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Short communication

Phylogenetic relationships of Mongolian *Babesia bovis* isolates based on the merozoite surface antigen (MSA)-1, MSA-2b, and MSA-2c genes

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ABSTRACT

We conducted a molecular epidemiological study on *Babesia bovis* in Mongolia. Three hundred blood samples collected from cattle grazed in seven different districts were initially screened using a previously established diagnostic polymerase chain reaction (PCR) assay for the detection of *B. bovis*-specific DNA. Positive samples were then used to amplify and sequence the hyper-variable regions of three *B. bovis* genes encoding the merozoite surface antigen (MSA)-1, MSA-2b, and MSA-2c. The diagnostic PCR assay detected *B. bovis* among cattle populations of all districts surveyed (4.4–26.0%). Sequences of each of the three genes were highly homologous among the Mongolian isolates, and found in a single phylogenetic cluster. In particular, a separate branch was formed only by the Mongolian isolates in the *MSA-2b* gene-based phylogenetic tree. Our findings indicate that effective preventative and control strategies are essential to control *B. bovis* infection in Mongolian cattle populations, and suggest that a careful approach must be adopted when using immunization techniques.

1. Introduction

Babesia bovis, a tick-transmitted hemoprotozoan parasite, is potentially one of the most pathogenic *Babesia* species to infect bovine hosts (McCosker, 1981). Infections with this parasite can result in anemia, fever, hemoglobinuria, and icterus, as well as the presence of nervous signs (Ristic, 1981). Sequestration of *B. bovis*-infected erythrocytes (RBCs) in the blood vessels of internal organs, including the brain, is responsible for the involvement of the nervous system (Everitt et al., 1986). In severe cases, bovine babesiosis caused by *B. bovis* often leads to the death of infected animals (McCosker, 1981). Despite the availability of tick control techniques and treatment strategies, the disease continues to be one of the leading causes of financial losses encountered in cattle farming operations, especially in tropical and sub-tropical regions of the world (Smith et al., 2000; Shkap et al., 2007). Live attenuated vaccines are currently used to immunize susceptible cattle populations in several countries (Dalrymple, 1992). However, outbreaks may still commonly occur after vaccination due to strain variation (Brown et al., 2006). Previous studies conducted using different geographical isolates, outbreak isolates, and vaccine strains concluded that polymorphism of essential antigenic components of the parasites is the major cause for vaccination failure (Berens et al., 2005; Bock et al., 1995). Hemoprotozoan parasites use such antigenic polymorphism as an effective mechanism to evade the host's immune response for their long-term survival (Katzer et al., 1994).

Glycosylphosphatidylinositol (GPI) anchor proteins of *B. bovis*, including the merozoite surface antigen (MSA-1),

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Table 1
Primer sets used in the current study.

Primers[a]	Name	Sequence	Annealing temperature (°C)	Product size (bp)	Reference
Screening	SBP-2 F	5′-CTGGAAGTGGATCTCATGCAACC-3′	53	1236	Modified from AbouLaila et al. (2010)
	SBP-2 R	5′-TCACGAGCACTCTACGGCTTTGCAG-3′			
	SBP-2 NF	5′-TGAATCTAGGCATATAAGGCATTTCG-3′	55	584	
	SBP-2 NR	5′-AACCCCTCCTAAGGTTGGCTAC-3′			
MSA-1	MSA-1 F	5′-ATGGCTACGTTTGCTCTTTTCATTTCAGC-3′	60	960	Present study
	MSA-1 R	5′-TTAAAATGCAGAGAGAACGAAGTAGCAGAG-3′			
MSA-2b	MSA-2b F	5′-TCTGTGTCTGCTTCTGAGGAATC-3′	60	777	Present study
	MSA-2b R	5′-GCAGAGAGAACGAAGTAGCAGAG-3′			
MSA-2c	MSA-2c F	5′-CATAATAACCGTTGCATTCTGCTCCATCC-3′	60	821	Present study
	MSA-2c R	5′-GCAGAGAGAACGAAGTAGCAGAGAGT-3′			

[a] Previously described primers were selected for the screening PCR, and the primers for the amplification of merozoite surface antigen genes were designed in the present study.

