ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 2010, p. 5012–5020 Vol. 54, No. 12
0066-4804/10/$12.00 doi:10.1128/AAC.01622-09

Efficacy of a Doxycycline Treatment Regimen Initiated during Three Different Phases of Experimental Ehrlichiosis▽

Jennifer C. McClure,[1] Michelle L. Crothers,[1] John J. Schaefer,[1] Patrick D. Stanley,[1] Glen R. Needham,[2] S. A. Ewing,[3] and Roger W. Stich[1]*

Department of Veterinary Pathobiology, University of Missouri, Columbia, Missouri 65211[1]; Department of Entomology, The Ohio State University, Columbus, Ohio 43210[2]; and Department of Veterinary Pathobiology, Oklahoma State University, Stillwater, Oklahoma 74078[3]

Received 16 November 2009/Returned for modification 8 February 2010/Accepted 26 September 2010

Doxycycline is the treatment of choice for canine monocytic ehrlichiosis (CME), a well-characterized disease and valuable model for tick-borne zoonoses. Conflicting reports of clearance of *Ehrlichia canis* after treatment with doxycycline suggested that the disease phase during which treatment is initiated influences outcomes of these treatments. The purpose of this study was to evaluate the efficacy of a 28-day doxycycline regimen for clearance of experimental *E. canis* infections from dogs treated during three phases of the disease. Ten dogs were inoculated with blood from *E. canis* carriers and treated with doxycycline during acute, subclinical, or chronic phases of CME. Daily rectal temperatures and semiweekly blood samples were monitored from each dog, and *Rhipicephalus sanguineus* ticks were acquisition fed on each dog for xenodiagnosis. Blood collected from dogs treated during acute or subclinical CME became PCR negative for *E. canis* as clinical parameters improved, but blood samples collected from dogs treated during chronic CME remained intermittently PCR positive. *R. sanguineus* ticks fed on dogs after doxycycline treatments became PCR positive for *E. canis*, regardless of when treatment was initiated. However, fewer ticks became PCR positive after feeding on two persistently infected dogs treated with doxycycline followed by rifampin, suggesting that antibiotic therapy can reduce tick acquisition of *E. canis*.

Tick-borne diseases attributable to the rickettsial family *Anaplasmataceae* have been subject to increasing interest from veterinary and public health perspectives. Several members of this group naturally infect dogs and have emerged as tick-borne zoonotic pathogens over recent decades (41, 59). Experimental studies of these diseases and their etiologic agents can be challenging, but *Ehrlichia canis*, the primary etiologic agent of canine monocytic ehrlichiosis (CME), offers a relatively unique opportunity to study interactions between a monocytotropic member of the family *Anaplasmataceae* and its invertebrate and vertebrate hosts (49).

CME canis is divided into acute, subclinical, and chronic phases, which were primarily defined through experimental infections of purpose-bred dogs that were infected by needle inoculation with *E. canis*-infected blood (7, 8, 14, 18, 26). The acute phase of experimental CME begins approximately 10 days postinoculation (dpi) and involves leukopenia, thrombocytopenia, fever, depression, and anorexia. Notably, reports of experimental infection with *E. canis* document that dogs presented signs of severe acute CME when they were inoculated with carrier blood (8, 20, 23, 24, 42, 57) while other investigators reported less severe disease when dogs were inoculated with *E. canis*-infected cell cultures (17, 20, 28, 30, 40). Clinical signs subside approximately 20 to 30 dpi, which is usually followed by a subclinical phase that can last from months to years. Although there are minimal clinical signs during the subclinical disease phase, hematologic signs such as leukopenia, anemia, and thrombocytopenia can still occur, with mild thrombocytopenia as the most consistent finding under experimental conditions (57). Chronic CME is the third phase, which may be mild to severe, with recurrent clinical and hematologic signs that include pancytopenia, hemorrhage, monocytosis, lymphocytosis, and weight loss (8, 10, 16, 18, 38, 45).

Tetracyclines are the antibiotics of choice for treatment of numerous tick-borne bacterial diseases, including those associated with rickettsial pathogens (41, 59). Similarly, oral doxycycline is commonly used for treatment of CME. Although doxycycline effectively ameliorates clinical CME, there are conflicting reports of the efficacy of the same treatment regimens for clearance of *E. canis* infection from these hosts. Some reports suggest persistence of infection following 7- to 85-day doxycycline regimens of naturally and experimentally infected dogs during postacute phases of CME (1, 23, 29, 58) while other reports indicate clearance of *E. canis* after 14- to 60-day doxycycline treatments of dogs during acute CME (4, 22). A 28-day course of oral doxycycline is the currently recommended protocol issued by a consensus statement of the American College of Veterinary Internal Medicine for treatment of CME (39).

We have observed similarly discrepant results with regard to persistence of *E. canis* infection in dogs following doxycycline regimens. *E. canis* infections were detected in peripheral blood and in *Rhipicephalus sanguineus* ticks acquisition fed as nymphs after a 14-day doxycycline regimen from dogs that were infected by tick transmission that resulted in mild acute-phase CME (43). Conversely, *E. canis* infections were not detected in peripheral blood after a 28-day doxycycline regimen from dogs

* Corresponding author. Mailing address: Department of Veterinary Pathobiology, University of Missouri, 210 Connaway Hall, Columbia, Missouri 65211. Phone: (573) 882-3148. Fax: (573) 884-5414. E-mail: stichrw@missouri.edu.

▽ Published ahead of print on 4 October 2010.

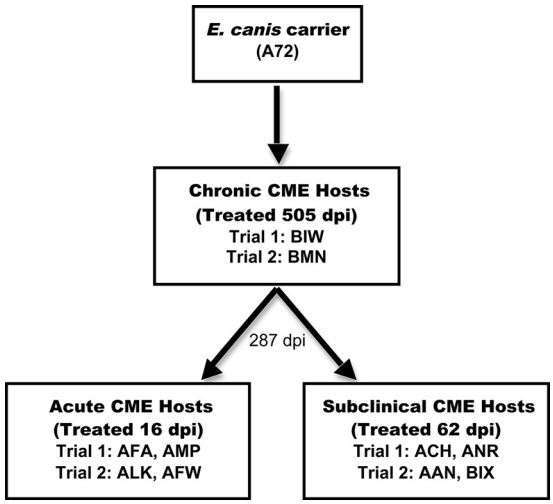

FIG. 1. Induction of experimental ehrlichiosis. Clinical CME was induced in dogs by inoculation of *E. canis* carrier blood. Dogs BIW and BMN were each simultaneously inoculated with 10 ml of heparinized blood from dog A72, and blood collected from dog BIW or BMN (287 dpi) was inoculated into dogs AFA, AMP, ACH, and AAN or into dogs ALK, AFW, ANR, and BIX, respectively. Doxycycline treatments were initiated 16 dpi for dogs AFA, AFW, ALK, and AMP, at 62 dpi for dogs ACH, AAN, ANR, and BIX, and at 505 dpi for dogs BIW and BMN.

that were infected by intravenous inoculation of carrier blood that resulted in severe acute-phase CME (42). Differences in experimental designs and results among these and the aforementioned studies collectively suggest that *E. canis* infections might be less likely to be cleared by doxycycline when they are transmitted by ticks, after mild acute CME, or as CME progresses through different phases. To test the last hypothesis, dogs were intravenously inoculated with *E. canis* carrier blood, to ensure severe acute-phase CME, and treated during three different clinical phases of CME. *E. canis* infections were monitored by PCR assays of host blood and ticks fed on each host after treatment.

MATERIALS AND METHODS

Experimental dogs. Ten adult purpose-bred beagle dogs were experimentally infected with *E. canis* by intravenous (i.v.) inoculation of heparinized *E. canis* carrier blood. Dogs BIW and BMN were each simultaneously inoculated with 10 ml of heparinized blood from dog A72, a persistently infected *E. canis* (Ebony isolate) carrier (47). At 287 dpi, dogs BIW and BMN served as sources of infectious blood for the remaining dogs used in this study. In the first trial, dogs AFA, AMP, ACH, and AAN were each simultaneously inoculated with 5 ml of heparinized blood from BIW. In the second trial, dogs ALK, AFW, ANR, and BIX were each simultaneously inoculated (i.v.) with 5 ml of heparinized blood from BMN. Doxycycline treatments were initiated (~10 mg/kg of body weight [kg_{bw}], *per os* [p.o.], once a day [q.d.], for 28 days) during the acute, subclinical, and chronic phases of CME at 16, 62, and 505 dpi, respectively (Fig. 1). To the best of our knowledge, dog A72, a long-term *E. canis* carrier, had not been exposed to doxycycline since experimental infection with *E. canis* via tick transmission (47). Likewise, dogs BMN and BIW, which were used as donors for the remaining dogs, were not exposed to doxycycline prior to the treatments used in the current study. Dogs were monitored for 2 weeks after receiving their final doses of antibiotic. Daily rectal temperatures were recorded for each dog, and semiweekly blood samples were collected for complete blood counts (CBCs) and PCR assays as described below. Blood samples used for hematological testing were collected in evacuated glass tubes containing EDTA (Kendall Monoject 311149), and CBC profiles were measured by the clinical pathology laboratory at the University of Missouri College of Veterinary Medicine with an Abbott Cell Dyn 3500 (Abbot Laboratories, Abbot Park, IL). Blood samples were collected for PCR assays in heparinized evacuated glass tubes (Vacutainer, item 366480; BD). Dogs used for this study were cared for in accordance with a protocol on file with the University of Missouri Columbia Animal Care and Use Committee.

