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Experimental transmission of *Babesia microti* by *Rhipicephalus haemaphysaloides*

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Abstract

Background: Human babesiosis is considered an emerging threat in China. Dozens of human infections with *Babesia microti* have been reported recently, especially in southern China. However, the transmission vectors of this parasite in these areas are not well understood. *Rhipicephalus haemaphysaloides,* which is one of the dominant tick species in southern China, is a major vector of bovine babesiosis in China. However, whether this tick has the potential to transmit *B. microti* has not been tested. The present study experimentally investigated the transmission competence of *B. microti* through *R. haemaphysaloides* ticks.

Methods: Larvae and nymphs of *R. haemaphysaloides* ticks were fed on laboratory mice infected by *B. microti*. The infection was detected by PCR at 4 weeks post-molting. BALB/c and NOD/SCID mice were infested by nymphs molting from larvae that ingested the blood of infective mice, and blood samples were then analyzed by PCR.

Results: Experimental transstadial transmission of *R. haemaphysaloides* for *B. microti* was proved in both the larvae to nymph and the nymph to adult transstadial routes. The positive rate of *B. microti* was 43.8 % in nymphs developed from larvae consumed infected mice and 96.7 % in adults developed from nymphs exposed to positive mice. Among the mice infested by infective nymphs, *B. microti* was detected in 16.7 % (2/12) of the BALB/c mice and in all of the NOD/SCID (6/6). However, the parasite was not observed to persist beyond more than one molt, and transovarial transmission did not occur.

Conclusions: This is the first study to demonstrate that *B. microti* can be transmitted artificially by *R. haemaphysaloides*. This tick species might be a potential vector of human babesiosis in southern China, which represents a public health concern.

Keywords: *Babesia microti*, *Rhipicephalus haemaphysaloides*, Transmission, Vector competence

Background

Babesia microti is a tick-transmitted, intraerythrocytic parasite that usually infects wild animals worldwide. This parasite is also known to be one of the major causative agents of human babesiosis and endemic mainly in the northeastern and upper midwestern United States [1]. Sporadic cases of *B. microti* or *B. microti*-like organisms have also been documented in places such as Germany, Japan, South Korea and Mongolia [1, 2]. In China, human cases of *B. microti* have been increasingly reported in recent years. In 2013, Zhou et al. reported 11 cases in Yunnan Province of southwestern China [3–5]. In 2015, Qiao et al. diagnosed a babesiosis patient and found 40 infected cases in colleagues of the patient in Guangxi Province of southern China [6]. Moreover, most medical staff are unaware of the risk, and investigation of this risk in the mass population is scarce. Hence, the number of human infections with this parasite might be largely underestimated. Together with the fact that human infections by other *babesia* species have also increased significantly in China [7, 8], it is believed that babesiosis is an emerging threat in the country [9].

Ticks of the genus *Ixodes* are considered the primary vector of *B. microti* [10]. The parasite is mainly transmitted to people by *Ixodes scapularis* in the United States. *Ixodes spinipalpis* is also a known vector, and still other

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Ixodes spp., including *I. angustus*, *I. eastoni* and *I. muris*, are suspected to transmit the parasite in North America. In many European countries, the primary vector of *B. microti* is usually *I. ricinus*, although *I. trianguliceps* is believed to play a more important role in England [11]. Infections by *I. persulcatus* have also been reported in Russia. In Asia, *B. microti* Hobetsu type have been detected in questing *I. ovatus* from Japan, and US type and a type related to *B. microti* Kobe have been detected in *I. persulcatus* [10, 12–15]. There are few studies on the vectors of *B. microti* in China, except in the northeastern regions, where *I. persulcatus* is considered the primary vector [13, 16]. However, competent vectors of *B. microti* in other areas of the country are not yet well understood [17].

Interestingly, *B. microti* was recently detected in ticks outside the *Ixodes* genus. Wójcik-Fatla and colleagues detected infections in questing *Dermacentor reticulatus* ticks from Poland [18], and the parasite was reported in questing *Haemaphysalis concinna* ticks from northeast China [16]. Therefore, more studies are needed on the transmission potentials of tick species from other genera.

