**FULL PAPER**  Parasitology

**Sequence and Phylogenetic Analysis of the Thrombospondin-Related Adhesive Protein (TRAP) Gene of *Babesia gibsoni* Isolates from Dogs in Taiwan**

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**ABSTRACT.** The genetic diversity of *Babesia gibsoni* strains worldwide is currently poorly defined. The aim of the present study was to characterize *B. gibsoni* strains in naturally infected dogs in Taiwan using a combination of polymerase chain reaction (PCR) and sequence analysis of both 18S rDNA and the gene encoding thrombospondin-related adhesive protein (TRAP). Genomic DNA was extracted from 29 parasitemic dogs, and the target genes were separately amplified, sequenced and aligned with corresponding sequences available in GenBank. All 18S rDNA sequences (1,262 bp) amplified from the Taiwanese isolates were identical to each other and had very high similarity (99.9–100%) with previously reported *B. gibsoni* sequences. These results provide the first molecular evidence showing infection of dogs with *B. gibsoni* from Taiwan. On the other hand, a phylogenetic analysis based on the deduced amino acid sequence of the TRAP gene demonstrated that the Taiwanese isolates were closely related to strains previously identified from Okinawa Island, Japan, but genetically distinct from strains found on Honshu in Japan and Jeju Island in South Korea. The divergence of TRAP among the geographically dispersed strains examined in this study and others supports the conclusion that this gene is useful for molecular genotyping of *B. gibsoni* strains.

**KEY WORDS:** *Babesia gibsoni*, PCR, phylogenetic analysis, TRAP gene.


*Babesia gibsoni* is a tick-borne intraerythrocytic protozoan parasite that causes piroplasmosis in dogs. The agent has a global distribution, and infections have been reported in many regions of Asia [5, 8, 9, 19], Africa [6, 15], United States [12, 14], Australia [17], Europe [3, 7] and the Americas [21]. Clinical manifestations of the disease often include remittent fever, progressive hemolytic anemia, hemoglobinuria and marked splenomegaly, while severe forms can lead to death [2, 22]. The disease has frequently been found in companion dogs, creating a significant clinical problem in some endemic regions [1, 9]. There is currently no vaccine available for *B. gibsoni*-derived canine piroplasmosis.

Despite the worldwide distribution of *B. gibsoni* and its veterinary importance, few data are available with respect to the genetic diversity and phylogeny of *B. gibsoni* strains from geographically distinct areas. To date, most molecular epidemiology studies of *B. gibsoni* have focused on the 18S ribosomal RNA gene (18S rDNA), while much less is known about the other genes. However, accumulated data show that molecular characterization of the 18S rDNA has provided little information on strain diversity and suggests a high level of conservation [7, 8, 15, 19]. This indicates that the 18S rDNA may not be an ideal target for study of the genetic diversity of *B. gibsoni*.

Recently, Jia et al. [11] analyzed and compared the thrombospondin-related adhesive protein (TRAP) gene sequences of *B. gibsoni* isolates originating from Japan and Jeju Island in South Korea and noted the presence of substantial variations in the TRAP sequences of these isolates. The TRAP gene encodes one of the leading immunoreactive proteins in *B. gibsoni* [23]. Its protein product has been shown to be a promising vaccine candidate for protection against *B. gibsoni* infection [5, 13]. Investigation into the extent of sequence variation in this antigen candidate may help elucidate the diversities of *B. gibsoni* strains and pave the way for development of effective serodiagnostic reagents as well as vaccines for canine babesiosis. Herein, we characterized *B. gibsoni* isolates from naturally infected dogs in Taiwan using a combination of polymerase chain reaction (PCR) and sequence analysis of the TRAP gene. Previous studies based on 18S rDNA have revealed that there are at least three genetically distinct small piroplasms infecting dogs: *B. gibsoni* Asian genotype, *Babesia conradae* and *Theileria annae* (formerly, *Babesia microti*-like piroplasm) [12, 24]. In order to define the genotypes of the isolates, the 18S rDNA was also analyzed.

