Experimental transmission of a novel relapsing fever group Borrelia harbored by Ornithodoros octodontus (Ixodida: Argasidae) in Chile

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Research Article

Keywords: Soft-tick-borne diseases, “Candidatus Borrelia octodonta”, MLST, phylogeny, vector competence, relapsing fever group

DOI: https://doi.org/

Additional Declarations: No competing interests reported.

Experimental transmission of a novel relapsing fever group *Borrelia* harbored by *Ornithodoros octodontus* (Ixodida: Argasidae) in Chile

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Abstract

Tick-borne relapsing fever spirochetes of genus *Borrelia* thrive in enzootic cycles involving *Ornithodoros* spp. (Argasidae) mainly, and rodents. The isolation of these spirochetes usually involves a murine model in which ticks are feed and the spirochetes detected in blood several days later. Such an experiment also demonstrates that a given species of tick is competent in the transmission of the bacteria. Here, soft ticks *Ornithodoros octodontus* were collected in Northern Chile with the objective to experimentally demonstrate its capacity to transmit a *Borrelia* sp. detected in a previous study. Two guienea pigs (*Cavia porcellus*) were used to feed nymphs and adults of *O. octodontus* and the spirochetes in blood were inspected by dark-field microscopy and nested PCR. Although spirochetes were not seen in blood, DNA was detected in only one animal 11 days after ticks were fed. Genetic sequences of *Borrelia flaB*, *clpX*, *pepX*, *recG*, *rplB*, and *uvrA* genes retrieved from DNA extraction of positive blood were employed to construct two phylogenetic analyses. On the one hand, the *flaB* tree showed the Borrelia sp. transmitted by *O. octodontus* clustering with *Borrelia* sp. Alcohuaz, which was previously detected in that same tick species. On the other hand, concatenated *clpX-pepX-recG-rplB-uvrA* demonstrated that the characterized spirochete branches together with "*Candidatus* Borrelia caatinga", a recently discovered species from Brazil. Based on the genetic profile presented in this study, the name "*Candidatus* Borrelia octodonta" is proposed for the species transmitted by *O. octodontus*. The fact that spirochete was not observed in blood of guinea pigs, may

reflect the occurrence of low spirochetemia, which could be explained because the succeptibilitysusceptibility of infection vary depending on the rodent species that is used in experimental models. Although the vertebrate reservoir of "Ca. *Borrelia octodonta*" is still unknown, *Octodon degus*, a rodent species that is commonly parasitized by *O. octodontus*, should be a future target to elucidate this issue.

Keywords: Soft-tick-borne diseases, "*Candidatus* Borrelia octodonta", MLST, phylogeny, vector competence, relapsing fever group.

Introduction

The genus *Borrelia* (*Spirochaetales*) encompasses a group of conical motile bacteria with helical shape (Margos et al. 2020). Spirochetes of this genus thrive in enzootic cycles infecting a wide range of vertebrate hosts and are primarily transmitted by ticks of the families Argasidae (soft ticks) and Ixodidae (hard ticks) (Wang 2015; Barbour 2018; Barbour and Gupta 2021). Three principal groups compose the genus *Borrelia*, but only two include species that pose a risk to human health: (i) the *Borrelia burgdorferi* sensu lato complex, some of which are known to cause Lyme borreliosis; and (ii) the relapsing fever group (RFG), which includes the causative agents of soft-tick-borne relapsing fever (STBRF), a zoonotic disease of global distribution transmitted by ticks of the genus *Ornithodoros* (Margos et al. 2018; Faccini-Martínez et al. 2022).

Ornithodoros spp. naturally transmit *Borrelia* spp. to vertebrate hosts while feeding (Barbour and Gupta 2021). As nidicolous ticks, they parasitize a limited range of vertebrate hosts, rendering the foci of RFG borreliae rather endemic (Barbour and Gupta 2021). In the tick, the spirochetes colonize the midgut and salivary glands and persist through subsequent molts and further bloodmeals (Lopez et al. 2021; Barbour and Gupta 2021). *Ornithodoros* spp. break their fast approximately every three months (Johnson et al. 2016); however, they can starve for years (Francıs 1938). Once colonized

by spirochetes, ticks remain infected throughout their lifespan (Francıs 1938; Johnson et al. 2016).

