantly parasitized by several parasite species [6-8], they can act as a bio-accumulators of parasites and may be a great importance in the transmission cycles of parasites in both wild ecotope and in the peridomicile of rural environments [6].

Piroplasmids and *Hepatozoon* Miller, 1908 are tick-borne apicomplexan protozoa that parasitize several vertebrate species, which may, in some situations,

cause diseases [9-11]. The Order Piroplasmida Wenyon, 1926 comprise four genera (*Babesia* Starcovici, 1893; *Rangelia* Carini and Maciel, 1914; *Theileria* Bettencourt et al., 1907; *Cytauxzoon* Neitz and Thomas, 1948) and are mainly transmitted by the saliva of ixodid ticks during blood feeding. These protozoa can infect different cells (erythrocytes, lymphocytes, monocytes and endothelial cells) depending on the host species involved [10, 12, 13].

Clinical signs associated with canine piroplasmosis may vary according to the involved agent and the immune status, ranging from subclinical to fatal infections. The main clinical and laboratorial findings associated with piroplasmid infection in canids are fever, anemia, anorexia, lethargy, thrombocytopenia, hemorrhages, and organ dysfunction [14]. These abnormalities are most seen in domestic dogs but reports of symptomatic wild canids have been described [15-20].

*Hepatozoon* is a genus composed of more than 340 species that parasitize mammal blood cells and tissues and is transmitted mainly by the ingestion of arthropod vectors containing mature oocysts, albeit other transmission routes (i.e. predation and transplacental transmission) have already been reported [9, 11, 21, 22]. Two species have been described in domestic dogs and wild canids: *Hepatozoon canis* James, 1905 and *Hepatozoon americanum* Vicent-Johnson et al., 1907 [11]. The former species causes mild, often subclinical infections and has been reported in different continents, while the latter causes more severe infections and has only been described in the USA since the presence of tick vector *Amblyomma maculatum* Koch, 1844 [11]. New 18S rRNA genotypes phylogenetically related to *H. americanum* have been detected in wild canids [23-29] from South America [23-29]. The main clinical signs associated with *Hepatozoon* infection in dogs are anorexia, weight loss, pale mucous membranes, pain, diarrhea, vomiting, gait abnormalities, fever, polyuria, polydipsia, anemia, glomerulonephritis, nephrotic syndrome, and thromboembolism [11, 30]. Few studies reported the occurrence of clinical signs of hepatozoonosis in wild canids [31, 32, 56-58]. In addition, a putative novel species unrelated to *H. canis* and *H. americanum* has been reported in wild canids from Brazil and Argentina [24, 25, 27].

In Brazil, piroplasmids and/or *Hepatozoon* spp. have been described in the following species of free-living or captive wild canids: crab-eating fox [7, 20, 23-26, 31, 33], bush dog (*Speothos venaticus* Lund, 1842) [24], maned wolf [*Chrysocyon*

*brachyurus* (Illiger, 1815)] [24, 36], Hoary fox [*Lycalopex vetulus* (Lund, 1842)] [24] and Pampas fox [*Lycalopex gymnocercus* (Fischer 1814)] [7, 17, 18, 23].

This study aimed to expand knowledge about the occurrence and diversity of piroplasmids and *Hepatozoon* spp. in *C. thous* captured in the Miranda Pantanal, Mato Grosso do Sul State, midwestern Brazil.

## 2. Materials and Methods

### 2.1. Wild canids captured and studied area

Twelve crab-eating foxes (*Cerdocyon thous*), including eight males and four females, were captured between August and December 2018 in the Pantanal of Miranda (20°05' S, 56°36' W), a city located in the state of Mato Grosso do Sul, Brazil (**Figure 1**). Five animals were adults, six were young and one was a puppy. Seven animals had a low body score and only one animal had pale mucous membranes.



**Figure 1.** Collection site of the *Cerdocyon thous* blood samples. The area marked in dark green represents the region of the Pantanal biome. The study area in Miranda, Mato Grosso do Sul, Brazil is represented by a red triangle, MS= Mato Grosso do Sul State.

The study area is an agroecosystem located on the banks of the Miranda River, made up of native vegetation of savanna and riparian forest, crops and exotic pastures. The box traps (tomahawk model 90 × 45 × 50; Equipos Fauna®, Brazil) were set at night and baited with chicken thighs, bacon or sardines. The captured animals were sedated with Zoletil 50 (8 mg/kg) or a combination of ketamine 15 mg/kg, xylazine 0.7 mg/kg and midazolam 0.5 mg/kg. A physical examination was carried out and blood was taken by puncturing the cephalic or jugular vein using tubes containing EDTA (Ethylenediamine Tetraacetic Acid). The animals were released at the same place as

they were captured, after they had fully recovered under sedation. The blood samples were placed into RNase/DNase microtubes and stored in an -20°C freezer until molecular analyses. The wild canid blood samples were collected by a veterinarian and kindly donated to our research group for molecular assays.

## **2.2. DNA extraction and PCR assay for the mammalian endogenous gene**

DNA was extracted from 200 µL of each blood sample using the DNAeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's instructions. DNA samples were subjected to a conventional PCR (cPCR) assay targeting the mammalian endogenous glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) gene [37]. Only the positive samples for the PCR described above were molecularly screened for piroplasmids and *Hepatozoon* spp. The DNA samples were registered under SISGEN #A9CCAD1 ("Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado").

## **2.3. PCR assays for piroplasmids and *Hepatozoon* spp., amplicon purification and sequencing**

DNA samples positive in the *gapdh*-based PCR were screened for piroplasmids using a nested(n) PCR assay targeting a fragment of approximately 800 bp of the 18S rRNA gene [38]. Positive samples were subjected to further molecular characterization using conventional PCR assays targeting different molecular markers: near-complete 18S rRNA gene (~1,500 bp) [39,40], *cox-1* (~800 bp) [41], *cox-3* (~600 bp) [42, 43], *hsp70* (~1,056 bp) [44], *cytB* (~1 kb) [43] and ITS-1 (~450 bp) [45].

Additionally, two distinct PCR assays were used aiming at amplifying different regions of 18S rRNA gene of *Hepatozoon* spp. based on Perkins and Keller (2001) (targeting a fragment of 800 bp) [46] and Ujvari et al. (2004) (targeting a fragment of 600 bp) [47] protocols. Subsequently, the samples that amplified only for one protocol were subjected to two other PCR assays aimed at amplifying a fragment of approximately 1,200-1,750 bp of the 18S rRNA gene to obtain a larger sequence [23, 47, 48].

A DNA sample obtained from a dog experimentally infected with *B. vogeli* (Jaboticabal strain) [49] and *Hepatozoon procyonis* Richards, 1961 obtained from coatis [50] were used as positive controls for piroplasmids and *Hepatozoon* spp. assays, respectively. Sterile ultrapure water (Life Technologies®, Carlsbad, CA, USA) was used as negative control. All primer sequences and thermal conditions used in the PCR assays are described in **Supplementary material 1**.

The PCR products were separated by horizontal electrophoresis on a 1% agarose gel stained with ethidium bromide (Life Technologies™, Carlsbad, CA, USA). The amplified products were purified using Wizard SV gel and PCR clean-up system (Promega, Madison, WI, USA) and sequenced by Sanger's method at the Centro de Pesquisa sobre Genoma Humano e Células Tronco – USP, São Paulo.

#### **2.4. Cloning and sequencing of piroplasmid near-complete 18S rRNA gene**

In order to obtain the near-complete fragment of the 18S rRNA gene of piroplasmids, the positive samples for the protocols that amplify approximately 1,500 bp were subjected to pGEM-T Easy (Promega® Madison, WI, USA) cloning, according to the manufacturer's recommendations. Four clones were selected from each positive sample according to the blue/white colony system. The colonies that had the gene fragment of interest confirmed by PCR were subjected to plasmid DNA extraction using the Wizard® Plus SV Minipreps DNA Purification Systems (Promega Madison, Wisconsin, USA). Subsequently, they were submitted for sequencing with primers M13 F (5'-CGCCAGGGTTTTCCCAGTCACGAC3') and M13 R (5'-GTCATAGCTGTTTCCTGTGTGA-3') [51] that flank the multiple cloning sites of the pGEM-T Easy plasmid to include the gene fragments analyzed. In addition, a pair of internal primers (forward: 5'-AGAAACGGCTACCACATCTA-3' and reverse: 5'-TTGAGTCAAATTAAGCCGCA-3') designed for the sequences of the present study were also used for sequencing to obtain better quality sequences. The sequencing was carried out as described above.

#### **2.5. BLASTn, phylogenetic and genotype analyses**

The sequences consensus sequences were obtained by Phred-Phrap software (version 23) [52]. The BLASTn program (available online: <https://blast.ncbi.nlm.nih.gov/Blast.cgi>) [53] was used to compare the obtained sequences with previously deposited sequences in the GenBank database [54] (Benson et al., 2002). The sequences alignments were made by MAFFT software [55] (Kato et al., 2019). For the *cox-1* gene, the amino acid sequences were used for alignment and subsequent phylogenetic analysis. W-IQ-Tree software was used for choosing the evolutionary model following BIC criterion as well as for phylogenetic analysis by the Maximum Likelihood method (available online: <http://iqtree.cibiv.univie.ac.at/>) [55].

Subsequently, new alignments were performed using the near-complete 18S rRNA, *cox-1* and *hsp70* sequences of piroplasmids obtained in the present study with the phylogenetically closest sequences and with a sequence detected in a maned wolf (*C. brachyurus*) for 18S rRNA and *cox-1* genes. These alignments were used in the distance analysis. A pairwise distance matrix was calculated using the *p*-distance method by MEGA X software [57, 58]. Additionally, a genetic diversity analysis were performed using the new near-complete 18S rRNA alignment by DnaSP v5 software to calculate the nucleotide diversity ( $\pi$ ), diversity of genotypes ( $D_g$ ), number of genotypes ( $g$ ) and the average number of nucleotide differences ( $K$ ) [59] and the genotype network was constructed by Population Analysis with Reticulate Trees (popART) software [60], using the TCS network method [61]. Finally, a pairwise distance analysis was also carried out to align the large *Hepatozoon* spp. sequences detected in *C. thous* with phylogenetically related sequences.

### **3. Results**

#### **3.1. PCR for the mammalian endogenous gene**

All 12 crab-eating foxes DNA samples were positive in the PCR targeting the mammalian endogenous glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) gene and were molecularly tested for piroplasmids and *Hepatozoon* spp.

#### **3.2. Piroplasmids**

Of the 12 animals analyzed, three (25%) were positive for piroplasmids based

| <i>Cerdocyon<br/>thous</i> ID | Piroplasmids             |                            |                      |                      |                      |                     |           | <i>Hepatozoon</i> spp. |                               |                               |
|-------------------------------|--------------------------|----------------------------|----------------------|----------------------|----------------------|---------------------|-----------|------------------------|-------------------------------|-------------------------------|
|                               | Screening                | Characterization           |                      |                      |                      |                     |           | Screening              | Characterization              |                               |
|                               | 18S<br>rRNA<br>(~800 bp) | 18S rRNA<br>(~1,500<br>bp) | <i>hsp70</i><br>gene | <i>cox-1</i><br>gene | <i>cox-3</i><br>gene | <i>cytB</i><br>gene | ITS-<br>1 | 18S rRNA<br>(~800 bp)  | 18S<br>rRNA<br>(~1,200<br>bp) | 18S<br>rRNA<br>(~1,750<br>bp) |
| #1                            | + (NS)                   | + (NS)                     | -                    | + (S)                | -                    | -                   | -         | + (S)                  | -                             | -                             |
| #2                            | + (S)                    | + (C, S)                   | + (S)                | + (S)                | -                    | -                   | -         | + (S)                  | + (NS)                        | -                             |
| #3                            | -                        |                            |                      |                      |                      |                     |           | + (NS)                 | -                             | -                             |
| #10                           | -                        |                            |                      |                      |                      |                     |           | + (S)                  | + (NS)                        | + (NS)                        |
| #12                           | -                        |                            |                      |                      |                      |                     |           | + (NS)                 | -                             | -                             |
| #13                           | -                        |                            |                      |                      |                      |                     |           | -                      |                               |                               |
| #15                           | -                        |                            |                      |                      |                      |                     |           | + (NS)                 | -                             | -                             |
| #17                           | -                        |                            |                      |                      |                      |                     |           | + (S)                  | + (NS)                        | -                             |
| #19                           | -                        |                            |                      |                      |                      |                     |           | + (S)                  | + (NS)                        | -                             |
| #24                           | -                        |                            |                      |                      |                      |                     |           | + (S)                  | + (S)                         | + (NS)                        |
| #25                           | + (NS)                   | + (C, S)                   | +<br>(NS)            | + (S)                | -                    | -                   | -         | + (S)                  | + (S)                         | -                             |
| #26                           | -                        |                            |                      |                      |                      |                     |           | + (S)                  | + (S)                         | + (S)                         |

on the 18S rRNA gene (fragment of 800 bp) and one sample was sequenced. These three samples were subjected to PCR assays based on six distinct target genes. Although all three samples were positive for the 18S rRNA gene (fragment of 1,500 bp), only two samples could be cloned, as one had a low band intensity on agarose gel electrophoresis. Two of these three samples (66.7%) were positive for the *hsp70* gene, but only one could be sequenced as well as all three samples were positive and sequenced for the *cox-1* gene. Unfortunately, no sample was positive for the *cox-3* and *cytB* genes or the intergenic region (ITS-1) (**Table 1**).

**Table 1.** PCR assays results obtained from *Cerdocyon thous* DNA samples.

\* += positive sample, - = negative sample, NS = non-sequenced; S= sequenced; C= cloned and sequenced

The BLASTn analyses showed that the obtained 18S rRNA (fragment of approximately 1,500 bp) and *hsp70* sequences had identities ranging from 95.96 to 96.14% and 94.29%, respectively, with *R. vitalii* sequences previously detected in dogs from South America. Additionally, the *cox-1* sequences showed identities ranging from 91.47 to 91.55% with *Babesia* sp. ‘Coco’ detected in dogs from USA (**Supplementary material 2**).

The Maximum Likelihood (ML) phylogenetic analysis based on a 1,749 bp alignment of the 18S rRNA gene sequences, using the GTR+I+G evolutionary model, showed that the sequences detected in this study were positioned as sister clade to *R. vitalii*, with bootstrap of 100% (**Figure 2**). The ML phylogenetic analysis based on the *hsp70* gene (905 bp alignment and GTR+I+G evolutionary model) also positioned the



sequence detected in the crab-eating fox studied as a sister clade to *R. vitalii*, with a bootstrap of 100% (**Figure 3**). The ML phylogenetic analysis performed with amino acid sequences from the *cox-1* gene (369 aa alignment and mtZOA+G evolutionary model) positioned the obtained sequences closely to *R. vitalii* detected in a dog from Brazil and *Babesia* sp. ‘Coco’, which was detected in a dog in USA, with a bootstrap of 97%. This clade was positioned closely to *Babesia bovis* (**Figure 4**).

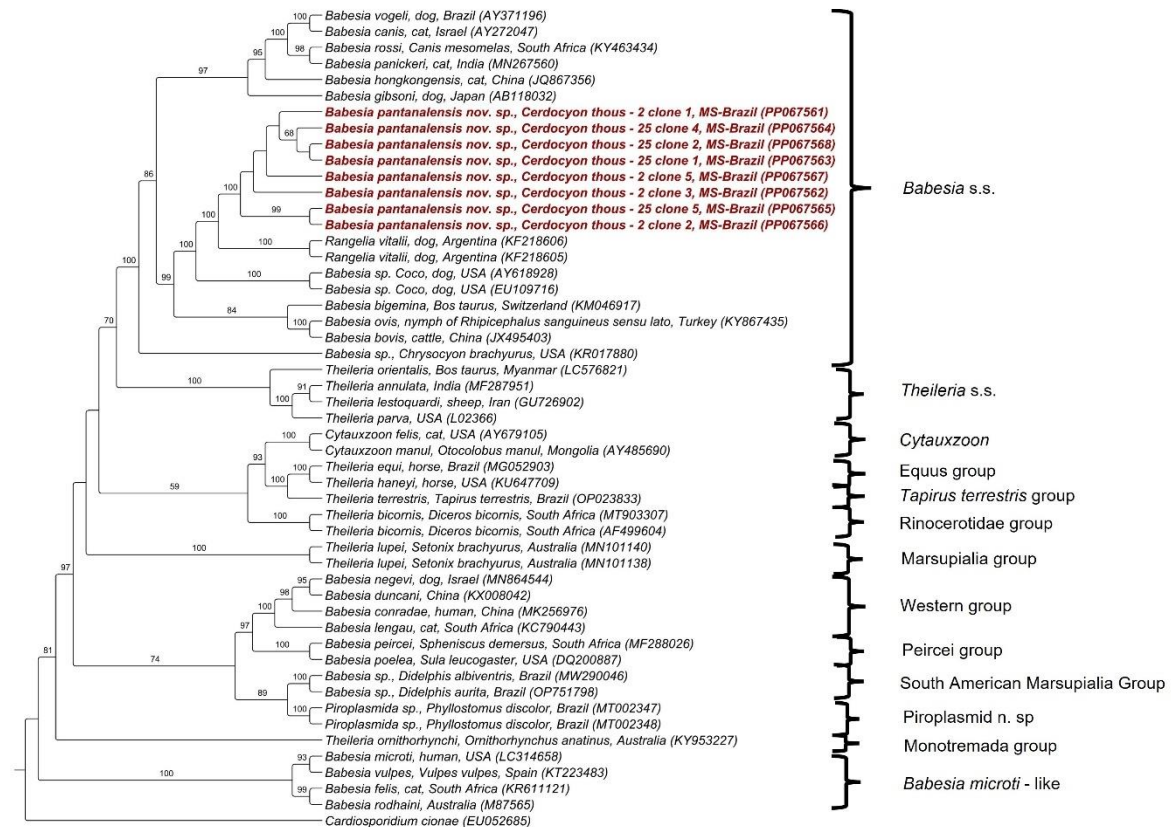


**Figure 2.** Phylogenetic analysis of Piroplasmids 18S rRNA sequences from an alignment of 1,749 bp generated by Maximum Likelihood and with GTR+I+G evolutionary model. *Cardiosporidium cionae* was used as an outgroup.

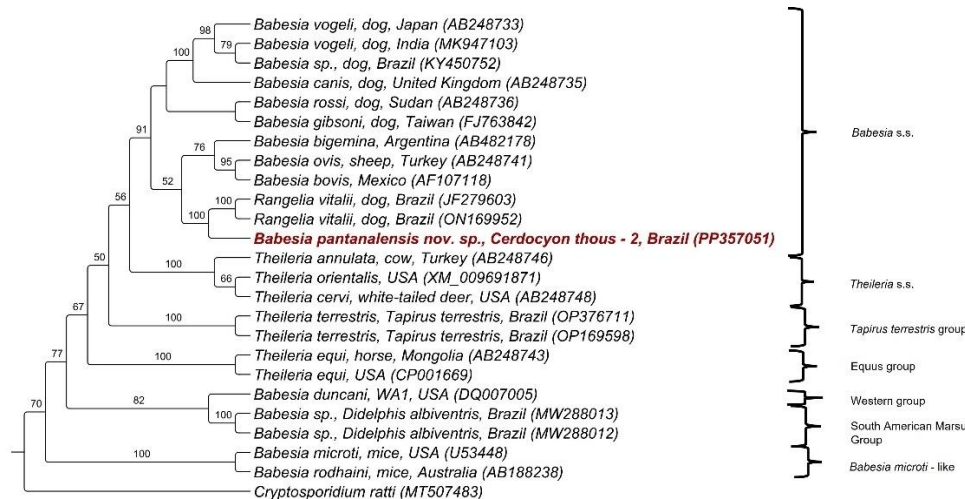


**Figure 3.** Phylogenetic analysis of Piroplasmids *hsp70* sequences from an alignment of 905 bp generated by Maximum Likelihood and with GTR+I+G evolutionary model. *Cryptosporidium ratti* was used as an outgroup.

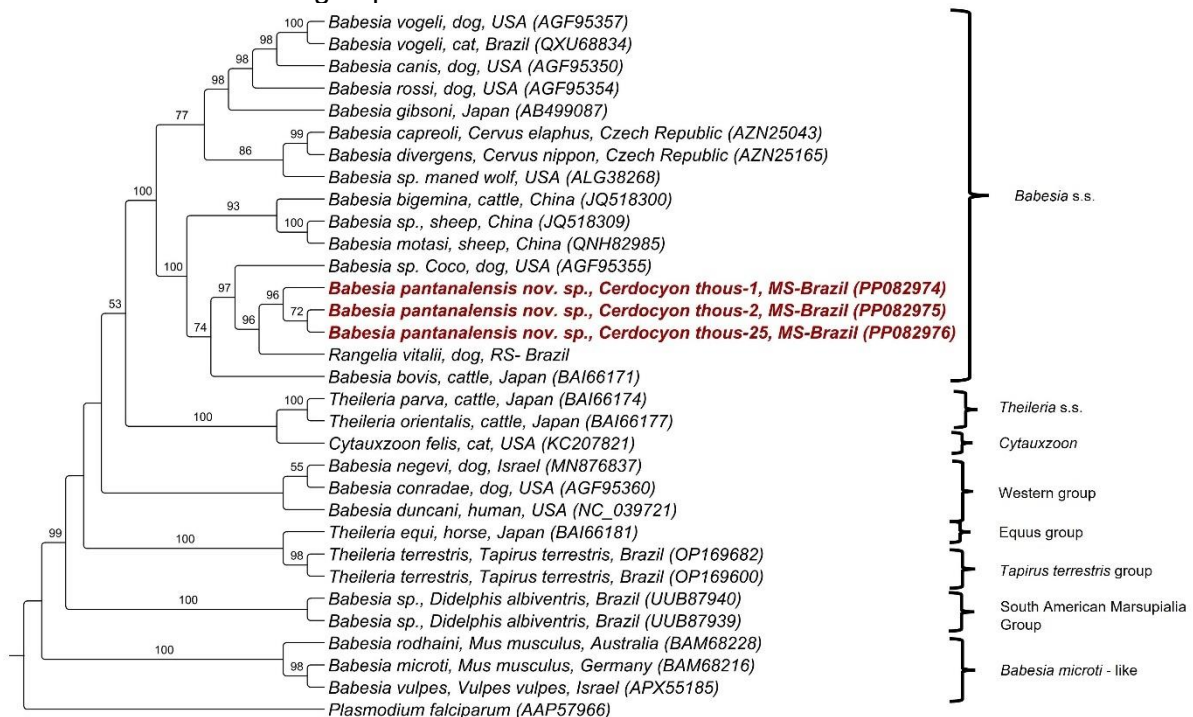


**Figure 4.** Phylogenetic analysis of Piroplasmids COX-1 amino acid sequences from an alignment of 369 amino acids generated by Maximum Likelihood and with mtZOA+G evolutionary model. *Plasmodium falciparum* was used as an outgroup.

Pairwise distance analyses were performed with the three genes, using the sequences of *Babesia* sp. obtained herein, the phylogenetically related sequences and a *Babesia* sp. sequence detected in a maned wolf. As results, the genetic divergence between the 18S rRNA sequences obtained herein ranged from 0 to 0.22%. On the

other hand, the divergence between the sequences detected in crab-eating foxes and *R. vitalii*, *Babesia* sp. 'Coco' and *Babesia* sp. detected in a maned wolf ranged from 4.17 to 4.76%, 5.25 to 5.62%, and 8.37 to 8.86%, respectively (**Figure 5**). For the *hsp70* gene, the divergences were 6.19 and 6.20% between the sequence detected in this study and *R. vitalii* sequences (**Supplementary material 3**). For the *cox-1* gene, the sequences obtained in this study did not differ from each other. However, the divergence ranged from 4.91 to 5.02% with *R. vitalii*, 9.17 to 9.25% with *Babesia* sp. 'Coco' and 20.14 to 20.35% with *Babesia* sp. detected in a maned wolf in the USA (**Supplementary material 4**).



**Figure 5.** A heatmap constructed using the distance matrix based on *p*-value between *Babesia* spp. 18S rRNA sequences detected in *Cerdocyon thous* and the phylogenetically related sequences. The numbers represent the percentage of divergence between the sequences.

The genotype analysis performed with 13 canid-associated Piroplasmida 18S rRNA sequences showed the existence of 11 different genotypes, most formed by a single sequence, of which: genotypes #1 and #2 composed of *R. vitalii* sequences, #3 and #4 by *Babesia* sp. 'Coco' sequences, #5 to #10 by cloned sequences obtained in the present study (genotypes #6 and #8 were formed by two sequences each) and #11 represented by *Babesia* sp. detected in a maned wolf. The genotype network showed that the Piroplasmida 18S rRNA genotypes were grouped according to canid species and locality and originated from median vectors (inferred ancestral nodes) and several mutational events (**Figure 6**). The genotype diversity ( $D_g$ ) was  $0.974 \pm 0.039$ , the nucleotide diversity ( $\pi$ ) was  $0.03474 \pm 0.00877$ , the average number of nucleotide differences ( $K$ ) was 46.29487, with 144 variable sites and 45.4% G+C content.

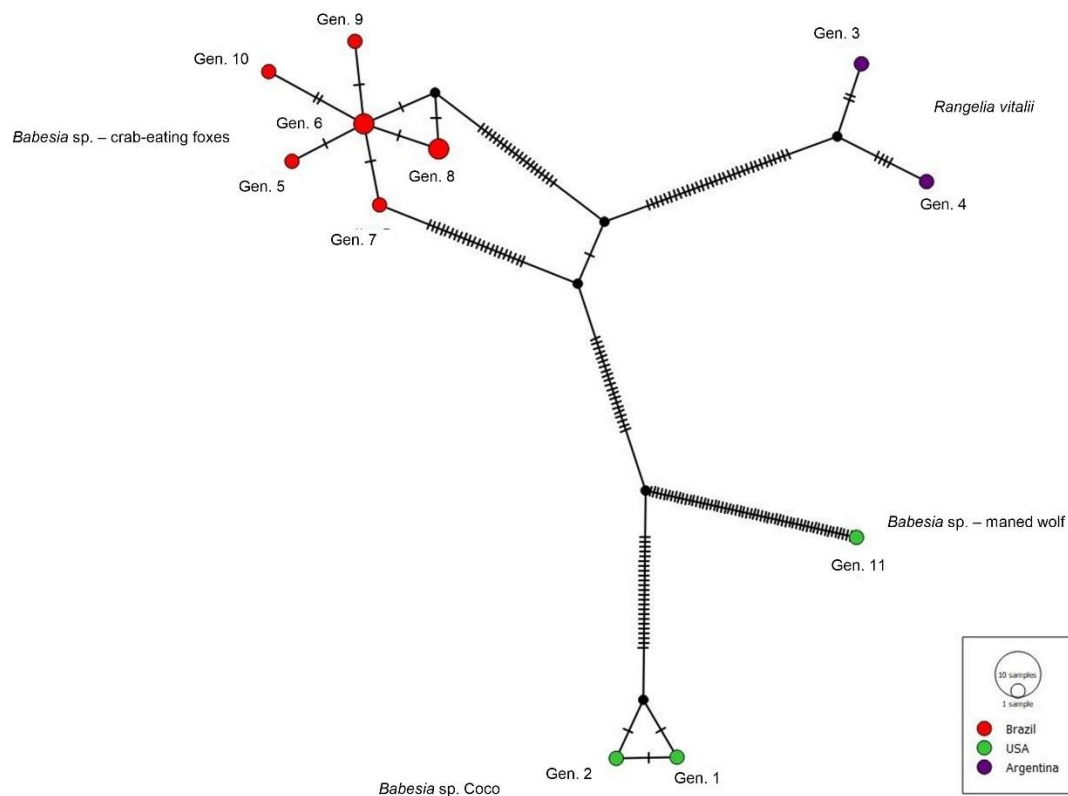


**Figure 6.** TCS network formed among *Babesia* spp. 18S rRNA sequences (1,344 bp) detected in wild and domestic canids. The size of the circles varies according to the number of sequences belonging to each genotype. Each color represents the location where each sequence was detected. The black vertical lines represent the mutational events that occurred between each genotype and the black circles represent median vectors.

### 3.3. *Hepatozoon* spp.

Eleven (91.67%) *C. thous* blood DNA samples were positive in the PCR for *Hepatozoon* spp. based on 18S rRNA (including the three animals positive for piroplasmids, indicating co-positivity for the studied agents) according to the protocol described by Perkins and Keller (2001) [70]. Eight out of 11 obtained amplicons were sequenced. None sample was positive in the PCR based on the Ujvari et al. (2004) protocol [71]. Of the eleven samples tested for the two protocols that amplify a large fragment of the *Hepatozoon* 18S rRNA gene, 7 (63.64%) and 3 (27.27%) were positive for the protocol that amplifies up to 1,200 bp [71, 72] and that one that amplifies up to 1,700 bp [46], respectively. Due to the intensity of the bands in the agarose gel electrophoresis, it was possible to sequence three samples for the first protocol and one sample for the second one (**Table 1**).

The BLASTn analysis showed that the short 18S rRNA sequences had identities ranging from 99.87 to 100% with *Hepatozoon* spp. detected in *C. thous* sampled in the state of Mato Grosso do Sul, midwestern Brazil (**Supplementary material 2**). The large sequences had an identity of approximately 98.9% with *Hepatozoon* sp. detected in a dog from Uruguay. Meanwhile, the largest sequence obtained (1,588 bp) had an identity of 97.66% with *Hepatozoon* sp. 'Curupira 2' detected in *C. thous* from Brazil (**Supplementary material 2**).

The ML phylogenetic analysis (991 bp alignment and TPM2u+I+G evolutionary model) positioned the short 18S rRNA sequences detected in this study in the same clade with *Hepatozoon* sp. sequences previously detected in *C. thous* sampled in the same Brazilian state, with a bootstrap of 93%. This clade was phylogenetically related to sequences of *Hepatozoon* sp. detected in *L. gymnocercus*, *Hepatozoon* sp. 'Curupira 2' and *H. americanum* (**Figure 7**). The ML analysis of the large 18S rRNA sequences (1,664 bp and TVM+I+G evolutionary model) corroborated the analysis of the short sequences, positioning the sequences obtained from *C. thous* in this study in the same clade, with a bootstrap of 93%, phylogenetically related to *Hepatozoon* sp. 'Curupira 2', *Hepatozoon* sp. detected in other canids in South America and *H. americanum* (**Figure 8**). The sequences previously detected in *C. thous* from the same state as this study were not added to this analysis due to their short size. Since animal #26 was positive for both protocols that amplified large fragments of the 18S rRNA gene, the largest consensus sequence was used in the phylogeny.

The pairwise distance analysis showed a divergence ranging from 0 to 0.63% between the *Hepatozoon* 18S rRNA large sequences obtained from *C. thous* in this study. In addition, the genetic divergence between the sequences detected herein and those of *H. americanum* (AF176836), *Hepatozoon* sp. Curupira 2 (AY461377), *Hepatozoon* sp. detected in *L. gymnocercus* (MZ230034) and *Hepatozoon* sp. detected in a dog (OR814214) were 2.58 to 2.81%, 1.43 to 2.06%, 0.79 to 1.49 and 0.79 to 1.42%, respectively (**Figure 9**).



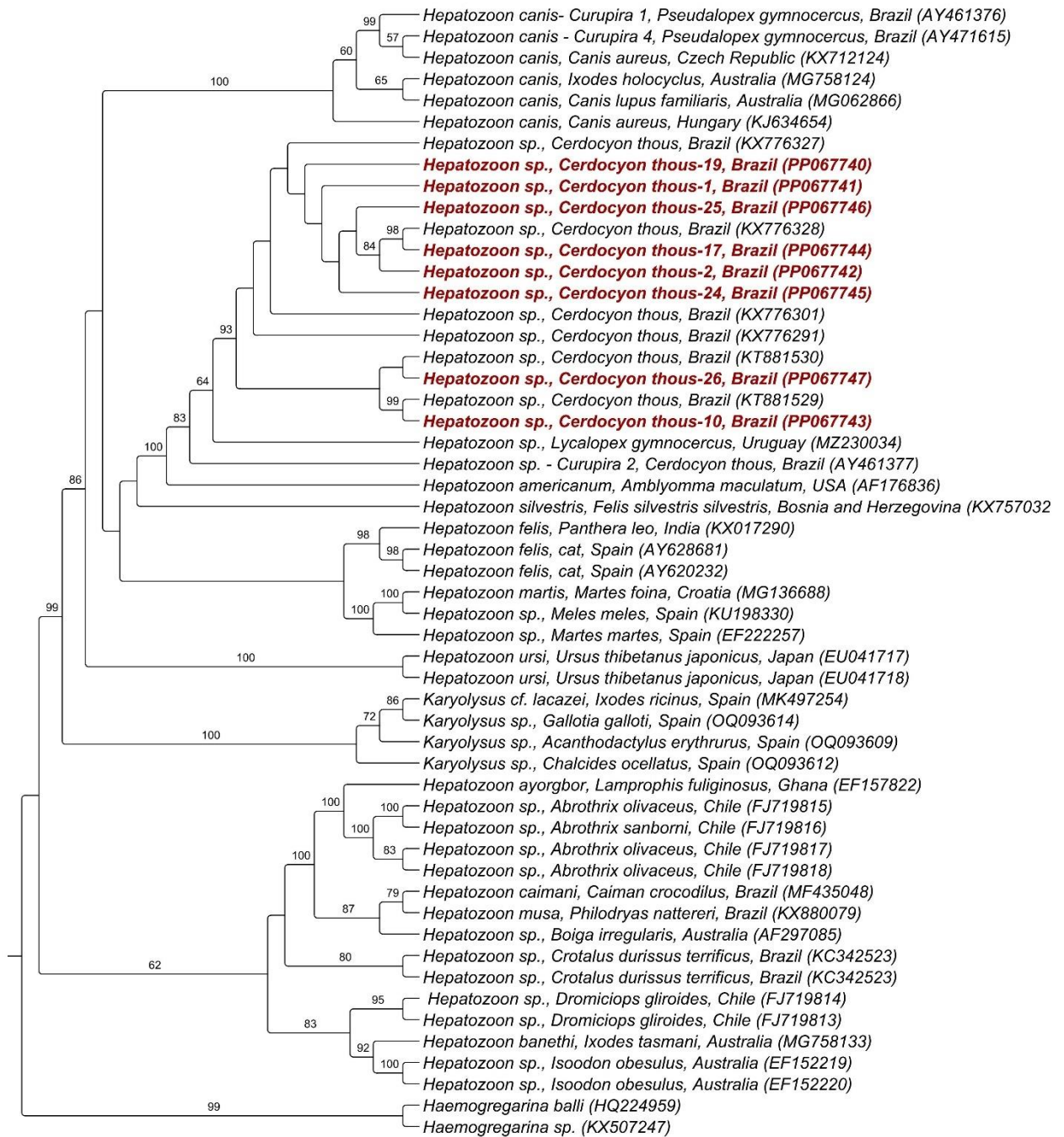


**Figure 7.** Phylogenetic analysis of *Hepatozoon* 18S rRNA short sequences from an alignment of 991 bp generated by Maximum Likelihood and with TPM2u+I+G evolutionary model. *Haemogregarina balli* and *Haemogregarina* sp. were used as an outgroup.

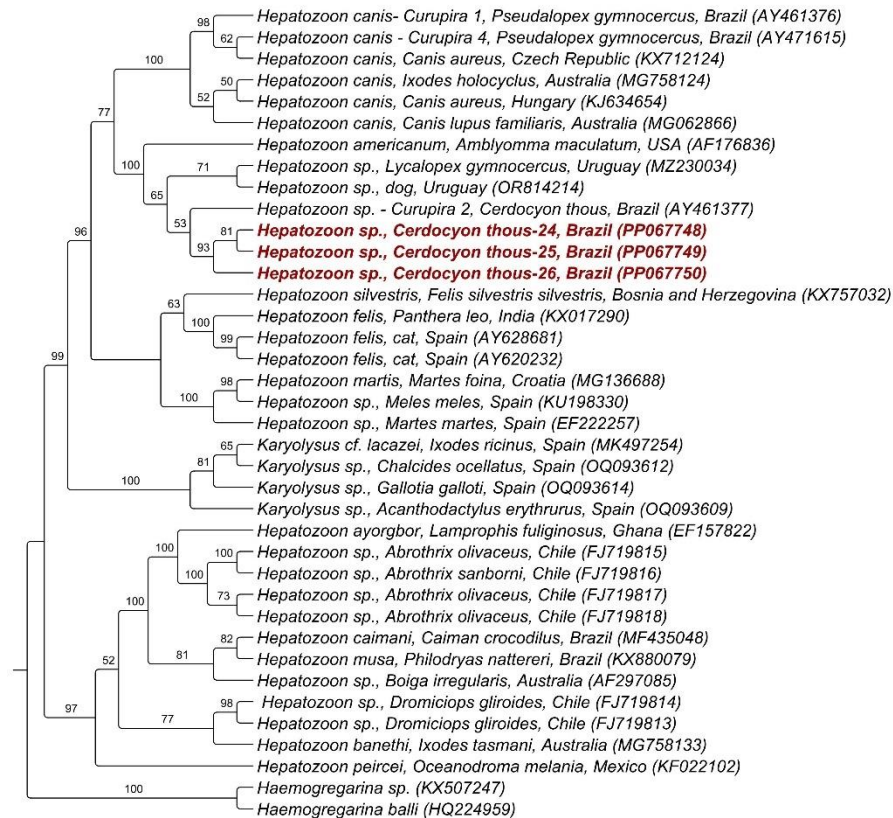


**Figure 8.** Phylogenetic analysis of *Hepatozoon* 18S rRNA large sequences from an alignment of 1,664 bp generated by Maximum Likelihood and with TVM+I+G evolutionary model. *Haemogregarina balli* and *Haemogregarina* sp. were used as an outgroup.



**Figure 9.** A heatmap constructed using the distance matrix based on *p*-value between *Hepatozoon* spp. 18S rRNA sequences detected in *Cerdocyon thous* and the phylogenetically related sequences. The numbers represent the percentage of divergence between the sequences.

### 3.4. Description of the new species

Family Babesiidae Poche, 1913

Genus *Babesia* Starcovici, 1893

***Babesia pantanalensis* nov. sp. Calchi and André**

**Type host:** crab-eating foxes (*Cerdocyon thous*) (Mammalia: Carnivora)

**Type locality:** Miranda (56° 36' W, 20° 05' S), Mato Grosso do Sul, Brazil

**Type material:** crab-eating foxes' blood and the genomic DNA extracted are stored in VBBL, UNESP, Jaboticabal, São Paulo, Brazil.

**DNA sequences:** DNA sequences amplified from the type material were deposited in GenBank under the accession numbers PP067561-PP067568 for 18S rRNA, PP082974-PP082976 for *cox-1* gene and PP357051 for *hsp70* gene.

**Vectors:** Unknown. It is assumed to be a local species of hard (Ixodida) tick.

**Etymology:** the new species is named *pantanalensis* in reference to Pantanal biome where the type of material was found.

**Zoobank registration:** Registration numbers will be added after the manuscript acceptance.

#### 4. Discussion

The present study reinforces the role of the crab-eating fox in the maintenance of piroplasmids in the wild environment. This result is consistent with the reported occurrence of these agents in wild canids by most studies performed in Brazil (range 18.18 to 26%) [7, 33, 34]. Conversely, a study also performed in the Pantanal region of the state of Mato Grosso do Sul [35] reported a low occurrence of piroplasmids (1.2% [1/78]) in *C. thous*. Previous studies performed in other countries, such as Iraq [62], Israel [63], Slovakia [64], Spain [65], Portugal [66], Italy [67], Hungary [68], China [69], South Africa [70], USA [71, 72] and Uruguay [73] reported piroplasmid occurrence ranging from 4.8% to 69.2% in the wild canids analyzed.

To date, nine different species of piroplasmids have been described circulating in canids: *Babesia canis* Piana and Galli-Valerio, 1895; *Babesia vogeli* Reichenow, 1937; *Babesia rossi* Nuttall, 1910; *Babesia gibsoni* Patton, 1910; *Babesia* sp. 'Coco' Birkenheuer et al., 2004; *Babesia conradae* Kjemtrup et al., 2006; *Babesia vulpes* (Baneth et al., 2015) (previously named *Babesia microti*-like and *Theileria annae*); *Babesia negevi* Baneth et al., 2020; and *Rangelia vitalii* (Pestana, 1910) [74-76]. At the molecular level, while most of them are grouped into the *Babesia sensu stricto* clade (including *R. vitalii*), *B. conradae* and *B. negevi* are grouped into the Western group, and *B. vulpes* in the *Babesia sensu lato* clade [74,77].



Strikingly, the large 18S rRNA sequences of piroplasmids detected in *C. thous* in this study were positioned within the *Babesia* s. s. clade, albeit grouping in a new sub-clade and closely related to *R. vitalii*. In addition, the BLASTn analysis showed low identities (95 to 96%) between the 18S rRNA sequences detected in *C. thous* and *R. vitalii*, strengthening the evidence that this is a putative novel *Babesia* species. When analyzing the genetic divergences between the Piroplasmida 18S RNA sequences detected in this study and those that were phylogenetically related, it was possible to notice that even when using the 18S rRNA gene, which is highly conserved, high divergences between the sequences were found (ranging from 4.17 to 4.76% with *R. vitalii*, 5.25 to 5.62% with *Babesia* sp. 'Coco' and 8.37 to 8.76% with *Babesia* sp. detected in a maned wolf). These percentages of genetic divergence corroborate with values reported for closely related but distinct canid-associated *Babesia* species, such as *B. vogeli* and *B. canis* (divergence of 1.60 to 2.43%), *B. rossi* and *B. vogeli* (5.31 to 5.57%), *B. rossi* and *B. canis* (4.77 to 5%) (data not shown). Finally, the genotype network clearly separated the distinct canid-associated *Babesia* spp. sensu stricto analyzed (*R. vitalii*, *Babesia* sp. 'Coco', *Babesia* sp. detected in *C. brachyurus* and *Babesia* sp. detected in *C. thous* herein), based on several mutational events and origins from median vectors. Additionally, the 18S rRNA-based genotype analysis showed the occurrence of different *Babesia* genotypes circulating in crab-eating foxes from Mato Grosso do Sul, Brazil, with low genetic divergences ranging from 0 to 0.22%.

In addition to the 18S rRNA gene, Piroplasmida *cox-1* amino acid sequences obtained herein from *C. thous* were phylogenetically associated with *R. vitalii* and *Babesia* sp. 'Coco'. BLASTn analysis showed low identity with both species, approximately 95% with *R. vitalii* (data not shown) and 91% with *Babesia* sp. 'Coco'. The pairwise distance analysis showed a divergence of 4.91 to 5.02 and 9.17 to 9.25% with each of them, respectively. The *hsp70* sequence obtained in the present work was also positioned closely to *R. vitalii*, with a low identity by BLASTn (94.29%) and a high divergence (6.19%) between both sequences. These results corroborate the findings for the 18S rRNA gene and reinforce the evidence of a novel *Babesia* species.

*Rangelia vitalii* is a piroplasmid restricted to South America, occurring mainly in the south and southeast regions of Brazil, Argentina, Uruguay, and Paraguay [33, 78-81], due to the presence of the vector *Amblyomma aureolatum* Pallas, 1772 [82]. This

Piroplasmida species is responsible for causing a severe disease in domestic dogs [33]. *Cerdocyon thous* is considered the most common host of *A. aureolatum* and *R. vitalli*'s reservoir [33, 83]. In Brazil, *A. aureolatum* occurs in the northeastern (Southern Bahia State), southeastern and southern regions in high-altitude areas within the Atlantic Forest and Pampa biomes [83]. This study was carried out in the Central West region of Brazil (Mato Grosso do Sul State), in the Pantanal biome, where this tick species, and consequently *R. vitalli*, do not occur, reinforcing the evidence that the species found is a novel *Babesia* species.

The lack of amplification of three additional molecular markers (*cox-3*, *cytB* and ITS-1) might be related to the low sensitivity of the PCR protocols used herein when dealing with low parasitemia, which was assumed to be the case of the present study. Alternatively, the primers used might have been unable to annealing to the target mitochondrial (*cox-3* and *cytB*) genes and intergenic region of the novel *Babesia* species described herein.

Concerning *Hepatozoon* spp. in wild canids, this study showed a high level of positivity in the animals sampled (91.67% [11/12]). This result corroborates the results reported by de Sousa et al. (2017) [26], who found 91% (71/78) positivity for *Hepatozoon* spp. among *C. thous* sampled in the Pantanal of Mato Grosso do Sul. Other studies carried out in other states of Brazil have also shown a high occurrence of *Hepatozoon* spp. among free-living crab-eating foxes (50 and 76.92%) [23, 25]. In contrast, a study carried out by André et al. (2010) [24] on wild canids kept in captivity found a low occurrence (only 5% [5/100]), which demonstrates the difference of infection between free-living and captive animals, likely associated with the use of ectoparasiticides in wild canids maintained in captivity in Brazilian zoos.

The phylogenetic analysis based on the 18S rRNA gene positioned the short sequences detected in *C. thous* sampled in Miranda, Mato Grosso do Sul, in the same clade as *Hepatozoon* sp. detected in the same host and in the same state by de Sousa et al. (2017) [26]. These genotypes showed to be closely related to *Hepatozoon* sp. 'Curupira 2' [23], *Hepatozoon* sp. detected in *L. gymnocercus* in Uruguay [29] and *H. americanum*, demonstrating the circulation of the same species among crab-eating foxes in the region. The phylogenetic inference performed with the large sequences of the 18S rRNA gene corroborated with that one based on short sequences and

positioned the sequences detected in this study in a single clade phylogenetically related to the *Hepatozoon* genotypes cited above.

Pairwise distance analysis showed low genetic divergence between the *C. thous*-associated sequences and those detected in *L. gymnocercus* and dog from Uruguay, with a range of 0.79 to 1.49% and 0.79 to 1.42%, respectively. On the other hand, higher genetic divergence (1.43 - 2.06%) was found between 18S rRNA *Hepatozoon* sequences detected herein and *Hepatozoon* sp. (Curupira-2 genotype also detected in *C. thous* samples from Rio Grande do Sul, Brazil). Nonetheless, higher genetic divergence was found between sequences detected in the present study when compared to *H. americanum* (2.58 to 2.81%). In addition, *H. americanum* showed a genetic divergence of 3.06 and 1.70% with *Hepatozoon* sp. Curupira-2 and *Hepatozoon* detected in canids from Uruguay, respectively. When comparing the divergence between *H. canis* sequences, the variation was between 0.07 and 1.15% (data not shown). The divergence between *H. americanum* and *H. canis* sequences ranged from 3.88 to 5.08% (data not shown). This indicates that a putative novel species related to *H. americanum* is probably circulating among canids in South America.

Previous studies carried out in South America have detected genotypes phylogenetically related to *H. americanum* in wild canids (*C. thous*, *C. brachyurus*, the South American grey fox [*Lycalopex griseus* (Gray, 1837)] and *L. gymnocercus*) [18, 23-27, 29]. Additionally, *H. canis* has already been detected in *C. thous*, *L. gymnocercus* and *C. brachyurus* from Brazil [23, 36]. While Almeida et al. (2013) [25] detected a genotype related to *Hepatozoon* sp. detected in reptiles in *C. thous* from Brazil, Millán et al. (2019) [27] detected a genotype related to *H. felis* circulating in South American grey foxes from Argentina.

Finally, the present study showed co-positivity by *Babesia* sp. and *Hepatozoon* sp. in three (25%) out of 12 sampled *C. thous*. Coinfection by piroplasmids and *Hepatozoon* has already been described in *V. vulpes* from Italy and Spain, where 12.41% (19/153) and 34% (30/89), respectively, of the animals were positive for *H. canis* and *B. vulpes* [65, 67, 25]. In Brazil, coinfection between *R. vitalii* and *Hepatozoon* sp. has already been described in *L. gymnocercus* [85] and *C. brachyurus* [19] in the states of Santa Catarina and Minas Gerais, respectively. The latter animal

presented severe anemia with jaundice and high parasitemia by *R. vitalii* observed in the cytoplasm of the endothelial cells of the small intestine [19]. The co-positivity by *Babesia* sp. and *Hepatozoon* sp. reported here reinforces that crab-eating fox may be considered a bio-accumulator of hemoparasites in the wild environment.

A limitation of this study was the lack of morphological characterization of the novel *Babesia* described. Similarly, a recent study described three new species of *Cytauxzoon* in European wild felids using only molecular data from mitochondrial and nuclear genes [86]. Although the description of morphological and morphometric features of piroplasmids are desirable, robust molecular data alone can be enough to describe a novel Piroplasmida species. The present study succeeded in amplifying the near-complete 18S rRNA gene, as well as a mitochondrial gene (*cox-1*) and the *hsp70* gene (which encodes the heat shock protein 70). The phylogenetic inferences based on three distinct molecular markers corroborated to each other and supported the unique phylogenetic positioning of this novel Piroplasmida.

## 5. Conclusion

The present study showed that crab-eating fox has a role in the maintenance of piroplasmids and *Hepatozoon* sp. at Pantanal of Mato Grosso do Sul, midwestern Brazil. Coinfection by both agents is reported for the first time in crab-eating foxes strongly suggesting that this wild canid species potentially acts as a bio-accumulator of hemoprotozoan. Phylogenetic, distance and genotype analyses based on three distinct genetic markers (near-complete 18S rRNA, *cox-1* and *hsp70*) supported the description of a novel *Babesia* sp. (*Babesia pantanalensis* nov. sp.), which is phylogenetically related to *R. vitalii* and *Babesia* sp. *Coco* in *C. thous*. *Hepatozoon* sp. phylogenetically related to *H. americanum* and *Hepatozoon* sp. detected in domestic and wild canids in South America was reported in *C. thous* from the Brazilian Pantanal.

## Ethical approval

All animal procedures and management protocols were approved by Instituto Chico Mendes for Conservation of Biodiversity (SISBIO number 31895) and by the Ethics Committees on Animal Use of the Federal University of Paraíba (protocol

number 7478200418 - ID 000109) and the School of Agricultural and Veterinarian Sciences (FCAV/UNESP) (protocol number 9412/2022).

### **Credit authorship contribution statement**

**Ana Cláudia Calchi:** Conceptualization, Methodology, Investigation, Formal analysis, Writing – Original Draft, Writing – Review & Editing. **Laíza de Queiroz Viana Braga:** Resources, Investigation, Writing – Review & Editing. **Ricardo Bassini-Silva:** Investigation, Writing – Review & Editing. **Ana Carolina Castro-Santiago:** Investigation, Writing – Review & Editing. **Heitor Miraglia Herrera:** Resources, Writing – Review & Editing. **João Fabio Soares:** Investigation, Writing – Review & Editing. **Darci Moraes Barros-Battesti:** Writing – Review & Editing. **Rosangela Zacarias Machado:** Writing – Review & Editing. **Fabiana Lopes Rocha:** Resources, Writing – Review & Editing. **Marcos Rogério André:** Conceptualization, Methodology, Resources, Writing – Original Draft, Writing – Review & Editing, Supervision.

### **Conflict of interest**

The authors declare no conflict of interest.

### **Data availability**

The datasets generated and analyzed during the current study are available in the NCBI Genbank Nucleotide platform (<https://www.ncbi.nlm.nih.gov/genbank/>) and can be accessed through accession numbers: PP067561-PP067568 and PP067740-PP067750 for 18S rRNA gene (piroplasmids and *Hepatozoon* sp., respectively), PP082974-PP082976 for *cox-1* gene and PP357051 for *hsp70* gene.

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## **CAPÍTULO 4\* - Genetic diversity of *Theileria* spp. in deer (Artiodactyla: Cervidae) from Brazil\***

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### **Abstract**

*Babesia* spp. and *Theileria* spp. are tick-borne apicomplexan protozoa that can cause disease in animals and humans. Deer are considered reservoirs for a wide variety of Piroplasmida species, including some potentially zoonotic. This study aimed to investigate the occurrence and genetic diversity of piroplasmids in wild deer sampled in four Brazilian states (São Paulo, Mato Grosso do Sul, Paraná and Goiás). For this purpose, extracted DNA samples from 181 deer buffy coat samples (138 *Blastocercus dichotomus*, 26 *Subulo gouazoubira*, 4 *Mazama jucunda*, 3 *Mazama rufa* and 10 *Ozotocercus bezoarticus*) were subjected to a nested PCR (nPCR) assay based on the 18S rRNA gene in order to perform a screening for piroplasmids and characterized based on the near-complete 18S rRNA, *hsp70* and *cox-3* genes. As a result, 75.14% (136/181) samples were positive for piroplasmids. Of these, 108 (79.41%), 101 (74.26%) and 67 (49.26%) were positive to near complete 18S rRNA, *hsp70* and *cox-*

3 genes, respectively. Phylogenetic analyses based on three molecular markers showed similar topology to each other. All sequences obtained in the present study were positioned into the *Theileria* sensu stricto clade, forming a distinct clade, albeit close to *T. cervi*. Most sequences grouped together into a large clade divided into subclades, which were often related to deer genus/species, showing that *Theileria* lineages seemed to show specificity according to deer genus/species. Two 18S rRNA sequences (one obtained from *S. gouazoubira* and another from *M. jucunda*) were positioned into a different clade, apart from other sequences detected in this study, indicating that different species of *Theileria* occur in deer from Brazil. Two subclusters were observed in the phylogenetic analysis based on the *hsp70* gene: the first containing only sequences detected in marsh deer and the second grouping sequences detected in brocket deer (*Mazama* spp. and *S. gouazoubira*). The latter was also divided into smaller clades that grouped *Theileria* genotypes according to deer species (*M. jucunda*, *M. rufa* and *S. gouazoubira*). This study provides the first molecular evidence of *Theileria* infection in *M. jucunda*, as well as co-infection by distinct *Theileria* (sub)species/genotypes in the same deer was evidenced. Finally, this study expanded the knowledge on the diversity of *Theileria* spp. infecting deer from South America.

**KeyWords:** marsh deer, brocket deer, protozoa, molecular diagnosis, phylogeny, genetic diversity

## Introduction

Deer (Artiodactyla: Cervidae) are medium to large herbivorous ruminants distributed on all continents, except Antarctica, Africa, and Oceania (Azevedo et al., 2021; Sandoval et al., 2022). They are ecologically important because they are prey for different species of carnivores, act in seed dispersal and soil movement (Peres et al., 2021a). In addition, these mammals play a role as hosts for different pathogens and different ectoparasite species such as tick, lice and flies (Böhm et al., 2007; Guglielmone et al., 2014; Shin et al. 2020; Wijburg et al., 2022). Brazil has a wide variety of neotropical deer species distributed across all biomes: the marsh deer, *Blastocerus dichotomus* (Illiger, 1815); the red brocket deer, *Mazama americana* (Erxleben, 1777); the southern red brocket deer, *Mazama rufa* (Illiger 1815); the small red brocket deer, *Mazama jucunda* (Thomas, 1913); the Brazilian dwarf brocket deer, *Mazama nana* (Hensel, 1872); the Pampas deer, *Ozotoceros bezoarticus* (L., 1758); the white-tailed deer, *Odocoileus virginianus* (Zimmermann, 1780); the Amazonian brown brocket deer, *Passalites nemorivagus* (Cuvier, 1817); and the gray brocket deer, *Subulo gouazoubira* (Azevedo et al., 2021; Mantellatto et al. 2021; Peres et al., 2021b;

Bernegossi et al., 2023; Morales-Donoso et al., 2023). According to the Red List of threatened species by the IUCN (International Union for Conservation of Nature), *O. bezoarticus* is considered a near threatened species, while *B. dichotomus*, *M. jucunda* and *M. nana* are considered vulnerable and the others as least concern or data deficient (IUCN, 2024).

*Babesia* spp. and *Theileria* spp. are tick-borne apicomplexan protozoa belonging to the Order Piroplasmida (Schnittger et al., 2012). While *Babesia* spp. infect only erythrocytes, with transovarial and transstadial transmission in ticks, *Theileria* spp. infect leukocytes and erythrocytes, and present only transstadial transmission in ticks (Jalovecka et al., 2018, 2019). Several piroplasmid species have been described infecting and potentially causing diseases in domestic and wild animals as well as humans (Alvarado-Rybak et al., 2016; Jalovecka et al. 2019; Karshima et al., 2022).

Different Piroplasmida species, including zoonotic species, have already been detected in deer, such as *Babesia odocoilei* (Holman et al., 2003; Mathieu et al., 2018; Calvente et al., 2021), *Babesia capreoli* (Tampieri et al., 2008; Zanet et al., 2014; Remesar et al., 2019; Kogler et al., 2021), *Babesia divergens* (García-Sanmartin et al., 2007; Gray et al., 2021; Kogler et al., 2021), *Babesia venatorum* (Zanet et al., 2014; Remesar et al., 2019), *Babesia bovis* (García-Vasquez et al., 2015), *Babesia bovis*-like (Ramos et al., 2010), *Babesia bigemina* (Cantu et al., 2007; Zanet et al., 2014; Remesar et al., 2019), *Babesia pecorum* (Jouglin et al., 2014), *Theileria cervi* (Chae et al. 1999; Olafson et al., 2020; Pavón-Rocha et al., 2020), *Theileria capreoli* (Hornok et al., 2017; Wang et al., 2019; Chen et al., 2024), *Theileria capreoli*-like (also known as *Theileria* sp. Sika 2) (He et al., 2012; Lee et al., 2019; Masatani et al., 2020), *Theileria annulata* (Liu et al., 2016), *Theileria luwenshuni* (Li et al., 2015; Han et al., 2017; Chung et al., 2023), *Theileria* sp. Thrivae (also known as *Theileria cervi*-like or *Theileria* sp. Sika 1) (He et al., 2012; Shibata et al., 2018; Lee et al., 2019), *Theileria* sp. OT3 (García-Sanmartin et al., 2007; Remesar et al., 2019) and *Theileria* sp. 3185/02 (García-Sanmartin et al., 2007; Remesar et al., 2019). Recently, a putative new species of *Babesia* spp. was described in the Southern Pudu, *Pudu puda* (Molina, 1782), from Chile (Santodomingo et al., 2022). Notably, *B. odocoilei*, *B. capreoli*, *B. divergens*, *B. venatorum* and *B. bigemina* have already been reported in humans (Kumar et al., 2021; Karshima et al., 2022).

In the American continent, *T. cervi* has been widely reported in cervids in the USA and Canada (Almazán et al., 2022). Additionally, some reports have also evidenced the occurrence of this *Theileria* species in Mexico, Brazil, and Argentina (da Silveira et al., 2011; 2013; Orozco et al., 2020; Pavón-Rocha et al., 2020; Sebastian et al., 2022). The white-tailed deer and the lone star tick, *Amblyomma americanum* (L., 1758) have been considered the main reservoir and tick vector, respectively, of *T. cervi* in North America (Kutler et al., 1967; Goddard et al., 2009). *Theileria cervi* shows low pathogenicity, albeit clinical signs such as fever, anemia, weakness, pale membranes, jaundice, liver failure and death have been described in deer under poor nutrition, co-infected with other parasites, presenting other associated diseases, exposed to stress factors, or translocated from non-endemic to endemic areas (Yabsley et al., 2005; Wood et al., 2013; Haus et al., 2018; Sebastian et al., 2022).

To date, few studies on the occurrence and diversity of piroplasmids in deer have been carried out in Brazil. *Theileria cervi* was detected in *B. dichotomus*, *M. americana*, *O. bezoarticus* and *S. gouazoubira* in the states of Minas Gerais (da Silveira et al., 2011), Mato Grosso do Sul (da Silveira et al., 2013) and Pará (Soares et al., 2017); *Theileria* sp. was detected in *S. gouazoubira* from Minas Gerais (da Silveira et al., 2011); *B. bigemina* was reported in *S. gouazoubira* and *O. bezoarticus* from Minas Gerais (da Silveira et al., 2011) and Mato Grosso do Sul (da Silveira et al., 2013), *B. bovis* was detected in *B. dichotomus* from Minas Gerais (da Silveira et al., 2011). In order to shed light on the diversity of piroplasmids in deer from Brazil, the present study aimed to investigate the occurrence and genetic diversity of these protozoa in deer sampled in four Brazilian states.

## **Materials and Methods**

### **Studied region and deer species sampled**

#### ***Blastocerus dichotomus* sampling**

One hundred and thirty-eight marsh deer were captured in an area close to the Porto Primavera hydroelectric power plant, located on the Paraná River, alongside the Aguapeí and Paranapanema rivers, in a location between the states of São Paulo and Mato Grosso do Sul. Sampling was performed into four stages: (1) MS01 (n= 99),

sampling performed in June, July and October 1998, before the first flooding in November 1998, in Mato Grosso do Sul State, between the cities of Anaurilândia and Bataguassu; (2) MS02 (n=14), sampling performed in the same locality, but in February 2001, after the flooding; (3) PX (n=9), sampling performed in November 1998 and December 2000 in the region of the Peixe River, São Paulo State; (4) AGUA (n=16), sampling performed from April 2001 to November 2002 alongside the Aguapeí River, São Paulo State (**Figure 1**).

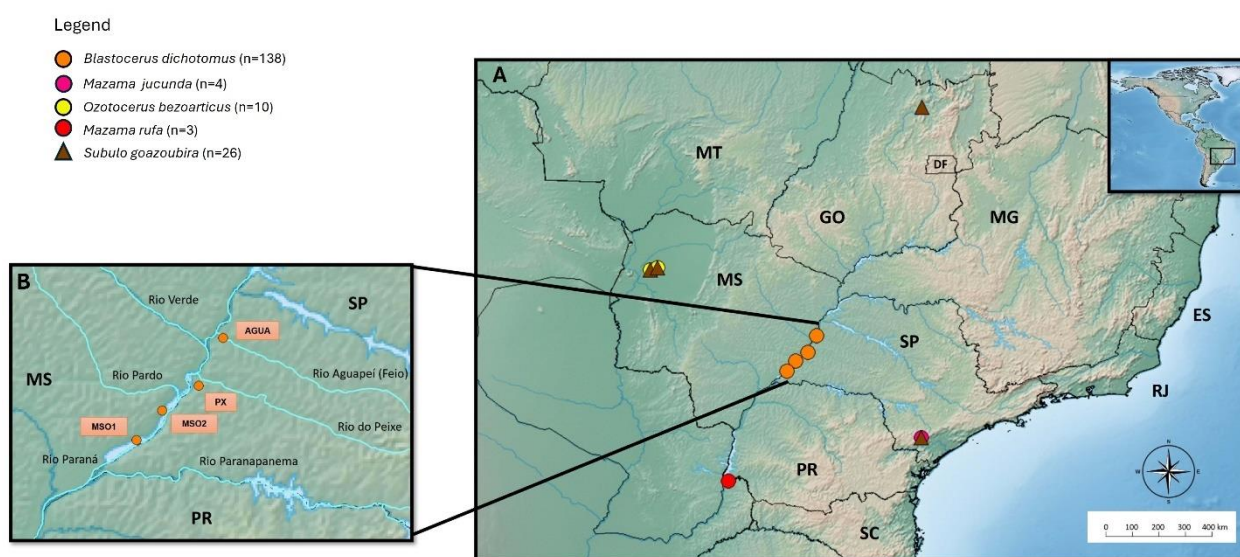


**Figure 1.** Collection site of the deer blood samples. **A** represents the capture and collection sites of all deer species. **B** represents the capture and collection sites and groups of *Blastocerus dichotomus*. SP= São Paulo; MS= Mato Grosso do Sul; GO= Goiás; MT= Mato Grosso; PR= Paraná; SC= Santa Catarina; MG= Minas Gerais; ES= Espírito Santo; RJ= Rio de Janeiro.

### ***Subulo gouazoubira*, *Mazama* spp. and *O. bezoarticus* sampling**

Deer blood samples were collected between 1996 and 2011 in different states of Brazil. In total, twenty *S. gouazoubira* and ten *O. bezoarticus* were captured in the Nhecolândia Pantanal, Mato Grosso do Sul State; four *S. gouazoubira* in the Serra da Mesa hydroelectric region, Goiás State; four *M. jucunda* and two *S. gouazoubira* in the Intervalles State Park, São Paulo State; and three *M. rufa* were captured in the Iguaçu National Park, Paraná State (**Figure 1**).

### **Blood collection**

After capturing the animals, they were chemically immobilized as described by Nunes et al. (2001) and blood was then collected by jugular venipuncture using vacutainer-type tubes with heparin. The tubes with the blood were centrifuged at 1,500 x *g* for 1 minute and the buffy coat with a small number of erythrocytes were aliquoted into a vial containing 4 mL of freezing medium (Hams F10, DMSO, PVP, Fetal Calf Serum), homogenized and kept at 4°C for four hours, after which the vials were submerged for 30 minutes in liquid nitrogen, followed by immersion and storage in the liquid nitrogen canister (-196°C) following the methodology described by Duarte et al. (1999). These samples were kindly provided to our research group for molecular assays for hemoparasites.

### **DNA extraction and PCR assay for mammalian endogenous gene**

DNA was extracted from 200 µL of buffy coat with a small number of erythrocytes samples using the Biopur Mini Spin Plus Kit (MoBius Life Science, PR, Brazil), according to the manufacturer's instructions. To verify the presence of amplifiable DNA, all samples were subjected to a PCR assay based on the mammalian endogenous glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) gene (Birkenheuer et al., 2003). Only the positive samples were subjected to specific PCR assays for piroplasmids. DNA samples were registered under SISGEN #A58995B ("Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado").

### **Molecular detection and characterization of piroplasmids**

DNA samples were screened for piroplasmids by a nested PCR (nPCR) assay based on the 18S rRNA gene (800 bp) (Jefferies et al., 2007). In addition, all samples were subjected to nPCR assays for *B. bovis* and *B. bigemina* targeting the *cytB* (195 bp) (Romero-Salas et al., 2016) and *rap-1a* (412 bp) (Terkawi et al., 2011) genes, respectively.

Positive samples for the 18S rRNA gene were characterized using different molecular markers: near-complete 18S rRNA gene (~1,500 bp) (Greay et al., 2018), *cox-1* (~800 bp) (Corduneanu et al., 2017), *cox-3* (~700 bp) (Schreeg et al., 2016;

Barbosa et al., 2019) and *hsp70* (~700 bp) (Soares et al., 2011). Primer sequences and thermal conditions are described in **Table 1**.

