2001

Infection and Transovarial Transmission of Rickettsiae in Dermacentor variabilis Acquired by Artificial Feeding

K. R. Macaluso

Daniel E. Sonenshine
Old Dominion University, dsonensh@odu.edu

Shane M. Ceraul
Old Dominion University

A. F. Azad

Final Version

Infection and transovarial transmission of rickettsiae in *Dermacentor variabilis* acquired by artificial feeding

Kevin R. Macaluso*, Daniel E. Sonenshine+, Shane M. Ceraul+, and Abdu F. Azad

Department of Microbiology and Immunology, School of Medicine, University of Maryland at Baltimore, 655 W. Baltimore Street, BRB13-009, Baltimore, MD 21201, U.S.A.

+Department of Biological Sciences, Old Dominion University, Norfolk, VA, 23529, U.S.A.

* Author to whom correspondence should be addressed.

E-mail: kmaca001@umaryland.edu

Phone: +1 410 706 7066

Facsimile: +1 410 706 0282

RH: INFECTION OF *DERMACENTOR VARIABILIS* WITH RICKETTSIAE

Abstract

In this study we examined the efficiency of an *in vitro* feeding technique using glass microcapillaries as a method of establishing rickettsiae-infected lines of ticks. To quantitate the volume ingested by ticks during microcapillary feeding, the incorporation of radiolabeled amino acids in tick gut and hemolymph was calculated. Fifteen of eighteen ticks consumed between 0.06 µl and 6.77 µl. However, ingestion of fluid was not correlated to weight gain during capillary feeding. Partially fed laboratory-reared females, *Dermacentor variabilis*, known uninfected, were exposed to either *Rickettsia montana*- or *R. rhipicephali*-infected Vero cells via microcapillary tubes, returned to rabbit hosts, and allowed to feed to repletion. All tissues collected from ticks allowed to feed overnight on rickettsia-infected fluids were found to be infected when examined by the immunofluorescence antibody assay (IFA). When rickettsia-infected and uninfected capillary-fed ticks were allowed to feed to repletion and lay eggs, no significant differences in mean engorgement weight or fecundity was observed. Assessing the efficiency of transovarial transmission of rickettsiae by ticks that imbibed rickettsia-infected solutions by the polymerase chain reaction (PCR) and IFA, infection was found in 85% of the eggs from ticks exposed to *R. montana* and 69% of the ticks exposed to *R. rhipicephali*. No rickettsial DNA was amplified in samples of the uninfected controls. 100% of egg samples from females exposed to rickettsia-infected fluids and 0% of the uninfected controls examined by IFA were found to be infected

Introduction

Rickettsiae are obligate intracellular bacteria that are transmitted to mammals by a number of arthropod vectors including mites, lice, fleas, and ticks. Most members of the spotted fever group (SFG) rickettsiae are transmitted by ixodid ticks. The SFG include both pathogenic and nonpathogenic rickettsiae. In most cases, the passage or development of tick-borne rickettsiae in a vertebrate host is not essential for survival of the bacteria because rickettsiae are maintained through transstadial and transovarial transmission (TOT) within the arthropod host. However, maintenance of rickettsiae in ticks via TOT may influence their virulence to vertebrate hosts.

To examine the tick-pathogen relationship, ticks must be deliberately infected with rickettsiae. Then, the spread of the bacteria throughout the tick's tissues can be investigated. Historically, the most widely used method to establish known rickettsia-infected ticks has been by allowing them to feed on rickettsemic animals (Burgdorfer 1988; Niebylski et al. 1999). However, important questions about the fate of ingested rickettsiae may be difficult to address due to uncertainty regarding the precise time **when** or number acquired during natural feeding.

Alternatively, ticks can be infected *in vitro* with cultured pathogens by artificial techniques. Artificial feeding offers important advantages, not the least of which is the ability to control the dose of microorganisms or the contents of the feeding medium. Several methods to artificially introduce microbial pathogens into ticks *in vitro* have been successful, including membrane feeding (Kemp et al. 1975; Howarth and Hokama 1983; Voigt et al. 1993; Abbasy et al. 1994; Young et al. 1996), percutaneous injection (Jongejan et al. 1980; Kocan et al. 1986), and capillary feeding (Chabaud 1950;

Burgdorfer 1957; Purnell and Joyner 1967; Rau and Hannoun 1968; Purnell 1970; Walker et al. 1979; Kurtenbach et al. 1994; Rechav et al. 1999).

