TRANSOVARIAL TRANSMISSION EFFICIENCY OF *BABESIA BOVIS* BY *RHIPICEPHALUS* (*BOOPHILUS) MICROPLUS*

By

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To the Faculty of Washington State University:

The members of the Committee appointed to examine the dissertation of JEANNE MARIE HOWELL find it satisfactory and recommend that it be accepted.

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Chair

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ABSTRACT

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Babesia bovis is a deadly disease of cattle transmitted by *Rhipicephalus* (*Boophilus*) ticks resulting in severe economic losses in vast regions of the world where it is endemic. Infected cattle develop high fever, depression, anemia, and often die. Although essentially eradicated from the U.S. in 1943, a quarantine zone remains along the U.S. border with Mexico. The endemicity of babesiosis in Mexico, in combination with the development of acaracide resistant *Boophilus* ticks, facilitates the reintroduction of this pathogen into the U.S. The parasite is acquired by feeding adult female *Boophilus* ticks, and is transmitted transovarially to developing larval offspring as the kinete stage. Infectious sporozoites develop within larval salivary glands and are transmitted when larvae commence feeding on susceptible cattle. The efficiency of female tick acquisition and transovarial transmission of *B. bovis* is poorly understood. In order to address the risk imposed on U.S. cattle, it is essential to examine the efficiency at which adult female *Boophilus* ticks acquire blood stages of *B. bovis* and how efficiently the kinete stage is passed transovarially to developing offspring. These studies were designed to first evaluate the efficiency of *B. bovis* transmission following acquisition feeding on splenectomized acutely parasitemic cattle, and secondly investigate the efficiency of transovarial transmission following acquisition feeding on spleen intact persistently infected calves.

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DEDICATION

This dissertation is dedicated to my parents, Thomas and Peggy Howell, for their unconditional love and support throughout this process.

CHAPTER 1

Transovarial transmission efficiency of *Babesia bovis* tick stages acquired by *Rhipicephalus* (*Boophilus) microplus* during acute infection

ABSTRACT

The protozoan parasite *Babesia bovis*, a reemerging threat to U.S. cattle, is acquired by adult female ticks of the subgenus *Boophilus*, and is transovarially transmitted as the kinete stage to developing larval offspring. Sporozoites develop within larvae and are transmitted during larval feeding on a bovine host. This study evaluated the efficiency of *B. bovis* infection within *Rhipicephalus* (*Boophilus) microplus* following acquisition feeding on acutely parasitemic cattle. Parasite levels were quantified in blood from experimentally infected cattle, and within hemolymph and larvae derived from acquisition fed female *B. microplu*s. There was a positive correlation between blood parasite levels in acutely parasitemic cattle and kinete levels in hemolymph of adult female *Boophilus* following acquisition feeding; however, there was no relationship between kinete levels in females and infection rates of larval progeny. *Boophilus microplus* females that acquisition fed produced larval progeny with infection rates of 12% to 48%. Importantly, larvae derived from replete females with very low levels of kinete infection as demonstrated by microscopy and PCR, had infection rates of 22% to 30% and transmitted *B. bovis* during a transmission feed. These data demonstrate that although hemolymph infection may be undetectable, transmission to larval progeny occurs at a level which assures transmission to the bovine host.

INTRODUCTION

The tick-borne apicomplexan protozoa, *Babesia bovis*, is the etiologic agent of bovine babesiosis. *Babesia bovis* is ranked as the most economically important arthropod transmitted pathogen of livestock worldwide (1). It is currently endemic in tropical and subtropical regions of the world including Australia, Africa, Asia, Southern Europe, and South and Central America where *Rhipicephalus* ticks of the subgenus *Boophilus* serve as vectors (1). Cattle infected with *B. bovis* develop high fever, depression, anemia, and in severe cases, a cerebral syndrome develops which is characterized by neurological lesions, marked behavioral changes, and death.

Although *Boophilus* spp. ticks were eradicated from the continental U.S. in 1943, a quarantine zone remains along the border with Mexico. The endemicity of babesiosis in Mexico in combination with the development of multi-acaracide resistant *Boophilus* spp. ticks, facilitates the reemergence of bovine babesiosis in the U.S. *Boophilus* tick infestations in both quarantined and free zones along the U.S. and Mexico border increased in 2005 above levels found in previous years (16). Acaracide resistant *B. microplus* are prevalent in Mexico (6-8) and have also been identified along the U.S.-Mexico border (11). Cattle north of the quarantine zone have not been previously exposed to *Babesia*, and thus there is no protective immunity at either the individual animal or population levels. Collectively, the risk imposed by the introduction of persistently infected cattle into the U.S., the lack of protective immunity in naive U.S. cattle, and difficulties in controlling the *Boophilus* vector underscore the need to quantify the risks associated with the reemergence of babesiosis in U.S. cattle. Specifically, research defining the efficiency at which *B. bovis* passes through tick vector stages, and the subsequent ability to transmit to

naïve bovine hosts, will assist in evaluating the risk of babesiosis confronting a susceptible U.S cattle population.

Development of *B. bovis* within an adult female *Boophilus* tick commences when the intraerythrocytic stage is acquired during acquisition feeding on an infected bovine host. Gametogenesis and zygote formation takes place within the female tick midgut lumen. The kinete stage is released from the midgut into hemolymph, and subsequently invades the tissues of the female tick, including the ovaries. The adult female does not directly transmit *B. bovis*, but rather passes the kinetes transovarially to larvae. Infectious sporozoites develop within larval salivary glands and are transmitted when larvae feed on susceptible animals, thus completing the cycle.

The efficiency of tick acquisition and transovarial transmission of *B. bovis* is poorly understood. It is unknown if the parasitemia level during adult female acquisition feeding affects the ability of the tick to pass kinetes on to the next generation. In the present work, we test three linked hypotheses regarding this process. The first hypothesis was that blood parasite levels are directly related to kinete levels found in replete females following acquisition feeding. To test this hypothesis we quantified parasite levels within bovine capillary and jugular blood as well as kinete levels in hemolymph of replete female ticks. Secondly, we hypothesized that kinete levels in hemolymph are directly associated with infection rates of larval progeny. We used nested PCR to define the relationship between kinete levels in hemolymph of female ticks and infection rates of larval progeny following transmission feeding. The third hypothesis tested was that parasite loads found in individual larval offspring are related to the numbers of kinetes found in hemolymph of females. Real-time PCR was utilized to quantify the number of parasites found in

individual transmission fed larvae. Furthermore, we tested the ability of larval progeny from females with very low kinete levels to transmit *B. bovis* to susceptible calves.

MATERIALS AND METHODS

Cattle, pathogen, and tick vector. Two splenectomized Holstein calves (numbers 1100 and 1099), approximately 4 months of age and determined to be babesiosis free by cELISA, were used for the acquisition feed of *B. bovis* by *B. microplus* as previously described (13). Four additional splenectomized calves of the same age and breed were used for transmission feeding of larval progeny from adult females. The La Minita strain of *B. microplus* was used. This strain originated from ticks collected off cattle on pasture in Starr County, Texas. Multiple generations of this strain (at least F28) have been reared in colonies and have been determined to be free of infection with *B. bovis*. For acquisition feeding, one gram (approximately 20,000 larvae) was placed within a cloth patch on each of the two calves, 1100 and 1099. Ticks are applied to calves as larvae, and as feeding occurs, larvae remain on the same host and molt via a nymphal stage to adults. When approximately 1% of ticks had molted to the unfed adult stage, the two calves were inoculated intravenously with approximately 1.4×10^8 *B. bovis* infected erythrocytes (T2Bo strain) so that the peak of infection would occur as the adult ticks were reaching repletion.

Determination of parasite levels in blood by light microscopy (LM) and real-time PCR. *B. bovis* localizes in capillaries (14,19) and therefore parasite levels in capillary blood most closely represent the levels ingested by engorging female ticks. Therefore, daily capillary blood was sampled from skin capillaries on the distal aspect of the tail to observe capillary parasites by LM. Blood smears were stained with Diff-Quik (Dade Behring, Deerfield, IL) and the percent parasitized erythrocytes (PPE) was calculated by

dividing the number of infected erythrocytes in five fields by the total number of erythrocytes in five fields counted at 100x under oil. The mean of three PPE values was used to calculate the $\log_{10}$ parasites/µl of capillary blood.

