Molecular screening for tick-borne bacteria and hematozoa in Ixodes cf. boliviensis and Ixodes tapirus (Ixodida: Ixodidae) from western highlands of Panama

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ABSTRACT

The first molecular screening for Rickettsia, Anaplasma, Ehrlichia, Borrelia, Babesia and Hepatozoon was carried out in questing Ixodes cf. boliviensis and Ixodes tapirus from Talamanca Mountains, Panama, using specific primers, sequencing and phylogeny. Phylogenetic analyses for the microorganisms in Ixodes cf. boliviensis confirmed the presence of Rickettsia sp. strain Ibr/CRC endosymbiont (26/27 ticks), three genotypes of the Borrelia burgdorferi sensu lato complex (4/27 ticks), Babesia odocoieli (1/27 ticks), and Hepatozoon sp. (2/27 ticks), tentatively designated Hepatozoon sp. strain Chiriquensis. Phylogenetic analyses for the microorganisms in I. tapirus revealed an undescribed Rickettsia sp., tentatively designated Rickettsia sp. strain Itapirus LQ (6/6 ticks), and Anaplasma phagocytophilum (2/6 ticks). To the best of our knowledge, this is the first report of B. burgdorferi (s.L) complex, A. phagocytophilum, B. odocoieli, and Hepatozoon sp. in Ixodes ticks from Central America, and also the first detection of Rickettsia spp. in Ixodes species in Panama. In light of the importance of these findings, further studies are needed focusing on the role of I. tapirus and I. cf. boliviensis as vectors, and the vertebrates acting as reservoirs.

1. Introduction

Ixodes (Ixodida: Ixodidae) is the most diverse genus of ticks, comprising nearly 260 species worldwide (Guglielmone et al., 2019; Saracho-Bottero et al., 2021). Of these, nearly 70 species have been reported parasitizing humans (Guglielmone & Robbins, 2018). From a public health standpoint, Ixodes spp. are considered among the most important arthropods, particularly in the Northern Hemisphere, because of their implications as vectors of Lyme disease (B. burgdorferi (s.L) complex), granulocytic anaplasmosis (A. phagocytophilum), and to a lesser extend of babesiosis and viral diseases (CDC, 2018). In the Neotropics, where nearly 47 Ixodes species occur (Guglielmone et al., 2019; Saracho-Bottero et al., 2021), parasitism in humans is rare. Indeed, only I. boliviensis, Ixodes pararicinus and Ixodes tropicalis have been reported feeding on humans in this region (Guglielmone & Robbins, 2018; Quintero et al., 2020).

In recent decades, a diverse group of microorganisms have been detected from Ixodes spp. of South America. These finding include the spotted fever group rickettsia (SFGR) “Candidatus Rickettsia andeanae” in I. boliviensis (see Blair et al., 2004), Rickettsia spp. endosymbionts in I. pararicinus, Ixodes fuscipes and Ixodes cf. affinis (Sebastian et al., 2020), and the basal group rickettsia Rickettsia bellii in Ixodes luricatus and I. tropicalis (see Melis et al., 2020, Quintero et al., 2020). In addition, different genotypes of B. burgdorferi (s.L) complex were reported from I. fuscipes (reported as Ixodes aragasi), Ixodes australis, Ixodes longiscutatus, Ixodes neustenensis, Ixodes paranaensis, I. pararicinus, Ixodes sigelos group, and Ixodes stilesi (see Barbiieri et al., 2013; Ivanova et al., 2014; Sebastian et al., 2016; Dall’Agno et al., 2017; Saracho-Bottero et al., 2021).

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et al., 2017; Verdugo et al., 2017; Flores et al., 2018; Cicuttin et al., 2019; 
Muñoz-Leal et al., 2019, 2020; Carvalho et al., 2020). Further, “Candidatus 
Neoehrlichia chilensis” and hemoparasites of the genus Hepatozoon 
were found in Ixodes sp. and I. sigelos group, respectively (Muñoz-Leal 
et al., 2019). Although these Ixodes spp. are not of public health impor-
tance (Guglielmone & Robbins, 2018), recognizing tick species that 
harbor DNA of possible pathogenic microorganisms constitutes a foun-
dational step to understand putative vector roles.

