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Experimental infection of *Rhipicephalus sanguineus* with *Ehrlichia chaffeensis*

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Abstract

Ehrlichia chaffeensis, the etiologic agent of human monocytic ehrlichiosis, is a tick-borne rickettsial pathogen that is infectious to a wide range of mammals, including dogs and people. *Amblyomma americanum*, the lone star tick, is considered the primary vector of *E. chaffeensis*, but this pathogen has been detected in other tick species, including the brown dog tick, *Rhipicephalus sanguineus*. We hypothesized that the Arkansas strain of *E. chaffeensis* is infective to *R. sanguineus*, and used a novel PCR assay to test for acquisition of this pathogen by *R. sanguineus* and *A. americanum* ticks that were simultaneously fed on experimentally infected dogs. Although *E. chaffeensis* was not frequently detected in peripheral blood of these dogs, the pathogen was detected in both tick species and in canine lung, kidney, lymph node, bone marrow and frontal lobe samples. One dog (AFL) was maintained for several years, and ticks again acquired *E. chaffeensis* from this dog 566 days after intradermal inoculation with *E. chaffeensis*, but the pathogen was not detected in ticks fed on the same dog at 764 or 1,086 days after the intradermal inoculation.

Keywords

Ehrlichia chaffeensis; *Rhipicephalus sanguineus*

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INTRODUCTION

Ehrlichia chaffeensis is a tick-borne rickettsial pathogen that is the etiologic agent of human monocytic ehrlichiosis, an emerging infectious disease predominantly found in the lower midwestern and southeastern United States (Paddock and Childs, 2003). Like most vector-borne zoonotic pathogens, *E. chaffeensis* infects a wide range of vertebrate hosts. Although there is strong evidence that *Amblyomma americanum* is the primary vector of *E. chaffeensis*, this pathogen was reported in naturally infected *Dermacentor variabilis* and *Ixodes pacificus* in the U.S.A. (Kramer *et al.*, 1999; Roland *et al.*, 1998). *E. chaffeensis* was also reported to infect *D. variabilis*, as well as *A. americanum*, under experimental conditions (delos Santos *et al.*, 2007; Ewing *et al.*, 1995). *E. chaffeensis* was reportedly detected in *A. testudinarium*, *Haemaphysalis longicornis*, *H. yeni*, *I. persulcatus* and *R. sanguineus* ticks in regions of the world where *A. americanum* are not found (Cao *et al.*, 2000; Kim *et al.*, 2003; Lee *et al.*, 2005; Ndip *et al.*, 2010). Among these, *R. sanguineus* is arguably the most widely distributed tick, because it is found circumglobally between latitudes 50° N and 30° S (Dantas-Torres, 2008).

Dogs, the primary hosts of *R. sanguineus*, are also naturally infected with *E. chaffeensis* (Breitschwerdt *et al.*, 1998; Murphy *et al.*, 1998). Because both *R. sanguineus* and dogs were reported to be naturally infected with *E. chaffeensis*, we simultaneously acquisition fed *R. sanguineus* and *A. americanum* on experimentally infected dogs to test the hypothesis that *E. chaffeensis* (Arkansas strain) is infective to both tick species. A novel PCR assay was used to detect experimental *E. chaffeensis* infections in canine tissues and in both tick species that were fed on these dogs as nymphs or adults.

MATERIALS AND METHODS

Canine hosts

Four Beagle dogs were inoculated with cell lines infected with *E. chaffeensis* (Arkansas strain) on two separate occasions, and these dogs were cared for in accordance with a protocol on file with the University of Missouri Columbia Animal Care and Use Committee. For inoculation 1, *E. chaffeensis* was cultivated in either the canine macrophage cell line DH82 at 37 °C or in the tick cell line ISE6 at 34 °C (Ganta *et al.*, 2007). Inoculum (1 ml) was injected intraperitoneally, intravenously and subcutaneously. Dogs AFL and AUW received infected ISE6 cell culture, and dogs AFY and AIA received infected DH82 cell culture. However, shortly after inoculation, dog AIA displayed signs consistent with an anaphylaxis, was treated with epinephrine and atropine, and recovered without further incident. Thus, dog AFY received 1.5 ml of inoculum subcutaneously and intraperitoneally, but did not receive intravenous inoculum in order to avoid the possible adverse reaction described for dog AIA.

