Utilizing the attached hard ticks as pointers to the risk of infection by *Babesia* and *Theileria* species in sika deer (*Cervus nippon yesoensis*), Japan

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Abstract

Ticks are hematophagous ectoparasites that have a significant impact on their animal hosts. Moreover, along with mosquitoes, they are the main arthropod vectors of disease agents in domestic animals, wildlife and humans. To investigate the occurrence and prevalence of piroplasmids in the ticks, DNA was extracted from 519 hard ticks collected from 116 hunted Hokkaido sika deer (*Cervus nippon yesoensis*). The success of the DNA extraction was confirmed by touchdown PCR targeting the mitochondrial 16S rDNA gene of ticks. Touchdown PCR and reverse line blot (RLB) hybridization targeting the 18S rRNA gene were used to detect 14 piroplasm species. All hard ticks parasitizing Hokkaido sika deer were identified as belonging to the genera *Ixodes* and *Haemaphysalis*. A total of 163 samples (31.4%) were positive for *Babesia* and *Theileria* spp. among different tick species according to RLB hybridization. The DNA from 27.0% (140/519), 10.6% (55/519), 1.7% (9/519), 0.6% (3/519), 0.4% (2/519), and 0.4% (2/519) of ticks hybridized to the *Theileria* sp. Thrivae, *Theileria capreoli*, *Babesia divergens*-like, *Babesia* sp. (Bab-SD), *Babesia microti* U.S., and *B. microti* Hobetsu oligonucleotide probes, respectively. The partial sequencing and phylogenetic analyses of the 18S rRNA gene confirmed the RLB hybridization results. Further investigations are needed to reveal the epidemiology and respective vectors of these pathogens.

Keywords

Babesia, Hard ticks, Reverse line blot, Sika deer, *Theileria*

Introduction

Ticks are hematophagous ectoparasites that have significant impacts on their animal hosts, such as by causing anemia and direct skin tissue damage. Moreover, along with mosquitoes, they are the main arthropod vectors of disease agents in domestic animals and humans (Dantas-Torres et al. 2012; Ferrolho et al. 2016; Masatani et al. 2017). Two major families of ticks, Ixodidae (hard ticks) and Argasidae (soft ticks), which include more than 900 tick species, have been recorded globally (Chen et al. 2014). In Japan, 47 tick species have been reported, of which 21 parasitize humans (Takano et al. 2014).

The incidence of tick-borne diseases (TBDs) and the emergence of novel TBD agents have been increasing worldwide during the past two decades (Kernif et al. 2016; Piesman and Eisen 2008). Hence, it is important to investigate the prevalence of tick-borne pathogens to develop effective control strategies against TBDs. Tick-borne apicomplexan parasites, such as *Babesia* and *Theileria* spp., are of great importance worldwide, as they cause diseases in domestic and wild animals. In addition, some *Babesia* spp., such as *Babesia microti*, *Babesia divergens*, and *B. divergens*-like, are considered zoonotic pathogens (Andersson et al. 2017).

In Japan, many studies have identified several *Babesia* and *Theileria* spp. in domestic animals (Fujinaga 1981; Kim et al. 2004; Minami and Ishihara 1980; Ota et al. 2009; Sivakumar et al. 2012), wild animals (Elbaz et al. 2017; Ikawa et al. 2011; Inokuma et al. 2004; Watanabe et al. 2016) and in humans (Wei et al. 2001). These studies have provided information about the potential vector hosts for *Babesia* and *Theileria* spp. in Japan. For example, *Haemaphysalis longicornis* and *Ixodes ovatus* act as vectors for *Babesia ovata* (Sivakumar et al. 2014), and *Ixodes persulcatus*, *Haemaphysalis megaspinosa*, *Haemaphysalis douglasi*, and *Ixodes ovatus* act as vectors for *Theileria orientalis* (Yokoyama et al. 2012); zoonotic *Babesia* spp. such as *B. microti* U.S. and Hobetsu strains have been detected in *I. ovatus* and *I. persulcatus*, respectively (Zamoto-Niikura et al. 2012), and *B. divergens*-like in *I. persulcatus* (Zamoto-Niikura et al. 2018). Other studies have identified piroplasm species from ticks feeding on animals (Inokuma et al. 2003; Iwakami et al. 2014; Masatani et al. 2017; Tateno et al. 2015).

Moreover, the same tick may be co-infected with different piroplasm species (Abdallah et al. 2017) or various other tick-borne pathogens that may have important implications for transmitting pathogens to the animal host (Moutailler et al. 2016). Disease severity may be enhanced if humans or animals are bitten by co-infected ticks, as reported for concurrent babesiosis and Lyme diseases (Golightly et al. 1989; Grunwaldt et al. 1983). This could also have serious consequences for the diagnosis and treatment of TBDs (Chen et al. 2014).

Sika deer (*Cervus nippon*) is a native cervid species in Japan (Ohdachi et al. 2009) that has recently expanded in range and number due to environmental and climate changes (Takatsuki 2009). Moreover, sika deer are one of the most important reproductive hosts for several tick species at all developmental stages, such as *H. longicornis*, *Haemaphysalis flava*, *Haemaphysalis yeni*, *H. megaspinosa*, *I. ovatus*, and *Amblyomma testudinarium*, which have been collected from western and central Japan (Inokuma et al. 2002; Tsukada et al. 2014). Although several piroplasm species have been detected in sika deer from Japan (Elbaz et al. 2017; Inokuma et al. 2004; Watanabe et al. 2016; Zamoto-Niikura et al. 2014), few molecular epidemiological studies have been conducted to detect piroplasm species in host-feeding ticks collected from sika deer. Two *Theileria* spp. have been reported in feeding *Haemaphysalis* spp. collected from sika deer in southern Japan (Masatani et al. 2017). One of these *Theileria* spp. is closely related to *Theileria sp.* Thrivae and the other to *T. capreoli*, both of which were detected previously in sika deer (Elbaz et al. 2017; Watanabe et al. 2016).

In our previous study, four piroplasm species were identified in Hokkaido sika deer blood samples: *B. divergens*-like, a novel *Babesia* species (here referred to as : *Babesia* sp. Bab-SD), *Theileria* sp. Thrivae, and *T. capreoli* (Elbaz et al. 2017). In addition, *Theileria* sp. (sika 1) was detected in sika deer collected from Hokkaido, Japan (Shibata et al. 2018) and was found to be different from *T. orientalis*. *Theileria* sp. Thrivae is a non-pathogenic *Theileria* sp. that has been reported from sika deer in Japan (Watanabe et al. 2016). A closely related *Theileria* sp. has been detected in China and named "*Theileria cerv*i" (Liu et al. 2016). However, *T. cervi* that was reported from white-tailed deer and elk (*Cervus canadensis*) in USA and Canada (Chae et al. 1999) is genetically separated from *Theileria* sp. Thrivae and *T. cervi* of Japan and China, respectively.

