

Distribution of *Ehrlichia chaffeensis* (Rickettsiales: Rickettsiaceae) in *Amblyomma americanum* in Southern Indiana and Prevalence of *E. chaffeensis*—Reactive Antibodies in White-Tailed Deer in Indiana and Ohio in 1998

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ABSTRACT To continue monitoring the prevalence and distribution of *Ehrlichia chaffeensis* (Rickettsiales: Rickettsiaceae) in southern Indiana, a total of 498 *Amblyomma americanum* (L.) ticks (262 adults and 292 nymphs) was collected from five southern Indiana counties during May and June 1998. Ticks were pooled and examined for the presence of *E. chaffeensis* using nested polymerase chain reaction and primers specific for the 16S rRNA gene of *E. chaffeensis*. The average minimum infection rate for adult ticks collected in 1998 was 3.8% (ranging from 0 to 7.7% in various counties) as compared with previous average minimum infection rates of 1.6% in 1995 and 4.9% in 1997. None of the pools of *A. americanum* nymphs tested positive. In addition, blood samples were collected from 325 white-tailed deer taken in Indiana and 327 taken in Ohio in November 1998. Serum samples were tested for the presence of *E. chaffeensis*-like organisms reactive to antibodies using an indirect immunofluorescence assay (IFA). Antibodies were found in deer from six Indiana counties where infection rates ranged from 42.6 to 66.7% and in four Ohio counties where infection rates ranged from 4.4 to 25%. The results of this study reconfirm that *E. chaffeensis* is well established in southern Indiana and also provide the first evidence of *E. chaffeensis*-like organisms infecting white-tailed deer in Ohio, suggesting the need to survey Ohio ticks for the presence of *Ehrlichia*.

KEY WORDS *Amblyomma americanum*, *Ehrlichia chaffeensis*, polymerase chain reaction, indirect immunofluorescence assay, human monocytic ehrlichiosis

HUMAN MONOCYtic EHRLICHIOSIS (HME) is a tick-borne infectious disease caused by the bacterium *Ehrlichia chaffeensis* (Anderson et al. 1991, 1992; Dawson et al. 1991). The disease causes a flu-like illness with wide-ranging symptoms that, if left untreated, can lead to hospitalization and, on rare occasions, ultimately to death (Fishbein et al. 1994). More than 1,500 cases have been reported worldwide from Europe, Africa, and the United States (Chen et al. 1997, Lockhart et al. 1997a). In Indiana, 24 confirmed cases have been reported from 11 counties since the first case was reported in 1994 (Indiana State Department of Health 1998). No cases have been reported from the neighboring state of Ohio (R. L. Berry, Department of Health, Columbus, OH, personal communication).

The distribution of HME cases generally coincides with the distribution of its principal vector, *Amblyomma americanum* (L.), the lone star tick (Lockhart et al. 1997a). The natural reservoir of *E. chaffeensis* is white-tailed deer, *Odocoileus virginianus* Zimmerman (Lockhart et al. 1997b), a preferred host of *A. americanum* now abundantly distributed throughout most of the tick's range. Previous studies in Indiana by Burket et al. (1998) and by Steiner et al. (1999) have

established the presence of *E. chaffeensis* in both lone star ticks and in white-tailed deer in southern Indiana.

The goals of this study were to provide annual data on the distribution and prevalence of *E. chaffeensis* in ticks in Indiana, to examine a greater number and wider distribution of deer serum samples in Indiana for the presence of antibodies to *E. chaffeensis*-like organisms, and to screen deer serum samples in Ohio for the antibodies that might support a similar recent invasion of *Ehrlichia* into Ohio. The study was performed by determining the *E. chaffeensis* minimum field infection rates of *A. americanum* adults and nymphs collected in southern Indiana using nested polymerase chain reaction (PCR), and by establishing the prevalence of *E. chaffeensis*-like organisms reactive to antibodies in blood samples collected from hunter-harvested deer in each state using an indirect immunofluorescence assay (IFA).

