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***Amblyomma americanum* ticks infected with *in vitro* cultured wild-type and mutants of *Ehrlichia chaffeensis* are competent to produce infection in naïve deer and dogs**

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Abstract

Monocytic ehrlichiosis in people caused by the intracellular bacterium, *Ehrlichia chaffeensis*, is an emerging infectious disease transmitted by the lone star tick, *Amblyomma americanum*. Tick transmission disease models for ehrlichiosis require at least two hosts and two tick blood feeding episodes to recapitulate the natural transmission cycle. One blood feeding is necessary for the tick to acquire the infection from an infected host and the next feeding is needed to transmit the bacterium to a naïve host. We have developed a model for *E. chaffeensis* transmission that eliminates the entire tick acquisition stage while still producing high numbers of infected ticks that are also able to transmit infections to naïve hosts. Fully engorged *A. americanum* nymphs were ventrally needle-infected, possibly into the midgut, and following molting, the unfed adult ticks were used to infect naïve deer and dogs. We have also described using the ticks infected by this method the transmission of both wild-type and transposon mutants of *E. chaffeensis* to its primary reservoir host, white tailed deer and to another known host, dog. The infection progression and IgG antibody responses in deer were similar to those observed with transmission feeding of ticks acquiring infection by natural blood feeding. The pathogen infections acquired by natural tick transmission and by feeding needle-infected ticks on animals were also similar to intravenous infections in causing persistent infections. Needle-infected ticks having the ability to transmit pathogens will be a valuable resource to substantially simplify the process of generating infected ticks and to study infection systems in vertebrate hosts where interference of other pathogens could be avoided.

Keywords

Tick; *Amblyomma americanum*; *Ehrlichia chaffeensis*; bacterial pathogenesis; tick-borne transmission; disease acquisition

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Introduction

The lone star tick, *Amblyomma americanum*, is an ixodid tick with increasing populations extending more northward and westward in the United States in recent years, primarily due to the increase in white-tailed deer in these areas (Paddock and Yabsley, 2007). As tick and deer populations have increased in the past several decades, the emergence of human and animal diseases attributed to this tick species has also increased. *A. americanum* is an obligate blood feeder that must consume blood meals in each of its motile life stages (larva, nymph and adult). Pathogen transmission occurs when bacteria, virus or protozoans are migrated from the midgut epithelium to the salivary glands and into the host during tick blood feeding. The tick's unique midgut and salivary glands have facilitated a diverse group of viral, bacterial and protozoan pathogens that can be transmitted by ticks—a greater diversity of pathogens than any other vector arthropod (Sonenshine, 1991). Disease agents and potentially pathogenic microorganisms transmitted by *A. americanum* ticks include *Francisella tularensis*, *Ehrlichia chaffeensis*, *Ehrlichia ewingii*, *Borrelia lonestari*, *Rickettsia amblyommii* and Heartland virus (Hopla, 1953; Taylor et al., 1991; Murphy et al., 1998; James et al., 2001; Apperson et al., 2008; Savage et al., 2013).

We adapted an infection model for *E. chaffeensis* whereby we can inoculate ticks with culture-grown bacteria during the nymphal state; and follow the infection progression to adult ticks that in turn serve as vectors of transmission to a naïve host. For the culture-grown protozoan, *Theileria parva*, such a model was attempted in the past for *Rhipicephalus appendiculatus* tick, but it did not result in producing infections in hosts (Jongejan et al., 1980). Goddard (2003) also successfully inoculated *A. americanum* ticks with a bacterial pathogen, *Rickettsia parkeri*, but were unable to produce infections in guinea pigs from those ticks. Karim et al. (2012) demonstrated that nymphal lone star ticks could be inoculated with culture-grown *E. chaffeensis* and the infection in ticks was greater than 80% when assessed after molting to the adult stage, although the authors did not describe its validity for the pathogen transmission. Recently, we validated this study in generating *E. chaffeensis* infected ticks by a needle inoculation method (Cheng et al., 2015).