Many of tick-borne pathogens (TBPs) are zoonotic (Moustafa et al., 2016); for examples, human granulocytic anaplasmosis, human babesiosis and human monocytic ehrlichiosis are caused by zoonotic tick-borne pathogens and both wildlife and domestic animals act as reservoir or amplifying hosts (Dantas-Torres et al. 2012). Thus, detecting pathogens in ticks collected from animals is crucial to predict their spread and emergence and to protect humans and animals. Hence, the present study investigated the occurrence and prevalence of piroplasmids in ticks collected from sika deer in Hokkaido.

Material and methods

Tick collection and identification

A total of 519 ticks were collected from 116 Hokkaido sika deer (*Cervus nippon yesoensis*) from May to November 2012, which were hunted for nuisance control procedures in two different regions on the Shiretoko Peninsula in Hokkaido Prefecture (Shari and Shibetsu). All collected ticks were either engorged with blood or questing on the surface of the sika deer (non-engorged). The collected ticks were identified under a microscope according to their morphological characteristics (Yamaguti et al. 1971). Subsequently, the ticks were separated into groups according to species and stage and stored in 70% ethanol at −20°C until DNA extraction.

DNA extraction

Total DNA was extracted from ticks individually using the Wizard ®Genomic DNA purification Kit (Promega, Madison, WI, USA) per the manufacturer's instructions with modifications (Taylor et al. 2013). The whole ticks were crushed in a 50/50 mixture of the included cell and nuclear lysis solutions supplemented with 17.5 µl proteinase K, followed by incubation at 60°C for 90 min. DNA concentration and quality were assessed by spectrophotometry (NanoDrop 2000, Thermo Fisher Scientific, Yokohama, Japan). The DNA was stored at −20°C until analysis.

Touchdown polymerase chain reaction (PCR) to validate tick DNA extraction

The success of DNA extraction was confirmed by touchdown PCR targeting the mitochondrial 16S rDNA gene (mt-*rrs*) of ticks. Touchdown PCR was conducted using the PCR System 9700 (Applied Biosystems, Foster City, CA, USA), KOD-Plus-Neo high fidelity DNA polymerase kit (Toyobo Co. Ltd., Tokyo, Japan), and Mt-rrs1 (5′- CTG CTC AAT GAT TTT TTA AAT TGC TGT GG-3′) and Mt-rrs2 (: 5′-CCG GTC TGA ACT CAG ATC AAG TA-3′) primers (Ushijima et al. 2003) to amplify a 401–416 bp fragment. The 25 µl PCR mixture consisted of 2.5 µl 10× KOD-Plus-Neo buffer, 2.5 µl dNTPs (2 mM), 1.5 µl 25 mM $MgSO_4$, 0.75 µl each primer (10 pmol/µl), 0.5 µl KOD-Plus-Neo DNA polymerase, 15.5 µl molecular biology-grade water, and 1 µl DNA. The PCR conditions were 94°C for 2 min, a touchdown step of 10 cycles at 94°C for 10 s, 65°C for 30 s, and 68°C for 30 s with the annealing temperature decreasing by 1°C every cycle. This was followed by 40 cycles of 98°C for 10 s, 55°C for 30 s, 68°C for 30 s, and a final extension of 68°C for 7 min. The PCR products (5 μl) were mixed with 1 μl loading buffer (Nippon Gene Co. Ltd., Tokyo, Japan) and examined by 1% agarose gel electrophoresis with ethidium bromide staining, followed by visualization using a UV illuminator.

Touchdown PCR and reverse line blot (RLB) hybridization

Touchdown PCR targeting the 18S rRNA gene of *Babesia* and *Theileria* spp. was performed using the PCR System 9700, KOD-Plus-Neo high fidelity DNA polymerase kit, and the RLB-F2 (5'-GAC ACAGGG AGG TAG TGA CAA G-3') and RLB-R2 (biotin-5'-CTA AGA ATT TCA CCT CTG ACA GT-3') primer pair (Gubbels et al. 1999; Matjila et al. 2004). The PCR mixture was used as described above to amplify a 460–540 bp segment. One microliter of molecular grade water and genomic DNA samples of *B. divergens*-like and *Theileria* sp. Thrivae were used as negative and positive controls for *Babesia* and *Theileria* spp., respectively. The PCR conditions were 94°C for 2 min, a touchdown step of 10 cycles at 94°C for 10 s, 67°C for 30 s, and 68°C for 30 s with a 1°C decrease in annealing temperature every cycle. This was followed by 40 cycles at 98°C for 10 s, 57°C for 30 s, 68°C for 30 s, and a final extension at 68°C for 7 min. The PCR products were examined by 1% agarose gel electrophoresis with ethidium bromide staining followed by visualization using a UV illuminator.