For inoculation 2, *E. chaffeensis* was cultivated in DH82 cells grown in Earl's minimum essential media supplemented with 5% tetracycline-free fetal bovine serum, 25 mM hepes, 2 mM L-glutathione, 0.1 mM nonessential amino acids, and 1 mM sodium pyruvate (HyClone Laboratories, South Logan UT), and each of the four dogs were again inoculated

(intradermally) with 5×10^6 DH82 cells/ml (60% infected) at 267 days after the first inoculation.

Daily rectal temperatures, clinical assessments and semiweekly blood samples were collected from 13 days prior to each inoculation to 64 days post-inoculation 1 (DPI₁) and 34 days post-inoculation 2 (DPI₂), as previously described elsewhere (McClure *et al.*, 2010). Heparinized blood (1.0 ml) was centrifuged at $1200 \times g$ for 30 min at 4°C, and buffy coats were collected for PCR template. Dogs AFY, AIA and AUW were euthanized via barbiturate overdose at 34 DPI₂, and liver, lung, brain frontal lobe, bone marrow, mesenteric lymph node, kidney and jejunum samples were aseptically collected and immediately placed in liquid nitrogen. Template was isolated from buffy coat samples using the High Pure Viral Nucleic Acid Kit (Roche, Indianapolis IN) as previously described (McClure *et al.*, 2010). Template was also isolated from 10 mg of spleen and 25 mg of other tissues with the DNeasy Blood & Tissue Kit (QIAGEN, Valencia CA) and eluted in 100 µl volumes.

For each PCR assay, every sample was assayed twice, followed by a third assay of samples with discrepant results between the initial trials. Buffy coat samples from the first inoculation were assayed with an *E. chaffeensis*-specific *p28*-based PCR assay as reported elsewhere (Wagner *et al.*, 2004). These samples were reassessed after the development of a novel real-time PCR assay based on an *E. chaffeensis* hypothetical outer membrane protein gene (*homp*). This new assay was first developed with SYBR green chemistry, using 25 µl reactions containing [1×] ABsolute QPCR SYBR® Green Mix (ABgene, Epsom Surrey, UK), and 1.8 µM each of forward primer ECH144762S (5'-ACCCTGCAACACCTACAAC-3') and reverse primer ECH144994A (5'-CCACCTACAGCACCTAAAGCA-3'), with an ABI 7300 real-time thermocycler (Applied Biosystems, Foster City, CA) at 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 sec, 65 °C for 30 sec, and 72 °C for 1 min, and followed by a dissociation step of 95 °C for 15 sec, 60 °C for 30 sec, and 95 consisting of °C for 15 sec. PCR products were electrophoresed on 1.5% agarose gel in 1× TBE (89 mM Tris, 89 mM boric acid, 2 mM EDTA) with ethidium bromide (0.5 µg/ml). Representative amplicons were extracted (Wizard Gel and PCR Clean-Up System, Promega, Madison, WI) and sequenced at the University of Missouri DNA Core Facility.

A fluorescent probe, ECH144816S (5'-FAM-TCTGTAACACCTATAGCATCTGCAACGC-BHQ-3') was designed with RealTimeDesign software (Biosearch Technologies, Novato, CA) for adaptation to a fluorogenic real time PCR assay with the *homp*-based primers. 25 µl volumes contained [1×] PCR buffer, 2 mM MgCl₂, 0.06 U/µl Platinum *Taq* DNA polymerase, 200 µM dNTP mix (Invitrogen, Carlsbad, CA), 1% DMSO, 1.8 µM each of primers 144762S and 144994A and 0.25 µM of probe 144816S, which were used to assay 2.5 µl of template. Fluorogenic assays were run on an ABI 7300 thermocycler at 95 °C for 2 min followed by 60 cycles of 95 °C for 15 sec and 61 °C for 1 min.

Infectivity to *R. sanguineus*

Ticks were purchased from the Oklahoma State University Medical Entomology Laboratory, and were placed in cloth stockinettes attached to dogs AFL, AIA and AFY as previously

described (McClure *et al.*, 2010). Dog AUW was excluded from tick feeding due to behavioral issues. Ticks were placed on these dogs at 24 DPI₂ and allowed to feed to repletion (nymphs) or for 10 days (males). Ticks were also fed on dog AFL at 566 (*R. sanguineus* males), 764 (*R. sanguineus* and *A. americanum* nymphs and males) and 1086 (*R. sanguineus* males and *A. americanum* nymphs) DPI₂. Acquisition-fed ticks were stored in a humidity chamber for 15 days at room temperature, 95–100% relative humidity (rh) and a 12:12 hr light:dark photoperiod, then incubated at 37 °C (100% rh) for 85 h, bisected along the sagittal plane, and stored at –80 °C in pools of 5 different tick halves. For template isolation, frozen tick halves were stored at –20 °C for 16 hours in RNeasy-ICE (Ambion, Austin, TX), centrifuged (12,000 × *g* for 15 min at 4°C), and used for template isolation and PCR assay as described for canine buffy coat samples.