Materials and Methods

Tick Collection and Preservation. During the final week of May 1998, ticks were collected from eight sites in five Indiana counties (Perry, Orange, Pike, Spencer, and Crawford). Ticks were collected from vegetation by dragging a 1 m² white corduroy cloth along trails and through fields, particularly at the periphery of wooded areas (Sonenshine 1979). After dragging for

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≈20 m (the distance varied inversely with the density of ticks), the cloth was examined and attached ticks were collected with forceps and placed into 50-ml screw-cap centrifuge tubes. An X was cut in the top of each lid so that ticks could be introduced into the tubes without removing the lid. The tubes were labeled and placed in a cooler for transportation to the laboratory where they were sorted by species, developmental stage, and collection site. The ticks were then pooled into groups of five adults or 10 nymphs. Each pool was appropriately labeled, placed in a heat-sealable plastic bag, and stored at -80°C until DNA extraction was performed.

Template Preparation and PCR Analysis. DNA was extracted from the pools of ticks or nymphs by homogenization in a high-salt/cetyltrimethylammonium-bromide (CTAB) solution (Doyle and Doyle 1990), which was followed with a phenol/chloroform extraction (Burket et al. 1998). Nested PCR amplifications were performed as previously described (Dawson et al. 1994b) in a Perkin Elmer 2400 thermal cycler (Norwalk, CT). The primers used in the initial PCR reaction were ECB and ECC, which amplify DNA common among all known species of *Ehrlichia* and a few other bacterial species (Dawson et al. 1994b). Subsequently, primers HE1 and HE3 were used for nested PCR amplification of the 16S ribosomal RNA (rRNA) gene specific to *E. chaffeensis* (Anderson et al. 1992). Specifics of the nested PCR protocol may be found in Steiner et al. (1999). As a positive control, 25 ng of pTD2 (which contains the 5' end [767 bp] of the *E. chaffeensis* 16S rRNA gene cloned into pUC19) was run with each reaction (provided by Rob Massung, CDC, Atlanta, GA).

Gel Electrophoresis. The final products of the nested PCR reactions were separated through a 1.5% agarose gel stained with ethidium bromide (50 ng/μl) as previously described (Steiner et al. 1999). Gels were photographed using the Gel Print 2000 Imaging System (Biophotonics, Pittsfield, MA).

Deer Serum Sample Collection. In November 1998, blood samples were obtained from 652 hunter-harvested deer brought to official check stations in southern Indiana and Ohio. All samples were labeled with the state and county from which the deer were harvested. A previously described procedure (Stallknecht and Davidson 1992, Steiner et al. 1999) was used to obtain dried blood samples from deer. The blood was collected from the thoracic cavity of the deer within several hours of its death by immersing a 1 by 2-cm filter paper strip into pooled blood for a few seconds. Samples were completely dried with a hair drier, cut into four equal pieces, and stored in 1.8-ml microcentrifuge tubes with holes in the lids. All tubes were placed in a desiccated container and stored at 4°C.

IFA Antigen Preparation and Assays. To prepare antigen for IFA, *E. chaffeensis* was cultured in DH82 canine macrophages using a modification of the procedure employed by Dawson et al. (1991). DH82 macrophage cells (American Type Culture Collection, Rockville, MD) were grown in sterile, vented-cap T75

cell culture flasks containing minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 1% L-glutamine (200 mM). Cultures were maintained at 5% CO₂ in a humid 37°C incubator.

Growth of cells and culture conditions were monitored daily with the aid of an inverted phase contrast microscope. The media was replaced every 4 d until the cell monolayer reached confluency. Once the DH82 macrophages had grown to the desired density, a 200-μl inoculum of *E. chaffeensis*-infected DH82 macrophages (provided by J. M. Lockhart, Valdosta State University, Valdosta, GA) was added to each flask. The flasks were monitored daily for evidence of infection. Six days after inoculum, cells began rounding up from the bottom of the flask, indicating that infection had occurred. Infection was confirmed by Gimenez staining specific for Rickettsiae in DH82 macrophages counterstained with malachite green (Gimenez 1964). Once infected, the cells were removed from the flask [≈1 × 10⁶ cells/ml] by a brief exposure to 0.05% trypsin-EDTA and distributed into 1.8-ml microcentrifuge tubes for storage at -80°C.