The PCR products were subjected to RLB hybridization as described previously (Kong and Gilbert 2006; Moustafa et al. 2016) with modifications. Briefly, a 15 × 15 cm Biodyne C membrane (Pall Life Science, Ann Arbor, MI, USA) was activated with 20 ml 16% 1-ethyl-3-(3-dimethyl-amino-propyl) carbodiimide (Sigma Aldrich, St. Louis, MO, USA) for 10 min at room temperature. The membrane was washed gently with MilliQ water for 2 min and placed in the Miniblotter MN45 (Immunetics, Boston, MA, USA). This study included 19 oligonucleotide probes: 17 previously published probes (Elbaz et al. 2017) and 2 probes designed in this study. The designed probes (*T. capreoli* 5'-ATACGAGTTTTTGCATTGTG-3' and *Babesia* sp. (Bab-SD) 5'-GTTGGCTTTTCTTATTACTTTGA-3') linked to a C6 amino linker were obtained from Sigma Aldrich (Tokyo, Japan) and reconstituted as 100 pmol/μl solutions by using Tris-EDTA (TE) buffer (PH 8.0) (Nippon Gene, Japan). Ten microliters of each 100 pmol/μl probe solution were diluted in 0.5 M $NaHCO_3$ to a final volume of 170 μl. The openings in the miniblotter were filled with 150 μl each diluted oligonucleotide, and the membrane was incubated for 5 min at room temperature. The membrane was inactivated in 250 ml 0.1 M NaOH with gentle shaking for exactly 8 min at room temperature, then washed with prewarmed 250 ml 2× SSPE/0.1% SDS at 60°C. Finally, the membrane was sealed in a plastic bag with 15 μl 20 mM EDTA for future use. Ten microliters of each PCR product were diluted in 2× SSPE/0.1% SDS to a final volume of 170 μl, denatured by heating at 100°C for 10 min, and immediately cooled on ice. The denatured mixtures were allowed to hybridize to the membrane at 60°C for 1 h. Afterwards, the membrane was washed twice in 250 ml 2× SSPE/0.5% SDS at 52°C for 10 min and then incubated with diluted peroxidase-labeled NeutrAvidin (Thermo Fisher Scientific, Waltham, MA, USA) at 42°C for 45–60 min. The NeutrAvidin was removed by washing twice with 250 ml 2× SSPE/0.5% SDS at 42°C for 10 min and with 2× SSPE twice at room temperature for 5 min. The membrane was exposed to 15 ml Immobilon™ Western Chemiluminescent HRP Substrate (Millipore, Tokyo, Japan) for 5 min and photographed using the Ez-Capture MG/ST (ATTO Corp. Tokyo, Japan). To remove the PCR products, the membrane was washed twice in pre-warmed 1% SDS at 90°C for

30 min and washed with 250 ml 20 mM EDTA. The membrane was sealed in a plastic bag with 15 ml 20 mM EDTA and stored at 4°C for future reuse.

Cloning and sequencing

The RLB results were confirmed by cloning and sequencing 12 randomly selected positive PCR products. A 460–540 bp fragment was amplified from the 18S rRNA gene of piroplasm species using the KOD-Plus-Neo high fidelity DNA polymerase kit with the abovementioned RLB-F2 and RLB-R2 primers and PCR conditions. The PCR products were examined by electrophoresis, and the band corresponding to 460–540 bp was excised for purification using the NucleoSpin® Gel and PCR clean up kit (Macherey-Nagel, Düren, Germany). The purified PCR products were attached to dA nucleotides by mixing 0.9 µl 10× KOD-Plus-Neo buffer, 0.9 µl 2 mM dNTPs, 0.54 µl 25 mM $MgSO_4$, 6.66 µl each purified PCR product, and 1 µl 10× A-attachment mix (Toyobo) at 60°C for 10 min. One microliter of each dA-attached product was cloned into the pMD20 T-vector (Takara Bio, Otsu, Japan). Five colonies per sample were selected and screened by EmeraldAmp MAX PCR (Takara Bio) as described in the manufacturer's manual. The purified PCR products were sequenced using the Big-Dye Terminator version 3.1 Cycle Sequencing Kit and purified using the BigDye® XTerminator™ Purification Kit (Applied Biosystems). Subsequently, the purified products were sequenced using the ABI PRISM™ 310 genetic analyzer (Applied Biosystems). BLASTn searches were performed to compare the sequences to reference sequences recorded in GenBank for identification.

Phylogenetic analysis

The 18S rRNA partial sequences were examined by BLASTn search against the *Babesia* and *Theileria* spp sequences in GenBank and submitted to the DNA Data Bank of Japan (DDBJ). The obtained sequences were aligned in MEGA software version 7 with representative 18S rRNA gene sequences of the previously identified *Babesia* and *Theileria* spp. in the GenBank to know its taxonomic position. The "Find Best DNA/Protein Models" option in MEGA 7 was used to select the most suitable substitution model for the phylogenetic analysis. The phylogenetic trees were constructed using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993) using the gamma distribution in the same software and a full 18S rRNA gene sequence for *Plasmodium falciparum* (XR_002966679) was used as an outgroup. Node support was assessed using 1,000 bootstrap replicates (Kumar et al. 2016).

Statistical analysis

Linear mixed models (LMM) was performed to investigate differences in the prevalence of *Theileria* sp. Thrivae and *T. capreoli*. We fitted LMM with predictive variables (tick species, stage, and status) in presence of sampling location as a random effect variable. We used "lmer" function in R package lme4 to perform the LMM. The correlations among the detected piroplasm species were investigated by the chi-square test. The statistical analyses were performed using SPSS version 21.0 (SPSS Inc., Chicago, IL, USA). A P-value < 0.05 was considered significant.

Results

Identifying the tick species and detecting tick DNA

The collected ticks were identified as belonging to the genera *Ixodes* and *Haemaphysalis*. The *Ixodes* ticks included 330 (63.6%), 40 (7.7%), 11 (2.1%) and 110 (21.2%) *I. persulcatus* nymph, larvae, adult and *I. ovatus* adults, respectively. The remaining collected ticks including 15 (2.9%), 11 (2.1) and 2 (0.4%) were identified as *H. japonica* adult, nymph and larvae, respectively. Of these ticks, 70 (18.4%) and 66 (60%) of the collected *I. persulcatus* and *I. ovatus* were engorged with blood; while 311 (81.6%), 44 (40%) and 28 (100%) of the collected *I. persulcatus*, *I. ovatus* and *H. japonica* were non-engorged, respectively.

Detection and identification of *Babesia* and *Theileria* spp. in tick samples by touchdown PCR/RLB hybridization

Touchdown PCR and RLB hybridization, used to detect a partial fragment of the 18S rRNA gene of *Babesia* and *Theileria* spp., showed that 163 of the 519 (31.7%) DNA samples examined were positive for *Babesia* and *Theileria* spp. including *Theileria* sp. Thrivae, *T. capreoli*, *B. divergens*-like, *Babesia* sp. (Bab-SD), *B. microti* U.S., and *B. microti* Hobetsu (Table 1).

Furthermore, the prevalence of *Theileria* sp. Thrivae was the highest among the detected piroplasms and was detected mostly in adult *I. ovatus*.. In addition, the highest prevalence *Theileria* sp. Thrivae was found in the nymph stage of *H. japonica* (45.5%; 5/11) and adult of *I. persulcatus* (36.4%; 4/11). (Table 1).

In addition, *T. carpreoli* was found in all tick species and stages except *H. japonica* larvae, and the highest prevalence were observed in the adult stages of *I. ovatus*: 22.7% (25/110) and *H. japonica*: 26.7% (4/15). A total of 7.5% (3/40) and 9.1% (1/11) of the DNA samples extracted from engorged *I. persulcatus* larvae and adult *I. persulcatus* were positive for *T. capreoli*, respectively.