Xenodiagnosis. Adult *R. sanguineus* male ticks were purchased from the Oklahoma State University Medical Entomology Laboratory. Ticks were placed into orthopedic stockinettes adhered to the surface of each dog and allowed to feed for 10 days, as previously described elsewhere (6). Heparinized blood was collected from dogs treated with doxycycline during the acute (2 ml per dog), subclinical (2 ml per dog), or chronic (5 ml per dog) CME phase, and 8 to 10 ml of pooled blood was inoculated into naïve dogs as an additional test for persistent infections in host blood.

PCR assays. Heparinized blood samples (1 ml) were centrifuged at 1,200 × *g* for 30 min, buffy coat layers were isolated (~200 μl), and DNA was extracted with a High Pure Viral Nucleic Acid Isolation kit (Roche, Indianapolis, IN) as previously described elsewhere (35). Two extra buffy coats were stored at −80 °C for each sample collected as replicates in the event that PCR-positive samples were observed after doxycycline treatment; samples were interpreted as negative when frozen replicates tested negative by PCR. In preparation for PCR, frozen buffy coats were overlaid with 1.5 ml of RNA*later*-ICE (Ambion Inc., Austin, TX) and stored (−20°C) for ≥18 h. These samples were centrifuged (12,000 × *g* for 15 min at 4°C), and supernatants were discarded prior to DNA isolation as described above.

Ticks were removed from dogs, grouped according to host, and stored in the same humidity chamber for 10 days at room temperature (RT) and 95 to 100% rH, with a 12:12-h light-dark photoperiod. Previous work has shown that anaplasmal pathogens incapable of infecting ticks are detectable for only 2 days after tick removal from the host (50). Thus, these ticks remained in the humidity chamber for 10 days to improve the likelihood of detection of *E. canis* in infected ticks rather than in remnants of blood meals from the canine hosts (6). After incubation to allow blood meal removal, these ticks were then incubated for another 85 h at 37°C (100% rH) prior to bisection and DNA isolation. New razor blades were used for ticks collected from each individual dog, and these blades were flame sterilized prior to bisection of each individual tick. Ticks collected before and after doxycycline treatments did not occupy humidity chambers at the same time. Tick halves were immediately placed on dry ice and stored at −80°C until DNA was extracted with the same protocol used for blood.

A *p30*-based PCR assay was used to test for the presence of *E. canis* DNA as previously described elsewhere, with some modifications (6, 35, 42, 43, 47). Briefly, 25-μl reaction mixtures were prepared with 2% dimethyl sulfoxide (DMSO), 2.0 mM $MgCl_2$, 200 μM of each deoxynucleoside triphosphate (dNTP), 0.3 μM (each) of primers ECA30-384S (ATAAACACGCTGACTTT ACTGTTCC) and ECA30-583A (GTGATGAGATAGAGCGCAGTACC), and 0.03 U/μl of Platinum *Taq* polymerase (Invitrogen, Carlsbad, CA). Samples were incubated at 94°C for 2 min, followed by 75 cycles at 94, 65, and 72°C for 30 s each, with a final extension for 7 min at 72°C. Positive controls consisted of 10 pg of DNA extracted from *E. canis*-infected DH82 cell cultures that were prepared as previously described (6, 35) while negative controls consisted of preinoculation canine blood and unexposed ticks. No-template controls served as tests for contamination for every assay. Every sample was assayed a minimum of two times, followed by a third assay of samples with discrepant results between the two trials. Amplicons of representative samples were excised from agarose gels, extracted with a Wizard SV Gel and PCR Clean Up System (Promega, Madison, WI), and submitted to the University of Missouri DNA Core Facility for sequencing of both DNA strands. Amplicon sequences were compared via multiple sequence alignment with CodonCode Aligner (CodonCode Corporation, Dedham, MA).

RESULTS

Efficacy of doxycycline for treatment of experimental CME. Reports of clearance of *E. canis* infections from experimentally infected hosts usually involved (i) needle inoculation (i.v.) with infected host cells and (ii) treatment during the acute phase of clinical CME (4, 12, 22). Thus, dogs used in the current study were inoculated with carrier blood (i.v.) to consistently induce overt signs of clinical CME including fever and pancytopenia that began 14 to 16 dpi (Fig. 2).

(i) Acute phase. Treatment regimens for dogs AFA, AFW, ALK, and AMP were initiated approximately 24 h after clinical

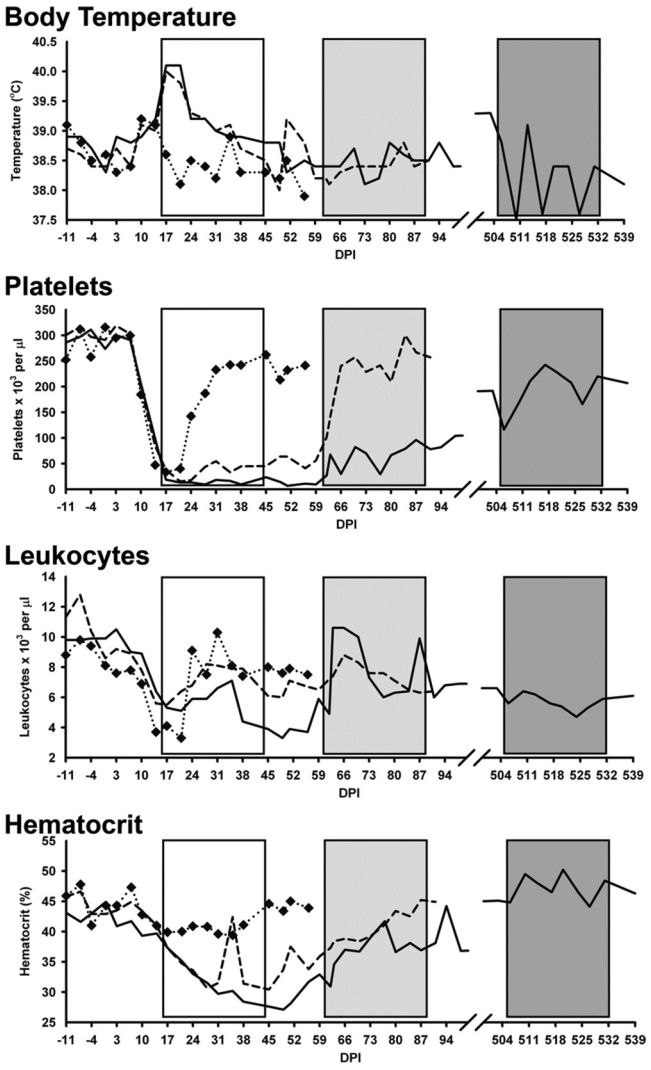

FIG. 2. Clinical responses to doxycycline treatment by dogs treated during different phases of ehrlichiosis. Dogs were inoculated with blood from a persistently infected *E. canis* carrier on day 0, and treatment with doxycycline was initiated during the acute (16 dpi), subclinical (62 dpi), or chronic (505 dpi) phase of ehrlichiosis. Each panel (top to bottom), respectively, illustrates rectal temperature, platelet, white blood cell, or hematocrit values. Dotted, dashed, or solid lines represent dogs treated during the acute, subclinical, or chronic phase, respectively. Open, light gray, and dark gray rectangles represent treatment periods during the acute, subclinical, and chronic phases, respectively.

and hematological signs of CME became apparent (16 dpi). Clinical signs of CME abated among all four dogs soon after treatment was initiated, and CBC parameters continually improved during and after the treatment period (Fig. 2). Rectal temperatures of this group increased from a preinoculation baseline of 38.8 ± 0.5°C (mean ± standard deviation [SD]) to a peak of 40.3 ± 0.2°C at 15 dpi, which recovered to 37.7 ± 0.3°C by 32 dpi, 16 days posttreatment initiation (dpti). Platelet counts of this group dropped from a baseline of $2.76 \times 10^5 \pm 0.65 \times 10^5$ to a nadir of $3.4 \times 10^4 \pm 3.0 \times 10^4$ at 16 dpi, which recovered to $2.62 \times 10^5 \pm 1.23 \times 10^5$ platelets per μl by 38 dpi (22 dpti). Similarly, leukocyte counts of this group dropped from a baseline of $8.30 \times 10^3 \pm 1.70 \times 10^3$ to a nadir of $3.30 \times 10^3 \pm 0.80 \times 10^3$ at 17 dpi, which recovered to $1.033 \times 10^4 \pm 0.291 \times 10^4$ leukocytes per μl by 28 dpi (12 dpti). Hematocrit values dropped from a baseline of 44.7 ± 3.8% to a nadir of 39.4 ± 4.0% at 31 dpi, which recovered to 45.0 ± 3.8% by 49 dpi (33 dpti).