Rhipicephalus haemaphysaloides, a three-host hard tick, is widely distributed in southern China and other countries in Southeast Asia [17, 19]. This species has been reported to be a major vector of bovine babesiosis in China [20] and can transmit the Kyasanur Forest disease virus [21]. As far as we are aware, the transmission of *B. microti* through *R. haemaphysaloides* has not been reported. To understand whether *R. haemaphysaloides* could play a role in the spread of *B. microti* in China, we performed a series of experiments to study the transmission competence of *B. microti* through a *R. haemaphysaloides* strain maintained in our laboratory.

Methods

Experimental mice and ticks

Specific-pathogen-free (SPF) female BALB/c or NOD/SCID mice weighing 16 to 18 grams were purchased from Shanghai Laboratory Animal Center, Chinese Academy of Sciences. All animal experiments were performed according to the protocols approved by the Ethics Committee at the National Institute of Parasitic Diseases, Chinese Center for Disease Control and Prevention based in Shanghai.

An engorged *Rhipicephalus haemaphysaloides* female was removed from a dog in a village of Tengchong County, Yunnan province, China, in 2014. The tick was then maintained in our laboratory in an incubator at 25 °C, with 85 % relative humidity and a 14/10 h light/dark photoperiod regimen. The larvae and nymphs of *R. haemaphysaloides* used in this study were the third generation of the colony.

Transmission experiments of *Babesia microti*

Three experiments were performed to study the transmission competence of *R. haemaphysaloides* ticks. The experimental procedures are shown in Figs. 1 and 2.

(i) Transmission of B. microti from mice to ticks

B. microti (Peabody mjr Strain, ATCC PRA-99) [22] were provided by Institute of Laboratory Animal Sciences, Chinese Academy of Sciences. The cryopreserved stabilates of *B. microti* were thawed to room temperature. Donor BALB/c mice were infected intraperitoneally with 100 μl of the stabilates. The resulting infections were then inoculated intraperitoneally into experimental animals at doses of 100 μl of blood with 50–60 % parasitemia.

Approximately 600 larvae were applied equally by brush to 2 mice with *B. microti* parasitemia of 20–30 % three days after incubation. Each mouse was collared with a plastic cap with central drilling to prevent grooming. In the same way, 4 infected mice were infested with nymphs by applying 30 nymphs to each animal. Mice infested with ticks were maintained over trays with water in the bottom from which detached engorged ticks were harvested.

Twenty engorged larvae (10 from each mouse) and 12 engorged nymphs (3 from each mouse) were placed in 70 % alcohol for storage before being analyzed for infection by PCR. The engorged ticks remaining were

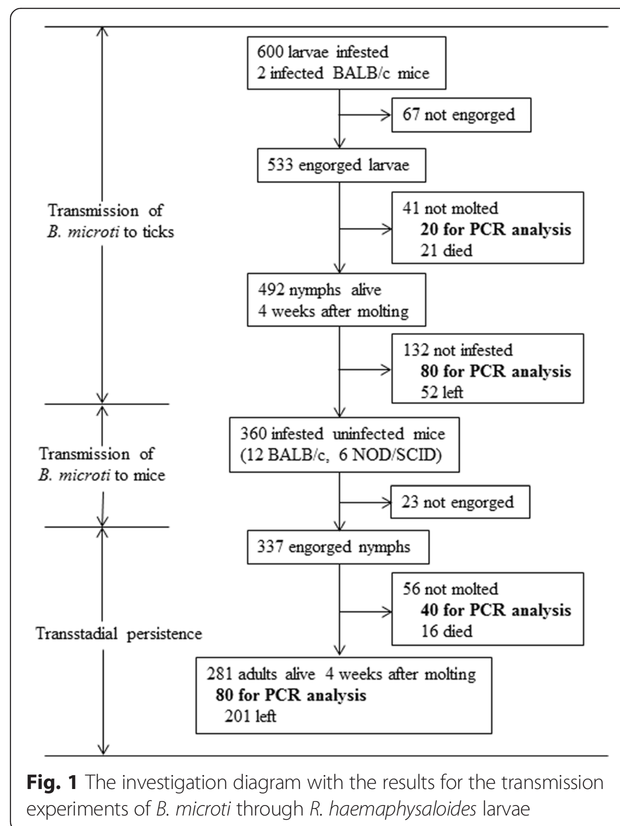

Fig. 1 The investigation diagram with the results for the transmission experiments of *B. microti* through *R. haemaphysaloides* larvae