IFA assays were used to test for the presence of antibodies to *E. chaffeensis*-like organisms using the antigen from the infected DH82 macrophages (Dawson et al. 1991). The reaction was optimized using a serum dilution of 1:40 in 0.1 M PBS (Steiner et al. 1999). Fluorescein isothiocyanate-labeled rabbit anti-deer immunoglobulin G (Kirkegaard and Perry, Gaithersburg, MD) was diluted 1:100 in 0.1 M PBS and used as the antibody conjugate.

Statistical Analysis. The results of the PCR analyses and the IFA assays were compared with our 1997 study (Steiner et al. 1999) using chi-square analysis with 1 degree of freedom and a confidence interval of 0.05.

Results

Tick Collection and DNA Analysis. A total of 498 ticks was collected from southern Indiana, including 262 *A. americanum* adults and 292 nymphs from Crawford, Orange, Perry, Pike, and Spencer counties. In total, 55 pools of adults (up to 5/pool) and 32 pools of nymphs (up to 10/pool) were examined using nested PCR extracted DNA. Fig. 1 shows the results of one set of reactions separated through a 1.5% agarose gel in 1 × TBE buffer [89 mM Tris base, 89 mM boric acid, 2 mM EDTA (pH 7.6)] at 50 V for 1.5 h. Lane 1 on the gel contains the 389 bp positive control product amplified from pTD2, lane 2 contains the molecular weight marker, and lane 3 is a negative control. Lanes 4–21 contain amplified samples from Orange and Crawford counties. These samples are all negative except for one positive adult *A. americanum* tick pool from Orange County run in lane 18 in which amplification of a 389 bp product is observed.

Table 1 presents the results of the nested PCR reactions for this study indicating that 10 of the tested 55 pools of adult ticks tested positive. Spencer County had the highest adult *A. americanum* infection rate of the Indiana counties with 7.7% or 1/13 positive pools. Crawford County was second highest with 7.4% (2/

1 3 5 7 9 11 13 15 17 19 21

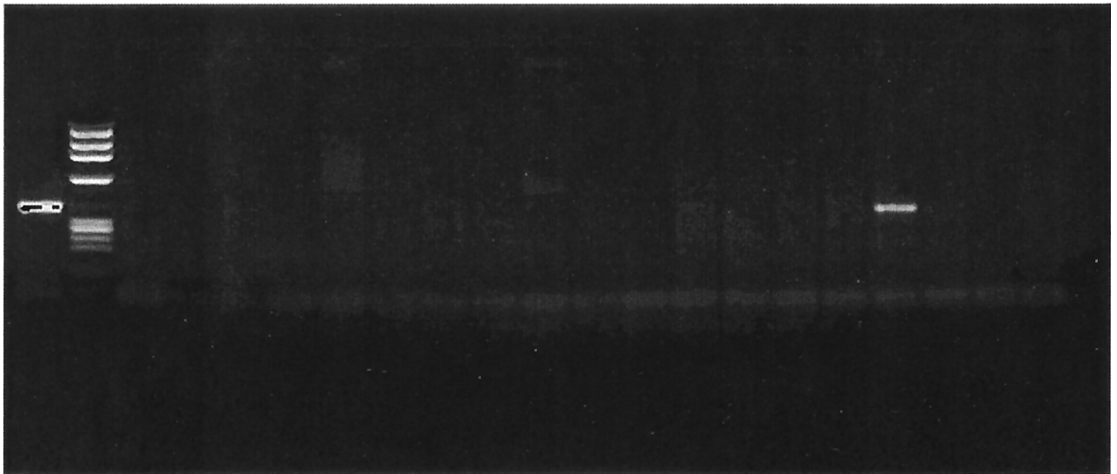


Fig. 1. Nested PCR products separated through a 1.5% agarose gel in 1 × TBE buffer. Lane 1 contains a 389 bp positive control. Lane 2 contains 0.6 µg of molecular weight marker ϕ X174/*Hae*III. Lane 3 is a negative control. Lanes 4–21 contain samples from Orange and Crawford counties. Lane 18 contains a positive adult *A. americanum* pool from Orange County, IN.