In the current study, we tested the hypothesis that the needle-inoculated *E. chaffeensis* infected ticks have the ability to transmit the pathogen to a naïve host via tick blood feeding. Such a system will be a valuable resource to substantially simplify the process of generating infected ticks and lead to a clean infection system where interference of other pathogens could be avoided. Here, we present the evidence that needle-inoculated ticks produce infections in white-tailed deer and in dogs that are similar to infections observed with those generated by the natural tick acquisition/transmission cycle.

Materials and Methods

In vitro cultivation of *E. chaffeensis*

E. chaffeensis Arkansas isolate (wild-type and transposon mutants) were cultivated in the macrophage like cell line (DH82) as described in Cheng and Ganta (2008).

Needle inoculation infection of *A. americanum* ticks with *E. chaffeensis* cultures

Newly engorged *A. americanum* nymphal ticks (within 24–48 h post blood meal) were obtained from the Oklahoma State University tick rearing facility and used for these studies. Fed nymphal ticks were needle injected with concentrated cultured *E. chaffeensis* (mutants or wild-type) containing approximately 5,000 infected cells by following the method described in Karim *et al.*, 2012. Inocula were prepared as described below. Briefly, a culture from a T25 flask having about 80% infection was harvested by centrifugation at $15,000 \times g$ for 10 min and the pellet was washed with phosphate buffered saline (PBS). The culture was then centrifuged one more time and the final pellet was resuspended in PBS to the concentration equivalent to 10^5 bacterial copies per microliter volume and used immediately for needle puncture inoculation (26-gauge needle) into the ventral side of the ticks possibly injecting into the midgut. In particular, given that the ticks were fully engorged nymphs, the injections most likely resulted in the mixing of the infected culture into blood meal, although the possibility of injection into the hemolymph cannot be ruled out. Ticks were then allowed to molt to adults at room temperature by exposure to 14 h light and 10 hour dark cycle in a 96% humidity chamber (Patrick and Hair, 1975). The fed nymphs molted to adults by about 6 weeks. Following molting, 14–21 randomly selected ticks from every group were assessed for the infection rates. Total genomic DNA from ticks was individually isolated (Wizard genomic DNA isolation kit, Promega, Madison, WI), resuspended into 100 μ l of nuclease-free water and 2 μ l each of the DNA was used as template for nested PCR analysis targeting to Ech_1136 gene by following the protocol described in Nair *et al.*, (2015). The infection with a specific mutant was also assessed as described in Cheng *et al.*, (2015).

Recovering *E. chaffeensis* from needle inoculation infected ticks

The culture recovery method of isolating viable *E. chaffeensis* from ticks was followed essentially as described earlier (Paddock *et al.*, 2010). Briefly, needle inoculation infected ticks were washed sequentially in 2% Micro-Chem Plus (National Chemical Laboratories of PA, Inc., Philadelphia, PA), 10.5% sodium hypochlorite, and 3% hydrogen peroxide. The ticks were agitated gently in each solution for about 5 min, rinsed in sterile distilled water after the final wash, blotted lightly on sterile filter paper, bisected longitudinally and minced with a sterile scalpel blade in 0.5 ml of complete tick cell culture media. All of the minced tick extract was inoculated onto a semi-confluent monolayer of ISE6 cells in a T25 tissue culture flask containing 5 ml of complete tick cell infection media with 100 U/ml penicillin, 100 μ g/ml streptomycin sulfate, and 0.25 μ g/ml amphotericin B. Cell cultures were incubated at 34°C without CO₂. After 24 h, the medium was replaced with 5.0 ml of fresh cell culture medium that did not contain the antibiotics. The medium replacement was repeated once a week thereafter. Cultures were monitored for evidence of infection by examining a cytospin slide.