The highest prevalence of *B. divergens*-like were 6.7% (1/15) in *H. japonica*. The *Babesia* sp. (Bab-SD) was detected only in *Ixodes* spp., with prevalence of 0.3% (1/330) and

1.8% (2/110) in *I. persulcatus* nymphs and *I. ovatus* adults, respectively. In addition, *B. microti* U.S. and Hobetsu were identified in *Ixodes* spp., with prevalence of 0.5% (2/381) in *I. persulcatus* and 1.8% (2/110) in *I. ovatus*, respectively.

Of the 163 ticks positive for piroplasm species, the co-infection rate was 26.4% (43/163) and included 39 double, 3 triple, and 1 quadruple infection cases. The prevalence of double co-infection were 79.1% (34/43), 9.3% (4/43), and 2.3% (1/43) between *Theileria* sp. Thrivae and *T. capreoli*, *Theileria* sp. Thrivae and *B. divergens*-like, and *T. capreoli* and *B. divergens*-like, respectively (Table 2). Of the three ticks with triple co-infection, two were infected with *Theileria* sp. Thrivae, *T. capreoli*, and *B. divergens*-like, and the third tick was infected with *Theileria* sp. Thrivae, *Babesia* sp. (Bab-SD), and *B. microti* (Hobetsu type). In addition, only one tick was infected with four pathogens: *Theileria* sp. Thrivae, *T. capreoli*, the *Babesia* sp. (Bab-SD), and *B. divergens*-like. The highest co-infection rate, 53.5% (23/43), was detected in adult ticks, whereas a 41.9% (18/43) co-infection rate was found in nymph ticks.

DNA sequencing and molecular characterization

The sequencing results yielded 12 partial 18S rRNA gene sequences (Table 3), which were classified as six piroplasm species: two *Theileria* spp. and four *Babesia* spp. (Figs. 1 and 2). The sequences from *I. persulcatus* (LC336717–LC336718), *I. ovatus* (LC336719), and *H. japonica* (LC336720) were identical to those from the uncultured *Theileria* spp. (LC215385–LC271213) and *Theileria* sp. Thrivae (AB981972), which were previously detected in sika deer in Japan and are 99% similar to *Theileria* sp. sequences (LC169094 and LC169091) found in *Haemaphysalis* sp. collected from sika deer and vegetation, respectively. Our sequences obtained from *I. persulcatus* (LC336721 and LC336724), a *H. japonica* (LC336722), and *I. ovatus* (LC336723) were identical to each other and were identical to the uncultured *Theileria* spp. sequence (LC271206) from sika deer in Japan and 99% similar to sequences of *T. capreoli* (KJ188219) from red deer in China and of *Theileria* sp. (LC169093) from a feeding *Haemaphysalis* sp. detected on sika deer in Japan.

The obtained sequence (LC336725) was identical to that of the *Babesia* sp. (Bab-SD) (LC215388), whereas the sequence LC336727 was identical to a *B. divergens*-like sequence (LC215387) detected in sika deer from Japan. The sequences (LC336726 and LC336728) were clustered with *B. microti*. The first sequence was identical to that of *B. microti* Hobetsu (AB050732) from a field rodent (*Apodemus speciosus*), while the other sequence was identical with that of the *B. microti* U.S. strain (LC127372) from *I. persulcatus* in Japan.

Prevalence of *Theileria* spp. according to tick variables

In this study, LMM test was used to analyze the results of *Theileria* spp., but not for *Babesia* spp. because of the low number of positive *Babesia* spp. samples. The prevalence of *Theileria* sp. Thrivae and *T. capreoli* were significantly higher in the engorged ticks than in the non-engorged ones (LMM: $P < 0.001$ and $P < 0.05$), respectively (Table 4 and Fig. 3). Additionally, a significant positive correlation between *Theileria* sp. Thrivae and *T. capreoli* was detected by chi-square test ($P < 0.01$) (Table 5).

Discussion

Ticks are obligate blood feeders and important vectors of a large number of pathogens infecting animals and humans (Azmi et al. 2016). Despite that many studies have reported piroplasm species from sika deer in Japan (Elbaz et al. 2017; Inokuma et al. 2004; Shibata et al.

2018; Watanabe et al. 2016; Zamoto-Niikura et al. 2014), only few published reports are available on piroplasm species from feeding ticks detected on sika deer in Hokkaido (Masatani et al. 2017).

Here, a total of six piroplasms, *Theileria* sp. Thrivae, *T. capreoli*, *B. divergens*-like, *Babesia* sp. (Bab-SD), *B. microti* U.S., and *B. microti* Hobetsu, were identified. Hokkaido sika deer are potential hosts for *Theileria* sp. Thrivae, *T. capreoli*, *B. divergens*-like, and the *Babesia* sp. (Bab-SD) (Elbaz et al. 2017). However, *B. microti* strains have not been reported in Hokkaido sika deer but were detected previously in rodents; therefore, the ticks infected with *B. microti* strains in this study may have been infected during previous life stages when those ticks fed on other animal hosts.

Our results revealed *Theileria* sp. Thrivae and *T. capreoli* are the most prevalent species in the ticks examined, and both organisms were detected in all examined stages of *Ixodes* nymphs and adults of *H. japonica*. This observation is in agreement with the high prevalence of *Theileria* sp. Thrivae in sika deer, indicating the persistence of infection in sika deer (Elbaz et al. 2017). Recently, a high prevalence of *Theileria* spp. in sika deer (97.8%; 89/91) was detected in Hokkaido, Japan (Shibata et al. 2018). Here, most of the ticks positive for *Theileria* spp. were engorged with sika deer blood, which may have caused this high prevalence. Although the prevalence of *T. capreoli* in Hokkaido sika deer is unknown, the results of this study suggest that sika deer could be the main animal host for this piroplasm species in the Hokkaido ecosystem. Moreover, the finding that the sequences from ticks collected in Kyushu and Hokkaido were almost identical suggests that *Theileria* sp. Thrivae and *T. capreoli* are distributed throughout Japan (Masatani et al. 2017). However, this study cannot confirm that these tick species are the vector hosts to the detected pathogens, because the blood meal from infected host could be the source of the positive results. It is required to examine salivary glands of tick species to confirm that these ectoparasites act as epidemiological vectors.