(ii) Subclinical phase. Treatment regimens for dogs AAN, ACH, ANR, and BIX were initiated at 62 dpi. Body temperatures of all four dogs returned to the normal range by 61 dpi, thus prior to initiation of treatment, and CBC parameters began to recover after the initial nadir at approximately 21 dpi (Fig. 2). Rectal temperatures of this group rose from a preinoculation baseline of 38.7 ± 0.4°C to a peak of 40.0 ± 0.6°C at 15 dpi, which recovered to 38.5 ± 0.3°C at initiation of doxycycline therapy (62 dpi) and to 37.9 ± 0.2°C by 67 dpi. Platelet counts of this group dropped from a baseline of $3.04 \times 10^5 \pm 0.48 \times 10^5$ to a nadir of $1.5 \times 10^4 \pm 1.1 \times 10^4$ at 21 dpi, which recovered to $1.0 \times 10^5 \pm 0.6 \times 10^5$ at 62 dpi and to $3.00 \times 10^5 \pm 0.70 \times 10^5$ platelets per μl by 84 dpi (22 dpti). Leukocyte counts of this group dropped from a baseline of $9.40 \times 10^3 \pm 2.50 \times 10^3$ to a nadir of $5.50 \times 10^3 \pm 0.39 \times 10^3$ at 17 dpi, which recovered to $7.2 \times 10^3 \pm 0.6 \times 10^3$ at 62 dpi and to $8.80 \times 10^3 \pm 1.86 \times 10^3$ leukocytes per μl by 66 dpi (4 dpti). Hematocrit values dropped from a baseline of 45.1 ± 4.6% to a nadir of 30.4 ± 2.7% at 45 dpi, which recovered to 36.4 ± 2.4% at 62 dpi and 45.6 ± 1.4% by 87 dpi (25 dpti).

(iii) Chronic phase. Treatment regimens for dogs BIW and BMN were initiated at 505 dpi. Both dogs exhibited signs of severe CME during the acute phase of the disease (Fig. 2). Rectal temperatures of this group changed from a preinoculation baseline of 38.7 ± 0.3°C to a peak of 40.2 ± 0.5°C at 19 dpi, which averaged 37.3 ± 0.6°C at 445 dpi (60 days before treatment) and were 39.3 ± 0.7°C at the time of treatment (505 dpi). Platelet counts dropped from a baseline of $2.97 \times 10^5 \pm 0.79 \times 10^5$ to a nadir of $7.0 \times 10^3 \pm 6.0 \times 10^3$ at 48 dpi, which recovered to $1.92 \times 10^5 \pm 0.64 \times 10^5$ platelets per μl at 503 dpi, 2 days prior to initiation of treatment, and further recovered to $2.43 \times 10^5 \pm 0.11 \times 10^5$ platelets per μl by 517 dpi (12 dpti). Leukocyte counts dropped from a baseline of $9.70 \times 10^3 \pm 2.40 \times 10^3$ to a nadir of $3.27 \times 10^3 \pm 1.36 \times 10^3$ at 44 dpi, which recovered to $1.059 \times 10^4 \pm 0.680 \times 10^4$ leukocytes per μl by 62 dpi (443 days before treatment) and were $4.8 \times 10^5 \pm 2.1 \times 10^5$ leukocytes per μl at 503 dpi. Hematocrit values dropped from a baseline of 43.3 ± 2.4% to a nadir of 27.1 ± 2.3% at 44 dpi, which recovered to 45.1 ± 3.2 by 503 dpi, 2 days prior to initiation of treatment, and further recovered to 50.2 ± 3.7% by 520 dpi (15 dpti).

Clearance of *E. canis* during different phases of CME. (i) Acute phase. Previous reports of clearance of *E. canis* from experimentally infected hosts usually involved initiation of treatment during the acute phase of CME (4, 12, 22). Thus, the working hypothesis for this experiment was that administration of a 28-day doxycycline regimen during the acute phase of CME would eliminate *E. canis* infections. To test this hypothesis, peripheral blood was collected before, during, and after doxycycline treatment of dogs AFA, AFW, ALK, and AMP and assayed for the presence of *E. canis*. Xenodiagnosis was also performed by allowing adult male *R. sanguineus* ticks to acquisition feed for 10 days on each dog, starting 11 days after the final doxycycline treatment (i.e., 57 to 68 dpi). Cohorts of

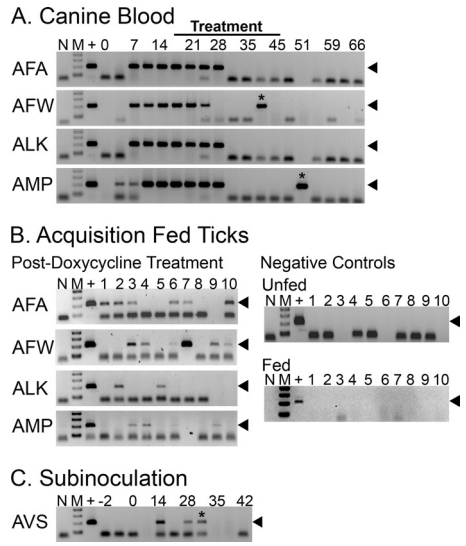

FIG. 3. Detection of *E. canis* in dogs treated during acute CME. (A) PCR assays of buffy coats collected semiweekly throughout the experiment. The bar at the top of this panel represents the treatment period from 16 to 44 dpi. (B) PCR assays of adult male *R. sanguineus* ticks fed on dogs AFA, AFW, ALK, and AMP at 13 days after the end of doxycycline treatment (57 dpi). Unfed male *R. sanguineus* ticks and cohorts fed on a naïve dog served as controls. Lanes 1 to 10 represent individual tick halves. (C) PCR assay of buffy coats from dog AVS which was inoculated with blood pooled from dogs AFA, AFW, ALK, and AMP after doxycycline treatment (114 dpi). All samples were tested with a single-step *E. canis*-specific *p30*-based PCR assay. Samples resulting in a 200-bp amplicon (arrowhead) were considered PCR positive. Asterisks denote samples with a frozen replicate that tested PCR negative. For each panel, no-template controls (N) served as tests for contamination, DNA (10 pg) extracted from *E. canis*-infected DH82 cells served as positive control (+), and lanes labeled 0 to 66 or −2 to 42 indicate dpi. The molecular size standard (M) was a 100-bp ladder.

these ticks were fed on a naïve dog to serve as controls. In addition, 2 ml of blood was collected from each dog at 114 dpi (98 dpti), pooled, and inoculated (i.v.) into a naïve dog (AVS) as a test of infectivity.

E. canis was not detected in blood prior to inoculation but was detected in all four dogs by 7 dpi. Blood samples from these dogs continued to test positive up to 24 to 28 dpi (8 to 12 dpti), and peripheral blood from these dogs remained PCR negative throughout the remainder of the study except for two samples collected from dogs AFW (38 dpi) and AMP (51 dpi) (Fig. 3A). Frozen replicates of these samples tested PCR negative. However, 2 to 7 of 10 *R. sanguineus* male ticks fed on these dogs tested PCR positive while both unfed and fed cohorts of these ticks were PCR negative (Fig. 3B).

Dog AVS, which was inoculated with pooled blood from this group, did not exhibit overt clinical signs of CME although the rectal temperature for dog AVS did rise from a preinoculation baseline of 38.4 ± 0.6°C to a peak of 39.9°C at 5 dpi. Platelet counts for this dog did not decrease following inoculation but instead rose from a baseline of $2.23 \times 10^5 \pm 0.44 \times 10^5$ to 2.43×10^5 at 28 dpi while leukocyte counts dropped from a baseline of $8.40 \times 10^3 \pm 0.40 \times 10^3$ to a nadir of 7.16×10^3 at 35 dpi, and hematocrit values dropped from a baseline of 47.5 ± 2.0% to a nadir of 43.3% at 39 dpi. Overall these parameters changed 4, 9, −15, and −9% from baseline values for rectal temperature, platelet count, leukocyte count, and hematocrit, respectively. However, *E. canis* was detected in peripheral blood collected from this dog at 14, 28, and 31 dpi (Fig. 3C), and the frozen backup samples for 14 and 28 dpi also tested positive by PCR.

(ii) Subclinical phase. Several reports suggested that *E. canis* infections can persist in dogs treated with doxycycline during postacute phases of CME (23, 29, 43, 58). Thus, the working hypothesis for this experiment was that the initiation of a 28-day doxycycline regimen during the subclinical phase of experimental CME would result in less efficacious elimination of *E. canis* infections than treatment during the acute phase of the disease. The 28-day doxycycline treatments were initiated for dogs ACH, AAN, ANR, and BIX at 62 dpi. Xenodiagnosis was also performed with acquisition-fed *R. sanguineus* both before and after doxycycline treatment at 22 and 123 dpi, respectively. Blood (2 ml) was collected from each dog at 135 dpi (73 dpti), pooled, and inoculated (i.v.) into dog AHS.

E. canis was not detected in blood prior to inoculation but was detected among this group as early as 3 dpi, and these dogs remained consistently PCR positive throughout the acute and subclinical phases of CME. However, these infections were not detected in peripheral blood within 7 dpti (Fig. 4A), except for three samples from dogs AAN (87 dpi) and ACH (98 and 101 dpi). Frozen backup buffy coats of these samples tested PCR negative. However, 2 to 4 of 10 *R. sanguineus* male ticks tested positive by PCR when fed prior to treatment while 1 to 5 of 10 ticks tested PCR-positive after doxycycline treatment (Fig. 4B).

