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SUSCEPTIBILITY OF RED AND GRAY FOXES TO INFECTION BY *EHRLICHIA CHAFFEENSIS*

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ABSTRACT: Red foxes (*Vulpes vulpes*) and gray foxes (*Urocyon cinereoargenteus*) were evaluated for their susceptibility to experimental infection with *Ehrlichia chaffeensis,* the causative agent of human monocytotropic ehrlichiosis. Two red foxes and three gray foxes were inoculated intravenously with *E. chaffeensis* (15B-WTD-GA strain) and were monitored at 7, 14, 21, and 28 days post inoculation (DPI) for evidence of infection using an indirect fluorescent antibody (IFA) assay, light microscopy, polymerase chain reaction (PCR), and cell culture methods. One red fox and one gray fox served as negative controls. Red foxes were susceptible to infection based on reisolation of *E. chaffeensis* from blood at 7 and 14 DPI, seroconversion by 7 DPI, and positive PCR assays on spleen and lymph nodes at 28 DPI. Morulae were not found in circulating leukocytes and clinical signs or lesions of ehrlichiosis were not observed. In contrast, gray foxes were refractory to infection based on negative results on all culture, PCR, serologic, and microscopic examinations. These findings imply that red foxes, but not gray foxes, are potential vertebrate reservoirs for *E. chaffeensis.* These findings also illustrate the need to verify serologic evidence of *E. chaffeensis* infection among wild animals.

Key words: Ehrlichia chaffeensis, experimental infection, gray fox, red fox, *Urocyon cinereoargenteus, Vulpes vulpes*.

INTRODUCTION

Human monocytotropic ehrlichiosis (HME) caused by *Ehrlichia chaffeensis* is one of two tick-borne ehrlichial zoonoses recognized in the United States subsequent to the mid-1980's. Current understanding of the epidemiology of HME indicates that the white-tailed deer (*Odocoileus virginianus*) is the principal reservoir host for *E. chaffeensis.* Antibodies to *E. chaffeensis* have been detected in deer from many locations (Dawson et al., 1994a; Lockhart et al., 1996), and deer have been experimentally infected with a human isolate (Arkansas strain) of *E. chaffeensis* (Dawson et al., 1994b). Naturally acquired infection with *E. chaffeensis* has been confirmed through polymerase chain reaction (PCR) or isolation from deer populations in Georgia (USA), Missouri (USA), and South Carolina (USA) (Little et al., 1997; Lockhart et al., 1997a, b). In addition, deer represent an important host for all stages of the lone-star tick (*Amblyomma americanum*) (Patrick and Hair 1978; Bloemer et al., 1986). The lone star tick has been incriminated as a vector for *E. chaffeensis* based on epidemiologic associations (Eng et al., 1990; Lockhart et al., 1995, 1996; Standaert et al., 1995), PCR detection of *E. chaffeensis* 16S rRNA in ticks (Anderson et al., 1992a, 1993; Lockhart et al., 1997a), and an experimental transstadial transmission study (Ewing et al., 1995).

Although deer and lone star ticks clearly are the critical components in the epidemiology of HME, there is field evidence that other species of mammals may have lesser roles in the perpetuation of *E. chaffeensis* (Lockhart et al., 1997a). Serologic testing of 10 species of wild rodents in the Southeast failed to detect antibodies (Lockhart, 1997a; 1998), but *E. chaffeensis*-reactive antibodies were found in other wild mammals from the eastern United

States including raccoons (*Procyon lotor*), opossums (*Didelphus virginiana*) (Lockhart et al., 1997a), rabbits (*Sylvilagus* spp.), and foxes (*Vulpes vulpes* and *Urocyon cinereoargenteus*) (J. E. Dawson, unpubl. data). Lone star ticks commonly parasitize all of these seropositive mammals (Bishop and Trembley, 1945; Koch and Dunn, 1980; Bloemer and Zimmerman, 1988). Domestic dogs also were susceptible to experimental *E. chaffeensis* infection (Dawson and Ewing, 1992) and are naturally infected (Dawson et al., 1996).