27), followed by Perry County with 3.4% (6/177), and Orange County with 2.7% (1/37). All pools of ticks from Pike County tested negative. *E. chaffeensis* was detected from a single tick pool taken from Orange County, a county that had tested negative in 1997. These data translate into an overall minimum infection rate of 3.8%. None of the 32 pools of *A. americanum* nymphs tested positive (data not shown).

The results of this survey were fairly consistent with our previous surveys in southern Indiana of 1997 (average infection rate of 1.6% for six counties) and 1995 (4.9% for six counties). Although eight counties have been examined during these surveys, only five or six counties were surveyed each year. The most heavily infected counties have been Crawford, Perry, Spencer, and Warrick (average infection rates for at least 2 of the 3 yr ranged from 2.6 to 4.0%).

IFA Results. A total of 325 blood samples from deer taken in 26 Indiana counties was collected by volunteers from local and state health departments, the Indiana Department of Natural Resources, and students from Ball State University, the University of Notre Dame, Indiana State University, and Purdue

University-North Central. A total of 327 blood samples from deer killed in 31 Ohio counties was collected by volunteers from the Ohio State Department of Health during the last 2 wk of November 1998. In Indiana, sera collected from 117 of 325 (36%) deer killed were found to contain *E. chaffeensis*-reactive antibodies. Positive IFA results were recorded from deer taken in 6 of 26 counties (Table 2). The highest percentage of positive samples, 66.7% (8/12), was observed in Orange County. Crawford was the second highest county with 64.0% positives (32/50), followed by Dubois with 58.1% (25/43), Spencer and Warrick with 50% (1/2 and 11/22, respectively), and Perry with 42.6% (40/94).

In Ohio overall, 3.4% of deer (11/327) tested positive for *E. chaffeensis*-reactive antibodies. The 11 positive deer came from four of the southern-most counties in the state. Antibodies were found in 25% (1/4)

Table 1. Minimum infection rates of pools of *E. chaffeensis*-infected adult *A. americanum* ticks from southern Indiana in 1998

County	Total ticks	Min. infection rate ^a
Crawford	27	7.4% (2/27)
Orange	37	2.7% (1/37)
Perry	177	3.4% (6/177)
Pike	8	0% (0/8)
Spencer	13	7.7% (1/13)
Total avr infection rate		3.8% (10/262)

^a Minimum infection rates were determined by dividing the number of positive pools of ticks by the total number of ticks examined.

Table 2. White-tailed deer positive for *E. chaffeensis*-reactive antibodies in various Indiana and Ohio counties

State	County	No. of deer tested	No. of positive deer	% positive IFA, %
Indiana	Crawford	50	32	64.0
	Dubois	43	25	58.1
	Orange	12	8	66.7
	Perry	94	40	42.6
	Spencer	2	1	50.0
	Warrick	22	11	50.0
Indiana total/average		223	117	52.5%
Ohio	Adams	4	2	4.4
	Gallia	4	1	25.0
	Jackson	37	6	16.2
	Lawrence	45	2	4.4
Ohio total/average		133	11	8.3%

