The PCR detection and phylogenetic characterization of *Babesia microti* in questing ticks in Mongolia

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ABSTRACT

Babesia microti is a tick-transmitted zoonotic hemoprotozoan parasite. In the present study, we investigated *B. microti* infection in questing ticks in Mongolia. A total of 219 questing ticks were collected from three different Mongolian provinces (Bayan-Olgii, Khovsgol, and Selenge). Of these, 63 from Selenge were identified as *Ixodes persulcatus*, while the remaining 156 (from all three provinces) were identified as *Dermacentor nuttalli*. When the tick DNA samples were screened using a *B. microti*-specific nested PCR, 19 (30.2%) of the 63 *I. persulcatus* ticks were found to be *B. microti*-positive. The parasite was not detected in *D. nuttalli*. Subsequently, the *18S rRNA*, *cox1*, and *tufA* sequences of *B. microti* were amplified, sequenced, and subjected to phylogenetic analyses. Sequencing analyses showed that the Mongolian *18S rRNA*, *cox1*, and *tufA* sequences were 99.6–100%, 96.7–97.2%, and 94.7–95.3% homologous, respectively, with *B. microti* R1 strain US-type sequences from humans. In the phylogenetic analyses, the Mongolian *cox1* and *tufA* sequences were found to be separate lineages, which formed sister-clades to the R1 strain sequences, while all of the Mongolian *B. microti 18S rRNA* sequences were clustered within US-type clade containing several other sequences of human origin. In conclusion, in addition to reporting the presence of *B. microti* for the first time in questing ticks in Mongolia, the present study found that Mongolian *I. persulcatus* ticks were infected with US-type *B. microti*. These findings warrant large-scale studies to detect and characterize *B. microti* in ticks, small mammals, and humans. Such studies should provide us with a better understanding of zoonotic *Babesia* epidemiology in Mongolia.

Keywords: *B. microti*, *cox1*, Mongolia, Ticks, *tufA*, *18S rRNA*

1. Introduction

Human babesiosis is a tick-transmitted zoonotic protozoan disease caused by several *Babesia* species, including *Babesia microti*, *B. divergens*, *B. duncani*, *Babesia* sp "venatorum" (EU1), and *Babesia* sp. KO1 [1–6]. Clinically, human babesiosis is characterized by a malaria-like illness, and the clinical manifestations may include fever, headache, myalgia, severe anemia, respiratory failure, and death [7]. Outside of North America, where human cases of *B. microti* infection are commonly found, human infections have been reported in Europe, Asia, and Australia [8–13]. The *B. microti* lifecycle involves tick vectors and their vertebrate hosts [4]. Vertebrates, including humans, are infected with *B. microti* through the bites of infected ticks. Although humans are a dead-end host for *B. microti*, people with asymptomatic infections can transmit the parasite to uninfected people through blood donations [14–16]. The transplacental and perinatal transmission of *B. microti* has also been reported in humans [17].

For the control of tick-borne pathogens, tick control methodologies must be employed [18,19]. The first step toward effective tick control begins with the identification of the species of ticks that transmit *B. microti*, because the risk of contracting human babesiosis caused by *B. microti* is associated with the distribution of its tick vectors [20]. In the last few decades, studies have investigated the prevalence of *B. microti* in ticks distributed in different geographical regions of the world. The parasite has been detected in several tick species, including *Ixodes scapularis* [21], *I. ricinus* [22], *I. persulcatus* [23], and *I. ovatus* [24]. *B. microti* parasites in ticks are usually detected by a PCR, after which DNA sequencing and phylogenetic analyses are used for confirmation [24,25]. In the recent past, in addition to the commonly used 18S ribosomal RNA sequences (*18S rRNA*), the genes encoding β -tubulin and chaperonin-containing t-complex polypeptide 1 (CCT7) have been used for the genetic

characterization of *B. microti* isolates [26,27]. Similarly to other apicomplexan parasites, *B. microti* contains three separate genomes: nuclear, mitochondrial, and plastid [28–30]. In addition to genes in the nuclear genome (e.g., *18S rRNA*), mitochondrial genes (e.g., cytochrome *c* oxidase subunit 1, *cox1*) and plastid genes (e.g., the apicoplast translation elongation factor tuA gene, *tufA*) have also been widely used for evolutionary studies on the plastid-containing organisms [31–34].