Blood samples were also obtained by jugular venipuncture to quantify parasite levels by LM and real-time PCR. DNA was isolated from daily blood samples using a blood DNA isolation kit (Gentra, Minneapolis, MN). Real-time PCR was performed on jugular blood samples following the development of a standard curve using dilutions of known numbers of an *msa-1* plasmid. The r^2 values of repeated assays ranged from 0.98 to 0.99. The single copy *msa-1* gene was amplified using primers (forward, 5'-GATGCGTTTGCACATGCTAAG-3'; reverse, 5'-TGAGAGCACCGAAGTACCCG-3') amplifying a 150 bp fragment between bases 604 and 754 (GenBank accession number AF275911). A Taqman assay utilizing a PE Applied Biosystems fluorogenic probe 5'-CACGCTCAAGTAGGAAATTTTGTTAAACCTGGA-3' annealing at bp 628 to 660 was performed under the following conditions: 95ºC for 10 min, 40 cycles of 95ºC for 30 s and 55.8ºC for 15 s, and extension at 72ºC for 1 min. A standard curve was prepared utilizing $10^2 - 10^6$ plasmid copies of *msa-1* (18) and all test samples were run in triplicate.

Quantification of kinetes in adult female tick hemolymph by LM. Replete adult females were collected daily from *B. bovis* infected calves beginning 6 days post inoculation as previously described (13). Briefly, engorged females were washed and placed in individual wells of 24 well tissue culture plates, and then into airtight containers with saturated KNO_3 solution at 26ºC to maintain 92.5% relative humidity. It has been previously demonstrated that a low proportion of eggs laid during the first five days of incubation are infected (3,10) and therefore eggs laid during the first 5 days of oviposition

were removed and discarded. Hemolymph from individual females was sampled at days 7, 8, 9, or 10 days post incubation by removing a distal leg segment and blotting the exuding droplet of hemolymph on a slide. Slides were stained with Diff-Quik and examined by LM. A minimum of 50 fields were observed per sample by LM and the mean number of kinetes/high powered field (hpf) was recorded for each individual female. A total of 435 females from both calves were rated as either 1) undetectable kinetes by LM (0 kinetes observed in entire hemolymph smear), 2) moderately infected (1-9 kinetes/hpf), or 3) > 10 kinetes/ hpf. Capillary tubes were used to collect the maximum amount of hemolymph possible from females in which kinetes were undetectable by LM. Hemolymph from these females was placed in 100µl of cell lysis solution (Gentra, Minneapolis, MN) with 2 mg/ml proteinase K (Invitrogen, Carlsbad, CA) and stored at -20ºC until DNA isolation was performed.

Kinete detection by nested PCR in hemolymph having undetectable kinetes by LM. Sixty-two hemolymph samples were obtained from females in which kinetes were undetectable in hemolymph smears by LM. DNA isolation was performed using a Gentra DNA isolation kit (Gentra, Minneapolis, MN) with the addition of 1 µl of glycogen (Fermentas, Hanover, MD) per sample. The final DNA pellet was resuspended in 10 µl of TE (Gentra, Minneapolis, MN). Samples were analyzed by nested PCR using primers specific to *msa-1* (external forward, 5'-TTCGACCAGACCAAATTGT-3', external reverse, 5'-CGCATCAAAAGACTCAACA-3') under the following conditions: 95ºC for 2 min; 30 cycles of 95ºC for 30 s, 55ºC for 30 s, and 72ºC for 1 min; final extension at 72ºC for 2 min. The total reaction volume was 25 µl containing 5 µl of a 1:10 dilution of DNA, a 10 µM concentration of each primer set, and 12.5 µl of PCR Master (Roche Diagnostics

Corp., Nutles, N.J.). Two µl of a 1:100 dilution of the first PCR product was used in a final reaction volume of 25 µl for nested PCR. The *msa-1* internal primers (forward, 5'-GCCCTGATCTATTTAATGCA-3', reverse, 5'-CCCCGTATAAACATGCTTC-3') were predicted to amplify a 212 bp product under the conditions described above with a 30s extension time. The sensitivity of nested PCR under the conditions specified was determined by spiking hemolymph DNA from uninfected ticks with dilutions of DNA isolated from known quantities of *B. bovis* merozoites. The sensitivity was determined to be in the range of 1 to 10 parasites (data not shown). The presence of amplifiable DNA was confirmed in all *B. bovis msa-1* negative samples using primers specific to the *B. microplus* calreticulin gene (GenBank accession number AY395254) (forward, 5'GGTTTGTAGCCTGCGTAG-3'; reverse, 5'-GTTCTTGCCCTTGTAGTTGA-3') which amplified a 478 bp product under the following conditions: 95ºC for 2 min; 40 cycles of 95ºC for 30 s, 60ºC for 30 s, and 72ºC for 30 s, with a final extension at 72ºC for 2 min.

Transmission feed and determination of tick infection rates. A previous study has shown that feeding larvae provides a better stimulus for sporozoite development compared to an incubation at 37ºC alone, and is therefore essential for determining accurate larval infection rates (5). Egg masses from individual female ticks within each of the four groups: >10 kinetes/hpf, 1-9 kinetes/hpf, undetectable kinetes by LM/PCR positive hemolymph, and undetectable kinetes by LM/PCR negative hemolymph were combined but kept as separate groups and incubated at 26ºC and 92.5% relative humidity for three weeks (10). Following larval hatching, they were kept at 14ºC and 92.5% relative humidity (5) for an additional 21 days. Approximately 1 g of larval progeny from females having undetectable kinetes by LM/PCR positive hemolymph, undetectable kinetes by LM/PCR

negative hemolymph, 1-9 kinetes/hpf, and 0.5 g of larval progeny from females having >10 kinetes/hpf (the total weight of larvae from this group) was used in transmission feedings. Each group of larval progeny was applied to a different splenectomized calf for a total of four calves. Research utilizing ticks reared from the same colony infected with the M07 strain of *B. bovis* showed that sporozoites can be isolated from 60 h fed larvae followed by a 12 h incubation (12,13). In an attempt to observe differences in infection rates between two time points during transmission feeding, we tested larvae by nested PCR immediately following 1 and 3 days of feeding. On these days, 50 larvae were removed and placed at -80ºC until DNA extraction was performed. Larvae were macerated with a plastic pestle in microcentrifuge tubes containing 100 µl cell lysis solution (Gentra, Minneapolis, MN), 2 mg/ml proteinase K (Invitrogen, Carlsbad, CA), and 1 µl glycogen (Fermentas, Hanover, MD) and then placed at 56ºC overnight. DNA was isolated using standard protocols (Gentra, Minneapolis, MN) and the final pellet resuspended in 20 µl TE (Gentra, Minneapolis, MN). Nested PCR on DNA obtained from larvae was performed using the same *msa-1* primers and conditions described above for hemolymph. DNA from uninfected larvae fed for 1 and 3 days were run as negative controls. Nested PCR sensitivity was determined by spiking DNA from uninfected larvae with dilutions of DNA isolated from known quantities of merozoite infected erythrocytes. The sensitivity was determined to be in the range of 1 to 10 parasites (data not shown). The presence of DNA was confirmed on 107 randomly selected *B. bovis msa-1* negative samples using primers specific to *B. microplus* calreticulin as described above. Infection rates were then calculated for both days by dividing the number of positive larvae by 50. Non-parametric statistical analysis was performed to compare infection rates among groups. Comparisons

were made using Kruskal-Wallis One-Way ANOVA followed by a Kruskal-Wallis Mulitple Comparison Z-value test with Bonferroni correction.

Parasite levels in individual larvae by real-time PCR. A standard curve based on 10^6 – 10^2 plasmid copies of *msa-1* was used to quantify *msa-1* copies in individual larvae that tested positive by nested PCR. A total of 10 larval progeny, 5 from day 1 and 5 from day 3, were tested from each of the four groups of females: >10 kinetes/hpf, 1-9 kinetes/hpf, undetectable kinetes by LM/PCR positive hemolymph, and undetectable kinetes by LM/PCR negative hemolymph. Five µl of a 1:10 dilution of DNA isolated from individual larvae was used in each reaction. DNA from uninfected *B. microplus* larvae was tested simultaneously as a negative control and each reaction was assayed in triplicate.