**Table 1.** Description of primers, amplicon sizes and thermal sequences used in conventional and nested PCR assays for detection and characterization of piroplasmids.

| Agent                                     | Gene/ name primer              | Primers sequences                   | Size (bp) | Thermal sequences                                                       | Reference                  |
|-------------------------------------------|--------------------------------|-------------------------------------|-----------|-------------------------------------------------------------------------|----------------------------|
| Endogenous gene                           | <i>gapdh</i>                   | 5'-TCCAACACCACCACTGAGATCGGAC -3'    | 400       | 95°C for 5 min;                                                         | Birkenheuer et al. (2003)  |
|                                           | -GAPDHF<br>-GAPDHR             | 5'-GTGAGAAGAAATCGGACTGGCC-3'        |           | 35 cycles: 95°C for 15s, 50°C for 30s and 72°C for 30s;                 |                            |
| Piroplasmids (screening -nPCR)            | 18S rRNA                       | 1 <sup>st</sup> Round               | 800       | 72°C for 5 min                                                          | Jefferies et al. (2007)    |
|                                           | 1 <sup>st</sup> Round          | 5'-GGCTCATTACAACAGTTATAG-3'         |           | 94°C for 3 min, 58°C for 1 min, 72°C for 2 min;                         |                            |
|                                           | -BTF1                          | 5'-CCCAAAGACTTTGATTTCTCTC-3'        |           | 45 cycles: 94°C for 30s, 58°C for 20s and 72°C for 30s;                 |                            |
|                                           | -BTR1                          | 2 <sup>nd</sup> Round               |           | 72°C for 7 min                                                          |                            |
|                                           | 2 <sup>nd</sup> Round          | 5'-CCGTGCTAATTGTAGGGCTAATAC-3'      |           | Annealing temperature 2 <sup>nd</sup> round =62°C                       |                            |
|                                           | -BTF2<br>-BTR2                 | 5'-GGACTACGACGGTATCTGATCG-3'        |           |                                                                         |                            |
| <i>Babesia bovis</i> (screening -nPCR)    | <i>cytB</i>                    | 1 <sup>st</sup> Round               | 195       | 95°C for 3 min;                                                         | Romero-Salas et al. (2016) |
|                                           | 1 <sup>st</sup> Round          | 5'-TGAACAAAGCAGGTATCATAGG-3'        |           | 30 cycles: 95°C for 30s, 59°C for 30s and 72°C for 30s;                 |                            |
|                                           | -oBb mit F                     | 5'-CCAAGGAGATTGTGATAATTCA-3'        |           | 72°C for 7 min                                                          |                            |
|                                           | -oBb mit R                     | 2 <sup>nd</sup> Round               |           | Annealing temperature 2 <sup>nd</sup> round =53°C                       |                            |
|                                           | 2 <sup>nd</sup> Round          | 5'-TCCACGATCTGTGATACGTCA-3'         |           |                                                                         |                            |
|                                           | -iBb mit F<br>-iBb mit R       | 5'-CAAATCCTTTGCAAACCTCCAA-3'        |           |                                                                         |                            |
| <i>Babesia bigemina</i> (screening -nPCR) | <i>rap-1a</i>                  | 1 <sup>st</sup> Round               | 412       | 95°C for 5 min;                                                         | Terkawi et al. (2012)      |
|                                           | 1 <sup>st</sup> Round          | 5'-GAGTCTGCCAAATCCTTAC-3'           |           | 35 cycles: 94°C for 1min, 55°C for 1min and 72°C for 1min;              |                            |
|                                           | -BbigRAP-1aF1                  | 5'-TCCTCTACAGCTGCTTCG-3'            |           | 72°C for 10 min                                                         |                            |
|                                           | -BbigRAP-1aR1                  | 2 <sup>nd</sup> Round               |           | Annealing temperature 2 <sup>nd</sup> round =66°C                       |                            |
|                                           | 2 <sup>nd</sup> Round          | 5'-AGCTTGCTTTCACAACTCGCC-3'         |           |                                                                         |                            |
|                                           | -BbigRAP-1aF2<br>-BbigRAP-1aR2 | 5'-TTGGTGCTTTGACCGACGACAT-3'        |           |                                                                         |                            |
| Piroplasmids (characterization)           | 18S rRNA                       | 5'-AAGCCATGCATGTCTAAGTATAAGCTTTT-3' | 1500      | 95°C for 5 min;                                                         | Greay et al. (2018)        |
|                                           | -Nbab_1F<br>-18SApiR           | 5'-GGATCACTCGATCGGTAGGAG-3'         |           | 50 cycles: 95°C for 30s, 60°C for 30s and 72°C for 2min; 72°C for 5 min |                            |
| Piroplasmids (characterization-nPCR)      | <i>cox-1</i>                   | 1 <sup>st</sup> Round               | 924       | 95°C for 1 min;                                                         | Corduneanu et al. (2017)   |
|                                           | 1 <sup>st</sup> Round          | 5'-ATWGGATTYTATATGAGTAT-3'          |           | 35 cycles; 95°C for 15s, 45°C for 30s and 72°C for 1 min                |                            |
|                                           | -Bab_for1                      | 5'-ATAATCWGGWATYCTCCTTGG-3'         |           | 72°C for 10 min                                                         |                            |
|                                           | -Bab_Rev1                      | 2 <sup>nd</sup> Round               |           | Annealing temperature 2 <sup>nd</sup> round =49°C                       |                            |
|                                           | 2 <sup>nd</sup> Round          | 5'-TCTCTWCATGGWTTAATTATGATAT-3'     |           |                                                                         |                            |
|                                           | -Bab_for2<br>-Bab_rev2         | 5'-TAGCTCCAATTGAHARWACAAAGTG-3'     |           |                                                                         |                            |



|                                            |              |                              |     |                                             |                                                   |
|--------------------------------------------|--------------|------------------------------|-----|---------------------------------------------|---------------------------------------------------|
| <b>Piroplasmids<br/>(characterization)</b> | <i>cox-3</i> |                              |     | 94°C for 5 min;                             | Schreeg et al.<br>(2016); Barbosa<br>et al.(2019) |
|                                            | -Cox3F       | 5'-ACTGTCAGCTAAAACGTATC-3'   | 700 | 50 cycles: 94°C for 20s, 55°C               |                                                   |
|                                            | -Cox3R       | 5'-ACAGGATTAGATACCCTGG-3'    |     | for 30s and 68°C por 45s;<br>72°C for 7 min |                                                   |
| <b>Piroplasmids<br/>(characterization)</b> | <i>hsp70</i> |                              |     | 95°C for 5 min;                             | Soares et al.<br>(2011)                           |
|                                            | -Hsp70F1     | 5'-CATGAAGCACTGGCCHTTCAA- 3' | 700 | 35 cycles: 95°C for 15s, 60°C               |                                                   |
|                                            | -Hsp70R1     | 5'-GBAGGTTGTTGTCCTTVGTCAT-3' |     | for 30s and 72°C for 30s;<br>72°C for 5 min |                                                   |

The PCR assays were performed using 5 µL of the DNA samples in a mixture containing 0.75 U Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA), 1 X PCR buffer (PCR buffer 10 X – 100 nM Tris-HCl, pH 9.0, 500 mM KCl), 0.2 mM deoxynucleotides (dATP, dTTP, dCTP, and dGTP) (Invitrogen, Carlsbad, CA, USA), 1.5 mM of Magnesium chloride (Invitrogen, Carlsbad, CA, USA), 0.5 µM of each primer (Invitrogen, Carlsbad, CA, USA), and sterile ultrapure water (Invitrogen, Carlsbad, CA, USA) comprising a total volume of 25 µL. In nPCR assays, 1 µL of the amplified product from the first PCR reaction was used as the target DNA in the second reaction. A DNA sample obtained from a dog experimentally infected with *Babesia vogeli* (Jaboticabal strain) (Furuta et al., 2009) and DNA samples obtained from cattle naturally infected with *B. bovis* (da Silva et al., 2013) and *B. bigemina* (da Silva et al., 2013) were used as positive controls. Sterile ultrapure water was used as negative control.

The PCR products were separated by horizontal electrophoresis on a 1% agarose gel stained with ethidium bromide (Life Technologies, Carlsbad, CA, USA) in TBE running buffer pH 8.0 at 100 V/150 mA for 50 min. The gels were examined under ultraviolet light illumination using ChemiDoc MP Imaging System (Bio Rad, Hercules, CA, USA) and photographed using Image Lab Software v.4.1 (Biorad, Hercules, CA, USA).

The amplified products that showed high band intensity on agarose gels were purified using Wizard SV gel and PCR clean-up system (Promega, Madison, WI, USA) and sequenced by Sanger's method using the BigDye Terminator v3.1 Cycle Sequencing kit (Thermo Fisher Scientific, Waltham, MA, USA) and the ABI PRISM 310 DNA Analyzer (Applied Biosystems, Foster City, CA, USA) at the "Centro de Recursos Biológicos e Biologia Genômica" (CREBIO/FCAV/UNESP).

## Cloning

Selected positive samples in the PCR protocols targeting the near-complete 18S rRNA, *hsp-70* and *cox-3* genes were subjected to pGEM-T Easy (Promega, Madison, WI, USA) cloning, according to the manufacturer's recommendations. Up to three clones were selected from each positive sample according to the blue/white colony system. The colonies containing the insert (confirmed by PCR) were subjected to plasmid DNA extraction using the Wizard® Plus SV Minipreps DNA Purification Systems (Promega, Madison, WI, USA). Plasmid DNA samples were submitted for sequencing with primers M13 F (5'-CGCCAGGGTTTCCCAGTCACGAC-3') and M13 R (5'-GTCATAGCTGTTTCCTGTGTGA-3') (Lau et al., 2010) that flank the multiple cloning site of the pGEM-T Easy plasmid. Internal primers for 18S rRNA (5'-CAACTAAGAACGGCCATGCACCA-3'), (5'-AAGACGAACTACTGCGAAAG-3') and (5'-AGGATAAACTCGATGAACGC-3') were designed and used for sequencing to obtain inner fragments of the sequences.

## BLASTn, phylogenetic and genotypes analyses

The obtained sequences were submitted to a quality-screening test using Phred-Phrap software version 23 (Ewing and Green, 1998; Ewing et al., 1998) to evaluate the quality of the electropherograms and to obtain the consensus sequences from the alignment of the sense and antisense sequences (only nucleotides with a Phred index above 20 were used). The BLASTn (Altschul et al., 1990) was used to compare the obtained sequences with previously deposited sequences in the GenBank database (Benson et al., 2002). The sequences were aligned with other homologous sequences of each gene retrieved from Genbank database, using the MAFFT software (Kato et al., 2019) and edited via Bioedit v. 7.0.5.3 (Hall, 1999). W-IQ-Tree software was used for choosing the evolutionary model following BIC criterion as well as for phylogenetic analysis inferred using the Maximum Likelihood method (available online: <http://iqtree.cibiv.univie.ac.at/>) (Trifinopoulos et al., 2016). Clade support indices were evaluated through bootstrap analyses of 1,000 repetitions. The phylogenetic trees were edited using Treegraph v. 2.0.56-381 beta software (Stover and Muller, 2010).

Additionally, genetic diversity analyses were also performed using the near-complete 18S rRNA alignment obtained only with *Theileria* spp. sequences detected in deer (sequences shorter than 1,329 bp were removed from the analysis), *hsp70* and *cox-3* alignments obtained with the sequences of the present study and those phylogenetically related by DnaSP v. 5 software to calculate the nucleotide diversity ( $\pi$ ), diversity of genotypes (Dg), number of genotypes (g) and the average number of nucleotide differences (K) (Librado and Rozas, 2009) and the genotype network was constructed by Population Analysis with Reticulate Trees (popART) v. 4.8.4 software (Leigh and Bryant, 2015), using the TCS network method (Clement et al., 2002).

## Results

DNA was extracted from 181 deer buffy coat samples collected in four Brazilian states. All samples were positive in the PCR assay based on the endogenous mammalian *gapdh* gene and were screened using a nPCR based on the 18S rRNA gene for piroplasmids, of which 136 (75.14%) were positive (101 *B. dichotomus*, 22 *S. gouazoubira*, 6 *O. bezoarticus*, 4 *M. jucunda* and 3 *M. rufa*). The number of positives per site and percentages are shown in **Table 2**. In addition, no sample was positive in the nPCR assays for *B. bovis* and *B. bigemina*.

**Table 2.** Number of positive animals for each molecular marker analyzed and their percentages according to the Brazilian states and the collections of each cervid species.

| Deer species (Brazilian state)          | Screening                              | Characterization                      |                   |                   |
|-----------------------------------------|----------------------------------------|---------------------------------------|-------------------|-------------------|
|                                         | 18S rRNA gene<br>(~800 bp<br>fragment) | 18S rRNA gene<br>(~1,500 bp fragment) | <i>hsp70</i> gene | <i>cox-3</i> gene |
| <i>Blastocerus dichotomus</i> (MS01-MS) | 69 (69.70%)                            | 53 (76.81%)                           | 47 (68.11%)       | 46 (66.67%)       |
| <i>Blastocerus dichotomus</i> (MS02-MS) | 14 (100%)                              | 11 (78.57%)                           | 11 (78.57%)       | 11 (78.57%)       |
| <i>Blastocerus dichotomus</i> (PX-SP)   | 9 (100%)                               | 9 (100%)                              | 9 (100%)          | 7 (77.78%)        |
| <i>Blastocerus dichotomus</i> (AGUA-SP) | 9 (56.25%)                             | 6 (66.67%)                            | 7 (77.78%)        | 3 (33.34%)        |
| <i>Subulo goazoubira</i> (MS)           | 19 (95%)                               | 19 (100%)                             | 16 (84.21%)       | 0                 |
| <i>Subulo goazoubira</i> (SP)           | 2(100%)                                | 2 (100%)                              | 1 (50%)           | 0                 |
| <i>Subulo goazoubira</i> (GO)           | 1 (25%)                                | 1 (100%)                              | 1(100%)           | 0                 |
| <i>Ozotocerus bezoarticus</i> (MS)      | 6 (60%)                                | 0                                     | 2 (33.33%)        | 0                 |
| <i>Mazama jucunda</i> (SP)              | 4 (100%)                               | 4 (100%)                              | 4 (100%)          | 0                 |
| <i>Mazama rufa</i> (PR)                 | 3 (100%)                               | 3 (100%)                              | 3 (100%)          | 0                 |
| Total                                   | 136                                    | 108                                   | 101               | 67                |

All 136 positive samples were submitted to molecular characterization assays based on different gene targets. Of these, 108 (79.41%) were positive for the 1,500 bp

fragment of the 18S rRNA gene (79 *B. dichotomus*, 22 *S. gouazoubira*, 4 *M. jucunda* and 3 *M. rufa*), 101 (74.26%) for the *hsp70* gene (74 *B. dichotomus*, 18 *S. gouazoubira*, 2 *O. bezoarticus*, 4 *M. jucunda* and 3 *M. rufa*) and 67 (49.26%) for the *cox-3* gene (67 *B. dichotomus*) (**Table 2**). All the samples were negative in the assay targeting the *cox-1* gene.

For the cloning, only amplicons showing high intensity unique bands in the agarose gel electrophoresis were selected. Twelve amplicons of the 18S rRNA gene (1,500 bp) were cloned. Amplicons of the *hsp70* (n=4) and *cox-3* (n=4) genes that showed multiple peaks in the electropherograms after sequencing were also cloned. As a result, 3 short 18S rRNA, 22 near-complete 18S rRNA, 12 *hsp70* and 11 *cox-3* readable sequences were obtained.

BLASTn analysis showed that the near-complete 18S rRNA gene sequences detected in marsh-deer had an identity ranging from 97.35 to 99.46% with *T. cervi* detected in the tick species *Dermacentor nitens* from the USA (MW008531). Most 18S rRNA gene sequences obtained from brocket deer had a higher identity (98.35 to 99.64%) with *T. cervi* detected in *Cervus elaphus canadensis* Erxleben, 1777 from the USA (AY735129). Near complete 18S rRNA gene sequences obtained from one *S. gouazoubira* and one *M. jucunda* had identities ranging from 99.05 to 99.11% with *Theileria* sp. from *O. virginianus* from the USA (AY735132). The *hsp70* sequences detected in marsh deer showed identities ranging from 85.36 to 95.95% with *T. orientalis* previously detected in cattle from Japan (XM\_009691871). The *hsp70* sequences detected in *S. gouazoubira* showed identity (83.27 to 84.37%) with *T. cervi* from *O. virginianus* from the USA (AB24874). The *hsp70* sequences detected in brockets showed identity (85.65-86.06%) with *Theileria* sp. previously detected in sheep from China (DQ278176). The *cox3* sequences detected in marsh deer showed identity with *T. annulata* (ON239748) detected in cattle from Algeria (identities ranging from 73.41 to 73.78%), *Theileria velifera* (NC\_070080; identity 77.56 and 77.76%) and *Theileria orientalis* (OR066189; 75.45) (**Supplementary material 1**).

The Maximum Likelihood phylogenetic analysis (ML) inferred with TIM3+I+G as evolutionary model and based on an alignment of 1,520 characters of the 18S rRNA gene positioned all sequences detected in this study into the *Theileria sensu stricto* clade. Most sequences grouped together forming a large clade with a sequence

previously detected in *B. dichotomus* from Argentina. This clade was positioned close to the *T. cervi* clade, with bootstrap of 99%. Two cloned sequences obtained from one *M. jucunda* and one *S. gouazoubira* were positioned into a clade with *Theileria* sequences previously detected in *Odocoileus virginianus* from the USA, with a bootstrap of 100% (**Figure 2**).

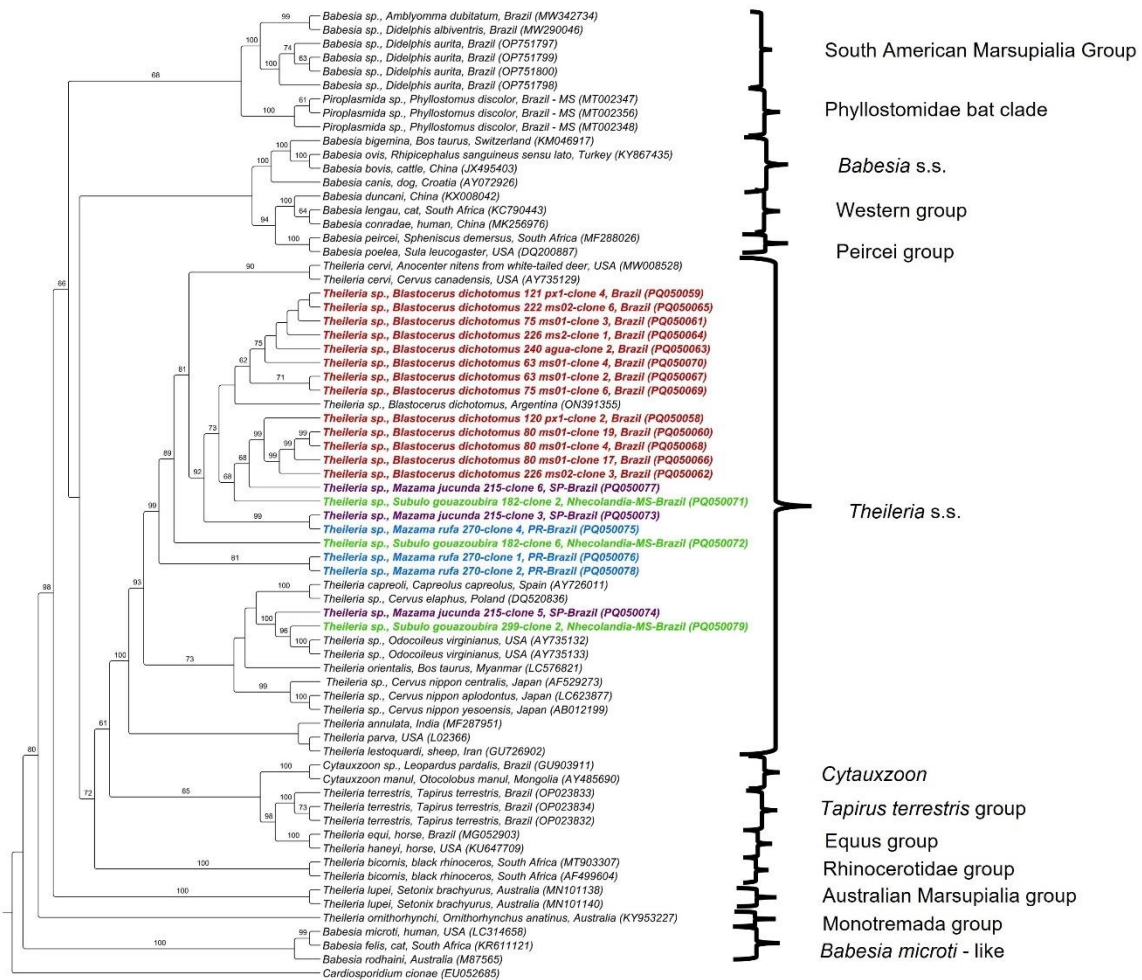


**Figure 2.** Phylogenetic analysis of Piroplasmids 18S rRNA sequences from an alignment of 1,520 characters generated by Maximum Likelihood and with TIM3+I+G evolutionary model. *Cardiosporidium cionae* was used as an outgroup.

To better understand the arrangement of the clades, an ML analysis based on the 1500 bp fragment of the 18S rRNA gene (1,520 characters were included in the alignment used to generate the phylogenetic tree) and using only sequences detected in deer worldwide was performed with TIM3+I+G as evolutionary model. As a result, eight main clades were evidenced, most of which were related to deer host species. The sequences obtained in the present study were distributed among three different

clades. Clade #1 comprised all sequences obtained from marsh deer from Brazil (present study) and Argentina, one sequence detected in *S. gouazoubira*, one from *M. rufa* and two detected in *M. jucunda*. Clade #2 comprised only sequences from the present study (two detected in *M. rufa* and one in *S. gouazoubira*). Clade #6 comprised one sequence detected in *S. gouazoubira*, one from *M. jucunda* and *Theileria* sequences previously detected in *O. virginianus* from the USA. While clades #1 and #2 were closer to *T. cervi* clade, clade #6 was closer to *T. capreoli* clade (**Figure 3**).

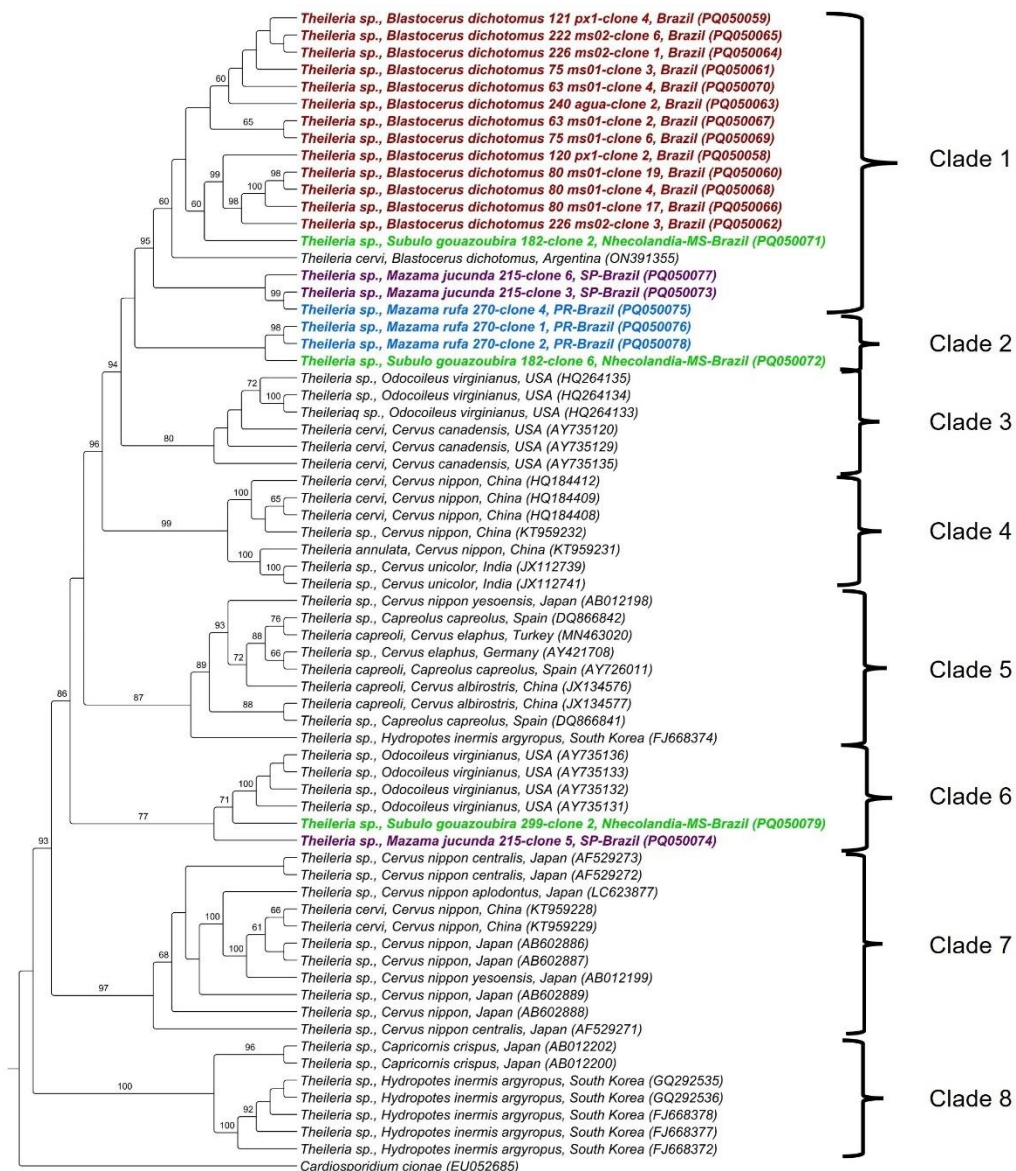


**Figure 3.** Phylogenetic analysis of 18S rRNA sequences detected only in deer from an alignment of 1,520 characters generated by Maximum Likelihood and with TIM3+I+G evolutionary model. *Cardiosporidium cionae* was used as an outgroup.



The Maximum Likelihood phylogenetic analyses based on the *hsp70* (490 characters were included in the alignment and SYM+G evolutionary model) (**Figure 4**) and *cox-3* (190 characters were included in the alignment and mtZOA+G evolutionary model) genes (**Figure 5**) showed topologies that corroborated to each other and positioned all sequences detected from deer in a single clade, within the large clade of *Theileria sensu stricto*, with bootstrap values of 100 and 73, respectively. Noteworthy, two subclusters were observed in the phylogenetic analysis based on the *hsp70* gene: the first containing only sequences detected in marsh deer and the second containing sequences detected in the brocket deer (*Mazama* spp. and *S. gouazoubira*). The latter was also divided into smaller clades that grouped *Theileria* genotypes according to deer species (*M. jucunda*, *M. rufa* and *S. gouazoubira*).

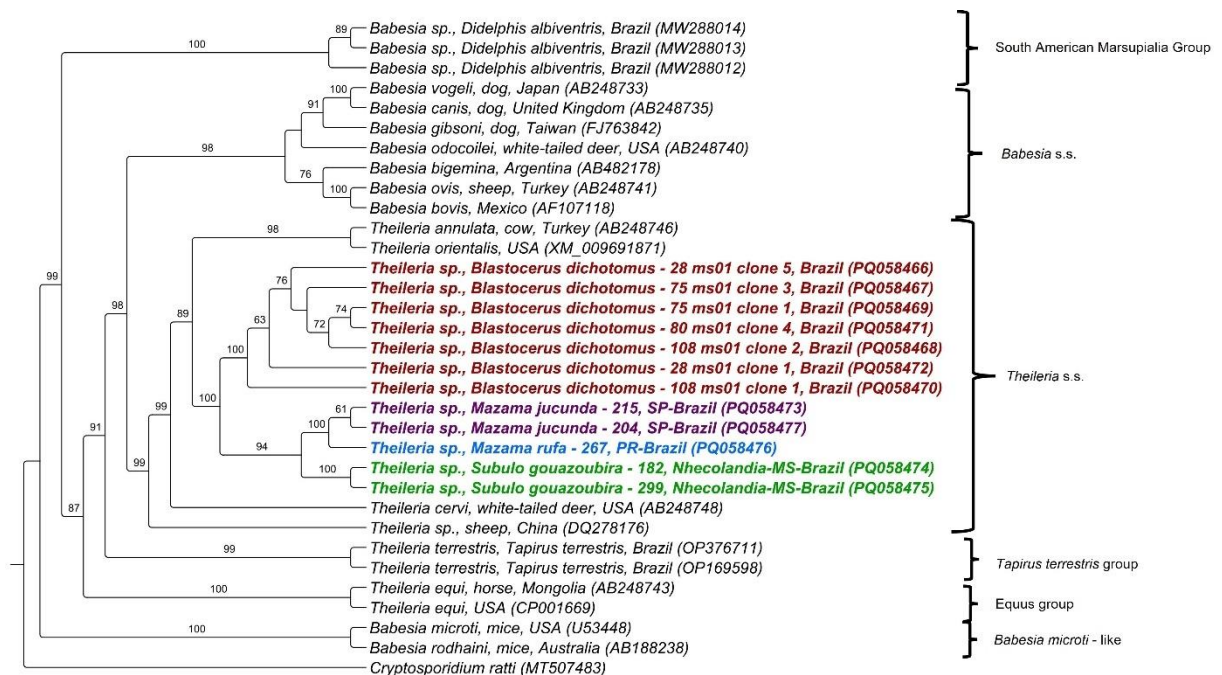


**Figure 4.** Phylogenetic analysis of piroplasmids *hsp70* sequences from an alignment of 490 characters generated by Maximum Likelihood and with SYM+G evolutionary model. *Cryptosporidium ratti* was used as an outgroup.

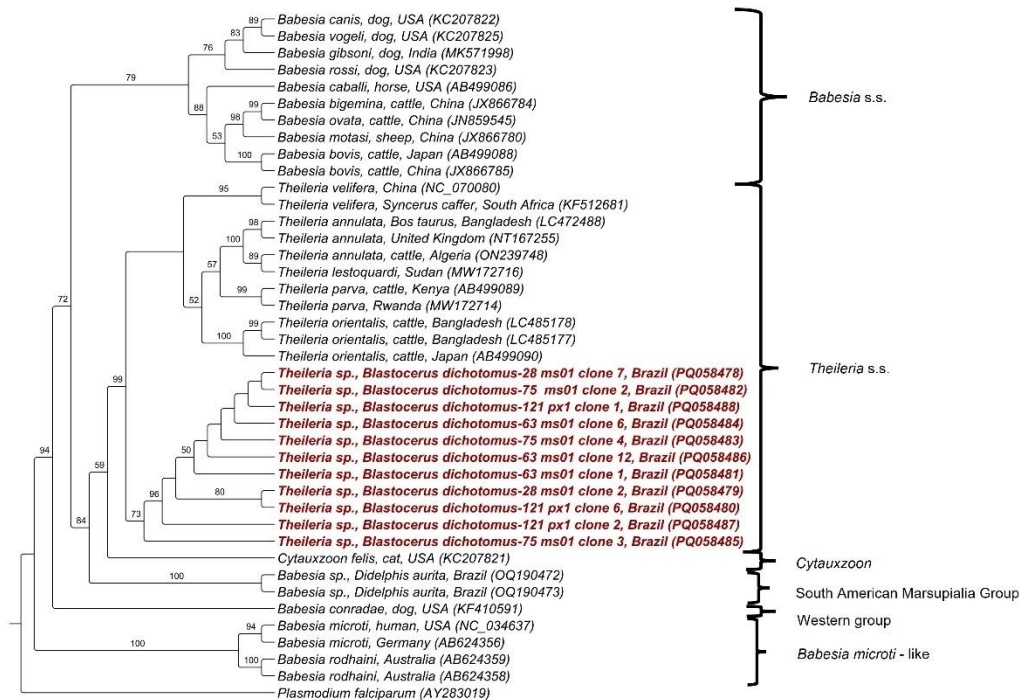


**Figure 5.** Phylogenetic analysis of Piroplasmids COX-3 amino acid sequences from an alignment of 190 characters generated by Maximum Likelihood and with mtZOA+G evolutionary model. *Plasmodium falciparum* was used as an outgroup.

The genotypes analysis performed with 59 near-complete 18S rRNA *Theileria* sequences from deer showed the existence of 47 different genotypes; 16 of which were formed only by sequences obtained in this study (genotypes #20, #21, #26, #27 to #35, #37 to #40). The genotypes grouped mirroring the ML phylogenetic inference. All the identified clusters originated from different median vectors (inferred ancestral nodes). In addition, only seven genotypes were formed by more than one sequence (#1, #4, #8, #15, #22, #28, #41) (**Figure 6**).



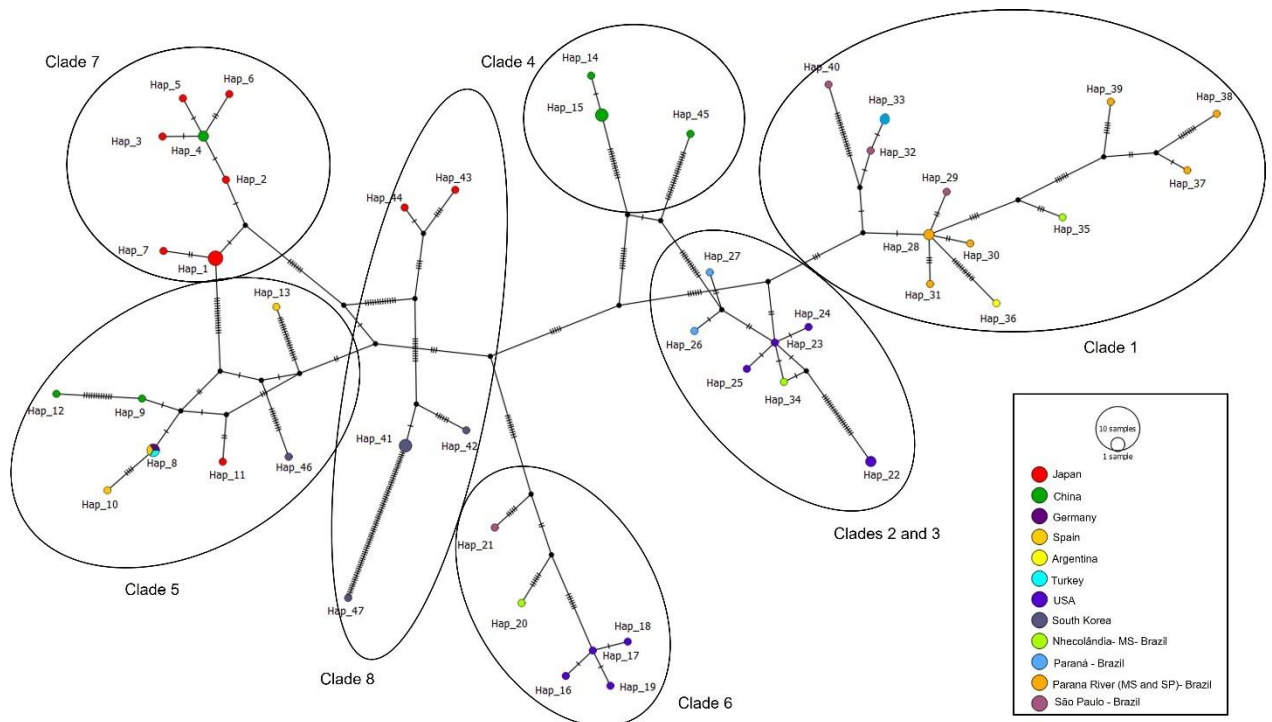


**Figure 6.** TCS network formed among 18S rRNA *Theileria* spp. sequences detected in deer (1,367 bp). The size of the circles varies according to the number of sequences belonging to each genotype. Each color represents the location where each sequence was detected. The black vertical lines represent the mutational events that occurred between each genotype and the black circles represent median vectors.

The genotype analysis of 15 *hsp70* (sequences detected in deer from this study, *T. cervi*, *T. annulata* and *T. orientalis*) showed the occurrence of 14 genotypes. All clusters originated from median vectors and several mutational events. Only genotype #4 comprised two cloned sequences obtained from different marsh deer specimens. In general, the obtained genotypes grouped according to the species of deer (**Figure 7**).

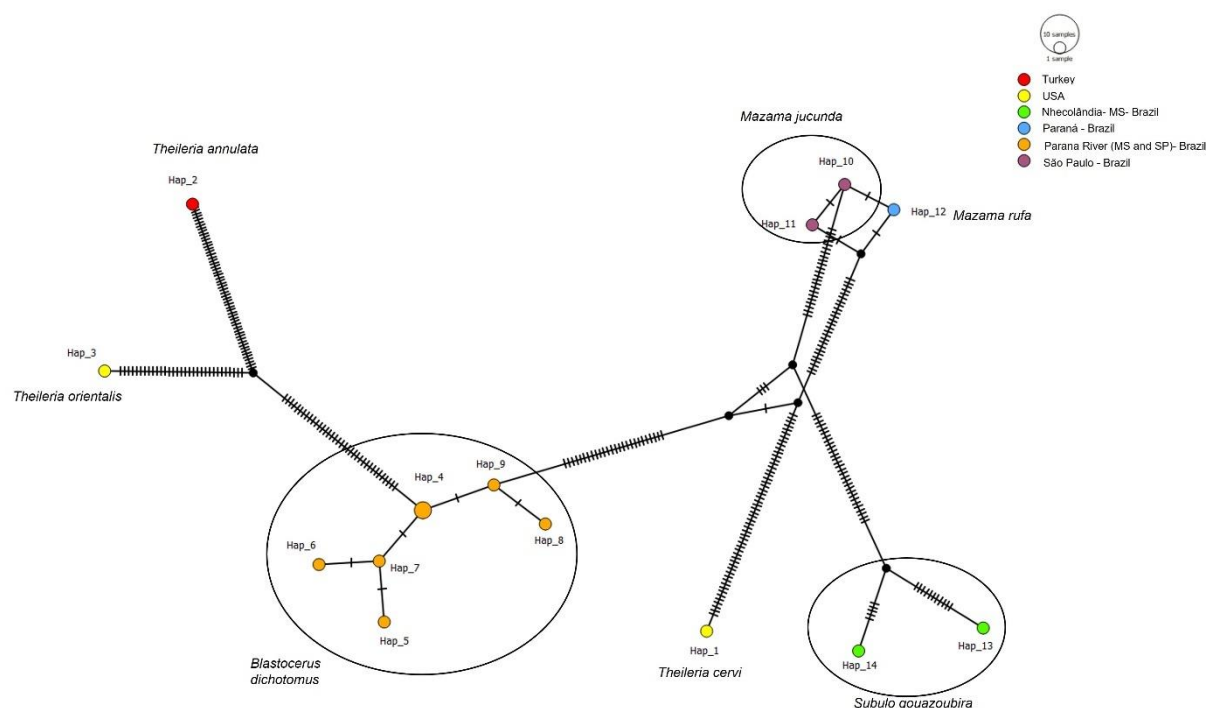


**Figure 7.** TCS network formed among *hsp70* sequences detected in deer (486 bp). The size of the circles varies according to the number of sequences belonging to each genotype. Each color represents the location where each sequence was detected. The black vertical lines represent the mutational events that occurred between each genotype and the black circles represent median vectors.

Finally, out of 11 *cox-3* nucleotide sequences analyzed, 10 genotypes were obtained. Only one genotype (#2) comprised more than one sequence from different clones obtained from the same marsh deer specimen. The parameters obtained in the genetic diversity analyses for each gene are shown in **Table 3**.

**Table 3.** Genetic diversity and polymorphisms of piroplasmids 18S rRNA, *hsp-70* and *cox-3* sequences.

| Gene         | bp    | N  | VS  | GC%  | h  | dh (mean±SD) | π (mean±SD)     | K        |
|--------------|-------|----|-----|------|----|--------------|-----------------|----------|
| 18S rRNA     | 1,367 | 59 | 252 | 45.9 | 47 | 0.989±0.006  | 0.02582±0,00209 | 34.31853 |
| <i>hsp70</i> | 486   | 15 | 160 | 54.3 | 14 | 0.998±0,028  | 0.11354±0,01914 | 55.18095 |
| <i>cox-3</i> | 572   | 11 | 18  | 28.2 | 10 | 0.982±0.046  | 0.00871±0.00079 | 4.98182  |

N= number of sequences analysed, VS=number of variable sites, GC%=C+G content, h=number of haplotypes, dh=diversity of haplotypes, SD=standard deviation, π=nucleotide diversity (per site), K=nucleotide difference number

## Discussion

The present study found a high positivity (75.14% [136/181]) for piroplasmids in deer sampled in different Brazilian states. A similar result was obtained by Da Silveira et al. (2011), who showed an occurrence of 71.4% of piroplasmids in deer (*B. dichotomus* and *S. gouazoubira*) sampled in the Minas Gerais State. On the other hand, 38% (23/60) *O. bezoarticus* sampled in the Pantanal of the Mato Grosso do Sul State showed to be positive for piroplasmids (da Silveira et al., 2013). Herein, 60% (6/9) *O. bezoarticus* sampled in the Pantanal showed to be positive for piroplasmids. Likewise, high occurrence for piroplasmids (ranging from 65.9 to 97.8%) was reported among deer sampled in other countries (Japan, South Korea, USA, Spain, and Italy) (Han et al., 2009; Galuppi et al., 2011; Shibata et al., 2018; Cauvin et al., 2019; Lee et al., 2019; Remesar et al., 2019; Masatani et al., 2020).

The phylogenetic analyses based on the near-complete 18S rRNA, *hsp70*, *cox3* genes showed similar topology to each other and positioned all *Theileria* sequences obtained from deer sampled in the present study within *Theileria* s.s. clade. In all phylogenetic inferences, except for two 18S rRNA sequences detected in *S. gouazoubira* and *M. jucunda*, the sequences detected herein were positioned into a single large clade, albeit in subclades with clear separation between sequences detected in marsh deer and those obtained from brocket deer. This clustering related to deer genus and/or species was also observed in the phylogenetic analysis performed only with deer-associated *Theileria* 18S rRNA sequences, showing specificity of *Theileria* genotypes according to deer hosts.

Although previous studies carried out in Brazil showed the occurrence of genotypes closely related to *T. cervi* in several deer species (*S. gouazoubira*, *B. dichotomus*, *O. bezoarticus* and *M. americana*), those works were based on the analysis of short fragments of the 18S rRNA gene (300-500 bp) (da Silveira et al., 2011; 2013; Soares et al., 2017). In the present study, phylogenetic inferences based on the near-complete 18S rRNA and *hsp70* genes also positioned the clades containing genotypes obtained from Neotropical deer close to *T. cervi* clade, however forming distinct clades. The same topology was observed in the genotype analysis based on both *hsp70* and *cox-3* genes. These findings might indicate the occurrence of novel *Theileria* species/subspecies circulating in Neotropical deer. The occurrence

of different *T. cervi* genotypes in deer (*O. virginianus* and *C. canadensis*) from the USA has already been reported in studies based on the V4 variable region of the small ribosomal subunits (Chae et al., 1999; Cauvin et al., 2019). Additional studies should be performed to confirm if these genotypes represent the same species or whether they are actually different species of *Theileria*.

To date, the only known vector of *T. cervi* is the tick *A. americanum*, which does not occur in Brazil. *Theileria cervi* DNA has previously been detected in *D. nitens* collected from deer in Texas, USA, but no experimental study has proved its vectorial capacity yet (Olafson et al., 2020). The tick species already found parasitizing deer species sampled in this study were *Amblyomma aureolatum* (Pallas, 1878), *Amblyomma brasiliense* Aragão, 1908, *Amblyomma cajennense* (Fabricius, 1787), *Amblyomma coelebs* Neumann, 1906, *Amblyomma dubitatum* Neumann, 1899, *Amblyomma incisum* Neumann, 1906, *Amblyomma longirostre* (Koch, 1844), *Amblyomma naponense* (Packard, 1869), *Amblyomma oblogoguttatum* Koch, 1844, *Amblyomma ovale* Koch, 1844, *Amblyomma parvum* Aragão, 1908, *Amblyomma pseudoconcolor* Aragão, 1908, *Amblyomma sculptum* Berlese, 1888, *Amblyomma tigrinum* Koch, 1844, *Amblyomma triste* Koch, 1844, *Dermacentor nitens* Neumann, 1897, *Haemaphysalis juxtakochi* Cooley, 1946, *Ixodes fuscipes* Koch, 1844 and *Rhipicephalus microplus* (Canestrini, 1888) (Guglielmone et al., 2021). The absence of the primary tick vector of *T. cervi* in Brazil reinforces the hypothesis that putative novel *Theileria* species might be circulating in deer in the country.

Two 18S rRNA sequences (one obtained from *S. gouazoubira* and another from *M. jucunda*) were positioned into a different clade from the other sequences detected in this study, indicating that different species of *Theileria* might occur in these animals. Similarly, previous studies performed in Japan showed the occurrence of two *Theileria* species in *Cervus nippon* (sika deer). While some of the 18S rRNA sequences detected in that study were positioned into a clade related to *T. cervi*, others were positioned into a clade related to *T. capreoli*. Based on the distinct phylogenetic positioning, authors named the two genotypes as *Theileria* sp. Sika 1 and *Theileria* sp. Sika 2 (Inokuma et al., 2004; Shibata et al., 2018; Lee et al., 2019). Herein, distinct 18S rRNA cloned sequences obtained from *M. jucunda* #215 were positioned into different clades while two cloned sequences grouped together forming a subclade

within the clade containing other sequences detected in the present study and a sequence detected in *B. dichotomus* from Argentina, another cloned sequence was positioned into a clade together with a genotype detected in *S. gouazoubira* and *Theileria* genotypes previously detected in *O. virginianus* from USA. These findings show that the same animal might have been co-infected with different *Theileria* species.

The analyses of near-complete 18S rRNA, *hsp70* and *cox-3* genotypes showed high genetic diversity among sequences detected in deer herein. Noteworthy, most genotypes were represented by unique sequences. Despite the conserved nature of the 18S rRNA gene, 47 different genotypes were found among 59 worldwide deer-associated sequences. Interestingly, cloning showed that distinct genotypes were found in the same animal. Previous studies have already reported co-infection with different *Theileria* genotypes in deer from Kenya (*Nanger granti*, Grant's gazelle) and USA (*O. virginianus*) (Hooge et al., 2015; Cauvin et al., 2019). Cauvin et al. (2019) demonstrated coinfection by distinct *T. cervi* genotypes in both farmed and wild white-tailed deer from Florida, USA. Interestingly, most genotypes found in farmed deer were also found in wild deer, but one genotype (called genotype x) was only found in wild deer. The authors also showed a lower occurrence of *Theileria* spp. in farmed deer when compared to wild deer, probably due to tick control applied on the farm.

To the best of authors' knowledge, although *T. cervi* have never been detected in cattle or buffaloes, this *Theileria* species has been reported in horses and a donkey from Mexico, this finding was described by a phylogenetic analysis with concatenated partial fragments of the 18S rRNA and *hsp70* genes (Bravo-Ramos et al., 2023). In Brazil, a previous study showed the occurrence of *Theileria* sp. phylogenetically related to *T. orientalis*, *T. buffeli* and *T. sinensis* in buffaloes sampled in the Amazon region (da Silveira et al., 2016). Therefore, considering that the deer-associated *Theileria* genotypes detected herein have never been reported in cattle from Brazil, one might assume that these piroplasmids are circulating only among deer, and do not seem to represent a risk for cattle herds.

In the present study, no deer buffy coat was positive in the PCR assays for *B. bovis* and *B. bigemina*. This result differs from those found by Da Silveira et al. (2011; 2013), who, despite the low occurrence (9 to 13%), detected *B. bovis* in *O. bezoarticus*

and *B. dichotomus*, and *B. bigemina* in *O. bezoarticus* and *S. gouazoubira* from Minas Gerais and Mato Grosso do Sul States, Brazil. The use of buffy coat with a small number of erythrocytes instead of whole blood samples might have hampered the proper molecular detection of *B. bovis* and *B. bigemina*, since these protozoa only infect erythrocytes and the number of erythrocytes present in the samples were probably not enough to detect these agents.

A previous study using the same marsh deer buffy coat samples reported 42.6% and 48.9% positivity rates for *Ehrlichia* sp. closely related to *E. chaffeensis* and *Anaplasma* sp. closely related to *Anaplasma platys*, respectively (Sacchi et al., 2012). Additionally, seropositivity for *Coxiella burnetii* was found in 5.32% of the animals (two *S. gouazoubira* and seven *B. dichotomus*) (Zanatto et al., 2019). Altogether, these studies show evidence of the circulation of distinct tick-borne pathogens in neotropical deer, highlighting their possible role as reservoirs for these agents.

In the present study, the percentage of deer positive for piroplasmids was higher for the MS02 (100%) and PX (100%) groups when compared to MS01 (69.7%). Deer from MS01 group were captured before the flooding process, inhabiting a more preserved area without much contact with farms. After the flooding, deer from the MS02, PX and AGUA groups were closer to farms and deer had to compete for space. In this new condition after flooding, the encroachment of habitats might have favored the infestation by ticks showing vector competence for *Theileria* transmission (Szabó et al. 2003). Similarly, higher occurrence for *E. chaffeensis*-like was found among deer from MS02 group (66.66%) when compared to MS01 (38.38%) (Sacchi et al., 2012).

Future studies are needed to elucidate if there is more than one *Theileria* species infecting deer from Brazil. Additionally, the search for tick vectors associated with transmission of *Theileria* spp. in deer from South America is much needed. Understanding the pathogenicity of the *Theileria* genotypes circulating in deer from Brazil is necessary to establish future actions regarding deer conservation.

## Conclusion

The present study showed a high occurrence of *Theileria* spp. among deer from Brazil. This work brings the first molecular evidence of *Theileria* infection in *M. jucunda*. Phylogenetic and genotype analyses indicated the occurrence of at least two *Theileria*

species/subspecies circulating in deer from Brazil. Co-infection by distinct *Theileria* (sub)species/genotypes in the same deer was evidenced. *Theileria* lineages detected herein seemed to be specific to deer genus/species, suggesting a possible co-evolution between the *Theileria* lineages and distinct deer species.

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### **Declarations**

### **Ethical approval**

All animal procedures and management protocols were approved by IBAMA (license number 10636-1) and the Ethics Committee on Animal Use of the School of Agricultural and Veterinarian Sciences (FCAV/UNESP) (protocol number 9412/2022).

### **Consent to participate and consent for publication**

Not applicable.

### **Conflict of interest**

The authors declare no conflict of interest.

### **Author contribution**

Conceptualization: Ana Cláudia Calchi and Marcos Rogério André; Methodology: Ana Cláudia Calchi, José Maurício Barbanti Duarte, Ana Carolina Castro-Santiago, Ricardo Bassini-Silva; Writing – Original Draft: Ana Cláudia Calchi, José Maurício Barbanti Duarte, Ana Carolina Castro-Santiago, Ricardo Bassini-Silva, Darci Moraes Barros-Battesti, Rosangela Zacarias Machado and Marcos Rogério

André; Writing – Review & Editing: Ana Cláudia Calchi and Marcos Rogério André; Funding acquisition: Marcos Rogério André; Supervision: Marcos Rogério André.

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## Data availability

The datasets generated and analyzed during the current study are available in the NCBI Genbank Nucleotide platform (<https://www.ncbi.nlm.nih.gov/genbank/>) and can be accessed through accession numbers: PQ050058-PQ050079 for 18S rRNA gene, PQ058466- PQ058477 for *hsp70* gene and PQ058478-PQ058488for *cox-3* gene.

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## **CAPÍTULO 5\* - Diversity of *Cytauxzoon* spp. (Piroplasmida: Theileriidae) in Wild Felids from Brazil and Argentina\***

\* Este capítulo corresponde ao artigo científico publicado na revista Pathogens 14:148, 2025. <https://doi.org/10.3390/pathogens14020148>

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**Abstract:** Domestic and wild felids are frequently parasitized by apicomplexan protozoa in the genus *Cytauxzoon*. Expanding species diversity has recently been described within this genus, with potential implications for epidemiology and pathogenesis. In light of these findings, this study assessed the genetic diversity of *Cytauxzoon* spp. in wild felids (n = 66) from different eco-regions of Brazil and Argentina. Of the 66 blood samples analyzed, 53 (80.3%) were 18S rRNA gene PCR-positive for *Cytauxzoon* spp., including 43 jaguars (*Panthera onca*) and 10 ocelots (*Leopardus pardalis*). *Panthera onca* specimens (100%, 43/43) were most frequently infected, followed by *Leopardus pardalis* (76.9%; 10/13). *Cytauxzoon* spp. were not detected in *Leopardus braccatus* (n = 1) or *Puma concolor* (n = 9). Phylogenetic analyses of fragments of the 18S rRNA, *cytB*, and *cox-1* gene sequences from jaguars were closely related to *Cytauxzoon felis*. In contrast, sequences from ocelots were more closely associated with *Cytauxzoon brasiliensis*. Distance and haplotype analysis further confirmed the circulation of at least two distinct genovariants of *C. felis* among jaguars, as evidenced by their close positioning and low genetic divergence (0–0.14% for 18S rRNA, 0.37–0.56% for *cytB*, and 0.08–0.74% for *cox-1*). Additionally, sequence data from ocelots suggested that multiple genovariants of *C. brasiliensis* are circulating among these cats in different Brazilian eco-regions. Our study provides evidence of two distinct *Cytauxzoon* organisms parasitizing free-ranging and captive jaguars and ocelots, respectively, in Brazil and Argentina.

**Keywords:** *Cytauxzoon brasiliensis*; *Cytauxzoon felis*; genovariants; jaguars; ocelots

## 1. Introduction

*Cytauxzoon* spp. (Piroplasmida: Theileriidae) are apicomplexan protozoa that parasitize erythrocytes, monocytes, and macrophages in mammals [1]. This genus was first described in 1948 by Neitz and Thomas, who identified the piroplasmid in the common duiker (*Sylvicapra grimmia*) in South Africa. Since schizonts of the parasite were not found in lymphocytes, as commonly observed in *Theileria* spp., a new genus was established [2,3]. Currently, the genus *Cytauxzoon* has been primarily described in domestic and wild felids [1,3–7], with limited reports in meerkats [8] and bears [9,10].

Six *Cytauxzoon* species have been described in felids to date: *Cytauxzoon felis*, *Cytauxzoon manul*, *Cytauxzoon europaeus*, *Cytauxzoon otrantorum*, *Cytauxzoon banethi*, and *Cytauxzoon brasiliensis* [4,6,7,11]. Phylogenetic inferences based on distinct molecular markers (18S rRNA, *cox-1*, and *cytB*) have identified two major geographically limited clades. The first clade includes *Cytauxzoon* species found in the New World (*C. felis* and *C. brasiliensis*), and the second clade consists of species detected in the Old World (*C. manul* in Asia, and *C. europaeus*, *C. otrantorum*, and *C. banethi* in Europe) [6,7].

Out of six *Cytauxzoon* species described so far, *C. felis* is the most pathogenic species infecting cats. Disease in domestic cats presents with fever, weight loss, lethargy, dehydration, dyspnea, anemia, pale mucous, hemolytic crises, jaundice, and even death [1,12]. In the past, mortality rates in cats in the USA were approximately 100%; however, mortality has decreased with advances in treatment and improved molecular diagnostics for earlier infection detection [12,13]. Disease has been reported in association with *Cytauxzoon*-infected cats in Europe; however, there is no substantial evidence that disease manifestation is related exclusively to *Cytauxzoon* infection [14–19]. The outcome of *Cytauxzoon* infection in free-ranging felids is largely unknown, as these animals are exposed to numerous infectious agents. Fatal cytauxzoonosis has been documented in captive lions (*Panthera leo*) [20] and in a jaguar (*Panthera onca*) [21] in Brazil, a captive Bengal tiger (*Panthera tigris*) in Germany [22], and a captive-reared white tiger (*Panthera tigris*) [23] and bobcat (*Lynx rufus*) in the USA [5].

The only experimentally confirmed vectors for *C. felis* are *Amblyomma americanum* and *Dermacentor variabilis*, which are exclusively found in the USA [24,25]. *Amblyomma sculptum* and *Ixodes ricinus* have been suggested as potential vectors for the species of *Cytauxzoon* found in Brazil and Europe, respectively [26–29].

In South America, descriptions of *Cytauxzoon* spp. have only been published in Brazil, where this piroplasmid has been detected in captive and free-ranging wild felids, including lions, jaguars, pumas (*Puma concolor*), ocelots (*Leopardus pardalis*), and little spotted cats (*Leopardus tigrinus*) [7,20,21,27,30–35], as well as in domestic cats [36–42]. The majority of published studies were based on a short fragment of the 18S

rRNA, precluding accurate phylogenetic assessment of the isolates that circulate among wild and domestic felids in the country. Using phylogenetic inferences based on the near-complete 18S rRNA and *cythochrome B* (*cytB*) genes, Duarte et al. (2024) [7] demonstrated the occurrence of a novel species (*C. brasiliensis*) in a little spotted cat sampled in central-western Brazil, suggesting a greater diversity of *Cytauxzoon* spp. in South America than previously documented. The purpose of this study was to analyze the genetic diversity of *Cytauxzoon* spp. in jaguars, pumas, and ocelots sampled in different eco-regions of Brazil and Argentina.

## 2. Materials and Methods

### 2.1. Sampling

Between 2015 and 2022, blood samples were collected from 66 wild felids (40 free-ranging and 26 captive felids), including *L. pardalis* (n = 13), *Leopardus braccatus* (n = 1), *P. concolor* (n = 9), and *P. onca* (n = 43). Cats were captured or maintained in different Brazilian states (the states of Amazonas, Bahia, Espírito Santo, Goiás, Maranhão, Mato Grosso, Mato Grosso do Sul, Minas Gerais, Paraná, Piauí, and Tocantins) and Argentina (the provinces of Misiones and Corrientes) (Figure 1). All sampled animals except the ocelots were part of conservation projects. Captures were performed in different ecoregions of Brazil, such as the Cerrado, Pantanal, Amazon Rainforest, and Atlantic Rainforest. Blood samples were collected by puncturing the cephalic or jugular veins using tubes containing EDTA (ethylenediamine tetra acetic acid) and stored at –20 °C until analysis. Samples, collected by different research groups, were provided for molecular screening. The table provided in Supplementary Table S1 shows the collection sites, sample origin, and those institutions responsible for collecting each sample used in this study.

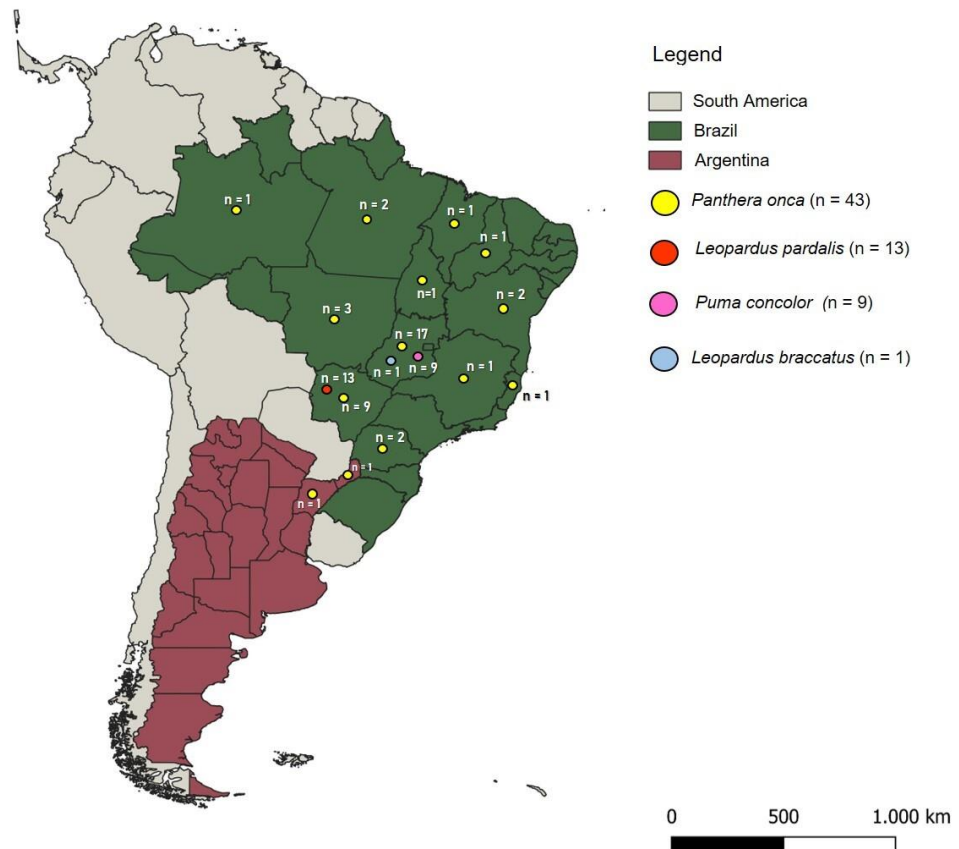


**Figure 1.** Map of South America illustrating the ecoregions where the free-living or captive felids were sampled.

Free-ranging ocelots (*L. pardalis*) were captured in box traps (90 × 45 × 50 cm; Equipos Fauna®, Criciúma, SC, Brazil) and sedated with zoletil 50 (8 mg/kg) or a combination of ketamine (15 mg/kg), xylazine (0.7 mg/kg), and midazolam (0.5 mg/kg). The free-ranging large wild felids were captured as described by May-Junior et al. (2021) [43] and Fagundes-Moreira et al. (2022) [27]. After their complete recovery from sedation, all animals were released at the capture sites. For the captive wild felids from the “Instituto Onça Pintada” (IOP), collected by the FCAV/UNESP research group, chemical restraint was performed using an intramuscular dart with a combination of medetomidine (0.08–0.1 mg/kg) and ketamine (5 mg/kg). After sample collection, anesthesia was reversed with atipamezole (0.25 mg/kg), and the animals were placed back in their enclosures.

## 2.2. DNA Extraction and PCR Assays

DNA was extracted from 200 µL of each blood sample using the DNeasy Blood & Tissue Kit (Qiagen, Valencia, CA, USA), following the manufacturer's instructions. The DNA samples were then subjected to a conventional PCR (cPCR) assay, targeting the mammalian endogenous glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) gene, to confirm the presence of amplifiable DNA [44]. Only the *gapdh*-positive samples were subsequently tested for *Cytauxzoon* spp. DNA. DNA samples were registered under SISGEN #A9CCAD1 ("Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado").

For *Cytauxzoon* spp. DNA detection, a PCR assay was performed based on the amplification of a 284 bp fragment of the 18S rRNA gene [45]. PCR-positive samples were further characterized using conventional or nested PCR assays targeting different molecular markers: the near-complete 18S rRNA gene (~1500 bp) [46–48], *cytB* (~1444 bp) [49], *cox-1* (~1656 bp) [6,50,51], *cox-3* (~600 bp) [51,52], and the 18S–5.8S rRNA (ITS-1) and 5.8S–28S rRNA (ITS-2) regions [53].

The assays were performed using 5 µL of DNA in a reaction mixture containing 0.75 U Platinum Taq DNA Polymerase (Invitrogen, Carlsbad, CA, USA), 10X PCR buffer (100 mM Tris-HCl, pH 9.0, 500 mM KCl), 0.2 mM of each deoxynucleotide (dATP, dTTP, dCTP, and dGTP) (Invitrogen, Carlsbad, CA, USA), 1.5 mM magnesium chloride (Invitrogen, Carlsbad, CA, USA), 0.5 µM of each primer (Invitrogen, Carlsbad, CA, USA), and sterile ultrapure water (Invitrogen, Carlsbad, CA, USA) to a total volume of 25 µL. For the nested PCR assays, 1 µL of the amplified product from the first PCR reaction was used as the target DNA in the second reaction. A DNA sample obtained from a cat that was naturally infected with a Brazilian *Cytauxzoon* sp. [39] and sterile ultrapure water (Life Technologies®, Carlsbad, CA, USA) were used as the positive and negative controls, respectively. All primer sequences and thermal conditions used in the PCR assays are described in Supplementary Table S2.

PCR products were separated by horizontal electrophoresis on 1% agarose gel stained with ethidium bromide (Life Technologies™, Carlsbad, CA, USA) in TEB running buffer (pH 8.0) at 100 V/150 mA for 50 minutes. The gels were examined under ultraviolet light using the ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA, USA) and photographed with Image Lab Software v.4.1 (Bio-Rad, Hercules, CA, USA). The amplified products were purified using the Wizard SV Gel and PCR Clean-Up System

(Promega, Madison, WI, USA) and bidirectionally sequenced by Sanger's method using the BigDye Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific, Waltham, MA, USA) and the ABI 3730 DNA Analyzer (Applied Biosystems, Foster City, CA, USA).

To obtain higher-quality sequences, internal primers were designed for the 18S rRNA, *cytB*, and *cox-1* genes, as follows: 18S-R (5'-TTGAGTCAAATTAAGCCGCA-3') [54], CytB-F1 (5'-TTGTTTTATTGGGCTGCTGA-3'—used in the jaguar samples), CytB-F2 (5'-GAGTTATTGGGGAGCAACAG-3'—used in the ocelot samples), CytB-Finn (5'-ACCTACTAAACCTTATTCAAGCRTT-3') [6], and COI-F (5'-TGCTGGTATTGCTAGTGCTT-3'). These primers were only used for sequencing.

### 2.3. Cloning and Sequencing of the Near-Complete 18S rRNA Gene

Three near-complete 18S rRNA amplicons obtained from ocelot blood samples were cloned, due to the presence of multiple peaks in the electropherograms. These amplicons were subjected to pGEM-T Easy (Promega®, Madison, WI, USA) cloning, following the manufacturer's instructions. At least four clones were selected from each positive sample using the blue/white colony-screening method. Colonies containing the gene fragment of interest, as confirmed by PCR, were subjected to plasmid DNA extraction using the Wizard® Plus SV Miniprep DNA Purification System (Promega, Madison, WI, USA). The purified plasmids were then sequenced using the M13 F (5'-CGCCAGGGTTTTCCAGTCACGAC-3') and M13 R (5'-GTCATAGCTGTTTCCTGTGTGA-3') primers [55], which flank the multiple cloning sites of the pGEM-T Easy plasmid, allowing sequencing of the inserted gene fragments. Additionally, a set of internal primers (forward: 5'-CGCGTAAATTACCCAATCCT-3' and reverse: 5'-TATGGTTAGGACTACGACGG-3') designed for the sequences of this study were also used to obtain higher-quality sequences. Sequencing was carried out as previously described.