Although artificial infection of ticks by capillary feeding has been reported for several different microbial pathogens, e.g., *Ehrlichia* spp. (Rechav et al. 1999), it is not known whether it is possible to infect ticks with *Rickettsia* spp by this method. Based on techniques used to infect tick vectors with other organisms, we attempted here to introduce known species of cultured rickettsiae into ticks using a microcapillary feeding tube. *Dermacentor variabilis* was chosen for these studies since it is a known vector of several rickettsial species (Schreifer and Azad 1994). We were especially interested in determining whether it was possible for female ticks to acquire rickettsiae during microcapillary feeding and transmit them to their eggs (TOT). Other objectives of the study were to quantitate the volume of solution ingested by ticks via capillary feeding and to confirm the dissemination of rickettsiae from the guts of orally infected ticks to the other body tissues. We report here a technique to introduce *in vitro* cultured *Rickettsia montana* and *R. rhipicephali* into ticks by capillary feeding and identify TOT of bacteria to the eggs by the polymerase chain reaction (PCR) and the immunofluorescence antibody assay (IFA).

Materials and Methods

Ticks. Pathogen-free adult *Dermacentor variabilis* ticks were from a colony maintained at Old Dominion University. Adult ticks were maintained in an Aminco Aire Incubator (American Instrument Co., Washington, D.C.) at 27 $\pm$ 1 °C, 92% RH, and a 16:8 (L:D) photoperiod. Adult ticks were fed on rabbits, *Oryctolagus cunniculus*, as described previously (Sonenshine 1993).

Bacteria. *R. montana* and *R. rhipicephali* were maintained in Vero cell monolayers. Vero cells were grown in high glucose (4.5 grams per liter) Dulbecco's Modified eagle Medium (DMEM;Gibco BRL, Grand Island, NY) containing 4% heat-inactivated fetal bovine serum at 37° C in a humidified 5% CO_2 atmosphere. Vero cells were infected with rickettsiae as previously described (Troyer et al. 1999). Briefly, frozen stock (~200 µl) of either *R. montana* or *R. rhipicephali* was quickly thawed at 37°C and used to infect one 75-cm^2 flask (Sarstedt Inc., Newton, NC) of a Vero cell monolayer. Infected monolayers were cultured for 7 d, and growth medium was changed daily. Prior to use in capillary feeding assay, cells were harvested and resuspended in growth medium at a concentration of 2.8 x 10^6 cells per milliliter. Staining with Diff-Quik (Dade Behring, New Castle, DE) showed that 90-95% of the cells were infected with either *R. montana* or *R. rhipicephali.*

Quantifying media ingested by capillary feeding. In order to quantify the volume of solution ticks were ingesting by capillary feeding, a ^{14}C (uniformly labeled) labeled amino acid mixture, **54.2 mCi/mmol** (New England Nuclear Corp., Boston, MA) was diluted in BSK with 6% rabbit serum (Sigma, St. Louis, MO) and fed to ticks in an experiment separate from the Vero cell feeding experiment. Female *D. variabilis*, 5 d post-attachment, were forcibly removed from the rabbit host and restrained ventral side up on double-sided sticky tape in the bottom of a large petri dish. Microcapillary tubes (10 µl) containing the feeding medium with the radiolabeled amino acids (0.0075 µCi/µl) were placed over the entire mouthparts of each tick. The microcapillary tubes were immobilized by embedding them in modeling clay that lined the perimeter of the petri dish. Ticks were allowed to feed from microcapillary tubes for up to 24 h in an incubator at 27° C and 92% RH. Following microcapillary tube feeding, the ticks were washed by

repeated immersion in water and subsequent washing of the mouthparts with brushes, to remove coagulated BSK from these body structures. Washing was repeated until no radioactive solution could be found contaminating the body surfaces of the treated ticks. Ticks were then bled by severing the coxa-trochanteral joint of leg 2, far removed from the mouthparts, and pressing gently. Hemolymph was collected with a 10 µl Drummond micropipette (Drummond Scientific Company, Broomall, PA), changing capillaries between specimens. The amount of hemolymph collected from each tick specimen was recorded. Each hemolymph sample was diluted in buffer and transferred to liquid scintillation vials containing liquid scintillation cocktail and counted for radioactivity. Following hemolymph collection, the ticks were dissected in 0.2 M PBS, and a sample of midgut removed, minced and diluted to 100 µl, then transferred to liquid scintillation vials. The radioactivity in the hemolymph and midgut samples was recorded using a liquid scintillation counter (LS-1700; Beckman Instruments Inc., Fullerton, CA) with quench correction. To determine the loss of radioactivity due to quench (as a result of the color from the midgut), a quench curve was prepared using midgut from ticks that were not exposed to radiolabeled amino acids. In addition to quench correction, the sample values were corrected for the differences between the ratio of the hemolymph sample volume versus the estimated total body hemolymph volume (Kaufman and Phillips 1973) and the ratio of the gut sample to the total weight of all midgut contents.

