The molecular epidemiology of bovine *Babesia* species in livestock animals in Mongolia

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モンゴルの家畜動物における牛バベシア種の分子疫学研究

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Abbreviations

%	Percentage
°C	Degree celsius
CI	Confidence interval
ama-1	Apical membrane protein 1
B. bigemina	*Babesia bigemina*
B. bovis	*Babesia bovis*
B. divergens	*Babesia divergens*
B. naoakii	*Babesia naoakii*
B. ovata	*Babesia ovata*
BLAST	Basic Local Alignment Search Tool
bp	Base pair
DDW	Double distilled water
DNA	Deoxyribonucleic acid
dNTP	Deoxyribose nucleoside triphosphates
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
GGU	Galbiin Gobiin Ulaan
HZ	Hos Zogdort
LB	Luria Bertani
MAFFT	Multiple Alignment using Fast Fourier Transform software
MatGAT	Matrix Global Alignment Tool software
MEGA	Molecular Evolutionary Genetics Analysis software
µL	Microliter
MNT	Mongolian Native Camel
mL	Milliliter
mM	Millimolar
No.	Number
OIE	The Office International des Epizooties
PCR	Polymerase chain reaction
rap-1	Rhoptry associated protein-1
RBCs	Red blood cells
sp.	Species
U	Unit
UV	Ultraviolet

General Introduction

1. Bovine babesiosis

Bovine babesiosis is a disease caused by species of genus *Babesia* in bovines (Bock et al., 2002). The clinical bovine babesiosis is most common among cattle, while in non-cattle hosts, the *Babesia* infections are usually asymptomatic (Jaimes-Dueñez et al., 2018). The clinical bovine babesiosis is caused mainly by four *Babesia* species, including *Babesia bovis*, *Babesia bigemina*, *Babesia naoakii*, and *Babesia divergens* (Fig. 1; Zintl et al., 2003; Bock et al., 2004; Sivakumar et al., 2018). The bovine babesiosis has a global distribution with a few exceptions, such as Japan, USA, New Zealand, and Canada (Jacob et al., 2020). The distribution of causative *Babesia* species coincides with that of their specific tick vectors (Bock et al., 2004). The bovine babesiosis results in huge economical losses due to cost for the treatment and control, production loss, and death of affected cattle (Guglielmone et al., 1992; He et al., 2021). In addition, international trade of cattle is also affected because of regulations that restrict the import of cattle from endemic countries (OIE, 2019). Therefore, control of bovine babesiosis is important for successful cattle farming. However, currently available control methods are often ineffective due to several reasons, such as lack of commercially available vaccines, side-effects and low efficacy of anti-babesial drugs, and emergence of tick strains resistance to acaricides (Bock et al., 2004; Abbas et al., 2014; Tuvshintulga et al., 2019). Additionally, in several endemic countries, lack of epidemiological data is a major stumbling block for designing strategies to control and prevent bovine babesiosis.

2. Lifecycle of bovine *Babesia* species

The lifecycle of *Babesia* species involves vertebrates and ticks as intermediate and definitive hosts, respectively (Hunfeld et al., 2008). The bovine *Babesia* species are transmitted

primarily by the competent tick vectors. In general, *Rhipicephalus* ticks transmit both *B. bovis* and *B. bigemina*, while *Ixodes ricinus* transmits *B. divergens* (Bock et al., 2004). Tick vectors that transmit *B. naoakii* are currently unknown. The *Babesia* species can also be transmitted mechanically via contaminated fomites or transplacentally to a lesser extent (Costa et al., 2016).

The infected ticks inject *Babesia* sporozoites when they feed on susceptible hosts (Fig. 2). The sporozoites invade red blood cells (RBCs), transform into merozoites, and undergo an asexual reproduction, known as merogony, to produce additional merozoites (Yokoyama et al., 2006). The merozoites released from the infected RBCs during egress invade non-infected RBCs, and continue to proliferate by the merogony (Bock et al., 2004).

Some of the merozoites will transform into gametocytes, which are acquired by the ticks when they feed on the infected animals (Bock et al., 2004). In the midgut of ticks, the gametocytes become gametes, which undergo a sexual reproduction and form zygotes (Mackenstedt et al. 1995; Gough et al., 1998). The zygotes invade the endothelial cells of midgut, where they transform into kinetes. Subsequently, the kinetes reach various internal organs of the ticks, including salivary glands and ovaries, via hemolymph (Mackenstedt et al. 1995). In the salivary glands of *I. ricinus* ticks, sporozoites are produced from the kinetes of *B. divergens,* and transmitted to cattle during the blood feeding of subsequent tick stages (Zintl et al., 2003). The persistence of infection from one tick stage to other is known as transstadial persistence (Bock et al., 2004). On the other hand, the kinetes of *B. bovis* and *B. bigemina* in females *Rhipicephalus* ticks are transovarially transmitted to their eggs, and then transstadially to larva, where the sporozoites are produced in their salivary glands (Bock et al., 2004). During their blood meal on cattle, the larvae transmit *B. bovis*, whereas the nymphs and adults transmit *B. bigemina* (Fig. 2; Riek,1966; Dalgliesh et al., 1978).

3. Clinical bovine babesiosis and the risk factors

In *Babesia*-infected cattle, the merogony result in a massive intravascular hemolysis, leading to severe anemia and other related clinical signs, which include fever, icterus, hemoglobinuria, loss of production, etc (Fig. 2; Mosqueda et al., 2012). However, not all of the infected animals experience such severe clinical signs. The outcome of infection largely depends on several risk factors, including the species of *Babesia*, age, management practices, acquired immunity, and cattle breed (Bock et al., 2004). Several species of *Babesia* are known to infect cattle, but only four of them cause clinical bovine babesiosis; *B. bovis*, *B. bigemina*, *B. naoakii*, and *B. divergens* (Fig. 1; Zintl et al., 2003; Bock et al., 2004; Sivakumar et al., 2018). However, infections with these virulent *Babesia* species do not necessarily result in clinical babesiosis. Young calves are typically resistant to clinical babesiosis because of their early innate immune response. The infected calves develop a long-lasting immunity, which protect the animals from clinical babesiosis, if they receive the subsequent infection as adult (Goff et al., 2001; Zintl et al., 2005). Under extensive management systems, most of the calves are exposed to *Babesia* infections in the endemic countries, leading to an endemically stable situation, where clinical babesiosis is rare (Bock et al., 2004). Cattle breed is also an important risk factor. The *Bos taurus* cattle are more susceptible to clinical babesiosis, as compared to *Bos indicus* animals (Bock et al., 1999). Therefore, clinical bovine babesiosis is common among non-immune adult cattle of *Bos taurus* breeds that are maintained under intensive management systems (Bock et al., 2004).

4. Diagnosis of bovine babesiosis

The diagnosis of clinical bovine babesiosis is usually based on the clinical signs and microscopic examination of stained thin blood smears (Mosqueda et al., 2012). The parasites can be easily identified on the smears prepared with collected blood from animals with acute

infections (Sivakumar et al., 2018). A careful microscopic examination may identify the causative *Babesia* species. *Babesia bovis* and *B. divergens* are classified as small type of *Babesia* species, and differentially diagnosed based on the location of their paired pyriforms; within the RBCs, *B. bovis* is centrally located, while *B. divergens* is peripherally located (Lempereur et al., 2017). On the other hand, *B. bigemina* and *B. naoakii* are both large type of *Babesia* species, and can be differentially diagnosed based on the angle between paired pyriforms; *B. bigemina* forms an acute angle, whereas *B. naoakii* forms an obtuse angle (Lempereur et al., 2017; Sivakumar et al., 2018).

Although the microscopy is a useful tool for detecting *Babesia* infections in cattle with clinical babesiosis, this technique is unsuitable for detecting the carrier animals with low parasitaemia (Alvarez et al., 2019). In the recent past, various molecular and serological assays have been developed, and widely used for detecting the *Babesia* infections. Molecular diagnostic tools, such as polymerase chain reaction (PCR) assay, loop-mediated isothermal amplification (LAMP) assay, and real-time PCR assays, have been proven to be useful for detecting the carrier animals in epidemiological surveys (Mosqueda et al., 2012; Alvarez et al., 2019). Similarly, serodiagnostic tools, such enzyme-linked immunosorbent assay (ELISA) and immunofluorescence antibody test (IFAT) are useful for determining the rate of animals exposed to *Babesia* infections (Mosqueda et al., 2012; Alvarez et al., 2019). Although serological tests are suitable for large-scale surveys, such assays cannot differentiate the animals between the current and past infections. For getting a better picture of the epidemiological status of bovine *Babesia* infections, use of a combination of molecular and serological surveys is recommended by OIE (OIE, 2019).

5. Control of bovine babesiosis

Treatment

Initiation of treatment with anti-babesial agents at the early stage of clinical bovine babesiosis is essential for recovery, whereas delayed treatment often leads to death of the affected animals (Sivakumar et al., 2018). Currently, only two kinds of anti-babesial drugs, including diminazene aceturate and imidocarb dipropionate, are used for treating clinical babesiosis (Mosqueda et al., 2012). Although they are highly effective for the treatment, their wide use is limited due to the toxic side effects, regulations in each endemic country, and emergence of drug-resistant strains (Tuvshintulga et al., 2019). Therefore, development of novel therapeutics is required for treating the bovine babesiosis. In the recent past, several compounds have been evaluated as anti-babesial agents *in vitro* and in mouse models (Mosqueda et al., 2012). However, further experiments have not been conducted to evaluate their actual efficacy against bovine babesiosis in the natural hosts.

Vaccination

Live attenuated vaccines have been used to immunize cattle against bovine babesiosis caused by *B. bovis* and *B. bigemina*, in some of the endemic countries (Shkap et al., 2007). The virulences of *B. bovis* and *B. bigemina* are attenuated by passaging the isolates through splenectomised calves (Bock et al., 2004). The *Babesia*-infected RBCs obtained after several passages are used to infect additional splenectomised calves, and the blood collected from them is used as live vaccines (de Vos and Bock, 2000). Alternatively, the attenuated strains can be grown *in vitro*, and the infected RBCs from the cultures can be used as the vaccines (Bock et al., 2004). A single immunization with the live attenuated vaccines offers a long-lasting immunity, which protects the cattle from clinical babesiosis (de Vos and Bock, 2000). However, a wide use of live attenuated vaccines is constrained by several factors, including

time-consuming production procedures, risk of contamination with other blood pathogens, and vaccine breakthrough due to strain variations (de Vos and Bock, 2000). Development of subunit vaccines may be important for overcoming these shortcomings. In the recent past, several *Babesia* antigens were characterized as the candidates for subunit vaccines (Suarez et al., 2019). However, no subunit vaccines are commercially available at present to immunize cattle against bovine babesiosis.

Tick control

Since the *Babesia* species are tick-borne parasites, control of ticks is vital for preventing bovine babesiosis. Several classes of acaricides with different mode of applications are commercially available for controlling the ticks that infest cattle (Graf et al., 2004). However, rapid development of acaricide resistance renders the tick control strategies less effective (Abbas et al., 2014). A subunit vaccine based on a tick midgut antigen, known as Bm86, has been used in some countries against *Rhipicephalus* ticks (de la Fuente et al., 2007). However, the vaccines had inconsistencies in its efficacy against different species of *Rhipicephalus* ticks (de Vos et al., 2001).

6. Importance of epidemiological surveys

A vast majority of cattle are chronically infected with bovine *Babesia* species in endemic countries (Alvarez et al., 2019). Although the chronic carriers appear as apparently healthy, they act as a source of infection. In other words, *Babesia* parasites can be transmitted from these animals by ticks to susceptible animals, where the infection may result in clinical bovine babesiosis (Calder et al., 1996). Due to this reason, epidemiological surveys have been conducted to identify the *Babesia*-infected cattle in several endemic countries (Alvarez et al., 2019). These studies identified not only the *Babesia* species infecting cattle in the surveyed regions, but also the risk factors associated with the infections, allowing the veterinary

authorities to design systematic control measures (Jacob et al., 2020). However, the epidemiological status of bovine babesiosis is currently unknown in many countries, especially in developing countries. As a result, there is no effective measures to control bovine babesiosis in these regions, and this may lead to severe economic consequences.

7. Epidemiology of bovine *Babesia* species in Mongolia

Mongolia is an agricultural country with large populations of livestock animals. As of 2021, the livestock population in Mongolia consists of about 67 million animals, including 31, 26, 4.1, 4, 0.9, and 0.5 million of sheep, goats, cattle, horses, yaks, and Bactrian camels, respectively (National statistics office of Mongolia, 2021). Despite of this large inventory of livestock, the profit from livestock farming remains low in Mongolia due to various reasons, including infectious diseases (World Bank, 2009). Presence of infectious diseases is the major reason why Mongolia is unable to export the livestock animals and their products to developed countries (World Bank, 2009).

A recent serological survey found that cattle throughout Mongolia were exposed to *B. bovis* and *B. bigemina*, highlighting that the cattle were at risk of bovine babesiosis in Mongolia (Battsetseg et al., 2018). On the other hand, molecular surveys were conducted to detect active infections with these two parasite species in cattle only in three Mongolian provinces (Altangerel et al., 2012; Sivakumar et al., 2012a). In addition, the recently discovered *B. naoakii*, which is capable of causing clinical bovine babesiosis, has never been surveyed in Mongolia, although this *Babesia* species has been reported in a number of host animals in several Asian countries (Sivakumar et al., 2020, 2022). Therefore, the current epidTickemiological status of bovine babesiosis and its causative agents are unclear in Mongolia.

In Mongolia, all livestock animals are reared together by an extensive management system from the time of their birth (Fig. 3; Suttie, 2005). As a result, tick species infesting one livestock animal often infest the others (Narankhajid et al., 2018). Therefore, there is a possibility that bovine *Babesia* species may be tick-transmitted from cattle to other livestock animals and vice versa in Mongolia (Fig. 4). If proved to be so, the control strategies for bovine babesiosis in cattle should also focus on minimizing *Babesia* infections in non-cattle carrier animals (Jaimes-Dueñez et al., 2018). In addition, the infections with the bovine *Babesia* species might also be of health significance in non-cattle hosts. For example, clinical babesiosis caused by *B. bigemina* has been described in yaks in India (Saud et al., 2005). Therefore, cattle and non-cattle livestock should be surveyed throughout Mongolia for *B. bovis*, *B. bigemina*, and *B. naoakii* infections, which can cause clinical bovine babesiosis.

