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Experimental Infection of White-tailed Deer (*Odocoileus virginianus*) with *Ehrlichia chaffeensis* by Different Inoculation Routes

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ABSTRACT: The infection dynamics of the tick-transmitted organism *Ehrlichia chaffeensis* were investigated in white-tailed deer (*Odocoileus virginianus*) using different routes of inoculation. Six deer were each inoculated with 5.4×10^6 DH82 cells infected with *E. chaffeensis* (Arkansas strain) by three different routes: intravenous ($n=2$), subcutaneous ($n=2$), and intradermal ($n=2$). Two control deer were inoculated with uninfected cells. Infections were monitored for 54 days and were continued in one deer from each *E. chaffeensis* inoculated group for an additional 31 days. All deer inoculated with *E. chaffeensis* seroconverted (≥1:64) and became 16S rDNA polymerase chain reaction and/or cell culture positive by post-inoculation day 15. There was no apparent difference in susceptibility to infection between deer inoculated by different routes for the first 50 days based on detection of *E. chaffeensis* infection by PCR assay of blood or culture isolation. These results demonstrate infection of deer by intradermal and subcutaneous routes for the first time.

Key words: Ehrlichia chaffeensis, experimental infection, *Odocoileus virginianus,* white-tailed deer.

Since first recognized in 1991, *Ehrlichia chaffeensis* has emerged as an important tick-transmitted human disease agent in the United States. An obligate intracellular bacterium, *E. chaffeensis* is the causative agent of human monocytic ehrlichiosis (HME). *Ehrlichia chaffeensis* is transmitted via the lone star tick, *Amblyomma americanum*; white-tailed deer (*Odocoileus virginianus*) serve as the principal vertebrate reservoir host (Dawson et al., 1994b; Ewing et al., 1995). White-tailed deer were first identified as a potential reservoir host for *E. chaffeensis* in 1994, when anti-*E. chaffeensis* antibodies were detected in 43% of deer from 17 states and experimental infection was demonstrated through PCR and seroconversion (Dawson et al., 1994a, b).

To our knowledge, intravenous (IV) inoculation has been the only route used in needle inoculation of deer with *E. chaffeensis* (Dawson et al., 1994b; Ewing et al., 1995; Kocan et al., 2000; Davidson et al., 2001). Because *E. chaffeensis* is naturally transmitted through the bite of a lone star tick, intravenous injection of organism directly into blood may not suitably illustrate the true course of infection. Experimental transmission by feeding *E. chaffeensis* infected ticks on deer has been successfully accomplished (Ewing et al., 1995), but captive deer are difficult to handle and controlled tick-feeding studies are challenging and often impracticable compared to needle inoculation. Subcutaneous (SQ) and intradermal (ID) inoculations are also easy to administer, but unlike IV inoculation, SQ and ID routes may more closely mimic transmission via tick feeding because they do not introduce organisms directly into a major vein, but rather near the surface of the skin. Here we describe the course of *E. chaffeensis* infection in deer following IV, SQ, and ID inoculation.

The Arkansas strain of *E. chaffeensis* was cultivated in the continuous canine macrophage cell line, DH82, grown in 75 cm^2 culture flasks supplemented with minimal essential media (MEM) with 10% fetal bovine serum (FBS). Cultures were harvested by detaching the cell monolayer when gross cytopathic effect (CPE) was evident. Collected cells were counted using a hemocytometer and the percent infected in the *E. chaffeensis* inoculum determined using a direct fluorescent anti-

body (FA) test specific for *E. chaffeensis*. Cells were diluted in MEM with 10% FBS for a total of 5.4×10^6 *E. chaffeensis* infected or noninfected cells and used to inoculate deer. Noninfected DH82 control cells were grown and harvested in the same manner.