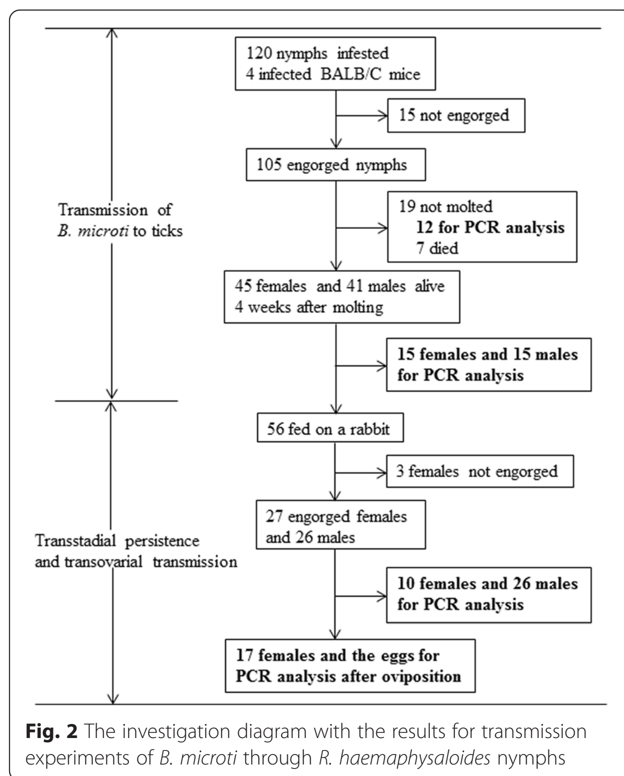

Fig. 2 The investigation diagram with the results for transmission experiments of *B. microti* through *R. haemaphysaloides* nymphs

maintained in 6 bottles at 25 °C, with 85 % relative humidity and a 14/10 h light/dark photoperiod regimen.

Twenty-eight days after the first molted tick was observed, 80 nymphs or 30 adults (15 females and 15 males) were analyzed for infection by PCR. The remaining ticks were used for the following experiments.

(ii) Transmission of B. microti to mice by infected nymphs

Twelve BALB/c mice or 6 NOD/SCID mice were each infested with 20 nymphs that were infected with *B. microti* as larvae. Approximately 50 μl tail blood of mice was collected for *B. microti* detection on every other day from day 7 to 30 after infestation. For each blood sample, a thin blood smear was made with 2 μl of blood, fixed with methanol, Giemsa-stained and examined using a microscope. Forty-five microliters of blood was used for DNA extraction, and PCR was performed to examine *B. microti* infection. Blood collection was stopped after the mice were proved to be infected by PCR. The engorged nymphs were used for the transstadial persistence experiment.

(iii) Transstadial persistence and transovarial transmission experiments

Forty engorged nymphs dropped from the uninfected BALB/c mice in the second experiment were stored for PCR analysis. The remaining ticks were maintained for molting as described above. Twenty-eight days after the first molted tick was observed, 80 adults harvested were analyzed by PCR to investigate transstadial persistence.

The infected adult ticks obtained in the first experiment were fed on the shaved ears of a laboratory rabbit. Ten engorged females and all of the fed males were stored for PCR. The other engorged females were maintained in bottles for oviposition and then stored for PCR analysis. Transovarial transmission was investigated by examining the resulting eggs and larvae by PCR.

DNA extraction

DNA was extracted from both tick samples and mouse blood using a DNeasy Blood & Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. DNA was extracted in pools of approximately 50 eggs from both the infected females and the resulting larvae and was extracted individually for other ticks.

