

## Research Paper

# Evaluation of a Prototype *Ehrlichia chaffeensis* Surveillance System using White-Tailed Deer (*Odocoileus virginianus*) as Natural Sentinels

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### ABSTRACT

The natural history of *Ehrlichia chaffeensis*, the causative agent of human monocytotropic ehrlichiosis, includes the lone star tick (LST, *Amblyomma americanum*) as a vector and white-tailed deer (WTD; *Odocoileus virginianus*) as both a natural reservoir of *E. chaffeensis* and a major host of LST. The goal of the current study was to implement and evaluate a prototype surveillance system to delineate the geographic distribution of *E. chaffeensis* using WTD as natural sentinels. To accomplish this goal, serologic testing using the indirect immunofluorescent antibody (IFA) test was performed on WTD serum samples, and to confirm serologic results, polymerase chain reaction (PCR) assays and culture isolation were conducted. Considerations relevant to the applicability of a surveillance system utilizing WTD were analyzed (e.g., age and gender relationships to serologic status, adequacy of sample sizes needed to distinguish between uninfected and infected populations, presence of LST, and ability to detect stability and spread of *E. chaffeensis* in WTD populations). Of 3275 WTD serologically tested, 549 (47%) from 17 of 18 states had antibodies reactive to *E. chaffeensis* (IFA titer  $\geq 1:128$ ). No difference between age groups or gender was noted with serologic testing, thus these variables would not be a concern for a surveillance system using WTD. Significantly more deer in younger age groups ( $\leq 1.5$  yr) were PCR and culture positive, and 46% of 122 seropositive WTD populations were confirmed positive by PCR or culture isolation. A significant association between LST infestation and *E. chaffeensis* seroreactivity was noted. Furthermore, the surveillance system was able to detect stability of *E. chaffeensis* within WTD populations and also spread to new populations, both of which were associated with LST status. These data clearly demonstrate that WTD are useful as natural sentinels for this emerging human pathogen, and establish a prototypical framework for a WTD surveillance system. **Key Words:** *Ehrlichia chaffeensis*—White-tail deer—Surveillance systems—*Amblyomma americanum*—Tick-borne—Serology. Vector-Borne Zoonotic Dis. 3, 195–207.

### INTRODUCTION

*Ehrlichia chaffeensis*, the causative agent of human monocytotropic ehrlichiosis (HME), is maintained in a zoonotic cycle involving white-tailed deer (WTD; *Odocoileus virginianus*) as the

primary vertebrate reservoir and lone star ticks (LST, *Amblyomma americanum*) as biological vectors (see Childs and Paddock, 2003). Many populations of white-tailed deer from the southeastern United States have been shown to have antibodies reactive to *E. chaffeensis* (Dawson et al.

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1994a), and experimental and field studies have proven that WTD are competent and persistently infected reservoirs (Dawson et al. 1994b; Lockhart et al. 1997a; Davidson et al. 2001; Yabsley et al. 2002). The LST was initially suspected to be a vector based on the geographic distribution of HME cases and this was confirmed by polymerase chain reaction (PCR) detection of *E. chaffeensis* DNA in LST (Anderson et al. 1993; Roland et al. 1998; Steiert and Gilfoy, 2002) and by experimental transstadial transmission between WTD (Ewing et al. 1995). In addition to being important reservoirs for *E. chaffeensis*, WTD are an important host for all three mobile stages of LST (Bloemer et al., 1988).

The potential utility of WTD as a sentinel for determining the distribution of *E. chaffeensis* was first suggested when antibodies reactive to *E. chaffeensis* were detected in WTD (Dawson et al. 1994a), and a prototype WTD serologic surveillance system for *E. chaffeensis* recently was applied statewide in Iowa (Mueller-Anneling et al. 2000). White-tailed deer have been utilized as sentinel animals to monitor numerous human and livestock pathogens including Jamestown Canyon virus (Boromisa and Grimstad 1987), Venezuelan equine encephalitis (Smart and Trainer 1975), Lyme disease (Gill et al. 1993), vesicular stomatitis virus (Fletcher et al. 1991), and bluetongue and epizootic hemorrhagic disease viruses (Stallknecht et al. 1991). The advantages associated with surveillance systems utilizing deer include (1) a distribution that includes 45 states allowing development of national, regional, or state-wide surveillance systems; (2) relatively high abundances allowing use as sentinels in most locations; (3) regulated harvests in all states enhancing the ease and reducing the cost of collecting samples from hunter-killed animals; (4) limited size of deer home ranges (mean approximately 400 ha) allowing identification of exposure location; (5) a much higher rate of exposure to tick vectors compared to humans or potential domestic animal sentinels; (6) relatively long life spans increasing opportunity for exposure; (7) opportunities to simultaneously monitor for presence of tick vectors; (8) occurrence in both natural habitats and areas proximal to human habitation; and (9) freedom from prior treatment with antibiotics or acaricides.

Field studies have demonstrated that serologic monitoring of deer would allow for fine-scale mapping of the distribution of *E. chaffeensis* (Lockhart et al. 1996; Mueller-Anneling et al. 2000). Using a sample size of only five animals per population, Lockhart et al. (1996) successfully discriminated between LST infested populations which had a 100% antibody prevalence and populations believed to be LST-free which had a seroprevalence of 6.7%. Although *E. chaffeensis*-reactive antibodies are reported for many WTD populations, confirmation of infection using PCR and/or culture has only been reported for 10 seropositive populations in Arkansas, Georgia, Kentucky, North Carolina, and South Carolina (Lockhart et al. 1997a, 1997b; Little et al. 1997, 1998; Yabsley et al. 2002).

