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

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ABSTRACT

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, we surveyed the Bactrian camels (*Camelus bactrianus*) in Mongolia for three clinically significant bovine *Babesia* species, including *Babesia bovis*, *B. bigemina*, and *Babesia* sp. Mymensingh, which had been detected previously in Mongolian cattle. We screened blood DNA samples from 305 Bactrian camels in six Mongolian provinces for these species, using parasite-specific PCR assays. Our 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 *Babesia* sp. Mymensingh were 32.1%, 21.6%, and 24.3%, respectively, whereas 52.5% of the surveyed animals were infected with at least one parasite species. We 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 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.

Keywords: *Babesia bovis*, *Babesia bigemina, Babesia* sp. Mymensingh, Bactrian camel, Mongolia, cattle

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 *Babesia* sp. Mymensingh are known to cause clinical 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, 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 erythrocytes (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 production of milk, wool, and meat, as well as for the transportation of people and goods. As of 2019, the estimated camel population was approximately 472,000 in Mongolia (National Statistics Office of Mongolia, 2019). 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 usually co-graze in pasture lands with other livestock, 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, we hypothesized that the *Babesia* species infecting cattle may also infect these camels. To test our hypothesis, we surveyed the Bactrian camels reared in various Mongolian provinces for *B. bovis*, *B. bigemina*, and *Babesia* sp. Mymensingh, which had been recently detected in Mongolian cattle (Otgonsuren et al., 2020).

2. Materials and methods

2.1. Blood sampling and DNA extraction

In July 2017 and April 2018, we collected blood samples randomly from 305 Bactrian camels reared in the following six Mongolian provinces: Bayan-Ulgii, Govi-Altai, Khovd, Uvs, and Zavkhan in Western Mongolia and Bayankhongor in Southern Mongolia (Fig. 1, Table 1).

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, we prepared thin blood smears on glass slides using the samples collected from 33, 24, and 12 animals in Khovd, Uvs, and Zavkhan provinces, respectively. We also prepared genomic DNA from each blood sample using a previously reported phenol:chloroform:isoamyl alcohol method (Sambrook and Russell, 2001). We then stored the DNA samples at −30°C. 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).

2.2. Microscopic examination for Babesia *parasites*

We fixed the thin blood smears with absolute methanol, stained with Giemsa, and then observed under a light microscope for detecting *Babesia* parasites within erythrocytes.

2.2. PCR screening for B. bovis, B. bigemina, *and* Babesia *sp. Mymensingh*

We screened all the DNA samples from the Bactrian camels using previously described PCR assays specific to *B. bovis, B. bigemina,* and *Babesia* sp. Mymensingh. 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 *Babesia* sp. Mymensingh (Sivakumar et al., 2018). We have described the primers, reaction mixtures, and cycling conditions for the PCR assays in our previous report (Otgonsuren et al., 2020). We used DNA samples extracted from *B. bovis* and *B. bigemina in vitro* cultures and from blood of a cow naturally infected with *Babesia* sp. Mymensingh (Sivakumar et al., 2018) 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.

We resolved the resultant PCR products 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 *Babesia* sp. Mymensingh if the band sizes were approximately 298, 211, and 371 bp, respectively (Otgonsuren et al., 2020).

2.3. Cloning and sequencing

We cloned and sequenced the selected amplicons obtained from each PCR assay 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). We then sequenced the inserted gene fragments using an ABI PRISM 3100 genetic analyzer (Applied Biosystems, Branchburg, NJ, USA). We analyzed the newly generated sequences using a basic local alignment search tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm the origins of the gene sequences and to determine the identity scores shared with the corresponding sequences from GenBank.

2.4. Statistical analyses

We calculated the 95% confidence intervals (CIs) for the positive rates based on Wilson's score interval (Wilson, 1927) using an OpenEpi online software (https://www.openepi.com/Proportion/Proportion.htm). We calculated the *P* values for the differences between the positive rates 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.

