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RESEARCH NOTE

# Transovarial persistence of *Babesia ovata* DNA in a hard tick, *Haemaphysalis longicornis*, in a semi-artificial mouse skin membrane feeding system

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## Abstract

Bovine piroplasmosis, a tick-borne protozoan disease, is a major concern for the cattle industry worldwide due to its negative effects on livestock productivity. Toward the development of novel therapeutic and vaccine approaches, tick-parasite experimental models have been established to clarify the development of parasites in the ticks and the transmission of the parasites by ticks. A novel tick-*Babesia* experimental infection model recently revealed the time course of *Babesia ovata* migration in its vector *Haemaphysalis longicornis*, which is a dominant tick species in Japan. However, there has been no research on the transovarial persistence of *B. ovata* DNA using this experimental infection model. Here we assessed the presence of *B. ovata* DNA in eggs derived from parthenogenetic *H. longicornis* female ticks that had engorged after semi-artificial mouse skin membrane feeding of *B. ovata*-infected bovine red blood cells. The oviposition period of the engorged female ticks was 21–24 days in the semi-artificial feeding. Total egg weight measured daily reached a peak by day 3 in all female ticks. Nested PCR revealed that 3 of 10 female ticks laid *B. ovata* DNA-positive eggs after the semi-artificial feeding. In addition, *B. ovata* DNA was detected at the peak of egg weight during oviposition, indicating that *B. ovata* exist in the eggs laid a few days after the onset of oviposition in the tick. These findings will contribute to the establishment of *B. ovata*-infected *H. longicornis* colonies under laboratory conditions.

## Keywords

Tick, *Haemaphysalis longicornis*, egg, *Babesia ovata*, β-tubulin, nested PCR

## Introduction

Bovine piroplasmosis causes economic losses in the livestock industry throughout the world (Marcelino *et al*. 2012). In Japan, the parasites *Babesia ovata* and *Theileria orientalis* are causative agents of bovine piroplasmosis and are transmitted by a dominant tick species, *Haemaphysalis longicornis* (Sivakumar *et al*. 2016; Watts *et al*. 2016). Although *B. ovata* and *T. orientalis* are both known to be benign, piroplasmosis caused by these parasites is a major concern for the cattle industry due to the adverse impacts of piroplasmosis on animal productivity. Since chemotherapeutics and a vaccine against bovine piroplasmosis are not available in Japan, usually a pour-on formulation of an acaricide, flumethrin, is used for the pre-

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vention and control of piroplasmosis there (Shimizu *et al*. 2000). It is noteworthy that the cost of the control of both ticks and tick-borne protozoan disease is estimated to be immeasurable not only in Japan but also in endemic areas worldwide.

Tick-parasite experimental models are essential to elucidate the transmission mechanism of parasites by ticks, toward the development of novel therapeutic and vaccine approaches. Tick-*Theileria* and tick-*Babesia* experimental models in cattle have been well established in Japan (Fujisaki *et al*. 1988; Higuchi *et al*. 1991; Ohta *et al*. 1996). To minimize the number of experimental animals, artificial feeding techniques have been developed using infected red blood cells (iRBCs) with their vector ticks (Bonnet *et al*. 2007; Inokuma and Kemp 1998; Voigt *et al*. 1993). A semi-artificial mouse skin membrane feeding technique was applied to establish an *H. longicornis*-*B. ovata* experimental infection model (Hatta *et al*., 2012; Maeda *et al*., 2016).

The *H. longicornis*-*B. ovata* experimental infection model revealed the time course of *B. ovata* migration in each organ of *H. longicornis* by a quantitative polymerase chain reaction (PCR) assay and the presence of *B. ovata* in the egg morphologically by immunostaining. However, the occurrence of *B. ovata* in the eggs of *H. longicornis* during oviposition remains largely unknown in this experimental model. Therefore, in the present study we performed a nested PCR assay to detect *B. ovata* DNA in *H. longicornis* eggs that were collected daily after a semi-artificial feeding of iRBCs.