Tick infection. Part-fed (4 d) virgin female ticks were forcibly detached from the rabbit host, individually **weighed using a Cahn Microbalance (Cahn Instruments, Inc., Cerritos, CA)** and restrained ventral side up in the same fashion as described earlier. Three groups of ticks were fed a solution consisting of growth medium alone, or

growth medium containing Vero cells infected with either *R. montana* or *R. rhipicephali*. Weights of partially fed ticks were taken prior to, and directly after, microcapillary feeding, to determine if weight gain occurred during microcapillary feeding. Five individuals from each treatment group were washed twice in 70% ethanol, dried, and dissected under a stereoscopic microscope and their tissues examined for evidence of rickettsial infection by IFA as described below. The remaining ticks from each treatment were placed back on the rabbit hosts, using individual rabbits for each treatment, and allowed to feed until repletion. After repletion, the ticks were weighed and held individually in numbered vials in an incubator at 27°C and 92% R.H. Seven days after the beginning of oviposition, the egg masses from five randomly-selected female ticks from each group were selected and weighed. Engorgement and egg mass weights recorded for ticks that were exposed to control treatment versus rickettsia-infected Vero cells were compared.

Detection and identification of rickettsiae. Detection and identification of rickettsial species in Vero cells and tick eggs was determined using PCR, and by analysis of restriction fragment length polymorphism (RFLP) of PCR products. Also, presence of rickettsiae in tick tissue was assessed by IFA. Detection of *R. montana- and R. rhipicephali*-specific DNA sequences in Vero cells and tick eggs was done by PCR using primers designed from the published sequences for *R. montana* (GenBank accession number AJ238760) and *R. rhipicephali* (accession number AJ238761) *metK*. In addition, amplification of *rompA* gene encoding the SFG-specific 190-kDa protein was also used for species identification. Genomic DNA from rickettsia-infected Vero cells or tick eggs (~100 eggs per tick) was obtained using a genomic DNA purification system (Promega,

Madison, WI). For the PCR of *metK*, 2 µl of purified genomic DNA extracted from Vero cells or tick eggs was added to a solution containing 18 µl of PCR Master mix (Roche, Mannhein, Germany), 1 µl each of forward [5' CGT GGA CAG TTA TGG AAG AAT GG 3'] and reverse [5' CCG AAA TGA CCG TAA GAA GCC 3'] primers, and 3 µl of sterile redistilled water. In a PRCSprint thermal cycler (Hybaid), each 25 µl of sample was heated to 94°C for 3 min, followed by 30 cycles of 94°C for 45 s, 60°C for 30 s, 72°C for 45 s, with an additional incubation period of 72°C for 5 min on the final cycle. The target PCR amplified DNA sequence was visualized by electrophoresis on a 1% agarose gel and stained with ethidium bromide.

PCR amplification of *rompA* DNA utilized primers Rr190.70p (Regnery et al. 1991) and Rr190.701 (Roux et al. 1999), with the following thermal cycler conditions: initial denaturation for 5 min at 94°C, followed by 35 cycles of 94°C for 30 s, 58°C for 1 min, 72°C for 2 min, with an additional incubation period of 72°C for 5 min on the final cycle. The PCR product was visualized on a 1% agarose gel stained with ethidium bromide, excised, and DNA recovered from the gel using a StrataPrep DNA extraction kit (Stratagene, La Jolla, CA) according to manufacturer's protocol. Enzymatic digestion of cleaned PCR product was accomplished by incubating 8 µl of DNA, 1 µl of enzyme buffer, and 1 µl (15 U) of RsaI (Stratagene) for 1 h at 37°C. Digested products were visualized on 8% TBE gels (Novex, San Diego, CA) stained with ethidium bromide.

Detection of rickettsia in eggs or the tissues of microcapillary fed ticks by IFA was done according to procedures described by Johns et al. (2000), with minor modifications. Egg samples from each egg mass were smeared onto silyated glass slides (CEL Associates, Houston, TX), air dried, acetone fixed, blocked overnight with 5%

dried milk/bovine serum albumin, and incubated with 10 µl of diluted (1:500) monoclonal antibody 7D11-A2 (kind gift of D. H. Walker, Galveston, TX) specific for rOmpA for 1 h. FITC-conjugated goat anti-mouse (Kirkegaard & Perry, Gaithersburg, MD) diluted 1:40 was added for 1 h, slides were washed with 0.2 M PBS between each step, and viewed by fluorescence microscopy.

Data analysis. Tick biology parameters were evaluated for statistical significance by 1-way ANOVA with Fisher's least significant difference mean separation test (Steel et al. 1997). A P-value ≤ 0.05 was considered significant.