8. Aims of the present study

Incomplete picture of the epidemiology of bovine *Babesia* species is a stumbling block for assessing the risks associated with bovine babesiosis, which is essential to design the control strategies in Mongolia. Since all livestock animals are reared together by an extensive management system in Mongolia (Fig. 3), I hypothesized that bovine *Babesia* species might be tick-transmitted between cattle and other livestock animals, including Bactrian camels and yaks (Fig. 4). Therefore, the aim of the present study was to survey the cattle and non-cattle livestock in Mongolia for the infections of bovine *Babesia* species that can cause clinical bovine babesiosis. The specific objectives were to survey the cattle, Bactrian camels, and yaks reared throughout Mongolia for the infections of *B. bovis*, *B. bigemina*, and *B. naoakii* using the parasite-specific PCR assays, as well as to investigate the risk factors associated with the *Babesia* infections. Moreover, an additional objective of the present study was to create epidemiological maps for these infections in the Mongolian livestock animals.

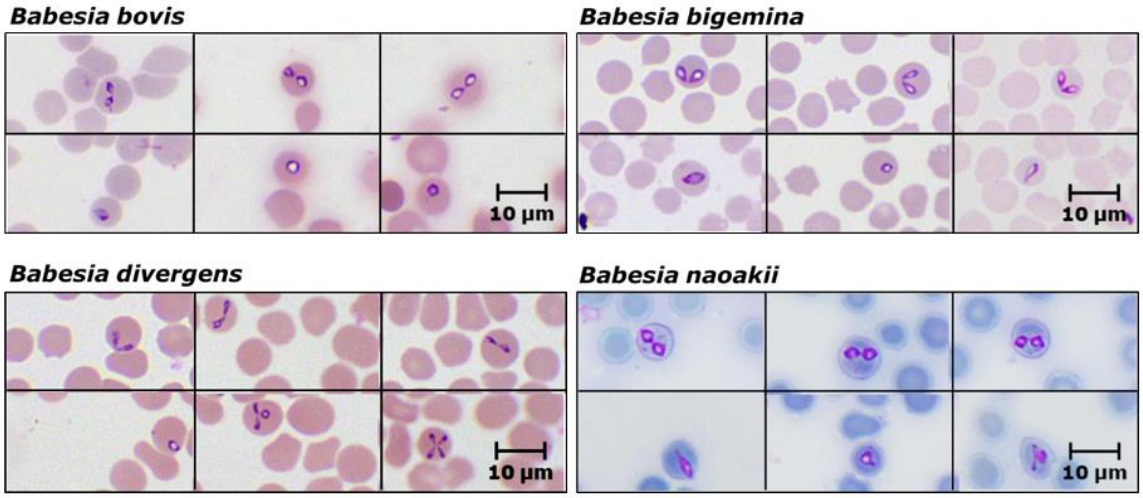

Fig. 1. Micrographs of *Babesia bovis*, *B. bigemina*, *B. divergens*, and *B. naoakii*. These images were retrieved from the website of the OIE reference laboratory for bovine babesiosis, National Research Center for Protozoan Diseases, Obihiro University of Agriculture, Japan (https://www.obihiro.ac.jp/facility/protozoa/en/oie-rl-bb-bb).

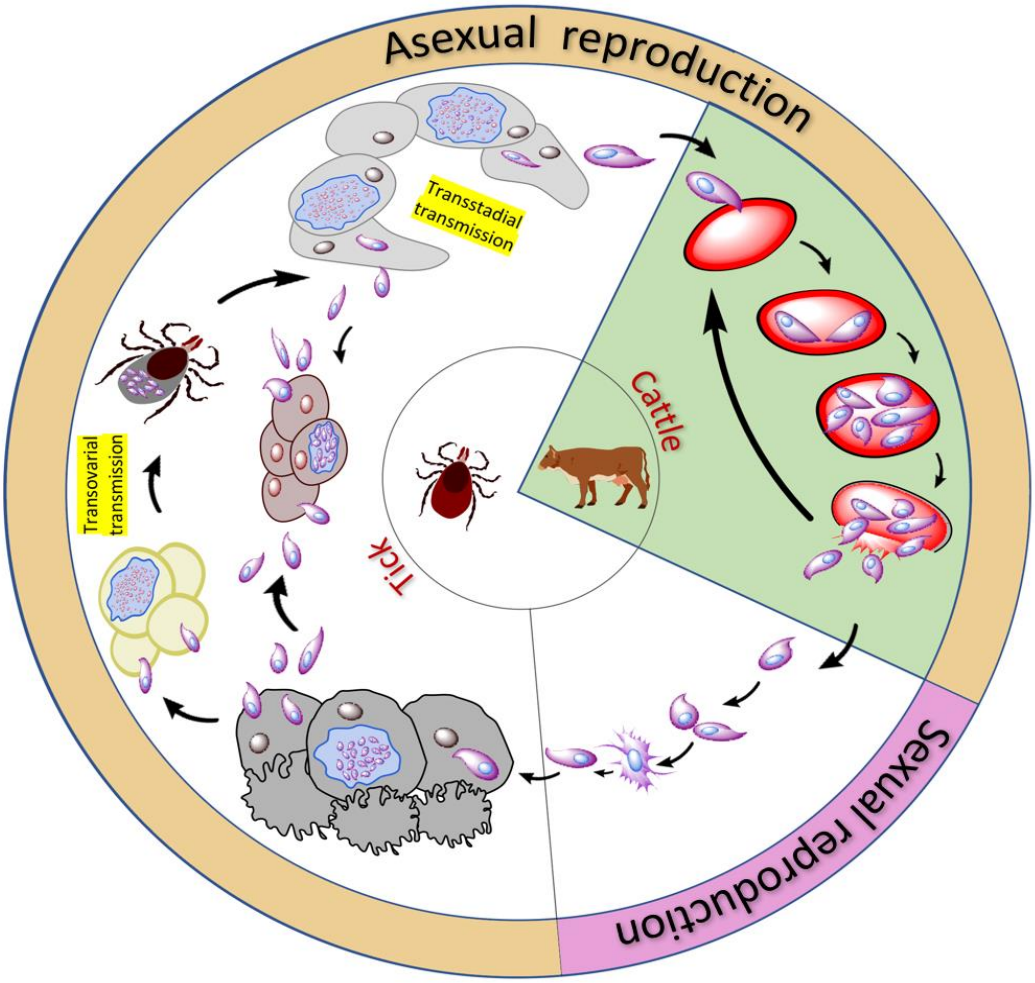

Fig. 2. Lifecycle of bovine *Babesia* species. The *Babesia* species sexually reproduce in the midgut of tick vector, while they asexually reproduce in tick salivary glands and host's red blood cells (RBCs). The asexual reproduction in RBCs results in a massive intravascular hemolysis, leading to severe anemia in the infected cattle.

Fig. 3. Grazing livestock in Mongolia. All livestock animals in Mongolia are reared together by an extensive management system.

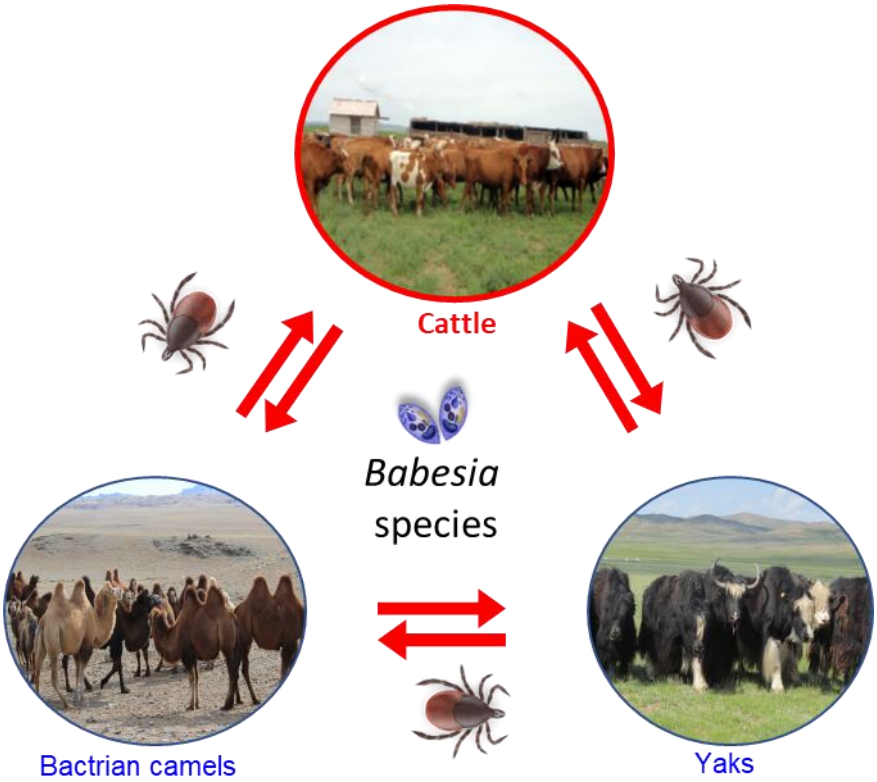

Fig. 4. Hypothesis of the present study. I hypothesized that bovine *Babesia* species might be tick-transmitted among cattle, Bactrian camels, and yaks.

Chapter 1

Molecular epidemiological survey of *Babesia bovis*, *Babesia bigemina*, and *Babesia naoakii* infections in Mongolian cattle

1-1. Introduction

Bovine babesiosis is an economically significant infectious disease of cattle caused by *Babesia* species, which are tick-borne hemoprotozoan parasites (Bock et al., 2004). *Babesia bovis* and *B. bigemina* in the tropics and sub-tropics and *B. divergens* in Europe are known to cause severe clinical bovine babesioses (Bock et al., 2004; Zintl et al., 2003). In a recent study, however, *Babesia naoakii*, formerly known as *Babesia* sp. Mymensingh, was also identified as an additional species of clinical significance in cattle (Sivakumar et al., 2018; 2022). Asexual reproduction of *Babesia* merozoites within bovine RBCs results in severe intravascular hemolysis, leading to clinical signs, such as fever, hemoglobinuria, anemia, and jaundice (Hunfeld et al., 2008; Homer et al., 2000). In addition to these symptoms, nervous and respiratory distress syndrome signs are often observed in cattle with *B. bovis*-induced babesiosis, because of the sequestration of infected RBCs in capillary beds (Everitt et al., 1986). Early diagnosis followed by treatment with anti-babesial agents are vital for recovery, while delayed treatment may result in the death of affected animals (Vial and Gorenflot, 2006; Mosqueda et al., 2012; Sivakumar et al., 2018). Cattle infected with *Babesia* species often remain as carriers, and detection of these carrier animals is important for risk assessment, as the parasites can be tick-transmitted to uninfected cattle (Alvarez et al., 2019). Therefore, many epidemiological surveys aimed at detecting the carrier animals have been conducted in several endemic countries (Giglioti et al., 2016; Jaimes-Dueñez et al., 2018; Tayebwa et al., 2018; Sivakumar et al., 2012b).

Mongolia is an agriculturally productive country, where the livestock industry plays a key role in the national economy. As of 2021, the cattle population of Mongolia was estimated to be about 4.1 million (National statistics office of Mongolia, 2021). However, cattle export from Mongolia remains low for various reasons, including the incidence of severe infectious diseases (World Bank, 2009). A recent seroepidemiological survey found that the cattle throughout Mongolia had been exposed to both *B. bovis* and *B. bigemina* (Battsetseg et al., 2018), although it is not known whether these sero-positive animals were infected with the parasites at the time of sampling. Furthermore, molecular epidemiological surveys, which can detect the current incidence rate of infections, have been conducted only in a few Mongolian provinces (Altangerel et al., 2012; Sivakumar et al., 2012a). In addition, the recently reported *B. naoakii*, a potential agent of clinical bovine babesiosis (Sivakumar et al., 2018), has not been surveyed in Mongolia. Therefore, the objective of my present study was to assess the prevalence of *B. bovis*, *B. bigemina*, and *B. naoakii* in cattle from various Mongolian provinces using the specific PCR assays.

1-2. Materials and methods

Ethical statement

All animal protocols were approved by the Animal Care and Use Committee of Obihiro University of Agriculture and Veterinary Medicine, Japan (Approval number: 28-45). All experiments were carried out in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Sample collection and DNA extraction

A total of 725 blood samples were collected from cattle (Fig. 5) in 16 Mongolian provinces in 2016 and 2017 (Fig. 6, Table 2). All animals were apparently healthy at the time of sampling. Approximately 3 ml of blood was collected from the jugular vein of each animal into a clean sterile vacutainer tube containing EDTA. Genomic DNA was prepared from each blood sample by a phenol:chloroform:isoamil alcohol (25:24:1, v/v) method, as previously described (Sambrook and Russell, 2001). The DNA samples were then stored at -30 °C until further use.

PCR screening for *B. bovis*, *B. bigemina*, and *B. naoakii*

The bovine blood DNA samples were subjected to PCR assays to detect *B. bovis*, *B. bigemina*, and *B. naoakii* DNAs using previously described species-specific primers (Table 1). A nested PCR assay based on rhoptry-associated protein 1 gene (*rap-1*) (Figueroa et al., 1993) was used to detect *B. bovis*, whereas single step PCR assays based on apical membrane antigen 1 gene (*ama1*) were used to screen for *B. bigemina* (Sivakumar et al., 2012a) and *B. naoakii* (Sivakumar et al., 2018). A 10-µl reaction mixture, containing 1 µl DNA, 200 µM of each dNTP (Applied Biosystems, Roche Molecular Systems, Branchburg, NJ, USA), 1× PCR buffer (Applied Biosystems), 0.5 µM of each forward and reverse primer, 0.5 units *Taq* DNA polymerase (Applied Biosystems), and 5.9 µl double distilled water (DDW), was prepared for each PCR assay. PCR cycling conditions were as follows: an initial pre-denaturation step at 95 °C for 5 min; 35 (*B. bovis*) or 40 (*B. bigemina* and *B. naoakii*) cycles of a denaturation step at 95 °C for 30 sec, an annealing step at the appropriate temperature (Table 1) for 1 min, and an extension step at 72 °C for 1 min; followed by a final elongation step at 72 °C for 7 min. For *B. bovis*, 1 µl of the first PCR product was transferred to new PCR tubes containing the reaction mixture, as described above except that the outer primers were replaced with inner primers,

and then subjected to cycling conditions similar to the first PCR assay. The resultant PCR products were resolved by agarose gel electrophoresis, stained with ethidium bromide, and then visualized under UV illumination. Detection of the bands of approximately 298, 211, and 371 bp indicated that the samples were infected with *B. bovis*, *B. bigemina*, and *B. naoakii*, respectively.

