Ticks and Tick-borne Diseases xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

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

Original article

Epidemiological survey of a cervine *Theileria* in wild deer, questing ticks, and cattle in Hokkaido, Japan

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ARTICLE INFO

Keywords:
Cattle
Deer
Japan
Theileria sp.
Ticks

ABSTRACT

Species of *Theileria* are tick-borne hemoprotozoan parasites of ruminants that can cause severe clinical disease. In this study, blood samples were obtained from 91 wild sika deer in various districts in Hokkaido, Japan. Samples were tested using a PCR assay designed to amplify a full-length major piroplasm surface protein (*MPSP*) gene of a cervine *Theileria* species, designated as *Theileria* sp. (sika 1). The amplicons of 57 out of 89 PCR-positive samples were cloned and sequenced. The sequences shared 99.1%–100% identity scores, indicating the *MPSP* gene of *Theileria* sp. (sika 1) is highly conserved. Next, a *Theileria* sp. (sika 1)-specific PCR assay was developed based on the newly-generated *MPSP* gene sequences and used to screen DNA samples from 671 questing ticks, collected from cattle pastures where wild sika deer are often observed, in Hamanaka, Shibecha, Shikaoi, Otofuke, Taiki, and Shin-Hidaka districts of Hokkaido. *Ixodes persulcatus* and *Haemaphysalis japonica* were infected with *Theileria* sp. (sika 1), while *Ixodes ovatus* and *Haemaphysalis megaspinosa* were negative. Furthermore, blood DNA samples collected from 767 cattle, grazing on the same pastures where the ticks had been collected, were negative for *Theileria* sp. (sika 1), using the same PCR assay. The *MPSP* gene from *Theileria* sp. (sika 1)-positive ticks was sequenced. The *Theileria* sp. (sika 1) *MPSP* gene sequences from ticks shared 99.1%–100% identity scores with those from the wild sika deer. In a phylogenetic analysis, *Theileria* sp. (sika 1) *MPSP* gene sequences from both deer and ticks clustered together and formed a monophyletic clade. Our findings infer that *I. persulcatus* and *H. japonica* are potential vectors for the transmission of *Theileria* sp. (sika 1) to sika deer but not cattle, though *I. persulcatus* and *H. japonica* are known to infest both cattle and wild sika deer in Hokkaido.

1. Introduction

Theileria species are tick-borne, hemoprotozoan parasites that infect ruminants (Bishop et al., 2004). *Theileria* infection persists in ticks transstadially as the tick stages molt, while transovarial transmission, which is commonly seen with *Babesia* species, is not observed in *Theileria* (Bock et al., 2004; Dolan, 1989). *Theileria* infection, acquired by the larval and nymphal stages of tick vectors during a blood meal on infected host animals, is transmitted to new host animals by the subsequent nymphal and adult stages, respectively (Mans et al., 2015). In host animals, *Theileria* sporozoites injected by the tick vectors invade the leukocytes, where the sporozoites transform into the schizont stage. Based on the behavior of schizont-containing leukocytes, *Theileria* parasites can be divided into transforming and non-transforming species. In transforming *Theileria*, such as *T. parva* and *T. annulata*, the infected leukocytes will be transformed in a way that enables them to proliferate indefinitely, together with the schizonts occupying them (Dobbelaere and Heussler, 1999). In contrast, leukocyte transformation and proliferation do not take place if a non-transforming *Theileria* species infects the host animal (Sugimoto and Fujisaki, 2002).

Although transforming *Theileria* parasites are known to be highly virulent, non-transforming *Theileria* species do occasionally induce severe clinical disease. For example, *T. orientalis*, which is a non-transforming *Theileria*, causes severe anemia in cattle, especially when the parasite is introduced to a non-endemic area (Kamau et al., 2011; Pulford et al., 2016). The recent outbreaks of hemolytic anemia of cattle associated with *T. orientalis* infection in Australia and New Zealand can be traced to the introduction of *T. orientalis* genotype 2, which had not been previously present in these countries (Eamens et al., 2013; Lawrence et al., 2018; McFadden et al., 2011; Perera et al., 2013). Non-transforming *Theileria* species are widespread in different host ruminant animals of tropical and subtropical regions (Mans et al., 2015;

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https://doi.org/10.1016/j.ttbdis.2018.05.006
Received 19 January 2018; Received in revised form 17 April 2018; Accepted 3 May 2018

Sivakumar et al., 2014).

