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Experimental evaluation of transovarial transmission efficiency of Babesia ovis by *Rhipicephalus bursa*

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Abstract

This study aimed to evaluate the transovarial transmission efficiency of *Babesia ovis* in *Rhipicephalus bursa*. An 8-month-old pathogen-free (Babesia spp.) lamb was infected with the stabilate. Following parasite inoculation, the lamb was infested with 25 unfed adult *R. bursa* ticks (10 females, 15 males). When the ticks detached from the lamb, 8 engorged females were incubated at 27 ± 1 °C with 70-80% Relative Humidity to allow oviposition. Following oviposition, the adult female ticks were removed and aseptically bisected. Five egg pools, each consisting of 100 eggs, were prepared from each of 8 females and maintained in the incubator. Following larval hatching, the larval pools were utilized for DNA extraction and PCR. One larval batch confirmed positive for *B. ovis* was used to infest a New Zealand rabbit. A total of ~ 1,200 larvae were applied, and a total of 372 engorged nymphs were collected, incubated, and allowed to molt into unfed adults, yielding 370 ticks (173 females, 197 males). From these, 150 unfed adults (75 males, 75 females) were randomly selected and individually screened for evidence of *B. ovis* DNA. Following inoculation with the parasite, the lamb was euthanized on day 15 due to severe clinical symptoms. Molecular analysis confirmed *B. ovis* infection in the lamb. All ticks tested positive for *B. ovis* DNA, confirming transovarial transmission. *Babesia ovis* DNA was detected in 135 out of 150 adults (90%), with infection rates of 90.6% in females and 89.3% in males. These results demonstrate highly efficient transovarial transmission of *B. ovis*.

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Introduction

Tick-borne protozoan *Babesia ovis* is the causative agent of ovine babesiosis, and is considered the most economically significant pathogen affecting sheep in endemic regions, including Northern Africa, Southern Europe, the Middle East, and Asia (1-6). *Babesia ovis* is widespread in sheep across nearly all regions of Türkiye and is known for its high pathogenicity (7). Sheep infected with *B. ovis* develop severe clinical infection, characterized by high fever, anemia, icterus, hemoglobinuria, and in some cases, death (8,9). The biology of *Babesia* protozoa, which consists of three main stages **(**gamogony**,** sporogony, and merogony), is completed between ixodid ticks and vertebrate hosts (10-12). *Babesia ovis* is transmitted by *Rhipicephalus bursa* through both transovarial (from parent ticks to their offspring via eggs) and transstadial (from one developmental stage to another) routes (6-17). The development of the parasite in adult female *Rhipicephalus bursa* ticks begins when the tick feeds on an infected sheep and ingests the piroplasms. Sexual development occurs in the lumen of the female tick's midgut. Then, the kinetes are released from the midgut into the hemolymph and subsequently invade the ovaries (6,16,18). As a result, the parasite is transmitted to the larvae that hatch from the eggs of the adult female ticks. Pre-adult stages of *R. bursa*, such as larvae and nymphs, do not directly transmit *B. ovis*; instead, the kinetes are passed on to the adult stage. Infectious sporozoites develop in the salivary glands of the

adult tick and are transmitted to susceptible sheep hosts during feeding (15,17). Although the mechanisms that allow parasites to persist within tick populations are not fully understood, transovarial transmission has been suggested to play a significant role in maintaining the parasite within endemic regions (14,15).

Despite this, the efficiency of tick acquisition of *B. ovis* and its transovarial transmission to subsequent generations remains insufficiently characterized. In this study, we aimed to evaluate the transovarial transmission capacity of *R. bursa* for *B. ovis* and to determine the rate of transmission of infection from engorged adult females to their larval progeny.

Materials and methods

Ethical approval

All experimental procedures involving animals were approved by the Ethics Committee of Fırat University (protocol no. 2023/03-02**)**.