Interpretation of *E. chaffeensis*-reactive antibody titers detected during indirect fluorescent antibody (IFA) surveys among potential wildlife hosts can be complicated by serologic cross-reactions (Dawson et al., 1994a). Particularly problematic is interpretation of IFA data from species such as red and gray foxes which are known to be susceptible to infection by *E. canis* (Amyx and Huxsoll, 1973), an ehrlichial species known to serologically cross-react with *E. chaffeensis.* The less studied *E. ewingii,* a granulocytic ehrlichial agent of canines, is a second cross-reacting organism (Anderson et al., 1992b; Rikihisa et al., 1992) that may be present among wild fox populations. The purpose of this study was to evaluate the susceptibility of red foxes and gray foxes to infection with *E. chaffeensis.*

MATERIALS AND METHODS

Experimental design and sample collection

Three 6-mo-old red foxes and four 5-mo-old gray foxes born and raised in captivity were obtained from the French Creek Game Farm (West Virginia Department of Natural Resources, French Creek, West Virginia, USA). Foxes were individually housed in stainless steel cages within a single indoor animal room in the Department of Animal Resources (College of Veterinary Medicine, The University of Georgia, Athens, Georgia, USA) and were provided a diet of dry commercial cat food and water *ad libitum.* None of the animals had a history of antibiotic therapy. Two red foxes (No. 60, female; No. 61, male) and three gray foxes (No. 57, female; No. 58, male; No. 59, male) were randomly selected as principals and one red fox (No. 62, female) and one gray fox (No. 63, female) were selected as uninfected controls. To facilitate experimental procedures and blood sample collection, foxes were sedated with an intramuscular injection of a mixture of 20mg/kg ketamine (Fort Dodge Laboratories, Inc., Fort Dodge, Iowa, USA) and 1mg/kg xylazine (Mobay Corporation, Shawnee, Kansas, USA). All foxes were acclimated to the research facility for 7 days prior to beginning the study. Two days before initiation of the study, all foxes were ear tagged, shaved along the lateroventral aspects of their necks, examined and found to be negative for tick infestations. A blood sample obtained from each animal was tested and found to be negative for *E. chaffeensis* infection based on IFA and PCR assays.

After the skin was wiped with 80% isopropanol, approximately 2 ml of blood was collected via jugular venipuncture on days 0, 7, 14, 21, and 28 days post inoculation (DPI). Approximately 1 ml of blood was placed in plain tubes for the collection of serum and 1 ml was placed in EDTA tubes for isolation of *Ehrlichia,* PCR, preparation of thin blood films, and hematology profiles. At each sampling date blood samples were analyzed for packed cell volume and total and differential leukocyte counts. Thin blood films were prepared, stained with Giesma stain, and a minimum of 200 mononuclear leukocytes examined for *E. chaffeensis* morulae.

Foxes were observed at 1 to 2 day intervals for clinical signs of infection. At 28 DPI, all foxes were sedated as described above and euthanized by an intracardiac injection of sodium pentobarbital (1.0 ml/kg; Butler Company, Columbus, Ohio, USA). Animals were necropsied within 2 hr and examined for lesions. Tissues collected for culture and PCR assays included prescapular, mesenteric, and parotid lymph nodes, spleen, and femur bone marrow. In addition to the above tissues, portions of brain, heart, lung, liver, kidney, adrenal, urinary bladder, pancreas, stomach, and small intestine were preserved in 10% neutral buffered formalin for histopathologic examination.

Inocula and isolation of organisms

A continuous canine macrophage cell line (DH82) was used as previously described to propagate the 15B-WTD-GA strain of *E. chaffeensis,* which had been originally isolated from a white-tailed deer in Georgia (Lockhart et al., 1997b). Infected cells were scraped from two 25 cm^2 tissue culture flasks and pooled. The cell suspension was centrifuged at 1,500 $\times$ *g* for 5 min, and the pellet was washed with 5 ml of minimum essential medium (MEM), resuspended in 2.5 ml of MEM, and the washed cells were counted using a Coulter counter

(Coulter Electronics, Inc., Haleah, Florida, USA). The proportion of infected cells was estimated by direct IFA (Dawson et al., 1994b). Each principal was inoculated intravenously with 1.12×10^6 infected cells suspended in 0.5 ml of MEM. To verify that the inoculum was infectious, 0.5 ml was inoculated onto DH82 cells. Control animals were inoculated intravenously with 4.7×10^6 uninfected DH82 cells suspended in 1 ml of MEM.

