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Genetic detection of *Babesia bigemina* from Mongolian cattle using *apical membrane antigen-1* gene-based PCR assay

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ABSTRACT

We developed a new nested PCR (nPCR) assay based on the *Babesia bigemina* apical membrane antigen-1 (*AMA-1*) gene sequence for parasite-specific detection. The primers were designed to amplify 738-bp and 211-bp fragments of the *AMA-1* gene by primary and nested PCRs, respectively. The assay was proven to be specific for the *B. bigemina*, whereas the previously established *Spe*I-*Ava*I nPCR assay amplified not only the target fragment of *B. bigemina* but also a homologous one from *Babesia ovata*. The AMA-1 nPCR assay was also evaluated using field DNA samples extracted from 266 bovine blood samples collected from Mongolia in 2010. In a comparative evaluation, 90 (33.8%) and 25 (9.4%) of the blood samples showed positive reactions for *B. bigemina* by the *Spe*I-*Ava*I nPCR and AMA-1 nPCR assays, respectively. The sequencing analysis of the nPCR products confirmed that the AMA-1 nPCR method had specifically detected the target *B. bigemina* DNA. However, 4 different kinds of sequences were determined among the *Spe*I-*Ava*I nPCR amplicons. Two of them were derived from *B. bigemina* and *B. ovata*, while the origins of the others were unknown. In the current study, the presence of *B. bigemina* was clearly demonstrated among Mongolian cattle populations by the current nPCR assay for the first time. Furthermore, our findings also indicate that the AMA-1 nPCR assay may be a useful diagnostic tool for the specific detection of *B. bigemina*.

1. Introduction

Bovine babesiosis is an economically important infectious disease affecting the cattle industry, especially in the tropical and sub-tropical regions of world, and two species of the *Babesia* parasites, *B. bovis* and *B. bigemina*, are known to be the major etiological agents of the disease (McCosker, 1981). They are transmitted by *Ixodid* ticks, and the lifecycle of *Babesia* parasites begins with the injection of sporozoites by the infected ticks (Bock et al., 2004). The disease in the host animals is often characterized by anemia, hemoglobinuria, fever, and jaundice (Ristic, 1981). In addition, nervous signs and respiratory distress syndrome might be observed when the babesiosis is associated with *B. bovis* infection (Everitt et al., 1986; Wright and Goodger, 1988). Although *B. bovis* is thought to be the most pathogenic among all of the *Babesia* parasites that affect the bovine hosts, the effect of the disease caused by *B. bigemina* also becomes serious if the disease goes untreated (Brown et al., 2006). Therefore, early and correct diagnosis is essential to initiate proper treatment for the disease.

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Mongolia is an agricultural country, and an important sub-sector of the Mongolian economy is the livestock industry. However, many infectious diseases threaten the farming activities, and enormous financial losses have been recently experienced in Mongolia. The status of tick-borne hemoprotozoan diseases among the cattle population was not clear in the past. Very recently, our studies of Mongolian cattle have indicated the presence of *B. bovis* and *Theileria orientalis* (AbouLaila et al., 2010; Altangerel et al., 2011, in press). However, no epidemiological studies of *B. bigemina* have been conducted. Therefore, we decided to survey the Mongolian cattle populations for the presence of *B. bigemina*.

The microscopic examination of Giemsa-stained thin blood smears is the gold standard technique to detect the *Babesia* parasites. However, the low sensitivity and difficulties encountered in species differentiation have limited the role of microscopy (Bose et al., 1995). Therefore, the technique might not be effectively used to conduct large-scale epidemiological studies, especially those aimed to detect the carrier animals (Almeria et al., 2001). Consequently, new species-specific diagnostic techniques with higher sensitivity have been desired for the detection of *Babesia* parasites. Many PCR-based diagnostic methods with high sensitivity have been developed and evaluated for the genetic detection of *B. bigemina* (Figueroa et al., 1993; Guerrero et al., 2007). Among them, a nested PCR (nPCR) assay based on a *Spe*I-*Ava*I restriction fragment of *B. bigemina* has been widely employed for the detection of the parasite from bovine blood samples (Figueroa et al., 1992; Oliveira-Sequeira et al., 2005; Silva et al., 2009; Tsai et al., 2011).

