UNIVERSIDADE ESTADUAL PAULISTA – UNESP

FACULDADE DE CIÊNCIAS AGRÁRIAS E VETERINÁRIAS

CAMPUS DE JABOTICABAL

DETECÇÃO E CARACTERIZAÇÃO MOLECULAR DE AGENTES ANAPLASMATACEAE, BARTONELLACEAE, COXIELLACEAE, MYCOPLASMATACEAE, RICKETTSIACEAE, BABESIIDAE E THEILERIIDAE EM JAVALIS DE VIDA LIVRE NO ESTADO DE SÃO PAULO, BRASIL

Matheus de Souza Santana

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Orientador: Prof. Dr. Marcos Rogério André

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Jaboticabal, 28 de março de 2022

DADOS CURRICULARES DO AUTOR

Matheus de Souza Santana – nascido na cidade de Campo Grande, Mato Grosso do Sul, em 28 de maio de 1996. Graduado em Medicina Veterinária (Bacharelado) pela Universidade Católica Dom Bosco (UCDB), Campo Grande, Mato Grosso do Sul, no ano de 2019. Durante a graduação, foi aluno de Iniciação Científica no laboratório de Engenharia Genética Animal do Departamento de Sanidade Animal, da Embrapa Gado de Corte (CNPGC) pelo programa PIBIC/CNPq, trabalhando na área de Imunologia com ênfase no desenvolvimento de Vacinas de DNA e Subunidade sob orientação das pesquisadoras Dra. Lenita Ramires do Santos e Dra. Grácia Maria Soares Rosinha. No primeiro semestre de 2016 realizou graduação sanduíche na Universidad Austral de Chile (UACH), na qual pôde estagiar no Laboratório Clínico Veterinário, sob orientação da Profa. Dra. Ananda Müller Pereira, na área de Biologia Molecular aplicada à Parasitologia Veterinária. Ingressou no curso de Mestrado em Medicina Veterinária na Universidade Estadual Paulista "Júlio de Mesquita Filho" – Faculdade de Ciências Agrárias e Veterinárias – FCAV/UNESP, Campus de Jaboticabal, em agosto de 2019, inicialmente com bolsa da Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES) e, depois, beneficiado pela bolsa da Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP Processo número 2019/24726-7).

EPÍGRAFE

“A poesia está guardada nas palavras — é tudo que eu sei. Meu fado é o de não saber quase tudo. Sobre o nada eu tenho profundidades. Não tenho conexões com a realidade. Poderoso para mim não é aquele que descobre ouro. Para mim poderoso é aquele que descobre as insignificâncias (do mundo e as nossas). Por essa pequena sentença me elogiaram de imbecil. Fiquei emocionado. Sou fraco para elogio.”

Poema “Tratado geral das grandezas do ínfimo” – Manoel de Barros

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SUMÁRIO

DETECÇÃO MOLECULAR DE AGENTES TRANSMITIDOS POR VETORES EM JAVALIS (*Sus scrofa*) E CARRAPATOS ASSOCIADOS NO BRASIL, COM EVIDÊNCIA DE POSSÍVEIS NOVOS GENÓTIPOS DE *Ehrlichia*, *Anaplasma* E HEMOPLASMAS

RESUMO - O presente estudo objetivou investigar, por meio de técnicas moleculares, a ocorrência de agentes Anaplasmataceae, Bartonellaceae, Rickettsiaceae, Mycoplasmataceae, Coxiellaceae e Babesiidae/Theileriidae em amostras de sangue de javalis selvagens e seus respectivos carrapatos amostrados no sudeste do Brasil. Para tal, foram analisadas 67 amostras de sangue e 265 carrapatos (264 *Amblyomma sculptum* e um *A. ovale*). Na triagem molecular para agentes Anaplasmataceae por meio de ensaio de PCR baseado no gene 16S rRNA, 5,97% amostras de sangue de javalis e 50,54% de carrapatos foram positivas. Em ensaio de PCR para *Ehrlichia* spp. baseado no gene *dsb*, 9,24% dos carrapatos foram positivos. Apesar da baixa ocorrência, um possível novo genótipo 16S rRNA de *Anaplasma* sp. foi detectado nos javalis amostrados. Com base nas análises filogenéticas baseadas nos genes *groEL*, *gltA, sodB* e região ITS (23S-5S), verificou-se que carrapatos *A. sculptum e A. ovale* coletados de javalis carreiam genótipos de *Ehrlichia* spp. filogeneticamente associados a *E. ewingii*, *E. ruminantium*, e novos genótipos de *Ehrlichia* previamente detectados em equinos, catetos e carrapatos coletados de onças-pintadas. Na triagem para hemoplasmas por meio de qPCR para o gene 16S rRNA, 88,06% das amostras de sangue e 8,69% de carrapatos mostraram-se positivas. *Mycoplasma suis, M. parvum* e um possível novo genótipo de hemoplasma foram detectados em javalis na região sudeste do Brasil. Na triagem para *Bartonella* spp. utilizando qPCR baseada no gene *nuoG,* 3,8% das amostras de carrapatos mostraram-se positivas. Inferências filogenéticas alocaram quatro sequências *nuoG* e uma *gltA* de *Bartonella* no mesmo clado de *Bartonella machadoae*. Nenhuma amostra de sangue e carrapato de javalis mostrou-se positiva na qPCR para *Coxiella burnetii* baseada no gene *IS111*. Por outro lado, apenas 1,6% dos carrapatos mostraram-se positivos no ensaio de nPCR para piroplasmídeos baseado no gene 18S rRNA. Uma sequência 18S rRNA detectada em *pool* de ninfas de *A. sculptum* posicionou-se filogeneticamente próxima às sequências *Cytauxzoon felis* detectadas em felinos nos Estados Unidos. *Rickettsia* sp. filogeneticamente associada à *R. belli* foi detectada em um *pool* de ninfas de *A. sculptum*. Trata-se da primeira detecção de hemoplasmas, *B. machadoae* e *Cytauxzoon* spp. em *A. sculptum*. Javalis e carrapatos associados parecem não participar do ciclo epidemiológico de *C. burnetii* na região estudada. Esta espécie de mamífero invasora pode atuar como potencial dispersor de carrapatos infectados com *Ehrlichia* spp., *Bartonella* spp., micoplasmas hemotrópicos e *Cytauxzoon,* podendo trazer implicações epidemiológicas importantes na transmissão de bartoneloses, erliquioses, hemoplasmoses e cytauxzoonose para seres humanos e animais, mais especificamente para equinos, roedores, suínos e gatos.

Palavras-chave: Anaplasmataceae, *Amblyomma* spp., *Bartonella* sp., *Coxiella burnetii, Cytauxzoon* sp., hemoplasmas, *Rickettsia* sp..

MOLECULAR DETECTION OF VECTOR-BORNE AGENTS IN WILD BOARS (*Sus scrofa*) AND ASSOCIATED TICKS FROM BRAZIL, WITH EVIDENCE OF PUTATIVE NEW GENOTYPES OF *Ehrlichia, Anaplasma* AND HEMOPLASMAS

ABSTRACT - The present study aimed to investigate, by molecular techniques, the occurrence of Anaplasmataceae, Bartonellaceae, Rickettsiaceae, Mycoplasmataceae, Coxiellaceae e Babesiidae/Theileriidae in blood samples of free-living wild boars and associated ticks in southeastern Brazil. For this purpose, 67 blood samples and 265 ticks (264 *Amblyomma sculptum* and one *A. ovale*) were analyzed. In the screening for Anaplasmataceae agents by a PCR assay based on the 16S rRNA gene, 5.97% blood samples and 50.54% ticks were positive. In the PCR assay for *Ehrlichia* spp. based on the *dsb* gene, 9.24% of ticks were positive. Despite the low occurrence, a possible new 16S rRNA genotype of *Anaplasma* sp. was detected in a wild boar's blood sample. Based on phylogenetic analyses based on the *groEL*, *gltA*, *sodB* genes and ITS (23S-5S) intergenic region, it was found that *A. sculptum* and *A. ovale* ticks collected from wild boars carry *Ehrlichia* genotypes phylogenetically associated with *E. ewingii, E. ruminantium*, and new *Ehrlichia* genotypes previously detected in horses, peccaries, and ticks collected from jaguars. In the screening for hemoplasmas by a qPCR based on the 16S rRNA gene, 88.06% of blood samples and 8.69% of ticks were positive. *Mycoplasma suis*, *M. parvum* and a possible new hemoplasma genotype were detected in wild boars in southeastern Brazil. In the screening for *Bartonella* spp. using a *nuoG*-based qPCR assy, 3.8% of tick samples were positive. Phylogenetic inferences positioned four *nuoG* and one for *gltA Bartonella* sequences into the same clade as *Bartonella machadoae*. No blood or tick samples from wild boars showed to be positive in the qPCR for *Coxiella burnetii* based on the *IS111* gene. On the other hand, only 1.6% of ticks was positive in the nested PCR assay for piroplasmids based on the 18S rRNA gene. An 18S rRNA sequence detected in a pool of *A. sculptum* nymphs was phylogenetically close to *Cytauxzoon felis* sequences previoously detected in cats from the United States. *Rickettsia* sp. closely related to *R. belli* was detected in a pool of *A. sculptum nymphs*. This is the first report of hemoplasmas, *B. machadoae* and *Cytauxzoon* spp. in *A. sculptum*. Wild boars and associated ticks do not seem to participate in the epidemiological cycle of *C. burnetii* in the region studied. This invasive mammal species may act as a potential disperser of ticks infected with *Ehrlichia* spp., *Bartonella* spp., hemotropic mycoplasmas, and *Cytauxzoon*, and may bring important epidemiological implications in the transmission of bartonelosis, ehrlichiosis, hemoplasmosis, and cytauxzoonosis to humans and animals, more specifically to horses, rodents, pigs, and cats.

Keys-word: Anaplasmataceae, *Amblyomma* spp., *Bartonella* sp., *Coxiella burnetii, Cytauxzoon* sp., hemoplasmas, *Rickettsia* sp..

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CAPÍTULO 1 – CONSIDERAÇÕES GERAIS

1. INTRODUÇÃO

Nativo da Eurásia e norte da África, o javali (*Sus scrofa*) foi introduzido na América do Sul no início do século XX (DEBERDT & SCHERER, 2007), para consumo da carne. Uma invasão a partir do Uruguai decorrente de um período de seca que possibilitou a travessia dos animais pelo rio em 1989 favoreceu a entrada do animal em território brasileiro (SALVADOR, 2012).

Na natureza, cruzou-se com o porco doméstico (*Sus scrofa domesticus*) resultando em híbridos férteis (GROSSI et al., 2006), chamado de "porco selvagem" ou “javaporco” (KEITER et al., 2016; MELLETTI e MEIJAARD, 2017). O javali está entre as espécies invasoras que causam maior impacto sobre os ecossistemas naturais e agrícolas e, em consequência disso, geram prejuízos no setor agropecuário (PEDROSA et al., 2015). Hoje, o javali é distribuído mundialmente devido à sua facilidade de adaptação a uma grande diversidade de condições climáticas (FUMACO et al., 2015).

No Brasil, foram introduzidos para consumo na região do Rio Grande do Sul e São Paulo na década de 1960. Após dispersão pelo país, atualmente são encontrados em 15 estados (BRASIL, 2017). Frente à grande expansão dessa espécie invasora no território brasileiro, foi criada a instrução normativa 03/2013 que permite a caça legal desses animais e seus híbridos para controle populacional. Considerados uma espécie exótica invasora, são responsáveis por causar problemas de âmbito sócio-econômico e ambiental, tais como destruição de lavouras e nascentes e extinção de espécies nativas por princípios de exclusão competitiva (HARDIN et al., 1960; LEE e LEE, 2019). Javalis representam não só preocupações ecológicas, como também sanitárias, já que podem ser portadores de patógenos transmissíveis para suínos domésticos de criações comerciais e outras espécies animais, inclusive os seres humanos (MENG, LINDSAY & SRIRANGANATHAN, 2009). Alguns estudos sugerem que estes animais agem como portadores assintomáticos de alguns patógenos, sem o desenvolvimento de sinais clínicos (HÄLLI et al., 2014), desta forma tornando-se uma fonte de infecção na natureza. Além disso, podem participar como hospedeiros na manutenção do ciclo biológico de ectoparasitas, tais como o

carrapato *Amblyomma sculptum*, espécie vetora de *Rickettsia rickettsii* (Rickettsiales: Rickettsiaceae) (LABRUNA et a., 2009; 2011; ORGZEWALSKA et al., 2012).

Entretanto, embora estejam amplamente distribuídos no Brasil, ainda são incipientes os estudos relacionados à sanidade desses animais. Dessa forma, faz-se importante a realização deste tipo de pesquisa, sobretudo pela proximidade desses mamíferos com animais domésticos e seres humanos, o que pode representar um risco de transmissão, introdução e manutenção de doenças emergentes e re-emergentes (CLEVELAND et al., 2017; HERREIRA, et al., 2008). No que diz respeito aos agentes patogênicos transmitidos por vetores em javalis, destacam-se aqueles pertencentes às famílias Anaplasmataceae, Bartonellaceae, Coxiellaceae, Mycoplasmataceae, Rickettsiaceae, Babesiidae e Theileriidae.

Sendo assim, considerando a relativa escassez de estudos sobre o tema, a presença de relatos de hemoparasitas de caráter zoonótico em javalis e a crescente preocupação com a interação do ser humano com carne de caça contaminada com patógenos, o presente estudo objetivou investigar a ocorrência molecular de agentes transmitidos por vetores artrópodes (Anaplasmataceae, Bartonellaceae, Mycoplasmataceae, Coxiellaceae, Rickettsiaceae e Babesiidae/Theileriidae) em javalis de vida livre e carrapatos associados amostrados no sudeste do Brasil. Os resultados aqui obtidos contribuirão para a aquisição de conhecimento a respeito dos hemoparasitas transmitidos por vetores em javalis de vida livre, bem como a respeito das espécies de ectoparasitas que infestam estes animais, favorecendo assim, a compreensão dos ciclos selvagens destes patógenos e ectoparasitas.

2. REVISÃO DE LITERATURA

2.1. Javalis invasores

O javali (*Sus scrofa*) é uma espécie nativa da Europa que foi introduzida em diversos locais do mundo a partir do século XVI, época em que as grandes viagens marítimas intensificaram a rota do comércio. O animal figura a lista das 100 piores espécies invasoras da União Internacional para Conservação da

Natureza (IUCN), devido ao seu alto potencial destruidor e à enorme dificuldade de manejo. Além disso, eles têm um efeito negativo sobre o ecossistema nativo, incluindo a predação, degradação do hábitat e competição com animais nativos (BREDA & WARD, 2017). No Brasil, possuem grande quantidade de alimentos disponíveis e, sem predadores naturais, encontram-se sempre em crescimento e expansão populacional (PEDROSA et al., 2015).

Além do prejuízo causado às culturas agrícolas, a presença constante de javalis em áreas de produção rural resulta em problemas sanitários graves para as propriedades rurais, sobretudo na criação de suínos (dada à proximidade genética), uma vez que estes animais funcionam como hospedeiros de diversos patógenos bacterianos, virais e parasitários e podem desempenhar um papel importante na transmissão de doenças para a suinocultura doméstica e comercial. Sem qualquer controle, tais enfermidades podem causar a infecção de rebanhos inteiros, prejudicando economicamente, em especial, os pequenos produtores (TOULOUDI et al., 2015; MENG, LINDSAY & SRIRANGANATHAN, 2009). Neste contexto, as pesquisas envolvendo espécies invasoras se tornam cada vez mais importantes, uma vez que o aparecimento de doenças emergentes e reemergentes demonstra que a complexidade da tríade hospedeiro patógeno-ambiente ainda é subestimada no que se refere à transmissão das doenças (PAPER, 2008).

Como já mencionado, desde 2013, a caça ao javali é permitida no Brasil, como medida de controle populacional. Entretanto, o que se verifica nos últimos anos é a não obtenção de resultados significativos na redução da densidade demográfica destes animais. Além disso, pode-se dizer que a caça também é problemática, uma vez que configura um elo entre os microrganismos patogênicos e as cidades, favorecendo a ruptura de ciclos naturalmente estabelecidos e gerando, como consequência, a emergência de enfermidades que permaneceriam longe das cidades, caso não houvesse esse contato.

