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BRIEF REPORT

First molecular detection of *Borrelia* sp. in tapirs (*Tapirus terrestris*)

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Abstract

Borrelia theileri is a tick-borne spirochete causative agent of fever, apathy and reduced food consumption in cattle. Molecular diagnosis has expanded the understanding of *Borrelia theileri* with new hosts and geographical locations being described. The present study aimed to describe the first molecular detection of *B. theileri* in wild tapirs (*Tapirus terrestris*) from South America. Blood DNA samples obtained from 99 tapirs sampled in Pantanal ($n=61$) and Cerrado ($n=38$) biomes were screened using a qPCR assay based on the 16 S rRNA gene of *Borrelia* sp. Positive samples in the qPCR assay were subjected to PCR assays to allow characterization of fragments from 16 S rRNA and *flaB* genes. Two (2/99; 2.0%) animals from Pantanal biome were positive in the qPCR and one sample presented bands of expected size for the *flaB* protocol. Amplicons from this sample were successfully cloned and sequenced. In the phylogenetic analysis, *Borrelia* sp. from *T. terrestris* grouped together with *B. theileri* sequences previously detected in *Rhipicephalus microplus* ticks and cattle from Minas Gerais State in Brazil, *Rhipicephalus geigyi* from Mali, and *R. microplus* and *Haemaphysalis sulcata* from Pakistan. This finding contributes to our knowledge regarding susceptible hosts species for *B. theileri*. More studies are necessary to understand the potential effects of *B. theileri* on tapir's health.

Keywords Borreliosis · PCR · Spirochetes · South America · Wildlife

Introduction

Bacteria from the genus *Borrelia* are vector-borne spirochetes that can cause disease in animals and humans. Since the first borrelial description in the early 20th century, different species and strains, with distinct epidemiological and clinical aspects, have been reported. Currently, these organisms are divided into three main groups: (1) The Lyme group (LG): represented by the *Borrelia burgdorferi sensu lato* and transmitted exclusively by *Ixodes* ticks; (2) Relapsing fever group (RFG): represented by species mostly vectored by Argasidae ticks, with some species being transmitted by Ixodidae ticks or *Pediculus humanus* louse; and (3) Echidna-Reptile group (ERG): a recently described group, phylogenetically distinct from the former two, and transmitted by Ixodidae ticks (Margos et al. 2018; Trevisan et al. 2021a, b).

Some *Borrelia* species within the RFG group are known to be transmitted by hard ticks. *Borrelia theileri* is transmitted by *Rhipicephalus* sp. ticks and initially reported in Africa, Australia and South America (Theiler 1905; Callow 1967). Although this pathogen was first described in 1904 as

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a causative of fever, apathy and anorexia in cattle (Theiler 1904), it has been also reported in horses (Theiler 1904; Van Heerden and Reyers 1984) and small ruminants (Theiler 1904, 1905; Auoadi et al. 2017).

Molecular diagnosis has expanded the understanding of the epidemiology of *B. theileri* infections, allowing the description of new hosts and geographical locations for this agent. Up to now, this agent has been molecularly detected in raccoon dogs (*Nyctereutes procyonoide*) in Korea (Kang et al. 2018), impalas (*Aepyceros melampus*) in Zambia (Qiu et al. 2021) and rodents (*Rattus rattus*) in Thailand (Takhampunya et al. 2021). Additionally, genotypes closely related to *B. theileri* were detected in sika deer (*Cervus nippon*), wild boars (*Sus scrofa*), and one raccoon (*Procyon lotor*) in Japan (Furuno et al. 2017). Regarding tick vectors, DNA from *B. theileri* was detected in a pool of *Amblyomma* and *Rhipicephalus* (*Boophilus*) ticks in Ethiopia (Cutler et al. 2012) and in *Rhipicephalus* spp. ticks from Pakistan (Khan et al. 2023).

