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# The vector potential of *Haemaphysalis longicornis* ticks for *Babesia microti* parasites under experimental condition

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## ABSTRACT

*Babesia microti* is a known zoonotic agent naturally transmitted by *Ixodes* ticks. *Haemaphysalis longicornis*, a widely distributed hard tick in East Asia and Australia, is one of the most important tick vectors of *Babesia* and *Theileria* parasites. Here, we experimentally evaluated the interaction of *B. microti* parasites and *H. longicornis* ticks after blood feeding on mice infected with *B. microti*. *B. microti* DNA was detected in engorged larvae and nymphs after blood feeding on mice infected with *B. microti* by nested polymerase chain reaction (nested PCR). In addition, we performed a transmission test of *B. microti* parasites from *H. longicornis* ticks to mice. Although, the transmission of *B. microti* parasites from *H. longicornis* ticks to mice was not confirmed, the parasites' DNA was detected in engorged nymphs fed on *B. microti*-free mice. These results indicate that *B. microti* parasites can infect to *H. longicornis* ticks, but the tick not a vector for *B. microti* parasites.

**Keywords**: *Babesia microti*, *Haemaphysalis longicornis*, transmission test, vector

## INTRODUCTION

*Babesia* parasites are obligate intraerythrocytic protozoa, which are capable of infecting a broad range of vertebrate hosts. The parasites initiate infection to mammalian hosts by sporozoites, which are transmitted through the bite of infected ticks. Subsequently, the merozoites invade and replicate within the infected erythrocytes, eventually leading to acute babesiosis (Uilenberg 2006).

*Babesia microti* is reported mainly in North America, Europe and Japan, where it is transmitted by *Ixodes* ticks (Vannier *et al*. 2008, Vannier and Krause 2012, Zamoto-Niikura *et al*. 2012). *B. microti* is an important public health concern because it can affect both animals and humans (Vannier and Krause 2012). In North America, *B. microti* is naturally transmitted by *I. scapularis*, but the female *I. scapularis* feeds mainly on white-tailed deer (*Odocoileus virginianus*), which do not serve as reservoirs for *B. microti* (Piesman *et al*. 1979, Spielman *et al*. 1985). The natural reservoir of *B. microti* is the white-footed mouse, *Peromyscus leucopus* (Spielman *et al*. 1985). The adult *I. scapularis* feeds on deer during fall and again in the next spring, after which the females lay eggs. The eggs hatch in summer, and the larvae feed primarily on white-footed mice. At that time, *I. scapularis* larvae can acquire *B. microti* parasites. These infected larvae withstand winter, and then molt to become nymphs in the spring. The nymphs feed on the hosts during summer. Finally, these nymphs molt to adults in the fall, completing their life cycle (Piesman *et al*. 1987).

The hard tick *Haemaphysalis longicornis*, distributed mainly in East Asia and Australia, is known as a vector of theileriosis and non-zoonotic babesiosis, including *B. ovata* (Hoogstraal *et al*. 1968, Ishihara 1968). However, to our knowledge, it has not been investigated whether *H. longicornis* can maintain *B. microti* and serve as a host.

Here, the interaction of *B. microti* and *H. longicornis*, a non-vector tick, was studied. In this study, *H. longicornis* ticks acquired *B. microti* parasites through blood feeding on mice infected with *B. microti*..

## MATERIALS AND METHODS

### Animals

Japanese white rabbits (Kyudo, Kumamoto, Japan) were used to maintain the ticks in this study. Four-week-old ICR mice (Kyudo) were used for the experimental infection with *B. microti* and subsequent *H. longicornis* infestation. Rabbits and mice were cared for according to the guidelines certified by the Animal Care and Use Committee of Kagoshima University (Approval number VM13031). They were maintained under regulated conditions throughout the experiments.

### Ticks and parasites

A parthenogenetic *H. longicornis* population (Okayama strain) was maintained by feeding on the ears of Japanese white rabbits in our laboratory (Fujisaki 1978).

