Molecular investigation of tick-borne diseases in Hokkaido wildlife

(北海道の野生動物におけるダニ媒介性感染症の分子疫学研究)

Mohamed Abdallah Mohamed Moustafa

Laboratory of Wildlife Biology and Medicine,

Graduate School of Veterinary Medicine,

Hokkaido University

January 2016

AUTHOR'S DECLARATION

This study is my original work and has not been presented at any other University for the award of a degree. A part of this thesis has been published in advance as follow:

Molecular characterization and specific detection of *Anaplasma* species (AP-sd) in sika deer and its first detection in wild brown bears and rodents in Hokkaido, Japan. **Moustafa MA**, Lee K, Taylor K, Nakao R, Sashika M, Shimozuru M, Tsubota T. Infect Genet Evol., 36:268-274, 2015.

DEDICATION

This thesis is dedicated to my family for always being there for me.

CONTENTS

LIST OF TABLES

LIST OF FIGURES

ABBREVIATIONS

CDC	Centers for Disease Control and Prevention
PCR	Polymerase chain reaction
DNA	Deoxyribonucleic acid
RLB	Reverse line blot
18S rRNA	18S ribosomal RNA
16S rRNA	16S ribosomal RNA
TBDs	Tick-borne diseases
TBPs	Tick-borne pathogens
HGA	Human granulocytic anaplasmosis
GLMs	Generalized Linear Models
USA	United States of America

PREFACE

Ticks and tick-borne diseases (TBDs) are considered an emerging public health threat to humans. After mosquitoes, ticks are the second major vectors of zoonotic diseases in the world (Parola and Raoult, 2001). They are capable of infesting on many vertebrate and invertebrate animals. During their life cycles, they can acquire and transmit many types of zoonotic pathogens.

Tick-borne pathogens (TBPs) are found in cycles which include ticks and animals. Accidently, these pathogens can be transmitted to humans (de la Fuente et al., 2008). Several species of small mammals are considered to be vertebrate reservoirs of tick-borne bacterial zoonoses such as human granulocytic ehrlichiosis (Barandika et al., 2007) and human granulocytic anaplasmosis (HGA) (Bown et al., 2003; Liz et al., 2000). In addition, many *Babesia* species and genotypes were identified (Gray et al., 2010; Yabsley and Shock, 2013), few of these were reported as zoonotic. The most prevalent *Babesia* spp. in humans are *Babesia microti*, *B. divergens*, *B. duncani* and *B. venatorum* (Leiby, 2011).

Anaplasma phagocytophilum is an obligate intracellular Gram-negative bacterium that causes HGA and belongs to the family *Anaplasmataceae*, order Rickettsiales. It infects the neutrophils of the reservoir hosts in the blood and organs. *A. phagocytophilum* is highly heterogeneous with many genetic variants having different virulence and host tropism.

In the USA, there are two major variants of *A. phagocytophilum*: Ap-V1 and Ap-ha (Chen et al., 1994). Both variants have the same geographical distribution, and could also be transmitted by the same vectors (Courtney et al., 2003), but, these two variants have different host tropism. The major reservoir host for Ap-V1 is the white-tailed deer (*Odocoileus virginianus*) (Massung et al., 2005; Reichard et al., 2009), while the white-footed mouse (*Peromyscus leucopus)* is the reservoir host for Ap-ha (Massung et al., 2003).

The situation in Japan is similar to that in the USA where there are several strains of *A. phagocytophilum*. An *A. phagocytophilum* strain is present, which has caused disease in 2 humans (Ohashi et al., 2013) and has been detected *in Ixodes ovatus* and *I. persulcatus* ticks in Hokkaido (Ybañez et al., 2012b); however, no mammalian reservoir host has been identified for this strain in Hokkaido. On the other hand, the *Anaplasma* sp. (AP-sd) detected in sika deer and cattle (Jilintai et al., 2009), is very common in Japan.

Human ehrlichiosis is an important emerging TBD in humans, as well as in domestic animals (Rikihisa, 1991; Wen et al., 1995). This disease is caused by *Ehrlichia* spp. such as *E. chaffeensis*, *E. canis* and *E. ewingii.* Another member of Genus *Ehrlichia* is *E. muris* which was first detected from small mammals in Japan (Kawahara et al., 1993; Wen et al., 1995). However, geographic distribution, reservoir host, vector host, and the exposure of humans of the described strain of *E. muris* were not fully understood (Kawahara et al., 1999). It is thought that *E. chaffeensis*, *E. canis* and *E. ewingii* are the only *Ehrlichia* spp. that can cause human diseases until *Ehrlichia* sp. closely related to *E. muris* was identified in 3 symptomatic patients in Wisconsin and 1 in Minnesota, USA (Pritt et al., 2011). In addition, *E. ruminantium* which causes a disease known as “heart water” in ruminants was detected in 3 fatal human cases in South Africa (Allsopp et al., 2005).

Neoehrlichiosis is a human disease caused mainly by *Candidatus* Neoehrlichia mikurensis which is a member of order Rickettsiales, family *Anaplasmataceae* (Kawahara et al., 2004). This pathogen was first reported from humans in 2010 (Fehr et al., 2010; von Loewenich et al., 2010). Although this pathogen was detected in many countries in Europe and Asia, the distribution and reservoir hosts of *Candidatus* N. mikurensis in Japan is not fully studied.

Human babesiosis is an intraerythrocytic zoonotic disease (Zhou et al., 2014), caused mainly by *B. microti*. Different rodent species are considered reservoir hosts for *B. microti* in

the northern parts of North America (Burkot et al., 2000; Watkins et al., 1991), Europe (Shortt and Blackie, 1965), and Eurasia (Shih et al., 1997). In Japan, the first case of symptomatic human babesiosis was found at Kobe City in Hyogo Prefecture. The strains found in Japan are genetically different from *B. microti* strains in the United States (Tsuji et al., 2001).

Molecular tools allow the detection and identification of these pathogens with higher specificity and sensitivity compared to microscopy and serology (Criado-Fornelio, 2007). One of these tools is the Reverse Line Blot (RLB) hybridization, which is a cheap and practical epidemiological technique to identify TBPs with high throughput (Tait and Oura, 2004). Using this method with a single multiplex polymerase chain reaction (PCR) can detect 43 pathogens in 43 PCR products in a single run (Kong and Gilbert, 2006). The nylon membrane with the oligonucleotide probes can be reused for 20 times. Moreover, novel pathogens can be detected and identified when RLB is combined with DNA sequencing (Nijhof et al., 2003; Nijhof et al., 2005; Oosthuizen et al., 2008).

The 16S ribosomal RNA (16S rRNA) and 18S ribosomal RNA (18S rRNA) genes are usually used to identify *Ehrlichia* and *Babesia* spp., respectively, by RLB hybridization and sequencing. The 16S rRNA and 18S rRNA genes have both conserved and variable regions, which makes them excellent in both detection and molecular differentiation of TBPs (Allsopp and Allsopp, 2006).

The studies which are included in this thesis provide information about a number of zoonotic TBPs in different wild animals in Hokkaido, Japan. In the first chapter, we determined the taxonomic position of *Anaplasma* sp. AP-sd and provided a molecular tool for its specific detection along with *A. phagocytophilum* and *A. bovis* among the most prominent wild ruminant, apex predator, and the most common rodent species in Hokkaido ecosystem.

In the second chapter, I surveyed the prevalence of 13 zoonotic TBPs in 6 different small mammals collected from two different regions in Hokkaido, Japan. In addition, I used the statistical modelling to examine the relationship between the infection rates and the explanatory variables. Finally, I provided a phylogenetic analysis for the detected TBPs based on the 16S rRNA and 18S rRNA gene sequences.

1. Chapter 1

Molecular characterization and specific detection of *Anaplasma* species (AP-sd) in sika deer and its first detection in wild brown bears and rodents in Hokkaido, Japan

1.1. INTRODUCTION

Anaplasma phagocytophilum (A. phagocytophilum) is a Gram-negative, intracellular bacterium, which causes human granulocytic anaplasmosis (HGA). According to the Centers for Disease Control and Prevention (CDC), anaplasmosis cases in the USA have increased from 348 to 1,761 cases between 2000 and 2010. In Japan, fever of unknown cause and rickettsiosis-like symptoms have occasionally been recorded in people. The first report of *A. phagocytophilum* in ticks in Japan was published in 2005 (Ohashi et al., 2005). However, little was known about human infection with *A. phagocytophilum* for many years. Recently, 2 cases of HGA were identified by retrospective study in Japan (Ohashi et al., 2013).

A. phagocytophilum is a highly heterogenic bacterium sharing genetic divergence. While these strains are closely related based on 16S rRNA gene sequences, some strains show unique host tropism (Massung et al., 2003). For example, Ap-Variant 1 in the USA, a strain associated with white-tailed deer, has not caused human disease and can be distinguished from strains that cause human disease by a few base differences in the 16S rRNA gene (Massung et al., 2006b). The situation in Japan is similar to that in the USA in that there are several genetically different strains of *A. phagocytophilum*. An *A. phagocytophilum* strain is present, which has caused disease in 2 humans (Ohashi et al., 2013) and has been detected in *Ixodes ovatus* and *I. persulcatus* ticks in Hokkaido (Ybañez et al., 2012b); however, no mammalian reservoir host has been identified for this strain in Hokkaido. On the other hand, the *Anaplasma* sp. (AP-sd) detected in sika deer and cattle (Jilintai et al., 2009), is very common in Japan. AP-sd has also been reported as a strain of *A. phagocytophilum* in Japan and China (Kawahara et al., 2006; Liu et al., 2012; Yang et al., 2013), deer type *A. phagocytophilum* (Wu et al., 2015) or an *Anaplasma* species closely related to *A. phagocytophilum* (Ybañez et al., 2013). Despite the last report showing high prevalence in

sika deer, 45% (10/ 22) (Jilintai et al., 2009), AP-sd has never been reported from humans. In addition, to date, no studies have detected *A. phagocytophilum* in sika deer in Japan.

The high similarity between AP-sd and *A. phagocytophilum* sequences in Japan could likely lead to miscalculation of *A. phagocytophilum* prevalence by both molecular and serological techniques. In fact, it was confirmed in the USA that there is strong antigenic cross-reactivity between *A. phagocytophilum* human agent (Ap-ha) and AP-Variant 1 strains of *A. phagocytophilum* (Massung et al., 2005). Previously, several polymerase chain reactions have been used to detect *A. phagocytophilum* and AP-sd. Unfortunately, this technique lacks specificity and is unable to distinguish the two genotypes of bacteria. Hence, there was a necessity for a molecular technique with high specificity to differentiate infections. Here I analyzed sequences of AP-sd to identify its taxonomic position. The reverse line blot hybridization (RLB) was then used for the specific detection of AP-sd and *A. phagocytophilum* among the most prominent wild ruminant, apex predator, and the most common rodent species in Hokkaido for the presence of each bacterium to contrast their ecologies in Hokkaido by examining potential hosts.

1.2. MATERIALS AND METHODS

1.2.1. Mammalian blood samples and DNA extraction

Whole blood samples were collected in EDTA.Na tubes from 250 sika deer (*Cervus nippon yesoensis*), 13 brown bears (*Ursus arctos yesoensis*) and 252 rodents including 138 (*Apodemus speciosus*), 45 (*Apodemus argenteus*), 42 (*Myodes rufocanus*) and 27 (*Myodes rutilus*) from Hokkaido, northern Japan, during 2010 to 2015. All sika deer and brown bears included in this study were killed by hunting and nuisance control culling. All rodent sampling was performed using methods approved by the Animal Care and Use Committee of Hokkaido University (Approval No. JU1105). DNA was extracted from the blood samples using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA). The obtained DNA samples were kept at -20°C until analysis.

1.2.2. Nested PCR and electrophoresis

DNA samples and positive control DNA from cultured *A. phagocytophilum* (Ameland Netherland strain) were screened for the presence of *Anaplasma* sp. by nested PCR using Takara Ex Taq (Takara Bio, Otsu, Japan). The primer pair EC9/ EC12A was used for the first PCR to amplify a 1462 bp segment of the 16S rRNA gene of all *Ehrlichia* and *Anaplasma* spp. (Kawahara et al., 2006). PCR products were used as a template for the second PCR to amplify a 770 bp segment of *Anaplasma* sp. (AP-sd) and *A. phagocytophilum* using the primer pair AP-f1/AP-r1 (Sashika et al., 2011). Resulting amplicons were stained by 10× Loading Buffer (Takara, Shiga, Japan) and visualized after electrophoresis on 1% agarose gel at 100-voltage for 20 min.

1.2.3. Cloning and sequencing of 16S rRNA and gltA genes

The 16S rRNA nested PCR positive products were purified with NucleoSpin® Gel and PCR clean up (Macherey-Nagel, GM & CO KG, Düren, Germany) and directly sequenced by

ABI PRISM™ 310 genetic analyzer, BigDye Terminator V3.1 Sequencing Kit and BigDye® XTerminator™ Purification Kit (Life Technologies Co., Grand Island, NY, USA). The obtained sequences were analyzed to identify the sequences with overlapping signals. Out of 20 sequences with overlapping signals, I selected 4 samples for cloning (Here referred to as C_AP1, C_AP2, C_AP3 and C_AP4). I performed nested PCR using KOD-PlusNeo high fidelity DNA polymerase kit (Toyobo Co. Ltd., Osaka, Japan). The same primers above were used to amplify a 770 bp segment of *Anaplasma* spp. 16S rRNA gene, while the primer pairs CS7F2/ HG1085R and F1B/CS1076R (Ybañez et al., 2012a) were used to amplify a 382 bp segment of gltA gene. Each PCR reaction consisted of 2.5 µl of 10× KOD-Plus-Neo buffer, 2.5 µl of dNTPs (2 mM each), 1.5 µl of 25 mM $MgSO_4$, 0.75 µl of each primer (10 pmol), 1 µl of DNA (100 ng/ µl) and 0.5 µl (1 U/ µl) of KOD-Plus-Neo DNA polymerase. The reaction conditions for the first PCR of both genes were 94°C for 2 min and 40 cycles of 98°C for 10 sec, 44°C for 30 sec and 68°C for 30 sec, followed by a final extension at 68°C for 7 min. The nested PCR reactions to amplify both target genes were similar to the first PCR with an annealing temperature of 52°C for 30 sec.

Nine microliter of each PCR product were incubated for 10 min at 60°C with 1 µl of 10× A-attachment mix (Toyobo Co. Ltd., Life Science Department, Osaka, Japan). The A-attached PCR products were cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA) as described in the manufacturer's quick protocol manual. Five colonies per sample were selected and screened by PCR for recombinants. Five microliter of each PCR product were purified by incubation with 4 µl Exosap mix (USB Co., Cleveland, OH) for 30 min at 37°C followed by 15 min at 80°C. The purified PCR products were subjected to sequencing reactions and cleaned by Agencourt CleanSEQ (Agencourt Bioscience Co., Beckman Coulter, Beverly, Massachusetts, USA) according to the manufacturer's instructions. The

cleaned products were sequenced by ABI Prism 3130 × genetic analyzer according to the manufacturers' instructions.

1.2.4. Sequence analysis

The obtained sequences were deposited to DDBJ. The accession numbers of the 16S rRNA gene sequences were: LC068730, LC068731, LC068732, LC068733, LC068734, LC068735 and LC068736. For gltA gene sequences, the accession numbers were: LC068819, LC068820, LC068821, LC068822, LC068823 and LC068824. These sequences were compared with the previously published 16S rRNA and gltA gene sequences of *Anaplasma* spp. available in GenBank. The stability of phylogenies was assessed by 1,000 bootstrap replications. Alignment and phylogenetic analyses were performed by MEGA software version 5.1 (Tamura et al., 2011) using ClustalW and Neighbor Joining Tree, respectively.

1.2.5. PCR and reverse line blot (RLB) hybridization

A total of 126 nested PCR positive sika deer, 13 brown bear and 252 rodent blood DNA samples were examined by PCR and RLB. The primers Ehr-F (5′-GGA ATT CAG AGT TGG ATC MTG GYT CAG-3′) (Schouls et al., 1999) and Ehr-R (5′-biotin-CGG GAT CCC GAG TTT GCC GGG ACT TYT TCT-3′) (Bekker et al., 2002) were used to amplify a fragment of 460– 520 bp from the 16S rRNA gene. PCR was performed by PCR System 9700 (Applied Biosystems, Foster City, CA) and Takara Ex Taq kits (Takara Bio, Otsu, Japan). PCR reactions consisted of 2.5 µl of 10× Ex Taq Buffer, 2 µl dNTP Mixture (2.5 mM each), 1 µl of each primer (10 pmol), 1 µl DNA and 0.125 µl TaKaRa Ex Taq (5 units/ µl). The volumes were made up to 25 µl with molecular biology grade water. DNA from cultured *A. phagocytophilum* (Ameland Netherland strain*), Ehrlichia chaffeensis*, *E. ruminantium* and double-distilled water were used as positive and negative controls, respectively. The amplification condition was 40 cycles of 30 sec at 94°C, 30 sec at 63°C and 1 min at 72°C.