The overall goal of this study was to implement and evaluate an extensive fine-scale (county) surveillance system for *E. chaffeensis* utilizing WTD as natural sentinels. Specific objectives to achieve this goal were to (1) combine current and prior WTD serologic data to construct an extensive regional database on *E. chaffeensis*-reactive antibodies in WTD; (2) use PCR assays and cell culture isolation testing to confirm *E. chaffeensis* infection in seropositive WTD populations; (3) document any age class or gender relationships to antibody prevalence and PCR detection; (4) evaluate sample sizes that would be functionally effective at distinguishing between infected and uninfected populations; (5) affirm that infection status of WTD populations corresponds with the presence of LST; and (6) investigate the ability of a WTD surveillance system to consistently discern the distribution or spread of *E. chaffeensis* among WTD populations over time.

## MATERIALS AND METHODS

### *Sample collections*

White-tailed deer samples specifically for this project were collected from sites selected to complement and further expand four previous studies where WTD in the south central and southeastern United States were tested for *E. chaffeensis*-reactive antibodies (Dawson et al. 1994a; Lockhart et al. 1996; Little et al. 1997; Yabsley et al. 2002). Blood samples were col-

lected from 2,101 WTD from 471 populations in 18 states (AL, AR, GA, FL, KS, KY, LA, MD, MO, MS, NC, NJ, OK, SC, TN, TX, VA, and WV) during Southeastern Cooperative Wildlife Disease Study herd health evaluation activities, other research projects, or in cooperation with state wildlife agency personnel from hunter-harvested animals. Blood samples were collected either aseptically from the heart, from the jugular vein, or from the body cavity. One exception was that blood samples from 40 of 79 deer from Missouri were collected on Nobuto strips and eluted in phosphate-buffered saline (PBS), pH 7.4 as described (Mueller-Anneling et al. 2000). Serum, plasma, or eluted blood samples were frozen at  $-20^{\circ}\text{C}$  until serologic testing. From a subset of WTD (368 from 112 seropositive populations and 61 from 20 seronegative populations), whole blood samples were collected and frozen at  $-20^{\circ}\text{C}$  for PCR testing. Ticks were collected from a second subset of 715 WTD (117 populations) and submitted to the National Veterinary Services Laboratories (U.S. Department of Agriculture, Ames, IA) for identification. Animals were handled and samples collected by procedures approved by the Animal Care and Use Committee at the University of Georgia (A3437-01).

#### Serologic assays

Serum, plasma, or eluted blood samples were tested for antibodies reactive to *E. chaffeensis* by the indirect immunofluorescent antibody (IFA) test as previously described (Dawson et al. 1994; Lockhart et al. 1996). Samples were screened at a dilution of 1:128 using *E. chaffeensis* antigen slides (Focus Technologies, Cypress, CA). A 1:50 dilution of fluorescein isothiocyanate-labeled rabbit anti-deer immunoglobulin G (Kirkegaard and Perry Laboratories, Gaithersburg, MD) was used as conjugate. Geometric mean titer (GMT) was computed for 152 seropositive deer.

#### Molecular assays

DNA from 300  $\mu\text{l}$  of 429 whole blood samples was extracted using the GFX Genomic Blood DNA Purification Kit (Amersham Pharmacia Biotech, Piscataway, NJ) following the manufacturer's protocol. Two gene targets, the

16S rRNA gene and the variable length PCR target (VLPT) antigen gene, were utilized for screening of whole blood for *E. chaffeensis* DNA using a nested PCR format. Primary outside amplification for the 16S rRNA gene consisted of 5  $\mu\text{L}$  of DNA (from whole blood) in a 25- $\mu\text{L}$  reaction containing 10 mM Tris-Cl (pH 8.3), 50 mM KCl, 1.5 mM  $\text{MgCl}_2$ , 0.2 mM each dNTP (Promega, Madison, WI), 2.5 units Taq DNA Polymerase (Promega), and 0.8  $\mu\text{M}$  of primers ECC and ECB (Dawson et al. 1994b, 1996a). For the nested PCR, 1  $\mu\text{L}$  of primary product was used as template in a 25- $\mu\text{L}$  reaction containing the same PCR components except *E. chaffeensis* specific primers, HE1 and HE3 (Anderson et al. 1992), were used.

Nested PCR for the VLPT antigen gene was conducted on 429 blood samples collected during this study as previously described (Sumner et al. 1999). In addition, 101 blood samples from an additional 10 populations previously tested using 16S rRNA primers (Yabsley et al. 2002) were tested for *E. chaffeensis* using the VLPT gene target. Amplified products were separated in 2% agarose gels, stained with ethidium bromide, and visualized with UV light. To confirm identity, representative secondary VLPT gene amplicons were purified with a Microcon spin filter (Amicon Inc., Beverly, MA), sequenced at MWG-BIOTECH (High Point, NC), and compared to published *E. chaffeensis* VLPT sequences in the GenBank database.

Stringent protocols and controls were utilized in all PCR assays to prevent and detect contamination, DNA extraction, primary amplification, secondary amplification, and product analysis were performed in separate dedicated laboratory areas. Two negative water controls were included in each set of DNA extractions and one water control was included in each set of primary and secondary PCR reactions.