Mouse erythrocytes infected with *B. microti* (Munich strain) were stored under liquid nitrogen until used. First, the mice were intraperitoneally injected with the erythrocytes infected with *B. microti*. As the parasitemia peaked, whole blood was passaged to other mice through intraperitoneal injection. Finally, when the parasitemia of the mouse reached about 70% (hemolytic start), the whole blood was passaged to the other mice at 50 µl each. When the parasitemia of the mice was near to 10%, infestation with larval or nymphal *H. longicornis* was commenced. The reason for this timing is that the parasitemia of the mice intraperitoneally injected with 50µl *B. microti*-infected erythrocytes will reach about 70% after 4 to 5 days of injection. The amount of blood ingested

by ticks greatly increases after 4 to 5 days from attachment. Therefore, we expect that by the time the ticks are in rapid feeding, the parasitemia is already high, ensuring high numbers of parasites ingested by the tick.

**Blood feeding of larval and nymphal ticks on mice infected with *B. microti***

To examine whether *B. microti* parasites can be acquired by larvae and nymphs during blood feeding, *B. microti* DNA was extracted from the whole bodies of engorged larvae and nymphs fed on mice infected with *B. microti* using nested PCR. Moreover, we also determined whether *B. microti* can pass through the midgut barrier by also detecting *B. microti* DNA in different internal organs of the tick to evaluate its vector potential for *B. microti*. Larvae or nymphs were fed on mice infected with *B. microti* until they were engorged. Ticks were kept in the incubator at 25°C until use. DNA samples were extracted from the whole body of two separate ticks collected on days 0, 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, and 30 after engorgement. Additionally, on days 13 and 15, the engorged larvae molted to nymphs and the nymphs to adult ticks, respectively. DNA samples were also extracted from the internal organs (salivary glands, midgut, ovary, fat body, synganglion, and hemolymph) of three engorged nymphs without pooling them at days 0, 10, 15, 20, and 30 after engorgement.

***B. microti* DNA extraction and nested PCR**

DNA extraction was performed according to the methods of Maeda *et al*. (2013). DNA extracted from *B. microti*-free *H. longicornis* were used as negative control (NC) and DNA extracted from mice blood infected with *B. microti* merozoites were used as a positive control (PC) for nested PCR analyses. Primers designed to amplify the 18S ribosomal RNA of *B. microti* (accession number AY789075; product size 240 bp) were used in detection of the parasite. An outer forward primer, “CRYPTOFL” (5'-AAC CTG GTT GAT CCT GCC AGT AGT CAT-3'), and an outer reverse primer, “CRYPTORN” (5'-GAA TGA TCC TTC CGC AGG TTC ACC TAC-3'), were used for outer PCR, and then an inner forward primer, “Bab 1” (5’-CTT AGT ATA AGC TTT TAT ACA GC-3’), and an inner reverse primer, “Bab 4” (5’-ATA GGT CAG AAA CTT GAA TGA TAC A-3’), were used for nested PCR (Pieniazek *et al*. 2006, Welc-Faleciak *et al*. 2007). The PCR thermal cycle profiles of the outer PCR are as follows: initial denaturation at 95°C for 15 min, 45 cycles of denaturation at 94°C for 0.5 min, annealing at 65°C for 0.5 min, extension at 72°C for 1.5 min, and a final extension at 72°C for 9 min. And then, the inner PCR thermal profiles as follow: initial denaturation at 94°C for 1 min, 35 cycles of denaturation at 94°C for 1 min, annealing at 58°C for 1 min, extension at 72°C for 2 min, and a final extension at 72°C for 7 min. Amplification was confirmed by analyzing 10 µl of PCR product on 2% TAE agarose gel. A band of 240 bp was observed after the nested PCR for *B. microti* DNA.

The ribosomal protein L23 of *H. longicornis* (HlL23; accession number DQ849041; product size 408 bp) and the 18S ribosomal RNA of mice (M18SrRNA; accession number NR_003278; product size 236 bp) were detected as internal control DNA in order to confirm the proper DNA extraction from tick and mice blood samples, respectively.

### *B. microti* transmission test

Larvae previously fed on mice infected with *B. microti* molted to nymphs at around 13 days after engorgement. They were further kept in the incubator for 6 weeks before being infested onto *B. microti*-free splenectomized mice. Approximately 10 μl blood was collected from each mouse and then DNA samples were prepared every other day from 0 to 40 days. Blood samples for day 0 were collected from mice just before the start of tick infestation. The blood smears were also prepared by fixing with methanol, and then staining with 3% Giemsa solution, at the same time of the preparation of DNA samples.