RESULTS

Canine hosts

Several investigators have reported the susceptibility of dogs to natural and experimental infection with *E. chaffeensis*, although usually in the absence of clinical disease (Dawson *et al.*, 1996; Dawson and Ewing, 1992; Kordick *et al.*, 1999; Murphy *et al.*, 1998; Zhang *et al.*, 2003). In the initial phase of the present study, dogs were inoculated with *E. chaffeensis*, within in tick or canine cell lines, through intraperitoneal, intravenous and subcutaneous routes, to determine if these treatments could exacerbate the infection or the disease. The dogs were observed daily for clinical signs and rectal temperatures, and complete blood counts were assessed semiweekly, through 64 DPI₁. Mild fever (>39.2 °C) and mild hematologic abnormalities were the only signs observed during this period. Dog AFL had a rectal temperature of 39.3 °C on 25, 36 and 57 DPI₁, and was leukopenic ($<6.02 \times 10^3$ WBC/ μ l) on 4 and 50 DPI₁ with values of 5.77×10^3 and 5.73×10^3 WBC/ μ l, respectively. Dog AFY had 39.5 and 39.9 °C temperatures on 36 and 54 DPI₁, respectively, and was leukopenic (5.92×10^3 WBC/ μ l) 38 DPI₁. Dog AIA had a 39.2 °C temperature at 62 DPI₁ and was thrombocytopenic ($<1.64 \times 10^5$ platelets/ μ l) with 6.08×10^4 platelets/ μ l 60 DPI₁. Dog AUW was leukopenic at 32–64 DPI₁ with values ranging from $4.57 - 5.46 \times 10^3$ WBC/ μ l. All of these semiweekly blood samples were PCR-negative with the *p28*-based assay, but *E. chaffeensis* was later detected in 3 DPI₁ samples of dogs AIA and AUW with the fluorogenic PCR assay.

In previous reports, intradermal inoculations were used to infect white-tailed deer with *E. chaffeensis* (Varela *et al.*, 2003), mice with *Ixodes ovatus Ehrlichia* (IOE) agent (Stevenson *et al.*, 2006) and cattle with *A. marginale* and *A. phagocytophilum* (Hoar *et al.*, 2008). Thus, dogs in the present study were challenge-exposed (intradermally) with *E. chaffeensis* (Arkansas strain) to determine the pathogen would be more readily detected in peripheral blood after this route of exposure. Clinical signs were not observed other than mild fevers up to 34 DPI₂. Body temperatures of 39.4 °C, 39.3 °C and 39.6 °C were observed at 14 DPI₂ for dogs AFL, AFY and AIA, respectively, and 39.3 °C for dog AIA at 18 DPI₂. Dog AFL was leukopenic with white blood cell counts of 4.94 and 5.07×10^3 WBC/ μ l on –4 and 0 DPI₂, respectively, indicating that these values were not attributable to the second inoculation. Dog AUW was also leukopenic with values ranging from 4.70 to 5.85×10^3

WBC/ μ l from 14 to 34 DPI₂. Mild thrombocytopenia (1.62×10^4 platelets/ μ l) was observed at 3 DPI₂ for dog AIA. A single buffy coat sample from dog AFL at 7 DPI₂ was PCR-positive with the *E. chaffeensis* *homp*-based PCR assay.

It is unclear how monocytotropic ehrlichial infections could be maintained in the mammalian host when they are undetectable within peripheral blood, but xenodiagnosis with ticks has proved more sensitive than buffy coat assays (delos Santos *et al.*, 2007; McClure *et al.*, 2010), suggesting that these pathogens might be more detectable in stationary tissues than in peripheral blood. Thus, liver, lung, brain frontal lobe, bone marrow, mesenteric lymph node, kidney and jejunum samples were aseptically collected from dogs AFY, AIA and AUW at 34 DPI₂, and tested with the *homp*-based PCR assay. Various soft organ tissues collected from each of these dogs were PCR-positive with SYBR green chemistry, all of which were confirmed with agarose electrophoresis and amplicon sequence analysis. The lung sample from dog AUW, mesenteric lymph node sample from dog AIA and frontal lobe sample from dog AFY were PCR-positive for *E. chaffeensis* with SYBR green chemistry. Spleen, liver and jejunum samples from all these dogs were PCR-negative. However, some amplicons had slightly different melting temperatures and slightly different molecular sizes than the *E. chaffeensis* positive control (data not shown). Interestingly, amplicon sequence analyses revealed that some of the smaller than expected amplicons contained the full *E. chaffeensis* target sequence. Thus, a fluorogenic probe was used to mitigate erroneous interpretation of results with SYBR green. Lung from dogs AIA and AUW, kidney from dogs AIA and AFY, mesenteric lymph node from dog AIA, bone marrow from dog AIA and frontal lobe from dog AFY were PCR-positive with the fluorogenic probe (Table 1).