Soft tick-borne RFG spirochetes seem to have evolved to depend on a specific tick vector for effective transmission, as notably seen in *Ornithodoros* spp., which transmit a specific species of *Borrelia* (Davis 1956; Schwan 1996; Krishnavajhala et al. 2018; Faccini-Martínez et al. 2022). For instance, in South America, some studies implemented murine models to demonstrate the competence of *Ornithodoros rudis* and *Ornithodoros* cf. *tabajara* as vectors of RFG spirochetes (Muñoz-Leal et al. 2018; Oliveira et al. 2023), meaning that the ticks can acquire, maintain, and successfully transmit an infectious agent (Sonenshine and Mather 1994). Intrinsic (genetic) tick factors that define host predilection, feeding time, tick-host-pathogen interactions, and susceptibility to infection modulate the competence of transmitting a given microorganism (Beerntsen et al. 2000; Sonenshine 2005; de la Fuente et al. 2017). It is worth mentioning that the susceptibility of infection with RFG spirochetes may vary depending on the rodent species that is used in experimental models (Burgdorfer and Mavros 1970; Bermúdez et al. 2021).

In Chile, 11 *Ornithodoros* spp. are distributed across the northern and central regions of the country (González-Acuña and Guglielmone 2005; Muñoz-Leal et al. 2016, 2020, 2023). However, evidence regarding RFG borreliae is still emerging, and currently limited to molecular detections in ticks (Muñoz-Leal et al. 2019a, b; Thompson et al. 2021) and one rodent species (Thomas et al. 2020). Noteworthy the diversity of *Borrelia* in Chile might be underestimated. Based on the report of a novel *Borrelia* genotype detected in *Ornithodoros octodontus*, a tick associated with *Octodon degus* (Rodentia: Octodontidae) (Thompson et al. 2021), the present study aimed to determine whether *O. octodontus* could transmit RFG borreliae under laboratory conditions, and to genetically characterize the detected spirochetes.

Methods

Tick collection and identification

In July of 2021, we visited two localities in the Coquimbo Region, northern Chile: (i) a residential area in El Guindo, Santa BertaOvalle Municipality (-30.651693, -71.091803; elevation 346 m) and (ii) a rural area in Illapel Municipality (-31.502081, -71.112455; 604 m) (Fig. 1). One hundred and one ticks were extracted from dens frequented by *O. degus*. Two females, six males, and 82 nymphs were collected at El Guindo, and three females, one male, and seven nymphs at Illapel. In the laboratory, two tubes with living ticks, each one from one locality, were maintained in an incubator at 25ºC and 80% humidity for four months before starting the experiment. All the specimens were identified as *O. octodontus* (Muñoz-Leal et al. 2020).

Feeding of ticks

To determine whether the collected ticks could transmit spirochetes, two guinea pigs (GP1 and GP2), were infested with each group of ticks. Ninety ticks from El Guindo were fed on GP1, while 11 ticks from Illapel were fed on GP2. Guinea pigs were anesthetized intramuscularly with a dexmedetomidine and ketamine solution (0.05 mg/kg and 5 mg/kg, respectively) (Hedley 2020), ventrally trichotomized, and ticks introduced into transparent plastic feeding chambers fixed to the abdomen of the animals with a skin-compatible adhesive (Kamar Products, Zionsville, IN, USA). Tick attachment and detachment was visually corroborated. After the meal, all ticks were recovered and placed in an incubator. Atipamezole (1 mg/kg) was inoculated subcutaneously to both guinea pigs to recover of anesthesia (Morrisey and Carpenter 2020). Procedures using laboratory animals were approved by the Ethics Committee of the Faculty of Veterinary Sciences of University of Concepción (CBE-24-2021).