Cloning, sequencing, and phylogenetic analyses

Amplicons from the *B. naoakii*-specific PCR assay were cloned and sequenced, as this parasite species has not been previously reported in Mongolia. Briefly, the PCR amplicons were gel-extracted using a commercial kit (NucleoSpin Gel and PCR Clean-up, Macherey-Nagel, Duren, Germany) and ligated to a PCR 2.1 plasmid vector (PCR 2.1-TOPO, Invitrogen, Carlsbad, CA, USA), and the inserted *ama-1* gene fragment was sequenced using an ABI PRISM 3100 genetic analyzer (Applied Biosystems). The newly generated gene sequences were analyzed using the basic local alignment search tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm their origin. A MatGAT software program (Campanella et al., 2003) was then used to calculate the identity scores shared by the newly determined Mongolian sequences with the following gene sequences retrieved from GenBank; *ama-1* sequences of *B. naoakii* (n=49) from various livestock animals in Sri Lanka (LC385893, LC385894, LC485990–LC485998 and LC486031), Philippines (LC485999–LC486013), Vietnam (LC486014–LC486020 and LC486032–LC486036), Uganda (LC486021–LC486023), and Argentina (LC486024–LC486030), *B. bigemina* (n=31), *B. ovata* (n=6), and *Babesia* sp. Hue-1 (n=5). For the construction of phylogeny, the newly determined sequences, *B. naoakii* sequences representing Sri Lanka, Philippines, Vietnam, Uganda, and Argentina, and *ama1* sequences of other *Babesia* and *Theileria* species retrieved from GenBank, were aligned using an MAFFT online software (https://mafft.cbrc.jp/alignment/server/), and the

resultant alignment was analyzed using the MEGA v7.0 software program (Kumar et al., 2016) to predict the best-fitting substitution model. Finally, a maximum likelihood phylogenetic tree based on a Kimura 2-parameter substitution model (Kimura, 1980) was constructed using the MEGA software.

Statistical analyses

The positive rates were analyzed using an OpenEpi online software (https://www.openepi.com/Proportion/Proportion.htm) to calculate 95% confidence intervals based on the Wilson score interval (Wilson, 1927). The *P* values to determine the statistical significance of the differences between positive rates were calculated using a "N-1" chi-squared test (Campbell, 2007; Richardson, 2011) (https://www.medcalc.org/calc/comparison_of_proportions.php). The differences were considered statistically significant if *P* values were <0.05.

1-3. Results

The PCR findings revealed that 346 (47.7%) of 725 surveyed cattle were infected with at least one *Babesia* species. The overall positive rates of *B. bovis*, *B. bigemina*, and *B. naoakii* among the surveyed animals were 27.9, 23.6, and 5.4%, respectively (Table 2). The positive rates of *B. bovis* and *B. bigemina* were comparable (*P*=0.0612) and significantly higher (*P*<0.0001) than that of *B. naoakii*. *Babesia bovis* and *B. bigemina* were detected in all 16 provinces surveyed in the present study, whereas *B. naoakii* was found in 11 provinces (Table 2 and Fig. 6). On a per province basis, the positive rates of *B. bovis*, *B. bigemina*, and *B. naoakii* ranged from 5.9–52.0, 9.1–76.3, and 0.0–35.7%, respectively (Table 2). Of the 346 animals infected with at least one *Babesia* species, 62 (17.9%) were co-infected with two or three

Babesia species (Table 3). In fact, co-infections with all possible combinations of *Babesia* species were detected.

I also compared the positive rates between the females (n=569) and males (n=156), as well as between the 1 to 3-year-old (n=284) and >3-year-old (n=441) age groups. The positive rate of all three *Babesia* species did not differ significantly between the female and male cattle (Table 4). Although the positive rates of *B. bigemina* and *B. naoakii* did not differ between the 1 to 3- and >3-year-old age groups, the *B. bovis*-positive rate was significantly higher (P=0.0403) in >3-year-olds, compared with that of 1 to 3-year-old cattle (Table 5).

As the *B. naoakii* had not been previously reported in Mongolia, I cloned and sequenced 15 randomly selected PCR amplicons, and the resulting *ama1* sequences (GenBank accession numbers: LC506531–LC506545) were subjected to sequencing and phylogenetic analyses. The newly determined *ama1* sequences of *B. naoakii* shared 99.5–100% identity scores among themselves and 99.2–100% identity with previously reported *B. naoakii* sequences from Sri Lanka, Philippines, Vietnam, Uganda, and Argentina. The Mongolian sequences also shared 80.9–81.7%, 79.0–80.6%, and 79.0–79.5% identity scores with the *ama-1* sequences of *Babesia* sp. Hue-1, *B. bigemina*, and *B. ovata*, respectively. In the phylogenic analysis, the Mongolian sequences clustered together with the *B. naoakii* sequences from Sri Lanka, Philippines, Vietnam, Uganda, and Argentina, to form a monophyletic clade (Fig. 7). These findings confirm the validity of PCR results.

1-4. Discussion

The livestock industry is a key part of the Mongolian economy (Shagdar, 2002). However, the international trade of live cattle and their products is limited, mainly because of severe infectious diseases (Shagdar, 2002). Therefore, the control of infectious diseases of

cattle is vital in Mongolia. In the present study, I investigated whether the cattle grazed in various Mongolian provinces harbored three serious protozoan pathogens, *B. bovis*, *B. bigemina*, and *B. naoakii*. I found that the cattle throughout Mongolia were infected with all three surveyed *Babesia* species.

I found that the overall positive rates of *B. bovis* and *B. bigemina* were comparable, suggesting that both the parasite species might be transmitted by the same tick vector species with the same competency. In contrast, the lower positive rate of *B. naoakii*, as compared with those of *B. bovis* and *B. bigemina*, may infer a transmission by different tick vectors. However, these assumptions will be confirmed only through the identification of tick vectors for *B. bovis*, *B. bigemina*, and *B. naoakii*, followed by experimental infections via the infected-tick bites.

Although *B. bovis* and *B. bigemina* were detected in all surveyed provinces, *B. naoakii* was detected in 11 provinces, excluding Bayan-Ulgii, Khovd, Khuvsgul, Omnogovi, and Tuv. However, the negative result obtained in these provinces does not guarantee that the cattle in these provinces were free from the infection, as only a limited number of animals were surveyed in each province. Similarly, the differences in the positive rates among the surveyed provinces do not represent a reliable comparison of infection rates because of the low sample size. Nevertheless, these findings suggest that cattle in all the surveyed provinces are at risk of clinical bovine babesiosis. In particular, low positive rates in some of the surveyed provinces indicates an endemically unstable situation, where clinical babesiosis is common in cattle (Mahoney, 1974; Bock et al., 2004). Until now, the occurrence of clinical bovine babesiosis had not been documented in Mongolia. Mongolian cattle herds, which are managed by extensive systems, typically consist of a large head of cattle. Therefore, animals with clinical babesiosis might not have been identified. Basing the monitoring of susceptible cattle populations on several risk factors, such as age of animals (Goff et al., 2001), management practices (Rubaire-Akiiki et al., 2004), cattle breed (Bock et al., 1999), and endemic stability

(Mahoney, 1974), might be helpful in detecting clinical babesiosis in Mongolia (Bock et al., 2004). As well as putting cattle at a risk for clinical babesiosis, the presence of *Babesia* infections is a stumbling block for the export of live cattle from Mongolia, as the World Organisation for Animal Health (OIE) places restrictions on the international trade of cattle from countries endemic for bovine babesiosis (OIE, 2019). Therefore, strategies to minimize *Babesia* infections in cattle would be highly beneficial to the Mongolian economy.

In the present study, I found that the co-infections with two or three *Babesia* species were common among the surveyed cattle. Further studies are necessary to investigate whether the co-infections are due to co-transmission by the same tick species or whether an infection by one *Babesia* species results in an immunity that is unable to protect the animals from the subsequent infections with other *Babesia* species.

To investigate whether the sex and age are risk factors for *Babesia* infections in cattle, I compared the positive rates between females and males, as well as between 1 to 3-year-old and >3-year-old cattle. I found that the positive rates of all three *Babesia* species were comparable between the females and males. Both the female and male cattle surveyed in the present study were reared in an extensive management system. Therefore, the animals were highly exposed to the infected ticks regardless of their gender, which may explain the comparable positive rates between females and males. On the other hand, the positive rates of *B. bigemina* and *B. naoakii* did not differ between the age groups, but the *B. bovis*-positive rate was higher in >3-year-olds than the 1 to 3-year-old cattle. *Babesia bigemina* usually persists in host animals for less than six months before the parasites are cleared by the host immune defenses, whereas *B. bovis* is known to persist for up to 4 years (Mahoney et al., 1973). This may be why the *B. bovis*-positive rate was higher in >3- than 1 to 3-year-old animals. Although the persistence of *B. naoakii* has not been investigated yet, the comparable positive rate

between the 1 to 3- and >3-year-old groups suggests that *B. naoakii* might also be cleared from the blood of infected animals earlier than *B. bovis*.

In conclusion, in addition to reporting *B. naoakii* for the first time in Mongolia, the present study found that the cattle populations throughout Mongolia are infected with three species of *Babesia* that are capable of causing clinical bovine babesiosis. Studies to investigate the prevalence of clinical bovine babesiosis must now be a priority in Mongolia.

1-5. Summary

Bovine babesiosis caused by *Babesia* species is an economically significant disease of cattle. Severe clinical babesiosis in cattle is caused by *Babesia bovis*, *B. bigemina*, and the recently discovered *B. naoakii*. Mongolia is an agricultural country with a large cattle inventory. Although previous studies have detected active infections of *B. bovis* and *B. bigemina* in Mongolian cattle, only a few provinces were surveyed. Additionally, the endemicity of *B. naoakii* remains unknown in Mongolia. In the present study, I screened blood DNA samples from 725 cattle reared in 16 of the 21 Mongolian provinces, using the *B. bovis*-, *B. bigemina*-, and *B. naoakii* -specific PCR assays. The overall positive rates of *B. bovis*, *B. bigemina*, and *B. naoakii* were 27.9% (n=202), 23.6% (n=171), and 5.4% (n=39), respectively. *Babesia bovis* and *B. bigemina* were detected in cattle from all surveyed provinces, whereas *B. naoakii* was detected in 11 of the 16 surveyed provinces. On a per province basis, the *B. bovis*-*B. bigemina*-, and *B. naoakii*-positive rates were 5.9–52.0%, 9.1–76.3%, and 0–35.7%, respectively. In conclusion, this is the first report of *B. naoakii* infection in Mongolia. In addition, we found that the species of *Babesia,* including *B. bovis*, *B. bigemina*, and *B. naoakii*, are widespread throughout the country.

□

Fig. 5. Cattle surveyed in Mongolia.

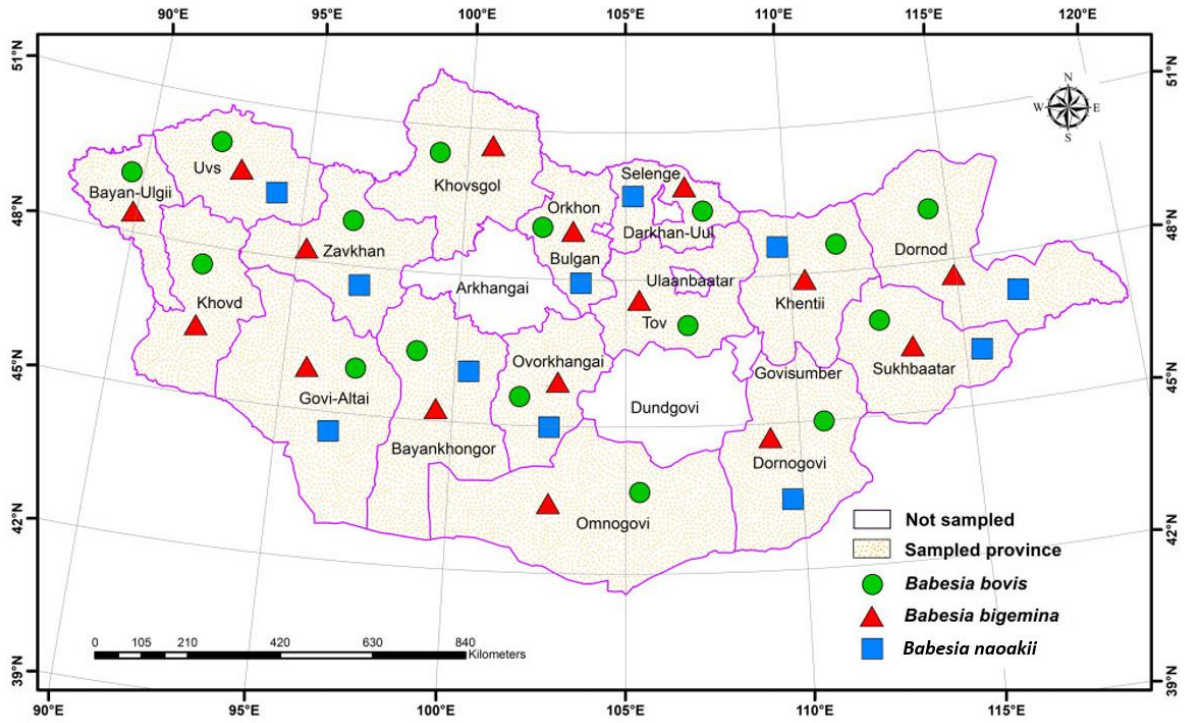

Fig. 6. Geographical distribution of three bovine *Babesia* species in Mongolia. Positive results for *Babesia bovis*, *B. bigemina*, and *B. naoakii* are indicated in the surveyed provinces by circles, triangles, and squares, respectively. Note that *B. bovis* and *B. bigemina* were detected in cattle from all 16 surveyed provinces, while *B. naoakii* was detected in 11 provinces.