Infectivity to *R. sanguineus*

Rhipicephalus sanguineus ticks were reportedly naturally infected with *E. chaffeensis* (Ndip *et al.*, 2010; Ndip *et al.*, 2007), but, to the best of our knowledge, it was not determined if this observation could be due to infectivity of the pathogen to this tick species or to uniquely co-adapted *E. chaffeensis* and *R. sanguineus* strains. Thus, to test the former hypothesis, *R. sanguineus* ticks were placed on dogs AFL, AFY and AIA at 24 DPI₂, and allowed to feed to repletion and detachment (nymphs) or for 10 days (male adults). *Amblyomma americanum* nymphs and adults were simultaneously fed on the same dogs to serve as controls.

Pools of *R. sanguineus* and of *A. americanum* fed on each dog were PCR-positive for *E. chaffeensis* (Table 2). For ticks fed at 24 DPI₂, dog AFY *R. sanguineus* pool 3 (of 3) was PCR-positive with both SYBR green and fluorogenic probe chemistries, while both of the dog AFY *A. americanum* pools were only positive with the fluorogenic probe. Dog AIA *R. sanguineus* pool 3 was PCR-positive with the probe-based assay, and the sole pool of *A. americanum* from this dog was PCR-positive with both SYBR green and fluorogenic probe. Dog AFL *R. sanguineus* pools 2 and 3 were PCR-positive with SYBR green, but *R. sanguineus* pool 1 and *A. americanum* pool 1 were PCR-positive only with the fluorogenic probe.

Ticks were repeatedly fed on dog AFL to determine if this host could continue to provide infectious blood meals to ticks over an extended time period. A single pool of *R. sanguineus* was PCR-positive with the probe-based at 566 DPI₂. Individual and pooled *R. sanguineus* and *A. americanum* fed on dog AFL at 764 or 1086 DPI₂ tested PCR-negative.

DISCUSSION

In this study, dogs were experimentally infected with *E. chaffeensis* and transmitted the pathogen to both *R. sanguineus* and *A. americanum* ticks, confirming that *E. chaffeensis* is infective to these divergent metastriate tick species. To the best of our knowledge, this is the first report of experimental infection of *R. sanguineus* with *E. chaffeensis*. Notably, these results corroborate previous reports of detection of *E. chaffeensis* in *R. sanguineus* collected from dogs in Cameroon (Ndip *et al.*, 2010; Ndip *et al.*, 2007).

PCR of buffy coat DNA was not a reliable method of detection in this study, because *E. chaffeensis* was only detected in buffy coat samples from dogs AIA and AUW at 3 DPI₁ and from dog AFL at 7 DPI₂. Clinical and hematologic signs were also mild in these dogs, similar to those reported from other studies (Dawson and Ewing, 1992; Zhang *et al.*, 2003). Interestingly, *E. chaffeensis* was detected in other soft organ tissue samples collected from all three dogs necropsied at 34 DPI₂. Moreover, *E. chaffeensis* was detected in at least one pool of *R. sanguineus* and of *A. americanum* ticks fed on dogs AFL, AIA and AFY at 24 DPI₂. *E. chaffeensis* was also not detected in blood samples from dogs that hosted naturally infected ticks in Cameroon (Ndip *et al.*, 2005; Ndip *et al.*, 2007), underscoring the utility of xenodiagnosis for *ante mortem* detection of this fastidious pathogen.