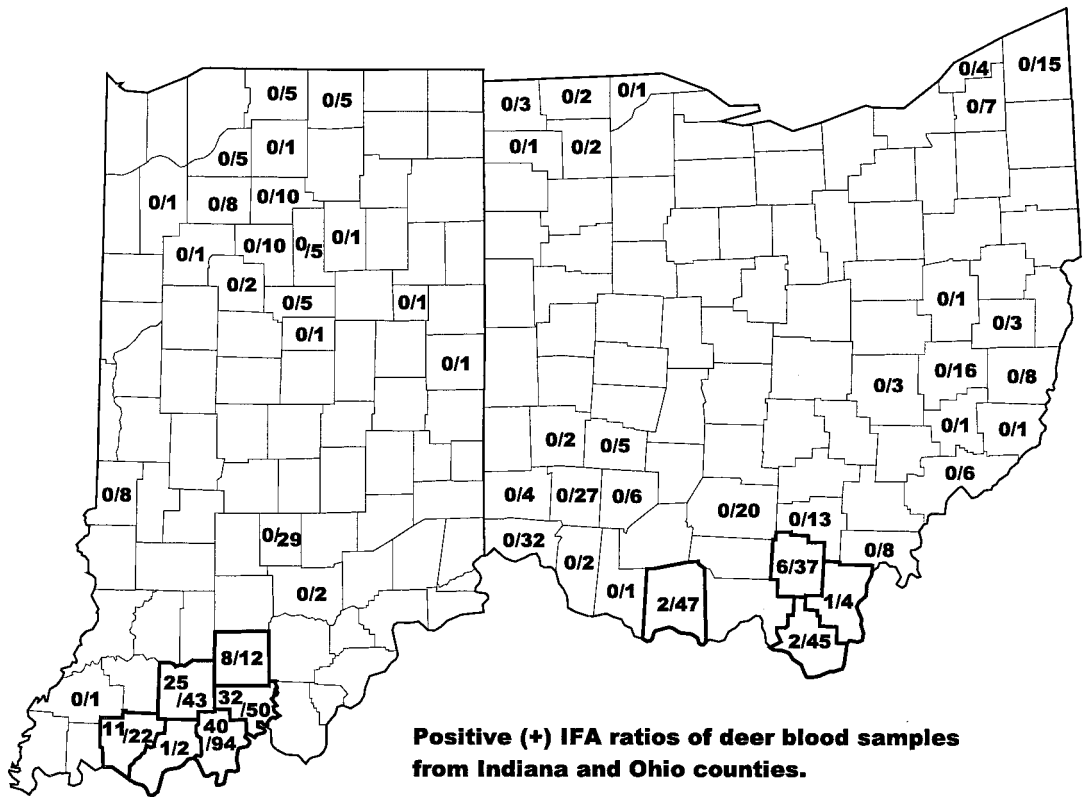


Fig. 2. Positive (+) IFA ratios of deer blood samples from Indiana and Ohio counties.

of deer killed in Gallia County. The second highest infection rate, 16.2% (6/37), was found in Jackson County, whereas Lawrence and Adams counties each had 4.4% infection rates (2/45 and 2/47, respectively). Fig. 2 illustrates the Indiana and Ohio counties having blood samples that tested positive via IFA assays and the ratio of positive samples over the total number of samples assayed is indicated.

Discussion

The presence of *E. chaffeensis* in adult *A. americanum* ticks collected from five counties in southern Indiana was demonstrated for a third year and confirms the endemicity of this agent in southern Indiana. The overall minimum infection rate for the counties surveyed in the current study was 3.8% (10/262), slightly lower (although not statistically significant; $P = 0.05$) than the previous value of 4.9% (21/430) reported by Burket et al. (1998) but significantly higher ($P = 0.05$) than the 1.6% (15/925) reported by Steiner et al. (1999). Comparing all sites sampled for at least 2 of the 3 yr yielded an average infection rate of 3.2% (48/1498) for southern Indiana.

Over the three survey years, the four counties bordering the Ohio River (Crawford, Perry, Spencer, and Warrick) have yielded the highest minimum infection rates. In 1998, the percentage of infected ticks in

Crawford and Warrick counties was at least 7.4 and 7.7%, respectively. In 1997, at least 5% of the ticks were infected in Perry County and in 1995, at least 4.9% were infected in Spencer County. It should be noted that as the percentage of positive tick pools in an area increases, it is more likely that the minimum infection rate underestimates the actual percentage of infected ticks because it is assumed only a single tick in a pool is infected.