Several *Babesia* parasites have been reported in Mongolia [35–37], a land-locked country located between Russia and China. Both of these bordering countries have reported the presence of *B. microti* in ticks and humans in their territories [12,23]. A recent study detected *B. microti* infections among Mongolian farmers, using PCR and serological methods [38]. However, the parasite infection in ticks is yet to be studied in this country. Thus, in the present study, we screened DNA samples from questing ticks collected in Mongolia for the presence of *B. microti*. Subsequently, we analyzed the *18S rRNA*, *cox1*, and *tufA* sequences amplified from the *B. microti*-positive DNA samples to confirm our findings.

2. Materials and methods

2.1 Tick and DNA samples

Questing ticks were collected in the mountain areas of Bayan-Olgii (westernmost) province and in the forest areas of Khovsgol (northernmost) and Selenge (northern) provinces of Mongolia in 2012 and 2013 by a flagging method. The collected ticks were morphologically identified as described previously [39,40]. After morphological identification, the collected ticks were subjected to DNA extraction. Briefly, individual ticks were cut into small pieces using sterile surgical blades, digested with a lysis buffer (20 mM Tris-HCl pH 8.0, 1 mM EDTA pH 7.5, 10 mM NaCl, 1% SDS, and 100 µg/ml Proteinase K), and then purified by a phenol/chloroform method. After ethanol precipitation, the DNA samples were dissolved in 50 µl of double distilled water (DDW), and then stored at –30°C until further use.

2.2 PCR detection of *B. microti*

All of the DNA samples were screened for the presence of *B. microti* using a previously described *B. microti*-specific nested PCR (Table 1) [41]. After gel electrophoresis and ethidium bromide staining, the PCR products were identified under UV light. **A Blood DNA sample sourced from a mouse experimentally infected with *B. microti* (Munich strain) was used as a positive control for the PCR.** In addition to using several negative controls, filtered tips and designated pipette sets for pre- and post-PCR handling were used for all of the PCR procedures described in this article to avoid possible contamination.

2.3 PCR amplification of 18S rRNA, *cox1* and *tufA*

B. microti-positive tick DNA samples were subjected to three separate PCR processes for the *18S rRNA*, *cox1*, and *tufA* genes. A nested PCR employing the previously described primer sets was used to amplify *18S rRNA* [42], while single step PCRs using the primers designed in the present study were used to amplify the *cox1* and *tufA* genes (Table 1). For the first round of *18S rRNA* amplification, 1 µl of tick DNA was added into 9 µl of the PCR reaction mixture containing 1 µl of 10 × PCR buffer (Applied Biosystems, Branchburg, NJ, USA), 1 µl of 2 mM dNTPs (Applied Biosystems), 0.2 µl of 10 µM Piro0F2 and Piro6R2 primers (Table 1), 0.1 µl of 5 U/µl Taq DNA polymerase (AmpliTaq Gold; Applied Biosystems), and 6.5 µl of DDW. For the *cox1* and *tufA* genes, the 9 µl reaction mixture included 5 µl of 2 × Ampdirect®plus PCR buffer (Shimadzu Corporation, Kyoto, Japan), 0.2 µl of 10 µM primers (Cox1F133 and Cox1R1130, and EFtuAF2 and EFtuAR2, respectively), 0.1 µl of 5 U/µl Taq DNA polymerase (ExTaq; Takara BIO INC, Shiga, Japan), and 3.5 µl of DDW. Finally, 1 µl of tick DNA was added to each reaction mixture before the PCR amplification. The initial enzyme activation step at 95°C for 5 min was followed by 40 (*18S rRNA*) or 45 (*cox1* and *tufA*) cycles, each containing a denaturing step at 94°C for 1 min, an annealing step at the relevant temperatures shown in Table 1 for 1 min, and an elongation step at 72°C for 90 s. After the final elongation step at 72°C for 10 min, the *cox1* and *tufA* PCR products were analyzed by gel electrophoresis. For *18S rRNA*, the PCR products (1 µl) were transferred to new tubes containing reaction mixtures with compositions that were similar to those used for the first amplification round, with the exception that the primers were replaced with Piro1F2 and Piro5R2, before being subjected to cycling conditions that were similar to those of the first round.