Results

Quantification of ingested media. Labeled amino acids diluted in BSK were ingested by 88% (15/18) of the ticks tested. The volume taken averaged 1.35 ± 2.15 µl, **including the 3 individuals that did not imbibe any fluid. For the 15 ticks that did imbibe fluid, the average ± S.D. was 1.63 ± 2.27 µl**. There was a great deal of variation in the amount taken up by different ticks, from 0.06 µl to 6.77 µl (Table 1). We followed passage of labeled amino acids from the midgut into hemocoel during microcapillary feeding. Of the ticks that did ingest the solution, 0.13 ± 0.24 µl of solution, representing ~9.6% of the total volume ingested during microcapillary feeding was in the hemolymph, while 1.23 ± 1.93 µl was **present** in the midgut. Based on the concentration of Vero cells used for tick infection, part fed ticks consumed between ~170 (0.06 µl) and ~18,500 (6.77 µl) Vero cells.

Establishment of infected tick lines. Changes in mean weights of ticks prior to versus post-microcapillary feeding varied between the groups. Ticks exposed to growth media alone had slightly decreased weights (-2.0%), while ticks exposed to *R. montana-*

infected Vero cells had slightly increased mean weights (+2.4%), after a 12 h period. **These differences were not statistically significant**. However, the mean weight of ticks exposed to *R. rhipicephali*-infected Vero cells (-4.6%) was significantly lower after microcapillary feeding (Table 2).

IFA showed that 40.0% of ticks fed either *R. montana*- or *R. rhipicephali*-infected Vero cells were positive for rickettsiae in gut, hemolymph, or salivary gland tissue. Tissues from ticks fed on uninfected growth medium had no evidence of rickettsial infection. Ticks exposed to rickettsiae-infected Vero cells had lower mean engorgement weights (*R. montana* = -16.2%; *R. rhipicephali* = -9.0%), however, the differences between the groups were not significantly different (Table 2). On day seven of oviposition, average weight of the egg masses produced by five ticks of each group was determined (Table 2). Although the ticks exposed to rickettsia-infected Vero cells had decreased average egg mass weights (*R. montana* = -16.1%; *R. rhipicephali* = -21.2%) compared to control ticks, the percent of egg mass weight relative to engorgement weight did not **differ significantly** between control ticks (36.7 ± 5.2 mg), ticks fed *R. montana*- (38.4 ± 12.0 mg), or *R. rhipicephali*-(32.4 ± 8.7 mg) infected Vero cells.

Transovarial transmission of rickettsiae. In order to determine if ticks exposed to rickettsia-infected Vero cells during microcapillary feeding were capable of transmitting rickettsiae to their progeny, a portion of eggs from individual ticks from each group were assessed by PCR and IFA. The presence of rickettsial DNA was confirmed by PCR using *metK* primers and/or *rompA* primers. Egg samples collected from rickettsiae-infected adult ticks contained amplified rickettsial *metK* fragments with different band sizes for *R. montana* (~350 bp) and *R. rhipicephali*, (~250 bp), indicating

that ticks exposed to rickettsiae via microcapillary feeding were capable of passing these organisms to their progeny (Figure 1). Amplification of rickettsial *rompA* resulted in a product for both rickettsial species (~630 bp), and *RsaI* digestion of the PCR products yielded fragments unique to each species (Figure 2). Three bands were observed for *R. montana* (~ 300, 200, and 100 bp), **as compared to two pairs of bands for *R. rhipicephali* (200 and 100 bp).** Transovarial transmission of *R. montana* was observed **in ~85% (11/13) of the ticks exposed to the *R. montana*-infected Vero cells versus 69% (11/16) of the ticks exposed to the *R. rhipicephali*-infected Vero cells.** Samples of eggs from individual ticks were also examined for the presence of rickettsiae by IFA. Egg pools from all the infected lines tested were positive for *R. montana* (11/11) and *R. rhipicephali* (3/3) by IFA. Control ticks remained negative throughout the experimentation.

Revised for consistency of sentence structur

Discussion

Ticks, *D. variabilis,* readily imbibed liquids from glass microcapillaries applied to their mouthparts, thereby resulting in rickettsial infection in these specimens. Moreover, infection was established in the tick's internal tissues within as little as 15 hours, as demonstrated by IFA of their salivary glands and hemolymph. This is the earliest reported translocation of ingested rickettsiae from the midgut to the tick's tissues.