### 2.4. BLASTn, Phylogenetic, and Haplotype Analyses

The quality of the bidirectional sequences and the consensus sequences were analyzed and obtained using Phred-Phrap software (version 23) [56,57]. The BLASTn program (available online: <https://blast.ncbi.nlm.nih.gov/Blast.cgi> accessed on November 26, 2024) [58] was used to compare the obtained sequences with those



previously deposited sequences in the GenBank database [59]. The sequences, saved in “FASTA” format, were aligned with homologous sequences retrieved from GenBank using the MAFFT software [60] and trimmed with Bioedit v. 7.0.5.3 [61]. For the *cox-1* gene, the amino acid sequences were used for alignment and subsequent phylogenetic analysis. The W-IQ-Tree software was employed to choose the evolutionary model based on the BIC criterion and for phylogenetic analysis using the maximum likelihood method (available online: <http://iqtree.cibiv.univie.ac.at/> accessed on November 26, 2024) [62]. The phylogenetic trees were edited using Treegraph 2.0.56-381 beta software [63].

The same alignments, excluding the outgroups, were used to construct genealogies via network analysis with the neighbor-net method, applying 100 bootstrap replicates using the Splitstree v4.11.3 software [64]. Additionally, a pairwise distance matrix was calculated using the *p*-distance method with MEGA X software [65–67].

New alignments were constructed using the near-complete 18S rRNA, *cytB*, and *cox-1* nucleotide sequences of *Cytauxzoon* obtained in the present study, along with the phylogenetically closest sequences in GenBank. These alignments were used in the genetic diversity analysis, conducted with DnaSP v5 software, to calculate nucleotide diversity ( $\pi$ ), haplotype diversity ( $H_d$ ), the number of haplotypes ( $h$ ), and the average number of nucleotide differences ( $K$ ) [68]. The haplotype network was constructed using population analysis with reticulate trees (popART) software [69], applying the TCS network method [70].

### 3. Results

All 66 blood DNA extractions were endogenous mammalian *gapdh* gene PCR-positive, of which 53 (80.3%) tested positive for *Cytauxzoon* spp., based on amplification of the 18S rRNA gene. All *P. onca* (43/43) specimens and 76.9% of *L. pardalis* specimens were 18S rRNA PCR-positive for *Cytauxzoon*. No *L. braccatus* or *P. concolor* felids were found to be positive for *Cytauxzoon* spp. Of the 53 short-fragment PCR-positive samples, readable sequences were obtained from 51 (96.2%) of the *Cytauxzoon* 18S rRNA gene (1500 bp), 50 (94.3%) of the *cox-1* and *cytB* *Cytauxzoon* spp. genes, and 48 (90.6%) of the ITS-1 and ITS-2 regions of *Cytauxzoon*

spp. There was no amplification of the *cox-3* gene. Table 1 summarizes the number of *Cytauxzoon* PCR-positive samples for each wild felid species and sampling location.

**Table 1.** Positivity of molecular tests for *Cytauxzoon* using different molecular markers in blood samples collected from free-ranging or captive felids from different eco-regions of Brazil and Argentina. Brazilian states where felids were sampled: MS = Mato Grosso do Sul, GO = Goiás State, MT = Mato Grosso State, MG = Minas Gerais State, ES = Espírito Santo State, PR = Paraná State, PA = Para State, PI = Piauí State, BA= Bahia State, MA = Maranhão State, AM = Amazonas State, and TO = Tocantins State.

| Wild Felids Species         | Collection site (number of samples) | Positive for <i>Cytauxzoon</i> sp. | Near-complete 18S rRNA (~1500 bp) | <i>cytB</i> (~1444 bp) | <i>cox-1</i> (~1656 bp) | ITS-1 | ITS-2 |
|-----------------------------|-------------------------------------|------------------------------------|-----------------------------------|------------------------|-------------------------|-------|-------|
| <i>Leopardus pardalis</i>   | MS (n = 13)                         | 10                                 | 9                                 | 10                     | 10                      | 10    | 10    |
| <i>Leopardus braccatus</i>  | GO (n = 1)                          | 0                                  | -                                 | -                      | -                       | -     | -     |
|                             | MS (n = 9)                          | 9                                  | 9                                 | 9                      | 9                       | 9     | 9     |
|                             | MT (n = 3)                          | 3                                  | 3                                 | 3                      | 3                       | 3     | 3     |
|                             | GO (n = 17)                         | 17                                 | 16                                | 15                     | 14                      | 13    | 13    |
|                             | MG (n = 1)                          | 1                                  | 1                                 | 1                      | 1                       | 1     | 1     |
|                             | ES (n = 1)                          | 1                                  | 1                                 | 1                      | 1                       | 1     | 1     |
|                             | PR (n = 2)                          | 2                                  | 2                                 | 2                      | 2                       | 1     | 1     |
| <i>Panthera onca</i> (n=43) | PA (n = 2)                          | 2                                  | 2                                 | 2                      | 2                       | 2     | 2     |
|                             | PI (n = 1)                          | 1                                  | 1                                 | 1                      | 1                       | 1     | 1     |
|                             | BA (n = 2)                          | 2                                  | 2                                 | 2                      | 2                       | 2     | 2     |
|                             | MA (n = 1)                          | 1                                  | 1                                 | 1                      | 1                       | 1     | 1     |
|                             | AM (n = 1)                          | 1                                  | 1                                 | 0                      | 1                       | 1     | 1     |
|                             | TO (n = 1)                          | 1                                  | 1                                 | 1                      | 1                       | 1     | 1     |
|                             | Argentina (n = 2)                   | 2                                  | 2                                 | 2                      | 2                       | 2     | 2     |
| <i>Puma concolor</i>        | GO (n = 9)                          | 0                                  | -                                 | -                      | -                       | -     | -     |
| Total                       | 66                                  | 53                                 | 51                                | 50                     | 50                      | 48    | 48    |

A subset of conventional PCR-positive samples was selected for DNA sequencing based on band quality (single, clear, and intense bands on agarose gel electrophoresis). High-quality consensus sequences were deposited in GenBank: the 18S rRNA gene (18 from jaguars and 3 from ocelots, with the latter cloned to yield 11 sequences; accession numbers PQ686785–PQ686813), *cytB* gene (20 from jaguars and 4 from ocelots; GenBank accession numbers PQ724014–PQ724037), *cox-1* gene (13 from jaguars and 1 from an ocelot; GenBank accession numbers PQ724000–PQ724013), and the ITS-1 (GenBank accession numbers PQ687019 and PQ687020) and ITS-2 regions from ocelots (GenBank accession numbers PQ687021 and PQ687022).

Based upon BLASTn analysis, the 18S rRNA sequences (~1500 bp) obtained from jaguar DNA blood samples were 99.78–99.86% identical to *C. felis* sequences from domestic cats from the USA (AF399930), while those from ocelots were 99.87–99.94% identical to *Cytauxzoon* sp. obtained from the ocelots from Brazil (GU903911). The *cytB* sequences from the jaguars exhibited 99.52%–99.56% identity with *C. felis* samples from a cat from the USA (KC207821), while those from ocelots were 99.03%–99.12% identical with *C. brasiliensis* detected in *L. tigrinus* samples from Brazil (PP588457). The *cox-1* sequences from the jaguars showed 99.42%–99.92% similarity with *C. felis* sequences from cats from the USA (MT916252), while the sequences obtained from ocelots showed 91.46% similarity with *C. felis* from domestic cats (MT916250). The ITS-1 sequences from ocelots were identical to a *Cytauxzoon* sp. sequence obtained from a domestic cat from Brazil (KP683154), while the ITS-2 sequences were 94.98 - 95.82% similar to a *Cytauxzoon* sp. sequence amplified from an ocelot from Brazil (FJ876458) (Supplementary Materials Table S3).

Maximum likelihood phylogenetic analyses of all molecular markers grouped the sequences obtained from jaguars into subclades closer to the *C. felis* clade from the USA. All sequences from ocelots formed a single clade, sister to the main clade of *C. felis* and jaguars, along with *C. brasiliensis*; all were supported by high bootstrap values (Figures 2–5). Based on the 18S rRNA gene phylogenetic inference (alignment of 1587 characters in the evolutionary model TN+G), six sequences obtained from jaguars (#2, #378, #172, #292, #180, and #351) clustered with *C. felis*, while the other 13 sequences obtained from these felid species formed a sister clade to that of *C. felis* (Figure 2). In contrast, phylogenies based on all *cytB* (alignment of 1082 bp, evolutionary model K3Pu+G) (Figure 3) and COX-1 (alignment of 417 aa; evolutionary model mtZOA+I) (Figure 4) genes clearly clustered with jaguar-associated *C. felis* sequences. The phylogeny based on the ITS-1 intergenic region (alignment of 558 bp; evolutionary model K2P) and ITS-2 (alignment of 252 bp; evolutionary model HKY) grouped the ocelot sequences with a sequence previously amplified from a sample taken from an ocelot in Brazil (Figure 5a and 5b).

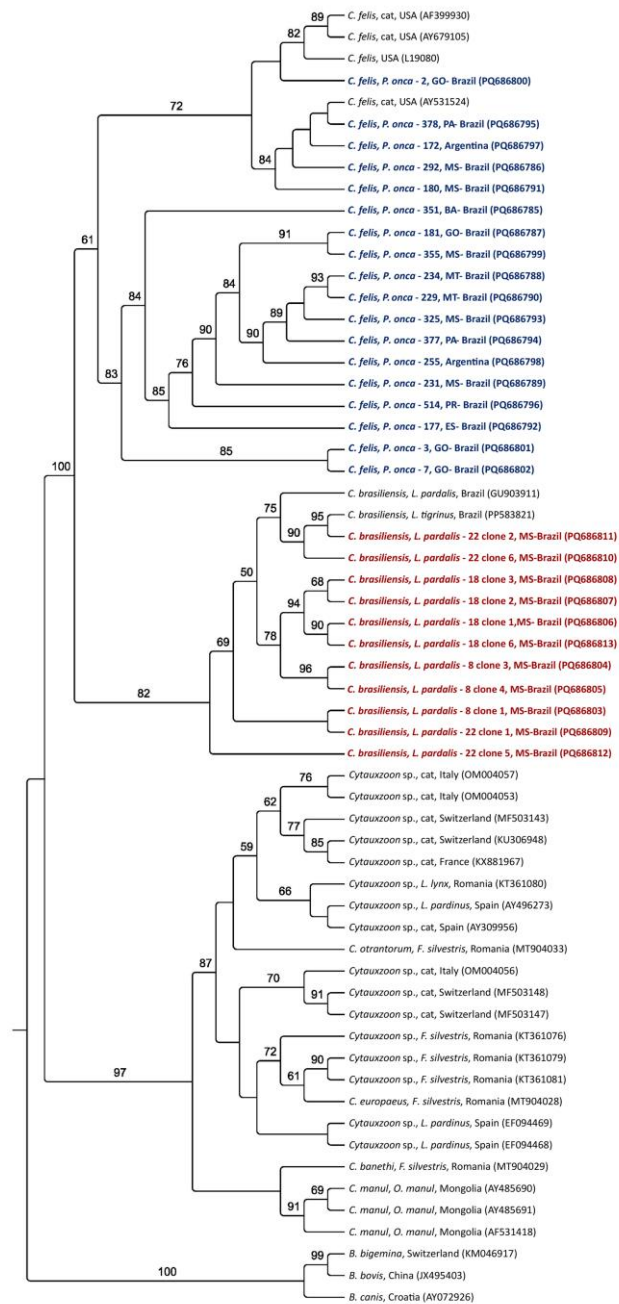


**Figure 2.** Phylogenetic analysis of *Cytauxzoon* spp. The 18S rRNA sequences from an alignment of 1587 characters were generated by maximum likelihood and with the TN+G evolutionary model. *B. bigemina*, *B. bovis*, and *B. canis* were used as an outgroup. Sequences obtained from jaguar DNA blood samples are in blue, whereas those obtained from ocelot DNA are in red.

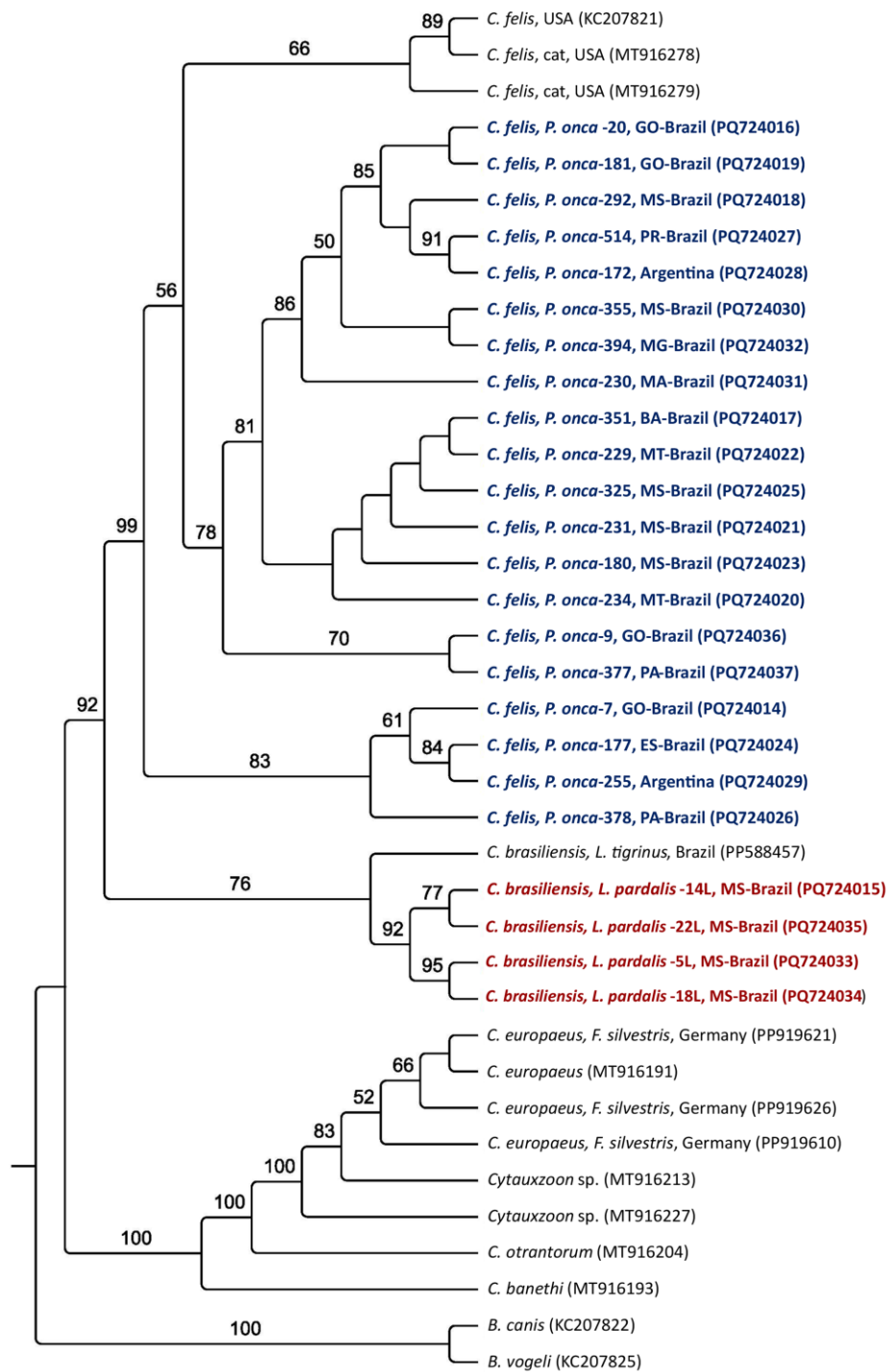


**Figure 3.** Phylogenetic analysis of *Cytauxzoon* spp. The *cytB* sequences from an alignment of 1082 bp were generated by maximum likelihood and with the K3Pu+G evolutionary model. *B. canis* and *B. vogeli* were used as an outgroup. Sequences obtained from jaguar DNA blood samples are in blue, whereas those obtained from ocelots are in red.

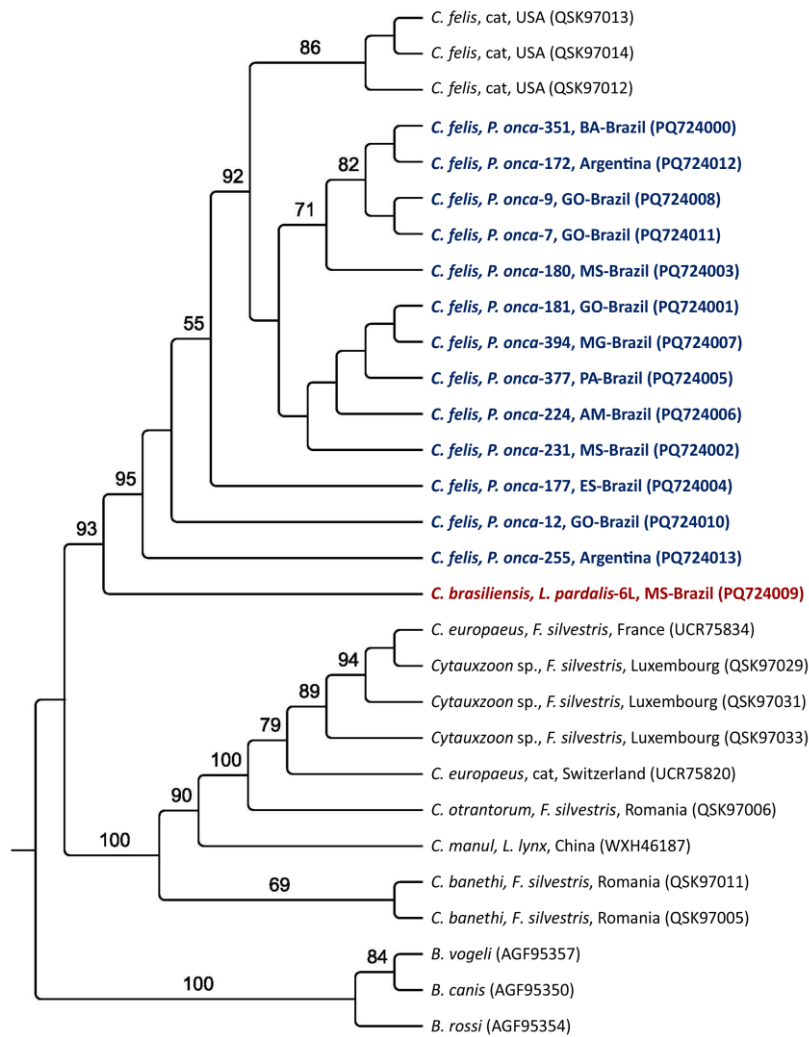
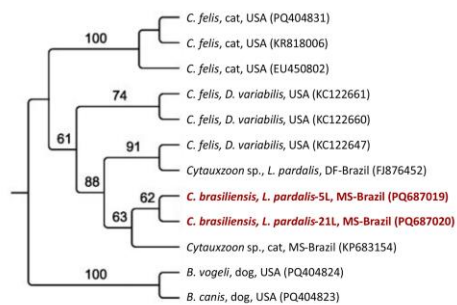


**Figure 4.** Phylogenetic analysis of *Cytauxzoon* spp. COX-1 amino acid sequences from an alignment of 417 amino acids were generated by maximum likelihood and with the mtZOA+I evolutionary model. *B. canis*, *B. vogeli*, and *Babesia rossi* were used as an outgroup. The sequences obtained from jaguar DNA blood samples are in blue, whereas those obtained from ocelot DNA are in red.

A



B

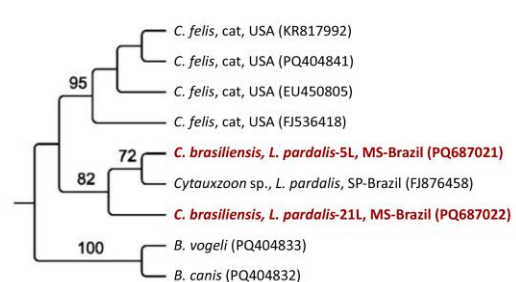


**Figure 5. A.** Phylogenetic analyses of *Cytauxzoon* spp. ITS-1 sequences from an alignment of 558 bp were generated by maximum likelihood and with the K2P evolutionary model. **B.** Phylogenetic analyses of *Cytauxzoon* spp. ITS-2 sequences were from an alignment of 252 characters generated by maximum likelihood and with the HKY evolutionary model. *Babesia canis* and *B. vogeli* were used as an outgroup. The sequences obtained from ocelot DNA samples are in red.

Distance analyses of the 18S rRNA (Figure 6a), *cytB* (Figure 7a), and COX-1 (Figure 8a) revealed a clear separation between the jaguar and ocelot sequences. The jaguar sequences were much closer to *C. felis*, while the ocelot sequences were nearer to *C. brasiliensis*. In addition, there is a clear relationship between the sequences detected in jaguars and *C. felis*, and a greater separation between the other *Cytauxzoon* species. In pairwise *p*-distance analyses, the divergence between *C. felis* and the jaguar 18S rRNA sequences ranged from 0 to 0.14%, that between *C. brasiliensis* and ocelots ranged from 0 to 0.07%, and that between the ocelot and jaguar sequences ranged from 0.34% to 0.51% (Supplementary Materials Table S4). For *CytB*, the divergence between *C. felis* and jaguar sequences ranged from 0.37% to 0.56%, from 0.88% to 0.97% between *C. brasiliensis* and ocelot sequences, and from 7.27% to 7.43% between the jaguar and ocelot sequences (Supplementary Materials Table S5). For *cox-1*, the divergence between the *C. felis* and jaguar sequences ranged from 0.08% to 0.74%, while the divergences between the jaguar and ocelot sequences ranged from 8.59% to 8.69% (Supplementary Material S6). No *cox-1* sequences for *C. brasiliensis* were available in GenBank.

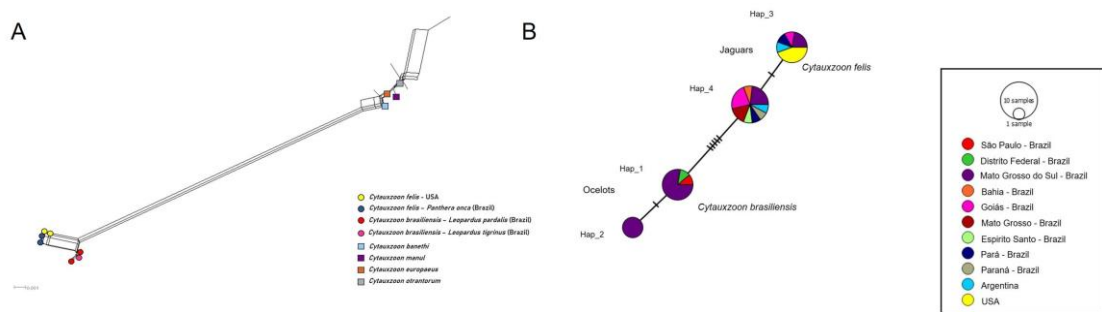


**Figure 6. A.** *Cytauxzoon* split-network distance analysis of 18S rRNA sequences. **B.** The TCS network formed among 18S rRNA sequences (1375 bp), as detected in wild and domestic cats. The size of the circles varies according to the number of sequences belonging to each haplotype. The color represents the geographic location where each sequence was detected. The black vertical lines represent the mutational events that occurred between each haplotype and the black circles represent median vectors.



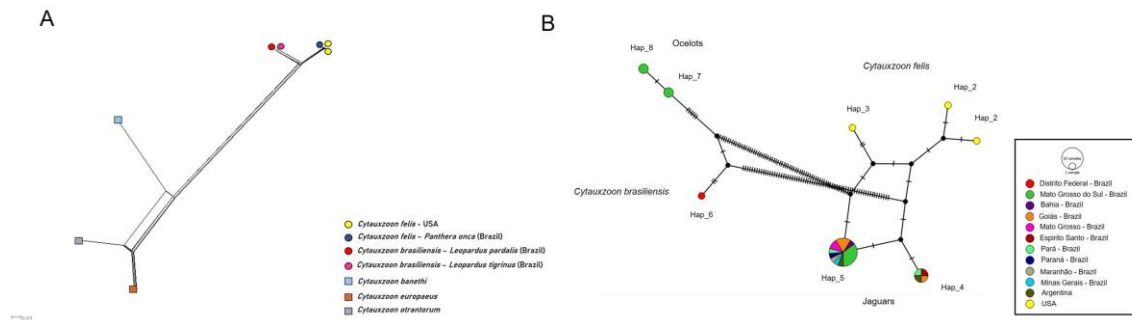


**Figure 7. A.** *Cytauxzoon* split-network distance analysis of *cytB* sequences. **B.** The TCS network formed among *cytB* sequences (1027 bp), as detected in wild and domestic cats. The size of the circles varies according to the number of sequences belonging to each haplotype. The color represents the geographic location where each sequence was detected. The black vertical lines represent the mutational events that occurred between each haplotype and the black circles represent median vectors.

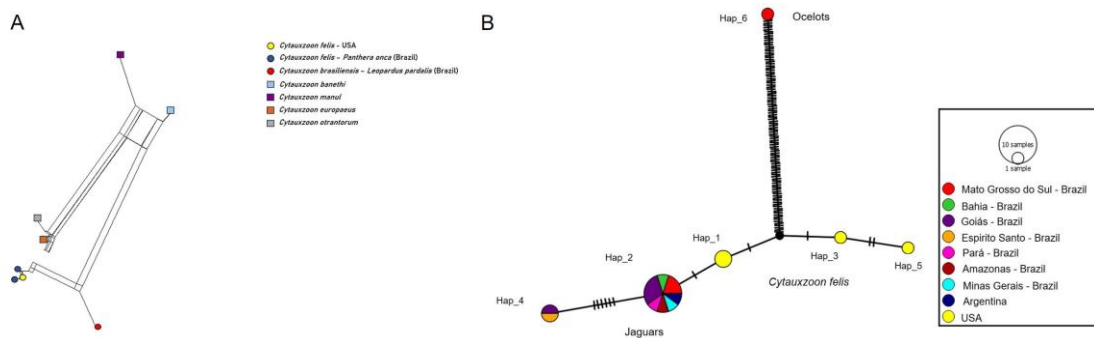


**Figure 8. A.** *Cytauxzoon* split-network distance analysis of COX-1 sequences. **B.** The TCS network formed among *cox-1* sequences (1219 bp), as detected in wild and domestic cats. The size of the circles varies according to the number of sequences belonging to each haplotype. The color represents the geographic location where each sequence was detected. The black vertical lines represent the mutational events that occurred between each haplotype and the black circles represent median vectors.

Haplotype analysis of 35 18S rRNA sequences identified 4 distinct haplotypes among the ocelots: Haplotype #1 was formed by seven sequences drawn from ocelots in this study, a sequence from an ocelot from São Paulo, Brazil, and *C. brasiliensis*; Haplotype #2 comprised four cloned sequences obtained from an ocelot (ID #18); Haplotype #3 included five sequences from jaguars and *C. felis* sequences from the USA; and Haplotype #4 consisted of 13 sequences from jaguars. All haplotypes were separated from each other by mutational events, without median vectors (inferred ancestral nodes). Five mutational events separated *C. felis* and the jaguar-associated haplotypes from *C. brasiliensis* and ocelot-associated haplotypes (Figure 6b).



The analysis of 29 *cytB* sequences identified 8 haplotypes: Haplotypes #1 to #3 were formed by *C. felis* sequences; Haplotypes #4 and #5 by four and 17 jaguar sequences, respectively; Haplotype #6 by a *C. brasiliensis* sequence; and Haplotypes #7 and #8 by two sequences each from ocelots in this study. While *C. felis* and the jaguar-associated haplotypes were separated by median vectors and few mutational events, *C. felis* and jaguar-associated haplotypes were separated from *C. brasiliensis* and ocelot-associated haplotypes were separated by median vectors and several mutational events. *Cytauxzoon brasiliensis* and the ocelot-associated haplotypes were separated by median vectors and few mutational events (Figure 7b). The haplotype network of the *cox-1* gene, based on 17 sequences, formed 6 haplotypes: Haplotypes #1, #3, and #5 comprised only *C. felis* sequences; Haplotypes #2 and #4 comprised 10 and 2 jaguar-associated sequences, respectively; and Haplotype #6 was an ocelot-associated sequence. A single median vector grouped the haplotypes into clusters (#1, 2, and 4; #3 and 5; #6), separated by mutational events. While few mutational events separated *C. felis* and the jaguar-associated haplotypes, the ocelot-associated haplotype was separated from *C. felis* and the jaguar-associated haplotypes by a median vector and several mutational events (Figure 8b). Table 2 shows the genetic diversity values for each analyzed gene.

**Table 2.** Genetic diversity and polymorphisms of *Cytauxzoon* 18S rRNA, *cytB*, and *cox-1* sequences.

| Gene         | Bp   | N  | VS  | GC%  | h | Hd (mean $\pm$ SD) | $\pi$ (mean $\pm$ SD) | K        |
|--------------|------|----|-----|------|---|--------------------|-----------------------|----------|
| 18S rRNA     | 1375 | 35 | 7   | 43.4 | 4 | 0.738 $\pm$ 0.033  | 0.00219 $\pm$ 0.00019 | 3.00504  |
| <i>cytB</i>  | 1027 | 29 | 83  | 29.7 | 8 | 0.645 $\pm$ 0.095  | 0.02242 $\pm$ 0.00659 | 23.02709 |
| <i>cox-1</i> | 1219 | 17 | 110 | 30.9 | 6 | 0.654 $\pm$ 0.122  | 0.01180 $\pm$ 0.00853 | 14.38235 |

N = number of sequences analyzed; VS = number of variable sites; GC% = G+C content; h = number of haplotypes; Hd = haplotype diversity; SD= standard deviation;  $\pi$  = nucleotide diversity (per site); K = nucleotide difference number.

#### 4. Discussion

In this study, all 43 jaguars tested were infected with a *Cytauxzoon* spp., corroborating previous reports carried out in Brazil. Previously, high molecular positivity for this protozoan in jaguars (96.5–100%), along with the persistence of positivity after recapture, suggested that this wild felid species might play a role as a reservoir host [27,32]. Additionally, consistent with the present study, which found a

high occurrence (76.9% [10/13]) in ocelots, Fagundes-Moreira et al. (2022) [27] reported 80% (4/5) positivity in ocelots sampled in midwestern Brazil. Interestingly, no ocelots sampled in the state of Rio Grande do Sul State tested *Cytauxzoon* PCR-positive [27]. Contrary to previous reports, we did not find any pumas infected with a *Cytauxzoon* spp. [27,30,35], perhaps due to the limited sampling numbers (n = 9).

It has been previously established that the haplotypes of *Cytauxzoon* spp. circulating in domestic and wild felids from Brazil are phylogenetically related to *C. felis* from the USA [32–34,37,39,40,42,71,72]. However, as most of these studies used only a short fragment of the 18S rRNA gene, the exact phylogenetic position remained uncertain. Additionally, domestic cats in Brazil do not exhibit the severe clinical signs commonly observed in cats from the USA [37,39,40] and, to date, schizonts have never been demonstrated in *Felis catus* in the Brazilian territory. In a recent study, a new species (*C. brasiliensis*) was described in a little spotted cat specimen from Brazil. This new species, molecularly identified using the near-complete 18S rRNA and *cytB* genes, forms a distinct clade, sister to the *C. felis* clade from the USA [7]. Our study documented the presence of *C. brasiliensis* in ocelots from Mato Grosso do Sul state, Brazil, and also detected haplotypes related to *C. felis* in jaguars.

Interestingly, one of the jaguars sampled in Argentina that tested positive for *C. felis* was born in captivity after the crossbreeding of two animals from Brazil. Although one study failed to demonstrate the occurrence of perinatal infection by *Cytauxzoon* sp. in domestic cats [73], other studies have reported the presence of *Cytauxzoon* spp. in domestic kittens and wild felid kittens [5,17,20,74]. However, none of the studies have confirmed whether this was due to vertical transmission or tick infestation. In this study, we were unable to determine whether the infection resulted from perinatal transmission or from the presence of tick vectors in the animal's enclosure in Argentina.

In this study, phylogenetic analysis based on the near-complete 18S rRNA and *cytB* genes documented a clear separation between the sequences obtained from ocelots, which were grouped within the *C. brasiliensis* clade, and those from jaguars, which clustered into subclades within the *C. felis* clade. These findings demonstrate the presence of at least two distinct *Cytauxzoon* species parasitizing free-ranging and captive felids in Brazil. Phylogenetic analysis based on the translated COX-1 protein sequence also separated ocelot and jaguar-associated *C. felis* sequences into

separate clades. However, the lack of *C. brasiliensis cox-1* sequences in GenBank and the limited number of readable sequences obtained in this study hampered further interpretation of phylogenetic inferences based on this molecular marker.

In addition, phylogenetic analysis of 13 near-complete 18S rRNA gene sequences from jaguars identified a sister clade to the *C. felis* clade detected in the USA, whereas 5 sequences were clustered within the *C. felis* clade. However, in the *cytB* and COX-1-based phylogenetic analyses, all jaguar-associated sequences formed a single clade, distinct from the *C. felis* clade from the USA. Panait et al. (2021) [6] described three new *Cytauxzoon* species (*C. europaeus*, *C. otrantorum*, and *C. banethi*) circulating in Europe, but the 18S rRNA gene alone was insufficient to differentiate these species, which did not reveal high genetic divergence. In contrast, due to their greater genetic variability, two mitochondrial genes (*cytB* and *cox-1*) more effectively differentiated these three species, highlighting the limitations of the 18S rRNA gene for distinguishing closely related taxa [6,51].

To assess whether the *Cytauxzoon* spp. infecting jaguars represent distinct haplotypes of *C. felis* or a possibly new species, distance and haplotype analyses based on three distinct molecular markers (near-complete 18S rRNA, *cytB*, and *cox-1*) were analyzed. The genealogies constructed using the neighbor-net method indicate that *C. felis* is closely related to the jaguar-associated *C. felis*, while the separation among other *Cytauxzoon* species is more pronounced. Pairwise *p*-distance analyses revealed that the divergence between the jaguar-associated *Cytauxzoon* sp. and *C. felis* was 0 to 0.14% for the near-complete 18S rRNA, 0.37 to 0.56% for *cytB*, and 0.08 to 0.74% for *cox-1*. In comparison, the divergence between *C. brasiliensis* and *C. felis* was approximately 0.4% for the 18S rRNA and 7% for *cytB* genes, while interspecific divergence among *C. europaeus*, *C. otrantorum*, and *C. banethi* was approximately 0.1% (18S rRNA) [6,7], 9 to 22% (*cytB*), and 6 to 14% (*cox-1*), values that are much higher than those found in this study. These results indicate infection with *C. felis* genovariants in jaguars rather than a novel *Cytauxzoon* species.

Haplotype analyses based on the *cytB* and *cox-1* genes indicated that the *Cytauxzoon* spp. sequences from jaguars were divided into two distinct haplotypes, although none of them were shared with *C. felis* from the USA. In contrast, the 18S rRNA-based haplotype analysis indicated that some jaguar-associated haplotypes

belonged to the same haplotype as *C. felis*, likely due to the high conservation of this molecular marker, which can make accurate analysis of genetic diversity difficult, as previously reported by Panait et al. (2021) [6]. In contrast, the haplotype analyses based on *cytB* and *cox-1* genes were consistent with one another, each identifying two haplotypes.

Regarding the ocelot-associated *Cytauxzoon* spp. sequences, two haplotypes were found for both the 18S rRNA and *cytB* genes. Only one sequence was obtained for *cox-1*, negating the phylogenetic analysis. While one of the 18S rRNA haplotypes was shared with *C. brasiliensis*, the *cytB* haplotypes detected in ocelots were separated from *C. brasiliensis* by median vectors, indicating the occurrence of distinct genovariants of this recently described *Cytauxzoon* species. Further studies are needed to fully explore the genetic diversity of *C. brasiliensis* in Brazil.

The ITS region has been used to demonstrate high *C. felis* genotypic diversity in cats from the USA [75-79]. Two complete ITS-1 and ITS-2 intergenic region sequences were obtained from the *Cytauxzoon* spp. infecting two ocelots in this study. Phylogenetic analysis based on the ITS-2 region positioned both sequences in a clade with another haplotype that was previously detected in an ocelot from Brazil. The ITS-1-based phylogenetic analysis grouped both the sequences detected in ocelots in this study and sampled in the state of Mato Grosso do Sul (MS) with a sequence previously amplified from a domestic cat, also from MS. These findings indicate that *C. brasiliensis* can infect little spotted cats, ocelots, and domestic cats in Brazil.

Given the known occurrence of distinct *Cytauxzoon* species in Brazil, including genovariants of *C. felis*, further studies are needed to determine which species commonly infect domestic cats and to identify the primary tick vectors. Although molecular and epidemiological evidence suggests *A. sculptum* as a putative tick vector for *Cytauxzoon* spp. in Brazil [27,28], experimental studies are necessary to assess vector competence. To date, fatal cases of *Cytauxzoon* spp. infection with the presence of schizonts in several tissues have been reported in wild felids [20,21], but not in domestic cats. Additional research is required to understand whether the circulating *Cytauxzoon* spp. haplotypes in Brazil are non-pathogenic or whether pathogenicity is only associated with stress, debilitation, or poor immunity [20,21]. Finally, considering that at least two *Cytauxzoon* species can be found in Brazil, future

studies should utilize the near-complete 18S rRNA and/or mitochondrial genes when investigating the phylogenetic positioning of these parasites in domestic and wild felids. Whether *C. brasiliensis* infects felids in Argentina and other South American countries is yet to be determined.

## 5. Conclusions

This study documented the presence of at least two distinct *Cytauxzoon* species in wild felids from South America: while *C. brasiliensis* was detected in ocelots from Brazil, genovariants of *C. felis* were detected in jaguars from Brazil and Argentina.

**Supplementary Materials:** The following supporting information can be downloaded at: [www.mdpi.com/xxx/s1](http://www.mdpi.com/xxx/s1), Table S1: Description of the locality, origin, and institutions responsible for sample collection; Table S2: Description of the primers, amplicon sizes, and thermal conditions used in conventional and nested PCR assays for the detection and characterization of *Cytauxzoon* spp.; Table S3: BLASTn results of the sequences obtained in the amplification of five distinct molecular markers from *Cytauxzoon* spp. from wild felid blood samples; Table S4: Pairwise analysis of the near-complete 18S rRNA sequences; Table S5: Pairwise analysis of the near-complete *cytB* sequences; Table S6: Pairwise analysis of the near-complete *cox-1* sequences.

**Author Contributions:** Conceptualization, A.C.C. and M.R.A.; methodology, A.C.C., J.A.M.J., V.B.S., L.B., R.F.M., R.M.B., L.Q.V.B., M.D.K., M.F.S., R.A.N.A., C.O.M., R.B.S., H.M.H., E.B.B., R.G.M., E.E., F.L.R., J.F.S., and M.R.A.; software, A.C.C.; validation, A.C.C.; formal analysis, A.C.C.; investigation, A.C.C., J.F.S., M.R.A., and R.Z.M.; resources, M.R.A., J.F.S., E.B.B., R.G.M., R.B.S., F.L.R., and H.M.H.; data curation, A.C.C.; writing—original draft preparation, A.C.C. and M.R.A.; writing—review and editing, A.C.C. and M.R.A.; visualization, A.C.C., M.R.A., and R.Z.M.; supervision, M.R.A.; project administration, M.R.A.; funding acquisition, M.R.A., J.F.S., E.B.B., R.G.M., F.L.R., and H.M.H. All authors have read and agreed to the published version of the manuscript.

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**Data Availability Statement:** The datasets generated and analyzed during the current study are available on the NCBI Genbank Nucleotide platform (<https://www.ncbi.nlm.nih.gov/genbank/>) and can be accessed through accession numbers: PQ686785–PQ686813 for the 18S rRNA gene; PQ724000–PQ724013 for the *cox-1* gene; PQ724014–PQ724037 for the *cytB* gene; PQ687019 and PQ687020 for the internal transcribed spacer 1 (ITS-1); PQ687021 and PQ687022 for the internal transcribed spacer 2 (ITS-2).

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## **CAPÍTULO 6 – Investigação molecular de piroplasmídeos em pequenos mamíferos e aves selvagens amostrados nos biomas Amazônico e Pantanal do Brasil**

### **Resumo**

Piroplasmídeos são protozoários apicomplexas e parasitos obrigatórios que infectam animais vertebrados, podendo causar doenças em animais e humanos. O grupo é formado por quatro gêneros *Babesia* spp., *Rangelia* spp., *Theileria* spp. e *Cytauxzoon* spp. O presente estudo teve como objetivo investigar a ocorrência molecular de Piroplasmida em 829 amostras biológicas de animais selvagens, a saber: baço de morcegos capturados no estado do Acre, bioma Amazônico (n=208; 30 espécies), sangue de pequenos roedores (n=113; 3 espécies) e marsupiais (n=7; 2 espécies) amostrados no Pantanal do Mato Grosso do Sul, e sangue de aves selvagens (n=500; 93 espécies) capturadas no Pantanal do Mato Grosso e Mato Grosso do Sul. Para isso, após extração de DNA, as amostras positivas nos ensaios de PCR realizados para controles endógenos (gene *gapdh* de mamíferos e  $\beta$ -*actin* de aves) foram triadas por meio de uma nested PCR baseada no gene 18S rRNA de piroplasmídeos. Nenhuma amostra mostrou-se positiva para o referido protocolo. A ausência de positividade para piroplasmídeos em amostras biológicas de morcegos, roedores, marsupiais e aves no presente estudo pode estar relacionada a diversos fatores, tais como: baixa parasitemia (a qual poderia estar abaixo do limiar de sensibilidade da nested PCR utilizada); inespecificidade dos oligonucleotídeos iniciadores, os quais, em diversas ocasiões se anelavam no DNA de aves; ausência de vetores competentes nas áreas estudadas; ou simplesmente devido à baixa susceptibilidade das espécies de aves e pequenos mamíferos amostrados a piroplasmídeos. Estudos futuros objetivando investigar a ocorrência de piroplasmídeos em aves e pequenos mamíferos selvagens de outros biomas e utilizando técnicas de maior sensibilidade (e.g. dPCR) são necessários.

**Palavras-chave:** Piroplasmídeos, quirópteros, roedores, marsupiais, aves



## 1. Introdução

Os piroplasmídeos são protozoários hemoparasitas apicomplexos que podem causar doenças em animais e humanos (Alvarado e Rybak, 2016; Krause et al., 2019; Schnittger et al., 2022). O grupo é composto pelas famílias Babesiidae (com os gêneros *Babesia* e *Rangelia*) e Theileriidae (com os gêneros *Theileria* e *Cytauxzoon*) (Solano-Gallego et al., 2011; Alvarado e Rybak, 2016). Esses parasitos infectam células sanguíneas, como eritrócitos, linfócitos, monócitos ou células endoteliais, dependendo do agente envolvido (Alvarado e Rybak, 2016).

A espécie mais comumente descrita em quirópteros até o momento é *Babesia vesperuginis*, a qual vem sendo detectada em morcegos não-hematófagos na Europa (Itália, Países Baixos, Reino Unido, Hungria, Romênia, República Tcheca, Áustria, Lituânia) e na Ásia (China) (Dionisi, 1899; Gardner and Molyneux, 1987; Concannon et al., 2005; Corduneanu et al., 2017; Han et al., 2018; Liu et al., 2018; Linhart et al., 2022; Sakalauskas et al., 2024). Adicionalmente, fragmento curto do gene 18S rRNA de *Babesia canis* foi detectado em amostras fecais de morcegos na Hungria (Hornok et al., 2015) e uma possível nova espécie de *Babesia*, pertencente ao grupo de *Babesia microti*, foi descrita em morcegos de Madagascar (Ranaivoson et al., 2019).

Na América do Sul, formas evolutivas de *Babesia* sp. foram detectadas em esfregaços sanguíneos de morcegos coletados na Colômbia. Os autores sugeriram trata-se, de acordo com a morfologia, de *B. vesperuginis*, embora sem confirmação molecular (Marinkelle, 1996). No Brasil, foram relatadas duas possíveis novas espécies de piroplasmídeos, uma infectando *Phyllostomus discolor*, que formou um clado distinto na filogenia dos piroplasmídeos (18S rRNA quase completo), e outra infectando *Artibeus* spp., filogeneticamente associada a *Babesia vogeli* (fragmento curto do 18S rRNA) (Ikeda et al., 2021). Em contraste, sequências de *Babesia* spp. e *Theileria* spp., posicionadas filogeneticamente nos clados “South American Marsupialia group”, “*Theileria sensu stricto*” e “*Tapirus terrestris*”, foram detectadas em morcegos hematófagos amostrados na região Norte do Brasil (de Mello et al., 2023a).

Pequenos roedores são considerados os principais hospedeiros de *Babesia microti* e *Babesia microti*-like, que são agentes zoonóticos (Yabsley et al., 2013; Rar et al., 2016; Karnchanabanthoeng et al., 2017). Na América do Sul, *Babesia microti*-

like foi descrita, até o momento, apenas no Chile, em *Phyllotis darwini*, um roedor da família Cricetidae (Santodomingo et al., 2022) e em humanos na Bolívia (Gabrielli et al., 2016). No Brasil, DNA de *Babesia* sp. filogeneticamente relacionada a *Babesia* sp. detectada em cuíca-de-rabo-curto (*Monodelphis domestica*) foi detectado em *Trichomys pachyurus* no Pantanal de Mato Grosso (Wolf et al., 2016). Além disso, sequências curtas do 18S rRNA filogeneticamente associadas à *Babesia vogeli* e *Theileria equi* foram detectadas em espécimes de *Trichomys fosteri* amostrados no Pantanal sul-matogrossense. Recentemente, uma possível nova espécie de piroplasmídeo foi descrita em *T. pachyurus* oriundos do estado do Mato Grosso; as sequências (18S rRNA quase completo) se alocaram em um novo clado denominado “South American Rodentia group”, clado-irmão àquele de “Phyllostomidae bat group” (Pacheco et al., 2025).

Novos genótipos e espécies de *Babesia* spp. vêm sendo descritas em marsupiais na América do Sul. Um novo clado que compreende sequências detectadas em gambás (*Didelphis albiventris*, *Didelphis aurita* e *Didelphis marsupialis*) foi descrito no Brasil (Gonçalves et al., 2021; de Oliveira et al., 2023; Braga et al., 2023). DNA de uma possível nova *Babesia* sp., posicionado no “Western Clade”, foi detectado em *D. marsupialis* na Guatemala (Thomas et al., 2023). Um estudo detectou a presença de *Babesia* sp., filogeneticamente associada à *Babesia* sp. detectada em *T. pachyurus*, em uma cuíca-de-rabo-curto (*Monodelphis domestica*) (50% [1/2]) amostrada no estado do Mato Grosso (Wolf et al., 2016).

Em relação aos piroplasmídeos que acometem aves, até o momento, 16 espécies de *Babesia* foram registradas circulando nesses animais (Peirce, 2000; Yabsley, et al., 2006; Jefferies et al., 2008; Yabsley, et al., 2009; Peirce and Parsons, 2012; Chavatte et al., 2017; Parsons et al., 2017), além de várias outras espécies não identificadas de *Babesia* spp. (Savage et al., 2009; Paparini et al., 2014; Martínez et al., 2015; Vanstreels et al., 2015; Montero et al., 2016; Parsons et al., 2016; Synman et al., 2020; Korobitsyn et al., 2021; Bonsergent et al., 2022). A maioria destas espécies foi descrita por meio de métodos morfológicos baseados em esfregaços sanguíneos ou histopatologia, com associação a hospedeiros vertebrados ou vetores, e à localização geográfica. Das espécies mencionadas, apenas sete foram detectadas por métodos moleculares e filogeneticamente posicionadas com base em fragmentos

quase completos do gene 18S rRNA. A análise filogenética revelou que esses agentes formam um grupo polifilético, dividido em três clados parafiléticos: o primeiro clado inclui *Babesia peircei*, *Babesia poelea*, *Babesia ardeae*, *Babesia uriae* e *Babesia ugwidensis*, que formam o “grupo Peircei”, sendo este um clado irmão ao “Western Clade”. O segundo clado é composto por *Babesia kiwiensis*, que está filogeneticamente relacionado ao clado de “*Babesia* spp. sensu stricto”. Já o terceiro clado é formado por *Babesia bennetti*, que está intimamente relacionado à *Babesia bovis* e *Babesia ovis*, embora haja suspeitas de que a sequência fornecida possa ser uma quimera e que a espécie não exista realmente (Chavatte et al, 2017; Yabsley et al., 2017; Malandrin et al., 2022). *Babesia shortii* e *Babesia uriae* são consideradas patogênicas para aves (Ebani and Mancianti, 2021).

No Brasil, poucos são os estudos relacionados à detecção desses agentes em aves. Quillfeldt et al. (2014) relataram a presença de *Babesia* spp. filogeneticamente associadas à *B. poelea* em atobá-pardo (*Sula leucogaster*) e atobá-grande (*Sula dactylatra*) coletados em ilhas costeiras brasileiras. Além disso, uma sequência 18S rRNA (693 pb) filogeneticamente associada à *B. vogeli* foi detectada em ganso-do-Orinoco (*Neochen jubata*) amostrado no estado de Goiás (Werther et al., 2017). Recentemente, sequência 18S rRNA (672 pb) de *Babesia* sp. foi detectada em um albatroz-de-bico-amarelo-do-Atlântico (*Thalassarche chlororhynchos*) amostrado em Santa Catarina e posicionou-se próxima à *B. kiwiensis* (Sgarioni et al., 2023).

O objetivo do presente estudo foi investigar a ocorrência molecular de piroplasmídeos em morcegos não-hematófagos no bioma Amazônico e em aves e pequenos mamíferos no bioma Pantanal.

## **2. Material e Métodos**

### **2.1. Amostras biológicas e locais de estudo**

#### **2.1.1. Morcegos não-hematófagos capturados no bioma Amazônico**

Foram utilizadas amostras de baço coletadas de 271 morcegos-hematófagos de 30 espécies diferentes amostrados em Rio Branco (n=192) e Xapuri (n=79) no Acre (**Tabela 1**). As amostras foram gentilmente cedidas pelo Dr. André Luiz Roque (IOC-FIOCRUZ, RJ). O processo de captura dos animais foi previamente publicado por dos Santos et al. (2017).

### 2.1.2. Roedores selvagens e marsupiais capturados no Pantanal sul-matogrossense

Amostras de sangue foram coletadas de 113 roedores selvagens de três espécies diferentes e sete marsupiais de duas espécies amostrados no Pantanal do Mato Grosso do Sul (**Tabela 1**). O processo de captura e coleta das amostras está descrito em do Amaral et al. (2022).

### 2.1.3. Aves selvagens capturadas no Pantanal matogrossense e sul-matogrossense

Aves foram capturadas por meio de redes de neblinas e aproximadamente 50 µL de amostras de sangue foram coletadas da veia braquial e armazenadas em cartões Filter Technology Application (FTA). As coletas foram realizadas nos estados de Mato Grosso e Mato Grosso do Sul nos municípios de Santo Antônio de Leverger (n=200), Poconé (n=100), Nossa Senhora do Livramento (n=99) e Corumbá (n=101) (Alabí-Cordova et al., 2024). Ao todo, foram coletadas 500 amostras de sangue de aves pertencentes a 14 ordens e 93 espécies (**Tabela 1**). As amostras foram gentilmente cedidas pelo Dr. Alan Fecchio.

**Tabela 1.** Espécies de animais e número de amostras analisados para piroplasmídeos.

| Ordem      | Espécie                          | Número de amostras |
|------------|----------------------------------|--------------------|
| Chiroptera | <i>Anoura caldifer</i>           | 1                  |
|            | <i>Artibeus lituratus</i>        | 47                 |
|            | <i>Artibeus obscurus</i>         | 4                  |
|            | <i>Artibeus planirostris</i>     | 54                 |
|            | <i>Carollia beikeith cf.</i>     | 1                  |
|            | <i>Carollia brevicauda</i>       | 9                  |
|            | <i>Carollia perspicillata</i>    | 69                 |
|            | <i>Chiroderma villosum</i>       | 3                  |
|            | <i>Dermanura cinerea</i>         | 12                 |
|            | <i>Gardnerycteris crenulatum</i> | 1                  |
|            | <i>Glossophaga soricina</i>      | 7                  |
|            | <i>Hsuyunyeris thomasi</i>       | 2                  |
|            | <i>Lophostoma silvicolu</i>      | 3                  |
|            | <i>Mesophylla macconnelli</i>    | 2                  |
|            | <i>Phyllostomus discolor</i>     | 4                  |
|            | <i>Phyllostomus elongatus</i>    | 10                 |

|                 |                                  |    |
|-----------------|----------------------------------|----|
|                 | <i>Phyllostomus hastatus</i>     | 6  |
|                 | <i>Platyrrhinus incarum</i>      | 3  |
|                 | <i>Platyrrhinus infuscus</i>     | 2  |
|                 | <i>Rhinophylla fischeriae</i>    | 4  |
|                 | <i>Rhinophylla pumilio</i>       | 4  |
|                 | <i>Saccopteryx leptura</i>       | 1  |
|                 | <i>Sturnira giannae</i>          | 2  |
|                 | <i>Sturnira tildae</i>           | 2  |
|                 | <i>Tonatia maresi</i>            | 1  |
|                 | <i>Trachops cirrhosus</i>        | 1  |
|                 | <i>Uroderma bilobatum</i>        | 13 |
|                 | <i>Uroderma bilobatum</i>        | 8  |
|                 | <i>Vampyressa thyoune</i>        | 1  |
|                 | <i>Vampyressa spectrum</i>       | 1  |
| Rodentia        | <i>Thrichomys fosteri</i>        | 71 |
|                 | <i>Clyomys laticeps</i>          | 4  |
|                 | <i>Oecomys mamorae</i>           | 38 |
| Didelphimorphia | <i>Thylamys macrurus</i>         | 4  |
|                 | <i>Monodelphis domestica</i>     | 3  |
| Accipitriformes | <i>Busarellus nigricollis</i>    | 1  |
| Apodiformes     | <i>Glaucis hirsutus</i>          | 1  |
|                 | <i>Hylocharis chrysura</i>       | 1  |
|                 | <i>Phaethornis nattereri</i>     | 1  |
|                 | <i>Phaethornis subochraceus</i>  | 1  |
| Charadriiformes | <i>Jacana jacana</i>             | 4  |
| Columbiformes   | <i>Columbina talpacoti</i>       | 5  |
|                 | <i>Leptotila verreauxi</i>       | 10 |
| Coraciiformes   | <i>Chloroceryle aenea</i>        | 1  |
|                 | <i>Chloroceryle amazona</i>      | 2  |
|                 | <i>Chloroceryle americana</i>    | 5  |
|                 | <i>Megaceryle torquata</i>       | 4  |
| Cuculiformes    | <i>Coccyzus melacoryphus</i>     | 1  |
|                 | <i>Crotophaga ani</i>            | 8  |
|                 | <i>Guira guira</i>               | 2  |
| Galbuliformes   | <i>Monasa nigrifrons</i>         | 1  |
| Galliformes     | <i>Crax fasciolata</i>           | 1  |
| Gruiformes      | <i>Aramides cajanea</i>          | 1  |
| Passeriformes   | <i>Agelaioides badius</i>        | 4  |
|                 | <i>Agelasticus cyanopus</i>      | 10 |
|                 | <i>Antilophia galeata</i>        | 1  |
|                 | <i>Arremon flavirostris</i>      | 3  |
|                 | <i>Arundinicola leucocephala</i> | 3  |
|                 | <i>Basileuterus flaveolus</i>    | 19 |
|                 | <i>Basileuterus hypoleucus</i>   | 1  |
|                 | <i>Cacicus cela</i>              | 7  |
|                 | <i>Cacicus solitarius</i>        | 2  |
|                 | <i>Campylorhynchus turdinus</i>  | 1  |
|                 | <i>Cantorchilus leucotis</i>     | 5  |

|                                       |     |
|---------------------------------------|-----|
| <i>Cercomacra melanaria</i>           | 9   |
| <i>Certhiaxis cinnamomeus</i>         | 12  |
| <i>Cnemotriccus fuscatus</i>          | 3   |
| <i>Coereba flaveola</i>               | 4   |
| <i>Conirostrum speciosum</i>          | 1   |
| <i>Coryphospingus cucullatus</i>      | 1   |
| <i>Cranioleuca vulpina</i>            | 3   |
| <i>Cyanocorax chrysops</i>            | 1   |
| <i>Cyanocorax cyanomelas</i>          | 3   |
| <i>Cyclarhis gujanensis</i>           | 1   |
| <i>Dendroplex picus</i>               | 4   |
| <i>Donacobius atricapilla</i>         | 3   |
| <i>Elaenia albiceps</i>               | 3   |
| <i>Elaenia spectabilis</i>            | 1   |
| <i>Eucometis penicillata</i>          | 5   |
| <i>Fluvicola albiventer</i>           | 6   |
| <i>Formicivora rufa</i>               | 1   |
| <i>Furnarius leucopus</i>             | 10  |
| <i>Furnarius rufus</i>                | 16  |
| <i>Gnorimopsar chopi</i>              | 1   |
| <i>Hemitriccus margaritaceiventer</i> | 2   |
| <i>Hemitriccus striaticollis</i>      | 4   |
| <i>Herpsilochmus longirostris</i>     | 1   |
| <i>Hylophilus pectoralis</i>          | 1   |
| <i>Hypocnemoides maculicauda</i>      | 6   |
| <i>Icterus cayanensis</i>             | 3   |
| <i>Icterus croconotus</i>             | 1   |
| <i>Legatus leucophaeus</i>            | 4   |
| <i>Lepidocolaptes angustirostris</i>  | 2   |
| <i>Machetornis rixosa</i>             | 4   |
| <i>Myiarchus ferox</i>                | 3   |
| <i>Myiophobus fasciatus</i>           | 2   |
| <i>Myiozetetes cayanensis</i>         | 6   |
| <i>Paroaria capitata</i>              | 26  |
| <i>Pipra fasciicauda</i>              | 7   |
| <i>Pitangus sulphuratus</i>           | 21  |
| <i>Poecilotriccus latirostris</i>     | 4   |
| <i>Progne tapera</i>                  | 2   |
| <i>Pseudoseisura unirufa</i>          | 9   |
| <i>Ramphocelus carbo</i>              | 107 |
| <i>Saltator coerulescens</i>          | 16  |
| <i>Sicalis flaveola</i>               | 2   |
| <i>Sporophila angolensis</i>          | 8   |
| <i>Sporophila caerulescens</i>        | 1   |
| <i>Sporophila collaris</i>            | 5   |
| <i>Stelgidopteryx ruficollis</i>      | 2   |
| <i>Synallaxis albilora</i>            | 5   |
| <i>Taraba major</i>                   | 5   |

|                |                                |   |
|----------------|--------------------------------|---|
|                | <i>Thamnophilus doliatus</i>   | 1 |
|                | <i>Thraupis palmarum</i>       | 2 |
|                | <i>Thraupis sayaca</i>         | 3 |
|                | <i>Thryothorus genibarbis</i>  | 1 |
|                | <i>Turdus amaurochalinus</i>   | 3 |
|                | <i>Turdus hauxwelli</i>        | 1 |
|                | <i>Turdus leucomelas</i>       | 7 |
|                | <i>Turdus rufiventris</i>      | 9 |
|                | <i>Tyrannus melancholicus</i>  | 5 |
|                | <i>Vireo olivaceus</i>         | 1 |
|                | <i>Volatinia jacarina</i>      | 3 |
| Pelicaniformes | <i>Butorides striata</i>       | 1 |
|                | <i>Tigrisoma lineatum</i>      | 3 |
| Piciformes     | <i>Pteroglossus castanotis</i> | 1 |
| Psitaciformes  | <i>Brotogeris chiriri</i>      | 5 |
| Tinamiformes   | <i>Crypturellus undulatus</i>  | 1 |

## 2.2. Extração de DNA e PCR para os genes endógenos *gapdh* de mamíferos e $\beta$ -actina de aves

A extração de DNA das amostras de sangue e tecidos dos animais amostrados foi realizada utilizando o Kit Biopur Mini Spin Plus (MoBius Life Science, Paraná, Brasil), de acordo com as recomendações do fabricante. Amostras de DNA extraído tiveram sua concentração e relações 260/280 e 260/230 mensuradas em aparelho espectrofotômetro (Nanodrop, Thermo Scientific®) e foram armazenadas a -20°C até a realização da PCR.

Para as amostras de sangue em cartões FTA foi realizado um pré-preparo antes da extração de DNA. Os cartões foram cortados e colocados em microtubos contendo 200 µL de solução salina tamponada com fosfato (PBS) pH 7,8 e 20µL de proteinase K e armazenados a 65°C por 4 horas (Oliveira et al., 2018). Posteriormente, a extração de DNA foi realizada de acordo com as recomendações do fabricante do kit, como descrito anteriormente (Alabí Córdova et al., 2024).

Para verificar a presença de DNA amplificável nas amostras de DNA extraídas, elas foram submetidas a uma PCR convencional para a amplificação do gene endógeno *gapdh* de mamíferos (Birkenheuer et al., 2003) e  $\beta$ -actina de aves (Hatai et al., 2008), respectivamente.

## 2.3. Detecção molecular de piroplasmídeos

Foi realizada uma triagem por meio de uma nested PCR (nPCR) baseada no gene 18S rRNA de piroplasmídeos (Jefferies et al., 2007) nas amostras positivas para os genes *gapdh* de mamíferos e *β-actina* de aves. A reação de amplificação foi realizada utilizando uma reação com volume total final de 25 µL, contendo uma mistura de 5 µL do DNA amostra, 0,2 mM de cada deoxinucleotídeos, 0,5 µM de cada oligonucleotídeo iniciador, 1,5 mM de Cloreto de Magnésio, 0,75 U de Platinum Taq DNA Polimerase, tampão da PCR e 25 µL de água ultra-pura esterilizada q.s.p.. Na segunda amplificação (nPCR) foi utilizado 1 µL do amplificado na primeira reação.

As sequências dos oligonucleotídeos iniciadores utilizadas na primeira reação foram: BTF1 (5'-GGCTCATTACAACAGTTATAG-3') e BTR1 (5'-CCCAAAGACTTTGATTTCTCTC-3'). Já na segunda reação foram utilizados: BTF2 (5'-CCGTGCTAATTGTAGGGCTAATAC-3') e BTR2 (5'-GGACTACGACGGTATCTGATCG-3'). A ciclagem térmica realizada foi de: 94°C por 3 min, 58°C por 1 min, 72°C por 2 min seguidos de 45 ciclos a 94°C por 30s, 58°C por 20s e 72°C por 30s e extensão final a 72°C por 7 min. Na segunda reação o mesmo ciclo térmico foi utilizado, porém a temperatura de anelamento foi de 62°C. Água esterilizada ultrapura e DNA de *Babesia vogeli* obtida de cão (Furuta et al., 2007) foi utilizada como controle negativo e positivo, respectivamente, em todos os ensaios de nPCR.

### 3. Resultados

Todas as amostras biológicas de aves, roedores e marsupiais mostraram-se positivas nos ensaios de PCR para os genes endógenos. Em contrapartida, das 271 amostras de DNA oriundas de baço de morcegos, 208 (76,8%) foram positivas na PCR baseada no gene *gapdh* de mamíferos.

Nenhuma das 829 amostras analisadas foram positivas na nPCR baseada no gene 18S rRNA de piroplasmídeos. Adicionalmente, o protocolo utilizado mostrou baixa especificidade para as amostras de DNA de aves, devido a ocorrência de amplificações inespecíficas por anelamento dos oligonucleotídeos iniciadores no DNA do hospedeiro.

### 4. Discussão



As taxas de positividade molecular para piroplasmídeos encontradas em estudos realizados no Brasil vêm se mostrando baixa para os grupos de animais aqui analisados. Para morcegos, a ocorrência obtida variou de 0 a 18,8% (Ikeda et al., 2021; de Mello et al., 2023a; de Mello et al., 2023b). De Mello et al. (2023b), não detectaram DNA de piroplasmídeos em amostras de fígado de morcegos hematófagos de distintas regiões do Brasil. Em contrapartida, 18,77% das amostras de DNA extraídas de baço de morcegos-hematófagos amostrados na região norte do país (de Mello et al., 2023a). O presente trabalho não obteve êxito na amplificação desses agentes ao se utilizar amostras de baço. De todos os estudos realizados no mundo, apenas um conduzido na Lituânia encontrou elevada positividade para piroplasmídeos, sendo de 35,2 (44/125) e 52,3% (45/86) em amostras de DNA extraídas a partir de sangue e tecidos, respectivamente, de morcegos não-hematófagos (Sakalauskas et al., 2024). Naquele estudo, utilizou-se qPCR baseado no gene 18S rRNA para a detecção de *B. vesperuginis*, método este que mostrou maior sensibilidade quando comparado à nPCR. Além disso, entre os tecidos analisados, o coração mostrou uma maior positividade, seguido de baço, rins, pulmões e fígado, porém sem significância estatística entre eles (Sakalauskas et al., 2024). Futuros estudos devem ser conduzidos utilizando métodos moleculares com maior sensibilidade (e.g. digital PCR e qPCR) e outros tecidos (e.g. coração), a fim de investigar a real prevalência de piroplasmídeos em morcegos em território brasileiro. Não obstante, estudos anteriores conduzidos pelo nosso grupo de pesquisa mostram a ocorrência de piroplasmídeos de distintas linhagens filogenéticas em quirópteros não-hematófagos amostrados no bioma Cerrado (Ikeda et al., 2021) e em espécimes de *Desmodus rotundus* amostrados no bioma Amazônico (de Mello et al., 2024).

