

Infection Rates of *Amblyomma americanum* (Acari: Ixodidae) by *Ehrlichia chaffeensis* (Rickettsiales: Ehrlichieae) and Prevalence of *E. chaffeensis*-Reactive Antibodies in White-Tailed Deer in Southern Indiana, 1997

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ABSTRACT To monitor the percentage and stability of *Ehrlichia chaffeensis*-infected ticks in southern Indiana over time, pools of *Amblyomma americanum* (L.) ticks were screened for infection in southern Indiana for a 2nd time. Nested polymerase chain reaction (with 6% DMSO included only in the 2nd reaction) was performed on 920 ticks in pools of 5 individuals from 9 sites (5 sites previously examined and 4 new ones) in 6 counties. The average minimum infection rate for all sites for 1997 was 1.6%, lower than that of 4.9% previously observed for 1995. However, when only the 5 sites that were positive for infected ticks in 1995 were reexamined, the average minimum infection rate was even more disparate (1.4% in 1997 and 5.1% in 1995). To correlate the presence of infected ticks with the presence of exposed deer, which serve as a reservoir, dried blood samples collected from hunter-killed deer at 2 locations in southern Indiana were tested for *E. chaffeensis*-reactive antibodies using an indirect immunofluorescent assay. Antibodies were detected in 45 and 47% of 98 samples examined from the 2 stations. These data provide support to our previous report of a population of *E. chaffeensis*-infected *A. americanum* in southern Indiana and the high proportion of deer previously exposed to *E. chaffeensis* suggests a stable maintenance of *E. chaffeensis* in this tick-vertebrate zoonotic system.

KEY WORDS *Ehrlichia chaffeensis*, *Amblyomma americanum*, white-tailed deer, infection rate, Indiana, polymerase chain reaction

Ehrlichia chaffeensis is the causative agent of human monocytic ehrlichiosis (HME). HME is a tick-borne disease that is transmitted primarily by the lone star tick, *Amblyomma americanum* (L.), that occurs in the south central and eastern United States (Dumler and Bakken 1998). Previous studies have determined the existence of a geographic relationship between *A. americanum* infestations and *E. chaffeensis*-reactive antibodies among white-tailed deer, *Odocoileus virginianus*, in the southeastern United States (Lockhart et al. 1996). Recent evidence (Lockhart et al. 1997) has confirmed white-tailed deer as a natural reservoir host for the bacterium.

We recently reported relatively high minimal infection rates of pools of *A. americanum* ticks by *E. chaffeensis* collected in 1995 in southern Indiana (Burket et al. 1998). The average minimal infection rate observed at that time (4.9%) was somewhat higher than reported for Missouri (1.18%), North Carolina (1.15%), and Kentucky (3.57%) (Anderson et al. 1993). The purposes of the current study were to compare the minimum infection rates in ticks for the same general region in 1997 (5 sites previously sampled were examined as well as 4 new sites). The combined data will add to a database being established on the minimal infection rates in various counties in southern Indiana, permitting us to report to physicians

and epidemiologists how the rates of infection fluctuate over time and changes in the distribution of infected ticks. If *E. chaffeensis*-infected ticks are being stably maintained in southern Indiana, we anticipated that deer in the area would serve as the reservoir host as has been seen previously by Lockhart et al. (1997). Therefore, we tested for the presence of *E. chaffeensis* antibodies in dried blood samples from deer collected in southern Indiana using an indirect fluorescent-antibody (IFA) assay and determined the percentage of deer previously exposed to the bacterium.

Materials and Methods

Indirect Fluorescent-Antibody Assays. Blood samples were collected from hunter-harvested deer brought to 2 deer checking stations in southern Indiana in November 1997: (1) Eckerty station in Crawford County, and (2) Boonville station in Warrick County. The deer sampled at Eckerty had been killed in 5 different southern Indiana counties: Crawford, Perry, Warren, Orange, and Dubois. The deer sampled at Boonville had been killed in Warrick and Spencer counties (for a map of Indiana counties, see Burket et al. [1998]).

