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THE FIRST SURVEY OF BOVINE *BABESIA* SPECIES INFECTING YAKS (*BOS GRUNNIENS*) IN MONGOLIA

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KEY WORDS

Babesia bigemina
Babesia bovis
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ABSTRACT

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, we demonstrated that *Babesia bovis*, *Babesia bigemina*, and *Babesia naoakii*—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 *Babesia* infections in this country. In the present study, we surveyed yaks in 8 Mongolian provinces: Bayankhongor, Bayan-Ulgii, Khovd, Khovsgol, Omnogovi, Ovorkhangai, Uvs, and Zavkhan. Blood samples were taken and deoxyribonucleic acid (DNA) was extracted from 375 yaks. 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 then tested for *B. bovis*, *B. bigemina*, and *B. naoakii* infection using specific polymerase chain reaction assays. We observed that 238 (63.5%) yaks in all surveyed provinces and 8 (2.1%) yaks in 3 provinces (Bayankhongor, Bayan-Ulgii, and Omnogovi) were positive for *B. bovis* and *B. bigemina*, respectively. However, all yaks tested were negative for *B. naoakii*. This epidemiological survey, the first to report *Babesia* infection in Mongolian yaks, suggests that disease management strategies for yaks in this country should further address bovine babesiosis.

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, 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 bovine babesiosis in cattle (Bock et al., 2004). Clinical bovine babesiosis is characterized by intravascular hemolytic anemia and associated symptoms such as fever, hemoglobinuria, and icterus caused by the parasites' asexual reproduction within and egress from red blood cells (RBCs; Homer et al., 2000). In noncattle hosts such as buffalo, infection by bovine *Babesia* species may be asymptomatic (Mahmmod, 2013). However, a previous study concluded that yaks infected with *Babesia* species were anemic and had lower hemoglobin concentration, hematocrit, and RBC counts compared with healthy yaks (Saud et al., 2005). Therefore, controlling bovine babesiosis in yaks is clinically important. Furthermore, because the infection can be transmitted from noncattle hosts to cattle via ticks (Jaimes-Dueñez et al., 2018),

