Ticks and Tick-borne Diseases 5 (2014) 841–847

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Original article

A relapsing fever group *Borrelia* sp. similar to *Borrelia lonestari* found among wild sika deer (*Cervus nippon yesoensis*) and *Haemaphysalis* spp. ticks in Hokkaido, Japan

Kyunglee Lee[a], Ai Takano[b], Kyle Taylor[c], Mariko Sashika[a], Michito Shimozuru[a], Satoru Konnai[d], Hiroki Kawabata[e], Toshio Tsubota[a,*]

[a] *Laboratory of Wildlife Biology and Medicine, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan*
[b] *Department of Veterinary Medicine, Joint Faculty of Veterinary Medicine, Yamaguchi University, Yamaguchi, Japan*
[c] *Department of Infectious Diseases and Pathology, College of Veterinary Medicine, University of Florida, FL, USA*
[d] *Laboratory of Infectious Disease, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo, Japan*
[e] *Department of Bacteriology-1, National Institute of Infectious Disease, Tokyo, Japan*

ARTICLE INFO

Article history:
Received 6 February 2014
Received in revised form 30 May 2014
Accepted 28 June 2014
Available online 6 August 2014

Keywords:
Borrelia lonestari-like
Haemaphysalis spp.
Sika deer
Ticks
Hokkaido

ABSTRACT

A relapsing fever *Borrelia* sp. similar to *Borrelia lonestari* (herein referred to as *B. lonestari*-like) was detected from wild sika deer (*Cervus nippon yesoensis*) and *Haemaphysalis* ticks in the eastern part of Hokkaido, Japan. The total prevalence of this *Borrelia* sp. in tested deer blood samples was 10.6% using conventional PCR and real-time PCR. The prevalence was significantly higher in deer fawns compared to adults (21.9% and 9.4%, respectively). Additionally, there was significant regional difference between our two sampling areas, Shiretoko and Shibetsu with 17% and 2.8% prevalence, respectively. Regional differences were also found in tick species collected from field and on deer. In the Shiretoko region, *Haemaphysalis* spp. were more abundant than *Ixodes* spp., while in Shibetsu, *Ixodes* spp. were more abundant. Using real-time PCR analysis, *B. lonestari*-like was detected from 2 out of 290 adult *Haemaphysalis* spp. ticks and 4 out of 76 pools of nymphs. This is the first report of a *B. lonestari*-like organism in *Haemaphysalis* spp. ticks, and the first phylogenetic analysis of this *B. lonestari*-like organism in Asia. Based on our results, *Haemaphysalis* spp. are the most likely candidates to act as a vector for *B. lonestari*-like; furthermore, regional variation of *B. lonestari*-like prevalence in sika deer may be dependent on the population distribution of these ticks.

Introduction

The genus *Borrelia* is comprised of three phylogenetic groups: Lyme disease (LD) borreliae, which include the agents of Lyme diseases, Relapsing fever (RF) borreliae, and Reptile-associated (REP) borreliae (Takano et al., 2010; Franke et al., 2012). LD and REP borreliae are transmitted by ixodid (hard-bodied) ticks while most RF borreliae are transmitted by argasid (soft-bodied) ticks, except for *Borrelia recurrentis*, which is transmitted by lice. Some RF borreliae such as *Borrelia theileri*, *Borrelia miyamotoi*, and *Borrelia lonestari*, however, use hard-bodied ticks as vectors: *Rhipicephalus* spp., *Ixodes* spp., or *Amblyomma* spp., respectively (Smith et al., 1978; Fukunaga et al., 1995; Armstrong et al., 1996; Barbour et al., 1996; Scoles et al., 2001; Barbour, 2005). *B. theileri* is the causative agent of bovine theileriosis (Smith et al., 1985). *B. miyamotoi* was originally isolated in Japan (Fukunaga et al., 1995), and was considered a non-pathogenic species until recently, when Platonov et al. (2011) reported the first evidence of human infections in Russia. This was followed by human case reports from the United States and Holland, including two patients who developed menigoencephalitis (Chowdri et al., 2013; Gugliotta et al., 2013; Hovius et al., 2013). The pathogenicity of *B. lonestari* in humans is still unclear (Feder et al., 2011), although it was once suspected to be the agent of Southern Tick-Associated Rash Illness, a disease with Lyme disease-like symptoms associated with *Amblyomma americanum* (Burkot et al., 2001; James et al., 2001; Stromdahl et al., 2003). Understanding the biology of these RF borreliae, which are transmitted by hard-bodied ticks, has advanced slowly due to the difficulty of cultivation.

