MOLECULAR EVIDENCE OF BABESIA EQUI TRANSMISSION IN HAEMAPHYSALIS LONGICORNIS

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Abstract. We studied the tick, Haemaphysalis longicornis, to determine the possibility of both transovarial and transstadial transmission of Babesia equi. We also studied the usefulness of the needle injection method for pathogenic tick-transmitted organisms including Babesia parasites. Erythrocytes infected with B. equi were injected into the midgut of engorged adults or nymphs using a hypodermic needle passed through the integument. DNA of B. equi in ticks was detected using nested polymerase chain reaction (PCR). B. equi DNA was present in adults, eggs, and larvae, indicating that transovarial transmission occurred. B. equi DNA was present in adults that developed from infected nymphs, and the B. equi antigen was present in their salivary glands, indicating that transstadial transmission occurred. These findings suggest that H. longicornis may play a role in the transmission of B. equi.

INTRODUCTION

Equine babesiosis, also known as biliary fever, is an acute, subacute, or chronic tick-borne disease of Equidae caused by infection with the hemoprotozoan parasites Babesia caballi or Babesia equi. The parasites, which are widely distributed in tropical and subtropical areas worldwide, result in significant economic loss for the horse industry. Babesiosis is generally characterized by fever, anemia, jaundice, and edema. It causes death in some cases.1-4 Infected horses often carry the parasites for long periods and act as sources of tick-borne infection of other horses.5 To date, there have been no reports of clinical cases of equine babesiosis in Japan,6 but there has been a long-term increase in the number of horses imported from foreign countries, including those where equine babesiosis is endemic. The presence of two tick vectors, Dermacentor reticulates and Rhipicephalus sanguineus, in Japan has also been reported.7 These conditions indicate that introduction of Babesia-infected horses poses a significant threat to the horse industry in Japan.

The hard tick, Haemaphysalis longicornis, is distributed mainly in East Asia and Australia, where it is known to transmit Babesia.8,9 H. longicornis is the most widespread tick species on wild and domestic animals, including horses, in Japan.8 Reproduction of this species is thelytokous.10 Moreover, H. longicornis was shown to be a capable biologic vector of equine B. caballi in an experimental study using non-obese, diabetic, and severe combined immune-deficient mice as the host model.11 B. equi is transmitted by tick species of the genera Dermacentor, Hyalomma, Rhipicephalus, and Boophilus.12-18 However, whether H. longicornis is a capable biologic vector of B. equi is unknown.

In this study, to elucidate the transmission system of B. equi, we studied transovarial and transstadial transmission of the parasite in H. longicornis by nested polymerase chain reaction (PCR) and show the tick as a potential biologic vector for the parasite.

MATERIALS AND METHODS

Haemaphysalis longicornis. The parthenogenetic Okayama strain of H. longicornis was obtained from the National Institute of Animal Health, Tsukuba, Japan, and was maintained in rabbits (Japan White, female; Crea Japan, Tokyo, Japan) in our laboratory for several generations.8 Tick colonies were kept in incubators at 25°C and 90-95% relative humidity under continuous darkness.

Parasite. US Department of Agriculture strains of B. equi were maintained in horse erythrocytes in continuous culture as previously described.19,20 The culture medium contained Medium 199 (M199; Sigma–Aldrich, St. Louis, MO) with 0.1 mmol/L hypoxanthine (Invitrogen, Carlsbad, CA). Horse erythrocytes at a packed cell volume of 10% in M199 supplemented with 40% (vol/vol) horse serum were cultured in 24-well culture plates (1-mL suspension/well) under a humidified atmosphere of 5% CO2 in air at 37°C. The culture medium was changed daily.

Transovarial transmission test. Twenty-two detached, engorged adult ticks were injected through the integument into the midgut with ~20 µL of a solution containing equal volumes of B. equi–infected erythrocytes with 19.0% parasitemia and M199. A hypodermic needle (MS needle 1/8; Tsuda, Saitama, Japan) was used for injection. The infected ticks were transferred into individual glass flasks and kept in an incubator at 25°C and ~85% relative humidity to allow oviposition. DNA samples were collected from adults after oviposition, from 200 eggs and from larvae hatched from the eggs.