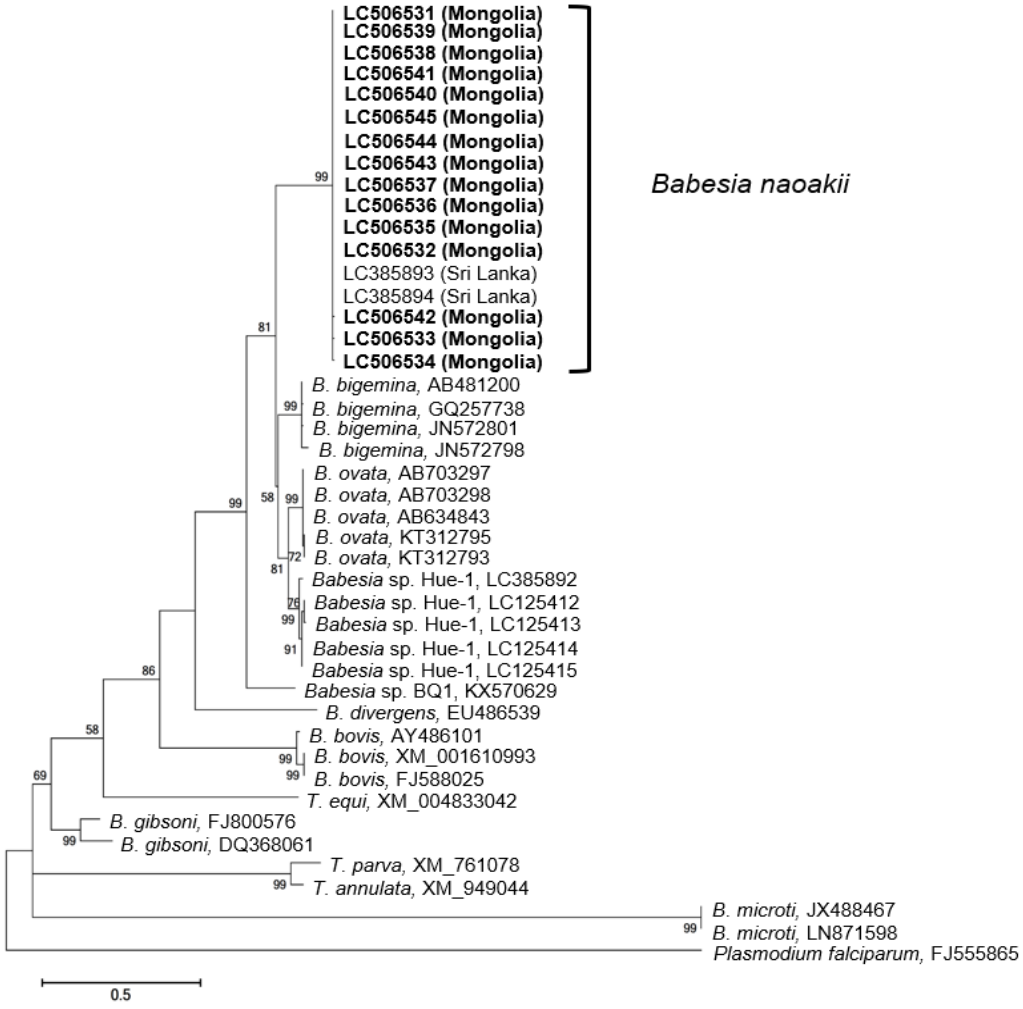

Fig. 7. Phylogenetic tree of the *ama-1* genes. A maximum likelihood phylogeny was constructed using *B. naoakii ama-1* gene sequences from Mongolia and other countries together with the *ama-1* sequences from other *Babesia* and *Theileria* species. The sequences determined in the present study are shown in boldface letters. Note that the Mongolian sequences clustered together with previously reported *B. naoakii ama-1* sequences to form a monophyletic clade.

Table 1. List of PCR primers

Species	Target gene	Primer (5'-3')	Annealing T (°C)	Product size (bp)	Reference
B. bovis	*rap-1*	Outer forward: cacgaggaaggaactaccgatgttga	55	356	(Figueroa et al. 1993)
		Outer reverse: ccaaggagcttcaacgtacgaggtca			
		Inner forward: tcaacaaggtactctatatggctacc	55	298	
		Inner reverse: ctaccgagcagaaccttcttcaccat			
B. bigemina	*ama1*	Forward: tactgtgacgaggacggatc	60	211	(Sivakumar et al. 2012)
		Reverse: cctcaaaagcagattcgagt			
B. naoakii	*ama1*	Forward: tactgtgacgaggacggatc	64	371	(Sivakumar et al. 2018)
		Reverse: cctcaaaagcagattcgagt			

Table 2. PCR detection of bovine *Babesia* species in 725 cattle from Mongolia

Province	No. sample	*B. bovis*		*B. bigemina*		*B. naoakii*	
		No. positive	% (CI[a])	No. positive	% (CI)	No. positive	% (CI)
Bayankhongor	75	39	52.0 (40.9-62.9)	12	16.0 (9.4-25.9)	6	8 (3.7-16.4)
Bayan-Ulgii	15	4	26.7 (10.9-52.0)	2	13.3 (3.7-37.9)	0	0.0 (0.0-20.4)
Bulgan	21	6	28.6 (13.8-50.0)	11	52.4 (32.4-71.7)	1	4.8 (0.9-22.7)
Dornod	111	36	32.4 (24.4-41.6)	13	11.7 (7.0-19.)	8	7.2 (3.7-13.6)
Dornogovi	40	8	20.0 (10.5-34.8)	11	27.5 (16.1-42.8)	2	5.0 (1.4-16.5)
Govi-Altai	84	25	29.8 (20.5-40.9)	14	16.7 (10.2-26.1)	3	3.6 (1.2-10.0)
Khentii	14	1	7.1 (1.3-31.5)	3	21.4 (7.6-47.6)	5	35.7 (16.4-61.2)
Khovd	78	13	16.7 (10.0-26.5)	17	21.8 (14.1-32.2)	0	0.0 (0.0-4.7)
Khovsgol	17	1	5.9 (1.1-27.0)	7	41.2 (21.6-64.0)	0	0.0 (0.0-18.4)
Omnogovi	3	1	33.3 (6.2-79.2)	1	33.3 (6.2-79.2)	0	0.0 (0.0-56.2)
Ovorkhangai	11	3	27.3 (9.7-56.6)	1	9.1 (1.6-37.7)	1	9.1 (1.6-37.7)
Selenge	17	4	23.5 (9.6-47.3)	6	35.3 (17.3-58.7)	1	5.9 (1.1-27.0)
Sukhbaatar	30	5	16.7 (7.3-33.6)	6	20.0 (9.5-37.3)	1	3.3 (0.6-16.7)
Tov	38	5	13.2 (5.8-27.3)	29	76.3 (60.8-87.0)	0	0.0 (0.0-9.2)
Uvs	119	39	32.8 (25.0-41.6)	22	18.5 (12.5-26.4)	4	3.4 (1.3-8.3)
Zavkhan	52	12	23.1 (13.7-36.1)	16	30.8 (19.9-44.3)	7	13.5 (6.7-25.3)
Total	725	202	27.9 (24.7-31.2)	171	23.6 (20.6-26.8)	39	5.4 (4.0-7.3)

[a] 95% confidence interval (CI)

Table 3. Co-infection of *Babesia* species in the surveyed cattle from Mongolia

Parasite species	**No. positive (%[a])**
B. bovis + *B. bigemina* + *B. naoakii*	4 (1.2)
B. bovis + *B. bigemina*	45 (13.0)
B. bovis + *B. naoakii*	4 (1.2)
B. bigemina + *B. naoakii*	9 (2.6)
Total	62 (17.9)

[a] Co-infection rates were expressed as the percentage of animals infected (n=346) with at least one *Babesia* species.

Table 4. Positive rates of *Babesia* species in female and male cattle

Parasites	Female (n=569)		Male (n=156)		*P* value
	No. positive	% (CI[a])	No. positive	% (CI)	
B. bovis	162	28.5 (24.9-32.3)	40	25.6 (19.4-33.02)	0.4745
B. bigemina	136	23.9 (19.4-33.0)	35	22.4 (16.6-29.6)	0.6960
B. naoakii	26	4.6 (3.1-6.6)	13	8.3 (4.9-13.7)	0.0702

[a] 95% confidence interval (CI)

Table 5. Positive rates of *Babesia* species in 1-3-year and >3-year age groups

Parasites	1-3-year (n=284)		>3-year (n=441)		*P* value
	No. positive	% (CI[a])	No. positive	% (CI)	
B. bovis	67	23.6 (19.0-28.9)	135	30.6 (26.5-35.1)	0.0403
B. bigemina	62	21.8 (17.4-27.0)	109	24.7 (20.9-29.0)	0.3695
B. naoakii	19	6.7 (4.2-10.4)	20	4.5 (3.0-6.9)	0.1996

[a] 95% confidence interval (CI)

Chapter 2

Molecular survey of bovine *Babesia* species in Bactrian camels (*Camelus bactrianus*) in Mongolia

2-1. Introduction

Bovine babesiosis is a serious disease affecting cattle populations, especially in tropical and subtropical regions of the world (Suarez et al., 2019). The disease is caused by species of intra-erythrocytic protozoan parasites belonging to the genus *Babesia*; these parasites are transmitted by ticks (Bock et al., 2004). The invasion, multiplication, and egress of *Babesia* cause a massive hemolysis, leading to severe anemia in the infected cattle (Hunfeld et al., 2008). Although several *Babesia* species infect cattle, only *Babesia bovis*, *B. bigemina*, *B. divergens*, and *B. naoakii* are known to cause clinical bovine babesiosis (Bock et al., 2004; Sivakumar et al., 2018; Zintl et al., 2003).

Bovine *Babesia* species infect not only cattle, but also other animals (Elsify et al., 2015; Jaimes-Dueñez et al., 2018; Sivakumar et al., 2013, 2014, 2020). These non-cattle hosts may act as reservoirs, from which the *Babesia* species can be transmitted to cattle via tick vectors (Jaimes-Dueñez et al., 2018). Therefore, in order to ensure effective control of bovine babesiosis, it is imperative to minimize *Babesia* infections not only in cattle but also in non-cattle hosts (Romero-Salas et al. 2016). However, only a few non-cattle hosts, including water buffalo (*Bubalus bubalis*), goats (*Capra aegagrus hircus*), sheep (*Ovis aries*), dromedary camels (*Camelus dromedaries*), horses (*Equus caballus*), and white-tailed deer (*Odocoileus virginianus*), have been studied to date (Cantu-C et al., 2009; Criado-Fornelio et al., 2009; Elsify et al., 2015; Fereig et al., 2017; Jaimes-Dueñez et al., 2018; Sivakumar et al., 2013, 2014,

2020). Among them, only water buffalo are considered to play a significant role in the epidemiology of bovine *Babesia* species (Jaimes-Dueñez et al., 2018). Conversely, the role of other host animals remains unclear. For instance, *in vitro* studies have demonstrated that *B. bovis* undergoes only limited asexual multiplication in caprine and ovine RBCs (Gaffar et al., 2003).

Bactrian camels (*Camelus bactrianus*) are found mostly in the desert regions of the countries in Central Asia, such as Mongolia, China, and Kazakhstan (Chuluunbat et al., 2014; Imamura et al., 2017; Ji et al., 2009). Recent studies have reported *Theileria equi* and *T. sinensis* infections in Bactrian camels (Li et al., 2019; Li et al., 2020). However, the infections with bovine *Babesia* species have not been investigated in this animal species.

In Mongolia, Bactrian camels are maintained primarily by nomads in the Gobi Desert region under harsh environmental conditions (Chuluunbat et al., 2014). They have long been used for the productions of milk, wool, and meat, as well as for the transportation of people and goods. As of 2019, the estimated camel population was approximately 454,000 in Mongolia (National Statistics Office of Mongolia, 2021). The four camel breeds in Mongolia include Mongolian native camel (MNT), Hos Zogdort (HZ), Galbiin Gobiin Ulaan (GGU), and Haniin Hetsiin Huren (HHH) breeds (Chuluunbat et al., 2014). These camels are usually co-grazed in pasture lands with other livestock animals, including cattle, as all farm animals are maintained under an extensive management system (Suttie, 2005).

Notably, all three tick species known to infest Bactrian camels, including *Dermacentor marginatus*, *D. nuttalli*, and *Hyalomma asiaticum*, also infest cattle in Mongolia (Narankhajid et al., 2018). Therefore, I hypothesized that the *Babesia* species infecting cattle may also infect these camels. To test this hypothesis, I surveyed the Bactrian camels reared in various Mongolian provinces for *B. bovis*, *B. bigemina*, and *B. naoakii*, which had been recently detected in Mongolian cattle (Otgonsuren et al., 2020).

2-2. Materials and methods

Ethical statement

All animal protocols were approved by the Animal Care and Use Committee of Obihiro University of Agriculture and Veterinary Medicine, Japan (Approval No. 28-45). All experiments were carried out in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Blood sampling and DNA extraction

In July 2017 and April 2018, blood samples were randomly collected from 305 Bactrian camels (Fig. 8) reared in the following six Mongolian provinces: Bayan-Ulgii, Govi-Altai, Khovd, Uvs, and Zavkhan in Western Mongolia and Bayankhongor in Southern Mongolia (Fig. 9, Table 6). The sampled animals included 84 females and 221 males and three breeds (MNT, n = 120; HZ, n = 177; and GGU, n = 8).

At the time of sampling, all animals were asymptomatic and apparently healthy. Approximately 5 ml of blood was collected from the jugular vein of each animal, and placed in a sterile vacutainer tube containing EDTA. Subsequently, thin blood smears were prepared on glass slides using the samples collected from 33, 24, and 12 animals in Khovd, Uvs, and Zavkhan provinces, respectively. Genomic DNA was extracted from each blood sample, using a previously reported phenol:chloroform:isoamyl alcohol method (Sambrook and Russell, 2001). The DNA samples were then stored at −30°C until further use.