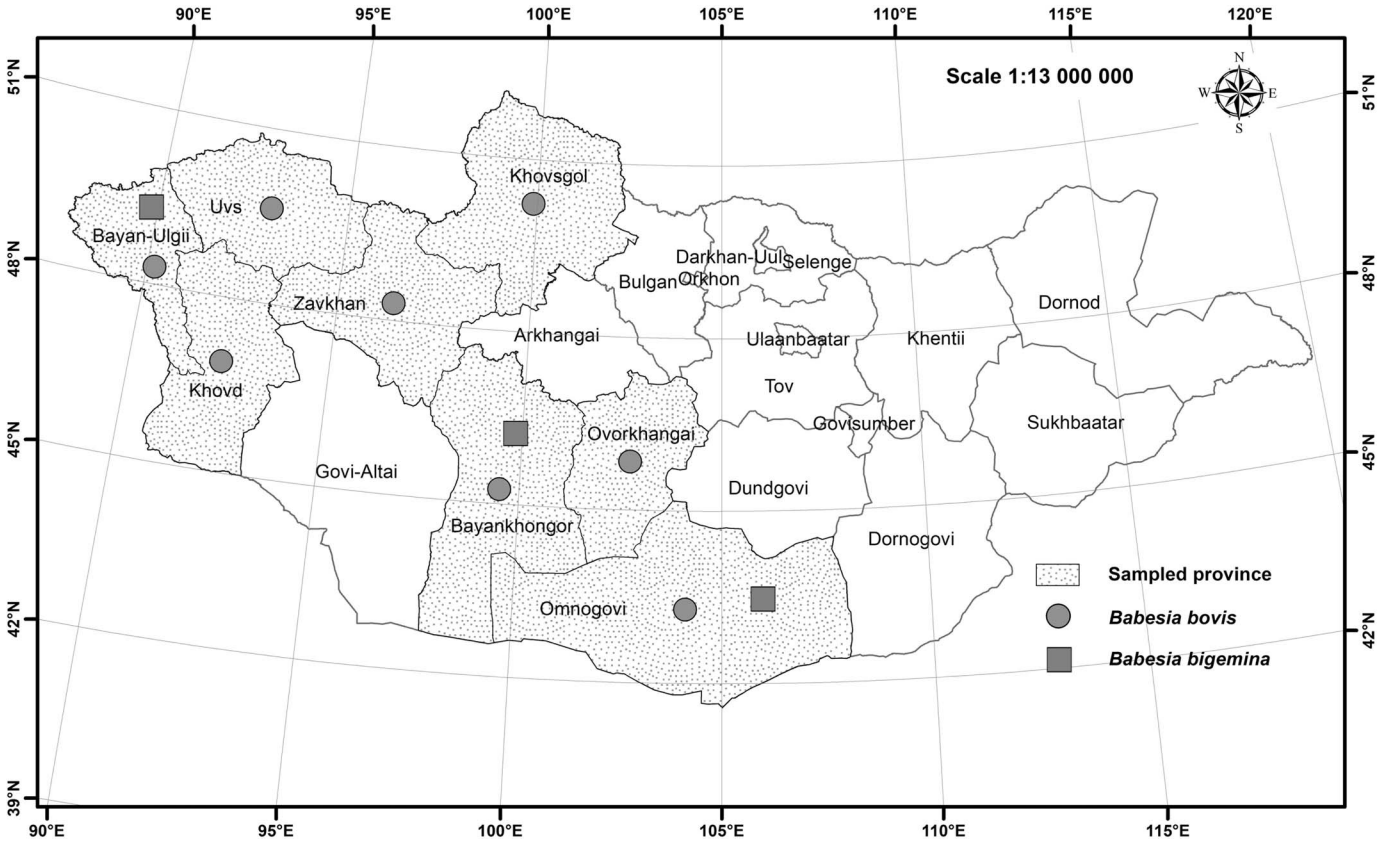

Figure 1. Mongolian map showing 8 provinces where yaks (*Bos grunniens*) were surveyed. The map was created using the ArcGIS v10.1 software program (Environmental Systems Research Institute, Redlands, California). The animals in all 8 provinces and those in 3 provinces surveyed were polymerase chain reaction (PCR) positive for *Babesia bovis* and *Babesia bigemina* infection, respectively.

minimizing *Babesia* infection in yaks may be critical in managing babesiosis in cattle in countries where cattle and yaks 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 they can survive in harsh environments, yaks are raised for meat, milk, and wool production 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 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, livestock animals graze together in Mongolia (Suttie, 2005). Therefore, tick species that infest 1 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 in Mongolia. According to a recent study, cattle in Mongolia were infected with *B. bovis*, *B. bigemina*, and *Babesia naoakii* (formerly known as *Babesia* sp. Mymensingh) (Otgonsuren et al., 2020; Sivakumar et al., 2022). A subsequent survey observed that Bactrian camels were also infected with these *Babesia* species (Otgonsuren et al., 2022). We therefore hypothesized that Mongolian yaks are likewise infected with the bovine *Babesia* species that infect cattle in Mongolia. To test this hypothesis, we conducted an epidemiological survey to detect infection with bovine *Babesia* species in Mongolian yaks.

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, we collected blood samples from 375 yaks grazing in 8 Mongolian provinces, namely Bayankhongor, Bayan-Ulgii, Khovd, Khovsgol, Omnogovi, Ovorkhangai, Uvs, and Zavkhan (Fig. 1, Table I). 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 ethylenediaminetetraacetic acid. Thin smears were prepared on glass slides using blood from 315 of the 375 animals sampled. Subsequently, deoxyribonucleic acid

Table I. Microscopic and polymerase chain reaction (PCR) detections of bovine *Babesia* species in Mongolian yaks (*Bos grunniens*).

	Microscopy			PCR assays				
					Babesia bovis		*Babesia bigemina*	
Province	No. sample	No. *Babesia*-positive	% (CI*)	No. sample	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)

* CI = 95% confidence interval.

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

Microscopic examination for *Babesia* parasites

The blood smears were fixed with absolute methanol and then stained with Giemsa. The stained smears were examined under a light microscope with a ×100 objective lens and immersion oil for the morphological detection of *Babesia* parasites as previously described (Lempereur et al., 2017).

Polymerase chain reaction screening for *B. bovis*, *B. bigemina*, and *B. naoakii* infections

We screened all 375 DNA samples from yaks using previously described *B. bovis*-, *B. bigemina*-, and *B. naoakii*-specific polymerase chain reaction (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* using 2 single-step PCR assays developed on the basis of their apical membrane antigen 1 gene (*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 from prepared in vitro cultures of *B. bovis* and *B. bigemina* and from a cow infected with *B. naoakii* were used as positive controls in the respective PCR assays, whereas 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 positive for *B. bovis*, *B. bigemina*, and *B. naoakii* infection if the band sizes in PCR assays were approximately 298, 211, and 371 base pairs, 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, California). Inserted gene fragments were sequenced using an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Branchburg, New Jersey). The newly generated sequences were analyzed using a basic local alignment search tool (Altschul et al., 1990) to confirm the origins of gene sequences and to determine the identity scores shared with corresponding sequences previously registered in the GenBank database.

Statistical analyses

The 95% confidence intervals were calculated for positive rates using OpenEpi online software (Dean et al., 2013) on the basis of Wilson's score interval (Wilson, 1927). The *P* values were calculated for the differences in the 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 statistically significant if $P < 0.05$.

RESULTS

This study surveyed a total of 375 yaks grazed in 8 Mongolian provinces for infection by 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. 2; Table I). Except for Khovsgol, all surveyed provinces had *Babesia*-positive yaks, with positive rates ranging from 4.7% to 16.1%.