RESULTS

In calves 1100 and 1099, parasites were detectable by LM in capillary blood two days before detection by LM in jugular blood samples (data not shown), consistent with the sequestration of *B. bovis* within small capillaries (14). The number of *B. bovis* detected in capillary blood were >10 fold higher than the number obtained by real-time PCR on jugular venous blood (Fig. 1). Parasites in jugular blood were undetectable by real-time PCR until days 6 and 9 post inoculation for calves 1100 and 1099, respectively (Fig. 1). Parasite levels ranged from $10^{0.56}$ parasites/µl (3.68 parasites/µl) to $10^{4.3}$ parasites/µl (2.0×10^4 parasites/µl) during tick acquisition feeding on calf 1100 and from $10^{0.58}$ parasites/µl (3.8 parasites/µl) to 10^4 parasites/µl (1.1×10^4 parasites/µl) in calf 1099. Peak parasitemia was $10^{4.3}$ on day 9 post inoculation and $10^{4.0}$ on day 12 post inoculation for calves 1100 and 1099, respectively (Fig. 1). Parasites were not detectable by LM in jugular blood smears until day 8 post inoculation in calf 1100 ($10^{3.7}$) and day 12 post inoculation in calf 1099

(10^4). As capillary PPE increased during acute infection with *B. bovis*, the number of replete females containing >10 kinetes/hpf in hemolymph also increased (Fig. 2) with a correlation coefficient (r) of 0.88 and 0.98 for females containing >10 kinetes/hpf replete on calves 1100 and 1099, respectively. There was also a positive correlation between increasing PPE and the number of females containing 1-9 kinetes/hpf with r = 0.85 for females feeding on calf 1100 and r= 0.89 for females feeding on calf 1099. A negative correlation was found between increasing PPE and the percentage of females with undetectable hemolymph kinete levels engorging on calves 1100 (r= -0.88) and 1099 (r= -0.90).

Hemolymph samples were collected between 7 and 10 days post repletion from a total of 62 females with undetectable kinete levels by LM. Of these 62 samples, 32 samples were positive by nested PCR (undetectable kinetes by LM/PCR positive hemolymph) using primers amplifying *msa-1* (Fig. 3). Twenty-two of the 32 PCR positive hemolymph samples were obtained from females that became replete between 7.70×10^3 and 1.20×10^5 capillary parasites/µl. The remaining 10 samples were from females that became replete during the very early stages of acute infection. Of the 30 samples that were negative by nested PCR (undetectable kinetes by LM/PCR negative hemolymph), 18 were obtained from females that became replete during the very early levels of parasitemia, undetectable by LM in capillary blood smears.

For transmission feeding, larvae derived from females with >10 kinetes/hpf, 1-9 kinetes/hpf, undetectable kinetes by LM/PCR positive hemolymph, and undetectable kinetes by LM/PCR negative hemolymph were each applied to one of a group of four splenectomized calves. Fifty ticks removed from each calf on days 1 and 3 were tested by

nested PCR for the presence of *B. bovis*. Infection rates were calculated as the number of PCR positive larvae divided by the total number tested and are shown in Figure 4. On day 1 of larval feeding there was a significant difference in infection rates between larvae derived from females having >10 kinetes/hpf and larvae derived from females with undetectable kinetes by LM/PCR positive hemolymph ($p= 0.014$). Similarly, on day 3 of tick feeding, the infection rates of larvae from females with >10 kinetes/hpf and larvae from females with undetectable kinetes by LM/PCR positive hemolymph were significantly different ($p=0.010$). There was no significant difference between infection rates on days 1 and 3 within any of the groups ($p >0.05$).

Previous studies utilize an estimate of approximately 20,000 larvae that will hatch from 1 gram of *Boophilus* eggs(17). A high level of larval mortality (approximately 40%) was observed on day 1 following application of larval progeny from females with >10 kinetes/hpf. In contrast, approximately 90% of larvae in all other groups attached and fed to completion. All groups of larvae transmitted *B. bovis*. Calves that received larvae derived from adult female ticks with >10 kinetes/hpf, 1-9 kinetes/hpf, and females with undetectable kinetes by LM/PCR positive hemolymph, all developed fever beginning at day 7 or 8 post application. The calf infested with larvae derived from adult females with undetectable LM/PCR negative hemolymph did not show an elevated temperature until day 12 post tick application. Parasites were first detectable in capillary blood smears on days 7, 9, 8, and 12 for calves receiving larvae derived from females with >10 kinetes/hpf, 1-9 kinetes/hpf, undetectable LM/PCR positive hemolymph, and undetectable LM/PCR negative hemolymph, respectively.

Real-time PCR was performed on 10 individual larvae from each group. Larval progeny from females having hemolymph with >10 kinetes/hpf ranged from 8.9×10^2 to 4.6×10^3 detectable parasites per tick on day 1 of feeding and from 4.8×10^1 to 1.2×10^5 parasites per tick on day 3 of feeding with one larvae that was below the detectable limits of real-time PCR and was not quantifiable (Table 1). Larvae obtained from females with 1-9 kinetes/hpf ranged from 1.2×10^2 to 1.2×10^3 parasites per tick with one larva below detection after feeding for 1 day, and on day 3 values ranged from 4.2×10^1 to 8.1×10^4 parasites per tick with one larva below detection. There were only two larvae above the threshold of detection by real-time PCR on both days 1 and 3 from females with undetectable kinetes by LM/PCR positive hemolymph (1.8×10^2 parasites per tick on day 1 and 1.8×10^3 parasites per tick on day 3). In the final group of larval progeny from females with undetectable kinetes by LM/PCR negative hemolymph, all ten of the larvae tested had parasite levels below detection by real-time PCR.

DISCUSSION

The acquisition of *B. bovis* by a feeding adult female *Boophilus* and transovarial passage of the parasite to developing offspring is required for transmission of this parasite, yet little is known about the efficiency of this process. The first hypothesis that blood parasite levels in the host animal are directly related to kinete levels in replete female ticks is accepted. There was a positive correlation between the highest parasite levels in the blood and the percent of engorged females containing high levels of kinetes in hemolymph samples. The inverse was true for females engorging during undetectable blood parasite levels (less than 10^3 parasites/µl).

The second hypothesis that kinete levels in hemolymph are associated with percent infection rates of larval progeny is rejected. The efficiency of transmission from hemolymph to larvae did not depend on the levels of kinetes present. Infection rates of fed larval progeny were highest (48% and 40%) from females with undetectable kinetes by LM/PCR positive hemolymph, and ranged from 12 to 30% in the other groups. Previous studies to determine infection rates have relied on Giemsa stained smears of either egg or larval squashes to identify infective forms by morphology alone (5,9). Using this method, the highest percentage of infected larvae were found at 1 day (5%) and 2 days (6%) post attachment (9). In our research, nested PCR amplifying *msa-1* was applied to individual larvae. The differences in sensitivity and specificity using this method may account for the higher infection rates found in all groups of this study.

A previous study demonstrated a negative yet statistically insignificant association between a high level of hemolymph infection and the percent infection rate of eggs as determined by smears of egg squashes (3). These eggs were not tested after hatching for determination of larval infection rates. Another study noted egg degeneration and a decrease in the total number of *B. ovis* infected eggs produced by female ticks with high hemolymph infections (2). Oliveira *et al.* found a reduction in larval hatching rate for increasing numbers of kinetes in hemolymph (15). In the current study, the infection rate of the larvae derived from females with the highest parasite load was the lowest of all groups, supporting the premise that very high levels of parasites are detrimental to some aspect of larval development. It is possible that the moderately and highly infected groups represent parasite loads at or above a detrimental level. As a result, those larvae containing kinete loads above this upper limit die, while those with loads below this level, and those

uninfected larvae, survive. High numbers of kinetes passing from females to eggs may result in a reduced hatching rate and/or a decrease in larval attachment. This is consistent with the observation of increased larval mortality seen one day post application for the larvae derived from females with >10 kinetes/hpf.