In South America, reports of *B. theileri* are restricted to ticks and cattle (Faccini-Martínez et al. 2022). Occurrence of this agent was molecularly confirmed in *Rhipicephalus microplus* collected from horses and cattle from southeastern Brazil (Yparraguirre et al. 2007; Cordeiro et al. 2018). Moreover, detection is also reported in cattle blood samples from southeastern, midwest and northern Brazil (Paula et al. 2023; Figueiroa et al. 2023), Argentina (Morel et al. 2019) and Colombia (Ramires-Hernández et al. 2022). To date, there are no reports of *B. theileri* in wildlife in South America. The present study aimed to describe the molecular detection of *B. theileri* in wild tapirs (*Tapirus terrestris*) from Pantanal region (Mato Grosso do Sul State) in Brazil.

Materials and methods

Sampling

Between 2013 and 2018, a total of 122 blood samples from free-living ($n=94$) and road-killed ($n=5$) wild tapirs were collected for health assessment purposes. Out of these animals, 61.6% (61/99) were sampled in Pantanal biome whereas 38.4% (38/99) were sampled in Cerrado biome (both biomes located in Mato Grosso do Sul State). All road-killed animals were sampled in Cerrado biome. Sampled animals were 49.5% (49/99) femalces and 50.5% (50/99) males, and 53.5% (53/99) adults (> 48 months old) and 46.5% (46/99) sub-adults (< 48 months old).

Sampling of free-living animals was performed during tapir anesthesia for the installation of GPS collars by professionals from the "Iniciativa Nacional para a Conservação da Anta Brasileira (INCAB-IPÊ)" (Lowland Tapir Conservation Initiative (LTCI-IPÊ). Detailed information about sampling procedures and study areas were described elsewhere (Mongruel et al. 2022a).

The 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 4558/20. The "Instituto Chico Mendes de Conservação da Biodiversidade (ICMBIO)" provided the required annual permits for the capture and immobilization of tapirs and collection of biological samples (SISBIO# 14,603). All protocols for the capture, anesthesia, handling, and sampling of tapirs have been reviewed and approved by the Veterinary Advisors of the Association of Zoos and Aquariums (AZA) — Tapir Taxon Advisory Group (TAG), and the Veterinary Committee of the IUCN SSC Tapir Specialist Group (TSG).

DNA extraction and amplification of mammals' endogenous gene

DNA extraction was performed individually on each tapir blood sample, without making pools, using a commercial kit (InstaGene™ Matrix, Biorad®, Hercules, CA, USA), following the manufacturers' instructions. Conventional PCR (cPCR) assays for the mammalian endogenous genes glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) (450 bp) (Birkenheuer et al. 2003) and interphotoreceptor retinoid-binding protein (*irpb*) (227 bp) (Ferreira et al. 2010) were performed to ensure DNA recovery from blood samples. Samples that did not yield amplicons in either of the PCR protocols were excluded from the subsequent analysis.

Molecular assays

Screening of positive samples was performed using a quantitative real-time (qPCR) assay based on the 16 S rRNA gene from *Borrelia* sp., with a detection limit of approximately 10–20 copies of the fragment (Parola et al. 2011). Each DNA sample was independently evaluated in duplicates and samples that presented differences in Cq values higher than 0.5 were retested in triplicate. For the construction of the standard curve of each reaction, serial dilutions were performed at different concentrations (2.0×10^7 to 2.0×10^1 copies) of a plasmid encoding a conservative fragment of the 16 S rRNA gene from *Borrelia* sp. (pIDTSMART; Integrated DNA Technologies, Coralville, IA, USA). These plasmids were also used as positive controls. The number of plasmid copies was determined by the formula (XG/µL DNA/ [Plasmid Length (BP) × 660]) × 6. 22×10^{23} × plasmid copies/µL. The amplification efficiency (E) was calculated according to the slope of the standard curve using the formula $E=10^{-1/slope}$ (Bustin et al. 2009). Ultra-purified sterilized water (Nuclease-Free Water, Promega®, Madison, Wisconsin,

United States) was used as a negative control for each reaction performed. The qPCR assays were carried out in a C1000-CFX96 thermocycler (BIORAD, Hercules, CA, USA).