#### Culture isolation

During 2001–2003, aseptically collected blood samples from 72 WTD from 15 LST positive populations were cultured in DH82 cells for isolation of *E. chaffeensis* as previously described (Lockhart et al. 1997a). Cultures were fed biweekly with MEM medium (Sigma, St.

Louis, MO) supplemented with 10% fetal bovine serum (Sigma) and monitored for evidence of cytopathic effect or for a maximum of 45 days. Cultures showing cytopathic effect and cultures completing the 45-day incubation period were harvested with a cell scraper and tested by direct fluorescent antibody assay as previously described (Lockhart et al. 1997a).

#### Data analysis

Serologic data from this study ( $n = 2,101$ ) were combined with data from previous studies conducted independently by SCWDS researchers ( $n = 425$ ; Lockhart et al. 1996; Little et al. 1997; Yabsley et al. 2002) or collaboratively with others ( $n = 749$ ; Dawson et al. 1994a) to construct a comprehensive map delineating the distribution of *E. chaffeensis* seroreactive WTD in the southeastern and south central United States. Some of the previous studies used different cutoff values for IFA testing (e.g., 1:64 or 1:128), but in order to standardize results from this study and previous studies, IFA assays were classified as positive only if a sample had a titer of  $\geq 1:128$ . To facilitate graphic presentation, data for each population were categorized by county or parish; if one or more deer with antibodies reactive to *E. chaffeensis* was detected, that county or parish was classified as seropositive.

To confirm serologic data, PCR and/or culture isolation were conducted on a total of 541 deer from 122 seropositive populations and on 61 deer from 20 seronegative populations. Chi-square analysis was used to test for differences of PCR prevalence between seropositive and seronegative populations.

Prevalence of antibodies reactive to *E. chaffeensis* among different age classes and gender categories were only determined for WTD in seropositive populations. Serologic data from Dawson et al. (1994), Lockhart et al. (1996), Little et al. (1997), Yabsley et al. (2002), and this study were combined for determining prevalence among age classes because analysis of WTD age data had not been previously conducted. PCR testing was not conducted by Dawson et al. (1994) and Lockhart et al. (1996); therefore, analysis of age and PCR positivity was conducted only on deer from Little et al. (1997), Yabsley et al. (2002), and this study. Chi

square analysis was used to test for differences in both seroprevalence and PCR positivity among age classes.

To accurately classify the serostatus of a population, an appropriate sample size needs to be tested. In previous studies, testing of five deer from a population consistently detected *E. chaffeensis*-reactive antibodies in deer populations infested with LST (Lockhart et al. 1997; Little et al. 1997, 1998), and based on Dawson et al. (1994) testing a mean of 21 deer failed to detect seroreactive deer in seronegative populations. However, additional evaluation of the sample sizes required to reliably classify the serologic status of populations was conducted in this study. To determine minimal number of samples needed to detect the presence of a seropositive deer in a population, the formula  $n = (1 - (1-a)^{1/D}) (N - (D - 1))/2$  was used as described (Thrusfield 1995). Because seronegative populations are more difficult to accurately classify, two methods were used to examine adequacy of sample size. Larger numbers of deer ( $n = 8 - 16$ ) were tested from nine seronegative populations to enhance detection of potentially low prevalences in those populations. Also, four populations with no history of LST infestation were repeatedly tested for several years.

To assess the predicted epidemiologic association between LST infestation and serologic status of WTD populations, chi-square analysis was used to test for an association between LST presence and seroreactors at the population level. Furthermore, 11 populations for which both tick and serologic data were available were evaluated during multiple years; three populations had LST infestation each sample year, four never had any evidence of LST infestation, and four were negative for LST during earlier sampling period (s) but became LST positive during subsequent test years. Chi-square analysis was used to test for differences between groups.

## RESULTS

### Regional serologic database for WTD

Among the 2,101 WTD collected specifically for this study, 984 (46.8%; CI95% = 44.7%,

48.9%) had antibodies reactive ( $\geq 1:128$  titer) to *E. chaffeensis* by IFA testing (Table 1). The mean prevalence of antibodies reactive to *E. chaffeensis* in seropositive populations was 73.8% (SD = 25.8%, range 20–100%). White-tailed deer with antibodies reactive to *E. chaffeensis* were detected in all states tested except West Virginia. The modal antibody titer for seropositive deer was 1:128 with a maximum titer of 1:4,096. The GMT of a subset of 152 seropositive WTD was 355.

The combined dataset of WTD serologically tested during this and four prior studies (Dawson et al. 1994a; Lockhart et al. 1996; Little et al. 1997; Yabsley et al. 2002) contained a total of 3,275 WTD from 18 southeastern and south central states. In the combined data set, 1,549 (47.3%) WTD examined had antibodies reactive to *E. chaffeensis* with seropositive populations detected in 17 of 18 states (Fig. 1).

The highest overall seroprevalences were in Arkansas and Missouri. Although high prevalences were detected in local WTD populations in most states, considerable variation in prevalence occurred within different regions of some states (e.g., Florida, Kansas, North Carolina,

Oklahoma, and Virginia). A western boundary for the distribution of *E. chaffeensis* was evident spanning the states of Kansas, Oklahoma, and Texas. Similarly, a southern boundary was noted across peninsular Florida. Clusters of seronegative populations were detected in southern Mississippi and Louisiana, along the lower Mississippi River floodplain and in the Appalachian Mountains from western Maryland to northern Alabama.