## RESULTS

### Detection of *B. microti* DNA in larvae and nymphs fed on mice infected with *B. microti*

In larvae, *B. microti* DNA was only detected during the first two days after engorgement but not thereafter (Fig. 1 and Table 1). Meanwhile, in engorged nymphs, the parasites' DNA was detected in the whole body until 6 days after engorgement, but gradually disappeared thereafter (Fig. 2 and Table 1). In the internal organs, the DNA bands were found in the midgut, salivary glands, fat body, and synganglion at 0 days after engorgement. Moreover, DNA bands were found consistently in the ovary and fat body at 10 days and occasionally in the hemolymph (Fig. 3 and Table 1). After 15 days, no DNA was detected in all organs.

**Table 1. Detection of *B. microti* DNA fed on mice infected with *B. microti***

| Days after engorgement | No. of positive samples / No. of samples examined | | | |
|---|---|---|---|---|
| | Larvae[a] | Nymphs[a] | | |
| | whole[b] | whole[b] | internal organs[b, c] | |
| 0 | 2 / 2 | 2 / 2 | 3 / 3 | MG, SG, FB, SY |
| 1 | 2 / 2 | 2 / 2 | | |
| 2 | 1 / 2 | 2 / 2 | | |
| 4 | 0 / 2 | 1 / 2 | | |
| 6 | 0 / 2 | 1 / 2 | | |
| 8 | 0 / 2 | 0 / 2 | | |
| 10 | 0 / 2 | 0 / 2 | 3 / 3 | HE, OV, FB |
| 12 | 0 / 2 | 0 / 2 | | |
| 13 | molting to nymph | — | | |
| 14 | 0 / 2 | 0 / 2 | | |
| 15 | — | molting to adult | 0 / 3 | molting to adult |
| 16 | 0 / 2 | 0 / 2 | | |
| 18 | 0 / 2 | 0 / 2 | | |
| 20 | 0 / 2 | 0 / 2 | 0 / 3 | |
| 22 | 0 / 2 | 0 / 2 | | |
| 24 | 0 / 2 | 0 / 2 | | |
| 26 | 0 / 2 | 0 / 2 | | |
| 28 | 0 / 2 | 0 / 2 | | |
| 30 | 0 / 2 | 0 / 2 | 0 / 3 | |

[a] Larval and nymphal ticks were used for detection of *B. microti* DNA.
[b] DNA was extracted from whole body or internal organs of tick.
[c] HE, hemolymph; MG, midgut; OV, ovary; SG, salivary glands; FB, fat body; SY, synganglion

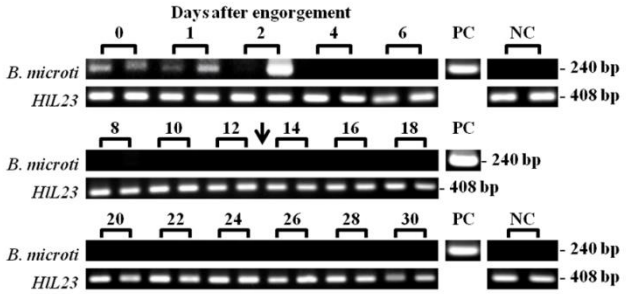


Fig. 1. Detection of *B. microti* DNA in the whole body of *H. longicornis* engorged larvae fed on mice infected with *B. microti*. *B. microti* DNA from engorged larvae was detected using nested PCR. Two engorged larvae were collected on each day after engorgement. *B. microti* indicates *B. microti* 18S ribosomal RNA, *HlL23* indicates *H. longicornis* ribosomal protein L23 for control DNA. PC, positive control; NC, negative control; arrow denotes molting (13 days). Day 0 indicates the day of engorgement and then the succeeding days after engorgement.