Em roedores selvagens, as ocorrências descritas no país variaram de 0 a 11,7% (Wolf et al., 2016; de Sousa et al., 2018; Colle et al., 2019; Gonçalves et al., 2021; Pacheco et al., 2025). DNA de piroplasmídeos também não foi detectado em estudos prévios realizados com 11 diferentes espécies de roedores (n=34) no estado do Mato Grosso (bioma Amazônico) (Colle et al., 2019) e com espécimes de *Mus musculus* (n=9) e *Rattus rattus* (n=39) amostrados no estado do Mato Grosso do Sul (bioma Cerrado) (Gonçalves et al., 2021) (Colle et al., 2019; Gonçalves et al., 2021). Além disso, em trabalho prévio realizado no Pantanal sul-matogrossense, apenas seis

de 110 roedores selvagens (5,5%) foram positivos para piroplasmídeos, evidenciando cinco genótipos (aproximadamente 700 pb do 18S rRNA) posicionados próximos à *B. vogeli* e um genótipo associado à *Theileria equi* (de Sousa et al., 2018). Em conjunto, tais os achados do presente estudo corroboram com estudos prévios, indicando baixa ocorrência de piroplasmídeos em roedores nos biomas Pantanal, Amazônia e Cerrado. Recentemente, um novo clado de piroplasmídeos (“South America Rodentia group”) foi descrito, incluindo genótipos 18S rRNA de Piroplasmida detectados em *T. pachyurus* capturados no bioma Cerrado (Pacheco et al., 2025).

Já a ocorrência molecular de piroplasmídeos em marsupiais reportada em estudos anteriores variou de 0 a 50% (sendo esta última positividade baseada em apenas dois animais amostrados) (Wolf et al., 2016; Soares et al., 2017; Colle et al., 2019; Gonçalves et al., 2021; Fava et al., 2022; Braga et al., 2023; de Oliveira et al., 2023). DNA de piroplasmídeos também não foi detectado em 30 marsupiais pertencentes à quatro espécies diferentes (incluindo *T. macrurus* e *M. domestica*) (de Sousa et al., 2018) capturados em uma fazenda vizinha à área de coleta do presente estudo, corroborando com nosso resultado. É digno de nota que o baixo número de amostras coletadas pode ter dificultado o encontro de amostras positivas em nosso estudo.

Um novo clado (“South American Marsupialia group”) composto por provavelmente uma nova espécie de *Babesia* spp. detectada em gambás no país foi descrito (Gonçalves et al., 2021; de Oliveira et al., 2023). A detecção de sequências 18S rRNA de piroplasmídeos posicionadas no referido clado em larvas de *Amblyomma* sp. e ninfas de *Amblyomma dubitatum* coletados de quatis e em ninfas de *A. dubitatum* coletadas de *D. albiventris* sugerem que essa espécie de carrapato possa atuar como possível vetor desse agente em marsupiais (Gonçalves et al., 2021; Perles et al., 2023), ainda que estudos de competência vetorial sejam necessários. Ainda, faz-se necessário compreender se espécies de piroplasmídeos (“South American Marsupialia group”) circulantes em gambás sinantrópicos (*Didelphis* spp.) poderiam infectar marsupiais selvagens de outros gêneros.

Outrossim, a ocorrência de piroplasmídeos em aves também é baixa (Ebani e Mancianti 2021). De fato, entre os poucos estudos realizados no Brasil, a positividade relatada foi baixa, variando de 0,7% a 14,3% (Quillfeldt et al., 2014; Werther et al.,

2017; Sgarioni et al., 2023). Além disso, uma das dificuldades encontradas no presente estudo foi a amplificação do DNA na nested PCR para piroplasmídeos, mostrando que os primers utilizados, que se mostram eficazes em detectar tais agentes em amostras biológicas de mamíferos, são menos eficientes em aves, o que torna ainda mais difícil obter uma detecção confiável.

Ainda que os vetores que transmitem as espécies de piroplasmídeos que infectam aves ainda sejam desconhecidos, suspeita-se que *Ornithodoros capensis* e *Ixodes uriae* possam estar envolvidos no ciclo de *B. peircei* e *B. ugwidiensis* (Peirce e Parsons, 2012; Yasbley et al., 2017), e que *Ornithodoros maritimus* atue como vetor de *Babesia* sp. YLG (pertencente ao “grupo Peircei”), a qual foi detectada em gaivotas-de-patas-amarelas (*Larus michahellis*) (Bonsergent et al., 2022; Bonsergent et al., 2023). No Brasil, as espécies de carrapatos mais encontradas parasitando aves do Pantanal foram ninfas de *Amblyomma nodosum*, *Amblyomma longirostre*, *Amblyomma sculptum*, *Amblyomma triste*, *Amblyomma calcaratum*, *Amblyomma ovale* e larvas de *Amblyomma* spp. e de *Ornithodoros mimom* (Ramos et al., 2015; Fecchio et al., 2020; Fecchio et al., 2023).

No geral, a ausência de positividade para piroplasmídeos em amostras biológicas de morcegos, roedores, marsupiais e aves no presente estudo pode estar relacionada a diversos fatores, tais como: baixa parasitemia (a qual poderia estar abaixo do limiar de sensibilidade da nested PCR utilizada); inespecificidade dos oligonucleotídeos iniciadores, os quais, em diversas ocasiões se anelavam no DNA de aves; ausência de vetores competentes nas áreas estudadas; ou simplesmente devido à baixa susceptibilidade das espécies de aves e pequenos mamíferos amostrados a piroplasmídeos. Estudos futuros objetivando investigar a ocorrência de piroplasmídeos em aves e pequenos mamíferos selvagens de outros biomas e utilizando técnicas de maior sensibilidade (e.g. dPCR) são necessários.

## 5. Conclusão

O presente estudo não detectou DNA de piroplasmídeos em morcegos amostrados no bioma Amazônico e roedores, marsupiais e aves amostrados no bioma Pantanal. Esse resultado pode estar associado a uma provável baixa parasitemia dos animais ou baixa ocorrência desses agentes nas espécies de animais amostrados.

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## **CAPÍTULO 7\* - Using digital PCR to unravel the occurrence of piroplasmids, *Bartonella* spp. and *Borrelia* spp. in wild animals from Brazil**

\* Este capítulo corresponde ao artigo científico publicado na revista Pathogens 14:567, 2025. <https://doi.org/10.3390/pathogens14060567>

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## Abstract

Piroplasmids (*Babesia* spp., *Rangelia* spp., *Theileria* spp., *Cytauxzoon* spp.) are tick-borne apicomplexan protozoa that infect, depending on the species, erythrocytes and leucocytes in a wide variety of mammals and birds. The genera *Bartonella* and *Borrelia* include vector-borne bacteria that can infect and cause disease in both animals and humans. Detection of hemotropic bacteria and piroplasmids in wild animals is often challenging due to low bacteremia or parasitemia. Digital (d)PCR has proven to be an effective modality for the detection and quantification of DNA of hemotropic pathogens with low parasitemia. This study compared dPCR results from 366 biological samples from seven different Brazilian wild animal groups (5 *Xenarthra* species, 5 deer species, 3 felid species, 1 canid species, 3 rodent species, 1 bat species, 1 tapir species, and 12 bird species) to two other molecular diagnostic techniques: quantitative real-time (qPCR) and nested (nPCR). For this study, DNA extracted from wild animal blood and spleen samples were subjected to a multiplex dPCR assay for piroplasmids, *Bartonella* spp., and *Borrelia* spp. For comparison, the same primers and probes for each agent were used in qPCR assays. Additionally, an nPCR based on the 18S rRNA gene for piroplasmids was performed. The proportions of positive results obtained using dPCR were 85.5% for piroplasmids, 33.6% for *Bartonella* spp., and 16.7% for *Borrelia* spp. For all tested agents, dPCR proved to be the technique with the highest sensitivity, making it a useful tool for screening vector-borne agents in biological samples from wild animals with low parasitemia.

**Key-Words:** PCR techniques; dPCR; hemoparasites; vector-borne agents; wild animals

### 1. Introduction

Arthropod-borne diseases currently account for 22.8% of emerging diseases [1]. Climate, environment, and ecology changes can disrupt the natural cycles of microorganisms, leading to the emergence of new diseases [2,3]. These changes also broaden the distribution of vectors and increase pathogen proximity among wildlife, domestic animals, and humans. As a result, closer interactions among reservoirs, vectors, and susceptible hosts are expected, facilitating the spread of diseases and presenting significant challenges to public and animal health [2,3].

*Bartonella* spp. (Hyphomicrobiales: Bartonellaceae) are Gram-negative fastidious facultative intracellular alphaproteobacteria that are primarily transmitted by arthropods (e.g., fleas, lice, ticks, and sand flies), as well as through scratches and bites [4–6]. *Borrelia* spp. (Spirochaetales: Spirochaetaceae) are spirochete bacteria transmitted by ixodid and argasid ticks and lice. Three distinct groups of *Borrelia* spp. are currently recognized: the Lyme group, the relapsing fever group, and the Echidna-

Reptile group [7–9]. Piroplasmids (Order Piroplasmida) are apicomplexan protozoa transmitted by ixodid ticks. This group consists of two families: Babesiidae (genera *Babesia* and *Rangelia*) and Theileriidae (genera *Theileria* and *Cytauxzoon*) [10,11]. These three pathogen groups contain zoonotic species that can cause disease in domestic and wild animals and human patients [7,8,12,13].

Brazil is a country with a highly diverse fauna [14]. Research into these agents among wild animals has increased substantially in recent years, resulting in the discovery of putative new species [15–36]. Despite numerous published studies, molecular detection of pathogen DNA in wild animals remains a diagnostic challenge due to the low parasitemia associated with these agents, which underestimates organism prevalence in epidemiological and clinical studies [3,11,19].

Currently, techniques known as third-generation PCR are being developed to increase the sensitivity of molecular assays. The most notable of these, digital PCR (dPCR), shares the same amplification principle as real-time PCR (qPCR) by using hydrolysis probes, but it allows samples to be fractionated into micro-well plates, capillaries (dPCR), or emulsions (digital droplet (dd)PCR) [37,38]. Fractionation enables separate amplification reactions in each partition, enhancing the detection of low concentrations of DNA, such as in samples from animals with low parasitemia or in the detection of rare genetic mutations [39–41]. Additionally, absolute quantification is achieved without the need for a standard curve using Poisson statistics to compare the number of positive and negative partitions [42–45]. Another advantage of this technique is its effectiveness in diluting out potential PCR inhibitors compared to qPCR [38,45–48]. The main disadvantages are its high cost and the inability to sequence dPCR-positive samples [39–42].

To date, dPCR has been used to enhance the molecular detection of different hemoparasites such as *Borrelia* spp. [49–51], *Bartonella* spp. [51,52], *Anaplasma phagocytophilum* [53], *Coxiella burnetii* [54], *Ehrlichia canis* [40], *Trypanosoma cruzi* [55], *Leishmania* spp. [56,57], *Plasmodium* spp. [58–60], and piroplasmids [51,61–63]. The use of this technique is still not widespread worldwide, including in Brazil. Due to the enhanced sensitivity, this study aimed to compare the positivity rates obtained using different PCR techniques (nested PCR–nPCR, qPCR, and dPCR) for piroplasmids, *Bartonella* spp., and *Borrelia* spp. in order to determine which technique

is most suitable for detecting the selected vector-borne agents in wild animal blood and spleen samples.

## **2. Material and Methods**

### **2.1. Samples analyzed**

In total, 366 blood ( $n = 116$ ), buffy coat ( $n = 174$ ), and spleen ( $n = 76$ ) DNA samples from wild animals in Brazil were analyzed. The animal species included: 174 deer (139 *Blastocerus dichotomus*, 6 *Ozotocerus bezoarticus*, 22 *Subulo gouazoubira*, 3 *Mazama americana*, and 4 *Mazama bororo*), 51 vampire bats (*Desmodus rotundus*), 11 *Cerdocyon thous*, 35 felids (12 *Leopardus pardalis*, 21 *Panthera onca*, and 2 *Puma concolor*), 44 Xenarthrans (17 *Myrmecophaga tridactyla*, 9 *Tamandua tetradactyla*, 7 *Dasypus novemcinctus*, 5 *Euphractus sexcinctus*, and 6 *Priodontes maximus*), 24 tapirs (*Tapirus terrestris*), 9 rodents (4 *Oecomys mamorae*, 4 *Thrichomys fosteri*, and 1 *Clyomis laticeps*), and 18 birds (1 *Pseudoseisura unirufa*, 3 *Saltator coerulescens*, 2 *Turdus leucomelas*, 1 *Cercomacra melanaria*, 2 *Ramphocelus carbo*, 1 *Basileuterus flaveolus*, 3 *Leptotila verreauxi*, 1 *Icterus cayanensis*, 1 *Lepidocolaptes angustirostris*, 1 *Fumarius rufus*, 1 *Fumarius leucopus*, and 1 *Cyamocorax chrysops*). All of the samples had been used in previously published studies [23,25,27,28,31–33,35].

### **2.2. Molecular assays**

#### **2.2.1. dPCR Assays (Multiplex for Piroplasmids, *Bartonella* spp., *Borrelia* spp., and housekeeping gene)**

A multiplex dPCR assay for piroplasmids, *Bartonella* spp., *Borrelia* spp., and the housekeeping gene was performed as previously described, with modifications (Maggi et al., 2021). All primer and probe sequences are described in **Table 1**.

**Table 1.** Description of primers, probes, and amplicon sizes of multiplex dPCR assays.

| Agent                  | Gene                             | Size       | Primer sequences                                     | Probe sequences                                             |
|------------------------|----------------------------------|------------|------------------------------------------------------|-------------------------------------------------------------|
| Piroplasmids           | 18S rRNA                         | 125-138 bp | Piro18S-238s<br>5'-TCGGTGATTGATAATAAACTRGCGAATCGC-3' | Piro18S-340probe<br>5'-CY5-GACGGTAGGGTATTGGCCTACCG-BHQ2-3'  |
|                        |                                  |            | Piro18S-380as<br>5'-AATCGAACCCCAATTCCCCGTTACCCG-3'   |                                                             |
| <i>Bartonella</i> spp. | 16S-23S rRNA (ITS)               | 90-120 bp  | BsppITS325s<br>5'-CCTCAGATGATGATCCCAAGCCTTCTGGCG-3'  | BsppITS500probe<br>5'-FAM-GTTAGAGCGCGCGCTTGATTAAG-BHQ1-3'   |
|                        |                                  |            | BsppITS543as<br>5'-AATTGGTGGGCTGGGAGGACTTG-3'        |                                                             |
| <i>Borrelia</i> spp.   | 16S-23S rRNA (ITS)               | 104 bp     | BobulITS120s<br>5'-AGGTCATTTGGGGGTTAGTCTAGTTGGCT-3'  | BobulITS160probe<br>5'-HEX-CGGCTTTGCAAGCCGAGGGTCAAG-BHQ2-3' |
|                        |                                  |            | BobulITS200as<br>5'-AATGGAGGTAA GGGACTCGAACCCT-3'    |                                                             |
| Housekeeping gene      | B-Raf Proto-Oncogene (BRAF) gene | 120 bp     | CaFeBRAF-15s<br>5'-TCAYGA AGACCTCACAGTAAAAATAGG T-3' | CaFeBRAF-50<br>5'-Cy5.5-GTCTAGCCACAGTGAAATCTCGATG-BHQ3-3'   |
|                        |                                  |            | CaFeBRAF-110as<br>5'-GATCCAGACAAC TGTTC AACTGATG -3' |                                                             |

The assays were performed using a QIAcuity Nanoplate 8.5k 96-well format (QIAGEN, Germantown, MD, USA). Each reaction (12 µL final volume) included 4.5 µL of molecular-grade water, 3 µL of 4X QIAcuity Probe PCR Master Mix (QIAGEN), 0.15 µL of each species-specific primer at 100 µM (IDT-DNA Technologies, Coralville, IA, USA), 0.05 µL of each species-specific probe at 100 µM, and 5 µL of DNA sample. Molecular-grade water and DNA from a negative dog blood sample were used as negative controls, while *Babesia microti*, *Bartonella henselae* SA1, and *Borrelia burgdorferi* B31 DNA were used as positive controls.

Amplification was performed on a QIAcuity One 5plex Platform System using 8.5k 96-well nanoplates under the following conditions: 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 20 s, and annealing/extension at 64 °C for 20 s. The detection of each species was assessed by reading the fluorescent signals (dots) using the following color codes: FAM (green) for *Bartonella* spp., HEX (yellow) for *Borrelia* spp., TAMRA (orange) for the housekeeping gene, and Cy5 (crimson) for piroplasmids. The fluorescent signal threshold was assessed as positive or negative for DNA amplification. The results were analyzed using QIAcuity Software Suite version 2.2.0.26. Partitions with fluorescence signals below the defined threshold were classified as negative, while those above the threshold were considered positive. Samples were deemed positive when they contained one or more positive partitions, provided the signal was clearly above the threshold, in order to minimize the risk of

false positive results. The number of copies per microliter (copies/ $\mu$ L) was calculated by the software based on the number of positive and negative partitions using Poisson statistics.

### **2.2.2. qPCR Assays (Multiplex for Piroplasmids, *Bartonella* spp., and *Borrelia* spp.)**

Real-time qPCR assays were performed using the same primers and probes described above for the detection of piroplasmids, *Bartonella* spp., and *Borrelia* spp. in order to compare the results obtained using both PCR techniques.

Multiplex real-time qPCR was performed using a reaction mixture with a final volume of 25  $\mu$ L per reaction containing: 0.25  $\mu$ L of each primer for *Bartonella* spp. and *Borrelia* spp., 0.375  $\mu$ L of each primer for piroplasmids at 100  $\mu$ M (IDT-DNA Technologies, Coralville, IA, USA), 0.125  $\mu$ L of 100  $\mu$ M of each species-specific probe, 12.5  $\mu$ L of 2X SsoAdvanced Probe Master Mix (Bio-Rad, Hercules, CA, USA), 5  $\mu$ L of template DNA, and 5.875  $\mu$ L of molecular-grade water (QIAGEN, Germantown, MD, USA). Molecular-grade water and DNA from a negative dog blood sample were used as PCR negative controls. *Babesia microti*, *Bartonella henselae* SA1, and *Borrelia burgdorferi* B31 DNA were used as positive controls.

Amplification was performed using a Bio-Rad CFX 96-well Opus PCR system under the following conditions: 95 °C for 3 min, followed by 45 cycles of denaturation at 94 °C for 10 s, annealing at 68 °C for 10 s, and extension at 72 °C for 10 s. Fluorescent signals were used to assess the detection of each species, using FAM (green) for *Bartonella* spp., HEX (yellow) for *Borrelia* spp., and Cy5 (crimson) for piroplasmids. The fluorescent signal threshold value (Cq) was recorded to determine positive and negative DNA amplification.

Additionally, positive samples were purified and sequenced by Sanger's method (Genewiz, Azenta, Research Triangle Park, NC, USA) and analyzed using SnapGene Version 7.0.2.

### **2.2.3. Nested (n)PCR assays for Piroplasmids detection**

nPCR was performed to amplify approximately an 800 bp fragment of the 18S rRNA gene of piroplasmids using the primers BTF1 (5'-

GGCTCATTACAACAGTTATAG-3') and BTR1 (5'-CCCAAAGACTTTGATTTCTCTC-3') in the first reaction, and BTF2 (5'-CCGTGCTAATTGTAGGGCTAATAC-3') and BTR2 (5'-GGACTACGACGGTATCTGATCG-3') in the second reaction, as described by Jefferies et al. (2007) [64].

The amplification reaction was carried out with a total final volume of 25  $\mu$ L containing the following components: 5  $\mu$ L of sample DNA, 0.2 mM of each deoxynucleotide, 0.5  $\mu$ M of each primer oligonucleotide, 1.5 mM of magnesium chloride, 0.75 U of Taq DNA polymerase, PCR buffer, and sterile ultrapure water to complete the volume. For the second amplification, 1  $\mu$ L of the product from the first reaction was used.

The thermal cycling conditions for the first reaction were the following: initial denaturation at 94 °C for 3 min, followed by 45 cycles of 94 °C for 30 s, 58 °C for 20 s, and 72 °C for 30 s, with a final extension at 72 °C for 7 min. For the second reaction, the same thermal conditions were used, with the exception that the annealing temperature was set to 62 °C. Ultrapure sterile water was used as a negative control in all PCR assays, while *Babesia vogeli* DNA (Jaboticabal sample) was used as a positive control.

### 2.3. Statistical Analysis

To compare the performance of the assays for detecting piroplasmids, Fleiss' Kappa test was initially used to assess the level of agreement between all tests (dPCR, qPCR, and nPCR), beyond what would be expected by chance [65]. As a post hoc analysis, agreement beyond chance between test pairs was evaluated using Cohen's Kappa test [66]. For the other agents (*Bartonella* spp. and *Borrelia* spp.), Cohen's Kappa test was applied.

The interpretation of Kappa ( $\kappa$ ) values was based on the criteria outlined by McHugh (2012) [67], which are as follows: values below zero indicate agreement below that expected by chance (disagreement); values between 0.00 and 0.20 suggest no agreement; values between 0.21 and 0.39 represent minimal agreement; values between 0.40 and 0.59 indicate weak agreement; values between 0.60 and 0.79 reflect moderate agreement; values between 0.80 and 0.90 indicate strong agreement; and values above 0.90 reflect almost perfect agreement.



To assess the difference between the proportions obtained by the tests, McNemar's test [68] was performed for each pair of tests. All statistical analyses were carried out using RStudio 4.2.3 software with the *psych* [69] and *irr* [70] packages.

The sensitivity and specificity of the assays evaluated (qPCR and nPCR for piroplasmids; qPCR for *Bartonella* spp. and *Borrelia* spp.) were determined using dPCR as the gold standard due to its superior sensitivity compared to the other assays [51]. Finally, the Matthews correlation coefficient (MCC) [71] was calculated for each pair, using dPCR as the gold standard, to assess the performance of the assays. The MCC values range from -1 to 1, with extreme values indicating maximum performance (1) or total failure in classification (-1). A value of zero indicates performance equivalent to random choice [71].

The formulas shown in Figure 1 were used to calculate sensitivity (Se), specificity (Sp), and MCC.

**A**

$$Se = \frac{TP}{TP + FN}$$

**B**

$$Sp = \frac{TN}{TN + FP}$$

**C**

$$MCC = \frac{(TP \times TN) - (FP \times FN)}{\sqrt{(TP + FP)(TP + FN)(TN + FP)(TN + FN)}}$$

**Figure 1.** Formulas used to calculate sensitivity (A), specificity (B), and Matthews correlation coefficient (C). In these formulas, TP represents the number of true positive samples (identified as positive by the gold standard), FN represents the number of false negative samples (identified as negative by the assay evaluated but as positive by the gold standard), TN represents the number of true negative samples (identified as negative by the gold standard), and FP represents the number of false positive samples (identified as positive by the assay evaluated but as negative by the gold standard).

### 3. Results

#### 3.1. Positivity for each technique

Table 2 depicts the number of positive animals for each group and species based on the agent analyzed (piroplasmids, *Bartonella* spp., and *Borrelia* spp.). For all agents, dPCR had a higher number of positive samples compared to the other

techniques, with the exception of piroplasmid detection in *Xenarthra* species, where nPCR detected one more positive sample ( $n = 22$ ) than dPCR ( $n = 21$ ).

**Table 2.** Number of animals positive for each agent and test performed.

| Wild Animal Group                    | Number of Positive Samples |             |                      |           |              |              |              |
|--------------------------------------|----------------------------|-------------|----------------------|-----------|--------------|--------------|--------------|
|                                      | <i>Bartonella</i> spp.     |             | <i>Borrelia</i> spp. |           | Piroplasmids |              |              |
|                                      | dPCR                       | qPCR        | dPCR                 | qPCR      | nPCR         | dPCR         | qPCR         |
| Deer (Total)                         | 56 (32.18%)                | 12 (6.9%)   | 25 (14.37%)          | 0         | 137 (78.73%) | 154 (88.51%) | 51 (29.31%)  |
| <i>Subulo gouazoubira</i>            | 6                          | 2           | 6                    | 0         | 22           | 19           | 2            |
| <i>Mazama rufa</i>                   | 0                          | 0           | 1                    | 0         | 3            | 2            | 0            |
| <i>Mazama jucunda</i>                | 2                          | 0           | 3                    | 0         | 4            | 4            | 0            |
| <i>Blastocerus dichotomus</i>        | 45                         | 10          | 15                   | 0         | 102          | 124          | 47           |
| <i>Ozotocerus bezoarticus</i>        | 3                          | 0           | 0                    | 0         | 6            | 5            | 2            |
| <i>Desmodus rotundus</i>             | 46 (90.2%)                 | 17 (33.33%) | 14 (27.45%)          | 2 (3.92%) | 42 (82.35%)  | 47 (92.15%)  | 16 (31.37%)  |
| <i>Cerdocyon thous</i> (Total)       | 3 (27.27%)                 | 1 (9.09%)   | 1 (9.09%)            | 0         | 3 (27.27%)   | 11 (100%)    | 9 (81.81%)   |
| Felids (Total)                       | 6 (17.14%)                 | 1 (2.86%)   | 7 (20%)              | 0         | 29 (82.86%)  | 34 (97.14%)  | 19 (54.29%)  |
| <i>Leopardus pardalis</i>            | 1                          | 0           | 1                    | 0         | 9            | 12           | 10           |
| <i>Panthera onca</i>                 | 4                          | 1           | 4                    | 0         | 18           | 21           | 9            |
| <i>Puma concolor</i>                 | 1                          | 0           | 2                    | 0         | 2            | 2            | 0            |
| Xenarthra (Total)                    | 6 (13.64%)                 | 6 (13.64%)  | 6 (13.67%)           | 0         | 22 (50%)     | 21 (47.73%)  | 14 (31.82%)  |
| <i>Myrmecophaga tridactyla</i>       | 0                          | 2           | 1                    | 0         | 12           | 10           | 6            |
| <i>Tamandua tetradactyla</i>         | 1                          | 0           | 1                    | 0         | 4            | 3            | 1            |
| <i>Euphractus sexcinctus</i>         | 3                          | 1           | 1                    | 0         | 1            | 3            | 2            |
| <i>Dasypus novemcinctus</i>          | 2                          | 2           | 2                    | 0         | 4            | 2            | 3            |
| <i>Priodontes maximus</i>            | 0                          | 1           | 1                    | 0         | 1            | 3            | 2            |
| Birds (Total)                        | 4 (22.22%)                 | 1 (5.56%)   | 2 (11.11%)           | 0         | 8 (44.44%)   | 13 (72.22%)  | 6 (33.33%)   |
| <i>Pseudoseisura unirufa</i>         | 0                          | 0           | 0                    | 0         | 1            | 1            | 1            |
| <i>Saltator coerulescens</i>         | 2                          | 0           | 1                    | 0         | 2            | 2            | 2            |
| <i>Turdus leucomelas</i>             | 1                          | 0           | 0                    | 0         | 1            | 1            | 0            |
| <i>Cercomacra melanaria</i>          | 0                          | 0           | 0                    | 0         | 1            | 1            | 0            |
| <i>Ramphocelus carbo</i>             | 0                          | 0           | 0                    | 0         | 1            | 0            | 0            |
| <i>Basileuterus flaveolus</i>        | 0                          | 0           | 0                    | 0         | 1            | 1            | 0            |
| <i>Leptotila verreauxi</i>           | 0                          | 1           | 0                    | 0         | 1            | 2            | 1            |
| <i>Icterus cayanensis</i>            | 0                          | 0           | 0                    | 0         | 0            | 1            | 1            |
| <i>Lepidocolaptes angustirostris</i> | 0                          | 0           | 1                    | 0         | 0            | 1            | 0            |
| <i>Fumarius rufus</i>                | 0                          | 0           | 0                    | 0         | 0            | 1            | 0            |
| <i>Fumarius leucopus</i>             | 0                          | 0           | 0                    | 0         | 0            | 1            | 0            |
| <i>Cyamocorax chrysops</i>           | 1                          | 0           | 0                    | 0         | 0            | 1            | 1            |
| <i>Tapirus terrestris</i> (Total)    | 4 (16.67%)                 | 0           | 4 (16.67%)           | 0         | 16 (66.67%)  | 24 (100%)    | 4 (16.67%)   |
| Rodents (Total)                      | 4 (44.44%)                 | 1 (11.11%)  | 2 (22.22%)           | 0         | 0            | 9 (100%)     | 4 (44.44%)   |
| <i>Oecomys mamorae</i>               | 3                          | 0           | 0                    | 0         | 0            | 4            | 3            |
| <i>Thrichomys fosteri</i>            | 1                          | 1           | 1                    | 0         | 0            | 4            | 1            |
| <i>Clyomys laticeps</i>              | 0                          | 0           | 1                    | 0         | 0            | 1            | 0            |
| <b>Total</b>                         | 129 (35.26%)               | 39 (10.66%) | 61 (16.67%)          | 2 (0.55%) | 257 (70.22%) | 313 (85.52%) | 123 (33.61%) |

To better understand the positivity rates and compare the number of positive samples across techniques, Venn diagrams were generated (Figure 2). These diagrams showed that the number of positive samples was higher when using the dPCR technique for all target agents. For *Bartonella* spp. (Figure 2A) and *Borrelia* spp. (Figure 2B), only 27 and 1 samples, respectively, were positive when both techniques were used (dPCR and qPCR). Additionally, 12 and 1 samples were exclusively positive when qPCR was used for *Bartonella* spp. and *Borrelia* spp., respectively.

In the case of piroplasmids (Figure 2C), 73 samples tested positive across all three techniques performed (dPCR, nPCR, and qPCR). A larger number of samples ( $n = 161$ ) was positive when both dPCR and nPCR were used, while 43 samples were positive when both dPCR and qPCR were used. Only 25 samples were positive using techniques other than dPCR—18 in nPCR alone, 2 in qPCR alone, and 5 in both nPCR and qPCR.

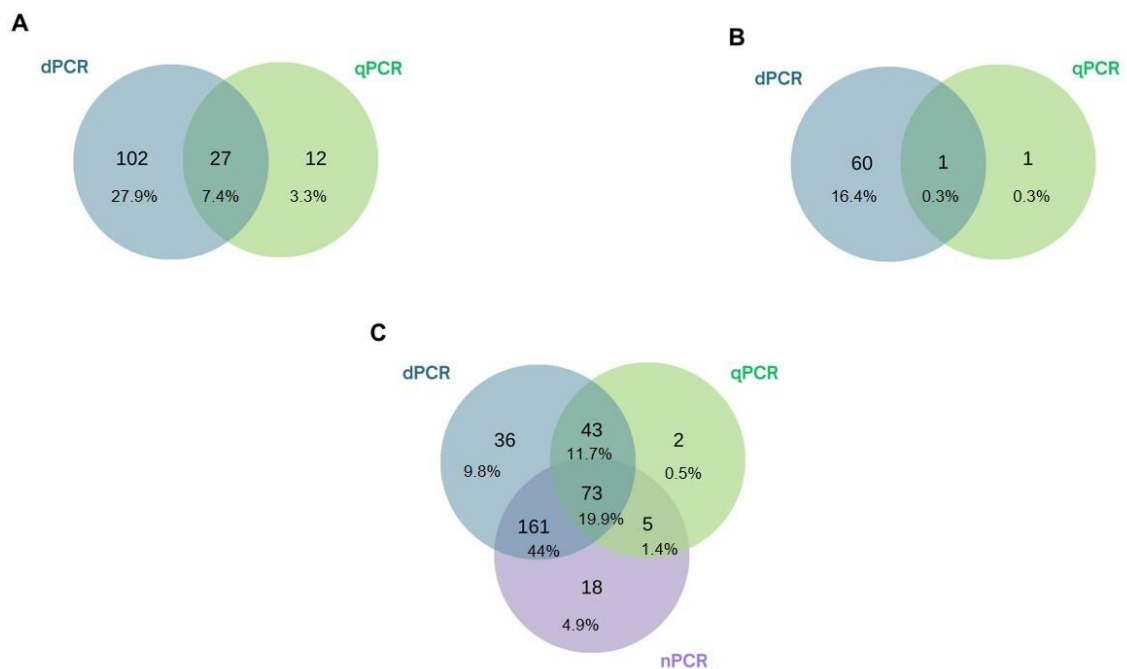


**Figure 2.** Venn diagram illustrating the samples positive for *Bartonella* spp. (A), *Borrelia* spp. (B), and piroplasmids (C), as identified by the tests analyzed in this study.

Given that the objective of this study was to compare the positivity between techniques, and considering the absence of standard calibration curves for qPCR, absolute quantification of the samples using this method was not performed. Nonetheless, the number of positive partitions, the concentration expressed as copies per microliter (copies/ $\mu$ L) of the target fragment obtained via dPCR, and the quantification cycle (C<sub>q</sub>) values derived from qPCR were duly documented.

For *Bartonella* spp. and *Borrelia* spp., most samples exhibited only a single positive partition in the dPCR assays ( $n = 50$  and  $n = 47$ , respectively) (Figures 3A,B). For *Bartonella* spp., the lowest concentration of the amplified 16S–23S rRNA gene fragment was 0.356 copies/ $\mu$ L, while the highest concentration reached 4150.8

copies/ $\mu\text{L}$  in the dPCR assays (Table S1). In the qPCR assays, the mean Cq value was 30.86, with a minimum of 14.62 and a maximum of 44.7 (Table S1). For *Borrelia* spp., dPCR detected a minimum of 0.356 copies/ $\mu\text{L}$  and a maximum of 2.636 copies/ $\mu\text{L}$  of the target fragment (Table S2). In the qPCR analyses, Cq values of 43.26 and 44.93 were obtained for the two amplified bat DNA samples (Table S2).

Regarding piroplasmids, the dPCR analyses revealed that the majority of positive samples ( $n = 207$ ) exhibited between 2 and 100 positive partitions, while 20 samples presented with more than 1000 positive partitions (Figure 3C). This technique was capable of detecting as few as 0.356 copies/ $\mu\text{L}$  of the 18S rRNA gene fragment, with the highest concentration recorded at 1784.1 copies/ $\mu\text{L}$  (Table S3). In the qPCR assays, the mean Cq value was 33.22, ranging from a minimum of 25.12 to a maximum of 43.57 (Table S3).

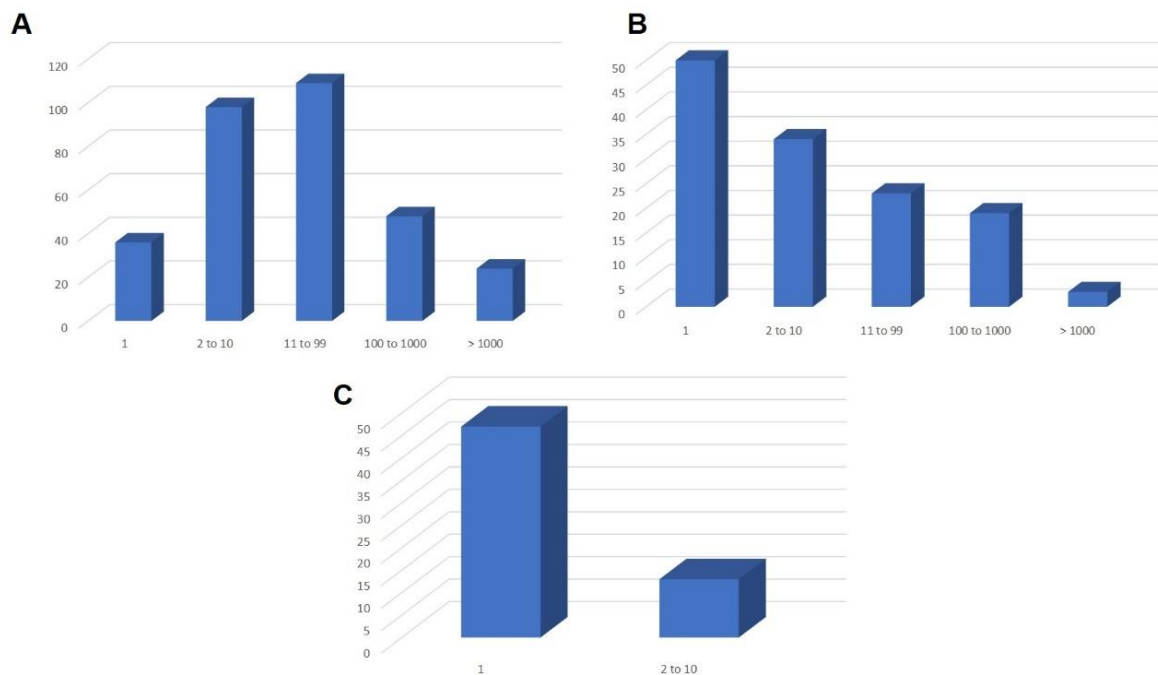


**Figure 3.** Number of samples positive for piroplasmids (A), *Bartonella* spp. (B), and *Borrelia* spp. (C) across different ranges of positive partitions. The x-axis represents the range of the number of positive partitions, while the y-axis shows the number of samples corresponding to each range of positive partitions.

With regard to the samples that tested positive using qPCR but negative using dPCR, the majority presented Cq values greater than 39. Among the seven piroplasmid samples that were positive only using qPCR, five exhibited Cq values

above 39. Similarly, of the twelve *Bartonella* spp. samples that were qPCR-positive only, eight had Cq values exceeding 39. For the two *Borrelia* spp. samples derived from bat DNA that tested positive exclusively using qPCR, both exhibited Cq values above 43.

Only samples with Cq values lower than 38 using qPCR were sent for sequencing to confirm that there was targeted amplification of the group of agents surveyed. In total, 94 and 29 samples were submitted for DNA sequencing for piroplasmids and *Bartonella* spp., respectively. Of these samples, 49 piroplasmid and 12 *Bartonella* spp. were successfully sequenced. The qPCR fragment obtained for piroplasmids, ranging from 50 to 70 bp, had high identity (97 to 100% similarity) with *Theileria* spp. or *Babesia* spp. For *Bartonella* spp., the sequences (approximately 100 bp) were mostly obtained from vampire bats (*Desmodus rotundus*) and were identical to a *Bartonella* spp. sequence deposited from the same host species in Guatemala (MN504749). A sequence obtained from *B. dichotomus* was 98.29% identical to a *Bartonella henselae* sequence found in a flea from Brazil (MT095054). A sequence obtained from *T. fosteri* was identical to a *Bartonella machadoae* sequence (CP087114).

Additional Venn diagrams were generated to compare the results obtained using each technique for the different target agents, considering dPCR-positive samples as those with one or more, two or more, and three or more positive partitions (Supplementary Material Figure S1). For all agents evaluated, the highest concordance between the techniques was observed when samples with one or more positive partitions were considered positive in the dPCR analyses.

## **3.2. Statistical Analyses**

### **3.2.1. Level of Agreement Between Tests**

#### **Piroplasmids**

The Fleiss' Kappa test indicated that the agreement among the three tests (dPCR vs. qPCR vs. nPCR) was lower than expected by chance; however, this difference was not statistically significant ( $p > 0.05$ ). When analyzing the agreement between pairs of tests, the Cohen's Kappa test revealed that the levels of agreement

were lower than expected by chance, or absent, but all were statistically significant ( $p < 0.05$ ). These results suggested that the agreement levels among assays varied depending upon which pair of tests was being compared. Among all comparisons, the lowest Kappa index was observed between qPCR and nPCR results ( $k = -0.09$ ), indicating a significant disagreement. The agreement between nPCR and dPCR results was considered minimal ( $k = 0.20$ ), while the agreement between dPCR and qPCR results was considered absent ( $k = 0.089$ ). Table 3 summarizes the results from both the Fleiss' Kappa and Cohen's Kappa tests.

**Table 3.** Results obtained in the Fleiss' Kappa and Cohen's Kappa analyses of the agreement between test results for piroplasmids.

| Test          | Comparison             | Kappa (k) | p-value           |
|---------------|------------------------|-----------|-------------------|
| Fleiss' Kappa | dPCR vs. qPCR vs. nPCR | -0.0517   | 0.0865            |
| Cohen's Kappa | qPCR vs. nPCR          | -0.0935   | <b>0.0185</b>     |
|               | nPCR vs. dPCR          | 0.2010    | <b>&lt;0.0001</b> |
|               | dPCR vs. qPCR          | 0.0896    | <b>0.0012</b>     |

p-value considered significant when  $< 0.05$ .

### ***Bartonella* sp. and *Borrelia* spp.**

For the detection of *Bartonella* spp., the Cohen's Kappa test indicated that the agreement between qPCR and dPCR was absent ( $k = 0.1887$ ) but statistically significant ( $p = <0.0001$ ;  $p < 0.05$ ). These results indicated a non-random disagreement between these molecular assays, with dPCR presenting superior performance for detecting *Bartonella* spp. compared to qPCR. For *Borrelia* spp., the Cohen's Kappa test showed that the agreement between qPCR and dPCR was absent ( $k = 0.0214$ ) and not statistically significant ( $p = 0.4261$ ;  $p > 0.05$ ).

### **3.2.2. Analysis of Differences Between Proportions**

#### **Piroplasmids**

The difference in proportions between the tests was analyzed in pairs using McNemar's test. All comparisons yielded statistically significant results ( $p < 0.05$ ), indicating that the PCR assays significantly differed in their ability to detect piroplasmid-positive samples. The most notable difference was observed between dPCR and qPCR ( $\chi^2 = 117.09$ ), reinforcing a discrepancy in positive results obtained using the two methods. Table 4 summarizes the results of McNemar's test.

**Table 4.** Analysis of the difference between the proportions obtained in the tests for detecting piroplasmids evaluated in this study.

| Comparison    | $\chi^2$ | p-value |
|---------------|----------|---------|
| qPCR vs. nPCR | 79.101   | <0.0001 |
| nPCR vs. qPCR | 30.447   | <0.0001 |
| dPCR vs. qPCR | 177.09   | <0.0001 |

p-value considered significant when  $< 0.05$ ;  $\chi^2$  = chi-squared value.

#### *Bartonella* spp. and *Borrelia* spp.

The difference in proportions between qPCR and dPCR, for both *Bartonella* spp. and *Borrelia* spp. detection, was statistically significant ( $p < 0.05$ ), indicating that qPCR and dPCR significantly differed in their ability to amplify the DNA of the targeted organisms in wild animal samples. Table 5 summarizes the results of McNemar's test for these assays.

**Table 5.** Analysis of the difference between the proportions obtained in the tests for detecting *Bartonella* spp. and *Borrelia* spp. evaluated in this study.

| Agent                  | Comparison    | $\chi^2$ | p-value |
|------------------------|---------------|----------|---------|
| <i>Bartonella</i> spp. | qPCR vs. dPCR | 69.482   | <0.0001 |
| <i>Borrelia</i> spp.   | qPCR vs. dPCR | 55.148   | <0.0001 |

p-value considered significant when  $< 0.05$ ;  $\chi^2$  = chi-squared value.

### 3.2.3. Sensitivity and Specificity

#### Piroplasmids

The sensitivity and specificity values for qPCR, compared to the gold standard (dPCR), were 36.82% and 86.27%, respectively. These values also indicated a low capacity to identify positive samples compared to dPCR. For nPCR, the sensitivity and specificity values were 74.60% and 54.90%, respectively, demonstrating an unsatisfactory ability to correctly identify negative results compared to dPCR.

### ***Bartonella* spp. and *Borrelia* spp.**

For the *Bartonella* spp. qPCR assay, the sensitivity and specificity values, compared to the gold standard (dPCR), were 20.93% and 94.94%, respectively. These results indicated a poor ability to detect positive samples compared to dPCR, but a high specificity in distinguishing *Bartonella* spp.-negative samples.

For the *Borrelia* spp. detection assay, the sensitivity and specificity values were 1.64% and 99.67%, respectively, indicating an extremely low capacity to detect positive samples when compared to the gold standard, but a high ability to identify negative samples.

### **3.2.4. Matthews Correlation Coefficient (MCC)**

#### **Piroplasmids**

The MCC was calculated to assess the degree of agreement between the qPCR and nPCR assays compared to the gold standard (dPCR). The value obtained for the comparison between qPCR and dPCR was 0.1693538, while the value for the comparison between nPCR and dPCR was 0.2240352. These values indicated that both qPCR and nPCR had a low correlation with dPCR, although nPCR had a slightly higher correlation.

### ***Bartonella* spp. and *Borrelia* spp.**

For the *Bartonella* spp. detection assay, the MCC value for the comparison between qPCR and dPCR was 0.24566722, indicating a low correlation with dPCR. For the *Borrelia* spp. detection assay, the MCC value was -0.0256786, indicating no correlation between the tests.

## **4. Discussion**



In this study, we evaluated the performance of dPCR, qPCR, and nPCR in detecting three vector-borne agents in biological samples from wild animals from Brazil, in which low parasitemia was expected. In general, there was no agreement between the molecular tests performed for piroplasmids, *Bartonella* spp., and *Borrelia* spp. nPCR and dPCR had slightly better agreement for the detection of piroplasmids, albeit the level of agreement was still low. The qPCR protocol used demonstrated low sensitivity in detecting the targeted agents investigated in the selected wild animal samples, whereas dPCR proved to be much more sensitive in detecting these selected agents compared to the other two techniques.

Some previous studies contradict our findings, reporting a high level of agreement between results obtained using ddPCR and qPCR [46,57,62]. These studies investigated the detection of *Cryptosporidium* oocysts in fecal samples from animals and humans previously confirmed positive by microscopy [46], *Cytauxzoon* spp. in blood samples from cats with acute cytauxzoonosis [62], and *Leishmania infantum* in spleen samples from dogs exhibiting clinical signs consistent with leishmaniasis [57]. Notably, none of these studies included samples from wild animals, and all analyzed specimens originated from individuals either displaying clinical signs or previously confirmed positive by other diagnostic methods. By contrast, a study published by Lashnits et al. (2021) [72], which compared the sensitivity of *Bartonella* diagnostic assays in dogs with hemangiosarcoma, found that ddPCR was significantly more sensitive than qPCR for the detection of *Bartonella* DNA in blood samples (36% vs. 0%,  $p < 0.0001$ ). Additionally, a study by Belmonte et al. (2016) [73] compared the effectiveness of ddPCR and qPCR in detecting low-abundance mitochondrial DNA (mtDNA) deletions in human samples. The results demonstrated that ddPCR was highly effective and preferable for samples with moderate deletion loads, whereas qPCR was only effective in samples with high deletion levels.

Furthermore, in previous studies targeting helminth DNA in liver samples from small mammals [74] and in sheep fecal samples [75], ddPCR demonstrated superior sensitivity compared to qPCR. For example, the positivity rate for *Echinococcus multilocularis* in small mammal liver samples was 31.13% using ddPCR, versus only 4.72% with qPCR [74]. In another study, the detection rates for *Trichuris* spp. in sheep fecal samples were 80.6% using ddPCR and 72.4% using qPCR [75]. According to the

authors, one possible explanation for these differences was the presence of PCR inhibitors in fecal samples, which may interfere with the amplification and detection of parasite DNA using qPCR. However, the relative abundance of the target DNA may also represent an equally important factor influencing detection sensitivity.

In the present study, the discrepancy between the results obtained using dPCR and qPCR, especially in the detection of *Bartonella* spp. and *Borrelia* spp., might be attributed to extremely low parasitemia in the samples, precluding detection of the target DNA using qPCR. Indeed, the low number of positive partitions in dPCR across most samples indicated low parasitemia. It has been demonstrated that ddPCR can quantify smaller amounts of DNA than the standard curves used in qPCR [61].

Additionally, ddPCR has been shown to identify a higher number of positive samples for *Bartonella* spp. and *Candida* spp. compared to qPCR [39,52]. This can be explained by the fact that DNA samples are fractionated and amplification reactions occur individually in each fraction in dPCR, which favors the detection of low copy numbers of the target DNA fragment. By contrast, qPCR measures fluorescent signals, which can sometimes be less sensitive for low-abundance targets [39–41,44].

Herein, some samples were positive using qPCR but negative using dPCR for the agents investigated. When analyzed individually, all of these samples had a C<sub>q</sub> value above 39, suggesting that the positive qPCR results were likely due to nonspecific amplification.

Regarding the detection of piroplasms, only 19.9% samples were positive using all three molecular techniques in this study, while 44% samples were only positive using both nPCR and dPCR, and 11.7% were positive using both dPCR and qPCR. The higher number of positive samples observed using nPCR compared to qPCR is noteworthy; however, when comparing the specificities for each test, nPCR yielded a specificity of 54.9%, while qPCR had a specificity of 86.3%. These findings likely indicate that nPCR might have generated false positive results through nonspecific amplification or due to a higher risk of amplicon contamination, as nPCR requires extensive sample processing between reactions.

An enrichment blood culture protocol for the detection of piroplasms targeting the 18S rRNA gene, applied to DNA extracted from human blood samples and from 7-, 14-, and 21-day enrichment blood cultures, also demonstrated greater sensitivity

using dPCR compared to qPCR. However, when qPCR protocols specifically designed for the amplification of species-specific ITS-1 and ITS-2 intergenic regions for *Babesia odocoilei*, *B. microti*, and *B. divergens* were employed, qPCR proved to be highly effective and sensitive for species detection, with the results confirmed by sequencing [76,77].

Most published studies have employed ddPCR rather than dPCR, with the primary distinction being that ddPCR is performed in an oil emulsion, while dPCR relies on micro-well plates or capillary systems [38]. In the present study, dPCR was conducted using the QIAcuity Digital PCR System (QIAGEN), utilizing 8.5k 96-well nanoplates in an effort to reduce costs while evaluating whether the method would offer sufficient sensitivity for detecting hemoparasite DNA in biological samples from wild animals. Although the use of 26k 24-well plates could potentially yield improved results—given that the fourfold increase in partition number may enhance assay sensitivity—the results obtained demonstrated that the chosen approach was both effective and suitable for the study's objectives.

There remains some disagreement among manufacturers of dPCR and ddPCR platforms regarding the minimum number of positive partitions or droplets required to consider a sample positive. In the present study, samples that had one or more positive partitions were utilized for the three agents studied. Although a low number of positive partitions could reflect false positive testing results, it was likely attributable to the low levels of parasitemia or bacteremia in the tested samples. This conclusion was supported by the low copy numbers of the target fragments obtained for all the agents analyzed using dPCR, with values ranging from as low as 0.356 copies/ $\mu$ L to a maximum of only 4150.8 copies/ $\mu$ L.

The detection of *Bartonella* spp. in deer blood samples was particularly noteworthy, marking it as the first report of this agent in deer from Brazil. Previously, *Bartonella ribC* genotypes closely related to *Bartonella schoenbuchensis* were detected in *Lipoptena mazamae* flies collected from deer in Rio Grande do Sul, Brazil [78]. Another interesting result was the detection of *Borrelia* spp. in the tested animals. Since all of the positive samples showed only one positive partition, this may help to explain why *Borrelia* spp. are rarely reported in wild animals and ectoparasites from Brazil [20,26,79,80]. A new genospecies of *Borrelia burgdorferi*, called 'Candidatus

*Borrelia paulista*’, was found in two cricetid rodents sampled in the state of São Paulo through qPCR and PCR characterization assays using different gene markers (16S rRNA, *flaB*, *ospC*, *clpA*, *nifS*, *pepX*, *pyrG*, *recG*, *rlpB*, and *uvrA*) [26]. Additionally, genotypes of *Borrelia* spp. phylogenetically related to *Borrelia* spp. detected in bats in Colombia were found circulating in five vampire bats sampled in the state of Ceará, Brazil, based on the 16S rRNA and *flaB* genes [80]. Our research group recently reported the occurrence of *Bartonella* spp. and *Borrelia theileri* in the same tapir samples reported in this study [34,81]. In these two previous tapir studies, positive rates of 8% and 2% were reported for *Bartonella* spp. and *Borrelia* spp., respectively, using qPCR assays. The present study yielded positive rates of >16% for both genera, reinforcing the greater sensitivity of dPCR in detecting these bacterial agents in wildlife.

High diversity of piroplasmids has been reported in wild animals from Brazil, with five new clades added to the phylogeny of Piroplasmida based on the detection of putative novel species in non-hematophagous bats [22], opossums [21,29], tapirs [25], capybaras [30], and wild rodents [36]. Additionally, previous studies using some of the samples analyzed in the present study have reported the detection of a new *Babesia* species, namely *Babesia pantanalensis*, in *C. thous* blood samples [32], as well as the identification of a new *Theileria* species (*Theileria terrestris*) in tapirs [25]. Moreover, at least two species of *Cytauxzoon*—*Cytauxzoon brasiliensis* and genovariants of *Cytauxzoon felis*—have been reported in ocelots and jaguars [35]. The dPCR protocol employed in this study proved to be both useful and sensitive for detecting the diverse range of piroplasmids circulating in the country, as it yielded positive results across all analyzed animal orders. Furthermore, this protocol detected the presence of piroplasmids in all rodent samples and in some bird samples that had tested negative using nPCR, highlighting its potential as a reliable method for detecting these parasites in wildlife hosts.

## 5. Conclusion

This study demonstrated that dPCR has high sensitivity and is a very useful tool for detecting vector-borne agents in biological samples from wild animals with parasitemia levels that range from very low to very high. Additionally, qPCR was not found to be a sensitive technique for the types of samples examined in this study.

nPCR exhibited high sensitivity but moderate specificity for detecting piroplasmid DNA when compared to dPCR.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/pathogens14060567/s1>, Table S1: Results obtained for each test carried out for *Bartonella* spp.; Table S2: Results obtained for each test carried out for *Borrelia* spp.; Table S3: Results obtained for each test carried out for piroplasmids; Figure S1: Venn diagrams comparing the positivity between different techniques for the detection of *Bartonella* spp. (A), *Borrelia* spp. (B), and piroplasmids (C), considering samples as positive using dPCR when presenting one or more, two or more, or three or more positive partitions in the assays performed.

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## Abbreviations

The following abbreviations are used in this manuscript:

dPCR    digital PCR

qPCR    quantitative PCR

nPCR    nested PCR

ddPCR    droplet digital PCR

MCC    Matthews correlation coefficient

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## **CAPÍTULO 8\* - Development of Multiplex Assays for the Identification of Zoonotic *Babesia* Species\***

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**Abstract:** More than one-hundred *Babesia* species that affect animals and humans have been described, eight of which have been associated with emerging and underdiagnosed zoonoses. Most diagnostic studies in humans have used serology or molecular assays based on the 18S rRNA gene. Because the 18S rRNA gene is highly conserved, obtaining an accurate diagnosis at the species level is difficult, particularly when the amplified DNA fragment is small. Also, due to its low copy number, sequencing of the product is often unsuccessful. In contrast, because the *Babesia* internal transcribed regions (ITS), between 18S rRNA and 5.8S rRNA, and between 5.8S rRNA and 28S rRNA, contain highly variable non-coding regions, the sequences in these regions provide a good option for developing molecular assays that facilitate differentiation at the species level. In this study, the complete ITS1 and ITS2 intergenic regions of different Piroplasmida species were sequenced to add to the existing GenBank database. Subsequently, ITS1 and ITS2 sequences were used to develop species-specific PCR assays and specific single-plex and multiplex conventional (c)PCR, quantitative real-time (q)PCR, and digital (d)PCR assays for four zoonotic *Babesia* species (*Babesia divergens*, *Babesia odocoilei*, *Babesia duncani*, and *Babesia microti*). The efficacy of the assay protocols was confirmed by testing DNA samples extracted from human blood or enrichment blood cultures. Primers were first designed based on the 18S rRNA-5.8S rRNA and 5.8S rRNA-28S rRNA regions to obtain the ITS1 and ITS2 sequences derived from different Piroplasmida species (*B. odocoilei*, *Babesia vulpes*, *Babesia canis*, *Babesia vogeli*, *Babesia gibsoni*, *Babesia lengau*, *Babesia*

*divergens*-like, *B. duncani*, *B. microti*, *Babesia capreoli*, *Babesia negevi*, *Babesia conradae*, *Theileria bicornis*, and *Cytauxzoon felis*). Subsequently, using these sequences, single-plex or multiplex protocols were optimized targeting the ITS1 region of *B. divergens*, *B. microti*, and *B. odocoilei*. Each protocol proved to be sensitive and specific for the four targeted *Babesia* sp., detecting  $10^{-2}$  (for *B. microti* and *B. odocoilei*) and  $10^{-1}$  (for *B. divergens* and *B. duncani*) DNA copies per microliter. There was no cross-amplification among the *Babesia* species tested. Using 226 DNA extractions from blood or enrichment blood cultures obtained from 82 humans, *B. divergens* (seven individuals), *B. odocoilei* (seven individuals), and *B. microti* (two individuals) were detected and identified as a single infection, whereas co-infection with more than one *Babesia* sp. was documented by DNA sequencing in six (7.3%) additional individuals (representing a 26.8% overall prevalence). These newly developed protocols proved to be effective in detecting DNA of four *Babesia* species and facilitated documentation of co-infection with more than one *Babesia* sp. in the same individual.

**Keywords:** babesiosis; diagnosis; zoonotic *Babesia*; tick-borne diseases

## 1. Introduction

Piroplasmids, apicomplexan protozoa transmitted by Ixodid ticks, can cause diseases in animals and humans. These protozoa form a polyphyletic group comprising four genera: *Babesia*, *Theileria*, *Cytauxzoon*, and *Rangelia* [1–4]. Currently, there are more than 100 *Babesia* (Phylum: Apicomplexa; Order: Piroplasmida) species that infect domestic, wildlife, and human hosts, with several new species and genotypes being identified every year [2,5–10]. Because of the worldwide economic relevance (especially affecting production animals) and due to the increasing impact on human health, the genus *Babesia* is of medical concern, particularly as an emerging zoonotic pathogen [1,11–15]. Human *Babesia* sp. transmission has been predominantly associated with tick bites [2], with *Ixodes scapularis* being the primary tick vector in the USA and Canada [14–24] and *Ixodes ricinus* and *Ixodes canisuga* being the primary vectors in Europe [25,26]. Other modes of transmission such as transfusion of *Babesia*-contaminated blood, organ transplantation, and oral and transplacental transmission have also been reported [27–35].

From over one-hundred *Babesia* species recognized to date, only eight species, confirmed by DNA sequencing, have been reported to cause infections in humans (Table S1): *Babesia bigemina*, *Babesia crassa* (including *B. crassa*-like), *Babesia divergens* (including *B. divergens*-like MO1), *Babesia duncani*, *Babesia microti*,

*Babesia motasi*, *Babesia odocoilei*, and *Babesia venatorum* [1,5,12,14,15,27,28,36–69]. Despite being reported as a worldwide infection in people [15,53], 95% of all human babesiosis cases have been reported from the USA and Canada [38,70].

Babesiosis symptoms in people vary from asymptomatic, to non-specific, to life-threatening hemolytic anemia, particularly in splenectomized or otherwise immunocompromised individuals [27,39–41,71,72]. Predominant symptoms and clinical findings include myalgia, fever, sweating, chills, profound fatigue, sleep disorders, and hepatosplenomegaly. Hemolytic anemia severity can be related to immunosuppressive factors, such as splenectomy and comorbidities (e.g., cancer, chronic heart, lung, renal, or liver disease, infection with HIV/AIDS, or patients receiving immunosuppressive drugs). Illness typically occurs after an incubation period of 1 to 4 weeks following tick transmission and can persist for several weeks prior to diagnosis and treatment [27,39–41,71,72].

Historically, diagnosis of animal and human babesiosis was primarily based on microscopical observation of intraerythrocytic *Babesia* merozoites/trophozoites in stained blood smears and serology. Unfortunately, merozoite/trophozoite visualization has poor sensitivity due to the low number of circulating infected erythrocytes. For example, less than 1% of erythrocytes can be infected with *B. microti* during the acute disease and chronic illness phase of human babesiosis. [2]. Serology does not confirm ongoing infection, and does not definitively determine the infecting species due to a high degree of cross-reactivity among closely related *Babesia* species (i.e., *B. divergens*, *B. odocoilei*, and *B. duncani*) [39,69]. Molecular methods, most often PCR targeting the highly conserved 18S rRNA gene, followed by DNA sequence analyses, have been a widely used method for *Babesia* sp. detection in clinical cases, which has facilitated species identification and, importantly, phylogenetic inferences at the taxon level (Table S1). In recent years, due to highly conserved 18S rRNA *Babesia* sequences (that in some instances impair speciation by DNA sequencing) and because the internal transcribed spacer (ITS) between the 18S rRNA, 5.8S rRNA, and 28S rRNA genes are more highly variable, the ITS regions have been increasingly used for the detection and identification of specific *Babesia* sp., such as *B. divergens*, *B. microti*, and *B. odocoilei* in people, as well as for different *Babesia* spp. infecting animals [1,2,37,42,43,73–75].

Because the regions between 18S rRNA and 5.8S rRNA (referred to as *Babesia* ITS1 in this manuscript) and the 5.8S rRNA and 28S rRNA genes (referred to as *Babesia* ITS2) had higher discriminatory power (when compared with amplification targets derived from the conserved 18S rRNA region), we developed conventional (cPCR), quantitative real-time (qPCR), and digital (dPCR) PCR assays for detecting and identifying *Babesia* at both genus and species levels (specifically aimed at *B. divergens*, *B. duncani*, *B. microti*, and *B. odocoilei*, the predominant or suspected *Babesia* spp. that infect humans in the USA [15,39,76–78]).

## 2. Material and Methods

### *Animal and Human Sample Sources*

*Babesia*-infected animal samples were kindly provided by Dr. Barbara Qurollo and Brittany Thomas from the Vector-Borne Disease Diagnostic Laboratory, College of Veterinary Medicine [79], North Carolina State University, and from Dr. Sam Telford, Cummings School of Veterinary Medicine, Tufts University.

All human study participants provided three blood and serum specimens collected within a 7-day period. These individuals were previously tested because of a history of arthropod or animal contact as a component of an Institutional Review Board (IRB)- approved study entitled “Detection of *Bartonella* Species in the Blood of People with Extensive Animal Contact” (North Carolina State University Institutional Review Board, IRB#s 4925-03 and 164-08-05). The culture and molecular testing approach used in this study was described in prior publications from our research group [1,80–82]. A total of 226 human DNA blood and enrichment blood culture samples from 82 patients from 7 countries (including 27 USA states) were tested for the presence of *Babesia* species DNA.

### *GenBank Reference Sequences Used in This Study*

The following 18S rRNA, 5.8S rRNA, and 28S rRNA gene reference sequences from representative *Babesia* species were retrieved from the GenBank database (<http://www.ncbi.nlm.nih.gov>, accessed on 1 October 2024). Using Clustal W multi-sequence alignment (AlignX, Vector NTI Advanced 10.3.0 from

Invitrogen, Waltham, MA, USA), the complete or partial regions between 18S and 28S rRNA from *B. bigemina* (HQ840960), *B. canis* (AY072926), *B. vogeli* (HQ148664), *Babesia* sp. Coco (AY618928, EU109720, EU109721), *B. conradae* (AF158702), *B. crassa* (MK240324, AY260176, KX590751), *B. divergens* (AY572456, EU182599, EU185801, EF458187, EU182603, EU182598), *B. duncani* (HQ289870, MH333111), *B. gibsoni* (CP141526), *B. microti* (AB112337, GU230755, LN871598, AB190435, MK609547, XR\_002459986), *B. motasi* (AY260179), *B. odocoilei* (AF158711, AY339747, AY339754, AY339756, AY339757, AY339759, AY345122, BOU16369, KC1622888, MF357056), *B. orientalis* (HQ840969), *B. poeala* (DQ200887), *B. rodhaini* (AF510201), *B. uriae* (FJ17705), and *B. venatorum* (GQ888709, MG344777, AY046575, KF724377, OP522105) were used as species reference genes for alignments.

#### *Sequence Analysis, Babesia Genus ITS1 and ITS2 Region Primer Design, DNA Amplification, and Sequence Analysis of Different Babesia Species from Naturally Infected Animals*

After DNA sequence alignment and homology analysis of GenBank reference genes between 18SrRNA and 5.8S rRNA, and between 5.8SrRNA and 28SrRNA (Figure 1A–D), oligonucleotides were designed: Api18S rRNA-1690s: 5' CTCCTACCGATCGAGTGATCCGGT 3' (selected from a conserved region at the 3' end of the *Babesia* 18S rRNA gene); Api5.8SrRNA-20as: 5' GCTGCGTCCTTCATCGTTGTGTGAG 3'; Api5.8SrRNA-20s: 5' CTCACACAACGATGAAGGACGCAGC 3' (selected from a conserved region in the *Babesia* 5.8S rRNA gene); and Api28SrRNA-1as: 5' CCGCTGAATTTAAGCATAAAAYTAAGCGG 3' (selected from a conserved region at the 5' end of the *Babesia* 28S rRNA gene). These PCR primers were used for DNA amplification, using cPCR and qPCR, followed by sequencing of the complete intergenic ITS1 and ITS2 regions of several *Babesia* species from infected animals (high-copy-number samples previously characterized by qPCR and dPCR targeting 18S rRNA, [79]), including *B. odocoilei* (from infected caribou [*Rangifer tarandus*]), *Babesia vulpes*, *B. canis*, *B. vogeli* and *Babesia gibsoni* (from infected dogs), *B. lengau* (from an infected cheetah [*Acinonyx jubatus*]), *B. divergens*-like

(isolate MO-1 from a infected rabbit [*Sylvilagus* sp.]), *B. duncani* (strain J3 from infected hamster [*Mesocricetus auratus*]),

*B. microti* (isolate GI from infected hamster), *Theileria bicornis* (from infected rhinoceros [*Diceros bicornis*]), and *Cytauxzoon felis* (from infected cat) (Table S2). The same conventional and qPCR primers sets were used for the amplification of *Babesia* ITS1 and *Babesia* ITS2 regions from each of the above *Babesia* species.