The prevalence of *Theileria* sp. Thrivae and *T. capreoli* in *H. japonica* in this study are higher than the previously reported rates of 6.3% (2/32) and 12.5% (4/32) (Masatani et al. 2017), respectively. This is possibly due to the higher sensitivity of the RLB technique compared with conventional PCR assays. In addition, most of ticks that were examined in this study were engorged with sika deer blood, which can lead to an increase in the prevalence of *Theileria* species. Interestingly, both *Theileria* spp. were detected in the larval stage of *I. persulcatus*; however, *Theileria* spp. are not known to be transmitted via a transovarian route (Bhattacharyulu et al. 1975). Positivity for *Theileria* spp. was likely because all larvae were engorged with infected sika deer blood.

The detected *Babesia* spp., *B. divergens*-like, *B. microti* U.S., and *B. microti* Hobetsu are zoonotic pathogens. The highest rate of *B. divergens*-like recovery was from an *I. persulcatus* nymph in this study and was in accordance with a previously published report on questing *I. persulcatus* (1.3%; 11/845) from Japan (Zamoto-Niikura et al. 2018).

In this study, the *Babesia* sp. (Bab-SD) was detected in *Ixodes* spp. only. Surprisingly, the *Babesia* sp. (Bab-SD) was found in a non-engorged *I. persulcatus* nymph, suggesting that the larvae of this tick were infected before molting into nymphs, either from an infected reservoir host or by transovarian transmission. However, not all larvae examined in this study were infected by the *Babesia* sp. (Bab-SD); thus, further study on this *Babesia* sp. is needed to understand its life cycle.

Sika deer are not hosts for *B. microti*; however, this study found that *Ixodes* spp. feeding on sika deer were positive for *B. microti* strains. Furthermore, the *B. microti* U.S. and Hobetsu types were detected in *I. persulcatus* and *I. ovatus*, respectively. This result is consistent with previous studies reporting that *I. persulcatus* and *I. ovatus* are vectors for *B. microti* U.S. and *B. microti* Hobetsu in Japan and Russia, respectively (Katargina et al. 2011; Rar et al. 2011; Rar et al. 2014; Zamoto-Niikura et al. 2012). The prevalence of *B. microti* U.S. type in *I. persulcatus* in this study was in accordance with the rates of those previously published reports from Japan and Russia (Katargina et al. 2011; Rar et al. 2011; Rar et al. 2014; Zamoto-Niikura et al. 2012).

The partial 18S rRNA gene sequences were identified as belong to two *Theileria* spp. and four *Babesia* spp. The two *Theileria* spp. clustered with *Theileria* sp. Thrivae and *T. capreoli*, which were detected in sika deer in Japan. The *Babesia* spp. sequences were associated with four branches. The first branch clustered with *B. divergens*-like sequences, the second branch with the *Babesia* sp. (Bab-SD) sequences detected in sika deer in Japan (Elbaz et al. 2017), and the remaining two with *B. microti* sequences, one with a previously published *B. microti* U.S. sequence from *I. persulcatus* in Japan (Zamoto-Niikura et al. 2016) and the other with *B. microti* Hobetsu type detected in a field rodent (*Apodemus speciosus*) (Wei et al. 2001).

The prevalences of both *Theileria* spp. detected in this study were significantly higher in the engorged ticks than in non-engorged ones, which could be due to the high infection rates of both *Theileria* spp. in sika deer blood, indicating the persistence of infection in sika deer.

Co-infection of ticks with more than one pathogen is not uncommon, and one tick can be infected with up to five pathogens (Eshoo et al. 2014; Moutailler et al. 2016; Reis et al. 2011). Multiple pathogens in individual ticks increases the risk of co-transmission to, and co-infections in, humans and animals (Moutailler et al. 2016). In this study, a significant co-infection correlation was shown between *Theileria* sp. Thrivae and *T. capreoli*, which may have occurred because both *Theileria* spp. have similar life cycles. This was supported by the phylogenic analysis showing that the two *Theileria* spp. belong to the same *Theileria* spp. cluster (Inokuma et al. 2008; Li et al. 2014). The association between *Theileria* and *Babesia* spp. reported in double, triple, or quadruple co-infection is likely due to shared reservoir hosts or co-feeding during different life stages. Additionally, the highest number of co-infection cases was observed in adult ticks, which may be because of the greater opportunity of adult ticks to receive more pathogens from host feeding via transstadial transmission.

Conclusion

Ticks and TBDs constitute a growing burden for human and animal health. This study revealed that ticks collected from sika deer are exposed to a high diversity of piroplasm species and demonstrated the ecology of these pathogens endemic to Hokkaido. The sika deer is an amplifying host for *Theileria* sp. Thrivae, *T. capreoli*, *B. divergens*-like, and *Babesia* sp. (Bab-SD). In addition, zoonotic TBPs, such as *B. divergens*-like and *B. microti* U.S. and Hobetsu, were detected in the ticks of this study. Because this is the first report of piroplasm species detected in feeding ticks collected from sika deer in Hokkaido, further investigations are needed to reveal the epidemiology and respective vectors of these pathogens.

Competing interests

The authors declare that they have no competing interests.

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English check

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Table 1 The prevalence of piroplasm species in ticks collected from Hokkaido sika deer by RLB

Tick samples				**Detected pirolplasm spp.**						
Species	**Stage**	**No.tested**		***Theileria* sp. Thrivae**	***T. capreoli***	***B. divergens*-like**	***Babesia* sp. (Bab-SD)**	***B. microti* U.S.**	***B. microti* Hobetsu**	***Co-infection***
		Engorged	**Non-engorged**							
I. persulcatus	Larvae	15	25	8 (20%)	3 (7.5%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2
	Nymph	53	277	74 (22.4%)	21 (6.4%)	7 (2.1%)	1 (0.3%)	1 (0.3%)	0 (0.0%)	18
	Adult	2	9	4 (36.4%)	1 (9.1%)	0 (0.0%)	0 (0.0%)	1 (10%)	0 (0.0%)	1
	Sub total	70	311	86 (22.6%)	25 (6.6%)	7 (1.8%)	1 (0.3%)	2 (0.5%)	0 (0.0%)	21
I. ovatus	Adult	66	44	45 (40.9%)	25 (22.7%)	1 (0.9%)	2 (1.8%)	0 (0.0%)	2 (1.8%)	20
	Sub total	66	44	45 (40.9%)	25 (22.7%)	1 (0.9%)	2 (1.8%)	0 (0.0%)	2 (1.8%)	20
Haemaphysalis japonica	Larvae	0	2	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0
	Nymph	0	11	5 (45.5%)	1 (9.1%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	0
	Adult	0	15	4 (26.7%)	4 (26.7%)	1 (6.7%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2
	Sub total	0	28	9 (32.1%)	5 (17.9%)	1 (3.6%)	0 (0.0%)	0 (0.0%)	0 (0.0%)	2
Total		519		140 (27%)	55 (10.6%)	9 (1.7%)	3 (0.6%)	2 (0.4%)	2 (0.4%)	43