Positive samples in the qPCR assay were subjected to cPCR assays to allow characterization of fragments from 16 S rRNA (Marti Ras et al. 1996) and *flaB* (flagellin) genes (Stromdahl et al. 2003). A positive sample for *Borrelia anserina* (Ataliba et al. 2007) was used as positive control and ultra-purified sterilized water (Nuclease-Free Water, Promega®, Madison, Wisconsin, United States) was used as a negative control for all reactions. Fragment sizes, primer sequences, qPCR hydrolysis probes and annealing temperature used herein are described in Table 1.

Cloning and sequencing assays

Amplicons obtained in the cPCR assays were cloned using pGEM®-T Easy System (Promega, Madison, WI, USA), following the manufacturer's recommendations. DNA and vector concentrations used in the ligation reaction were determined to obtain an insert: vector ratio of 3:1. The DNA-binding reaction consisted of adding 40 ng of insert (amplicon), 5 µL buffer, 1 µL of pGEM-T Easy vector, 1 µL of T4 Ligase enzyme and sterilized water q.s.p. Solutions were kindly mixed and incubated at 4 °C for 16 h. Then, 50 µL of competent *Escherichia coli* DH5α cells (10^9–10^{10} CFU/ng DNA) was added to the 10 µL ligation reaction. The mix was kept in ice for 30 min, followed by thermal shock, where microtubes were placed into a water bath at 42 °C for 2 min. Then, 100 µL of SOC (Super Optimal broth with Catabolite repression – Tryptone; Yeast Extract; NaCl; KCl; MgCl2; MgSO4; glucose [ThermoFisher Scientific, Waltham, MA, USA]) was added, and cells were incubated at 37 °C for 1.5 h, under the agitation of 200 rpm. Subsequently, 250 µL of this medium was added to Petri plates containing agar LB (Luria Bertani medium - Tryptone; Yeast Extract; NaCl; distilled water q.s. [ThermoFisher Scientific, Waltham, MA, USA]) medium prepared with 100 µg/mL ampicillin, 40 µl X-gal (5-bromo-4-chloro-3-indolyl-β-Dgalactoside; 0.026%) and 20 µL IPTG (isopropylthio-β-galactoside; 0.82 mM). Plates were incubated at 37 °C for approximately 20 h.

Colonies of bacteria containing the inserts (white colonies) were transferred to tubes containing 5 mL of broth LB medium and incubated at 37 °C for 20 h, followed by plasmid DNA extraction using Wizard® Plus SV Minipreps DNA (Promega, Madison, WI, USA). Purified plasmids were sequenced using the BigDye™ Terminator v3.1 Cycle Sequencing Kit (Thermo Fisher Scientific™, Waltham, MA, USA) and ABI PRISM 3730 DNA Analyzer (Applied Biosystems™, Foster City, CA, USA) (Sanger et al. 1977), at the "Centro de Recursos Biológicos e Biologia Genômica - CREBIO" (Faculdade de Ciências Agrárias e Veterinárias/FCAV, UNESP, Jaboticabal, SP, Brazil). For sequencing, primers GTAAAACGACGGCCAG-3′) and M13-R(5′-CAGGAAACAGCTATGAC-3′) flanking the multiple cloning site (M13) of the pGEM®-T Easy plasmid (Promega), which includes the target gene inserts, were used (Lau et al. 2010).

Phylogenetic reconstruction

Obtained sequences were first submitted to a screening test using Geneious 11.1.3 software (hhtp://www.geneious.com) to evaluate the electropherogram quality and generate the consensus sequences. The BLASTn online program (National Center for Biotechnology Information, Bethesda, MD, USA (Altschul et al. 1990) was used to analyze the nucleotide sequences aiming to browse and compare with

Table 1 Description of gene and size fragment, primer sequences, annealing temperature and reference of the qPCR and cPCR assays used in the present study