The use of radiolabeled amino acids provided a unambiguous measure of fluid uptake by the feeding ticks. In contrast, measuring weight changes before and after feeding does not allow for weight loss due to concomitant salivary secretion. As ticks feed, they alternately suck fluid and salivate, often in an erratic pattern (Gregson 1960). For example, *Theileria parva*-infected *Rhipicephalus appendiculatus* that were fed for 4

days on rabbits secreted infective stage *T. parva* into capillary tubes after a period of 2 hours (Purnell and Joyner 1967). In addition, virgin female *Hyalomma asiaticum*, detached after 7-8 days and forced to salivate into glass capillaries, secreted as much as 9 µl in 30 minutes (Balashov 1972). Such large volumes of salivary secretion would be likely to compromise any attempt to measure fluid consumption by ticks feeding from fluid-filled capillaries. Therefore, we believe that the volumes shown in Table 1 based on radioisotopic assay are more reliable than similar measurements of artificial feeding based solely on weight changes. Further, attempts to measure tick fluid uptake by measuring fluid loss from the capillaries from which the ticks were imbibing must also consider evaporation from the end of the capillaries. This may occur even in a humidified environment. The use of radioassay measurements eliminates this concern. However, other losses such as spillage of contents around the mouthparts would not have been counted with either method.

The volume of fluid imbibed by *D. variabilis* females, as measured by radioassay, is much less than that reported for *Amblyomma americanum* and *D. variabilis* by Burgdorfer (1957) or Rechav et al. (1999), approximately 10 µl per tick, based on weight determinations or fluid displacement. Direct comparisons cannot be made since the latter workers used unfed females. However, 83% of the ticks used in this experiment imbibed some fluid, slightly more than the 71.8% for *Amblyomma americanum* and considerably more than the 61% for *D. variabilis* feeding success reported by Rechav et al. (1999). Another noteworthy finding was the very great degree of variability in the amounts imbibed by different ticks, from as little as 0.06 µl to as much as 6.77 µl; only 6 individuals consumed more than 1 µl. Nevertheless, regardless of how much they

consumed, even very small amounts were sufficient to establish infection, as shown by the very high percent success of TOT. Other disparities between the results reported here and that of other workers could be due to the manner of capillary placement. We inserted the capillaries over the mouthparts, including the palps, whereas others inserted them over the hypostome, spreading the palps. However, differences in capillary placement did not modify the feeding ability of *R. appendiculatus* (Purnell and Joyner 1967).

In addition to measuring weight gain, capillary feeding allowed us to examine the influence of rickettsial infection on biological parameters of ticks, specifically engorgement weight and fecundity. We observed no significant difference in either engorgement weight or egg mass **weight** between control and infected female ticks. Positive confirmation of rickettsial infection in the eggs post-oviposition supported the hypothesis that the females were infected. These results differ from those reported by Niebylski et al. (1999), in which reduced fecundity was observed in ticks naturally infected with *R. montana* or *R. rhipicephali*, as compared to uninfected ticks. These differences may be due to the fact that the ticks used in the experiments in this study were infected during feeding, while the ticks studied by Niebylski et al. (1999) were infected prior to feeding. Similar findings may arise upon engorgement and egg laying of the progeny of ticks used in this study.

The volume of rickettsia-infected medium imbibed by the ticks did not appear to influence infection success. The percent of egg mass infection for *R. montana* (85%) as determined by PCR was almost identical to the percent of ticks that fed (88%), even though 60% imbibed less than 1 µl. Monitoring of rickettsial infection directly after microcapillary feeding identified a minimum infection rate of 40%. Compared to the

higher percentage of TOT observed in this study, the data suggest that infection due to co-feeding on the rabbit host likely occurred. Clearly, ticks will become infected even if they only imbibe very small amounts of rickettsia-infected media. The reason for the lower percentage success with *R. rhipicephali*, 69%, is unknown, but it may be because *D. variabilis* has not been proven to be a natural vector for this rickettsial species (Schreifer and Azad 1994) originally isolated from the brown dog tick, *Rhipicephalus sanguineus* (Hayes and Burgdorferi 1979).

The association between arthropods and rickettsiae is the result of an evolutionary relationship in which highly adapted rickettsiae coexist with their arthropod host. Tick-borne rickettsiae are somewhat unique in that the bacteria are maintained through transstadial and transovarial transmission, and typically have no significant deleterious effects on the arthropod host itself. Exceptions exist in which the bacteria are capable of manipulating cellular functions of the host (Werren 1997), or even result in mortality of the arthropod host, as in the case of *R. prowazekii* in its vector, the human body louse (Azad 1988).

The presence of SFG rickettsia in many tick surveys is to be expected due to the mechanisms of maintenance that occurs within tick populations. One aspect of this relationship that is of interest is the higher occurrence of nonpathogenic rickettsia reported in ticks, compared to *R. rickettsii* in the U.S. The relationship, or competition, of rickettsiae within the arthropod vector is of interest not only from an evolutionary standpoint, but from a pathogen control aspect as well. To date, the mechanisms of intraspecific competition between rickettsiae within the tick vector is unclear.