Eight, 3-mo-old, captive-reared white-tailed deer from Clarke County, Georgia (USA; 33°95.19′N, 83°36.60′W) were kept in a climate-controlled animal housing facility at the College of Veterinary Medicine, University of Georgia (Athens, Georgia). Prior to inoculation, all deer were determined to be seronegative to *E. chaffeensis* and *Anaplasma phagocytophilum* (the agent of human granulocytic ehrlichiosis) by indirect fluorescent-antibody test (Dawson et al., 1991), and free of detectable *E. chaffeensis*, the HGE agent and the *Ehrlichia*-like agent of deer, by nested polymerase chain reaction (PCR) (Little et al., 1998). Six deer were inoculated with 5.4×10^6 *E. chaffeensis* infected DH82 cells by one of three routes: intravenous (IV; $n=2$), subcutaneous (SQ; $n=2$), and intradermal (ID; $n=2$). Two deer served as negative controls and were inoculated intravenously with uninfected DH82 cells. Blood samples were collected for serology, nested PCR (ethylenediaminetetraacetic acid [EDTA] tube), and complete blood count (CBC; EDTA tube) immediately prior to inoculation, and on days 8, 15, 22, 29, 36, 43, and 50 post-inoculation (DPI) for the first 54 days of the trial. One deer exposed to *E. chaffeensis* from each inoculation group was maintained for an additional 31 days and euthanatized on DPI-85. Blood samples from these three deer were collected for nested PCR on DPI-61, 68, 78, and 85; the other three deer were removed for use in a separate study. Blood samples for culture isolation of *E. chaffeensis* were collected on all of the above days except DPI-22 and 36. All deer were inoculated and blood samples collected while under manual restraint. Deer were monitored for clinical signs of infection throughout the study period.

For serology, deer were pre-screened for the presence of *E. chaffeensis*-reactive and HGE agent-reactive antibodies at a serum dilution of 1:64 using *E. chaffeensis* and HGE antigen slides obtained from Focus Technologies (formerly MRL Diagnostics, Cypress, California, USA). After inoculation, anti-*E. chaffeensis* antibodies were measured by an indirect fluorescent-antibody test as previously described (Dawson et al., 1991). Samples were screened at a serum dilution of 1:64 and positive samples were serially diluted 2-fold. For both pre- and post inoculation samples, a 1:50 dilution of fluorescein isothiocyanate-labeled rabbit anti-deer immunoglobulin G was used as the conjugate (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Maryland, USA).

DNA was extracted from 100 μl EDTA-anti-coagulated whole blood using a GFX Genomic Blood Purification Kit (Amersham Pharmacia Biotech, Piscataway, New Jersey, USA). Extracted DNA was used as the template to test for the presence of *Ehrlichia* spp. 16S rDNA in a nested PCR (Little et al., 1998). *Ehrlichia*-wide primers ECC and ECB were used with 10 μl template DNA in a primary reaction, and 1 μl of these products was amplified using *E. chaffeensis* specific primers HE1 and HE3 in a secondary reaction. For detection of the HGE agent and *Ehrlichia*-like deer agent in pre-screening samples, primers GA1UR and GE9F were used in the secondary reaction. Amplified products were visualized on ethidium bromide stained 2% agarose gels via UV trans-illumination. To prevent contamination, DNA extraction, primary amplification, secondary amplification, and product analysis were performed in separate laboratories. A negative water control was included in DNA extraction and for each set of reactions in primary and secondary PCR.

For isolation of *E. chaffeensis*, 5–7 ml of aseptically collected whole blood in EDTA was transferred into sterile 50 ml centrifuge tubes containing 30 ml of ACE lysing buffer (150 mM NH_4Cl, 0.7 mM KH_2PO_4,

3 mM EDTA-Na_2). Tubes were gently inverted to lyse red blood cells and then centrifuged at 2,500 rpm for 10 min to obtain a white blood cell (WBC) pellet. Supernatant was discarded and the pellet was washed in 15 ml of fresh ACE lysing buffer and centrifuged again. The washed WBC pellet was re-suspended in 1 ml of cell growth medium (MEM supplemented with 10% FBS) and overlaid on a confluent culture of DH82 cells in a 12.5 cm^2 flask with 5 ml medium. Cultures were given fresh medium twice weekly and monitored for evidence of CPE, or for a maximum of 45 days. Cultures showing CPE and cultures negative after 45 days were harvested with a cell scraper and tested by direct FA test as previously described (Lockhart et al., 1997b).