PCR results were examined by electrophoresis through 1% agarose gel stained with ethidium bromide and visualized by UV illuminator.

RLB was performed on the PCR products as described before (Kong and Gilbert, 2006) with modifications. Briefly, a 15 × 15 cm Biodyne C membrane (Pall Life Sciences, Ann Arbor, MI, USA) was activated by 20 ml 16% 1-ethy-3-(3-dimethyl-amino-propyl) carbodiimide (EDAC) (Sigma Aldrich, St. Louis, MO, USA) for 10 min at room temperature. Subsequently, the membrane was washed gently with milliQ water for 2 min and placed in Miniblotter MN45 (Immunetics, Boston, MA, USA). Oligonucleotide probes with C6 amino linker (Table 1-1) were obtained from Sigma Aldrich Co., LLC, Japan. Five microliter of each 100 pmol/ µl probes were diluted in 0.5 M $NaHCO_3$ to a final volume of 170 µl. The slots of the miniblotter were filled with the 150 µl of each diluted oligonucleotide and the membrane was incubated at room temperature for 5 min. The membrane was inactivated in 250 ml 0.1 M NaOH with gentle shaking for 8 min at room temperature, then washed with prewarmed 250 ml of 2×SSPE/0.1% SDS for 5 min at 60°C. Then the membrane was directly used or it was stored in a sealed plastic bag with 15 µl of 20 mM EDTA, pH 8.0.

A volume of 10 µl of each PCR product was diluted in 2× SSPE/0.1% SDS to a final volume of 170 µl and heat-denatured in boiling water for 10 min and immediately cooled on ice. The PCR products were introduced into the miniblotter, which contained the prepared membrane, and hybridized at 60°C for 1 hr. The membrane was washed twice in 250 ml of 2× SSPE/0.5% SDS for 10 min at 60°C and then incubated with diluted peroxidase labeled NeutrAvidin (Thermo Fisher Scientific, Walyham, MA, USA) for 45 min at 42°C. The membrane was washed twice with 2× SSPE/0.5% SDS for 10 min at 42°C. Finally, the membrane was incubated with 15 ml ImmobilonTM Western Chemiluminescent HRP Substrate (Milipore, Japan) for 5 min at room temperature, and the result was detected by Chemiluminescent imager (Billerica, MA, USA). To strip the membrane for reuse, it was

washed twice in prewarmed 1% SDS at 90°C for 30 min and washed with 250 ml of 20 mM EDTA. The membrane was stored in a plastic bag with 15 ml of 20 mM EDTA at 4°C until reuse.

1.2.6. Culture

Whole blood from 14 sika deer was inoculated into *Ixodes scapularis* ISE6, the human promyelocytic HL60 and canine monocytic DH82 cell lines. The success of isolation was examined every other week by nested PCR for 3 months.

1.3. RESULTS

1.3.1. Prevalence in sika deer, brown bears and rodents

The prevalence of *Anaplasma* spp. in sika deer, brown bear and rodent samples by nested PCR was 51.2% (128/250), 15.4% (2/13) and 2.4% (6/252), respectively. As the nested PCR showed low specificity in the differentiation between *A. phagocytophilum* and AP-sd, the prevalence was confirmed by direct sequencing of 75, 2 and 6 16S rRNA gene nested PCR positive products from sika deer, brown bears and rodents, respectively.

1.3.2. Molecular characterization

Both brown bear and rodent positive 16S rRNA sequences were identical to that of *Anaplasma* sp. (JN055357). A total number of 20 sequences from sika deer positive samples had mixed signals. Four of these mixed sequences were resolved by cloning of the 16S rRNA and gltA gene segments.

The obtained sequences of the 16S rRNA gene were mainly divided into two *Anaplasma* species that clustered with selected *A. bovis* sequences from the GenBank. Our sequences (LC068730) from C_AP2, (LC068735) from C_AP4 and [(LC068736) from C_AP3 were 99% similar to those of *Anaplasma* spp. (JN055357 and AY570539), 98-99% to *A. bovis* (JN558829 and HQ913645) and 98% similar to *A. phagocytophilum* (HG916767 and CP000235). Whereas, our sequences (LC068731) from C_AP1, (LC068732) from C_AP4, (LC068733) from C_AP3 and LC068734 from C_AP3 were 99% similar to that of *A. bovis* (GU937020, EU181143 and AY144729) and 96% similar to that of *A. phagocytophilum* (CP000235) (Fig. 1-1). The 16S rRNA nucleotide polymorphisms and substitutions are listed in Tables 1-2 and 1-3.

The obtained gltA gene partial sequences were also divided into two different *Anaplasma* species. One cluster that included the sequence (LC068821) from C_AP2 was 100%, 83%

and 71% similar to those of *Anaplasma* spp. (JN055362) from sika deer in Hokkaido, (AY570541) from dog in South Africa and *A. phagocytophilum* (CP000235), respectively. The other cluster, which included LC068819 from C_AP1, (LC068820) from C_AP2, (LC068822) from C_AP2, (LC068823) from C_AP4 and (LC068824) from C_AP4 was 95-98% similar to that of *A. bovis* (JN588561) from raccoon in Hokkaido (Fig. 1-2).

1.3.3. Anaplasma sp. (AP-sd) specific detection by RLB hybridization

In the present study, 4 different oligonucleotide probes were used to detect several *A. phagocytophilum* variants. However, only one probe was used to detect AP-sd (Table 1-1). A total of 64% (81/126) of AP-sd nested PCR positive sika deer samples were positive by RLB and the prevalence in brown bears and rodents was, 15% (2/13) and 2.4% (6/252), respectively. This method successfully differentiated between the 16S rRNA gene partial sequences of the *Anaplasma phagocytophilum* positive control and the other *Anaplasma* sp. (AP-sd) from sika deer, brown bear and rodent positive samples (Fig. 1-3).

All of the sika deer, brown bear or rodent samples tested in our study were negative for the *Anaplasma phagocytophilum* specific probes by the reverse line blot hybridization technique (Fig. 1-3).

1.3.4. Culture

Culture of the 14 deer blood samples for 3 months resulted in no growth even from 10 deer that were positive by nested PCR.

1.4. DISCUSSION

The unclear epidemiology of *A. phagocytophilum* in Japan is further confused by the presence of genetically different bacterial strain, different reservoir hosts and unknown pathology. It is thought that there are 2 genetically different strains of *A. phagocytophilum*. The first one was detected from human beings and ticks (Ohashi et al., 2005), but its reservoir host is still unknown, whereas AP-sd has been detected in ruminants and ticks (Kawahara et al., 2006), but not in humans.

Here, I identified two more potential reservoir hosts for AP-sd: brown bears and rodents. This suggests that AP-sd may have a broader host range than *A. phagocytophilum* in the Hokkaido ecosystem. Previous studies have indicated that *A. phagocytophilum* and AP-sd share the same vector ticks in Hokkaido (Ybañez et al., 2012b). However, none of the deer, brown bears or rodents tested in our study were infected with the *A. phagocytophilum* strains.

Our nested PCR was unable to identify *A. phagocytophilum*, *A. bovis*, and AP-sd to species in sika deer and rodents. In the case of sika deer, *A. bovis* and AP-sd were detected by using the same primer sets for both 16S rRNA and gltA genes. In contrast, AP-sd, *Candidatus* Neoehrlichia mikurensis and *Ehrlichia muris* were detected in the rodent samples by using the primer sets for the 16S rRNA gene (data not shown).

In sika deer, two *Anaplasma* spp. were detected, *A. bovis* and AP-sd, based on the 16S rRNA partial sequence analysis of this study. Despite the fact that AP-sd was considered to be a strain of *A. phagocytophilum* in Japan (Kawahara et al., 2006; Wu et al., 2015) and China (Liu et al., 2012) or a strain related to *A. phagocytophilum* in Japan (Ybañez et al., 2012a) and South Africa (Inokuma et al., 2005), our analysis revealed that AP-sd is clustered with *A. bovis* sequences selected from GenBank. Unfortunately, there is little genetic difference between *Anaplasma* spp. especially in the 16S rRNA genes (Dumler et al., 2001).

Adding to this, the lack of additional genetic information of *A. bovis*, because it has not been cultivated *in vitro*, makes identifying the taxonomic position of AP-sd difficult. To overcome this issue, I included several strains of AP-sd to our phylogenetic analysis to establish a robust position of this bacterium. Based on the 16S rRNA gene sequences, the similarity between AP-sd and the previously published *A. bovis* sequences is less than 99%. Therefore, AP-sd is likely a different species from *A. bovis*. However, it is more related to *A. bovis* than any other *Anaplasma* spp. including *A. phagocytophilum*. Studying the pathology of AP-sd is required to identify which host blood cells they are infecting to further clarify relationship with the other *Anaplasma* species.

Both *A. bovis* and AP-sd 16S rRNA gene partial sequences obtained from this study showed nucleotide polymorphisms (Tables 1-2 & 1-3). In total, 4 and 3 genetic variants were detected for *A. bovis* and AP-sd in this study, respectively. This indicates that both *A. bovis* and AP-sd are similar to *A. phagocytophilum* in having genetically diverse populations (Shukla et al., 2007).

The partial sequences of gltA gene divided into two separate clusters. One cluster was closely related to the previously published *Anaplasma* sp. sequences from South Africa and Japan. The second cluster was similar to an *Anaplasma* sp. which was previously reported as *A. bovis* found in raccoons (*Procyon lotor*) from Hokkaido, Japan (Ybañez et al., 2014). This is the first report of this *Anaplasma* sp. sequence from sika deer. This result supports the phylogenetic analysis based on the 16S rRNA gene sequences.

Sometimes AP-sd 16S rRNA gene sequences clustered with *A. bovis* (Inokuma et al., 2005; Ybañez et al., 2014); however, the gltA gene sequences were not available for *A. bovis* until it was found in raccoons (*Procyon lotor*) from Hokkaido, Japan (Ybañez et al., 2014). Afterwards, our study showed that AP-sd is placed in a different cluster from *A. bovis* gltA

gene sequences from raccoons (Ybañez et al., 2014) and sika deer of our study. This suggests that AP-sd is separate from *A. bovis*. Therefore, the bacterial morphology of AP-sd is needed to elucidate its taxonomic position and physiological needs. *A. phagocytophilum* strains are able to grow in HL-60 and ISE6 cell lines (Massung et al., 2006a; Zhan et al., 2010). However, *A. bovis*, which infects some mammalian monocytes, has not been isolated *in vitro*. The failure of cultivation of AP-sd *in vitro* using three different cell lines suggests that it might have different physiologic needs from *A. phagocytophilum*.

In this study, I used a RLB technique to specifically detect *A. phagocytophilum* and AP-sd. In order to account for *A. phagocytophilum* genetic diversity, I selected 4 different probes for its detection. Little was known about AP-sd population genetic diversity in Hokkaido, so that I could design only one probe for its detection. All brown bear and rodent nested PCR positive samples tested positive by RLB. According to sequencing results, they have an AP-sd variant with a similar attachment site to the AP-sd oligonucleotide probe used in this study. However, the total number of RLB positive sika deer samples is lower than that of the number of nested PCR positive samples. This is likely due to the detection of 3 AP-sd and 4 *A. bovis* strains by nested PCR. All *A. bovis* and one AP-sd strains have different probe attachment site sequences (Table 1-3). RLB in this study may not detect *A. bovis* and AP-sd genetic variants with nucleotide substitutions at the attachment sites to our AP-sd oligonucleotide probe. Previously, several oligonucleotide probes have been designed to detect *A. phagocytophilum* strains, the sequence difference between these probes was only one nucleotide (Table 1-1) (Matjila et al., 2008). Therefore, it is suggested that species-specific probes should be designed to detect all AP-sd and *A. bovis* strains in Hokkaido ecosystem.

Unlike nested PCR, using the RLB technique enabled us to specifically detect AP-sd. This could likely facilitate proper estimation of prevalences of *A. phagocytophilum*, *A. bovis* and

AP-sd which have very similar 16S rRNA sequences. This can be used to investigate the ecologies of these bacteria and their potential threat to humans in Japan.

Whereas Ap-sd has a broad infection range, with a number of prominent mammals in the Hokkaido ecosystem, I found no evidence of *A. phagocytophilum* infection. The most important question then remains: what is the host for *A. phagocytophilum* in Hokkaido? An equal or greater prevalence of *A. phagocytophilum* than Ap-sd has been reported in ticks (Ybañez et al., 2012b), so although all of these animals are likely exposed to both of the bacteria, they appear to be only susceptible to AP-sd.

1.5. CONCLUSION

This study provides information on the host ranges and genetic diversities of *Anaplasma* spp. in Hokkaido. Brown bears and rodents were identified to be carriers of AP-sd, although minor compared to sika deer, and this suggests a broader host range than is seen for *A. phagocytophilum*. By using a RLB technique I was able to specifically differentiate AP-sd from *A. phagocytophilum*. This high throughput method should be useful for better understanding the ecologies of these *Anaplasma* spp. in Hokkaido, where genetic material from both bacteria have been detected.

Table 1-1. List of *Anaplasma* spp. and their oligonucleotide probes used for its detection.

Target	Oligonucleotide probe	Sequence	Reference
A. phagocytophilum	*A. phagocytophilum 1*	TTGCTATAAAGAATAATTAGTGG	(Matjila et al., 2008)
	A. phagocytophilum 3	TTGCTATGAAGAATAATTAGTGG	
	A. phagocytophilum 5	TTGCTATAAAGAATAGTTAGTGG	
	A. phagocytophilum 7	TTGCTATAGAGAATAGTTAGTGG	
***Anaplasma* sp. (AP-sd)**	AP-sd	GCTACGGGGATAATTAGTGG	This study
All *Ehrlichia/Anaplasma* spp.	*Ehrlichia/Anaplasma* catch-all	GGGGGAAAGATTTATCGCTA	(Matjila et al., 2008)
	All *Ehrlichia* & *Anaplasma*	CTATAGCTGGTCTGAGAG	This study

Table 1-2. Nucleotide polymorphism in AP-sd and *A. bovis* based on their 16S rRNA gene partial sequences.

DDBJ Acc. number		Nucleotides and its position according to *A. phagocytophilum* [GenBank: CP000235]																						
		289	**290**	**293**	**294**	**295**	**296**	**297**	**298**	**362**	**443**	**450**	**464**	**465**	**514**	**630**	**651**	**747**	**752**	**756**	**814**	**823**	**909**	**916**
AP-sd	LC068730	T	A	A	G	A	T	A	A	A	A	A	G	A	T	G	C	C	C	A	T	A	A	G
	LC068735	C	G	G	G	A	T	A	A	A	A	A	G	A	T	A	T	C	C	A	T	A	A	G
	LC068736	C	G	G	G	A	T	A	A	A	A	A	G	A	T	G	C	T	T	T	G	G	T	A
A. bovis	LC068731	C	G	G	A	T	C	A	A	G	G	T	A	G	C	G	C	T	T	T	G	A	T	A
	LC068732	C	G	G	G	A	T	A	T	A	G	T	A	G	C	G	C	C	C	A	T	G	A	G
	LC068733	C	A	G	G	A	T	G	A	G	G	T	A	G	C	G	C	T	T	T	G	A	A	G
	LC068734	C	G	G	G	A	T	A	A	G	G	T	A	G	C	G	C	C	C	A	T	G	A	G

Table 1-3. Polymorphisms in AP-sd and *A. bovis* 16S rRNA gene partial sequences at AP-sd probe attachment site.