2.2. Agentes transmitidos por vetores artrópodes em javalis

Pode-se dizer que a pandemia causada pelo Coronavírus 19 ajudou a lançar luz sobre temas profundamente relevantes nas áreas de estudo em vigilância epidemiológica e Saúde Pública como um todo. O modo como os seres

humanos utilizam os recursos naturais e a maneira nociva como os exploram, além de ter como consequência as mudanças climáticas, modificam o curso natural do ambiente remodelando a interação entre patógenos, vetores, hospedeiros e seres humanos. Estas interações, que já são tipicamente complexas, passam a gerar efeitos negativos na dinâmica do ecossistema (CROWL et al., 2008). Do ponto de vista de Saúde Pública, devemos estar sempre atentos àas espécies exóticas invasoras, uma vez que estas, além de afetar drasticamente o ecossistema local, podem trazer consigo patógenos que não existiam nessa nova área, ou passar a fazer parte do ciclo silvestre de patógenos e ectoparasitas locais. Como consequência, potencializa-se a transmissão de patógenos causadores de enfermidades graves aos seres humanos (CHINCHIO et al., 2020).

De acordo com a Organização Mundial da Saúde (OMS), 60% dos patógenos existentes que afetam os seres humanos são de caráter zoonótico, os quais são responsáveis pela ocorrência de 75% das enfermidades emergentes; destas, 22,8% são carreadas por vetores artrópodes (TAYLOR et al., 2001; JONES et al, 2008; ZANELLA, 2016; MACHADO, 2017). Dessa forma, a compreensão da dinâmica de transmissão e manutenção de enfermidades transmitidas por vetores permite estabelecer medidas de prevenção. Javalis podem atuar como reservatórios de agentes zoonóticos, além de poderem participar como hospedeiros na manutenção do ciclo biológico de ectoparasitas, tais como do carrapato *Amblyomma sculptum*, espécie vetora de *Rickettsia rickettsii* (Rickettsiales: Rickettsiaceae) (LABRUNA et a., 2009; 2011; ORGZEWALSKA et al., 2012).

No que diz respeito a agentes patogênicos transmitidos por vetores em javalis, destacam-se aqueles pertencentes às famílias Anaplasmataceae, Bartonellaceae, Coxiellaceae, Mycoplasmataceae, Rickettsiaceae, Babesiidae e Theileriidae.

2.2.1. Agentes Anaplasmataceae

A família Anaplasmataceae pertencence à ordem Rickettsiales e é formada pelos gêneros *Anaplasma*, *Ehrlichia*, *Neorickettsia*, *Wolbachia* e '*Candidatus Neoehrlichia*'. Compreendem alfa-proteobactérias Gram-negativas

intracelulares obrigatórias, que formam mórulas em vacúolos intracitoplasmáticos de neutrófilos, monócitos, macrófagos, plaquetas e células endoteliais de mamíferos, a depender da espécie. Tais agentes são transmitidos, em sua maioria, por artrópodes, sobretudo carrapatos, e podem causar doenças em seres humanos e animais (DUMLER et al., 2001; INOKUMA et al., 2001; AGUIAR et al., 2014).

A manutenção da infecção destes agentes dos gêneros *Ehrlichia* e *Anaplasma* ocorre pela interação de vetores artrópodes com os hospedeiros vertebrados no ecossistema. Sendo assim, carrapatos, ao se alimentarem de um hospedeiro infectado, ingerem as bactérias, que penetram no epitélio do intestino médio do invertebrado e realizam a primeira replicação. Posteriormente, as mesmas migram até as glândulas salivares, invadem as células epiteliais e passam pelo segundo ciclo de replicação; em seguida, são eliminadas na secreção salivar e transmitidas a outro hospedeiro durante o repasto sanguíneo do carrapato (UETI et al., 2009; RAR e GOLOVLJOVA, 2011).

A detecção de DNA de *Anaplasma phagocytophilum* (Rickettsiales: Anaplasmataceae), agente etiológico da anaplasmose granulocítica animal e humana, vem sendo relatada em javalis. Na Romênia (4,48% [39/870]), por meio de ensaio de nested PCR (nPCR) tendo como alvo o gene 16S rRNA (KISS et al., 2014), como também na Alemanha (12,5% [3/24]) (SILAGHI; PFISTER; OVERZIER, 2014) e Bélgica (0,97% [5/513]) (NAHAYO et al., 2014). Já na Eslovênia, ocorrência de 4,03% (10/248) (STRAEK SMRDEL et al., 2009) foi relatada, com base em ensaios de nPCR baseados no gene 16S rRNA e operon *groESL*. Por outro lado, na Eslováquia, Reiterová et al. (2016) relataram ocorrência de 2,7% (3/113), por meio de ensaio de qPCR (PCR em tempo real quantitativa) baseada no gene *msp2* e nPCR para o gene 16S rRNA e operon *groESL*. Ainda na Eslováquia, Kazimírová et al. (2018) detectaram ocorrência de 2,7% (3/113) por meio de ensaios de qPCR baseados nos genes *msp2* e *groEL,* e de nPCR baseado no gene 16S rRNA. Na República Tcheca, Hrazdilová et al. (2020) detectaram ocorrência de 5,1% (28/550), por meio de ensaios de nPCR baseados no operon *groESL* e gene *groEL*. Inferências filogenéticas agruparam as sequências *groEL* de *A. phagocytophilum* detectadas em javalis na República Tcheca em um grande clado composto por genótipos detectados previamente na Europa e estreitamente relacionadas com genótipos norte-americanos. Três

sequências posicionaram-se em um subclado com sequências detectadas em humanos da Europa e sequências previamente detectadas em cães e cavalos. Por fim, outras três sequências posicionaram-se em diferentes grupos, os quais eram compostos em sua maioria por genótipos previamente detectados em gado e veados-vermelhos (*Cervus elaphus*) (HRAZDILOVÁ et al., 2020). Na Malásia Peninsular, Koh et al. (2016), baseados em ensaios de PCR baseados no gene 16S rRNA, detectaram *Anaplasma bovis* em 7/10 javalis. Por meio de qPCR baseada no gene *groEL*, Hornok et al. (2022) detectaram *A. phagocytophilum* em 19 carrapatos coletados de javalis na Hungria: 0,6% [1/165] *Dermacentor reticulatus*, 10,34% [3/29] *Haemaphysalis concinna* e 1,6% [15/90] *Ixodes ricinus*).

2.2.2. *Bartonella* spp.

O gênero *Bartonella* pertence à Ordem Rhizobiales e Família Bartonellaceae (BIRTLES et al., 1999). O gênero *Bartonella* foi proposto há mais de um século quando Alberto Barton identificou o agente etiológico da doença de Carrión, analisando bactérias intracelulares em esfregaços de sangue de pacientes com febre de Oroya em 1905. As espécies de *Bartonella* são bactérias Gram-negativas intracelulares facultativas que promovem bacteremia intraeritrocítica prolongada em hospedeiros de mamíferos em todo o mundo (BIRTLES et al., 1999; HARMS and DEHIO, 2012).

Atualmente, 37 espécies de *Bartonella* e três subespécies já foram descritas infectando principalmente roedores, felídeos, canídeos, ruminantes e humanos ao redor do mundo. As espécies de *Bartonella* são principalmente transmitidas por artrópodes hamatófagos de mamíferos. Vários artrópodes, tais como como pulgas, piolhos, moscas e carrapatos, estão associados à transmissão *Bartonella* sp. (BILLETER et al., 2008; CHOMEL et al., 2009; OKARO et al., 2017).

Nos Estados Unidos, Beard et al. (2011) identificaram *Bartonella* (cinco sequências da região intergênica ITS 16S-23S rRNA), *Bartonella koehlerae* (sete sequências ITS) e *Bartonella vinsonii* subsp. *berkhoffi* (três sequências ITS) (Rhizobiales: Bartonellaceae) em 19,7% (15/76) dos porcos ferais avaliados.

2.2.3 Micoplasmas hemotrópicos

Micoplasmas hemotrópicos (hemoplasmas) são bactérias pertencentes à classe Mollicutes, ordem Mycoplasmatales, família Mycoplasmataceae e ao gênero *Mycoplasma* (TULLY et al., 1993). Inicialmente faziam parte dos gêneros *Haemobartonella* e *Eperythrozoon*, os quais foram extintos (MESSICK, 2004). No entanto, análises moleculares a partir do gene 16S rRNA permitiram alocar as espécies *Haemobartonella felis, Haemobartonella muris, Eperythrozoon suis e Eperythrozoon wenyonii* dentro do gênero *Mycoplasma* (NEIMARK et al., 2001). Micoplasmas são bactérias fastidiosas caracterizadas por possuírem formato cocóide, inexistência de parede celular e alta plasticidade em sua membrana externa. No hospedeiro, hemoplasmas são encontrados ligadas à superfície das hemácias ou circulantes no plasma. Quando aderidas às hemácias podem gerar deformidades na superfície celular (EDWARD & FREUNDT, 1967; NEIMARK et al., 2001).

Devido à dificuldade de se realizar cultivos *in vitro*, grande parte das espécies de micoplasmas hemotrópicos carregam em seu status taxonômico a denominação “Candidatus” (VOLOKHOV et al., 2012; OREN et al., 2020). Acredita-se que tal dificuldade no cultivo seja decorrente da dependência dessas bactérias aos eritrócitos e aos promotores de crescimento provenientes do hospedeiro, que em sua maioria são desconhecidos. Dessa forma, cabe ressaltar que as classificações de espécies não estão ainda definidas (CITTI & BLANCHARD, 2013; GUIMARÃES et al., 2014).

Hemoplasmas são bactérias que estão distribuídas mundialmente, com exceção da Antártica (BIONDO et al., 2009). Essa dispersão é decorrente da sua habilidade de fixar-se à superfície dos glóbulos vermelhos e infectar diversas espécies de animais vertebrados, inclusive seres humanos (MESSICK, 2004; DOS SANTOS et al., 2008).

O mecanismo de transmissão de micoplasmas hemotrópicos ainda é incerto. Porém, acredita-se que ele ocorra predominantemente por meio de vetores artrópodes hematófagos (pulgas, carrapatos e piolhos) (WILLI et al., 2007). Sendo assim, embora a espécie de carrapato *Rhipicephalus sanguineus* sensu lato seja incriminada como vetor e reservatório de *Mycoplasma*

haemocanis, estudos de infecção experimental devem ser conduzidos a fim de confirmar essa afirmação (NOVACCO et al., 2010; VIEIRA et al., 2015).

Na Alemanha, Hoelzle et al. (2010) detectaram, por ensaio de qPCR baseado no gene 16S rRNA, DNA de *M. suis* em 10% dos 359 javalis amostrados. Quando realizada a análise filogenética das sequências, observou-se que as mesmas foram divididas em dois grandes grupos: enquanto o grupo A foi composto por nove sequências, formando um clado único e alocadas próximas a *M. suis* detectado em Illinois, nos Estados Unidos, o grupo B foi composto por cinco sequências e formaram um clado único, alocado próximo ao clado irmão da sequência de *M. suis* de Guangdong, China. No Brasil, Dias et al. (2019) detectaram a ocorrência de hemoplasmas em 50% (7/14) dos javalis amostrados no estado de São Paulo, com base em qPCR baseada no gene 16S rRNA. Recentemente, Fernandes et al. (2021), também por meio de qPCR, detectaram DNA de micoplasmas hemotróficos em 58,5% (38/65) dos javalis amostrados no estado de Goiás, centro-oeste brasileiro.

2.2.4. *Coxiella burnetii*

Coxiella burnetii é uma bactéria pertencente à ordem Legionellales e família Coxiellaceae (SESHADRI et al., 2003). Anteriormente era denominada *Rickettsia burnetii,* devido à sua semelhança morfológica com agentes da ordem Rickettsiales. Entretanto, análises moleculares baseadas no gene 16S rRNA demonstraram que essas bactérias pertenciam à família Coxiellaceae e classe das Gama-proteobactérias (DRANCOURT & RAOULT, 1994). Trata-se de bactéria intracelular obrigatória e agente causador da Febre Q (*Query Fever*), enfermidade zoonótica de caráter global. Quando infectado, o indivíduo apresenta manifestações febris, podendo evoluir para manifestações clínicas mais severas (DOS SANTOS et al., 2007; DE LEMOS et al., 2011).

Morfologicamente, possuem forma de cocobacilo pleomórfico com membrana celular similar à de outras bactérias Gram-negativas. Por outro lado, apresentam características únicas, como a capacidade de se multiplicar no interior do fagolisossoma e alta capacidade de sobrevivência extracelular e resistência devido ao processo de esporulação (HOWE & MALLAVIA, 2000).

Coxeilla burnetii infecta uma grande variedade de animais domésticos e selvagens (MAURIN e RAOULT, 1999; CDC 2018). Recentemente, Lim et al. (2020) relataram a presença de bactérias endossimbiontes do gênero *Coxiella* spp. em 16,6% (5/32) das amostras de DNA de carrapatos *Haemaphysalis hystricis* coletados de javalis de vida livre na Malásia. *Coxiella burnetii* é capaz de infectar uma ampla variedade de animais selvagens e domésticos. No Brasil, DNA do agente já foi detectado em amostras de placenta de caprinos (OLIVEIRA et al., 2018) e de baço de roedores selvagens (ROZENTAL et al., 2017) e morcegos (FERREIRA et al., 2018). Evidências sorológicas de exposição ao agente já foram detectadas em bovinos e cervídeos no Brasil (ZANATTO et al., 2019a; 2019b; DE SOUZA RAMOS et al., 2020). Recentemente, DNA do agente foi detectado em amostras de queijo minas frescal no sudeste brasileiro (NASCIMENTO et al., 2021), enfatizando a necessidade de estudos mais aprofundados acerca da epidemiologia da Febre Q no Brasil, onde casos humanos já foram descritos (MARES-GUIA et al., 2016; ROZENTAL et al., 2018; LEMOS et al., 2018)

2.2.5. *Rickettsia* spp.

O gênero *Rickettsia* engloba bactérias Gram-negativas e intracelulares obrigatórias. Pertencem ao Filo das Proteobactérias, classe Alphaproteobactérias, ordem Ricketssiales, e família Rickettsiaceae (GARRITY et al., 2004). Morfologicamente são classificadas como microrganismos procarióticos, caracterizadas por possuírem formas cocobacilares, bacilos ou de pequenos bastonetes. Essas bactérias infectam vários animais e seres humanos, sendo que a sua transmissão ocorre por meio de vetores artrópodes hematófagos (pulgas, piolhos e carrapatos). *Amblyomma sculptum* é incriminado como o principal vetor na transmissão de *Rickettsia rickettsii* no Brasil (LABRUNA et a., 2009; 2011; ORGZEWALSKA et al., 2012).

Nos vetores, possuem predileção pelas glândulas salivares e ovários, como também são capazes de infectar e multiplicarem-se em células intestinais, túbulos de Malpighi e hemolinfa (BABALIS, 1994; BURGDORFER, 1970; BILLINGS et al., 1998; WALKER, 2003).

Recentemente, Kmetiuk et al. (2021) realizaram análise sorológica para *Rickettsia* spp. em amostras de javalis, cães de caça e caçadores, em áreas de Mata Atlântica e Cerrado. Os resultados mostraram exposição de 72,5% (58/80) dos javalis, 14,1% (24/170) dos cães de caça e 14,7% (5/34) dos caçadores a *Rickettsia* spp.. Em Córsega, ilha francesa no mar mediterrâneo, Defaye et al. (2021) detectaram a presença de DNA de *Rickettsia* spp., *R. slovaca* e *R. aeschlimannii* (PCR baseado no gene *gltA*) em 79,6% (90/113) de carrapatos das espécies *Dermacentor marginatus* e *Hyalomma marginatum* coletados de javalis. Já Sgroi et al. (2020) identificaram, na Itália, a partir de análise de fragmento do gene *ompA*, DNA de rickettsias do grupo da febre maculosa em 9% (16/173) e 0,6% (1/173) em carrapatos *Dermacentor marginatus* e *Ixodes ricinus*, respectivamente. A análise das sequências mostrou tratarem-se das espécies *Rickettsia slovaca* e *Rickettsia raoultii*. Apenas uma amostra de baço de javali, dentre 93 amostrados, foi positivo para *Rickettsia slovaca*. A análise filogenética baseada no gene *ompA* posicionou as sequências de *R. slovaca* detectadas na Itália no mesmo clado de sequências previamente detectadas em carrapatos *D. marginatus* e javalis, na Turquia, China, Itália e Portugal. No sudoeste da China, Wang et al. (2020) analisaram a presença de DNA de *Rickettsia* em 1197 carrapatos ixodídeos adultos coletados de javalis. A partir de PCR baseada no gene *ompA*, foi detectada a presença de DNA de '*Candidatus* Rickettsia laoensis' em 1,16% (14/1197) dos carrapatos analisados. Ademais, na Espanha, Castilho-Contreras et al. (2021), utilizando ensaio de PCR baseado no gene *gltA*, detectaram à ocorrência de três espécies de *Rickettsia* (*Rickettsia massiliae, R. slovaca* e *R. raoultii*) em *Dermacentor marginatus, Rhipicephalus sanguineus* e *Hyalomma lusitanicum* coletados de 261/438 (59,58%) javalis.