**Maintenance of *B. microti* from larvae to nymphs and *B. microti* parasites' transmission from *H. longicornis* to *B. microti*-free mice**

To investigate whether *B. microti* is maintained after molting from larvae to nymphs (transstadial transmission) and subsequently can transmit *B. microti* to *B. microti*-free splenectomized mice, a further experiment was performed. *B. microti* DNA was detected in larvae fed on *B. microti*-infected mice (Table 2). These larvae were allowed to moult into nymphs, and were kept 6 weeks after molting for starvation. The parasites' DNA, however, was not detected from 6-week-old nymphs (Table 2). Nevertheless, the 6-week-old nymphs were infested on *B. microti*-free splenectomized mice and allowed to engorge. Interestingly, *B. microti* DNA was detected from detached nymphs on day 6 after blood feeding started (Table 3), but not from the blood samples of the mice infested with the 6-week-old nymphs (data not shown). *B. microti* parasites were also not found in the blood smears (data not shown). *B. microti* mice (No. 2 ~ 4) and the control mouse (No. 2) died early in the experiment.

**Table 2. Detection of *B. microti* DNA from pre-transmission test ticks**

| Tick stages of transmission test | No. of positive samples / No. of samples examined | | |
|---|---|---|---|
| | Engorged Larvae[a] | → molting | 6-weeks-old Nymphs[b] |
| *B. microti* mouse No.1[c] | 3 / 10 | → | 0 / 10 |
| *B. microti* mouse No.2[c] | 2 / 10 | → | 0 / 10 |

[a] The larvae fed on *B. microti*-infected mice and engorged.
[b] The nymphs were collected at 6 weeks after molting from the engorged larvae fed on *B. microti*-infected mice.
[c] *B. microti* mouse indicate the mouse infected witn *B. microti*.

**Table 3. Detection of *B. microti* DNA from transmission test ticks**

| Days after infestation start[a] | No. of positive samples / No. of samples examined | | | No. of Succeeding Engorgement[b] |
|---|---|---|---|---|
| | Day 4 | Day 5 | Day 6 | |
| mouse No.1[c] | 0 / 29 | 0 / 7 | 0 / 1 | 37 / 40 |
| mouse No.2[c] | 0 / 18 | 0 / 12 | 0 / 2 | 32 / 40 |
| mouse No.3[c] | 0 / 17 | 0 / 8 | 0 / 9 | 34 / 40 |
| mouse No.4[c] | 0 / 10 | 0 / 13 | 1 / 5 | 28 / 40 |
| mouse No.5[c] | 0 / 13 | 0 / 11 | 1 / 3 | 26 / 40 |

[a] Days indicate the detachment days that take the number of day from ticks' infestation start.
[b] Collected No. of engorged nymphs / infesting No. of 6-weeks-old nymphs
[c] Mouse indicate *B. microti*-free and splenectomized mouse.

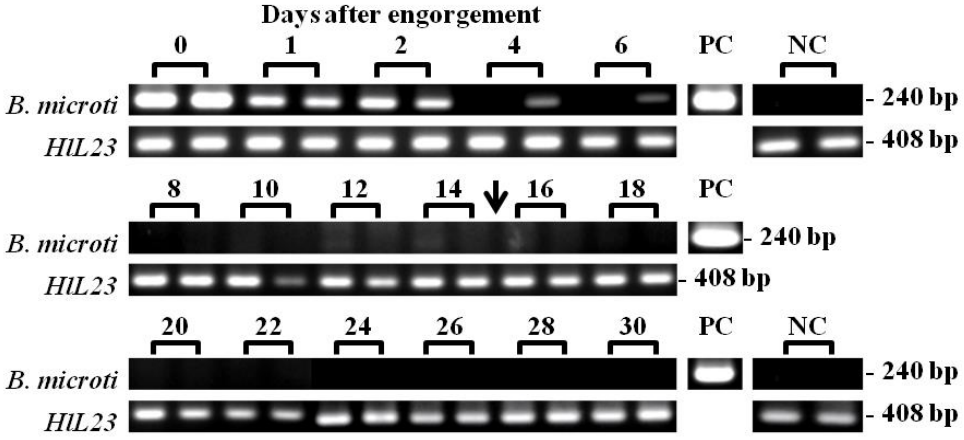


Fig. 2. Detection of *B. microti* DNA in the whole body of *H. longicornis* engorged nymphs fed on mice infected with *B. microti*. *B. microti* DNA from engorged nymphs was detected using nested PCR. Two engorged nymphs were collected on each day after engorgement. *B. microti* indicates *B. microti* 18S ribosomal RNA, *HlL23* indicates *H. longicornis* ribosomal protein L23 for control DNA. PC, positive control; NC, negative control; arrow denotes molting (15 days). Day 0 indicates the day of engorgement and then the succeeding days after engorgement.