Ehrlichia chaffeensis was isolated from the blood of each deer at least twice during the initial 54 day study period and as late as day 85 in both SQ and IV inoculated deer maintained for the 31 additional days (Table 1). Positive cell cultures evident by gross CPE and negative cultures showing no CPE for 45 days were consistently confirmed using the direct FA test. Polymerase chain reaction evidence of circulating *E. chaffeensis* was found at least three times in each inoculated deer during the initial 54 day trial but was only detected in the SQ inoculated deer beyond day 50 of the trial. There was no apparent difference in conversion to positive by PCR detection in blood or cell culture isolation of *E. chaffeensis*, between the ID, IV, or SQ groups of inoculated deer for the first 54 days of the infection.

All six deer exposed to *E. chaffeensis* by SQ, ID, or IV routes seroconverted (≥1:64) by DPI-15 and remained seropositive in weekly tests during the first 50 days of the study. Antibodies (1:128) were detected in one IV and one ID inoculated deer on DPI-8. Peak geometric mean antibody titer (GMT=1,024) for IV and SQ routes occurred on DPI-29 and 36, respectively, and on DPI-36 and 43 for deer inoculated intradermally (Fig. 1). Deer that were in-

TABLE 1. Detection of *E. chaffeensis* by polymerase chain reaction and cell culture isolation[a] for six white-tailed deer inoculated by three routes[b].

Route[c]	WTD number	Day post-inoculation										
		8	15	22	29	36	43	50	61	68	78	85
SQ	119	+ (−)	+ (−)	+	− (+)	−	− (+)	+ (−)	− (−)	+ (+)	+ (+)	+ (+)
	118	+ (+)	+ (+)	+	+ (c)[d]	+	+ (+)	− (−)	ND[d]	ND	ND	ND
ID	116	+ (+)	+ (−)	+	− (+)	−	− (+)	− (c)	− (−)	− (−)	− (−)	− (−)
	121	+ (+)	+ (−)	+	+ (c)	−	+ (+)	+ (−)	ND	ND	ND	ND
IV	122	+ (+)	+ (+)	+	+ (c)	−	− (−)	− (−)	− (−)	− (−)	− (−)	− (+)
	123	+ (+)	+ (+)	+	+ (+)	+	+ (−)	− (+)	ND	ND	ND	ND

[a] Results of cell culture isolation are shown in parentheses, when attempted.
[b] Two control deer (numbers 82 and 117) were negative on all tests through DPI 50 at which time they were removed from the study.
[c] Route: SQ = subcutaneously, ID = intradermally, IV = intravenously.
[d] c = culture contaminated; ND = not done (deer were removed for separate study).

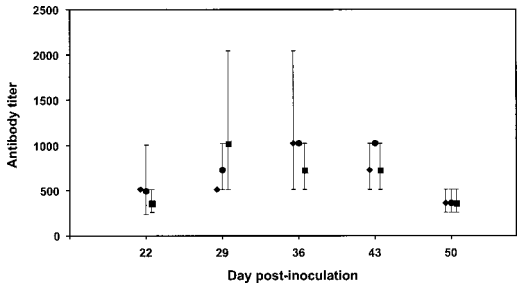

FIGURE 1. Indirect fluorescent antibody (IFA) titers for six white-tailed deer inoculated subcutaneously (n=2; ♦), intradermally (n=2; ●), and intravenously (n=2; ■). For DPI-15 through DPI-50, geometric mean titers (GMT) for both deer are shown as symbols for each inoculation group, except for the SQ group on DPI-15 when only one deer sample was available for titering; the other SQ deer was seropositive at 1:64 but a titer was not determined. Individual deer titers, if different from GMT, are shown as standard error bars. For DPI-8, GMTs were not calculated because all deer were negative except one ID and one IV inoculated deer; symbols at DPI-8 represent results for individual deer.

oculated with uninfected DH82 cells were consistently seronegative, culture negative, and PCR negative. No clinical signs (such as decrease in appetite, lethargy, fever) or hematologic abnormalities were observed in any deer during the trial. All deer were seronegative and PCR negative for all organisms tested for during pre-screening.