## Materials and Methods

### Ticks, animals, and bovine blood

Female ticks of parthenogenetic *H. longicornis* (Okayama strain) were used. They were maintained at the National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine (OUAVM), feeding on the ears of Japanese white rabbits (Japan SLC, Shizuoka, Japan) by the cotton bag method (Fujisaki 1978). For the semi-artificial mouse skin membrane feeding, 3-week-old SPF BALB/c mice were purchased from CLEA Japan (Tokyo). The rabbits and mice were cared for in accord with the guidelines approved by the OUAVM Animal Care and Use Committee (approval nos. 28–34 and 28–35). Bovine blood was collected from a healthy Holstein cow from an OUAVM cow, under approval by the Animal Care and Use Committee of OUAVM (approval no. 25–78). The blood was defibrinated by shaking with glass beads and then depleted of white blood cells after centrifugation at 4°C for 500 × g for 5 min. The remaining RBCs were stored at 4°C as described (Igarashi *et al*. 1994).

### *Babesia ovata*

The Miyake strain of *B. ovata* was cultured *in vitro* as described (Igarashi *et al*. 1994). Briefly, *B. ovata* were cultivated with adult bovine RBCs in M199 medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 40% fetal bovine serum (Biological Industries, Beit Haemek, Israel) in 24-well plates under a low-oxygen atmosphere, 5% $O_2$, 5% $CO_2$ and 90% $N_2$ at 37°C.

### Semi-artificial mouse skin membrane feeding

Unfed female ticks of *H. longicornis* were used to feed *B. ovata*-iRBCs or *B. ovata*-free RBCs by artificial feeding. According to the method established by Hatta *et al*. (2012) and Maeda *et al*. (2016), the mouse skin membrane was prepared for 2–3 female ticks per mouse. Artificial feeding devices were made at the beginning of the ticks' rapid feeding phase. A mixture of 300 μl of *B. ovata*-iRBCs (0.9%–1.4% parasitemia) or *B. ovata*-free RBCs and 700 μl of bovine serum (Biological Industries) which had been inactivated and sterilized was pre-warmed at 30°C and then poured into each device. The devices were kept in a plastic container at 30°C with saturated humidity in continual darkness. The RBC and serum mixture was changed at every 12 h. A total of 10 engorged females was transferred to individual wells of 24-well microplates, and then kept in a plastic container at 25°C with saturated humidity in continual darkness for oviposition.

### DNA extraction

Eggs that each engorged female had laid were collected daily from the onset to the end of oviposition. The eggs laid daily were transferred to a 1.5-ml tube within 3 days after oviposition and kept at −30°C until use. The total DNA of each egg sample was extracted by a standard procedure for Bo_02, Bo_03, Bo_04, Bo_05, Bo_08, and Bo_11 (Hatta *et al*., 2013) or using NucleoSpin® Tissue (Takara Bio, Shiga, Japan) for Bo_06, Bo_07, Bo_09, Bo_10, and egg samples of female ticks fed *B. ovata*-free RBCs. For the PCR's positive control, *in vitro*-cultured *B. ovata* in bovine RBCs were subjected to DNA extraction using NucleoSpin® Blood (Takara Bio). All DNA samples were treated with RNase A (Life Technologies, Carlsbad, CA) to avoid RNA contamination.

### PCR and nested PCR assays

For the 1st amplification, 1 μl of each DNA sample was added to a 19-μl reaction mixture that contained 2 μl of 10 × PCR Buffer for KOD -Plus- Neo, 2 μl of 2 mM dNTP, 1.2 μl of 25 mM $MgSO_4$, 0.6 μl of 10 μM forward and reverse *B. ovata* β-tubulin specific primers (Sivakumar *et al*., 2014), 0.4 μl of KOD -Plus- Neo (1 U/μl) (Toyobo, Osaka, Japan), and 12.2 μl of autoclaved, distilled water. The reaction mixtures were subjected to an initial denaturation at 94°C for 2 min, followed by 30 cycles of denaturation at 98°C for 10 sec, annealing at 60°C for 30 sec, and extension at 68°C for 15 sec. For the nested PCR, the reaction mixture of 20 μl contained 2 μl of 10 × PCR Buffer for KOD -Plus- Neo, 2 μl of 2 mM dNTP, 1.2 μl of