2.2.6. Piroplasmídeos

Os gêneros *Babesia*, *Cytauxzoon*, *Rangelia* e *Theileria* englobam protozoários transmitidos por carrapatos que infectam diversos mamíferos, inclusive seres humanos e, em menor proporção, aves. São hemoprotozoários intracelulares obrigatórios, pertencentes ao filo Apicomplexa e ordem Piroplasmida. Seu nome é originário do *Grego,* devido ao formato de pera

apresentado pelo parasito no estágio intraeritrocitário (UILENBERG 2006; SCHNITTGER et al., 2012). Entretanto, quando realizadas análises filogenéticas baseadas no gene 18S rRNA, notou-se que os agentes anteriormente citados representam um conjunto polifilético que requer nova revisão taxonômica (SCHNITTGER et al., 2012).

No hospedeiro, as infecções por *Babesia* acometem unicamente os eritrócitos, enquanto que *Cytauxzoon* e *Theileria* infectam além dessas células vermelhas e leucócitos (monócitos, macrófagos e linfócitos-T) (KAKOMA e MEHLHORN 1994; MEHLHORN et al., 1994). Protozoários do gênero *Rangelia*, por sua vez, infectam eritrócitos, leucócitos e células endoteliais (SOARES et al., 2012). Ademais, cabe ressaltar que existem dois diferentes tipos de transmissões: a primeira, denominada como perpetuação transestadial, que ocorre exclusivamente em representantes do grupo de *Babesia* sensu lato e *Theileria*. Por meio deste processo, após a ingestão primária de sangue infectado por larvas e ninfas de carrapatos, ocorre perpetuação do protozoário durante o período de transição de um estágio para o outro (larva para ninfa e ninfa para adulto) (FLORIN-CHRISTENSEN e SCHNITTGER 2009). Nos integrantes do grupo de *Babesia* sensu stricto, cinetos primários atingem ovários e ovos de carrapatos, caracterizando a chamada transmissão transovariana (FLORIN-CHRISTENSEN et al. 2021).

Com relação à ocorrência de piroplasmídeos em javalis, recentemente, na Hungria, Hornok et al. (2022) detectaram *Babesia canis* e *Babesia* cf. *crassa* em quatro (2,42% [4/165]) *Dermacentor reticulatus* e um (3,4% [1/29]) *Haemaphysalis concinna* parasitando javalis, respectivamente, utilizando ensaio de cPCR baseado no gene 18S rRNA. Filogeneticamente, as sequências de *Babesia* sp. agruparam-se com genótipos do extremo oriente. Já na República Tcheca baseados em ensaio de nPCR, um genótipo do gene 18S rRNA com 99,8% de identidade com *Babesia divergens* (Piroplasmida: Babesiidae) foi detectado em 1 (0,18%) dentre 550 javalis amostrados (HRAZDILOVÁ et al., 2020). Enquanto que, Morikawa et al (2021) detectaram, por meio de cPCR com base no gene 18S rRNA, 63,9% (140/219) de javalis positivos para piroplasmídeos no Japão. As inferências filogenéticas baseadas no gene 18S rRNA alocaram as sequências de *Babesia* sp. dos javalis em um clado único, junto das sequências previamente detectadas em *Amblyomma testudinarium* do

Japão. Já as sequências obtidas a partir da região intergênica ITS (obtidas por meio de cPCR) formaram um único clado, alocado próximo às sequências de *Babesia gibsoni* de cães da China, India e USA. Por outro lado, sequências baseadas no gene *Cob* (obtidas por meio de cPCR) também formam um clado único, o qual se alocou próximo ao clado irmão de *Babesia gibsoni* de cães da China e Japão.

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CAPÍTULO 2 – Molecular detection of vector-borne agents in wild boars (*Sus scrofa*) and associated ticks from Brazil, with evidence of putative new genotypes of *Ehrlichia, Anaplasma* and hemoplasmas

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Abstract

The present study aimed to investigate, by molecular techniques, the occurrence of Anaplasmataceae, Bartonellaceae, Rickettsiaceae, Mycoplasmataceae,

Coxiellaceae e Babesiidae/Theileriidae agents in blood samples of free-living wild boars (*Sus scrofa*) and associated ticks in southeastern Brazil. For this purpose, 67 blood samples and 265 ticks (264 *Amblyomma sculptum* and one *A. ovale*) were analyzed. In the screening for Anaplasmataceae agents by a PCR assay based on the 16S rRNA gene, 5.97% blood samples and 50.54% ticks were positive. In the PCR assay for *Ehrlichia* spp. based on the *dsb* gene, 9.24% of ticks were positive. Despite the low occurrence, a possible new 16S rRNA genotype of *Anaplasma* sp. was detected in a wild boar's blood sample. According to phylogenetic analyses based on the *groEL*, *gltA*, *sodB* genes and ITS (23S-5S rRNA) intergenic region, it was found that *A. sculptum* and *A. ovale* ticks collected from wild boars carry *Ehrlichia* genotypes phylogenetically associated with *E. ewingii, E. ruminantium*, and new *Ehrlichia* genotypes previously detected in horses, peccaries, and ticks collected from jaguars. In the screening for hemoplasmas by a qPCR based on the 16S rRNA gene, 88.06% of blood samples and 8.69% of ticks were positive. *Mycoplasma suis*, *M. parvum* and a possible new hemoplasma genotype were detected in wild boars in southeastern Brazil. In the screening for *Bartonella* spp. using a *nuoG*-based qPCR assay, 3.8% of tick samples were positive. Phylogenetic inferences positioned four *nuoG* and one r *gltA Bartonella* sequences into the same clade as *Bartonella machadoae*. No blood or tick samples from wild boars showed to be positive in the qPCR for *Coxiella burnetii* based on the *IS1111* gene. On the other hand, only 1.6% of ticks was positive in the nested PCR assay for piroplasmids based on the 18S rRNA gene. A 18S rRNA sequence detected in a pool of *A. sculptum* nymphs was phylogenetically close to *Cytauxzoon felis* sequences previously detected in cats from the United States. *Rickettsia* sp.

closely related to *R. bellii* was detected in a pool of *A. sculptum nymphs*. This is the first report of hemoplasmas, *B. machadoae* and *Cytauxzoon* spp. in *A. sculptum*. Wild boars and associated ticks do not seem to participate in the epidemiological cycle of *C. burnetii* in the region studied. This invasive mammal species may act as a potential disperser of ticks infected with *Ehrlichia* spp., *Bartonella* spp., hemotropic mycoplasmas, and *Cytauxzoon*, and may bring important epidemiological implications in the transmission of bartonelosis, ehrlichiosis, hemoplasmosis, and cytauxzoonosis to humans and animals, more specifically to horses, rodents, pigs, and cats.

KeyWords: Anaplasmataceae, *Amblyomma* spp., *Bartonella* sp., *Coxiella burnetii, Cytauxzoon* sp., hemoplasmas, *Rickettsia* sp.

1. Introduction

Wild boars (*Sus scrofa*) belong to the Suidae family and are native to Eurasia. In Brazil, they were introduced for consumption in the states of Rio Grande do Sul and São Paulo in the 1960's. After dispersing across the country, they are currently found in 15 states (Brasil, 2017). Due to the great expansion of this invasive species in the Brazilian territory, the normative instruction 03/2013 was created, which allows the legal control of these animals and their hybrids for population control. Considered an invasive alien species, they are responsible for causing socio-economic and environmental problems, such as destruction of crops and springs (Hardin et al., 1960; Lee and Lee, 2019).

According to the World Health Organization, 60% of the described pathogens that affect humans are zoonotic, which are responsible for 75% of the emerging diseases; out of these, 22.8% are transmitted by arthropod vectors

(Taylor et al., 2001; Jones et al, 2008; Zanella, 2016; Machado, 2017). Understanding the dynamics of transmission and maintenance of vector-borne diseases will allow the adoption of prevention measures.

Regarding vector-borne pathogens in wild boars, those belonging to the families Anaplasmataceae, Rickettsiaceae, Bartonellaceae, Coxiellaceae, Mycoplasmataceae, Babesiidae and Theileriidae are of major importance. The presence of DNA of *Anaplasma phagocytophilum* (Rickettsiales: Anaplasmataceae), the causative agent of human and animal granulocytic anaplasmosis, has already been reported in wild boars sampled in Europe (Strasek Smrdel et al., 2009; Kiss et al., 2014; Silaghi; Pfister; Overzier, 2014; Nahayo et al., 2014; Reiterová et al., 2016; Kazimírová et al., 2018; Hrazdilová et al., 2020). In the United States, Beard et al. (2011) detected *Bartonella henselae*, *Bartonella koehlerae*, and *Bartonella vinsonii* subsp. *berkhoffii* (Rhizobiales: Bartonellaceae) in feral pigs evaluated. Hoelzle et al. (2010) detected *Mycoplasma suis* (Mycoplasmatales: Mycoplasmataceae) in wild boars sampled in Germany. Regarding the detection of Coxiellaceae agents (Order Leigionellales), Lim et al. (2020) reported the presence *Coxiella* sp. DNA in *Haemaphysalis hystricis* ticks collected from wild boars in Malaysia. Rickettsia sp. has been molecularly detected in ticks collected from wild boars from France (Defaye et al., 2021), Italy (Sgroi et al., 2020), China (Wang et al., (2020), and Spain (Castillo-Contreras et al., 2021). Piroplasmids (Piroplasmida) were detected in wild boars sampled from the Czech Republic (Hrazdilová et al., 2020) and Japan (Morikawa et al., 2021).

On the other hand, studies reporting the occurrence of arthropod vector-borne agents in wild boars in Brazil are scarce. For instance, the presence of anti-

Rickettsia spp. antibodies in wild boars, hunting dogs, and hunters in the southern and central-western Brazil (Kmetiuk et al. (2019). Hemoplasmas were molecularly detected in wild boars from southeastern (Dias et al., 2019) and central-western Brazil (Fernandes et al., 2021).

The present study aimed to investigate the molecular occurrence of arthropod vector-borne agents (Anaplasmataceae, Rickettsiaceae, Bartonellaceae, Mycoplasmataceae, Coxiellaceae, and Babesiidae/Theileriidae) in free-living boars and associated ticks sampled in southeastern Brazil.

2. Material and Methods

2.1 Sampling

Between the years 2018 and 2020, wild boar captures were conducted with the assistance of legalized hunters registered by the Brazilian Institute of Environment and Renewable Natural Resources (IBAMA). After the wild boars were slaughtered, blood samples were obtained by cardiac puncture and ticks were collected after external inspection of the animals' bodies. Blood samples were collected from wild boars by intracardiac route and ticks (when possible) in the municipalities of Barretos, Colina, Guaraci, Jaboticabal, Monte Alto, Monte Azul Paulista, Morro Agudo, Olímpia and Torrinha (**Figure 1**).

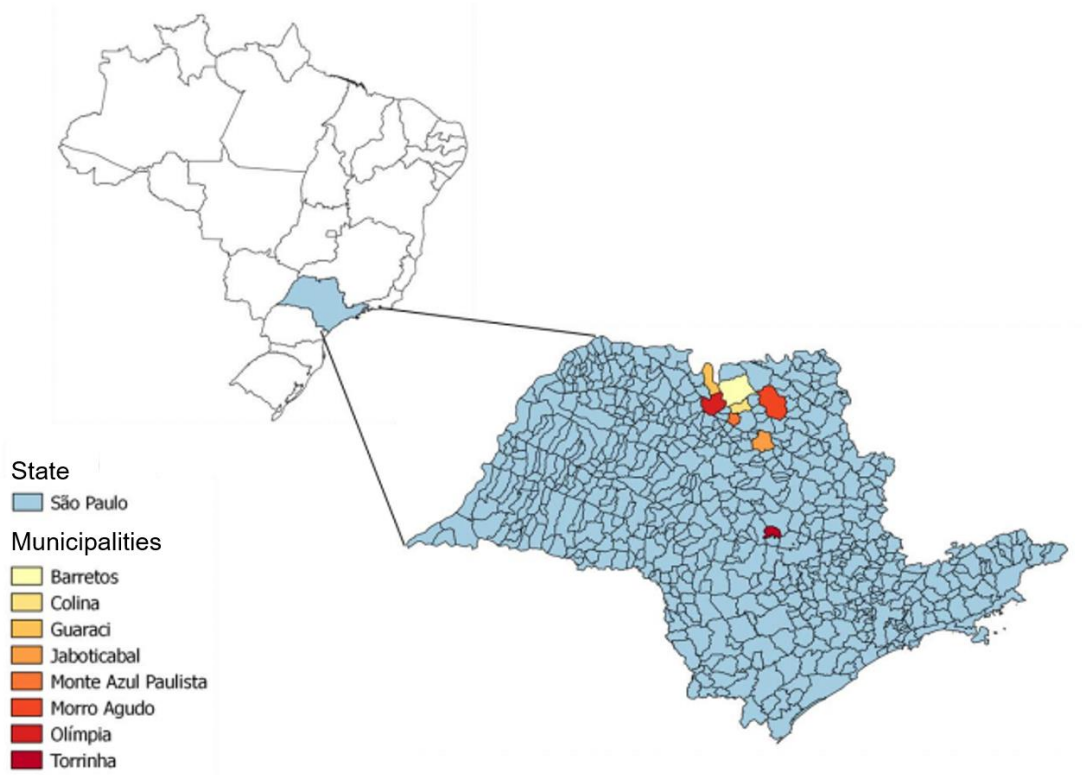

Figure 1. Regions where blood samples and tick were collected from free-ranging wild boars in São Paulo state, southeastern Brazil.

2.2 Taxonomic identification of collected ticks

Taxonomic identification of the collected ticks was performed using a stereomicroscope (Olympus Corporation, Tokyo, Japan) and following previously described taxonomic keys for adult ticks (Barros-Battesti et al., 2006; Martins et al., 2016) and *Amblyomma* nymphs (Martins et al., 2010).

2.3 Molecular assays

2.3.1 DNA extraction and endogenous reaction control

DNA was extracted from blood samples and tick specimens collected from wild boars using Instagene Matrix kits (Bio-Rad, Hercules, California, USA) and

Biopur Extraction Mini Spin Plus (Mobius Life Science, Pinhais, Paraná, BR), respectively, following the manufacturers' instructions. While tick nymphs had their DNA extracted in pools of up to five individuals, adults were individually submitted to DNA extraction, totaling 184 samples of ticks. Subsequently, DNA blood samples were submitted to a conventional PCR (PCR) targeting the endogenous mammalian *gapdh* (glyceraldehyde-3-phosphate dehydrogenase) gene (Birkenheuer et al., 2003), in order to ascertain the presence of amplifiable DNA. Tick DNA samples were submitted to a PCR assay based on the 16S rRNA gene (Black and Piesman, 1994). DNA samples positive in the abovementioned PCR assays were submitted to PCR assays for *Anaplasma* spp., *Ehrlichia* spp., *Bartonella* spp., hemotropic *Mycoplasma* spp., *Coxiella* spp., *Babesia/Theileria* spp., and *Rickettsia* spp. (ticks only) (**Figure 2**).