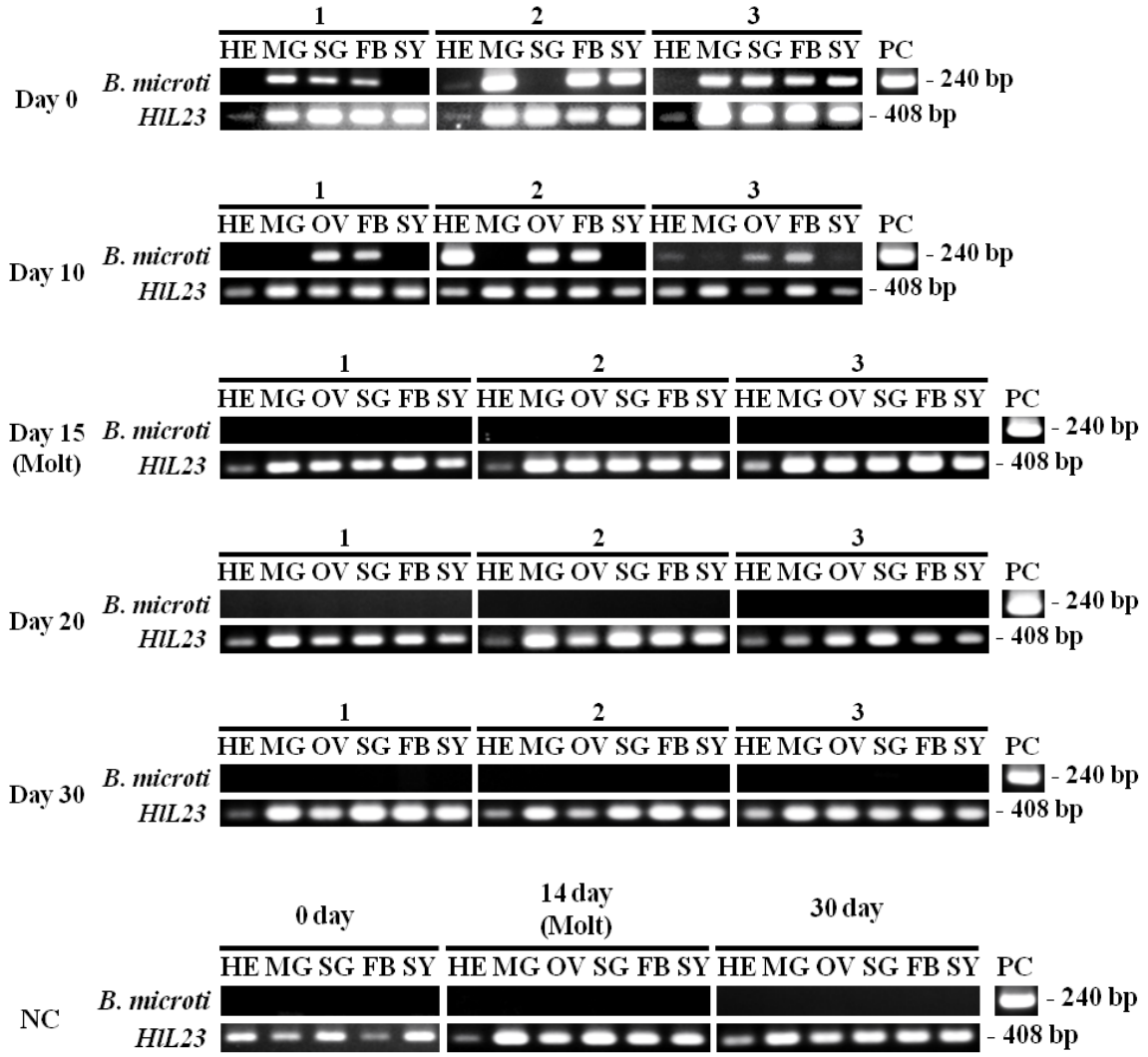


Fig. 3. Detection of *B. microti* DNA in different internal organs from *H. longicornis* engorged nymphs fed on mice infected with *B. microti*. *B. microti* DNA from different organs of engorged nymphs was detected using nested PCR. Three engorged nymphs were collected on each day after engorgement. Molt indicates nymphs molted to adult ticks after engorgement. *B. microti* indicates *B. microti* 18S ribosomal RNA, *HlL23* indicates *H. longicornis* ribosomal protein L23 for control DNA. PC, positive control; NC, negative control; HE, hemolymph; MG, midgut; OV, ovary; SG, salivary glands; FB, fat body; SY, synganglion. Day 0 indicates the day of engorgement and then the succeeding days after engorgement.