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**Table I.** Biological parameter of individual engorged female *H. longicornis* in artificial feeding of *B. ovata*-infected red blood cells

| Biological parameter | Tick ID | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | **Bo_02** | **Bo_03** | **Bo_04** | **Bo_05** | **Bo_06** | **Bo_07**† | **Bo_08**† | **Bo_09** | **Bo_10** | **Bo_11**† |
| Body weight of engorged females (mg)[a] | 295.0 | 295.2 | 235.8 | 265.4 | 259.2 | 278.1 | 321.5 | 271.3 | 291.2 | 243.4 |
| Oviposition period (days) | 24 | 23 | 21 | 21 | 23 | 18 | 15 | 22 | 25 | 18 |
| Total egg weight during oviposition (mg)[b] | 182.3 | 186.3 | 120.5 | 112.6 | 146.6 | 153.7 | 170.4 | 162.1 | 170.5 | 144.6 |
| Ratio of egg weight/body weight (%)* | 61.8 | 63.1 | 51.1 | 42.4 | 56.6 | 55.3 | 53.0 | 59.7 | 58.6 | 59.4 |

*The ratio of egg weight/body weight was calculated using the body weight values of engorged females (a) and the total egg weight during oviposition (b)
†Female ticks of Bo_07, Bo_08, and Bo_11 accidentally died by piercing with tweezers during oviposition

25 mM $MgSO_4$, 0.6 µl of 10 µM forward primer (the same as that used in the 1st PCR) and reverse inner primer (*B. ovata*_tubulin_Rn2: 5'-CCTCAGCCTCCTTGCGTA-CAACATCAAG-3'), 0.4 µl of KOD -Plus- Neo (1 U/µl) (Toyobo), 12.2 µl of autoclaved, distilled water, and 1 µl of 50-fold-diluted 1st PCR products. The amplification conditions of the nested PCR were 30 cycles of denaturation at 98°C for 10 sec, annealing at 60°C for 30 sec, and extension at 68°C for 15 sec. On the other hand, to amplify ribosomal DNA internal transcribed spacer region 2 (ITS2) of *H. longicornis*, the PCR reaction mixture containing forward primer (HlITS2f-2: 5'-GGTGCTCGAGACTCGTTTTGAC-3') and reverse primer (HlITS2r: Hatta *et al*., 2013) was prepared as described above and were then subjected to an initial denaturation at 94°C for 2 min, followed by 30–40 cycles of denaturation at 98°C for 10 sec, annealing at 63°C for 30 sec, and extension at 68°C for 15 sec.

The PCR products were detected by ultraviolet illumination after agarose gel electrophoresis and ethidium bromide staining. The amplicon size of *B. ovata β-tubulin* after the 1st PCR and the nested PCR were 444 bp and 349 bp, respectively. The nested PCR products were cloned into a pGEM®-T easy vector (Promega, Madison, WI), and each ligation reaction mixture was used to transform ECOS™-competent *Escherichia coli* DH5α (Nippon Gene, Tokyo). Plasmid DNA was purified from each positive transformant using NucleoSpin® Plasmid EasyPure (Takara Bio). Each plasmid was subjected to DNA sequencing (Fasmac, Kanagawa, Japan).