Microscopic examination for *Babesia* parasites

Thin blood smears were fixed with absolute methanol, stained with Giemsa, and then observed under a light microscope for detecting *Babesia* parasites within the infected RBCs.

PCR screening for *B. bovis*, *B. bigemina*, and *B. naoakii*

All the DNA samples from the Bactrian camels were screened, using previously described PCR assays specific to *B. bovis*, *B. bigemina*, and *B. naoakii*. A single-step PCR assay using the inner forward and reverse primers of a previously described nested PCR assay based on rhoptry-associated protein 1 gene (*rap-1*), was used to detect *B. bovis* (Figueroa et al., 1993), while two single-step PCR assays based on apical membrane antigen 1 genes (*ama-1*) were used to detect *B. bigemina* (Sivakumar et al., 2012) and *B. naoakii* (Sivakumar et al., 2018). The primers, reaction mixtures, and cycling conditions for the PCR assays have been described in a previous report (Otgonsuren et al., 2020). DNA samples extracted from *in vitro* cultures of *B. bovis* and *B. bigemina* and from blood of a cow naturally infected with *B. naoakii* (Sivakumar et al., 2018) were used as positive controls in the respective PCR assays. A DNA sample from a non-infected cow was used as a negative control, while a no template control was used to monitor the cross-contamination.

The resultant PCR products were resolved by agarose gel electrophoresis, stained with ethidium bromide, and then visualized under UV light. The samples were considered to be positive for *B. bovis*, *B. bigemina*, and *B. naoakii* infections, if the band sizes were approximately 298, 211, and 371 bp, respectively (Otgonsuren et al., 2020).

Cloning and sequencing

The selected amplicons obtained from each PCR assay were cloned after extracting these amplicons, using a commercial kit (QIAquick Gel Extraction Kit, Qiagen, Hilden,

Germany) and ligating them to a PCR 2.1 plasmid vector (PCR 2.1-TOPO, Invitrogen, Carlsbad, CA, USA). The inserted gene fragments were then sequenced, using an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Branchburg, NJ, USA). The newly generated sequences were analysed, using a basic local alignment search tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm the origins of gene sequences and to determine the identity scores shared with the corresponding sequences from GenBank.

Statistical analyses

The 95% confidence intervals (CIs) for the positive rates were calculated based on Wilson's score interval (Wilson, 1927) using an OpenEpi online software (https://www.openepi.com/Proportion/Proportion.htm). The *P* values for the differences between positive rates were calculated, using an "*N*−1" chi-square test (https://www.medcalc.org/calc/comparison_of_proportions.php) (Campbell, 2007; Richardson, 2011). The differences were considered to be statistically significant, if the *P* values were <0.05.

2-3. Results

The microscopic examination revealed that 22 (31.9%) of 69 examined camels, including 10 of 33, 8 of 24, and 4 of 12 animals in Khovd, Uvs, and Zavkhan provinces, respectively, were positive for *Babesia* parasites (Fig. 10). Although the species differentiation was not possible in microscopy, the subsequent PCR results demonstrated that the camels were infected with all three *Babesia* species surveyed (Fig. 9, Table 6). All microscopy-positive animals were also positive by the PCR assays. Furthermore, the infections were particularly common in camels, considering the fact that 160 (52.5%) of the 305 Bactrian camels surveyed

were infected with at least one *Babesia* species. The overall positive rates were 32.1%, 21.6%, and 24.3% for *B. bovis*, *B. bigemina*, and *B. naoakii* infections, respectively (Table 6). Bactrian camels from all surveyed provinces, except for the Bayan-Ulgii, were positive for at least one *Babesia* species (Fig. 9, Table 6). Co-infections with two or three *Babesia* species were also common among the surveyed camels. Of 160 animals infected with at least one *Babesia* species, 66 (41.3%) had co-infections (Table 7). *Babesia bovis*, *B. bigemina*, and *B. naoakii* were identified in three (Uvs, Khovd, and Zavkhan), five (Uvs, Zavkhan, Khovd, Govi-Altai, and Bayankhongor), and four provinces (Uvs, Khovd, Zavkhan, and Govi-Altai), respectively. The positive rates of *B. bovis*, *B. bigemina*, and *B. naoakii* in the provinces where they were detected, ranged 16.8–67.5%, 14.8–37.5%, and 9.1–53.3%, respectively (Table 6).

In the present study, the positive rates of *B. bovis* and *B. bigemina* in Bactrian camels were comparable between the 1−4-year and >4-year age groups, but the *B. naoakii*-positive rate was higher ($P = 0.0027$) in the >4-year age group (28.0%) than that in the 1−4-year age group (9.7%) (Table 8). Comparison of the positive rates between female and male animals revealed that the rates for all three tested *Babesia* species were significantly higher ($P < 0.0001$) in females than those in males (Table 9). Furthermore, I also analyzed the differential positive rates among the camel breeds (MNT, HZ, and GGU) (Table 10). The comparative analysis indicated that the positive rates of *B. bovis*, *B. bigemina*, and *B. naoakii* infections were significantly higher in MNT than in HZ ($P < 0.0001$, 0.0285, < 0.0001, respectively). I sampled only eight GGU Bactrian camels, and therefore did not consider this breed in our analysis.

I cloned and sequenced nine, eight, and nine randomly selected amplicons from *B. bovis*, *B. bigemina*, and *B. naoakii* PCR assays, respectively. The newly determined *B. bovis rap-1* sequences (LC598509–LC598517) shared 99.7–100% identities with a sequence reported from Egypt (AB917246), while the *B. bigemina ama-1* sequences (LC598518−LC598525) shared 99.5–100% identity scores with the sequences from Sri Lanka

(LC438499), Israel (KU557538), Turkey (KP000033), South Africa (KF626599), and Italy (GQ257740). Similarly, the *ama-1* sequences generated from the amplicons of *B. naoakii* PCR assay (LC598526−LC598534) shared 99.2–100% identities with the sequences from several countries, including Mongolia (LC506534), Vietnam (LC486036), Argentina (LC486028), and Uganda (LC486021). These findings confirmed that the respective PCR assays specifically detected the targeted *Babesia* species.

2-4. Discussion

I designed the present study to investigate whether Bactrian camels in Mongolia were infected with bovine *Babesia* species, including *B. bovis*, *B. bigemina*, and *B. naoakii*, which are capable of causing clinical babesiosis in cattle. Bactrian camels are reared mainly in Western and Southern Mongolia. However, except for Bayankhongor, cattle farming is not very common in Southern Mongolia. Therefore, the present survey was conducted only in Western Mongolia and Bayankhongor province. The sampling was carried out during the tick-active season in Mongolia (March to September), and therefore tick infestation was common among the sampled camels (Fig.8).

The PCR findings indicated that Bactrian camels in all surveyed provinces, except for the Bayan-Ulgii, were infected with bovine *Babesia* species. The negative results obtained in the PCR assays do not necessarily mean that the camel population in Bayan-Ulgii is free from *Babesia* infections, as only 10 animals were surveyed. There were differences in the positive rates of each *Babesia* species among the surveyed provinces. However, a fair comparison among the positive rates in different provinces was not possible because of the low sample numbers in each area surveyed. Therefore, large-scale epidemiological surveys of bovine *Babesia* species are required in Bactrian camels.

The *B. bovis*- and *B. bigemina*- positive rates in Bactrian camels (32.4% and 21.6%, respectively) were comparable to those in cattle (27.9% and 23.6%, respectively) in Mongolia (Otgonsuren et al., 2020). In addition, the positive rate of *B. naoakii* (24.3%) was higher than that in cattle (5.4%) (Otgonsuren et al., 2020). These observations indicate that the Bactrian camels are an important host for bovine *Babesia* species. As reported in previous study, the tick species infesting camels (*D. marginatus, D. nuttalli,* and *H. asiaticum*) also infest cattle in Mongolia (Narankhajid et al., 2018), and therefore, tick transmission of bovine *Babesia* species might be possible between these two host animals. However, this assumption can be confirmed, only if additional experiments demonstrate that the tick species infesting camels are capable of transmitting the bovine *Babesia* species, as their vector competence is currently unknown.

I found that *B. bovis*- and *B. bigemina*-positive rates in Bactrian camels were comparable between the age groups. As shown in a previous study, the positive rates of hemoprotozoan parasites may increase with rising age, if the infections persist (Rüegg et al., 2007). In a recent PCR-based longitudinal study conducted in Sri Lanka, most of the *B. bovis*- and *B. bigemina*-positive cattle turned negative within 3 months, which suggests that the infection persistence of these parasite species might be less pronounced (Sivakumar et al., 2016). In agreement with this observation, my previous study found that the positive rates of *Babesia* species were comparable between the different age groups of cattle in Mongolia (Otgonsuren et al., 2020). Therefore, the non-persistence of infections may be a reason why the positive rates of *B. bovis* and *B. bigemina* in Bactrian camels were comparable between the age groups. In contrast, *B. naoakii*-positive rate was higher in the older animals, as compared to that of younger ones. These findings suggest that in Bactrian camels, the persistence of *B. naoakii* infection might be pronounced, as compared to those of *B. bovis* and *B. bigemina*. However, this argument is inconclusive, as the findings might have been confounded by several factors, such as parasitemia levels and period between the time of infection and sampling.

I also found that the positive rates of all three *Babesia* species were higher in females and MNT breed than those in males and HS breed, respectively. The observed discrepancies could be associated with the differences in the tick burden. Therefore, further studies investigating the tick burden and the predisposing factors of tick infestation, such as coat and skin characteristics (Ibelli et al., 2012; Shyma et al., 2015) in the females and males, as well as in the MNT and HZ breeds, may elucidate the differences in the positive rates of tested *Babesia* species. *Babesia* infections in cattle are known to be influenced by grazing management practices (Rubaire-Akiiki et al., 2004). In the present study, the comparison of the positive rates based on management practices was not possible, because all Bactrian camels are managed extensively from their birth in Mongolia.

The bovine *Babesia* species tend to have a minimum clinical significance in water buffalos (Benitez et al. 2018). However, the clinical significance of these species is not clear in Bactrian camels. Therefore, experimental infections are essential to determine the clinical relevance of bovine *Babesia* species in Bactrian camel. Even if the detected parasites are of clinical significance in Bactrian camels, the clinical babesiosis may be a relatively uncommon condition in Mongolia, considering that the high positive rates may indicate an endemically stable situation (Bock et al., 2004; Mahoney and Ross, 1972). It is imperative to conduct a serological survey of the bovine *Babesia* species in Mongolian Bactrian camels in order to confirm the assumption related to the endemic stability.

In conclusion, Bactrian camels in Mongolia are commonly infected with at least three bovine *Babesia* species that are capable of causing clinical babesiosis in cattle. The present study is possibly the first to report the existence of bovine *Babesia* species in Bactrian camels.

2-5. Summary

Bovine babesiosis, which is caused by species of genus *Babesia*, is a leading cause of considerable economic losses to the cattle industry each year. Bovine *Babesia* species have frequently been detected in non-cattle hosts, such as water buffalo (*Bubalus bubalis*), from which the parasites can be transmitted by ticks to cattle. Therefore, *Babesia* infections should be minimized not only in cattle, but also in non-cattle carriers. In the present study, I surveyed the Bactrian camels (*Camelus bactrianus*) in Mongolia for three clinically significant bovine *Babesia* species, including *Babesia bovis*, *B. bigemina*, and *B. naoakii*, which had been detected previously in Mongolian cattle. I screened blood DNA samples from 305 Bactrian camels in six Mongolian provinces for these species, using the parasite-specific PCR assays. The findings showed that the Bactrian camels in Mongolia were infected with all three *Babesia* species surveyed. The overall positive rates of *B. bovis*, *B. bigemina*, and *B. naoakii* infections were, 32.1%, 21.6%, and 24.3%, respectively, whereas 52.5% of the surveyed animals were infected with at least one parasite species. I also found that the female Bactrian camels and the Mongolian native camel breeds had significantly higher *Babesia*-positive rates than the male Bactrian camels and the Hos Zogdort breed. In Mongolia, cattle and Bactrian camels usually share common pasture lands for grazing; furthermore, tick species infesting cattle also infest Bactrian camels. Our findings, together with these observations, suggest that the tick transmission of bovine *Babesia* species might be possible between the cattle and Bactrian camels. Therefore, strategies for the control of bovine babesiosis in Mongolia should include methods to minimize bovine *Babesia* species infections in Bactrian camels.

Fig. 8. Survey of Bactrian camels for bovine *Babesia* species. Tick infestations (indicated by an arrow) were commonly found among the surveyed Bactrian camels.