Tick and parasite

The sterile, laboratory-reared adult *R. bursa* ticks used in this study were obtained from a colony maintained in the laboratory, as described previously (19). The *B. ovis*-free colony was established at our department (Parasitology, Veterinary Faculty, Fırat University, Elazığ, Türkiye). In this study, the *B. ovis*-Alacakaya stabilate was used as the source of infection to obtain infected adult *Rhipicephalus bursa* ticks. The isolation and molecular characterization of the stabilate have been described in previously published studies (9,17,20,21).

Selection of the lamb

An 8-month-old tick-borne pathogens-free Akkaraman lamb was used for the experimental infection. To ensure that the lamb was free of infections caused by Babesia, Theileria, and Anaplasma species, nested polymerase chain reaction (PCR) was performed as previously described (20). In addition, the presence of antibodies against *B. ovis* in the lamb was assessed by an indirect ELISA using the recombinant B. ovis spherical body protein 4 (rBoSBP4) as the antigen, as previously reported (22). As no ELISA kits are available for ovine tick-borne pathogens (Theileria spp. and Anaplasma spp.), serology was not conducted.

Experimental infection of the splenectomized lamb

In previous studies aimed at determining the vector competence of blood parasites such as Babesia and Theileria, splenectomy has been commonly used to induce high parasitemia and thereby enhance vector infection efficiency (20,23). In this study, the lamb was also splenectomized at the Fırat University Animal Hospital under general anesthesia using standard surgical techniques before experimental infection. The cryopreserved *B. ovis*-Alacakaya stabilate containing 5% parasitized erythrocytes (PPE) was thawed in a 37°C water bath, and 15 ml was intravenously injected into the lamb.

The acquisition of *B. ovis* by adult *R. bursa* from the experimentally infected lamb

Before the experimental infection, a feeding capsule made of EVA foam was attached to the shaved posterior thoracic area of the lamb (19,24,25). Twenty-five unfed adult *R. bursa* (10 females and 15 males) were placed in the feeding capsule. Following tick infestation, the lamb was monitored daily for clinical findings of babesiosis, as well as the duration of tick engorgement and detachment from the host. All fully engorged ticks that dropped into the capsule were collected in a plastic container. Fully engorged female ticks were maintained under controlled conditions at 27 ± 1 °C with 70-80% Relative Humidity (RH) to facilitate oviposition. The carcasses of females that had completed oviposition were retrieved from the incubator. They were aseptically bisected using a sterile scalpel. Each female tick carcass was placed into an individual Eppendorf tube and kept at -20 °C for subsequent use in DNA isolation and nested polymerase chain reaction (nested PCR). When the color of the eggs began to change, five separate egg pools, each consisting of 100 eggs, were prepared from the egg batches derived from individual engorged female ticks. After larval emergence was complete from these egg pools, the larval pools were kept at -20 °C until DNA isolation.

Feeding of *R. bursa* Larvae on the rabbit and obtaining unfed adults

One of the larval batches confirmed by nested PCR to be infected with Babesia ovis was randomly selected and used to experimentally infect a New Zealand rabbit. A total of ~ 2.000 viable larvae were introduced into EVA foam capsules affixed to the rabbit, following the methodology previously described (24) and further refined in recent studies (19,25,26). All engorged nymphs collected from the rabbit were maintained under controlled environmental conditions (27 ± 1 °C and 70 ± 10% RH) and allowed to molt into unfed adult ticks. Engorged nymphs kept in the incubator were monitored daily. Upon completion of molting, the total number of unfed adults was recorded, and they were sorted into females and males. Of these, 150 unfed adults (75 males, 75 females) were randomly selected and stored in a deep freezer for subsequent DNA extraction and molecular analysis.

Microscopic detection of *B. ovis*

Thin blood smears were methanol-fixed and subsequently stained using a 10% Giemsa solution. The prepared slides were then examined under a light microscope at 1000X magnification to detect intraerythrocytic forms of *Babesia* spp. To determine the percentage of parasitized erythrocytes (PPE), at least 20 fields of view were assessed, following the methodology described in an earlier study (27).