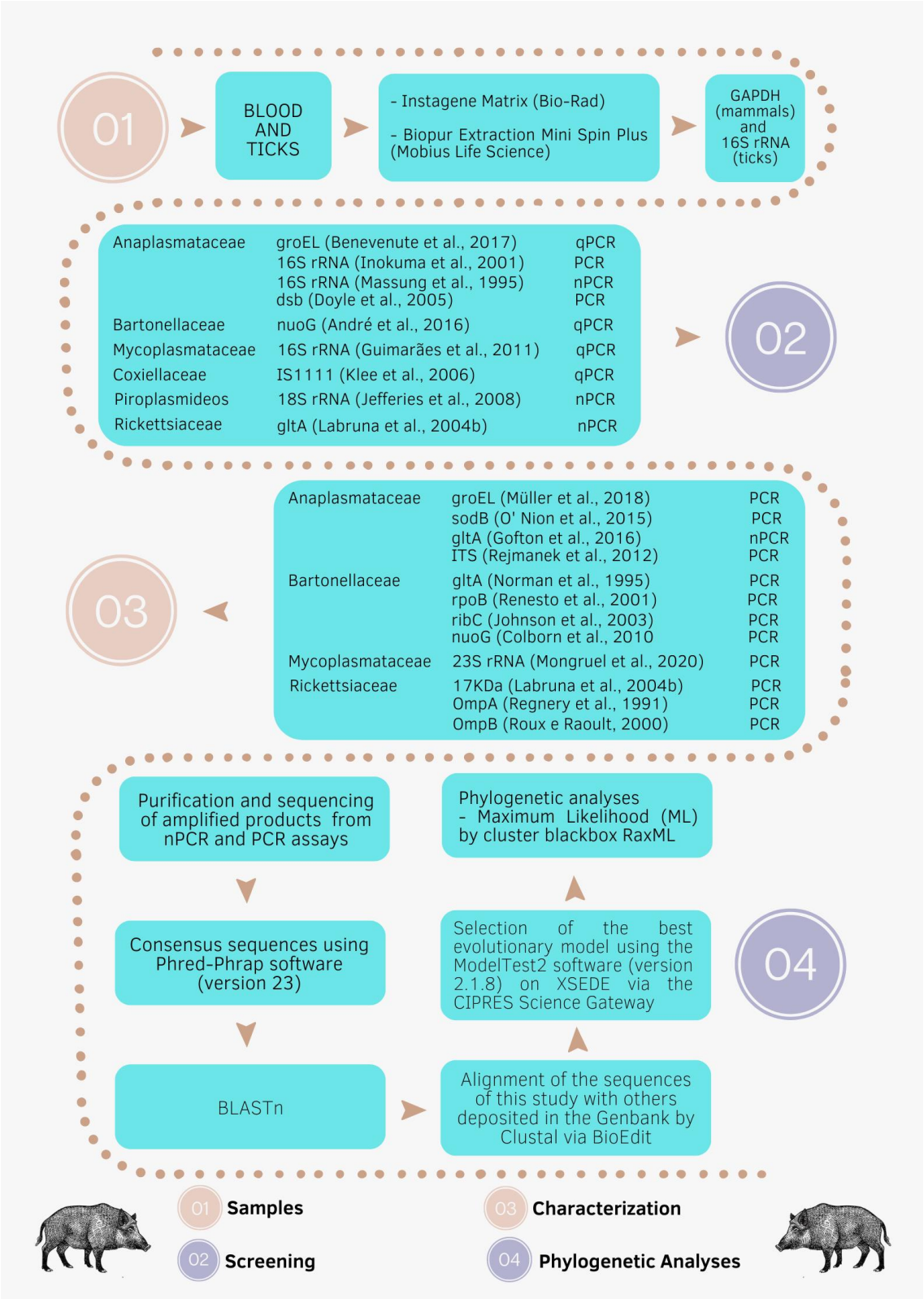

Figure 2. Molecular and phylogenetic analyses used in the present study to detected and characterize Anaplasmataceae agents, *Bartonella* spp., hemoplasmas, *Coxiella burnetii, Rickettsia* spp. (only ticks) and piroplasmids in wild boars' blood and tick samples.

2.3.2 PCR assays for Anaplasmataceae agents

Blood and tick samples were screened by PCR assays targeting fragments of the 16S rRNA gene for Anaplasmataceae agents (Inokuma et al., 2001) and *Anaplasma* spp. (Massung et al., 1998), as well as for the *dsb* gene of *Ehrlichia* spp. (Doyle et al., 2005). Additionally, DNA samples were submitted to a multiplex quantitative real-time PCR (qPCR) assay for *Ehrlichia* spp. and *Anaplasma* spp. based on the *groEL* gene (Benevenute et al., 2017). Positive samples for the abovementioned PCR assays performed were molecularly characterized using additional PCR assays based on the *groEL* (Müller et al., 2018) and *gltA* (Gofton et al., 2016) genes, and 23S-5S intergenic region (Rejmanek et al., 2012) of *Anaplasma* spp. and *Ehrlichia* spp., and to a PCR assay for *Ehrlichia* spp. based on the *sodB* gene (O'Nion et al., 2015) (**Table 1**).

Table 1. Description of primers and probes used in quantitative real-time PCR assays (qPCR) for *Ehrlichia* spp., *Anaplasma* spp., *Bartonella* spp., hemoplasmas and *Coxiella burnetii*.

Agent (Target Gene)	PCR	Primer sequences	TaqMan Hydrolysis probe	Fragment size	Reference
Ehrlichia spp. (*groEL*)*	qPCR	F-Ehr (5'-GCGAGCATAATTAC TCAGAG-3)' R-Ehr – (5' CAGTATGGAGCAT GTAGTAG-3')	TET- 5'-CATTGGCTCTTGCTATTGC TAAT3'[BHQ2a-Q]3'	83 bp	Benevenute et al., 2017
Anaplasma spp. (*groEL*)*	qPCR	F-Anap (5'-TTATCGTTACATTG AGAAGC-3') R-Anap (5'-GATATAAAGTTAT TAAAAGTATAAAGC-3')	Cy-5- 5'-CCACCTTATCATTACACTG AGACG3'[BHQ2a-Q]3'	83 bp	Benevenute et al., 2017
Bartonella spp. *(nuoG)*	qPCR	F-Bart (5'-CAATCTTCTTTTGCTT CACC-3') e R-Bart (5'-TCAGGGCTTTATGTGA ATAC-3')	TexasRed-5'-TTYGTCATTTGAACA CG-3'[BHQ2a-Q]3'	83 bp	André et al., 2015
Hemotropic *Mycoplasma* spp. (16S rRNA)	qPCR	Fsuis-F (5′-CCC TGA TTG TAC TAA TTG AAT AAG-3') Rsuis-R (5′-GCG AAC ACT TGT	MGBsuis2: 5 ′ FAM-TGR ATA CAC AYT TCA G -MGBNFQ 3 ′	157 bp	Guimarães et al., 2011

		TAA GCA AG-3')			
Coxiella burnetti (*IS1111*)	qPCR	Cox-F (5'GTCTTAAGGTGGGC TGCGTG-3') Cox-R (5'-CCCCGAATCTCATT GATCAGC-3')	Cox-TM = FAM-AGCGAACCATTGGT ATCGGACGTT-TAMRA-TATGG	295 bp	Klee et al., 2006

*qPCR multiplex

2.3.3 PCR assays for hemoplasmas

A molecular screening for hemoplasmas was performed by using a qPCR based on the 16S rRNA gene (Guimarães et al., 2011) (**Table 2**). DNA samples positive in the abovementioned qPCR with an estimated quantification higher than 10^4 copy numbers of a 16S rRNA gene fragment per microliter (Gatto et al., 2019) were additionally characterized by a PCR based on the 23S rRNA gene (Mongruel et al., 2020) (**Table 1**).

2.3.4. Cloning

The hemoplasma 23S rRNA amplicons for which the obtained sequences presented electropherograms containing multiple peaks were submitted to cloning, using pGEM-T Easy (Promega® Madison, WI, USA), according to the manufacturer's recommendations. Two clones were selected from each positive sample according to the blue/white colony system. Colonies were subjected to plasmid DNA extraction using the alkaline lysis method (Sambrook & Russell, 2006 et al., 2006). Subsequently, a PCR assay was performed using the primers M13 F (5′-CGCCAGGGTTTTCCCAGTCACGAC3′) and M13 R (5′-GTCATAGCTGTTTCCTGTGTGA-3′) (Lau et al., 2010), which flank the multiple cloning site of the pGEM-T Easy plasmid, in order to include the analyzed gene

fragments. The obtained amplicons were purified and sent for sequencing as described later.

2.3.5 PCR assays for *Bartonella* spp.

A *nuoG* gene-based qPCR for *Bartonella* spp. was performed following a previously described protocol (André et al., 2015a) (**Table 2**). Samples positive in the qPCR were molecularly characterized by conventional PCR assays based on the *nuoG* (400 bp) (Colborn et al., 2010), *gltA* (750 bp) (Norman et al., 1995), *rpoB* (458 bp) (Renesto et al., 2001) and *ribC* (540 bp) (Johnson et al., 2003) genes.

2.3.6 PCR assays for *Coxiella burnetii*

A qPCR assay based on the repetitive element *IS1111* for *Coxiella burnetti* was performed following a previously described protocol (Klee et al., 2006) (**Table 2**).

2.3.7 PCR assays for *Rickettsia* spp.

The molecular screening of ticks for *Rickettsia* spp. was performed using a PCR based on the *gltA* gene (Labruna et al., 2004a). Positive samples were submitted to PCR assays based on the *ompA* (Regnery, Spruill & Plikaytis, 2006), *ompB* (Choi et al., 2005), and 17KDa (Labruna et al., 2004b) genes (**Table 1**).

2.3.8. PCR assays for piroplasmids

A molecular screening for piroplasmids was performed by a nested PCR (nPCR) assay based on the 18S rRNA gene (Jefferies et al., 2008) (**Table 1**).

2.3.9. PCR assay reaction conditions

All qPCR assays were performed with a total final volume of 10 µL, containing a mixture of 1 µL (approximately 50 ng) of the sample DNA, 0.2 µM of each primer oligonucleotide and hydrolysis probe, PCR buffer (IQ Multiplex Power Mix, BioRad®, Hercules, California, United States) and sterile ultrapure water (Nuclease-Free Water, Promega®, Madison, Wisconsin, United States). The amplification reactions were conducted in a CFX96 Thermal Cycler (BioRad®, Hercules, California, United States). The quantification of the target DNA copy number/µL was performed using plasmids (IDT psmart, Integrated DNA Technologies®) containing the target sequences. Serial dilutions were made in order to build standards with different concentrations of plasmid DNA containing the target sequence (2.0×10^7 copies/µL to 2.0×10^0 copies/µL), in order to obtain the efficiency and correlation coefficient of the reactions. The plasmid copy number was determined according to the formula (Xg/µL DNA/ [plasmid size (bp) x 660]) x 6.022x1023 x plasmid copies/µL. Assays were performed including duplicates of each DNA sample. All duplicates showing Cq difference values greater than 0.5 were retested in triplicate. Amplification efficiency (E) was calculated from the slope of the standard curve in each run using the following formula ($E = 10^{-1/slope}$) (Bustin et al., 2009). The reactions performed followed the standards established by the MIQE Guidelines (Bustin et al., 2009).

All conventional and nested PCR assays were performed using 5 µL (approximately 70-120 ng) of the DNA samples in a mixture containing 1.25 U Platinum Taq DNA Polymerase (Invitrogen®, Carlsbad, California, United

States), PCR Buffer (PCR buffer 10 X - 100nM 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 Magnesium Chloride (Invitrogen®, Carlsbad, California, United States), 0.5 µM of each primer oligonucleotide (Invitrogen®, Carlsbad California, United States) and sterile ultrapure water (Invitrogen®, Carlsbad, California, United States) q. s.p. 25µL. In the nested PCR assays, 1 µL of the amplified product from the first PCR reaction was used as a template. DNA samples of *Ehrlichia canis* (Jaboticabal strain) (Nakaghi et al., 2010) and *A. phagocytophilum* (kindly provided by Prof. Dr. John Stephen Dumler, Uniformed Services University of the Health Sciences, Bethesda, Maryland, USA) were used as positive controls in the PCR assays for Anaplasmataceae agents. DNA samples of *Babesia vogeli* (de Sousa et al., 2018) and *Mycoplasma suis* (Sonálio et al., 2020) were used as positive controls in PCR assays for piroplasmids and hemotrophic mycoplasmas, respectively. Sterile ultrapure water (Nuclease-Free Water, Promega Corporation, Madison, USA) was used as a negative control in all PCR assays. *Rickettsia vini* DNA, kindly provided by Prof. Dr. Marcelo Labruna (University of São Paulo), was used as a positive control in the PCR assays for *Rickettsia* spp. DNA sample from the Jaboticabal strain of *Bartonella henselae* isolated from cat blood samples (Furquim et al., 2021) was used as a positive control in the qPCR/PCR assays for *Bartonella* spp.

Amplified products were separated by electrophoresis in 1% agarose gels stained with ethidium bromide (Life Technologies™, Carlsbad, California, USA) under 100 V/150 mA electric current for 50 minutes. The agarose gels were

subjected to ultraviolet light (ChemiDoc MP Imaging System, Bio Rad, Hercules, California, USA) and photographed using Image Lab Software version 4.1.

Table 2. Description of primers used in conventional (PCR) and nested (nPCR) PCR assays used in this study.

Agents (Target gene)	PCR technique	Primer sequences	Fragment size	Reference
Hemotropic *Mycoplasma* spp. (23S rRNA)	PCR	23S_HAEMO_F (5'- TGAGGGAAAGAGCCCAGAC-3') 23S_HAEMO_R (5'-GGACAGAATTTACCTGACAAGG-3')	800 bp	Mongruel et al, 2020
Ticks (16 rRNA)	PCR	Cox16SF1 (5'-CGTAGGAATCTACCTTRTAGWGG-3') Cox16SR1 (5'-ACTYYCCAACAGCTAGTTCTCA-3') Cox16SF2 (5'-TGAGAACTAGCTGTTGGRRAGT-3') Cox16SR2 (5'-GCCTACCCGCTTCTGGTACAATT-3')	1321-1416 bp	Duron et al, 2014
Piroplasmada (18S rRNA)	nPCR	BTF1 (5'-GGCTCATTACAACAGTTATAGCCCAAAGACTTTGATTTCTCTC-3') BTR1 (5'-CCGTGCTAATTGTAGGGCTAATACGGACTACGACGGTATCTGATCG-3') BTF2 (5'-GGCTCATTACAACAGTTATAGCCCAAAGACTTTGATTTCTCTC-3') BTR2 (5'-CCGTGCTAATTGTAGGGCTAATACGGACTACGACGGTATCTGATCG-3')	930 bp 800 bp	Jefferies et al., 2008
Ehrlichia spp. (*dsb*)	PCR	DSB-330 (F) (5′-GATGATGTCTGAAGATATGAAACAAAT-3 ′) DSB-728 (R) (5′-CTGCTCGTCTATTTTACTTCTTAAAGT-3')	409 bp	Doyle et al., 2005
Anaplasma spp. (16S rRNA)	nPCR	ge3a (5′-CACATGCAAGTCGAACGGATTATTC-3') ge10r 5'-TTCCGTTAAGAAGGATCTAATCTCC-3') ge9f (5′-AACGGATTATTCTTTATAGCTTGCT-3') ge2 (5′-CTGGCAGCAGTAA-3')	932 bp 546 bp	Massung et al., 1998
Anaplasmataceae (16S rRNA)	PCR	EHR16SD (5'GGTCCYACAGAAGTCC3') EHRSR (5'TAGCACTCATCGTTTACAGC3')	345 bp	Inokuma et al., 2001
Ehrlichia spp. *(sodB)*	PCR	sodbEhr1600-F (5′-ATGTTTACTTTACCTGAACTTCCATATC-3′) sodbEhrl600-R (5′-ATCTTTGAGCTGCAAAATCCCAATT-3′)	600 bp	O'nion et al., 2015
Anaplasma spp. (ITS - 23S-5S)	PCR	ITS2F (5′-AGGATCTGACTCTAGTAACGAG-3 ′) ITS2R (5′-CTCCCATGTCTTAAGACAAAG-3 ′)	300 bp	Rejmanek et al., 2012
Bartonella spp. (*nuoG*)	PCR	F-nuoG (5'-GGCGTGATTGTTCTCGTTA-3') R-nuoG (5'- CACGACCACGGCTATCAAT-3')	400 bp	Colborn et al., 2010
Bartonella spp. (*gltA*)	PCR	CS443f (5′-GCTATGTCTGCATTCTATCA-3′) CS1210r (5′-GATCYTCAATCATTTCTTTCCA-3′)	750 bp	Norman et al., 1995

Bartonella spp. *(rpoB)*	nPCR	400F (5'-CGCATTGGCTTACTTCGTAT-3') 2300R (5'-GTAGACTGATTAGAACGCTG G-3') 2300R (5'-GTAGACTGATTAGAACGCTGG-3') 1596F (5'-CGCATTATGGTCGTATTTGTCC-3')	800 bp	Renesto et al., 2001
Bartonella spp. *(ribC)*	PCR	BARTON-1 (5'-TAACCGATATTGGTTGTGTTGAAG-3') BARTON-2 (5'-TAAAGCTAGAAAGTCTGGCAACATAACG-3')	540 bp	Johnson et al., 2003
Rickettsia spp. (*gltA*)	PCR	CS-239 (5-GCTCTTCTCATCCTATGGCTATTAT-3) CS-1069 (5-CAGGGTCTTCGTGCATTTCTT-356)	834 bp	Labruna et al., 2004a
Rickettsia spp. (*17KDa*)	PCR	17KD1 (5'-ACTTGGTTCTCAATTCGGTCAC-3') 17kD2 (5'-GACACTTGCACCGATTTGTCC-3')	440 bp	Labruna et al., 2004b
Rickettsia spp. (*ompA*)	PCR	Rr190.70p (5′–ATGGCGAATATTTCTCCAAAA-3′) Rr190.602n (5′–AGTGCAGCATTCGCTCCCCCT-3′)	530 bp	Regnery, Spruill & Plikaytis et al., 1991
Rickettsia spp. (*ompB*)	PCR	120-M59 (5'- CCGCAGGGTTGGTAACTGC-3') 120–807 (5'-CCTTTTAGATTACCGCCTAA-3)	862 bp	Roux e Raoult, 2000

2.4. Amplicon Purification and Sequencing

The products of the PCR assays were purified using the ExoSAP-IT PCR Product Cleanup Reagent enzyme (Applied Biosystems TM) and sequenced using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific™, Waltham, MA, USA) and the ABI PRISM 310DNA Analyzer (Applied Biosystems™, Foster City, CA, USA) (Sanger, 1997).

