Parasitology International 64 (2015) 527–532

Contents lists available at ScienceDirect

Parasitology International

journal homepage: www.elsevier.com/locate/parint

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

Bumduuren Tuvshintulga [a,b], Thillaiampalam Sivakumar [a], Badgar Battsetseg [b], Sandag-ochir Narantsatsaral [b], Batsaikhan Enkhtaivan [b], Banzragch Battur [b], Kyoko Hayashida [a], Kazuhiro Okubo [a], Takahiro Ishizaki [a], Noboru Inoue [a], Ikuo Igarashi [a], Naoaki Yokoyama [a,*]

[a] National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Inada-cho, Obihiro, Hokkaido 080-8555, Japan
[b] Laboratory of Molecular Genetics, Institute of Veterinary Medicine, Mongolian State University of Agriculture, Ulaanbaatar, Mongolia

ARTICLE INFO

Article history:
Received 27 February 2015
Received in revised form 22 July 2015
Accepted 24 July 2015
Available online 28 July 2015

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

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.

1. Introduction

Human babesiosis is a tick-transmitted zoonotic protozoan disease caused by several *Babesia* species, including *Babesia microti*, *Babesia divergens*, *Babesia 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], *Ixodes ricinus* [22], *Ixodes persulcatus* [23], and *Ixodes 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*)

* Corresponding author.
E-mail address: yokoyama@obihiro.ac.jp (N. Yokoyama).

http://dx.doi.org/10.1016/j.parint.2015.07.007
1383-5769/

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. A 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].

Table 1
PCR primers.

Primers	Target genes	Sequences (5′-3′)	Amplicon sizes (bp)	Annealing temperatures	References
Bab1	18S rRNA	CTTAGTATAAGCTTTTATACAGC	238	54	[40]
Bab4		ATAGGTCAGAAACTTGAATGATACA			
Bab2		GTTATAGTTTATTTGATGTTCGTTT	154		
Bab3		AAGCCATGCGATTCGCTAAT			
Piro0F2	18S rRNA	GCCAGTAGTCATATGCTTGTCTTA	~1702	61	[41]
Piro6R2		CTCCTTCCTTTAAGTGATAAGGTTCAC			
Piro1F2		CCATGCATGTCTTAGTATAAGCTTTTA	~1670		
Piro5R2		CCTTTAAGTGATAAGGTTCACAAAACTT			
Cox1F133	cox1	GGAGAGCTAGGTAGTAGTGGAGATAGG	1023	56	This study
Cox1R1130		GTGGAAGTGAGCTACCACATACGCTG			
EFtuAF2	tufA	ATGATAGTAGGTGTAACTCAAATGG	823	55	This study
EFtuAR2		CACCTGGCATAGCCATTTTTAATTC			