Transstadial transmission test. Twenty detached, engorged nymphs were injected through the integument into the midgut with ~4 µL of a solution containing equal volumes of B. equi–infected erythrocytes with 10.9% parasitemia and M199. All nymphs were injected using a hypodermic needle (Tsuda). Five nymphs were collected for DNA analysis on Days 0, 10, 20, and 30 after injection.

Migration of the B. equi parasite to adults was monitored as follows. Fifty detached, engorged nymphs were injected through the integument into the midgut with 4 µL of a solution containing equal volumes of B. equi–infected erythrocytes with 8.8% parasitemia and M199. All nymphs were in-
ected using a hypodermic needle (Tsuda). The infected nymphs were allowed to molt. After molting, the ticks were allowed to feed on rabbits for 3 days to develop sporoblasts. Twenty of these adult ticks were dissected, and DNA was collected from salivary glands and carcass tissues for analysis by nested PCR. The remaining 30 adults were used for indirect immunofluorescence antibody tests (IFATs).

DNA preparation and nested PCR assay. Babesia-free H. longicornis adults were used as negative controls for PCR, and cultured B. equi merozoites were used as positive controls. DNA extraction of samples from ticks was performed using previously reported methods. Extracted DNA was stored in 50 μL TE buffer (10 mmol/L Tris-HCl, 1 mmol/L EDTA) at 4°C until use. Four oligonucleotide primers specific for B. equi were used for the PCR and nested PCR. Primers were derived from the merozoite antigen-1 (EMA-1) gene of B. equi. Forward primer EMA5 (5′-TCGACTTCCAGTTGGAGTCC-3′) and reverse primer EMA6 (5′-AGCTGCCACCACTATTACAC-3′) were used in the first amplification reaction. Primers EMA7 (5′-ATTGACCCACGTCCATCGA-3′) and EMA8 (5′-GTCCCTCTTGGAGAAGGT-3′) were used in nested PCR. The 50-μL reaction mixture contained 1 μL of template DNA, 5 μL of 10 × PCR buffer containing 15 mmol/L MgCl₂, 5 μL of the respective 2 mmol/L dNTP mix (Applied Biosystems), 1.25 U Taq DNA polymerase (Applied Biosystems), and 50 pmol of B. equi-specific primers for PCR. Amplification conditions were as follows: 94°C for 4 minutes, 40 cycles of denaturation at 94°C for 1 minute, primer annealing at 60°C for 1 minute, and amplification at 72°C for 4 minutes, followed by final extension at 72°C for 5 minutes. The product was chilled to 4°C. The final PCR products were subjected to electrophoresis in a 2.0% agarose gel with TBE buffer (89 mmol/L Tris-base, 89 mmol/L boric acid, 2 mmol/L EDTA). B. equi DNA produced visible bands at 268 bp in the first PCR and 218 bp in the second. The amplified DNA was cloned into a PCR 2.1-TOPO vector using a TOPO TA cloning kit (Invitrogen). The entire ligation reaction was used to transform Escherichia coli DH5α-competent cells. Plasmid DNA from two positive transformants was used for DNA sequencing of the insert. The plasmid containing the gene was isolated and subjected to DNA sequence analysis.

Anti-B. equi mouse serum. The purified B. equi merozoites used to immunize mice were treated by infecting erythrocytes from in vitro cultures with lysis solution (10 mmol/L KHCO₃, 155 mmol/L NH₄Cl, 0.1 mmol/L EDTA) for 5 minutes at room temperature, centrifugation (11,000 rpm, 4°C), three washes of the resulting pellet with cold phosphate-buffered saline (PBS), and suspension in PBS. Purified B. equi merozoites (1 × 10⁶) in Freud complete adjuvant (Difco Laboratories, Detroit, MI) were injected intraperitoneally into mice (BALB/c mice; age, 7 weeks). The same antigen in Freund incomplete adjuvant (Difco Laboratories) was injected intraperitoneally into the mice on Days 14, 28, and 42. Sera were collected from immunized mice 10 days after the last immunization.