Dogs are often overlooked as reservoirs of zoonotic rickettsial infections, perhaps in part because it is assumed that ticks that feed on dogs would not subsequently feed on human hosts or that dogs do not develop rickettsemias of sufficient magnitude to infect ticks. This report, along with reports of naturally infected *R. sanguineus* (Ndip *et al.*, 2010; Ndip *et al.*, 2007), the established roles of *R. sanguineus* vectors and canine reservoirs of *Rickettsia conorii* in the Mediterranean region (Gilot *et al.*, 1990) and reports indicating transmission of *R. rickettsii* by *R. sanguineus* in the southwestern United States (Demma *et al.*, 2005), all underscore the need to reexamine this paradigm with regard to the role of *R. sanguineus*, as well as more anthrophilic tick species, in the maintenance of zoonotic rickettsial pathogens in canine host populations. Whether the possible ecological role of dogs has been overlooked, or changes in vector ecology are resulting in new tick-host dynamics, experimental studies are warranted to explore the epidemiologic roles of these often overlooked invertebrate and vertebrate hosts of important zoonotic pathogens.

In summary, the ability of *E. chaffeensis* to infect divergent metastriate ticks, *A. americanum* and *R. sanguineus*, was demonstrated. These results suggest that *E. chaffeensis* is adapted to divergent ixodid tribes, a feature which appears to be unique among the Anaplasmataceae. Moreover, *R. sanguineus* may have a role in maintenance and propagation of this zoonotic pathogen among relatively asymptomatic canine hosts. Further studies are warranted to examine the epidemiological role of *R. sanguineus* in the maintenance of *E. chaffeensis* in canine populations, and to understand how *E. chaffeensis* is infective to divergent tick

species while closely related pathogens appear to have a much narrower invertebrate host range.

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E. chaffeensis hypothetical outer membrane protein gene-based real time PCR assays of canine tissue samples with SYBR green and fluorogenic probe chemistries.

Table 1

Tissue*											
Dog	Spleen		Liver		Lung		Mesenteric LN**				
	SYBR	Probe	SYBR	Probe	SYBR	Probe	SYBR	Probe	SYBR	Probe	
AFY	-	-	-	-	-	-	-	-	-	-	-
AIA	-	-	-	-	-	+	+	+	+	+	+
AUW	-	-	-	-	+	+	-	-	-	-	-
	Frontal Lobe		Kidney		Bone Marrow		Jejunum				
	SYBR	Probe	SYBR	Probe	SYBR	Probe	SYBR	Probe	SYBR	Probe	
AFY	+	+	-	+	-	-	-	-	-	-	-
AIA	-	-	-	+	-	+	-	-	-	-	-
AUW	-	-	-	-	-	-	-	-	-	-	-

* Samples collected from experimentally infected dogs 34 DPI₂.

** Lymph node

Table 2

PCR assay of acquisition-fed ticks.

24 DPI ₂						
Dog	Ticks	SYBR	Probe	Dog	Ticks	DPI ₂ Probe
AIA	<i>R. sanguineus</i> (N ^d) P1 ^b	-	-	AFL	<i>R. sanguineus</i> (M ^c) P1	566 -
	<i>R. sanguineus</i> (N) P2	-	-		<i>R. sanguineus</i> (M) P2	566 -
	<i>R. sanguineus</i> (N) P3	-	+		<i>R. sanguineus</i> (M) P3	566 -
	<i>A. americanum</i> (N) P1	+	+		<i>R. sanguineus</i> (M) P4	566 +
AFL	<i>R. sanguineus</i> (N) P1	-	+		<i>R. sanguineus</i> (M) P5	566 -
	<i>R. sanguineus</i> (N) P2	+	-		<i>R. sanguineus</i> (M) P6	566 -
	<i>R. sanguineus</i> (N) P3	+	-		<i>R. sanguineus</i> (M) P11-10	764 -
	<i>A. americanum</i> (N) P1	-	+		<i>A. americanum</i> (M) P11-10	764 -
AFY	<i>A. americanum</i> (N) P2	-	-		<i>R. sanguineus</i> (N) P1-10	764 -
	<i>R. sanguineus</i> (N) P1	-	-		<i>R. sanguineus</i> (N) P1-10	764 -
	<i>R. sanguineus</i> (N) P2	-	-		<i>A. americanum</i> (N) P1-10	764 -
	<i>R. sanguineus</i> (N) P3	+	+		<i>A. americanum</i> (N) P1-20	1086 -
	<i>A. americanum</i> (N) P1	-	+		<i>R. sanguineus</i> (M) P1-6	1086 -
	<i>A. americanum</i> (N) P2	-	+			

^a Acquisition fed as nymphs and assayed as adult male ticks^b Groups represent 5 pooled tick halves (P) or individual (I) ticks acquisition fed on experimentally infected dogs^c Adult male ticks (M) fed for 10 days on dog AFL and then assayed after holding in a humidity chamber for an additional 10 days