#### PCR and culture validation of *E. chaffeensis* serologic data

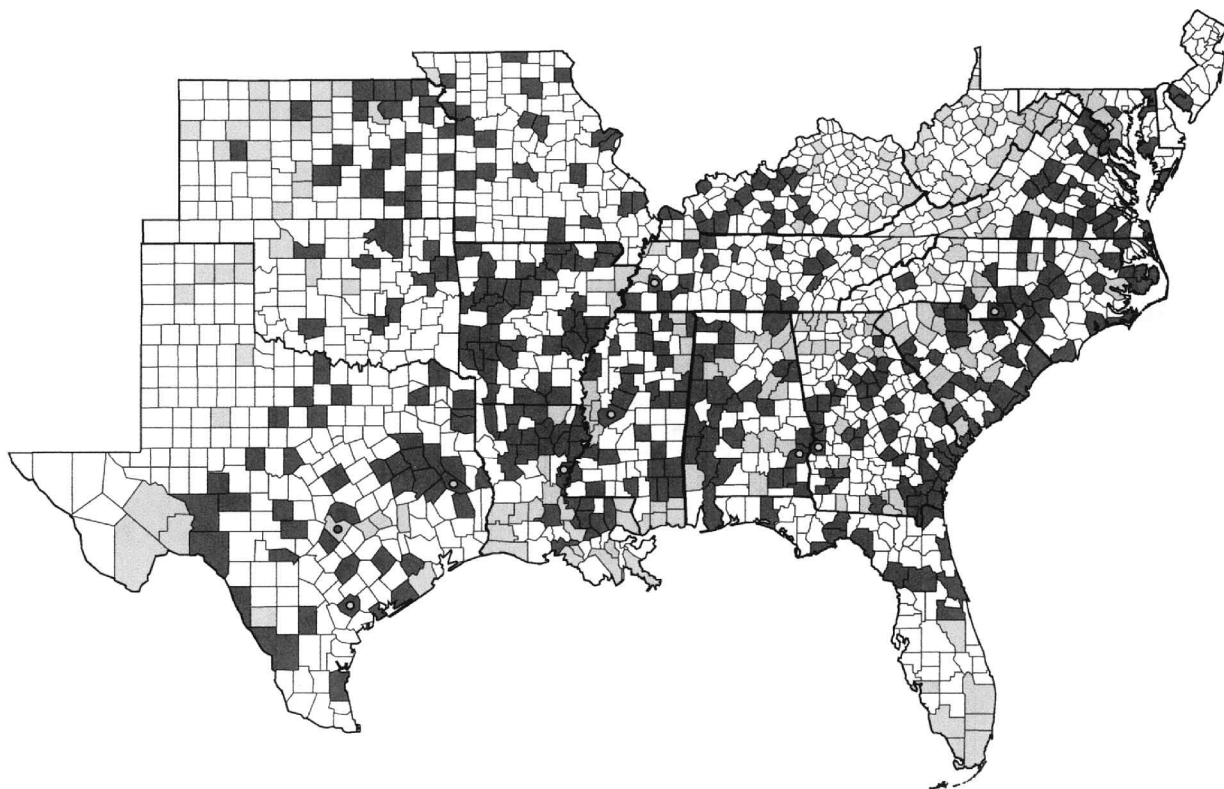
Eighty-six of 368 (23.4%) WTD from 50 seropositive populations were PCR positive for *E. chaffeensis* by either 16S or VLPT PCR. Of 101 WTD previously tested by 16S PCR (Yabsley et al. 2002), nine deer from seven populations were positive by VLPT PCR. Collectively for this study and Yabsley et al. (2002), 95 of 469 (20.3%) WTD from 56 of 122 (46%) seropositive populations have been confirmed using PCR (Table 1); 70 of 95 deer with both gene targets, 2 of 95 for only the 16S rRNA gene, and 23 of 95 only for the VLPT gene. In contrast, 16S

TABLE 1. RESULTS OF SEROLOGIC AND POLYMERASE CHAIN REACTION TESTING FOR *EHRlichia chaffeensis* IN WHITE-TAILED DEER FROM 18 STATES<sup>a</sup>

State	Years	Counties tested by IFA test	Number IFA positive/ no. tested (%)	Number PCR positive/ no. tested (%)
Alabama	1981–2001	33	59/141 (42)	NT <sup>b</sup>
Arkansas	1982–2002	29	111/154 (72)	7/46 (15)
Florida	1983–2002	15	43/104 (40)	9/36 (25)
Georgia	1973–2002	50	119/243 (49)	26/98 (27)
Kansas	1998–2002	38	39/76 (51)	3/26 (12)
Kentucky	1983–2001	27	26/99 (26)	2/23 (9)
Louisiana	1981–2001	34	54/166 (33)	1/18 (6)
Maryland	1999–2002	5	12/25 (48)	1/7 (14)
Missouri	1994–2002	19	57/79 (72)	3/23 (13)
Mississippi	1995–2001	38	80/152 (53)	1/19 (5)
North Carolina	1982–2002	33	75/131 (57)	15/72 (21)
New Jersey	2001	2	6/10 (60)	0/5
Oklahoma	1985–2002	13	30/50 (60)	0/3
South Carolina	1991–2001	26	52/124 (42)	4/60 (7)
Tennessee	1981–2001	20	54/100 (54)	0/10
Texas	1992–2002	33	73/123 (59)	0/13
Virginia	1983–2002	37	94/168 (56)	23/57 (40)
West Virginia	1977–2001	18	0/156	0/14
Total		471	984/2101 (47)	95/530 (18)

<sup>a</sup>Includes PCR results for the VLPT antigen gene for 101 WTD previously tested by PCR for the 16S rRNA gene (Yabsley et al. 2002).