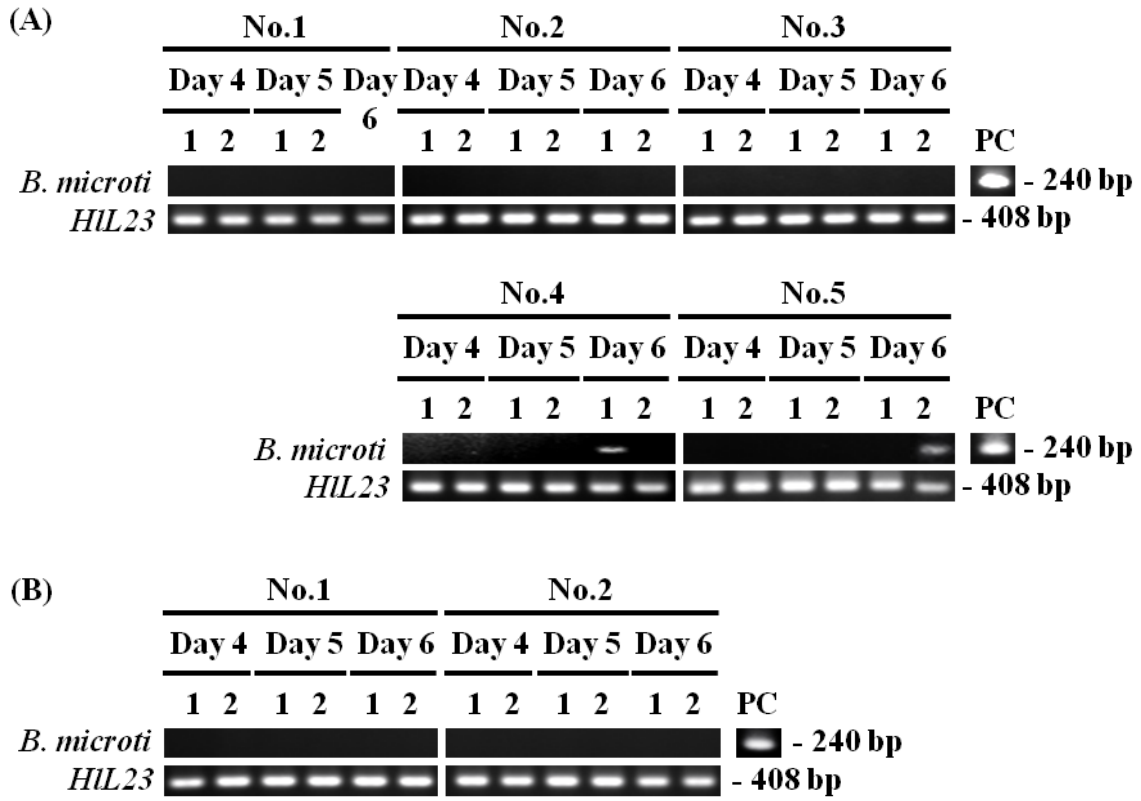


Fig. 4. Detection of *B. microti* DNA in the whole body of *H. longicornis* engorged nymph fed on *B. microti*-free and splenectomized mice. *B. microti* DNA from nymphs was detected using nested PCR. Two nymphs were collected each day from 0 days after engorgement, fed on *B. microti*-infected mice during larval stage (A) and on *B. microti*-free mice (B). *B. microti* indicates *B. microti* 18S ribosomal RNA; *HlL23* indicates *H. longicornis* ribosomal protein L23 for control DNA. Days indicate the detachment days that take the number of day from ticks' infestation start. Numbers above the bar indicate mouse numbers. Numbers under the bar indicate tick sample numbers. PC, positive control.