Dog AHS, which was inoculated with pooled blood from this group, did not become PCR positive or exhibit signs of disease attributable to *E. canis* (Fig. 4C). Rectal temperatures for dog AHS rose from a preinoculation baseline of 38.3 ± 0.5°C to a peak of 39.4°C at 7 dpi. Platelet counts for this dog did not decrease following inoculation and instead rose from a baseline of $2.60 \times 10^5 \pm 0.13 \times 10^5$ to 2.73×10^5 at 11 dpi. Leukocyte counts for AHS dropped from a baseline of $6.50 \times 10^3 \pm 0.30 \times 10^3$ to a nadir of 5.95×10^3 at 32 dpi. Hematocrit values dropped from a baseline of 45.4 ± 1.7% to a nadir of 40.1% at 35 dpi. Overall, these parameters changed 5, 8, −12, and −3% from baseline values for rectal temperature, platelet count, leukocyte count, and hematocrit, respectively.

(iii) Chronic phase. In previous work, infections were cleared from peripheral blood of dogs treated during subclinical CME if treatment was initiated while values of CBC parameters were low (16, 42). Conversely, infections persisted after doxycycline therapy of dogs with values of clinical and hematological parameters that were closer to the normal range (43, 58), and similar results were observed for naturally infected dogs with higher CBC values (1, 5, 58). Therefore, the working hypothesis for this experiment was that persistent *E. canis* infections in dogs with CBC values in the normal range are more resistant to clearance by doxycycline therapy. Two dogs, BIW and BMN, were treated with doxycycline from 505 to 533 dpi. *R. sanguineus* ticks were acquisition fed on these dogs both before and after doxycycline treatment at 434 and 549 dpi, respectively. Additionally, 5 ml of blood was collected from each dog at 629 dpi (96 dpti), pooled, and inoculated (i.v.) into dog BJW. Dogs BMN and BIW were also treated

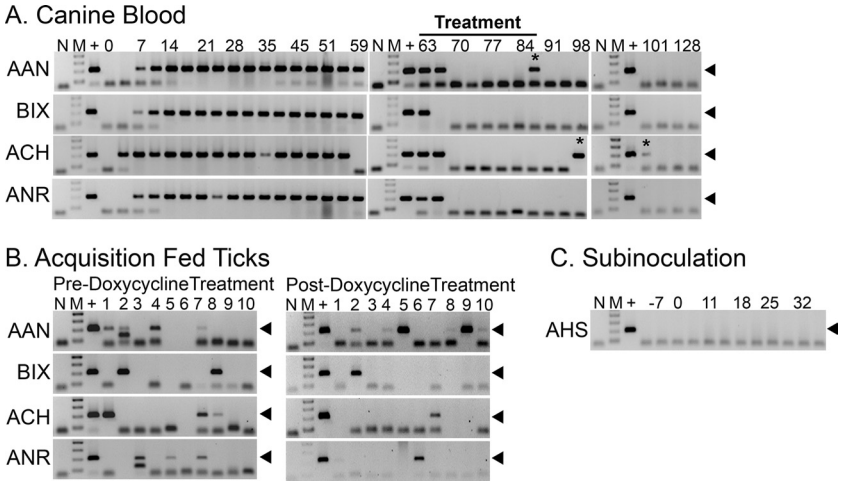

FIG. 4. Detection of *E. canis* in dogs treated during subclinical CME. (A) PCR assays of buffy coats collected semiweekly throughout the experiment. The bar at the top of this panel represents the treatment period from 62 to 90 dpi. (B) PCR assays of adult male *R. sanguineus* ticks fed on dogs AAN, BIX, ACH, and ANR before (22 dpi) and after (123 dpi) treatment. Lanes 1 to 10 represent individual ticks. (C) PCR assays of buffy coats from dog AHS, which was inoculated with blood pooled from dogs AAN, BIX, ACH, and ANR after doxycycline treatment (129 dpi). All samples were tested with a single-step *E. canis*-specific *p30*-based PCR assay. Samples resulting in a 200-bp amplicon (arrowhead) were considered PCR positive. Asterisks indicate samples with a frozen replicate that tested PCR negative. For each panel, no-template controls (N) served as tests for contamination, template DNA (10 pg) extracted from *E. canis*-infected DH82 cells served as a positive control (+), and lanes labeled 0 to 128 or −14 to 35 indicate dpi. The molecular size standard (M) was a 100-bp ladder.

with rifampin (~15 mg/kg$_{bw}$, p.o., twice a day [b.i.d.]) from 701 to 708 dpi, which was followed by *R. sanguineus* acquisition feeding at 715 dpi.

E. canis was not detected in blood prior to inoculation but was consistently detected in peripheral blood of both dogs from 9 dpi until initiation of antibiotic treatment. Although clinical signs of CME were no longer manifest prior to treatment, both dogs were still PCR positive when treatment was initiated (Fig. 5A). *E. canis* was intermittently detected in peripheral blood of BMN and BIW during and after doxycycline treatments. Frozen backups of these samples also tested positive by PCR. Notably, 5 and 8 of 10 ticks fed on dogs BMN and BIW, respectively, tested PCR positive prior to doxycycline treatment while 7 and 9 of 10 ticks tested PCR positive when fed on the same dogs after doxycycline treatments (Fig. 5B). However, only 1 of 10 ticks fed on BMN and none of the 10 ticks fed on BIW tested PCR positive for *E. canis* after these dogs were treated with rifampin.

Dog BJW, which was inoculated with pooled blood from BMN and BIW after doxycycline treatment, did not exhibit signs of disease. Rectal temperatures for dog BJW rose from a preinoculation baseline of 37.9 ± 0.3°C to a peak of 38.4°C at 17 dpi. Interestingly, platelet counts for BJW decreased from a baseline of $3.45 \times 10^5 \pm 0.45 \times 10^5$ to a nadir of 2.29×10^4 at 31 dpi. Platelet aggregation was not observed in the corresponding blood smear. However, this drop in platelets was seen in only a single sample, with the next lowest value of 3.09×10^5. Leukocyte counts for dog BJW dropped from a baseline of $1.06 \times 10^4 \pm 0.08 \times 10^4$ to a nadir of 8.70×10^3 at 10 dpi. Hematocrit values dropped from a baseline of 50.6 ± 3.4% to a nadir of 45.1% at 10 dpi. Overall these parameters changed 1, −93, −18, and −11% from baseline values for rectal temperature, platelet count, leukocyte count, and hematocrit, respectively. Dog BJW tested PCR positive once at 10 dpi (Fig. 5C), which corresponded with the nadirs in leukocyte and hematocrit values for this dog, and this result was confirmed when the 10-dpi frozen backup buffy coat sample also tested PCR positive.

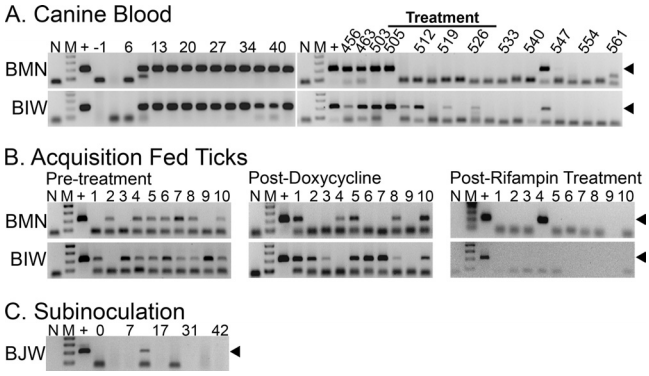

FIG. 5. Detection of *E. canis* in dogs treated during chronic CME. (A) PCR assays of buffy coats collected semiweekly throughout the experiment. The bar at the top of this panel represents the treatment period at 505 to 533 dpi. (B) PCR assays of adult male *R. sanguineus* ticks fed on dogs BMN and BIW before (434 dpi) and after (549 dpi) doxycycline treatment and of *R. sanguineus* ticks fed after treatment with rifampin (715 dpi). Lanes 1 to 10 represent individual tick halves. (C) PCR assay of buffy coats collected weekly from dog BJW, which was inoculated with blood pooled from both dogs after doxycycline treatment (629 dpi). Samples were tested with a single-step *E. canis*-specific *p30*-based PCR assay, and those with a 200-bp amplicon (arrowhead) were considered PCR positive. For each panel, no-template controls (N) served as tests for contamination, template DNA (10 pg) extracted from *E. canis*-infected DH82 cells served as a positive control (+), and lanes labeled −1 to 554 or 0 to 42 indicate dpi. The molecular size standard (M) was a 100-bp ladder.

Amplicon sequence analysis. Representative buffy coat and tick samples that tested PCR positive after doxycycline treatments were reassayed in order to excise and sequence the 200-bp bands for comparison to the *E. canis p30* target sequence. One amplicon generated from a tick specimen was 99% identical to the target sequence while remaining amplicons were 100% identical to the target sequence (Fig. 6).