2.4 DNA cloning and sequencing

The *18S rRNA*, *cox1*, and *tufA* amplicons were cloned and sequenced, using previously described methods with minor modifications [43]. Briefly, after gel electrophoresis, PCR amplicons were purified using a NucleoSpin® Gel and PCR Clean-up Kit (MACHEREY-NAGEL, Düren, Germany), ligated to a PCR 2.1 plasmid vector (TOPO, Invitrogen, Carlsbad, CA, USA), transformed into *Escherichia coli* cells (TOP 10, Invitrogen), and then plated onto Luria-Bertani agar plates (Invitrogen). Three clones were picked for each PCR amplicon and then subjected to DNA sequencing, using an ABI PRISM 3100 genetic analyzer (Applied Biosystems).

2.5 Sequence and phylogenetic analyses

The *18S rRNA*, *cox1*, and *tufA* DNA sequences were initially analyzed by the basic local alignment search tool (BLAST) (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>), and then analyzed by MatGAT 2.02 software [44] to calculate their identity scores. Subsequently, three separate phylogenetic trees were constructed for the *18S rRNA*, *cox1*, and *tufA* sequences, using the sequences that were generated in the present study and the homologous gene sequences that are available in GenBank. An MEGA software version 6.06 [45] was used to construct maximum likelihood phylogenetic trees, based on Tamura 3-parameter (*18S rRNA*) [46] or General Time Reversible (*cox1* and *tufA*) substitution models [47].

3. Results

A total of 219 questing ticks were collected from three different Mongolian provinces. Out of the 219 ticks, 102, 16, and 101 were collected from the Bayan-Olgii, Khovsgol, and Selenge provinces, respectively. All of the ticks collected from the Bayan-Olgii and Khovsgol provinces were identified as *Dermacentor nuttalli*, while 63 *I. persulcatus* and 38 *D. nuttalli* were identified from Selenge. A *B. microti*-specific nested PCR assay detected the parasite DNA in 19 of the tick DNA samples. All of the *B. microti*-positive DNA samples were sourced from *I. persulcatus*, indicating that the parasite-positive rate was 30.2% among this tick species, whereas none of the DNA derived from *D. nuttalli* was positive for this species of parasite.

When all of the *B. microti*-positive DNA samples were subjected to three separate PCR processes (which targeted the *18S rRNA*, *cox1*, and *tufA* genes [Table 1]), these gene sequences were amplified in 9, 16, and 13 DNA samples, respectively. The differences in the sensitivities of the individual PCR processes, based on the amplicon size and the copy numbers of the target genes, might explain these discrepancies. Sequencing analyses showed that the sizes of the amplified *18S rRNA*, *cox1*, and *tufA* fragments were 1,667–1,668, 1,023, and 823 bp, respectively. For each PCR amplicon, three clones were sequenced, and the analyses showed that the sequences often varied among the clones. As a result, 21 (LC005752–LC005772), 36 (LC005778–LC005813), and 23 (LC005814–LC005836) of the *18S rRNA*, *cox1*, and *tufA* sequences, respectively, were registered with GenBank. The identity scores among the *18S rRNA*, *cox1*, and *tufA* nucleotide sequences were 99.3–100%, 98.6–99.9%, respectively. Moreover, the similarities among the *cox1* and *tufA* amino acid sequences were 97.4–100% and 96.7–100%, respectively. Additionally, the *18S rRNA* sequences were highly homologous (99.6–100%) with the *18S rRNA* sequences from the *B.*

microti R1 strain (FO082874). Similarly, *cox1* and *tufA* nucleotide sequences were 96.7–97.2% and 94.7–95.3% homologous, respectively, with those from the R1 strain (FO082868 and LK028575). In addition, the *cox1* and *tufA* amino acid sequences shared 98.5–100% and 93.5–95.3% similarities, respectively, with the R1 strain sequences.