Gene and total fragment size		Primer	Sequence	Annealing temperature (°C)	Reference
16 S rRNA (qPCR) 148 bp		Bor16S3-F	5'-AGCCTTTAAAGCTTCGCTTGTAG-3'	60	
		Bor16S3-R	5'-GCCTCCCGTAGGAGTCTGG-4'		Parola et al. 2011
		Probe Bor16S3P	[6FAM] CCGGCCTGAGAGGGTGAACGG		
16 S rRNA (cPCR) 1489 bp	1st Round	FD3 (F)	5'-AGAGTTTGATCCTGGCTTAG-3'	55	
		T50 (R)	5'-GTTACGACTTCACCCTCCT-3'		
	2nd Round	FD3 (F)	5'-AGAGTTTGATCCTGGCTTAG-3'	56	
		16s-1 (R)	5'-TAGAAGTTCGCCTTCGCCTCTG-3'		Marti Ras et al. 1996
	3rd Round	16s-2 (F)	5'-TACAGGTGCTGCATGGTTGTCG-3'	56	
		T50 (R)	5'-GTTACGACTTCACCCTCCT-3'		
	4th Round	Rec4 (F)	5'-ATGCTAGAAACTGCATGA-3'	54	
		Rec9 (R)	5'-TCGTCTGAGTCCCCATCT-3'		
flaB (cPCR) 665 bp		FlaRL (F)	5'-GCAATCATAGCCATTGCAGATTGT-3'	55	Stromdahl et al. 2003
		FlaLL (R)	5'-ACATATTCAGATGCAGACAGAGGT-3'		

sequences from GenBank international database (https://www.ncbi.nlm.nih.gov/genbank/). Consensus sequences obtained in the current study and those retrieved from GenBank were aligned using a MAFFT online software (https://mafft.cbrc.jp/alignment/server/) (accessed on 3 March 2024). Phylogenetic inferences were based on Maximum Likelihood (ML) which was carried out using the IQ-TREE on ACCESS tool via CIPRES portal (https://www.phylo.org, accessed on 3 March 2024) (Miller et al. 2010) and for both best-fit model (following Bayesian Inference Criteria) determination and tree reconstruction. Clades' support was evaluated using bootstrap analysis of 1000 repetitions. Trees were recovered and edited using FigTree v1.3.1. software (Rambaut 2010).

Results

All DNA blood samples successfully amplified at least one of the tested endogenous genes and were considered suitable for the further analysis. Two (2/99; 2.0%) animals from Pantanal biome were positive in the qPCR for *Borrelia* spp. The Efficiency, R^2, Y-intercept and Slope values of the qPCR assays ranged from 92.3 to 99.8%, 0.997 to 1.000, 36.612 to 38.896 and − 3.349 to -3.520, respectively. Quantification values obtained for positive samples were described in Table 2.

The two positive samples in the qPCR assay were negative for the cPCR 16 S rRNA assay tested herein. Regarding the *flab* assay, only one sample (animal ID WE-P-1) amplified bands of expected size. Amplicons from this sample were successfully cloned and sequenced. A sequence of 593 bp from *Borrelia* spp. *flaB* gene was obtained and compared with sequences deposited in the GenBank database using the BLASTn software. The tapir-related sequence showed identity values of 99.66% (query cover: 99%; E-value: 0.0) with a sequence from *B. theileri* detected in *R. microplus* collected from a horse in Brazil (EF141022), 99.65% (Query cover: 96%; E-value: 0.0) with *B. theileri* from *R. microplus* collected from cattle in Colombia (PP262609) and 98.65% (Query cover: 100%; E-value: 0.0) with *B. theileria* from cattle in Goiás State, Brazil (ON191583). The sequence identified in the present study was deposited in the GenBank database under access number PP259188. Results from the molecular assays conducted here were summarized in Table 2.

A ML tree was constructed based on a total alignment of 615 bp containing 37 homologue *flaB* sequences and TPM3 + F + G4 as an evolutionary model. Sequences of representatives from LG (*B. burgdorferi* – DQ016625) and REG (*Borrelia turcica* - AB109246; *Borrelia tachyglossi* - KY586966) were used as outgroups. Sequence obtained from *T. terrestris* grouped with *B. theileri* sequences detected in *R. microplus* ticks in Brazil (MG601737, EF141022), cattle from Brazil (OQ344270, OQ344269, OQ344268, ON191583), *R. geigyi* from Mali (KF569936), *R. microplus* from Pakistan (OR574986), *R. microplus* from cattle in Colombia (PP262609) and *Haemaphysalis sulcata* from Pakistan (OR574985). The *B. theileri*-clade presented a separation in two minor sub-clades with high bootstrap values (100) (Fig. 1).