In vitro isolation from blood followed the procedures of Dawson et al. (1994b) with slight modifications. Briefly, 1 ml of EDTA blood was transferred to sterile plastic tubes containing 40 ml of lysing solution (150 mM NH_4Cl, 0.7 mM KH_2PO_4, and 3 mM EDTA-Na_2) and gently inverted. After 5 min at room temperature, the suspensions were centrifuged at $160 \times g$ for 5 min and the supernatant discarded; this procedure was repeated twice. The pellet was resuspended in 1 ml of MEM, and then 0.5 ml of this suspension was inoculated into a 12.5 cm^2 tissue culture flask containing uninfected DH82 cells. Isolation attempts from tissues obtained at necropsy were as described by Lockhart et al. (1997a, b). Cultures were monitored weekly for 60 days by direct FA for evidence of infection (Dawson et al., 1994b).

Serologic tests

Serum harvested from clotted blood was tested for *E. chaffeensis*-reactive antibodies using an IFA assay (Dawson et al., 1994a). A fluorescein isothiocyanate (FITC)-labeled goat anti-dog commercial antibody conjugate (Kirkegaard and Perry Laboratories Inc., Gaithersburg, Maryland, USA) was used. Sera were screened at a dilution of 1:64 in 0.01M phosphate buffered saline (PBS) on spot slides of *E. chaffeensis*-infected DH82 cells. Samples giving positive results at 1:64 were tested to determine titer endpoints using serial two-fold dilutions.

Molecular techniques

A portion of cells harvested from EDTA blood were processed for use in a nested PCR assay as described by Dawson et al. (1994b) with modifications. Briefly, lysing solution was mixed at a 1:5 ratio with EDTA whole blood. The mixture was incubated at room temperature for 5 min, centrifuged at $7{,}000 \times g$ for 5 min, and the supernatant discarded. An additional 1.5 ml of lysing solution was added to the pellet and the process repeated. Pelleted cells were washed once in 0.5 ml distilled water and centrifuged as above. The pellet was resuspended in 0.5 ml PBS, and DNA was extracted using Instagene™ Purification Matrix (BIO-RAD, Hercules, California, USA) as per manufacturer's directions. In the initial amplification, 10 μl of each sample was placed in a 100 μl reaction containing 10 mM of Tris-Cl, 50 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM each dNTP, 5 μM tetramethylammonium chloride (TMAC), 2.5 units *Taq* DNA polymerase (Promega, Madison, Wisconsin, USA), and 0.8 μM each of primer ECB (5′-CGTATTACCGCGGCTGCTGGCA-3′) and ECC (5′-AGAACGAACGCTGGCGGCAAGCC-3′), which amplify a DNA fragment common among all known species of *Ehrlichia* and a few other bacterial species (Dawson et al., 1994b). The temperature profile for the initial amplification was 40 cycles of 1 min at 94 C, 2 min at 45 C, and 30 sec at 72 C. For the nested PCR amplification, 1 μl of the product from the first amplification was run in a 100 μl reaction as above using the primers HE1 (5′-CAATTGCTTATAACCTTTTGGTTATAAAT-3′) and HE3 (5′-TATAGGTACCGTCATTATCTTCCCTAT-3′), which amplify DNA only from *E. chaffeensis* (Dawson et al., 1994b). The temperature profile for the nested reaction was the same as the first reaction except that the annealing temperature was 55 C and the extension time was 15 sec. Amplification products were electrophoresed in 1.5% agarose and were detected by staining with ethidium bromide.

RESULTS

Both red foxes inoculated with *E. chaffeensis* seroconverted with antibody titers ≥1:64 by 7 DPI and by 28 DPI had titers of 1:256 and 1:1024 (Table 1). Although most culture attempts from blood samples of red foxes were lost due to bacterial contamination before completion of the full 60 day culture period, *E. chaffeensis* was reisolated from the blood of red fox 61 at 7 and 14 DPI. A PCR assay conducted on cell culture samples from red fox 60 at DPI 7 also was positive but this cell culture was lost to extraneous bacterial contamination before completion of the full culture period. PCR assays of blood samples were negative for both red foxes on all sample dates; however, positive PCR results were obtained from the spleen of red fox 60 and from the parotid and mesenteric lymph nodes of red fox 61 at 28 DPI. Morulae were not found in the blood films of any of the animals. The control red fox was negative on all tests at all sample