These results are in contrast to a study where no relationship was found between parasite levels in peripheral blood during tick feeding and the infection rates of fed larval offspring tested by larval squashes (10). Similar to the current study, replete females and blood samples were collected daily from splenectomized calves during acquisition feeding. The authors speculate that blood collected at several time points during each 24 h engorgement period may have given a more accurate representation of what the female ticks are actually ingesting. In the current study, daily capillary measurements appear to be adequate for splenectomized animals as the parasitemia consistently increases daily following inoculation and does not fluctuate as is in the case of jugular blood samples from spleen intact animals (4). We attribute differences in results to the use of capillary parasitemia levels and to an improvement in detection through the use of nested PCR.

Significantly, the larval progeny derived from females that had undetectable kinetes by LM/PCR negative hemolymph were capable of transmitting to a naïve host. It can be concluded that PCR negative hemolymph samples either did not accurately represent the total hemolymph present in that female, or the time at which the sample was tested was too early or too late for detection of kinetes passing through the hemolymph to invade the ovaries. In order to limit the number of false negatives due to the possibility that kinetes are not equally distributed in hemolymph, we collected as much hemolymph as possible from each female.

The third hypothesis tested was parasite levels in larval offspring are associated with kinete levels in hemolymph of adult females. This hypothesis is accepted, as parasite levels in larval progeny of the >10 kinetes/hpf females were highest of all progeny groups tested. There was more variation following 3 days of feeding in larvae derived from females with either >10 kinetes/hpf or 1-9 kinetes/hpf. This variation can be explained by the different time points at which individual larvae attach and commence feeding, and also by the differences in rates at which larvae inject sporozoites into the host. With the exception of one larvae on each of the days tested, larval progeny from females with undetectable kinetes by LM/PCR positive hemolymph, and all of the larval progeny from females with undetectable kinetes by LM/PCR negative hemolymph were below the threshold level of detection by real-time PCR. This can be explained by low levels of infection in these groups of larvae, detectable only by a more sensitive method such as nested PCR.

This research quantified the passage of *B. bovis* through multiple stages of development beginning with acquisition during acute infection by females and ending with transmission by infected larval progeny. The data described show a correlation between parasite levels during acquisition and kinete levels in hemolymph of replete females but no direct relationship between kinete levels in hemolymph and infection rates of larval progeny. In addition, females having very low kinete loads in hemolymph, undetectable by light microscopy and by nested PCR, are capable of passing the parasite to their larvae which can subsequently transmit *B. bovis* to a naïve animal. These results imply that ticks feeding on cattle with persistent infections characterized by very low blood parasite levels

are capable of passing parasites to their larval offspring. We propose to address this question in the next stage of investigation.

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Figure 1a.

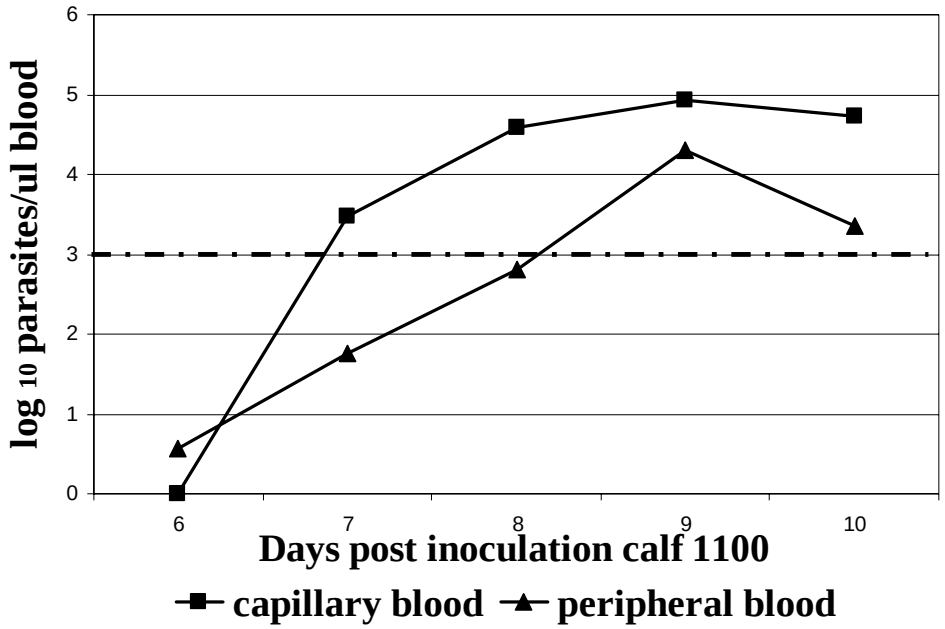

Figure 1b.

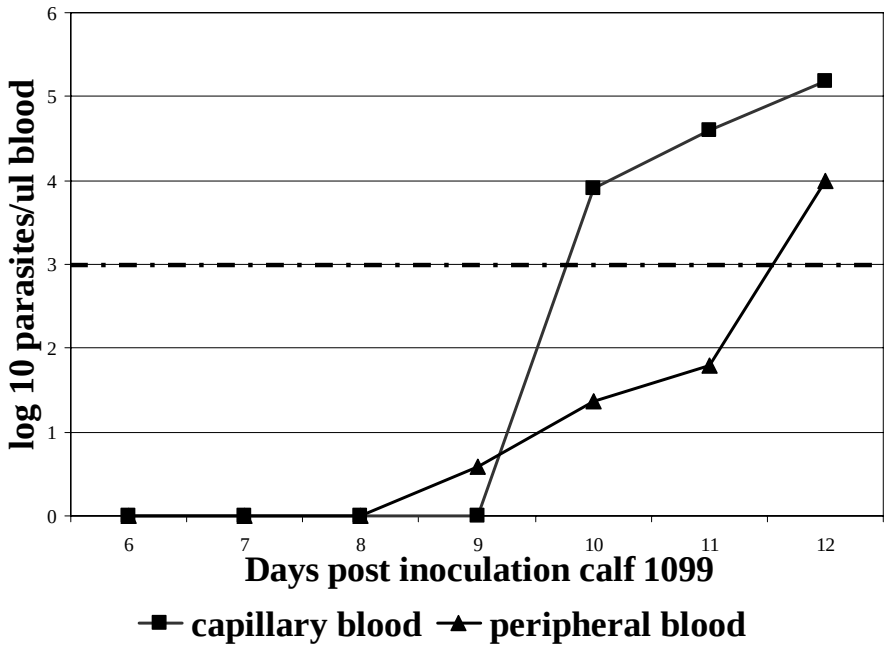

FIG. 1. *B. bovis* levels detected in tail capillary and jugular venous blood samples of calves 1100 (a) and 1099 (b) during ascending and at peak parasitemia as detected by light microscopy in capillary blood and by real-time PCR in jugular blood. The dotted line represents the lower limit of infected erythrocytes detectable by light microscopy (1×10^3 infected erythrocytes per µl).

Figure 2a.

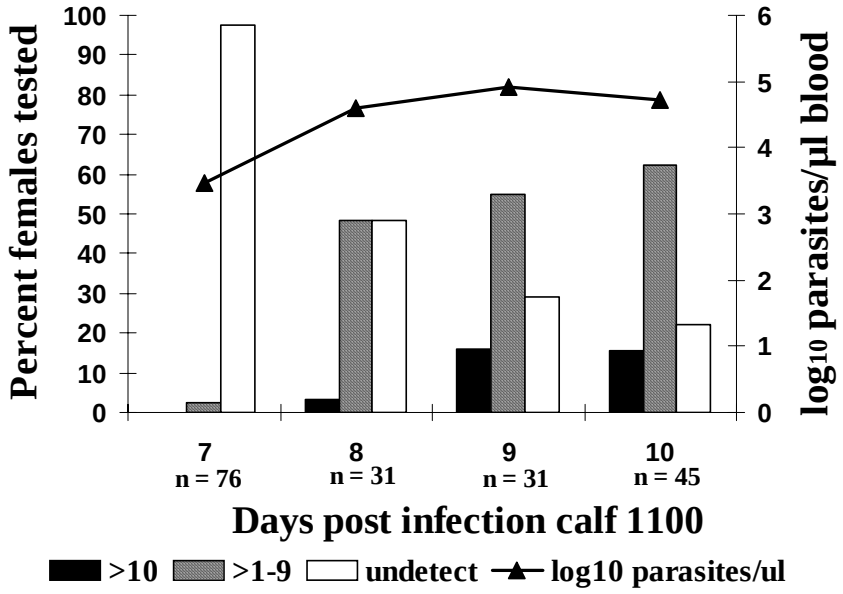

Figure 2b.