NT, not tested.



**FIG. 1.** Indirect immunofluorescent antibody results for *Ehrlichia chaffeensis* in white-tailed deer. Results are from the current study and from previously published work obtained in collaboration with SCWDS. Darkly shaded counties positive and lightly shaded counties negative for antibodies ( $\geq 1:128$ ) reactive for *E. chaffeensis*. Previous serologic results that differed from the results of the current study are shown as small circles within the county. ●, previously positive for antibodies ( $\geq 1:128$ ) reactive for *E. chaffeensis*, ○, previously negative for antibodies reactive for *E. chaffeensis*.

rRNA and VLPT PCR assays on 61 deer from 20 seronegative populations were all negative. Presence of PCR positive deer was strongly associated with seropositive populations ( $\chi^2 = 11.6$ ,  $p < 0.001$ ).

Of 72 culture attempts, 34 (44.2%) were lost to contamination with bacteria and/or *Trypanosoma cervi*. Seven of the remaining 38 cultures (18.4%) were positive for *E. chaffeensis*. Positive cultures were obtained from two WTD from Greene Co., AR; two from Woodruff Co., AR; and one each from Clarke Co., GA; Anson Co., NC; and Georgetown Co., SC. All isolates were confirmed as *E. chaffeensis* by PCR of the 16S rRNA and VLPT gene targets.

#### *Relationship of age class and gender to antibody prevalence and PCR detection*

Age was available on 1,882 deer tested serologically; 1,086 deer from this study, 517 from Dawson et al. (1994), 165 from Lockhart et al.

(1996), five from Little et al. (1997), and 109 from Yabsley et al. (2002). For this combined data set, differences were not noted ( $\chi^2 = 5.65$ ,  $p = 0.46$ ) in seroprevalance among age classes (Fig. 2A). Similarly, seroprevalance did not differ between males and females (69.3% vs. 69.6%;  $\chi^2 = 0.016$ ,  $p > 0.05$ ).

Age was available for 393 deer from seropositive populations that were tested by PCR. Higher proportions of deer were PCR positive in the younger age classes ( $\chi^2 = 71.7$ ,  $p < 0.001$ ). Among deer  $< 0.75$  years old, 53.1% were PCR positive, within the next age group (0.76–1.5 years) the prevalence decreased significantly to 27% ( $\chi^2 = 14.7$ ,  $p < 0.001$ ), and less than 8% of deer  $> 1.5$  years old were PCR positive (Fig. 2B). The mean age of PCR positive WTD was 1.1 years (range 0.25–4 years). Insufficient culture isolates were made to examine age-related associations; however, the mean age for culture positive deer was 3.2 years (range 0.3–9.5 years). Similar to antibody