TABLE 1. Results of cell culture isolation attempts and polymerase chain reaction (PCR) assays from blood and indirect fluorescent antibody (IFA) tests for red foxes inoculated with *Ehrlichia chaffeensis.*

Parameter	Fox[a]	Day post inoculation				
		0	7	14	21	28
Culture	R60	Con/33[b]	Con/19	Con/5	Con/36	Con/29
	R61	Con/26	Pos/15	Pos/15	Con/13	Con/33
	R62	Con/25	Con/22	Con/5	Neg/61	Con/29
Culture PCR[c]	R60	ND[d]	Pos	ND	Neg	Neg
	R61	ND	Pos	ND	ND	Neg
	R62	ND	Neg	ND	ND	Neg
Blood PCR	R60	Neg	Neg	Neg	Neg	Neg
	R61	Neg	Neg	Neg	Neg	Neg
	R62	Neg	Neg	Neg	Neg	Neg
IFA Titer	R60	<64	256	512	512	1024
	R61	<64	64	128	256	256
	R62	<64	<64	<64	<64	<64

[a] Animals R60 and R61 inoculated with *E. chaffeensis*; animal R62 uninoculated control.
[b] Con = contaminated prior to full 60 day culture period; Pos = positive; Neg = negative; number following slash mark is the post-culture day on which final status was assigned.
[c] PCR results on cells harvested from cell culture flasks on day that final cell culture status was assigned.
[d] ND = not done.

dates. None of the red foxes exhibited clinical signs of infection, gross lesions, or alterations in hematologic parameters. All three red foxes had mildly hyperplastic lymphoid follicles in one or more lymph nodes. *Helicobacter heilmannii*-like spirochetes were abundant in gastric glands of all three red foxes, and foxes 61 and 62 had mild multifocal lymphoplasmacytic gastritis which is often associated with these organisms. Fox 60 had mild multifocal hepatic microabscesses. Fox 62 had multifocal chronic active cystitis with variable degrees of hemorrhage and these lesions contained cross sections of nematodes and nematode eggs tentatively identified as *Capillaria plica.* Similar cystitis lesions but no nematodes or eggs were present in fox 61.

The three inoculated gray foxes and the control gray fox were negative on all serologic, culture, PCR, microscopic, hematologic, and pathologic assays for evidence of infection by *E. chaffeensis.* Foxes 57, 58, and 63 had chronic mild multifocal cystitis similar to that attributed to *C. plica* but no nematodes or eggs were present in tissue sections. All four gray foxes had mild multifocal lymphoplasmacytic gastritis, and *H. heilmannii*-like spirochetes were present in all four animals. Fox 59 had a mild chronic multifocal interstitial nephritis, and fox 63 had moderately severe focal bronchitis and mild multifocal submucosal lymphoid hyperplasia in the duodenum. Potential causative agents were not detected in any of these lesions.

DISCUSSION

The finding that red foxes and gray foxes differed in susceptibility to infection with *E. chaffeensis* was unexpected. Reasons for anticipating similar results for both species included (1) red and gray foxes are close relatives (Nowak and Paradiso, 1983); (2) both species are susceptible to infection with *E. canis* (Amyx and Huxsoll, 1973), the species of *Ehrlichia* most closely related to *E. chaffeensis* (Walker and Dumler, 1996); (3) domestic dogs, the only other species of Canidae that has been evaluated, are susceptible to *E. chaffeensis* (Dawson and Ewing, 1992); (4) *E. chaffeensis* is known to infect taxonomically divergent mammals within the Orders Rodentia (Telford and Dawson, 1996; Lockhart et al., 1998), Artiodactyla (Dawson et al., 1994b; Ewing et al., 1995; Lockhart et al.,

1997b), Carnivora (Dawson and Ewing, 1992), and Primates (Anderson et al., 1991; Walker and Dumler, 1996); and (5) *E. chaffeensis*-reactive antibodies had been detected in red and gray foxes from a single location in the wild (J. E. Dawson, unpubl. data). Despite these reasons to expect similar results, there are examples where red and gray foxes differ markedly in their susceptibilities to common canine diseases. Gray foxes, but not red foxes, are very susceptible to canine distemper whereas red foxes, but not gray foxes, are very susceptible to sarcoptic mange (Davidson et al., 1992; Little et al., 1998).