A previously described procedure (Stallknecht and Davidson 1992) was modified and used to obtain dried

blood samples from the deer. Briefly, blood was collected from the thoracic inlet or cavity within 3–4 h of death by immersing a paper strip (1 by 2 cm) in blood for a short time. Samples were dried at room temperature for 4–7 h, then completely dried with a hair dryer at low setting. Strips were stored in individual, sealed plastic tubes at 4°C, no longer than 2 mo. Elution of antibodies was performed as previously described (Stallknecht and Davidson 1992).

The IFA assay was used to test for the presence of antibodies to *E. chaffeensis* (Dawson et al. 1991). Antigen consisting of *E. chaffeensis*-infected DH82 canine macrophages (supplied by L. M. Lockhart, Valdosta State University, Valdosta, GA) was used at a 10-fold greater concentration (as per L. M. Lockhart) than reported by Dawson et al. (1991). The optimized antigen dilution for screening the samples was determined by testing samples at 1/20, 1/40, and 1/80. We found background fluorescence high with the 1/20 dilution and had more difficulty detecting positive samples at a 1/80 dilution. Therefore, all subsequent samples used a 1:40 dilution in 0.01 M PBS (similar to the optimal dilution of 1/40 reported by Stallknecht and Davidson 1992). Fluorescein isothiocyanate-labeled rabbit anti-deer immunoglobulin G (Kirkegaard and Perry, Gaithersburg, MD) diluted 1:100 in 0.01 M PBS was used as the antibody conjugate. A positive control serum was provided by M. L. Lockhart. Control dried blood samples presumed to be negative for antibodies were obtained from 6 hunter-killed deer from northern Indiana, outside the normal range of *A. americanum* (R.R.P., unpublished data). All preparations were examined by epifluorescence microscopy. Positive samples showed pinhead size fluorescing bodies inside cellular membranes, whereas no fluorescence or only faint background fluorescence was observed in negative preparations.

Tick Collection. A total of 925 *A. americanum* ticks was collected during the 1st and 2nd wk of May 1997. The counties surveyed were Pike, Orange, Perry, Warrick, Spencer, and Crawford. Ticks were collected from all sites previously sampled that were positive for infected ticks in 1995 (Burket et al. 1998) and from an additional 4 sites in neighboring counties.

Template Preparation and PCR Analysis. DNA was extracted from pools of ticks using the modified CTAB procedure described previously (Burket et al. 1998). PCR amplifications were performed in a Perkin Elmer 2400 thermal cycler (Foster City, CA). Extracted DNA was used as a template for nested PCR amplification of the 16S ribosomal RNA gene of *E. chaffeensis* as previously described (Dawson et al. 1994). The PCR reaction was optimized as follows: 5 μ l of extracted DNA was included in a 50 μ l reaction mixture containing 10 mM Tris-HCl (pH 9.0) 50 mM KCl, 0.1% Triton X-100, 200 μ M each of dNTPs, 2.5 mM MgCl₂, 1.25 U *Taq* DNA polymerase (Promega, Madison WI) and 0.6 μ M each primer. The primers used in the initial reaction were ECB (5'-CGTATTACCGCGGCTGCTGGCA-3') and ECC (5'-AGAACGAACGCTGGCGGCAAGCC-3'). These primers amplify a DNA fragment common among all known species of *Ehrlichia*

Table 1. IFA results of dried blood samples from 2 southern Indiana deer checking stations

Station	Counties of origin	No. of deer dried blood samples tested	% IFA positive
Eckerty	Crawford, Perry, Orange, Warren, Dubois	55	45%
Boonville	Warrick, Spencer	34	47%

and a few other bacterial species (Dawson et al. 1994). The following temperature profile was run for 30 cycles: 15 s at 94°C, 30 s at 48°C, and 30 s at 72°C, with a final extension of 5 min at 72°C.