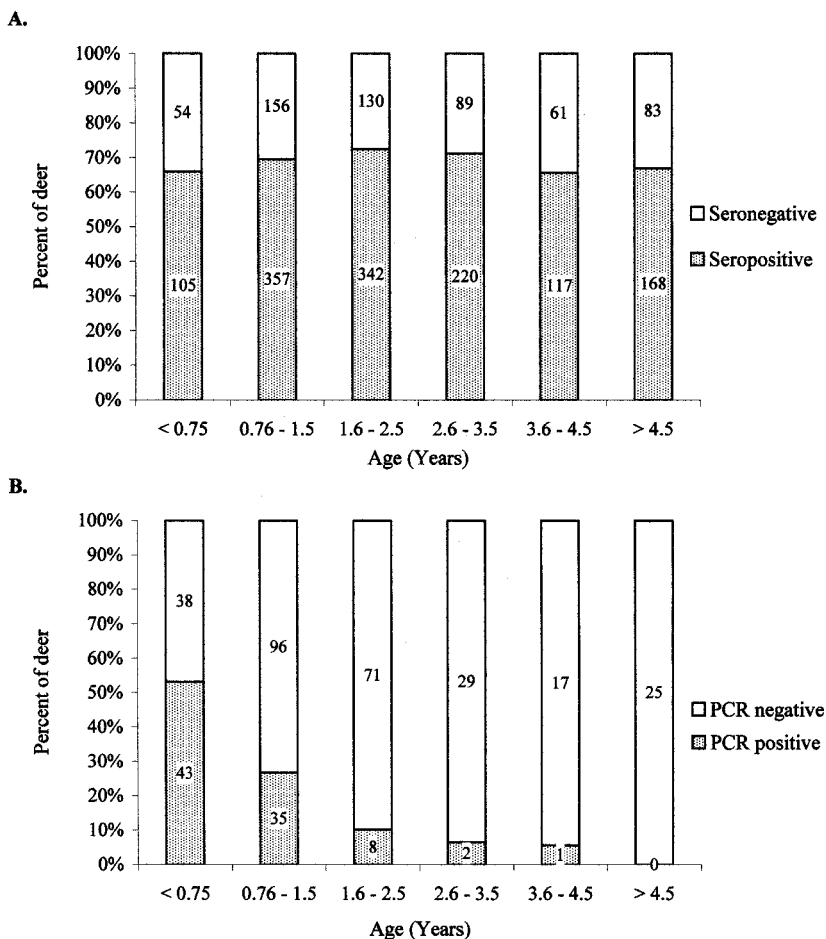


FIG. 2. (A) Prevalence of antibodies reactive to *E. chaffeensis* in WTD among age classes. Numbers in bars represent number of deer tested. (B) Prevalence of PCR-positive WTD among age classes. Numbers in bars represent number of deer tested and different letters represent significant differences at  $p < 0.05$ .

prevalence, PCR positivity did not differ between males and females (19% vs. 22.8%;  $\chi^2 = 0.89, p > 0.05$ ).

*Evaluation of sample size adequacy*

Regarding sample size for seropositive populations, post hoc statistical analysis utilizing a mean 73% seroprevalence indicated that testing of only three or four deer gives a high probability (95% and 99%, respectively) of detecting a positive population (Thrusfield, 1995). Although validation of seronegative populations is more problematic, testing of larger numbers of deer per population and repeated testing of selected populations both produced consistent negative results. In a combined dataset from Dawson et al. (1994a) and this study, 250 deer from 17 seronegative populations (9–30 deer

per population) were negative for antibodies to *E. chaffeensis* with means of 20.8 and 10.4 deer tested per seronegative population, respectively. Similarly, none of the 131 deer from four populations consistently negative for LST infestation had antibodies reactive to *E. chaffeensis*. The mean number of WTD tested per population in this study was 6.2 (SD = 6.1, range 1–58) and data for populations with sample sizes less than four deer were used only if a seropositive animal was detected.

*Association between LST and serologic status at population level*

Of 117 WTD populations examined for ticks, 82 were infested with LST, 29 with *A. maculatum* (Gulf Coast tick), four with *Ixodes affinis*, and 13 with *I. scapularis* (blacklegged tick).

Eighty of the 82 (97.6%) populations infested with LST contained at least one seropositive WTD, while only four of 35 (11.4%) populations not infested with LST contained seropositive deer. One LST-negative population (Harris Co., GA) was both seronegative and tick negative in 1986; however, in 2002 the deer population was seropositive but the LST status was not evaluated. The prevalence of antibodies reactive to *E. chaffeensis* was significantly higher among LST infested populations of WTD than populations not infested by LST ( $\chi^2 = 89.9, p < 0.001$ ). None of the six populations infested only with *A. maculatum* contained seropositive deer; there were insufficient populations with *I. affinis* or *I. scapularis* alone to permit evaluation.

#### Stability and spread of *E. chaffeensis* and LST among WTD populations

Fifty-seven populations which were tested during previous studies (Dawson et al. 1994a; Lockhart et al. 1996) were retested during this study. The serologic status for 50 (88%) of these populations remained unchanged; 35 seropositive populations remained seropositive and 15 seronegative populations remained seronegative. Six populations which previously tested seronegative were seropositive during this

study. Three of these representing Anson Co., NC, Haywood Co., TN, and Stewart Co., GA had documented invasion of LST between the first reported testing and this study. One Texas population (Travis Co.) was seropositive ( $n = 7, 71.4\%$ ) when tested in 1992 (Dawson et al. 1994a) but was seronegative when sampled ( $n = 10$ ) in 2002 for this study. The LST status for this county was not known for either sampling period.

Among the 11 populations where LST infestation and serologic status were monitored repeatedly over time, there was complete concordance between LST presence and seropositive deer (Table 2). All three LST positive populations were consistently seropositive each year tested. The mean seroprevalence for these three populations was 83%, and two of these populations (Jones Co., GA and Ashley Co., AR) were confirmed PCR positive for *E. chaffeensis*. None of the four populations where LST was consistently absent contained seropositive deer at any sampling time. Among the four populations where LST status changed from absent to present, seropositive deer were only detected following the appearance of LST. The mean prevalence of antibodies reactive to *E. chaffeensis* in the most recently tested year was significantly higher for populations infested with LST for multiple years (mean = 100%)

TABLE 2. SEROLOGIC AND TICK SURVEY RESULTS FROM SEVEN POPULATIONS OF WTD COLLECTED DURING MULTIPLE TIME PERIODS AND TESTED FOR ANTIBODIES REACTIVE FOR *E. CHAFFEENSIS*

Site	Years	No. of years sampled	Tick status (years)	No. of deer tested	Mean IFA prevalence, % (range)
Sites positive for ticks					
Jones Co., GA	1979–2001	6	+ (6)	41	92 (80–100)
Ashley Co., AR	1982–2001	7	+ (7)	37	84 (71–100)
Franklin Co., FL	1984–1996	5	+ (5)	25	68 (40–100)
Sites negative for ticks					
Floyd Co., GA	1973–2001	6	– (6)	58	0
Tyler Co., WV	1985–2000	4	– (4)	21	0
Hardy Co., WV	1977–1999	7	– (7)	37	0
Monroe Co., FL	1992–2001	4	– (4)	15	0
Sites where tick status changed					
Anson Co., NC	1987, 2001	2	–, + <sup>a</sup>	10	0, 40 <sup>b</sup>
Concordia Pa., LA	1986, 1991, 1999	3	–, +, +	22	0, 38, 60
Haywood Co., TN	1989, 1994, 1998	3	–, –, +	16	0, 0, 20
Stewart Co., GA	1986, 1998	2	–, +	10	0, 20

<sup>a</sup>Tick status at sequential sampling periods.