2.5 Phylogenetic Analyses

The obtained sequences were submitted to a screening test in Phred-Phrap version 23 software (Ewing & Green, 1998; Ewing et al., 1998) in order to evaluate the quality of the electropherograms and to obtain consensus sequences from the alignment of sense and antisense sequences. Subsequently, the BLASTn analysis tool (Altschul, 1990) was used to analyze the

nucleotide sequences by comparing them with sequences previously deposited in the GenBank database (Benson et al., 2017).

The obtained consensus sequences were aligned together with other sequences of the same gene previously deposited in GenBank using MAFFT software (Katoh and Standley, 2002). Phylogenetic inferences based on the Maximum Likelihood method were performed using IQTree analysis software (Trifinopoulos et al., 2016). The bootstrap was accessed with 1,000 replicates and the best evolutionary model was selected by the software according to the BIC (Bayesian Information Criterion) criterion, based on the characteristics of each sequence group. Editing and rooting (via external groups) of the phylogenetic trees were performed using Treegraph 2.0.56-381 beta software (Stover & Müller, 2010).

3. Results

3.1. Identification of ticks

Out of 97 boars sampled, ticks were collected, by convenience, from 34 (35.05%) animals. Out of 265 ticks collected, 264 (99.51%) were identified as *A. sculptum* (100 nymphs and 164 adults [116 males and 48 females]) and 1 (0.48%) as a female of *Amblyomma ovale*.

3.2. PCR assays for mammal and tick endogenous genes

Out of 97 wild boar blood samples, 67 (69.07%) were positive in the PCR for the endogenous mammalian *gapdh* gene. All the 184 tick DNA samples were positive in the PCR for the 16S rRNA tick endogenous gene.

3.2.1. Occurrence of Anaplasmataceae agents DNA in wild boars' blood and tick samples

In the PCR-based screening assays for Anaplasmataceae agents based on the 16S rRNA gene, 5.97% (4/67) of wild boars' blood samples and 50.54% of ticks (93/184 [7 pools of five nymphs and 85 adults of *A. sculptum*, and one female of *A. ovale*]) were positive. Additionally, 9.24% (17/184) of the tick samples were positive for *Ehrlichia* spp. in the *dsb*-based PCR. None sample was positive in the nPCR based on the 16S rRNA gene of *Anaplasma* spp.

One hundred tick samples positive in the screening PCR assays based on the 16S rRNA and *dsb* genes were submitted to additional PCR assays for molecular characterization: 24 (24% [24/100]), 27 (27%[27/100]), 60 (60% [60/100]), and 43 (43% [43/100]) were positive in the molecular assays based on the *groEL*, *gltA*, 23S-5S intergenic region and *sodB* genes, respectively.

One to two samples positive in the molecular assays for 16S rRNA, *dsb*, *sodB* genes and the 23S-5S intergenic region were chosen for sequencing. The samples were chosen according to the intensity of the bands on the agarose gel electrophoresis and the location. Despite attempts, successful sequencing of *groEL* and *gltA* amplicons, which showed faint bans in the agarose gel electrophoresis, was not achieved.

All the 67 wild boars' blood and 184 tick samples were negative in the multiplex qPCR assays for *Ehrlichia* and *Anaplasma* based on the *groEL* gene.

Table 3 - Parameters (range and mean of efficiency, R2, slope, and y-intercept) of qPCR assays for Anaplasmataceae (*groEL* gene), haemoplasmas (16S rRNA gene), *Bartonella* spp. (*nuoG* gene), and *Coxiella burnetii* (1S1111 gene) used in this study for screening wild boars' blood and associated ticks DNA samples for the studied agents

Agent (target gene)	Samples Analyzed	Efficiency	R^2	Slope	Y-intercept
Ehrlichia spp. *(groEL)*	Blood	98.9%	0.098	-3.349	38.273
	Ticks	86.5% to 113.4% (mean=102.88%)	0.872 to 0.998 (mean=0.972)	-3.694 to -3.038 (mean= -3.279)	36.295 to 39.735 (mean=37.493)
Anaplasma spp. *(groEL)*	Blood	101.2%	0.993	-3.293	37.895
	Ticks	91.6 to 101.5% (mean=97.44%)	0.994 to 0.999 (mean=0.990)	-3.542 to -3.287 (mean=-3.389)	40.185 to 43.96 (mean=42.462)
Hemoplasmas (16S rRNA)	Blood	85.5-95.0% (mean=90.25)	0.996-0.984 (mean=0.990)	-3.448 - -3.725 (mean=-3.585)	41.908-42.390 (mean=42.149)
	Ticks	94.8% to 104.1% (mean= 99.17%)	0.914 to 0.999 (mean= 0.97)	-3.228 to -3.452 (mean= -3.344)	38.044 to 40.900 (mean= 39.158)
Bartonella spp. (*nuoG*)	Blood	101.8 and 105% (mean=105.9)	0.999	-3.103 and -3.28 (mean=-3.191)	37.17 and 39.01 (mean=37.17)
	Ticks	95.5% to 108.7% (mean= 102.3%)	0.998 to 1 (mean= 0.999)	-3.129 to -3.335 (mean= -3.251)	36.624 to 39.007 (mean= 37.11)
Coxiella burnetii (1S1111 gene)	Blood	100.7% to 104.3% (mean= 102.5%)	0.978 to 0.984 (mean= 0.981)	-3.305 to -3.224 (mean= -3.264)	44.267 to 45.247 (mean= 44.759)
	Ticks	99% to 109.8% (mean= 104.5%)	0.991 to 0.998 (mean=0.996)	3.107 to -3.343 (mean= -3.222)	38.179 to 39.272 (mean=39.193)

3.2.2. Occurrence of hemoplasmas DNA in wild boars' blood and tick samples

Out of the 67 wild boars' blood samples analyzed, 88.06% (59/67) were positive in the qPCR for hemoplasmas based on the 16S rRNA gene. The copy number of a fragment of the 16S rRNA gene ranged from 1.66 x 10^1 to 6.90 x 10^5, with an average of 1.93 x 10^4 copies per microliter of DNA. Out of 59 positive blood samples, only 8 (13.55%) showed estimated quantification higher than 10^4 copies and were submitted to a PCR assay based on the 23S rRNA gene for additional molecular characterization. As a result, 24% (3/8) samples were positive in the abovementioned PCR assay. Considering that the obtained 23S rRNA sequences showed double peaks in electropherogram, they were submitted to cloning using the pGEM T-easy system (Promega).

Regarding the tick samples analyzed, 16 (8.69% [16/184]) samples were positive and had their quantification estimated in the qPCR based on the 16S rRNA gene for porcine hemoplasmas. The estimated quantification ranged from 3.41 x 10^0 to 9.49 x 10^1, with an average of 2.8 x 10^1 copies of a fragment of the 16S rRNA gene per microliter of DNA. Furthermore, 69 samples (37.5% [58/184 - 10 pools of five nymphs and 47 adults of *A. sculptum*, and one adult female of *A. ovale*]) were also positive, but could not be quantified, due to the low amount of initial DNA of hemoplasmas in the sample tested (Monte Carlo effect) (Bustin et al., 2009). Since no tick samples showed estimated quantification of a fragment of the 16S rRNA gene of porcine hemoplasmas per microliter higher than 10^1, these samples were not additionally characterized by the PCR assay based on the 23S rRNA gene. The parameters (efficiency, R^2, slope and Y-intercept) of

qPCR assays for porcine hemoplasmas followed Bustin et al. (2009) and are showed in **Table 3**.

3.2.3. Occurrence of *Bartonella* DNA in wild boars' blood and tick samples

All 67 wild boars' blood samples analyzed were negative in the qPCR assay for *Bartonella* spp. based on the *nuoG* gene.

Out of the 184 tick DNA samples analyzed, 3.8% (7/184 [7 adults of *A. sculptum*]) were positive in the qPCR assay for *Bartonella* spp. based on the *nuoG* gene. Two samples were not amenable to quantification due to the low amount of initial *Bartonella* DNA in the sample tested (Monte Carlo effect). Copy numbers ranged from 1.60×10^0 to 1.97×10^1, with an average of 1.32×10^1 copies per microliter of DNA. The parameters (efficiency, R^2, slope and Y-intercept) of qPCR assays for *Bartonella* spp. followed Bustin et al. (2009) and are showed in **Table 3**.

Among the seven tick-DNA samples positive in the qPCR for *Bartonella* spp. based on the *nuoG* gene, four and one were positive the conventional PCR assays for *Bartonella* spp. based on the *nuoG* and *gltA* genes, respectively. On the other hand, all the seven tick samples were negative in PCR assays based on the *rpob* and *ribC* genes and on the 16S-23S rRNA intergenic region (ITS).

3.2.4. Occurrence of *Coxiella burnetii* DNA in wild boars' blood and tick samples

Out of the 67 blood samples analyzed, all showed to be negative in the qPCR assays for *C. burnetii* based on the *1S1111* gene. The 184 tick DNA

samples analyzed were also negative in qPCR assays for *C. burnetii* based on the *1S111* gene. The parameters (efficiency, R^2, slope and Y-intercept) of qPCR assays for *C. burnetii* followed Bustin et al. (2009) and are showed in **Table 3**.

3.2.5. Occurrence of *Rickettsia* DNA in ticks collected from wild boars

Out of the 184 tick samples analyzed, 0.54% (1/184 [a pool of five *A. sculptum* nymphs]) tested positive in the conventional PCR assay for *Rickettsia* spp. based on the *gltA* gene, but negative in the PCR assays based on the *ompA, ompB,* and *17KDa* genes.

3.2.6. Occurrence of piroplasmids in wild boars' blood and tick samples

All 67 wild boars' blood samples were negative in the nPCR assay for piroplasmids based on the 18S rRNA gene.

Out of 184 tick samples analyzed, 1.6% (3/184 [1 adult and 2 pools of five *A. sculptum* nymphs]) were positive in the 18S rRNA gene-based nPCR assay for Piroplasmida. Based on the intensity of the bands obtained on the agarose gel electrophoresis, only one sample was submitted for sequencing.

3.3. Co-positivity for vector-borne agents in wild boars' blood and tick samples

None of the wild boars' samples were positive for both Anaplasmataceae agent and hemotropic mycoplasmas simultaneously. On the other hand, *A. sculptum* ticks showed the following co-positivities: 1.08% (1/92 [1 adult]) for Anaplasmataceae/Piroplasmida; 55.43% (51/92 [47 adults and 4 nymphs])

Anaplasmataceae/hemoplasmas; 1.08% (1/92 [1 adult]) Anaplamastaceae/*Bartonella* spp. ; 3.26% (3/92 [1 adult and 2 pools of five nymphs]) hemoplasmas/Piroplasmida; 2.17% (2/92 [2 adults]) hemoplasmas/*Bartonella* spp.; 1.08% (1/92 [1 pool of five nymphs]) hemoplasmas/*Rickettsia* spp.; 2.17% (2/92 [2 adults]) Anaplasmataceae/hemoplasmas/Piroplasmida; 2.17% (2/92 [2 adults]) Anaplasmataceae/hemoplasmas/*Bartonella* spp. The female of *A. ovale* was positive for both Anaplasmataceae and hemoplasmas.

3.4. BLASTn analyses

The BLASTn results for all agents and target genes are shown in **Table 4.**

Table 4. Percentage of BLASTn-associated identity of sequences of Anaplasmataceae, Rickettsiaceae, Bartonellaceae, hemoplasmas and piroplasmids detected in wild boars and associted ticks sampled in southeastern Brazil.

Species /ID	**Target Agents**	**Target gene**	**Query Lenght (pb)**	**Query-coverage (%)**	***E-value***	**Identity (%)**	**GenBank accession numbers**
Sus scrofa/ 19	Anaplasmataceae	16S rRNA	235	100	1X10^{-112}	98.72	*A. phagocytophilum* detected in *Haemophysalis longicornis* in China (KX810088)
A. sculptum/ 29B	Anaplasmataceae	16S rRNA	230	100	7X10^{-115}	100	*Ehrlichia sp.* detected in *A. sculptum* in Brazil (MT514732)
A. sculptum/ 25K	Anaplasmataceae	16S rRNA	318	100	7X10^{-163}	100	*Ehrlichia* sp. detected in *A. sculptum* in Brazil (MT514732)
A. sculptum/ 34D	*Ehrlichia* sp.	*dsb*	347	89	1X10^{-159}	100	*Ehrlichia* sp. detected in *Amblyomma* sp. in Brazil (HQ388287)
A. sculptum/ 35O	*Ehrlichia* sp.	*dsb*	346	89	1X10^{-159}	100	*Ehrlichia* sp. detected in *Amblyomma* sp. in Brazil (HQ388287)
A. sculptum/ 1D	*Ehrlichia* sp.	ITS-23S-5S	390	100	0	95,69	*E. chaffeensis* detected in a human in the USA (CP007480)
A. sculptum/ 26D	*Ehrlichia* sp.	ITS-23S-5S	401	100	0	96,78	*E. chaffeensis* detected in a human in the USA (CP007480)
A. ovale/ 24C	*Ehrlichia sp.*	ITS-23S-5S	304	100	2 X10^{-136}	96,09	*E. chaffeensis* detected in a human in the USA (CP007480)
A. sculptum/ 34L	*Bartonella* sp.	*nuoG*	257	100	1 X10^{-124}	98,84	*Bartonella machadoae* sp. nov. isolated

							from wild rodents in the
A. sculptum/ 35N	*Bartonella* sp.	*nuoG*	223	100	5 X10^{-97}	96,41	Pantanal wetland (CP087114.1) *Bartonella machadoae* sp. nov. isolated from wild rodents in the
A. sculptum/ 35R	*Bartonella* sp.	*nuoG*	252	96	2 X10^{-101}	93,98	Pantanal wetland (CP087114.1) *Bartonella machadoae* sp. nov. isolated from wild rodents in the
A. sculptum/ 35S	*Bartonella* sp.	*nuoG*	258	74	3 X10^{-70}	92,67 %	Pantanal wetland (CP087114.1) *Bartonella machadoae* sp. nov. isolated from wild rodents in the
A. sculptum/ 35S	*Bartonella* sp.	*gltA*	686	100	0	97.96	Pantanal wetland *(CP087114.1) Bartonella machadoae* sp. nov. isolated from wild rodents in the
A. sculptum/ 35S	*Rickettsia* sp.	*gltA*	219	100	1 X10^{-108}	100	Pantanal wetland *(CP087114.1) Rickettsia bellii* detected in ticks in Colombia (MT174170)
A. sculptum/ 4A	*Ehrlichia* sp.	*sodB*	228	100	2 X10^{-110}	99,12	*Ehrlichia* sp. detected in an horse in Nicaragua (KJ434180)
Sus scrofa/ 3	*Mycoplasma* sp.	23S rRNA	721	100	0	88,78	*Mycoplasma parvum* detected in pigs in Brazil (MT530439)

Sus scrofa/ 6	*Mycoplasma* sp.	23S rRNA	722	100	0	99,86	*Mycoplasma parvum* detected in pigs in Brazil (MT232831)
Sus scrofa/ 80-clone1	*Mycoplasma* sp.	23S rRNA	559	100	0	99.64	*M. suis* detectado detected in pigs in USA (NR_103970)
Sus scrofa/ 80-clone2	*Mycoplasma* sp.	23S rRNA	550	97	0	99,63	*Mycoplasma parvum* detected in pigs in Brazil (MT530439)
A. sculptum/ 36A	Piroplasmida	18S rRNA	733	100	0	99,86	*Cytauxzoon* sp. detected in cats in USA (MT904037)

3.5. Phylogenetic analyses

3.5.1. Phylogenetic analyses of the 16S rRNA, *dsb*, *sodB* genes and 23S-5S (ITS) sequences of *Anaplasma* spp. and *Ehrlichia* spp.