Although 2/99 tapirs showed positive results in the qPCR protocol for *Borrelia* spp., amplification and sequencing of additional genes, and consequently confirmation of the agent, were possible for only one (1.01%; 1/99) positive tapir.

Discussion

We described herein the first evidence of *B. theileri* occurring in a wild animal in Brazil. The lowland tapir is considered the largest terrestrial mammal from Brazil and the last representative of Neotropical mega-herbivores (Thoisy et al. 2010). Although this mammal species is currently found in four different biomes in the country, it is classified as vulnerable for extinction due factors linked to human activities (e.g. illegal hunting, wildfires, road-kills and land competition) (Medici et al. 2012).

Recently, lowland tapirs have been reported as hosts for different blood-borne and vector-borne agents, including *Trypanosoma terrestris* (Acosta et al. 2013), '*Candidatus* Mycoplasma haematoterrestris', '*Candidatus* Mycoplasma haematotapirus' (Mongruel et al. 2022a), piroplasmids (Gonçalves et al. 2020; Silva et al. 2021; Mongruel et al. 2022b) and *Bartonella* sp. (Mongruel et al. 2023). This is the first detection of *Borrelia* spp. in animals of the genus *Tapirus*. Although serological studies conducted in Germany evidenced the exposure to *B. burgdorferi* sensu lato

Table 2 Information (ID and location) of tapirs positive for *Borrelia* sp. and results obtained in qPCR for 16 S rRNA gene and cPCR for *flaB* gene

Animal ID	Location	Sex/Age	qPCR 16 S rRNA	qPCR Quantification (fragment of 16 S rRNA copy numbers/µL)	Quantification cycle (Cq)	cPCR flaB
WE-P-1	Pantanal	Female/sub-adult	+	1.020×10^1 9.412×10^0	32.86 32.98	+
NAO-P	Pantanal	Male/adult	+	1.358×10^0 1.673×10^0	38.43 38.11	negative

