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Short communication

Genetic diversity of two selected antigen loci in *Babesia gibsoni* Asian genotype obtained from Japan and Jeju island of South Korea

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ABSTRACT

Previous reports have shown that the secreted antigen 1 of *Babesia gibsoni* (BgSA1) and the thrombospondin-related adhesive protein of *B. gibsoni* (BgTRAP) are promising diagnostic reagents and vaccine candidates. Therefore, we determined the extent of nucleotide sequence variation in the *BgSA1* and *BgTRAP* genes, obtained from eight isolates of *B. gibsoni* got from clinically infected dogs in geographically distinct areas of Japan and one isolate from Jeju island of South Korea. Sequence analyses have revealed that nucleotide diversity is lower in *BgSA1* than that in *BgTRAP*. The mean number of non-synonymous (dn) nucleotide substitutions was significantly greater than that of synonymous (ds) ones per site in region II of *BgTRAP*. Overall, the results predict more allele-specific immunity to BgTRAP than that to BgSA1, which could be useful in designing and testing efficacy of diagnostic reagents as well as vaccine candidates for the *B. gibsoni* isolates from Japan and Jeju island of South Korea.

1. Introduction

Babesia gibsoni is a small, tick-borne intraerythrocytic protozoan parasite, which is distributed worldwide (Boozer and Macintire, 2003). During the asexual stage in its natural hosts, *B. gibsoni* causes serious clinical problems, including remittent fever, progressive anemia, hemoglobinuria, and sometimes death (Casapulla et al., 1998). Because of the lack of effective control methods, *B. gibsoni* infection has become a significant problem in some endemic regions. Therefore, to be able to control the disease, there is need for development of both specific and sensitive diagnostic tests as well as protective vaccine.

In previous studies, several enzyme-linked immunosorbent assays (ELISAs) and immunochromatographic tests (ICTs) based on recombinant proteins, including *B. gibsoni* secreted antigen 1 (BgSA1) and thrombospondin-related adhesive protein (BgTRAP), have been established by our group (Jia et al., 2006, 2007; Goo et al., 2008). These methods have shown some advantages and comparable results with a semi-nested polymerase chain reaction (PCR) when used to detect the antibodies directed at *B. gibsoni*. In addition, BgTRAP has been shown to be a promising vaccine candidate for protection against *B. gibsoni* infection (Zhou et al., 2006). However, the accumulation and frequent switch of mutation in these genes under high immune pressure might lead to their

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incorporation when they are used as diagnostic agents or vaccine candidates. To date, there is no sequence available to document the variation in these genes among *B. gibsoni* isolates derived from different geographic origins of Japan. Here, we analyzed the sequence variations in the two leading antigens, BgSA1 and BgTRAP, of the parasite isolates from naturally infected dogs in Japan. A previous report based on small subunit ribosome RNA (*ssurRNA*) suggested that there are at least three genetically distinct small piroplasms from dogs (Kjemtrup et al., 2000). In order to define the genotype of the isolates, the *ssurRNA* locus was analyzed in this study.

2. Materials and methods

2.1. Blood samples

Nine blood samples were collected from domestic dogs with clinical signs consistent with *B. gibsoni* infection. These samples were positive for *B. gibsoni* infection when analyzed using both semi-nested PCR and ELISAs (Goo et al., 2008). The positive antibody responses against both BgSA1 and BgTRAP determined by the ELISAs suggested their expression in the isolates. Therefore, we used the PCR instead of RT-PCR to conduct the genetic analysis. The sample sources covered four geographically separate areas, Honshu ($n = 2$), Kyushu ($n = 2$), Shikoku ($n = 2$), and Okinawa ($n = 2$) in Japan and Jeju island ($n = 1$) in South Korea. In addition, to obtain a reference nucleotide sequence, the DNA of *B. gibsoni* NRCPD strain (Fukumoto et al., 2001), which was isolated from a hunting dog in the Hyougo Prefecture of Japan, was used as a template for PCR amplifications and subsequent DNA sequencing.