## Results and Discussion

Ten female ticks dropped from the artificial feeding device and then laid eggs (Table I). The mean body weight of the engorged females and the mean ratio of egg weight/body weight were 275.6 ± 26.1 mg and 56.1 ± 6.1%, respectively. There was no significant difference between the parameters and those of *B. ovata*-free female ticks (249.5 ± 16.1 mg and 58.9 ± 2.9%; n = 4). The oviposition period of the ticks was 21–24 days except for the female ticks of Bo_07, Bo_08 and Bo_11, which were accidentally killed by tweezer-pierces. The weight of laid eggs per day of all female ticks reached a peak by day 3 and declined day by day (Fig. 1). The decline in oviposition was generally observed in *B. ovata*-free female ticks. These results indicate that the ovipositional ability of *H. longicornis* was unaffected by the ingestion of *B. ovata*-infected RBCs in

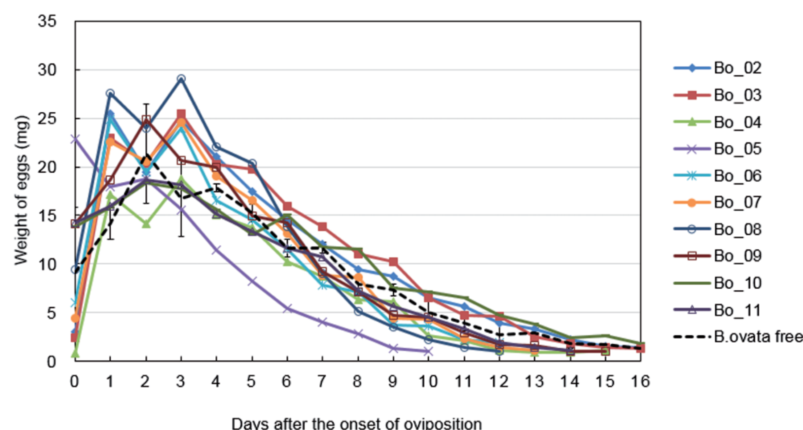


**Fig. 1.** Total egg weight in daily oviposition. The total egg weight from each *H. longicornis* was measured daily. The values >1.0 mg were plotted. Each ID of female ticks which ingested *B. ovata*-iRBCs is shown as "Bo_XX". Dashed line ("*B. ovata* free") indicates average values of eggs from female ticks feed on *B. ovata* free-RBCs