The maximum likelihood (ML) phylogenetic analysis based on the 16S rRNA gene of Anaplasmataceae family (335 bp) and TIM2+I+G evolutionary model positioned the sequence detected in a wild boar blood sample (ID #19 - OM842840) in a single clade, close to the other clades comprising *Anaplasma* species, with a clade support of 70%. On the other hand, the two sequences obtained from a male (25K - OM842839) and a female (29B - OM842838) *A. sculptum* were positioned into different *Ehrlichia* clades. While the sequence 29B was closely positioned to *E. ewingii*, the sequence 25K was closely related to *Ehrlichia* sp. detected in *A. sculptum* collected from a horse in the Pantanal wetland, state of Mato Grosso do Sul, and *E. ruminantium* (**Figure 3**).

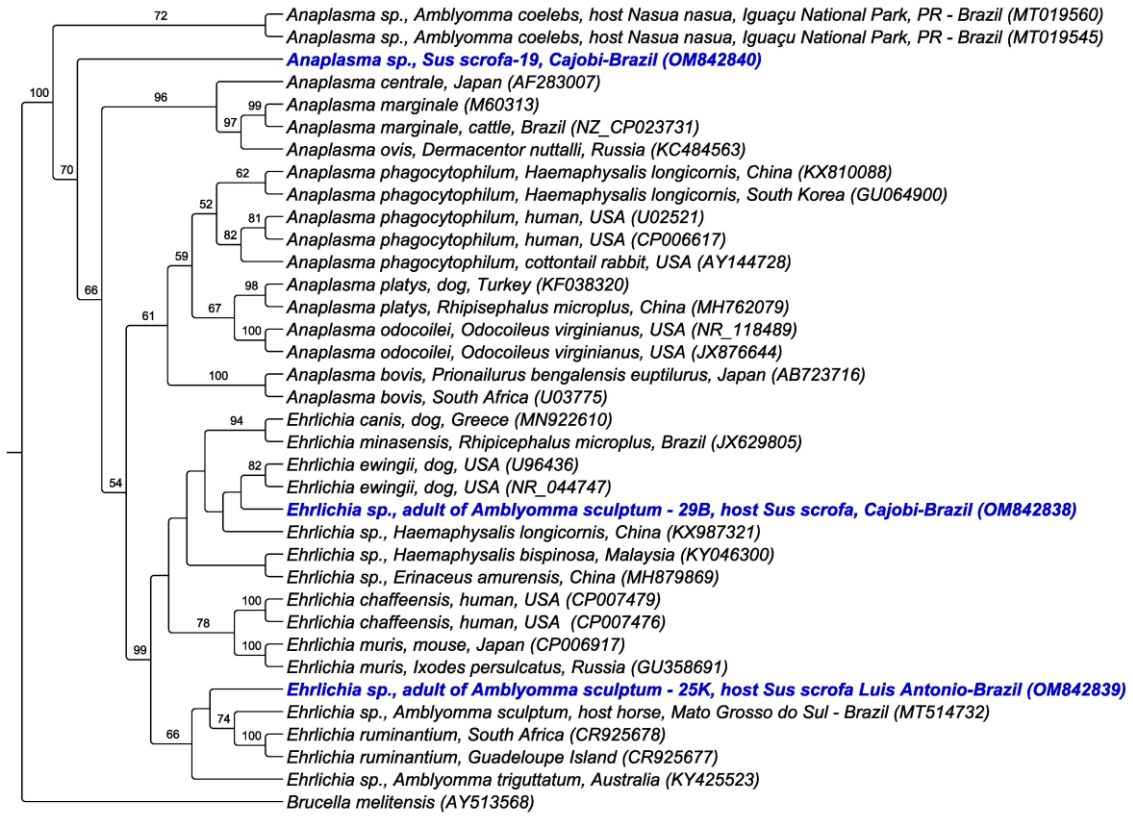

Figure 3. Phylogenetic analysis of 16S rRNA sequences (335 bp alignment) of Anaplasmataceae agents inferred by Maximum Likelihood method and TIM2+I+G evolutionary model. *Brucella melitensis* was used as an outgroup.

The ML phylogenetic analysis based on the *dsb* gene of *Ehrlichia* spp. (339 bp) and evolutionary model TN+F+I grouped two sequences obtained from two males of *A. sculptum* (34D-OM863958 and 35O-OM863959) into a clade that showed to be sister to the that one comprising *Ehrlichia* genotypes previously detected in *Amblyomma* sp. nymphs collected from a jaguar (*Panthera onca*) from Mato Grosso do Sul State, central-western Brazil, with a clade support of 96%. These sequences were separated from those previously detected in horses from the state of Paraná (Southern Brazil) and peccaries (*Tayassu pecari*) from the state of Pará (northern Brazil), with 81% and 66% support (**Figure 4**).

Figure 4. Phylogenetic analysis of *dsb* sequences (339 bp alignment) of *Ehrlichia* spp. inferred by Maximum Likelihood method and TN+F+I evolutionary model. *Ehrlichia ruminantium* was used as an outgroup.

The ML phylogenetic analysis using the evolutionary model HKY+G for the 23S-5S intergenic region (406 bp) grouped two *Ehrlichia* sequences detected in *A. sculptum* (1D - OM863955 and 26D - OM863956) in a clade that showed to be a sister clade to that one comprising an *Ehrlichia* genotype detected in adult *A. ovale* (24C - OM863957), with a support of 94% (**Figure 5**). These two clades were grouped separately from those containing sequences of other *Ehrlichia* species.

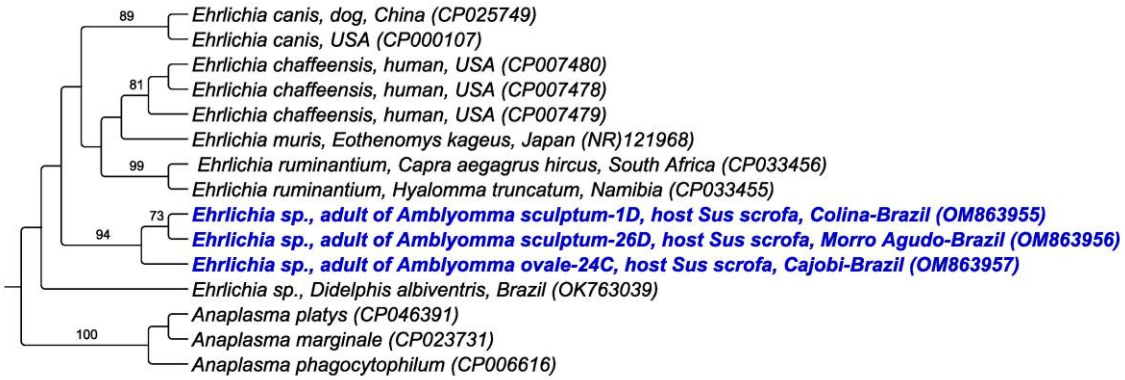

Figure 5. Phylogenetic analysis of 23S-5S sequences (406 bp alignment) of *Ehrlichia* spp. inferred by Maximum Likelihood method and HKY+G evolutionary model. *Anaplasma platys, Anaplasma phagocytophilum* and *Anaplasma marginale* were used as an outgroup.

The ML phylogenetic inference based on the *sodB* gene (534 bp) and the TPM3+G as evolutionary model positioned the *Ehrlichia* sequences detected in *A. ovale* (OM863960) and *A. sculptum* (OM863961) in the same clade, together with *Ehrlichia* genotypes previously detected in horses from the state of Paraná and Nicaragua, in a clade that showed to be sister to that one comprising *E. ruminantium*, with a clade support of 95% (**Figure 6**).

Figure 6. Phylogenetic analysis of *sodB* sequences (534 bp alignment) of *Ehrlichia* spp. inferred by Maximum Likelihood method and TPM3+G evolutionary model. *Anaplasma phagocytophilum* was used as an outgroup.

3.5.2. Phylogenetic analyses of *nuoG* and *gltA* gene sequences of *Bartonella* spp.

Phylogenetic analyses (ML) based on the *nuoG* (363 bp) (**Figure 7**) and *gltA* (712 bp) (**Figure 8**) genes from *Bartonella* spp. with evolutionary models TNe+I+G and TPM+I+G, respectively, positioned the sequences detected in *A. sculptum* specimens collected from wild boars into the same clade containing *Bartonella machadoae*, a *Bartonella* species recently described in wild rodents in the Brazilian Pantanal, with clade support rates of 95 and 57%, respectively.

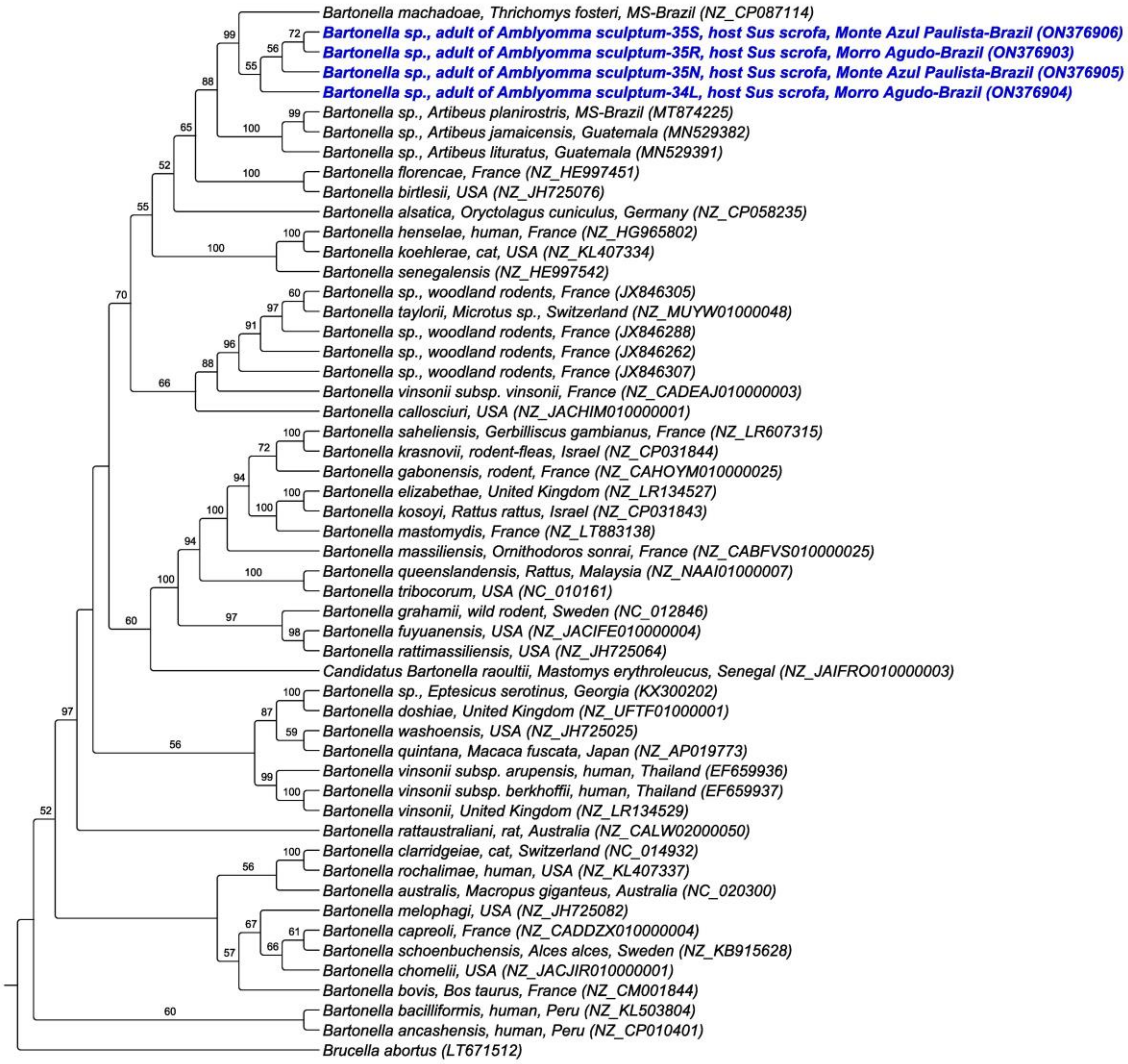

Figure 7. Phylogenetic analysis of *nuoG* sequences (363 bp alignment) of *Bartonella* spp. inferred by Maximum Likelihood method and TNe+I+G evolutionary model. *Brucella abortus* was used as an outgroup.

Figure 8. Phylogenetic analysis of *gltA* sequences (712 bp alignment) of *Bartonella* spp. inferred by Maximum Likelihood method and TPM+I+G evolutionary model. *Brucella abortus* was used as an outgroup.

3.5.3. Phylogenetic analysis of *gltA* gene sequence of *Rickettsia* spp.

The Maximum Likelihood (ML) phylogenetic analysis based on the *gltA* gene (634 bp alignment) of *Rickettsia* spp. and evolutionary model K3PU+F+G4

positioned the sequence 36E detected in an pool of *A. sculptum* nymphs within the *R. bellii* clade, with 91% clade support **(Figure 9).**

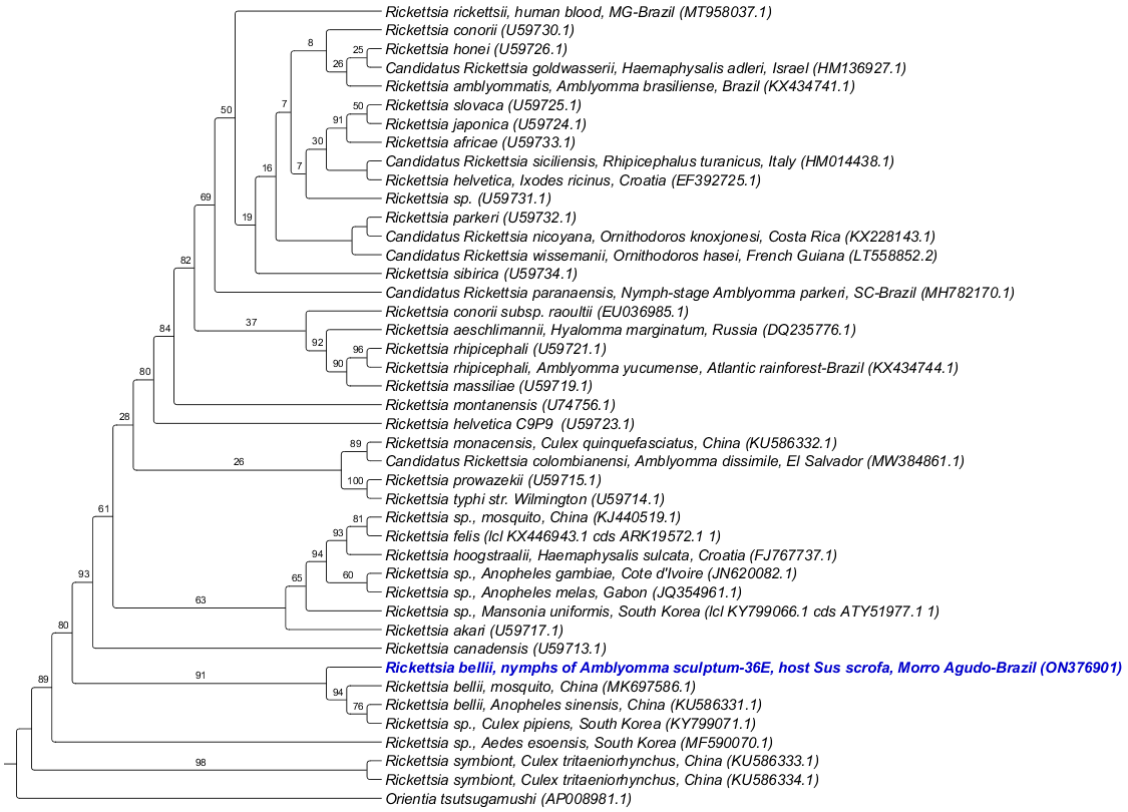

Figure 9. Phylogenetic analysis of *gltA* sequences (634 bp alignment) of *Rickettsia* spp. inferred by Maximum Likelihood method and K3PU+F+G4 evolutionary model. *Orientia tsutsugamushi* was used as an outgroup.

