Quantitative PCR-Based Parasite Burden Estimation of Babesia gibsoni in the Vector Tick, Haemaphysalis longicornis (Acari: Ixodidae), Fed on an Experimentally Infected Dog

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ABSTRACT. Most causative agents of babesiosis, Babesia parasites, are transmitted transovarially in ixodid ticks. In this study, B. gibsoni, the causative agent of canine babesiosis which has transovarial transmission, was detected in tissues of the vector tick, Haemaphysalis longicornis using a modified quantitative PCR assay. Conventional PCR results showed that the newly designed primer set, which amplifies a 143-bp fragment of rhoptry-associated protein-1 (BgRAP-1) gene in B. gibsoni, was 100 times more sensitive than primers targeting P18 gene encoding 18 kDa protein of B. gibsoni, which was recently renamed as thrombospondin related adhesive protein (BgTRAP) gene, in an artificially generated sample solution containing metagenomic DNA (B. gibsoni DNA extracted from infected dog blood mixed with tick DNA). The TaqMan probe-based quantitative PCR (qPCR) for BgRAP-1 could also detect infected RBCs (iRBCs) at levels of 3.5 × 10^3 to 3.5 × 10^4/µl, a range that is broader than that of a past SYBR Green-based qPCR method for P18/BgTRAP which had a detection limit of 3.5 × 10^2 iRBC/µl. Using this qPCR assay, we attempted to quantify the B. gibsoni burden in tick ovaries and embryonated eggs. Levels of infection were normalized to the copy number of tick’s genomic DNA fragment of ribosomal DNA internal transcribed spacer region 2 (ITS2) for the standardization. According to this, low levels of parasite burden were quantified in ovaries and eggs. This detection system is sensitive and is recommended as a tool for elucidating the biological interactions between the vector tick H. longicornis and the parasite, B. gibsoni.

KEY WORDS: Babesia gibsoni, Haemaphysalis longicornis, parasite burden, quantitative PCR, transovarial transmission.


Ticks are important arthropod vectors of many pathogens, including Babesia spp. Detection and quantification of Babesia parasites in tick tissues such as hemolymph, egg, and the larval body have mainly been undertaken by microscopy [1, 2, 11–13, 21]. Recently, we established an experimental model for the babesial transovarial transmission [17] involving a dog experimentally infected with B. gibsoni, and its vector tick Haemaphysalis longicornis [20]. Previously, we reported a sensitive and accurate quantitative fluorogenic-detection PCR assay with SYBR Green dye for quantifying the number of tick-stage B. gibsoni based on its P18 gene which was recently renamed as thrombospondin related adhesive protein (BgTRAP) gene (GenBank accession no. AB053292 [5, 22]) [17, 19]. However, this method was not sensitive enough to quantify low concentrations of target DNA in metagenomic samples, including genomic DNA of both blood and ticks. Further, levels of infection normalized to an internal control DNA marker would be more suitable in quantifying the Babesia parasite burden in tick organs or in the whole tick described previously [9, 10, 17, 19]. Thus, the present study was undertaken to analyze parasite burden of B. gibsoni in tick tissues using a modified TaqMan probe-based qPCR system that targets parasite DNA in combination with the SYBR Green dye system for tick DNA. The method described here provides a way to determine B. gibsoni burden in tick tissues and should be useful for elucidating biological interactions between the vector tick, H. longicornis, and parasite, B. gibsoni.

MATERIALS AND METHODS

Parasites and tick: The parthenogenetic Okayama strain of the ixodid tick, H. longicornis, reared by feeding on rabbits as described previously [18] and B. gibsoni NRCPD strain [5] were maintained at the Laboratory of Parasitic Diseases, National Institute of Animal Health (Tsukuba, Ibaraki, Japan). Animals employed for the tick maintenance (rabbits) and for B. gibsoni maintenance (beagle dog) were acclimated to the experimental conditions for 2 weeks prior to the experiment and treated in accordance with protocols approved by the Animal Care and Use Committee, NIAH (Approval nos. 441, 508, 578).
B. gibboni DNA extraction from infected dog blood: One splenectomized dog was infected with B. gibboni prior to the start of the tick infestation experiment. Blood samples were collected daily, and the number of infected RBCs (iRBCs), and parasitemia were determined with light microscopic examination as described previously [5]. Genomic DNA was extracted from 200 µl of fresh blood using the Illustra blood genomicPrep mini spin kit (GE Healthcare Bio-Sciences AB, Uppsala, Sweden) according to the manufacturer’s instructions.