The course of infection in red foxes paralleled the course of *E. chaffeensis* infection in needle-exposed young domestic dogs (Dawson and Ewing, 1992). In both hosts, rickettsemia was first confirmed by culture by 7 DPI and persisted at least through 14 DPI, seroconversion occurred at 7 to 14 DPI, morulae were not detected in blood films, and the animals remained clinically normal. Although attempts were not made to infect ticks by feeding them on red foxes, the duration of rickettsemia probably would have been sufficient to infect ticks based on transmission studies conducted with white-tailed deer (Ewing et al., 1995).

When combined with serologic data from wild red foxes, these findings imply, but do not prove, that red foxes are involved in the epidemiology of *E. chaffeensis.* Antibodies in wild foxes could represent serologic cross-reactions because both red and gray foxes are known to be susceptible to infection with *E. canis* (Amyx and Huxsoll, 1973) which serologically cross-reacts with *E. chaffeensis* (Anderson et al., 1991; Dawson and Ewing, 1992). Although wild foxes are infrequently parasitized by *Rhipicephalus sanguineus* (Bishopp and Trembley, 1945; Bloemer and Zimmerman, 1988; Forrester, 1992), the presumed primary vector of *E. canis* (Mathew et al., 1996), they frequently are parasitized by *Dermacentor variabilis* (Samuel and Nelson, 1982; Bloemer and Zimmerman, 1988; Forrester, 1992), a known but less studied *E. canis* vector (Mathew et al., 1996). In addition, *E. ewingii,* the serologically cross-reactive agent of canine granulocytic ehrlichiosis (Anderson et al., 1992b), also occurs in the southeastern USA and is transmitted by the lone star tick (Anziani et al., 1990) which commonly parasitizes foxes (Bishopp and Trembley, 1945; Bloemer and Zimmerman, 1988; Forrester, 1992). Thus, the *E. chaffeensis*-reactive antibodies detected among wild red and gray foxes potentially could be the result of exposure to multiple species of *Ehrlichia,* including currently unknown species. Confirmation of natural *E. chaffeensis* infection in wild red foxes will require isolation of the organism in culture or positive *E. chaffeensis*-specific PCR assays.

Since the recognition of HME in 1986 (Maeda et al., 1987) and later the isolation and description of *E. chaffeensis* as the causative agent (Dawson et al., 1991; Anderson et al., 1991), considerable progress has been made in delineating the natural history of this tick-borne zoonosis. A series of field and experimental studies has provided convincing evidence that white-tailed deer and the lone star tick are the principal vertebrate host and vector, respectively, for *E. chaffeensis* (Dawson et al., 1994a, b; Lockhart et al., 1995, 1996, 1997a, b; Anderson et al., 1992a, 1993; Ewing et al., 1995). However, the potential for transmission by other ticks or for infection among other hosts presently should not be excluded (Lockhart et al., 1997a). In addition to dogs and possibly red foxes, there is serologic evidence that other medium sized mammals that are commonly parasitized by nymph or adult lone star ticks also may be naturally infected (Dawson et al., 1996; Lockhart et al., 1997b). Rodents, which are common hosts for lone star tick larvae, infrequently hosts for nymphs, and rarely hosts for adults, do not appear to have significant involvement in the epidemiology of *E. chaffeensis* (Lockhart et al., 1998). If

shown to be naturally infected, red foxes and certain other seropositive medium sized mammals such as raccoons and opossums could play roles in the epidemiology of HME that deer would not readily fulfill. These medium sized mammals could serve as bridges enabling infection of other ticks such as *D. variabilis,* which rarely parasitizes deer. A similar bridging role has been suggested for domestic dogs (Dawson et al., 1996). The earlier detection of *E. chaffeensis* DNA in an adult *D. variabilis* removed from an opossum (Anderson et al., 1992a, 1993) may represent such a bridging scenario. These species of medium sized mammals also might sustain *E. chaffeensis* in some urban and suburban settings where deer are rare or absent.

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