This evidence of high levels of infection supports the view that the *A. americanum* population has existed longer in the southern-most counties (most likely for many years) and has only more recently expanded northward. The northward expansion of *A. americanum* is believed to be a consequence of the increase in the state's deer population, which began to grow in the 1950s and achieved exponential growth beginning \approx 1980. More liberal issuing of deer hunting permits in the 1990s has resulted in a leveling off of the deer populations in most counties as measured by the number of deer harvested by hunters annually in Indiana (Weaver and Mitchell 1998).

Antibodies reacting with *E. chaffeensis* antigen were detected in white-tailed deer blood samples from Indiana and Ohio via IFA. In those Indiana counties from which at least one positive deer blood sample was obtained, the overall average infection rate was 52% (117/223). Previous research reported that 43–70% of

deer sampled in the eastern United States were seropositive for *E. chaffeensis*—reactive antibodies and even higher values were observed in areas heavily infested with *A. americanum* (Dawson et al. 1994a). The 52% (117/223) value found in this study is slightly higher (although not significantly; $P = 0.05$) than the 46% (41/89) previously reported by Steiner et al. (1999) for southern Indiana.

In Indiana, the counties with deer positive for *E. chaffeensis*-reactive antibodies are counties known to harbor high populations of *A. americanum* ticks infested with *E. chaffeensis* (Burket et al. 1998, Steiner et al. 1999). It is assumed that the introduction of *E. chaffeensis* into southern Indiana occurred some time after the reintroduction of deer into the state which occurred in 1934 (Mumford and Whitaker 1982). Annual fluctuations in the rates of infection of *A. americanum* with *E. chaffeensis* can be expected to vary with the density and distribution of infected deer.

In the four Ohio counties where we found evidence of infected deer, an average of only 8.3% (11/133) of the deer had *E. chaffeensis*-reactive antibodies. This is significantly lower ($P = 0.05$) than the percentage of infected deer in the positive counties in southern Indiana (117/223 or 52%). This may be because the southern-most counties in Ohio are somewhat further north than the southern-most Indiana counties or because *A. americanum* is a more recent invader in Ohio. Although specimens of *A. americanum* were sometimes encountered in Ohio during the 1980s, they were widely and randomly distributed throughout the state and viewed as temporary residents, only arriving on migratory birds, interstate travelers, or their pets (Pretzman et al. 1990, R. L. Berry, personal communication). It was not until 1993 that *A. americanum* larvae were found in Jackson County, OH, indicating the presence of an established population (R. M. Berry, personal communication).

Although there is still no record of a confirmed case of HME originating in Ohio (R. M. Berry, personal communication), this report of *E. chaffeensis*-reactive antibodies occurring in deer suggests that cases could arise at any time. Although there may be fewer ticks to spread *E. chaffeensis* among white-tailed deer in Ohio, there appears to be a potential focus of infection along the Ohio River that bears monitoring.

It is possible that a cross reaction of the antibody to bacterial species other than *E. chaffeensis* may be occurring (Dawson et al. 1994b). However, based on the results of these studies, it is recommended that Indiana and Ohio state and local health department officials distribute brochures to the public and medical personnel describing the risks associated with *A. americanum*, *E. chaffeensis*, and HME. Physicians, especially in the southern one-third of Indiana and the Ohio counties bordering the Ohio River, should likewise be encouraged to educate their patients, staffs, and colleagues about this emerging disease. The importance of providing more information to the public and physicians concerning the risk of acquiring HME from tick bites in southern Indiana was highlighted recently in the popular science magazine *Discover* (Weaver

1999). During 1999, a 33% increase in HME (six new cases) was seen in southern Indiana, making a total of 24 reported cases since 1994. Although Ohio has not had a confirmed case of HME, the discovery of *E. chaffeensis*-reactive antibodies in the white-tailed deer blood samples should serve as a warning to state officials that human cases of HME are possible based on our findings.

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