Considering the need for increased knowledge concerning the interaction of rickettsiae within the arthropod vector, known rickettsia-infected lines of ticks must be established.

We report here a capillary technique to infect partially-fed *D. variabilis* ticks with either *R. montana* or *R. rhipicephali*, and furthermore report TOT of rickettsiae in the eggs as detected by PCR and IFA. Rickettsiae-infected tick lines have now been established and propagation of these lines is in progress. Having known infected lines of ticks, a technique to infect ticks with a relatively high amount of efficiency, and the ability to differentiate between species using PCR and RFLP analysis, will allow for studies to examine intraspecific competition within the tick vector.

Acknowledgments

This project is supported by NIH grant AI-_____. We thank Jacqueline Macaluso for valuable assistance and support during this study.

References Cited

Abbassy, MM, Stein, KJ, Osman, M. New artificial feeding technique for experimental infection of *Argas* ticks (Acari: Argasidae). J Med Entomol 1994; 31:202-205.

Andersson, JO, Andersson, SG. Genome degradation is an ongoning process in Rickettsia. Mol Biol Evol 1999; 16:1178-1191.

Azad, AF. Relationship to vector biology and epidemiology of louse and flea-borne rickettsioses. In: Walker, DH, ed. *Biology of rickettsial diseases*. Florida: CRC Press; 1988:52-62.

Balashov, YuS. Bloodsucking ticks (Ixodoidea) - vectors of disease of man and animals (English Translation). Misc Publ Entomol Soc Amer 1972; 8:163-376.

Burgdorfer, W. Artificial feeding of ixodid ticks for studies on the transmission of disease agents. J Infect Dis 1957; 100:212-214.

Burgdorfer, W. The spotted fever group diseases. In: Steel, JH, ed. *CRC Handbook in Zoonoses. Section A. Bacterial, Rickettsial, and Mycotic Diseases*. Vol.2. Florida: CRC Press; 1988:279-301.

Chabaud, AG. Sur la nutrition artificelle des tiques. Ann Parasitol Hum Comp 1950; 25:42-47.

Gregson, J.D. Morphology and functioning of the mouthparts of *Dermacentor andersoni* Stiles. *Acta Tropica* 17: 48 –79.

Hayes, SF, Burgdorfer, W. Ultrastructure of *Rickettsia rhipicephali*, a new member of the spotted fever group rickettsiae in tissues of the host vector *Rhipicephalus sanguineus*. J Bacteriol 1979; 137:605-613.

Howarth, JA, Hokama, Y. Artificial feeding of adult and nymphal *Dermacentor andersoni* (Acari: Ixodidae) during studies on bovine anaplasmosis. J Med Entomol 1983; 20:248-256.

Johns, R, Sonenshine, DE, Hynes, WL. Response of the tick *Dermacentor variabilils* (Acari: Ixodidae) to hemocelic incoulation of *Borrelia burgdorferi* (Spirochetales). J Med Entomol 2000; 37:265-270.

Jongejan, F, Perie, NM, Franssen, FFJ, Uilenberg, G. Artificial infection of *Rhipicephalus appendiculatus* with *Theileria parva* by percutaneous injection. Res Vet Sci 1980; 29:320-324.

Kaufman, WR, Phillips JE. Ion and water balance in the ixodid tick, *Dermacentor andersoni*. I. Routes of ion and water excretion. J Exp Biol 1973; 58:523-536.

Kemp, DH, Koudstaal, D, Roberts, JA, Kerr, JD. Feeding of *Boophilus microplus* larvae on a partially defined medium through thin slices of cattle skin. Parasitology 1975; 70:243-254.

Kocan, KM, Wickwire, KB, Hair, JA, Ewing, SA, et al. Percutaneous infection of nymphal *Dermacentor andersoni* with *Anaplasma marginale*. Am J Vet Res 1986; 47:1662-1664.

Kurtenbach, K, Dizij A, Seitz HM, Margos G, et al. Differential immune responses to *Borrelia burgdorferi* in European wild rodent species influence spirochete transmission to *Ixodes ricinus* L. (Acari: Ixodidae). Infect Immun 1994; 62:5344-5352.

Niebylski, ML, Peacock, MG, Schwan, TG. Lethal effect of *Rickettsia rickettsii* on its tick vector (*Dermacentor andersoni*). Appl Environ Microbiol 1999; 65:773-778.

Purnell, RE. Infection of the tick *Rhipicephalus appendiculatus* with *Theileria parva* using an artificial feeding technique. Res Vet Sci 1970; 11:403-405.