In Central America, of the 18 reported species of Ixodes, the only 
information on associated microorganisms corresponds to SFGR in 
I. boliviensis, Ixodes minor and Ixodes affinis (Troyo et al., 2014; Ogr-
zewalska et al., 2015; Lopes et al., 2016; Polsomboon et al., 2017; 
Bermúdez et al., 2021). Similar to South America, Ixodes spp. in 
Central America are not a public health concern (Guglielmone & 
Robbins, 2018). Nevertheless, serological evidence indicates exposure 
to Lyme disease in people and dogs from Costa Rica (Villalobos-Zúñiga 
& Somogyi, 2012; Montenegro et al., 2017), but this infection has not 
been confirmed.

Eleven species of Ixodes occur in Panama (Bermúdez et al., 2018) and 
there are limited data about their microorganisms (Bermúdez et al., 
2021). In this study, we performed genetic screening for detection of 
Rickettsiales (Rickettsia and Anaplasma), B. burgdorferi (s.l.) complex, and 
tick-borne hematozoa (Babesia and Hepatozoon) in two species of Ixodes 
collected on vegetation in the Talamanca Mountains in Panama.

Fig. 1. A General view of Panama with Volcan Baru National Park and La Amistad International Park (black rectangle). B Ixodes cf. boliviensis, female. C Ixodes tapirus, female. D Ixodes cf. boliviensis, male. E Ixodes tapirus, male
2. Materials and methods

2.1. Sites of collection, tick collection and identification

During September 2019, prospections were performed in the Chiriqui Province, within the Las Nubes station (Volcan Baru National Park, VBNP) and in Los Quetzales trail (La Amistad International Park, LAIP), at an elevation of 2,300 and 2,500 m, respectively (Fig. 1). Both sites belong to the Talamancan mountain range, which represents the highest mountains of the country, with elevations above 3,000 m, and are among the most important hot spots of diversity in Central America, and are also close to one of the main agricultural production areas in Panama. According to the Köppen-Geiger climatic classification (Kottek et al., 2006), this region corresponds to tropical wet climate (Am), but due to the high elevation, subtropical temperatures (0–20°C), and high humidity prevail (Anonymous, 2007, 2012).

Free-living ticks were collected using white cloth dragging and by visual search over the vegetation and preserved in 80% ethanol. The ticks were identified using a taxonomical key (Bermúdez et al., 2018).

2.2. DNA extraction and analysis

Individual ticks were bisected longitudinally using sterile scalpels and washed with distilled water to remove ethanol. DNA was extracted using the commercial kit GeneJET Genomic DNA Purification Kit (Thermo Scientific, Lithuania) following the manufacturer’s instructions.

To compare with other Neotropical *Ixodes* spp., ticks were analyzed via PCR amplification of a ~460-bp fragment of the tick mitochondrial 16S rRNA gene (Mangold et al., 1998). The identity and distances for these sequences were calculated using the Sequence Identity and Similarity calculator (Anonymous, 2021).

Extracted DNA was tested by a battery of PCR protocols targeting *Rickettsia*, family *Anaplasmataceae*, *Borrelia*, *Babesia* and *Hepatozoon*, using specific primers and published protocols for each agent (Table 1). In all PCR assays, distilled water was used as a negative control, and *Rickettsia parkeri* strain Toledo, *Ehrlichia canis* isolate P1091, *Borrelia anserina* PL and *Babesia bovis* Paysandú were included as positive controls. Five microliters of PCR amplicons were separated by electrophoresis in a 1.5% agarose gel, stained with GoodView TM Nucleic Acid Stain (Beijing SBS Genetech Co., Ltd, China), and examined under UV transillumination. Amplicons were purified using GeneJET PCR purification kit (Thermo Fisher Scientific, Lithuania).

2.3. Analyses of sequences and phylogenies

Amplicons of expected size were purified and Sanger sequenced (Macrogen, Korea). Sequences were assembled and trimmed using Geneious software (Kearse et al., 2012). Consensus sequences were submitted to BLASTn analyses to compare with sequences available on GenBank. An alignment of the newly generated sequences and GenBank-retrieved homologues was built for each microorganism group with MAFFT (Katoh et al., 2002). Bayesian analyses were performed in MrBayes v3.1.2 (Husonbeck & Ronquist, 2001) employing four independent Markov chains, 1,000,000 metropolis-coupled MCMC generations and sampling trees every 100th generation. The first 25% of the trees were discarded as “burn-in”, and the remaining trees were used to calculate the Bayesian posterior probability.