Table 2. A summary of co-infections between piroplasm species in ticks collected from Hokkaido sika deer by RLB

Co- infection	**Piroplasms**	***I. persulcatus***	***I. ovatus***	***H. japonica***	**Total**
Double infection	*Theileria* sp. Thrivae and *T. capreoli*	15	18	1	34
	Theileria sp. Thrivae and *B. divergens* like	3	1	0	4
	T. capreoli and *B. divergens* like	0	0	1	1
Triple infection	*Theileria* sp. Thrivae, *T. capreoli* and *B. divergens* like	2	0	0	2
	Theileria sp. Thrivae, *Babesia* sp. (Bab-SD) and *B. microti* Hobetsu	0	1	0	1
Quadruple infection	*Theileria* sp. Thrivae, *T. capreoli, Babesia* sp. (Bab-SD) and *B. divergens* like	1	0	0	1

Table 3 Samples of sequencing

Number	Sample I.D.	Accession number	RLB results
1	130308 I.P.N	LC336717	*Theileria* sp. Thrivae
2	130304 I.P.A	LC336718	
3	120619D3 I.O.A	LC336719	
4	120618D21 H.N	LC336720	
5	120925D2 I.P.L	LC336721	*Theileria capreoli*
6	120610D14 H.A	LC336722	
7	120219D13 I.O.A	LC336723	
8	120219D3 I.P.N	LC336724	
9	12012D3 I.O.A	LC336725	*Babesia* sp. (Bab-SD)
10	120618D13 I.O.A	LC336726	*Babesia microti* Hobetsu
11	120928N2 I.P.N	LC336727	*Babesia divergens*-like
12	130305 I.P.A	LC336728	*Babesia microti* U.S.

I.P.N: *I. persulcatus* nymph, I.P.A: *I. persulcatus* adult, I.P.L: *I. persulcatus* larvae, I.O.A: *I. ovatus* adult, H.N: *H. japonica* nymph and H.A: *H. japonica* adult

Table 4. The final models for the prevalence of *Theileria* sp. Thrivae and *T. capreoli* in ticks collected from Hokkaido sika deer.

		Prevalence of *Theileria* sp. Thrivae					**Prevalence of *Theileria capreoli***				
Random	Predictors	Estimate	Std. Err	df	t value	Pr(>|t|)	Estimate	Std. Err	df	t value	Pr(>|t|)
Location	(Intercept)	0.44	0.12	40.98	3.68	0.00 ***	0.29	0.07	141.83	3.92	0.00 ***
	Species_*I. ovatus*	0.04	0.11	512.22	0.38	0.71	-0.04	0.07	434.92	-0.47	0.64
	Species_*I. persulcatus*	-0.11	0.10	509.42	-1.11	0.27	-0.08	0.07	503.20	-1.14	0.26
	Stages_Larvae	-0.07	0.12	512.99	-0.57	0.57	-0.10	0.08	512.99	-1.21	0.23
	Stages_Nymph	0.06	0.10	512.67	0.58	0.56	-0.09	0.07	499.98	-1.20	0.23
	Status_Non-engorged	-0.18	0.05	512.25	-3.82	0.00 ***	-0.08	0.03	512.60	-2.36	0.02 *

Significance codes: 0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Table 5 Number of Ticks infected with *Theileria* sp. Thrivae and/or *T. capreoli*.

		T. capreoli		
		Positive	Negative	Total
***Theileria* sp. Thrivae**	Positive	37*	103	140
	Negative	18	361	379
	Total	55	464	519

***** Correlation between *Theileria* sp. Thrivae and *T. capreoli* was significant positive (P<0.01) using chi-square (χ^2) test in SPSS

Fig. 1 Phylogenetic tree based on nucleotide 18S rRNA gene sequences of *Babesia* spp. from GenBank. Sequences from the present study are labeled in bold. The DNA sequences from this study was deposited to DDBJ as accession number from LC336725 to LC336728. Phylogenetic trees were constructed using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0.2599)). The analysis involved 22 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 174 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al. 2016).

Fig. 2 Phylogenetic tree based on nucleotide 18S rRNA gene sequences of *Theileria* spp. from GenBank. Sequences from the present study are labeled in bold. The DNA sequences from this study was deposited to DDBJ as accession number from LC336717 to LC336724. Phylogenetic trees were constructed using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei 1993). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. A discrete Gamma distribution was used to model evolutionary rate differences among sites (5 categories (+G, parameter = 0. 3910)). The analysis involved 31 nucleotide sequences. All positions containing gaps and missing data were eliminated. There was a total of 177 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar et al., 2016).

Fig. 3 Descriptive analysis of the prevalence of *Theileria* sp. Thrivae and *T. capreoli* in (a) engorged and non-engorged ticks and (b) the developmental stages of ticks

Declarations

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Availability of data and material (The DNA sequence data were registered at the DNA DataBank of Japan (DDBJ) under the accession numbers LC336717 - LC336728)

Code availability (Not applicable)