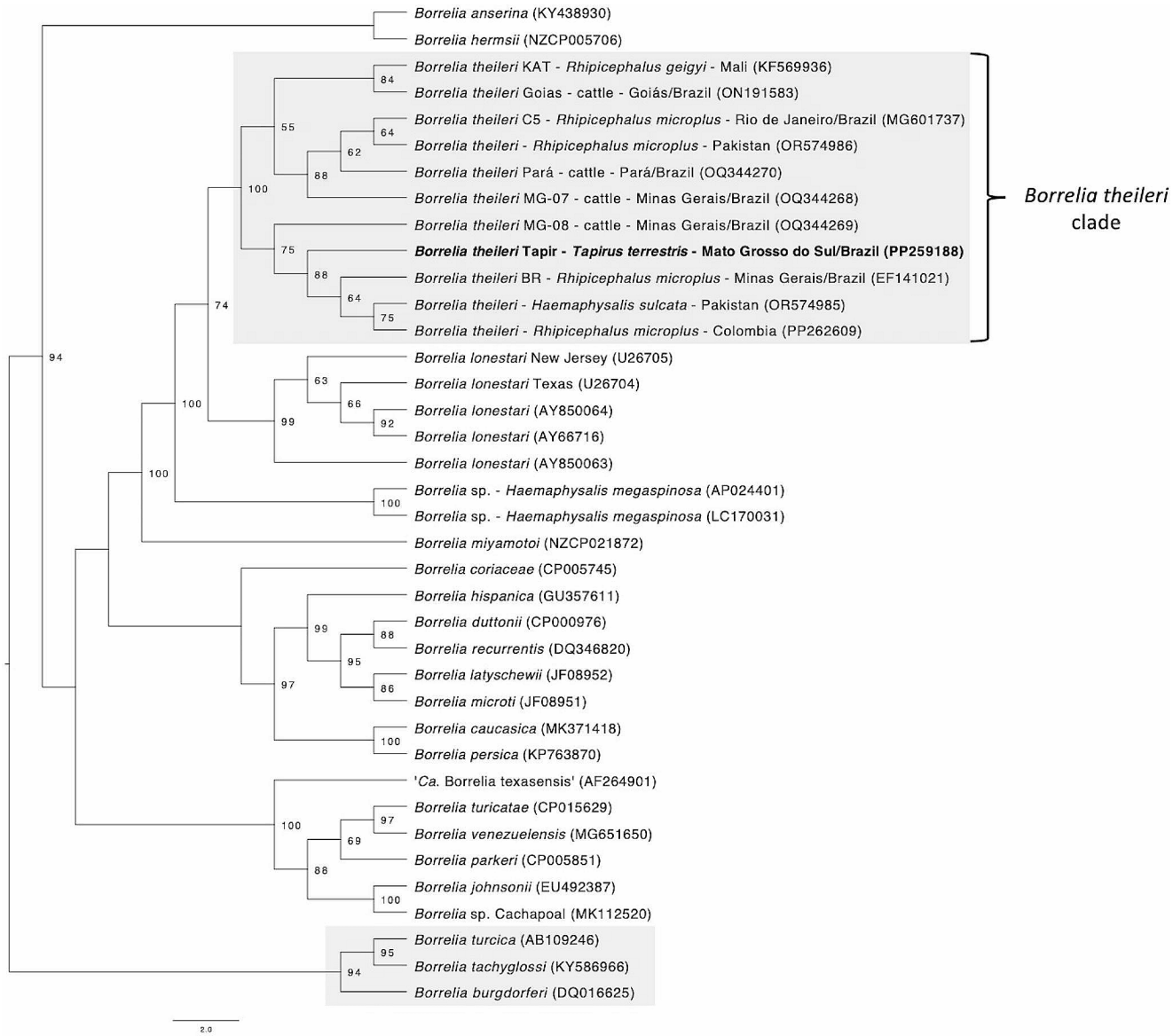

Fig. 1 A Maximum Likelihood tree constructed based on a 615 bp fragment of the *flaB* gene and TPM3u + F + G evolutionary model. Sequence from the present study is highlighted in bold. The clade formed containing *Borrelia theileri* sequences is highlighted in green. Sequences of *B. burgdorferi* (DQ016625), *B. turcica* (AB109246) and *B. tachyglossi* (KY586966) were used as outgroups (highlighted in blue). Only bootstraps values > 50 are shown

(s.l.) in captive *T. terrestris* (Stoebel et al. 2003), the occurrence of cross-reactivity between antibodies produced by *B. theileri* and whole-cell *B. burgdorferi* antigens is described (Rogers et al. 1999) and must be considered during the interpretation of serological results using crude antigens. In this scenario, evidence of *B. theileri* exposure may be misinterpreted. In South America, the molecular screening of *B. burgdorferi* s.l. in *Amblyomma* spp. ticks collected from Andean tapirs (*Tapirus pinchaque*) from Ecuador resulted in negative results (Pesquera et al. 2015).

Molecular detection of *B. theileri* in Brazil has only been reported previously in cattle and *R. microplus* ticks. The 1.01% prevalence found in the present study is within the range reported by previous studies, including 1.0-1.52% for cattle (Paula et al. 2023; Figueiroa et al. 2023), and 0.25-2.0% for *R. microplus* collected from horses and cattle (Yparraguirre et al. 2007; Cordeiro et al. 2018). Furthermore, attempts to characterize additional target genes through cPCR was successful for only one positive sample and one molecular marker (*flaB*). These results might be due the low levels of bacteremia found in the sampled animals, evidenced by the low quantification values estimated by the qPCR assay.

Clinical manifestations associated with *B. theileri* infection are usually non-specific. Classically, this pathogen has been described as a causative of fever (Theiler 1904) and other non-specific clinical signs, such as apathy, rough hair coat, and pallor of mucous membranes in cattle (Sharma et al. 2000; Cordeiro et al. 2018). A study with cattle from Cameroon demonstrated a statically significant correlation between *B. theileri* infection and anemia (Abanda et al. 2019). Moreover, cattle presenting reduced milk production and food consumption were found to be positive for *B. theileri* in Brazil (Figueiroa et al. 2023). However, animals with subclinical infection have also been reported (Paula et al. 2023). Co-infection with *B. theileri* and other vector-borne agents have been reported reported favoring clinical alterations (Abanda et al. 2019; Figueiroa et al. 2023). The *B. theileri*-positive blood sample from the present study also amplified fragments from hemotropic *Mycoplasma* spp. (hemoplasmas) 16 S rRNA (Mongruel et al. 2022a), but the presence of clinical signs due to this coinfection is unknown.