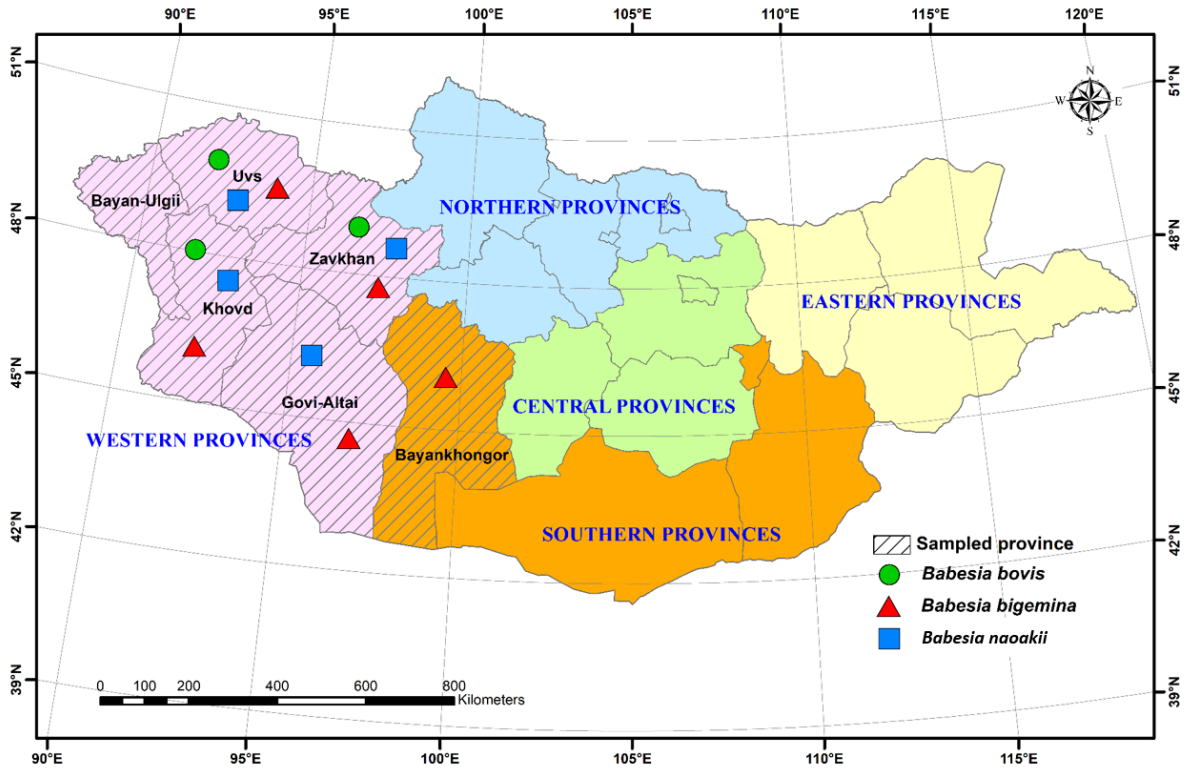

Fig. 9. Map showing the Mongolian provinces where the Bactrian camels were surveyed for *Babesia bovis*, *B. bigemina*, and *B. naoakii* infections. The map was created with an ArcGIS v10.1 software program (Environmental Systems Research Institute, Redlands, CA, USA). Among the six provinces surveyed (marked with lines), five had animals infected with the tested *Babesia* species.

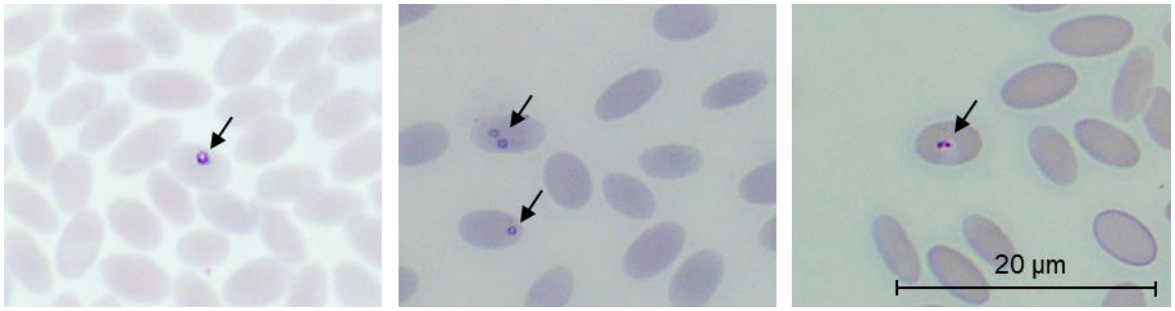

Fig. 10. Micrographs of *Babesia* parasites detected in Bactrian camels. Giemsa-stained thin blood smears prepared from Bactrian camels were observed under a light microscope for detecting *Babesia* parasites (indicated by arrows) within the infected RBCs.

Table 6. PCR detections of *B. bovis*, *B. bigemina*, and *B. naoakii* in 305 Bactrian camels from Mongolia

Province	No.	*B. bovis*		*B. bigemina*		*B. naoakii*	
		No. positive	% (CI[a])	No. positive	% (CI)	No. positive	% (CI)
Bayankhongor	8	0	0.0 (0.0–32.4)	3	37.5 (13.7–69.4)	0	0.0 (0.0–32.4)
Bayan-Ulgii	10	0	0.0 (0.0–34.5)	0	0.0 (0.0–34.5)	0	0.0 (0.0–34.5)
Govi-Altai	22	0	0.0 (0.0–14.9)	7	31.8 (16.4–52.7)	2	9.1 (2.5–27.8)
Khovd	155	26	16.8 (11.7–23.4)	23	14.8 (10.1–21.3)	15	9.7 (6.0–15.4)
Uvs	30	18	60.0 (42.3–75.4)	5	16.7 (7.3–33.6)	16	53.3 (36.1–69.8)
Zavkhan	80	54	67.5 (56.6–76.8)	28	35.0 (25.5–45.9)	41	51.3 (40.5–61.9)
Total	305	98	32.1 (27.1–37.6)	66	21.6 (17.4–26.6)	74	24.3 (19.8–29.4)

[a]95% confidence interval.

Table 7. Co-infection of *Babesia* species in Bactrian camels surveyed in Mongolia

Parasite species	No. positive (%[a])
B. bovis + *B. bigemina* + *B. naoakii*	12 (7.5)
B. bovis + *B. bigemina*	17 (10.6)
B. bovis + *B. naoakii*	32 (20.0)
B. bigemina + *B. naoakii*	5 (3.1)
Total	66 (41.3)

[a]Co-infection rates are expressed as the percentage of the number of animals infected (*n* = 160) with at least one *Babesia* species.

Table 8. Positive rates of *Babesia* species in 1−4-year and > 4-year age groups

Parasites	1−4-year (*n* = 62)		>4-year (*n* = 243)		*P* value
	No. positive	% (CI[a])	No. positive	% (CI)	
B. bovis	17	27.4 (17.9–39.6)	81	33.3 (27.7–39.5)	0.3752
B. bigemina	15	24.2 (15.3–36.2)	51	21 (16.3–26.5)	0.5856
B. naoakii	6	9.7 (4.5–19.6)	68	28 (22.7–33.9)	0.0027

[a]95% confidence interval.

Table 9. Positive rates of *Babesia* species in female and male Bactrian camels

Parasites	Female (*n* = 84)		Male (*n* = 221)		*P* value
	No. positive	% (CI[a])	No. positive	% (CI)	
B. bovis	58	69.1 (58.5–77.9)	40	18.1 (13.6–23.7)	< 0.0001
B. bigemina	45	53.6 (43.0–63.9)	21	9.5 (6.3–14.1)	< 0.0001
B. naoakii	48	57.1 (46.5–67.2)	26	11.8 (8.2–16.7)	< 0.0001

[a]95% confidence interval.

Table 10. Positive rates of *Babesia* species in three Bactrian camel breeds in Mongolia

Parasites	MNT[a] (*n* = 120)		HZ (*n* = 177)		GGU (*n* = 8)	
	No. positive	% (CI[b])	No. positive	% (CI)	No. positive	% (CI)
B. bovis	72	60.0 (51.1–68.3)	26	14.7 (10.2–20.7)	0	0.0 (0.0–32.4)
B. bigemina	33	27.5 (20.3–36.1)	30	16.9 (12.1–23.2)	3	37.5 (13.7–69.4)
B. naoakii	57	47.5 (38.8–56.4)	17	9.6 (6.1–14.8)	0	0.0 (0.0–32.4)

[a]Three Bactrian camel breeds, including Mongolian native camel (MNT), Hos Zogdort (HZ), and Galbiin Gobiin Ulaan (GGU), were sampled.

[b]95% confidence interval.

Chapter 3

The first survey of bovine *Babesia* species infecting yaks (*Bos grunniens*) in Mongolia

3-1. Introduction

Yaks (*Bos grunniens*) are high-altitude bovines found in several Asian countries, including Afghanistan, Bhutan, China, India, Kyrgyzstan, Mongolia, Nepal, Pakistan, Russia, and Tajikistan (Joshi et al., 2020). Yaks are important to the local economy, because they provide meat, milk, fiber, and hide (Wiener et al., 2013). However, various infectious diseases caused by bacteria, viruses, and parasites pose a threat to the health of yaks (RangaRao et al., 1994; Mauroy et al., 2008; Han et al., 2013). Tick infestations and tick-borne pathogens, including *Babesia* species, have further been reported in yaks (Li et al., 2020; He et al., 2021). Previous studies conducted in India and China reported that yaks were infected with *Babesia bovis* and *Babesia bigemina* (Saravanan et al., 2013; Qin et al., 2015; He et al., 2021), which cause clinical babesiosis in cattle (Bock et al., 2004). Clinical bovine babesiosis is characterized by intravascular hemolytic anemia and the associated symptoms, such as fever, hemoglobinuria, and icterus caused by the parasites' asexual reproduction within and egress from the infected RBCs (Homer et al., 2000). In non-cattle hosts, such as buffalo, infection with bovine *Babesia* species may be asymptomatic (Mahmmod, 2013). However, a previous study concluded that yaks infected with the *Babesia* species were anemic and had lower hemoglobin concentration, hematocrit, and RBC counts, as compared with those of healthy yaks (Saud et al., 2005). Therefore, controlling bovine babesiosis is clinically important in yaks. Furthermore, because the infection can be transmitted from the non-cattle hosts to cattle via tick vectors (Jaimes-Dueez et al., 2018), minimizing *Babesia* infection in yaks may be

critical in managing the babesiosis in cattle in the countries where these livestock animals coexist.

A livestock-rich agricultural country, Mongolia had 67.4 million livestock animals, including 901,295 yaks, registered by 2021 (www.1212.mn/stat.aspx). Because yaks can survive in harsh environments, they are raised for meat, milk, and wool productions in mountainous and forested areas in 13 of Mongolia's 21 provinces (Magash, 2003). Yaks also serve as a mode of transportation in Mongolia's mountainous regions. Therefore, yaks are considered as an important part of Mongolia's economy.

Nomadic farmers in Mongolia often maintain mixed farms consisting of various livestock animals, including cattle, horses, yaks, Bactrian camels, sheep, and goats (Suttie, 2005). Reared under an extensive management system since birth, these livestock animals are grazed together in Mongolia (Suttie, 2005). Therefore, tick species that infest one animal type may infest others (Narankhajid et al., 2018). For example, tick species that infest cattle further infest Bactrian camels in Mongolia (Narankhajid et al., 2018). As a result, *Babesia* species that infect cattle have the potential to infect other livestock animals in Mongolia. According to a recent study, cattle in Mongolia were infected with *B. bovis*, *B. bigemina*, and *Babesia.* sp Mymensingh (formerly known as *B. naoakii*) (Otgonsuren et al., 2020; Sivakumar et al., 2022). The subsequent survey observed that Bactrian camels, which are reared together with cattle were also infected with these *Babesia* species in Mongolia (Otgonsuren et al., 2022). I, therefore, hypothesized that Mongolian yaks are likewise infected with these bovine *Babesia* species, which infect cattle in Mongolia. To test this hypothesis, I conducted an epidemiological survey to detect the infection with bovine *Babesia* species in Mongolian yaks.

3-2. Materials and methods

Ethical statement

All animal protocols were approved by the Animal Care and Use Committee of Obihiro University of Agriculture and Veterinary Medicine, Japan (Approval No. 22–10). All experiments were carried out in accordance with the Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Blood sampling and DNA extraction

From 2014 to 2017, I collected blood samples from 375 yaks (Fig. 11) grazed in eight Mongolian provinces, namely Bayankhongor, Bayan-Ulgii, Khovd, Khovsgol, Omnogovi, Ovorkhangai, Uvs, and Zavkhan (Fig. 12, Table 11). Among the sampled animals, 290 were females, and 85 were males. All animals appeared healthy during the sampling period. From the jugular vein of each animal, approximately 5 ml of blood was collected into a sterile vacutainer tube containing EDTA. Thin smears were prepared on glass slides, using blood from 315 of the 375 animals sampled.

Subsequently, DNA from each blood sample was extracted using a phenol:chloroform: isoamyl alcohol method (Sambrook et al., 1989). The DNA samples were stored at −30°C until they were used.

Microscopic examination for *Babesia* parasites

The blood smears were fixed with an absolute methanol, and then stained with Giemsa solution. The stained smears were examined under a light microscope with a 100 × objective

lens and emersion oil for the morphological detection of *Babesia* parasites, as previously described (Lempereur et al., 2017).

PCR screening for *B. bovis*, *B. bigemina*, and *B. naoakii*

I screened all 375 DNA samples from yaks, using previously described *B. bovis*-, *B. bigemina*-, and *B. naoakii*-specific PCR assays. Briefly, the samples were screened for *B. bovis* infection using a nested PCR assay targeting the rhoptry-associated protein 1 gene (*rap-1*) (Figueroa et al., 1993). The samples were also screened for *B. bigemina* and *B. naoakii* infections, using two single-step PCR assays developed based on their apical membrane antigen 1 genes (*ama-1*) (Sivakumar et al., 2012, 2018). The primers, reaction mixtures, and cycling conditions for the PCR assays have been described in a previous report (Otgonsuren et al., 2020). DNA samples prepared from *in vitro* cultures of *B. bovis* and *B. bigemina* and from a cow infected with *B. naoakii* were used as the positive controls in the respective PCR assays, while a reaction mixture without a DNA template was used as a negative control (Sivakumar et al., 2018).

The PCR products were separated by agarose gel electrophoresis, stained with ethidium bromide, and then visualized under ultraviolet light. The samples were considered to be positive for *B. bovis*, *B. bigemina*, and *B. naoakii* infections, if the band sizes in PCR assays were approximately 298, 211, and 371 bp, respectively (Figueroa et al., 1993; Sivakumar et al., 2012, 2018).

Cloning and sequencing

Randomly selected amplicons from each PCR assay were extracted from the agarose gel, using a commercial kit (QIAquick Gel Extraction Kit, Qiagen, Hilden, Germany), and then ligated to a PCR 2.1 plasmid vector (PCR2.1®-TOPO®, Invitrogen, Carlsbad, CA, USA). The

inserted gene fragments were sequenced using an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Branchburg, NJ, USA). The newly generated sequences were analyzed with a basic local alignment search tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm the origins of gene sequences and to determine the identity scores shared with the corresponding sequences previously registered in the GenBank database.