DISCUSSION

The results of this investigation confirmed the efficacy of doxycycline for amelioration of clinical CME during the acute phase of the disease and potentially for clearance of *E. canis* from peripheral blood when dogs are treated during the acute and subclinical phases of disease. However, these results also indicate that *E. canis* can persist in clinically normal dogs, even after an extensive doxycycline treatment regimen. These results have important implications with regard to possible recrudescence of ehrlichiosis due to such persistent infections and indicate that ehrlichiosis patients should be continually monitored even after a clinical response to antibiotic therapy. All of the dogs used for doxycycline treatment in this investigation exhibited signs of severe acute CME, and all of the parameters measured returned to normal levels after initiation of doxycycline treatments. To the best of our knowledge, this is the first report of attempted xenodiagnosis following a rifampin treatment regimen, and this result suggests that a treatment regimen that includes rifampin could be more efficacious for control of vector infection. However, it should be noted that the rifampin regimen used in the current study was subsequent to the doxycycline regimen. Furthermore, we have since observed persistent *E. canis* infections in two dogs after treatment with rifampin in the absence of any other antibiotic (data not shown). Therefore, the results of the rifampin regimen used in this report should be interpreted with caution.

Doxycycline treatment effectively mitigated the clinical signs associated with acute CME. Values of CBC parameters for dogs treated during the acute phase dropped to 12.3, 39.8, and 88.1% of baseline for platelets, leukocytes, and hematocrit, respectively, and the same respective parameters recovered to 94.9, 124.5, and 100.7% of baseline values after treatment was initiated. Recovery from acute CME was not complete among dogs prior to treatment at 62 dpi, until after initiation of doxycycline treatment of these dogs. Collectively, values of CBC parameters dropped to 5, 58.5, and 67.4% and, after treatment was initiated, recovered to 98.7, 93.6, and 101.1% of baseline for platelets, leukocytes, and hematocrit, respectively. Rectal temperatures had fully recovered while hematological values had increased but not fully recovered among dogs allowed to reach the chronic phase of CME. Further recovery was observed after initiation of treatments at 505 dpi. For this group, platelet, leukocyte, and hematocrit values, respectively, dropped to 2.3, 34.0, and 61.2%; recovered to 64.6, 109.3, and 104.0% of baseline; were at 36, 51, and 104% of baseline prior to treatment (503 dpi); and reached 81.8, 68, and 113.3% of baseline values after treatment was initiated. The lower average leukocyte value observed for this group prior to treatment is partially due to leukocytosis observed for dog BIW at two time points during the subclinical phase for this group, skewing

```
E. canis ATAAACACGCTGACTTTACTGTTCCAAACTATTCGTTCAGATACGAGAAC
ECF1     ATAAACACGCTGACTTTACTGTTCC-------------------------
AFA-T    ATAAACACGCTGACTTTACTGTTCCAAACTATTCGTTCAGATACGAGAAC
AFW-T    ATAAACACGCTGACTTTACTGTTCCAAACTATTCGTTCAGATACGAGAAC
ALK-T    ATAAACACGCTGACTTTACTGTTCCAAACTATTCGTTCAGATACGAGAAC
AMP-T    ATAAACACGCTGACTTTACTGTTCCAAACTATTCGTTCAGATACGAGAAC
AAN-T    ATAAACACGCTGACTTTACTGTTCCAAACTATTCGTTCAGATACGAGAAC
BIX-T    ATAAACACGCTGACTTTACTGTTCCAAACTATTCGTTCAGATACGAGAAC
ACH-T    ATAAACACGCTGACTTTACTGTTCCAAACTATTCGCTCAGATACGAGAAC
ANR-T    ATAAACACGCTGACTTTACTGTTCCAAACTATTCGTTCAGATACGAGAAC
BMN-T    ATAAACACGCTGACTTTACTGTTCCAAACTATTCGTTCAGATACGAGAAC
BIW-T    ATAAACACGCTGACTTTACTGTTCCAAACTATTCGTTCAGATACGAGAAC
ACH.BC   ATAAACACGCTGACTTTACTGTTCCAAACTATTCGTTCAGATACGAGAAC
BMN.BC   ATAAACACGCTGACTTTACTGTTCCAAACTATTCGTTCAGATACGAGAAC
BIW.BC   ATAAACACGCTGACTTTACTGTTCCAAACTATTCGTTCAGATACGAGAAC
ECR1     --------------------------------------------------

E. canis AATCCATTTCTAGGGTTTGCAGGAGCTATCGGTTACTCAATGGGTGGCCC
ECF1     --------------------------------------------------
AFA-T    AATCCATTTCTAGGGTTTGCAGGAGCTATCGGTTACTCAATGGGTGGCCC
AFW-T    AATCCATTTCTAGGGTTTGCAGGAGCTATCGGTTACTCAATGGGTGGCCC
ALK-T    AATCCATTTCTAGGGTTTGCAGGAGCTATCGGTTACTCAATGGGTGGCCC
AMP-T    AATCCATTTCTAGGGTTTGCAGGAGCTATCGGTTACTCAATGGGTGGCCC
AAN-T    AATCCATTTCTAGGGTTTGCAGGAGCTATCGGTTACTCAATGGGTGGCCC
BIX-T    AATCCATTTCTAGGGTTTGCAGGAGCTATCGGTTACTCAATGGGTGGCCC
ACH-T    AATCCATTTCTAGGGTTTGCAGGAGCTATCGGTTACTCAATGGGTGGCCC
ANR-T    AATCCATTTCTAGGGTTTGCAGGAGCTATCGGTTACTCAATGGGTGGCCC
BMN-T    AATCCATTTCTAGGGTTTGCAGGAGCTATCGGTTACTCAATGGGTGGCCC
BIW-T    AATCCATTTCTAGGGTTTGCAGGAGCTATCGGTTACTCAATGGGTGGCCC
ACH.BC   AATCCATTTCTAGGGTTTGCAGGAGCTATCGGTTACTCAATGGGTGGCCC
BMN.BC   AATCCATTTCTAGGGTTTGCAGGAGCTATCGGTTACTCAATGGGTGGCCC
BIW.BC   AATCCATTTCTAGGGTTTGCAGGAGCTATCGGTTACTCAATGGGTGGCCC
ECR1     --------------------------------------------------

E. canis AAGAATAGAATTCGAAATATCTTATGAAGCATTCGACGTAAAAAGTCCTA
ECF1     --------------------------------------------------
AFA-T    AAGAATAGAATTCGAAATATCTTATGAAGCATTCGACGTAAAAAGTCCTA
AFW-T    AAGAATAGAATTCGAAATATCTTATGAAGCATTCGACGTAAAAAGTCCTA
ALK-T    AAGAATAGAATTCGAAATATCTTATGAAGCATTCGACGTAAAAAGTCCTA
AMP-T    AAGAATAGAATTCGAAATATCTTATGAAGCATTCGACGTAAAAAGTCCTA
AAN-T    AAGAATAGAATTCGAAATATCTTATGAAGCATTCGACGTAAAAAGTCCTA
BIX-T    AAGAATAGAATTCGAAATATCTTATGAAGCATTCGACGTAAAAAGTCCTA
ACH-T    AAGAATAGAATTCGAAATATCTTATGAAGCATTCGACGTAAAAAGTCCTA
ANR-T    AAGAATAGAATTCGAAATATCTTATGAAGCATTCGACGTAAAAAGTCCTA
BMN-T    AAGAATAGAATTCGAAATATCTTATGAAGCATTCGACGTAAAAAGTCCTA
BIW-T    AAGAATAGAATTCGAAATATCTTATGAAGCATTCGACGTAAAAAGTCCTA
ACH.BC   AAGAATAGAATTCGAAATATCTTATGAAGCATTCGACGTAAAAAGTCCTA
BMN.BC   AAGAATAGAATTCGAAATATCTTATGAAGCATTCGACGTAAAAAGTCCTA
BIW.BC   AAGAATAGAATTCGAAATATCTTATGAAGCATTCGACGTAAAAAGTCCTA
ECR1     --------------------------------------------------

E. canis ATATCAATTATCAAAATGACGCGCACAGGTACTGCGCTCTATCTCATCAC
ECF1     --------------------------------------------------
AFA-T    ATATCAATTATCAAAATGACGCGCACAGGTACTGCGCTCTATCTCATCAC
AFW-T    ATATCAATTATCAAAATGACGCGCACAGGTACTGCGCTCTATCTCATCAC
ALK-T    ATATCAATTATCAAAATGACGCGCACAGGTACTGCGCTCTATCTCATCAC
AMP-T    ATATCAATTATCAAAATGACGCGCACAGGTACTGCGCTCTATCTCATCAC
AAN-T    ATATCAATTATCAAAATGACGCGCACAGGTACTGCGCTCTATCTCATCAC
BIX-T    ATATCAATTATCAAAATGACGCGCACAGGTACTGCGCTCTATCTCATCAC
ACH-T    ATATCAATTATCAAAATGACGCGCACGGTACTGCGCTCTATCTCATCAC
ANR-T    ATATCAATTATCAAAATGACGCGCACAGGTACTGCGCTCTATCTCATCAC
BMN-T    ATATCAATTATCAAAATGACGCGCACAGGTACTGCGCTCTATCTCATCAC
BIW-T    ATATCAATTATCAAAATGACGCGCACAGGTACTGCGCTCTATCTCATCAC
ACH.BC   ATATCAATTATCAAAATGACGCGCACAGGTACTGCGCTCTATCTCATCAC
BMN.BC   ATATCAATTATCAAAATGACGCGCACAGGTACTGCGCTCTATCTCATCAC
BIW.BC   ATATCAATTATCAAAATGACGCGCACAGGTACTGCGCTCTATCTCATCAC
ECR1     ---------------------------GGTACTGCGCTCTATCTCATCAC
```

FIG. 6. Sequences of selected PCR products amplified from canine and acarine hosts after treatment with doxycycline. Multiple sequence alignment of amplicons from PCR-positive tick and buffy coat samples collected after doxycycline treatment of dogs. Sequences of 200-bp bands excised from agarose gels were aligned with the *p30* target sequence (*E. canis*) and primers ECA30-384S (ECF1) and the reverse complement of ECA30-583A (ECR1). For the remaining sequences, identification codes of the treated dogs are followed by letters indicative of amplicons from posttreatment buffy coat (BC) samples that were confirmed with PCR-positive frozen replicates or from ticks (T) allowed to feed on dogs after doxycycline treatment. The sequences were aligned and compared by using the Codoncode and Boxshade programs. Black letters surrounded by white represent bases that are identical in each sequence of the alignment, and white letters in a black box indicate residues that did not match remaining bases in the alignment.

the average peak leukocyte value prior to initiation of doxycycline treatment during the chronic phase.