3. Results and discussion

149 We designed the present study to investigate whether Bactrian camels in Mongolia were
150 infected with *Babesia* species, including *B. bovis*, *B. bigemina*, and *Babesia* sp. Mymensingh,
151 that are capable of causing clinical babesiosis in cattle. Bactrian camels are reared mainly in
152 Western and Southern Mongolia. However, except for Bayankhongor, cattle farming is not
153 very common in Southern Mongolia. Therefore, the present survey was conducted only in
154 Western Mongolia and Bayankhongor province (Fig. 1). The sampling was carried out during
155 the tick season in Mongolia (March to September), and therefore tick infestation was common
156 among the sampled camels (data not shown).

157 Our microscopic examination revealed that 22 (31.9%) of 69 examined camels,
158 including 10 of 33, 8 of 24, and 4 of 12 animals in Khovd, Uvs, and Zavkhan provinces,
159 respectively, were positive for *Babesia* parasites (Fig. 2). Although species differentiation was
160 not possible with microscopy, our PCR results demonstrated that the camels were infected with
161 all three *Babesia* species surveyed (Fig. 1, Table 1). All microscopy-positive animals were also
162 positive by PCR assays. Furthermore, the infections were particularly common in camels
163 considering the fact that 160 (52.5%) of the 305 Bactrian camels surveyed were infected with
164 at least one *Babesia* species. The overall positive rates were 32.1%, 21.6%, and 24.3% for *B.*
165 *bovis*, *B. bigemina,* and *Babesia* sp. Mymensingh, respectively (Table 1).

166 We cloned and sequenced nine, eight, and nine randomly selected amplicons from *B.*
167 *bovis*, *B. bigemina*, and *Babesia* sp. Mymensingh PCR assays, respectively. The newly
168 determined *B. bovis rap-1* sequences (LC598509–LC598517) shared 99.7–100% identities
169 with a sequence reported from Egypt (AB917246), while the *B. bigemina ama-1* sequences
170 (LC598518−LC598525) shared 99.5–100% identity scores with the sequences from Sri Lanka
171 (LC438499), Israel (KU557538), Turkey (KP000033), South Africa (KF626599), and Italy
172 (GQ257740). Similarly, the *ama-1* sequences generated from the amplicons of *Babesia* sp.
173 Mymensingh PCR assays (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.

A previous study found that cattle in all provinces surveyed in our present investigation were infected with bovine *Babesia* species (Otgonsuren et al., 2020). Similarly, Bactrian camels from all surveyed provinces, except for the Bayan-Ulgii, were positive for at least one *Babesia* species (Fig. 1, Table 1). The negative results obtained in the PCR assays do not necessarily mean that camel population in Bayan-Ulgii is free from *Babesia* infections, as only 10 animals were surveyed. *Babesia bovis*, *B. bigemina,* and *Babesia* sp. Mymensingh 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 *Babesia* sp. Mymensingh in the provinces where they were detected ranged 16.8–67.5%, 14.8–37.5%, and 9.1–53.3%, respectively (Table 1). 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 in Bactrian camels are required.

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 *Babesia* sp. Mymensingh (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 the bovine *Babesia* species we surveyed. As reported in previous study, the tick species infesting camels (*D. marginatus*, *D. nuttalli*, and *H. asiaticum*) also infest cattle in Mongolia, and therefore, tick transmission of bovine *Babesia* species might be possible between these two host animals. However, our assumption can be confirmed only if additional experiments demonstrate that the tick species infesting camels are

capable of transmitting bovine *Babesia* species, as their vector competence is currently unknown.

Co-infections with two or three *Babesia* species were common among the surveyed camels. Of 160 animals infected with at least one *Babesia* species, 66 (41.3%) had co-infections (Table 2). Further studies are now essential to investigate whether the co-infections were due to shared tick vectors (Cutler et al., 2021).

As shown in a previous study, 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 is less pronounced (Sivakumar et al., 2016). In agreement with this observation, our 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). Similarly, 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 *Babesia* sp. Mymensingh-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 3). These findings suggest that in Bactrian camels, the persistence of *Babesia* sp. Mymensingh infection might be pronounced as compared to that of *B. bovis* and *B. bigemina*. However, this argument is inconclusive, as our findings might have been confounded by several factors, such as parasitemia levels and period between the time of infection and sampling.