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### Table 1

| Targeted microorganism | Gene | Primer name | Sequence | Length (bp) | Reference |
|------------------------|------|-------------|----------|-------------|-----------|
| Tick (mitochondrial)   | 16S rRNA | 16S + 1 | CCGTCTGAACCTGACATCAAG | 460 | Mangold et al. (1998) |
| *Rickettsia* sp.       | 16S   | 16S-1 | GCCAATGATTITTTTAAATTTGCTG | 401 | Labruna et al. (2004) |
| *Rickettsia* sp.       | 16S   | CS-78 | GAACTATGCCTGAGGATGTAAT | 834 | Labruna et al. (2004) |
| *Rickettsia* sp.       | 16S   | CS-239 | GCCCTCTCACCTGTTGGCATATT | 511 | Roux et al. (1996) |
| *Rickettsia* spotted fever group | ompA | Rr190.70p | ATGGCGAATATTTCTCCAAAA | 420 | Choi et al. (2005) |
| *Rickettsia* sp.       | ompB  | ompB-0 | GCCATACCTGCTCTAAACCA | 1500 | Inokuma et al. (2001) |
| *Anaplasmataceae*      | 16S   | EHR166D | AGATTTCTGGCTGCATCAC | 409 | Doyle et al. (2005) |
| *Ehrlichia* sp.        | Dab   | Dab-230 | GTATATCGCAATGACGACAAAT | 1297 | Lotric-Furlan et al. (1998) |
| *Ehrlichia* sp.        | groEl | HS1a | ATGGGCTGGTATGAAAT | 869 | Cyr et al. (2005) |
| *Borrelia* spp.        | 16S rRNA | LoneTop | CTGGCAGTTGGCTTCATAGCA | 665 | Barbour et al. (1996) |
| *Borrelia* spp.        | flaB  | FLb LL | ACCATCTTCATAGGACGAGGG | 354 | Barbour et al. (1996) |
| *Firoplasmid*          | 18S   | BAB 140-167 | CGGTGCTAATGGCTGCTAGG | 551 | Soares et al. (2017) |
Since *I. boliviensis* corresponds to a taxon described in South America, this difference may indicate two different tick species; therefore, here we name the specimens from Panama provisionally as *Ixodes cf. boliviensis*. Our study also provides the first DNA sequences of *I. tapirus*. The newly generated 16S rDNA sequences for the two tick species are available on GenBank under the accession numbers MW717930 and MW717931 (*I. tapirus* females), MW717932 and MW717934 (*I. cf. boliviensis* female and male from VBNP), and MW717934 and MW717935 (*I. cf. boliviensis* female and male from LAIP). Voucher materials (15 *I. cf. boliviensis* and 6 *I. tapirus*) and 6 females of *I. tapirus* from highlands of western Panama were in adults in questing phases, the presence of these microorganisms was detected in adults in questing phases, the presence of these microorganisms detected in *I. tapirus* strain Itapirus LQ, after the species of tick and the name of the trail (Los Quetzales).

**Table 2**

| Microorganism | LAIP | VBNP |
|---------------|------|------|
|               | *Ixodes cf. boliviensis* | *Ixodes cf. boliviensis* | *Ixodes tapirus* |
|               | d (%) | d (%) | q (%) |
| (n = 17)      | (n = 6) | (n = 3) | (n = 1) | (n = 6) |
| Ixodes sp. strain IbR/CRC | 15 | 6 | 1 | 0 | 0 |
| Ixodes sp. strain Itapirus LQ | 0 | 0 | 0 | 0 | 6 (100) |
| Babesia odocoilei | 0 | 0 | 0 | 0 |
| Hepatozoon sp. strain Chiriquensis | 2 (11.8) | 0 | 0 | 0 | 0 |

**Table 3**

| Microorganism | LAIP | VBNP |
|---------------|------|------|
|               | *Ixodes cf. boliviensis* | *Ixodes cf. boliviensis* | *Ixodes tapirus* |
|               | d (%) | d (%) | q (%) |
| (n = 17)      | (n = 6) | (n = 3) | (n = 1) | (n = 6) |
| Babesia odocoilei | 0 | 0 | 0 |