Peripheral blood samples from dogs treated during the acute or subclinical phase of CME became PCR negative and remained so following treatment. However, intermittent PCR-positive samples indicated that doxycycline treatments did not clear *E. canis* from the peripheral blood of dogs treated during chronic CME even though hematologic parameters improved after treatments were initiated. Frozen backup samples were assayed to confirm PCR-positive samples detected after doxycycline treatments, and samples were interpreted as negative when corresponding frozen replicates tested PCR negative. However, it is conceivable that the templates were degraded in the frozen replicates, resulting in reduced sensitivity of the PCR assay. Some frozen replicates tested positive, as was the case for samples from BMN and BIW. Furthermore, *E. canis* was sporadically detected in blood collected from dogs AVS and BJW, which were subinoculated with blood pooled from dogs treated during acute and chronic phases of CME. Surprisingly, *R. sanguineus* ticks tested PCR positive for *E. canis* after feeding on dogs subjected to all three doxycycline treatment scenarios, but only a single tick tested PCR positive after feeding on BMN and BIW post-rifampin treatment.

The results of this investigation corroborate in some measure several studies which collectively suggest that the phase of CME can affect the efficacy of doxycycline for clearance of *E. canis* infections. For example, earlier reports indicated that CME was resolved and that *E. canis* was not detected in peripheral canine blood when doxycycline therapy was initiated during acute and subclinical phases of the disease (12, 22, 42). Breitschwerdt et al. (4) reported successful clearance of infection from eight of eight dogs with acute experimental CME after 14 days of treatment, while Harrus et al. (22) reported successful clearance of infection from five of five dogs after 16 of 60 days of treatment. Conversely, other studies indicated that *E. canis* infections could persist among dogs treated during the postacute phases of CME (23, 29, 43, 58). Iqbal and Rikihisa (29) reported reisolation of *E. canis* from three of five experimentally infected dogs with subclinical CME that were treated for 7 days. Harrus et al. (23) reported persistent *E. canis* infection in one of six dogs after a 42-day doxycycline regimen.

However, the hypothesis that *E. canis* infections become more resistant to clearance by doxycycline as CME progresses was not fully supported by the results of this study, because infections appeared more resistant when treatments were initiated during the acute phase (16 dpi) than when treatments were initiated during the subclinical phase (62 dpi). Notably, the persistence of acute-phase infections during treatment was reminiscent of an earlier study where a 14-day doxycycline regimen failed to clear *E. canis* from dogs (43), indicating that the 28-day regimen is warranted (39).

There is evidence that *E. canis* can persist after doxycycline treatment of naturally infected hosts. Breitschwerdt et al. (5) reported detection of *E. canis* by PCR assay in one of four dogs tested 6 months after a 28-day doxycycline regimen prescribed after diagnosis with ehrlichiosis attributable to *E. canis*. However, persistence was not distinguished from reinfection of naturally exposed dogs in this study. Interestingly, the single host with a detectable infection had the highest platelet count at diagnosis (2.22×10^5 platelets/μl), suggesting a postacute phase of the disease, while two of the remaining three dogs, which cleared the infections, were thrombocytopenic at initiation of treatment. Wen et al. (58) screened 105 canine blood samples from Texas and Arizona, 84% of which had been treated with doxycycline for 1 to 24 months, suggesting that the majority of these dogs were likely to be subclinical, and *E. canis* was detected in 44% of these samples.

Several reports indicate that other *Anaplasmataceae* can persist in hosts treated with tetracyclines. For example, *Ehrlichia chaffeensis* was detected several times after doxycycline treatment of 3 of 12 dogs diagnosed with ehrlichiosis (5). Notably, this work was conducted with naturally exposed dogs in the southeastern United States, where persistent exposure to *Amblyomma americanum*, the primary vector of *E. chaffeensis*, suggested reinfection as an alternative explanation for those results. In another study, *Ehrlichia ruminantium*, which is also closely related to *E. canis* and *E. chaffeensis*, was recovered from the blood of ruminants after treatment with oxytetracycline (27), and different strains of the same pathogen were detected by xenodiagnosis in four of five experimentally infected sheep after tetracycline therapy (2). Notably, one of the sheep described in the latter study was tested 3.5 years after initial infection and therapy, and this sheep was subclinical at the time of that study. Oxytetracycline (20 mg/kg_{bw}, intramuscularly [i.m.]), injected three times at 3-day intervals, was reported to clear natural infections of *Anaplasma marginale*, an intraerythrocytic rickettsial pathogen of cattle that is closely related to *E. canis*, as evidenced by failure of blood from treated cattle to induce clinical anaplasmosis in splenectomized calves (52). However, more recent studies indicate that *A. marginale* infections can persist after various oxytetracycline regimens, which was detected by both PCR assay and subinoculation of blood from treated hosts into susceptible calves (11).

The results of the current study also indicate that the ability of ticks to acquire *E. canis* is not always associated with detectable infections within peripheral blood and that xenodiagnosis with ticks is the most sensitive method for detection of such infections. Similar results were reported when *Dermacentor variabilis* and *A. americanum* ticks acquired *E. chaffeensis* from calves with PCR-negative blood (15). Similarly, *E. ruminantium* was detected in adult *Amblyomma variegatum* ticks acquisition fed as nymphs on experimentally infected, but PCR-negative, sheep (2).

A satisfactory explanation for the mechanism responsible for detection of *E. canis* in ticks fed on dogs with PCR-negative blood is lacking at this time. It is conceivable that an undetectably low level of the infection persists in the host blood and that these infections are biologically amplified through replication within infected ticks. This principle underlies the long-standing process of xenodiagnosis for detection of vector-borne infections (36). Similar results were reported for unrelated tick-borne parasites that replicate within the vector. For example, *Hepatozoon americanum* was detected in ticks allowed to feed on dogs with infections that are undetectable by muscle biopsy, the most reliable method currently available *ante mortem* (19). Similarly, *Borrelia burgdorferi*, an extracellular spirochete, was reported in *Ixodes scapularis* after acquisition from mice experimentally treated with doxycycline (3). In

this case, cryptic *B. burgdorferi* infections were later described in collagen layers of skin of doxycycline-treated mice (25). In addition to low-level infections in the blood, it is also conceivable that there is an undefined stage of *E. canis* infections that is not detectable in peripheral blood. For example, some reports suggest that *Anaplasmataceae* or their primary vertebrate host cells might be closely associated with endothelium. *A. marginale* and *Anaplasma phagocytophilum* are reportedly infective for endothelial cells *in vitro* (37, 55, 56) as well as *in vivo* (9). Both *E. canis* and *E. chaffeensis* were reported to infect a human endothelial cell line derived from dermal microvasculature *in vitro*, but these infections apparently did not persist under those culture conditions, and thus further work is needed to confirm that these monocytotropic *Ehrlichia* spp. are indeed infectious to endothelium *in vitro* and *in vivo* (13). Alternatively, monocytes infected with *E. canis* were observed in close association with pulmonary endothelial cells (44), suggesting that they are not limited to peripheral blood.

Additional work is needed to determine if tick infections acquired from doxycycline-treated dogs are transmissible to naïve hosts. Infection alone does not demonstrate pathogen transmissibility by ticks, and there is evidence that tick-transmissible *Anaplasmataceae* are adapted to infect and pass through at least two tick organ systems, the midgut and salivary glands (31, 32, 48, 51, 53, 54). Furthermore, infectivity for vertebrate hosts may not be shared by all stages of *Anaplasmataceae* that are found in their invertebrate hosts. Such was the case for *A. marginale* infections described in ticks, where infectivity of tick stages of these organisms for cattle was associated with pathogen morphology (33). Other investigators have also reported *I. scapularis* acquisition of *B. burgdorferi* from doxycycline-treated mice, and these spirochetes could not be cultured and were not infectious to naïve mice (3). It remains to be determined if doxycycline could similarly attenuate members of the *Rickettsiales*, and this hypothesis should be tested before bolder conclusions are drawn with regard to acquisition and dissemination of these pathogens by ticks that feed on hosts after antibiotic treatment.