DNA extraction from tick samples: Thirty adult ticks were placed in a cotton bag attached to the back of the infected splenectomized dog with parasitemia around 0.5 to 1.0%. Parasitemia gradually increased and reached about 30% in 5 to 6 days. Eight ticks of those were mechanically crushed by dog’s scratch during their feeding process. Therefore, remaining 22 engorged ticks that detached spontaneously, were collected and incubated in a moist chamber with >95% relative humidity at 25°C. Of those, 14 ticks were randomly selected for dissection with 2 ticks dissected daily from 0 to 6 days post-engorgement (DPE) in sterilized phosphate-buffered saline (PBS) to collect the ovaries. The remaining 8 ticks were monitored until the end of the oviposition. Ten eggs were collected from each egg mass laid daily by each of eight ticks during the oviposition period (12–16 days) and incubated for 11 days for embryogenesis. Genomic DNA was extracted from the ovaries and eggs using standard procedures. DNA concentrations were estimated by measuring absorbance at 260 nm. All samples were stored at −80°C prior to use.

Construction of plasmids for the qPCR assay: Primers (DQ386864F and DQ386864R) targeting part of the B. gibboni rholtryp-associated protein-1 gene (BgRAP-1, GenBank accession no. DQ386864) were purchased from Nihon Gene Research Laboratories Inc. (nGRL, Miyagi, Japan). The P18d3 and P18d4 primer set [5] targeting P18/BgTRAP and the HIITS2f and HIITS2r primer set targeting H. longicornis ribosomal DNA internal transcribed spacer region 2 (HIITS2, GenBank accession no. AF271286) designed using Primer3 [15] were purchased from Sigma-Aldrich Japan (DQ386864TP) and labeled with 5’FAM (6-carboxyfluorescein) and 3’TAMRA (Table 1) was purchased from nGRL.

The amplified BgRAP-1 and HIITS2 DNA fragments were detected using SYBR Green dye. For BgRAP-1, the PCR mixture (20 µl) contained 0.5 µM of each primer, 200 nM of probe, reaction enzyme mix (LightCycler TaqMan Master, Roche), and 1 µl of template DNA (10-fold serial dilution of metagenomic DNA, 10-fold serial dilution of plasmid control DNA, sample DNA, 50 ng of tick genomic DNA as a negative control, or B. gibboni-infected dog blood DNA as a positive control). Amplification was carried out under the following conditions: an initial denaturation at 95°C for 4 min, followed by 35 cycles of denaturation at 95°C for 5 sec, annealing at 65°C (for P18/BgTRAP) or 60°C (for BgRAP-1) for 20 sec, and extension at 72°C for 20 sec. These cycles were followed by an extension at 72°C for 20 sec. PCR products were separated by electrophoresis in a 1% agarose gel and visualized by ethidium bromide staining.

Quantitative PCR: Quantitative PCR was performed with LightCycler® 1.5 (Roche Diagnostics, Mannheim, Germany) using standardized conditions and was analyzed using LightCycler® software version 3.5 (Roche). A double-dye hydrolysis TaqMan probe targeting BgRAP-1 (DQ386864TP) and labeled with 5’FAM (6-carboxyfluorescein) and 3’TAMRA (Table 1) was purchased from nGRL. The amplified P18/BgTRAP and HIITS2 DNA fragments were used as templates for creating standard curves for qPCR.

Conventional PCR: According to the previous report on the multiplicity of babesial RAP-1 gene in the genome [3], we verified that the primer set targeting BgRAP-1 was more sensitive than the set targeting P18/BgTRAP. Conventional PCR was performed using 10-fold serial dilutions of a metagenomic DNA sample: B. gibboni-infected dog blood DNA (parasitemia, 9.9%; iRBCs, 3.5 × 10⁵/µl) mixed with non-infected naïve H. longicornis genomic DNA. The number of iRBCs was calculated based on counts made on 1 µl of blood using a Burker-Turk hemocytometer (System Stage, Tokyo, Japan). The reaction mixture (20 µl) contained 1.0 µM of the primer set, 0.2 mM dNTP, 1 mM MgCl₂, 1.5 U of GoTaq® DNA polymerase (Promega, Madison, WI, U.S.A.) and 1 µl of template DNA. Cycling conditions were an initial denaturation at 95°C for 4 min, followed by 35 cycles of denaturation at 95°C for 5 sec, annealing at 65°C (for P18/BgTRAP) or 60°C (for BgRAP-1) for 20 sec, and extension at 72°C for 20 sec. These cycles were followed by an extension at 72°C for 20 sec. PCR products were separated by electrophoresis in a 1% agarose gel and visualized by ethidium bromide staining.