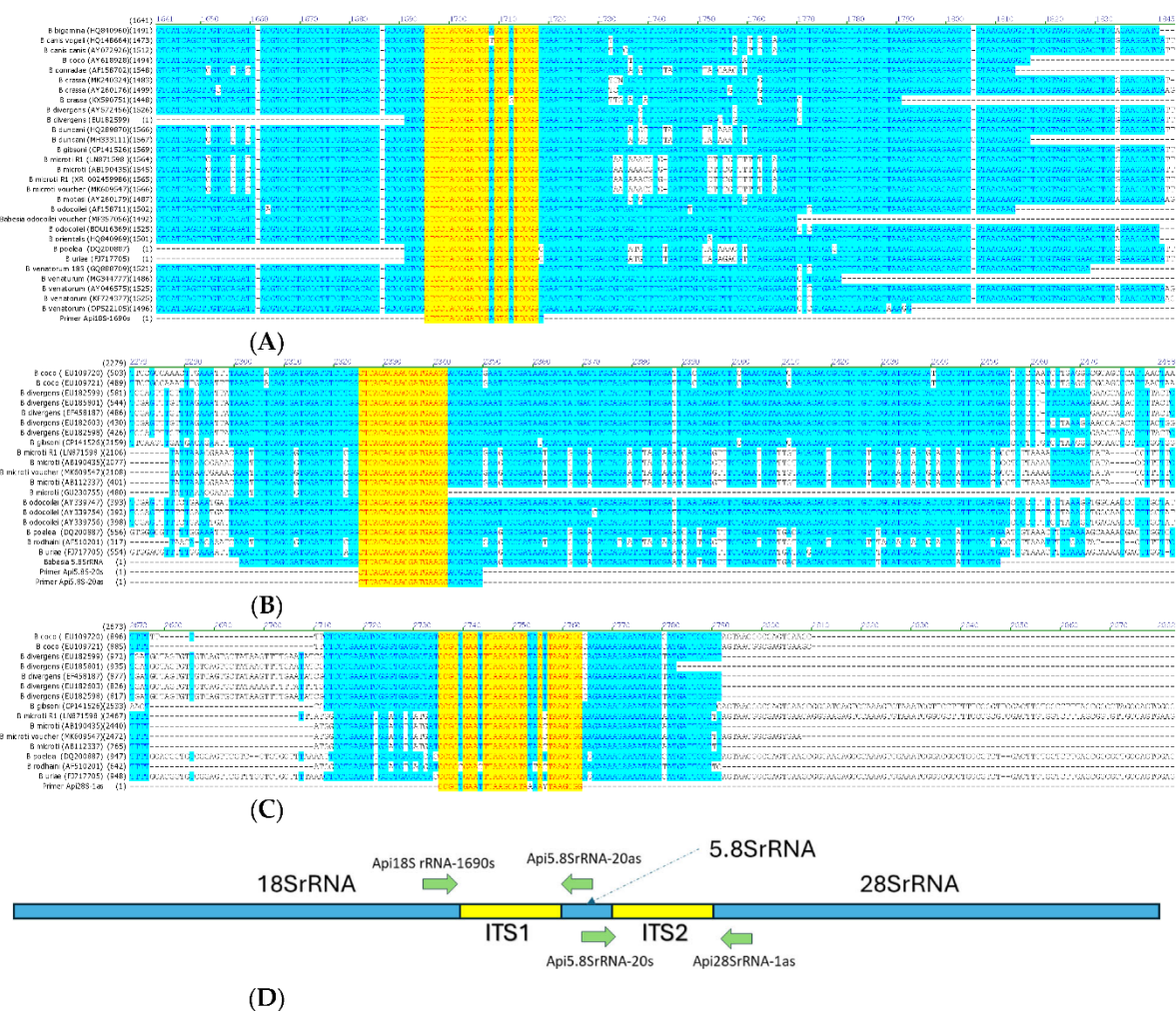


**Figure 1.** (A): Region in yellow of the conserved downstream 18S rRNA for primer Api18S-1690s. (B): Region in yellow of the conserved downstream 5.8S rRNA for primer Api5.8S-20s and Api5.8S-20as. (C): Region in yellow of the conserved downstream 28S rRNA for primer Api28S-1as. (D): Schematic diagram of the intergenic transcribed spacer 1 (ITS1) and 2 (ITS2) DNA regions targeted for amplification and sequencing of several *Babesia* species.

For conventional cPCR, amplification was performed with a master-mix reaction, at 25 µL final volume per reaction, comprising 7.3 µL of molecular-grade water (QIAGEN Germantown, MD, USA), 12.5 µL of 2× My Taq HS Red Mix (Bioline, Memphis, TN, USA), 0.1 µL of 100 µM each of Api18S rRNA-1690s and Api5.8SrRNA-20as (for *Babesia* ITS1 region) or Api5.8SrRNA-20s and Api28SrRNA-1as (for *Babesia* ITS2 region) as forward and reverse primers (IDT-DNA Technologies, Coralville, IA, USA), and 5µL of extracted DNA. Molecular-grade water and DNA extracted from the blood of a naive dog were used as negative controls. Amplification was performed using a CFX Opus Real-Time PCR System (Bio Rad, Hercules, CA, USA) under the following conditions: 95 °C for 3 min, followed by 45 cycles of denaturing at 94 °C for 10 s, annealing at 66 °C for 15 s, and extension at 72 °C for 20 s. The products obtained in PCR assays were separated by horizontal electrophoresis on 2% agarose gel stained with gelGreen (Thermofisher Scientific, Greenville, NC, USA), visualized under ultraviolet light illumination using a ChemiDoc MP Imaging System (Bio Rad, Hercules, CA, USA) and photographed using Image Lab Software v.3.01 3.01 (Bio Rad, Hercules, CA, USA).

Similarly, real-time qPCR amplification of *Babesia* ITS1 and *Babesia* ITS2 was performed as above with minor modifications: 7.5 µL of molecular-grade water (QIAGEN, German- town, MD, USA), 12.5 µL of 2× SsoAdvanced SYBR green master mix (Bio Rad, Hercules, CA, USA), 0.2 µL of 100 µM each of oligonucleotide primers Api18S rRNA-1690s and Api5.8SrRNA-20as (for detection of *Babesia* ITS1) and Api5.8SrRNA-20s and Api28SrRNA- 1as (for detection of *Babesia* ITS2), and 5µL of extracted DNA. As above, molecular-grade water and DNA extracted from naive dog blood samples were used as PCR negative con- trols. Amplification was performed in a Bio-Rad CFX 96-well Opus PCR system machine under the following conditions: 95 °C for 3 min, followed by 40 cycles of denaturing at 94 °C for 15 s, annealing at 68 °C for 15 s, and extension at 72 °C for 15 s. An additional gradient cycle from 65 °C to 95 °C was performed for melting curve analysis. Quantification cycle (Cq) values were determined and melting

temperature analysis was performed by reading fluorescent signals using the FAM/Syber channel.

For both cPCR and qPCR assays, amplified products were purified and sequenced by Sanger's method. The obtained sequences were assessed for quality, analyzed using Clustal W multi-sequence alignment (AlignX, Vector NTI Advanced 10.3.0 from Invitrogen), and compared to sequences previously deposited in the GenBank database [1].

*Species-Specific qPCR Amplification, Limit of Detection (LOD), and Cross-Amplification Assessment for the Detection of B. microti, B. duncani, B. divergens, and B. odocoilei ITS1 Region*

For each representative *Babesia* species (*B. divergens*-like isolate MO-1, *B. duncani* isolate J3, *B. microti* isolate GI, and *B. odocoilei* isolate VB19-09386), the entire *Babesia* ITS1–ITS2 region PCR amplicon obtained using oligonucleotides Api18SrRNA-1690s and Api28SrRNA-1as as forward and reverse primers, respectively, was cloned into plasmid pGEM-T Easy (Promega® Madison, WI, USA), transformed into *E. coli* DH5- $\alpha$  strain-competent cells, and purified using Qiagen plasmid miniprep kits. Plasmid insert DNA was sequenced using M13 primers for species verification and quantified using Qubit Flex Fluorometric Quantification (QIAGEN, Germantown, MD, USA).

Serial dilutions in molecular-grade water, ranging from  $10^7$  to  $10^{-3}$  copies/ $\mu$ L, were used as templates to assess the limit of detection (LOD) by real-time PCR (both using Taqman probes and SYBR Green followed melting curve analysis).  
Single-plex species-specific real-time PCR: Real-time PCR was performed using reaction mixture at a 25  $\mu$ L final volume per reaction. The PCR reaction included 7.3  $\mu$ L of molecular-grade water (QIAGEN, Germantown, MD, USA), 12.5  $\mu$ L of 2 $\times$  SsoAdvanced SYBR green master mix (Bio Rad, Hercules, CA, USA), 0.1  $\mu$ L of each species-specific primer at 100  $\mu$ M (IDT-DNA Technologies, Coralville, IA, USA), and 5  $\mu$ L of DNA sample. Molecular-grade water and DNA extracted from naive dogs and human blood specimens were used as PCR negative controls. Amplification was performed in a Bio-Rad CFX 96- well Opus PCR system machine under the following conditions: 95 °C for 3 min, followed by 40 cycles of



denaturing at 94 °C for 10 s, annealing at 68 °C for 10 s, and extension at 72 °C for 10 s. An additional gradient temperature cycle from 65 °C to 95 °C at 0.2 °C per second was performed for melting curve analysis. Quantification cycle (Cq) values were determined and melting temperature analysis was performed by reading fluorescent signals using the FAM/Syber channel. All PCR-positive samples were sequenced by Sanger's method and analyzed using Clustal W multi-sequence alignment as described above.

#### *Multiplex Species-Specific qPCR*

qPCR was performed using the reaction mixture at 25 µL final volume per reaction. PCR amplification included 7.3 µL of molecular-grade water (QIAGEN, Germantown, MD, USA), 12.5 µL of 2× SsoAdvanced probe master mix (Bio Rad, Hercules, CA, USA), 0.1 µL of each species-specific primer at 100 µM (IDT-DNA Technologies Coralville, IA, USA), 0.1 µL of 100 µM (IDT-DNA Technologies Coralville, IA, USA) of each species-specific probe, and 5 µL of DNA sample. Molecular-grade water and DNA extracted from naive dogs and human blood specimens were used as PCR negative controls. Amplification was performed in a Bio-Rad CFX 96-well Opus PCR system machine under the following conditions: 95 °C for 3 min, followed by 40 cycles of denaturing at 94°C for 10 s, annealing at 68 °C for 10 s, and extension at 72 °C for 10 s. Reading of fluorescent signals for detection of each *Babesia* species was performed during the annealing phase of amplification using FAM (green) for *B. odocoilei*, HEX (yellow) for *B. divergens*, CalFluo590 (red) for *B. microti*, and Cy5 (crimson) for *B. duncani*. Recording of the fluorescent signal quantification value (Cq value) was used to assess positive and negative DNA amplification. As above, all PCR-positive samples were sequenced by Sanger's method (Genewiz from Azenta, Research Triangle Park, NC, USA) and analyzed using Clustal W multi-sequence alignment (AlignX, Vector NTI Advance 10.3.0 from Invitrogen) to confirm species identification.

### **3. Results**

Using Api18S rRNA-1690s and Api5.8SrRNA-20as (as forward and reverse primers), cPCR DNA amplification of the *Babesia* ITS1 region generated a single 557–650 bp band consistent with the expected DNA sizes (depending on species) for all *Babesia* species investigated herein. Importantly, due to a high copy number, all amplicons generated using the *Babesia* ITS1 region assay rendered a high-quality DNA sequence chromatogram, facilitating clear and distinctive differentiation between species and strains (i.e., different *B. odocoilei* strains were identified from different cervid species and from infected humans). Similarly, Api5.8SrRNA-20s and Api28SrRNA-1as (as forward and reverse primers) targeting the *Babesia* ITS2 regions generated a single 385–436 bp band consistent with the expected *Babesia* ITS2 region DNA size (depending on *Babesia* spp.) for *Babesia* species investigated herein (Table S2). Using the qPCR assay method, similar results were obtained for all animal samples tested, generating only a single signal with melting curve temperatures depending on the *Babesia* sp. *Babesia* species ITS1 and ITS2 sequences (amplified by either cPCR or qPCR) were deposited in GenBank (Table S2).

#### *Comparison of Babesia Genus Detection in Human Clinical Samples by qPCR and dPCR Targeting Babesia 18S rRNA Region vs. qPCR Targeting Babesia ITS1 and ITS2 Regions*

Previously, our research group described the development of a digital PCR assay targeting the Piroplasmida 18S rRNA region, to detect piroplasmids, at the genus level, in animals and human patients [79]. Despite the higher sensitivity and specificity of the digital PCR assay when compared with real-time PCR targeting the same gene region (including primers and probes) [79], species identification using digital PCR was not possible due to the inability to concentrate target DNA for sequence analysis. Therefore, 226 human blood and enrichment blood culture DNA samples, obtained from 82 individuals previously tested in our laboratory [1,36,80–84], were used to assess the relative sensitivity and specificity of four amplification modalities (18S rRNA qPCR, 18S rRNA dPCR, *Babesia* ITS1 qPCR, and *Babesia* ITS2 qPCR). These samples were analyzed to determine the frequency of positive

DNA amplification and to assess whether the sequence quality (available for qPCR testing only) was adequate to obtain a readable DNA sequence.

From the 226 human DNA blood and enrichment blood culture samples tested, 19 (8.4%) were positive by qPCR targeting Piroplasmida 18S rRNA; 70 (31%) were positive by dPCR targeting Piroplasmida 18S rRNA; 29 (12.8%) were positive by qPCR targeting *Babesia* ITS1; and 40 (17.7%) were positive by qPCR targeting *Babesia* ITS2 (Table 1).

**Table 1.** Prevalence of *Babesia* species as positive DNA amplification using qPCR and dPCR aiming at 18S rRNA gene and qPCR aiming at the 18S rRNA-5.8S rRNA intergenic (ITS1) region, in 226 human DNA blood and enrichment blood culture samples.

|                  | Piroplasmida 18S rRNA |      | <i>Babesia</i> ITS1 |
|------------------|-----------------------|------|---------------------|
|                  | qPCR                  | dPCR | qPCR                |
| <b>Negatives</b> | 207                   | 156  | 197                 |
| <b>Positives</b> | 19                    | 70   | 29                  |

DNA sequence analysis (using Clustal W multi-sequence alignment; AlignX, Vector NTI Advance 10.3.0 from Invitrogen) was not successful for any of the 18S rRNA qPCR-positive samples, precluding species identification, whereas *Babesia* DNA was successfully sequenced from 26 of 29 *Babesia* ITS1 qPCR and 20 of 40 *Babesia* ITS2 qPCR samples, respectively.

In most instances, *Babesia* ITS1 qPCR rendered higher-quality DNA sequences (using both forward and reverse primers) compared to the *Babesia* ITS2 qPCR for the same DNA extraction. Based upon these comparative ITS1 and ITS2 results, the *Babesia* ITS1 region was selected for the detection of Piroplasmida at the genus level and for the design of species-specific primers and probes for the detection of *B. divergens*, *B. duncani*, *B. microti*, and *B. odocoilei*, which are the most frequently documented human *Babesia* spp. pathogens in the USA.

#### *Development of B. divergens, B. duncani, B. microti, and B. odocoilei ITS1 Species-Specific Primers and Probes*

After DNA sequence analysis of PCR amplicons obtained from *B. odocoilei*-positive animal blood specimens (Figure 2), from *B. divergens*-like isolate MO-I (Figure

3), from *B. duncani* isolate J3 (Figure 4), and from *B. microti* isolate GI (Figure 5), the following species-specific primers and probes for amplification of the ITS1 region were designed and subsequently tested for amplification specificity:

*Babesia divergens* ITS1 target region (225 bp):

Primer BdivergensITS1-25s: 5' CTCGGCTTCGACATTTACGTTGTGTAAGCT 3'

Primer BdivergensITS1-150as: 5' CAACTACAGTAGTTACACCGYAGTAARCATAC

3' Probe BdivergensITS1-70: 5' HEX CTTTTKGTGGTTTCGTATTTGYCGTTG-BHQ2 3'

*Babesia duncani* ITS1 target region (170 bp):

Primer BduncaniITS1-1s: 5' GTGTTTAAACCGCGCTTATGCGCAGGTC 3'

Primer BduncaniITS1-130as: 5' CTGCACTGGCGGGGTGAAAAGTAAC 3'

Probe BduncaniITS1-80: 5' Cy5-TGGCTTTGCGGTTTCGCCGTACGGCCCC-BHQ3 3'

*Babesia microti* ITS1 target region (185 bp):

Primer BmicrotiITS1-25s: 5' TATCAGAGTTCTTTGTATCCCATTGTTGGTTA 3'

Primer BmicrotiITS1-160as: 5' GAAAATACCTTGGGAGTGAGAACGCCCCGT 3'

Probe BmicrotiITS1-70: 5' CalFluoRed590-AGAAGAGTGGCCTTGGACGTAG-BHQ2 3'

*Babesia odocoilei* ITS1 target region (150 bp):

Primer BodocolITS1a-100s: 5' CTGTTGCACTTTTGTGCTTGACGTTGT 3' Primer

BodocolITS1a-255as: 5' CAAGCGCAGGGATGGAAACGGA 3'

Probe BodocolITS1a-200probe: 5' FAM-GGCCTCGTCATGGCGACGTGGT-BHQ1 3'

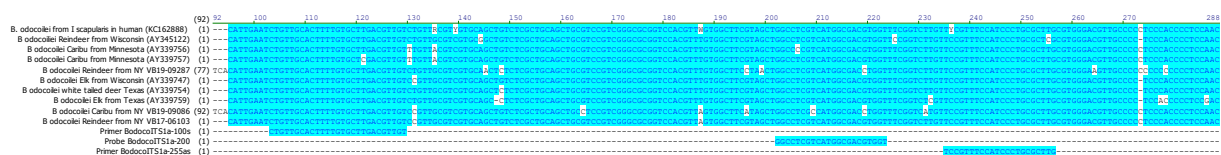


**Figure 2.** Alignments of *B. odocoilei* ITS1 sequence region



**Figure 3.** Alignments of *B. divergens* ITS1 region.



**Figure 4.** Alignments of *B. duncani* ITS1 region. No *B. duncani* ITS1 region available from Genbank database.

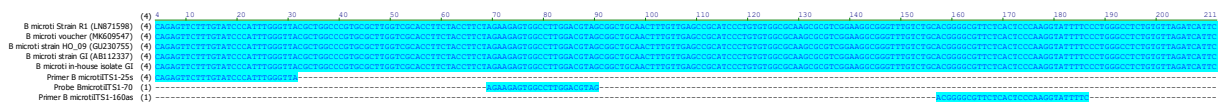


**Figure 5.** Alignments of *B. microti* ITS1 region.

For both TaqMan probes and SYBR Green-based qPCR, species-specific ITS1 amplification was detectable at levels of  $10^{-2}$  (for *B. microti* and *B. odocoilei*) and  $10^{-1}$  (for *B. divergens* and *B. duncani*) copies per microliter for each of the four targeted *Babesia* spp. Amplicons of the lowest DNA concentration generated good-quality chromatograms with sequences that matched the targeted *Babesia* sp. Melting curve analyses for each species by SYBR Green qPCR generated peaks at 89.5 °C to 90 °C for *B. odocoilei*, 86.5 °C for *B. divergens*, 89 °C for *B. duncani*, and 88.5 °C for *B. microti*.

#### *Assessment of Species-Specific Amplification for Cross-Amplification with Non-Target Babesia spp.*

There was no cross-amplification using either TaqMan probes or SYBRGreen-based qPCR assays, when *B. divergens*, *B. duncani*, *B. microti*, and *B. odocoilei* species-specific primers and probes were tested against DNA extracted from the blood of animals infected with *B. vulpes*, *B. odocoilei*, *B. canis*, *B. vogeli*, *Babesia* sp. Coco, *B. gibsoni*, *B. lengau*, *B. felis*, pr *B. conradae* or when tested against *B. microti*, *B. divergens*, *B. odocoilei*, or *B. duncani* plasmid-based DNA (Table S3).

#### *Detection of Babesia spp. Infection in Human Samples*

Of the 82 human research subjects tested during this assay validation study, 22 (26.8%) were infected with one or more *Babesia* species. Amplicon DNA sequence analysis of qPCR products targeting the *Babesia* ITS1 and/or *Babesia* ITS2 regions identified single infection with *Babesia divergens* in seven (8.5%) individuals, *B. odocoilei* in seven (8.5%) individuals, or *B. microti* in two (2.4%) individuals (Table S3). As a result of analyzing different samples (different time points within 7-day blood

collections or blood vs. 7-, 14-, or 21-day enrichment culture samples) from the same individual, *Babesia* spp. co-infection was detected in six (7.3%) individuals, as well as two (2.4%) individuals infected with *B. divergens* and *B. microti*, three (3.7%) individuals infected with *B. divergens* and *B. odocoilei*, and one (1.2%) individual infected with *B. microti* and *B. odocoilei* (Table 2).

**Table 2.** *Babesia* species detected in 22 of 82 individuals tested as a component of this study.

| Species Detected and Sequenced              | Individuals |
|---------------------------------------------|-------------|
| <i>B. divergens</i>                         | 7           |
| <i>B. microti</i>                           | 2           |
| <i>B. odocoilei</i>                         | 7           |
| <i>B. divergens</i> and <i>B. microti</i>   | 2           |
| <i>B. divergens</i> and <i>B. Odocoilei</i> | 3           |
| <i>B. microti</i> and <i>B. Odocoilei</i>   | 1           |

For many of these individuals, the same *Babesia* species was detected at different blood sampling time points. Sequence analysis of some qPCR products obtained from people where co-infection was documented indicated the presence of mixed sequencing chromatograms (usually double peaks with one chromatogram as a low signal, as shown in Figure 6), indicating the potential for alternative allele sequences. To assess if these amplicons indicated co-infection with more than one *Babesia* sp. (beyond the species previously identified by qPCR and DNA sequencing), a single-plex species-specific real-time PCR targeting the *Babesia* ITS1 region was developed. Using the qPCR single-plex assay targeting ITS-1 of the four selected zoonotic *Babesia* sp., co-infection with more than one *Babesia* sp. was confirmed by DNA sequencing, as these patient samples contained alternative allele sequences. For other individuals, the single-plex assay amplified sequences of the same species (i.e., *B. divergens* or *B. odocoilei*) previously identified by the *Babesia* ITS1 or ITS2 amplification. *Babesia odocoilei* was the “secondary” co-infecting species identified by species-specific ITS1 region amplification in all eight individuals, with amplifications of very high threshold cycle (Ct) values (>37 to over 40 total cycles), indicating a very low-level parasitemia. Co-infection with *B. divergens* and *B. odocoilei* was most frequently detected (seven of eight individuals), as illustrated

by the patient depicted in Figure 6 for enrichment culture sample 20468C21. Another individual was co-infected with *B. microti* and *B. odocoilei*.

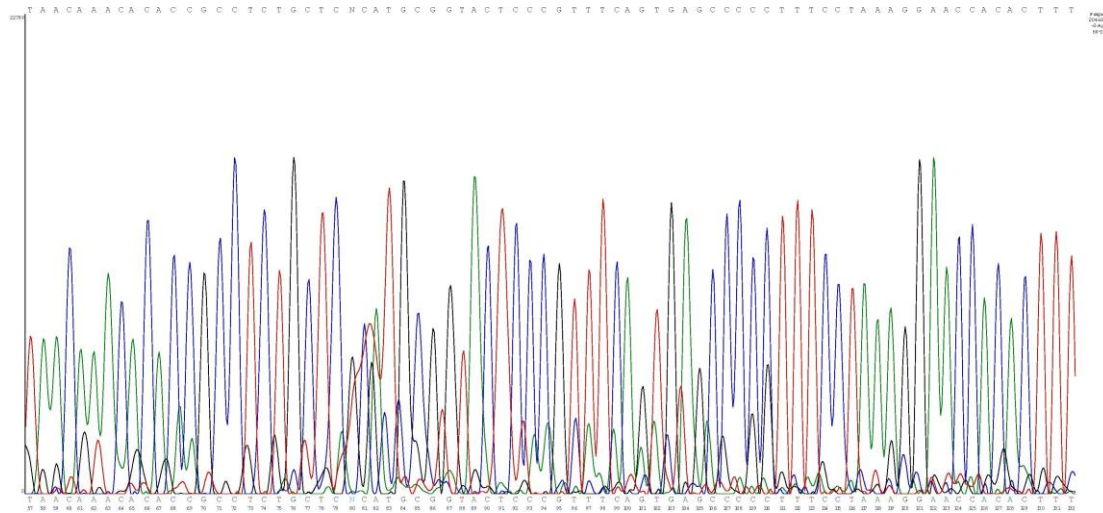


**Figure 6.** Overlapping sequencing chromatograms (double peaks) obtained from the enrichment blood culture sample 20468C21 amplicon from a human patient obtained by *Babesia* ITS1 amplification, indicating potential alternative allele sequences due to co-infection, which was confirmed as *B. divergens* and *B. odocoilei* using species-specific ITS1 probes.

*Babesia* sequences of amplicons (ITS1 and ITS2) obtained from these individuals have been deposited in the GenBank database under the following accession numbers: *B. odocoilei*: PP693407, PP693408, PP693409, PQ452565, PQ452566, PQ452567, PQ452568, PQ452569, PQ452570, PP550653, PP550654, PP550655, PP550656, PP550657, PP550658, PP550659, PP550660, PP550661, PP592351, PP550644, PP550645, PP550646, PP550647, PP550648, PP550649, PP550650, PP550651, PP550652, PQ452561, PQ452562, PQ452563, and PQ452564; *B. divergens*: PQ404846, PQ404847, PQ404848, PP693420, PP693421, PP693422, PP693423, PP693424, PP693425, PP693426, PQ452546, PQ452547, PQ452548, PQ394580, PQ459266, PQ459267, PQ459268, PQ459269, PQ459270, PQ459271, PQ459272, PQ459273, PQ459274, and PQ459275; *B. microti*: PQ459008, PQ459009, PQ459010, PQ459011, PQ459012, PP693410, PP693411, PP693412, PP693413, PP693414, PP693415, PQ452571, PQ452572, PP693416, PP693417, PP693418, PP693419, PQ452549, PQ452550, PQ452551, PQ452552,

PQ452553, PQ452554, PQ452555, PQ452556, PQ452557, PQ452558, PQ452559, PQ452560, PQ459293, PQ459262, PQ459263, PQ459264, and PQ459265.

*Babesia duncani* DNA was not amplified as a single or co-infection in any of the 226 human patient samples analyzed in this study.

#### 4. Discussion

Using blood specimens from animals naturally infected with piroplasmids, experimentally infected animals, and human clinical samples derived from research studies, specific qPCR assays were designed and validated for amplification of the *Babesia* intergenic region (ITS1) at the genus, species-specific, and strain levels, specifically targeting *B. odocoilei*, *B. duncani*, *B. divergens*, and *B. microti*. In previously published human babesiosis case reports confirmed by DNA sequencing, the diagnosis was most often confirmed by targeting the highly conserved 18S rRNA gene, which is a good marker for phylogenetic inferences, especially at the taxon level. However, failure to obtain a confirmatory DNA sequence from positive 18S rRNA gene amplicons occurs frequently, which negatively impacts the possibility of achieving a definitive species identification. Failure to successfully sequence an 18S rRNA qPCR amplicon is most often due to low DNA quantity, a finding consistent with testing results reported in this study, as most samples rendered very high qPCR threshold cycle (Ct) values (>37 over 40 total cycles). In addition, non-specific amplification or co-infection with two *Babesia* species can also contribute to sequencing failure.

The *Babesia* spp. intergenic spacer regions have greater evolutionary variation when compared to the conserved 18S rRNA gene. This genetic variation is associated with the facilitation of more rapid genetic change, making the ITS regions useful for defining species and microbial populations at a more refined specific species or strain level [12,13,38]. The ITS region has proven useful for diagnosing closely related species of *Cystoisospora* [12], as well as being used as a PCR target for dinoflagellates [15], fungi [14,39], Thalassiosirales [38], and nematodes [40,41]. Previously, Wilson et al. (2005) [65] developed qPCR and ddPCR protocols based on the ITS1 region of *B. microti* and *B. duncani*.

The primers and probes developed in this study proved to be specific for the four *Babesia* spp. investigated, with no cross-amplification with any other tested



Piroplasmids species. The ITS1 TaqMan probe and SYBRGreen-based assays proved to be sensitive, being able to detect a minimum of  $10^{-1}$  copies per  $\mu\text{L}$ . The use of more sensitive and specific assays, primarily aimed at detecting zoonotic *Babesia* species, favors a more accurate, species-specific diagnosis, which could impact treatment decisions and preventive strategies. In addition, the use of sensitive and specific species primers or probes facilitates the detection of *Babesia* spp. co-infections, as reported in this study, which may go undetected when targeting the more evolutionarily conserved 18S rRNA gene. In the initial studies carried out by our research group, DNA extracted from the blood of cervids was positive by conventional 18S rRNA PCR, and DNA sequencing confirmed infection with *Theileria cervi*. These same cervid blood samples were also positive using the *B. odocoilei*-specific ITS1 qPCR assay, with species confirmation determined by DNA sequencing. Thus, co-infection was confirmed in reservoir hosts, results that would not have been obtained by solely targeting the 18S rRNA gene (Figures S1–S3).

Currently, babesiosis is increasingly being diagnosed in humans, often in association with a diagnosis of Lyme borreliosis, transmitted by *Ixodes* ticks [2,5,36,85]. As reviewed, the initial recognition and molecular characterization of different *Babesia* species infecting humans is also increasingly reported in the worldwide medical literature [43]. The most recent descriptions and sequencing confirmation were of *B. odocoilei* detected in symptomatic humans from Canada and the USA [1,42]. With the advent of molecular assays, a more accurate diagnosis of the specific *Babesia* species infecting animals and human patients has become possible, confirming substantially greater diversity of species circulating in and among animals with the possibility of spillover to humans. In the past, most human *Babesia* studies were based on direct microscopic visualization or serological assays, so most cases of babesiosis were mainly attributed to *B. microti* [28,44,45,47,49–52,86,87]. However, the possibility of serological cross-reactivity among *Babesia* spp. cannot be ruled out, as well as co-infection with more than one *Babesia* species that would not likely be diagnosed clinically. More specific diagnostic tests, such as those targeting the ITS1 *Babesia* genus and species-specific assays developed in this study, will favor diagnosis at the species level and will facilitate documentation of *Babesia* and Piroplasmida co-infections diagnostically and in epidemiological studies. The bite

of an infected tick is described as the main route of *Babesia* spp. transmission, followed by blood transfusion and tissue transplants [27,53,88]. However, other potential transmission routes should not be ruled out. For example, there is evidence of *B. gibsoni* being transmitted through fighting among American Staffordshire (Pitbull) terriers [69], indicating the possibility of direct transmission between animals and humans.

In addition to the development of targeted *Babesia* spp. qPCR assays through this research, we generated and submitted to GenBank complete sequences of the ITS-1 and ITS-2 regions of different Piroplasmida species (*B. vulpes*, *B. canis*, *B. vogeli*, *Babesia* sp. Coco, *B. gibsoni*, *B. lengau*, *B. felis*, *B. odocoilei*, *B. divergens*, *B. capreoli*, *B. negevi*, *T. bicornis* and *C. felis*), including many ITS regions that had never been previously sequenced. Access to these sequences will contribute to future studies aimed at developing and validating molecular assays targeting these regions.

Based on the use of PCR amplification and sequencing of the *Babesia* ITS1 region (both at genus and species levels), we were able to identify not just *B. odocoilei*, *B. divergens*, and *B. microti* in studied participants, most of whom reported chronic and often non-specific symptoms, but also co-infections with two *Babesia* species in a small subgroup of individuals. Interestingly, *B. duncani* DNA was not detected as either single or co-infection in any of the 226 human samples analyzed in this study. As the purpose of this study was to describe improvements in methodology for the detection and molecular characterization of *Babesia* spp., medical details regarding the 82 study participants will be reported in future publications, as these assays were used to generate *B. odocoilei* DNA sequences for a recent publication [1].

## 5. Conclusions

Using the intergenic spacer regions between the 18S rRNA and the 5.8S rRNA (known as ITS1) and between the 5.8S rRNA and the 28S rRNA genes (known as ITS2), we designed a set of highly sensitive molecular primers and probes that facilitate the amplification, detection, and characterization of *Babesia* spp. that infect humans and animals. The primers and probes designed for species-specific detection

(here targeting *B. divergens*, *B. duncani*, *B. microti*, and *B. odocoilei*) will facilitate the diagnosis of single and co-infections with *Babesia* spp. in animals and human patients.

**Supplementary Materials:** The following supporting information can be downloaded at: <https://www.mdpi.com/article/10.3390/pathogens13121094/s1>, Figure S1: Electropherogram of an 18S rRNA gene sequence showing the overlapping peaks; Figure S2: Part of the 18S rRNA alignment of *T. cervi*, *B. odocoilei* and deer sequences showing great divergence between *T. cervi* and *B. odocoilei*; Figure S3: Alignment of ITS-1 sequences; Table S1: *Babesia* species currently identified by DNA sequence infecting people worldwide; Table S2: Piroplasmida species, animal source, case identification and GenBank Accession numbers generated in this study for the intergenic transcribed spacer regions between 18S rRNA and 5.8S rRNA (ITS1) and 5.8S rRNA and 28S rRNA (ITS2). Note: N/A: not amplified; Table S3: Assessment of *Babesia* species specificity and cross-amplification with other *Babesia* species for the species-specific ITS1 assay. Note: original refers to DNA extracted from blood from pre-characterized animal clinical samples.

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**Institutional Review Board Statement:** Part of this study was conducted in accordance with the Declaration of Helsinki and approved by the Institutional Review Board (or Ethics Committee) of North Carolina State University (protocol code IRB#s 4925-03 and 164-08-05, “Detection of *Bartonella* Species in the Blood of People with Extensive Animal Contact”).

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**Data Availability Statement:** Data on reference human results are unavailable due to privacy and ethical restrictions (IRB#s 4925-03 and 164-08-05). All other data are available upon request.

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**Conflicts of Interest:** E. B. Breitschwerdt is a co-founder, shareholder and Chief Scientific Officer for Galaxy Diagnostics (Research Triangle Park, NC, USA), a company that provides advanced diagnostic testing for the detection of *Bartonella* spp. infections. Ricardo Maggi is a co-founder and the Chief Technical Officer for Galaxy Diagnostics Inc. All other authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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## CAPÍTULO 9 – CONSIDERAÇÕES FINAIS

O presente estudo contribuiu para o avanço acerca da ocorrência e diversidade genética de piroplasmídeos em diferentes grupos de animais selvagens no Brasil.

No que diz respeito à detecção de piroplasmídeos em mamíferos da Superordem Xenarthra, foi encontrada uma positividade de 5,5% (25/455). DNA de piroplasmídeos foi detectado em tamanduás-bandeiras, tamanduás-mirins, tatus-pebas, tatus-canastra e tatus-galinha. As sequências curtas para o gene 18S rRNA obtidas posicionaram-se em diferentes clados da filogenia. Enquanto as sequências de tatus-Canastra se alocaram no clado “South American Marsupialia group”, a sequência obtida em tatu-galinha se posicionou em um clado juntamente com uma sequência previamente obtida na mesma espécie, formando um clado irmão ao de *Theileria* sensu stricto. Tais achados indicam que esse grupo de animais pode ser parasitado por *Babesia* spp. e *Theileria* spp. Sequências de ITS-1 foram obtidas a partir de amostras de DNA de tamanduás, as quais formaram um novo clado na filogenia. Nossos resultados indicam que grande diversidade desses protozoários em Xenarthra.

Uma nova espécie de *Babesia* sp., nomeada *Babesia pantanalensis* nov. sp., foi descrita em cachorros-do-mato amostrados no Pantanal do Mato Grosso do Sul. A descrição desta nova espécie foi baseada em análises filogenéticas com alvo em diferentes marcadores moleculares (fragmento quase completo do gene 18S rRNA, *hsp-70* e *cox-1*), as quais posicionaram as sequências obtidas em um clado dentro do grupo de *Babesia* sensu stricto, irmão ao clado de *Rangelia vitalii*.

Duas espécies de *Cytauxzoon* spp. foram relatadas circulando em felinos selvagens amostrados em diferentes localidades no Brasil: *Cytauxzoon brasiliensis* foi detectado em jaguatiricas amostradas no Pantanal do Mato Grosso do Sul e genovariantes de *Cytauxzoon felis* foram detectadas em onças-pintadas amostradas em diferentes estados do país e na Argentina. Tais achados evidenciam uma diversidade de espécies circulando nesses animais no Brasil maior do que se pensava anteriormente. Além disso, todas as onças amostradas mostraram-se positivas para o referido agente, o que corrobora com outros estudos que apontam essa espécie como possível reservatório de *Cytauxzoon* spp. no país.

Uma alta positividade para piroplasmídeos foi encontrada em cervídeos neotropicais: 73,2% (101/138) em cervos-do-Pantanal, 100% (4/4) em veados-mateiro (*Mazama jucunda* – 4/4), 100% (3/3) em veados-mateiro-do-Ecuador (*Mazama rufa*), 60% (6/10) em veados-campeiro e 84,6% (22/26) em veados-catingueiro amostrados em diferentes estados do país. As sequências obtidas dos genes *hsp70*, *cox-3* e 18S rRNA quase-completo posicionaram-se dentro do clado de “*Theileria sensu stricto*”, alocando-se proximamente à *Theileria cervi* e *Theileria capreoli*.

Amostras biológicas de morcegos da Amazônia brasileira, e de roedores selvagens, marsupiais e aves do Pantanal foram negativas nos ensaios moleculares para piroplasmídeos. Tais achados indicam baixas parasitemia (abaixo do limiar de detecção da técnica de nPCR utilizada) ou susceptibilidade das espécies de vertebrados amostradas à infecção por piroplasmídeos.

Ao comparar diferentes técnicas de PCR (dPCR, qPCR e nPCR) em algumas das amostras biológicas de animais selvagens aqui descritas, foi possível notar que dPCR se mostrou a melhor técnica para um diagnóstico mais confiável da infecção por piroplasmídeos nesses animais. De fato, a dPCR detectou positividade em 36 amostras que haviam sido negativas na nPCR. Apenas nove amostras dos roedores selvagens foram testadas na dPCR e as nove mostraram-se positivas, o que pode indicar que provavelmente a parasitemia desses animais é baixa e inviabilizou a detecção por nPCR. Porém, o número amostral deveria ser maior para uma conclusão mais assertiva.

Adicionalmente, o presente trabalho também contribuiu para o diagnóstico de espécies zoonóticas de *Babesia* spp., uma vez que desenvolvemos protocolo de qPCR/dPCR multiplex para detecção de *B. divergens*, *B. odocoilei*, *B. duncani* e *B. microti*. O protocolo de qPCR mostrou-se eficaz, amplificando de  $10^{-1}$  a  $10^{-2}$  cópias dos fragmentos-alvo, a depender da espécie de piroplasmídeo. Ademais, o protocolo não mostrou amplificação cruzada entre várias espécies de piroplasmídeos testadas. Os ensaios testes realizados com amostras de tecido, sangue ou cultura enriquecida de humanos oriundos dos EUA, Canadá e México mostraram-se promissores, sendo capazes de identificar coinfeção com diferentes espécies de *Babesia* spp. em uma mesma amostra.



Em suma, a detecção de piroplasmídeos na fauna selvagem amplia a compreensão acerca de hospedeiros, espécies que ocorrem no país e distribuição desses agentes. Os resultados apresentados no trabalho em tela contribuem para o entendimento da diversidade deste grupo de hemoparasitos, além de fornecer informações cruciais para o desenvolvimento de estratégias de monitoramento e controle de doenças associadas no futuro. A grande diversidade genética de piroplasmídeos apresentada neste trabalho evidencia a complexidade dos ecossistemas brasileiros e enfatiza a necessidade de um esforço contínuo na pesquisa sobre a interação entre patógenos, hospedeiros e ambiente. A continuidade da pesquisa nesse campo é essencial para o aprimoramento das estratégias de conservação da fauna selvagem e manejo de doenças, promovendo o equilíbrio ecológico e a saúde tanto da fauna quanto da população humana.

A partir dos resultados obtidos no presente trabalho, as seguintes perspectivas futuras podem ser traçadas:

- identificação dos possíveis vetores das espécies de piroplasmídeos detectadas no presente estudo;
- inferências filogenéticas baseadas em fragmentos longos do 18S rRNA elucidarão, de maneira mais robusta, o posicionamento filogenético de piroplasmídeos associados aos mamíferos da Superordem Xenartha;
- estudos adicionais em outras áreas do país e em outras espécies de animais permitirão uma melhor compreensão acerca da diversidade dos referidos agentes, seu possível impacto conservação dos animais selvagens e potencial zoonótico dos isolados;
- implementação de técnicas moleculares com maior sensibilidade, tais como a dPCR, auxiliará na detecção de piroplasmídeos e outros agentes transmitidos por vetores que demonstram baixa parasitemia.

## **APÊNDICES**

### **APÊNDICE A – Material suplementar citado no Capítulo 1**

**Tabela Suplementar 2.** Estudos realizados no Brasil sobre a detecção e ocorrência de piroplasmídeos em animais selvagens no país.

| Ordem            | Família  | Espécie do hospedeiro                             | Técnica (Gene alvo)                   | Tamanho das sequências obtida ou do fragmento amplificado (pb) | Tipo de amostra analisada e positiva na triagem | Nº de animais positivos na triagem/nº de animais analisados (%) | Espécies de Piroplasmídeo e/ou posicionamento filogenético                      | Origem da Amostra estado do Brasil (Cativeiro-C ou Vida-livre-VL) | Sinais clínicos e/ou lesões | Referências            |
|------------------|----------|---------------------------------------------------|---------------------------------------|----------------------------------------------------------------|-------------------------------------------------|-----------------------------------------------------------------|---------------------------------------------------------------------------------|-------------------------------------------------------------------|-----------------------------|------------------------|
| <b>Mamíferos</b> |          |                                                   |                                       |                                                                |                                                 |                                                                 |                                                                                 |                                                                   |                             |                        |
| Artiodactyla     | Cervidae | <i>Blastocerus dichotomus</i> (cervo-do-Pantanal) | nPCR (18S rRNA)                       | 360 a 420                                                      | Sangue                                          | 2/4 (50%)                                                       | <i>T. cervi</i> (n=1)<br><i>B. bovis</i> (n=1)                                  | MG (C)                                                            | Não                         | Silveira et al. (2011) |
|                  |          | <i>Mazama americana</i> (veado-mateiro)           | cPCR (18S rRNA)                       | 434                                                            | Pulmão e fígado                                 | 2/2                                                             | <i>Theileria</i> sp. filogeneticamente relacionada a <i>T. cervi</i>            | PA (VL)                                                           | -                           | Soares et al. (2017)   |
|                  |          | <i>Ozotoceros bezoarticus</i> (veado-campeiro)    | nPCR (18S rRNA)                       | 360 a 450                                                      | Sangue                                          | 23/60 (38,3%)                                                   | <i>T. cervi</i> (n=12)<br><i>B. bovis</i> (n=3)<br><i>B. bigemina</i> (n=2)     | MS (VL)                                                           | -                           | Silveira et al. (2013) |
|                  |          |                                                   | cPCR                                  | 1684                                                           | Sangue                                          | 1/1                                                             | Sem sequência                                                                   | GO e MG (C)                                                       | Não                         | Fava et al. (2022)     |
|                  |          | <i>Subulo gouazoubira</i> (veado-catingueiro)     | Esfregaço sanguíneo e nPCR (18S rRNA) | 360 a 420                                                      | Sangue                                          | 7/12 (58,3%) – esfregaços sanguíneos 13/17 (76,5%) - PCR        | <i>T. cervi</i> (n=9)<br><i>Theileria</i> sp. (n=3)<br><i>B. bigemina</i> (n=1) | MG (n=15 VL e n= 2 C)                                             | Sim                         | Silveira et al. (2011) |
|                  |          |                                                   | Esfregaço sanguíneo e nPCR (18S rRNA) | 439 e 820                                                      | Fígado, cérebro e linfonodo                     | 4/8 (50%) – esfregaços sanguíneos 5/9 (55,6%) – nPCR            | <i>Theileria</i> sp. filogeneticamente relacionada a <i>T. cervi</i>            | MG (VL)                                                           | Não                         | Silveira et al. (2014) |
|                  |          |                                                   | Esfregaço sanguíneo e cPCR            | 561                                                            | Sangue                                          | 1/6 (16,7%) – esfregaços sanguíneos                             | <i>Theileria cervi</i>                                                          | GO e MG (C)                                                       | Não                         | Fava et al. (2022)     |

|           |                |                                             |                                         |                                           |                       |                           |                                                                           |                          |                   |                        |                               |                          |
|-----------|----------------|---------------------------------------------|-----------------------------------------|-------------------------------------------|-----------------------|---------------------------|---------------------------------------------------------------------------|--------------------------|-------------------|------------------------|-------------------------------|--------------------------|
|           | Hippopotamidae | <i>Hippopotamus amphibius</i> (hipopótamo)* | nPCR (18S rRNA)                         | 430                                       | Sangue                | 1/6 - PCR<br>1/1          | Sem sequência                                                             | MG (C)                   | Não               | Castillo et al. (2024) |                               |                          |
|           | Tayassuidae    | <i>Pecari tacaju</i> (caititu)              | Esfregaço sanguíneo                     | -                                         | Sangue                | 1/1 – esfregaço sanguíneo | -                                                                         | GO e MG (C)              | Não               | Fava et al. (2022)     |                               |                          |
|           |                | <i>Tayassu pecari</i> (queixada)            | cPCR (18S rRNA)                         | 514                                       | Pulmão e fígado       | 1/9 (11,1%)               | <i>Babesia</i> sp. filogeneticamente relacionada a <i>Babesia</i> sp. WA1 | PA (VL)                  | -                 | Soares et al. (2017)   |                               |                          |
| Carnivora | Canidae        | <i>Cerdocyon thous</i> (cachorro-do-mato)   | cPCR (18S rRNA)                         | 549                                       | Sangue e/ou tecidos   | 6/20 (30%)                | <i>R. vitalii</i>                                                         | RS (n=5) e SP (n=1) (VL) | Não               | Soares et al. (2014)   |                               |                          |
|           |                |                                             | cPCR (18S rRNA)                         | 500                                       | Fígado                | 1 (Relato de caso)        | <i>R. vitalii</i>                                                         | RS (VL)                  | Sim               | Fredo et al. (2015)    |                               |                          |
|           |                |                                             | nPCR (18S rRNA)                         | 739                                       | Sangue                | 1/78 (1,3%)               | <i>Babesia</i> sp. filogeneticamente relacionada a <i>B. caballi</i>      | MS (VL)                  | Não               | De Sousa et al. (2018) |                               |                          |
|           |                |                                             | Esfregaço sanguíneo e nPCR (18S rRNA)   | 593                                       | Sangue                | 1 (Relato de caso)        | <i>R. vitalii</i>                                                         | RS (VL)                  | Sim               | Copat et al. (2019)    |                               |                          |
|           |                |                                             | cPCR (18S rRNA)                         | 500                                       | Sangue                | 7/27 (25,9%)              | <i>R. vitalii</i>                                                         | RS (VL)                  | Não               | De Souza et al. (2019) |                               |                          |
|           |                |                                             |                                         | <i>Chrysocyon brachyurus</i> (lobo-guará) | qPCR ( <i>hsp70</i> ) | 180                       | Tecidos                                                                   | 8/31 (25,8%)             | <i>R. vitalii</i> | RS (VL)                | Não                           | De Lorenzo et al. (2021) |
|           |                |                                             | Esfregaço sanguíneo e cPCR              |                                           | 1684                  | Sangue                    | 5/10 (50%)-esfregaços sanguíneos<br>4/10 (40%)-PCR                        | Sem sequência            | GO e MG (C)       | Não                    | Fava et al. (2022)            |                          |
|           |                |                                             | qPCR ( <i>hsp70</i> ) e cPCR (18S rRNA) |                                           | 495                   | Sangue                    | 2/3 (66,7%)                                                               | <i>R. vitalii</i>        | SC (VL)           | Não                    | De Souza Júnior et al. (2023) |                          |
|           |                |                                             | Esfregaço sanguíneo                     |                                           | -                     | Sangue                    | 1 (Relato de caso)                                                        | <i>B. canis</i>          | DF (VL)           | Sim                    | Cansi et al. (2012)           |                          |
|           |                |                                             | cPCR ( <i>hsp70</i> )                   |                                           | 614                   | Linfonodo mesentérico     | 1 (Relato de caso)                                                        | <i>R. vitalii</i>        | MG (VL)           | Sim                    | Silveira et al. (2016)        |                          |

|         |                                                        |                                       |              |                 |                                                     |                                                                |                         |     |                          |
|---------|--------------------------------------------------------|---------------------------------------|--------------|-----------------|-----------------------------------------------------|----------------------------------------------------------------|-------------------------|-----|--------------------------|
|         |                                                        | cPCR                                  | 1684         | Sangue          | 1/6 (16,7%)                                         | Sem sequência                                                  | GO e MG (C)             | Não | Fava et al. (2022)       |
|         | <i>Lycalopex gymnocercus</i> (graxaim-do-campo)        | cPCR (18S rRNA)                       | 492          | Sangue          | 1 (Relato de caso)                                  | <i>R. vitalii</i>                                              | SC (VL)                 | Sim | De Quadros et al. (2015) |
|         |                                                        | cPCR (18S rRNA)                       | 500          | Fígado          | 1 (Relato de caso)                                  | <i>R. vitalii</i>                                              | RS (VL)                 | Sim | Fredo et al. (2015)      |
|         |                                                        | Esfregaço sanguíneo e nPCR (18S rRNA) | 1307         | Sangue          | 1 (Relato de caso)                                  | <i>R. vitalii</i>                                              | SC (VL)                 | Não | Da Silva et al. (2018)   |
|         |                                                        | cPCR (18S rRNA)                       | 500          | Sangue          | 1/17 (5,9%)                                         | <i>R. vitalii</i>                                              | RS (VL)                 | Não | De Souza et al. (2019)   |
|         | <i>Lycalopex vetulus</i> (raposinha-do-campo)          | Esfregaço sanguíneo e cPCR            | 1684         | Sangue          | 1/5 (20%)-esfregaço sanguíneo 3/5 (60%) - PCR       | Sem sequência                                                  | GO e MG (C)             | Não | Fava et al. (2022)       |
| Felidae | <i>Leopardus braccatus</i> (gato-palheiro-do-Pantanal) | nPCR (18S rRNA)                       | 790          | Sangue          | 1/5 (20%)                                           | <i>Babesia</i> sp. (97 a 99% de identidade com <i>B. leo</i> ) | SP (C)                  | Não | André et al. (2011)      |
|         |                                                        | nPCR (18S rRNA)                       | 480          | Sangue          | 2/2                                                 | Sem sequência                                                  | MG (C)                  | Não | Castillo et al. (2024)   |
|         | <i>Leopardus pardalis</i> (jaguaririca)                | cPCR (18S rRNA)                       | ~400         | Sangue          | 6/29 (20,7%)                                        | <i>Cytauxzoon</i> sp.                                          | DF (n=2) e SP (n=4) (C) | Não | André et al. (2009)      |
|         |                                                        | cPCR (18S rRNA)                       | 1598         | Sangue          | 1/16 (6,2%)                                         | <i>Cytauxzoon brasiliensis</i>                                 | SP (C)                  | Não | Filoni et al. (2012)     |
|         |                                                        | cPCR (18S rRNA)                       | 508          | Pulmão e fígado | 1/1                                                 | <i>Cytauxzoon</i> sp.                                          | PA (VL)                 | -   | Soares et al. (2017)     |
|         |                                                        | Esfregaço sanguíneo nPCR (18S rRNA)   | ~250 ou ~750 | Sangue          | 1/7 (14,3%) – esfregaço sanguíneo 3/7 (42,9%) – PCR | <i>Cytauxzoon</i> sp.                                          | MS (VL)                 | Não | De Sousa et al. (2018)   |

|                                            |                                                       |                                       |         |                                                   |                                |                                    |     |                                |
|--------------------------------------------|-------------------------------------------------------|---------------------------------------|---------|---------------------------------------------------|--------------------------------|------------------------------------|-----|--------------------------------|
|                                            | nPCR (18S rRNA)                                       | 721                                   | Baço    | 1/4 (25%)                                         | <i>Cytauxzoon</i> sp.          | MT (VL)                            | Não | Silva et al. (2021)            |
|                                            | cPCR (18S rRNA)                                       | 284                                   | Sangue  | 4/5 (80%)                                         | <i>Cytauxzoon</i> sp.          | MT e MS (VL)                       | Não | Fagundes-Moreira et al. (2022) |
|                                            | Esfregação sanguíneo e cPCR                           | 574                                   | Sangue  | 1/3 (33,3%)—<br>esfregação sanguíneo<br>3/3 - PCR | <i>Cytauxzoon</i> sp.          | GO e MG (C)                        | Não | Fava et al. (2022)             |
| <i>Leopardus tigrinus</i> (gato-macambira) | cPC (18S rRNA)                                        | 1202                                  | Sangue  | 1 (Relato de caso)                                | <i>Cytauxzoon</i> sp.          | RJ (C)                             | -   | Amaral (2006)                  |
|                                            | Esfregação sanguíneo e nPCR (18S rRNA e <i>cytB</i> ) | 1459 – 18S rRNA<br>1097 - <i>cytB</i> | Sangue  | 1 (Relato de caso)                                | <i>Cytauxzoon brasiliensis</i> | DF (VL)                            | Não | Duarte et al. (2024)           |
| <i>Panthera leo</i> (leão)*                | Sinais clínicos, necropsia e histopatológico          | -                                     | Tecidos | 2 (Relato de caso)                                | <i>Cytauxzoon</i> sp.          | RJ (C)                             | Sim | Peixoto et al. (2007)          |
| <i>Panthera onca</i> (onça pintada)        | cPC (18S rRNA)                                        | 947                                   | Sangue  | 1 (Relato de caso)                                | <i>Cytauxzoon</i> sp.          | RJ (C)                             | -   | Amaral (2006)                  |
|                                            | cPCR (18S rRNA)                                       | ~400                                  | Sangue  | 1/9 (11,1%)                                       | <i>Cytauxzoon</i> sp.          | DF (C)                             | Não | André et al. (2009)            |
|                                            | cPCR (18S rRNA)                                       | 330                                   | Sangue  | 28/29 (96,5%)                                     | <i>Cytauxzoon</i> sp.          | GO (n=4), MS (n=22), TO (n=3) (VL) | Não | Furtado et al. (2017)          |
|                                            | cPCR (18S rRNA – ITS1)                                | 525                                   | Tecidos | 1 (Relato de caso)                                | <i>Cytauxzoon</i> sp.          | MS (C)                             | Sim | Guizelini et al. (2021)        |
|                                            | nPCR (18S rRNA)                                       | 721                                   | Baço    | 3/6 (50%)                                         | <i>Cytauxzoon</i> sp.          | MT (C)                             | -   | Silva et al. (2021)            |
|                                            | cPCR (18S rRNA)                                       | 284                                   | Sangue  | 45/45                                             | <i>Cytauxzoon</i> sp.          | MT e MS (VL)                       | Não | Fagundes-Moreira et al. (2022) |

|             |                                   |                                       |           |        |                                   |                                                                                                     |                       |     |                                |
|-------------|-----------------------------------|---------------------------------------|-----------|--------|-----------------------------------|-----------------------------------------------------------------------------------------------------|-----------------------|-----|--------------------------------|
|             |                                   | nPCR (18S rRNA)                       | 430       | Sangue | 1/1                               | <i>Cytauxzoon</i> sp.                                                                               | MG (C)                | Não | Castillo et al. (2024)         |
|             |                                   | qPCR (18S rRNA)                       | ~220      | Baço   | 2/2                               | <i>Cytauxzoon</i> sp.                                                                               | MS (VL – atropelados) | -   | Alves et al. (2025)            |
|             | <i>Puma concolor</i> (onça-parda) | Esfregaço sanguíneo                   | -         | Sangue | 2/7 (28,6%)                       | <i>Cytauxzoon</i> sp.                                                                               | MS (VL e C)           | Não | Juliano et al. (2004)          |
|             |                                   | cPCR (18S rRNA)                       | ~400      | Sangue | 2/9 (22,2%)                       | <i>Cytauxzoon</i> sp.                                                                               | DF (C)                | Não | André et al. (2009)            |
|             |                                   | Esfregaço sanguíneo                   | -         | Sangue | 1 (Relato de caso)                | <i>Cytauxzoon</i> sp.                                                                               | MS (VL)               | Não | Antunes et al. (2018)          |
|             |                                   | Esfregaço sanguíneo e cPCR (18S rRNA) | 600       | Sangue | 6/11 (54,5%)                      | <i>Cytauxzoon</i> sp.                                                                               | MS (C)                | Não | Silva et al. (2020)            |
|             |                                   | nPCR (18S rRNA)                       | 721       | Sangue | 2/8 (25%)                         | <i>Cytauxzoon</i> sp.                                                                               | MT (VL)               | -   | Silva et al. (2021)            |
|             |                                   | Esfregaço sanguíneo e cPCR (18S rRNA) | 455       | Sangue | 1 (Relato de caso)                | <i>Cytauxzoon</i> sp.                                                                               | GO (VL)               | Não | De Paula et al. (2022)         |
|             |                                   | cPCR (18S rRNA)                       | 284       | Sangue | 3/3                               | <i>Cytauxzoon</i> sp.                                                                               | MT e MS (VL)          | Não | Fagundes-Moreira et al. (2022) |
|             |                                   | Esfregaço sanguíneo                   | -         | Sangue | 2/12 (16,7%)                      | -                                                                                                   | GO e MG (C)           | Não | Fava et al. (2022)             |
| Viverridae  | <i>Genetta genetta</i> (gineta)*  | nPCR (18S rRNA)                       | 526       | Sangue | 1/2 (50%)                         | <i>Babesia</i> sp. (97 a 99% de identidade com <i>B. leo</i> )                                      | SP (C)                | Não | André et al. (2011)            |
| Mustelidae  | <i>Eira arbara</i> (irara)        | cPCR (18S rRNA)                       | 1684      | Sangue | 1/1                               | Sem sequência                                                                                       | GO e MG (C)           | Não | Fava et al. (2022)             |
| Procyonidae | <i>Nasua nasua</i> (quati)        | Esfregaço sanguíneo                   | 450 a 564 | Sangue | 1/31 (3,2%) – esfregaço sanguíneo | <i>Theileria</i> sp. filogeneticamente relacionada <i>Theileria</i> sp. detectada em gato no Brasil | MS (VL)               | Não | De Sousa et al. (2018)         |
|             |                                   | nPCR (18S rRNA)                       | ~380      | Sangue | 3/209 (1,4%)                      | <i>Babesia</i> sp. filogeneticamente                                                                | MG (VL)               | Não | Estevam et al. (2020)          |

|            |                |                                                                |                            |             |                 |                                              |                                                                                                                                                                                                    |                                    |     |                        |
|------------|----------------|----------------------------------------------------------------|----------------------------|-------------|-----------------|----------------------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------|-----|------------------------|
|            |                |                                                                | cPCR (18S rRNA)            | 1684        | Sangue          | 2/2                                          | relacionada a <i>B. bigemina</i><br>Sem sequência                                                                                                                                                  | GO e MG (C)                        | Não | Fava et al. (2022)     |
|            |                | <i>Procyon cancrivorus</i> (mão-pelada)                        | Esfregaço sanguíneo e cPCR | 1684        | Sangue          | 1/2 (50%) – esfregaço sanguíneo<br>2/2 – PCR | Sem sequência                                                                                                                                                                                      | GO e MG (C)                        | Não | Fava et al. (2022)     |
| Chiroptera | Phyllostomidae | <i>Artibeus lituratus</i> (morcego-de-cara-branca)             | nPCR e cPCR (18S rRNA)     | 708         | Sangue          | 1/37 (2,7%)                                  | <i>Piroplasmid</i> sp. (Clado de “ <i>Babesia</i> sensu stricto”)                                                                                                                                  | MS (VL)                            | -   | Ikeda et al. (2021)    |
|            |                | <i>Artibeus planirostris</i> (morcego-da-frutas-de-face-plana) | nPCR e cPCR (18S rRNA)     | 741         | Sangue e baço   | 4/33 (12,1%)                                 | <i>Piroplasmid</i> sp. (Clado de “ <i>Babesia</i> sensu stricto”)                                                                                                                                  | MS (VL)                            | -   | Ikeda et al. (2021)    |
|            |                | <i>Desmodus rotundus</i> (morcego-vampiro)                     | nPCR e cPCR (18S rRNA)     | ~1500       | Baço            | 42/228 (18,4%)                               | <i>Babesia</i> sp. (Clado “South American Marsupialia Group”)<br><i>Theileria</i> sp. (Clado “ <i>Theileria</i> sensu stricto”)<br><i>Theileria</i> sp. (Clado “ <i>Tapirus</i> terrestres group”) | AP (n=1), PA (n=37), RR (n=4) (VL) | -   | De Mello et al. (2023) |
|            |                | <i>Diaemus youngi</i> (morcego-vampiro-de-asas-brancas)        | nPCR e cPCR (18S rRNA)     | -           | Baço            | 1/1                                          | Sem sequência                                                                                                                                                                                      | PA (VL)                            | -   | De Mello et al. (2023) |
|            |                | <i>Platyrrhinus lineatus</i> (morcego-de-linha-branca)         | nPCR e cPCR (18S rRNA)     | -           | Baço            | 2/23 (8,7%)                                  | Sem sequência                                                                                                                                                                                      | MS (VL)                            | -   | Ikeda et al. (2021)    |
|            |                | <i>Phyllostomus discolor</i>                                   | nPCR e cPCR (18S rRNA)     | 1455 a 1570 | Sangue e Baço   | 10/15 (66,7%)                                | <i>Piroplasmid</i> sp. (Clado “Phyllostomatidae bat Group”)                                                                                                                                        | MS (VL)                            | -   | Ikeda et al. (2021)    |
|            | Dasypodidae    | <i>Dasypus novemcinctus</i> (tatu-galinha)                     | cPCR (18S rRNA)            | 473         | Pulmão e fígado | 3/32 (9,4%)                                  | <i>Theileria</i> sp. filogeneticamente relacionada a <i>Theileria</i> sp. detectada em cutia                                                                                                       | MT (n=1) e PA (n=2) (VL)           | -   | Soares et al. (2017)   |



|                 |             |                                                       |                                                                                          |                                                                         |                       |                                                           |                                                                                                         |                          |                |                           |
|-----------------|-------------|-------------------------------------------------------|------------------------------------------------------------------------------------------|-------------------------------------------------------------------------|-----------------------|-----------------------------------------------------------|---------------------------------------------------------------------------------------------------------|--------------------------|----------------|---------------------------|
|                 |             |                                                       | nPCR (18S rRNA),                                                                         | 731                                                                     | Sangue ou baço        | 3/10 (30%)                                                | <i>Theileria</i> sp. filogeneticamente relacionada a <i>Theileria</i> sp. detectada no mesmo hospedeiro | MS (n=1) e SP (n=2) (VL) | -              | Calchi et al. (2023)      |
| Didelphimorphia | Didelphidae | <i>Didelphis albiventris</i> (gambá-de-orelha-branca) | Esfregaço sanguíneo nPCR e cPCR (18S rRNA), nPCR ( <i>cox-1</i> ), cPCR ( <i>hsp70</i> ) | 1463 – 18S rRNA<br>434 a 575 – <i>hsp70</i><br>665 e 708 – <i>cox-1</i> | Sangue                | 10/67 (14,9%) – esfregaços sanguíneos 22/67 (32,8%) - PCR | <i>Babesia</i> sp. (clado “South American Marsupialia Group”)                                           | DF (n=24) MS (n=43) (VL) | -              | Gonçalves et al. (2021)   |
|                 |             |                                                       | Esfregaço sanguíneo e cPCR (18S rRNA)                                                    | 1684                                                                    | Sangue                | 5/26 (19,2%) – esfregaços sanguíneos 4/26 (15,4%) - PCR   | Sem sequência                                                                                           | GO e MG (C)              | Não            | Fava et al. (2022)        |
|                 |             | <i>Didelphis aurita</i> (gamba-de-orelha-preta)       | Esfregaço sanguíneo, nPCR e cPCR (18S rRNA) e cPCR ( <i>cox-3</i> )                      | 1569 e 1609 – 18S rRNA<br>771 e 776 – <i>cox-3</i>                      | Sangue e medula óssea | 2/15 (13,3%) – Esfregaço sanguíneo 5/15 (33,3%) - PCR     | <i>Babesia</i> sp. (clado “South American Marsupialia Group”)                                           | RJ (VL)                  | Sim (1 animal) | De Oliveira et al. (2023) |
|                 |             | <i>Didelphis marsupialis</i> (gambá-comum)            | cPCR (18S rRNA)                                                                          | 524                                                                     | Pulmão e fígado       | 1/17 (5,9%)                                               | <i>Babesia</i> sp. filogeneticamente relacionada a <i>Babesia</i> sp. detectada em cuiça                | PA (VL)                  | -              | Soares et al. (2017)      |
|                 |             |                                                       | cPCR (18S rRNA)                                                                          | 525                                                                     | Sangue                | 2/31 (6,5%)                                               | <i>Babesia</i> sp. filogeneticamente relacionada a <i>Babesia</i> sp. detectada em gambás               | MT (VL)                  | -              | Colle et al., 2019        |
|                 |             |                                                       | nPCR (18S rRNA)                                                                          | 769                                                                     | Sangue                | 1/45 (2,2%)                                               | <i>Babesia</i> sp. (clado “South American Marsupialia Group”)                                           | MA (VL)                  | -              | Braga et al. (2023)       |