Three separate phylogenetic trees were constructed using the *18S rRNA*, *cox1*, and *tufA* sequences, together with the homologous sequences that were retrieved from GenBank. In the phylogenetic tree based on the *18S rRNA* sequences, all of the Mongolian sequences were clustered within the US-type clade (Fig. 1). This clade contains sequences from several other countries, including Zaire (AB190459), the United States (AF231348), Japan (AB190435), and Germany (EF413181). For the *cox1* and *tufA* genes, only the sequences from the R1 and Munich strains were available in GenBank; hence these, together with the Mongolian sequences, were used for phylogenetic construction (Fig. 2 and Fig. S1). In both of the *cox1*- and *tufA*-based phylogenetic trees, the Mongolian sequences were identified as separate lineages, which formed sister-clades to the R1 strain sequences within the major clades of *B. microti*.

4. Discussion

Babesia microti, which is primarily transmitted by biting ticks, causes human babesiosis. Babesiosis caused by *B. microti* has also been reported among travelers in endemic areas [48,49]. Hence, effective strategies to control the tick vectors of babesiosis are of paramount importance. In the present study, we investigated questing ticks in Mongolia to determine the prevalence of *B. microti* in these insects. The ticks collected from three provinces included *D. nuttalli*, which is the most abundant tick species in Mongolia. The *I. persulcatus* species, which was previously reported in the Khovsgol and Selenge provinces [50], was not detected in Khovsgol in the present study. The low number of samples from Khovsgol might explain this discrepancy. The presence of *B. microti*-DNA was only detected from *I. persulcatus*. In agreement with the present findings, the parasite has also been identified in *I. persulcatus* in previous studies in Russia and China; both countries neighbor Mongolia [23,51].

Babesia microti refers to a group of closely related subpopulations, which are usually classified by the analysis of their *18S rRNA* sequences [52,53]. Based on the phylogeny of the *18S rRNA* sequences of *B. microti*, the parasite population can be divided into four major types: Munich, Kobe, U.S. (US-type), and Hobetsu types [52]. The phylogeny constructed in the present study revealed that all of the Mongolian *18S rRNA* sequences corresponded to the US-type. In addition, the Mongolian *18S rRNA* sequences shared high identity scores with the sequence from *B. microti* R1 strain, which is a well-studied US-type human isolate [28–30]. These findings suggest that the parasites detected in the present study might be infective to humans. However, this assumption is not conclusive at this point, as the US-type also includes sequences sourced from other host animals in addition to those that are sourced from humans. Furthermore, in a laboratory-based investigation, a US-type *B. microti* isolate was

successfully transmitted by the *I. persulcatus* to hamsters [24]. Therefore, the detection of the US-type in *I. persulcatus* suggests that this tick species might be a vector of *B. microti* in Mongolia. *Babesia microti* was very recently detected in a human blood sample from Khutul city in Selenge province, Mongolia by molecular and serological tests [38]. The authors found that the 18S rRNA sequences were similar to either Chinese (KF410827) or Russian (AY144693) *B. microti* sequences. The Chinese sequence (KF410827) can be classified as the Kobe-type according to the phylogenetic trees that were constructed in the present study (Fig. 1); however, the Russian sequence (AY144693) was not considered, as the length of this sequence (1,254 bp) was relatively shorter than the sequences that we analyzed in the present study. At this point, we are not able to conclude that the *B. microti* detected in humans in Mongolia can be classified as the Kobe-type, as these recently determined Mongolian sequences are not available in a public database.