In the present study, severe acute-phase CME was consistently induced among all of the dogs tested; thus, differences observed among these groups were not attributable to severity of acute CME. In addition, tick acquisition feeding was utilized for more sensitive pathogen detection and to evaluate the efficacies of these treatment scenarios for control of vector infection. However, although the majority of similar investigations utilized needle inoculation of anaplasmal and ehrlichial agents for consistent induction of the associated experimental diseases, tick transmission is the most likely infection scenario in nature (21, 34, 46, 49). Previous reports of *E. canis* persistence after doxycycline treatment also involved dogs that were experimentally infected via tick transmission (43) or that were naturally infected, presumably through tick transmission (1, 5, 58). Thus, further work is warranted to compare persistence of *E. canis* among dogs experimentally infected via needle injection versus tick transmission feeding.

In conclusion, dogs were intravenously inoculated with blood from *E. canis* carriers and consistently developed overt clinical and hematologic signs of severe acute-phase CME. The doxycycline regimen used in this study effectively ameliorated CME among dogs treated at 16 or 62 dpi, and blood from dogs treated during the corresponding CME phases became PCR negative after doxycycline treatment. However, blood collected from dogs with long-term infections intermittently tested PCR positive during and after doxycycline treatment. Ticks were able to acquire *E. canis* from all of the dogs subjected to doxycycline treatment. Although these xenodiagnostic results suggest that doxycycline alone may not block tick acquisition of *E. canis* from dogs, regardless of when the treatment is initiated, reductions in tick acquisition rates after rifampin treatment suggested that a treatment regimen can be developed to better control tick acquisition of *E. canis* and possibly other rickettsial agents.

ACKNOWLEDGMENTS

This work was supported by NIH R01 AI47932 and the University of Missouri Department of Veterinary Pathobiology.

We thank A. J. Littles, A. A. Louton, M. J. Roth, S. K. Stege, A. R. Woolery, M. C. McClure, and R. T. Stoffel for technical assistance.

REFERENCES

1. **Bartsch, R. C., and R. T. Greene.** 1996. Post-therapy antibody titers in dogs with ehrlichiosis: follow-up study on 68 patients treated primarily with tetracycline and/or doxycycline. J. Vet. Intern. Med. **10:**271–274.
2. **Bekker, C. P., S. de Vos, A. Taoufik, O. A. Sparagano, and F. Jongejan.** 2002. Simultaneous detection of *Anaplasma* and *Ehrlichia* species in ruminants and detection of *Ehrlichia ruminantium* in *Amblyomma variegatum* ticks by reverse line blot hybridization. Vet. Microbiol. **89:**223–238.
3. **Bockenstedt, L. K., J. Mao, E. Hodzic, S. W. Barthold, and D. Fish.** 2002. Detection of attenuated, noninfectious spirochetes in *Borrelia burgdorferi*-infected mice after antibiotic treatment. J. Infect. Dis. **186:**1430–1437.
4. **Breitschwerdt, E. B., B. C. Hegarty, and S. I. Hancock.** 1998. Doxycycline hyclate treatment of experimental canine ehrlichiosis followed by challenge inoculation with two *Ehrlichia canis* strains. Antimicrob. Agents Chemother. **42:**362–368.
5. **Breitschwerdt, E. B., B. C. Hegarty, and S. I. Hancock.** 1998. Sequential evaluation of dogs naturally infected with *Ehrlichia canis*, *Ehrlichia chaffeensis*, *Ehrlichia equi*, *Ehrlichia ewingii*, or *Bartonella vinsonii*. J. Clin. Microbiol. **36:**2645–2651.
6. **Bremer, W. G., J. J. Schaefer, E. R. Wagner, S. A. Ewing, Y. Rikihisa, G. R. Needham, S. Jittapalapong, D. L. Moore, and R. W. Stich.** 2005. Transstadial and intrastadial experimental transmission of *Ehrlichia canis* by male *Rhipicephalus sanguineus*. Vet. Parasitol. **131:**95–105.
7. **Buhles, W. C., Jr., D. L. Huxsoll, and P. K. Hildebrandt.** 1975. Tropical canine pancytopenia: role of aplastic anaemia in the pathogenesis of severe disease. J. Comp. Pathol. **85:**511–521.
8. **Buhles, W. C., Jr., D. L. Huxsoll, and M. Ristic.** 1974. Tropical canine pancytopenia: Clinical, hematologic, and serologic response of dogs to *Ehrlichia canis* infection, tetracycline therapy, and challenge inoculation. J. Infect. Dis. **130:**357–367.
9. **Carreno, A. D., A. R. Alleman, A. F. Barbet, G. H. Palmer, S. M. Noh, and C. M. Johnson.** 2007. In vivo endothelial cell infection by *Anaplasma marginale*. Vet. Pathol. **44:**116–118.
10. **Codner, E. C., and L. L. Farris-Smith.** 1986. Characterization of the subclinical phase of ehrlichiosis in dogs. J. Am. Vet. Med. Assoc. **189:**47–50.
11. **Coetzee, J. F., M. D. Apley, K. M. Kocan, F. R. Rurangirwa, and J. Van Donkersgoed.** 2005. Comparison of three oxytetracycline regimes for the treatment of persistent *Anaplasma marginale* infections in beef cattle. Vet. Parasitol. **127:**61–73.
12. **Davidson, D. E., Jr., G. S. Dill, Jr., M. Tingpalapong, S. Premabutra, P. L. Nguen, E. H. Stephenson, and M. Ristic.** 1978. Prophylactic and therapeutic use of tetracycline during an epizootic of ehrlichiosis among military dogs. J. Am. Vet. Med. Assoc. **172:**697–700.
13. **Dawson, J. E., F. J. Candal, V. G. George, and E. W. Ades.** 1993. Human endothelial cells as an alternative to DH82 cells for isolation of *Ehrlichia chaffeensis*, *E. canis*, and *Rickettsia rickettsii*. Pathobiology **61:**293–296.
14. **de Castro, M. B., R. Z. Machado, L. P. de Aquino, A. C. Alessi, and M. T. Costa.** 2004. Experimental acute canine monocytic ehrlichiosis: clinicopathological and immunopathological findings. Vet. Parasitol. **119:**73–86.
15. **delos Santos, J. R., K. Boughan, W. G. Bremer, B. Rizzo, J. J. Schaefer, Y. Rikihisa, G. R. Needham, L. A. Capitini, D. E. Anderson, M. Oglesbee, S. A. Ewing, and R. W. Stich.** 2007. Experimental infection of dairy calves with *Ehrlichia chaffeensis*. J. Med. Microbiol. **56:**1660–1668.
16. **Eddlestone, S. M., P. P. Diniz, T. M. Neer, S. D. Gaunt, R. Corstvet, D. Cho, G. Hosgood, B. Hegarty, and E. B. Breitschwerdt.** 2007. Doxycycline clearance of experimentally induced chronic *Ehrlichia canis* infection in dogs. J. Vet. Intern. Med. **21:**1237–1242.