DNA isolation and nested PCR

Eight engorged female ticks, a total of 40 separate larval pools (n = approximately 400 larvae; 5 larval pools from each of the 8 engorged female ticks), and 150 unfed adults (75 males, 75 females) were individually homogenized in liquid nitrogen using sterile pestles and subsequently processed for genomic DNA extraction (27,28). Genomic DNA from blood and tick specimens was isolated using the PureLink™ Genomic DNA Mini Kit (Invitrogen, Carlsbad, CA, USA) according to the kit protocol. The extracted DNA was utilized as a template in nested PCR assays to detect the 18S rRNA gene of *B. ovis* using two primer sets and the protocol previously reported in the literature (29,30). For the amplification of *B. ovis*, the first PCR was performed using the Nbab1F/Nbab1R primers (30), followed by a second nested PCR using the Bbo-F/ Bbo-R primers (29). Each PCR reaction included both positive (genomic DNA of *B. ovis* confirmed by DNA sequencing, GenBank accession no. EF092454) and negative (DNase/RNase-free water) controls. PCR amplifications were performed using a thermal cycler (Labcycler Gradient, Göttingen, Germany). Ten microliters of each PCR product were separated by electrophoresis on a 1.4% agarose gel for 30 minutes and then visualized with a Quantum Vilber Lourmat gel documentation system (Marne-la-Vallée, France). To confirm the presence of *R. bursa* DNA, PCR was performed using the 16S +1 and 16S -1 primers (31).

Results

Experimental infection of *B. ovis* in the lamb

Following the inoculation of *B. ovis*-Alacakaya stabilate, clinical and parasitological monitoring revealed a response consistent with acute babesiosis characterized by high fever, anemia, jaundice, hemoglobinuria, anorexia, and abdominal breathing. Intracellular piroplasms of the parasite were first detected in peripheral blood smears on day 6 post-inoculation. This observation coincided with a marked rise in rectal temperature, reaching 42.3°C. Parasitemia progressively increased, reaching a maximum level of 10% on day 15 post-infection (Figure 1). The prepatent period overlaps with the active tick attachment and feeding phase (days 6 to 15), which is considered the most favorable timeframe for the efficient acquisition of *B. ovis* by adult *R. bursa* ticks. Due to the severity of clinical signs and the poor prognosis, the animal was humanely euthanized on day 15, after the collection of all engorged female ticks. PCR analysis followed by DNA sequencing confirmed that the lamb was infected with *B. ovis*.

Acquisition of *B. ovis* by adult *R. bursa* fed on the clinically infected lamb.

Out of 25 adult *R. bursa* ticks (comprising 10 females and 15 males) applied to the lamb, a total of 8 females and 12 male ticks successfully completed their feeding. They were recovered as engorged individuals between the 6th and 9th days post-infestation (Figure 1b). Following incubation, engorged females began oviposition on day 5. Egg hatching began between days 28 and 34 post-oviposition and continued for 6 to 13 days. Post-oviposition, all engorged female tick carcasses tested positive for *B. ovis* via nested PCR analysis (Figure 2, lanes 1-8). Likewise, parasite DNA was detected in all larval pools derived from these females (Figure 2, lanes 9-10). These findings confirm that female adult *R. bursa* ticks acquired *B. ovis* during feeding on the infected lamb, and were capable of transmitting the pathogen to their offspring through transovarial transmission.