* Corresponding author at: Laboratory of Wildlife Biology and Medicine, Graduate School of Veterinary Medicine, Hokkaido University, Kita 18, Nishi 9, Kita-ku, Sapporo 060-0818, Japan. Tel.: +81 11 706 5101; fax: +81 11 706 5569.
E-mail address: tsubota@vetmed.hokudai.ac.jp (T. Tsubota).

http://dx.doi.org/10.1016/j.ttbdis.2014.06.006
1877-959X/

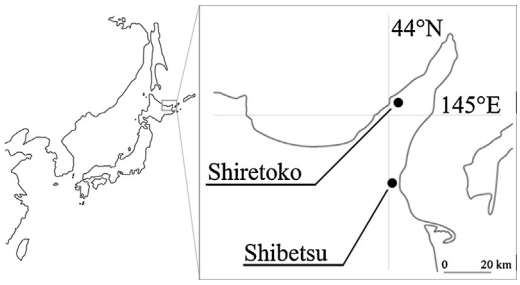

Fig. 1. Map of sampling locations.

In the United States, there is strong evidence implicating the white-tailed deer (*Odocoileus virginianus*) as the main reservoir of *B. lonestari* (Moore et al., 2003; Moyer et al., 2006; Varela-Stokes, 2007). *B. lonestari* DNA prevalence in *A. americanum* from 29 sites in 4 states was 2.5%, in total (Mixson et al., 2006). The prevalence of *B. lonestari* antibody in deer was overall 15% throughout 20 eastern states, and there was regional difference, with higher prevalence in southern states (17.5%) than in northern states (9.2%) (Murdock et al., 2009). There have been few reports of *B. lonestari* outside of the United States. In Brazil, a RF *Borrelia* sp. closely related to *B. lonestari* and *B. theileri* was detected from a *Rhipicephalus microplus* feeding on a horse (Yparraguirre et al., 2007). Takano et al. (2012) reported a RF *Borrelia* sp. from the *Amblyomma geoemydae* collected in Okinawa prefecture, the most southern part of Japan, who's sequences clustered with *B. lonestari* and *B. miyamotoi* by phylogenetic analysis. These findings suggested the possibility that unknown *Borrelia* spp. exist worldwide.

In a previous survey of *Borrelia* spp. among wild animals in Hokkaido, a northern island of Japan, borrelial DNA fragments which were similar to *B. lonestari* (Taylor, 2013) were found among blood samples from sika deer (*Cervus nippon yesoensis*). However, *Amblyomma* spp., *Rhipicephalus* spp., or soft ticks, which are the heretofore known vectors of RF borreliae, have never been reported in Hokkaido (Yamaguti et al., 1971; Shimada et al., 2003; Taylor, 2013; Yamauchi et al., 2013). To understand how this borrelial organism is maintained in the ecosystem, in this study, we conducted surveillance of wild sika deer and ticks in Hokkaido, Japan.

Materials and methods

Sampling from field

To examine the infection rate of *Borrelia* spp. among sika deer, we surveyed deer samples from hunting and nuisance control culling held in the eastern part of Hokkaido from July 2011 to August 2013. Two regions were selected for sample collection: Shiretoko and Shibetsu, which are separated by approximately 40 km and the Shiretoko mountain range (Fig. 1). Blood samples were collected from veins or heart and dispensed into EDTA·Na tubes and plain tubes. EDTA blood was kept at 4 °C until DNA extraction, which was performed within 2 days. Buffy coat or unspun plasma were collected on the day of sampling and kept in −20 °C until DNA extraction, which was performed within 1 week. Deer were identified to sex, and individuals were grouped into fawns (lesser than 1 year old) and adults (1 year or older) based on a tooth formula (Koike and Ohtaishi, 1985). Sampling was divided into two seasons based on snow covering from November through April (winter) and from May through October (summer). When possible, a portion of an entire ear from the dead deer was collected and kept at −20 °C for tick counts and species and stage identification.