Target	**DDBJ accession number**	**Oligonucleotide probe attachment site sequence**																			
AP-sd probe sequence		**G**	**C**	**T**	**A**	**C**	**G**	**G**	**G**	**G**	**A**	**T**	**A**	**A**	**T**	**T**	**A**	**G**	**T**	**G**	**G**
AP-sd	LC068730	-	-	-	-	T	A	-	A	-	-	-	-	-	-	-	-	-	-	-	-
	LC068735	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	LC068736	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Anaplasma bovis	LC068731	-	-	-	-	-	-	-	-	A	T	C	-	-	-	-	-	-	-	-	-
	LC068732	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-
	LC068733	-	-	-	-	-	A	-	-	-	-	-	G	-	-	-	-	-	-	-	-
	LC068734	-	-	-	-	-	-	-	-	-	-	-	-	T	-	-	-	-	-	-	-

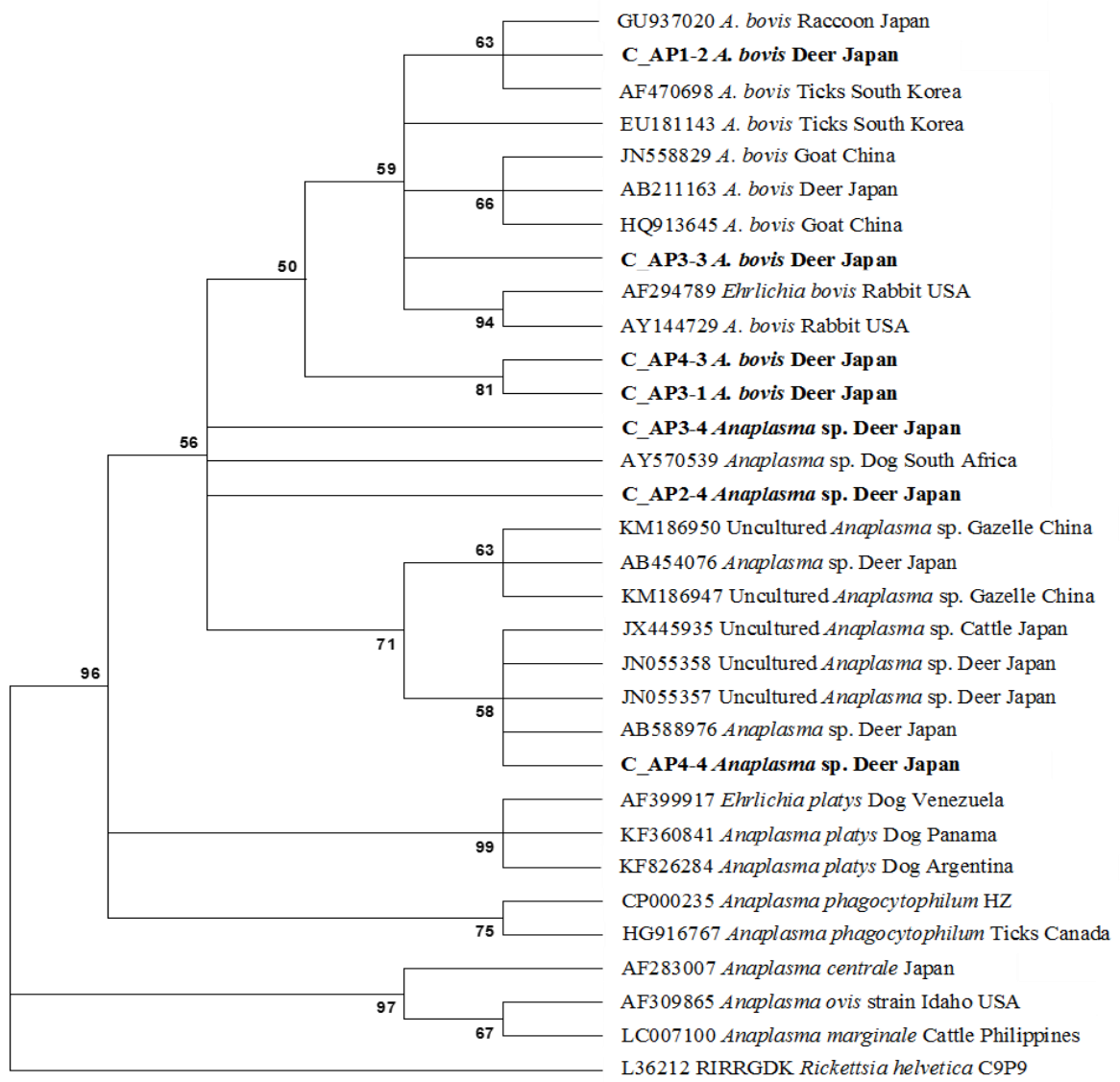

Figure 1-1. Phylogenetic relationship between 16S rRNA gene sequences of *Anaplasma* and *Ehrlichia* species. Sequences derived from the present study are represented in bold letters. This Neighbor Joining Tree was built by MEGA software version 5.1 and the Bootstrap values over 50% are shown. It shows the relationship between a 679 bp segment of the 16S rRNA gene of *Anaplasma* spp. obtained from this study and related *Anaplasma* and *Ehrlichia* spp. sequences from the GenBank®. This study DNA sequences were deposited to DDBJ as accession numbers: (LC068730, LC068731, LC068732, LC068733, LC068734, LC068735 and LC068736) for clone numbers: (C_AP2-4, C_AP1-2, C_AP4-3, C_AP3-3, C_AP3-1, C_AP4-4 and C_AP3-4), respectively.

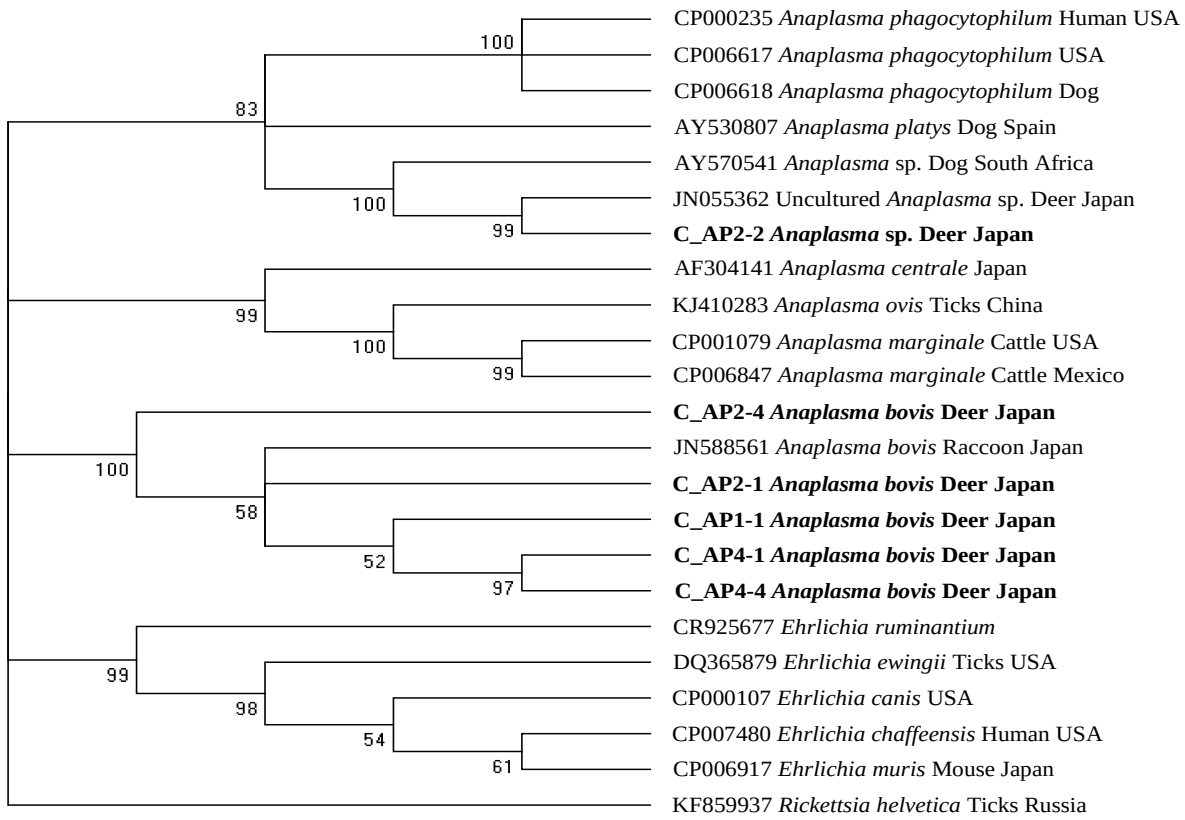

Figure 1-2. Phylogenetic relationship between gltA gene sequences of *Anaplasma* and *Ehrlichia* species. Sequences derived from the present study are represented in bold letters. This Neighbor Joining Tree was built by MEGA software version 5.1 and the Bootstrap values over 50% are shown. It shows the relationship between a 380 bp segment of the gltA gene of *Anaplasma* spp. obtained from this study and related *Anaplasma* and *Ehrlichia* spp. sequences from the GenBank®. This study DNA sequences were deposited to DDBJ as accession numbers: (LC068819, LC068820, LC068821, LC068822, LC068823 and LC068824) for clone numbers: (C_AP1-1, C_AP2-1, C_AP2-2, C_AP2-4, C_AP4-1 and C_AP4-4), respectively.

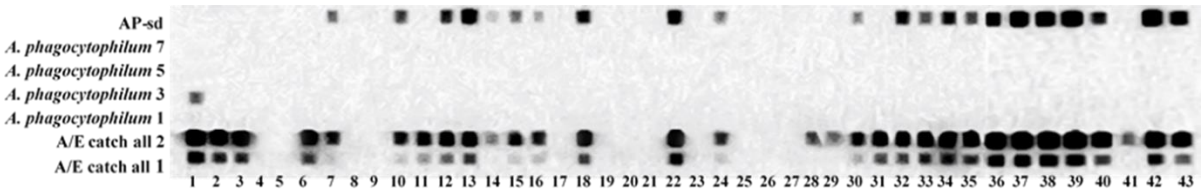

Figure 1-3. A representative RLB. 1: *A. phagocytophilum* positive control (Ameland, Netherland); 2: *Ehrlichia chaffeensis* positive control; 3: *Ehrlichia ruminantium* positive control 4: negative control; 5-43: Sika deer blood derived 16S rRNA partial sequences.

2. Chapter 2

Molecular epidemiology of *Candidatus* Neoehrlichia mikurensis, *Ehrlichia muris*, *Anaplasma* species AP-sd and *Babesia microti* in Hokkaido small mammals

2.1. INTRODUCTION

Tick-borne pathogens are maintained in lifecycles that include ticks and animals. Occasionally, these pathogens can be transmitted to humans (de la Fuente et al., 2008). In Japan, several tick-borne pathogens including members of *Anaplasma*, *Ehrlichia* and *Babesia* species have been detected (Kawahara et al., 1999; Kawahara et al., 2004; Kawahara et al., 1993; Zamoto-Niikura et al., 2012). These obligate intracellular pathogens are capable of infecting both vertebrates and invertebrates (Naitou et al., 2006). Many members of these species are known to be zoonotic and have been detected in humans. For example, human granulocytic anaplasmosis, human monocytic ehrlichiosis and human babesiosis are caused by the zoonotic tick-borne pathogens *A. phagocytophilum*, *E. chaffeensis* and *B. microti*, respectively.

Many of the emerging and re-emerging infectious diseases are zoonotic and have their origin in wildlife, which plays an important role in their maintenance in the ecosystem (Parola and Raoult, 2001). Despite considerable research on tick-borne diseases, there is relatively little information about the ecology of these pathogens in Japan. The scarcity of information may be in part due to the lack of molecular techniques with high specificity and throughput to detect these pathogens. Using molecular assays in the identification of these pathogens show higher specificity and sensitivity than microscopical or serological examinations (Criado-Fornelio, 2007). Reverse line blot (RLB) is a practical epidemiological tool that can identify those pathogens (Oura et al., 2004) through examination of a large number of samples for many parasitic microbes simultaneously (Kong and Gilbert, 2006).

In this study we identified tick-borne pathogens of public health concern in Hokkaido, Japan by using multiplex PCR and RLB. The detected pathogens were molecularly characterized and phylogenetically analysed.

2.2. MATERIALS AND METHODS

2.2.1. Small mammal blood samples and DNA extraction

During May through September 2010 to 2012, blood samples were collected from the heart in Eppendorf tubes from 459 small mammals including 219 (*Apodemus speciosus*), 86 (*Apodemus argenteus*), 85 (*Myodes rufocanus*), 51 (*Myodes rutilus*), 11 (*Myodes rex*) and 7 (*Sorex unguiculatus*) from Furano and Shari sites in Hokkaido, Japan. Species identification was performed as previously described by Abe (1994) and Ohdachi et al. (2009). These small mammals were classified by age into adult and sub-adult groups as previously described (Taylor et al., 2013). The sampling methods were approved by the Animal Care and Use Committee of Hokkaido University (Approval No. JU1105). DNA was extracted from the blood samples using the Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA). The obtained DNA samples were kept at -20°C until analysis.

2.2.2. Multiplex PCR and reverse line blot (RLB) hybridization

Blood DNA samples from a total of 459 small mammals were examined by multiplex PCR and RLB. The primers Ehr-F (5′-GGA ATT CAG AGT TGG ATC MTG GYT CAG-3′) (Schouls et al., 1999), Ehr-R (5′-biotin-CGG GAT CCC GAG TTT GCC GGG ACT TYT TCT-3′) (Bekker et al., 2002), 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’) (Gubbels et al., 1999; Matjila et al., 2004) were used to amplify a fragment of 460–520 bp from the 16S rRNA gene of *Anaplasma* and *Ehrlichia* and 460–540 bp from the 18S rRNA gene of *Babesia* species. Multiplex PCR was performed using the PCR System 9700 (Applied Biosystems, Foster City, CA, USA) and QIAGEN Multiplex PCR kits (Qiagen, Hilden, Germany). PCR reactions consisted of 25 µl of 2x QIAGEN Multiplex PCR Master Mix, 1.25 µl of each primer (10 pmol), 2 µl DNA and 18 µl RNase-free water. DNAs from cultured *A.*

phagocytophilum (Ameland Netherland strain), *Ehrlichia chaffeensis*, *E. ruminantium*, previously sequenced *Anaplasma* sp. Ap-sd, *Candidatus* Neoehrlichia mikurensis and *Babesia microti* were used as positive controls and double-distilled water was used as a negative control. The amplification conditions included an initial activation step of 15 min at 94°C followed by a touch down step of 12 cycles of 30 sec at 94°C, 90 sec at 69°C and 90 sec at 72°C with the annealing temperature decreasing every cycle by 2°C. This was followed by 40 cycles of 30 sec at 94°C, 90 sec at 57°C and 90 sec at 72°C and a final extension step of 10 min at 72°C.

PCR products were examined by electrophoresis through 1% agarose gel stained with ethidium bromide and visualized by an UV illuminator.

RLB was performed on the PCR products as previously described (Kong and Gilbert, 2006) with modifications. Briefly, a 15 × 15 cm Biodyne C membrane (Pall Life Sciences, Ann Arbor, MI, USA) was activated by 20 ml of 16% 1-ethy-3-(3-dimethyl-amino-propyl) carbodiimide (EDAC, Sigma Aldrich, St. Louis, MO, USA) for 10 min at room temperature. Subsequently, the membrane was washed gently with milliQ water for 2 min and placed in Miniblotter MN45 (Immunetics, Boston, MA, USA). Oligonucleotide probes with C6 amino linker (Table 2-1) were obtained from Sigma Aldrich Co., LLC, Japan. Five microliters of each 100 pmol/ µl probes were diluted in 0.5 M $NaHCO_3$ to a final volume of 170 µl. The slots of the miniblotter were filled with 150 µl of each diluted oligonucleotide and the membrane was incubated at room temperature for 5 min. The membrane was inactivated in 250 ml of 0.1 M NaOH with gentle shaking for 8 min at room temperature, then washed with prewarmed 250 ml of 2× SSPE/0.1% SDS for 5 min at 60°C. Then the membrane was directly used or it was stored in a sealed plastic bag with 15 ml of 20 mM EDTA, pH 8.0.

A volume of 10 µl of each PCR product was diluted in 2× SSPE/0.1% SDS to a final volume of 170 µl, heat-denatured in boiling water for 10 min and immediately cooled on ice. The PCR products were introduced into the miniblotter, which contained the prepared membrane, and hybridized at 60°C for 1 hr. The membrane was washed twice in 250 ml of 2× SSPE/0.5% SDS for 10 min at 60°C and then incubated with diluted peroxidase labeled NeutrAvidin (Thermo Fisher Scientific, Walyham, MA, USA) for 45 min at 42°C. The membrane was washed twice with 2× SSPE/0.5% SDS for 10 min at 42°C. Finally, the membrane was incubated with 15 ml ImmobilonTM Western Chemiluminescent HRP Substrate (Milipore, Japan) for 5 min at room temperature, and the result was detected by a chemiluminescent imager (Billerica, MA, USA). To strip hybridized PCR products from the membrane for reuse, it was washed twice in prewarmed 1% SDS at 90°C for 30 min and washed with 250 ml of 20 mM EDTA. The membrane was stored in a plastic bag with 15 ml of 20 mM EDTA at 4°C until reuse.