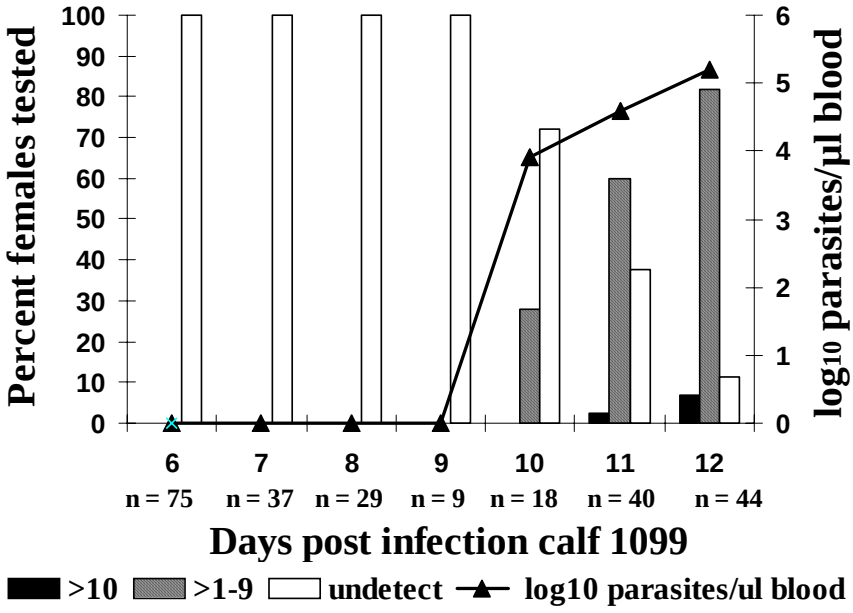

FIG. 2. The percent of engorged females containing >10 kinetes/hpf, 1-9 kinetes/hpf, or undetectable kinetes by light microscopy during acute parasitemia versus parasite levels in capillary blood. Percent of females tested are shown as bars on various days post inoculation for calves 1100 (a) and 1099 (b) where n is the total number of females in all categories. Capillary mean $\log_{10}$ parasites/µl is indicated as a line overlaying the bar graph. The correlation coefficient (r) between the number of females with >10 kinetes/hpf and increasing capillary parasite levels was 0.88 (calf 1100) and 0.98 (calf 1099). An inverse correlation was seen between the number of females having undetectable hemolymph kinete levels by light microscopy and increasing capillary parasite levels -0.88 (calf 1100) and -0.90 (calf 1099).

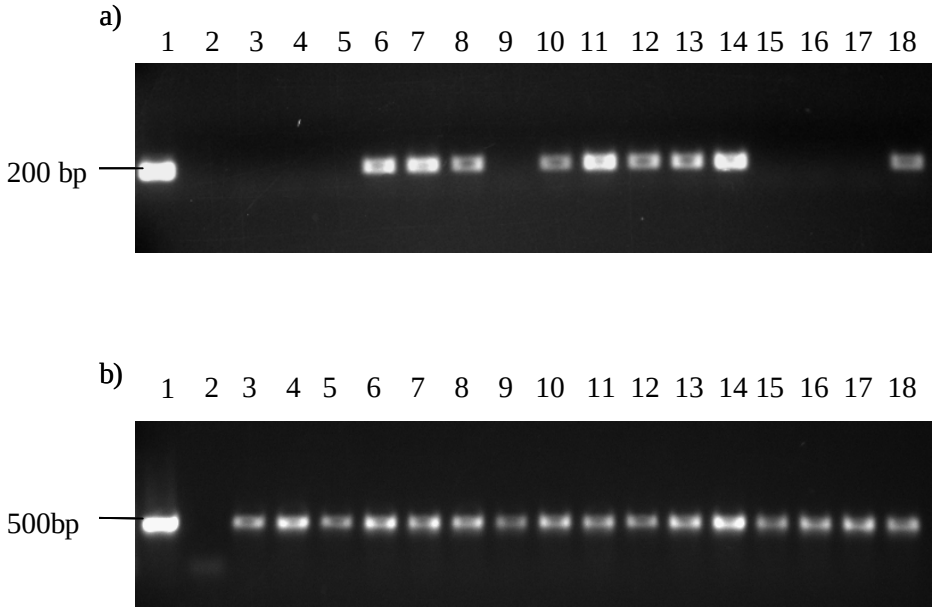

FIG. 3. (a) Detection of *B. bovis* by nested PCR amplifying *msa-1* in hemolymph collected from females having no detectable kinetes by light microscopy. Lane 1: *B. bovis* gDNA positive control. Lane 2: no DNA template negative control. Lanes 3 – 18 designate amplicons of the predicted 212 bp in hemolymph DNA from individual females with undetectable levels of kinetes when analyzed by light microscopy. (b) Confirmation of the presence of DNA in the same hemolymph samples shown in (a) using PCR amplifying *Boophilus* calreticulin. Lane 1: *B. microplus* gDNA positive control. Lane 2: no DNA template negative control. Lanes 3 -18 represent amplicons of the predicted 478 bp.

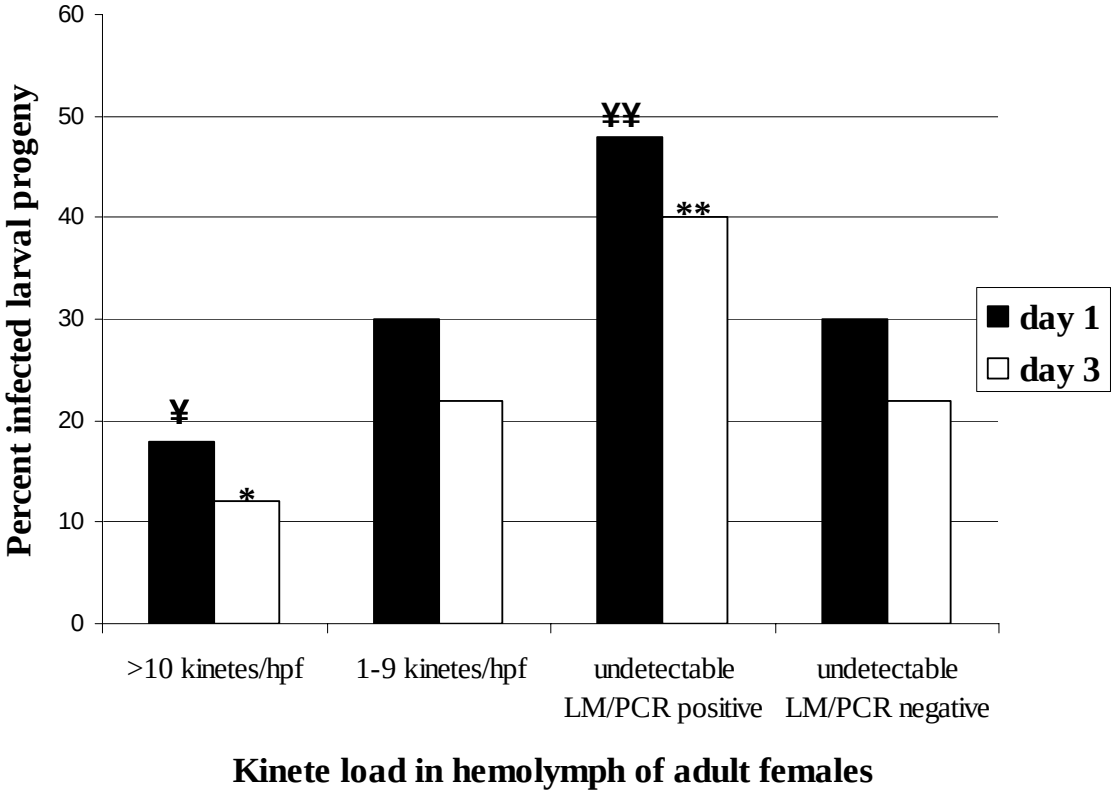

FIG. 4. Percent infected larvae derived from females with >10 kinete/hpf, 1-9 kinetes/hpf, undetectable kinetes by light microscopy/PCR positive hemolymph and undetectable kinetes by light microscopy/PCR negative hemolymph after 1 and 3 days of feeding. Significant differences (indicated by ¥ and *) were seen on days 1 and 3 between larvae derived from females with >10 kinetes/hpf and undetectable kinetes by light microscopy/PCR positive hemolymph.