<table>
<thead>
<tr>
<th>Gene name (GenBank ID)</th>
<th>Primer or probe</th>
<th>Sequence (5’–3’)(a)</th>
<th>Nucleotide position</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>P18/BgTRAP (AB055292)</td>
<td>primer d3(b)</td>
<td>TCCGTTCACCAACACACCACGC</td>
<td>1417–1436</td>
<td>182 bp</td>
</tr>
<tr>
<td></td>
<td>primer d4(b)</td>
<td>TCCCTCTATCTATATCTATTCG</td>
<td>1577–1598</td>
<td></td>
</tr>
<tr>
<td>BgRAP-1 (DQ386864)</td>
<td>DQ386864F</td>
<td>GCTCTTGCTCATCATCTTTTC</td>
<td>815–835</td>
<td>143 bp</td>
</tr>
<tr>
<td></td>
<td>DQ386864R</td>
<td>GTTTCCATGTAGTCGATGTAC</td>
<td>936–957</td>
<td></td>
</tr>
<tr>
<td></td>
<td>DQ386864TP</td>
<td>(6-Fam)AATGCGTGCTACGTTGTATCCAA(Tamra)</td>
<td>894–919</td>
<td></td>
</tr>
<tr>
<td>HIITS2 (AF271286)</td>
<td>HIITS2f</td>
<td>GCCGTTGAGGAGATTCAAAT</td>
<td>200–219</td>
<td>196 bp</td>
</tr>
<tr>
<td></td>
<td>HIITS2r</td>
<td>CGCGTGTACAGGAAAGT</td>
<td>377–396</td>
<td></td>
</tr>
</tbody>
</table>

a) Fukumoto et al., 2001 [5].
RESULTS

Sensitivity and specificity of qPCR for the detection of *B. gibsoni* DNA: Conventional PCR assay showed that both primer sets targeting *P18/BgTRAP* and *BgRAP-1* from *B. gibsoni* DNA amplified the expected fragment sizes (182- and 143-bp, respectively), but no amplification occurred from tick DNA (Fig. 1A and 1B). The nucleotide sequences of both amplicons were found to be identical to those of the respective genes [5, 22, 23] (data not shown). In particular, the *BgRAP-1* sequence was identical to *BgRAP-1a* [16]. The primer set targeting *BgRAP-1* was about 100 times more sensitive than that targeting *P18/BgTRAP*, and this set was sensitive enough to detect parasite DNA, indicating that it was useful for the detection of *B. gibsoni* DNA in tick samples with very low levels of infection. Modified qPCR based on the TaqMan probe targeting *BgRAP-1* DNA was also conducted and quantitatively compared to qPCR for *P18/BgTRAP*. The assays were performed in triplicate with 10-fold serial dilutions of samples (3.5 × 10^−6 to 3.5 × 10^1 iRBCs/µl) and showed typical amplification curves from 3.5 × 10^5 to 3.5 × 10^1 (for *P18/BgTRAP*) and from 3.5 × 10^5 to 3.5 × 10^3 (for *BgRAP-1*) (Fig. 1C and 1D). Melting curve analysis of *P18-SYBR Green assay showed that an initial quantity of iRBCs equivalent to ≤ 3.5 × 10^3/µl was determined as non-quantifiable because of primer-dimerization (Fig. 1E). According to the linear standard curve, the mean cycle threshold (Ct) value ranged from 16.9 ± 0.45 to 23.64 ± 0.08 with a two-log dynamic range for *P18* and 19.58 ± 0.012 to 32.77 ± 0.41 with a four-log dynamic range for *BgRAP-1* (Fig. 1F), indicating that qPCR for *BgRAP-1* is more sensitive than that for *P18/BgTRAP*.

Parasite burden in ovaries and embryonated eggs: Standardized qPCR for *BgRAP-1* was used to estimate parasite burden in various samples of ovary at different days of engorgement. *BgRAP-1* DNA was detected on the day of engorgement (0 DPE), when parasite burden of 2.44 × 10^−3 ± 0.028 × 10^−3 and 2.04 × 10^−3 ± 1.09 × 10^−3, respectively, was observed (Fig. 2). The highest parasite burden (60.22 × 10^−3 ± 1.05 × 10^−3) was detected in 1 of the 2 ovaries on 3 DPE (Fig. 2).

According to the conventional PCR findings using embryonated egg samples, 1 of 8 egg batches (1 batch was derived from 1 adult female tick), namely batch c, showed the highest infection rate with 12 positives out of 16 pools (75.0%) (Fig. 3A). In this same batch, 11 of the 16 days post-engorgement-start (DPO) showed positive parasite burden by qPCR starting at 6 DPO, showing variable burden to 13 DPO and then consistently high parasite burden at 14 to 16 DPO (Fig. 3B). Indirect immunofluorescent staining of eggs from the most densely infected pools of eggs (14–16 DPO) shows spherical forms of parasites (Fig. 3C and 3D). These forms did not appear in the naïve eggs deposited by ticks fed on a naïve dog (data not shown). Considering that this form was previously observed in tick eggs infected with other *Babesia* spp. [6, 12, 13, 21], we suggest that the circular structure is the egg stage parasite of *B. gibsoni*.