2.2.3. Cloning and sequencing of 16S rRNA and 18S rRNA genes

The result of the RLB was confirmed through cloning of randomly selected 6 and 12 positive PCR products for *Anaplasma*/*Ehrlichia* and *Babesia* spp., respectively. I performed PCR using the KOD-Plus-Neo high fidelity DNA polymerase kit (Toyobo Co. Ltd., Osaka, Japan). The same primers above were used to amplify a segment of *Anaplasma*/*Ehrlichia* spp. 16S rRNA and *Babesia* spp. 18S rRNA. Each PCR reaction consisted of 2.5 µl of 10× KOD-Plus-Neo buffer, 2.5 µl of dNTPs (2 mM), 1.5 µl of 25 mM $MgSO_4$, 0.75 µl of each primer (10 pmol), 1 µl of DNA (100 ng/ µl) and 0.5 µl (1 U/ µl) of KOD-Plus-Neo DNA polymerase. The reaction conditions were 94°C for 2 min and 40 cycles of 98°C for 10 sec, 57°C for 30 sec and 68°C for 30 sec, followed by a final extension at 68°C for 7 min.

Nine microliters of each PCR product were incubated for 10 min at 60°C with 1 µl of 10× A-attachment mix (Toyobo Co. Ltd., Life Science Department, Osaka, Japan). The A-attached PCR products were cloned into pGEM-T Easy Vector (Promega, Madison, WI, USA) as described in the manufacturer's quick protocol manual. Five colonies per sample were selected and screened by PCR for recombinants. Five microliters of each PCR product were purified by incubation with 4 µl Exosap mix (USB Co., Cleveland, OH, USA) for 30 min at 37°C followed by 15 min at 80°C. The purified PCR products were subjected to sequencing reactions and cleaned by Agencourt CleanSEQ (Agencourt Bioscience Co., Beckman Coulter, Beverly, MA, USA) according to the manufacturer's instructions. The cleaned products were sequenced by ABI Prism 3130 × genetic analyzer according to the manufacturers' instructions.

2.2.4. Sequence analysis

The obtained sequences from the *Anaplasma*, *Ehrlichia* and *Babesia* spp. PCR products were compared with previously published 16S rRNA and 18S rRNA gene sequences of each. The stability of phylogenies was assessed by 1,000 bootstrap replications. Alignment and phylogenetic analyses were performed by MEGA software version 5.1 (Tamura et al., 2011) using ClustalW and Neighbor Joining Tree, respectively.

2.2.5. Statistical analysis

We used Generalized Linear Models (GLMs) with a binomial error distribution to assess differences in pathogen infections detected by RLB from the two sampling sites of Shari and Furano. The binary response variable was the presence or absence of pathogen DNA in small mammal blood. While, small mammal species, gender, age, year, month of sampling and the presence of other infections were selected as fixed explanatory variables. Starting from the full model including all explanatory variables, I carried out a model selection test based on

Akaike information criterion (AIC). Variance inflation factors and conditional boxplot were applied to assess collinearity. Statistical analyses were performed using the R statistical system (R v3.1.1; RStudio v0.98.1079).

2.3. RESULTS

2.3.1. Descriptive analysis of the prevalence in small mammal blood DNA samples

A total of 459 blood DNA samples was examined by multiplex PCR and RLB for the presence of zoonotic tick-borne pathogens (Table 2-1). The RLB results (Fig. 2-1) revealed that 19 (4.1%), 5 (1.1%), 56 (12.2%) and 95 (20.7%) of the small mammals' blood DNA samples were positive for *Anaplasma* sp. AP-sd, *E. muris*, *C.* N. mikurensis and *B. microti*, respectively (Table 2-2). *B. microti* showed higher infection rates in 2012 than observed in other years. The prevalence of all detected pathogens was higher in adults than in sub-adults. In addition, males appeared to have higher infection rates than females. There were no differences in the infection rates of all detected pathogens between collection sites. *Apodemus* spp. exhibited relatively low prevalence of *B. microti*, however, *C.* N. mikurensis was more prevalent among *A. speciosus* than any other small mammal species (Fig. 2-2).

2.3.2. The epidemiology of the detected tick-borne pathogens in the small mammals blood DNA samples

A GLM model with binomial error for the probability of infection was fitted to my data. Based on Akaike information criterion (AIC), I selected a minimal model for *B. microti*, *C.* N. mikurensis and *Anaplasma* sp. AP-sd. For *E. muris*, no model was used to analyse its prevalence due to too few positive samples (Table 2-1).

In the case of *B. microti*, the minimal model included year, small mammal species and the presence of *C.* N. mikurensis as explanatory variables. The model showed that the prevalence of *B. microti* was significantly higher in 2012 than in the other years *($P < 0.001$)*. Its infection rates were also significantly higher in *M. rufocanus ($P < 0.01$)* and *M. rex ($P < 0.05$)* than in other small mammal species. Interestingly, *B. microti* infection rates were

significantly higher in small mammals also infected with *C.* N. mikurensis *(P < 0.001)* (Tables 2-3 & 2-4, Fig. 2-3).

The selected minimal model for *C.* N. mikurensis included small mammal species, gender, age and mixed infection with *B. microti* as explanatory variables. Prevalence was significantly higher in *A. speciosus (P < 0.001)* and *S. unguiculatus (P < 0.05)* than in other small mammal species. In addition, *C.* N. mikurensis prevalence was significantly higher in males than in females *(P < 0.01).* The statistical model showed significantly lower infection rates in sub-adults than in adult small mammals *(P < 0.001)* but significantly higher in small mammals co-infected with *B. microti (P < 0.05)* (Tables 2-5 & 2-6, Fig. 2-4).

For *Anaplasma* sp. AP-sd, the selected minimal model included year, month and animal species as explanatory variables. Its infection rates were significantly lower in *A. speciosus* than in other small mammal species *(P < 0.05)* (Tables 2-7 & 2-8, Fig. 2-5).

2.3.3. Molecular characterization

Partial gene sequences of the obtained 16S rRNA were mainly divided into one *Anaplasma* sp. and two *Ehrlichia* species. Our sequence (EH2-2) was 100% similar to *C.* N. mikurensis (JQ359045) from a human case in China, and our sequence (EH5-1) was 99% similar to *E. muris* (NR_121714) from wild mouse in Japan. In addition, the obtained sequence (EH8-1) was 100% and 99% similar to *Anaplasma* sp. AP-sd (LC068736) from sika deer in Japan and *Anaplasma* sp. (AY570539) from South African dog, respectively (Fig. 2-6).

The obtained 18S rRNA gene partial sequences were all clustered with *Babesia microti*. One strain sequence (BabC4-1) was 100% similar to *Babesia microti* (AB190435) from rodent in Hokkaido, Japan. The other strain sequence (BabC5-1) was 100% similar to *Babesia microti* (AB243679) from rodents Hyogo, Japan (Fig. 2-7).

2.3.4. Co-infection

A total of 24, two triple and 22 double, co-infection cases were detected in this study (5.2%). The most frequent co-infection cases occurred between *C.* N. mikurensis and *B. microti* (66.7%). Co-infection between *Anaplasma* sp. AP-sd and *B. microti* was also present (12.5%), while the triple coexistence of *Anaplasma* sp. AP-sd, *C.* N. mikurensis and *B. microti* was 8.3% of the total number of co-infection cases. Each co-infection of *E. muris* + *C.* N. mikurensis, *E. muris* + *B. microti* and *Anaplasma* sp. AP-sd + *C.* N. mikurensis was observed in 4.2% of the total co-infection numbers (Table 2-9).

Most of co-infections occurred in *A. speciosus* (66.7%) and a total of 19/24 (79.1%) co-infection cases were detected in males. In addition, eighteen (75%) co-infections occurred in August and September and 16/24 (66.7%) observed in sub-adults.

2.4. DISCUSSION

In order to identify tick-borne pathogens with public health threat in small mammal reservoir hosts of Hokkaido, I examined 6 small mammal species for the infection with 13 different zoonotic *Anaplasma*, *Ehrlichia* and *Babesia* species by multiplex PCR and RLB. The present study detected 4 zoonotic tick-borne pathogens; *Anaplasma* sp. AP-sd, *E. muris*, *C.* N. mikurensis and *B. microti*. Despite the fact that *Anaplasma* sp. AP-sd was not previously detected in humans, I included this in the category of zoonotic pathogens in this study because it has broad host range (Moustafa et al., 2015) and molecularly close to *A. phagocytophilum* (Ybañez et al., 2012a) which makes it a potential threat to human beings.

Babesia microti and *C.* N. mikurensis infection rates in small mammals were obviously predominant with a prevalence of 20.7% and 12.2%, respectively. The prevalence of *B. microti* detected in this study is higher than the previously published one from the northern parts of Japan (Zamoto et al., 2004). However, *C.* N. mikurensis infection rates were similar to those detected from wild rodents in Shimane prefecture, Japan (Tabara et al., 2007).

The prevalence of *Anaplasma* sp. AP-sd and *E. muris* was lower than 5% and they were not detected in all examined animal species. The absence of both *Anaplasma* sp. AP-sd and *E. muris* in *M. rex* and *S. unguiculatus* is likely due to the relatively small sample size (n = 11 and n = 7, respectively) in addition to their low prevalence.

In comparison to its prevalence in sika deer (*Cervus nippon yesoensis*), *Anaplasma* sp. AP-sd infection rates in small mammals of this study were low but similar to the previously published data from Hokkaido rodents (Moustafa et al., 2015).

Previously, *Ehrlichia muris* DNA fragments were detected in Japanese rodents (Kawahara et al., 1999; Muramatsu et al., 2005). The present study results showed a relatively low prevalence of this pathogen in Hokkaido small mammals but higher than its prevalence in

Hokkaido sika deer populations (Tamamoto et al., 2007). This suggests that small mammals are likely to be the main reservoir hosts for *E. muris* in Hokkaido ecosystem.

The RLB results were confirmed through sequencing of 5 clones from each randomly selected 6 and 12 samples for *Anaplasma*/*Ehrlichia* and *Babesia* spp., respectively. The phylogenetic analysis of the 16S rRNA sequences revealed the existence of *C.* N. mikurensis, *Anaplasma* sp. AP-sd and *E. muris* genetic materials. Interestingly, the detected strain of *C.* N. mikuresis is identical to that from 7 human cases in China (Li et al., 2012) and was previously detected in rodents of Nagano, Japan (Naitou et al., 2006). Only one genetic variant of *Anaplasma* sp. AP-sd and *E. muris* were detected in our study. Previously, these variants were reported from Japanese wildlife but not from humans (Moustafa et al., 2015; Thirumalapura et al., 2014).

The 18S rRNA gene partial sequences divided into two *B. microti* genetic variants. One strain is identical to the US strain of *B. microti* that was previously reported from Hokkaido and the other is identical to the Ot4 strain which was previously detected in Hyogo, Japan (Saito-Ito et al., 2007).

Out of the explanatory variables that were included in this study, the small mammal species variation was significantly correlated with the infection rates of *Anaplasma* sp. AP-sd, *C.* N. mikurensis and *B. microti*. The explanation of this host-pathogen relationship was different for each pathogen. Although it is difficult to evaluate this interaction, the results of this study suggest that the role played by each small mammal species in maintaining TBPs is specific. Although *B. microti* was previously reported to have higher infection rates in *A. speciosus* (Saito-Ito et al., 2007; Wei et al., 2001), the results of this study suggest that the prevalence of this pathogen is significantly higher in *M. rufocanus* and *M. rex* than in *A. speciosus*. However, there are many genetically different strains of *B. microti* in Japan and

each strain may have different reservoir host preferences (Saito-Ito et al., 2007). Unfortunately, 2012 is a special year in that it was the only year that *A. speciosus*, which was most common during the other years, was exceptionally rare. However, excluding this year from my statistical analysis did not affect the significantly lower prevalence of *B. microti* in *A. speciosus.*

The significant higher prevalence of *B. microti* in 2012 ($P < 0.001$) is probably because in this year sampling was only performed in May, in which most of the samples collected were adults because they have made it through the winter and are just starting to breed. Whereas, *B. microti* is known to cause persistent infection in rodents (Bown et al., 2008) , which would lead to increase infection rates in adults due to the concentration effect.

For *C.* N mikurensis and *Anaplasma* sp. AP-sd, there are no enough published data on their distribution in the small mammals of Japan. Here, this study showed that *C.* N mikurensis has significantly higher tropism to *A. speciosus*, While, *Anaplasma* sp. AP-sd prevalence was significantly higher in *A. argenteus* than in any other small mammal species of this study.

Babesia microti, *C.* N mikurensis and *Anaplasma* sp. AP-sd have broad range of host tropism and can infect many mammalian species. However, many vertebrates are considered insufficient reservoirs for each pathogen. The reservoir host competence differs according to the age, gender, life history traits, the population density and body mass of this host (Ostfeld et al., 2014). For example, it was reported that reservoir host competence for *B. microti* is more related to the smaller body mass and that for *A. phagocytophilum* is more dependent on higher population density (Ostfeld et al., 2014).

Our data description showed higher prevalence of all pathogens in males more than in females. This was more apparent in case of *C.* N mikurensis infection rates in this study

samples. The higher susceptibility of males to specific microbes than females was observed in many parasites including *Plasmodium berghei* (Kamis and Ibrahim, 1989), *P. chabaudi* (Cernetich et al., 2006; Zhang et al., 2000), *P. falciparum* (Landgraf et al., 1994), *Leishmania major, Leishmania mexicana* (Alexander, 1988), *Trypanosoma cruzi* (do Prado junior et al., 1998). Previously, testosterone appeared to increase the susceptibility of male animals to the infection by some pathogens such as *B. microti* because, sometimes, it represses the immune functions (Sasaki et al., 2013). Another possible reason is that males have larger body mass than females, which make them preferable targets for ticks (Arneberg, 2002; Moore and Wilson, 2002). In this study, males are more susceptible to the infection with *C.* N. mikurensis, *Anaplasma* sp. AP-sd, *E. muris* and *B. microti.* This information is important because when more males become infected, their role in the transmission of these pathogens will be greater.

Interestingly, a significant positive correlation between *B. microti* and *C.* N. mikurensis infections was observed in this study, since *B. microti* infection rates are significantly higher in those animals infected with *C.* N. mikurensis *($P < 0.001$).* This could be due to sharing the same host competence requirements. Moreover, both *B. microti* and *C.* N. mikurensis are transmitted mainly by *Ixodes ovatus* and *I. persulcatus* ticks (Pan et al., 2003; Saito-Ito et al., 2004; Shpynov et al., 2006), which may explain the high number of co-infection cases with these two pathogens in this study. Further study is needed to understand whether this correlation has an immunological and etiological advantage.

2.5. CONCLUSION

In conclusion, my study elucidates the highest prevalent potentially zoonotic TBPs in the most common small mammals in Hokkaido, Japan. *Babesia microti* and *C.* N. mikurensis were widely distributed and their prevalence was linked to variations in animal species, gender and age. Interestingly, an ecological interaction between these two pathogens was observed, although this interaction needs to be further examined to understand its mechanisms. The reservoir host preferences appeared to be different for each TBP, this information could be helpful to understand the ecologies of these pathogens in relation to the distribution of small mammals in Hokkaido ecosystem. The existence of these zoonotic TBPs in Hokkaido is irrelevant to the rare report of symptomatic human cases. Therefore, human investigations for TBPs in Hokkaido are recommended to understand their epidemiology.