For the 2nd PCR amplification in the nested protocol, 1 μ l of the product from the 1st amplification was run in a 50 μ l reaction mixture as described above but with the addition of 6% dimethylsulfoxide (DMSO) and 0.3 μ M of primers HE1 and HE3. These primers amplify a 389-bp fragment specific to *E. chaffeensis* DNA (Anderson et al. 1993), which is contained within the larger PCR product of the 1st amplification. The identity of the PCR products were confirmed by restriction fragment-length polymorphism (RFLP) analysis (Lockhart et al. 1997). A positive control provided by Rob Massung (CDC, Atlanta, GA) was included (25 ng of pTD2 which contains the 5' end [767 bp] of the 16S rRNA gene cloned into pUC19).

Results

Indirect Fluorescent-Antibody Assays. A total of 55 and 34 dried blood samples was collected at Eckerty and Boonville stations, respectively (see Table 1). Antibodies to *E. chaffeensis* were detected in 25 (45%) of the samples collected at Eckerty and 16 (47%) of the samples collected at Boonville.

Polymerase Chain Reaction Determinations of Minimum Infection Rates. A PCR analysis followed by Southern blotting was used to calculate the minimum infection rate in pools of ticks collected in southern Indiana in 1995 (Burket et al. 1998). The calculations assumed that only a single tick was positive in any tick pool yielding an amplified band of appropriate size. In the current study a nested PCR approach was used to determine minimum infection rates in 4 new sites (Pike 1, 2.1%; Pike 2, 0%; Orange, 0%; Perry, 4.0%). In addition, the minimum infection rates of 5 previously examined sites was determined (Warrick 1, 0%; Warrick 2, 2.1%; Spencer 1, 0%; Spencer 2, 2.5%; and Crawford, 0%). (See Table 2 for a comparison of minimum infection rates per county per year sampled).

Preliminary experiments suggested that the addition of 6% DMSO to PCR reactions yielded clearer amplification products. The addition of DMSO is believed to enhance PCR reactions by facilitating strand separation of the template and disrupting base pairing that can lead to secondary structures (Frackman et al. 1998). Fig. 1 shows the results of an experiment designed to examine the efficacy of inclusion of DMSO in our PCR reactions. Four reactions were compared for amplification: (1) a 2-step nested PCR using both

Table 2. Comparison of counties surveyed and minimum infection rates of *E. chaffeensis* from pools of *A. americanum* in southern Indiana for 1995 and 1997

County	1997				1995
	Total no. of ticks	No. of pools	No. of positive pools	Min. infection rate	Min. infection rate
Warrick 1	40	8	0	0	5/52 (9.4%)
Warrick 2	340	68	7	7/340 (2.1%)	8/174 (4.6%)
Spencer 1	45	9	0	0	2/25 (8.0%)
Spencer 2	40	8	1	1/40 (2.5%)	2/57 (3.5%)
Crawford	100	20	0	0	4/105 (3.8%)
Pike 1	95	19	2	2/95 (2.1%)	-
Pike 2	90	18	0	0	-
Orange	70	14	0	0	-
Perry	100	20	5	5/100 (5.0%)	-
Harrison	-	-	-	-	0/11 (0%)
Posey	-	-	-	-	0/5 (0%)
Total average infection rate	-	-	-	15/920 (1.6%)	21/430 (4.9%)
Avg infection rate for 5 sites sampled both years	-	-	-	8/565 (1.4%)	21/413 (5.1%)

sets of primers without DMSO, (2) a reaction with 6% DMSO added only to the 2nd amplification using HE1/HE3 primers, (3) a reaction with 6% DMSO added to both amplification steps, and (4) a reaction in which 6% DMSO was added only to the initial amplification using the ECB/ECC primers. The conditions that produced the clearest 389-bp product were the inclusion of 6% DMSO only in the amplification using HE1/HE3. Therefore, all subsequent reactions to determine infection rates were done in this manner.