Table 1. Quantification of *B. bovis* in individual larvae by real-time PCR

	Number of parasites[a]	
Female hemolymph rating	day 1 post application	day 3 post application
>10 kinetes/hpf	4.60E+03	1.20E+05
	1.60E+03	1.16E+05
	1.46E+03	7.90E+04
	1.20E+03	4.80E+01
	8.88E+02	NQ*
1-9 kinetes/hpf	1.20E+03	8.60E+01
	6.76E+02	8.10E+04
	1.40E+02	4.20E+01
	1.22E+02	5.00E+01
	NQ	NQ
undetectable LM / PCR positive	1.80E+02	1.80E+03
	NQ	NQ
	NQ	NQ
	NQ	NQ
	NQ	NQ
undetectable / PCR negative	NQ	NQ
	NQ	NQ
	NQ	NQ
	NQ	NQ
	NQ	NQ

[a]Total number of parasites in individual ticks detected by real-time PCR amplifying *msa-1*

*NQ=not quantifiable by real time PCR; below detectable limits

CHAPTER 2

Persistently infected calves serve as reservoirs for the acquisition and efficient transovarial transmission of *Babesia bovis* by *Rhipicephalus* (*Boophilus) microplus*

ABSTRACT

Babesia bovis is a deadly disease of cattle resulting in severe economic losses in the vast regions of the world where it is endemic. If re-introduced into the United States, babesiosis would cause significant mortality in the naïve cattle population. In order to address the risk to U.S. cattle, it is essential to quantify the transovarial transmission efficiency in adult female *Boophilus microplus* following acquisition feeding on persistently infected cattle. This study tested the hypothesis that infection rates are the same for larval progeny derived from females fed to repletion during persistent or acute infection. Increasing parasite levels during acute infection correlated with an increasing number of females harboring kinetes detectable in hemolymph (r= 0.9). Percent infected larvae ranged from 0-20% when derived from females fed to repletion on persistently infected calves and from 4-6% when derived from females fed to repletion during acute parasitemia. There was no significant difference in infection rates of larval progeny implying that the risk associated with the introduction of either persistently infected or acutely infected cattle is equal. Parasite levels ranged from 2.4×10^2 to 1.9×10^5 in three day fed larvae derived from females fed to repletion on persistently infected cattle. One group of larvae failed to transmit the parasite suggesting that a threshold level of parasites must be obtained by larval progeny via transovarial transmission in order for larvae to deliver sufficient parasites to infect a naïve host.

INTRODUCTION

Bovine babesiosis, also known as Texas Cattle Fever, is endemic to tropical and subtropical regions of the world including Central and South America, Asia, Australia, and Africa and is ranked as the most economically important arthropod transmitted pathogen of cattle (1). The disease is caused by the tick-borne apicomplexan protozoa *Babesia bovis* and is characterized by anemia, fever, and in severe cases multi-organ failure resulting in death. Young calves are relatively resistant to severe disease and have the potential to recover but remain persistent carriers with no clinical signs of disease (8,18). While parasite levels in the persistent state are often undetectable (4-6), ticks may still be capable of acquiring infection from these animals. Thus, persistently infected cattle are potentially an important facet in the maintenance of *B. bovis* in nature, as well as in the introduction and spread of the parasite to non-endemic areas where competent vectors are present.

Following outbreaks that resulted in devastating economic losses to the U.S. cattle industry, the major tick vectors of *B. bovis*, *Rhipicephalus* (*Boophilus*) *microplus* and *Rhipicephalus* (*Boophilus*) *annulatus*, were eradicated from the continental United States by 1943 (2,7,10). Today there remains a quarantine zone along the border between Texas and Mexico that extends from Brownsville to Del Rio. In Mexico, both the parasite and vector remain prevalent and acaracide resistant *Boophilus* ticks are increasingly common (15-17). There is no serological testing of cattle within the quarantine zone, and therefore movement of cattle is not restricted based on *B. bovis* infection status. Due to the increase in acaracide resistant ticks and the lack of clinical signs in persistent cattle, the introduction of infected ticks and/or cattle into the U.S. is likely. The introduction of babesiosis into the

previously unexposed cattle population outside the limits of the quarantine zone would result in significant mortality.

When an adult female *Boophilus* tick feeds on an infected bovine host, the merozoite stage of *B. bovis* is acquired. Following gametogenesis and zygote formation within the lumen of the midgut, the kinete stage is released into the hemolymph of the female. The kinete stage can be detected in the hemolymph of the tick during migration from the midgut to ovaries. After invasion of the ovaries, kinetes are transovarially transmitted to developing larvae. Within developing larvae, *B. bovis* invades salivary glands and develops into infective sporozoites, which are subsequently transmitted when larvae commence feeding on a bovine host.

Determining the efficiency of transmission is crucial to developing strategies to prevent the re-introduction of *B. bovis* into the United States. If the efficiency of transovarial transmission is equivalent in females acquiring the parasite from either acutely or persistently infected cattle, and should emerging acaracide resistance lead to the re-establishment of *B. microplus* in the U.S., then the absence of serological screening of cattle entering the U.S. is a definite oversight. In the current study we began to address this issue by examining the transovarial transmission efficiency of female *B. microplus* fed to repletion on persistently infected calves. We hypothesized that infection rates of larval progeny from these females would be the same as infection rates of larval progeny from females fed to repletion during acute parasitemia. We examined hemolymph kinete levels in females by light microscopy and nested PCR, determined infection rates of transmission fed larval progeny by nested PCR, and quantified parasite levels in transmission fed larvae using real-time PCR.

MATERIALS AND METHODS

Acquisition of *B. bovis* by *B. microplus*. For acquisition feeding of adult female *Boophilus* on persistently infected spleen intact calves, two Holstein calves (designated 1144 and 1158) approximately 4 months of age were inoculated intravenously with 1.4 x 10^8 infected erythrocytes (T2Bo strain). At 123 days post inoculation for calf 1144 and 73 days post inoculation for calf 1158, unexposed larvae hatched from 1g of *B. microplus* (La Minita strain) eggs or the equivalent of approximately 20,000 *B. microplus* larvae (14), were applied beneath cloth patches placed on the back of each calf. At the time of larval application, both calves were determined to be persistently infected using a RAP-1 cELISA (9). A third calf designated 1167 was inoculated with 1.4 x 10^8 infected erythrocytes (T2Bo strain) 13 days following larval application when approximately 1% of larvae had molted to the unfed adult stage to ensure that adult ticks were feeding during the acute phase of infection. Peripheral blood samples from the jugular vein and capillary samples taken from the distal aspect of the tail were collected daily from each calf beginning at 1% larval molt. To verify *B. bovis* infection in persistently infected calves following acquisition feeding, nested PCR amplifying *msa-1* was performed on jugular blood, brain, skin, and spleen samples following euthanasia.

Transovarial transmission of *B. bovis* in *B. microplus* females. Replete *Rhipicephalus* females were collected daily from calves 1144, 1158, and 1167 as previously described (11). Briefly, females were rinsed in water and placed in individual wells of tissue culture plates. Females were incubated at 26°C and 92.5% relative humidity during egg production. It has been previously reported that a low proportion of eggs laid during the first five days of oviposition are infected, and therefore these eggs were removed and

discarded (3,13). Hemolymph from individual females was sampled on day 10 post repletion as previously described (11). A minimum of 50 fields were observed by light microscopy and the average number of kinetes identified per field was recorded. Total hemolymph was collected from a subset of females having undetectable kinetes by light microscopy. Nested PCR was performed on these hemolymph samples using primers specific to *msa-1* as previously described (11). Eggs produced by females that were hemolymph checked by light microscopy were pooled from each calf resulting in two pools of eggs from the persistently infected calves and one pool of eggs from the acutely infected calf. Nine weeks after the eggs were pooled, larvae were applied to naïve hosts for transmission feeding.