Table 2-1. List of *Anaplasma*, *Ehrlichia* and *Babesia* spp. and their oligonucleotide probes used for its detection

Target	Oligonucleotide probe	Sequence	Reference
All *Ehrlichia Anaplasma* spp.	*Anaplasma/Ehrlichia* all	CTATAGCTGGTCTGAGAG	(Moustafa et al., 2015)
All *Babesia & Theileria* spp.	*Babesia/Theileria* all	TAATGGTTAATAGGARCRGTWG	This study
All *Babesia* spp.	*Babesia* all 1	ATTAGAGTGTTTCAAGCAGAC	(Matjila et al., 2008)
	Babesia all 2	ACTAGAGTGTTTCAAACAGGC	(Matjila et al., 2008)
A. phagocytophilum	*Anaplasma 1*	TTGCTATAAAGAATAATTAGTGG	(Matjila et al., 2008)
	Anaplasma 3	TTGCTATGAAGAATAATTAGTGG	
	Anaplasma 5	TTGCTATAAAGAATAGTTAGTGG	
	Anaplasma 7	TTGCTATAGAGAATAGTTAGTGG	
Anaplasma sp. (AP-sd)	AP-sd	GCTACGGGGATAATTAGTGG	(Moustafa et al., 2015)
Ehrlichia chaffeensis	*E. chaffeensis*	ACCTTTTGGTTATAAATAATTGTT	(Matjila et al., 2008)
Ehrlichia ruminantium	*E. ruminantium*	AGTATCTGTTAGTGGCAG	(Matjila et al., 2008)
Ehrlichia muris	*E. muris C*	GCTATAGGTTCGCTATTAG	(Alekseev et al., 2001)
	E. muris T	AGCTATAGGTTTGCTATTAGT	(Alekseev et al., 2001)
Ehrlichia ewingii	*E. ewingii*	TTCCTAAATAGTCTCTGACTATTT	This study
Ehrlichia canis & E. ovina	*E. canis-ovi*	TCTGGCTATAGGAAATTGTTA	(Schouls et al., 1999)
Candidatus Neoehrlichia mikurensis	*C.*N. mik	GTTTACTATAGTTRCAGTTAGTGGC	This study
Babesia microti	*B. microti*	GRCTTGGCATCWTCTGGA	(Matjila et al., 2008)
Babesia divergens & B. capreoli	*B. divergens*	GGTGTTAATATTGACTRATGTCGAG	This study
Babesia venatorum	*B. venatorum*	GAGTTATTGACTCTTGTCTTTAA	(Gigandet et al., 2011)
Babesia rodhaini	*B. rodhaini*	CTTTGTGGATTAGTGCGC	This study
Babesia duncani	*B. duncani*	AGTTGAACTTCTGCCGCTT	This study

Table 2-2. Prevalence of *Anaplasma* sp. AP-sd, *E. muris*, *C.* N. mikurensis and *B. microti* in blood samples among small mammal species

Animal species	No. tested	*Anaplasma* sp. AP-sd		*E. muris*		*C.* N. mikurensis		*B. microti*	
A. argenteus	86	6	(7.0%)	1	(1.2%)	4	(4.7%)	13	(15.1%)
A. speciosus	219	7	(3.2%)	2	(0.9%)	40	(18.3%)	30	(13.7%)
M. rex	11	0	(0%)	0	(0%)	1	(9.1%)	6	(54.6%)
M. rufocanus	85	3	(3.5%)	1	(1.2%)	6	(7.1%)	28	(32.9%)
M. rutilus	51	3	(5.9%)	1	(2.0%)	2	(3.9%)	11	(21.6%)
S. unguiculatus	7	0	(0%)	0	(0%)	3	(42.8%)	7	(100%)
Total	459	19	(4.1%)	5	(1.1%)	56	(12.2%)	95	(20.7%)

Table 2-3. Analysis of deviance for *B. microti* in blood samples among small mammal species.

Explanatory variables	Df	Deviance	Residual Df	Residual Deviance	Pr(>Chi)
Year	2	18.855	456	441.07	8.05e-05 ***
Species	5	20.148	451	420.92	0.001172 **
***C.* N. mikurensis**	1	10.498	450	410.42	0.001195 **

Significance Codes: '*' 0.001 '**' 0.01 '*' 0.05**

Table 2-4. Model coefficients for *B. microti* in blood samples among small mammal species.

Explanatory variables	Estimate	Std. Error	z value	Pr(>|z|)
Year2011	0.4018	0.3554	1.131	0.258151
Year2012	1.7556	0.4885	3.594	0.000326 ***
Species *A. speciosus*	0.1439	0.4166	0.345	0.729825
Species *M. rex*	1.7027	0.7265	2.344	0.019101 *
Species *M. rufocanus*	1.3117	0.4183	3.135	0.001716 **
Species *M. rutilus*	0.5293	0.4776	1.108	0.267756
Species *S. unguiculatus*	1.5648	0.8623	1.815	0.069562 ¤
C. N. mikurensis	1.1763	0.3517	3.344	0.000825 ***

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

Table 2-5. Analysis of deviance for *C.* N. mikurensis in blood samples among small mammal species.

Explanatory variables	Df	Deviance	Resid. Df	Resid. Dev	Pr(>Chi)
Species	5	20.614	453	315.90	0.000958 ***
Gender	1	9.8651	452	306.04	0.001684 **
Age	1	11.898	451	294.14	0.000562 ***
B.microti	1	5.8504	450	288.29	0.015574 *

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

Table 2-6. Model coefficients for *C.* N. mikurensis in blood samples among small mammal species

Explanatory variables	Estimate	Std. Error	z value	Pr(>|z|)
Species *A. speciosus*	2.0396	0.5738	3.554	0.000379 ***
Species *M. rex*	0.5293	1.2215	0.433	0.664767
Species *M. rufocanus*	0.6031	0.6880	0.877	0.380701
Species *M. rutilus*	-0.409	0.8983	-0.455	0.649219
Species *S. unguiculatus*	2.2024	1.0238	2.151	0.031460 *
Gender Male	0.9345	0.3386	2.760	0.005785 **
Age sub-adult	-1.098	0.3321	-3.31	0.000940 ***
B.microti	0.8787	0.3549	2.476	0.013299 *

Significance codes: 0 ‘*’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘¤’ 0.1 ‘ ’ 1**

Table 2-7. Analysis of deviance for *Anaplasma* sp. AP-sd in blood samples among small mammal species.

Explanatory variables	Df	Deviance	Resid. Df	Resid. Dev	Pr(>Chi)
Year	2	5.0284	438	151.64	0.08093 ¤
Month	4	8.6753	434	142.96	0.06975 ¤
Species	3	5.8745	431	137.09	0.11788

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

Table 2-8. Model coefficients for *Anaplasma* sp. AP-sd in blood samples among small mammal species.

Explanatory variables	Estimate	Std. Error	z value	Pr(>|z|)
Year2011	1.95902	1.07139	1.828	0.06748 ¤
Year2012	0.63127	1.79690	0.351	0.72536
Month July	-2.60498	1.09339	-2.382	0.01720 *
Month June	-0.97696	0.65638	-1.488	0.13664
Month May	-1.08552	1.13092	-0.960	0.33713
Month September	-1.30994	0.80593	-1.625	0.10408
Species *A. speciosus*	-1.41416	0.64702	-2.186	0.02884 *
Species *M. rufocanus*	-1.15773	0.76163	-1.520	0.12849
Species *M. rutilus*	-0.06972	0.77413	-0.090	0.92824

Significance codes: 0 ‘*’ 0.001 ‘**’ 0.01 ‘*’ 0.05 ‘¤’ 0.1 ‘ ’ 1**

Table 2-9. Summary of the detected co-infections

Pathogen	*E. muris*	*C. N. mikurensis*	*Anaplasma sp. AP-sd*	*B. microti*
E. muris		1	0	1
C. N. mikurensis	1		1 2	16
Anaplasma sp. AP-sd	0	1 2		3
B. microti	1	16	3	

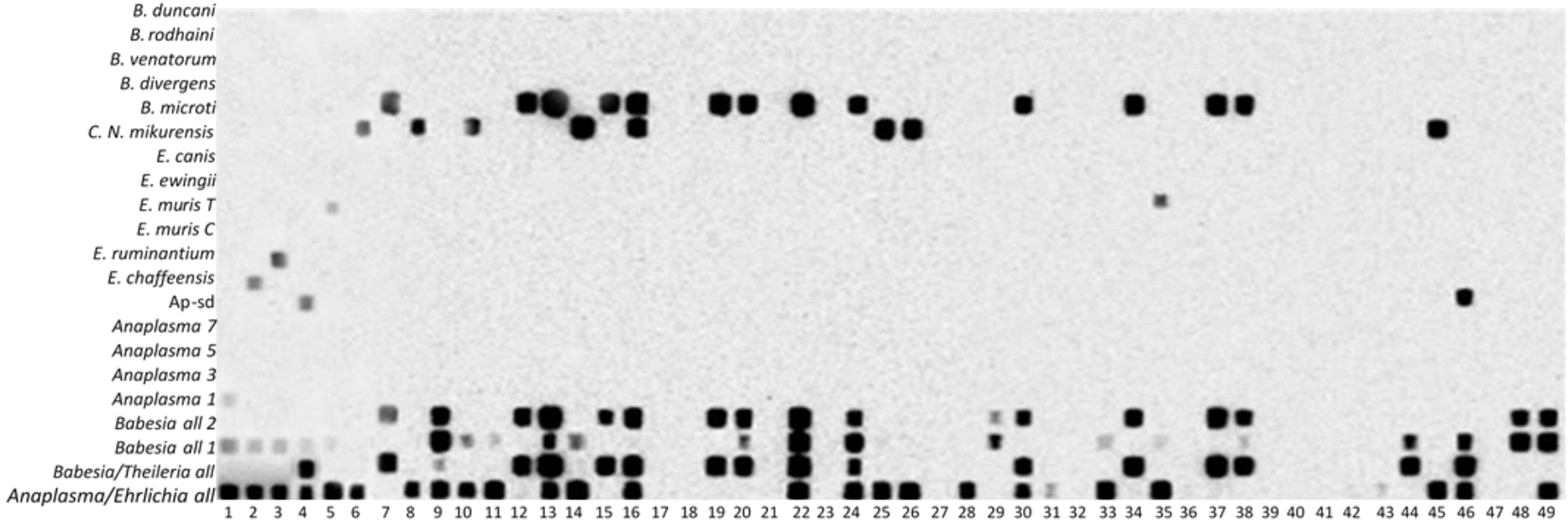

Figure 2-1. A representative RLB. 1: *A. phagocytophilum* positive control; 2: *Ehrlichia chaffeensis* positive control; 3: *Ehrlichia ruminantium* positive control 4: *Anaplasma* sp. AP-sd positive control; 5: *Ehrlichia muris* positive control; 6: *Candidatus* Neoehrlichia mikurensis positive control; 7: *Babesia microti* positive control; 8-49: small mammals' blood derived 16S rRNA and 18S rRNA gene amplicons.

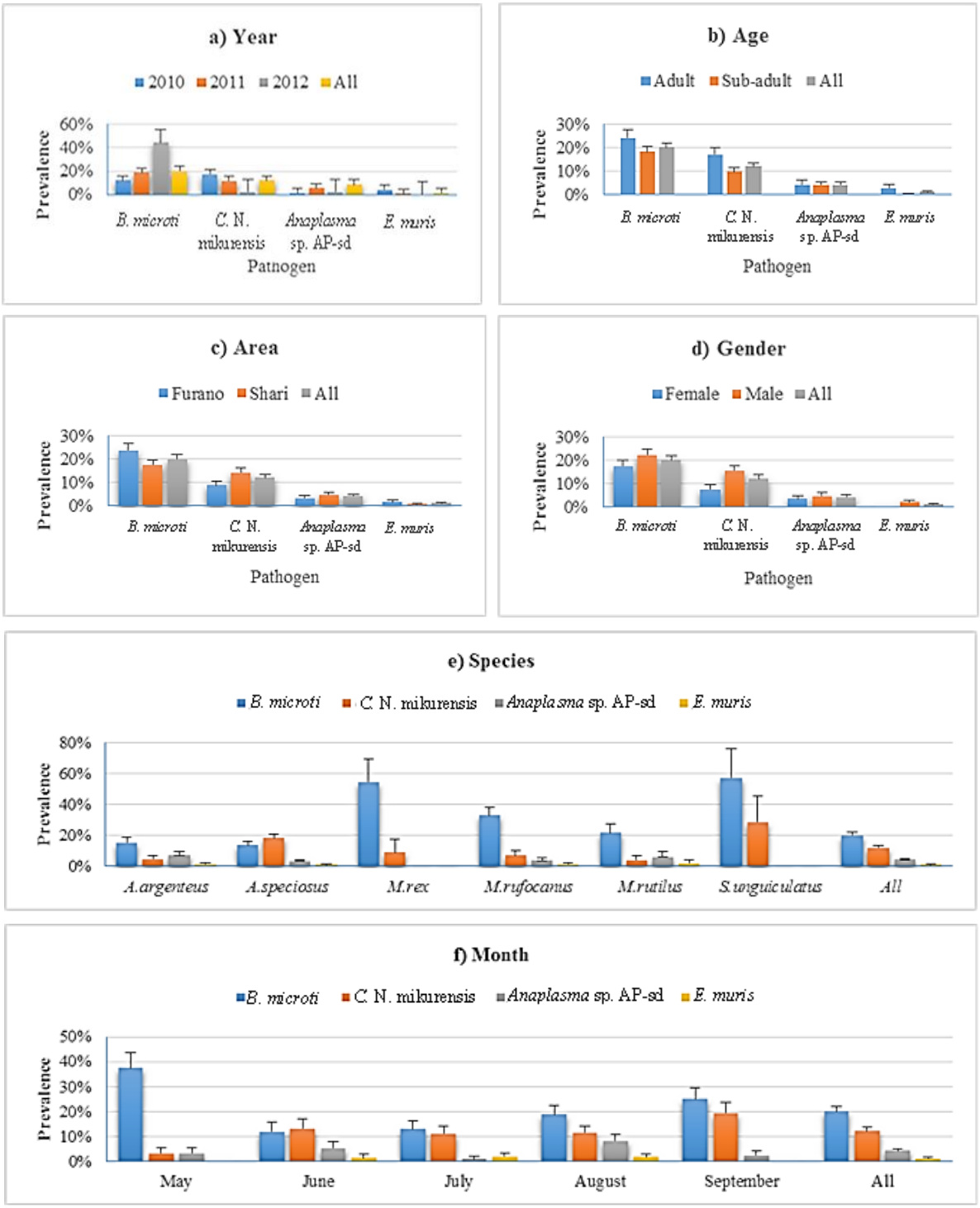

Figure 2-2. Descriptive analysis of the prevalence of *B. microti*, *C.* N. mikurensis, *Anaplasma* sp. AP-sd and *E. muris* in small mammals according to different year, age, area, gender, species and month.

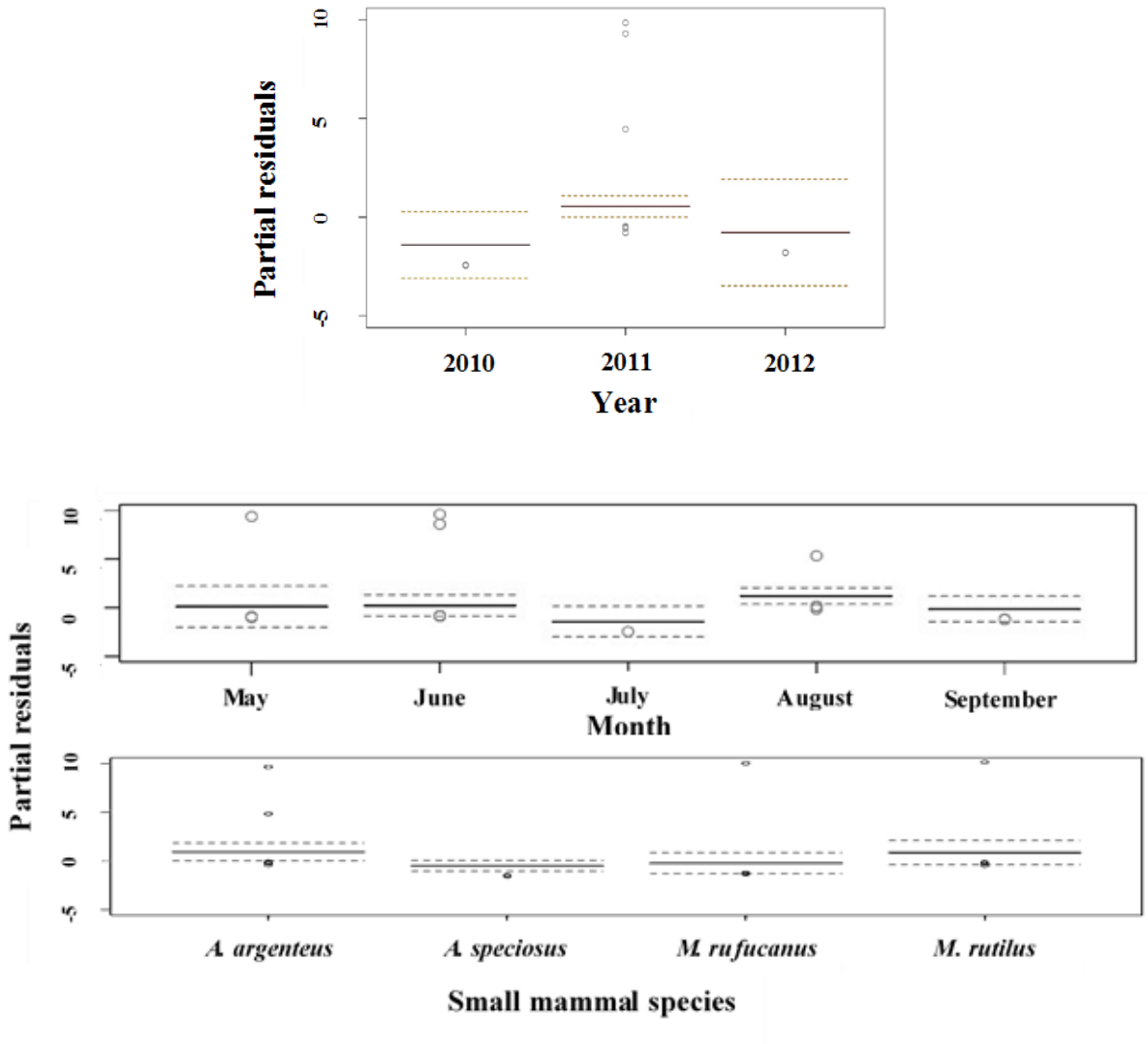

Figure 2-3. The relationship between *Anaplasma* sp. AP-sd response with year, month and small mammal species variation based on the minimal GLM model coefficients.