**4. Discussion**

*Ixodes cf. boliviensis* and *I. tapirus* have been reported in highlands of Panama since the middle of the 20th century (Fairchild et al., 1966); however, little is known about these species. Both species are common to collect in trails of VBNP and LAIP, and crawl actively in the underbrush vegetation, possibly due to the behavior of their hosts. In Panama, *I. cf. boliviensis* feeds mainly on wild and domestic carnivores, but also other mammals and humans are included as sporadic hosts (Fairchild et al., 1966; Bermúdez et al., 2018). Since *I. cf. boliviensis* could be a species different from *I. boliviensis*, this fact is relevant for public health, because the only reports of *I. boliviensis* parasitizing humans have been registered in Mexico and Central America, but not in South America (Guglielmo & Robbins, 2018). Regarding *I. tapirus*, to our knowledge, tapis (Tapirus pinchaque and Tapirus bairdii) are the only hosts of this species (Fairchild et al., 1966; Apanaskevich et al., 2017).

To the best of our knowledge, this is the first study to molecularly detect multiple microorganisms in questing *I. cf. boliviensis* and *I. tapirus*. Despite the small numbers of ticks analyzed, it is interesting to note the wide variety of microorganisms found in both species. Since these findings were in adults in questing phases, the presence of these microorganisms must be a result of transstadial transmission.
Rickettsia sp. strain IbR/CRC was initially found in I. boliviensis from Costa Rica (Troyo et al., 2014), therefore our finding was to be expected, considering that Talamanca Mountains extend to Costa Rica and represent similar environments. Our phylogenetic analysis showed that Rickettsia strain Itapirus LQ differs from Rickettsia sp. strain IbR/CRC; therefore, the degree of nucleotide dissimilarity (0.1–0.2% for gltA and 0.2–0.4% for ompA) allows these to be considered as two different endosymbionts, and not a genetic variety. Regarding the ompB gene, the use of a highly conserved fragment prevented the separation of these Rickettsia genotypes. Considering Fournier et al. (2003) and Fournier and Raoult (2009) about the differences in the homologous sequences of gltA, ompA and ompB genes, and the present phylogenetic analysis, both

**Fig. 2.** Bayesian phylogenetic trees for Rickettsia spp. based on the gltA (A) and ompA (B) genes. Bayesian posterior probabilities are shown at the nodes (only values > 0.95 are shown). Rickettsia sibirica and Rickettsia parkeri were used as the outgroup. The newly generated sequences are indicated in bold.

**Fig. 3.** Bayesian phylogenetic trees for Anaplasma phagocytophilum based on the 16S rDNA (A) and groEL (B) genes. Bayesian posterior probabilities are shown at the nodes (only values > 0.95 are shown). Ehrlichia ruminantium was used as the outgroup. The newly generated sequences are indicated in bold.
Rickettsiae represent endosymbionts of the genus Rickettsia not yet described. Rickettsia endosymbionts can be transmitted vertically and show a high prevalence in tick populations (Socolovschi et al., 2009; Kurtti et al., 2015), a fact that explains our findings in I. cf. boliviensis (96%) and I. tapirus (100%). Other Rickettsia endosymbionts from Central American Ixodes spp. include Rickettsia sp. strain Barva in I. minor from Costa Rica (Ogrzewalska et al., 2015) and Rickettsia spp. in I. affinis from Belize and Panama (Lopes et al., 2016; Polsomboom et al., 2017; Bermúdez et al., 2021). Springer et al. (2018) reported Rickettsia monacensis in I. boliviensis from Costa Rica based on multi-locus typing of seven loci; however, our phylogenetic analyses showed that Rickettsia sp. IbR/CRC strain from Costa Rica and Panama share, albeit with low support, the same clade with other Rickettsia endosymbionts reported in Ixodes spp. of Central and South America. This clade is separate from R. monacensis; therefore, because this pathogen is reported from Ixodes ricinus complex in Europe, its records from Ixodes spp. in Central America must be considered with caution.

This is the first report of A. phagocytophilum in I. tapirus. Phylogenetic results demonstrated various genotypes of A. phagocytophilum around the world, which have been detected in Ixodes spp. or from different groups of mammals (Brouqui & Matzumoto, 2007). This pathogen affects domestic mammals such as horses, ruminants and carnivores, and also causes human granulocytic anaplasmosis, a disease reported in some countries of Europe and in the USA (Grzeszczuk et al., 2007). In the Neotropics, information of Anaplasma spp. includes reports in mammals and ticks from Mexico (Ojeda-Chi et al., 2019) and South America (Santos et al., 2013; Felix et al., 2020). So far, genotypes closely related to A. phagocytophilum were reported in Amblyomma multitunicatum collected from T. pinchaque and vegetation (Pesquera et al., 2015). The finding of Anaplasma spp. in A. multipunctum and I. tapirus may represent a potential ticks-tapirs relationship to investigate. Since A. phagocytophilum is a pathogen of medical and veterinary importance, its relevance in Panama should be considered.