In Japan, *Theileria* parasites have been reported in cattle and wild sika deer, *Cervus nippon* (Elbaz et al., 2017; Ikawa et al., 2011; Ota et al., 2009; Yokoyama et al., 2011). *T. orientalis* infection has been detected in cattle reared all over the country, and often results in clinical disease, leading to huge economic loss (Ota et al., 2009; Yokoyama et al., 2011, 2012). Wild sika deer in Japan are known to harbor yet unnamed *Theileria* species. Based on the genetic analyses of *18S rRNA* and major piroplasm surface protein (*MPSP*) gene sequences, one study identified the presence of two different *Theileria* species in sika deer in Japan, designated in the present study as *Theileria* sp. (sika 1) and *Theileria* sp. (sika 2) (Inokuma et al., 2004, 2008). The phylogenetic analyses also suggested that these parasite species were different from *T. cervi*, which is a previously described cervine *Theileria* (Chae et al., 1999; Inokuma et al., 2004, 2008). *Theileria* sp. (sika 1) was detected in several prefectures including Hokkaido, whereas the sika deer in Yamaguchi prefecture were infected with *Theileria* sp. (sika 2) (Inokuma et al., 2008). The sika deer in Hokkaido (*Cervus nippon yesoensis*) are frequently observed in close proximity to cattle pastures. The tick species suspected as the vectors of *T. orientalis* are also known to infest sika deer (Lee et al., 2014; Yokoyama et al., 2012). The question now is whether the *Theileria* sp. (sika 1) can be transmitted to cattle via the same tick species. In the present study, we developed a PCR assay specific to *Theileria* sp. (sika 1), to determine the prevalence in questing ticks collected from cattle pastures in this prefecture. In addition, the PCR assay was also used to determine the prevalence of *Theileria* sp. (sika 1) in cattle grazing in the same locations as the questing ticks.

2. Materials and methods

2.1. Cervine and bovine blood samples and DNA extraction

A total of 91 blood samples were collected from wild sika deer (*C. n. yesoensis*) in different districts of Hokkaido prefecture. In total, 62 blood samples were collected from deer in Akan (n = 51), Urahoro (n = 1), Hiroo (n = 4), Erimo (n = 2), Samani (n = 3), and Urakawa (n = 1) districts, and 29 blood samples were from deer in unknown locations within Hokkaido (Fig. 1). Thin blood smears were prepared on glass slides from samples that had been collected from the deer in Akan district, stained with Giemsa, and then observed under a microscope. Blood samples were also collected from 596 cattle in Hamanaka (n = 50), Shikaoi (n = 300), Shibecha (n = 100), and Taiki (n = 146) districts. All blood samples underwent DNA extraction using a commercial kit (Qiagen Blood Mini Kit, Qiagen, Hilden, Germany), according to the manufacturer's instructions. In addition, 171 archived bovine DNA samples that had been prepared from blood collected in Otofuke district in 2008 (n = 20, Ota et al., 2009) and 2010 (n = 57, Yoshinari et al., 2013) and in Shin-Hidaka district in 2009 (n = 94, Sivakumar et al., 2012), were also used in the present study. The procedures for blood sampling from deer and cattle were approved by the Committee on the Ethics of Animal Experiments, Obihiro University of Agriculture and Veterinary Medicine (Approval numbers: 29-3 and 29-4).