Animal infestations

Animal experiments with deer and dogs were performed in compliance with the Public Health Service (PHS) Policy on the Humane Care and Use of Laboratory Animals, the US Department of Agriculture's (USDA) Animal Welfare Act & Regulations (9CFR Chapter 1, 2.31), and with approvals of the Oklahoma State University (OSU) and Kansas State

University (KSU) Institutional Animal Care and Use Committees (IACUC), and as per the guidelines of the protocols. Laboratory-reared deer (n=2) and purebred laboratory reared dogs (n=4) were used for conducting infestation experiments. Deer identification numbers and specific *E. chaffeensis* inoculi used for these experiments along with the days that were tested post-tick infestation are shown in Table 1. Table 2 indicates the dog identification numbers, *E. chaffeensis* inoculi utilized and the days tested for infection post-tick infestation. Protocols for rearing deer were described in Cheng et al., (2013). Three- to four-month old beagle dogs of either sex were obtained from Covance Research Products (Denver, PA). For each animal, a tick containment cell was affixed to the shorn back of the host. Unfed adult ticks in pairs were placed in the tick containment cell and permitted to feed for six to seven days before removal from the host. Six to seven days feeding was sufficient to ensure that infective organisms were injected into a host during tick feeding and also short enough to avoid the rapid engorgement phase for the ticks where larger volumes of blood could negatively impact subsequent PCR and culture assays (Schwartz et al., 1997). Needle-inoculated ticks were used to produce infections in two deer and four dogs. In particular, we used two groups of needle infected ticks for infections in deer. The first group of ticks included an *E. chaffeensis* transposon mutant (Ec_0480) which is known to persist similar to wild-type *E. chaffeensis* (Cheng et al., 2015) and the second group of ticks received five equal molar mixed transposon mutants containing two mutants which persist in deer (Ech_0284 and Ech_0480) and three mutants which clear rapidly (Ech_0202, Ech_0379, and Ech_0660) in deer and dogs (Cheng et al., 2013; Cheng et al., 2015). We performed a tick-transmission experiment to test if the needle-infected ticks cause similar infections as those seen with natural transmission in deer (Nair et al., 2014). For deer infestations, we used 20 ticks (10 females and 10 males; for dog infestations, we used fifty ticks per dog (25 females and 25 males).

Detection of *E. chaffeensis* by culture or by nested PCR

About 3 ml of deer or dog blood was collected in EDTA blood collection tubes on day zero (before infestation) and on different days post-infestation from all groups of animals. Blood samples were centrifuged at 3,000 rpm in a Clay Adams Sero-fuge (Becton Dickinson, Sparks, MD) for 5 min. Plasma was removed and about 1 ml of buffy coat each was transferred to a 15 ml sterile falcon centrifuge tube containing 10 ml red blood cell lysis buffer (155 mM NH₄Cl, 10 mM KHCO₃ and 0.1 mM EDTA) and mixed several times until complete lysis of erythrocytes occurred. The samples were then centrifuged at 5,000 × g for 5 min to pellet the white blood cells. The pellet from each sample was then resuspended in 200 µl of sterile 1X PBS. One hundred µl of the buffy coat cell suspension from deer or dog blood was used for isolating total genomic DNA using the Wizard SV Genomic DNA purification kit as per the manufacturer's instructions (Promega, Madison, WI); purified DNA from each sample was resuspended in 100 µl of buffer containing 10 mM Tris-HCl and 1 mM EDTA (pH 8.0) (TE buffer). The DNAs were used to assess *E. chaffeensis* infection status by performing semi-nested PCR targeting the Ech_1136 gene. Briefly, 2 µl of genomic DNA from deer or dog blood was used for the first round PCRs in a 25 µl reaction volume using Platinum Taq DNA polymerase as per the manufacturer's instructions (Life Technologies, Carlsbad, CA). The primers utilized for these reactions were as follows: forward primer 5'-GCAATAGCAGATAAGAAATATG; reverse primer 5'-

GAGCTCCTTCTAATACTAC; and nested reverse primer 5'-AACTCCGTGGTAGTATCCTCC. The PCR reactions were performed with the following temperature cycles: 94°C for 4 min, followed by 35 cycles of 94°C for 30 s, 52°C for 30 s, and 72°C for 1 min and 1 cycle of 72°C for 3 min. The second round of PCR was performed using the same PCR conditions as the first round PCR and the templates for the second round included 2 µl of 1:100 diluted products from the first PCR and with nested PCR primer set. The PCR products were resolved on 1.5% agarose gel to identify specific size products (Sambrook and Russell, 2000). Primer sets for each of the specific mutants are found in Cheng et al. (2015).