An example of a gel showing the results of nested amplification with the *E. chaffeensis*-specific HE1/

HE3 primers and using the conditions just described can be seen in Fig. 1. In addition, the restriction digests of these PCR products producing 338- and 51-bp products as predicted for the *E. chaffeensis* 16S r RNA gene are shown in Fig. 2.

Discussion

This study corroborates the role of white-tailed deer as a reservoir host for *E. chaffeensis* in southern Indiana. Previous work has shown that 43–70% of deer sampled were seropositive for *E. chaffeensis*-reactive antibodies and even higher values were observed in

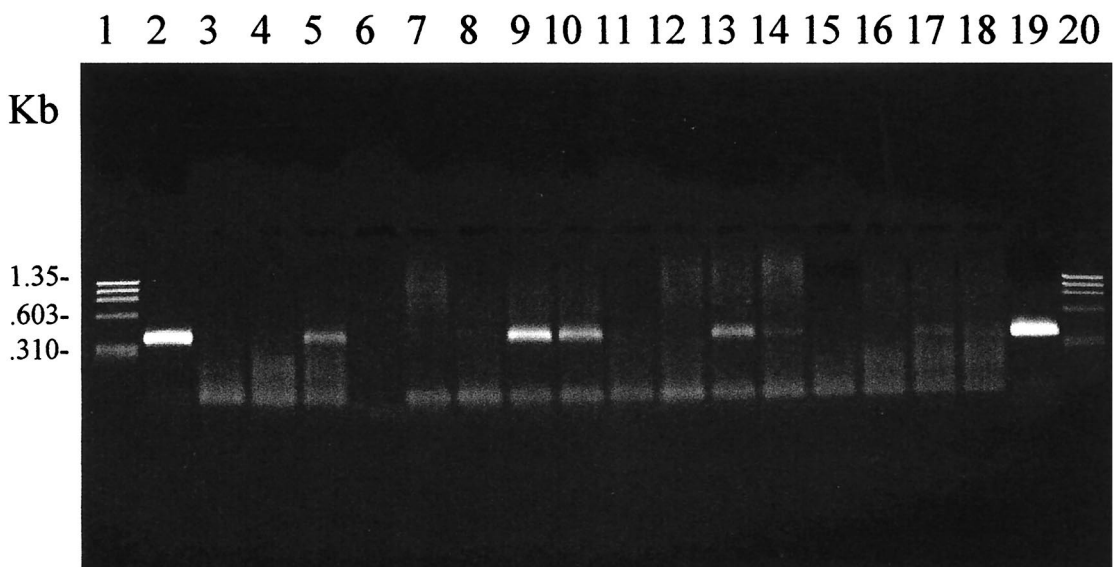


Fig. 1. Agarose gel electrophoresis of nested PCR products (15 of 50 μ l reactions) amplified from 3 tick pools (9, 39, and 49) collected from Warrick County. Lane 1: 0.5 μ g ϕ X174/*Hae*III marker. Lane 2: positive control. Lane 3: a negative control (no DNA) without DMSO. Lanes 4–6: no DMSO in amplifications performed on the 3 pools. Lane 7: a negative control with DMSO only in the 2nd PCR reaction. Lanes 8–10: DMSO only in the 2nd PCR reaction of the 3 pool. Lane 11: a negative control with DMSO in both PCR reactions. Lanes 12–14: DMSO in both PCR reactions of the 3 pools. Lane 15: a negative control with DMSO only in the 1st PCR reaction. Lanes 16–18: DMSO only in the 1st PCR reaction of the 3 pools. Lanes 19–20: repeated positive control and marker.