In previous studies of *E. chaffeensis*, needle injection of organisms into experimental vertebrate hosts, such as white-tailed deer or dogs, has relied on the IV route (Dawson and Ewing, 1992; Dawson et al., 1994b; Kocan et al., 2000; Davidson et al., 2001; Felek et al., 2001). Natural transmission of *E. chaffeensis*, however, occurs through the bite of a lone star tick (Anderson et al., 1993; Lockhart et al., 1997b), a route that may be more closely simulated by intradermal or subcutaneous inoculation. However, our data show no difference in the time course of *E. chaffeensis* rickettsemia between the artificial routes used; IV, ID, or SQ inoculation routes should all be appropriate for investigating the infection dynamics of *E. chaffeensis* in white-tailed deer.

In a previous study investigating the persistence of *E. chaffeensis* infection in intravenously inoculated deer, detection of *E. chaffeensis* organisms was intermittent over the 9 mo study period, evident by inconsistent PCR results and culture isolation of organisms (Davidson et al., 2001). Other experimental studies also detected *E. chaffeensis* inconsistently in needle-inoculated deer over time (Dawson et al., 1994b; Ewing et al., 1995). Similar findings were observed in this study for deer, regardless of inoculation route. Although *E. chaffeensis* was regularly detectable by at least PCR assay through DPI-22, evidence of circulating organisms became sporadic in some deer after that time. This was most noticeable in one intradermally inoculated deer that remained blood PCR negative on all sample days beyond DPI-22, and in a single IV and single ID deer that were negative on at least three sampling dates before *E. chaffeensis* was again detected by PCR of blood. Culture isolation was similarly variable and was not consistent with PCR results. The intradermally inoculated deer that was sampled only for 50 days was also negative for one sample day, DPI-36, despite showing evidence of *E. chaffeensis* on all other sample days.

Although infected deer in this study were monitored for only 54 or 85 days, findings presented in this study lend further support to existence of a recrudescent rickettsemia in deer (Davidson et al., 2001) and typical of ehrlichial infections in other vertebrate reservoirs (Rikihisa, 1991). The findings that antibodies were present (≥1:64) after DPI-15 in all deer exposed to *E. chaffeensis* throughout the 85 day study, and that antibody titers rose and fell 1–2 mo post-infection (Fig. 1), are similar to the serologic response previously described for deer experimentally infected with a high dose inoculum via the intravenous route (Davidson et al., 2001). Because this was observed in deer inoculated by SQ, IV, and ID routes, it is likely that this phenomenon occurs independent of

the means by which these organisms reach the blood.

Discrepancies between PCR detection and cell culture isolation, such as those observed in this study, have been previously encountered (Ewing et al., 1995; Davidson et al., 2001). One reason for this may be inherent difficulty in culturing ehrlichiae (Walker and Dumler, 1996). Although DH82 cells, a canine macrophage cell line, are commonly used to isolate *E. chaffeensis* in both human cases of HME and infected deer (Dawson et al., 1991, 1994b; Dumler et al., 1995; Ewing et al., 1995; Lockhart et al., 1997a, b; Little et al., 1998; Davidson et al., 2001), variable success has been encountered when using DH82 cells for isolation from experimentally infected deer (Dawson et al., 1994b; Ewing et al., 1995). The possibility that PCR assay may have detected non-viable *E. chaffeensis* in blood samples must also be considered. In comparison to cell culture, PCR only tests 10 μl of extracted blood, whereas 5–7 ml of blood are used to isolate *E. chaffeensis* in cell culture. Thus, the chances of including infected monocytes, the blood cell that *E. chaffeensis* invades, are greater in cell culture assays.

Results from this study show that IV, SQ, and ID routes of inoculation will cause infection in the principal vertebrate host of *E. chaffeensis,* white-tailed deer, suggesting that any of these routes is suitable for future studies investigating infection in deer. However, results of cell culture isolation and PCR detection should be interpreted with caution, considering the limitations of both assays. Comparison of the course of infection in deer via tick transmission is essential to determine whether any of the artificial routes are a suitable model to understanding natural infection in white-tailed deer.

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