Purnell, RE, Joyner, LP. Artificial feeding technique for *Rhipicephalus appendiculatus* and the transmission of *Theileria parva* from the salivary secretion. Nature 1967; 216:484-485.

Rau, U, Hannoun, C. The use of a capillary-tube technique for artifically feeding *Argas reflexus reflexus* ticks. Bull World Health Organ 1968; 39:332-333.

Rechav, Y, Zyzak, M, Fielden, LJ, Childs, JE. Comparison of methods for introducing and producing artificial infection of ixodid ticks (Acari: Ixodidae) with *Ehrlichia chaffeensis*. J Med Entomol 1999; 36:414-419.

Regnery, RL, Spruill, CL, Plikaytis, BD. Genotypic identification of rickettsiae and estimation of intraspecies sequence divergence for portions of two rickettsial genes. J Bacteriol 1991; 173:1576-1589.

Roux, V, Fournier, P, Raoult, D. Differentiation of spotted fever group rickettsiae by sequencing and analysis of restriction fragment length polymorphism of PCR-amplified DNA of the gene encoding the protein rOmpA. J Clin Microbiol 1996; 34:2058-2065.

Schreifer, ME, Azad, A. Changing ecology of Rocky Mountain spotted fever. In: Sonenshine, DE, Mather, TN, eds. *Ecological Dynamics of Tick-borne Zoonoses.* New York: Oxford University Press; 1994:314-326.

Sonenshine, DE. Biology of ticks, vol. 2. New York: Oxford University Press; 1993:465pp.

Steel, RD, Torrie, JH, Dickey, DA. Principals and procedures of statistics: A biometrical approach. New York: McGraw-Hill; 1997.

Troyer, JM, Radulovic, S, Azad, AF. Green fluorescent protein as a marker in *Rickettsia typhi* transformation. Infect Immun 1999; 67:3308-3311.

Voigt, WP, Young, AS, Mwaura, SN, Nyaga, SG, et al. *In vitro* feeding of instars of the ixodid tick *Amblyomma variegatum* on skin membranes and its application to the transmission of *Theileria mutans* and *Cowdria ruminantium*. Parasitology 1993; 107:257-263.

Walker, AR, Brown, CGD, Bell, LJ, McKellar, SB. Artificial infection of the tick *Rhipicephalus appendiculatus* with *Theileria parva*. Res Vet Sci 1979; 26:264-265.

Werren, JH. Biology of Wolbachia. Annu Rev Entomol 1997; 42:587-609.

Young, AS, Waladde, SM, Morzaria, SP. Artificial feeding systems for ixodid ticks as a tool for study of pathogen transmission. Annals NY Acad Sci 1996; 791:211-218.

Table 1. Amount (μl) of radiolabeled (^{14}C) amino acids in BSK medium ingested by *D. variabilis* via microcapillary feeding*

Tick #	Hemolymph	Midgut	Total
1	0.37	2.32	2.69
2	0.07	0.78	0.84
4	0.02	0.10	0.13
5	0.04	0.57	0.61
6	0.32	2.90	3.22
7	0.00	0.06	0.06
8	0.00	0.08	0.08
9	0.41	6.10	6.51
10	0.01	0.08	0.08
11	0.02	0.16	0.18
12	0.01	0.12	0.14
13	0.93	5.84	6.77
15	0.00	0.06	0.06
17	0.03	1.86	1.89
18	0.07	1.05	1.11
Mean ± S.D.	0.15 ± 0.26	1.47 ± 2.04	1.63 ± 2.27

* Partially fed ticks were exposed to 14C-labeled amino acids in BSK for ~24 h. radioactivity present in the hemolymph and midgut was measured, and volume of solution ingested was calculated for individual ticks.

TABLE 2. Mean weight (mg) ± standard deviation for the biological parameters of *D. variabilis* used in capillary feeding experiment*

	Capillary feeding[a]		Engorgement[b]	Egg mass[b]
Group	Pre-fed weight	Post-fed weight	weight	weight
R. montana	23.9 ± 8.6	24.5 ± 9.3	521.0 ± 141.6 a	189.1 ± 51.7 a
R. rhipicephali	25.1 ± 7.8	**23.9 ± 7.4**	565.4 ± 182.0 a	177.6 ± 59.0 a
Control	19.5 ± 8.4	19.1 ± 8.1	621.5 ± 107.8 a	225.3 ± 31.3 a

* Partially fed ticks were exposed to growth medium alone (control), or growth medium containing either *R. montana*- or *R. rhipicephali*-infected Vero cells via microcapillary feeding for ~12 h. Weights of ticks were taken prior to, and directly after, microcapillary feeding, and ticks were returned to rabbit host. Post-microcapillary fed weight values in bold are significantly different than pre-fed weights ($P \leq 0.05$). After feeding to repletion, weight of engorged ticks and egg mass (seven day post-oviposition) were recorded. In each column, means followed by the same letter are not statistically significant ($P \geq 0.05$).