Monitoring of guinea pigs

To detect spirochetes, blood samples were taken within 24 hours after tick exposure and for 20 days thereafter (21 days in total) (Muñoz-Leal et al. 2018; Oliveira et al. 2023). Two drops of blood were drawn puncturing the animals' ear pinna using a 25-gauge needle (Williams and Kendall 2015). One drop of blood (≈2.5 µL) was expressed onto a slide and 100 fields were observed at 400X using dark-field microscopy. The other drop (≈50 µL) was stored in a microtube at -80°C until processing. Guinea pigs that did not display motile spirochetes within the 21 days of experiment were considered negative and no subsequent bleeding was performed. At the end of the experiment, the animals were sacrificed according to the American Veterinary Medical Association (AVMA) Guidelines for the Euthanasia of Animals (AVMA 2020).

DNA extraction, gene amplification and sequencing

After 21 days of experiment, stored samples underwent DNA extraction using the DNeasy Blood & Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and DNA was eluted in 25 µL of buffer AE (10 mM Tris–Cl; 0.5 mM ethylenediaminetetraacetic acid [EDTA], pH 9.0). Subsequently, DNA was quantified and assessed for quality using an Epoch™ Microplate Spectrophotometer (BioTek Instruments, Inc., Winooski, VT, USA). Samples with an A260/A280 DNA ratio ranging from 1.6-2.0 were deemed suitable for PCR amplification protocols (Khare et al. 2014). A conventional PCR assay targeting the mammalian gene glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was used as an internal extraction control (Birkenheuer et al. 2003).

Borrelia screenings were carried out through a nested PCR protocol targeting the flagellin (*flaB*) gene following Stromdahl et al. (2003). Additionally, attempts to amplify the housekeeping genes *clpA*, *clpX*, *pepX*, *pyrG*, *recG*, *nifS*, *rplB,* and *uvrA* using

degenerate primers from the *Borrelia* MLST database (https:// pubmlst.org/borrelia) were performed as well. DNA of *Borrelia anserina* PL was employed as a positive control. All PCR reactions were performed in a thermal cycler (ProFlex™ Base 32 × 3; Applied Biosystems, Thermo Fisher Scientific, Waltham, MA, USA). Each reaction had a final volume of 25 µL, consisting of 12.5 µL DreamTaq Green PCR Master Mix (Thermo Fisher Scientific), 1 µL of each primer (0.4 µM), 8.5 µL of ultra-pure water and 2 µL of template DNA. Ultra-pure water was used as a negative control in all cases. PCR products were stained with GelRed® (Biotum, Tehran, Iran), separated by electrophoresis in 2% agarose gels, and then visualized using an ENDURO™ GDS UV transilluminator (Labnet International, Edison, NJ, USA). Amplicons displaying bands of the expected size were purified and Sanger-sequenced in both directions at Macrogen (Seoul, South Korea).

Sequence and phylogenetic analyses

To generate consensus sequences, AB1 files were visualized, quality-checked, and edited with Geneious Prime® version (v) 2021.2.2 (www.geneious.com). Base calls with Phred values ≥ 20 were considered suitable for subsequent analyses (Ewing and Green 1998; Ewing et al. 1998). The consensus sequences were then compared with the BLASTn (https://blast.ncbi.nlm.nih.gov/Blast.cgi) tool to identify orthologous sequences.

Orthologous sequences recovered from GenBank were aligned with the sequences obtained in this study with MAFFT using default parameters (Katoh and Standley 2013). Subsequently, the alignments were curated with Block Mapping and Gathering with Entropy (BMGE) using default parameters to map informative regions for phylogenetics inferences (Criscuolo and Gribaldo 2010).

Phylogenies were constructed with Maximum likelihood (ML, Felsenstein 1981) and Bayesian inference (BI, Rannala and Yang 1996; Yang and Rannala 1997) methods in IQ-TREE v. 1.6.12 (Nguyen et al. 2015) and MrBayes v. 3.2.6 (Ronquist et al. 2012), respectively. As protein-coding genes exhibit distinct nucleotide exchange rates at the first, second, and third codon positions (heterogeneity) (Yang 1996; Ronquist et al. 2012), datasets were partitioned into three codon positions (position-1, position-2, and position-3) (Yang 1996; Ronquist et al. 2012; Lanfear et al. 2012; Kainer and Lanfear 2015).