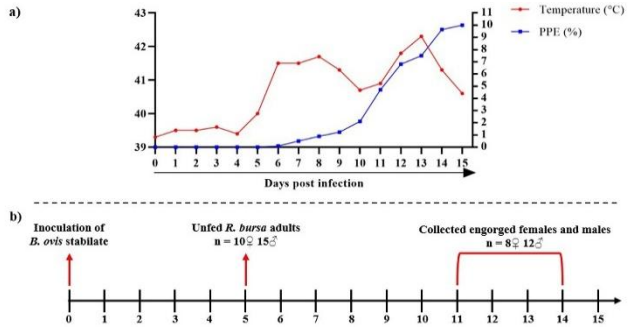

Figure 1: Progression of clinical infection and adult *R. bursa* infestation in the lamb. a) Progression of parasitemia (percentage of parasitized erythrocytes, PPE) and rectal temperature (°C) throughout the course of infection in the lamb (the lamb was euthanized on day 15 post-infection due to the development of acute babesiosis). b) Timeline of tick infestation in the lamb (GraphPad Prism v8 (GraphPad Software, San Diego, CA) was used to create time-course and scatter plots).

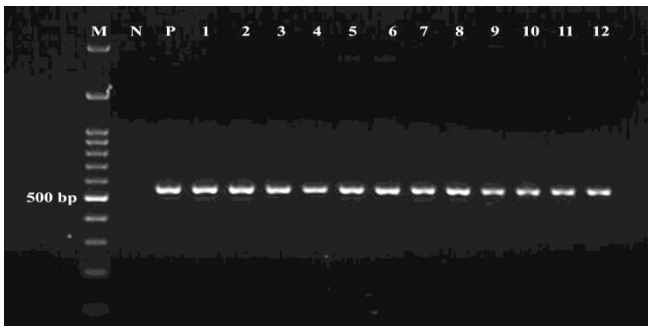

Figure 2: Gel electrophoresis image illustrating nested PCR amplification of *B. ovis* DNA (549 bp) from engorged female ticks, larval pools, and unfed adult *Rhipicephalus bursa* collected from a clinically infected lamb. M: 100 bp marker, N: negative control (distilled water), P: positive control (*B. ovis* confirmed by DNA sequence analysis, GenBank accession no. EF092454), 1-8: Engorged female carcasses, 9-10: representative positive larval pools, 11-12: representative positive unfed adults.

Transovarial transmission efficiency of *B. ovis* in *R. bursa*

The attached larvae, having derived from engorged female *R. bursa* ticks that imbibed *B. ovis*-containing blood from the infected lamb, completed their feeding and detached from the rabbit as engorged nymphs between the 12th and 14th days post-infestation. A total of 372 engorged nymphs were collected from the rabbit and incubated at 27±1°C with 70–80% RH to allow molting into the adult stage, resulting in 370 (173 females, 197 males) unfed adult *R. bursa* (Figure 3).

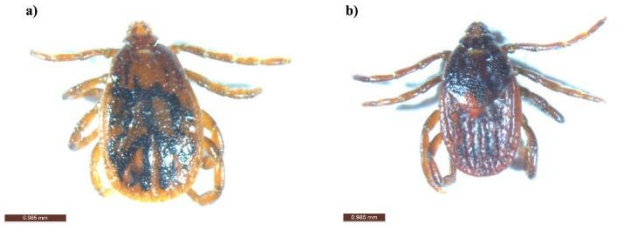

Figure 3: Unfed adults *R. bursa* infected with *B. ovis*, male (a) and female (b)

From this population, a representative subset of 150 unfed adult ticks (75 females and 75 males) was randomly selected and screened for the evidence of *B. ovis* DNA using nested PCR (Table 1). The results showed that B. ovis DNA was amplified in 135 of 150 ticks tested, corresponding to an overall infection rate of 90%. This finding indicates that adult female *R. bursa* ticks acquired *B. ovis* during the feeding period on the infected lamb and transmitted it transovarially to the subsequent generation. These findings demonstrate a high efficiency of transovarial transmission of *B. ovis* in *R. bursa*. Specifically, the infection prevalence was 90.6% in female and 89.3% in male ticks (Table 1).