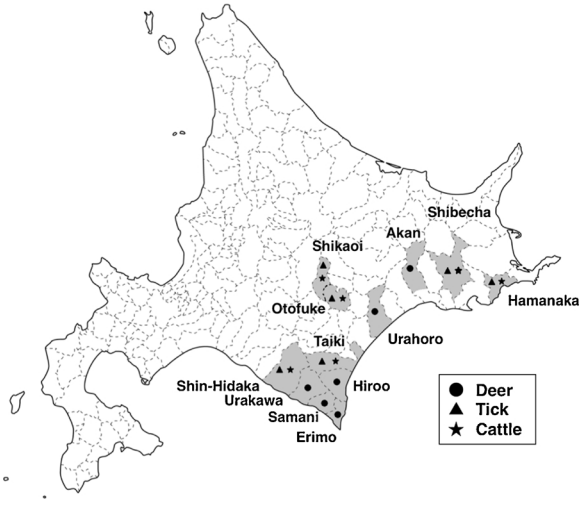

Fig. 1. The sampling locations. The wild deer, questing ticks, and grazing cattle were sampled in various districts in Hokkaido prefecture.

2.2. Tick DNA samples

Using a flagging method (i.e., by waving a cotton flag through vegetation) (Dantas-Torres et al., 2013), 377 questing ticks were collected in and around the cattle pastures in Hamanaka (n = 30), Shibecha (n = 231), and Shikaoi (n = 116) districts. The ticks were identified based on morphology (Yamaguti et al., 1971) and DNA was extracted as described previously (Yokoyama et al., 2012). In addition, archived DNA samples (Yokoyama et al., 2012) that had been prepared from 294 questing ticks in Otofuke (n = 42), Taiki (n = 188), and Shin-Hidaka (n = 64) districts, were also used in the present study.

2.3. PCR amplification, cloning, and sequencing of a full-length Theileria sp. (sika 1) MPSP gene

The genomic DNA extracted from the blood sampled from wild sika deer was subjected to the PCR assay developed to amplify a full-length (867-bp) *MPSP* gene. A forward primer (5′-AAATCGCCAGTGTAAAGC TAATTG-3′), located 51-bp upstream from the start codon and a reverse primer (5′-GTTATTTAACAGGCGAGATAGTTGC-3′), located 75-bp downstream from the stop codon were designed based on a reference sequence that had been determined by next generation sequencing of a blood DNA sample from a deer, which was positive for *Theileria* by microscopy in Toyokoro district (unpublished data). One μl of cervine DNA was added to a 24 μl reaction mixture containing 1 × PCR buffer (Toyobo, Osaka, Japan), 400 μM of each dNTPs (Toyobo), 0.6 μM of each forward and reverse primers, 0.5 μl of 1 U/μl KOD FX Neo DNA polymerase (Toyobo), and 3 μl of distilled water. After a pre-denaturation step at 94 °C for 2 min, the reaction mixture underwent 45 cycles of which each contained a denaturation step at 98 °C for 10 s, an annealing step at 62 °C for 30 s, and an extension step at 68 °C for 1 min. The PCR products were analyzed by 1.5% agarose electrophoresis and after staining with ethidium bromide visualized by UV illumination. A band with an approximate size of 993 bp was considered positive. The PCR products were gel-extracted, cloned into a PCR 2.1 plasmid vector (TOPO, Invitrogen, Carlsbad, CA, USA), and the integrated DNA fragment was sequenced. The sequences obtained were analyzed using a basic local alignment tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) to confirm whether the sequences were derived from *Theileria* sp. (sika 1). The identity and similarity scores between the sequences were analyzed using a MatGAT software program (Campanella et al., 2003).

2.4. Development of a PCR assay specific to Theileria sp. (sika 1)

The analyzed *MPSP* gene sequences of *Theileria* sp. (sika 1), together with those reported previously for different species of *Theileria,* were subjected to multiple alignment. A set of forward (5′-AGCCAATGCAG CTGGTTCAGCTG-3′) and reverse (5′-CATGAAGATCCTTGTTGGCTTCG GAT-3′) primers were designed, based on the regions specific to the *Theileria* sp. (sika 1). The specificity of the PCR assay was examined using DNA samples from *Theileria* sp. (sika 1)-positive deer as determined in the aforementioned sequencing analyses (Section 2.3). In addition, DNA samples from *T. orientalis* genotypes 1–5, *T. annulata*, *T.*