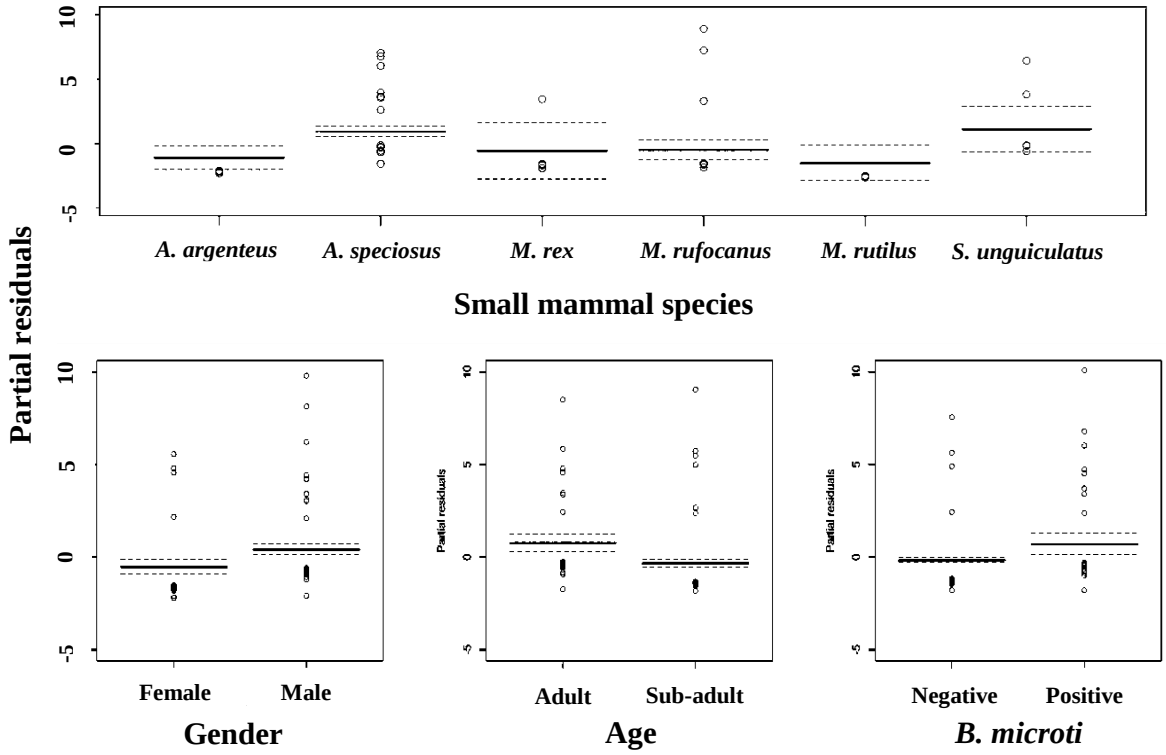

Figure 2-4. The relationship between *C.* N. mikurensis response with animal species, gender, age and *B. microti* infection variation based on the minimal GLM model coefficients.

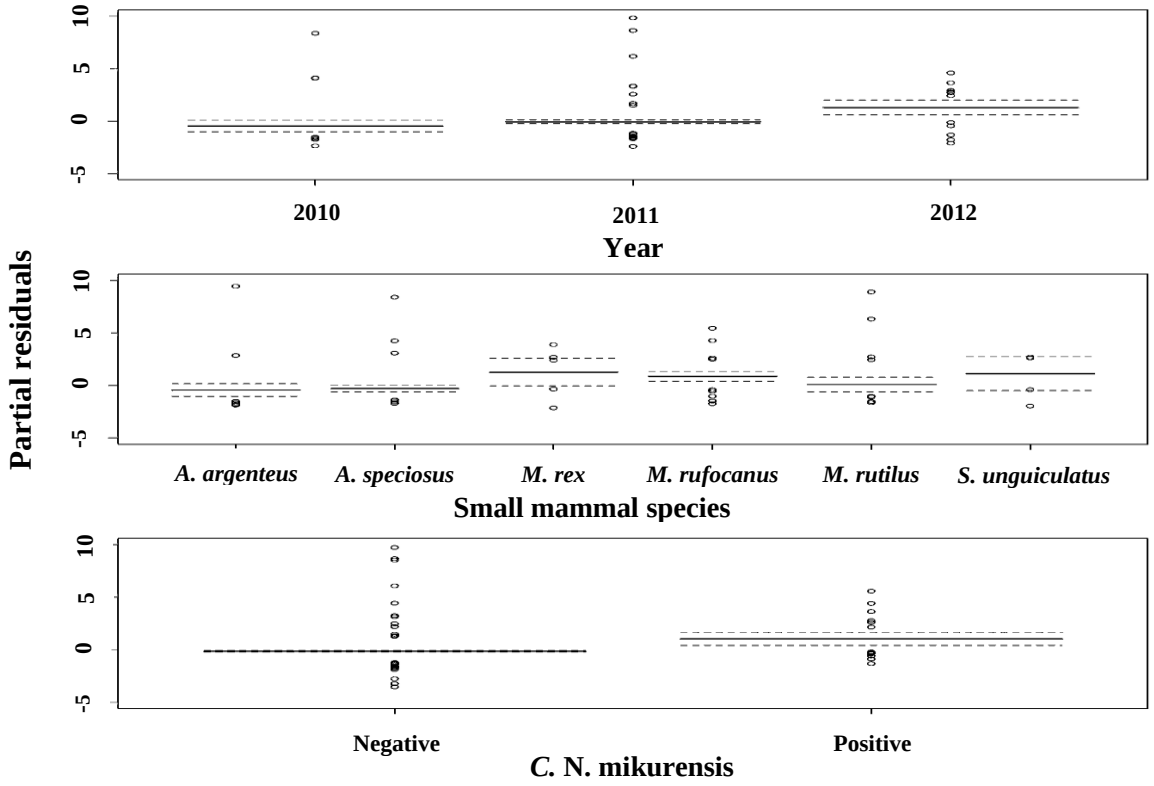

Figure 2-5. The relationship between *B. microti* response with year, animal species and *C.* N. mikurensis infection variation based on the minimal GLM model coefficients.

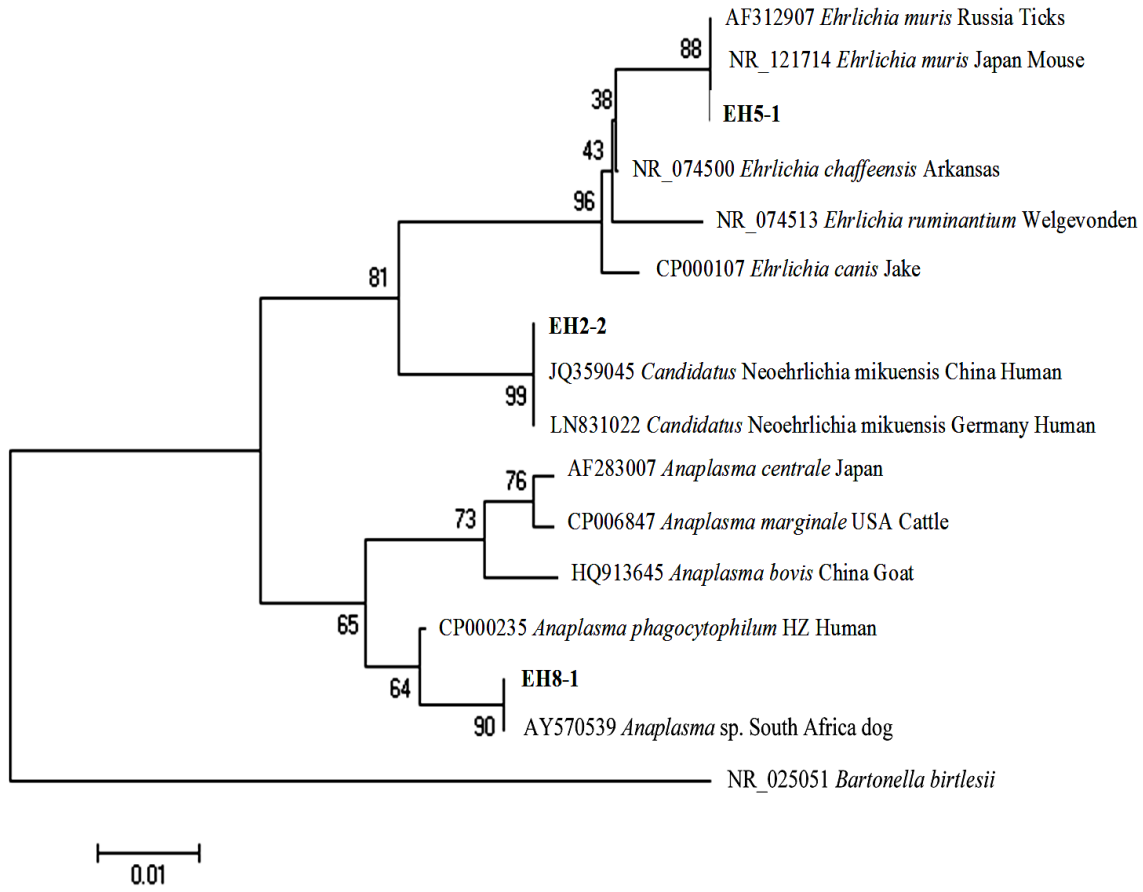

Figure 2-6. Phylogenetic relationship between 16S rRNA gene sequences of *Anaplasma* and *Ehrlichia* spp. from GenBank. Sequences derived from the present study are written in bold letters. This Neighbor Joining Tree was built by MEGA software version 5.1 with 1000 Bootstrap replications. It shows the relationship between a 460-520 bp segment of the 16S rRNA gene of *Anaplasma* and *Ehrlichia* spp. obtained from this study and related *Anaplasma* and *Ehrlichia* spp. sequences from the GenBank®.

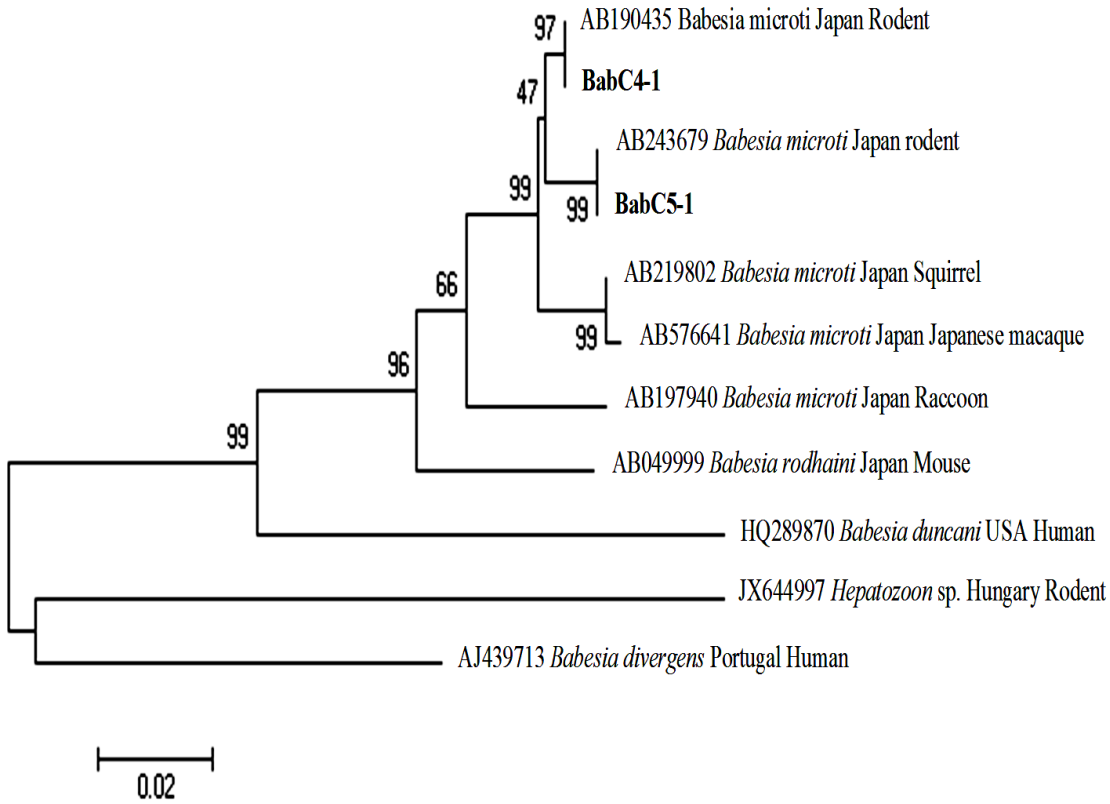

Figure 2-7. Phylogenetic relationship between 18S rRNA gene sequences of *Babesia* spp. from GenBank. Sequences derived from the present study are written in bold letters. This Neighbor Joining Tree was built by MEGA software version 5.1 with 1000 Bootstrap replications. It shows the relationship between a 460-520 bp segment of the 18S rRNA gene of *Babesia* spp. obtained from this study and related *Babesia* spp. sequences from the GenBank®.

ACKNOWLEDGEMENTS

I am greatly thankful to my advisors who reviewed this thesis; Professor Chihiro Sugimoto, Dr. Satoru Konnai and Dr. Michito Shimozuru.

I would like to express my deepest gratitude and appreciation to Professor Toshio Tsubota, Laboratory of Wildlife Biology and Medicine, Graduate School of Veterinary Medicine, Hokkaido University, my deepest thanks and gratitude for his valuable supervision, valuable and critical suggestions, scientific acumen perspicacious remarks, In addition, he was always accessible and willing to help his students during their research. As a result, research life became smooth and rewarding for me.

I am grateful to Dr. Michito Shimozuru, Laboratory of Wildlife Biology and Medicine, Graduate School of Veterinary Medicine, Hokkaido University, for his kindness, advice, encouragement, and guidance, tireless editing; supplying the facilities and continuous encouragement through this work and his perpetual energy and in research problems on the local and international fields. I feel extremely fortunate to be counted as one of his students and all words are unable to express my thanks.

I acknowledge Dr. Kyle Taylor, Department of Infectious Diseases and Pathology, University of Florida, Gainesville, Florida, USA. He taught me the morality and necessity of science and I owe him my deepest gratitude. He provided me with samples and taught me laboratory techniques and how to use different instruments.

I would like to express my deepest thanks to Dr. Ryo Nakao, assistant professor Laboratory of Parasitology, Department of Disease Control, Graduate School of Veterinary Medicine Hokkaido University, for his great assistance to me through helping at the laboratory work and scientific advices which helped me a lot to finish this study.