Table 1: PCR analysis outcomes demonstrating the acquisition and transovarial retention efficiency of *Babesia ovis* in *Rhipicephalus bursa* ticks fed on the experimentally infected lamb

Tick stage	No. of ticks (pools/or individual)	No. PCR-positive ticks/no. Ticks tested (% infection)
Engorged adult female	20	20/20 (%100)
Unfed larva	40*	40/40 (%100)
Unfed male	75	67/75 (89.3%)
Unfed female	75	68/75 (90.6%)
Total unfed adult ticks	150	137/150 (90%)

* Pool tick sample (approximately 100 larvae in each pool).

Discussion

Variation in tick-borne diseases is influenced by multiple factors, including global climate change, urbanization, and human activity (16). In addition, key biological mechanisms, such as transovarial transmission by tick vectors and the competence of reservoir hosts, also play crucial roles (32). Research on the transovarial transmission of pathogens by ixodid ticks helps elucidate mechanisms underlying the circulation, persistence, and epidemiology of tick-borne microorganisms in endemic foci (16,33). *Babesia ovis* is a tick-transmitted protozoan parasite that causes ovine babesiosis, a disease of significant economic impact in tropical and subtropical areas (1,34). Despite *Rhipicephalus bursa* being a known biological vector of *B. ovis*, the dynamics of vertical (transovarial) transmission are poorly understood. This study provides compelling experimental evidence supporting the transovarial transmission of *B. ovis* by *R. bursa* under controlled laboratory conditions. The successful acquisition of *B. ovis* by adult female ticks, followed by the detection of parasite DNA in their progeny (larvae and unfed adults), underscores the vectorial competence of *R. bursa* for this economically significant hemoparasite of sheep.

The experimental infection model employed in a splenectomized lamb effectively induced a high parasitemia, peaking at 10% parasitized erythrocytes. The experimental model used in the present study is consistent with previous studies, which used splenectomy to enhance parasite amplification and thereby optimize conditions for vector infection (20,23,35). The overlap between the peak parasitemia phase and the active feeding period of adult *R. bursa* likely facilitated the efficient acquisition of the parasite. Our findings revealed that 8 engorged female *R. bursa* ticks tested positive for *B. ovis* by nested PCR, and more importantly, parasite DNA was also detected in their larval progeny. These results confirm not only the successful acquisition but also the vertical transmission of *B. ovis* through the transovarial pathway. This is consistent with earlier reports suggesting the possibility of vertical transmission of *Babesia* spp. in particular tick species (13,17,36,37).

Furthermore, larvae derived from PCR-positive females were able to develop through the nymph stage and molt into adult ticks, with a high percentage (90%) of these unfed adults testing positive for *B. ovis*. This is a particularly significant observation, as it demonstrates R. bursa's capacity not only to transmit B. ovis transovarially but also to maintain the infection across developmental stages (transstadial persistence). The observed infection rates were slightly higher in female ticks (90.6%) compared to males (89.3%). However, the difference was not statistically significant, suggesting that both sexes are competent carriers in the absence of differential susceptibility. These results are consistent with previous findings (15), which reported that both male and female *R. bursa* play an essential role in the transmission of *B. ovis*.

The high transovarial transmission efficiency observed in this study suggests that *R. bursa* populations could serve not

only as vectors but also as long-term reservoirs of *B. ovis*, thereby contributing to the persistence of infection in the absence of active transmission cycles involving infected hosts. This is particularly relevant in endemic foci where seasonal fluctuations in tick activity and host availability may interrupt horizontal transmission dynamics. Previous findings indicated that while up to 95% of vertically infected female ticks were capable of producing infected eggs, less than 30% of these eggs were actually infected (38). In contrast, our study demonstrated a significantly higher infection rate of 90% in unfed adult ticks, suggesting substantially greater vertical transmission efficiency under our experimental conditions. This notable difference may be attributed to variations in parasite strains, the genetic background of the tick populations, or methodological approaches. Overall, our results provide strong evidence for the effective and stable maintenance of *B. ovis* through vertical transmission, even in the absence of selective pressure.