<sup>b</sup>Prevalence of IFA seropositive deer at sequential sampling periods.



compared to populations only recently infested (mean = 53.8%,  $\chi^2 = 15.5$ ,  $p < 0.001$ ). One recently infested population (Anson Co., NC) was confirmed by isolation of *E. chaffeensis* from a single WTD in 2001.

## DISCUSSION

The overarching goal of this study was to implement and evaluate a prototype surveillance system using WTD as natural sentinels to determine the geographic distribution of *E. chaffeensis* across most of the suspected HME endemic region of the United States. To this end, the data obtained demonstrate the following critical attributes: (1) serologic findings reflect *E. chaffeensis* infection, (2) effective surveillance can be achieved with small sample sizes, (3) any deer >6 month old is suitable for surveillance purposes, (4) enzootic and consistently negative locales were identified repeatedly across time, (5) surveillance of deer has the ability to detect spread to new locales, and (6) serologic, molecular, and culture diagnostic findings among deer could be related to presence of the principal tick vector, *A. americanum*. These findings compliment and expand on a recent WTD serologic surveillance effort within Iowa (Mueller-Anneling et al. 2000); however, that study exclusively used serologic assays and did not include either confirmatory microbiological diagnostics or contemporaneous tick vector monitoring. Collectively, these two studies confirm the effectiveness of this prototypical surveillance system for monitoring the distribution of this emerging human pathogen.

Although several studies have examined the prevalence and distribution of *E. chaffeensis* antibodies among many southeastern and south central WTD populations (Dawson et al. 1994a; Lockhart et al. 1996; Little et al. 1997; Yabsley et al. 2002), the extensive additional testing filled many geographic gaps and provide a more complete fine scale distribution of *E. chaffeensis* among WTD throughout the regions of the United States with the highest HME risk (Paddock and Childs 2003). The combined data evaluated herein disclosed a wide distribution of seropositive WTD populations across all of the 18 states represented except for West Vir-

ginia; however, some distributional limits also were discerned including a western boundary extending across Kansas, Oklahoma, and Texas, a southern boundary across peninsular Florida, and a large cluster of many seronegative populations centered along the Appalachian Mountains (Fig. 1). Other prior studies of WTD have documented an apparent northern range limit for *E. chaffeensis* in southern portions of Iowa, Illinois, Indiana, and Ohio (Dawson et al. 1994a; Irving et al. 2000; Mueller-Anneling et al. 2000). Along the East coast, surveys have indicated that *A. americanum* and *E. chaffeensis* are present as far north as Connecticut, Maine, and Rhode Island (Keirans and Lacombe 1998; Ijdo et al. 2000).

The accuracy of diagnostic assays utilized is critical to any pathogen surveillance system. Infection with *E. chaffeensis* was confirmed in nearly half (46%) of 122 seropositive WTD populations tested by PCR and was supplemented by isolation from others. In contrast, PCR evidence of infection was not detected in any of 20 seronegative WTD populations. Thus, PCR and culture testing applied at the population level were effective at confirming infection in many seropositive populations and demonstrating a strong association between the serologic status of populations and infection with *E. chaffeensis*.

Because WTD in these regions are known to be infected with three other related rickettsiae (*E. ewingii*, *A. phagocytophilum*, and an *Anaplasma* sp. [WTD-agent]) in addition to *E. chaffeensis* (Dawson et al. 1996b; Little et al. 1998; Brandsma et al. 1999; Magnarelli et al. 1999; Yabsley et al. 2002), the potential for serologic cross-reaction is an important consideration. Although antibodies reactive with *E. chaffeensis* in WTD from Maryland have been confirmed by immunoblotting using the 28- to 29-kDa antigens of *E. chaffeensis* (Walls et al. 1998), the extent of serologic cross-reactions among these four species is not fully understood (Dumler and Walker 2001). However, data from other studies suggests that infection with these other rickettsiae may not commonly result in production of antibodies that cross-react with *E. chaffeensis*. Cross-reactions between *E. chaffeensis* and *E. ewingii* have been noted in infected humans and dogs; however, not all *E. ewingii*

infected dogs or humans develop antibodies to *E. chaffeensis* antigens (Murphy et al. 1998; Paddock et al. 2001). Similarly, a WTD fawn experimentally infected with *E. ewingii* did not develop antibodies reactive with *E. chaffeensis* (Yabsley et al. 2002). Cross-reactions between *E. chaffeensis* and *A. phagocytophilum* have been reported in humans and may complicate diagnosis (Comer et al. 1999). Far fewer WTD in the southeastern states have antibodies reactive to *A. phagocytophilum* (<25%) than to *E. chaffeensis* antigens; however, although some deer reacted to both *E. chaffeensis* and *A. phagocytophilum* at or above the 1:128 cut-off, many also reacted to only one of these antigens at titers  $\geq 1:128$  (Little et al. 1998; Walls et al. 1998; V.G. Dugan and M.J. Yabsley, unpublished data). The high percentage of populations where seropositive or seronegative status was confirmed by PCR or culture, together with data from these other studies, provide considerable evidence that the seroreactivity reported herein largely represents specific seroconversion to *E. chaffeensis*.