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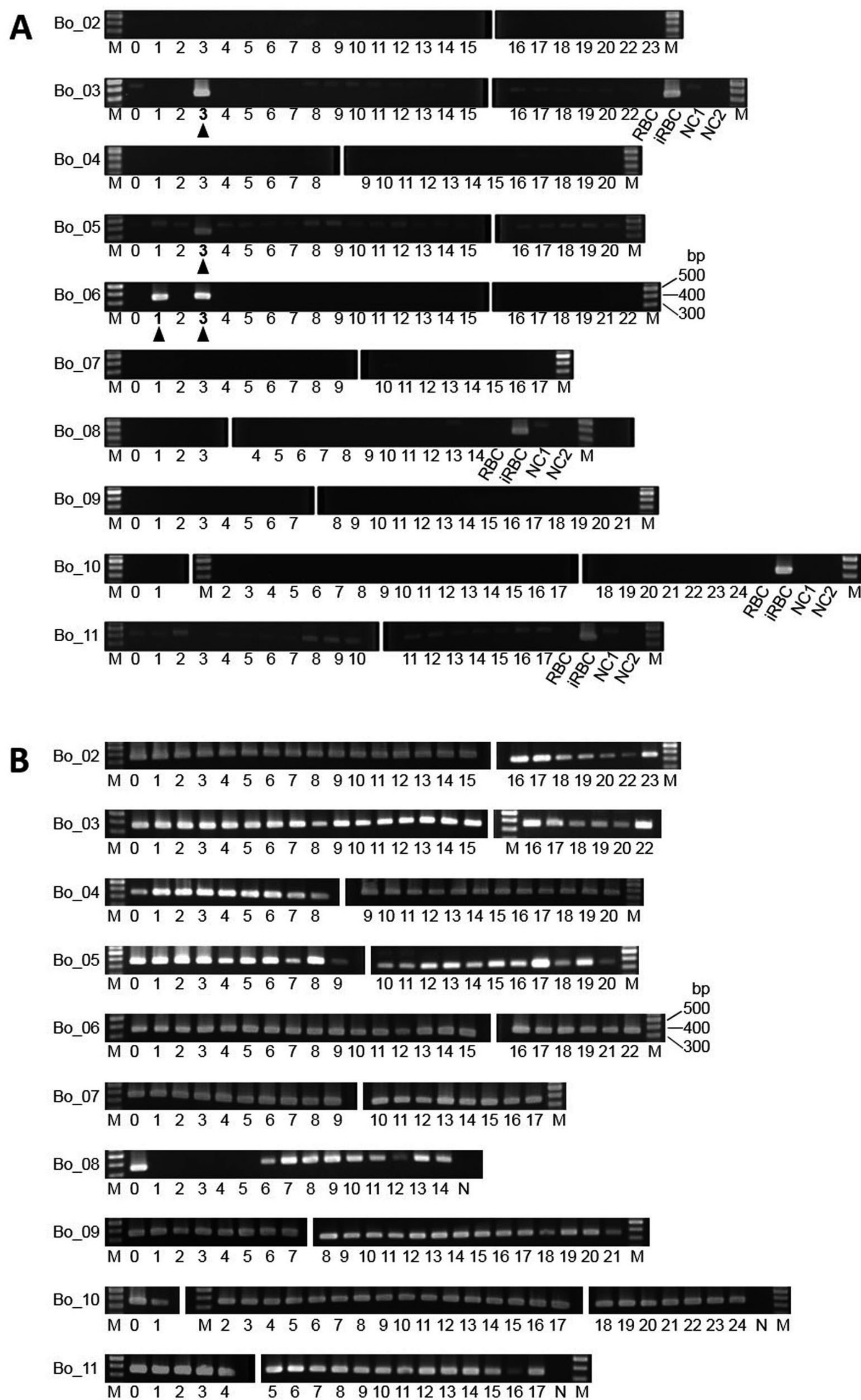

**Fig. 2.** (A) Nested PCR amplification of *B. ovata* DNA from total egg DNA samples derived from 10 female *H. longicornis*. Each tick ID is shown as "Bo_XX" like Fig.1. Numbers indicate days after the onset of oviposition. The nested PCR was conducted using 1 µl of 50-fold-diluted 1st PCR products as templates. Each DNA extracted from *B. ovata*-iRBCs (iRBC) and uninfected RBCs (RBC) was used as *B. ovata*-positive and negative controls, respectively. First-PCR products without template DNA were used as a negative control (NC1). The nested PCR reaction mixture without the template DNA was used as a negative control for the nested PCR (NC2). Black arrowheads indicate *B. ovata*-positive samples. (B) PCR amplification of ITS2 from total egg DNA samples derived from 10 female *H. longicornis*. Tick IDs and numbers were same as (A). PCR products without template DNA were used as a negative control (N). M, marker showing three bands of 300, 400, and 500 bp

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our study. Two egg-weight peaks were observed at day 1 and day 3 in six (Tick IDs: Bo-02, Bo_03, Bo_04, Bo_06, Bo_07, and Bo_08) of the 10 female ticks.

No amplification for *B. ovata β-tubulin* was detected by the 1st PCR in any of the egg samples. By the subsequent nested PCR, visible bands of the expected size of 349 bp were detected in egg samples from three of the 10 female ticks (Day 3 in Bo_03, Day 3 in Bo_05, and Days 1 and 3 in Bo_06) (Fig. 2A). Sequence analyses then confirmed that these bands showing the expected molecular weight (349 bp) were *B. ovata β-tubulin* (data not shown). Some approx. 450-bp bands observed in gels (e.g., Days 8 and 9 in Bo_05) did not show *B. ovata β-tubulin* by sequencing; these were nonspecific bands as detected in lanes of NC1. Although we tried to optimize PCR conditions and use other primers for such samples several times, the bands were found. The samples that the nonspecific bands were detected had been prepared by a standard procedure as described in the Materials and Methods section. Moreover, as shown Fig. 2B, *H. longicornis* ITS2 gene was successfully amplified by PCR in egg samples except for days 1 to 5 in Bo_08. Therefore, it is better to extract DNA from egg samples using a DNA extraction kit for PCR and nested PCR. Furthermore, the target gene in all egg samples was undetectable by a real-time PCR developed previously (Maeda *et al*., 2016) (data not shown), suggesting that the eggs collected within 3 days after oviposition contained a low copy number of *B. ovata β-tubulin* gene.