For ML analyses, the best-fit evolutionary models and the optimal partition scheme were computed using the ModelFinder command "-m TESTNEWONLYMERGE -mrate G" (Kalyaanamoorthy et al. 2017). To assess the robustness of the inferred tree, we employed rapid hill-climbing and stochastic disturbance methods with 1000 ultrafast bootstrapping pseudo-replicates. Ultrafast-bootstrap values < 70%, 70–94%, and ≥ 95% were interpreted as representing non-significant, moderate, and strong statistical support, respectively (Minh et al. 2013). Best partition schemes for BI were calculated by ModelFinder and MrBayes command "lset = mixed rates = invgamma" (Huelsenbeck et al. 2004; Ronquist et al. 2012; Lanfear et al. 2012). Two independent tests of 20 x 10^6 generations and four Markov Chain Monte Carlo (MCMC) chains were conducted, sampling every cycle until convergence, which was assessed using Tracer v. 1.7.1 (Rambaut et al. 2018). Nodes with Bayesian posterior probabilities (BPP) > 0.70 were considered high statistical support (Huelsenbeck and Rannala 2004). All best-fit models and partitions schemes were selected based on Bayesian Information Criteria (BIC) (Schwarz 1978). Trees were visualized and edited using FigTree v. 1.4.1 (http://tree.bio.ed.ac.uk/software/figtree/) and Inkscape v. 1.1 (https://inkscape.org/es/). Consensus trees for both ML and BI were generated for each dataset following the approach of Santodomingo et al. (2022).

Results

Experimental transmission

Not all the ticks introduced into the feeding chamber attached to the hosts' skin. Indeed, only nymphs were engorged and the adults did not fed. The tick feeding time did not exceed 180 minutes. Following tick exposure Spirochetes were not detected during the 21 days of blood examination with dark-field microscopy.

Borrelia detection

Stored blood samples were analyzed at the end of the transmission experiment. PCRs targeting the *GAPDH* gene yielded amplicons of the expected size, confirming successful DNA extraction for both guinea pigs. All DNA extractions yielded a concentration ranging from ~10 to 15 ng/µL, along with an A260/A280 quality ratio of 1.8 to 2.0. In GP1, the PCR targeting the *flaB* gene yielded amplicons of the expected size (462 bp) between day 11^{th} to 18^{th} posterior to tick exposure. GP2 was negative for *Borrelia* detection during all the period. One sample yielding marked amplicons for *flaB* PCR was submitted to the eight-gene MLST characterization. Expected amplicons and good quality sequences were obtained for five of them (*clpX*, *pepX*, *recG*, *rplB,* and *uvrA*). After BLASTn comparisons, the sequence of *flaB* matched with 95.44% identity (440/461 bp, 99% query cover, 0 gaps, 0 E-value) the uncultured *Borrelia* sp. clone Omi2MT (MT076262), detected from *Carios mimon* in Brazil (Muñoz-Leal et al. 2021). Subsequent pairwise comparisons of our *Borrelia flaB* genotype with *Borrelia* sp. Alcohuaz (MW981443), detected in *O. octodontus* by Thompson et al. (2021) in Chile, revealed 99.64% similarity (279/280 bp, 99% query cover, 0 gaps, 6e-150 E-value).

Phylogenies inferred from *flaB* and MLST genes positioned the sequenced *Borrelia* genotype into the RFG borreliae clade. Notably, *Borrelia* sp. Alcohuaz formed a monophyletic group with the *Borrelia* sp. detected in this study. This clade is related

to borreliae genotypes characterized from rodents in Chile, including one genotype detected in an undetermined *Ornithodoros* sp. from Bolivia. On the other hand, the concatenated MLST phylogenetic analysis showed the *Borrelia* sp. detected in this study branching independently within a clade including *Borrelia* sp. FMV_PCST_FN from Brazil, *Borrelia recurrentis* and other STBRF borreliae (*Borrelia hispanica*, *Borrelia duttonii*, *Borrelia crocidurae*, *Borrelia persica*), indicating that the characterized species belongs to the relapsing fever group. Given that phylogenetic analyses support the detection of a putative new species, we provisionally propose the name "*Candidatus* Borrelia octodonta" for the spirochete detected in *O. octodontus* (Fig. 2). The name is proposed in allusion to the epithet of the tick species from which the spirochete was transmitted.