The results of PCR screening indicated that the surveyed yaks were infected with *B. bovis* and *B. bigemina* (Fig. 1; Table I). 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 infected yaks, with positive rates ranging from 22.8% to 95.0% (Table I). In contrast, *B. bigemina* was detected in only 8 (2.1%) animals in 3 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 (98.0–100.0%) identity scores 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 sequences

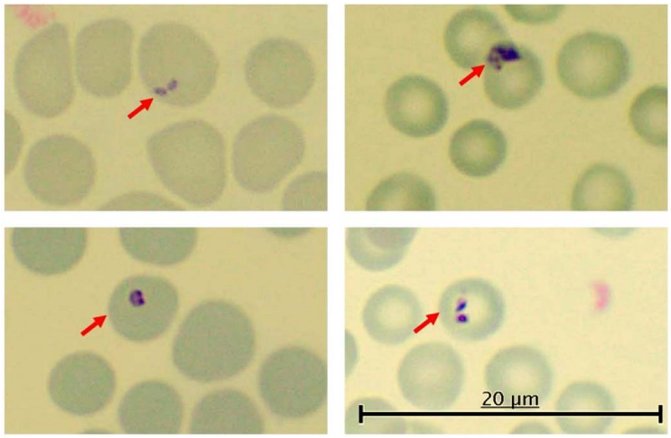

Figure 2. Microscopic images of *Babesia* parasites observed in blood smears prepared from Mongolian yaks (*Bos grunniens*). Thin blood smears prepared from yaks were stained with Giemsa and then observed under a light microscope. Detected *Babesia* parasites are indicated by arrows.

previously determined in Sri Lanka (LC438499), Israel (KU557538), Turkey (KP000033), South Africa (KF626599), and Italy (GQ257740).

We further analyzed the positive rates of *B. bovis* and *B. bigemina* infection in yaks on the basis of sex and age group. We observed that positive rates of these parasite species did not differ by sex nor by the 1–3-yr and >3-yr age groups (Tables II, III).

DISCUSSION

The findings of the present study demonstrated that yaks in Mongolia are infected with *B. bovis* and *B. bigemina*. The PCR findings were confirmed by the sequencing analyses of amplicons. Our previous studies conducted in Mongolia found that *B. bovis*- and *B. bigemina*-positive rates were comparable in cattle, Bactrian camels, and between cattle and Bactrian camels (Otgonsuren et al., 2020, 2022). The diagnostic sensitivities of the PCR assays for *B. bovis* and *B. bigemina* used in these studies have not been compared previously. If the sensitivities of both PCR assays are similar, the comparable positive rates of *B. bovis* and *B. bigemina* in cattle and Bactrian camels suggest a possibility that both parasite species are transmitted by the same tick vectors in Mongolia. In contrast to 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 to *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 light on differential susceptibility.

Our findings indicate that the rate of positive results obtained through microscopy was lower than that obtained through PCR assays, implying a higher prevalence of chronic infections in yaks compared with acute infections. This finding is not unexpected, as chronic infections with *B. bovis* and *B. bigemina*, which exhibit low rates of positive results through microscopy, are also common in cattle (Romero-Salas et al., 2016). Previous research has demonstrated that ticks can acquire and transmit *B. bovis* from both chronically and acutely infected cattle at comparable levels (Howell et al., 2007). Therefore, despite being chronically infected, yaks are likely to serve as reservoirs for ticks, facilitating further transmission.

The most common tick species responsible for transmitting bovine *Babesia* species, *Boophilus microplus* (Bock et al., 2004), was not reported in Mongolia (Dash, 2018; Narankhajid et al., 2018). This observation suggests the involvement of other tick species in the transmission of *B. bovis* and *B. bigemina* in the country. Among the tick species reported in Mongolia, *Dermacentor nuttalli* is the most prevalent and known to infest a wide range of host animals, including cattle and yaks (Narankhajid et al., 2018). Previous studies conducted in Mongolia detected *Babesia caballi* in *D. nuttalli* (Battsetseg et al., 2001, 2002), identifying this tick species as a potential vector of *Babesia* species in this country. Future research should, therefore, investigate whether *D. nuttalli* is capable of transmitting *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.

Like *B. bovis* and *B. bigemina* infection, infection by the recently discovered *B. naoakii* may cause severe clinical bovine babesiosis (Sivakumar et al., 2018, 2022). In Mongolia, *B. naoakii* infection has been confirmed in 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*. Further studies using a large number of yak samples and experimental infection in yaks are essential to confirm our assumption.

We found that *B. bovis*- and *B. bigemina*-positive rates were comparable between males and females, as well as between 1–3-yr and >3-yr age groups. These findings are consistent with previous research, 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, given that all livestock animals are extensively reared from birth, they have similar exposure to tick vectors regardless of their age or sex (Suttie, 2005); this may explain why 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 management strategies for infectious diseases given that various bacterial and viral infections are common among Mongolian yaks (Odontsetseg et al., 2005;

Table II. Positive rates of *Babesia* species in female and male yaks (*Bos grunniens*).

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

* CI = 95% confidence interval.

Table III. Positive rates of *Babesia* species in 1–3-yr-old and >3-yr-old yaks (*Bos grunniens*).

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

* CI = 95% confidence interval.

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 disease management strategies for yaks should further address bovine babesiosis in Mongolia.

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