Larval transmission of *B. bovis* by *B. microplus*. For transmission feeding of larval progeny, three splenectomized Holstein calves approximately 4 months of age and designated 1170, 1174, and 5304, were used. Calves received larvae hatched from 1 g of eggs pooled from females that were hemolymph checked by light microscopy after feeding to repletion on persistently infected calf 1144 (calf 1170), persistently infected calf 1158 (1174) or acutely infected calf 1167 (calf 5304). Fifty larvae were removed from each calf for DNA isolation at 1 and 3 days post application and individual larvae were tested by nested PCR for detection of *msa-1* as previously described (11). Larval infection rates were calculated by dividing the number of positive larvae by 50. Non-parametric statistical analysis was performed using Kruskal-Wallis One-Way ANOVA followed by Kruskal-Wallis Multiple Comparison Z-value test with Bonferroni correction.

Quantification of parasite levels in blood and individual larvae by real-time PCR. Daily blood samples and individual larvae that were positive by nested PCR were subsequently quantified by real-time PCR. DNA was isolated using a blood DNA isolation kit (Gentra, Minneapolis, MN). A standard curve was developed using dilutions of known numbers of *msa-1* plasmid. Amplification of a 150 bp fragment between bases 604 and 754 of msa-1 (GenBank accession number AF275911) was performed using *msa-1* specific primers: forward, 5'-GATGCGTTTGCACATGCTAAG-3'; reverse, 5'-TGAGAGCACCGAAGTACCCG-3'. A TaqMan assay was performed utilizing a PE Applied Biosystems fluorogenic probe 5'-CACGCTCAAGTAGGAAATTTTGTTAAACCTGGA-3' annealing at bp 628 to 660 under the following conditions: 95.0°C for 10 min, 70 cycles of 95°C for 30 sec and 55.8°C for 20 sec, and a final extension at 72.0°C for 1 min.

RESULTS

Quantification of parasites during acute infection. Jugular blood was positive by nested PCR on 6 of 6 days that replete females were collected during acute infection. Infection levels shown in Figure 1 were lowest on the ninth day post inoculation (1.15×10^3 parasites/ml) and were highest on the 12^{th} day post inoculation (1.9×10^5 parasites/ml). Parasites were not detectable by light microscopy in tail capillary smears until the final day of female collection (PPE = 0.13%). Brain and skin samples obtained from acutely infected calf 1167 were positive for *msa*-1 by nested PCR.

Acquisition during persistent infection. Replete females were removed over a period of 5 days at 144 -148 days post inoculation from calf 1144 and for 6 days at 94-99 days post inoculation from calf 1158 (Table 1). There were no parasites detected by light microscopy

in tail capillary smears from either of the persistently infected calves during female tick acquisition. *B. bovis* was detected in jugular blood by nested PCR on 2 of 5 days that females were collected from calf 1144 and on 5 of 6 days from calf 1158. The sensitivity of nested PCR was determined by making dilutions of a known number of infected erythrocytes and was found to be between 1 and 10 parasites (data not shown). Nested PCR positive jugular blood samples from persistently infected calves were tested by real-time PCR and were below the threshold of quantification using this method. Brain, skin, and spleen samples obtained from calf 1158 post euthanasia were positive by nested PCR. However, three samples each of brain, skin, and spleen from calf 1144 were negative by nested PCR.

Kinete levels in hemolymph. Hemolymph from females fed to repletion on acutely and persistently infected calves were examined by light microscopy. The majority of the 195 females replete during acute parasitemia had no detectable kinetes (n=137) or 0-1 kinetes/hpf (n=53) in their hemolymph by light microscopy. However, there were 2 females observed to have 2-4 kinetes/hpf and 2 females containing 10 or more kinetes/hpf. There was a positive correlation (r= 0.9) between increasing parasitemia in jugular blood during acute infection quantified by real-time PCR and the proportion of females having detectable kinetes in hemolymph (Figure 1). Parasites were detected in 5 of the 18 light microscopy negative samples from females that fed to repletion during acute infection. None of the female ticks checked from persistently infected calves 1144 (n=187) and 1158 (n=154) had detectable kinetes by light microscopy. Nested PCR performed on light microscopy negative hemolymph samples detected parasites in 12 of 26 and 0 of 18 samples from females fed on persistently infected calves (Table 1).

Infection rates of larval progeny. Larval progeny were tested by nested PCR on days 1 and 3 post application. Infection rates were lowest (0.02 and 0) on day one post application for larval groups derived from persistently infected calves (Table 1). Infection rates of larvae derived from females replete during acute infection were not significantly different than those derived from either persistently infected calf ($p= 0.368$). There also was no significant difference among groups on different days of transmission. The highest infection rate, 0.2, was obtained on day 3 post application from larvae derived from females fed to repletion during persistent infection.

Infection levels of larval progeny. There were 19 out of a total 300 larvae derived from females fed to repletion on persistent and acutely infected calves that were positive by nested PCR following one or three days of transmission feeding. These positive larvae were subjected to real-time PCR and results are shown in Table 2. Twelve of the 19 total larvae evaluated by real-time PCR were below detection limits for this assay. The highest *msa-1* copy numbers were detected in larval progeny from females fed to repletion on a persistently infected calf (1.9×10^5 parasites) as well as females fed to repletion an acutely infected calf (2.3×10^4). The average number of parasites per larvae quantified by real-time PCR was 3.1×10^4.

Transmission by larval progeny. Larvae obtained from females fed to repletion during acute infection were capable of transmitting infection to calf 5304 as indicated by the presence of fever (105.3°F) 8 days post larval application, detection of merozoites by light microscopy 9 days post larvae application, and detection of *msa-1* by nested PCR in jugular blood. Also, larvae derived from females that fed to repletion on persistently infected calf 1158 were capable of transmitting infection to calf 1174 as evident by the

presence of fever (104.9°F) 10 days post tick application, detection of parasites by light microscopy in tail capillary smears at 10 days post larvae application, and detection of *msa-1* by nested PCR. However, larval progeny derived from females fed to repletion on persistently infected calf 1144 did not transmit following application on calf 1170. *B. bovis* DNA was not detected in jugular blood samples, or in brain, skin, hemal nodes, or kidney samples from calf 1170. Parasites were also not detected on daily tail capillary smears. A cELISA performed using serum obtained 29 days after larval application was negative. An additional splenectomized calf, 1177, was infested with 10g of the same batch of larvae derived from hemolymph checked females fed to repletion on persistently infected calf 1144. These larvae were six weeks older than those applied to calf 1170. Again, the presence of clinical infection was evaluated in calf 1177 for 21 days post infestation using daily capillary blood smears and nested PCR on daily jugular blood samples. Nested PCR was also performed on brain, skin, hemal nodes, and kidney samples post euthanasia. There were no parasites detected by light microscopy in capillary smears or by nested PCR in blood and tissues obtained from calf 1177. A cELISA performed using serum obtained 21 days post larval application was negative.

DISCUSSION

A detailed understanding of the efficiency of *B. bovis* acquisition and transmission by its *Rhipicephalus* tick vector is critical for evaluating the potential risk of re-emergence in U.S cattle. In a recent study, it was determined that blood parasite levels in acutely infected splenectomized calves were directly related to kinete levels in replete females and parasite levels in individual larvae were associated with kinete levels in hemolymph of adult females (11). The current study was designed to further evaluate the dynamics of

transmission by testing the hypothesis that females fed to repletion on persistently infected calves produce larval progeny with similar infection rates to females fed to repletion on acutely infected calves.

Similar to data in the previous study, a positive relationship (r = 0.9) was found between increasing blood parasite levels in acute infection during acquisition feeding and the number of *Rhipicephalus* females that had detectable kinetes in their hemolymph. Of the females tested, 29% had kinetes detectable in their hemolymph by light microscopy. This percentage is in agreement with a study performed by Mahoney *et al.* in which kinetes were detectable by light microscopy in hemolymph of 22-30% of engorged females fed on spleen-intact acutely infected calves (12). Blood parasite levels in persistently infected calves were below the level of quantification by real-time PCR during tick acquisition feeding but could be detected by nested PCR on several days. This is consistent with observations that detection of *B. bovis* using conventional PCR is sporadic (4-6), most likely due to sequestration of the parasite in capillaries.