I received incredible support from the past and present members of the Laboratory of Wildlife biology and Medicine. In particular, I would like to thank Dr. Mariko Sashika and

Dr. Kyunglee Lee for their generosity in teaching me how to do things right and for assisting in sampling collection and data analysis. I am so grateful to fellow students, past and present, for making my stay here easy and happy.

I gratefully acknowledge Professor Kazuhiko Ohashi, Dr Satoru Konnai (Laboratory of Infectious Diseases, Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University) and Professor Chihiro Sugimoto (Director of Research Center for Zoonosis Control, Hokkaido University) for providing me an access to their laboratories. I also acknowledge Dr Naftaly Githaka (International Livestock Research Institute, Kenya), Yongjin Qiu and May June Thu (Research Center for Zoonosis Control, Hokkaido University) for their assistance in the molecular work and sequencing.

I acknowledge Professor Annapaola Rizzoli and Roberto Rosà, Gruppo Ricerca Ecologia Animale, Fondazione Edund Mach, Italy. Thank you both for teaching me statistical modelling and helping me to interpret my data results.

Obviously, I would not be typing this acknowledgement without expressing my deepest gratitude, thanking and appreciation to my family for their unflagging love and support throughout my life, I owed my family, much of what I have become.; this thesis is simply impossible without them. I would like to express my great love, thanking and appreciation to my mother for her support and encouragement; I have no suitable words that can fully describe her everlasting love to me. I would like to express my deepest love, appreciation and thanking to my wife, Wessam, for her patience, encouragement and support, she was the constant source of inspiration throughout the course of my study and research work; this thesis would not have been possible without her help.

REFERENCES:

1. Abe H., 1994. A Pictoral Guide to the Mammals of Japan. Tokyo: Tokai University Press. 90–109, 171–183.
2. Alekseev, A.N., Dubinina, H.V., Van De Pol, I., Schouls, L.M., 2001. Identification of *Ehrlichia* spp. and *Borrelia burgdorferi* in *Ixodes* ticks in the Baltic regions of Russia. Journal of clinical microbiology 39, 2237-2242.
3. Alexander, J., 1988. Sex differences and cross-immunity in DBA/2 mice infected with L. mexicana and L. major. Parasitology 96, 297-302.
4. Allsopp, M.T., Allsopp, B.A., 2006. Molecular sequence evidence for the reclassification of some *Babesia* species. Annals of the New York Academy of Sciences 1081, 509-517.
5. Allsopp, M.T., Louw, M., Meyer, E.C., 2005. *Ehrlichia ruminantium*--an emerging human pathogen. South African medical journal = Suid-Afrikaanse tydskrif vir geneeskunde 95, 541.
6. Arneberg, P., 2002. Host population density and body mass as determinants of species richness in parasite communities: Comparative analyses of directly transmitted nematodes of mammals. Ecography 25, 88-94.
7. Barandika, J.F., Hurtado, A., Garcia-Esteban, C., Gil, H., Escudero, R., Barral, M., Jado, I., Juste, R.A., Anda, P., Garcia-Perez, A.L., 2007. Tick-borne zoonotic bacteria in wild and domestic small mammals in northern Spain. Applied and environmental microbiology 73, 6166-6171.
8. Bekker, C.P., de Vos, S., Taoufik, A., Sparagano, O.A., Jongejan, F., 2002. Simultaneous detection of *Anaplasma* and *Ehrlichia* species in ruminants and detection of *Ehrlichia ruminantium* in *Amblyomma variegatum* ticks by reverse line blot hybridization. Veterinary microbiology 89, 223-238.

9. Bown, K.J., Begon, M., Bennett, M., Woldehiwet, Z., Ogden, N.H., 2003. Seasonal dynamics of *Anaplasma phagocytophila* in a rodent-tick (*Ixodes trianguliceps*) system, United Kingdom. Emerging infectious diseases 9, 63-70.

10. Bown, K.J., Lambin, X., Telford, G.R., Ogden, N.H., Telfer, S., Woldehiwet, Z., Birtles, R.J., 2008. Relative importance of *Ixodes ricinus* and *Ixodes trianguliceps* as vectors for *Anaplasma phagocytophilum* and *Babesia microti* in field vole (*Microtus agrestis*) populations. Applied and environmental microbiology 74, 7118-7125.

11. Burkot, T.R., Schneider, B.S., Pieniazek, N.J., Happ, C.M., Rutherford, J.S., Slemenda, S.B., Hoffmeister, E., Maupin, G.O., Zeidner, N.S., 2000. *Babesia microti* and *Borrelia bissettii* transmission by *Ixodes spinipalpis* ticks among prairie voles, *Microtus ochrogaster*, in Colorado. Parasitology 121 Pt 6, 595-599.

12. Cernetich, A., Garver, L.S., Jedlicka, A.E., Klein, P.W., Kumar, N., Scott, A.L., Klein, S.L., 2006. Involvement of gonadal steroids and gamma interferon in sex differences in response to blood-stage malaria infection. Infection and immunity 74, 3190-3203.

13. Chen, S.M., Dumler, J.S., Bakken, J.S., Walker, D.H., 1994. Identification of a granulocytotropic *Ehrlichia* species as the etiologic agent of human disease. Journal of clinical microbiology 32, 589-595.

14. Courtney, J.W., Dryden, R.L., Montgomery, J., Schneider, B.S., Smith, G., Massung, R.F., 2003. Molecular characterization of *Anaplasma phagocytophilum* and *Borrelia burgdorferi* in *Ixodes scapularis* ticks from Pennsylvania. Journal of clinical microbiology 41, 1569-1573.

15. Criado-Fornelio, A., 2007. A review of nucleic-acid-based diagnostic tests for *Babesia* and *Theileria*, with emphasis on bovine piroplasms. Parassitologia 49 Suppl 1, 39-44.

16. de la Fuente, J., Estrada-Pena, A., Venzal, J.M., Kocan, K.M., Sonenshine, D.E., 2008. Overview: Ticks as vectors of pathogens that cause disease in humans and animals. Frontiers in bioscience : a journal and virtual library 13, 6938-6946.

17. do Prado junior, J.C., Leal Mde, P., Anselmo-Franci, J.A., de Andrade juniur, H.F., Kloetzel, J.K., 1998. Influence of female gonadal hormones on the parasitemia of female Calomys callosus infected with the "Y" strain of *Trypanosoma cruzi*. Parasitology research 84, 100-105.

18. Dumler, J.S., Barbet, A.F., Bekker, C.P., Dasch, G.A., Palmer, G.H., Ray, S.C., Rikihisa, Y., Rurangirwa, F.R., 2001. Reorganization of genera in the families *Rickettsiaceae* and *Anaplasmataceae* in the order Rickettsiales: unification of some species of *Ehrlichia* with *Anaplasma*, *Cowdria* with *Ehrlichia* and *Ehrlichia* with *Neorickettsia*, descriptions of six new species combinations and designation of *Ehrlichia* equi and 'HGE agent' as subjective synonyms of *Ehrlichia phagocytophila*. International journal of systematic and evolutionary microbiology 51, 2145-2165.

19. Fehr, J.S., Bloemberg, G.V., Ritter, C., Hombach, M., Luscher, T.F., Weber, R., Keller, P.M., 2010. Septicemia caused by tick-borne bacterial pathogen *Candidatus* Neoehrlichia mikurensis. Emerging infectious diseases 16, 1127-1129.

20. Gigandet, L., Stauffer, E., Douet, V., Rais, O., Moret, J., Gern, L., 2011. Prevalence of three zoonotic *Babesia* species in *Ixodes ricinus* (Linne, 1758) nymphs in a suburban forest in Switzerland. Vector borne and zoonotic diseases 11, 363-366.

21. Gray, J., Zintl, A., Hildebrandt, A., Hunfeld, K.P., Weiss, L., 2010. Zoonotic babesiosis: overview of the disease and novel aspects of pathogen identity. Ticks and tick-borne diseases 1, 3-10.

22. Inokuma, H., Oyamada, M., Kelly, P.J., Jacobson, L.A., Fournier, P.E., Itamoto, K., Okuda, M., Brouqui, P., 2005. Molecular detection of a new *Anaplasma* species closely

related to *Anaplasma phagocytophilum* in canine blood from South Africa. Journal of clinical microbiology 43, 2934-2937.

23. Jilintai, Seino, N., Hayakawa, D., Suzuki, M., Hata, H., Kondo, S., Matsumoto, K., Yokoyama, N., Inokuma, H., 2009. Molecular survey for *Anaplasma bovis* and *Anaplasma phagocytophilum* infection in cattle in a pastureland where sika deer appear in Hokkaido, Japan. Japanese journal of infectious diseases 62, 73-75.
24. Kamis, A.B., Ibrahim, J.B., 1989. Effects of testosterone on blood leukocytes in *plasmodium berghei*-infected mice. Parasitology research 75, 611-613.
25. Kawahara, M., Ito, T., Suto, C., Shibata, S., Rikihisa, Y., Hata, K., Hirai, K., 1999. Comparison of *Ehrlichia muris* strains isolated from wild mice and ticks and serologic survey of humans and animals with E. muris as antigen. Journal of clinical microbiology 37, 1123-1129.
26. Kawahara, M., Rikihisa, Y., Isogai, E., Takahashi, M., Misumi, H., Suto, C., Shibata, S., Zhang, C., Tsuji, M., 2004. Ultrastructure and phylogenetic analysis of *'Candidatus* Neoehrlichia mikurensis' in the family *Anaplasmataceae*, isolated from wild rats and found in *Ixodes ovatus* ticks. International journal of systematic and evolutionary microbiology 54, 1837-1843.
27. Kawahara, M., Rikihisa, Y., Lin, Q., Isogai, E., Tahara, K., Itagaki, A., Hiramitsu, Y., Tajima, T., 2006. Novel genetic variants of *Anaplasma phagocytophilum*, *Anaplasma bovis*, *Anaplasma centrale*, and a novel *Ehrlichia* sp. in wild deer and ticks on two major islands in Japan. Applied and environmental microbiology 72, 1102-1109.
28. Kawahara, M., Suto, C., Rikihisa, Y., Yamamoto, S., Tsuboi, Y., 1993. Characterization of ehrlichial organisms isolated from a wild mouse. Journal of Clinical Microbiology 31, 89-96.

29. Kong, F., Gilbert, G.L., 2006. Multiplex PCR-based reverse line blot hybridization assay (mPCR/RLB)--a practical epidemiological and diagnostic tool. Nature protocols 1, 2668-2680.

30. Landgraf, B., Kollaritsch, H., Wiedermann, G., Wernsdorfer, W.H., 1994. Parasite density of *Plasmodium falciparum* malaria in Ghanaian schoolchildren: evidence for influence of sex hormones? Transactions of the Royal Society of Tropical Medicine and Hygiene 88, 73-74.

31. Leiby, D.A., 2011. Transfusion-Transmitted *Babesia* spp.: Bull's-Eye on *Babesia microti*. Clinical microbiology reviews 24, 14-28.

32. Li, H., Jiang, J.F., Liu, W., Zheng, Y.C., Huo, Q.B., Tang, K., Zuo, S.Y., Liu, K., Jiang, B.G., Yang, H., Cao, W.C., 2012. Human Infection with *Candidatus* Neoehrlichia mikurensis, China. Emerging infectious diseases 18, 1636-1639.

33. Liu, Z., Ma, M., Wang, Z., Wang, J., Peng, Y., Li, Y., Guan, G., Luo, J., Yin, H., 2012. Molecular survey and genetic identification of *Anaplasma* species in goats from central and southern China. Applied and environmental microbiology 78, 464-470.

34. Liz, J.S., Anderes, L., Sumner, J.W., Massung, R.F., Gern, L., Rutti, B., Brossard, M., 2000. PCR detection of granulocytic *ehrlichiae* in *Ixodes ricinus* ticks and wild small mammals in western Switzerland. Journal of clinical microbiology 38, 1002-1007.

35. Massung, R.F., Courtney, J.W., Hiratzka, S.L., Pitzer, V.E., Smith, G., Dryden, R.L., 2005. *Anaplasma phagocytophilum* in white-tailed deer. Emerging Infectious Diseases 11, 1604-1606.

36. Massung, R.F., Levin, M.L., Munderloh, U.G., Silverman, D.J., Lynch, M.J., Kurtti, T.J., 2006a. Isolation of *Anaplasma phagocytophilum* strain Ap-variant 1 in a tick-derived cell line. Annals of the New York Academy of Sciences 1078, 541-544.

37. Massung, R.F., Mather, T.N., Levin, M.L., 2006b. Reservoir competency of goats for the Ap-Variant 1 strain of *Anaplasma phagocytophilum.* Infection and immunity 74, 1373-1375.

38. Massung, R.F., Priestley, R.A., Miller, N.J., Mather, T.N., Levin, M.L., 2003. Inability of a variant strain of *Anaplasma phagocytophilum* to infect mice. The Journal of infectious diseases 188, 1757-1763.

39. Matjila, P.T., Leisewitz, A.L., Jongejan, F., Penzhorn, B.L., 2008. Molecular detection of tick-borne protozoal and ehrlichial infections in domestic dogs in South Africa. Veterinary parasitology 155, 152-157.

40. Moore, S.L., Wilson, K., 2002. Parasites as a viability cost of sexual selection in natural populations of mammals. Science (New York, N.Y.) 297, 2015-2018.

41. Moustafa, M.A., Lee, K., Taylor, K., Nakao, R., Sashika, M., Shimozuru, M., Tsubota, T., 2015. Molecular characterization and specific detection of *Anaplasma* species (AP-sd) in sika deer and its first detection in wild brown bears and rodents in Hokkaido, Japan. Infect Genet Evol 36, 268-274.

42. Muramatsu, Y., Ikeda, E., Morita, C., Tamura, Y., 2005. Detection of ehrlichial DNA in small rodents captured in a woodland area of Hokkaido, the northernmost island of Japan, where Lyme disease is endemic. Japanese journal of infectious diseases 58, 316-319.

43. Naitou, H., Kawaguchi, D., Nishimura, Y., Inayoshi, M., Kawamori, F., Masuzawa, T., Hiroi, M., Kurashige, H., Kawabata, H., Fujita, H., Ohashi, N., 2006. Molecular identification of *Ehrlichia* species and *'Candidatus* Neoehrlichia mikurensis' from ticks and wild rodents in Shizuoka and Nagano Prefectures, Japan. Microbiol Immunol 50, 45-51.

44. Nijhof, A.M., Penzhorn, B.L., Lynen, G., Mollel, J.O., Morkel, P., Bekker, C.P.J., Jongejan, F., 2003. *Babesia bicornis* sp. nov. and *Theileria bicornis* sp. nov.: Tick-Borne Parasites Associated with Mortality in the Black Rhinoceros (*Diceros bicornis*). Journal of clinical microbiology 41, 2249-2254.

45. Nijhof, A.M., Pillay, V., Steyl, J., Prozesky, L., Stoltsz, W.H., Lawrence, J.A., Penzhorn, B.L., Jongejan, F., 2005. Molecular characterization of *Theileria* species associated with mortality in four species of African antelopes. Journal of clinical microbiology 43, 5907-5911.

46. Ohashi, N., Gaowa, Wuritu, Kawamori, F., Wu, D., Yoshikawa, Y., Chiya, S., Fukunaga, K., Funato, T., Shiojiri, M., Nakajima, H., Hamauzu, Y., Takano, A., Kawabata, H., Ando, S., Kishimoto, T., 2013. Human granulocytic anaplasmosis, Japan. Emerging infectious diseases 19, 289-292.

47. Ohashi, N., Inayoshi, M., Kitamura, K., Kawamori, F., Kawaguchi, D., Nishimura, Y., Naitou, H., Hiroi, M., Masuzawa, T., 2005. *Anaplasma phagocytophilum*-infected ticks, Japan. Emerging infectious diseases 11, 1780-1783.

48. Oosthuizen, M.C., Zweygarth, E., Collins, N.E., Troskie, M., Penzhorn, B.L., 2008. Identification of a novel *Babesia* sp. from a sable antelope (*Hippotragus niger* Harris, 1838). Journal of clinical microbiology 46, 2247-2251.

49. Ostfeld, R.S., Levi, T., Jolles, A.E., Martin, L.B., Hosseini, P.R., Keesing, F., 2014. Life history anddemographic drivers of reservoir competence for three tick-borne zoonotic pathogens. PLoS ONE 9.

50. Oura, C.A., Bishop, R.P., Wampande, E.M., Lubega, G.W., Tait, A., 2004. Application of a reverse line blot assay to the study of haemoparasites in cattle in Uganda. International journal for parasitology 34, 603-613.