The amplifications of *B. ovata β-tubulin* were detected at the peak weights of the laid eggs (25.5 mg in Bo_03 on day 3, 15.6 mg in Bo_05 on day 3, and 24.9 mg and 24.0 mg in Bo_06 on days 1 and 3, respectively) (Table I, Figs 1, 2). In a previous study, using ticks fed on *B. ovata*-infected cattle, microscopic observations revealed that the number of *B. ovata* appearing in the eggs was largest 10 days after engorgement (corresponding to 4 days after the onset of oviposition) (Higuchi *et al*. 1991). In addition, after infestation on *B. bovis*-infected calves, the eggs from each engorged *Rhipicephalus* (*Boophilus*) *microplus* female tick were collected daily until the fifth day and then used to prepare smears. The microscopic observation revealed that 9.5% of the total number of eggs was infected with *B. bovis* (Cafrune *et al*. 1995).

The Cafrune *et al*. study also showed that the eggs laid during the first 72 h of the period of oviposition contained no *B. bovis*; however, the maximum infection was found on the seventh day of oviposition, when the bulk of the eggs had already been laid. Similarly to these reports, in our study *B. ovata* DNA was detected at the peak of oviposition in *H. longicornis*, indicating that *B. ovata* exists in eggs laid a few days after the initiation of oviposition in the tick. Our semi-artificial feeding system could help substitute for the cyclical development of *Babesia* in tick vectors and host animals.

The dynamics of *Babesia* infection in tick eggs is affected by embryonic development (Büscher *et al*. 1988). In the case of *B. ovis* in *R. bursa*, the ratio of infected eggs was elevated at day 4 and the peak was at day 9 of incubation after oviposition. In a study by Hatta *et al*. (2013), eggs laid daily by *B. gibsoni*-infected ticks were incubated for 11 days after oviposition for embryogenesis and then subjected to DNA extraction. In those embryonated eggs, *B. gibsoni* DNA was amplified by conventional PCR in all egg batches and was also detected consistently in some samples during oviposition. Oliveira *et al*. (2005) investigated the rate of *Babesia* spp. infection in eggs laid by female ticks that had engorged on cattle in an endemic area for babesiosis. The mass of eggs laid within the 6th to 15th days was used to prepare smears, and the rate of *Babesia* spp. infection was approx. 30%–50%.

We collected eggs within 3 days after oviposition to avoid masking of *Babesia* gene by increasing the amount of embryonic tissue in the present study. The frequency of *B. ovata* in *H. longicornis* eggs during embryonic development will be investigated in the future; however, our present findings provide helpful information for the effective selection of eggs with *B. ovata* for the establishment of *B. ovata*-infected *H. longicornis* colonies under laboratory conditions. Without any selection, the infection rate in the tick colony would decrease to almost 0% within a few generations, as in the case of a *B. ovis*-*R. bursa* system{Friedhoff, 1981, Transmission of Babesia by ticks}.

In the present study, *B. ovata* (which hide in eggs of *H. longicornis*) were successfully detected at the molecular level. Conventional PCR is a useful method for molecular confirmation of tick-borne pathogens in blood samples of animals (Singla *et al*. 2016; Takeet *et al*. 2017), while our results suggest that nested PCR is more suitable for detection of the target gene in tick eggs. Our findings will contribute to the establishment of *B. ovata*-infected *H. longicornis* colonies under laboratory conditions.

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