On the other hand, *B. ovata* is also known as one of the bovine *Babesia* parasites and has been reported in Japan, Korea, and China (Bai et al., 1990; Minami and Ishihara, 1980; Suh, 1987). Although the parasite is less pathogenic for the hosts than *B. bigemina* (Minami and Ishihara, 1980), the morphological differentiation between the two *Babesia* parasites is often difficult (Bose et al., 1995). A recent study found that *B. ovata* locates close to *B. bigemina* in the 18S RNA gene sequence-based phylogram (Luo et al., 2005). However, none of the PCR techniques previously developed for the detection of *B. bigemina* has been evaluated against *B. ovata* for specificity.

A gene encoding the apical membrane antigen (AMA-1) of *B. bigemina* has recently been published (Torina et al., 2010). The gene sequences obtained from GenBank and Sanger sequencing indicate that the *AMA-1* genes are highly conserved, at least among Argentine, Italian, and Australian isolates of *B. bigemina*. Although the genomes of other *Babesia* parasites contain their original AMA-1 sequences that are homologous to the *B. bigemina* AMA-1, a certain degree of sequence diversity among different species of *Babesia* parasites was also observed (Tonukari, 2010). Therefore, in the present study, we developed an nPCR method for *B. bigemina*-specific detection based on the *AMA-1* gene sequences and compared the results with the specificity of the previously established *Spe*I-*Ava*I nPCR assay, as the latter one had not been evaluated for specificity against *B. ovata* (Figueroa et al., 1993). Subsequently, the Mongolian cattle populations were screened using nPCR assays to clarify the presence of *B. bigemina* in Mongolia and to evaluate the field utility of the newly developed nPCR assay.

2. Materials and methods

2.1. DNA samples

The Argentina strain (S1A) of *B. bigemina* (Hotzel et al., 1997), the Texas strain of *B. bovis* (Hines et al., 1992), and the Miyake strain of *B. ovata* (Minami and Ishihara, 1980) were maintained in *in vitro* cultures using previously established continuous micro-aerophilous stationary-phase culture systems (Igarashi et al., 1998; Vega et al., 1985). The bovine red blood cells (RBCs) infected with the parasites were washed in cold phosphate-buffered saline (PBS) three times; when the parasitemia reached 5%, the parasite DNAs were then extracted from the infected RBCs using a Qiagen Blood Mini Kit (Qiagen, Hilden, Germany). The extracted DNA samples were kept at −30 °C until use. *T. orientalis* DNA was prepared from the blood of cattle experimentally infected with the parasite. *Trypanosoma brucei gambiense* maintained by an Axenic culture system (Hirumi and Hirumi, 1989) was kindly provided from Dr. Noboru Inoue (National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Japan), and the parasite DNA was also extracted as described above. Bovine DNA samples were obtained from normal blood collected from a healthy cow. Qiagen Blood Mini Kit (Qiagen) was employed to extract DNA from 200 μl of each of 266 bovine blood samples collected from three different provinces (Khentii, Uvs, and Uvurkhangai) of Mongolia in 2010 (Table 2) (Altangerel et al., 2011).

2.2. Primer designs and establishment of new PCR assays

The *AMA-1* gene sequences of *Babesia* and *Theileria* parasites were obtained from GenBank (GenBank accession numbers, *B. bigemina*: GQ257738–GQ257740, HM543726–HM543730, AB481200, *B. bovis*: AY486101, FJ588028–FJ588028, XM001610993, *B. divergens*: EU486539, *T. parva*: XM761078, *T. annulata*: XM949044). The specifically conserved regions among the *AMA-1* gene sequences of different *Babesia* parasites were identified by multiple alignments using Multalin interface, an online software program (Corpet, 1988), and a pair of forward (5′-tggaccaggtacatgatcaagt-3′) and reverse primers (5′-aatcatcgtgctgacgacccttc-3′) was designed to amplify a part of the *AMA-1* homologous gene of *B. ovata* in the present study. In addition, *B. bigemina*-specific and highly conserved regions were also identified after the multiple alignments together with the newly determined *B. ovata AMA-1* gene sequence. Based on the sequence data, two pairs of primers were designed for the primary and nested PCR assays (Table 1).

For the AMA-1 nPCR assay, 1 μl of the template DNA sample was added to a 24 μl reaction mixture, which contained 2.5 μl of a 10 × PCR buffer (Applied Biosystems, Branchburg, NJ, USA), 200 μM of each dNTP (Applied Biosystems), 0.8 μM of the outer forward and reverse primers (BI-AMA-FO and BI-AMA-RO), 1 unit of Taq polymerase (Applied Biosystems), and double-distilled water