From May through September of 2012 and 2013, questing ticks on vegetation were collected by flagging with a 1 m^2 white flannel sheet in Shiretoko and Shibetsu. Sampling was implemented in several locations over nature trails and pasture. Collected ticks were identified to species and stage, and were kept at −20 °C until DNA extraction.

DNA extraction

DNA from deer blood was extracted using the Wizard® genomic DNA purification kit (Promega, Madison, WI) by the recommended protocol using 3 ml of whole blood or unspun plasma or buffy coat from 3 ml of blood sample. Tick DNA was extracted by using ammonium hydroxide (NH_4OH) as described in Barbour et al. (2009) with minor modification. Harvested DNA samples were stored at −20 °C until analysis.

Conventional PCR and sequencing

All deer blood DNA samples were examined using nested PCR to detect the *Borrelia* spp. flagellin gene (*flaB*) with the primer set of BflaPAD and BflaPDU for first PCR and BflaPBU and BflaPCR for nested PCR as previously described (Takano et al., 2010) with the GeneAmp® + PCRSystem9700 (Applied Biosystems, Foster City, CA). PCR was done with Takara Ex *Taq* (Takara Bio, Otsu, Japan) and the first PCR condition was 25 cycles of 20 s at 94 °C, 30 s at 55 °C and 30 s at 72 °C, and nested PCR was performed at 30 cycles with the same conditions. Contamination and amplicon carryover were carefully checked by using distilled water as blank control in each experiment. After gel electrophoresis, the PCR product (323 bp) was purified with the NucleSpin®Gel and PCR clean-up kit (Macherey-Nagel, Duren, Germany) according to the manufacturer's instructions. The forward primer of the nested PCR was used for direct sequencing of amplicon DNA using 27 cycles of 15 s at 96 °C, 5 s at 50 °C and 4-min at 60 °C with the BigDye® Terminator v1.1 Cycling Sequencing Kit. The sequenced results were analyzed on an ABI PRISM 310 Genetic Analyser (Applied Biosystem), and were compared in GenBank for identification to species.

Real-time PCR and quantification of borrelial DNA copy number

To confirm the positive cases of deer with *Borrelia* sp. (preliminarily designated *B. lonestari*-like), and to quantify the copy number of the borrelial genome in the blood, 16S rRNA gene detection by real-time PCR was performed on all deer blood samples for which *flaB* nested PCR was performed, with the exception of dried out samples.

(i) Construction of real-time PCR

To construct the real-time PCR, a part of the 16S rRNA gene (1363 bp) of *B. lonestari*-like was amplified by sets of primers 16S-F1 and 16S-R4 (Takano et al., 2010) for the first PCR and 16SMF (5′-GCGAACGGGTGAGTAACG-3′) and 16SMR (5′-CCTCCCTTACGGGTTAGAA-3′) for nested PCR using 3 borrelial *flaB* PCR positive samples. The PCR condition was 30 cycles of 10 s at 95 °C, 30 s at 55 °C (first PCR) or 58 °C (nested PCR) and 90 s at 72 °C, using Takara Ex *Taq* (Takara Bio.). After sequencing of the 16S rRNA gene as described above for *flaB* nested PCR, the sequences were compared with the 16S rRNA gene of *B. miyamotoi* and *B. lonestari*-like using Sequencher 5.1 (Gene Codes Corporation, MI, USA). In this study, we used a real-time PCR protocol previously reported by Barbour et al. (2009) with minor modification to the probe. Forward and reverse primers were, respectively, 16S RT-F (5′-GCTGTAAACGATGCACACTTGGT-3′) and 16S RT-R (5′-GGCGGCACACTTAACACGTTAG-3′) and the dye-labeled probe was modified by 1 bp from the VIC probe described by Barbour et al. (2009) as follows: BS-16S (FAM 5′-CGGTACTAATCTTTCGATTA-3′) with the 3′ end modified with a minor groove binding protein (Applied Biosystems). The real-time PCR was performed using the