Discussion

In the present study, we determined that *O. octodontus* transmitted an RFG *Borrelia* to a guinea pig under laboratory conditions. The fact that only nymphs attached and engorged, confirms the hypothesis that adults of *O. octodontus* this species do not feed, or are very specific on choosing their hosts (Muñoz-Leal et al. 2020). It is well-documented that *Ornithodoros* spp. are proficient at transmitting RFG spirochetes while feeding (Johnson et al. 2016; Jakab et al. 2022). Once ticks are attached, RFG spirochetes in the salivary glands are inoculated and swiftly penetrate the host's skin to reach the bloodstream (Lopez et al. 2021). In this study, *Borrelia* DNA was detected in blood only eleven days after the ticks fed. These findings suggest that the bacterium not only reached the bloodstream but it also was detectable in peripheral blood of the ear pinna, underscoring its dissemination capacity (Liang et al. 2020). Interestingly, our results are in the line with those of Oliveira et al. (2023), who also used guinea pigs as experimental model to recover spirochetes and observed the first peak of spirochetemia between 9 and 11 days after ticks fed on the animals. Collectively, our results suggest

that *O. octodontus* is a competent vector of a novel species of *Borrelia* (Sonenshine and Mather 1994; Beerntsen et al. 2000; de la Fuente et al. 2017).

Our incapacity to detect spirochetes in the blood of GP1 using dark-field microscopy might suggest a low spirochetemia that this novel *Borrelia* sp. could display in *C. porcellus*. Indeed, the propensity of borreliae to infect a given host varies depending on the host species (Burgdorfer and Mavros 1970; Bermúdez et al. 2021). On the other hand, even though the GP2 was negative using both nested PCR and dark-field microscopy, we cannot rule out that *O. octodontus* from Illapel do not harbor any *Borrelia* sp., because the number of specimens fed on GP2 was rather low.

The use of an experimental murine model to demonstrate tick vectorial competence for borreliae species is a widely used method (Schwan et al. 2012; Muñoz-Leal et al. 2018; Bermúdez et al. 2021; Oliveira et al. 2023). However, research has shown that not all murine models develop infections for a given *Borrelia* sp., even those rodent species sympatric or phylogenetically related with a competent host, a fact that reflects the complexities of the spirochetes' transmission cycles (Burgdorfer and Mavros 1970; Bermúdez et al. 2021). In this context, identifying the competent vertebrate hosts is crucial to gain a more comprehensive understanding of the epidemiological cycle of a given *Borrelia* sp. (Sonenshine 2005).

Once colonized by RFG borreliae, ticks of the genus *Ornithodoros* remain infectious throughout their lifespan (Francıs 1938; Johnson et al. 2016; Jakab et al. 2022). In this sense, the fact that the collected ticks fasted for four months until the beginning of the experiment, and transmitted a spirochete to one guinea pig, undoubtedly suggests that at least one tick from El Guindo locality was infected (Francıs 1938; Johnson et al. 2016). However, it is possible that more infected ticks were present among the fed specimens, since experimental studies have demonstrated that at least three ticks per mouse are necessary to transmit spirochetes (Boyle et al. 2014; Stewart et al. 2022). Our results support the hypothesis by Thompson et al. (2021) pointing that

O. octodontus is involved in the transmission of RFG borreliae. Considering that larvae of this tick species feed on the rodent *O. degus* and adult ticks and nymphs are found in their burrows (Muñoz-Leal et al. 2020; Thompson et al. 2021), it is likely that this rodent is involved in the enzootic cycle of this spirochete.