To assess for infection with *E. chaffeensis* by culturing, 100 µl of the buffy coat cell suspension from each dog blood sample was transferred into a T25 flask containing 5 ml DH82 cell suspension with about 80% confluence. The cultures were grown by following the detailed culture protocols reported earlier (Cheng and Ganta, 2008) and infection was monitored twice a week by microscopically examining the Hema3 stained cytospin slides for up to 8–10 weeks to determine if a sample was positive or negative for *E. chaffeensis* infection.

Enzyme-linked immunosorbent assay (ELISA)

Host cell-free *E. chaffeensis* lysate was prepared and used for the ELISA analysis (Nair et al., 2014). Plasma samples from deer or dogs collected prior to infection and on specific days following infestations were assessed by ELISA for the presence of the *E. chaffeensis*-specific IgG (Nair et al., 2014).

Results

We recently presented data that ticks infected by needle inoculations of *E. chaffeensis* into the engorged nymphal stage retain the pathogen infection through the molting to adults (Cheng et al. 2015). We used the artificially infected ticks for transmission feeding to deer; deer were infected with ticks that had received an injection with a pool of five equal-molar mixed mutants (Ech_0202, Ech_0284, Ech_0379, Ech_0480, and Ech_0660) or with a single mutant injection (Ech_0480) as described in (Cheng et al 2015). Deer were monitored for infection for 6 weeks after infestations by assessing the infection status initially by nested PCR (not shown), and then, by mutant-specific PCR (Table 1). As in our previous study by intravenous infection transmission (Cheng et al., 2013 and 2015), the mutants Ech_0284 and Ech_0480 were also detected up to 6 weeks when deer received infections from the ticks artificially infected with the pool of five mutants, while clearing Ech_0202, Ech_0379 and Ech_0660 mutants rapidly. Likewise, another deer infested with artificially infected ticks containing Ech_0480 mutant alone also exhibited persistent infection when assessed for 6 weeks (Table 1).

Dogs were also challenged using ticks infected with *E. chaffeensis* by the needle inoculation method. In this experiment, we used needle-infected ticks with either the wild-type *E. chaffeensis* or the persistent mutant, Ech_0480, that is similar to wild-type *E. chaffeensis* in causing persistent infection in deer and dogs (Cheng et al. 2013 and 2015). Both groups of artificially infected ticks transmitted the pathogen to dogs and the infections were detected

by nested PCR when assessed in blood sampled for about 6 weeks following tick feeding (Table 2). Infection was also assessed in these dogs by the culture recovery method; the culture positives were observed only on day 14 post infestation.

All tick infestations with needle-inoculated ticks in deer and dogs also induced specific IgG antibody response against *E. chaffeensis* antigens (Fig 1A and 1B). For white tailed deer, the IgG responses rose and peaked at day 28 and then dropped slightly by day 42 (Figure 1A). The IgG antibody responses in deer in the current study were similar to responses reported earlier for animals receiving infection transmission from naturally infected ticks (i.e., ticks receiving infection by acquisition feeding on needle infected animals) or by intravenous needle infection (Nair et al., 2014).

Dogs receiving infection from artificially infected ticks also developed pathogen-specific IgG responses (Fig. 1B). Dog antibody responses were highly variable among the four dogs. Variability in antibody responses was also noted in dogs in our previous studies where animals received infection by intravenous infection (Nair et al. 2014).

Discussion

To our knowledge, this is the first study documenting animal infections resulting from needle inoculation of ticks with cultured pathogens. Artificial infection methods have been employed for generating infected ticks using *Theileria*-infected blood (Schreuder and Uilenberg, 1976; FAO, 1977; Walker et al., 1979; Jongejan et al., 1980). Kocan et al. (1986) also described infections in cattle with ticks needle-inoculated with *Anaplasma marginale* infected blood, another tick-transmitted rickettsial pathogen. These studies demonstrated success in generating infected ticks utilizing blood from infected animals. Research on cultured organisms injected into ticks has been less successful for generating animal infections. Such a method was investigated for *Theileria* microorganisms transmitted by ticks as early as 1980 by Jongejan et al. In that study, the authors needle-infected *Rhipicephalus appendiculatus* ticks with cultured *Theileria parva* strains, but could not demonstrate the method's value in transmitting the parasite to naïve cattle possibly due to the incorporation of toxic substances like DMSO in the cell cultures. Work directed at injecting *E. chaffeensis* into ticks was attempted by Rechav et al. in 1999; however, the survival of these injected ticks was rather low.