The distribution of antibodies within WTD age and gender categories was investigated because these variables are an important consideration for a WTD natural sentinel system. Only a limited investigation of geometric mean titers among age categories has been reported for WTD (Lockhart et al. 1995), and any significant influence of age or gender on the occurrence of antibodies would require sampling stratified according to these host attributes. Importantly, differences in antibody prevalence were not noted among age or gender categories indicating that all WTD, particularly animals  $\geq 6$  mo of age, are suitable for use in a WTD surveillance system. In contrast to the high, stable prevalence of antibodies among age classes (Fig. 2A), PCR evidence of rickettsemia declined with age (Fig. 2B). This pattern conforms to proposed infection dynamics in naturally infected WTD whereby animals from approximately 6 months to 1.5 years of age are more likely to be rickettsemic than older adults (Paddock and Childs 2003; Dawson et al. 1994; Lockhart et al. 1997a; Davidson et al. 2001). In the present study, the prevalence of PCR positivity dropped dramatically from over half (53%) of WTD younger than 0.75 years to less than 8% of WTD older than 1.5 years. The ma-

ajority of deer tested from some seropositive populations were >1.5 years and only deer >1.5 years from other populations were available for PCR testing which may explain why not all seropositive populations were confirmed by PCR. Because the probability of a WTD being rickettsemic declines with age, serology represents a better surveillance tool than PCR, and under natural conditions of re-exposure to ticks, titers likely do not decline over time as often occurred in single exposure experimental infections of WTD (Dawson et al. 1994b; Ewing et al. 1995; Davidson et al. 2001).

Previous serologic surveys of *E. chaffeensis* in WTD (Dawson et al. 1994a; Lockhart et al. 1996; Little et al. 1997; Yabsley et al. 2002) have utilized relatively small sample sizes per population but, except for one (Mueller-Anneling et al. 2000), have not addressed the adequacy of these sample sizes to correctly classify infected and uninfected populations. Data from the current and prior studies (Dawson et al. 1994a; Lockhart et al. 1996; Little et al. 1997) have shown that infected WTD populations in the southeastern and southcentral United States typically have high (>70%) seroprevalences. Calculations (Thrusfield 1995) based on a mean 73% seroprevalence indicated that use of a sample size of five WTD per population should reliably detect nearly all positive populations. Although it was beyond the scope of this study to test enough deer to detect very low population prevalences, larger sample sizes and repeated sampling of seronegative populations did not reveal seropositive populations that would have been misclassified as seronegative based on smaller sample sizes. A chance of misclassifying newly infected populations of WTD exists because seroprevalence may be low initially; however, field evidence shows that antibody prevalence can increase rapidly once a population becomes infected with *E. chaffeensis* (Lockhart et al. 1995). Utilizing per county sample sizes equivalent to this study, statewide serotesting of WTD from Iowa demonstrated a clear north-south gradient of seroprevalence that corresponded to key epidemiologic factors (e.g., distributions of ticks, deer, and habitats) (Mueller-Anneling et al. 2000).

Because the distributions of pathogens often are reflected by some combination of both sta-

tic and changing geographic distributions, an important attribute of an effective surveillance system is the ability to identify both persistently enzootic locales and spread to new locations. Testing of WTD from LST infested populations over the span of 20 years showed that once this biologically important vector is present and WTD populations are infected with *E. chaffeensis*, there is a high probability that the populations will remain infected and that WTD sentinels reflect these enzootic conditions. Conversely, populations with no history of LST infestation remained consistently seronegative when tested multiple times. These results are compatible with a strong site-specific association between *E. chaffeensis* antibodies and the presence of LST (Lockhart et al. 1996). Of interest, each of two populations (Choctaw Co., AL and Bolivar Co., MS) that were earlier reported as seropositive but LST negative (Lockhart et al. 1996) were shown in this study to be both LST positive and seropositive for *E. chaffeensis*. The rare failure to find LST in seropositive WTD populations may have occurred because many collections were made during the fall hunting season when LST infestations are undergoing rapid seasonal declines (Allan 2001).

Four instances of *E. chaffeensis* spread to new locations were detected by monitoring WTD for multiple years (Table 2). At each of these locations, the change in population serologic status from negative to positive coincided with documented appearance of LST. These findings are very similar to those from a 12-year-long monitoring of a WTD population that demonstrated the introduction of *E. chaffeensis* was attributable to the establishment of LST (Lockhart et al. 1995). Because all of these changes to seropositive status were linked to the principal vector, they provide further evidence that this surveillance system reflects important epidemiologic factors and that it should discern changing local HME risks.

In summary, the biological reasons a sentinel WTD surveillance system functions effectively are that the force of transmission of *E. chaffeensis* is focused on WTD and that aspects of WTD biology allow attribution of infection to a specific geographic location. From an operational perspective, the system has the desirable at-

tributes of applicability on a wide geographic scale and logistic feasibility by using "free" (hunter-harvested) samples. The present work was done in a research context; however, it is possible to implement sentinel WTD serologic surveillance for *E. chaffeensis* in an operational public health context as clearly demonstrated previously in Iowa (Mueller-Anneling et al. 2000).

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