17. **Eddlestone, S. M., S. D. Gaunt, T. M. Neer, C. M. Boudreaux, A. Gill, E. Haschke, and R. E. Corstvet.** 2007. PCR detection of *Anaplasma platys* in blood and tissue of dogs during acute phase of experimental infection. Exp. Parasitol. **115:**205–210.
18. **Ewing, S. A.** 1969. Canine ehrlichiosis. Adv. Vet. Sci. Comp. Med. **13:**331–353.
19. **Ewing, S. A., R. J. Panciera, and J. S. Mathew.** 2003. Persistence of *Hepatozoon americanum* (Apicomplexa: Adeleorina) in a naturally infected dog. J. Parasitol. **89:**611–613.
20. **Gaunt, S. D., R. E. Corstvet, C. M. Berry, and B. Brennan.** 1996. Isolation of *Ehrlichia canis* from dogs following subcutaneous inoculation. J. Clin. Microbiol. **34:**1429–1432.
21. **Groves, M. G., G. L. Dennis, H. L. Amyx, and D. L. Huxsoll.** 1975. Transmission of *Ehrlichia canis* to dogs by ticks (*Rhipicephalus sanguineus*). Am. J. Vet. Res. **36:**937–940.
22. **Harrus, S., M. Kenny, L. Miara, I. Aizenberg, T. Waner, and S. Shaw.** 2004. Comparison of simultaneous splenic sample PCR with blood sample PCR for diagnosis and treatment of experimental *Ehrlichia canis* infection. Antimicrob. Agents Chemother. **48:**4488–4490.
23. **Harrus, S., T. Waner, I. Aizenberg, and H. Bark.** 1998. Therapeutic effect of doxycycline in experimental subclinical canine monocytic ehrlichiosis: evaluation of a 6-week course. J. Clin. Microbiol. **36:**2140–2142.
24. **Harrus, S., T. Waner, I. Aizenberg, J. E. Foley, A. M. Poland, and H. Bark.** 1998. Amplification of ehrlichial DNA from dogs 34 months after infection with *Ehrlichia canis*. J. Clin. Microbiol. **36:**73–76.
25. **Hodzic, E., S. Feng, K. Holden, K. J. Freet, and S. W. Barthold.** 2008. Persistence of *Borrelia burgdorferi* following antibiotic treatment in mice. Antimicrob. Agents Chemother. **52:**1728–1736.
26. **Huxsoll, D. L., H. L. Amyx, I. E. Hemelt, P. K. Hildebrandt, R. M. Nims, and W. S. Gochenour, Jr.** 1972. Laboratory studies of tropical canine pancytopenia. Exp. Parasitol. **31:**53–59.
27. **Ilemobade, A. A.** 1978. The persistence of *Cowdria ruminantium* in the blood of recovered animals. Trop. Anim. Health Prod. **10:**170.
28. **Iqbal, Z., W. Chaichanasiriwithaya, and Y. Rikihisa.** 1994. Comparison of PCR with other tests for early diagnosis of canine ehrlichiosis. J. Clin. Microbiol. **32:**1658–1662.
29. **Iqbal, Z., and Y. Rikihisa.** 1994. Reisolation of *Ehrlichia canis* from blood and tissues of dogs after doxycycline treatment. J. Clin. Microbiol. **32:**1644–1649.
30. **Jouret-Gourjault, S., D. Parzy, and B. Davoust.** 2006. Experimental infections in dogs with *Ehrlichia canis* strain Borgo 89. Ann. N. Y. Acad. Sci. **1078:**470–475.
31. **Kocan, K. M.** 1986. Development of *Anaplasma marginale* in ixodid ticks: coordinated development of a rickettsial organism and its tick host, p. 472–505. *In* J. R. Sauer and J. A. Hair (ed.), Morphology, physiology and behavioral ecology of ticks. Horwood, Chichester, England.
32. **Kocan, K. M., R. W. Stich, P. L. Claypool, S. A. Ewing, J. A. Hair, and S. J. Barron.** 1990. Intermediate site of development of *Anaplasma marginale* in feeding adult *Dermacentor andersoni* ticks that were infected as nymphs. Am. J. Vet. Res. **51:**128–132.
33. **Kocan, K. M., T. N. Yellin, P. L. Claypool, S. J. Barron, S. A. Ewing, and J. A. Hair.** 1990. Development and infectivity of *Anaplasma marginale* in *Dermacentor andersoni* nymphs. Am. J. Vet. Res. **51:**1292–1294.
34. **Lewis, G. E., Jr., M. Ristic, R. D. Smith, T. Lincoln, and E. H. Stephenson.** 1977. The brown dog tick *Rhipicephalus sanguineus* and the dog as experimental hosts of *Ehrlichia canis*. Am. J. Vet. Res. **38:**1953–1955.
35. **McClure, J. C., M. L. Crothers, J. J. Schaefer, P. D. Stanley, and R. W. Stich.** 2009. Rapid screening and cultivation of *Ehrlichia canis* from refrigerated carrier blood. Clin. Microbiol. Infect. **15**(Suppl. 2)**:**72–73.
36. **Mourya, D. T., M. D. Gokhale, and R. Kumar.** 2007. Xenodiagnosis: use of mosquitoes for the diagnosis of arboviral infections. J. Vector Borne Dis. **44:**233–240.
37. **Munderloh, U. G., M. J. Lynch, M. J. Herron, A. T. Palmer, T. J. Kurtti, R. D. Nelson, and J. L. Goodman.** 2004. Infection of endothelial cells with *Anaplasma marginale* and *A. phagocytophilum*. Vet. Microbiol. **101:**53–64.
38. **Mylonakis, M. E., A. F. Koutinas, E. B. Breitschwerdt, B. C. Hegarty, C. D. Billinis, L. S. Leontides, and V. S. Kontos.** 2004. Chronic canine ehrlichiosis (*Ehrlichia canis*): a retrospective study of 19 natural cases. J. Am. Anim. Hosp. Assoc. **40:**174–184.
39. **Neer, T. M., E. B. Breitschwerdt, R. T. Greene, and M. R. Lappin.** 2002. Consensus statement on ehrlichial disease of small animals from the infectious disease study group of the ACVIM. American College of Veterinary Internal Medicine. J. Vet. Intern. Med. **16:**309–315.
40. **Or, M., M. Samish, T. Waner, and S. Harrus.** 2009. Attenuation of *Ehrlichia canis* by multiple passages in two different cultures. Clin. Microbiol. Infect. **15**(Suppl. 2)**:**74–75.
41. **Parola, P., and D. Raoult.** 2001. Ticks and tickborne bacterial diseases in humans: an emerging infectious threat. Clin. Infect. Dis. **32:**897–928.
42. **Schaefer, J. J., J. Kahn, G. R. Needham, Y. Rikihisa, S. A. Ewing, and R. W. Stich.** 2008. Antibiotic clearance of *Ehrlichia canis* from dogs infected by intravenous inoculation of carrier blood. Ann. N. Y. Acad. Sci. **1149:**263–269.
43. **Schaefer, J. J., G. R. Needham, W. G. Bremer, Y. Rikihisa, S. A. Ewing, and R. W. Stich.** 2007. Tick acquisition of *Ehrlichia canis* from dogs treated with doxycycline hyclate. Antimicrob. Agents Chemother. **51:**3394–3396.
44. **Simpson, C. F.** 1974. Relationship of *Ehrlichia canis*-infected mononuclear cells to blood vessels of lungs. Infect. Immun. **10:**590–596.
45. **Skotarczak, B.** 2003. Canine ehrlichiosis. Ann. Agric. Environ. Med. **10:**137–141.
46. **Smith, R. D., D. M. Sells, E. H. Stephenson, M. R. Ristic, and D. L. Huxsoll.** 1976. Development of *Ehrlichia canis*, causative agent of canine ehrlichiosis, in the tick *Rhipicephalus sanguineus* and its differentiation from a symbiotic Rickettsia. Am. J. Vet. Res. **37:**119–126.
47. **Stich, R. W., Y. Rikihisa, S. A. Ewing, G. R. Needham, D. L. Grover, and S. Jittapalapong.** 2002. Detection of *Ehrlichia canis* in canine carrier blood and in individual experimentally infected ticks with a *p30*-based PCR assay. J. Clin. Microbiol. **40:**540–546.
48. **Stich, R. W., J. R. Sauer, J. A. Bantle, and K. M. Kocan.** 1993. Detection of *Anaplasma marginale* (Rickettsiales: Anaplasmataceae) in secretagogue-induced oral secretions of *Dermacentor andersoni* (Acari: Ixodidae) with the polymerase chain reaction. J. Med. Entomol. **30:**789–794.
49. **Stich, R. W., J. J. Schaefer, W. G. Bremer, G. R. Needham, and S. Jittapalapong.** 2008. Host surveys, ixodid tick biology and transmission scenarios as related to the tick-borne pathogen, *Ehrlichia canis*. Vet. Parasitol. **158:**256–273.
50. **Stiller, D., and M. E. Coan.** 1995. Recent developments in elucidating tick vector relationships for anaplasmosis and equine piroplasmosis. Vet. Parasitol. **57:**97–108.
51. **Stiller, D., K. M. Kocan, W. Edwards, S. A. Ewing, and J. A. Barron.** 1989. Detection of colonies of *Anaplasma marginale* in salivary glands of three *Dermacentor* spp. infected as nymphs or adults. Am. J. Vet. Res. **50:**1381–1385.
52. **Swift, B. L., and G. M. Thomas.** 1983. Bovine anaplasmosis: elimination of the carrier state with injectable long-acting oxytetracycline. J. Am. Vet. Med. Assoc. **183:**63–65.
53. **Ueti, M. W., D. P. Knowles, C. M. Davitt, G. A. Scoles, T. V. Baszler, and G. H. Palmer.** 2009. Quantitative differences in salivary pathogen load during tick transmission underlie strain-specific variation in transmission efficiency of *Anaplasma marginale*. Infect. Immun. **77:**70–75.
54. **Ueti, M. W., J. O. Reagan, Jr., D. P. Knowles, Jr., G. A. Scoles, V. Shkap, and G. H. Palmer.** 2007. Identification of midgut and salivary glands as specific and distinct barriers to efficient tick-borne transmission of *Anaplasma marginale*. Infect. Immun. **75:**2959–2964.
55. **Waghela, S. D., D. Cruz, R. E. Droleskey, J. R. DeLoach, and G. G. Wagner.** 1997. In vitro cultivation of *Anaplasma marginale* in bovine erythrocytes co-cultured with endothelial cells. Vet. Parasitol. **73:**43–52.
56. **Wamsley, H. L., and A. F. Barbet.** 2008. In situ detection of *Anaplasma* spp. by DNA target-primed rolling-circle amplification of a padlock probe and intracellular colocalization with immunofluorescently labeled host cell von Willebrand factor. J. Clin. Microbiol. **46:**2314–2319.
57. **Waner, T., S. Harrus, H. Bark, E. Bogin, Y. Avidar, and A. Keysary.** 1997. Characterization of the subclinical phase of canine ehrlichiosis in experimentally infected beagle dogs. Vet. Parasitol. **69:**307–317.
58. **Wen, B., Y. Rikihisa, J. M. Mott, R. Greene, H.-Y. Kim, N. Zhi, G. C. Couto, A. Unver, and R. Bartsch.** 1997. Comparison of nested PCR with immunofluorescent-antibody assay for detection of *Ehrlichia canis* infection in dogs treated with doxycycline. J. Clin. Microbiol. **35:**1852–1855.
59. **Woody, B. J., and J. D. Hoskins.** 1991. Ehrlichial diseases of dogs. Vet. Clin. North Am. Small Anim. Pract. **21:**75–98.