PCR assay

Nested PCR was used to test for infection using a set of primers highly specific for *B. microti*. Partial fragments of 18S rRNA gene were amplified using the primer sets Bab1A (5′- GTCTTAGTATAAGCTTTTATACAGCG-3′) and Bab4A (5′-GATAGGTCAGAAACTTGAATGATACATCG-3′) for the first round and Bab2A (5′-CAGTTATAGTTTATTTGATGTTCGTTTTAC-3′) and Bab3A (5′-CGGCAAAGCCATGCGATTCGCTAAT-3′) for the second round, as described by Zamoto et al. [23]. Negative and positive controls were always used in each PCR reaction. One negative control was used in each row of a 96-well PCR plate or 8-tube strip to exclude contamination. PCR was performed in a C1000 Touch™ Thermal Cycler (Bio-Rad laboratories Incorporation, California, USA) starting with a pre-PCR heat step of 3 min at 95 °C, followed by 35 cycles of 94 °C for 40 s, 57 and 72 °C for 30 s, and ending at 72 °C for 5 min. Samples with 154-bp PCR products were recognized as *B. microti* - positive.

Results

Transmission of *B. microti* to ticks by infected mice

Among the 600 larvae of *R. haemaphysaloides* infesting mice that were infected with *B. microti*, 533 were engorged and collected (Fig. 1). Nineteen of the 20 (95.5 %) engorged larvae were positive by PCR, which demonstrates that the ticks had ingested the parasite (Table 1). Eighty of the 492 molted nymphs were analyzed for infection 4 weeks after molting, and 35 (43.8 %) were positive.

A total of 105 engorged nymphs were collected from infected mice (Fig. 2). All 12 of the engorged nymphs tested by PCR were positive (Table 1). Eighty-six adults were developed from the remaining nymphs. Thirty adult ticks (15 females and 15 males) were selected and analyzed for infection 4 weeks after molting. All 15

Table 1 Infection of *R. haemaphysaloides* by exposure to mice infected with *B. microti*

Tick stage applied	No. of engorged ticks analyzed	No. of positive engorged ticks (%)	No. of ticks analyzed 4 week post molting	No. of positive molted ticks (%)*
Larva	20	19 (95.5)	80	35 (43.8)
Nymph	12	12 (100)	30	29 (96.7)

*$P < 0.01$

Table 3 Transstadial persistence of *B. microti* in *R. haemaphysaloides* ticks

Stage of infected ticks	No. of blood-feeding ticks analyzed	Positive blood-feeding ticks, *n* (%)	No. of ticks detected 4 week post molting/ oviposition	Positive ticks post molting/ oviposition, *n* (%)
Nymph	40	5 (12.5)	80	0 (0)
Female adult	10	1 (10.0)	17	2 (11.8)

(100 %) females and 14 of the 15 (93.3 %) males were positive ($P > 0.05$).

Transmission of *B. microti* to mice by infected nymphs

BALB/c and NOD/SCID mice were infested with infected nymphs by applying 20 nymphs to each mouse as described above. *B. microti* was detected by PCR in 2 (16.7 %) blood samples of 12 BALB/c mice and in all samples of the 6 NOD/SCID mice ($P < 0.01$, shown in Table 2). Parasitemia was earlier in NOD/SCID mice (7 to 11 days after infestation) than in BALB/c mice (13 to 17 days). However, the parasite was not found in any of the blood smears.

Transstadial persistence and transovarial transmission

Infective nymphs were fed on BALB/c or NOD/SCID mice free of *B. microti*, and 337 were engorged and harvested (Fig. 1). Five of the 40 engorged nymphs detected were still *B. microti* positive (positive rate: 12.5 %, shown in Table 3). Eighty of the adult ticks that developed from these nymphs were subsequently analyzed for infection four weeks after molting, and none of them were positive. These results indicated that *B. microti* infection could not persist in the *R. haemaphysaloides* tick beyond one instar and that infection of the tick must occur at the instar preceding the transmitting stage.