IFAT. Salivary glands from adult ticks were prepared on slides, dried, fixed in cold methanol–acetone (1:1) for 20 minutes, and incubated in anti-B. equi mouse serum at 37°C for 1 hour. The slides were washed with PBS for 10 minutes and incubated with fluorescein-conjugated goat anti-mouse IgM + IgG + IgA (H+L; Southern Biotechnology, Birmingham, AL) with 5 μg/mL Hoechst 33258 (Polysciences, Warrington, PA) at 37°C for 1 hour. The slides were washed with PBS for 10 minutes and mounted in 90% glycerol for microscopic observation. Fluorescence microscopy and digital image collection were performed using a Nikon Eclipse E600 fluorescence-DIC microscope (Tokyo, Japan) and a Pixera cooled CCD camera (Penguin 600CL) equipped with InStudio software from Pixera (Los Gatos, CA).

RESULTS

Transovarial transmission of B. equi in H. longicornis. Of the 22 adults that were injected with B. equi–infected erythrocytes, 2 died immediately. The 20 surviving adults laid ~400 or more eggs each, and eggs from 10 of the 20 adults hatched. DNA was extracted from 20 adults after they had laid eggs, from pooled samples of 200 eggs each from the 20 adults, and from pooled samples of larvae hatched from eggs laid by the 10 adults. Nested PCR revealed the presence of 218-bp bands in extracts from adults, eggs, and larvae (Figure 1). B. equi DNA was amplified from 14 of the adults injected with B. equi.
**DISCUSSION**

We injected engorged adult or nymph *H. longicornis* with *B. equi*-infected erythrocytes and used nested PCR to show the presence of parasite DNA in adults after oviposition in eggs, larvae, and in adults after molting. These findings suggest that transovarial and transstadial transmission of *B. equi* occurs in *H. longicornis*.

*Babesia* species are generally transmitted transovarially through the eggs of adults to their offspring. However, the normal mode of transmission of *B. equi* in ticks is considered to be transstadial. The recent detection of *B. equi* DNA in eggs and subsequent larvae from field isolates of *Dermacentor nuttalli* and *Boophilus microplus* suggests that transovarial transmission of this parasite does occur. In our study, when engorged adults were injected with *B. equi*-infected erythrocytes, *B. equi* DNA was detected in the adults and in the eggs and larvae. After injection of *B. equi*-infected erythrocytes into detached, engorged nymphs, *B. equi* DNA was also detected in the nymphs and in the adults after molting. Moreover, the salivary glands of adults that developed from injected nymphs were positive for *B. equi* DNA, and salivary gland cells reacted to anti-*B. equi* mouse serum. These results show that *B. equi* can be transmitted experimentally by transovarial and transstadial routes and suggest that the route of transmission of *B. equi* differs from that of most other species of *Babesia*. In addition, *B. equi* may be transmitted by *H. longicornis* as well as known vectors (tick species of the genera *Dermacentor*, *Hyalomma*, *Rhipicephalus*, and *Boophilus*). Although no clinical cases of *B. equi* infection in Japan have been reported to date, our results indicate that horses in Japan are at risk of contracting babesiosis through transmission of *B. equi* from imported horses by *H. longicornis*.

Ticks are second only to mosquitoes as vectors of disease-causing agents in humans and are the most important arthropod that transmits pathogens to domestic and wild animals (e.g., *Babesia* and *Theileria* protozoa, *Rickettsia* rickettsia, *Borrelia* bacteria, and tick-borne encephalitis virus as flavivirus). Our injection method is relatively simple to perform. The needle injection method can probably be applied with other pathogenic tick–transmitted organisms, including rickettsia, bacteria, and viruses, which would be useful for studying transmission methods and interactions of pathogenic organisms and ticks.

In conclusion, we developed a method for transovarial and transstadial transmission of *B. equi* parasites to *H. longicornis* using engorged adults or nymphs injected with *B. equi*-infected erythrocytes. Our findings show that *B. equi* can be transmitted transovarially and transstadially in *H. longicornis*.
indicating that it is not unlikely that *H. longicornis* will become a transmission vector for *B. equi*. Further studies are necessary to determine whether this tick species is able to infect horses with *B. equi* under field conditions.

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