Comparison of the positive rates between the male and female animals revealed that the rates for all three tested *Babesia* species were significantly higher ($P < 0.0001$) in females than in males (Table 4). Furthermore, we also analyzed the differential positive rates among the camel breeds (MNT, HZ, and GGU) (Table 5). A comparative analysis indicated that the positive rates of *B. bovis*, *B. bigemina*, and *Babesia* sp. Mymensingh were significantly higher

224 in MNT than in HS ($P < 0.0001$, 0.0285, < 0.0001, respectively). We sampled only eight GGU
225 Bactrian camels and therefore did not consider this breed in our analysis. The observed
226 discrepancies could be associated with the differences in the tick burden. Therefore, further
227 studies investigating the tick burden and the predisposing factors of tick infestation, such as
228 coat and skin characteristics (Ibelli et al., 2012; Shyma et al., 2015) in males and females as
229 well as in MNT and HZ breeds, may elucidate the differences in the positive rates of the tested
230 *Babesia* species. *Babesia* infections in cattle are known to be influenced by grazing
231 management practices (Rubaire-Akiiki et al., 2004). In the present study, the comparison of
232 the positive rates based on management practices was not possible because all Bactrian camels
233 in Mongolia are managed extensively from birth.

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

243 **4. Conclusion**

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

247 **Declarations of interest:** none

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403 **Figure legends**

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

409

410 **Fig. 2.** Micrographs of *Babesia* parasites detected in Bactrian camels. Giemsa-stained thin
411 blood smears prepared from Bactrian camels were observed under a light microscope for
412 detecting *Babesia* parasites (indicated by arrows) within erythrocytes.

Table 1. PCR detection of *B. bovis*, *B. bigemina*, and *Babesia* sp. Mymensingh in 305 Bactrian camels from Mongolia

Province	No.	*B. bovis*		*B. bigemina*		*Babesia* sp. Mymensingh	
		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 2. Co-infection of *Babesia* species in Bactrian camels surveyed in Mongolia

Parasite species	No. positive (%[a])
B. bovis + *B. bigemina*+ *Babesia sp.* Mymensingh	12 (7.5)
B. bovis + *B. bigemina*	17 (10.6)
B. bovis + *Babesia sp.* Mymensingh	32 (20.0)
B. bigemina + *Babesia sp.* Mymensingh	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 3. Positive rate 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
Babesia sp. Mymensingh	6	9.7 (4.5–19.6)	68	28 (22.7–33.9)	0.0027

[a]95% confidence interval.

Table 4. 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
Babesia sp. Mymensingh	48	57.1 (46.5–67.2)	26	11.8 (8.2–16.7)	< 0.0001