I lost the labeled for this figure ⟵

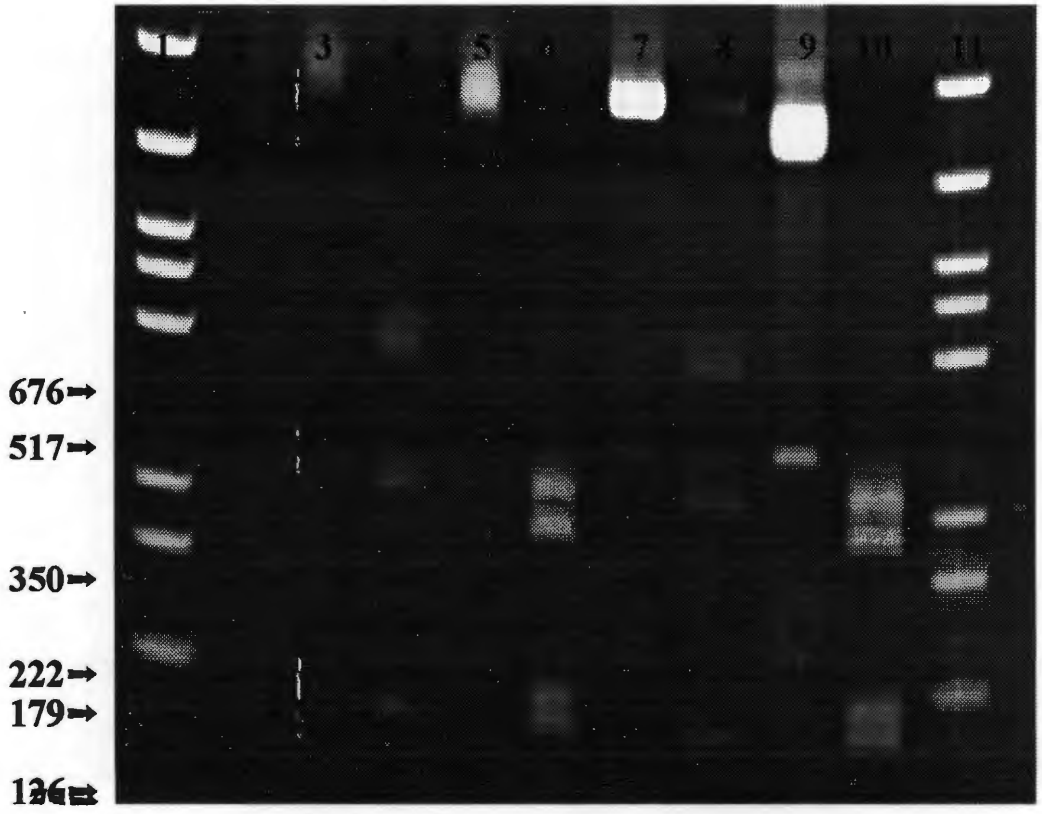

Figure Legends

Figure 1. Representative polymerase chain reaction (PCR) of rickettsial *metK* in eggs from capillary fed *Dermacentor variabilis* ticks. Lane 1, 100-bp marker ladder; Lane 2, uninfected *D. variabilis* eggs; Lane 3, *R. montana*-infected *D. variabilis* eggs; Lane 4, *R. rhipicephali*-infected *D. variabilis* eggs; Lane 5, *R. rhipicephali*-infected Vero cells; Lane 6, *R. montana*-infected Vero cells; Lane 7, H_2O PCR negative control. Numbers on the left are in base pairs.

Figure 2. Representative polymerase chain reaction (PCR) of a 630-bp amplification product of rickettsial *rompA*, and digest with RsaI, in eggs from capillary fed *Dermacentor variabilis* ticks. Lane 1 and 11, D-15 DNA marker (Novex); Lane 2, uninfected *D. variabilis* eggs; Lanes 3 and 4, purified *rompA* amplification product from *R. montana*-infected *D. variabilis* eggs and *Rsa* I digest, respectively; Lanes 5 and 6, purified *rompA* amplification product from *R. rhipicephali*-infected *D. variabilis* eggs and *Rsa* I digest, respectively; Lanes 7 and 8, *rompA* amplification product from *R. montana*-infected Vero cells and *Rsa* I digest, respectively; Lanes 9 and 10, *rompA* amplification product from *R. rhipicephali*-infected Vero cells and *Rsa* I digest, respectively. Numbers on the left are in base pairs.