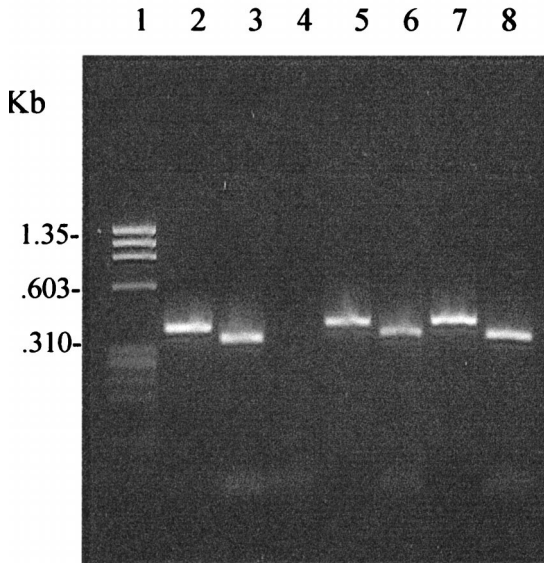


Fig. 2. RFLP analysis using *Hae*III digestion of DNA isolated from tick pools obtained in 2 counties. Analysis was performed by agarose gel electrophoresis of digested, nested PCR products (10 of a 50 μ l reaction) obtained after amplification with primers ECB and ECC in the 1st round and primers HE1 and HE2 (with 6% DMSO) in the 2nd round. Lane 1: 0.5 μ g ϕ X174/*Hae*III marker. Lane 2: positive control (undigested). Lane 3: positive control digested. Lane 4: pool #126 of Pike County, digested (because this sample was negative for *E. chaffeensis*, it served as a negative control). Lane 5: pool #124 of Pike county, undigested. Lane 6: pool #124 of Pike County, digested. Lane 7: pool #170 of Perry County, undigested. Lane 8: pool #170 of Perry County, digested.

areas heavily infested with *A. americanum* (Dawson et al. 1994). The seropositive rates we observed in southern Indiana during this study (45–47%) are within this range and the location of positive samples correlates well with the established geographic distribution of *E. chaffeensis*-infected *A. americanum* ticks (Burket et al. 1998) and with the occurrence of HME in Indiana (Indiana Department of Health 1997).

The use of dried blood for serologic testing is well documented (Stallknecht and Davidson 1992, Fiscus et al. 1998). Here we apply this technique to study antibodies to *E. chaffeensis* in deer. There are several advantages to using this technique for blood collection. Samples are easy to collect, a minimum of personnel training is required, and samples can be collected from dead animals (Stallknecht and Davidson 1992). Although the blood present in the animal thoracic cavity is often mixed with other body fluids, antibodies were readily detected in the samples, as described in this study.

Variations in the minimum infection rates were observed both between sites in different counties in a single sampling year and from the same sampling site examined in 1995 as compared with 1997. The average minimum infection rate observed in this study was lower than that found in 1995 when all sites are com-

pared (15/925 = 1.6% versus 21/430 = 4.9%) (see Table 2). This might be anticipated because 4 new sites were included that were nearby but were not known to have infected ticks. However, comparing just the 5 sites (Warrick 1 and 2, Spencer 1 and 2, and Crawford) sampled both years also yielded a lower average minimum infection rate in 1997 as compared with 1995 (8/565 = 1.4% versus 21/413 = 5.1%). These differences could be explained by natural variations in infection of ticks/deer by bacteria from one year to the next, the low proportion of total ticks examined, or by the clustering of infected ticks and the random sampling method employed. One purpose of the repeated sampling of previously positive sites was to determine the extent of yearly variation (including our ability to measure it). It should also be pointed out that the lower 1997 minimum infection rates are more similar to those previously reported in other nearby states (Anderson et al. 1993). The confirmation of *E. chaffeensis*-infected *A. americanum* present in the region a 2nd yr at 4 of the 9 sites sampled, in addition to the observation that a significant number of deer sampled had been previously exposed to the bacterium, support our contention that a permanent infestation of the bacterium in *A. americanum* has been established in southern Indiana. These data provide baseline information for ongoing studies examining changes in the distribution or density of infection in the region.

The change to nested PCR was not responsible for the variation observed in comparing 1995 and 1995 minimal infection rates. The addition of 6% DMSO to the nested step (Fig. 1) increased the sensitivity of the technique. We found that nested PCR in the presence of DMSO was comparable in sensitivity to our previous approach which used a single round of PCR followed by Southern hybridization but that the former was less time consuming (results not shown), providing more rapid analysis of tick pools.