DISCUSSION

In this study, sensitive qPCR assay for molecular detection of *B. gibsoni* was developed using the infected-dog blood and was also examined to estimate the parasite burden in the tissues and eggs of the vector tick, *H. longicornis* based on the LightCycler system with a TaqMan probe targeting *BgRAP-1*. This system has proved useful for quantifying *B. gibsoni* DNA in ticks, especially for low levels of parasite burden.

The presence of a miniscule parasite burden in the ovaries dissected on the day of engorgement (0 DPE) indicated that the migration of *B. gibsoni* from the midgut to the ovary via the hemocoel might occur on that day. Higuchi et al. [8] conducted a morphological observation of *B. gibsoni* parasites in the hemolymph and reproductive organs of *H. longicornis* to clarify the possibility of transition and transovarial transmission of *B. gibsoni* by this tick species and detected the kinetes form of *B. gibsoni* in the hemolymph on 10 DPE. They also described the existence of ovary-stage *B. gibsoni* on 5 DPE [7]. These observations suggest that only a fraction of the *B. gibsoni* acquired by ticks migrates from the midgut to the ovary during feeding.

Conventional PCR results confirmed transovarial trans-
Fig. 1. Sensitivity and dynamic range of PCR and qPCR for the detection of *B. gibsoni*. (A) Sensitivity and specificity of the P18/BgTRAP-conventional PCR assay using serial dilutions (iRBCs/µl). Lane 1, 3.5 × 10⁵; Lane 2, 3.5 × 10⁴; Lane 3, 3.5 × 10³; Lane 4, 3.5 × 10²; Lane 5, 3.5 × 10¹; Lane 6, 3.5; Lane 7, 3.5 × 10⁻¹; Lane 8, 3.5 × 10⁻²; Lane 9, 3.5 × 10⁻³; Lane 10, 3.5 × 10⁻⁴; Lane 11, 3.5 × 10⁻⁵; HI, naïve *H. longicornis* genomic DNA; Bg, *B. gibsoni* DNA extracted from infected dog blood; M, 100-bp DNA marker. The sample in Lane 4 (3.5 × 10² iRBCs/µl) is at the minimum detection limit. (B) Sensitivity and specificity of the BgRAP-1-conventional PCR assay with the same samples as in A. The sample in Lane 6 (3.5 iRBCs/µl) is at the minimum detection limit. (C) Selected amplification curves of the P18/BgTRAP-qPCR assay. The number shows the same sample as in A. (D) Selected amplification curves of the BgRAP-1-qPCR assay. The number shows the same sample as in A. (E) Selected melting curves of the P18/BgTRAP-qPCR assay. The number shows the same sample as in A. The Tₘ value of the P18/BgTRAP PCR product is 84.0°C. That of the primer dimer is about 89.5°C. (F) Standard curves for the P18/BgTRAP- and BgRAP-1-qPCR assays. The dynamic range of the BgRAP-1-assay is wider and more sensitive than that of the P18/BgTRAP-assay.

Fig. 2. Parasite burden of *B. gibsoni* in the ovary. DNA extracted from ovaries collected from dissections of ticks at each day post-engorgement was assayed by PCR and qPCR. Mean parasite burden is indicated as a percentage of the fragment copy number of amplified BgRAP-1 relative to that of tick HIITS2. Error bars depict the positive SD.
the infection of tick eggs with *Babesia* parasites is variable.

The pattern of parasite burden in eggs sampled throughout the oviposition period would suggest that densely parasitized eggs tend to be laid towards the end of the laying period. This is similar to the oviposition dynamics of *B. bovis*-infected eggs laid by *R/Bo. microplus* [2] and *B. bigemina*-infected eggs of *Bo. annulatus* [14]. Therefore, the vector-parasite interactions between *H. longicornis* and *B. gibsoni* might be similar to those of bovine *Babesia* and its vector tick in terms of transovarial transmission dynamics.

The present study is a preliminary report of a modified qPCR method based on the TaqMan probe that detects and quantifies tick-stage *B. gibsoni* burden in tick tissues, including ovaries and embryonated eggs that become infected following transovarial transmission of the parasite. This assay could be used to monitor and quantify the infection levels of tick-stage *Babesia* parasites in studies that elucidate the biological interactions between the vector tick *H. longicornis* and *B. gibsoni*.

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REFERENCES


153–158. [Medline]