Conclusion

This study confirms that *R. bursa* can acquire B. ovis from an infected lamb and transmit the parasite transovarially to its offspring with high efficiency. These results highlight the potential role of *R. bursa* as both a vector and a reservoir of *B. ovis*, emphasizing the need to include this species in integrated control strategies targeting ovine babesiosis in endemic areas. It is also noteworthy that molecular confirmation of *B. ovis* was achieved using a sensitive and specific nested PCR targeting the 18S rRNA gene. The use of multiple life stages (engorged female, larvae, and unfed adults) enhanced the reliability of the findings. However, detecting parasite DNA does not necessarily confirm the viability or infectivity of the organisms, and future studies should include attempts to isolate viable parasites from progeny or to demonstrate transmission to susceptible hosts.

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التقييم التجريبي لكفاءة انتقال بابيزيا الاغنام عبر المبيض بواسطة *Rhipicephalus bursa*

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الخلاصة

هدفت هذه الدراسة إلى تقييم كفاءة انتقال بابيزيا الاغنام عبر المبيض في *Rhipicephalus bursa*. تم إصابة خروف خالٍ من مسببات الأمراض (*Babesia spp*) يبلغ من العمر ٨ أشهر باللقاح المستقر. بعد تلقيح الطفيليات، أصيب الحمل بـ ٢٥ قرادًا بالغًا غير مغذي من نوع *R. bursa* (١٠ إناث و١٥ ذكرًا). عندما انفصل القراد عن الحمل، تم تحضين ٨ إناث محتقنة عند ٢٧ ± ١ درجة مئوية مع رطوبة نسبية ٧٠-٨٠% للسماح بوضع البيض. بعد وضع البيض، تمت إزالة القراد الإناث البالغة وتقطيعها بطريقة معقمة. تم تحضير خمس مجموعات بيض، كل منها تحتوي على ١٠٠ بيضة، من كل من الإناث الثماني، وتم الاحتفاظ بها في الحاضنة. بعد فقس اليرقات، تم استخدام تجمعات اليرقات لاستخراج الحمض النووي وتفاعل البوليميراز المتسلسل. تم استخدام دفعة واحدة من اليرقات التي تم تأكيد إصابتها بكتيريا بابيزيا الاغنام لإصابة أرنب نيوزيلندي. تم تطبيق ما مجموعه ~ ١٢٠٠ يرقة، وتم جمع ما مجموعه ٣٧٢ حورية محتقنة واحتضانها والسماح لها بالتساقط إلى بالغين غير مغذيين، مما أدى إلى إنتاج ٣٧٠ قرادًا (١٧٣ أنثى، ١٩٧ ذكرًا). ومن بين هؤلاء، تم اختيار ١٥٠ بالغًا غير مغذيين (٧٥ ذكرًا و٧٥ أنثى) بشكل عشوائي وتم فحصهم بشكل فردي بحثًا عن أدلة على وجود الحمض النووي لبابيزيا الاغنام. بعد التطعيم بالطفيلي، تم إعدام الحمل في اليوم الخامس عشر بسبب الأعراض السريرية الشديدة. أكد التحليل الجزيئي وجود عدوى بابيزيا الاغنام في الحمل. كانت نتيجة اختبار جميع القراد إيجابية بالنسبة للحمض النووي لبابيزيا الاغنام، مما يؤكد انتقال العدوى عبر المبيض. تم اكتشاف الحمض النووي لبابيزيا الاغنام في ١٣٥ من أصل ١٥٠ بالغًا (٩٠%)، وبلغت معدلات الإصابة ٩٠,٦% عند الإناث و٨٩,٣% عند الذكور. تظهر هذه النتائج انتقالًا عالي الكفاءة عبر المبيض لبابيزيا الاغنام.