Infective adult ticks were fed on a rabbit, and 27 engorged female ticks were collected (Fig. 2). Only 10 % (1/10) of the engorged females detected without oviposition were *B. microti* positive (Table 3). Two (11.8 %) of the 17 females were positive after oviposition. All the eggs and larvae were negative.

Discussion

Dozens of human infections of *B. microti* have been reported recently, especially in southern China [6, 9]. However, the transmission vectors of this parasite in these areas are not well understood. Moreover, *Rhipicephalus haemaphysaloides* ticks are very common and widespread in southern China [17]. Thus, investigating the transmission capability of *R. haemaphysaloides* for *B. microti* is needed.

As far as we are aware, this study is the first to investigate the transmission of *B. microti* using laboratory experiments in ticks of the genus *Rhipicephalus*. The present study confirmed the experimental transstadial transmission of *R. haemaphysaloides* for *B. microti* in two transstadial routes, i.e. larva to nymph (43.8 %) and nymph to adult (96.7 %). The transmission efficiency of *R. haemaphysaloides* from infected animals is comparable to that of *Ixodes scapularis* and *I. ricinus*, which are the primary vectors of *B. microti* in the United States and Europe [24, 25]. The results also showed that the transmission efficiency from nymph to adult was higher than that from larva to nymph (Table 1). This could be explained by nymphs ingesting much more infected blood than larvae do during feeding [26].

Consistent with other studies [24, 25], this study shows that the parasite of *B. microti* does not persist beyond more than one molt (Table 3) and that transovarial transmission does not occur. Interestingly, we also found that positive rate of tick infection declined dramatically after blood feeding (from 43.8 to 12.5 % for infected nymphs and from 100 to 10.0 % for female adults, as shown in Tables 1 and 3). The results suggest that blood-feeding induces immune response to pathogen in ticks such as other blood-sucking vectors [27, 28]. However, more investigations should be performed to understand the mechanisms underlying this phenomenon and to investigate the vector-parasite interaction in ticks.

The severity of babesiosis depends primarily on the immune status of the hosts [1]. Severe *B. microti* illness is usually seen among immunocompromised patients. Similarly, the present study showed that *B. microti* infection was much easier to establish from infective ticks in immunodeficient NOD/SCID mice than in immunocompetent BABL/C mice.

There are several limitations in our study. First, we did not witness any parasites by blood smear evaluation because the monitoring of the parasite was stopped after the first positive PCR result. Furthermore, no tick

Table 2 Infection of mice with *B. microti* by infected nymphs

Mice	No. of mice applied	No. (%) of infected mice*	Prepatent period (days after infestation)
BALB/c	12	2 (16.7)	13–17
NOD/SCID	6	6 (100)	7–11

*$P < 0.01$

salivary glands were tested for infection in our study. These weak points reduced the confidence that our study proved the ability of *R. haemaphysaloides* as a natural vector, as nested PCR is prone to contamination. Secondly, the transmission competence of *R. haemaphysaloides* might be challenged in natural environments due to the brief duration of patent infections in wild animals as opposed to the prolonged parasitaemias produced by repeated syringe passage into inbred laboratory mice [26]. Thirdly, the transmission of *B. microti* from ticks to natural hosts of this parasite was not investigated, and their susceptibility may be quite different from laboratory animals [29]. Finally, transovarial transmission is probably more likely to occur when engorging female ticks are exposed to infection, but this cannot occur in nature because *R. haemaphysaloides* adult females do not engorge on the small rodent reservoir hosts of *B. microti*. To further understand the role of *R. haemaphysaloides* in transmission of *B. microti* in nature, further investigations are needed on the infection of questing ticks and on the transmission to *R. haemaphysaloides* ticks by chronic infections.

Conclusion

In conclusion, our demonstration of *R. haemaphysaloides* transmission to mice suggests that this tick is a potential vector of human babesiosis in areas where it occurs and may be a matter of public health concern.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

LHL designed and performed the experiments and drafted the manuscript. DZ and CCZ maintained ticks in the laboratory and performed the DNA extraction and PCR assay. XNZ and YZ conceived the study and revised the manuscript. All authors read and approved the final manuscript. Written consent to publish was obtained.

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