## DISCUSSION

In the present study, *B. microti* DNA was detected in the whole body of larval and nymphal *H. longicornis* ticks fed on mice infected with *B. microti*. The midgut is generally known as the first barrier for orally introduced pathogens transmitted by vector ticks. Interestingly, *B. microti* DNA was also amplified by nested PCR from the internal organs of nymphs, such as the salivary glands, ovary, and fat body. These results suggest that *B. microti* parasites passed through the midgut barrier of *H. longicornis*. This could be further explained by the hypothesis that the *Babesia* parasites can spread rapidly and invade the internal organs of the tick after passing through the tick's midgut barrier, because ticks have an open blood-vascular system and *Babesia* parasites do not have an affinity for a specific organ of the ticks. *B. bigemina* parasites invade the Malpighian tubule or myofiber in the vector tick (Hodgson 1992), which may explain how *B. microti* parasites invade the fat body or trachea of engorged nymphs after passing through the midgut barrier, as observed in this study. *B.*

*microti* DNA was also detected in some internal organs 10 days after engorgement, but not in the whole body. In this regard, it is also noteworthy that *Babesia* parasites are generally digested in the midgut of the tick after ingestion (Chauvin *et al*. 2009). The short duration of parasites' DNA detection may be due to the digestion of *B. microti* parasites in the midgut lumen, leading to a small number of the parasites that can pass through the midgut barrier, and low number undetectable by nested PCR. However, the pre-gametocyte, which is one of the parasite stages in the vertebrate host, can escape digestion in the midgut of the tick, resulting in further development and invasion of the midgut cells (Chauvin *et al*. 2009). Alternatively, *Babesia* parasites can avoid digestion in the midgut and develop to the kinete stage. Finally, these kinetes invade salivary glands cells and then develop from the sporoblast to the sporozoite stage (Chauvin *et al*. 2009). Furthermore, *B. microti* do not seem to be transovarially transmitted by I. scapularis, the primary vector tick (Piesman *et al*. 1979). In this study, *B. microti* was not transmitted from female *H. longicornis* fed on mice infected with *B. microti* to their eggs and the hatched larvae (data not shown).

In general, *B. microti* parasites are known to be naturally transmitted by ticks belonging to the *Ixodes* genus, particularly *I. scapularis*. Adult *I. scapularis* feed primarily on deer, which do not serve as reservoirs for *B. microti* parasites (Spielman *et al*. 1985), and the parasites are also not transovarially transmitted by *I. scapularis*, which is a primary vector tick (Piesman *et al*. 1979). Accordingly, *B. microti* parasites are considered unable to develop further in engorged adult ticks because of the low possibility of feeding on other hosts. Thus, transmission in the larval and nymphal stages is a rare opportunity for *B. microti* parasites to maintain their life cycle (Homer *et al*. 2000).

The transmission of the parasite from nymphs that molted from larvae fed on mice infected with *B. microti* to *B. microti*-free splenectomized mice did not occur, although *B. microti* DNA was unexpectedly detected in the engorged nymphs. These results suggest *B. microti* may infect *H. longicornis*, but the tick cannot maintain *B. microti* parasites. In previous studies, the injection of *B. microti* infected *I. ovatus* salivary glands homogenate to hamster induced high parasitemia and *I. persulucatus* also transstadially transmitted *B. microti* from nymph to adult (Zamoto-Niikura *et al*. 2012). Taken together, the maintenance of *B. microti* might occur in *H. longicornis* from larvae to nymphs, but transmission to mice from *H. longicornis* seems not to occur. Other studies have shown that *Theileria equi* were transmitted to the eggs and found in the salivary glands of unfed female *H. longicornis* after injection with horse blood infected with *T. equi* (Ikadai *et al*. 2007), and *B. gibsoni* parasites were transmitted to the ovary and salivary glands of unfed adult female *Ornithodoros moubata* ticks after injection with canine blood infected with *B. gibsoni* (Battsetseg *et al*. 2007). These results indicate that, for successful transmission, *Babesia* parasites must pass through the midgut barrier in vector ticks. Our results showed that *B. microti* may be able to pass through the midgut barrier of *H. longicornis* and subsequently lead to transstadial transmission.

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