The tick species *R. microplus* is described as the primary vector for *B. theileri* in South America (Faccini-Martínez et al. 2022). In fact, *R. microplus* collected from cattle and horses from Minas Gerais (Yparraguirre et al. 2007) and Rio de Janeiro (Cordeiro et al. 2018) states and infested cattle from Goiás (Paula et al. 2023), Minas Gerais and Pará states (Figueiroa et al. 2023) were reported to be infected by *B. theileri* in Brazil. Moreover, this tick species has also been reported infesting tapirs from Pantanal, as a consequence of land sharing between tapirs and cattle (Labruna et al. 2021).

The phylogenetic analysis confirmed the identity of the sequence detected within the *B. theileri* clade. Moreover, this clade was divided in two minor clades, with high support value (100): one containing *B. theileri* strain C5 (MG601737) and related sequences, and other one containing *B. theileri* strain BR (EF1401022) and related sequences, including the sequence obtained from a tapir. The sequence from strain *B. theileri* C5 was obtained from an engorged female *R. microplus* hemolymph. The tick was collected from apparently healthy bovines maintained in Rio de Janeiro State, Brazil (Cordeiro et al. 2018). Sequence from *B. theileri* BR strain was obtained from *R. microplus* collected from a horse in Minas Gerais State, Brazil (Yparraguirre et al. 2007). This pattern of two major clades being formed was also observed in phylogenetic reconstructions inferred before using this same gene fragment (Paula et al. 2023; Figueiroa et al. 2023; Khan et al. 2023). More studies are necessary to understand if there are any differences among strains regarding the division of the *B. theileri* clade observed here.

We report the first documented occurrence of *B. theileri* in a wild tapir from South America. This finding contributes to our knowledge regarding susceptible hosts species for *B. theileri*. More studies are necessary to understand the potential effects of *B. theileri* on tapir's health.

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Author contributions Authors Contribution statement Anna Claudia Baumel Mongruel: Conceptualization, Methodology, Investigation, Formal analysis, Writing – Original Draft, Writing – Review & Editing. Emília Patrícia Medici: Methodology, Conceptualization, Resources, Writing – Review & Editing. Ariel da Costa Canena: Methodology, Writing – Review & Editing. Rosangela Zacarias Machado: Investigation, Writing – Review & Editing. Keith Clay: Methodology, Resources, Writing – Review & Editing, Supervision. Marcelo Bahia Labruna: Writing – Review & Editing. Marcos Rogério André: Conceptualization, Methodology, Resources, Writing – Review & Editing, Supervision.

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Data availability The dataset generated and analyzed during the current study is available in the NCBI GenBank Nucleotide platform (https://www.ncbi.nlm.nih.gov/genbank/) and can be accessed through accession number: PP259188.

Declarations

Ethics approval The 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 4558/20. The "Instituto Chico Mendes de Conservação da Biodiversidade (ICMBIO)" provided the required annual permits for the capture and immobilization of tapirs and collection of biological samples (SISBIO# 14,603). All protocols for the capture, anesthesia, handling, and sampling of tapirs have been reviewed and approved by the Veterinary Advisors of the Association of Zoos and Aquariums (AZA) — Tapir Taxon Advisory Group (TAG), and the Veterinary Committee of the IUCN SSC Tapir Specialist Group (TSG). Tapir

blood DNA samples from the present study were registered in the Brazilian National System for Management of Genetic Heritage and Associated Traditional Knowledge (Sistema Nacional de Gestão do Patrimônio Genético e do Conhecimento Tradicional Associado – SISGEN) under register number AE4CC0C.

Competing interests The authors declare no competing interests.

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