|                |                 |                                                    |                                                                             |                                                                          |                       |                                                          |                                                                                                       |                          |     |                           |
|----------------|-----------------|----------------------------------------------------|-----------------------------------------------------------------------------|--------------------------------------------------------------------------|-----------------------|----------------------------------------------------------|-------------------------------------------------------------------------------------------------------|--------------------------|-----|---------------------------|
|                |                 | <i>Monodelphis domestica</i> (cuica-de-rabo-curto) | cPCR (18S rRNA)                                                             | 556                                                                      | Sangue, baço e fígado | 1/2 (50%)                                                | <i>Babesia</i> sp. filogeneticamente relacionado a <i>Babesia</i> sp. detectado em <i>Thrichomys</i>  | MT (VL)                  | -   | Wolf et al. (2016)        |
| Perissodactyla | Rhinocerotidae  | <i>Ceratotherium simum</i> (rinoceronte-branco)*   | nPCR (18S rRNA)                                                             | 430                                                                      | Sangue                | 1/2 (50%)                                                | Sem sequência                                                                                         | MG (C)                   | Não | Castillo et al. (2024)    |
|                | Tapiridae       | <i>Tapirus terrestris</i> (anta)                   | cPCR (18S rRNA)                                                             | 414                                                                      | Sangue                | 1 (Relato de caso)                                       | <i>Theileria</i> sp. 98% idêntica a <i>T. equi</i>                                                    | MS (VL)                  | Não | Da Silveira et al. (2017) |
|                |                 |                                                    | Esfregaço sanguíneo e cPCR (18S rRNA)                                       | 392 a 475                                                                | Sangue                | 7/19 (36,8%) – esfregaços sanguíneos 11/19 (57,9%) – PCR | <i>T. equi</i>                                                                                        | AM (n=1) e PA (n=18) (C) | Não | Gonçalves et al. (2020)   |
|                |                 |                                                    | nPCR (18S rRNA)                                                             | 776                                                                      | Baço e Sangue         | 6/17 (35,3%)                                             | <i>Theileria</i> sp. filogeneticamente relacionada a <i>Theileria</i> sp. detectada em gato no Brasil | MT (VL)                  | -   | Silva et al. (2021)       |
|                |                 |                                                    | Esfregaço sanguíneo e cPCR                                                  | 553                                                                      | Sangue                | 1/2 (50%) – esfregaço sanguíneo 1/2 – PCR                | <i>Theileria</i> sp. filogeneticamente próximo a <i>T. equi</i>                                       | GO e MG (C)              | Não | Fava et al. (2022)        |
|                |                 |                                                    | Esfregaço sanguíneo e nPCR (18S rRNA e <i>cox1</i> ), cPCR ( <i>hsp70</i> ) | 1200 a 1500 – 18S rRNA 687 a 782 – <i>hsp70</i> 354 a 410 – <i>cox-1</i> | Sangue                | 56/99 (56,7%) – PCR                                      | <i>T. terrestris</i> (Clado <i>Tapirus terrestris</i> )                                               | MS (VL)                  | -   | Mongruel et al. (2022)    |
| Pilosa         | Myrmecophagidae | <i>Myrmecophaga tridactyla</i> (tamanduá-bandeira) | Esfregaço sanguíneo e cPCR (18S rRNA)                                       | 1684                                                                     | Sangue                | 6/21 (28,6%) – esfregaços sanguíneos 2/21 (9,5%) – PCR   | Sem sequência                                                                                         | GO e MG (C)              | Não | Fava et al. (2022)        |

|          |             |                                                           |                                                                                |                                                                               |                    |                                                                          |                                                                                |             |     |                                                       |
|----------|-------------|-----------------------------------------------------------|--------------------------------------------------------------------------------|-------------------------------------------------------------------------------|--------------------|--------------------------------------------------------------------------|--------------------------------------------------------------------------------|-------------|-----|-------------------------------------------------------|
|          |             | <i>Tamandua tetradactyla</i><br>(tamanduá-mirim)          | Esfregaço<br>sanguíneo e<br>cPCR (18S<br>rRNA)                                 | 1684                                                                          | Sangue             | 5/11(45,5%) –<br>esfregaço<br>sanguíneo<br>2/11<br>(18,2%) -<br>PCR      | Sem sequência                                                                  | GO e MG (C) | Não | Fava et al.<br>(2022)                                 |
| Primates | Atelidae    | <i>Alouatta caraya</i><br>(bugio-preto)                   | Esfregaço<br>sanguíneo e<br>cPCR (18S<br>rRNA)                                 | 1684                                                                          | Sangue             | 2/5 (40%) –<br>esfregaço<br>sanguíneo<br>1/5 (20%) –<br>PCR              | Sem sequência                                                                  | GO e MG (C) | Não | Fava et al.<br>(2022)                                 |
|          |             | <i>Ateles</i> spp.<br>(macaco-aranha)                     | nPCR (18S<br>rRNA)                                                             | 430                                                                           | Sangue             | 1/1                                                                      | Sem sequência                                                                  | MG (C)      | Não | Castillo et al.<br>(2024)                             |
|          | Cebidae     | <i>Callithrix penicillata</i><br>(sagui-de-tufos-pretos)* | Esfregaço<br>sanguíneo e<br>cPCR                                               | 1684                                                                          | Sangue             | 11/23<br>(47,8%) –<br>esfregaço<br>sanguíneo<br>4/23<br>(17,4%) –<br>PCR | Sem sequência                                                                  | GO e MG (C) | Não | Fava et al.<br>(2022)                                 |
|          |             | <i>Sapajus apella</i><br>(macaco-prego)                   | nPCR (18S<br>rRNA)                                                             | 430                                                                           | Sangue             | 1/2 (50%)                                                                | Sem sequência                                                                  | MG (C)      | Não | Castillo et al.<br>(2024)                             |
|          | Hominidae   | <i>Gorilla gorilla</i><br>(gorila)*                       | nPCR (18S<br>rRNA)                                                             | 430                                                                           | Sangue             | 2/3 (66,7%)                                                              | Sem sequência                                                                  | MG (C)      | Não | Castillo et al.<br>(2024)                             |
| Rodentia | Caviidae    | <i>Hydrochoerus hydrochaeris</i><br>(capivara)            | cPCR (18S<br>rRNA)                                                             | 1714                                                                          | Sangue             | 2/14<br>(14,3%)                                                          | <i>Babesia</i> sp.<br>Capybara                                                 | RS (VL)     | -   | Criado-<br>Fornelio et<br>al. (2009)                  |
|          |             |                                                           | cPCR (18S<br>rRNA)                                                             | 1684                                                                          | Sangue             | 1/2 (50%)                                                                | Sem sequência                                                                  | GO e MG (C) | Não | Fava et al.<br>(2022)                                 |
|          |             |                                                           | Esfregaço<br>sanguíneo,<br>cPCR (18S<br>rRNA, <i>hsp70</i> ,<br><i>cox-3</i> ) | 1519 a 1561<br>-18S rRNA<br>825 a 887 –<br><i>hsp70</i><br>590 – <i>cox-3</i> | Sangue             | 1/28<br>(3,6%) –<br>esfregaço<br>sanguíneo<br>11/28<br>(39,3%) –<br>PCR  | <i>B. goianiaensis</i><br>(clado “Capybara<br>group)                           | GO (VL)     | -   | Krawczak et<br>al. (2023);<br>Neves et al.,<br>(2023) |
|          | Cuniculidae | <i>Cuniculus paca</i><br>(paca)                           | cPCR (18S<br>rRNA)                                                             | 537                                                                           | Pulmão e<br>fígado | 4/33<br>(12,1%)                                                          | <i>Babesia</i> sp.<br>filogeneticamente<br>relacionada a<br><i>Babesia</i> sp. | MT (VL)     | -   | Soares et al.<br>(2017)                               |

|                   |                                                                  |                                                                            |                                                   |                       |                |                                                                                                                                |                                                                     |         |                        |                        |
|-------------------|------------------------------------------------------------------|----------------------------------------------------------------------------|---------------------------------------------------|-----------------------|----------------|--------------------------------------------------------------------------------------------------------------------------------|---------------------------------------------------------------------|---------|------------------------|------------------------|
|                   |                                                                  |                                                                            |                                                   |                       |                | detectada em capivara                                                                                                          |                                                                     |         |                        |                        |
| Dasyproctidae     | <i>Dasyprocta</i> sp. (cutia)                                    | cPCR (18S rRNA)                                                            | 314                                               | Pulmão e fígado       | 1/1            | <i>Theileria</i> sp. filogeneticamente relacionada a <i>Theileria</i> sp. detectada em tatu                                    | MT (VL)                                                             | -       | Soares et al. (2017)   |                        |
| Echimyidae        | <i>Thrichomys fosteri</i>                                        | cPCR (18S rRNA)                                                            | ~720                                              | Sangue                | 6/77 (7,8%)    | <i>Babesia</i> sp. filogeneticamente relacionada a <i>Babesia vogeli</i> e <i>Theileria</i> sp. relacionada com <i>T. equi</i> | MS (VL)                                                             | Não     | De Sousa et al. (2018) |                        |
|                   | <i>Thrichomys pachyurus</i>                                      | cPCR (18S rRNA)                                                            | 532                                               | Sangue, baço e fígado | 3/11 (27,3%)   | <i>Babesia</i> sp. filogeneticamente relacionado a <i>Babesia</i> sp. detectada em <i>Monodelphis</i>                          | MT (VL)                                                             | -       | Wolf et al. (2016)     |                        |
|                   |                                                                  | cPCR (18S rRNA e <i>cytB</i> )<br>nPCR (18S rRNA)                          | 1200 a 1230 – 18S rRNA<br>722 a 830 - <i>cytB</i> | Sangue, pulmão e baço | 17/93 (18,3%)  | <i>Piroplasmida</i> sp. (Clado “South American Rodentia Group”)                                                                | MT (VL)                                                             | -       | Pacheco et al. (2025)  |                        |
| Erinaceinae       | <i>Coendou prehensilis</i> (ouriço-cacheiro)                     | cPCR (18S rRNA)                                                            | 1684                                              | Sangue                | 7/10 (70%)–PCR | Sem sequência                                                                                                                  | GO e MG (C)                                                         | Não     | Fava et al. (2022)     |                        |
|                   | <i>Sphiggurus spinosus</i> (porco-espinho-peludo-anão-paraguaio) | Esfregaço sanguíneo                                                        | -                                                 | Sangue                | 1/1            | -                                                                                                                              | GO e MG (C)                                                         | Não     | Fava et al. (2022)     |                        |
| Aves              |                                                                  |                                                                            |                                                   |                       |                |                                                                                                                                |                                                                     |         |                        |                        |
| Anseriformes      | Anatidae                                                         | <i>Neochen jubata</i> (pato-corredor)                                      | nPCR (18S rRNA)                                   | 693                   | Sangue         | 6/62 (9,7%)                                                                                                                    | <i>Babesia</i> sp. filogeneticamente relacionado a <i>B. vogeli</i> | GO (VL) | -                      | Werther et al. (2017)  |
| Procellariiformes | Diomedidae                                                       | <i>Thalassarche chlororhynchos</i> (albatroz-de-bico-amarelo-do-Atlântico) | cPCR (18S rRNA)                                   | 672                   | Sangue         | 1/15 (6,7%)                                                                                                                    | <i>Babesia</i> sp. (Clado <i>Babesia</i> sensu stricto)             | SC (VL) | -                      | Sgarioni et al. (2023) |

|            |         |                                          |                    |      |        |                   |                                      |                                        |   |                             |
|------------|---------|------------------------------------------|--------------------|------|--------|-------------------|--------------------------------------|----------------------------------------|---|-----------------------------|
| Suliformes | Sulidae | <i>Sula dactylatra</i><br>(atobá-grande) | cPCR (18S<br>rRNA) | -    | Sangue | 22/168<br>(13,1%) | Sem sequência                        | Ilhas no<br>Nordeste do<br>Brasil (VL) | - | Quillfeldt et<br>al. (2014) |
|            |         | <i>Sula leucogaster</i><br>(atobá-pardo) | cPCR (18S<br>rRNA) | 1450 | Sangue | 12/172<br>(7,0%)  | <i>Babesia</i> sp. (Clado<br>Percei) | Ilhas no<br>Nordeste do<br>Brasil (VL) | - | Quillfeldt et<br>al. (2014) |

\* Animais exóticos no Brasil.

**APÊNDICE B – Material suplementar citado no Capítulo 3**

**Phylogenetic inferences based on distinct molecular markers reveals a novel *Babesia* (*Babesia pantanalensis* nov. sp.) and a *Hepatozoon americanum*-related genotype in crab-eating foxes (*Cerdocyon thous*)\***

\* Material correspondente ao artigo científico publicado na revista Experimental Parasitology 262:108786, 2024. <https://doi.org/10.1016/j.exppara.2024.108786>

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**Supplementary material 3.** Description of primers, amplicon sizes and thermal conditions used in conventional and nested PCR assays for detection and characterization of piroplasmids and *Hepatozoon* spp.

| Agent                           | Gene/ name primer     | Primers sequences                  | Size (bp) | Thermal sequences                                        | Reference                 |
|---------------------------------|-----------------------|------------------------------------|-----------|----------------------------------------------------------|---------------------------|
| Endogenous gene                 | <i>gapdh</i>          | 5'TCCAACACCACCACTGAGATCGGAC -3'    | 400       | 95°C for 5 min;                                          | Birkenheuer et al. (2003) |
|                                 | -GAPDHF               | 5'- GTGAGAAGAAATCGGACTGGCC3'       |           | 35 cycles: 95°C for 15s, 50°C for 30s and 72°C for 30s;  |                           |
|                                 | -GAPDHR               |                                    |           | 72°C for 5 min                                           |                           |
| Piroplasmids (screening -nPCR)  | 18S rRNA              |                                    | 800       | 94°C for 3 min, 58°C for 1 min, 72°C for 2 min;          | Jefferies et al.(2007)    |
|                                 | 1 <sup>st</sup> Round | 5'GGCTCATTACAACAGTTATAG-3'         |           | 45 cycles: 94°C for 30s, 58°C for 20s and 72°C for 30s;  |                           |
|                                 | -BTF1                 | 5'CCCAAAGACTTTGATTCTCTC-3'         |           | 72°C for 7 min                                           |                           |
|                                 | -BTR1                 |                                    |           | Annealing temperature 2nd round =62°C                    |                           |
|                                 | 2 <sup>nd</sup> Round | 5'CCGTGCTAATTGTAGGGCTAATAC-3'      |           |                                                          |                           |
|                                 | -BTF2                 | 5'GGACTACGACGGTATCTGATCG-3'        |           |                                                          |                           |
|                                 | -BTR2                 |                                    |           |                                                          |                           |
| Piroplasmids (characterization) | 18S rRNA              | 5'- AAGCCATGCATGTCTAAGTATAAGCTTTT- | 1500      | 95°C for 5 min;                                          | Greay et al. (2018)       |
|                                 | -Nbab_1F              | 3'                                 |           | 50 cycles: 95°C for 30s, 60°C for 30s and 72°C for 2min; |                           |
|                                 | -18SApiR              | 5'-GGATCACTCGATCGGTAGGAG-3'        |           | 72°C for 5 min                                           |                           |



|                        |          |                               |       |                                   |              |
|------------------------|----------|-------------------------------|-------|-----------------------------------|--------------|
|                        | 18S rRNA |                               |       | 95°C for 3 min;                   |              |
| <i>Hepatozoon</i> spp. | -HAM-1   | 5' -GCCAGTAGTCATATGCTTGTC -3' |       | 40 cycles: 95°C for 1min,         | Criado-      |
| (characterization)     | -HPF-2   | 5' -GACTTCTCCTTCGTCTAAG-3'    | 1,750 | 56°C for 1min and 72°C for 1 min; | Fornelio et. |
|                        |          |                               |       | 72°C for 7 min                    | al (2006)    |

**Supplementary material 2.** BLASTn results of the sequences obtained in the PCR assays for piroplasmids and *Hepatozoon* spp. from *Cerdocyon thous* blood samples from Pantanal of Miranda, Mato Grosso do Sul, Brazil.

| <i>Cerdocyon<br/>thous</i> ID | Agent        | Gene                              | Sequence<br>size (bp) | Query-<br>cover<br>(%) | E-<br>value | Identity<br>(%) | GenBank<br>sequence<br>(access<br>number)                                       |
|-------------------------------|--------------|-----------------------------------|-----------------------|------------------------|-------------|-----------------|---------------------------------------------------------------------------------|
| # 2                           | Piroplasmids | 18S<br>rRNA<br>(short<br>segment) | 675                   | 99                     | 0           | 99.70           | <i>Rangelia vitalii</i><br>detected in dog<br>from Paraguai<br>(MH100726)       |
| # 2 clone 1<br>(PP067561)     | Piroplasmids | 18S<br>rRNA<br>(large<br>segment) | 1465                  | 100                    | 0           | 95.93           | <i>Rangelia vitalii</i><br>detected in a<br>dog from<br>Argentina<br>(KF218606) |
| # 2 clone 2<br>(PP067566)     | Piroplasmids | 18S<br>rRNA<br>(large<br>segment) | 1470                  | 100                    | 0           | 96.01           | <i>Rangelia vitalii</i><br>detected in a<br>dog from<br>Argentina<br>(KF218606) |
| # 2 clone 3<br>(PP067562)     | Piroplasmids | 18S<br>rRNA<br>(large<br>segment) | 1467                  | 100                    | 0           | 95.87           | <i>Rangelia vitalii</i><br>detected in a<br>dog from<br>Argentina<br>(KF218606) |
| # 2 clone 5<br>(PP067567)     | Piroplasmids | 18S<br>rRNA<br>(large<br>segment) | 1484                  | 100                    | 0           | 95.98           | <i>Rangelia vitalii</i><br>detected in a<br>dog from<br>Argentina<br>(KF218606) |
| # 25 clone 1<br>(PP067563)    | Piroplasmids | 18S<br>rRNA<br>(large<br>segment) | 1342                  | 99                     | 0           | 96.07           | <i>Rangelia vitalii</i><br>detected in a<br>dog from                            |



|                            |                           |                                   |      |     |   |       |                                                                                 |
|----------------------------|---------------------------|-----------------------------------|------|-----|---|-------|---------------------------------------------------------------------------------|
|                            |                           |                                   |      |     |   |       | Argentina<br>(KF218606)                                                         |
| # 25 clone 2<br>(PP067568) | Piropasmids               | 18S<br>rRNA<br>(large<br>segment) | 1342 | 99  | 0 | 96.14 | <i>Rangelia vitalii</i><br>detected in a<br>dog from<br>Argentina<br>(KF218606) |
| # 25 clone 4<br>(PP067564) | Piropasmids               | 18S<br>rRNA<br>(large<br>segment) | 1376 | 100 | 0 | 96.03 | <i>Rangelia vitalii</i><br>detected in a<br>dog from<br>Argentina<br>(KF218606) |
| # 25 clone 5<br>(PP067565) | Piropasmids               | 18S<br>rRNA<br>(large<br>segment) | 1377 | 100 | 0 | 95.96 | <i>Rangelia vitalii</i><br>detected in a<br>dog from<br>Argentina<br>(KF218606) |
| # 2<br>(PP357051)          | Piropasmids               | <i>hsp70</i>                      | 980  | 100 | 0 | 94.29 | <i>Rangelia vitalii</i><br>detected in a<br>dog from<br>Brazil<br>(JF279603)    |
| # 1<br>(PP082974)          | Piropasmids               | <i>cox-1</i>                      | 768  | 100 | 0 | 91.55 | <i>Babesia</i> sp.<br>Coco detected<br>in a dog from<br>EUA<br>(KC207824)       |
| # 2<br>(PP082975)          | Piropasmids               | <i>cox-1</i>                      | 773  | 100 | 0 | 91.47 | <i>Babesia</i> sp.<br>Coco detected<br>in a dog from<br>EUA<br>(KC207824)       |
| # 25<br>(PP082976)         | Piropasmids               | <i>cox-1</i>                      | 774  | 100 | 0 | 91.48 | <i>Babesia</i> sp.<br>Coco detected<br>in a dog from<br>EUA<br>(KC207824)       |
| #1<br>(PP067741)           | <i>Hepatozoon</i><br>spp. | 18S<br>rRNA<br>(short<br>segment) | 700  | 100 | 0 | 100   | <i>Hepatozoon</i> sp.<br>detected in <i>C.</i><br><i>thous</i> from             |

|                   |                           |                                   |       |     |   |       |                                                                                             |
|-------------------|---------------------------|-----------------------------------|-------|-----|---|-------|---------------------------------------------------------------------------------------------|
|                   |                           |                                   |       |     |   |       | Brazil<br>(KX776327)                                                                        |
| #2<br>(PP067742)  | <i>Hepatozoon</i><br>spp. | 18S<br>rRNA<br>(short<br>segment) | 794   | 97  | 0 | 99.87 | <i>Hepatozoon</i> sp.<br>detected in <i>C.</i><br><i>thous</i> from<br>Brazil<br>(KX776291) |
| #10<br>(PP067743) | <i>Hepatozoon</i><br>spp. | 18S<br>rRNA<br>(short<br>segment) | 767   | 100 | 0 | 100   | <i>Hepatozoon</i> sp.<br>detected in <i>C.</i><br><i>thous</i> from<br>Brazil<br>(KT881529) |
| #17<br>(PP067744) | <i>Hepatozoon</i><br>spp. | 18S<br>rRNA<br>(short<br>segment) | 721   | 100 | 0 | 100   | <i>Hepatozoon</i> sp.<br>detected in <i>C.</i><br><i>thous</i> from<br>Brazil<br>(KX776328) |
| #19<br>(PP067740) | <i>Hepatozoon</i><br>spp. | 18S<br>rRNA<br>(short<br>segment) | 616   | 100 | 0 | 100   | <i>Hepatozoon</i> sp.<br>detected in <i>C.</i><br><i>thous</i> from<br>Brazil<br>(KX776327) |
| #24<br>(PP067745) | <i>Hepatozoon</i><br>spp. | 18S<br>rRNA<br>(short<br>segment) | 760   | 100 | 0 | 100   | <i>Hepatozoon</i> sp.<br>detected in <i>C.</i><br><i>thous</i> from<br>Brazil<br>(KX776301) |
| #25<br>(PP067746) | <i>Hepatozoon</i><br>spp. | 18S<br>rRNA<br>(short<br>segment) | 766   | 100 | 0 | 100   | <i>Hepatozoon</i> sp.<br>detected in <i>C.</i><br><i>thous</i> from<br>Brazil<br>(KX776327) |
| #26<br>(PP067747) | <i>Hepatozoon</i><br>spp. | 18S<br>rRNA<br>(short<br>segment) | 816   | 100 | 0 | 100   | <i>Hepatozoon</i> sp.<br>detected in <i>C.</i><br><i>thous</i> from<br>Brazil<br>(KT881530) |
| #24<br>(PP067748) | <i>Hepatozoon</i><br>spp. | 18S<br>rRNA<br>(large<br>segment) | 1,276 | 100 | 0 | 98.98 | <i>Hepatozoon</i> sp.<br>detected in dog<br>from Uruguay<br>(OR814214)                      |

|                   |                           |                                   |       |     |   |       |                                                                                                           |
|-------------------|---------------------------|-----------------------------------|-------|-----|---|-------|-----------------------------------------------------------------------------------------------------------|
| #25<br>(PP067749) | <i>Hepatozoon</i><br>spp. | 18S<br>rRNA<br>(large<br>segment) | 1,295 | 100 | 0 | 98.92 | <i>Hepatozoon</i> sp.<br>detected in dog<br>from Uruguay<br>(OR814214)                                    |
| #26<br>(PP067750) | <i>Hepatozoon</i><br>spp. | 18S<br>rRNA<br>(large<br>segment) | 1,588 | 99  | 0 | 97.66 | <i>Hepatozoon</i> sp.<br>Curupira 2<br>detected in <i>C.</i><br><i>thous</i> from<br>Brazil<br>(AY461377) |

|                                                       |          |          |          |
|-------------------------------------------------------|----------|----------|----------|
|                                                       | PP357051 | ON169952 | JF279603 |
| <b>PP357051 <i>Babesia pantanalensis</i> nov. sp.</b> |          |          |          |
| ON169952 <i>Rangelia vitalii</i>                      | 6.20%    |          |          |
| JF279603 <i>Rangelia vitalii</i>                      | 6.19%    | 0.37%    |          |

**Supplementary material 3.** A heatmap constructed using the distance matrix based on *p*-value between *hsp70* sequence detected in *Cerdocyon thous* and *hsp70* of *Rangelia vitalii*. The number represents the percentage of divergence between the sequences.

|                                                |          |                         |          |          |          |          |
|------------------------------------------------|----------|-------------------------|----------|----------|----------|----------|
|                                                | KR017881 | <i>Rangelia vitalii</i> | PP082974 | PP082976 | PP082975 | KC207824 |
| KR017881 <i>Babesia</i> sp.                    |          |                         |          |          |          |          |
| <i>Rangelia vitalii</i>                        | 19.27%   |                         |          |          |          |          |
| PP082974 <i>Babesia pantanalensis</i> nov. sp. | 20.14%   | 4.91%                   |          |          |          |          |
| PP082976 <i>Babesia pantanalensis</i> nov. sp. | 20.35%   | 5.02%                   | 0.00%    |          |          |          |
| PP082975 <i>Babesia pantanalensis</i> nov. sp. | 20.35%   | 5.02%                   | 0.00%    | 0.00%    |          |          |
| KC207824 <i>Babesia</i> sp. Coco               | 20.00%   | 9.52%                   | 9.17%    | 9.24%    | 9.25%    |          |

**Supplementary material 4.** A heatmap constructed using the distance matrix based on *p*-value between *Babesia* spp. *cox-1* sequences detected in *Cerdocyon thous* and the phylogenetically related sequences. The numbers represent the percentage of divergence between the sequences.

APÊNDICE C – Material suplementar citado no Capítulo 4

Genetic diversity of *Theileria* spp. in deer (Artiodactyla: Cervidae) from Brazil\*

\*Material correspondente ao artigo científico publicado na revista Parasitology Research 123:384, 2024. <https://doi.org/10.1007/s00436-024-08398-5>

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**Supplementary material 1.** BLASTn results of the sequences obtained in the amplification of target genes for piroplasmids from deer buffy coat samples from Brazil.

| Species/ID*                                     | Brazilian State | Gene                          | Length (bp) | Query-cover (%) | E-value | Identity (%) | GenBank sequence                                                                 |
|-------------------------------------------------|-----------------|-------------------------------|-------------|-----------------|---------|--------------|----------------------------------------------------------------------------------|
| <i>Blastocerus dichotomus</i> / 24 MS01         | MS              | 18S rRNA (~800 bp fragment)   | 739         | 100             | 0       | 99.59        | <i>Theileria</i> sp. detected in a cat from Brazil (KP402163)                    |
| <i>Blastocerus dichotomus</i> / 49 MS01         | MS              | 18S rRNA (~800 bp fragment)   | 392         | 100             | 0       | 100          | <i>Theileria</i> sp. detected in a cat from Brazil (KP402164)                    |
| <i>Subulo gouazoubira</i> / 209                 | MS              | 18S rRNA (~800 bp fragment)   | 854         | 100             | 0       | 99.30        | <i>Theileria cervi</i> detected in <i>Dermacentor nitens</i> from USA (MW008531) |
| <i>Blastocerus dichotomus</i> / 63 clone 2 MS01 | MS              | 18S rRNA (~1,500 bp fragment) | 1578        | 99              | 0       | 99.43        | <i>Theileria cervi</i> detected in <i>Dermacentor nitens</i> from USA (MW008528) |
| <i>Blastocerus dichotomus</i> / 63 clone 4 MS01 | MS              | 18S rRNA (~1,500 bp fragment) | 1489        | 100             | 0       | 99.46        | <i>Theileria cervi</i> detected in <i>Dermacentor</i>                            |

|                                                            |    |                                        |      |     |   |       |                                                                                               |
|------------------------------------------------------------|----|----------------------------------------|------|-----|---|-------|-----------------------------------------------------------------------------------------------|
|                                                            |    |                                        |      |     |   |       | <i>nitens</i> from USA<br>(MW008528)                                                          |
| <i>Blastocerus<br/>dichotomus</i> /<br>75 clone 3<br>MS01  | MS | 18S rRNA<br>(~1,500<br>bp<br>fragment) | 1387 | 100 | 0 | 99.21 | <i>Theileria cervi</i><br>detected in<br><i>Dermacentor<br/>nitens</i> from USA<br>(MW008528) |
| <i>Blastocerus<br/>dichotomus</i> /<br>75 clone 6<br>MS01  | MS | 18S rRNA<br>(~1,500<br>bp<br>fragment) | 1209 | 100 | 0 | 99.42 | <i>Theileria cervi</i><br>detected in<br><i>Dermacentor<br/>nitens</i> from USA<br>(MW008528) |
| <i>Blastocerus<br/>dichotomus</i> /<br>80 clone 4<br>MS01  | MS | 18S rRNA<br>(~1,500<br>bp<br>fragment) | 1581 | 99  | 0 | 98.17 | <i>Theileria cervi</i><br>detected in<br><i>Dermacentor<br/>nitens</i> from USA<br>(MW008528) |
| <i>Blastocerus<br/>dichotomus</i> /<br>80 clone 17<br>MS01 | MS | 18S rRNA<br>(~1,500<br>bp<br>fragment) | 1578 | 99  | 0 | 99.43 | <i>Theileria cervi</i><br>detected in<br><i>Dermacentor<br/>nitens</i> from USA<br>(MW008528) |
| <i>Blastocerus<br/>dichotomus</i> /<br>80 clone 19<br>MS01 | MS | 18S rRNA<br>(~1,500<br>bp<br>fragment) | 1128 | 100 | 0 | 97.35 | <i>Theileria cervi</i><br>detected in<br><i>Dermacentor<br/>nitens</i> from USA<br>(MW008528) |
| <i>Blastocerus<br/>dichotomus</i> /<br>120 clone 2<br>PX01 | SP | 18S rRNA<br>(~1,500<br>bp<br>fragment) | 1169 | 100 | 0 | 98.38 | <i>Theileria cervi</i><br>detected in<br><i>Dermacentor<br/>nitens</i> from USA<br>(MW008528) |
| <i>Blastocerus<br/>dichotomus</i> /<br>121 clone 4<br>PX01 | SP | 18S rRNA<br>(~1,500<br>bp<br>fragment) | 1163 | 100 | 0 | 99.23 | <i>Theileria cervi</i><br>detected in<br><i>Dermacentor<br/>nitens</i> from USA<br>(MW008528) |
| <i>Blastocerus<br/>dichotomus</i> /<br>222 clone 6<br>MS02 | MS | 18S<br>rRNA<br>(~1,500                 | 1578 | 99  | 0 | 99.43 | <i>Theileria cervi</i><br>detected in<br><i>Dermacentor</i>                                   |

|                                                             |    | bp<br>fragment)                        |      |     |   |       | <i>nitens</i> from USA<br>(MW008528)                                                                  |
|-------------------------------------------------------------|----|----------------------------------------|------|-----|---|-------|-------------------------------------------------------------------------------------------------------|
| <i>Blastocerus<br/>dichotomus</i> /<br>226 clone 3<br>MS02  | MS | 18S rRNA<br>(~1,500<br>bp<br>fragment) | 1379 | 100 | 0 | 98.33 | <i>Theileria cervi</i><br>detected in<br><i>Dermacentor<br/>nitens</i> from USA<br>(MW008528)         |
| <i>Blastocerus<br/>dichotomus</i> /<br>226 clone 10<br>MS02 | MS | 18S rRNA<br>(~1,500<br>bp<br>fragment) | 1159 | 100 | 0 | 99.31 | <i>Theileria cervi</i><br>detected in<br><i>Dermacentor<br/>nitens</i> from USA<br>(MW008528)         |
| <i>Blastocerus<br/>dichotomus</i> /<br>240 clone 2<br>Água  | SP | 18S rRNA<br>(~1,500<br>bp<br>fragment) | 1381 | 100 | 0 | 99.28 | <i>Theileria cervi</i><br>detected in<br><i>Dermacentor<br/>nitens</i> from USA<br>(MW008528)         |
| <i>Subulo<br/>gouazoubira</i><br>182 clone 2                | MS | 18S rRNA<br>(~1,500<br>bp<br>fragment) | 1587 | 100 | 0 | 99.17 | <i>Theileria cervi</i><br>detected in <i>Cervus<br/>elaphus<br/>canadensis</i> from<br>USA (AY735129) |
| <i>Subulo<br/>gouazoubira</i><br>182 clone 6                | MS | 18S rRNA<br>(~1,500<br>bp<br>fragment) | 1594 | 100 | 0 | 99.29 | <i>Theileria cervi</i><br>detected in <i>Cervus<br/>elaphus<br/>canadensis</i> from<br>USA (AY735129) |
| <i>Mazama<br/>bororo</i> / 215<br>clone 3                   | SP | 18S rRNA<br>(~1,500<br>bp<br>fragment) | 1592 | 100 | 0 | 99.53 | <i>Theileria cervi</i><br>detected in <i>Cervus<br/>elaphus<br/>canadensis</i> from<br>USA (AY735129) |
| <i>Mazama<br/>bororo</i> / 215<br>clone 5                   | SP | 18S rRNA<br>(~1,500<br>bp<br>fragment) | 1590 | 100 | 0 | 99.05 | <i>Theileria</i> sp.<br>detected in<br><i>Odocoileus<br/>virginianus</i> from<br>USA (AY735132)       |
| <i>Mazama<br/>bororo</i> / 215<br>clone 6                   | SP | 18S rRNA<br>(~1,500<br>bp<br>fragment) | 1596 | 100 | 0 | 98.35 | <i>Theileria cervi</i><br>detected in <i>Cervus<br/>elaphus</i>                                       |

|                                                 |    |                               |      |     |                      |       |                                                                                         |
|-------------------------------------------------|----|-------------------------------|------|-----|----------------------|-------|-----------------------------------------------------------------------------------------|
|                                                 |    |                               |      |     |                      |       | <i>canadensis</i> from USA (AY735129)                                                   |
| <i>Mazama americana</i> / 270 clone 1           | PR | 18S rRNA (~1,500 bp fragment) | 1585 | 100 | 0                    | 99.64 | <i>Theileria cervi</i> detected in <i>Cervus elaphus canadensis</i> from USA (AY735129) |
| <i>Mazama americana</i> / 270 clone 2           | PR | 18S rRNA (~1,500 bp fragment) | 1579 | 100 | 0                    | 99.58 | <i>Theileria cervi</i> detected in <i>Cervus elaphus canadensis</i> from USA (AY735129) |
| <i>Mazama americana</i> / 270 clone 4           | PR | 18S rRNA (~1,500 bp fragment) | 1581 | 100 | 0                    | 99.46 | <i>Theileria cervi</i> detected in <i>Cervus elaphus canadensis</i> from USA (AY735129) |
| <i>Subulo gouazoubira</i> / 299 clone 2         | MS | 18S rRNA (~1,500 bp fragment) | 1586 | 100 | 0                    | 99.11 | <i>Theileria</i> sp. detected in <i>Odocoileus virginianus</i> from USA (AY735132)      |
| <i>Blastocerus dichotomus</i> / 28 clone 1 MS01 | MS | <i>hsp70</i>                  | 650  | 99  | 0                    | 85.36 | <i>Theileria orientalis</i> detected in cattle from Japan (XM_009691871)                |
| <i>Blastocerus dichotomus</i> / 28 clone 3 MS01 | MS | <i>hsp70</i>                  | 644  | 99  | 0                    | 85.60 | <i>Theileria orientalis</i> detected in cattle from Japan (XM_009691871)                |
| <i>Blastocerus dichotomus</i> / 75 clone 3 MS01 | MS | <i>hsp70</i>                  | 606  | 99  | $3 \times 10^{-180}$ | 85.95 | <i>Theileria orientalis</i> detected in cattle from Japan (XM_009691871)                |
| <i>Blastocerus dichotomus</i> / 75 clone 3 MS01 | MS | <i>hsp70</i>                  | 648  | 99  | 0                    | 85.85 | <i>Theileria orientalis</i> detected in cattle from Japan (XM_009691871)                |

|                                                            |    |              |     |     |                      |       |                                                                                                         |
|------------------------------------------------------------|----|--------------|-----|-----|----------------------|-------|---------------------------------------------------------------------------------------------------------|
| <i>Blastocerus<br/>dichotomus</i> /<br>80 clone 4<br>MS01  | MS | <i>hsp70</i> | 650 | 98  | 0                    | 85.69 | <i>Theileria orientalis</i><br>detected in cattle<br>from Japan<br>(XM_009691871)                       |
| <i>Blastocerus<br/>dichotomus</i> /<br>108 clone1<br>MS01  | MS | <i>hsp70</i> | 647 | 99  | 0                    | 85.69 | <i>Theileria orientalis</i><br>detected in cattle<br>from Japan<br>(XM_009691871)                       |
| <i>Blastocerus<br/>dichotomus</i> /<br>108 clone 2<br>MS01 | MS | <i>hsp70</i> | 648 | 99  | 0                    | 85.38 | <i>Theileria orientalis</i><br>detected in cattle<br>from Japan<br>(XM_009691871)                       |
| <i>Subulo<br/>gouazoubira</i> /<br>182                     | MS | <i>hsp70</i> | 561 | 100 | $2 \times 10^{141}$  | 83.27 | <i>Theileria cervi</i><br>detected in<br><i>Odocoileus</i><br><i>virginianus</i> from<br>USA (AB248748) |
| <i>Mazama<br/>bororo</i> / 204                             | SP | <i>hsp70</i> | 599 | 72  | $1 \times 10^{123}$  | 85.65 | <i>Theileria</i> sp.<br>detected in sheep<br>from China<br>(DQ278176)                                   |
| <i>Mazama<br/>bororo</i> / 215                             | SP | <i>hsp70</i> | 620 | 73  | $5 \times 10^{-133}$ | 86.06 | <i>Theileria</i> sp.<br>detected in sheep<br>from China<br>(DQ278176)                                   |
| <i>Mazama<br/>americana</i> /<br>267                       | PR | <i>hsp70</i> | 543 | 68  | $4 \times 10^{-104}$ | 85.83 | <i>Theileria</i> sp.<br>detected in sheep<br>from China<br>(DQ278176)                                   |
| <i>Subulo<br/>gouazoubira</i> /<br>299                     | MS | <i>hsp70</i> | 627 | 99  | $1 \times 10^{-169}$ | 84.37 | <i>Theileria cervi</i><br>detected in<br><i>Odocoileus</i><br><i>virginianus</i> from<br>USA (AB248748) |
| <i>Blastocerus<br/>dichotomus</i> /<br>28 clone 2<br>MS01  | MS | <i>cox-3</i> | 643 | 98  | $6 \times 10^{-118}$ | 77.59 | <i>Theileria velifera</i><br>detected in China<br>(NC_070080)                                           |
| <i>Blastocerus<br/>dichotomus</i> /<br>28 clone 7<br>MS01  | MS | <i>cox-3</i> | 650 | 84  | $1 \times 10^{-49}$  | 73.78 | <i>Theileria annulata</i><br>detected in cattle<br>from Algeria<br>(ON239748)                           |



|                                                  |    |              |     |    |                      |       |                                                                      |
|--------------------------------------------------|----|--------------|-----|----|----------------------|-------|----------------------------------------------------------------------|
| <i>Blastocerus dichotomus</i> / 63 clone 1 MS01  | MS | <i>cox-3</i> | 647 | 84 | $1 \times 10^{-47}$  | 73.60 | <i>Theileria annulata</i> detected in cattle from Algeria (ON239748) |
| <i>Blastocerus dichotomus</i> / 63 clone 6 MS01  | MS | <i>cox-3</i> | 647 | 99 | $7 \times 10^{-104}$ | 77.76 | <i>Theileria velifera</i> detected in China (NC_070080)              |
| <i>Blastocerus dichotomus</i> / 63 clone 12 MS01 | MS | <i>cox-3</i> | 647 | 84 | $1 \times 10^{-47}$  | 73.60 | <i>Theileria annulata</i> detected in cattle from Algeria (ON239748) |
| <i>Blastocerus dichotomus</i> / 75 clone 2 MS01  | MS | <i>cox-3</i> | 646 | 84 | $2 \times 10^{-49}$  | 73.78 | <i>Theileria annulata</i> detected in cattle from Algeria (ON239748) |
| <i>Blastocerus dichotomus</i> / 75 clone 3 MS01  | MS | <i>cox-3</i> | 650 | 83 | $4 \times 10^{-62}$  | 75.45 | <i>Theileria orientalis</i> detected in cattle from India (OR066189) |
| <i>Blastocerus dichotomus</i> / 75 clone 4 MS01  | MS | <i>cox-3</i> | 646 | 84 | $2 \times 10^{-49}$  | 73.78 | <i>Theileria annulata</i> detected in cattle from Algeria (ON239748) |
| <i>Blastocerus dichotomus</i> / 121 clone 1 PX01 | SP | <i>cox-3</i> | 647 | 84 | $2 \times 10^{-49}$  | 73.72 | <i>Theileria annulata</i> detected in cattle from Algeria (ON239748) |
| <i>Blastocerus dichotomus</i> / 121 clone 2 PX01 | SP | <i>cox-3</i> | 647 | 84 | $1 \times 10^{-47}$  | 73.60 | <i>Theileria annulata</i> detected in cattle from Algeria (ON239748) |
| <i>Blastocerus dichotomus</i> / 121 clone 6 PX01 | SP | <i>cox-3</i> | 572 | 90 | $3 \times 10^{-42}$  | 73.41 | <i>Theileria annulata</i> detected in cattle from Algeria (ON239748) |

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## APÊNDICE D – Material suplementar citado no Capítulo 5

### Diversity of *Cytauxzoon* spp. (Piroplasmida: Theileriidae) in Wild Felids from Brazil and Argentina\*

\* Material correspondente ao artigo científico publicado na revista Pathogens 14:148, 2025. <https://doi.org/10.3390/pathogens14020148>

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**Table S1:** Description of the locality, origin, and institutions responsible for sample collection.

| Specie                     | Sample ID | Locality    | Origin       | Intitution responsible for collection |
|----------------------------|-----------|-------------|--------------|---------------------------------------|
| <i>Leopardus pardalis</i>  | L5        | Miranda-MS  | Free-ranging | UFPB                                  |
|                            | L6        | Miranda-MS  | Free-ranging | UFPB                                  |
|                            | L7        | Miranda-MS  | Free-ranging | UFPB                                  |
|                            | L8        | Miranda-MS  | Free-ranging | UFPB                                  |
|                            | L9        | Miranda-MS  | Free-ranging | UFPB                                  |
|                            | L11       | Miranda-MS  | Free-ranging | UFPB                                  |
|                            | L14       | Miranda-MS  | Free-ranging | UFPB                                  |
|                            | L16       | Miranda-MS  | Free-ranging | UFPB                                  |
|                            | L18       | Miranda-MS  | Free-ranging | UFPB                                  |
|                            | L20       | Miranda-MS  | Free-ranging | UFPB                                  |
|                            | L21       | Miranda-MS  | Free-ranging | UFPB                                  |
|                            | L22       | Miranda-MS  | Free-ranging | UFPB                                  |
|                            | L23       | Miranda-MS  | Free-ranging | UFPB                                  |
| <i>Leopardus braccatus</i> | 18        | Mineiros-GO | Captive      | UNESP-FCAV                            |
| <i>Panthera onca</i>       | 2         | Mineiros-GO | Captive      | UNESP-FCAV                            |
|                            | 5         | Mineiros-GO | Captive      | UNESP-FCAV                            |
|                            | 4         | Mineiros-GO | Captive      | UNESP-FCAV                            |
|                            | 7         | Mineiros-GO | Captive      | UNESP-FCAV                            |
|                            | 9         | Mineiros-GO | Captive      | UNESP-FCAV                            |
|                            | 12        | Mineiros-GO | Captive      | UNESP-FCAV                            |

|          |                                          |              |                                                                       |
|----------|------------------------------------------|--------------|-----------------------------------------------------------------------|
| 14       | Mineiros-GO                              | Captive      | UNESP-FCAV                                                            |
| 15       | Mineiros-GO                              | Captive      | UNESP-FCAV                                                            |
| 16       | Mineiros-GO                              | Captive      | UNESP-FCAV                                                            |
| 17       | Mineiros-GO                              | Captive      | UNESP-FCAV                                                            |
| 19       | Mineiros-GO                              | Captive      | UNESP-FCAV                                                            |
| 20       | Mineiros-GO                              | Captive      | UNESP-FCAV                                                            |
| 21       | Mineiros-GO                              | Captive      | UNESP-FCAV                                                            |
| 22       | Mineiros-GO                              | Captive      | UNESP-FCAV                                                            |
| 23       | Mineiros-GO                              | Captive      | UNESP-FCAV                                                            |
| bPon351  | Cocos-BA                                 | Free-ranging | UFRGS                                                                 |
| bPon292  | Aquidauna-MS                             | Free-ranging | UFRGS                                                                 |
| bPon325  | Miranda-MS                               | Free-ranging | UFRGS                                                                 |
| bPon355  | Miranda-MS                               | Free-ranging | UFRGS                                                                 |
| bPon394  | MG                                       | Free-ranging | UFRGS                                                                 |
| bPon321  | Miranda-MS                               | Free-ranging | UFRGS                                                                 |
| bPon412  | Miranda-MS                               | Free-ranging | UFRGS                                                                 |
| bPon410  | Miranda-MS                               | Free-ranging | UFRGS                                                                 |
| bPon350  | Miranda-MS                               | Free-ranging | UFRGS                                                                 |
| bPon 172 | Parque Nacional<br>Iguazu,<br>Argentina  | Free-ranging | Augustin Paviolo                                                      |
| bPon 177 | Reserva Natural<br>Vale, Linhares-<br>ES | Free-ranging | Ana Carolina<br>Srbek-Araújo                                          |
| bPon 180 | Taquarussu-MS                            | Captive      | Refúgio Biológico<br>Bela Vista, Itaipu<br>Binacional/Pedro<br>Telles |
| bPon 181 | Mineiros-GO                              | Captive      | Refúgio Biológico<br>Bela Vista, Itaipu<br>Binacional/Pedro<br>Telles |
| bPon 188 | Alvorada-TO                              | Free-ranging | Naturatins/Angélica<br>Beatriz Corrêa<br>Gonçalves                    |
| bPon 224 | Atalaia do Norte-<br>AM                  | Captive      | Associação Mata<br>Ciliar/ Cristina<br>Adania                         |
| bPon 229 | Sorriso-MT                               | Free-ranging | NEX no Extinction                                                     |
| bPon 230 | Falcão-MA                                | Free-ranging | NEX no Extinction                                                     |
| bPon 231 | Poconé-MS                                | Free-ranging | NEX no Extinction                                                     |
| bPon 234 | Guarantã do<br>Norte-MT                  | Free-ranging | NEX no Extinction                                                     |
| bPon 255 | Corrientes -<br>Argentina                | Captive      | Fundación<br>Rewilding<br>Argentina/<br>Sebastián Di<br>Martino       |
| bPon 377 | Rondon do Pará-<br>PA                    | Free-ranging | CENAP/ICMBio                                                          |
| bPon 378 | Rondon do Pará-<br>PA                    | Free-ranging | CENAP/ICMBio                                                          |
| bPon 395 | Parque Nacional<br>do Iguaçu-PR          | Free-ranging | CENAP/ICMBio                                                          |
| bPon 407 | Alta Floresta-MT                         | Captive      | CENAP/ICMBio                                                          |

|                      |          |                              |              |                                           |
|----------------------|----------|------------------------------|--------------|-------------------------------------------|
|                      | bPon 411 | Cocos-BA                     | Free-ranging | Instituto Biotropicos/Edsel Amorim Junior |
|                      | bPon 461 | Coronel José Dias-PI         | Free-ranging | CENAP/ICMBio                              |
|                      | bPon 502 | Mimoso de Goiás-GO           | Free-ranging | NEX no Extinction                         |
|                      | bPon 514 | Parque Nacional do Iguaçu-PR | Free-ranging | Projeto Onças do Iguaçu/Yara Barros       |
| <i>Puma concolor</i> | 1        | Miranda-MS                   | Captive      | UNESP-FCAV                                |
|                      | 5        | Miranda-MS                   | Captive      | UNESP-FCAV                                |
|                      | 6        | Miranda-MS                   | Captive      | UNESP-FCAV                                |
|                      | 8        | Miranda-MS                   | Captive      | UNESP-FCAV                                |
|                      | 10       | Miranda-MS                   | Captive      | UNESP-FCAV                                |
|                      | 11       | Miranda-MS                   | Captive      | UNESP-FCAV                                |
|                      | 13       | Miranda-MS                   | Captive      | UNESP-FCAV                                |
|                      | 24       | Miranda-MS                   | Captive      | UNESP-FCAV                                |
|                      | 25       | Miranda-MS                   | Captive      | UNESP-FCAV                                |

**Table S2.** Description of primers, amplicon sizes and thermal conditions used in conventional and nested PCR assays for detection and characterization of *Cytauxzoon* spp.

| Agent                              | Gene/ name primer | Primers sequences            | Size (bp) | Thermal sequences        | Reference |
|------------------------------------|-------------------|------------------------------|-----------|--------------------------|-----------|
| Endogenous gene                    | <i>gapdh</i>      | 5'- TCCAACACCACCACTGAGATCGG  | 400       | 95°C for 5 min;          | [44]      |
|                                    | -GAPDHF           | AC -3'                       |           | 35 cycles: 95°C for 15s, |           |
|                                    | -GAPDHR           | 5'- GTGAGAAGAAATCGGACTGGCC-  |           | 50°C for 30s and 72°C    |           |
|                                    |                   | 3'                           |           | for 30s;                 |           |
|                                    |                   |                              |           | 72°C for 5 min           |           |
| <i>Cytauxzoon</i> spp. (screening) | 18S rRNA          | 5'-GCGAATCGCATTGCTTTATGCT-   | 300       | 95°C for 5 min;          | [45]      |
|                                    | -CytF             | 3'                           |           | 40 cycles: 95°C for 45s, |           |
|                                    | -CytR             | 5'- CCAAATGATACTCCGAAAGAG-3' |           | 59°C for 45s and 72°C    |           |
|                                    |                   |                              |           | for 1 min;               |           |
|                                    |                   |                              |           | 72°C for 5 min           |           |
| Piropasmids (characterization)     | 18S rRNA          | 5'- AAGCCATGCATGTCTAAGTATAA  | 1500      | 95°C for 5 min;          | [48]      |
|                                    | -Nbab_1F          | GCTTTT-3'                    |           | 50 cycles: 95°C for 30s, |           |
|                                    | -18SApiR          | 5'-GGATCACTCGATCGGTAGGAG-    |           | 60°C for 30s and 72°C    |           |
|                                    |                   | 3'                           |           | for 2min;                |           |
|                                    |                   |                              |           | 72°C for 5 min           |           |
| Piropasmids (characterization)     | 18S rRNA          | 5'-GGATAACCGTGC TAATTGT-3'   | 1500      | 95°C for 10 min;         | [46,47]   |
|                                    | -NBAB1Bab         | 5'-AAAGGGCAGGGA CGTAATC-3'   |           | 40 cycles: 95°C for 30s, |           |
|                                    | -Hep1615R         |                              |           | 55°C for 30s and 72°C    |           |
|                                    |                   |                              |           | for 90s;                 |           |
|                                    |                   |                              |           | 72°C for 10 min          |           |

|                                                       |                       |                             |      |  |  |  |  |  |
|-------------------------------------------------------|-----------------------|-----------------------------|------|--|--|--|--|--|
| <b>Cytauxzoon spp.</b><br>(characterization)          | <i>cytB</i>           |                             |      |  |  |  |  |  |
|                                                       | -                     |                             |      |  |  |  |  |  |
| <b>Cytauxzoon spp.</b><br>(characterization)          | Cytaux_cy             | 5'-CTTAACCCAACTCACGTACC-3'  |      |  |  |  |  |  |
|                                                       | tb_F1                 | 5'-                         |      |  |  |  |  |  |
|                                                       | -                     | GGTTAATCTTTCCTATTCCTTACG-3' | 1434 |  |  |  |  |  |
|                                                       | Cytaux_cy             |                             |      |  |  |  |  |  |
|                                                       | tb_R3                 |                             |      |  |  |  |  |  |
| <b>Cytauxzoon spp.</b><br>(characterization<br>-nPCR) | <i>cox-1</i>          | 5'- TGGYTKGCTTATTGGTTTGG-3' |      |  |  |  |  |  |
|                                                       | 1 <sup>st</sup> round | 5'- ACTTTGAACACACTGCTCG-3'  |      |  |  |  |  |  |
|                                                       | -Th-for2              |                             |      |  |  |  |  |  |
|                                                       | -Piro mt R1           | 5'- TGGYTKGCTTATTGGTTTGG-3' | 1656 |  |  |  |  |  |
|                                                       | 2 <sup>nd</sup> round | 5'-                         |      |  |  |  |  |  |
|                                                       | -Th-For2              | AATTCCCATCTCGCTATCACTTTC-3' |      |  |  |  |  |  |
|                                                       | -Cytaux 260R          |                             |      |  |  |  |  |  |
| <b>Piropasmids</b><br>(characterization)              | <i>cox-3</i>          |                             |      |  |  |  |  |  |
|                                                       | -Cox3F                | 5'-ACTGTCAGCTAAAACGTATC-3'  | 600  |  |  |  |  |  |
|                                                       | -Cox3R                | 5'-ACAGGATTAGATACCCTGG-3'   |      |  |  |  |  |  |
| <b>Piropasmids</b><br>(characterization)              | 18S – 5.8S            |                             |      |  |  |  |  |  |
|                                                       | rRNA region           | 5'-                         |      |  |  |  |  |  |
|                                                       | - Api18S              | CTCCTACCGATCGAGTGATCCGGT    |      |  |  |  |  |  |
|                                                       | rRNA-1690s            | - 3'                        |      |  |  |  |  |  |
|                                                       | -                     | 5'-                         |      |  |  |  |  |  |
|                                                       | Api5.8SrRNA           | GCTGCGTCCTTCATCGTTGTGTGA    | 600  |  |  |  |  |  |
|                                                       | -20as                 | G-3'                        |      |  |  |  |  |  |
| <b>Piropasmids</b><br>(characterization)              | 5.8S – 28S            |                             |      |  |  |  |  |  |
|                                                       | rRNA region           | 5'-                         |      |  |  |  |  |  |
|                                                       | -                     | CTCACACAACGATGAAGGACGCA     |      |  |  |  |  |  |
|                                                       | Api5.8SrRNA           | GC-3'                       |      |  |  |  |  |  |
|                                                       | -20s                  | 5'-                         |      |  |  |  |  |  |
|                                                       | -                     | CCGCTTARTTTTATGCTTAAATTCA   | 400  |  |  |  |  |  |
|                                                       | Api28SrRNA-1as        | GCGG-3'                     |      |  |  |  |  |  |

**Table S2.** BLASTn results of the sequences obtained in the amplification of five distinct molecular markers from *Cytauxzoon* spp. from wild felids blood samples.

| Wild Felines                               |           |                                    |                       |                        |             |                 | GenBank                                                   |                    |
|--------------------------------------------|-----------|------------------------------------|-----------------------|------------------------|-------------|-----------------|-----------------------------------------------------------|--------------------|
| ID<br>(GenBank<br>access<br>number)        | Locality  | Gene                               | Sequence<br>size (bp) | Query-<br>cover<br>(%) | E-<br>value | Identity<br>(%) | sequence<br>(access<br>number)                            | Genotype<br>number |
| <i>Panthera onca</i><br>#172<br>(PQ686797) | Argentina | 18S rRNA<br>(~1500 bp<br>fragment) | 1381                  | 100                    | 0           | 99.86           | <i>Cytauxzoon</i><br><i>felis</i> detected<br>in cat from | #3                 |

|                                            |                                   |                                    |      |     |   |       |                                                                                |    |
|--------------------------------------------|-----------------------------------|------------------------------------|------|-----|---|-------|--------------------------------------------------------------------------------|----|
|                                            |                                   |                                    |      |     |   |       | USA<br>(AY531524)                                                              |    |
| <i>Panthera onca</i><br>#177<br>(PQ686792) | Espírito<br>Santo-<br>Brazil      | 18S rRNA<br>(~1500 bp<br>fragment) | 1388 | 100 | 0 | 99.78 | <i>Cytauxzoon</i><br><i>felis</i> detected<br>in cat from<br>USA<br>(AY531524) | #4 |
| <i>Panthera onca</i><br>#180<br>(PQ686791) | Mato<br>Grosso do<br>Sul - Brazil | 18S rRNA<br>(~1500 bp<br>fragment) | 1381 | 100 | 0 | 99.86 | <i>Cytauxzoon</i><br><i>felis</i> detected<br>in cat from<br>USA<br>(AY531524) | #3 |
| <i>Panthera onca</i><br>#181<br>(PQ686787) | Goiás -<br>Brazil                 | 18S rRNA<br>(~1500 bp<br>fragment) | 1381 | 100 | 0 | 99.78 | <i>Cytauxzoon</i><br><i>felis</i> detected<br>in cat from<br>USA<br>(AY531524) | #4 |
| <i>Panthera onca</i><br>#229<br>(PQ686790) | Mato<br>Grosso -<br>Brazil        | 18S rRNA<br>(~1500 bp<br>fragment) | 1379 | 100 | 0 | 99.78 | <i>Cytauxzoon</i><br><i>felis</i> detected<br>in cat from<br>USA<br>(AY531524) | #4 |
| <i>Panthera onca</i><br>#231<br>(PQ686789) | Mato<br>Grosso do<br>Sul - Brazil | 18S rRNA<br>(~1500 bp<br>fragment) | 1382 | 100 | 0 | 99.78 | <i>Cytauxzoon</i><br><i>felis</i> detected<br>in cat from<br>USA<br>(AY531524) | #4 |
| <i>Panthera onca</i><br>#234<br>(PQ686788) | Mato<br>Grosso -<br>Brazil        | 18S rRNA<br>(~1500 bp<br>fragment) | 1379 | 100 | 0 | 99.78 | <i>Cytauxzoon</i><br><i>felis</i> detected<br>in cat from<br>USA<br>(AY531524) | #4 |
| <i>Panthera onca</i><br>#255<br>(PQ686798) | Argentina                         | 18S rRNA<br>(~1500 bp<br>fragment) | 1381 | 100 | 0 | 99.78 | <i>Cytauxzoon</i><br><i>felis</i> detected<br>in cat from<br>USA<br>(AY531524) | #4 |

|                                            |                             |                                 |      |     |   |       |                                                             |    |
|--------------------------------------------|-----------------------------|---------------------------------|------|-----|---|-------|-------------------------------------------------------------|----|
| <i>Panthera onca</i><br>#292<br>(PQ686786) | Mato Grosso do Sul - Brazil | 18S rRNA<br>(~1500 bp fragment) | 1380 | 100 | 0 | 99.86 | <i>Cytauxzoon felis</i> detected in cat from USA (AY531524) | #3 |
| <i>Panthera onca</i><br>#325<br>(PQ686793) | Mato Grosso do Sul - Brazil | 18S rRNA<br>(~1500 bp fragment) | 1379 | 100 | 0 | 99.78 | <i>Cytauxzoon felis</i> detected in cat from USA (AY531524) | #4 |
| <i>Panthera onca</i><br>#351<br>(PQ686785) | Bahia - Brazil              | 18S rRNA<br>(~1500 bp fragment) | 1392 | 100 | 0 | 99.78 | <i>Cytauxzoon felis</i> detected in cat from USA (AY531524) | #4 |
| <i>Panthera onca</i><br>#355<br>(PQ686799) | Mato Grosso do Sul - Brazil | 18S rRNA<br>(~1500 bp fragment) | 1381 | 100 | 0 | 99.78 | <i>Cytauxzoon felis</i> detected in cat from USA (AY531524) | #4 |
| <i>Panthera onca</i><br>#377<br>(PQ686794) | Para - Brazil               | 18S rRNA<br>(~1500 bp fragment) | 1380 | 100 | 0 | 99.78 | <i>Cytauxzoon felis</i> detected in cat from USA (AY531524) | #4 |
| <i>Panthera onca</i><br>#378<br>(PQ686795) | Para - Brazil               | 18S rRNA<br>(~1500 bp fragment) | 1380 | 100 | 0 | 99.86 | <i>Cytauxzoon felis</i> detected in cat from USA (AY531524) | #3 |
| <i>Panthera onca</i><br>#514<br>(PQ686796) | Paraná - Brazil             | 18S rRNA<br>(~1500 bp fragment) | 1389 | 100 | 0 | 99.78 | <i>Cytauxzoon felis</i> detected in cat from USA (AY531524) | #4 |
| <i>Panthera onca</i><br>#2<br>(PQ686800)   | Goiás - Brazil              | 18S rRNA<br>(~1500 bp fragment) | 1465 | 100 | 0 | 99.86 | <i>Cytauxzoon felis</i> detected in cat from USA (AF399930) | #3 |

|                                                                  |                                   |                                    |      |     |   |       |                                                                                                |    |
|------------------------------------------------------------------|-----------------------------------|------------------------------------|------|-----|---|-------|------------------------------------------------------------------------------------------------|----|
| <i>Panthera onca</i><br>#3<br>(PQ686801)                         | Goiás -<br>Brazil                 | 18S rRNA<br>(~1500 bp<br>fragment) | 1466 | 100 | 0 | 99.80 | <i>Cytauxzoon</i><br><i>felis</i> detected<br>in cat from<br>USA<br>(AF399930)                 | #4 |
| <i>Panthera onca</i><br>#7<br>(PQ686802)                         | Goiás -<br>Brazil                 | 18S rRNA<br>(~1500 bp<br>fragment) | 1466 | 100 | 0 | 99.80 | <i>Cytauxzoon</i><br><i>felis</i> detected<br>in cat from<br>USA<br>(AF399930)                 | #4 |
| <i>Leopardus</i><br><i>pardalis</i> #8<br>clone 1<br>(PQ686803)  | Mato<br>Grosso do<br>Sul - Brazil | 18S rRNA<br>(~1500 bp<br>fragment) | 1578 | 99  | 0 | 99.94 | <i>Cytauxzoon</i><br><i>brasiliensis</i><br>detected in<br>ocelot from<br>Brazil<br>(GU903911) | #1 |
| <i>Leopardus</i><br><i>pardalis</i> #8<br>clone 3<br>(PQ686804)  | Mato<br>Grosso do<br>Sul - Brazil | 18S rRNA<br>(~1500 bp<br>fragment) | 1578 | 99  | 0 | 99.87 | <i>Cytauxzoon</i><br><i>brasiliensis</i><br>detected in<br>ocelot from<br>Brazil<br>(GU903911) | #1 |
| <i>Leopardus</i><br><i>pardalis</i> #8<br>clone 4<br>(PQ686805)  | Mato<br>Grosso do<br>Sul - Brazil | 18S rRNA<br>(~1500 bp<br>fragment) | 1578 | 99  | 0 | 99.87 | <i>Cytauxzoon</i><br><i>brasiliensis</i><br>detected in<br>ocelot from<br>Brazil<br>(GU903911) | #1 |
| <i>Leopardus</i><br><i>pardalis</i> #18<br>clone 1<br>(PQ686806) | Mato<br>Grosso do<br>Sul - Brazil | 18S rRNA<br>(~1500 bp<br>fragment) | 1545 | 100 | 0 | 99.87 | <i>Cytauxzoon</i><br><i>brasiliensis</i><br>detected in<br>ocelot from<br>Brazil<br>(GU903911) | #2 |
| <i>Leopardus</i><br><i>pardalis</i> #18<br>clone 2<br>(PQ686807) | Mato<br>Grosso do<br>Sul - Brazil | 18S rRNA<br>(~1500 bp<br>fragment) | 1572 | 99  | 0 | 99.87 | <i>Cytauxzoon</i><br><i>brasiliensis</i><br>detected in<br>ocelot from                         | #2 |



|                                                  |                             |                              |      |     |   |       |                                                                          |    |
|--------------------------------------------------|-----------------------------|------------------------------|------|-----|---|-------|--------------------------------------------------------------------------|----|
|                                                  |                             |                              |      |     |   |       | Brazil<br>(GU903911)                                                     |    |
| <i>Leopardus pardalis</i> #18 clone 3 (PQ686808) | Mato Grosso do Sul – Brazil | 18S rRNA (~1500 bp fragment) | 1543 | 99  | 0 | 99.87 | <i>Cytauxzoon brasiliensis</i> detected in ocelot from Brazil (GU903911) | #2 |
| <i>Leopardus pardalis</i> #18 clone 6 (PQ686813) | Mato Grosso do Sul - Brazil | 18S rRNA (~1500 bp fragment) | 1545 | 100 | 0 | 99.87 | <i>Cytauxzoon brasiliensis</i> detected in ocelot from Brazil (GU903911) | #2 |
| <i>Leopardus pardalis</i> #22 clone 1 (PQ686809) | Mato Grosso do Sul - Brazil | 18S rRNA (~1500 bp fragment) | 1578 | 99  | 0 | 99.94 | <i>Cytauxzoon brasiliensis</i> detected in ocelot from Brazil (GU903911) | #1 |
| <i>Leopardus pardalis</i> #22 clone 2 (PQ686811) | Mato Grosso do Sul – Brazil | 18S rRNA (~1500 bp fragment) | 1546 | 100 | 0 | 99.94 | <i>Cytauxzoon brasiliensis</i> detected in ocelot from Brazil (GU903911) | #1 |
| <i>Leopardus pardalis</i> #22 clone 6 (PQ686810) | Mato Grosso do Sul - Brazil | 18S rRNA (~1500 bp fragment) | 1548 | 100 | 0 | 99.94 | <i>Cytauxzoon brasiliensis</i> detected in ocelot from Brazil (GU903911) | #1 |
| <i>Panthera onca</i> #172 (PQ724028)             | Argentina                   | <i>cytB</i>                  | 1046 | 100 | 0 | 99.52 | <i>Cytauxzoon felis</i> detected in cat from USA (KC207821)              | #4 |
| <i>Panthera onca</i> #177 (PQ724024)             | Espírito Santo - Brazil     | <i>cytB</i>                  | 1076 | 100 | 0 | 99.54 | <i>Cytauxzoon felis</i> detected in cat from                             | #4 |

|                                            |                                   |             |      |     |   |       |                                                                                |    |
|--------------------------------------------|-----------------------------------|-------------|------|-----|---|-------|--------------------------------------------------------------------------------|----|
|                                            |                                   |             |      |     |   |       | USA<br>(KC207821)                                                              |    |
| <i>Panthera onca</i><br>#180<br>(PQ724023) | Mato<br>Grosso do<br>Sul - Brazil | <i>cytB</i> | 1077 | 100 | 0 | 99,54 | <i>Cytauxzoon</i><br><i>felis</i> detected<br>in cat from<br>USA<br>(KC207821) | #5 |
| <i>Panthera onca</i> #181<br>(PQ724019)    | Goiás -<br>Brazil                 | <i>cytB</i> | 1075 | 100 | 0 | 99.53 | <i>Cytauxzoon</i><br><i>felis</i> detected<br>in cat from<br>USA<br>(KC207821) | #5 |
| <i>Panthera onca</i><br>#229<br>(PQ724022) | Mato<br>Grosso -<br>Brazil        | <i>cytB</i> | 1077 | 100 | 0 | 99.54 | <i>Cytauxzoon</i><br><i>felis</i> detected<br>in cat from<br>USA<br>(KC207821) | #5 |
| <i>Panthera onca</i><br>#230<br>(PQ724031) | Maranhão<br>- Brazil              | <i>cytB</i> | 1076 | 100 | 0 | 99.54 | <i>Cytauxzoon</i><br><i>felis</i> detected<br>in cat from<br>USA<br>(KC207821) | #5 |
| <i>Panthera onca</i><br>#231<br>(PQ724021) | Mato<br>Grosso do<br>Sul - Brazil | <i>cytB</i> | 1077 | 100 | 0 | 99.54 | <i>Cytauxzoon</i><br><i>felis</i> detected<br>in cat from<br>USA<br>(KC207821) | #5 |
| <i>Panthera onca</i><br>#234<br>(PQ724020) | Mato<br>Grosso -<br>Brazil        | <i>cytB</i> | 1077 | 100 | 0 | 99.54 | <i>Cytauxzoon</i><br><i>felis</i> detected<br>in cat from<br>USA<br>(KC207821) | #5 |
| <i>Panthera onca</i><br>#255<br>(PQ724029) | Argentina                         | <i>cytB</i> | 1075 | 100 | 0 | 99.53 | <i>Cytauxzoon</i><br><i>felis</i> detected<br>in cat from<br>USA<br>(KC207821) | #5 |