Our findings of three genotypes of the B. burgdorferi (s.l.) complex in I. cf. boliviensis, indicate that the Talamanca strain A is a sister group of B. burgdorferi (s.s.), while strains Talamanca B and C represent different lineages. Considering the diversity of this complex in South American Ixodes spp., our results indicate that B. burgdorferi (s.l.) complex may be widely distributed in Talamanca Mountains. Since B. burgdorferi (s.l.) complex includes more than 20 species of pathogens and endosymbionts (Carvalho et al., 2020), our findings do not necessarily indicate a risk to public health.

![Bayesian phylogenetic trees for Borrelia spp. based on the flaB gene.](image)
even though *Ixodes cf. boliviensis* is a synanthropic and anthropophilic tick in the highlands of Panama (Bermúdez et al., 2016, 2018).

*Babesia odocoilei* is a parasite of deer in North America and has been related to *Ixodes scapularis* (see Milnes et al., 2019). Criado-Fornelio et al. (2003) pointed out that *B. odocoilei* belongs to the “true” *Babesia* group, along with *Babesia canis*, *Babesia gibsoni* and *Babesia divergens*, and Vannier and Krausse (2009) indicated that *Babesia venatorum* is a species closely related to *B. odocoilei*.

According to Smith (1996), the genus *Hepatozoon* includes more than 300 species. Reports for *Hepatozoon* spp. from Panama include *Hepatozoon muris* and *Hepatozoon procyonis*, diagnosed by microscopy of blood samples from rats and raccoons, respectively (Schneider, 1968), and an undescribed *Hepatozoon* sp. from a blood sample form the pit viper *Bothrops asper* (see Quintero et al., 2021). Phylogenetic analyzes indicate that the *Hepatozoon* strain Chiriquensis detected here represents a putative new species.

Fig. 5. Bayesian phylogenetic tree for *Hepatozoon* spp. based on the 18S rRNA gene. Bayesian posterior probabilities are shown at the nodes (only values > 0.95 are shown). *Adelina grylli* was used as the outgroup. The newly generated sequence is indicated in bold.
5. Conclusions

In comparison to other genera of the family Ixodidae, such as *Amblyomma* or *Rhipicephalus*, there are few studies of *Ixodes* spp. from Panama and Central America (Bermúdez et al., 2016; Bermúdez & Troyo, 2018). Our results present novel data for different microorganisms associated with two species of *Ixodes*, showing the importance that these ticks may have in enzootic cycles for pathogens such as *A. phagocytophilum*, but also in the maintenance of species whose pathogenic potential remains unknown, such as *Rickettsia* spp. strains Ibr/CRC and *Rickettsia* sp. strain Itapirú LQ, *Borrelia* sp. strains Talamanca, *B. odocoieli*, and *Hepatozoon* sp. strain Chiriquensis. Therefore, further studies are necessary to demonstrate the ecology of these microorganisms in the Talamanca Mountains range.

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CRediT author statement

SEBC and JMV conceived the study. SEBC, LD, NK and JMV collected and performed the laboratory work and molecular analyses. SM-L and JMV performed phylogenetic analyses. SEBC and JMV drafted the manuscript. All authors contributed to reviewing the manuscript, and read and approved the final version.

Data availability

Partial gene sequences generated in this study are deposited in the GenBank database: Rickettsia sp. strain Ibf/C (gb: MW696688; ompA: MW699697-MW699700, MW731468; ompB: MW699702, MW699703, MW699706-MW699709); Rickettsia sp. strain Itapizar LQ (gb: MW699694, MW699695; ompA: MW699696, MW699701; ompB: MW699704, MW699705); A. phagocytophilum (16S rDNA: MW677507, MW775708; groEL: MW699686, MW699687); B. burgdorferi (s.l.) complex Talamanca A (MW699712, MW699713); B. burgdorferi (s.l.) complex Talamanca B (MW699714); B. burgdorferi (s.l.) complex Talamanca C (MW699711); B. odocoilei (MW724527); and Hepatozoon sp. strain Chiriquensis (MW724528).

Declaration of competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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