Phylogenies of the *flaB* and MLST genes showed that the detected spirochete forms an independent lineage within the RFG species and is herein named as "Ca. *Borrelia octodonta*". Expectedly, a phylogenetic analysis of *flaB* gene denoted that "Ca. *Borrelia octodonta*" is closely related with *Borrelia* sp. Alcohuaz (MW981443) (Thompson et al. 2021), that was characterized from *O. octodontus* as well, but from a different locality at least 60 km towards the north of El Guindo. This fact aligns with the evidence that *Ornithodoros* species are specific vectors for *Borrelia* species (Davis 1956; Schwan 1996; Krishnavajhala et al. 2018). Consequently, "Ca. *Borrelia octodonta*" and *Borrelia* sp. Alcohuaz are likely the same *Borrelia* species, and the single nucleotide polymorphism in their *flaB* sequences suggests population structuring, which may be influenced by geographical barriers between both locations (Hellgren et al. 2011).

The clade encompassing the borreliae *flaB* sequences retrieved from rodents and ticks in South America exhibited a close relationship with the clade of borreliae associated with *Ornithorodos* ticks of the Old World (*B. hispanica*, *B. duttonii*, *B. crocidurae*, *B. persica*) supporting previous topologies (Fig. 2) (Thompson et al. 2021; Oliveira et al. 2023). In contrast, the MLST phylogeny clearly shows a lack of genetic data for *flaB* genotypes reported in RFG borreliae across South America. It's worth noting that both "*Candidatus* Borrelia caatinga" (Oliveira et al. 2023) and "Ca. *Borrelia octodonta*" stand out as the sole RFG spirochetes from South America in the MLST phylogeny (Fig. 2). To date, our results provide the most comprehensive genetic characterization of a *Borrelia* of the RFG in Chile.

Challenges associated with amplifying the genes in the MLST scheme of RFG borreliae from South America have been previously highlighted (Oliveira et al. 2023). Intriguingly, similar to our findings, Oliveira et al. (2023) could not amplify the *clpA* and *nifS* genes for "Ca. *Borrelia caatinga*". This suggests that the available primers designed upon RFG borreliae sequences of the Northern Hemisphere may not capture the genetic variability of South American RFG borreliae. Such difficulties underscore the need to generate genomic data to design novel primers and improve the MLST characterization of South American borreliae.

The name "Ca. *Borrelia octodonta*" is herein proposed based on the genomic profiling of the species. However, the isolation, culture, and complete genome sequencing, is needed to formally describe this novel species. Finally, the confirmation that "Ca. *Borrelia octodonta*" is transmitted by *O. octodontus*, poses a probable risk to humans, particularly because *O. octodontus* is associated with *O. degus*, and this rodent establishes their colonies close to human dwellings (Yefi-Quinteros et al. 2018). This fact challenges the boundaries between wild and peridomestic cycles, and while cases of tick-borne relapsing fever do not exist in Chile, attention should be paid as vector ticks are identified.

Acknowledgements We thank Fidel Castro Reboredo and Lleretny Rodriguez Alvarez for their collaboration in the laboratory work.

Author contributions All authors contributed to the study conception and design, material preparation, and data collection. Analysis was performed by [Adriana Santodomingo], [Richard Thomas], and [Sebastian Muñoz Leal]. The first draft of the manuscript was written by [Adriana Santodomingo] and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

Funding This study was funded by the “Fondo Nacional de Desarrollo Científico y Tecnológico (FONDECYT)” No. 11220177 and by the ANID BECAS/Scholarship Program/DOCTORADO NACIONAL/2019–21190078 and 2020–21200182. Funders had no role in the study design, data collection, analysis, preparation of the manuscript, and decision to publish.

Data Availability The datasets generated during and/or analyzed during the current study are available from

the supplementary information.

Declarations

Ethics approval Procedures performed in this study were verified and approved by the Bioethics Committee of School of Veterinary Sciences, University of Concepción CBE-24-2021.

Consent to participate Not applicable.

Consent to publish Not applicable.

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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Figures

Figure 1

Figure 2

Figure 3

Supplementary Files

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- TableS2.STNumberofMLSTphylogeny.xlsx
- TableS1.GenBankaccessionnumberofflaBphylogeny.xlsx
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