**MATERIALS AND METHODS**

**Blood samples and microscopic examination:** One hundred and twenty-five blood samples were collected from dogs exhibiting clinical signs compatible with canine piroplasmosis that were presented to the Veterinary Teaching Hospital of National Chung Hsing University and nine veterinary clinics throughout Taiwan from February 2008 to December 2009. Blood was collected into sterile tubes with anticoagulant (EDTA) and held at 4°C until arrival at the laboratory for further processing. Giemsa-stained blood smears were examined by light microscopy (by observing 100 microscopic fields at 1,000 × magnification) for the presence of *B. gibsoni* merozoites. Genomic DNA was
Nucleic acid was eluted into 100 µl of elution buffer and stored at –20°C until further use.

**PCR amplification:** The oligonucleotide primers used for amplification and sequencing of *B. gibsoni* genes (18S rDNA and TRAP) were designed using primer design software (PrimerSelect; DNASTAR, Madison, WI, U.S.A.) and related sequence information available in the GenBank database (accession Nos. AF175300-1 and AB053292) [23, 24]. All of the primers used in this study are shown in Table 1. For each PCR amplification, 5 µl of extracted DNA was used as a template in a 25 µl reaction mixture containing 1 × PCR buffer (Promega, Madison, WI, U.S.A.), 2.5 mM MgCl₂, 0.2 mM deoxynucleotide triphosphates (dNTPs), 0.8 µM each of primers and 0.125 U Taq DNA polymerase (Promega). PCRs were performed in an ABI#2700 thermocycler (Applied Biosystems, Foster City, CA, U.S.A.) according to the following parameters: 94°C for 3 min followed by 35 cycles of 94°C for 1 min, 58°C for 1 min and 72°C for 1.5 min, ending with an extension step of 72°C for 5 min.

**Cloning and sequencing:** The resulting PCR products were electrophoresed on a 1.2% agarose gel stained with ethidium bromide to check the size of amplified fragments by comparison to a DNA molecular weight marker (1 kb Plus DNA Ladder, Promega). In each case, the single amplicon of the expected size was column-purified using a QIAquick PCR Purification Kit (Promega) and ligated into pGEM-T vector (Promega) for subsequent transformation in DH5α competent cells. For each PCR amplicon, at least 3 recombinant clones were picked and purified individually using a QIAprep Spin Miniprep Kit (Qiagen) and then sequenced by using an ABI PRISM 3730 capillary sequencer (Applied Biosystems) and a Dye Terminator Cycle Sequencing Kit (Applied Biosystems) with a vector primer (T7 or SP6) and an appropriate internal sequencing primer (see Table 1). Both the sense and antisense strands of each PCR amplicon were sequenced. Sequences were manually edited to resolve ambiguities. A consensus sequence was obtained for each amplicon by comparing both the sense and antisense sequences.

**Sequence and phylogenetic analyses:** The BLAST program (http://www.ncbi.nlm.nih.gov/BLAST) was used for comparison and analysis of sequence data obtained in this study versus those previously deposited in GenBank. Multiple sequence alignment was performed using the program AlignX (Vector NTI Suite V5.5, InforMax, North Bethesda, MD, U.S.A.) with an engine based on the ClustalW algorithm [20]. Phylogenetic relationships between isolates were assessed by the neighbor-joining method using the MEGA 4 suite of programs [18, 20]. The distance matrix of amino acid divergences was calculated according to Kimura’s two parameter model furnished by MEGA.

**Nucleotide sequence accession numbers:** Representative nucleotide sequences for the *B. gibsoni* genes reported here have been deposited in GenBank. The selected isolates are as follows: the TWN2 isolate (FJ769386 for 18S rDNA, and GU447229 for TRAP), the TWN3 isolate (FJ769387 for 18S rDNA, and GU447230 for TRAP), and the TWN4 isolate (FJ769388 for 18S rDNA, and GU447231 for TRAP).

### RESULTS

The merozoites of *B. gibsoni* were detected by microscopic examination in 29 of the 125 blood smears (23.2%) of dogs exhibiting clinical signs compatible with canine piroplasmosis. Positive blood samples from these parasitic dogs were subjected to DNA extraction, amplification and sequencing. All 18S rDNA sequences of 1,262 base pairs (bp) derived from the 29 samples were found to be identical to each other, even though the samples were obtained from different locations. The sequence was 100% identical to the corresponding sequences from *B. gibsoni* strains in Japan (GenBank accession Nos. AF205636, AB478328-9 and AB118032), United States (AF205636, AF396748-9, DQ184507 and EU084677), Italy (EJ554534), Germany (AF175300) and Australia (AY102164) and 99.9% identical to eleven other Japanese isolates.
strains (AB478318-27 and AB478330) and four Korean strains (AB478320-3). Evidently, the sequencing data revealed that the identity of the detected pathogen was a *B. gibsoni* Asian genotype.