In addition to 18S rRNA, mitochondrial, and plastid gene sequences have been extensively used in evolutionary studies [31-34,54-56]. Hence, in the present investigation, we used the *cox1* (mitochondrial gene) and *tufA* (plastid gene) to characterize the *B. microti* species that we detected in ticks. All of the Mongolian sequences clustered together in the respective phylogenetic trees, and formed sister-clades to those of the R1 strain sequences. The common ancestors of the Mongolian and R1 strain sequences appeared as sister-clades to the *cox1* and *tufA* sequences from the Munich strain. These observations indicate that the *cox1* and *tufA* sequences in the present study are from *B. microti*. Mitochondrial genes are known to evolve rapidly in comparison to nuclear genes [54]. Thus, the *cox1*-based phylogeny may have divided the sequences into more distinct clades than those of the nuclear gene-based phylogenetic trees. This might explain why the *cox1* sequences from the R1 and Mongolia strains were clearly separated in the phylogeny, despite the fact that their 18S rRNA sequences were found in a monophyletic clade. In addition, the conserved nature of *cox1* among the four

distinct mitochondrial genomes of *B. microti*, [32], makes it an excellent target for PCR amplification. The present study also infers that the *tufA*-based phylogeny separates the *B. microti* sequences, similar to the *cox1* phylogeny. These findings collectively suggest that both *cox1* and *tufA* are useful targets for the phylogenetic characterization of *B. microti*. However, large numbers of gene sequences from different geographical regions should be analyzed to confirm this assumption.

In summary, the analysis of questing ticks in Mongolia found that *I. persulcatus* ticks are infected with *B. microti*, and that the detected parasites can be classified as *B. microti* US-type, based on the *18S rRNA* sequences. The results further suggest that *cox1* and *tufA* are potentially useful markers for phylogenetic characterization of *B. microti*. Having identified *B. microti* in Mongolian ticks, our findings warrant large-scale surveys to detect and characterize *B. microti* in small mammals, ticks, and humans. Surveys of this type would provide a more comprehensive understanding of the epidemiology of this zoonotic parasite in Mongolia.

Supplementary data

Supplementary Fig. S1

Acknowledgments

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Figure legends

Fig. 1. A phylogenetic tree of the *18S rRNA* sequences. The Mongolian *B. microti* sequences are shown in boldface letters. Bootstrap values are provided at the beginning of each branch. Note that all of the Mongolian sequences fall within the US-type.

Fig. 2. A phylogenetic tree of the *cox1* sequences. The Mongolian *B. microti* sequences are shown in boldface letters. Bootstrap values are provided at the beginning of each branch. Note that the Mongolian sequences clustered together and formed a sister-clade to the R1 strain sequences.

Graphical Abstract

Phylogenetic tree based on *B. microti* 18S rRNA sequences

Fig. 1

Fig. 2

Table 1. PCR primers.

Primers	Target genes	Sequences (5' - 3')	Amplicon sizes (bp)	Annealing temperatures	References
Bab1 Bab4 Bab2 Bab3	<i>18S rRNA</i>	CTTAGTATAAGCTTTTATACAGC ATAGGTCAGAACTTGAATGATACA GTTATAGTTTATTTGATGTTTCGTTT AAGCCATGCGATTTCGCTAAT	238 154	54	[40]
Piro0F2 Piro6R2 Piro1F2 Piro5R2	<i>18S rRNA</i>	GCCAGTAGTCATATGCTTGTCTTA CTCCTTCCTTTAAGTGATAAGGTTTCAC CCATGCATGTCTTAGTATAAGCTTTTA CCTTTAAGTGATAAGGTTTCACAAAACCTT	~1702 ~1670	61	[41]
Cox1F133 Cox1R1130	<i>cox1</i>	GGAGAGCTAGGTAGTAGTGGAGATAGG GTGGAAGTGAGCTACCACATACGCTG	1023	56	This study
EFtuAF2 EFtuAR2	<i>tufA</i>	ATGATAGTAGGTGTAAC TCAAATGG CACCTGGCATAGCCATTTTTAATTC	823	55	This study