Ticks are biological vectors of a wide range of organisms including viruses, bacteria and protozoans. The tick midgut is filled with a large microbiome (Narasimhan and Fikrig, 2015). Information on the impact of many endosymbionts and pathogenic bacteria found within the tick midgut is sparse. Circumventing some of the tick's intrinsic contamination by tipping the scale in favor of one pathogenic organism should greatly improve our outcomes for deciphering pathogenesis/virulence factors attributable to the tick environment. Using our needle infection method of generating infected ticks, one should be able to generate cleaner and more controlled pathogen infections compared to infections achieved by acquisition infection of a pathogen by blood feeding on a host. Using the needle injection method, we have already documented that we can follow various *E. chaffeensis* mutants within a tick (Cheng et al., 2015).

Natural transmission studies utilizing ticks and pathogens that have been previously reported by us and others (Kocan et al., 1985; Bremer et al., 2005; Ueti et al., 2008; Jaworski et al., 2013). In these studies, vertebrate hosts are infected by inoculation with a pathogen; and then, ticks are permitted to acquire pathogens during blood feeding as nymphs or adult males. Acquisition-fed immatures molt to the next life stage and become competent to feed again or acquisition-fed males are permitted to re-feed in a shorter interval of several days. While it is possible to achieve high numbers of infected ticks by these methods, tick infections generated by these methods vary widely for different hosts and ticks and for the type of a pathogen transmitted. With the needle inoculation infection method, we can produce infected ticks that are able to transmit the pathogen to a naïve host. We have demonstrated that the progression of the infection produced from needle-inoculated ticks closely resembles infections produced in a host by a tick naturally acquiring infection by blood feeding on a host. This method completely eliminates the tick acquisition phase. Moreover, tick infection experiments become less limited by time, the numbers of ticks permitted to feed on a host or by other host constraints formerly required for ticks to acquire an infection via tick feeding. Further, one can easily sample needle-inoculated tick populations and choose those populations that have the highest infections or are the most likely to produce infection in a vertebrate host. In addition, one can better able to control characteristics of a pathogen for tick inoculations and subsequent infestations.

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Fig. 1A.

Fig. 1B.

Fig. 1.

Ehrlichia chaffeensis specific IgG responses of deer and dogs post infestation with unfed adult ticks infected as engorged nymphs by needle inoculation. Fig. 1A. IgG response in deer (n=2) infested with *E. chaffeensis* needle-inoculated ticks. Fig. 1B. IgG response in dogs (n=4) infested with *E. chaffeensis* needle-inoculated ticks

Table 1

Deer infection status in blood after infesting with needle-inoculated ticks

deer ID	days post infestation										
	Inoculum	0	7	13	21	28	35	42			
115	Ech_0480	–	+	–	+	–	+	+			
217	Ech_0202	–	–	–	–	–	–	–			
	Ech_0284	–	–	–	–	–	–	+			
	Ech_0379	–	–	–	–	–	–	–			
	Ech_0480	–	–	+	+	+	+	+			
	Ech_0660	–	–	–	–	–	–	–			

Table 2
Dog infection status in blood and in cultures after infesting with needle-inoculated ticks

dog ID	Inoculum	days post infection									
		0	3	7	10	14	17	24	31	38	
Fed08-15	WT_Ech	-	-	-	-	+	-	-	+	-	
Fed09-15	WT_Ech	-	-	-	-	+	+	-	-	-	
Fed10-15	Ech_0480	-	-	-	-	+	-	-	-	-	
Fed11-15	Ech_0480	-	+	-	-	-	+	-	+	-	

⁺PCR and culture
⁺PCR only