In conclusion, we detected antibodies in deer to *E. chaffeensis* by using IFA on dried blood samples collected from hunter-killed deer, an extremely useful approach when the facilities to collect serum from numerous live deer are not available. The high percentage of deer positive for antibodies (46%) in addition to average minimum infection rates of ticks in 1995 of 4.9% and in 1997 of 1.7% provide evidence of the stable maintenance of *E. chaffeensis* in a tick-vertebrate zoonotic system in southern Indiana. Obtaining data such as these periodically is important for state officials and health care practitioners to monitor the distribution of infected ticks and to evaluate the potential for human disease.

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References Cited

- Anderson, B. E., K. G. Sims, J. G. Olson, J. E. Childs, J. F. Piesman, C. M. Happ, G. O. Maupin, and B. J. Johnson. 1993. *Amblyomma americanum*: a potential vector of human ehrlichiosis. *Am. J. Trop. Med. Hyg.* 49: 239–244.
- Burket, C. T., C. N. Vann, R. R. Pinger, C. L. Chatot, and F. E. Steiner. 1998. Minimum infection rate of *Amblyomma americanum* (Acari: Ixodidae) by *Ehrlichia chaffeensis* (Rickettsiales: Ehrlichieae) in southern Indiana. *J. Med. Entomol.* 35: 653–659.
- Dawson, J. E., B. E. Anderson, D. B. Fishbein, J. L. Sanchez, C. S. Goldsmith, K. H. Wilson, and C. W. Duntley. 1991. Isolation and characterization of an *Ehrlichia* sp. from a patient diagnosed with human ehrlichiosis. *J. Clin. Microbiol.* 29: 2741–2745.
- Dawson, J. E., D. E. Stallknecht, E. W. Howerth, C. Warner, K. Biggie, W. R. Davidson, J. M. Lockhart, V. F. Nettles, J. G. Olson, and J. E. Childs. 1994. Susceptibility of white-tailed deer (*Odocoileus virginianus*) to infection with *Ehrlichia chaffeensis*, the etiologic agent of human ehrlichiosis. *J. Clin. Microbiol.* 32: 2725–2728.
- Dumler, J. S., and J. S. Bakken. 1998. Human ehrlichiosis: newly recognized infections transmitted by ticks. *Annu. Rev. Med.* 49: 201–213.
- Fiscus, S. A., D. Brambilla, L. Grosso, J. Schock, and M. Cronin. 1998. Quantitation of human immunodeficiency virus type 1 RNA in plasma by using blood dried on filter paper. *J. Clin. Microbiol.* 36: 258–260.
- Frackman, S., G. Kobs, D. Simpson, and D. Storts. 1998. Betaine and DMSO: enhancing agents for PCR. *Promega Notes* 65: 27–29.
- Indiana State Department of Health. 1997. Indiana report of diseases of public health interest. Indiana State Department of Health, Indianapolis, IN.
- Lockhart, J. M., W. R. Davidson, D. E. Stallknecht, and J. E. Dawson. 1996. Site-specific geographic association between *Amblyomma americanum* (Acari: Ixodidae) infestations and *Ehrlichia chaffeensis*-reactive (Rickettsiales: Ehrlichieae) antibodies in white-tailed deer. *J. Med. Entomol.* 33: 153–158.
- Lockhart, J. M., W. R. Davidson, D. E. Stallknecht, J. E. Dawson, and E. W. Howerth. 1997. Isolation of *Ehrlichia chaffeensis* from white-tailed deer (*Odocoileus virginianus*) confirms their role as natural reservoir hosts. *J. Clin. Microbiol.* 35: 1681–1686.
- Stallknecht, D. E., and W. R. Davidson. 1992. Antibodies to blue tongue and epizootic hemorrhagic disease viruses from white-tailed deer blood samples dried on paper strips. *J. Wildl. Dis.* 28: 306–310.

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