Statistical analyses

The 95% confidence intervals (CI) were calculated for the positive rates using an OpenEpi online software (https://www.openepi.com/Proportion/Proportion.htm) based on Wilson's score interval (Wilson, 1927). The *P* values were calculated for the differences among positive rates, using the N − 1 chi-square test (https://www.medcalc.org/calc/comparison_of_proportions.php) (Campbell, 2007; Richardson, 2011). Differences were considered to be statistically significant, if *P* values were less than 0.05.

3-3. Results

The present study surveyed a total of 375 yaks grazed in eight Mongolian provinces for the infections with bovine *Babesia* species. Microscopic examination of blood smears prepared from 315 of the surveyed yaks revealed the presence of intraerythrocytic *Babesia* parasites in 34 (10.8%) of the animals (Fig. 13 and Table 11). Except for Khovsgol, all surveyed provinces had the *Babesia*-positive yaks, with the positive rates ranging from 4.7% to 16.1%.

The results of PCR assays indicated that the Mongolian yaks were infected with *B. bovis* and *B. bigemina* (Fig. 12 and Table 11). However, all samples tested negative for *B. naoakii* infection. *Babesia bovis* was detected in a large number of animals; 238 (63.5%) of

375 yaks were positive. All surveyed provinces had the infected yaks with the positive rates ranging from 22.8% to 95.0% (Table 11). In contrast, *B. bigemina* was detected in only eight (2.1%) animals in three provinces (Bayankhongor, Bayan-Ulgii, and Omnogovi). To validate the PCR results, 27 and 4 randomly selected amplicons from *B. bovis*- and *B. bigemina*-PCR assays, respectively, were sequenced. The resulting *B. bovis rap-1* sequences (GenBank accession no: LC721051–LC721077) shared high identity scores (98.0–100.0%) with those previously reported in Mongolia (LC598517), Egypt (AB917246), Sri Lanka (LC438493), Brazil (KC964615), Cuba (JF279443), Argentina (AF030056), and Mexico (AF027149). Similarly, the newly generated *B. bigemina ama-1* sequences (LC721078–LC721081) shared 99.1–100.0% identity with the sequences previously determined in Sri Lanka (LC438499), Israel (KU557538), Turkey (KP000033), South Africa (KF626599), and Italy (GQ257740).

I further analyzed the positive rates of *B. bovis* and *B. bigemina* infections in yaks based on the sex and age group. I observed that the positive rates of these parasite species did not differ by sex nor by the 1–3-year and > 3-year age groups (Tables 12 and 13).

3-4. Discussion

The findings of the present study demonstrated that yaks are infected with *B. bovis* and *B. bigemina* in Mongolia. The PCR findings were confirmed by the sequencing analyses of amplicons. My previous studies conducted in Mongolia found that *B. bovis*- and *B. bigemina*-positive rates were comparable in cattle, as well as in Bactrian camels (Otgonsuren et al., 2020, 2022). The positive rates of these parasite species were also comparable between the cattle and Bactrian camels (Otgonsuren et al., 2020, 2022). These findings suggested a possibility that both the *B. bovis* and *B. bigemina* might be transmitted by the same tick vectors in Mongolia. In contrast to the cases of cattle and camels, yaks had a higher positive rate of *B. bovis* infection

and a lower positive rate of *B. bigemina* infection. Taken together, these observations suggest that yaks might be more susceptible to *B. bovis* infection than *B. bigemina* infection. *In vitro* cultivation of *B. bovis* and *B. bigemina* in yak RBCs and experimental infections of these parasite species in yaks may shed some lights on the differential susceptibilities.

Tick species that infest Mongolian yaks have not been identified to date. In the present study, blood samples were collected during the tick-active season in Mongolia. Therefore, tick infestations were common among the surveyed yaks. Unfortunately, however, ticks were not collected during the sampling. Tick species, such as *Haemaphysalis qinghaiensis*, *Dermacentor nuttalli*, *Dermacentor silvarum*, and *Dermacentor everestianus*, have been identified in yaks in other countries (Li et al., 2015; Hao et al., 2020). Among these species, *D. nuttalli* and *D. silvarum* infest cattle in Mongolia (Unpublished data); future research should investigate whether these tick species infest Mongolian yaks and transmit *B. bovis* and *B. bigemina*. Findings from such studies may reveal whether *B. bovis* and *B. bigemina* are transmissible via ticks from cattle to yaks and contrariwise from yaks to cattle.

Similar to *B. bovis* and *B. bigemina* infections, infection with the recently discovered *B. naoakii* may also cause severe clinical bovine babesiosis (Sivakumar et al., 2018, 2022). The previous surveys detected the *B. naoakii* in cattle, buffalo, dromedary and Bactrian camels, sheep, and goats (Otgonsuren et al., 2020; Sivakumar et al., 2020; Salman et al., 2022). In Mongolia, the *B. naoakii* infection has been confirmed in both cattle and Bactrian camels (Otgonsuren et al., 2020, 2022). Therefore, the negative results in this investigation might imply that yaks are not a host animal for *B. naoakii* infection. Further studies using a large number of yak samples and experimental infection in yaks are essential to confirm this assumption.

I found that the *B. bovis* and *B. bigemina*-positive rates were comparable between females and males, as well as between 1–3-year and > 3-year age groups. These findings are

consistent with previous researches, in which the positive rates of *B. bovis* and *B. bigemina* infections in cattle and Bactrian camels were unrelated to the sex or age of animals (Otgonsuren et al., 2020; 2022). In Mongolia, since all livestock animals are extensively reared from their birth, they have similar exposure to tick vectors regardless of their age or sex (Suttie, 2005); this may explain why the positive rates did not differ by sex or age group.

The yak population has declined dramatically in Mongolia over the last decade (Rao et al., 2015). Efforts to preserve the yak population should also include the management strategies for infectious diseases given that various bacterial and viral infections are common among Mongolian yaks (Odontsetseg et al., 2005; Ochirkhuu et al., 2018). As previously demonstrated, yaks are susceptible to clinical bovine babesiosis (Saud et al., 2005). This epidemiological survey, the first to report *Babesia* infections in Mongolian yaks, indicates that the disease management strategies for yaks should further address bovine babesiosis in Mongolia.

3-5. Summary

Yak (*Bos grunniens*) farming is an important part of Mongolia's livestock industry. Yaks survive in harsh mountain environments, provide meat, milk, and wool, and serve as a mode of transportation. In Mongolia, yaks are frequently raised alongside other livestock animals, such as cattle, Bactrian camels, sheep, goats, and horses. Recently, I demonstrated that *Babesia bovis*, *Babesia bigemina*, and *Babesia naoakii*, which are hemoprotozoan parasites with the potential to cause clinical bovine babesiosis, infect not only cattle, but also Bactrian camels in Mongolia. However, yaks have never been surveyed for the *Babesia* infections in this country. In the present study, I surveyed yaks in eight Mongolian provinces: Bayankhongor, Bayan-Ulgii, Khovd, Khovsgol, Omnogovi, Ovorkhangai, Uvs, and Zavkhan. Blood samples were taken from 375 yaks and DNA was extracted. Furthermore, Giemsa-

stained thin smears were prepared from 315 of the 375 blood samples, and then examined for the microscopic detection of *Babesia* parasites. Microscopy revealed that 34 (10.8%) of 315 blood smears were positive for *Babesia* parasites. All 375 DNA samples were tested for *B. bovis, B. bigemina*, and *B. naoakii* infections, using the specific PCR assays. I observed that 238 (63.5%) yaks in all surveyed provinces and eight (2.1%) yaks in three provinces (Bayankhongor, Bayan-Ulgii, and Omnogovi) were positive for *B. bovis* and *B. bigemina* infections, respectively. However, all yaks tested were negative for *B. naoakii*. This epidemiological survey, the first to report *Babesia* infections in Mongolian yaks, suggests that the disease management strategies for yaks should further address bovine babesiosis in this country.

Fig. 11. Yaks surveyed in Mongolia.

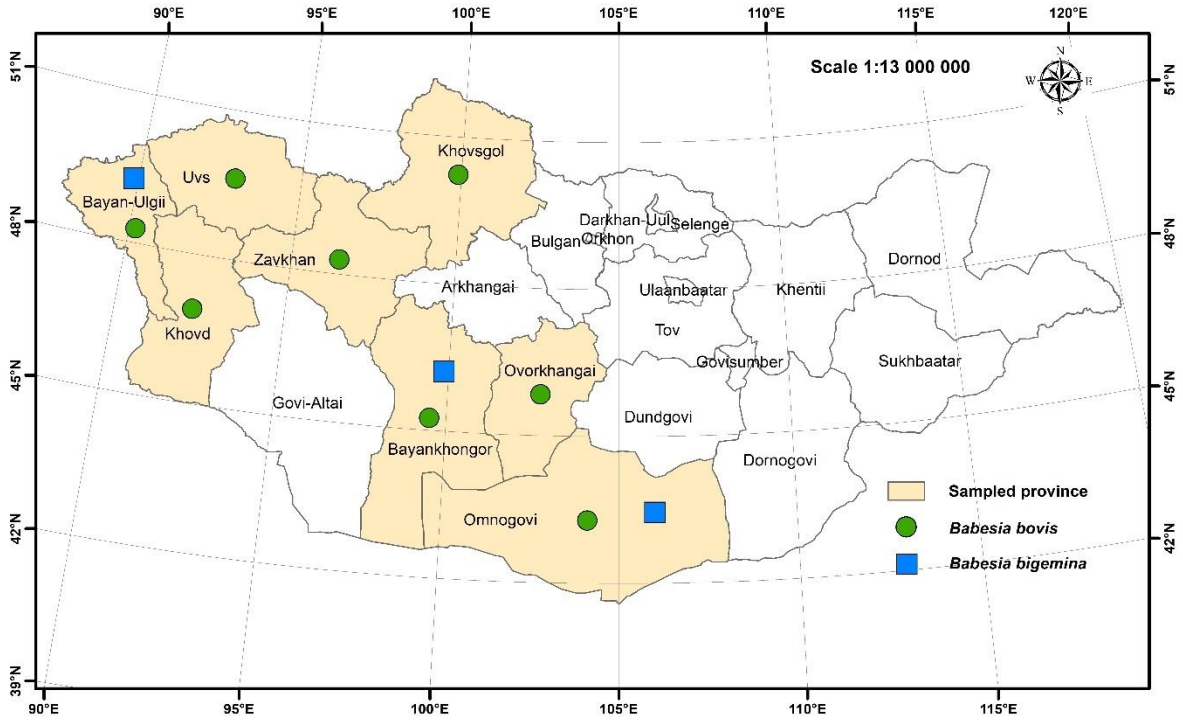

Fig. 12. Mongolian map showing eight provinces where yaks were surveyed. The map was created using the ArcGIS v10.1 software program (Environmental Systems Research Institute, Redlands, CA, USA). The animals in all eight provinces and those in three provinces surveyed were PCR-positive for *B. bovis* and *B. bigemina* infections, respectively.

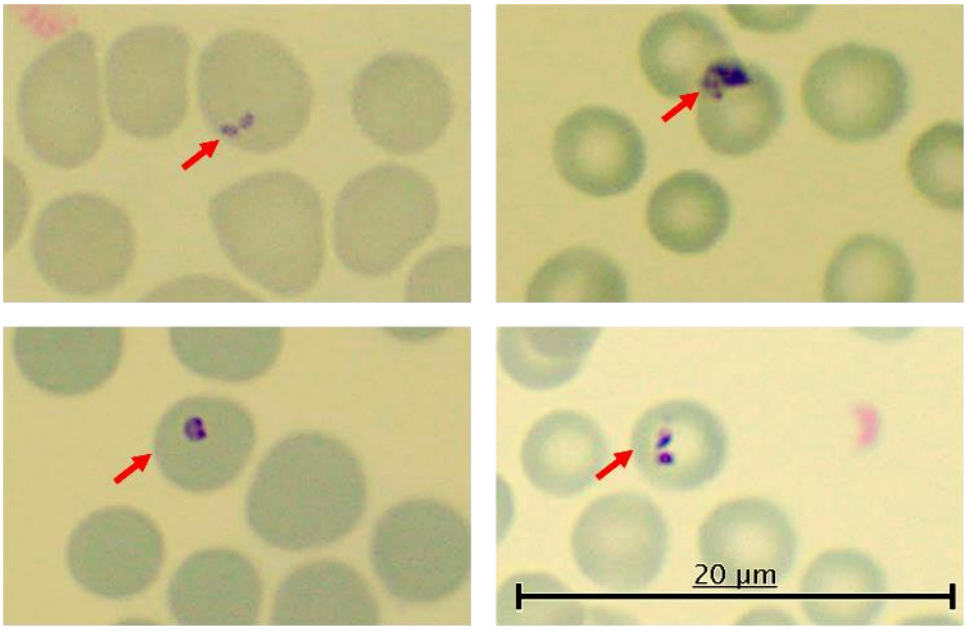

Fig. 13. Microscopic images of *Babesia* parasites observed in blood smears prepared from Mongolian yaks. Thin blood smears prepared from yaks were stained with Giemsa, and then observed under a light microscope. Detected *Babesia* parasites are indicated by arrows.

Table 11. Microscopic and PCR detections of bovine *Babesia* species in Mongolian yaks.

Province	Microscopy			PCR assays				
	No. sample	No. *Babesia*-positive	% (CI*)	No. sample	*B. bovis*		*B. bigemina*	
					No. positive	% (CI)	No. positive	% (CI)
Bayankhongor	63	3	4.7 (1.6-13.1)	63	34	54.0 (41.8-65.7)	1	1.6 (0.3-8.4)
Bayan-Ulgii	70	11	15.7 (9.0-26.0)	112	87	77.7 (69.1-84.4)	3	2.7 (0.9-7.6)
Khovd	26	3	11.5 (4.0-29.0)	26	23	88.5 (71.0-96.0)	0	0.0 (0.0-12.9)
Khovsgol	11	0	0 (0.0 -25.9)	11	10	90.9 (62.3-98.4)	0	0 (0.0-25.9)
Omnogovi	42	4	9.5 (3.8-22.1)	42	20	47.6 (33.4-62.3)	4	9.5 (3.8-22.0)
Ovorkhangai	31	5	16.1 (7.1-32.6)	31	29	93.5 (79.3-98.2)	0	0.0 (0.0-11.0)
Uvs	20	1	5.0 (0.9-23.6)	20	19	95.0 (76.4-99.1)	0	0.0 (0.0-16.1)
Zavkhan	52	7	13.4 (6.7-25.3)	70	16	22.8 (14.6-33.9)	0	0.0 (0.0-5.2)
Total	**315**	**34**	**10.8 (7.8-14.7)**	**375**	**238**	**63.5 (58.5-68.2)**	**8**	**2.1 (1.1-4.1)**

* 95% confidence interval

Table 12. Positive rates of *Babesia* species in female and male yaks.