2.2. PCR assay

The DNA was extracted from 100 μl of whole blood using the QIAamp DNA Blood Mini Kit (QIAGEN, Germany) according to the instructions of the manufacturer. Extracted DNAs were stored at 4 °C until use. The primers for *BgSA1* (BgSA1F: 5′ TCTGGATCCCAAATAACAACACCTGTG 3′ and BgSA1R: 5′ CACCTCGAGCGCACCATACTAGAAAAC 3′) and *BgTRAP* (BgTARPF: 5′ GCGAATTCTGAGAATGAAGATGGCGATGGA 3′ and BgTRAPR: 5′ GCCTCGAGTTACTTG TACTCCCAGAAAAGAGG3′) were designed to amplify the full open reading frame excluding the signal peptides (Jia et al., 2006; Zhou et al., 2006). In addition, a set of specific universal primer designed from ssuRNA of canine *Babesias* was used to amplify a 1665 bp of *ssuRNA* sequence (Ikadai et al., 2004). All extracted DNA samples were used for all three PCRs. The PCRs were performed in a 50 μl-reaction mixture, which contained 2 μl of the extracted DNA, 20 pmol of each primer, 200 μM of each deoxynucleoside triphosphate, and 1 U of Taq Gold DNA polymerase (Applied Biosystems, USA) in a 1× buffer (10 mM Tris–HCl, 1.5 mM $MgCl_2$, 0.001% gelatin). To amplify the open reading frames of *BgSA1* and *BgTRAP*, the PCRs were performed for 10 min at 95 °C to activate the Taq Gold DNA polymerase. Thereafter, the reactions were repeatedly run for 35 cycles under the following conditions: 1 min of denaturation at 95 °C, 1 min of annealing at 54 °C, and 1 min of extension at 72 °C. A 7-min extension at 72 °C was performed after the last cycle, in the reactions, with both sets of primers. To amplify the *BgSA1* gene, the semi-nested PCRs were performed using specific primers (BgSA1F and BgSA1R-1: 5′ TACTCGAGGAAATAACCAGTAGCCTTGCG 3′; BgSA1F-1: 5′ AAGGACAAGATAAATGCTGG 3′ and BgSA1R) under the same conditions.

2.3. DNA sequencing

The PCR products were purified with the QIAquick Gel Extraction Kit (QIAGEN, Germany), and ligated into the pGEM-T vector (Promega, USA) for subsequent transformation in the *Escherichia coli* DH5α competent cells. One plasmid vector having the insert was purified from each clone using the QIAprep Spin Miniprep Kit (QIAGEN, Germany), and then sequenced by using the Dye Terminator Cycle Sequencing Kit (Applied Biosystems, USA) with the amplification primers and additional internal sequencing primers. The computer software of GENETYX version 7.0 (Software Development, Japan) was used for preliminary sequence alignment and sequence comparison.

2.4. Sequence analyses

Haplotype diversity, nucleotide diversity (π), average number of pairwise differences of nucleotides (k), and minimum number of recombination events were analyzed using the DnaSP version 4 (Rozas et al., 2003). A phylogenetic tree was constructed using the neighbor joining method with bootstrap confidence (Saitou and Nei, 1987). The rates of nucleotide substitution in pairwise comparisons between alleles were computed by the numbers of synonymous substitutions per synonymous site (ds) and non-synonymous substitutions per non-synonymous site (dn) (Nei and Gojobori, 1986) using the MEGA version 4.0 (Tamura et al., 2007).

3. Results

3.1. Sequence analysis of ssuRNA

All sequences of the nine isolates were found to be very similar (99–100%) at the *ssuRNA* loci and form a unique group with the *B. gibsoni* Asian genotype, but the sequence was distinctly different from those of other *Babesia* species and closely related apicomplexan species (data not shown).

3.2. Sequence variations in BgSA1 and BgTRAP

All of the sequences of the selected gene loci were obtained from the nine samples except for a sample from Kyushu, in which the *BgTRAP* sequence could not be amplified. This might be caused by substitution on the primer binding site or the sensitivity of the primers. The overall haplotype diversity, or the probability that two randomly chosen haplotypes were different in the population of *BgSA1* and *BgTRAP*, was 1.000 (S.D. = 0.0045) and 1.000 (S.D. = 0.052), respectively. The nucleotide diversity per site, π, and average number of nucleotide differences, k, are shown in Table 1. *BgSA1* showed the lowest nucleotide