3.5.4. Phylogenetic analysis of the 23S rRNA gene sequences of hemotropic mycoplasmas

The maximum likelihood (ML) phylogenetic analysis based on the 23S rRNA gene (800 bp alignment) of hemotropic *Mycoplasma* spp. and the TIM+G as evolutionary model positioned all hemoplasma sequences detected in wild boars' blood samples in the upper two clusters of the dendogram, with clade support of 93% and 50%, respectively, along with the other sequences obtained from wild boars and pigs sampled in Brazil and the United States. While the

sequences detected in samples #3 (OM868237) and #6 (OM868238) were grouped into the *Mycoplasma parvum* clade, sequence #6 was positioned in a sub-clade that showed to be sister to a clade of a hemoplasma sequence detected in a pig from the state of Goiás (central-western Brazil) (MT232828), demonstrating that it is phylogenetically closer to the latter than to sequence #3 obtained in this study. The other sequences of hemotropic *Mycoplasma* sp. were positioned into the *Mycoplasma suis* clade of the dendogram. These sequences indeed corresponded to genotypes generated by two clones that were obtained from sample #80, which curiously positioned separated from each other: while the sequence corresponding to clone 1 (OM868239) showed closer phylogenetic proximity with *M. suis* strain Illinois (NR103970) detected in swine from the United States, clone 2 (OM868240) comprised a sister group to a *M. suis* sequence detected in swine from Brazil (MT530439) (**Figure 10**).

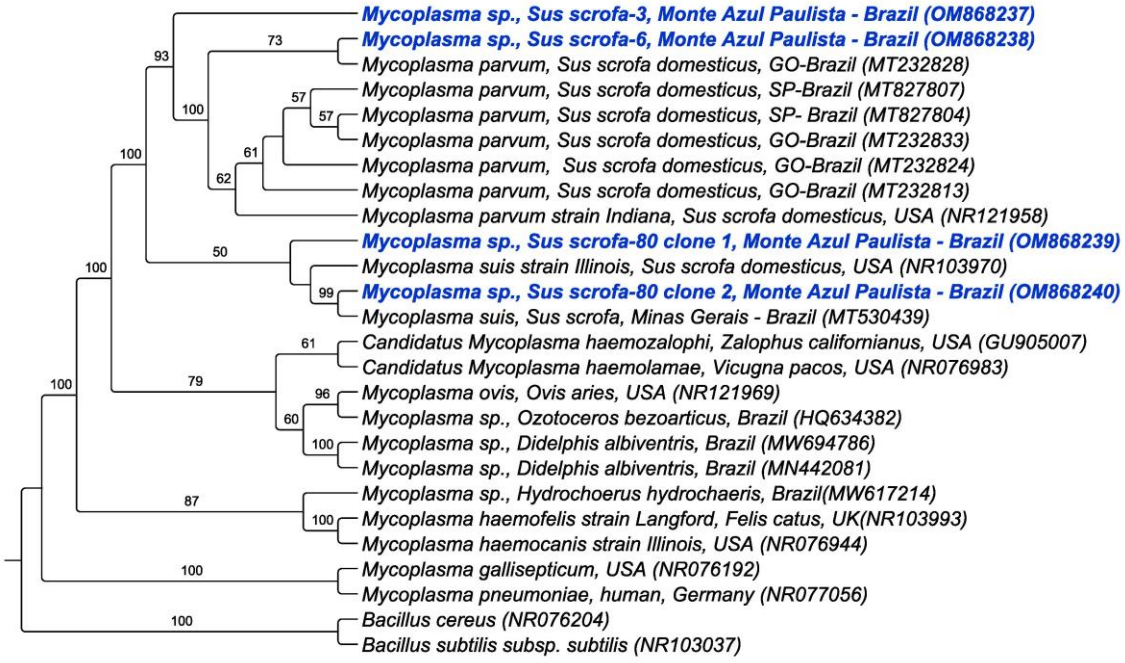

Figure 10. Phylogenetic analysis of 23S rRNA sequences (800 bp alignment) of hemotropic *Mycoplasma* spp. inferred by Maximum Likelihood method and TIM+G evolutionary model. *Bacillus cereus* and *Bacillus subtilis* was used as an outgroup.

3.5.5. Phylogenetic analysis of the 18S rRNA gene sequence of Piroplasmida

Phylogenetic analysis based on a piroplasmid 18S rRNA gene alignment (709 bp) and TN+G evolutionary model positionedd the sequence detected in a pool of *A. sculptum* nymphs (OM842841) close to *Cytauxzoon felis* sequences detected in cats from the United States **(Figure 11).**

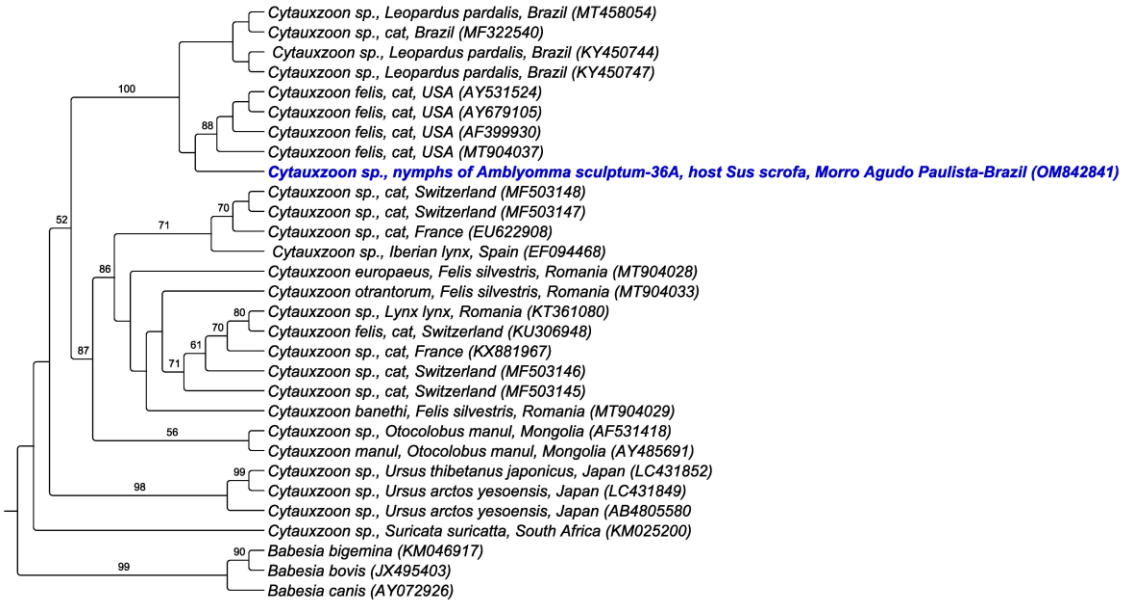

Figure 11. Phylogenetic analysis of 18S rRNA sequences (709 bp alignment) of *Cytauxzoon* spp. inferred by the Maximum Likelihood method and TN+G evolutionary model. *Babesia bigemina*, *Babesia bovis* and *Babesia canis* were used as an outgroup.

4. Discussion

The present study showed low molecular occurrence for Anaplasmataceae agents in wild boars sampled in the southeastern region of Brazil. Out of 67 wild boars' blood samples, 5.97% (4/67) were positive in the molecular assay for Anaplasmataceae agents based on the 16S rRNA gene. On

the other hand, ticks collected from these animals showed high occurrence (50.54% [93/184]) for these agents. The *Anaplasma* sp. sequence detected in a wild boar blood sample in the present study was positioned closely to two *Anaplasma* sp. sequences detected in *A. coelebs* ticks collected from coatis (*Nasua nasua*), in the Iguazu National Park, Paraná, southern region of Brazil. On the other hand, in Peninsular Malaysia, Koh et al. (2016), using a PCR assay based on the 16S rRNA gene, detected *Anaplasma bovis* in 7/10 boars. According to Galindo et al. (2012), although pigs are susceptible to *A. phagocytophilum*, such animals are able to control infection through activation of the innate immune response, as well as by processes of phagocytosis and autophagy. Although *Anaplasma* sp. detected in the present study is a possible new species yet to be described based on isolation and Whole Genome Sequencing techniques, the low prevalence for the agent in question could be related to the control of *Sus scrofa* infection for agents of the genus *Anaplasma*.

The detection of *Anaplasma* sp. in wild boars in the present study (1.49% [1/67]) corroborates previous studies conducted in other parts of the world. *Anaplasma phagocytophilum* DNA has already been reported in wild boars sampled in Romania (4,48% [39/870] by nPCR based on 16S rRNA gene) (Kiss et al., 2014), Germany (12,5% [3/24] by PCR based on 16S rRNA gene) (Silaghi; Pfister; Overzier, 2014), Belgium (0,97% [5/513] by nPCR based on 16S rRNA gene) (Nahayo et al., 2014); Slovenia (4,03% [10/248] by nPCR based on *groESL* operon) (Strasek Smrdel et al., 2009), Slovakia (28,2% [11/39] by PCR based on 16S rRNA gene) (Kazimírová et al 2018), and Czech Republic (5,1% [28/550] by nPCR based on the *groEL* gene) (Hrazdilová et al., 2020). Recently, Hornok et al. (2022), using a qPCR assay based on the *groEL* gene, detected *A.*

phagocytophilum DNA in 19 ticks collected from boars in Hungary: 0.6% (1/165) in *Dermacentor reticulatus*, 10.34% (3/29) *Haemaphysalis concinna* and 1.6% (15/90]) *Ixodes ricinus*.

While *Anaplasma* sp. was detected only in blood samples from wild boars in the present study, *Ehrlichia* spp. was detected in specimens of *A. sculptum* and *A. ovale* collected from the sampled animals. In the phylogenetic analysis based on the 16S rRNA gene, two genotypes of *Ehrlichia* spp. were identified in *A. sculptum* ticks: while one genotype grouped with *E. ewingii*, the other genotype grouped with *Ehrlichia* sp. previously detected in horse-associated *A. sculptum* in the state of Mato Grosso do Sul and *E. ruminantium*. Interestingly, *Ehrlichia dsb* sequences detected in *A. sculptum* adult ticks collected from wild boars in the present study were positioned into a large clade containing *Ehrlichia* sp. genotypes previously detected in jaguars (*Panthera onca*)-associated *Amblyomma* nymphs in the state of Mato Grosso do Sul and an *Ehrlichia* sp. genotype detected in a peccary in the Pará state (Soares et al., 2017). In the phylogeny based on the *dsb* gene, *Ehrlichia* sp. previously detected in horses from Paraná State (Vieira et al., 2016) represented a sister group to the sequences detected herein.

The *Ehrlichia sodB* sequence detected in adults specimens of *A. sculptum* and *A. ovale* collected from wild boars in the present study was shown to be phylogenetically related to *Ehrlichia* sequences previously detected in horses from Brazil and Nicaragua (Vieira et al., 2018), albeit separated from them with 95% clade support. Finally, two sequences of the ITS region (23S-5S) of *Ehrlichia* obtained from *A. sculptum* and one from *A. ovale* collected from wild boars in the present study were positioned into the same clade, separated from the other

clades containing other *Ehrlichia* species. It is important to point out that ITS (23S-5S rRNA) sequences of *Ehrlichia* genotypes detected in horses, peccaries and jaguar-associated ticks were not available in Genbank at the time this manuscript was written. Interestingly, 16S rRNA genotypes of *Ehrlichia* detected in *A. sculptum* collected from horses in the state of Mato Grosso do Sul (Muraro et al., 2021) and from wild boars in the present showed proximity to *E. ruminantium*. Collectively, the results of phylogenetic analyses based on fragments of the 16S rRNA, *dsb*, *sodB* genes and ITS (23S-5S) intergenic region genes suggest the occurrence of *Ehrlichia* genotypes in *Amblyomma* species collected from wild boars that are closely related to those previously detected in horses, peccaries, and ticks collected from jaguars. Considering that *A. sculptum* is a tick species with low host specificity, especially in the larvae and nymph stages, the *Ehrlichia* genotypes detected in ticks carried by wild boars in the present study might have acquired these genotypes from other hosts, such as wild felids and horses.

Even though qPCR assays are considered to show higher sensitivity than conventional PCR assays, all wild boars' blood and tick samples in this study were negative in the multiplex qPCR assays for *Ehrlichia* spp. and *Anaplasma* spp. based on the *groEL* gene. This result could be explained by the fact that the referred qPCR protocol was designed to catch four *Ehrlichia* species (*E. canis*, *E. chaffeensis, E. muris, Ehrlichia* sp. "Anan") and six *Anaplasma* species (*A. phagoytophilum, A. platys, A. bovis, A. ovis, A. centrale,* and *A. marginale*). Therefore, the primers and probes used might have not been able to hybridize to *groEL* gene of the putative novel genotypes of *Ehrlichia* and *Anaplasma* found in this body of work. Similar results were found when screening biological samples

of armadillos, sloths, anteaters (Calchi et al., 2020) and bats (Ikeda et al., 2021) for *Ehrlichia* and *Anaplasma* using this qPCR protocol. On the other hand, *Ehrlichia* and *Anaplasma* genotypes were detected in biological samples from wild rodents (Benevenute et al., 2017) and wild birds (Sacchi et al., 2021) using the referred qPCR assay.

Regarding the occurrence of hemoplasmas, the qPCR assay for porcine hemoplasmas based on the 16S rRNA gene identified 59 (59/67 [88.05%]) wild boars' blood samples and 16 (16/184 [8.69%]) ticks positive for hemotropic mycoplasmas. Previously, Dias et al. (2019), using the same qPCR assay, detected hemoplasmas DNA in 50% (7/14) of the wild boars sampled in the state of São Paulo. Fernandes et al. (2021), by using a qPCR based on the 16S rRNA gene, detected DNA of hemotropic mycoplasmas in 58.5% (38/65) of the wild boars sampled in the state of Goiás, central-western Brazil. The high occurrence of hemoplasmas in wild boars in Brazil differs from that found in Slovakia, where Hoelzle et al. (2010) detected, by using a qPCR assay based on the 16S rRNA gene, *M. suis* DNA in 10% of 359 sampled boars.