Subsequently, the *TRAP* gene fragments of 29 Taiwanese *B. gibsoni* isolates were amplified, cloned and sequenced to characterize the isolates. Computer-aided analysis with the resultant sequences revealed the presence of three different haplotypes of *TRAP*; they were provisionally designated as T WN 2 (n=10), T WN 3 (n=6) and T WN 4 (n=13). Both T WN 2 and T WN 3 had full-length sequences of 2,285 bp, including an open reading frame encoding a protein of 735 amino acid residues, while that of T WN 4 was 2,306 bp, including an open reading frame encoding a protein of 742 amino acids. Comparisons of these *TRAP* sequences derived from *B. gibsoni* genomic DNA with the reported *TRAP* cDNA sequence of the NRCPD strain (GenBank accession No. AB053292) [23] revealed interruption of the coding region by an 80-bp intron, starting at nucleotide position 68 and ending at position 69 of the cDNA sequence.

The nucleotide and deduced amino acid sequences of those *B. gibsoni* isolates were compared with those of nine published strains identified from Japan and Jeju Island in Korea [11]. As seen in Table 2, the Taiwanese isolates had nucleotide sequence identities of between 98.9% (T WN 3 and T WN 4) and 99.9% (T WN 2 and T WN 3) with each other and between 91.3% (T WN 4 and Honshu-2) and 99.9% (T WN 2 and Okinawa-1) with non-Taiwanese isolates. The Taiwanese isolates had amino acid sequence identities of between 98.6% (T WN 3 and T WN 4) and 99.7% (T WN 2 and T WN 3) with each other and between 89.7% (T WN 4 and Jeju-1) and 99.9% (T WN 2 and Okinawa-1) with non-Taiwanese isolates.

The deduced amino acid sequences of the *TRAP* genes of the aforementioned isolates were aligned for further comparison. In the first 430 residues, all Taiwanese isolates had amino acid changes at residues 33 (from K to H), 43 (from H to Y), 44 (from E to S), 45 (from Q to E) and 47 (from L to Q), respectively, in comparison with the NRCPD strain (data not shown). Most variations were observed in the remaining half of the protein, as shown in Fig. 1. Notably, most of the amino acid substitutions in this region are dimorphic, in which only two different amino acids are found at positions wherever substitutions occur. Also worth noting was the finding of the T WN 4 sequence, which uniquely contains an insertion of 7 amino acids (EAEETEE) between residues 587 and 588. Conversely, four reported *TRAP* sequences derived from strains Shikoku-2, Honshu-1, Honshu-2 and Jeju-1 lost 47 amino acids between residues 447 and 495.

Phylogenetic analysis of the deduced amino acid sequence of the full *TRAP* gene was performed to identify the evolutionary relationship between the detected Taiwanese isolates and other reported strains (Fig. 2). In the phylogenetic tree, the twelve isolates analyzed were grouped into two distinct clusters. The Honshu-1, Honshu-2 and Shikoku-2 strains (Japan), along with the Jeju-1 strain (South Korea), formed a separate group, whereas three representative Taiwanese isolates (TWNs 2-4) formed another group that also included the Okinawa-1, Okinawa-2, NRCPD, Shikoku-1 and Kyushu-2 strains (Japan). The results suggested that currently circulating *B. gibsoni* isolates in Taiwan may have a common ancestor originating in Japan.