Parasites	Female (*n* = 290)		Male (*n* = 85)		*P* value
	No. positive	% (CI*)	No. positive	% (CI)	
B. bovis	188	64.8 (59.1 -70.1)	50	58.8 (48.2 -68.7)	0.3131
B. bigemina	6	2.0 (0.9 -4.4)	2	2.3 (0.6 -8.2)	0.8426

* 95% confidence interval

Table 13. Positive rates of *Babesia* species in 1−3-year old and > 3-year old yaks.

Parasites	1−3-year (*n* = 145)		>3-year (*n* = 230)		*P* value
	No. positive	% (CI*)	No. positive	% (CI)	
B. bovis	94	64.8 (56.8 -72.1)	144	62.6 (56.2 -68.6)	0.667
B. bigemina	4	2.7 (1.0 -6.9)	4	1.7 (0.7 -4.4)	0.505

* 95% confidence interval

General Discussion

Successful livestock farming is vital for ensuring the food security. However, in addition to the other causes, infectious diseases pose a direct threat to livestock farming all over the world. Therefore, control of infectious diseases with animal health and economic significance is important. Among the infectious diseases, tick-borne diseases, such as babesiosis, theileriosis, and anaplasmosis, have a wide distribution, and are among the leading causes of economic losses in livestock industry. In particular, bovine babesiosis is a major concern in cattle farming, since this is a serious disease of adult cattle unlike the most of infectious diseases that mainly affect young calves. Therefore, in addition to treatment costs, economic losses are usually associated with loss of productions, such as milk and meat, and death of the adult cattle. In the endemic countries, therefore, control of bovine babesiosis is important based on the epidemiology of its causative *Babesia* species and associated risk factors.

Previous studies identified Mongolia as a country endemic for the bovine babesiosis. However, the epidemiology of *Babesia* species infecting Mongolian cattle is still unclear, undermining the efforts to develop control methods for bovine babesiosis in this country. In the present series of studies, I surveyed cattle, Bactrian camels, and yaks reared in various Mongolian provinces for *B. bovis*, *B. bigemina*, and *B. naoakii* infections.

I found that *B. bovis* and *B. bigemina* infections are common among cattle, Bactrian camels, and yaks, whereas *B. naoakii* is common among cattle and Bactrian camels in the surveyed Mongolian provinces. My findings of the present studies have implications for the control of bovine babesiosis not only in Mongolia, but also globally. I found that cattle throughout Mongolia were infected with all the three virulent *Babesia* species, including *B. naoakii*, which has not been previously reported in Mongolia. Since the animals are maintained

under an extensive system in Mongolia, one may expect that most of calves could have received the *Babesia* infections and developed immunity, and therefore clinical babesiosis may be uncommon in adult cattle in this country. However, a recent large-scale survey in Mongolia found that only 18.0% and 22.4% of the animals were seropositive to *B. bovis* and *B. bigemina* infections, respectively, suggesting a possibility that the endemic stability has not been achieved yet. Therefore, cattle reared throughout Mongolia should be monitored for clinical bovine babesiosis.

My study is the first to report bovine *Babesia* species, including *B. bovis*, *B. bigemina*, and *B. naoakii*, in Bactrian camels. The infection rates of these parasite species were comparable to those found in cattle, suggesting that Bactrian camels might not be an accidental host, but a host well-adapted to the bovine *Babesia* species. The *Babesia* parasites infected in Bactrian camels could therefore be potentially transmitted to cattle, since tick vectors that infest former also infest the later. Moreover, the present findings also warrant additional studies to investigate the clinical significance of bovine *Babesia* species in Bactrian camels.

I found that yaks in Mongolia were infected with *B. bovis* and *B. bigemina*, but not with *B. naoakii*. The negative results obtained for *B. naoakii* may exclude the yaks as a host for this recently discovered *Babesia* species. However, a large-scale yak survey is essential to confirm my assumption. The positive rates of *B. bovis* and *B. bigemina* infections in yaks were comparable to those I found in cattle and Bactrian camels, suggesting that yaks are a well-adapted host for these bovine *Babesia* species, similar to the case of Bactrian camels. Indeed, detections of *B. bovis* and *B. bigemina* in Mongolian yaks was not entirely unexpected, because both the parasite species have already been reported in yaks in other countries. Unlike Bactrian camels, clinical significance of bovine *Babesia* species in yaks has been documented in previous studies. Therefore, minimizing the *B. bovis* and *B. bigemina* infections in yaks is important for controlling bovine babesiosis in yaks, as well as in cattle.

Epidemiological mapping of infectious agents is very important to identity the high-risk areas. The availability of epidemiological maps may allow the relevant authorities to efficiently manage the resources available for disease control programs. Therefore, I created epidemiological maps illustrating the prevalence of *B. bovis*, *B. bigemina*, and *B. naoakii* in cattle and Bactrian camels and *B. bovis* and *B. bigemina* in yaks in Mongolia. I expect that these maps will now facilitate the Mongolian veterinary authorities to create awareness among the farmers and to design risk-based programs to minimizing *Babesia* infections in cattle, Bactrian camels, and yak.

Globally, Bactrian camel and yak populations are on decline due to various reasons, including climatic changes and infectious diseases. The present findings demonstrated that the infections with *Babesia* species capable of causing clinical disease are common among the Bactrian camels and yaks. The management strategies aimed at protecting Bactrian camels and yaks in Mongolia and other regions should also include the methods to control *Babesia* infections. However, very little is known about the *Babesia* infections in these two animal species. Especially, the tick vectors transmitting bovine *Babesia* species to Bactrian camels and yaks are currently unknown. The risk factors associated with clinical bovine babesiosis, such as age, immunity, management practices, and breed, have been extensively studied in cattle. However, such risk factors have never been investigated in Bactrian camels and yaks. The findings from the future studies investigating these issues will be useful for designing highly effective strategies to control the bovine babesiosis in Bactrian camels and yaks.

In short, findings of my studies and epidemiological maps that I created will be useful for designing and implementing the methods to control and prevent bovine babesiosis in Mongolia and globally.

General summary

Bovine babesiosis, which is a hemoprotozoan disease with clinical and economical significance, has a global distribution. The first step toward designing methods to control bovine babesiosis is identifying the causative *Babesia* species in cattle. In addition, the *Babesia* species that infect cattle should preferably be surveyed in non-cattle livestock, especially if they are reared together. The findings from such studies will equip the veterinary authorities with necessary epidemiological data to develop and implement effective disease management strategies. In Mongolia, the *Babesia* species infecting cattle have not been well investigated. Moreover, although all livestock animals are grazed together, the non-cattle livestock have never been surveyed in this country. Therefore, I conducted a series of the present studies to investigate the prevalence of three clinically significant bovine *Babesia* species, including *B. bovis*, *B. bigemina*, and *B. naoakii*, in cattle, as well as in Bactrian camels and yaks.

In chapter 1, I surveyed 725 cattle in 16 of the 21 Mongolian provinces, and found that 27.9%, 23.6%, and 5.4% of them were PCR-positive for *B. bovis*, *B. bigemina*, and *B. naoakii* infections, respectively. *Babesia bovis* and *B. bigemina* were detected in all 16 provinces surveyed, while *B. naoakii* was detected in cattle from 11 provinces. Detection of *B. naoakii* was one of the key findings, since this parasite species has not been previously reported in Mongolia. My findings of the present study highlight the importance of monitoring the cattle for clinical bovine babesiosis, since I found that the species of *Babesia* that can cause bovine clinical babesiosis, including *B. bovis*, *B. bigemina*, and *B. naoakii*, are widespread in Mongolia.

In chapter 2, I surveyed 305 Bactrian camels in six Mongolian provinces (Bayan-Ulgii, Govi-Altai, Khovd, Uvs, and Zavkhan, and Bayankhongor) for *B. bovis*, *B. bigemina*, and *B. naoakii* infections, using PCR assays, since I have detected these *Babesia* species in cattle in

this country. In common with cattle, all three surveyed *Babesia* species were detected in the surveyed Bactrian camels. The overall positive rates of *B. bovis*, *B. bigemina*, and *B. naoakii* infections were 32.1%, 21.6%, and 24.3%, respectively, and were comparable to those previously obtained for cattle. Tick species infesting Bactrian camels in Mongolia also infest cattle in this country. Therefore, the present findings suggest that the bovine *Babesia* species might be transmitted from cattle to Bactrian camels and vice versa. Therefore, minimizing the *Babesia* infections in Bactrian camels is vital for controlling bovine babesiosis in cattle in Mongolia.

In chapter 3, I surveyed 375 yaks in eight Mongolian provinces, including Bayankhongor, Bayan-Ulgii, Khovd, Khovsgol, Omnogovi, Ovorkhangai, Uvs, and Zavkhan, for *B. bovis*, *B. bigemina*, and *B. naoakii* infections, using the specific PCR assays. I found that Mongolian yaks were infected with the *B. bovis* and *B. bigemina*, but not with *B. naoakii*. Of 375 yaks surveyd, 238 (63.5%) in all surveyed provinces and eight (2.1%) in three provinces (Bayankhongor, Bayan-Ulgii, and Omnogovi) were positive for *B. bovis* and *B. bigemina* infections, respectively. The present study, which is the first to report the *Babesia* infections in Mongolian yaks, highlights that control of *Babesia* infections in yaks is important for managing bovine babesiosis in yaks, as well as in cattle.

Taken together, the abovementioned investigations found that the infections with bovine *Babesia* species, which can cause clinical bovine babesiosis, are widespread in cattle, Bactrian camels, and yaks in Mongolia. The findings from my studies generated epidemiological data, which may have implications for the control of bovine babesiosis in Mongolia and globally.

和文要旨

牛バベシア症は、獣医臨床学的に重要なマダニ媒介性の原虫病であり、その原因原虫種は世界に広く分布して経済的被害を引き起こしている。牛バベシア症の対応策を構築するためには、まずその原因となる牛バベシア種を同定することが重要となる。また、牛バベシア種の場合、牛のみならず、牛と一緒に飼育されている他の家畜動物種もその感染を調査する必要がある。このような調査研究から得られた知見は、汚染国の獣医当局が効果的な疾病管理の戦略を立案・実施する上で必要な疫学的データとなる。モンゴルでは、牛に感染する牛バベシア種は十分に解明されていない。また、様々な家畜動物種が同じエリアで一緒に放牧されているが、モンゴルの牛以外の家畜動物種についてもこれまで調査されてこなかった。そこで、獣医臨床学的に重要な 3 種の高病原性牛バベシア種（*Babesia bovis*、*Babesia bigemina*、及び *Babesia naoakii*）におけるモンゴルの牛、ラクダ、及びヤクの感染実態を調査するために、下記に示す一連の分子疫学研究を実施した。

第 1 章では、モンゴル 21 県のうち 16 県で放牧されている計 725 頭の牛を、PCR 法を用いて調査を行った。その結果、*B. bovis*、*B. bigemina*、及び *B. naoakii* の感染率が、それぞれ 27.9％、23.6%、及び 5.4%であった。*B. bovis* と *B. bigemina* は調査した 16 県すべてで検出されたのに対して、*B. naoakii* は 11 県の牛から確認された．*B. naoakii* はモンゴルで初めて確認された牛バベシア種となった。モンゴルでは、牛バベシア症の原因となる *B. bovis*、*B. bigemina*、及び *B. naoakii* の 3 種の牛バベシアが広範囲に分布している実態が明らかとなり、牛バベシア症の疫学研究の重要性が示された。

第 2 章では、モンゴル 6 県（Bayan-Ulgii 県、Govi-Altai 県、Khovd 県、Uvs 県、Zavkhan 県、及び Bayankhongor 県）で放牧されている計 305 頭のラクダについて、この国の牛から検出された 3 種の牛バベシア種（*B. bovis*、*B. bigemina*、及び *B. naoakii*）の感染疫学調査を、PCR 法を用いて行った。 3 種のバベシア種は、すべての県のラクダから検出され、*B. bovis*、*B. bigemina*、及び *B. naoakii* の全体の感染率はそれぞれ 32.1％、21.6％、及び 24.3％を示した。モンゴルのラクダに吸血するマダニ種は、同国の放牧牛にも寄生することから、牛バベシア種が牛とラクダの間で感染伝播している可能性が示唆された。モンゴルにおける牛バベシア症を制圧するためには、ラクダの牛バベシア感染を最小限に抑えることも重要となる。

第 3 章では、モンゴル 8 県（Bayankhongor 県、Bayan-Ulgii 県、Khovd 県、Khovsgol 県、Omnogovi 県、Ovorkhangai 県、Uvs 県、及び Zavkhan 県）で放牧されているヤク計 375 頭を対象に、 3 種の牛バベシア種（*B. bovis*、*B. bigemina*、及び *B. naoakii*）の感染について、PCR 法による分子疫学調査を行った。その結果、全県の 238 頭（63.5％）と 3 県（Bayankhongor 県、Bayan-Ulgii 県、及び Omnogovi 県）の 8 頭（2.1％）からそれぞれ *B. bovis* と *B. bigemina* が検出され、モンゴルのヤクは *B. bovis* と *B. bigemina* に感染していることが判明した。一方の *B. naoakii* はモンゴルのヤクでは感染が確認されなかった。モンゴルのヤクにおける牛バベシア種の感染は本研究が初めての報告となり、ヤクにおける牛バベシア感染の制御が、牛と同様に重要となることが浮き彫りとなった。

以上の分子疫学研究の成果から、獣医臨床学的に重要な牛バベシア症を引き起こす牛バベシア種が、モンゴルでは牛、ラクダ、及びヤクに広く感染していることが明らかになった。本研究で得られた疫学的データは、モンゴルのみならず世界的な牛バベシア症の制圧につながる有用な知見となった。

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