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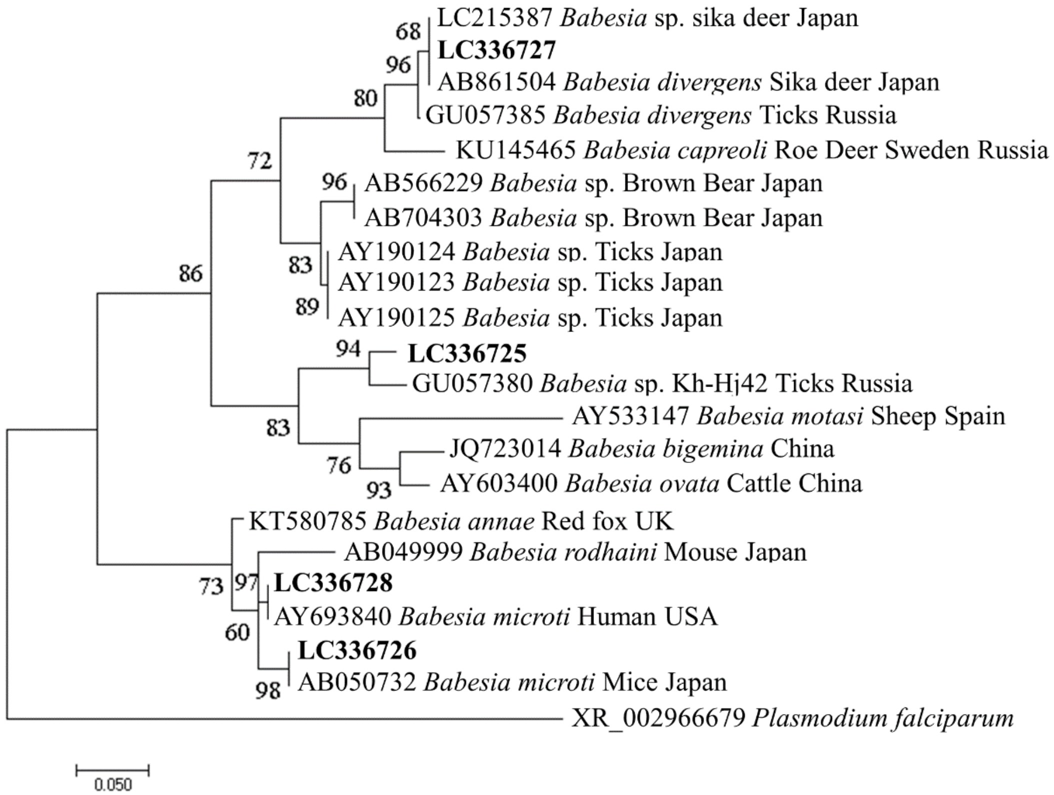
Fig. 1



68
96
80
72
86
96
83
89
94
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76
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73
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LC215387 *Babesia* sp. sika deer Japan
LC336727
AB861504 *Babesia divergens* Sika deer Japan
GU057385 *Babesia divergens* Ticks Russia
KU145465 *Babesia capreoli* Roe Deer Sweden Russia
AB566229 *Babesia* sp. Brown Bear Japan
AB704303 *Babesia* sp. Brown Bear Japan
AY190124 *Babesia* sp. Ticks Japan
AY190123 *Babesia* sp. Ticks Japan
AY190125 *Babesia* sp. Ticks Japan
LC336725
GU057380 *Babesia* sp. Kh-Hj42 Ticks Russia
AY533147 *Babesia motasi* Sheep Spain
JQ723014 *Babesia bigemina* China
AY603400 *Babesia ovata* Cattle China
KT580785 *Babesia annae* Red fox UK
AB049999 *Babesia rodhaini* Mouse Japan
LC336728
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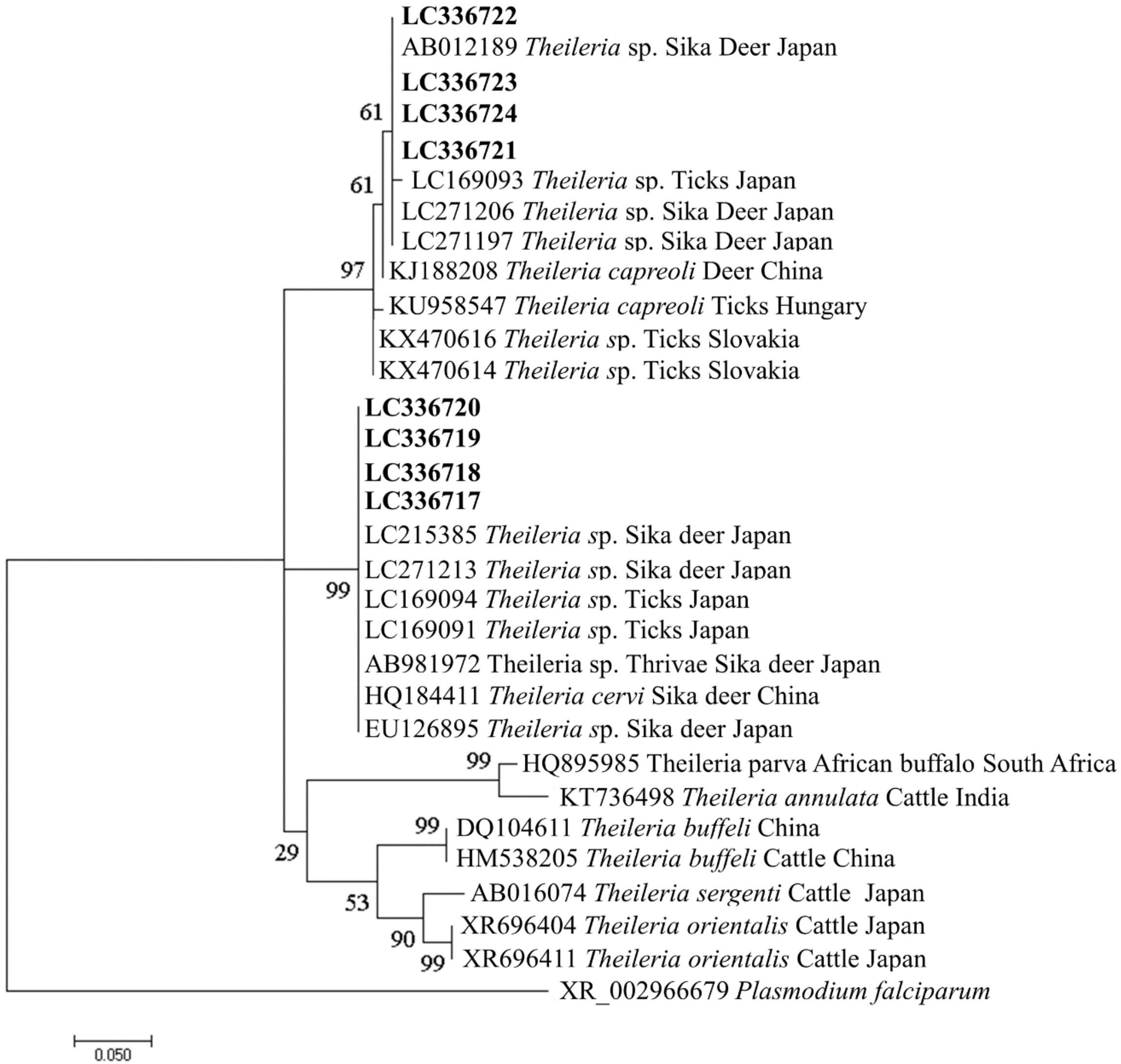
LC336726
AB050732 *Babesia microti* Mice Japan
XR_002966679 *Plasmodium falciparum*
0.050