|                                             |                                   |             |      |     |   |       |                                                                          |    |
|---------------------------------------------|-----------------------------------|-------------|------|-----|---|-------|--------------------------------------------------------------------------|----|
| <i>Panthera onca</i><br>#292<br>(PQ724018)  | Mato<br>Grosso do<br>Sul – Brazil | <i>cytB</i> | 1075 | 100 | 0 | 99.53 | <i>Cytauxzoon<br/>felis</i> detected<br>in cat from<br>USA<br>(KC207821) | #5 |
| <i>Panthera onca</i><br>#325<br>(PQ724025)  | Mato<br>Grosso do<br>Sul - Brazil | <i>cytB</i> | 1077 | 100 | 0 | 99.54 | <i>Cytauxzoon<br/>felis</i> detected<br>in cat from<br>USA<br>(KC207821) | #5 |
| <i>Panthera onca</i><br>#351<br>(PQ724017)  | Bahia -<br>Brazil                 | <i>cytB</i> | 1077 | 100 | 0 | 99.54 | <i>Cytauxzoon<br/>felis</i> detected<br>in cat from<br>USA<br>(KC207821) | #5 |
| <i>Panthera onca</i><br># 355<br>(PQ724030) | Mato<br>Grosso do<br>Sul - Brazil | <i>cytB</i> | 1076 | 100 | 0 | 99.54 | <i>Cytauxzoon<br/>felis</i> detected<br>in cat from<br>USA<br>(KC207821) | #5 |
| <i>Panthera onca</i><br>#377<br>(PQ724037)  | Para -<br>Brazil                  | <i>cytB</i> | 1087 | 100 | 0 | 99.54 | <i>Cytauxzoon<br/>felis</i> detected<br>in cat from<br>USA<br>(KC207821) | #5 |
| <i>Panthera onca</i><br>#378<br>(PQ724026)  | Para -<br>Brazil                  | <i>cytB</i> | 1079 | 100 | 0 | 99.54 | <i>Cytauxzoon<br/>felis</i> detected<br>in cat from<br>USA<br>(KC207821) | #4 |
| <i>Panthera onca</i><br>#394<br>(PQ724032)  | Minas<br>Gerais -<br>Brazil       | <i>cytB</i> | 1076 | 100 | 0 | 99.54 | <i>Cytauxzoon<br/>felis</i> detected<br>in cat from<br>USA<br>(KC207821) | #5 |
| <i>Panthera onca</i><br>#514<br>(PQ724027)  | Paraná -<br>Brazil                | <i>cytB</i> | 1044 | 100 | 0 | 99.52 | <i>Cytauxzoon<br/>felis</i> detected<br>in cat from<br>USA<br>(KC207821) | #5 |

|                                                        |                                   |             |       |     |   |       |                                                                                                                             |    |
|--------------------------------------------------------|-----------------------------------|-------------|-------|-----|---|-------|-----------------------------------------------------------------------------------------------------------------------------|----|
| <i>Panthera onca</i><br>#7<br>(PQ724014)               | Goiás -<br>Brazil                 | <i>cytB</i> | 1077  | 100 | 0 | 99.54 | <i>Cytauxzoon</i><br><i>felis</i> detected<br>in cat from<br>USA<br>(KC207821)                                              | #4 |
| <i>Panthera onca</i><br>#9<br>(PQ724036)               | Goiás -<br>Brazil                 | <i>cytB</i> | 1131  | 100 | 0 | 99.56 | <i>Cytauxzoon</i><br><i>felis</i> detected<br>in cat from<br>USA<br>(KC207821)                                              | #5 |
| <i>Panthera onca</i><br>#20<br>(PQ724016)              | Goiás -<br>Brazil                 | <i>cytB</i> | 1075  | 100 | 0 | 99.53 | <i>Cytauxzoon</i><br><i>felis</i> detected<br>in cat from<br>USA<br>(KC207821)                                              | #5 |
| <i>Leopardus</i><br><i>pardalis</i> #5L<br>(PQ724033)  | Mato<br>Grosso do<br>Sul - Brazil | <i>cytB</i> | 1,087 | 94  | 0 | 99.03 | <i>Cytauxzoon</i><br><i>brasiliensis</i><br>detected in<br><i>Leopardus</i><br><i>tigrinus</i> from<br>Brazil<br>(PP588457) | #8 |
| <i>Leopardus</i><br><i>pardalis</i> #14L<br>(PQ724015) | Mato<br>Grosso do<br>Sul - Brazil | <i>cytB</i> | 1,077 | 95  | 0 | 99.12 | <i>Cytauxzoon</i><br><i>brasiliensis</i><br>detected in<br><i>Leopardus</i><br><i>tigrinus</i> from<br>Brazil<br>(PP588457) | #7 |
| <i>Leopardus</i><br><i>pardalis</i> #18L<br>(PQ724034) | Mato<br>Grosso do<br>Sul - Brazil | <i>cytB</i> | 1085  | 94  | 0 | 99.03 | <i>Cytauxzoon</i><br><i>brasiliensis</i><br>detected in<br><i>Leopardus</i><br><i>tigrinus</i> from<br>Brazil<br>(PP588457) | #8 |
| <i>Leopardus</i><br><i>pardalis</i> #22L<br>(PQ724035) | Mato<br>Grosso do<br>Sul - Brazil | <i>cytB</i> | 1086  | 94  | 0 | 99.12 | <i>Cytauxzoon</i><br><i>brasiliensis</i><br>detected in<br><i>Leopardus</i><br><i>tigrinus</i> from                         | #7 |

|                                            |                                   |              |      |     |   |       |                                                                                          |    |
|--------------------------------------------|-----------------------------------|--------------|------|-----|---|-------|------------------------------------------------------------------------------------------|----|
|                                            |                                   |              |      |     |   |       | Brazil<br>(PP588457)                                                                     |    |
| <i>Panthera onca</i><br>#180<br>(PQ724003) | Mato<br>Grosso do<br>Sul - Brazil | <i>cox-1</i> | 1223 | 100 | 0 | 99.92 | <i>Cytauxzoon felis</i> detected<br>in <i>Felis silvestris</i> from<br>USA<br>(MT916252) | #2 |
| <i>Panthera onca</i><br>#181<br>(PQ724001) | Goiás-<br>Brazil                  | <i>cox-1</i> | 1255 | 100 | 0 | 99.92 | <i>Cytauxzoon felis</i> detected<br>in <i>Felis silvestris</i> from<br>USA<br>(MT916252) | #2 |
| <i>Panthera onca</i><br>#172<br>(PQ724012) | Argentina                         | <i>cox-1</i> | 1221 | 100 | 0 | 99.92 | <i>Cytauxzoon felis</i> detected<br>in <i>Felis silvestris</i> from<br>USA<br>(MT916252) | #2 |
| <i>Panthera onca</i><br>#177<br>(PQ724004) | Espírito<br>Santo -<br>Brazil     | <i>cox-1</i> | 1252 | 100 | 0 | 99.44 | <i>Cytauxzoon felis</i> detected<br>in <i>Felis silvestris</i> from<br>USA<br>(MT916252) | #4 |
| <i>Panthera onca</i><br>#224<br>(PQ724006) | Amazonas<br>- Brazil              | <i>cox-1</i> | 1254 | 100 | 0 | 99.92 | <i>Cytauxzoon felis</i> detected<br>in <i>Felis silvestris</i> from<br>USA<br>(MT916252) | #2 |
| <i>Panthera onca</i><br>#231<br>(PQ724002) | Mato<br>Grosso do<br>Sul - Brazil | <i>cox-1</i> | 1254 | 100 | 0 | 99.92 | <i>Cytauxzoon felis</i> detected<br>in <i>Felis silvestris</i> from<br>USA<br>(MT916252) | #2 |

|                                            |                             |              |      |     |   |       |                                                                                          |    |
|--------------------------------------------|-----------------------------|--------------|------|-----|---|-------|------------------------------------------------------------------------------------------|----|
| <i>Panthera onca</i><br>#255<br>(PQ724013) | Argentina                   | <i>cox-1</i> | 1040 | 100 | 0 | 99.42 | <i>Cytauxzoon felis</i> detected<br>in <i>Felis silvestris</i> from<br>USA<br>(MT916252) | -  |
| <i>Panthera onca</i><br>#351<br>(PQ724000) | Bahia -<br>Brazil           | <i>cox-1</i> | 1222 | 100 | 0 | 99.92 | <i>Cytauxzoon felis</i> detected<br>in <i>Felis silvestris</i> from<br>USA<br>(MT916252) | #2 |
| <i>Panthera onca</i><br>#377<br>(PQ724005) | Pará -<br>Brazil            | <i>cox-1</i> | 1254 | 100 | 0 | 99.92 | <i>Cytauxzoon felis</i> detected<br>in <i>Felis silvestris</i> from<br>USA<br>(MT916252) | #2 |
| <i>Panthera onca</i><br>#394<br>(PQ724007) | Minas<br>Gerais -<br>Brazil | <i>cox-1</i> | 1254 | 100 | 0 | 99.92 | <i>Cytauxzoon felis</i> detected<br>in <i>Felis silvestris</i> from<br>USA<br>(MT916252) | #2 |
| <i>Panthera onca</i><br>#7<br>(PQ724011)   | Goiás -<br>Brazil           | <i>cox-1</i> | 1219 | 100 | 0 | 99.92 | <i>Cytauxzoon felis</i> detected<br>in <i>Felis silvestris</i> from<br>USA<br>(MT916252) | #4 |
| <i>Panthera onca</i><br>#9<br>(PQ724008)   | Goiás -<br>Brazil           | <i>cox-1</i> | 1222 | 100 | 0 | 99.92 | <i>Cytauxzoon felis</i> detected<br>in <i>Felis silvestris</i> from<br>USA<br>(MT916252) | #2 |
| <i>Panthera onca</i><br>#12<br>(PQ724010)  | Goiás -<br>Brazil           | <i>cox-1</i> | 1222 | 100 | 0 | 99.43 | <i>Cytauxzoon felis</i> detected<br>in <i>Felis silvestris</i> from<br>USA<br>(MT916252) | #2 |



**Table S5:** Pairwise analysis of the near-complete *cytB* sequences.

[illegible]

Para melhor visualização acessar  
<https://www.mdpi.com/article/10.3390/pathogens14020148/s1>

**Table S6:** Pairwise analysis of the near-complete *cox-1* sequences.

[illegible]

Para melhor visualização acessar  
<https://www.mdpi.com/article/10.3390/pathogens14020148/s1>



## APÊNDICE E – Material suplementar citado no Capítulo 7

### Using digital PCR to unravel the occurrence of piroplasmids, *Bartonella* spp. and *Borrelia* spp. in wild animals from Brazil

\* Material correspondente ao artigo científico publicado na revista Pathogens 14:567, 2025. <https://doi.org/10.3390/pathogens14060567>

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**Table S5:** Results obtained for each test carried out for *Bartonella* spp.

| Animal species                 | ID    | Result   | dPCR                |                   | qPCR     |       |
|--------------------------------|-------|----------|---------------------|-------------------|----------|-------|
|                                |       |          | Positive partitions | Conc. (copies/μL) | Result   | CQ    |
| <i>Subulo gouazoubira</i>      | 182M  | Negative | 0                   | 0                 | Negative | N/A   |
| <i>Mazama juncunda</i>         | 215M  | Negative | 0                   | 0                 | Negative | N/A   |
| <i>Mazama rufa</i>             | 270M  | Negative | 0                   | 0                 | Negative | N/A   |
| <i>Blastocerus dichotomus</i>  | 63B   | Positive | 1246                | 602.2             | Positive | 44.53 |
| <i>Blastocerus dichotomus</i>  | 75B   | Positive | 7                   | 2.655             | Negative | N/A   |
| <i>Blastocerus dichotomus</i>  | 120B2 | Positive | 1                   | 0.378             | Negative | N/A   |
| <i>Blastocerus dichotomus</i>  | 121B2 | Negative | 0                   | 0                 | Negative | N/A   |
| <i>Blastocerus dichotomus</i>  | 222B3 | Negative | 0                   | 0                 | Negative | N/A   |
| <i>Blastocerus dichotomus</i>  | 226B3 | Negative | 0                   | 0                 | Negative | N/A   |
| <i>Desmodus rotundus</i>       | 91MO  | Positive | 36                  | 16.25             | Negative | N/A   |
| <i>Desmodus rotundus</i>       | 8MO   | Positive | 1                   | 0.375             | Negative | N/A   |
| <i>Cerdocyon thous</i>         | 2L    | Negative | 0                   | 0                 | Negative | N/A   |
| <i>Leopardus pardalis</i>      | 8L    | Negative | 0                   | 0                 | Negative | N/A   |
| <i>Leopardus pardalis</i>      | 22L   | Negative | 0                   | 0                 | Negative | N/A   |
| <i>Cerdocyon thous</i>         | 25L   | Negative | 0                   | 0                 | Negative | N/A   |
| <i>Myrmecophaga tridactyla</i> | 59T   | Negative | 0                   | 0                 | Negative | N/A   |
| <i>Myrmecophaga tridactyla</i> | 62T   | Negative | 0                   | 0                 | Positive | 40.71 |
| <i>Tamandua tetradactyla</i>   | 71T   | Negative | 0                   | 0                 | Negative | N/A   |
| <i>Dasypus novemcinctus</i>    | 16T   | Negative | 0                   | 0                 | Negative | N/A   |
| <i>Priodontes maximus</i>      | 153T  | Negative | 0                   | 0                 | Positive | 43.95 |
| <i>Priodontes maximus</i>      | 159T  | Negative | 0                   | 0                 | Negative | N/A   |

|                                |      |          |     |       |          |       |
|--------------------------------|------|----------|-----|-------|----------|-------|
| <i>Priodontes maximus</i>      | 160T | Negative | 0   | 0     | Negative | N/A   |
| <i>Ozotocerus bezoarticus</i>  | 06O  | Negative | 0   | 0     | Negative | N/A   |
| <i>Ozotocerus bezoarticus</i>  | 07O  | Positive | 1   | 0.387 | Negative | N/A   |
| <i>Ozotocerus bezoarticus</i>  | 08O  | Positive | 1   | 0.395 | Negative | N/A   |
| <i>Ozotocerus bezoarticus</i>  | 09O  | Negative | 0   | 0     | Negative | N/A   |
| <i>Ozotocerus bezoarticus</i>  | 10O  | Positive | 1   | 0.396 | Negative | N/A   |
| <i>Subulo gouazoubira</i>      | 154M | Negative | 0   | 0     | Negative | N/A   |
| <i>Subulo gouazoubira</i>      | 155M | Negative | 0   | 0     | Negative | N/A   |
| <i>Subulo gouazoubira</i>      | 156M | Negative | 0   | 0     | Negative | N/A   |
| <i>Subulo gouazoubira</i>      | 180M | Negative | 0   | 0     | Negative | N/A   |
| <i>Desmodus rotundus</i>       | 13MO | Negative | 0   | 0     | Negative | N/A   |
| <i>Desmodus rotundus</i>       | 24MO | Positive | 13  | 5.079 | Positive | 30.2  |
| <i>Desmodus rotundus</i>       | 25MO | Positive | 31  | 11.79 | Negative | N/A   |
| <i>Desmodus rotundus</i>       | 33MO | Positive | 189 | 72.42 | Negative | N/A   |
| <i>Desmodus rotundus</i>       | 34MO | Positive | 10  | 3.900 | Negative | N/A   |
| <i>Desmodus rotundus</i>       | 40MO | Positive | 98  | 38.81 | Negative | N/A   |
| <i>Desmodus rotundus</i>       | 50MO | Positive | 233 | 93.61 | Positive | 27.81 |
| <i>Desmodus rotundus</i>       | 58MO | Positive | 13  | 5.152 | Negative | N/A   |
| <i>Desmodus rotundus</i>       | 65MO | Positive | 1   | 0.398 | Negative | N/A   |
| <i>Desmodus rotundus</i>       | 68MO | Positive | 1   | 0.405 | Negative | N/A   |
| <i>Cerdocyon thous</i>         | 1L   | Negative | 0   | 0     | Negative | N/A   |
| <i>Leopardus pardalis</i>      | 4L   | Negative | 0   | 0     | Negative | N/A   |
| <i>Leopardus pardalis</i>      | 5L   | Negative | 0   | 0     | Negative | N/A   |
| <i>Leopardus pardalis</i>      | 11L  | Negative | 0   | 0     | Negative | N/A   |
| <i>Leopardus pardalis</i>      | 14L  | Negative | 0   | 0     | Negative | N/A   |
| <i>Leopardus pardalis</i>      | 20L  | Negative | 0   | 0     | Negative | N/A   |
| <i>Leopardus pardalis</i>      | 21L  | Negative | 0   | 0     | Negative | N/A   |
| <i>Panthera onca</i>           | 2P   | Positive | 2   | 0.805 | Negative | N/A   |
| <i>Panthera onca</i>           | 3P   | Negative | 0   | 0     | Negative | N/A   |
| <i>Panthera onca</i>           | 4P   | Positive | 1   | 0.402 | Negative | N/A   |
| <i>Panthera onca</i>           | 5P   | Positive | 1   | 0.409 | Negative | N/A   |
| <i>Panthera onca</i>           | 7P   | Negative | 0   | 0     | Negative | N/A   |
| <i>Panthera onca</i>           | 9P   | Negative | 0   | 0     | Negative | N/A   |
| <i>Panthera onca</i>           | 11P  | Negative | 0   | 0     | Negative | N/A   |
| <i>Panthera onca</i>           | 12P  | Negative | 0   | 0     | Negative | N/A   |
| <i>Panthera onca</i>           | 13P  | Negative | 0   | 0     | Negative | N/A   |
| <i>Panthera onca</i>           | 14P  | Negative | 0   | 0     | Negative | N/A   |
| <i>Myrmecophaga tridactyla</i> | 51T  | Negative | 0   | 0     | Negative | N/A   |
| <i>Myrmecophaga tridactyla</i> | 64T  | Negative | 0   | 0     | Negative | N/A   |
| <i>Tamandua tetradactyla</i>   | 66T  | Negative | 0   | 0     | Negative | N/A   |
| <i>Myrmecophaga tridactyla</i> | 69T  | Negative | 0   | 0     | Negative | N/A   |
| <i>Tamandua tetradactyla</i>   | 72T  | Negative | 0   | 0     | Negative | N/A   |

|                                |       |          |      |        |          |       |
|--------------------------------|-------|----------|------|--------|----------|-------|
| <i>Myrmecophaga tridactyla</i> | 01T   | Negative | 0    | 0      | Negative | N/A   |
| <i>Myrmecophaga tridactyla</i> | 03T   | Negative | 0    | 0      | Negative | N/A   |
| <i>Myrmecophaga tridactyla</i> | 05T   | Negative | 0    | 0      | Positive | 39.02 |
| <i>Myrmecophaga tridactyla</i> | 06T   | Negative | 0    | 0      | Negative | N/A   |
| <i>Tamandua tetradactyla</i>   | 12T   | Negative | 0    | 0      | Negative | N/A   |
| <i>Pseudoseisura unirufa</i>   | 18C   | Negative | 0    | 0      | Negative | N/A   |
| <i>Saltator coerulescens</i>   | 27C   | Negative | 0    | 0      | Negative | N/A   |
| <i>Turdus leocomelas</i>       | 97C   | Positive | 1    | 0.398  | Negative | N/A   |
| <i>Cercomacra melanaria</i>    | 98C   | Negative | 0    | 0      | Negative | N/A   |
| <i>Ramphocelus carbo</i>       | 404C  | Negative | 0    | 0      | Negative | N/A   |
| <i>Basileuterus flaveolus</i>  | 405C  | Negative | 0    | 0      | Negative | N/A   |
| <i>Saltator coerulescens</i>   | 452C  | Positive | 1    | 0.399  | Negative | N/A   |
| <i>Leptotila verreauxi</i>     | 453C  | Negative | 0    | 0      | Negative | N/A   |
| <i>Tapirus terrestris</i>      | 1A    | Negative | 0    | 0      | Negative | N/A   |
| <i>Tapirus terrestris</i>      | 2A    | Positive | 1    | 0.390  | Negative | N/A   |
| <i>Tapirus terrestris</i>      | 10A   | Negative | 0    | 0      | Negative | N/A   |
| <i>Tapirus terrestris</i>      | 11A   | Positive | 1    | 0.391  | Negative | N/A   |
| <i>Tapirus terrestris</i>      | 63A   | Negative | 0    | 0      | Negative | N/A   |
| <i>Tapirus terrestris</i>      | 64A   | Negative | 0    | 0      | Negative | N/A   |
| <i>Tapirus terrestris</i>      | 75A   | Negative | 0    | 0      | Negative | N/A   |
| <i>Tapirus terrestris</i>      | 82A   | Negative | 0    | 0      | Negative | N/A   |
| <i>Tapirus terrestris</i>      | 90A   | Negative | 0    | 0      | Negative | N/A   |
| <i>Tapirus terrestris</i>      | 110A  | Negative | 0    | 0      | Negative | N/A   |
| <i>Tapirus terrestris</i>      | 112A  | Negative | 0    | 0      | Negative | N/A   |
| <i>Tapirus terrestris</i>      | 114A  | Negative | 0    | 0      | Negative | N/A   |
| <i>Tapirus terrestris</i>      | 118A  | Negative | 0    | 0      | Negative | N/A   |
| <i>Tapirus terrestris</i>      | 119A  | Positive | 2    | 0.754  | Negative | N/A   |
| <i>Desmodus rotundus</i>       | 176MO | Positive | 1    | 0.369  | Negative | N/A   |
| <i>Desmodus rotundus</i>       | 182MO | Positive | 3869 | 4150.8 | Positive | 24.64 |
| <i>Desmodus rotundus</i>       | 214MO | Negative | 0    | 0      | Negative | N/A   |
| <i>Desmodus rotundus</i>       | 215MO | Positive | 7    | 2.656  | Negative | N/A   |
| <i>Desmodus rotundus</i>       | 216MO | Positive | 1    | 0.357  | Negative | N/A   |
| <i>Desmodus rotundus</i>       | 218MO | Positive | 107  | 40.79  | Positive | 30.92 |
| <i>Desmodus rotundus</i>       | 219MO | Positive | 59   | 22.76  | Negative | N/A   |
| <i>Desmodus rotundus</i>       | 220MO | Positive | 96   | 36.38  | Positive | 33.74 |
| <i>Desmodus rotundus</i>       | 221MO | Positive | 79   | 29.87  | Positive | 29.79 |
| <i>Desmodus rotundus</i>       | 231MO | Positive | 49   | 18.54  | Positive | 32.59 |
| <i>Desmodus rotundus</i>       | 205MO | Positive | 381  | 145.5  | Negative | N/A   |
| <i>Desmodus rotundus</i>       | 19MO  | Positive | 461  | 626.1  | Positive | 12.26 |
| <i>Desmodus rotundus</i>       | 27MO  | Positive | 839  | 572.6  | Positive | 28.25 |
| <i>Desmodus rotundus</i>       | 35MO  | Positive | 128  | 48.87  | Positive | 30.21 |
| <i>Desmodus rotundus</i>       | 76MO  | Positive | 442  | 1038.9 | Positive | 25.75 |

|                                |       |          |    |       |          |       |
|--------------------------------|-------|----------|----|-------|----------|-------|
| <i>Desmodus rotundus</i>       | 82MO  | Negative | 0  | 0     | Negative | N/A   |
| <i>Desmodus rotundus</i>       | 115MO | Positive | 5  | 1.958 | Negative | N/A   |
| <i>Desmodus rotundus</i>       | 90MO  | Positive | 27 | 10.55 | Negative | N/A   |
| <i>Desmodus rotundus</i>       | 170MO | Positive | 37 | 14.44 | Negative | N/A   |
| <i>Desmodus rotundus</i>       | 179MO | Positive | 46 | 18.21 | Positive | 31.59 |
| <i>Desmodus rotundus</i>       | 207MO | Positive | 17 | 6.641 | Negative | N/A   |
| <i>Desmodus rotundus</i>       | 63MO  | Positive | 17 | 6.641 | Negative | N/A   |
| <i>Desmodus rotundus</i>       | 70MO  | Positive | 1  | 0.356 | Negative | N/A   |
| <i>Desmodus rotundus</i>       | 88MO  | Positive | 2  | 0.753 | Negative | N/A   |
| <i>Desmodus rotundus</i>       | 92MO  | Positive | 7  | 2.646 | Negative | N/A   |
| <i>Leopardus pardalis</i>      | 9L    | Negative | 0  | 0     | Negative | N/A   |
| <i>Panthera onca</i>           | 15P   | Negative | 0  | 0     | Positive | 42.86 |
| <i>Panthera onca</i>           | 16P   | Negative | 0  | 0     | Negative | N/A   |
| <i>Panthera onca</i>           | 17P   | Positive | 1  | 0.395 | Negative | N/A   |
| <i>Panthera onca</i>           | 19P   | Negative | 0  | 0     | Negative | N/A   |
| <i>Panthera onca</i>           | 20P   | Negative | 0  | 0     | Negative | N/A   |
| <i>Panthera onca</i>           | 21P   | Negative | 0  | 0     | Negative | N/A   |
| <i>Panthera onca</i>           | 22P   | Negative | 0  | 0     | Negative | N/A   |
| <i>Panthera onca</i>           | 23P   | Negative | 0  | 0     | Negative | N/A   |
| <i>Panthera onca</i>           | 24P   | Negative | 0  | 0     | Negative | N/A   |
| <i>Panthera onca</i>           | 25P   | Positive | 3  | 1.135 | Negative | N/A   |
| <i>Myrmecophaga tridactyla</i> | 17T   | Negative | 0  | 0.756 | Negative | N/A   |
| <i>Euphractus sexcinctus</i>   | 32T   | Negative | 0  | 0     | Negative | N/A   |
| <i>Myrmecophaga tridactyla</i> | 50T   | Negative | 0  | 0     | Negative | N/A   |
| <i>Dasypus novemcinctus</i>    | 76T   | Negative | 0  | 0     | Positive | 44.7  |
| <i>Dasypus novemcinctus</i>    | 79T   | Negative | 0  | 0     | Positive | 13.03 |
| <i>Myrmecophaga tridactyla</i> | 81T   | Negative | 0  | 0     | Negative | N/A   |
| <i>Dasypus novemcinctus</i>    | 149T  | Positive | 1  | 0.404 | Negative | N/A   |
| <i>Tapirus terrestris</i>      | 122A  | Negative | 0  | 0     | Negative | N/A   |
| <i>Tapirus terrestris</i>      | 124A  | Negative | 0  | 0     | Negative | N/A   |
| <i>Subulo gouazoubira</i>      | 196M  | Positive | 1  | 0.392 | Negative | N/A   |
| <i>Mazama juncunda</i>         | 204M  | Positive | 1  | 0.390 | Negative | N/A   |
| <i>Mazama juncunda</i>         | 208M  | Positive | 5  | 1.909 | Negative | N/A   |
| <i>Subulo gouazoubira</i>      | 209M  | Negative | 0  | 0     | Negative | N/A   |
| <i>Mazama juncunda</i>         | 210M  | Negative | 0  | 0     | Negative | N/A   |
| <i>Mazama rufa</i>             | 267M  | Negative | 0  | 0     | Negative | N/A   |
| <i>Mazama rufa</i>             | 268M  | Negative | 0  | 0     | Negative | N/A   |
| <i>Subulo gouazoubira</i>      | 298M  | Positive | 3  | 1.216 | Negative | N/A   |
| <i>Subulo gouazoubira</i>      | 299M  | Negative | 0  | 0     | Negative | N/A   |
| <i>Subulo gouazoubira</i>      | 300M  | Positive | 3  | 1.225 | Positive | 33.26 |
| <i>Subulo gouazoubira</i>      | 301M  | Positive | 1  | 0.405 | Negative | N/A   |
| <i>Subulo gouazoubira</i>      | 302M  | Positive | 1  | 0.400 | Positive | 31.03 |
| <i>Subulo gouazoubira</i>      | 303M  | Negative | 0  | 0     | Negative | N/A   |

|                               |      |          |     |       |          |       |
|-------------------------------|------|----------|-----|-------|----------|-------|
| <i>Subulo gouazoubira</i>     | 307M | Positive | 1   | 0.394 | Negative | N/A   |
| <i>Subulo gouazoubira</i>     | 313M | Negative | 0   | 0     | Negative | N/A   |
| <i>Subulo gouazoubira</i>     | 314M | Negative | 0   | 0     | Negative | N/A   |
| <i>Subulo gouazoubira</i>     | 315M | Negative | 0   | 0     | Negative | N/A   |
| <i>Subulo gouazoubira</i>     | 316M | Negative | 0   | 0     | Negative | N/A   |
| <i>Subulo gouazoubira</i>     | 317M | Negative | 0   | 0     | Negative | N/A   |
| <i>Subulo gouazoubira</i>     | 318M | Negative | 0   | 0     | Negative | N/A   |
| <i>Subulo gouazoubira</i>     | 319M | Positive | 1   | 0.401 | Negative | N/A   |
| <i>Subulo gouazoubira</i>     | 320M | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 35B  | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 71B  | Positive | 1   | 0.399 | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 78B  | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 79B  | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 94B  | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 104B | Positive | 2   | 0.759 | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 24B  | Positive | 4   | 1.561 | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 25B  | Positive | 163 | 64.43 | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 26B  | Positive | 183 | 72.37 | Positive | 13.74 |
| <i>Blastocerus dichotomus</i> | 28B  | Positive | 65  | 25.91 | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 29B  | Positive | 7   | 2.798 | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 37B  | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 38B  | Positive | 1   | 0.394 | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 40B  | Negative | 0   | 0     | Positive | 31.07 |
| <i>Blastocerus dichotomus</i> | 41B  | Negative | 0   | 0     | Positive | 34.9  |
| <i>Blastocerus dichotomus</i> | 43B  | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 45B  | Positive | 1   | 0.388 | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 46B  | Positive | 3   | 1.113 | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 48B  | Negative | 0   | 0     | Positive | 15.91 |
| <i>Blastocerus dichotomus</i> | 49B  | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 51B  | Positive | 10  | 3.775 | Positive | 14.62 |
| <i>Blastocerus dichotomus</i> | 52B  | Positive | 1   | 0.388 | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 55B  | Positive | 1   | 0.383 | Positive | 42.86 |
| <i>Blastocerus dichotomus</i> | 64B  | Positive | 2   | 0.760 | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 68B  | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 76B  | Positive | 372 | 160.9 | Positive | 19.04 |
| <i>Desmodus rotundus</i>      | 36MO | Positive | 544 | 202.3 | Negative | N/A   |
| <i>Desmodus rotundus</i>      | 43MO | Positive | 1   | 0.366 | Negative | N/A   |
| <i>Desmodus rotundus</i>      | 44MO | Negative | 0   | 0     | Negative | N/A   |
| <i>Desmodus rotundus</i>      | 47MO | Negative | 0   | 0     | Negative | N/A   |
| <i>Desmodus rotundus</i>      | 54MO | Positive | 56  | 20.30 | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 77B  | Positive | 5   | 1.801 | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 78B  | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 80B  | Positive | 4   | 1.453 | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 81B  | Positive | 1   | 0.356 | Negative | N/A   |

|                               |       |          |     |       |          |       |
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| <i>Blastocerus dichotomus</i> | 83B   | Positive | 12  | 4.379 | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 84B   | Positive | 4   | 1.447 | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 86B   | Positive | 26  | 9.500 | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 87B   | Positive | 4   | 1.424 | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 88B   | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 92B   | Positive | 2   | 0.714 | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 93B   | Positive | 251 | 91.81 | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 98B   | Positive | 10  | 4.526 | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 100B  | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 102B  | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 103B  | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 106B  | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 107B  | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 108B  | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 110B  | Positive | 5   | 1.783 | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 112B  | Positive | 66  | 23.62 | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 128B  | Negative | 0   | 0     | Positive | 43.51 |
| <i>Blastocerus dichotomus</i> | 130B  | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 131B  | Positive | 147 | 52.80 | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 134B  | Positive | 8   | 2.919 | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 136B  | Positive | 47  | 16.77 | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 138B  | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 25B1  | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 50B1  | Positive | 1   | 0.357 | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 58B1  | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 65B1  | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 74B1  | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 82B1  | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 95B1  | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 129B1 | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 101B1 | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 32B2  | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 72B2  | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 85B2  | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 97B2  | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 109B2 | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 113B2 | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 114B2 | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 115B2 | Positive | 1   | 0.360 | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 116B2 | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 117B2 | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 118B2 | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 122B2 | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i> | 201B3 | Negative | 0   | 0     | Negative | N/A   |

|                                |       |          |     |       |          |       |
|--------------------------------|-------|----------|-----|-------|----------|-------|
| <i>Blastocerus dichotomus</i>  | 205B3 | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i>  | 209B3 | Positive | 1   | 0.356 | Negative | N/A   |
| <i>Blastocerus dichotomus</i>  | 207B3 | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i>  | 208B3 | Positive | 1   | 0.358 | Negative | N/A   |
| <i>Blastocerus dichotomus</i>  | 206B3 | Negative | 0   | 0     | Positive | 41.01 |
| <i>Blastocerus dichotomus</i>  | 212B3 | Positive | 1   | 0.357 | Negative | N/A   |
| <i>Blastocerus dichotomus</i>  | 213B3 | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i>  | 208MO | Positive | 459 | 168.0 | Positive | 28.24 |
| <i>Blastocerus dichotomus</i>  | 217B3 | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i>  | 218B3 | Positive | 1   | 0.358 | Negative | N/A   |
| <i>Blastocerus dichotomus</i>  | 227B3 | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i>  | 228B3 | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i>  | 231B3 | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i>  | 237B3 | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i>  | 240B3 | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i>  | 245B3 | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i>  | 246B3 | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i>  | 234B3 | Positive | 1   | 0.357 | Negative | N/A   |
| <i>Blastocerus dichotomus</i>  | 284B3 | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i>  | 139B3 | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i>  | 236B3 | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i>  | 264B3 | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i>  | 67B2  | Negative | 0   | 0     | Negative | N/A   |
| <i>Desmodus rotundus</i>       | 202MO | Negative | 0   | 0     | Negative | N/A   |
| <i>Cerdocyon thous</i>         | 3L    | Positive | 2   | 0.712 | Positive | 38.02 |
| <i>Leopardus pardalis</i>      | 7L    | Negative | 0   | 0     | Negative | N/A   |
| <i>Cerdocyon thous</i>         | 10L   | Negative | 0   | 0     | Negative | N/A   |
| <i>Cerdocyon thous</i>         | 13L   | Negative | 0   | 0     | Negative | N/A   |
| <i>Cerdocyon thous</i>         | 15L   | Negative | 0   | 0     | Negative | N/A   |
| <i>Leopardus pardalis</i>      | 16L   | Positive | 1   | 0.356 | Negative | N/A   |
| <i>Cerdocyon thous</i>         | 17L   | Positive | 1   | 0.357 | Negative | N/A   |
| <i>Cerdocyon thous</i>         | 19L   | Positive | 2   | 0.713 | Negative | N/A   |
| <i>Leopardus pardalis</i>      | 23L   | Negative | 0   | 0     | Negative | N/A   |
| <i>Cerdocyon thous</i>         | 24L   | Negative | 0   | 0     | Negative | N/A   |
| <i>Cerdocyon thous</i>         | 26L   | Negative | 0   | 0     | Negative | N/A   |
| <i>Puma concolor</i>           | 06P   | Negative | 0   | 0     | Negative | N/A   |
| <i>Puma concolor</i>           | 08P   | Negative | 0   | 0     | Negative | N/A   |
| <i>Puma concolor</i>           | 18P   | Negative | 0   | 0     | Negative | N/A   |
| <i>Myrmecophaga tridactyla</i> | 97T   | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i>  | 21B   | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i>  | 23B   | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i>  | 25B   | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i>  | 27B   | Negative | 0   | 0     | Negative | N/A   |
| <i>Blastocerus dichotomus</i>  | 30B   | Positive | 1   | 0.361 | Negative | N/A   |

|                                |      |          |   |       |          |     |
|--------------------------------|------|----------|---|-------|----------|-----|
| <i>Blastocerus dichotomus</i>  | 31B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 32B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 33B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 34B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 36B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 42B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 44B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 53B  | Positive | 1 | 0.356 | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 54B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 57B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 59B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 60B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 66B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 89B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 90B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 99B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 105B | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 111B | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 113B | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 117B | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 123B | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 124B | Positive | 1 | 0.361 | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 126B | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 127B | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 135B | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 160B | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 229B | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 230B | Positive | 1 | 0.357 | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 235B | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 238B | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 241B | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 265B | Negative | 0 | 0     | Negative | N/A |
| <i>Tapirus terrestris</i>      | 3A   | Negative | 0 | 0     | Negative | N/A |
| <i>Tapirus terrestris</i>      | 5A   | Negative | 0 | 0     | Negative | N/A |
| <i>Tapirus terrestris</i>      | 6A   | Negative | 0 | 0     | Negative | N/A |
| <i>Tapirus terrestris</i>      | 21A  | Negative | 0 | 0     | Negative | N/A |
| <i>Tapirus terrestris</i>      | 95A  | Positive | 1 | 0.361 | Negative | N/A |
| <i>Tapirus terrestris</i>      | 106A | Negative | 0 | 0     | Negative | N/A |
| <i>Tapirus terrestris</i>      | 104A | Negative | 0 | 0     | Negative | N/A |
| <i>Tapirus terrestris</i>      | 107A | Negative | 0 | 0     | Negative | N/A |
| <i>Myrmecophaga tridactyla</i> | 163T | Negative | 0 | 0     | Negative | N/A |
| <i>Myrmecophaga tridactyla</i> | 172T | Negative | 0 | 0     | Negative | N/A |
| <i>Myrmecophaga tridactyla</i> | 202T | Negative | 0 | 0     | Negative | N/A |



|                                      |       |          |      |       |          |       |
|--------------------------------------|-------|----------|------|-------|----------|-------|
| <i>Myrmecophaga tridactyla</i>       | 178T  | Negative | 0    | 0     | Negative | N/A   |
| <i>Euphractus sexcinctus</i>         | 100T  | Positive | 3    | 1.080 | Positive | 40.61 |
| <i>Euphractus sexcinctus</i>         | 35T   | Negative | 0    | 0     | Negative | N/A   |
| <i>Euphractus sexcinctus</i>         | 25T   | Positive | 2    | 0.720 | Negative | N/A   |
| <i>Tamandua tetradactyla</i>         | 101T  | Positive | 1    | 0.360 | Negative | N/A   |
| <i>Tamandua tetradactyla</i>         | 133T  | Negative | 0    | 0     | Negative | N/A   |
| <i>Tamandua tetradactyla</i>         | 115T  | Negative | 0    | 0     | Negative | N/A   |
| <i>Tamandua tetradactyla</i>         | 57T   | Negative | 0    | 0     | Negative | N/A   |
| <i>Tamandua tetradactyla</i>         | 73T   | Negative | 0    | 0     | Negative | N/A   |
| <i>Priodontes maximus</i>            | 135T  | Negative | 0    | 0     | Negative | N/A   |
| <i>Priodontes maximus</i>            | 145T  | Negative | 0    | 0     | Negative | N/A   |
| <i>Euphractus sexcinctus</i>         | 150T  | Positive | 1    | 0.360 | Negative | N/A   |
| <i>Priodontes maximus</i>            | 154T  | Negative | 0    | 0     | Negative | N/A   |
| <i>Dasypus novemcinctus</i>          | 41T   | Positive | 2    | 0.714 | Negative | N/A   |
| <i>Dasypus novemcinctus</i>          | 42T   | Negative | 0    | 0     | Negative | N/A   |
| <i>Dasypus novemcinctus</i>          | 88T   | Negative | 0    | 0     | Negative | N/A   |
| <i>Oecomys mamorae</i>               | 58R   | Positive | 214  | 77.40 | Negative | N/A   |
| <i>Oecomys mamorae</i>               | 18R   | Positive | 31   | 11.21 | Negative | N/A   |
| <i>Oecomys mamorae</i>               | 48R   | Positive | 11   | 3.932 | Negative | N/A   |
| <i>Oecomys mamorae</i>               | 84R   | Negative | 0    | 0     | Negative | N/A   |
| <i>Thrichomys fosteri</i>            | 7R    | Negative | 0    | 0     | Negative | N/A   |
| <i>Clyomis laticeps</i>              | 42R   | Negative | 0    | 0     | Negative | N/A   |
| <i>Thrichomys fosteri</i>            | 43R   | Positive | 1721 | 694.1 | Positive | 31.21 |
| <i>Thrichomys fosteri</i>            | 13R   | Negative | 0    | 0     | Negative | N/A   |
| <i>Thrichomys fosteri</i>            | 60R   | Negative | 0    | 0     | Negative | N/A   |
| <i>Leptotila verreauxi</i>           | 107C  | Negative | 0    | 0     | Positive | 43.84 |
| <i>Icterus cayanensis</i>            | 72C   | Negative | 0    | 0     | Negative | N/A   |
| <i>Lepidocolaptes angustirostris</i> | 59C   | Negative | 0    | 0     | Negative | N/A   |
| <i>Leptotila verreauxi</i>           | 78C   | Negative | 0    | 0     | Negative | N/A   |
| <i>Ramphocelus carbo</i>             | 90C   | Negative | 0    | 0     | Negative | N/A   |
| <i>Turdus leocomelas</i>             | 85C   | Negative | 0    | 0     | Negative | N/A   |
| <i>Fumarius rufus</i>                | 119C  | Negative | 0    | 0     | Negative | N/A   |
| <i>Saltator coerulescens</i>         | 120C  | Positive | 1    | 0.356 | Negative | N/A   |
| <i>Fumarius leucopus</i>             | 117C  | Negative | 0    | 0     | Negative | N/A   |
| <i>Cyanocorax chrysops</i>           | 124C  | Positive | 1    | 0.356 | Negative | N/A   |
| <i>Desmodus rotundus</i>             | 162MO | Positive | 11   | 3.922 | Positive | 1.87  |
| <i>Desmodus rotundus</i>             | 180MO | Positive | 951  | 359.8 | Negative | N/A   |
| <i>Desmodus rotundus</i>             | 158MO | Positive | 536  | 197.3 | Positive | 29.59 |
| <i>Desmodus rotundus</i>             | 223MO | Positive | 1    | 0.357 | Positive | 36.56 |
| <i>Desmodus rotundus</i>             | 200MO | Positive | 2    | 0.713 | Negative | N/A   |
| <i>Desmodus rotundus</i>             | 224MO | Positive | 15   | 5.355 | Positive | 36.19 |
| <i>Desmodus rotundus</i>             | 222MO | Positive | 4    | 1.429 | Negative | N/A   |
| <i>Desmodus rotundus</i>             | 201MO | Positive | 139  | 49.98 | Negative | N/A   |

**Table S6:** Results obtained for each test carried out for *Borrelia* spp.

| Animal species                 | ID    | Result   | dPCR                |                        | qPCR     |     |
|--------------------------------|-------|----------|---------------------|------------------------|----------|-----|
|                                |       |          | Positive Partitions | Conc (copies/ $\mu$ L) | Result   | CQ  |
| <i>Subulo gouazoubira</i>      | 182M  | Positive | 1                   | 0.413                  | Negative | N/A |
| <i>Mazama jucunda</i>          | 215M  | Positive | 1                   | 0.378                  | Negative | N/A |
| <i>Mazama rufa</i>             | 270M  | Positive | 2                   | 0.759                  | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 63B   | Negative | 0                   | 0                      | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 75B   | Negative | 0                   | 0                      | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 120B2 | Negative | 0                   | 0                      | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 121B2 | Negative | 0                   | 0                      | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 222B3 | Negative | 0                   | 0                      | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 226B3 | Negative | 0                   | 0                      | Negative | N/A |
| <i>Desmodus rotundus</i>       | 91MO  | Negative | 0                   | 0                      | Negative | N/A |
| <i>Desmodus rotundus</i>       | 8MO   | Positive | 1                   | 0.375                  | Negative | N/A |
| <i>Cerdocyon thous</i>         | 2L    | Negative | 0                   | 0                      | Negative | N/A |
| <i>Leopardus pardalis</i>      | 8L    | Negative | 0                   | 0                      | Negative | N/A |
| <i>Leopardus pardalis</i>      | 22L   | Negative | 0                   | 0                      | Negative | N/A |
| <i>Cerdocyon thous</i>         | 25L   | Negative | 0                   | 0                      | Negative | N/A |
| <i>Myrmecophaga tridactyla</i> | 59T   | Negative | 0                   | 0                      | Negative | N/A |
| <i>Myrmecophaga tridactyla</i> | 62T   | Negative | 0                   | 0                      | Negative | N/A |
| <i>Tamandua tetradactyla</i>   | 71T   | Negative | 0                   | 0                      | Negative | N/A |
| <i>Dasypus novemcinctus</i>    | 16T   | Negative | 0                   | 0                      | Negative | N/A |
| <i>Priodontes maximus</i>      | 153T  | Negative | 0                   | 0                      | Negative | N/A |
| <i>Priodontes maximus</i>      | 159T  | Negative | 0                   | 0                      | Negative | N/A |
| <i>Priodontes maximus</i>      | 160T  | Negative | 0                   | 0                      | Negative | N/A |
| <i>Ozotocerus bezoarticus</i>  | 06O   | Negative | 0                   | 0                      | Negative | N/A |
| <i>Ozotocerus bezoarticus</i>  | 07O   | Negative | 0                   | 0                      | Negative | N/A |
| <i>Ozotocerus bezoarticus</i>  | 08O   | Negative | 0                   | 0                      | Negative | N/A |
| <i>Ozotocerus bezoarticus</i>  | 09O   | Negative | 0                   | 0                      | Negative | N/A |
| <i>Ozotocerus bezoarticus</i>  | 10O   | Negative | 0                   | 0                      | Negative | N/A |
| <i>Subulo gouazoubira</i>      | 154M  | Negative | 0                   | 0                      | Negative | N/A |
| <i>Subulo gouazoubira</i>      | 155M  | Negative | 0                   | 0                      | Negative | N/A |
| <i>Subulo gouazoubira</i>      | 156M  | Negative | 0                   | 0                      | Negative | N/A |
| <i>Subulo gouazoubira</i>      | 180M  | Negative | 0                   | 0                      | Negative | N/A |
| <i>Desmodus rotundus</i>       | 13MO  | Negative | 0                   | 0                      | Negative | N/A |
| <i>Desmodus rotundus</i>       | 24MO  | Positive | 2                   | 0.781                  | Negative | N/A |
| <i>Desmodus rotundus</i>       | 25MO  | Negative | 0                   | 0                      | Negative | N/A |
| <i>Desmodus rotundus</i>       | 33MO  | Negative | 0                   | 0                      | Negative | N/A |
| <i>Desmodus rotundus</i>       | 34MO  | Positive | 1                   | 0.390                  | Negative | N/A |
| <i>Desmodus rotundus</i>       | 40MO  | Negative | 0                   | 0                      | Negative | N/A |
| <i>Desmodus rotundus</i>       | 50MO  | Negative | 0                   | 0                      | Negative | N/A |
| <i>Desmodus rotundus</i>       | 58MO  | Positive | 1                   | 0.396                  | Negative | N/A |
| <i>Desmodus rotundus</i>       | 65MO  | Positive | 1                   | 0.398                  | Negative | N/A |
| <i>Desmodus rotundus</i>       | 68MO  | Negative | 0                   | 0                      | Negative | N/A |
| <i>Cerdocyon thous</i>         | 1L    | Positive | 1                   | 0.401                  | Negative | N/A |
| <i>Leopardus pardalis</i>      | 4L    | Negative | 0                   | 0                      | Negative | N/A |
| <i>Leopardus pardalis</i>      | 5L    | Negative | 0                   | 0                      | Negative | N/A |
| <i>Leopardus pardalis</i>      | 11L   | Negative | 0                   | 0                      | Negative | N/A |
| <i>Leopardus pardalis</i>      | 14L   | Negative | 0                   | 0                      | Negative | N/A |

|                                |       |          |   |       |          |       |
|--------------------------------|-------|----------|---|-------|----------|-------|
| <i>Leopardus pardalis</i>      | 20L   | Negative | 0 | 0     | Negative | N/A   |
| <i>Leopardus pardalis</i>      | 21L   | Negative | 0 | 0     | Negative | N/A   |
| <i>Panthera onca</i>           | 2P    | Positive | 1 | 0.402 | Negative | N/A   |
| <i>Panthera onca</i>           | 3P    | Positive | 3 | 1.219 | Negative | N/A   |
| <i>Panthera onca</i>           | 4P    | Negative | 0 | 0     | Negative | N/A   |
| <i>Panthera onca</i>           | 5P    | Positive | 1 | 0.409 | Negative | N/A   |
| <i>Panthera onca</i>           | 7P    | Negative | 0 | 0     | Negative | N/A   |
| <i>Panthera onca</i>           | 9P    | Negative | 0 | 0     | Negative | N/A   |
| <i>Panthera onca</i>           | 11P   | Positive | 1 | 0.401 | Negative | N/A   |
| <i>Panthera onca</i>           | 12P   | Positive | 2 | 0.789 | Negative | N/A   |
| <i>Panthera onca</i>           | 13P   | Negative | 0 | 0     | Negative | N/A   |
| <i>Panthera onca</i>           | 14P   | Negative | 0 | 0     | Negative | N/A   |
| <i>Myrmecophaga tridactyla</i> | 51T   | Negative | 0 | 0     | Negative | N/A   |
| <i>Myrmecophaga tridactyla</i> | 64T   | Negative | 0 | 0     | Negative | N/A   |
| <i>Tamandua tetradactyla</i>   | 66T   | Positive | 1 | 0.403 | Negative | N/A   |
| <i>Myrmecophaga tridactyla</i> | 69T   | Negative | 0 | 0     | Negative | N/A   |
| <i>Tamandua tetradactyla</i>   | 72T   | Negative | 0 | 0     | Negative | N/A   |
| <i>Myrmecophaga tridactyla</i> | 01T   | Negative | 0 | 0     | Negative | N/A   |
| <i>Myrmecophaga tridactyla</i> | 03T   | Positive | 1 | 0.401 | Negative | N/A   |
| <i>Myrmecophaga tridactyla</i> | 05T   | Negative | 0 | 0     | Negative | N/A   |
| <i>Myrmecophaga tridactyla</i> | 06T   | Negative | 0 | 0     | Negative | N/A   |
| <i>Tamandua tetradactyla</i>   | 12T   | Negative | 0 | 0     | Negative | N/A   |
| <i>Pseudoseisura unirufa</i>   | 18C   | Negative | 0 | 0     | Negative | N/A   |
| <i>Saltator coerulescens</i>   | 27C   | Negative | 0 | 0     | Negative | N/A   |
| <i>Turdus leocomelas</i>       | 97C   | Negative | 0 | 0     | Negative | N/A   |
| <i>Cercomacra melanaria</i>    | 98C   | Negative | 0 | 0     | Negative | N/A   |
| <i>Ramphocelus carbo</i>       | 404C  | Negative | 0 | 0     | Negative | N/A   |
| <i>Basileuterus flaveolus</i>  | 405C  | Negative | 0 | 0     | Negative | N/A   |
| <i>Saltator coerulescens</i>   | 452C  | Negative | 0 | 0     | Negative | N/A   |
| <i>Leptotila verreauxi</i>     | 453C  | Negative | 0 | 0     | Negative | N/A   |
| <i>Tapirus terrestris</i>      | 1A    | Negative | 0 | 0     | Negative | N/A   |
| <i>Tapirus terrestris</i>      | 2A    | Positive | 1 | 0.390 | Negative | N/A   |
| <i>Tapirus terrestris</i>      | 10A   | Negative | 0 | 0     | Negative | N/A   |
| <i>Tapirus terrestris</i>      | 11A   | Negative | 0 | 0     | Negative | N/A   |
| <i>Tapirus terrestris</i>      | 63A   | Negative | 0 | 0     | Negative | N/A   |
| <i>Tapirus terrestris</i>      | 64A   | Negative | 0 | 0     | Negative | N/A   |
| <i>Tapirus terrestris</i>      | 75A   | Positive | 2 | 1.052 | Negative | N/A   |
| <i>Tapirus terrestris</i>      | 82A   | Negative | 0 | 0     | Negative | N/A   |
| <i>Tapirus terrestris</i>      | 90A   | Negative | 0 | 0     | Negative | N/A   |
| <i>Tapirus terrestris</i>      | 110A  | Negative | 0 | 0     | Negative | N/A   |
| <i>Tapirus terrestris</i>      | 112A  | Negative | 0 | 0     | Negative | N/A   |
| <i>Tapirus terrestris</i>      | 114A  | Positive | 1 | 0.383 | Negative | N/A   |
| <i>Tapirus terrestris</i>      | 118A  | Negative | 0 | 0     | Negative | N/A   |
| <i>Tapirus terrestris</i>      | 119A  | Negative | 0 | 0     | Negative | N/A   |
| <i>Desmodus rotundus</i>       | 176MO | Positive | 1 | 0.369 | Positive | 43.26 |
| <i>Desmodus rotundus</i>       | 182MO | Negative | 0 | 0     | Negative | N/A   |
| <i>Desmodus rotundus</i>       | 214MO | Negative | 0 | 0     | Negative | N/A   |
| <i>Desmodus rotundus</i>       | 215MO | Negative | 0 | 0     | Negative | N/A   |
| <i>Desmodus rotundus</i>       | 216MO | Negative | 0 | 0     | Negative | N/A   |
| <i>Desmodus rotundus</i>       | 218MO | Negative | 0 | 0     | Negative | N/A   |
| <i>Desmodus rotundus</i>       | 219MO | Negative | 0 | 0     | Positive | 44.93 |
| <i>Desmodus rotundus</i>       | 220MO | Positive | 2 | 0.754 | Negative | N/A   |

|                                |       |          |   |       |          |     |
|--------------------------------|-------|----------|---|-------|----------|-----|
| <i>Desmodus rotundus</i>       | 221MO | Positive | 2 | 0.753 | Negative | N/A |
| <i>Desmodus rotundus</i>       | 231MO | Positive | 1 | 0.377 | Negative | N/A |
| <i>Desmodus rotundus</i>       | 205MO | Negative | 0 | 0     | Negative | N/A |
| <i>Desmodus rotundus</i>       | 19MO  | Negative | 0 | 0     | Negative | N/A |
| <i>Desmodus rotundus</i>       | 27MO  | Negative | 0 | 0     | Negative | N/A |
| <i>Desmodus rotundus</i>       | 35MO  | Negative | 0 | 0     | Negative | N/A |
| <i>Desmodus rotundus</i>       | 76MO  | Positive | 1 | 0.386 | Negative | N/A |
| <i>Desmodus rotundus</i>       | 82MO  | Negative | 0 | 0     | Negative | N/A |
| <i>Desmodus rotundus</i>       | 115MO | Negative | 0 | 0     | Negative | N/A |
| <i>Desmodus rotundus</i>       | 90MO  | Negative | 0 | 0     | Negative | N/A |
| <i>Desmodus rotundus</i>       | 170MO | Negative | 0 | 0     | Negative | N/A |
| <i>Desmodus rotundus</i>       | 179MO | Negative | 0 | 0     | Negative | N/A |
| <i>Desmodus rotundus</i>       | 207MO | Negative | 0 | 0     | Negative | N/A |
| <i>Desmodus rotundus</i>       | 63MO  | Negative | 0 | 0     | Negative | N/A |
| <i>Desmodus rotundus</i>       | 70MO  | Negative | 0 | 0     | Negative | N/A |
| <i>Desmodus rotundus</i>       | 88MO  | Negative | 0 | 0     | Negative | N/A |
| <i>Desmodus rotundus</i>       | 92MO  | Positive | 1 | 0.378 | Negative | N/A |
| <i>Leopardus pardalis</i>      | 9L    | Negative | 0 | 0     | Negative | N/A |
| <i>Panthera onca</i>           | 15P   | Negative | 0 | 0     | Negative | N/A |
| <i>Panthera onca</i>           | 16P   | Negative | 0 | 0     | Negative | N/A |
| <i>Panthera onca</i>           | 17P   | Positive | 1 | 0.395 | Negative | N/A |
| <i>Panthera onca</i>           | 19P   | Negative | 0 | 0     | Negative | N/A |
| <i>Panthera onca</i>           | 20P   | Negative | 0 | 0     | Negative | N/A |
| <i>Panthera onca</i>           | 21P   | Negative | 0 | 0     | Negative | N/A |
| <i>Panthera onca</i>           | 22P   | Negative | 0 | 0     | Negative | N/A |
| <i>Panthera onca</i>           | 23P   | Negative | 0 | 0     | Negative | N/A |
| <i>Panthera onca</i>           | 24P   | Negative | 0 | 0     | Negative | N/A |
| <i>Panthera onca</i>           | 25P   | Negative | 0 | 0     | Negative | N/A |
| <i>Myrmecophaga tridactyla</i> | 17T   | Negative | 0 | 0     | Negative | N/A |
| <i>Euphractus sexcinctus</i>   | 32T   | Negative | 0 | 0     | Negative | N/A |
| <i>Myrmecophaga tridactyla</i> | 50T   | Negative | 0 | 0     | Negative | N/A |
| <i>Dasypus novemcinctus</i>    | 76T   | Negative | 0 | 0     | Negative | N/A |
| <i>Dasypus novemcinctus</i>    | 79T   | Negative | 0 | 0     | Negative | N/A |
| <i>Myrmecophaga tridactyla</i> | 81T   | Negative | 0 | 0     | Negative | N/A |
| <i>Dasypus novemcinctus</i>    | 149T  | Negative | 0 | 0     | Negative | N/A |
| <i>Tapirus terrestris</i>      | 122A  | Negative | 0 | 0     | Negative | N/A |
| <i>Tapirus terrestris</i>      | 124A  | Negative | 0 | 0     | Negative | N/A |
| <i>Subulo gouazoubira</i>      | 196M  | Positive | 2 | 0.784 | Negative | N/A |
| <i>Mazama jucunda</i>          | 204M  | Positive | 1 | 0.390 | Negative | N/A |
| <i>Mazama jucunda</i>          | 208M  | Positive | 1 | 0.381 | Negative | N/A |
| <i>Subulo gouazoubira</i>      | 209M  | Negative | 0 | 0     | Negative | N/A |
| <i>Mazama jucunda</i>          | 210M  | Negative | 0 | 0     | Negative | N/A |
| <i>Mazama rufa</i>             | 267M  | Negative | 0 | 0     | Negative | N/A |
| <i>Mazama rufa</i>             | 268M  | Negative | 0 | 0     | Negative | N/A |
| <i>Subulo gouazoubira</i>      | 298M  | Positive | 1 | 0.405 | Negative | N/A |
| <i>Subulo gouazoubira</i>      | 299M  | Negative | 0 | 0     | Negative | N/A |
| <i>Subulo gouazoubira</i>      | 300M  | Negative | 0 | 0     | Negative | N/A |
| <i>Subulo gouazoubira</i>      | 301M  | Positive | 1 | 0.405 | Negative | N/A |
| <i>Subulo gouazoubira</i>      | 302M  | Negative | 0 | 0     | Negative | N/A |
| <i>Subulo gouazoubira</i>      | 303M  | Negative | 0 | 0     | Negative | N/A |
| <i>Subulo gouazoubira</i>      | 307M  | Negative | 0 | 0     | Negative | N/A |
| <i>Subulo gouazoubira</i>      | 313M  | Negative | 0 | 0     | Negative | N/A |

|                               |      |          |   |       |          |     |
|-------------------------------|------|----------|---|-------|----------|-----|
| <i>Subulo gouazoubira</i>     | 314M | Positive | 1 | 0.385 | Negative | N/A |
| <i>Subulo gouazoubira</i>     | 315M | Negative | 0 | 0     | Negative | N/A |
| <i>Subulo gouazoubira</i>     | 316M | Negative | 0 | 0     | Negative | N/A |
| <i>Subulo gouazoubira</i>     | 317M | Negative | 0 | 0     | Negative | N/A |
| <i>Subulo gouazoubira</i>     | 318M | Negative | 0 | 0     | Negative | N/A |
| <i>Subulo gouazoubira</i>     | 319M | Negative | 0 | 0     | Negative | N/A |
| <i>Subulo gouazoubira</i>     | 320M | Positive | 1 | 0.405 | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 35B  | Positive | 1 | 0.400 | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 71B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 78B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 79B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 94B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 104B | Positive | 1 | 0.379 | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 24B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 25B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 26B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 28B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 29B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 37B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 38B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 40B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 41B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 43B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 45B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 46B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 48B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 49B  | Positive | 1 | 0.381 | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 51B  | Positive | 1 | 0.377 | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 52B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 55B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 64B  | Positive | 1 | 0.380 | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 68B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 76B  | Positive | 7 | 2.636 | Negative | N/A |
| <i>Desmodus rotundus</i>      | 36MO | Positive | 2 | 0.719 | Negative | N/A |
| <i>Desmodus rotundus</i>      | 43MO | Negative | 0 | 0     | Negative | N/A |
| <i>Desmodus rotundus</i>      | 44MO | Negative | 0 | 0     | Negative | N/A |
| <i>Desmodus rotundus</i>      | 47MO | Negative | 0 | 0     | Negative | N/A |
| <i>Desmodus rotundus</i>      | 54MO | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 77B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 78B  | Positive | 1 | 0.357 | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 80B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 81B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 83B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 84B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 86B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 87B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 88B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 92B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 93B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 98B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 100B | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i> | 102B | Negative | 0 | 0     | Negative | N/A |



|                                |       |          |   |       |          |     |
|--------------------------------|-------|----------|---|-------|----------|-----|
| <i>Blastocerus dichotomus</i>  | 284B3 | Positive | 1 | 0.356 | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 139B3 | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 236B3 | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 264B3 | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 67B2  | Negative | 0 | 0     | Negative | N/A |
| <i>Desmodus rotundus</i>       | 202MO | Negative | 0 | 0     | Negative | N/A |
| <i>Cerdocyon thous</i>         | 3L    | Negative | 0 | 0     | Negative | N/A |
| <i>Leopardus pardalis</i>      | 7L    | Negative | 0 | 0     | Negative | N/A |
| <i>Cerdocyon thous</i>         | 10L   | Negative | 0 | 0     | Negative | N/A |
| <i>Cerdocyon thous</i>         | 13L   | Negative | 0 | 0     | Negative | N/A |
| <i>Cerdocyon thous</i>         | 15L   | Negative | 0 | 0     | Negative | N/A |
| <i>Leopardus pardalis</i>      | 16L   | Negative | 0 | 0     | Negative | N/A |
| <i>Cerdocyon thous</i>         | 17L   | Negative | 0 | 0     | Negative | N/A |
| <i>Cerdocyon thous</i>         | 19L   | Negative | 0 | 0     | Negative | N/A |
| <i>Leopardus pardalis</i>      | 23L   | Positive | 1 | 0.356 | Negative | N/A |
| <i>Cerdocyon thous</i>         | 24L   | Negative | 0 | 0     | Negative | N/A |
| <i>Cerdocyon thous</i>         | 26L   | Negative | 0 | 0     | Negative | N/A |
| <i>Puma concolor</i>           | 06P   | Negative | 0 | 0     | Negative | N/A |
| <i>Puma concolor</i>           | 08P   | Negative | 0 | 0     | Negative | N/A |
| <i>Puma concolor</i>           | 18P   | Negative | 0 | 0     | Negative | N/A |
| <i>Myrmecophaga tridactyla</i> | 97T   | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 21B   | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 23B   | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 25B   | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 27B   | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 30B   | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 31B   | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 32B   | Positive | 1 | 0.362 | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 33B   | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 34B   | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 36B   | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 42B   | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 44B   | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 53B   | Positive | 1 | 0.356 | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 54B   | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 57B   | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 59B   | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 60B   | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 66B   | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 89B   | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 90B   | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 99B   | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 105B  | Positive | 2 | 0.712 | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 111B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 113B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 117B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 123B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 124B  | Positive | 1 | 0.361 | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 126B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 127B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 135B  | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>  | 160B  | Negative | 0 | 0     | Negative | N/A |

|                                      |      |          |   |       |          |     |
|--------------------------------------|------|----------|---|-------|----------|-----|
| <i>Blastocerus dichotomus</i>        | 229B | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>        | 230B | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>        | 235B | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>        | 238B | Positive | 2 | 0.713 | Negative | N/A |
| <i>Blastocerus dichotomus</i>        | 241B | Negative | 0 | 0     | Negative | N/A |
| <i>Blastocerus dichotomus</i>        | 265B | Negative | 0 | 0     | Negative | N/A |
| <i>Tapirus terrestris</i>            | 3A   | Negative | 0 | 0     | Negative | N/A |
| <i>Tapirus terrestris</i>            | 5A   | Negative | 0 | 0     | Negative | N/A |
| <i>Tapirus terrestris</i>            | 6A   | Negative | 0 | 0     | Negative | N/A |
| <i>Tapirus terrestris</i>            | 21A  | Negative | 0 | 0     | Negative | N/A |
| <i>Tapirus terrestris</i>            | 95A  | Negative | 0 | 0     | Negative | N/A |
| <i>Tapirus terrestris</i>            | 106A | Positive | 1 |       | Negative | N/A |
| <i>Tapirus terrestris</i>            | 104A | Negative | 0 | 0     | Negative | N/A |
| <i>Tapirus terrestris</i>            | 107A | Negative | 0 | 0     | Negative | N/A |
| <i>Myrmecophaga tridactyla</i>       | 163T | Negative | 0 | 0     | Negative | N/A |
| <i>Myrmecophaga tridactyla</i>       | 172T | Negative | 0 | 0     | Negative | N/A |
| <i>Myrmecophaga tridactyla</i>       | 202T | Negative | 0 | 0     | Negative | N/A |
| <i>Myrmecophaga tridactyla</i>       | 178T | Negative | 0 | 0     | Negative | N/A |
| <i>Euphractus sexcinctus</i>         | 100T | Negative | 0 | 0     | Negative | N/A |
| <i>Euphractus sexcinctus</i>         | 35T  | Negative | 0 | 0     | Negative | N/A |
| <i>Euphractus sexcinctus</i>         | 25T  | Negative | 0 | 0     | Negative | N/A |
| <i>Tamandua tetradactyla</i>         | 101T | Negative | 0 | 0     | Negative | N/A |
| <i>Tamandua tetradactyla</i>         | 133T | Negative | 0 | 0     | Negative | N/A |
| <i>Tamandua tetradactyla</i>         | 115T | Negative | 0 | 0     | Negative | N/A |
| <i>Tamandua tetradactyla</i>         | 57T  | Negative | 0 | 0     | Negative | N/A |
| <i>Tamandua tetradactyla</i>         | 73T  | Negative | 0 | 0     | Negative | N/A |
| <i>Prionomys maximus</i>             | 135T | Negative | 0 | 0     | Negative | N/A |
| <i>Prionomys maximus</i>             | 145T | Negative | 0 | 0     | Negative | N/A |
| <i>Euphractus sexcinctus</i>         | 150T | Positive | 1 | 0.360 | Negative | N/A |
| <i>Prionomys maximus</i>             | 154T | Positive | 1 | 0.357 | Negative | N/A |
| <i>Dasypus novemcinctus</i>          | 41T  | Positive | 1 | 0.357 | Negative | N/A |
| <i>Dasypus novemcinctus</i>          | 42T  | Negative | 0 | 0     | Negative | N/A |
| <i>Dasypus novemcinctus</i>          | 88T  | Positive | 1 | 0.356 | Negative | N/A |
| <i>Oecomys mamorae</i>               | 58R  | Negative | 0 | 0     | Negative | N/A |
| <i>Oecomys mamorae</i>               | 18R  | Negative | 0 | 0     | Negative | N/A |
| <i>Oecomys mamorae</i>               | 48R  | Negative | 0 | 0     | Negative | N/A |
| <i>Oecomys mamorae</i>               | 84R  | Negative | 0 | 0     | Negative | N/A |
| <i>Thrichomys fosteri</i>            | 7R   | Negative | 0 | 0     | Negative | N/A |
| <i>Clyomys laticeps</i>              | 42R  | Positive | 1 | 0.356 | Negative | N/A |
| <i>Thrichomys fosteri</i>            | 43R  | Negative | 0 | 0     | Negative | N/A |
| <i>Thrichomys fosteri</i>            | 13R  | Negative | 0 | 0     | Negative | N/A |
| <i>Thrichomys fosteri</i>            | 60R  | Positive | 1 | 0.358 | Negative | N/A |
| <i>Leptotila verreauxi</i>           | 107C | Negative | 0 | 0     | Negative | N/A |
| <i>Icterus cayanensis</i>            | 72C  | Negative | 0 | 0     | Negative | N/A |
| <i>Lepidocolaptes angustirostris</i> | 59C  | Positive | 1 | 0.357 | Negative | N/A |
| <i>Leptotila verreauxi</i>           | 78C  | Negative | 0 | 0     | Negative | N/A |
| <i>Ramphocelus carbo</i>             | 90C  | Negative | 0 | 0     | Negative | N/A |
| <i>Turdus leocomelas</i>             | 85C  | Negative | 0 | 0     | Negative | N/A |
| <i>Fumarius rufus</i>                | 119C | Negative | 0 | 0     | Negative | N/A |
| <i>Saltator coerulescens</i>         | 120C | Positive | 1 | 0.356 | Negative | N/A |
| <i>Furnarius leucopus</i>            | 117C | Negative | 0 | 0     | Negative | N/A |



|                            |       |          |   |       |          |     |
|----------------------------|-------|----------|---|-------|----------|-----|
| <i>Cyanocorax chryrops</i> | 124C  | Negative | 0 | 0     | Negative | N/A |
| <i>Desmodus rotundus</i>   | 162MO | Negative | 0 | 0     | Negative | N/A |
| <i>Desmodus rotundus</i>   | 180MO | Positive | 1 | 0.356 | Negative | N/A |
| <i>Desmodus rotundus</i>   | 158MO | Negative | 0 | 0     | Negative | N/A |
| <i>Desmodus rotundus</i>   | 223MO | Negative | 0 | 0     | Negative | N/A |
| <i>Desmodus rotundus</i>   | 200MO | Negative | 0 | 0     | Negative | N/A |
| <i>Desmodus rotundus</i>   | 224MO | Negative | 0 | 0     | Negative | N/A |
| <i>Desmodus rotundus</i>   | 222MO | Negative | 0 | 0     | Negative | N/A |
| <i>Desmodus rotundus</i>   | 201MO | Positive | 3 | 1.070 | Negative | N/A |