Larval infection rates obtained in this study ranged from 0 to 20% and were lower than in the previous study (12-48%) which utilized splenectomized acutely infected calves (11). We attribute these differences to lower parasite levels during acquisition feeding. The current study utilized spleen-intact persistently infected calves which harbored dramatically lower parasite levels in peripheral blood, resulting in lower kinete levels in replete females and subsequently lower larval infection rates. The percent infected larvae derived from females fed to repletion on acutely infected calves (4 – 6%) was within the range described by Mahoney *et al.* using light microscopy: up to 14.5%, with lower percentages mostly observed (12). Larvae obtained from field conditions reported by

Mahoney *et al.* had much lower infection percentages of 0.04% (12). This level is lower than three out of four data points we obtained using persistently infected calves (2% on day 1 and 6% on day 3 from calf 1170; 20% on day 3 from calf 1158). We attribute these differences to higher specificity and sensitivity in the method of detection used in this study and variations in experimental design.

There were several differences between the persistently infected calves used for acquisition. First, the numbers of days post inoculation during which females fed to repletion. Calf 1144 had a longer time of persistence (144 days) compared to calf 1158 (94 days). Our infection rate results indicate that this extended period of persistence had no statistically significant effect on infection rates of larval progeny from females that acquired *B. bovis* on this calf. Secondly, *msa-1* was amplified on fewer days during acquisition in calf 1144 jugular blood compared to calf 1158. Again, this did not result in significant differences in infection rates of larval progeny. However, we were unable to demonstrate transmission by larvae derived from females fed to repletion on calf 1144. Furthermore, a log increase in the number of larvae from the same females applied to a separate splenectomized calf also did not result in transmission. It is possible that the differences between our acquisition calves (i.e. a longer period of persistence and lower number of days infection was detectable in peripheral blood during acquisition feeding) could have resulted in a situation where parasites were detected in larvae but the number of parasites transmitted was below a threshold required for infection. Of note is a study performed by Mahoney *et al.* in which only 3 out of 5 calves each infested with 100 larvae and 4 out of 5 calves each infested with 200 larvae with an estimated 2% infection rate actually became clinically infected (12). Despite the discrepancy in the number of larvae

applied between this study and ours, it supports the hypothesis of a threshold number of parasites required for the development of clinical disease.

The level of parasites detected within individual larvae supports this hypothesis although low infection rates resulted in only 19 larvae to test by real-time PCR and 12 of those tested were below quantifiable levels. However, it was observed that following 3 days of transmission feeding, parasite levels in larvae that did transmit the parasite ranged from 2.4×10^2 to 1.9×10^5, values comparable to those found in the previous study in groups of larvae derived from females harboring elevated levels of kinetes in their hemolymph (11). On the other hand, only 1 of 4 larvae that did not transmit the parasite was quantifiable by real-time PCR, and its level (4.3×10^2) is comparable to those found in our previous study derived from females with no detectable kinetes by light microscopy and PCR positive hemolymph. Overall, the lower number of parasites quantified by real-time PCR in transmission fed larvae may be related to the lower parasitemia during acquisition feeding.

This study tested the hypothesis that females fed to repletion on persistently infected spleen intact calves produce larval progeny with similar infection rates as females fed to repletion on acutely infected spleen intact calves. This hypothesis is accepted, as the percent of infected larvae derived from females fed on persistently infected calves and larvae derived from females fed during acute infection, were not significantly different. This data suggests that females fed on persistent carriers, despite low blood parasite levels, are capable of acquiring the parasite and passing it transovarially to larval offspring. The fact that larval infection rates were not significantly different implies that the risk of transmission from introduction of a persistently infected animal into the U.S is comparable

to the risk associated with introduction of an acutely infected animal. We have shown that *B. microplus* is capable of transmitting *B. bovis* following acquisition on a clinically normal host, suggesting the need for more vigorous serological screening in the quarantine zone. Our data also suggests that there is a threshold limit below which larvae are not capable of transmission. Additional experiments are necessary to further characterize this threshold level.

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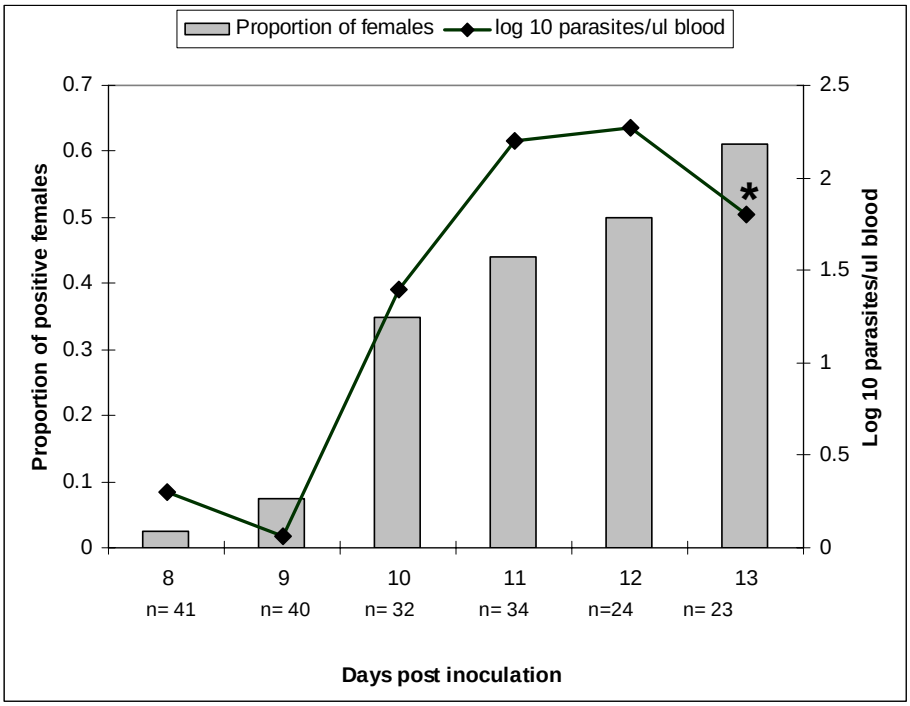

Figure 1. *B. bovis* levels quantified by real-time PCR in daily jugular blood samples from acutely infected calf 1167 and the proportion of *B. microplus* females having detectable kinetes in hemolymph by light microscopy. The * indicates the only day merozoites were detectable by light microscopy in a capillary tail smear ($10^{3.7}$ parasites/µl blood).

Table 1. Detection of *B. bovis* following acquisition and transovarial transmission

	Acquisition calf infection status		
	Persistent		Acute
	# 1144	# 1158	# 1167
days post inoculation replete females obtained	144 - 148	94 - 99	8 - 13
days detected in jugular blood[a] / days females collected	2 / 5	5 / 6	6 / 6
no. females positive by LM / no. females checked	0 / 187	0 / 154	57 / 195
no. positive females[a] / no. LM negative females tested	12 / 26	0 / 18	5 / 18
number infected larval progeny day 1	1/50 (2%)	0/50 (0%)	3/50 (6%)
number infected larval progeny day 3	3/50 (6%)	10/50 (20%)	2/50 (4%)
transmission of clinical disease by larval progeny	no[b]	yes	yes
nPCR on transmission host tissues	negative[b]	positive	positive

[a] by nested PCR amplifying *msa-1*

LM= light microscopy; nPCR= nested PCR

[b]no transmission with either 1g or 10g

Table 2. Quantification of *B. bovis* in individual larvae by real-time PCR

Transmission calf number (larval progeny)	# larvae quantifiable / # larvae tested	number of parasites [a]	day of transmission feed
5304 (acute larvae)	1/5	2.30E+04	day 1
1174 (persistent larvae)	5/10	2.40E+02	day 3
		6.40E+02	day 3
		1.40E+03	day 3
		1.50E+03	day 3
		1.90E+05	day 3
1170 (persistent larvae)	1/4	4.30E+02	day 3

[a] per individual larvae tested