51. Pan, H., Liu, S., Ma, Y., Tong, S., Sun, Y., 2003. *Ehrlichia*-like organism gene found in small mammals in the suburban district of Guangzhou of China. Annals of the New York Academy of Sciences 990, 107-111.

52. Parola, P., Raoult, D., 2001. Ticks and tickborne bacterial diseases in humans: an emerging infectious threat. Clinical infectious diseases 32, 897-928.

53. Pritt, B.S., Sloan, L.M., Johnson, D.K., Munderloh, U.G., Paskewitz, S.M., McElroy, K.M., McFadden, J.D., Binnicker, M.J., Neitzel, D.F., Liu, G., Nicholson, W.L., Nelson, C.M., Franson, J.J., Martin, S.A., Cunningham, S.A., Steward, C.R., Bogumill, K., Bjorgaard, M.E., Davis, J.P., McQuiston, J.H., Warshauer, D.M., Wilhelm, M.P., Patel, R., Trivedi, V.A., Eremeeva, M.E., 2011. Emergence of a new pathogenic *Ehrlichia* species, Wisconsin and Minnesota, 2009. The New England journal of medicine 365, 422-429.

54. Reichard, M.V., Roman, R.M., Kocan, K.M., Blouin, E.F., de la Fuente, J., Snider, T.A., Heinz, R.E., West, M.D., Little, S.E., Massung, R.F., 2009. Inoculation of white-tailed deer (*Odocoileus virginianus*) with Ap-V1 Or NY-18 strains of *Anaplasma phagocytophilum* and microscopic demonstration of Ap-V1 In *Ixodes scapularis* adults that acquired infection from deer as nymphs. Vector borne and zoonotic diseases 9, 565-568.

55. Rikihisa, Y., 1991. The tribe *Ehrlichieae* and ehrlichial diseases. Clinical microbiology reviews 4, 286-308.

56. Saito-Ito, A., Kasahara, M., Kasai, M., Dantrakool, A., Kawai, A., Fujita, H., Yano, Y., Kawabata, H., Takada, N., 2007. Survey of *Babesia microti* infection in field rodents in Japan: records of the Kobe-type in new foci and findings of a new type related to the Otsu-type. Microbiology and immunology 51, 15-24.

57. Saito-Ito, A., Yano, Y., Dantrakool, A., Hashimoto, T., Takada, N., 2004. Survey of Rodents and Ticks in Human Babesiosis Emergence Area in Japan: First Detection of *Babesia microti*-Like Parasites in *Ixodes ovatus*. Journal of clinical microbiology 42, 2268-2270.

58. Sasaki, M., Fujii, Y., Iwamoto, M., Ikadai, H., 2013. Effect of sex steroids on *Babesia microti* infection in mice. The American journal of tropical medicine and hygiene 88, 367-375.

59. Sashika, M., Abe, G., Matsumoto, K., Inokuma, H., 2011. Molecular survey of *Anaplasma* and *Ehrlichia* infections of feral raccoons (*Procyon lotor*) in Hokkaido, Japan. Vector borne and zoonotic diseases 11, 349-354.

60. Schouls, L.M., Van De Pol, I., Rijpkema, S.G., Schot, C.S., 1999. Detection and identification of *Ehrlichia, Borrelia burgdorferi sensu lato,* and *Bartonella* species in Dutch *Ixodes ricinus* ticks. Journal of clinical microbiology 37, 2215-2222.

61. Shih, C.M., Liu, L.P., Chung, W.C., Ong, S.J., Wang, C.C., 1997. Human babesiosis in Taiwan: asymptomatic infection with a *Babesia microti*-like organism in a Taiwanese woman. Journal of clinical microbiology 35, 450-454.

62. Shortt, H.E., Blackie, E.J., 1965. An account of the genus *Babesia* as found in certain small mammals in Britain. The Journal of tropical medicine and hygiene 68, 37-42.

63. Shpynov, S., Fournier, P.E., Rudakov, N., Tarasevich, I., Raoult, D., 2006. Detection of members of the genera *Rickettsia, Anaplasma,* and *Ehrlichia* in ticks collected in the Asiatic part of Russia. Annals of the New York Academy of Sciences 1078, 378-383.

64. Shukla, S.K., Aswani, V., Stockwell, P.J., Reed, K.D., 2007. Contribution of polymorphisms in ankA, gltA, and groESL in defining genetic variants of *Anaplasma phagocytophilum*. Journal of clinical microbiology 45, 2312-2315.

65. Tabara, K., Arai, S., Kawabuchi, T., Itagaki, A., Ishihara, C., Satoh, H., Okabe, N., Tsuji, M., 2007. Molecular survey of *Babesia microti*, *Ehrlichia* species and *Candidatus* Neoehrlichia mikurensis in wild rodents from Shimane Prefecture, Japan. Microbiology and immunology 51, 359-367.

66. Tait, A., Oura, C., 2004. Reverse line blotting: a new technique for the sensitive detection of tick borne pathogens. Archives de l'Institut Pasteur de Tunis 81, 47-50.

67. Tamamoto, C., Seino, N., Suzuki, M., Kaji, K., Takahashi, H., Inokuma, H., 2007. Detection of *Ehrlichia muris* DNA from sika deer (*Cervus nippon yesoensis*) in Hokkaido, Japan. Veterinary parasitology 150, 370-373.

68. Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular biology and evolution 28, 2731-2739.

69. Taylor, K.R., Takano, A., Konnai, S., Shimozuru, M., Kawabata, H., Tsubota, T., 2013. *Borrelia miyamotoi* infections among wild rodents show age and month independence and correlation with *Ixodes persulcatus* larval attachment in Hokkaido, Japan. Vector borne and zoonotic diseases 13, 92-97.

70. Thirumalapura, N.R., Qin, X., Kuriakose, J.A., Walker, D.H., 2014. Complete genome sequence of *Ehrlichia muris* strain AS145T, a model monocytotropic *Ehrlichia* strain. Genome announcements 2(1), e01234-13.

71. Tsuji, M., Wei, Q., Zamoto, A., Morita, C., Arai, S., Shiota, T., Fujimagari, M., Itagaki, A., Fujita, H., Ishihara, C., 2001. Human babesiosis in Japan: epizootiologic survey of rodent reservoir and isolation of new type of *Babesia microti*-like parasite. Journal of clinical microbiology 39, 4316-4322.

72. von Loewenich, F.D., Geissdorfer, W., Disque, C., Matten, J., Schett, G., Sakka, S.G., Bogdan, C., 2010. Detection of "*Candidatus* Neoehrlichia mikurensis" in two patients with severe febrile illnesses: evidence for a European sequence variant. Journal of clinical microbiology 48, 2630-2635.

73. Watkins, R.A., Moshier, S.E., O'Dell, W.D., Pinter, A.J., 1991. Splenomegaly and reticulocytosis caused by *Babesia microti* infections in natural populations of the montane vole, *Microtus montanus*. The Journal of protozoology 38, 573-576.

74. Wei, Q., Tsuji, M., Zamoto, A., Kohsaki, M., Matsui, T., Shiota, T., Telford, S.R., Ishihara, C., 2001. Human Babesiosis in Japan: Isolation of *Babesia microti*-like parasites from an asymptomatic transfusion donor and from a rodent from an area where babesiosis is endemic. Journal of clinical microbiology 39, 2178-2183.

75. Wen, B., Rikihisa, Y., Mott, J., Fuerst, P.A., Kawahara, M., Suto, C., 1995. *Ehrlichia muris* sp. nov., identified on the basis of 16S rRNA base sequences and serological, morphological, and biological characteristics. International journal of systematic bacteriology 45, 250-254.

76. Wu, D., Wuritu, Yoshikawa, Y., Gaowa, Kawamori, F., Ikegaya, A., Ohtake, M., Ohashi, M., Shimada, M., Takada, A., Iwai, K., Ohashi, N., 2015. Molecular and serological survey of Rickettsiales bacteria in wild sika deer (*Cervus nippon nippon*) in Shizuoka prefecture, Japan: High prevalence of *Anaplasma* species. Japanese journal of infectious diseases.

77. Yabsley, M.J., Shock, B.C., 2013. Natural history of zoonotic *Babesia*: Role of wildlife reservoirs. International journal for parasitology. Parasites and wildlife 2, 18-31.

78. Yang, J., Liu, Z., Guan, G., Liu, Q., Li, Y., Chen, Z., Ma, M., Liu, A., Ren, Q., Luo, J., Yin, H., 2013. Prevalence of *Anaplasma phagocytophilum* in ruminants, rodents and ticks in Gansu, north-western China. Journal of medical microbiology 62, 254-258.

79. Ybañez, A.P., Matsumoto, K., Kishimoto, T., Inokuma, H., 2012a. Molecular analyses of a potentially novel *Anaplasma* species closely related to *Anaplasma phagocytophilum* detected in sika deer (*Cervus nippon yesoensis*) in Japan. Veterinary microbiology 157, 232-236.

80. Ybañez, A.P., Matsumoto, K., Kishimoto, T., Yokoyama, N., Inokuma, H., 2012b. Dual presence of *Anaplasma phagocytophilum* and its closely related *Anaplasma* sp. in *ixodid* ticks in Hokkaido, Japan, and their specific molecular detection. The Journal of veterinary medical science 74, 1551-1560.

81. Ybañez, A.P., Sashika, M., Inokuma, H., 2014. The phylogenetic position of *Anaplasma bovis* and inferences on the phylogeny of the genus *Anaplasma*. The Journal of veterinary medical science 76, 307-312.

82. Ybañez, A.P., Tagawa, M., Matsumoto, K., Kishimoto, T., Yokoyama, N., Inokuma, H., 2013. Specific molecular detection of *Anaplasma* sp. closely related to *Anaplasma phagocytophilum* in *ixodid* ticks and cattle in a pastureland in Hokkaido, Japan. Vector borne and zoonotic diseases 13, 6-11.

83. Zamoto-Niikura, A., Tsuji, M., Qiang, W., Nakao, M., Hirata, H., Ishihara, C., 2012. Detection of two zoonotic *Babesia microti* lineages, the Hobetsu and U.S. lineages, in two sympatric tick species, *Ixodes ovatus* and I*xodes persulcatus*, respectively, in Japan. Applied and environmental microbiology 78, 3424-3430.

84. Zamoto, A., Tsuji, M., Kawabuchi, T., Wei, Q., Asakawa, M., Ishihara, C., 2004. U.S.-type *Babesia microti* isolated from small wild mammals in eastern Hokkaido, Japan. The Journal of veterinary medical science 66, 919-926.

85. Zhan, L., Cao, W.C., Jiang, J.F., Zhang, X.A., Liu, Y.X., Wu, X.M., Zhang, W.Y., Zhang, P.H., Bian, C.L., Dumler, J.S., Yang, H., Zuo, S.Q., Chu, C.Y., Liu, W.,

Richardus, J.H., Habbema, J.D.F., 2010. *Anaplasma phagocytophilum* from rodents and sheep, China. Emerging infectious diseases 16, 764-768.

86. Zhang, Z., Chen, L., Saito, S., Kanagawa, O., Sendo, F., 2000. Possible modulation by male sex hormone of Th1/Th2 function in protection against *Plasmodium chabaudi chabaudi* AS infection in mice. Experimental parasitology 96, 121-129.

87. Zhou, X., Xia, S., Huang, J.L., Tambo, E., Zhuge, H.X., Zhou, X.N., 2014. Human babesiosis, an emerging tick-borne disease in the People's Republic of China. Parasites & Vectors 7, 509.

SUMMARY

The emerging infectious diseases are mainly originated in wildlife and many of them are caused by vector-borne pathogens. Ticks are considered one of the most important vectors for zoonotic diseases in the world. They can transmit many zoonotic pathogens to both domestic and wild animal species. Humans are accidently infected by TBDs such as HGA, human ehrlichiosis, neoehrlichiosis and babesiosis. In Japan, many studies have been conducted on TBPs and they focused mainly on their molecular characterization and reservoir host identification. In this study, my main goal was to understand the ecology of potentially zoonotic TBPs in Hokkaido wildlife. I used the PCR-RLB molecular technique to specifically detect the genetic materials of these pathogens in many wild animal species. By using the species specific oligonucleotide probes, this technique can help to survey several TBPs with high specificity and throughput.

In chapter 1, I investigated a previously undescribed *Anaplasma* species (AP-sd), which has been detected in sika deer, cattle and ticks in Japan. Despite being highly similar to some strains of *A. phagocytophilum*, AP-sd has never been detected in humans. Its ambiguous epidemiology and the lack of tools for its specific detection make it difficult to understand and interpret the prevalence of this *Anaplasma* species. I developed a method for specific detection, and examined AP-sd prevalence in Hokkaido wildlife. My study included 250 sika deer (*Cervus nippon yesoensis*), 13 brown bears (*Ursus arctos yesoensis*) and 252 rodents including 138 (*Apodemus speciosus*), 45 (*Apodemus argenteus*), 42 (*Myodes rufocanus*) and 27 (*Myodes rutilus*) were collected from Hokkaido island, northern Japan, during 2010 to 2015. A 770 bp and 382 bp segment of the 16S rRNA and gltA genes, respectively, were amplified by nested PCR. Results were confirmed by cloning and sequencing of the positive PCR products. A reverse line blot hybridization (RLB) based on the 16S rRNA gene was then developed for the specific detection of AP-sd. The prevalence of AP-sd by nested PCR in

sika deer was 51% (128/250). We detected this *Anaplasma* sp. for the first time in wild brown bears and rodents with a prevalence of 15% (2/13) and 2.4% (6/252), respectively. The sequencing results of the 16S rRNA and gltA gene amplicons were divergent from the selected *A. phagocytophilum* sequences in GenBank. Using a newly designed AP-sd specific probe for RLB has enabled us to specifically detect this *Anaplasma* species. Besides sika deer and cattle, wild brown bears and rodents were identified as potential reservoir hosts for AP-sd. This study provided a high throughput molecular method that specifically detects AP-sd, and which can be used to investigate its ecology and its potential as a threat to humans in Japan.

In chapter 2, a combination of newly designed oligonucleotide probes and previously published ones were used to investigate the prevalence of 13 potentially zoonotic TBPs in 6 small mammal species in Hokkaido ecosystem. Generally, fever of unknown cause and rickettsiosis-like symptoms are reported from humans in Japan. Unfortunately, the causative agents of these symptoms are rare to be discovered. In addition, the ecology of many zoonotic TBPs is still not fully understood in Japan where many zoonotic TBPs were previously detected in ticks, domestic and wild animals. I believed that the co-existence of these pathogens in Hokkaido ecosystem could be driven by many variables such as age, gender, season and reservoir host preferences. The objective of this study was to examine the prevalence of potentially zoonotic TBPs in small mammal populations in Hokkaido, in relation to variations in many extrinsic factors that might affect the infection rates of these pathogens. In this study, we examined 459 small mammals including 219 (*Apodemus speciosus*), 86 (*Apodemus argenteus*), 85 (*Myodes rufocanus*), 51 (*Myodes rutilus*), 11 (*Myodes rex*) and 7 (*Sorex unguiculatus*) from Furano and Shari sites in Hokkaido, Japan, during 2010 to 2012. A fragment of 460–520 bp from the 16S rRNA gene of *Anaplasma* and *Ehrlichia* and 460–540 bp from the 18S rRNA gene of *Babesia* species was amplified by

multiplex PCR and then screened by RLB hybridization, which was developed to specifically detect 2, 6 and 5 potentially zoonotic *Anaplasma*, *Ehrlichia* and *Babesia* species, respectively. The result of the RLB was confirmed through cloning of randomly selected 6 and 12 positive PCR products for *Anaplasma*/*Ehrlichia* and *Babesia* spp., respectively. The prevalence of *Anaplasma* sp. AP-sd, *E. muris*, *C.* N. mikurensis and *B. microti* was 19 (4.1%), 5 (1.1%), 56 (12.2%) and 95 (20.7%), respectively. The infection rates of *B. microti* and *C.* N. mikurensis were significantly higher in males and adults. The prevalence of the detected TBPs was significantly correlated with the small mammal species variation. A total of 24, two triple and 22 double, co-infection cases were detected in this study (5.2%). The most frequent co-infection cases occurred between *C.* N. mikurensis and *B. microti* (66.7%). The existence of these zoonotic TBPs in Hokkaido is irrelevant to the rare report of symptomatic human cases. Therefore, human investigations for TBPs in Hokkaido are recommended to understand their epidemiology.