MSA-2b, and MSA-2c, play a key role in the invasion of RBCs by the parasite (Yokoyama et al., 2006). These surface antigens, which belong to the variable merozoite surface antigen (VMSA) family, are expressed in both sporozoites and merozoites (Suarez et al., 2000; Mosqueda et al., 2002). RBC attachment of sporozoites and merozoites was effectively inhibited by the presence of anti-sera raised against the recombinant forms of these surface antigens (Mosqueda et al., 2002). Therefore, these antigens had potential as a recombinant vaccine against *B. bovis* infection (Brown et al., 2006). However, later studies have pointed out that gene fragments encoding the surface-exposed epitopes of these antigens exhibited remarkable polymorphism, which might enable the *B. bovis* parasites to evade the host's defense system (Carcy et al., 2006). Analysis of the polymorphic genes revealed that the parasites form well-separated phylogenetic clusters, and therefore, these genes became novel molecular makers for epidemiological studies on geographically different isolates of *B. bovis* (Genis et al., 2008, 2009; Borgonio et al., 2008). Apart from the genes coding VMSAs, the small subunit ribosomal RNA (*ssrRNA*) gene has also been considered as a marker for phylogenetic analysis (Genis et al., 2008). However, previous investigators suggest that the *ssrRNA* gene lacks adequate discriminatory power because the gene is highly conserved between isolates (Genis et al., 2008).

The Mongolian agricultural sector has been severely affected by various infectious diseases, such as foot-and-mouth disease and anthrax, and the impact of infectious diseases on the national economy is currently a pertinent issue. Therefore, understanding the epidemiology of these infectious diseases is essential to avoid devastating impacts on the industry. Our recent study indicated the presence of *Theileria orientalis*, which is also a hemoprotozoan parasite in cattle, in Mongolia (Altangerel et al., in press). Although we previously described the presence of *B. bovis* in Mongolia, the purpose was to evaluate a diagnostic technique for the parasite infection, and only limited numbers of bovine blood samples were used in that study (AbouLaila et al., 2010). Therefore, in the present study we have undertaken extensive analysis of bovine blood DNA samples collected from cattle to understand the distribution of *B. bovis* in Mongolia. We also determined the phylogenetic relationships of the Mongolian isolates based on the *MSA-1*, *MSA-2b*, and *MSA-2c* genes.

2. Materials and methods

2.1. Bovine blood samples

Blood samples were collected from cattle populations grazed in seven selected districts of three different provinces (Uvurkhangai, Uvs, and Khentii) in Mongolia, in May 2010 (Table 2) (Altangerel et al., in press). Approximately 2 ml of blood from the jugular vein of each animal was drawn into an EDTA-contained plastic vacutainer tube, and then stored at −20 °C until the subsequent DNA extraction.

2.2. DNA extraction

Blood DNA was extracted using a commercial Qiagen Blood Mini Kit according to the manufacturer's protocol (Qiagen, Hilden, Germany). Extracted DNA was eluted with 100 μl of the kit elution buffer, and then stored at −30 °C until use (Altangerel et al., in press).

2.3. Screening for the presence of B. bovis

Extracted DNA samples were screened using PCR analysis to test for the presence of *B. bovis*. A recently developed diagnostic nested PCR assay based on the *B. bovis* spherical body-2 (SBP-2) gene was used for screening with modified primer sets (Table 1) and conditions (AbouLaila et al., 2010). One microliter of the extracted DNA was added to a 9 μl of reaction mix, which contained 1 μl of 10× reaction buffer (Applied Biosystems, New Jersey, USA), 200 μM dNTPs (Applied Biosystems), 0.1 μM outer forward and reverse primers, 0.5 unit of Taq polymerase (Applied Biosystems), and double distilled water (DDW), before being amplified on a Veriti thermocycler (Applied Biosystems). Cycling conditions consisted of an initial activation step at 95 °C for 5 min, followed by 35 cycles of 95 °C denaturation for 30 s, and 53 °C annealing and 72 °C extension for 1 min each. A final extension step was performed at 72 °C for 7 min. After the first round of amplification, 1 μl of the PCR product was then used as the template for nested PCR amplification. A set of the inner nested forward and reverse primers was used for the amplification under cycle conditions similar to above, except that the annealing temperature was increased to 55 °C (Table 1). Amplification of the 584-bp