Fig. 2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LC336723
61
LC336724
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LC271197 Theileria sp. Sika Deer Japan
97
KJ188208 Theileria capreoli Deer China
KU958547 Theileria capreoli Ticks Hungary
KX470616 Theileria sp. Ticks Slovakia
KX470614 Theileria sp. Ticks Slovakia
LC336720
LC336719
LC336718
LC336717
LC215385 Theileria sp. Sika deer Japan
LC271213 Theileria sp. Sika deer Japan
99
LC169094 Theileria sp. Ticks Japan
LC169091 Theileria sp. Ticks Japan
AB981972 Theileria sp. Thrivae Sika deer Japan
HQ184411 Theileria cervi Sika deer China
EU126895 Theileria sp. Sika deer Japan
99
HQ895985 Theileria parva African buffalo South Africa
KT736498 Theileria annulata Cattle India
99
DQ104611 Theileria buffeli China
29
HM538205 Theileria buffeli Cattle China
AB016074 Theileria sergenti Cattle Japan
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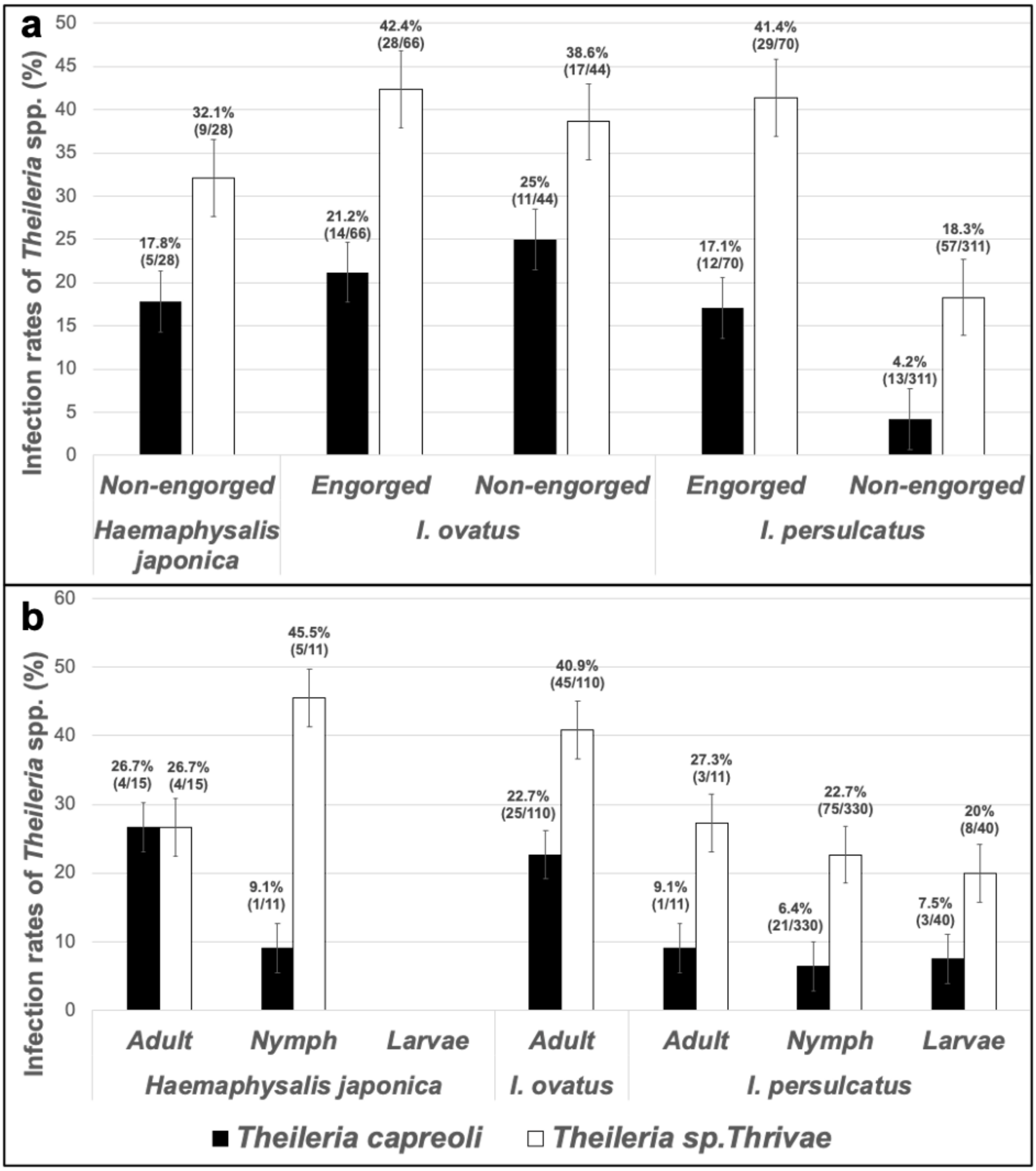
XR696404 Theileria orientalis Cattle Japan
90
99
XR696411 Theileria orientalis Cattle Japan
XR_002966679 Plasmodium falciparum
0.050

Fig. 3


a
b
Infection rates of Theileria spp. (%)
17.8% (5/28)
32.1% (9/28)
21.2% (14/66)
42.4% (28/66)
25% (11/44)
38.6% (17/44)
17.1% (12/70)
41.4% (29/70)
4.2% (13/311)
18.3% (57/311)
Non-engorged
Engorged
Non-engorged
Engorged
Non-engorged
Haemaphysalis japonica
I. ovatus
I. persulcatus
26.7% (4/15)
26.7% (4/15)
9.1% (1/11)
45.5% (5/11)
22.7% (25/110)
40.9% (45/110)
9.1% (1/11)
27.3% (3/11)
6.4% (21/330)
22.7% (75/330)
7.5% (3/40)
20% (8/40)
Adult
Nymph
Larvae
Adult
Adult
Nymph
Larvae
Haemaphysalis japonica
I. ovatus
I. persulcatus
Theileria capreoli
Theileria sp.Thrivae