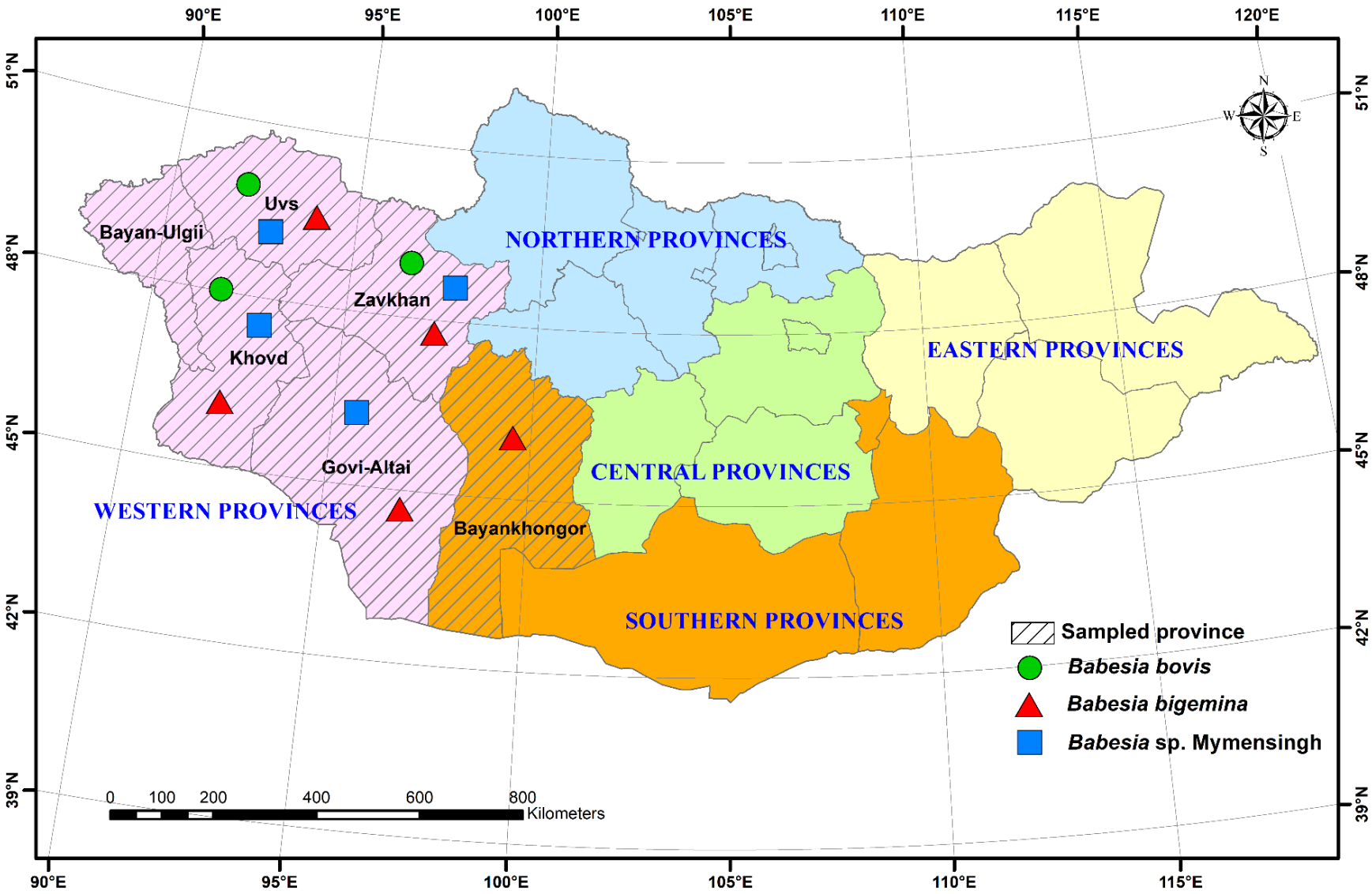
[a]95% confidence interval.

Table 5. 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)
Babesia sp. Mymensingh	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.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Babesia bovis
Babesia bigemina
Babesia sp. Mymensingh

Fig. 1

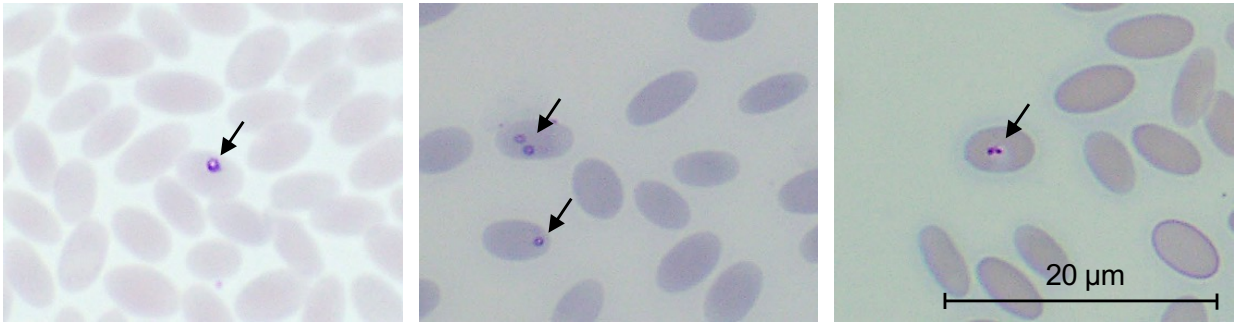

Fig. 2