The mean quantification of a fragment of the 16S rRNA gene of porcine hemoplasmas estimated by qPCR in wild boars' blood DNA samples (mean =1.93 $x10^4$ copies per microliter of DNA) was shown to be higher than the mean obtained from tick DNA samples (mean=2.8$x10^1$). Since there are no previous reports of *M. parvum* and *M. suis* detection in ticks, hemoplasma DNA detected in ticks could represent residual blood from the ticks hematophagy on the sampled boars. Nevertheless, Shi et al. (2019) demonstrated that *Rhipicephalus microplus* ticks were able to transmit '*Candidatus* Mycoplasma haemobos' from sheep and goats in China to murine models (BALBc). These authors also showed

the occurrence of transstadial transmission and transovarian transmission of this hemoplasma species in *R. microplus*. Thus, the real role of ticks in hemoplasma transmission should be further investigated among free-living boars. In domestic swine, mechanical transmission of hemotrophic mycoplasmas occurs through surgical materials, such as contaminated needles, or directly via excretions and blood from wounds caused through hierarchical fights between animals (Dietz et al., 2014; Henderson et al., 1997). Recently, detection of *M. suis* in piglets, prior to lactation, indicates that this pathogen can also be transmitted transplacentally (Stadler et al., 2019). Also, the detection of *M. suis* DNA in *Haematopinus suis* lice might indicate a possible role of this arthropod as a mechanical vector in the transmission of this agent (Acosta, Ruiz & Sanchez, 2019).

Based on the phylogeny for the 23S rRNA gene of *Mycoplasma* spp. it was possible to infer the occurrence of *M. parvum*, *M. suis* and a putative new genotype of hemoplasma (detected in *Sus scrofa* #3). While most of the sequences detected in the present study showed identity values higher than 99% with *M. suis* and *M. parvum* sequences previously detected in Genbank, the 23S rRNA sequence of hemoplasma detected in boar #3 showed 88.7% identity with *M. parvum*. Recently, Fernandes et al. (2021) also detected 16S rRNA sequences of hemoplasmas in wild boars in the state of Goiás and Paraná with identity values of 91.29% and 92.83% for *M. parvum* and *M. suis*, respectively. Due to the low identity values, the authors suggested the occurrence of new hemoplasma species in the studied population of wild boars. On the other hand, hunting dogs were parasitized by *M. haemocanis*, while hunters were negative for hemoplasmas (Fernandes et al., 2021). Since *M. suis* has been detected in dogs from Argentina (Mascarelli et al., 2016) and in pig farms workers from China

(Yuan et al., 2009), future studies are needed to investigate whether wild boars can act as a source of infection for *M. suis* for hunting dogs and hunters.

Interestingly, 23S rRNA sequences from hemoplasmas referring to clones from sample #8, were positioned separated from each other in the phylogenetic analysis: while the sequence corresponding to clone 1 showed to be closer to *M. suis* (strain lllinois) previously detected in pigs in the United States, clone 2 formed a sister group with a sequence from *M. suis* previosuly detected in a swine from Brazil (MT530439). These results suggest the occurrence of more than one hemoplasma genotype in the same wild boar, as previously demonstrated by Sonálio et al. (2020) in commercial swine farms in central-western Brazil.

With regard to the detection of *Bartonella* spp. in wild boars in this study, all blood samples were negative in the *nuoG* gene-based qPCR assay. On the other hand, Beard et al. (2011) identified sequences from the ITS intergenic region (16S-23S rRNA) of *B. henselae* (5 sequences), *B. koehlerae* (7 sequences) and *B. vinsonii* subsp. *berkhoffi* (3 sequences) in 19.7% (15/76) of feral pigs in North Carolina, United States. Future studies should be conducted aiming at submitting wild boars' blood samples to the diagnostic platform that combines BAPGM liquid cultures (*Bartonella* Alpha Proteobacteria Growth Medium) and solid cultures on blood or chocolate agar, followed by real-time and conventional PCR assays, in order to increase the chances of detection of *Bartonella* spp., as recently demonstrated in blood samples from cats from southeastern Brazil (Furquim et al., 2021) and rodents from central-western Brazil (do Amaral et al., 2022).

In contrast, 3.80% (7/184) of the ticks collected from wild boars in the present study were positive for *Bartonella* spp.. The phylogenetic inferences

based on both *nuoG* and *gltA* genes positioned the sequences obtained from *A. sculptum* collected from wild boars in the same clade as *Bartonella machadoae*, a species recently described by our research group in wild rodents in the Brazilian Pantanal (do Amaral et al., 2022), with a clade support of 95 and 55%, respectively. To the best of authors' knowledge, this is the first report of *Bartonella* sp. DNA in *A. sculptum* ticks. Since *Bartonella machadoae* has so far been detected only in wild rodents and all wild boars sampled herein were negative for this agent, one would suggest that *A. sculptum* nymphs might have acquired bartonellae when feeding on wild rodents in surrounding regions, and infection by this *Bartonella* species has been transstadially transmitted. Future studies aiming at investigating the vector competence of *A. sculptum* in the transmission of *B. machadoae* are much needed. Taking into account the low specificity of *A. sculptum* and parasitism on humans, the role of this tick species in the transmission of *Bartonella* spp. should be further investigated. Indeed, the role of ticks in the transmission of *Bartonella* spp. has been investigated in recent years. In this regard, 9.8% (9/92 [three nymphs and six adults]) of *I. ricinus* ticks collected from vegetation in France were positive for *Bartonella* spp. by a seminested PCR assay based on the *gltA* gene. The sequence obtained from an adult tick showed 96% identity with *B. schoenbuschensis* (Halos et al., 2005). Cotté et al. (2008) demonstrated, by using an artificial membrane feeding system, the ability of infection and transstadial transmission of *B. henselae* in *I. ricinus* collected in France. Similar results were found by Wechtaisong et al. (2020) when using *Rhipicephalus sanguineus* as an experimental model.

All blood and tick samples collected from wild boars in the present study were negative in the *IS1111* gene-based qPCR assay for *C. burnetii*. Recently,

Lim et al. (2020) reported the presence of *Coxiella* endosymbionts DNA in 16.6% (5/32) of *Haemaphysalis hystricis* ticks collected from free-ranging wild boars in Malaysia. *Coxiella burnetii* is capable of infecting a wide variety of wild and domestic animals. In Brazil, DNA of this agent has already been detected in placenta samples from goats (De Oliveira et al., 2018) and spleens from wild rodents (Rozental et al., 2017) and bats (Ferreira et al., 2018). Serological evidence of exposure to this agent has already been reported in cattle and deer from Brazil (Zanatto et al., 2019a; 2019b; De Souza Ramos et al., 2020). Recently, *C. burnetii* DNA has been detected in fresh cheese samples in southeastern Brazil (Nascimento et al., 2021), emphasizing the need for further studies about the epidemiology of Q fever in Brazil, where human cases have already been described (Mares-Guia et al., 2016; Rozental et al., 2018; Lemos et al., 2018).

Regarding the occurrence of piroplasmids, none wild boar sampled in the present study was positive in the 18S rRNA gene-based molecular assay. An 18S rRNA sequence detected in a pool of *A. sculptum* nymphs was positioned closely to *Cytauxzoon felis* sequences detected in cats in the United States. We hypotethized that *Amblyomma sculptum* nymphs might have been infected in the larval stage when feeding on wild felids. To the best of authors' knowledge, wild boars are not susceptible hosts for *Cytauxzoon* sp., which has been detected so far only in domestic (André et al., 2015b) and wild felids (André et al., 2008; De Sousa et al., 2017), bears (Moustafa et al., 2020) and meerkats (Leclaire, Menard and Berry, 2014). In fact, tracks of wild felids (ocelots [*Leopardus pardalis*] and jaguars [*Puma concolor*]) were found on the sites where the animals were sampled (personal communication, Prof. Dr. Estevam Guilherme Lux Hoppe;

Supplementary material). It is noteworthy that the present study detected, for the first time, the presence of *Cytauxzoon* sp. DNA in *A. sculptum* ticks in Brazil. Based on these findings, we suggest that this tick species may play a role as a vector for *Cytauxzoon* sp. in Brazil. Indeed, *Amblyomma sculptum* can parasitize wild felids (Widmer et al., 2011), which in turn may play a role as reservoirs of *Cytauxzoon* sp. in Brazil (André et al., 2018; De Sousa et al., 2017). Apart from the results obtained in the present regarding the occurrence of piroplasmids in wild boars, Hornok et al. (2022) recently detected *Babesia canis* and *Babesia* cf. *crassa* in 4 (2.42% [4/165]) *D. reticulatus* and one (3.4% [1/29]) *H. concinna* collected from wild boars in Hungary, respectively, using a 18S rRNA gene-based PCR assay.

Herein, out of 184 tick samples submitted to a *gltA* gene-based PCR assay for *Rickettsia* spp., only one (0.54% [1/184]) *A. sculptum* was positive. The sequence obtained in the present study was positioned in the same clade as *R. bellii*, with a clade support of 91%. The occurrence of *R. bellii* has been reported in 25 different tick species in America, demonstrating breadth of infection by this *Rickettsia* species in different tick species (Costa et al., 2017). Although this *Rickettsia* species has been recognized as a non-pathogenic rickettsial agent, Snellgrove et al. (2021) demosntrated that guinea pigs experimentally infected with *R. bellii* intra-peritoneally showed fever, orchitis and dermatitis. The detection of co-infection by *R. bellii* and *R. amblyommatis* in *Amblyomma calcaratum* and *Amblyomma longirostre* ticks, and by *R. bellii* and *R. parkeri*-like in *Amblyomma* sp. (haplotype Nazareth) collected from wild birds in Brazil, draws attention to how underestimated coinfection of ticks by two or more species of

Rickettsia is treated, emphasizing the possible role of *R. bellii* in the bio-ecology of *Rickettsia* spp. transmitted by arthropod vectors (Abreu et al, 2019).

Indeed, the role of wild boars as carriers of ticks infected by *Rickettsia* spp. has already been demonstrated on the island of Corsica (France) (*R. slovaca* and *R. aeschlimannii*) (Defaye et al., 2021), Italy (*R. slovaca* and *R. raoultii*) (Sgroi et al., 2020), China ('*Candidatus* Rickettsia laoensis') (Wang et al., 2020), Japan (*Rickettsia japonica*, *Rickettsia tamurae*) (Motoi et al., 2013, Someya et al., 2015), and Spain (*Rickettsia massiliae*, *R. slovaca* and *R. raoultii*) (Castillo-Contreras et al., 2021). In Brazil, Kmetiuk et al. (2019) detected the presence of anti-*Rickettsia* spp. antibodies (*Rickettsia bellii*, *R. rickettsii*, and *R. rhipicephali*) in 72.5% (58/80) wild boars, 14.1% (24/170) hunting dogs, and 14.7% (5/34) hunters in the southern and central-western region of Brazil, emphasizing the possible role of wild boars as carriers of zoonotic *Rickettsia* species.

Considering that wild boars have a broad geographic dispersion during their lifetime (Hartley et al., 2014), the data obtained in this body of work suggests that the studied population of wild boars may act as spreaders of ticks infected with pathogens, such as *Ehrlichia* spp., *Bartonella* spp., hemoplasmas and *Cytauxzoon* spp..

5. Conclusions

High and low occurrence for hemoplasmas and Anaplasmataceae agents, respectively, was detected in the wild boars sampled in the present study. Wild boars are parasitized, despite the low occurrence, by a putative new genotype of *Anaplasma* sp. *Amblyomma sculptum* ticks carried by wild boars were shown to be infected with *Ehrlichia* genotypes phylogenetically

associated with *E. ewingii, E. ruminantium*, and new *Ehrlichia* genotypes previously detected in horses, pecaries, and ticks collected from jaguars. *Mycoplasma suis, M. parvum* and a putative new hemoplasma genotype were detected in wild boars in the southeast region of Brazil. *Rickettsia bellii* was detected in *A. sculptum* nymphs collected from wild boars. This is the first molecular detection of hemoplasmas, *Bartonella machadoae* and *Cytauxzoon* sp. in *A. sculptum*. Coinfection by more than one vector-borne agent was reported in ticks collected from wild boars in the present study. Wild boars and associated ticks do not seem to participate in the epidemiological cycle of *C. burnetii* in the studied region. Wild boars in addition to be susceptible to infection by *Anaplasma* sp. and hemotropic mycoplasmas, may act as potential dispersers of ticks infected with *Ehrlichia* spp., *Bartonella* spp, hemotropic mycoplasmas and *Cytauxzoon* in Brazil. In a nutshell, the results found herein have important epidemiological implications in the transmission of bartonellosis, erlichiosis, hemoplasmosis and cytauxzoonosis to humans and animals, more specifically to horses, rodents, domesticated pigs and cats.

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Ethics Statement

This study was approved by the “Ethics Committee for Animal Experimentation” of FCAV/UNESP (Faculty of Agricultural and Veterinary Sciences of the São Paulo State University) under protocol number #014836/18. The “Instituto Chico Mendes de Conservação da Biodiversidade (ICMBIO)” provided the required annual permits for the capture of wild boars and collection of blood samples and ticks (#62641-2 and #66626-1).

Conflicts of interest

The authors declare no conflict of interest.

Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article. Accession numbers of the generated sequences are available at Genbank database.

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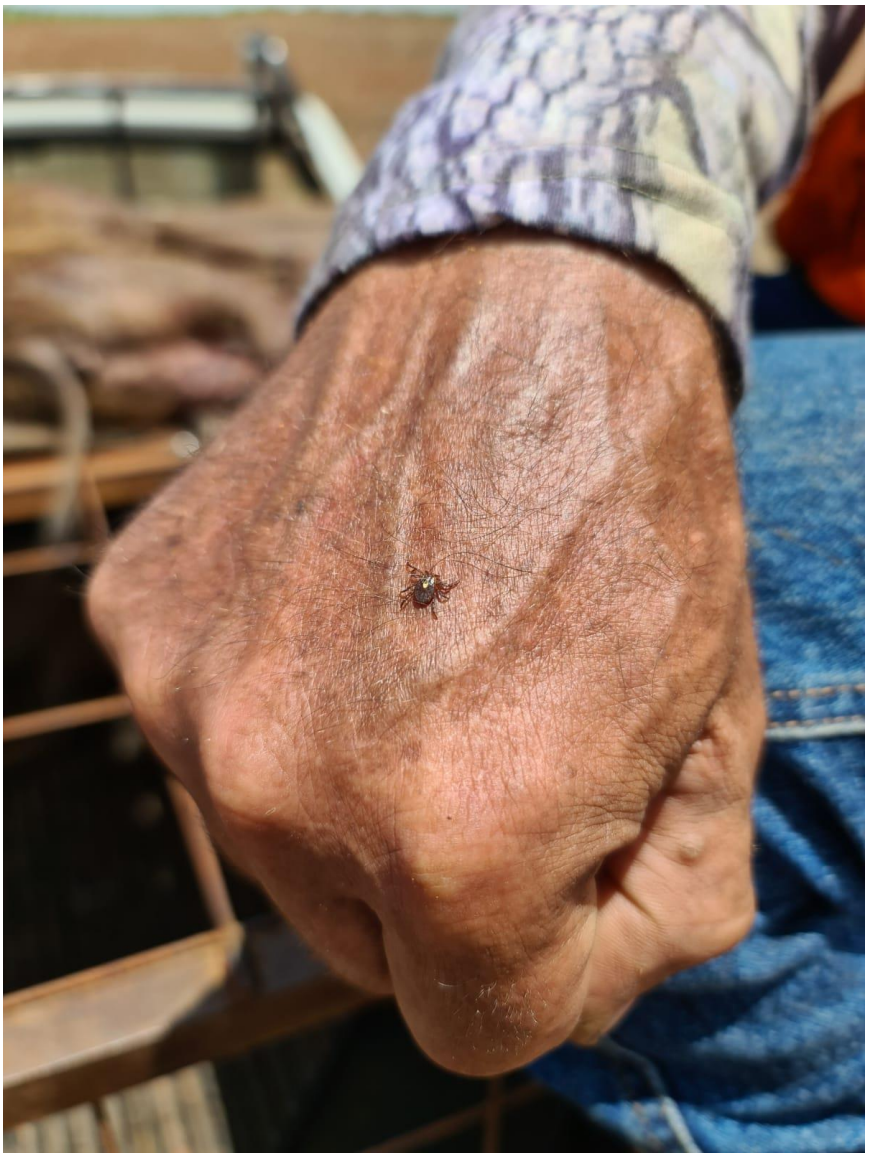
Supplementary Material

SM1. Footprint of a wild cat in the Morro Agudo Paulista, one of the locations where wild boars and ticks were sampled.



SM2. Female specimen of *Amblyomma sculptum* parasitizing the hand of a hunter.