**DISCUSSION**

Although *B. gibsoni* infection has long been considered enzootic throughout Taiwan, that conclusion is based on diagnoses that rely on clinical signs, hematological abnormalities and microscopic examination of Giemsa-stained peripheral blood smears. These diagnoses are often ambiguous and may fail to identify the parasitic species involved. In the present study, the 1,262-bp nearly complete 18S rDNAs of *B. gibsoni* from 29 infected dogs were amplified and sequenced, providing the first molecular evidence that this etiologic agent is involved in canine disease in Taiwan. Although the generated 18S rDNA sequences showed very low diversity compared with those from other geographically dispersed *B. gibsoni* strains, additional analysis of the

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**Percent similarity—nucleotide**
Fig. 1. Multiple alignment of deduced amino acid sequences of the TRAP gene of *B. gibsoni* strains. The TRAP sequences of *B. gibsoni*, including three representative isolates from Taiwan (TWNs 2–4), eight strains from Japan (NRCPD, Honshu-1, Honshu-2, Kyushu-1, Shikoku-1, Shikoku-2, Okinawa-1 and Okinawa-2) and one strain from South Korea (Jeju-1), were analyzed. Shown here is only the sequence of the carboxyl-terminal region where most variations were observed; numbering starts at the 421st residue of TRAP. Amino acids shaded in grey represent residues divergent from the sequence of the NRCPD strain, and a dash represents a gap. The GenBank accession No. for each individual sequence is given at the end of the sequence.
antigen-encoding gene TRAP did help to elucidate the genetic diversity of *B. gibsoni*.

Based on the sequence of the TRAP gene, the *B. gibsoni* strains reported in this study and others [11, 23] can be divided into two groups, one including the TWN 2, TWN 3, TWN 4, Okinawa-1, Okinawa-2, NRCPD, Kyushu-1 and Shikoku-1 strains, and the other containing the Shikoku-2, Honshu-1, Honshu-2 and Jeju-1 strains. The most remarkable difference between these two groups was the presence or absence of the 141-bp region encoding a 47-amino-acid domain (residues 447 to 495; see Fig. 1). It is worthwhile mentioning that this region was encompassed in the primer target of two published *B. gibsoni*-specific polymerase chain reaction (PCR) methods, including both conventional PCR and real-time PCR [4, 16]. In terms of the sequence divergence shown in Fig. 3, two primers, d3 (5'-TCC GTT CCC ACA ACA CCA GC-3') and d4 (5'-TCC TCA TCA TCG TGG TCG-3'), previously designed according to a cDNA clone of the TRAP gene (formerly known as the *P18* gene) of the NRCPD strain should be unable to amplify the expected 182-bp fragment from certain *B. gibsoni* strains, such as Shikoku-2, Honshu-1, Honshu-2 or Jeju-1. The incidence of canine *B. gibsoni* infections might have been underestimated in PCR-based epidemiological surveys.
using that primer set. The development of a widely applicable serologically and molecularly based diagnostics as well as an effective vaccine for B. gibsoni-derived canine babesiosis is undoubtedly dependent on an understanding of genetic differences that may exist in geographically dispersed strains of B. gibsoni, particularly with respect to the genes coding for immunodominant antigens, such as the TRAP gene. Subsequently, it would also be important to explore whether notable differences in the TRAP sequence of several B. gibsoni strains are involved in any antigenic variability.

On the other hand, the sequence divergence in the TRAP gene among geographically distributed B. gibsoni isolates examined in this study and others has provided useful information on a new target for genotyping of the organism. The results presented here also expand our knowledge about the genetic variability of B. gibsoni and encourage further research for analysis of genetic variation of B. gibsoni strains worldwide using additional samples. Further studies with more global distributed B. gibsoni isolates may enable the inference of the phylogeographic patterns of these strains. Considering that the clinical outcome of B. gibsoni infection varies widely from death to an asymptomatic chronic carrier state in untreated animals [1, 2, 10, 22], it would be of interest to assess the relationship between genetic variation in TRAP and strain virulence of B. gibsoni.

In conclusion, the present work is the first to document infection of dogs with B. gibsoni from Taiwan using molecular methods. The results of this study suggest that the TRAP gene of B. gibsoni, notably a 900-bp region near to its 3’ end, is an ideal target for phylogenetic analysis of the organism. Using this approach, we found that isolates identified in Taiwan were closely related to strains previously identified from Okinawa, Japan, but were phylogenetically distant from strains found on Honshu in Japan and Jeju Island in South Korea. Furthermore, analysis suggested that a strain of B. gibsoni bearing a novel TRAP genotype (namely TWN 4) was also present in sick Taiwanese dogs. Such molecular typing of currently circulating B. gibsoni isolates will facilitate improvement of existing diagnostic methods.

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REFERENCES