**Table S7:** Results obtained for each test carried out for piroplasmids.

| Animal species                 | ID    | Result   | dPCR                |                        | qPCR     |       | nPCR     |
|--------------------------------|-------|----------|---------------------|------------------------|----------|-------|----------|
|                                |       |          | Positive Partitions | Conc (copies/ $\mu$ L) | Result   | CQ    | Result   |
| <i>Subulo gouazoubira</i>      | 182M  | Positive | 20                  | 8.262                  | Negative | N/A   | Positive |
| <i>Mazama juncunda</i>         | 215M  | Positive | 22                  | 8.331                  | Negative | N/A   | Positive |
| <i>Mazama rufa</i>             | 270M  | Positive | 13                  | 4.934                  | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i>  | 63B   | Positive | 476                 | 186                    | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i>  | 75B   | Positive | 36                  | 13.68                  | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i>  | 120B2 | Positive | 121                 | 46.02                  | Positive | 28.51 | Positive |
| <i>Blastocerus dichotomus</i>  | 121B2 | Positive | 55                  | 19.97                  | Positive | 29.25 | Positive |
| <i>Blastocerus dichotomus</i>  | 222B3 | Positive | 125                 | 47.65                  | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i>  | 226B3 | Positive | 12                  | 4.290                  | Negative | N/A   | Positive |
| <i>Desmodus rotundus</i>       | 91MO  | Positive | 8                   | 2.849                  | Negative | N/A   | Positive |
| <i>Desmodus rotundus</i>       | 8MO   | Positive | 2                   | 0.750                  | Negative | N/A   | Positive |
| <i>Cerdocyon thous</i>         | 2L    | Positive | 16                  | 6.065                  | Positive | 31.55 | Positive |
| <i>Leopardus pardalis</i>      | 8L    | Positive | 3333                | 1830.1                 | Positive | 25.52 | Positive |
| <i>Leopardus pardalis</i>      | 22L   | Positive | 162                 | 63.38                  | Negative | N/A   | Positive |
| <i>Cerdocyon thous</i>         | 25L   | Positive | 4                   | 1.560                  | Positive | 33.27 | Positive |
| <i>Myrmecophaga tridactyla</i> | 59T   | Positive | 1                   | 0.392                  | Negative | N/A   | Positive |
| <i>Myrmecophaga tridactyla</i> | 62T   | Negative | 0                   | 0                      | Negative | N/A   | Positive |
| <i>Tamandua tetradactyla</i>   | 71T   | Positive | 5                   | 1.951                  | Positive | 34.61 | Positive |
| <i>Dasypus novemcinctus</i>    | 16T   | Positive | 112                 | 44.69                  | Positive | 29.11 | Positive |
| <i>Priodontes maximus</i>      | 153T  | Positive | 1                   | 0.357                  | Positive | 34.71 | Positive |
| <i>Priodontes maximus</i>      | 159T  | Positive | 4                   | 1.562                  | Negative | N/A   | Positive |
| <i>Priodontes maximus</i>      | 160T  | Positive | 2                   | 1.453                  | Negative | N/A   | Positive |
| <i>Ozotocerus bezoarticus</i>  | 06O   | Positive | 2                   | 0.770                  | Negative | N/A   | Positive |
| <i>Ozotocerus bezoarticus</i>  | 07O   | Positive | 18                  | 6.974                  | Negative | N/A   | Positive |
| <i>Ozotocerus bezoarticus</i>  | 08O   | Positive | 1                   | 0.395                  | Negative | N/A   | Positive |

|                                |      |          |      |        |          |       |          |
|--------------------------------|------|----------|------|--------|----------|-------|----------|
| <i>Ozotocerus bezoarticus</i>  | 09O  | Positive | 7    | 2.733  | Negative | N/A   | Positive |
| <i>Ozotocerus bezoarticus</i>  | 10O  | Positive | 4    | 1.584  | Negative | N/A   | Positive |
| <i>Subulo gouazoubira</i>      | 154M | Positive | 1    | 0.403  | Negative | N/A   | Positive |
| <i>Subulo gouazoubira</i>      | 155M | Positive | 2    | 0.801  | Negative | N/A   | Positive |
| <i>Subulo gouazoubira</i>      | 156M | Negative | 0    | 0.399  | Negative | N/A   | Positive |
| <i>Subulo gouazoubira</i>      | 180M | Positive | 3    | 1.075  | Negative | N/A   | Positive |
| <i>Desmodus rotundus</i>       | 13MO | Positive | 5    | 1.813  | Negative | N/A   | Positive |
| <i>Desmodus rotundus</i>       | 24MO | Positive | 8    | 3.125  | Positive | 33.05 | Positive |
| <i>Desmodus rotundus</i>       | 25MO | Positive | 72   | 27.45  | Negative | N/A   | Positive |
| <i>Desmodus rotundus</i>       | 33MO | Positive | 75   | 28.54  | Negative | N/A   | Positive |
| <i>Desmodus rotundus</i>       | 34MO | Positive | 12   | 4.680  | Negative | N/A   | Positive |
| <i>Desmodus rotundus</i>       | 40MO | Positive | 58   | 22.92  | Negative | N/A   | Positive |
| <i>Desmodus rotundus</i>       | 50MO | Positive | 35   | 13.89  | Negative | N/A   | Positive |
| <i>Desmodus rotundus</i>       | 58MO | Positive | 1    | 0.396  | Negative | N/A   | Positive |
| <i>Desmodus rotundus</i>       | 65MO | Positive | 17   | 6.781  | Negative | N/A   | Positive |
| <i>Desmodus rotundus</i>       | 68MO | Positive | 56   | 22.74  | Positive | 29.49 | Positive |
| <i>Cerdocyon thous</i>         | 1L   | Positive | 6    | 2.407  | Negative | N/A   | Positive |
| <i>Leopardus pardalis</i>      | 4L   | Positive | 363  | 286.7  | Positive | 27.1  | Positive |
| <i>Leopardus pardalis</i>      | 5L   | Positive | 2150 | 987.5  | Positive | 25.55 | Positive |
| <i>Leopardus pardalis</i>      | 11L  | Positive | 1255 | 486.7  | Positive | 26.3  | Positive |
| <i>Leopardus pardalis</i>      | 14L  | Positive | 1751 | 769.6  | Positive | 27.51 | Positive |
| <i>Leopardus pardalis</i>      | 20L  | Positive | 1135 | 477.7  | Positive | 26.42 | Positive |
| <i>Leopardus pardalis</i>      | 21L  | Positive | 3489 | 1784.1 | Positive | 25.12 | Positive |
| <i>Panthera onca</i>           | 2P   | Positive | 133  | 53.97  | Negative | N/A   | Positive |
| <i>Panthera onca</i>           | 3P   | Positive | 147  | 60.26  | Negative | N/A   | Positive |
| <i>Panthera onca</i>           | 4P   | Positive | 966  | 413.8  | Negative | N/A   | Positive |
| <i>Panthera onca</i>           | 5P   | Positive | 64   | 26.29  | Negative | N/A   | Positive |
| <i>Panthera onca</i>           | 7P   | Positive | 387  | 160.9  | Negative | N/A   | Positive |
| <i>Panthera onca</i>           | 9P   | Positive | 1018 | 435.7  | Negative | N/A   | Positive |
| <i>Panthera onca</i>           | 11P  | Positive | 44   | 17.68  | Negative | N/A   | Positive |
| <i>Panthera onca</i>           | 12P  | Positive | 1049 | 443.1  | Negative | N/A   | Positive |
| <i>Panthera onca</i>           | 13P  | Positive | 63   | 24.22  | Positive | 34.49 | Positive |
| <i>Panthera onca</i>           | 14P  | Positive | 911  | 372.3  | Negative | N/A   | Positive |
| <i>Myrmecophaga tridactyla</i> | 51T  | Negative | 0    | 0      | Positive | 22.49 | Positive |
| <i>Myrmecophaga tridactyla</i> | 64T  | Negative | 0    | 0      | Negative | N/A   | Positive |
| <i>Tamandua tetradactyla</i>   | 66T  | Negative | 0    | 0      | Negative | N/A   | Positive |
| <i>Myrmecophaga tridactyla</i> | 69T  | Negative | 0    | 0      | Negative | N/A   | Positive |
| <i>Tamandua tetradactyla</i>   | 72T  | Negative | 0    | 0      | Negative | N/A   | Positive |
| <i>Myrmecophaga tridactyla</i> | 01T  | Positive | 7    | 2.837  | Positive | 35.54 | Positive |
| <i>Myrmecophaga tridactyla</i> | 03T  | Positive | 4    | 1.606  | Positive | 34.89 | Positive |
| <i>Myrmecophaga tridactyla</i> | 05T  | Negative | 0    | 0      | Positive | 36.84 | Positive |

|                                |       |          |     |       |          |       |          |
|--------------------------------|-------|----------|-----|-------|----------|-------|----------|
| <i>Myrmecophaga tridactyla</i> | 06T   | Positive | 1   | 0.398 | Negative | N/A   | Positive |
| <i>Tamandua tetradactyla</i>   | 12T   | Negative | 0   | 0     | Negative | N/A   | Positive |
| <i>Pseudoseisura unirufa</i>   | 18C   | Positive | 3   | 1.071 | Positive | 35.51 | Positive |
| <i>Saltator coerulescens</i>   | 27C   | Positive | 14  | 5.331 | Positive | 33.51 | Positive |
| <i>Turdus leocomelas</i>       | 97C   | Positive | 80  | 32.00 | Negative | N/A   | Positive |
| <i>Cercomacra melanaria</i>    | 98C   | Positive | 115 | 45.37 | Negative | N/A   | Positive |
| <i>Ramphocelus carbo</i>       | 404C  | Negative | 0   | 0     | Negative | N/A   | Positive |
| <i>Basileuterus flaveolus</i>  | 405C  | Positive | 2   | 0.795 | Negative | N/A   | Positive |
| <i>Saltator coerulescens</i>   | 452C  | Negative | 0   | 0     | Negative | N/A   | Positive |
| <i>Leptotila verreauxi</i>     | 453C  | Positive | 1   | 0.401 | Negative | N/A   | Positive |
| <i>Tapirus terrestris</i>      | 1A    | Positive | 37  | 14.63 | Negative | N/A   | Positive |
| <i>Tapirus terrestris</i>      | 2A    | Positive | 345 | 145.8 | Positive | 33.56 | Positive |
| <i>Tapirus terrestris</i>      | 10A   | Positive | 47  | 16.80 | Negative | N/A   | Positive |
| <i>Tapirus terrestris</i>      | 11A   | Positive | 21  | 8.231 | Negative | N/A   | Positive |
| <i>Tapirus terrestris</i>      | 63A   | Positive | 258 | 100.9 | Negative | N/A   | Positive |
| <i>Tapirus terrestris</i>      | 64A   | Positive | 8   | 3.047 | Negative | N/A   | Positive |
| <i>Tapirus terrestris</i>      | 75A   | Positive | 13  | 6.844 | Negative | N/A   | Positive |
| <i>Tapirus terrestris</i>      | 82A   | Positive | 1   | 0.381 | Negative | N/A   | Positive |
| <i>Tapirus terrestris</i>      | 90A   | Positive | 41  | 15.55 | Negative | N/A   | Positive |
| <i>Tapirus terrestris</i>      | 110A  | Positive | 15  | 5.707 | Positive | 35.59 | Positive |
| <i>Tapirus terrestris</i>      | 112A  | Positive | 72  | 27.70 | Positive | 34.84 | Positive |
| <i>Tapirus terrestris</i>      | 114A  | Positive | 12  | 4.595 | Negative | N/A   | Positive |
| <i>Tapirus terrestris</i>      | 118A  | Positive | 22  | 8.341 | Negative | N/A   | Positive |
| <i>Tapirus terrestris</i>      | 119A  | Positive | 15  | 5.659 | Negative | N/A   | Positive |
| <i>Desmodus rotundus</i>       | 176MO | Positive | 7   | 2.585 | Negative | N/A   | Positive |
| <i>Desmodus rotundus</i>       | 182MO | Positive | 12  | 4.615 | Negative | N/A   | Positive |
| <i>Desmodus rotundus</i>       | 214MO | Negative | 0   | 0     | Negative | N/A   | Positive |
| <i>Desmodus rotundus</i>       | 215MO | Positive | 75  | 28.58 | Positive | 29.49 | Positive |
| <i>Desmodus rotundus</i>       | 216MO | Positive | 24  | 8.578 | Negative | N/A   | Positive |
| <i>Desmodus rotundus</i>       | 218MO | Positive | 16  | 6.065 | Positive | 34.69 | Positive |
| <i>Desmodus rotundus</i>       | 219MO | Positive | 3   | 1.154 | Negative | N/A   | Positive |
| <i>Desmodus rotundus</i>       | 220MO | Positive | 6   | 2.261 | Positive | 36.06 | Positive |
| <i>Desmodus rotundus</i>       | 221MO | Positive | 3   | 1.129 | Negative | N/A   | Positive |
| <i>Desmodus rotundus</i>       | 231MO | Positive | 38  | 14.37 | Positive | 35.33 | Positive |
| <i>Desmodus rotundus</i>       | 205MO | Positive | 9   | 3.358 | Positive | 34.29 | Positive |
| <i>Desmodus rotundus</i>       | 19MO  | Positive | 32  | 11.95 | Negative | N/A   | Positive |
| <i>Desmodus rotundus</i>       | 27MO  | Positive | 10  | 3.776 | Positive | 38.36 | Positive |
| <i>Desmodus rotundus</i>       | 35MO  | Positive | 22  | 8.345 | Positive | 35.38 | Positive |
| <i>Desmodus rotundus</i>       | 76MO  | Positive | 10  | 3.866 | Positive | 35.2  | Positive |
| <i>Desmodus rotundus</i>       | 82MO  | Positive | 8   | 3.115 | Negative | N/A   | Positive |
| <i>Desmodus rotundus</i>       | 115MO | Positive | 1   | 0.392 | Negative | N/A   | Positive |
| <i>Desmodus rotundus</i>       | 90MO  | Positive | 1   | 0.390 | Negative | N/A   | Positive |
| <i>Desmodus rotundus</i>       | 170MO | Positive | 3   | 1.168 | Negative | N/A   | Positive |
| <i>Desmodus rotundus</i>       | 179MO | Positive | 32  | 12.66 | Positive | 35.19 | Positive |

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|--------------------------------|-------|----------|------|--------|----------|-------|----------|
| <i>Desmodus rotundus</i>       | 207MO | Positive | 2    | 0.713  | Negative | N/A   | Positive |
| <i>Desmodus rotundus</i>       | 63MO  | Positive | 812  | 484.3  | Positive | 35.31 | Positive |
| <i>Desmodus rotundus</i>       | 70MO  | Positive | 5    | 1.781  | Positive | 36.06 | Positive |
| <i>Desmodus rotundus</i>       | 88MO  | Positive | 3    | 1.129  | Negative | N/A   | Positive |
| <i>Desmodus rotundus</i>       | 92MO  | Negative | 0    | 0      | Negative | N/A   | Positive |
| <i>Leopardus pardalis</i>      | 9L    | Positive | 2302 | 1156.5 | Positive | 25.87 | Positive |
| <i>Panthera onca</i>           | 15P   | Positive | 1826 | 1295.7 | Positive | 27.74 | Positive |
| <i>Panthera onca</i>           | 16P   | Positive | 38   | 14.81  | Negative | N/A   | Positive |
| <i>Panthera onca</i>           | 17P   | Positive | 1583 | 818.6  | Negative | N/A   | Positive |
| <i>Panthera onca</i>           | 19P   | Positive | 1351 | 1265.0 | Positive | 28.17 | Positive |
| <i>Panthera onca</i>           | 20P   | Positive | 1167 | 567.9  | Negative | N/A   | Positive |
| <i>Panthera onca</i>           | 21P   | Positive | 50   | 19.99  | Positive | 30.21 | Positive |
| <i>Panthera onca</i>           | 22P   | Positive | 51   | 19.93  | Positive | 30.26 | Positive |
| <i>Panthera onca</i>           | 23P   | Positive | 58   | 22.84  | Positive | 29.87 | Positive |
| <i>Panthera onca</i>           | 24P   | Positive | 67   | 26.20  | Positive | 29.41 | Positive |
| <i>Panthera onca</i>           | 25P   | Positive | 38   | 14.41  | Positive | 30.17 | Positive |
| <i>Myrmecophaga tridactyla</i> | 17T   | Positive | 1    | 0.378  | Positive | 41.27 | Positive |
| <i>Euphractus sexcinctus</i>   | 32T   | Positive | 7    | 2.724  | Positive | 35    | Positive |
| <i>Myrmecophaga tridactyla</i> | 50T   | Negative | 0    | 0      | Negative | N/A   | Positive |
| <i>Dasypus novemcinctus</i>    | 76T   | Negative | 0    | 0      | Positive | 39.78 | Positive |
| <i>Dasypus novemcinctus</i>    | 79T   | Negative | 0    | 0      | Positive | 38.36 | Positive |
| <i>Myrmecophaga tridactyla</i> | 81T   | Positive | 3    | 1.192  | Negative | N/A   | Positive |
| <i>Dasypus novemcinctus</i>    | 149T  | Positive | 1    | 0.404  | Negative | N/A   | Positive |
| <i>Tapirus terrestris</i>      | 122A  | Positive | 9    | 3.616  | Negative | N/A   | Positive |
| <i>Tapirus terrestris</i>      | 124A  | Positive | 7    | 2.787  | Negative | N/A   | Positive |
| <i>Subulo gouazoubira</i>      | 196M  | Positive | 14   | 5.489  | Negative | N/A   | Positive |
| <i>Mazama juncunda</i>         | 204M  | Positive | 16   | 6.242  | Negative | N/A   | Positive |
| <i>Mazama juncunda</i>         | 208M  | Positive | 7    | 2.671  | Negative | N/A   | Positive |
| <i>Subulo gouazoubira</i>      | 209M  | Positive | 2    | 0.766  | Negative | N/A   | Positive |
| <i>Mazama juncunda</i>         | 210M  | Positive | 7    | 2.729  | Negative | N/A   | Positive |
| <i>Mazama rufa</i>             | 267M  | Negative | 0    | 0      | Negative | N/A   | Positive |
| <i>Mazama rufa</i>             | 268M  | Positive | 6    | 2.365  | Negative | N/A   | Positive |
| <i>Subulo gouazoubira</i>      | 298M  | Positive | 3    | 1.216  | Negative | N/A   | Positive |
| <i>Subulo gouazoubira</i>      | 299M  | Positive | 22   | 8.842  | Negative | N/A   | Positive |
| <i>Subulo gouazoubira</i>      | 300M  | Negative | 0    | 0      | Positive | 17.37 | Positive |
| <i>Subulo gouazoubira</i>      | 301M  | Positive | 11   | 4.462  | Negative | N/A   | Positive |
| <i>Subulo gouazoubira</i>      | 302M  | Positive | 3    | 1.201  | Negative | N/A   | Positive |
| <i>Subulo gouazoubira</i>      | 303M  | Negative | 0    | 0      | Negative | N/A   | Positive |
| <i>Subulo gouazoubira</i>      | 307M  | Positive | 6    | 2.362  | Negative | N/A   | Positive |
| <i>Subulo gouazoubira</i>      | 313M  | Positive | 3    | 1.077  | Negative | N/A   | Positive |
| <i>Subulo gouazoubira</i>      | 314M  | Positive | 4    | 1.541  | Negative | N/A   | Positive |
| <i>Subulo gouazoubira</i>      | 315M  | Positive | 9    | 3.480  | Negative | N/A   | Positive |
| <i>Subulo gouazoubira</i>      | 316M  | Positive | 1    | 0.395  | Negative | N/A   | Positive |

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| <i>Subulo gouazoubira</i>     | 317M | Positive | 4    | 1.610  | Positive | 43.57 | Positive |
| <i>Subulo gouazoubira</i>     | 318M | Positive | 2    | 0.713  | Negative | N/A   | Positive |
| <i>Subulo gouazoubira</i>     | 319M | Positive | 5    | 2.007  | Negative | N/A   | Positive |
| <i>Subulo gouazoubira</i>     | 320M | Positive | 27   | 10.94  | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 35B  | Positive | 3    | 1.201  | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 71B  | Negative | 0    | 0      | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 78B  | Positive | 4    | 1.588  | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 79B  | Positive | 3    | 1.067  | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 94B  | Positive | 2    | 0.718  | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 104B | Positive | 1    | 0.379  | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 24B  | Positive | 8    | 3.122  | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 25B  | Positive | 231  | 91.70  | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 26B  | Positive | 221  | 87.58  | Positive | 28.99 | Positive |
| <i>Blastocerus dichotomus</i> | 28B  | Positive | 67   | 26.71  | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 29B  | Positive | 40   | 16.02  | Positive | 34.11 | Positive |
| <i>Blastocerus dichotomus</i> | 37B  | Positive | 3    | 1.200  | Positive | 31.82 | Positive |
| <i>Blastocerus dichotomus</i> | 38B  | Positive | 274  | 116.1  | Positive | 29.08 | Positive |
| <i>Blastocerus dichotomus</i> | 40B  | Positive | 16   | 6.245  | Positive | 18.9  | Positive |
| <i>Blastocerus dichotomus</i> | 41B  | Positive | 3498 | 2273.2 | Positive | 26.96 | Positive |
| <i>Blastocerus dichotomus</i> | 43B  | Positive | 17   | 6.056  | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 45B  | Positive | 240  | 86.94  | Positive | 28.43 | Positive |
| <i>Blastocerus dichotomus</i> | 46B  | Positive | 4    | 1.484  | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 48B  | Positive | 109  | 41.10  | Positive | 28.91 | Positive |
| <i>Blastocerus dichotomus</i> | 49B  | Positive | 740  | 412.2  | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 51B  | Positive | 3    | 1.132  | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 52B  | Positive | 172  | 67.51  | Positive | 28.51 | Positive |
| <i>Blastocerus dichotomus</i> | 55B  | Positive | 3    | 1.150  | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 64B  | Positive | 8260 | 2.496  | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 68B  | Positive | 2    | 0.756  | Positive | 35.76 | Positive |
| <i>Blastocerus dichotomus</i> | 76B  | Positive | 29   | 10.94  | Negative | N/A   | Positive |
| <i>Desmodus rotundus</i>      | 36MO | Positive | 54   | 19.47  | Negative | N/A   | Positive |
| <i>Desmodus rotundus</i>      | 43MO | Positive | 1    | 0.366  | Negative | N/A   | Positive |
| <i>Desmodus rotundus</i>      | 44MO | Positive | 23   | 8.221  | Negative | N/A   | Positive |
| <i>Desmodus rotundus</i>      | 47MO | Positive | 57   | 20.41  | Positive | 35.87 | Positive |
| <i>Desmodus rotundus</i>      | 54MO | Positive | 3    | 1.084  | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 77B  | Positive | 4    | 1.441  | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 78B  | Positive | 1082 | 414.9  | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 80B  | Positive | 275  | 101.6  | Positive | 31.19 | Positive |
| <i>Blastocerus dichotomus</i> | 81B  | Positive | 10   | 3.561  | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 83B  | Positive | 32   | 11.69  | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 84B  | Positive | 9    | 3.256  | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 86B  | Positive | 47   | 17.20  | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 87B  | Positive | 7    | 2.492  | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 88B  | Positive | 1396 | 546.4  | Positive | 38.14 | Positive |

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| <i>Blastocerus dichotomus</i> | 92B   | Positive | 46   | 16.46 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 93B   | Positive | 74   | 26.77 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 98B   | Positive | 7    | 3.168 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 100B  | Positive | 103  | 36.94 | Positive | 35.15 | Positive |
| <i>Blastocerus dichotomus</i> | 102B  | Positive | 91   | 32.51 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 103B  | Positive | 115  | 41.31 | Positive | 32.24 | Positive |
| <i>Blastocerus dichotomus</i> | 106B  | Positive | 9    | 3.204 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 107B  | Positive | 33   | 11.78 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 108B  | Positive | 39   | 13.92 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 110B  | Positive | 26   | 9.284 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 112B  | Positive | 1131 | 433.5 | Positive | 38.81 | Positive |
| <i>Blastocerus dichotomus</i> | 128B  | Positive | 257  | 92.84 | Positive | 29.66 | Positive |
| <i>Blastocerus dichotomus</i> | 130B  | Positive | 16   | 5.718 | Positive | 38.8  | Positive |
| <i>Blastocerus dichotomus</i> | 131B  | Positive | 60   | 21.44 | Positive | 35.72 | Positive |
| <i>Blastocerus dichotomus</i> | 134B  | Positive | 21   | 8.231 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 136B  | Positive | 19   | 6.767 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 138B  | Positive | 8    | 2.854 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 25B1  | Positive | 45   | 16.08 | Positive | 32.82 | Positive |
| <i>Blastocerus dichotomus</i> | 50B1  | Positive | 247  | 89.43 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 58B1  | Positive | 1    | 0.356 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 65B1  | Positive | 6    | 2.139 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 74B1  | Positive | 16   | 5.727 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 82B1  | Positive | 9    | 3.221 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 95B1  | Positive | 23   | 8.220 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 129B1 | Positive | 168  | 60.57 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 101B1 | Positive | 9    | 3.206 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 32B2  | Positive | 48   | 17.19 | Positive | 35.32 | Positive |
| <i>Blastocerus dichotomus</i> | 72B2  | Positive | 153  | 55.00 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 85B2  | Positive | 20   | 7.145 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 97B2  | Positive | 3    | 1.068 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 109B2 | Positive | 13   | 4.636 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 113B2 | Positive | 83   | 29.66 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 114B2 | Positive | 217  | 78.28 | Positive | 29    | Positive |
| <i>Blastocerus dichotomus</i> | 115B2 | Positive | 1226 | 479.4 | Positive | 27.2  | Positive |
| <i>Blastocerus dichotomus</i> | 116B2 | Positive | 159  | 57.44 | Positive | 29.74 | Positive |
| <i>Blastocerus dichotomus</i> | 117B2 | Positive | 52   | 18.60 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 118B2 | Positive | 35   | 12.62 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 122B2 | Positive | 84   | 30.05 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 201B3 | Positive | 5    | 1.782 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 205B3 | Positive | 1    | 0.357 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 209B3 | Positive | 65   | 23.22 | Positive | 41.89 | Positive |
| <i>Blastocerus dichotomus</i> | 207B3 | Positive | 80   | 28.60 | Positive | 36.49 | Positive |
| <i>Blastocerus dichotomus</i> | 208B3 | Positive | 90   | 32.37 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i> | 206B3 | Positive | 12   | 4.303 | Negative | N/A   | Positive |

|                                |       |          |     |       |          |       |          |
|--------------------------------|-------|----------|-----|-------|----------|-------|----------|
| <i>Blastocerus dichotomus</i>  | 212B3 | Positive | 12  | 4.283 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i>  | 213B3 | Positive | 42  | 15.29 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i>  | 208MO | Negative | 0   | 0     | Negative | N/A   | Negative |
| <i>Blastocerus dichotomus</i>  | 217B3 | Positive | 22  | 7.878 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i>  | 218B3 | Positive | 11  | 3.943 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i>  | 227B3 | Positive | 51  | 18.18 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i>  | 228B3 | Negative | 0   | 0     | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i>  | 231B3 | Positive | 13  | 4.637 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i>  | 237B3 | Negative | 0   | 0     | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i>  | 240B3 | Positive | 7   | 2.511 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i>  | 245B3 | Positive | 22  | 7.867 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i>  | 246B3 | Positive | 116 | 41.67 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i>  | 234B3 | Negative | 0   | 0     | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i>  | 284B3 | Positive | 3   | 1.068 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i>  | 139B3 | Positive | 11  | 3.930 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i>  | 236B3 | Positive | 4   | 1.423 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i>  | 264B3 | Positive | 7   | 2.495 | Negative | N/A   | Positive |
| <i>Blastocerus dichotomus</i>  | 67B2  | Positive | 1   | 0.356 | Negative | N/A   | Positive |
| <i>Desmodus rotundus</i>       | 202MO | Positive | 1   | 0.357 | Positive | 36.13 | Negative |
| <i>Cerdocyon thous</i>         | 3L    | Positive | 3   | 1.069 | Negative | N/A   | Negative |
| <i>Leopardus pardalis</i>      | 7L    | Positive | 4   | 1.432 | Negative | N/A   | Negative |
| <i>Cerdocyon thous</i>         | 10L   | Positive | 3   | 1.071 | Positive | 37.06 | Negative |
| <i>Cerdocyon thous</i>         | 13L   | Positive | 4   | 1.423 | Positive | 35.39 | Negative |
| <i>Cerdocyon thous</i>         | 15L   | Positive | 4   | 1.427 | Positive | 36.25 | Negative |
| <i>Leopardus pardalis</i>      | 16L   | Positive | 6   | 2.136 | Positive | 37.74 | Negative |
| <i>Cerdocyon thous</i>         | 17L   | Positive | 2   | 0.713 | Positive | 36.13 | Negative |
| <i>Cerdocyon thous</i>         | 19L   | Positive | 1   | 0.357 | Positive | 37.07 | Negative |
| <i>Leopardus pardalis</i>      | 23L   | Positive | 5   | 1.782 | Positive | 38.62 | Negative |
| <i>Cerdocyon thous</i>         | 24L   | Positive | 4   | 1.425 | Positive | 38.39 | Negative |
| <i>Cerdocyon thous</i>         | 26L   | Positive | 1   | 0.357 | Positive | 37.2  | Negative |
| <i>Puma concolor</i>           | 06P   | Positive | 52  | 18.62 | Negative | N/A   | Negative |
| <i>Puma concolor</i>           | 08P   | Positive | 75  | 26.93 | Negative | N/A   | Negative |
| <i>Puma concolor</i>           | 18P   | Positive | 56  | 20.05 | Positive | 35.24 | Negative |
| <i>Myrmecophaga tridactyla</i> | 97T   | Negative | 0   | 0     | Negative | N/A   | Negative |
| <i>Blastocerus dichotomus</i>  | 21B   | Negative | 0   | 0     | Negative | N/A   | Negative |
| <i>Blastocerus dichotomus</i>  | 23B   | Negative | 0   | 0     | Positive | 40.62 | Negative |
| <i>Blastocerus dichotomus</i>  | 25B   | Positive | 554 | 206.2 | Positive | 29.38 | Negative |
| <i>Blastocerus dichotomus</i>  | 27B   | Negative | 0   | 0     | Negative | N/A   | Negative |
| <i>Blastocerus dichotomus</i>  | 30B   | Positive | 653 | 243.9 | Positive | 29.11 | Negative |
| <i>Blastocerus dichotomus</i>  | 31B   | Positive | 297 | 108.4 | Positive | 29.58 | Negative |
| <i>Blastocerus dichotomus</i>  | 32B   | Positive | 2   | 0.723 | Negative | N/A   | Negative |
| <i>Blastocerus dichotomus</i>  | 33B   | Positive | 214 | 83.69 | Positive | 29.75 | Negative |
| <i>Blastocerus dichotomus</i>  | 34B   | Positive | 106 | 38.18 | Positive | 31.41 | Negative |
| <i>Blastocerus dichotomus</i>  | 36B   | Negative | 0   | 0     | Negative | N/A   | Negative |

|                                |      |          |      |        |          |       |          |
|--------------------------------|------|----------|------|--------|----------|-------|----------|
| <i>Blastocerus dichotomus</i>  | 42B  | Negative | 0    | 0      | Negative | N/A   | Negative |
| <i>Blastocerus dichotomus</i>  | 44B  | Positive | 138  | 49.77  | Positive | 39.16 | Negative |
| <i>Blastocerus dichotomus</i>  | 53B  | Positive | 325  | 118.1  | Positive | 30.57 | Negative |
| <i>Blastocerus dichotomus</i>  | 54B  | Negative | 0    | 0      | Negative | N/A   | Negative |
| <i>Blastocerus dichotomus</i>  | 57B  | Positive | 5    | 1.782  | Negative | N/A   | Negative |
| <i>Blastocerus dichotomus</i>  | 59B  | Negative | 0    | 0      | Negative | N/A   | Negative |
| <i>Blastocerus dichotomus</i>  | 60B  | Positive | 1    | 0.357  | Negative | N/A   | Negative |
| <i>Blastocerus dichotomus</i>  | 66B  | Positive | 83   | 29.74  | Positive | 32.4  | Negative |
| <i>Blastocerus dichotomus</i>  | 89B  | Positive | 4678 | 2921.6 | Positive | 32.94 | Negative |
| <i>Blastocerus dichotomus</i>  | 90B  | Positive | 520  | 191.1  | Positive | 30.46 | Negative |
| <i>Blastocerus dichotomus</i>  | 99B  | Negative | 0    | 0      | Negative | N/A   | Negative |
| <i>Blastocerus dichotomus</i>  | 105B | Positive | 9    | 3.204  | Negative | N/A   | Negative |
| <i>Blastocerus dichotomus</i>  | 111B | Positive | 2105 | 869.4  | Positive | 29.37 | Negative |
| <i>Blastocerus dichotomus</i>  | 113B | Positive | 2076 | 856.1  | Positive | 29.36 | Negative |
| <i>Blastocerus dichotomus</i>  | 117B | Positive | 83   | 29.74  | Positive | 32.51 | Negative |
| <i>Blastocerus dichotomus</i>  | 123B | Positive | 148  | 53.29  | Positive | 32.61 | Negative |
| <i>Blastocerus dichotomus</i>  | 124B | Positive | 1    | 0.361  | Negative | N/A   | Negative |
| <i>Blastocerus dichotomus</i>  | 126B | Negative | 0    | 0      | Negative | N/A   | Negative |
| <i>Blastocerus dichotomus</i>  | 127B | Positive | 2    | 0.713  | Positive | 39.71 | Negative |
| <i>Blastocerus dichotomus</i>  | 135B | Negative | 0    | 0      | Positive | 40.53 | Negative |
| <i>Blastocerus dichotomus</i>  | 160B | Negative | 0    | 0      | Negative | N/A   | Negative |
| <i>Blastocerus dichotomus</i>  | 229B | Positive | 163  | 58.70  | Positive | 29.13 | Negative |
| <i>Blastocerus dichotomus</i>  | 230B | Positive | 1133 | 435.1  | Positive | 32    | Negative |
| <i>Blastocerus dichotomus</i>  | 235B | Positive | 631  | 234.6  | Positive | 29.58 | Negative |
| <i>Blastocerus dichotomus</i>  | 238B | Positive | 323  | 117.5  | Positive | 30.58 | Negative |
| <i>Blastocerus dichotomus</i>  | 241B | Positive | 1754 | 713.4  | Positive | 27.43 | Negative |
| <i>Blastocerus dichotomus</i>  | 265B | Positive | 1    | 0.357  | Negative | N/A   | Negative |
| <i>Tapirus terrestris</i>      | 3A   | Positive | 15   | 5.346  | Negative | N/A   | Negative |
| <i>Tapirus terrestris</i>      | 5A   | Positive | 386  | 153.8  | Positive | 37.33 | Negative |
| <i>Tapirus terrestris</i>      | 6A   | Positive | 104  | 37.27  | Negative | N/A   | Negative |
| <i>Tapirus terrestris</i>      | 21A  | Positive | 14   | 4.989  | Negative | N/A   | Negative |
| <i>Tapirus terrestris</i>      | 95A  | Positive | 61   | 21.79  | Negative | N/A   | Negative |
| <i>Tapirus terrestris</i>      | 106A | Positive | 5    | 1.782  | Negative | N/A   | Negative |
| <i>Tapirus terrestris</i>      | 104A | Positive | 11   | 3.920  | Negative | N/A   | Negative |
| <i>Tapirus terrestris</i>      | 107A | Positive | 6    | 2.138  | Negative | N/A   | Negative |
| <i>Myrmecophaga tridactyla</i> | 163T | Positive | 12   | 4.276  | Positive | 36.1  | Negative |
| <i>Myrmecophaga tridactyla</i> | 172T | Positive | 8    | 2.848  | Negative | N/A   | Negative |
| <i>Myrmecophaga tridactyla</i> | 202T | Positive | 9    | 3.204  | Negative | N/A   | Negative |
| <i>Myrmecophaga tridactyla</i> | 178T | Positive | 18   | 6.415  | Negative | N/A   | Negative |
| <i>Euphractus sexcinctus</i>   | 100T | Positive | 1    | 0.357  | Positive | 37.01 | Negative |
| <i>Euphractus sexcinctus</i>   | 35T  | Negative | 0    | 0      | Negative | N/A   | Negative |
| <i>Euphractus sexcinctus</i>   | 25T  | Negative | 0    | 0      | Negative | N/A   | Negative |

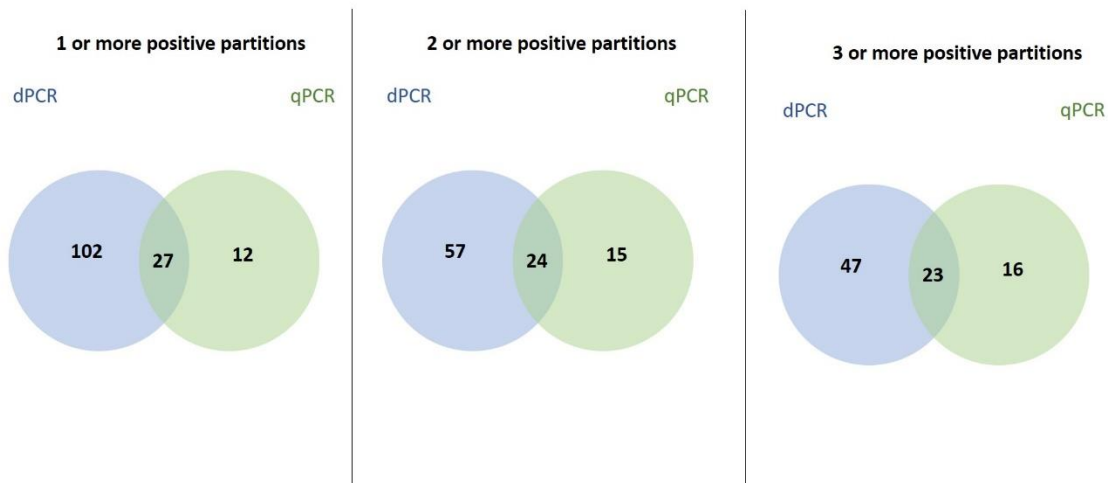
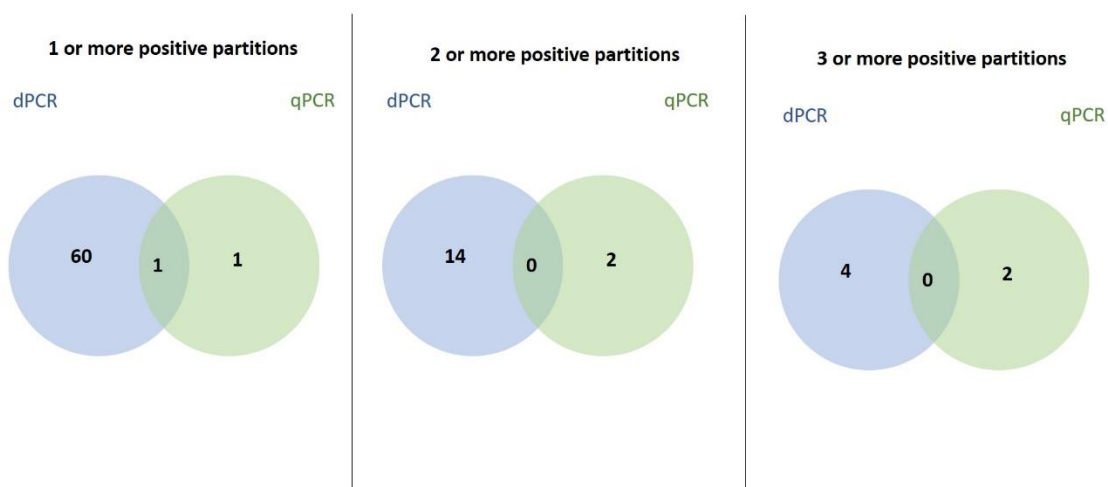


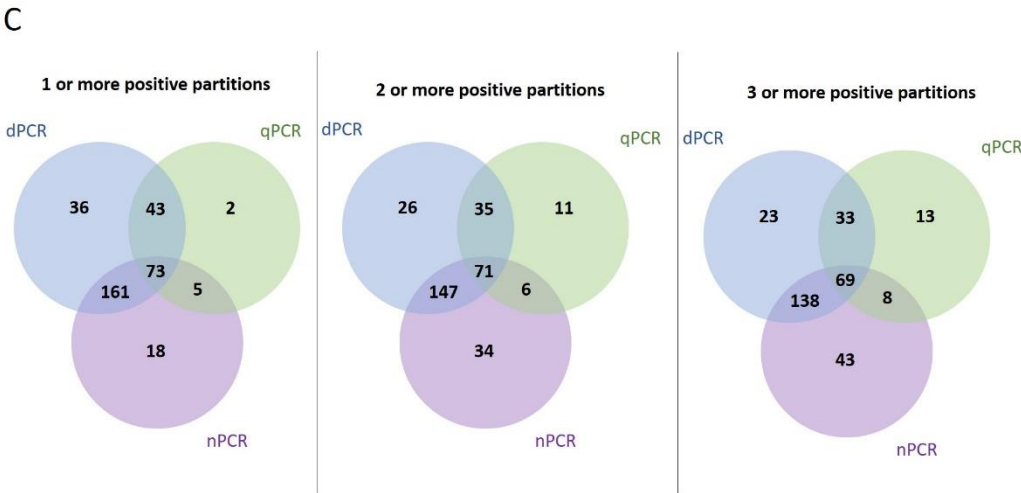
|                                      |       |          |     |       |          |       |          |
|--------------------------------------|-------|----------|-----|-------|----------|-------|----------|
| <i>Tamandua tetradactyla</i>         | 101T  | Negative | 0   | 0     | Negative | N/A   | Negative |
| <i>Tamandua tetradactyla</i>         | 133T  | Positive | 1   | 0.356 | Positive | 43.63 | Negative |
| <i>Tamandua tetradactyla</i>         | 115T  | Negative | 0   | 0     | Negative | N/A   | Negative |
| <i>Tamandua tetradactyla</i>         | 57T   | Negative | 0   | 0     | Negative | N/A   | Negative |
| <i>Tamandua tetradactyla</i>         | 73T   | Negative | 0   | 0     | Negative | N/A   | Negative |
| <i>Priodontes maximus</i>            | 135T  | Negative | 0   | 0     | Negative | N/A   | Negative |
| <i>Priodontes maximus</i>            | 145T  | Positive | 1   | 0.356 | Negative | N/A   | Negative |
| <i>Euphractus sexcinctus</i>         | 150T  | Positive | 1   | 0.360 | Negative | N/A   | Negative |
| <i>Priodontes maximus</i>            | 154T  | Positive | 1   | 0.357 | Negative | N/A   | Negative |
| <i>Dasypus novemcinctus</i>          | 41T   | Negative | 0   | 0     | Negative | N/A   | Negative |
| <i>Dasypus novemcinctus</i>          | 42T   | Negative | 0   | 0     | Negative | N/A   | Negative |
| <i>Dasypus novemcinctus</i>          | 88T   | Negative | 0   | 0     | Negative | N/A   | Negative |
| <i>Oecomys mamorae</i>               | 58R   | Positive | 108 | 38.81 | Positive | 35.42 | Negative |
| <i>Oecomys mamorae</i>               | 18R   | Positive | 56  | 20.05 | Positive | 34.86 | Negative |
| <i>Oecomys mamorae</i>               | 48R   | Positive | 32  | 11.45 | Positive | 35.49 | Negative |
| <i>Oecomys mamorae</i>               | 84R   | Positive | 68  | 24.35 | Negative | N/A   | Negative |
| <i>Thrichomys fosteri</i>            | 7R    | Positive | 91  | 32.58 | Negative | N/A   | Negative |
| <i>Clyomys laticeps</i>              | 42R   | Positive | 124 | 44.53 | Negative | N/A   | Negative |
| <i>Thrichomys fosteri</i>            | 43R   | Positive | 45  | 16.09 | Positive | 38.03 | Negative |
| <i>Thrichomys fosteri</i>            | 13R   | Positive | 44  | 15.90 | Negative | N/A   | Negative |
| <i>Thrichomys fosteri</i>            | 60R   | Positive | 14  | 5.013 | Negative | N/A   | Negative |
| <i>Leptotila verreauxi</i>           | 107C  | Positive | 1   | 0.360 | Positive | 40.74 | Negative |
| <i>Icterus cayanensis</i>            | 72C   | Positive | 1   | 0.357 | Positive | 37.42 | Negative |
| <i>Lepidocolaptes angustirostris</i> | 59C   | Positive | 1   | 0.357 | Negative | N/A   | Negative |
| <i>Leptotila verreauxi</i>           | 78C   | Negative | 0   | 0     | Negative | N/A   | Negative |
| <i>Ramphocelus carbo</i>             | 90C   | Negative | 0   | 0     | Negative | N/A   | Negative |
| <i>Turdus leocomelas</i>             | 85C   | Negative | 0   | 0     | Negative | N/A   | Negative |
| <i>Fumarius rufus</i>                | 119C  | Positive | 2   | 0.714 | Negative | N/A   | Negative |
| <i>Saltator coerulescens</i>         | 120C  | Positive | 1   | 0.356 | Positive | 38.9  | Negative |
| <i>Furnarius leucopus</i>            | 117C  | Positive | 2   | 0.713 | Negative | N/A   | Negative |
| <i>Cyanocorax chrysops</i>           | 124C  | Positive | 1   | 0.356 | Positive | 40.71 | Negative |
| <i>Desmodus rotundus</i>             | 162MO | Negative | 0   | 0     | Negative | N/A   | Negative |
| <i>Desmodus rotundus</i>             | 180MO | Negative | 0   | 0     | Negative | N/A   | Negative |
| <i>Desmodus rotundus</i>             | 158MO | Positive | 2   | 0.712 | Negative | N/A   | Negative |
| <i>Desmodus rotundus</i>             | 223MO | Positive | 1   | 0.357 | Negative | N/A   | Negative |
| <i>Desmodus rotundus</i>             | 200MO | Positive | 3   | 1.070 | Positive | 40.9  | Negative |
| <i>Desmodus rotundus</i>             | 224MO | Positive | 1   | 0.357 | Negative | N/A   | Negative |
| <i>Desmodus rotundus</i>             | 222MO | Positive | 4   | 1.429 | Negative | N/A   | Negative |
| <i>Desmodus rotundus</i>             | 201MO | Positive | 3   | 1.070 | Negative | N/A   | Negative |

#### Supplementary Material 4

Venn diagrams comparing the positivity between different techniques for the detection of *Bartonella* spp. (A), *Borrelia* spp. (B), and piroplasmids (C), considering samples as

positive in dPCR when presenting one or more, two or more, or three or more positive partitions in the assays performed.

**A****B**



**APÊNDICE F – Material suplementar citado no Capítulo 8**

**CAPÍTULO 8\* - Development of Multiplex Assays for the Identification of Zoonotic *Babesia* Species\***

\* Material correspondente ao artigo científico publicado na revista Pathogens 13:1094, 2024. <https://doi.org/10.3390/pathogens13121094>

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**Table S1.** *Babesia* species currently identified by DNA sequence infecting people worldwide.

| <i>Babesia</i> sp     | Gene     | GenBank accession  | Country     | Reference |
|-----------------------|----------|--------------------|-------------|-----------|
| <i>B. bigemina</i>    | 18S rRNA | OQ607820           | Ecuador     | [4]       |
| <i>B. bigemina</i>    | 18S rRNA | MW227627           | Colombia    | [25]      |
| <i>B. crassa-like</i> | 18S rRNA | MW504968           | France      | [26]      |
| <i>B. crassa-like</i> | 18S rRNA | KX590751, KX590750 | China       | [6]       |
| <i>B. crassa-like</i> | 18S rRNA | MK240324           | Slovenia    | [27]      |
| <i>B. divergens</i>   | 18S rRNA | KF533077           | Spain       | [9]       |
| <i>B. divergens</i>   | 18S rRNA | LC167305           | Netherlands | [8]       |

|                          |                               |                                                                                                                                                                                  |          |          |
|--------------------------|-------------------------------|----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------|----------|
| <i>B. divergens</i>      | 18S rRNA                      | MK510929                                                                                                                                                                         | Russia   | [28]     |
| <i>B. divergens</i>      | 18S rRNA                      | Not available                                                                                                                                                                    | France   | [29]     |
| <i>B. divergens</i>      | 18S rRNA                      | HM355854                                                                                                                                                                         | China    | [30]     |
| <i>B. divergens</i>      | 18S rRNA                      | AJ439713                                                                                                                                                                         | Portugal | [31]     |
| <i>B. divergens</i>      | 18S rRNA                      | GU945501                                                                                                                                                                         | Finland  | [26]     |
| <i>B. divergens</i>      | 18S rRNA                      | MK256977                                                                                                                                                                         | China    | [32]     |
| <i>B. divergens-like</i> | 18S rRNA                      | AY274114                                                                                                                                                                         | USA      | [33]     |
| <i>B. duncani</i>        | 18 SrRNA                      | HQ289870                                                                                                                                                                         | USA      | [34]     |
| <i>B. duncani</i>        | 18S rRNA, ITS2                | AF158700,<br>AF158701,<br>AY027815,<br>AY027816,<br>AF15873,<br>AF158705,<br>AF158704,<br>AY998763,<br>AY998762,<br>AY998761,<br>AY965740,<br>AY965741,<br>AY998766,<br>AY998765 | USA      | [35]     |
| <i>B. microti</i>        | 18S rRNA                      | KT271759                                                                                                                                                                         | Spain    | [36]     |
| <i>B. microti</i>        | suc                           | -                                                                                                                                                                                | USA      | [37]     |
| <i>B. microti</i>        | CT $\eta$ , 18S rRNA          | KT318131,<br>KT318132,<br>KT844553–<br>KT844568                                                                                                                                  | Bolivia  | [22]     |
| <i>B. microti</i>        | 18S rRNA                      | EF413181                                                                                                                                                                         | Germany  | [38]     |
| <i>B. microti</i>        | 18S rRNA                      | Not reported                                                                                                                                                                     | Denmark  | [39]     |
| <i>B. microti</i>        | 18S rRNA                      | KT429729                                                                                                                                                                         | Poland   | [40]     |
| <i>B. microti</i>        | 18S rRNA                      | KJ783438                                                                                                                                                                         | Poland   | [41]     |
| <i>B. microti</i>        | coxA, BMN1-11                 | MW665112-<br>MW665117                                                                                                                                                            | USA      | [21]     |
| <i>B. microti</i>        | 18S rRNA                      | MZ727029                                                                                                                                                                         | USA      | [42, 43] |
| <i>B. microti</i>        | 8S rRNA, <i>cox1</i> ,<br>ITS | MK609547,<br>MK609548                                                                                                                                                            | USA      | [32]     |
| <i>B. microti</i>        | 18S rRNA                      | MH048838                                                                                                                                                                         | Mexico   | [44]     |

*B. microti* ITS1 GU230755 Nicaragua [31]

**Table S2.** Piroplasmida species, animal source, case identification and GenBank Accession numbers generated in this study for the intergenic transcribed spacer regions between 18S rRNA and 5.8S rRNA (ITS1) and 5.8S rRNA and 28S rRNA (ITS2). Note: N/A: not amplified.

| sp.                        | Host                  | Case ID     | ITS1 | GenBank<br>Accession<br>number | ITS2 | GenBank<br>Accession<br>number |
|----------------------------|-----------------------|-------------|------|--------------------------------|------|--------------------------------|
| <i>Babesia canis</i>       | Dog                   | VB20-04614  | Yes  | PQ404823                       | Yes  | PQ404832                       |
| <i>Babesia capreoli</i>    | <i>Ixodes ricinus</i> | UKHSA       | Yes  | PQ404828                       | Yes  | PQ404838                       |
| <i>Babesia</i> sp.<br>Coco | Dog                   | VB21-00891  | Yes  | PQ415085                       | Yes  | PQ415090                       |
| <i>Babesia conradae</i>    | Dog                   | VB23-011673 | No   | N/A                            | Yes  | PQ404842                       |
| <i>Babesia divergens</i>   | Rabbit                | MO-1        | Yes  | PQ404827                       | Yes  | PQ404837                       |
| <i>Babesia duncani</i>     | Hamster               | WA-1        | Yes  | PQ415093                       | No   | N/A                            |
| <i>Babesia felis</i>       | Cat                   | VB17-02733  | Yes  | PQ415084                       | Yes  | PQ415089                       |
| <i>Babesia gibsoni</i>     | Dog                   | VB20-03041  | Yes  | PQ404826                       | Yes  | PQ404836                       |
| <i>Babesia gibsoni</i>     | Dog                   | VB20-05838  | Yes  | PQ404830                       | Yes  | PQ404840                       |
| <i>Babesia lengau</i>      | Cat                   | VB18-01695  | Yes  | PQ415083                       | Yes  | PQ404835                       |
| <i>Babesia microti</i>     | Hamster               | GI-1        | Yes  | PQ404825                       | Yes  | PQ404834                       |
| <i>Babesia negevi</i>      | Dog                   | R2024a      | Yes  | PQ415086                       | Yes  | PQ415091                       |
| <i>Babesia odocoilei</i>   | Caribou               | VB19-09086  | Yes  | PQ404843                       | Yes  | PQ411178                       |
| <i>Babesia odocoilei</i>   | Reindeer              | VB19-09287  | Yes  | PQ404844                       | Yes  | PQ411179                       |
| <i>Babesia odocoilei</i>   | Reindeer              | VB19-09670  | Yes  | PQ404845                       | Yes  | PQ411180                       |
| <i>Babesia vogeli</i>      | Dog                   | VB21-01537  | Yes  | PQ404824                       | Yes  | PQ404833                       |
| <i>Babesia vogeli</i>      | Dog                   | R2024b      | Yes  | PQ404829                       | Yes  | PQ404839                       |

|                           |            |             |     |          |     |          |
|---------------------------|------------|-------------|-----|----------|-----|----------|
| <i>Babesia vulpes</i>     | Dog        | VB21-010325 | Yes | PQ415082 | Yes | PQ415088 |
| <i>Cytauxzoon felis</i>   | Cat        | VB20-03823  | Yes | PQ404831 | Yes | PQ404841 |
| <i>Theileria bicornis</i> | Rhinoceros | VB19-09934  | Yes | PQ415087 | Yes | PQ415092 |

**Table S3.** Assessment of *Babesia* species specificity and cross-amplification with other *Babesia* species for the species-specific ITS1 assay. Note: original refers to DNA extracted from blood from pre-characterized animal clinical samples.

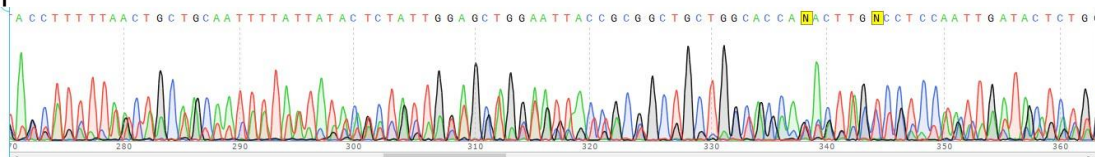
| Piroplasma sp.      | Case ID     | Samples        | qPCR specific assays (Cq) |                     |                   |                   |
|---------------------|-------------|----------------|---------------------------|---------------------|-------------------|-------------------|
|                     |             |                | <i>B. odocoilei</i>       | <i>B. divergens</i> | <i>B. duncani</i> | <i>B. microti</i> |
| <i>B. odocoilei</i> | VB19-09386  | Original       | 23.5                      | Neg                 | Neg               | Neg               |
| <i>B. odocoilei</i> | VB19-09386  | Diluted 1:10   | 27.1                      | Neg                 | Neg               | Neg               |
| <i>B. odocoilei</i> | VB19-09386  | Diluted 1:100  | 31.0                      | Neg                 | Neg               | Neg               |
| <i>B. Divergens</i> | R-1         | Diluted 1:100  | Neg                       | 33.9                | Neg               | Neg               |
| <i>B. Divergens</i> | R-1         | Diluted 1:1000 | Neg                       | 36.9                | Neg               | Neg               |
| <i>B. duncani</i>   | WA-1        | Original       | Neg                       | Neg                 | 26.9              | Neg               |
| <i>B. duncani</i>   | WA-1        | Diluted 1:10   | Neg                       | Neg                 | 31.0              | Neg               |
| <i>B. duncani</i>   | WA-1        | Diluted 1:100  | Neg                       | Neg                 | 35.6              | Neg               |
| <i>B. duncani</i>   | WA-1        | Diluted 1:1000 | Neg                       | Neg                 | 39.3              | Neg               |
| <i>B. microti</i>   | GI-2        | Original       | Neg                       | Neg                 | Neg               | 26.0              |
| <i>B. microti</i>   | GI-2        | Diluted 1:10   | Neg                       | Neg                 | Neg               | 24.5              |
| <i>B. microti</i>   | GI-2        | Diluted 1:100  | Neg                       | Neg                 | Neg               | 37.3              |
| <i>B. microti</i>   | GI-2        | Diluted 1:1000 | Neg                       | Neg                 | Neg               | 33.9              |
| <i>B. canis</i>     | VB18-07290  | Original       | Neg                       | Neg                 | Neg               | Neg               |
| <i>B. canis</i>     | VB20-01614  | Original       | Neg                       | Neg                 | Neg               | Neg               |
| <i>B. coco</i>      | VB20-06987  | Original       | Neg                       | Neg                 | Neg               | Neg               |
| <i>B. coco</i>      | VB17-04969  | Original       | Neg                       | Neg                 | Neg               | Neg               |
| <i>B. vulpes</i>    | VB21-010325 | Original       | Neg                       | Neg                 | Neg               | Neg               |
| <i>B. vulpes</i>    | VB21-01000  | Original       | Neg                       | Neg                 | Neg               | Neg               |
| <i>B. vulpes</i>    | VB22-003333 | Original       | Neg                       | Neg                 | Neg               | Neg               |
| <i>B. vulpes</i>    | VB22-008037 | Original       | Neg                       | Neg                 | Neg               | Neg               |

|                    |             |               |     |     |     |     |
|--------------------|-------------|---------------|-----|-----|-----|-----|
| <i>B. Conradae</i> | VB23-011673 | Diluted 1:100 | Neg | Neg | Neg | Neg |
| <i>B. lengau</i>   | VB18-01695  | Original      | Neg | Neg | Neg | Neg |
| <i>B. gibsoni</i>  | VB20-08351  | Original      | Neg | Neg | Neg | Neg |
| <i>B. gibsoni</i>  | VB20-05838  | Original      | Neg | Neg | Neg | Neg |

### Supplementary Material 5

In preliminary studies, DNA samples were extracted from deer blood and analyzed. First, PCR tests targeting the 18S rRNA gene were conducted. During sequence analysis, a significant number of overlapping peaks were observed, suggesting possible co-infection (Figure S1). The sequences were then trimmed, and the cleanest portion exhibited 99.73% identity with *Theileria cervi* by BLASTn.

**Figure S1.** Electropherogram of an 18S rRNA gene sequence showing the overlapping peaks.



A specific qPCR assay targeting the ITS-1 region of *B. odocoilei* was performed, as described in this manuscript. Some animals tested positive, including one that had a sequence similar to *T. cervi* in the 18S rRNA gene (here named as Deer 4). The PCR product from this animal was sequenced, and the sequence was identical to *B. odocoilei*.

An alignment comparing the 18S rRNA sequences of *T. cervi*, *B. odocoilei*, and the deer samples revealed that the sequences from the deer were indeed closer to *T. cervi* (Figure S2). In contrast, the alignment based on the ITS-1 intergenic region confirmed the presence of *B. odocoilei* (Figure S3).

**Figure S2.** Part of the 18S rRNA alignment of *T. cervi*, *B. odocoilei* and deer sequences. Showing great divergence between *T. cervi* and *B. odocoilei*.

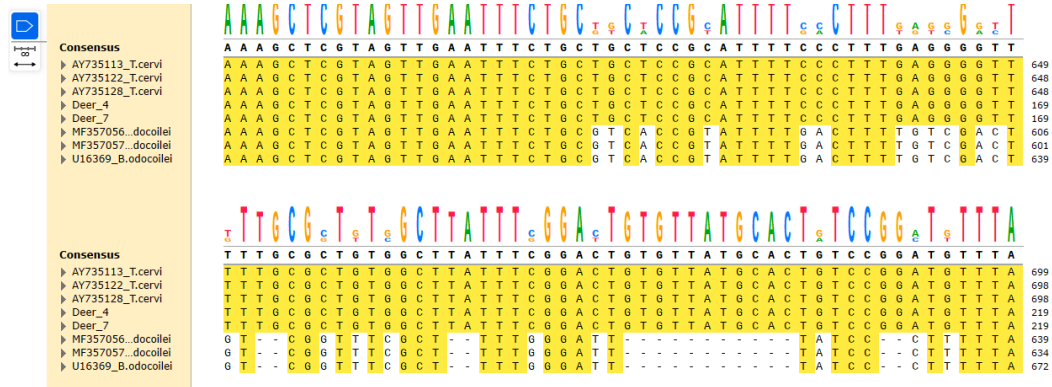


**Figure S3.** Alignment of ITS-1 sequences.

