Prevalence of Granulocytic *Ehrlichia* Infection among White-Tailed Deer in Wisconsin

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Human granulocytic ehrlichiosis (HGE) is caused by an agent that is nearly indistinguishable from the veterinary pathogens *Ehrlichia equi* and *Ehrlichia phagocytophila*. The deer tick, *Ixodes scapularis*, is a vector of the HGE agent, and the white-tailed deer is the primary host for adult *Ixodes* ticks. We assessed the distribution of granulocytic *Ehrlichia* infection among deer living within (Wisconsin) and outside (western and southern Iowa) the geographic range of *I. scapularis*. Whole-blood samples were tested for HGE 16S ribosomal DNA (rDNA) by PCR, and *E. equi* antibody was detected by indirect immunofluorescence assay (IFA). Antibody titers of ≥1:64 were defined as positive, and all positive samples were retested with a second lot of substrate antigen. *E. equi* antibody was present in 14 (8%) of 187 Wisconsin deer and 0 of 60 Iowa specimens (rate ratio undefined; $P = 0.025$). An additional 30 serum samples from Wisconsin deer were excluded because IFA results were discrepant between substrate lots. The reciprocal antibody titers ranged from 64 to 512 (geometric mean, 141) for positive samples. PCR results were positive for 27 (15%) of 181 Wisconsin deer. The prevalence of infection in northwestern Wisconsin deer was not significantly different from that in central Wisconsin deer, as determined by IFA and PCR. In two samples that were sequenced, the 16S rDNA was nearly identical to that of the granulocytic *Ehrlichia* species but distinct from that of *Anaplasma marginale*. The DNA sequences of the samples differed from the published sequences for *E. equi*, *E. phagocytophila*, and the HGE agent by 1 or 2 nucleotides (≥99.1% homology) at phylogenetically informative sites. Granulocytic *Ehrlichia* organisms in deer are widely distributed within the geographic range of *I. scapularis* in Wisconsin. Deer may serve as useful sentinels for areas where HGE transmission to humans may occur.

Human infection with a granulocytic *Ehrlichia* species was first reported in 1994 among residents of Wisconsin and Minnesota by researchers at the Duluth Clinic in northeastern Minnesota (3). The disease was subsequently identified in areas of the northeastern United States where Lyme disease is endemic (7, 24). Human granulocytic ehrlichiosis (HGE) patients typically present with a nonspecific febrile illness, often with thrombocytopenia, leukopenia, and elevated hepatic enzymes. Amplification and sequencing of the HGE agent's specific 16S ribosomal DNA (rDNA) have demonstrated that it is nearly identical (≥99.8%) to the known sequences for *Ehrlichia phagocytophila* and *Ehrlichia equi* (8). These three organisms are included in a single genogroup that is distinct from *Ehrlichia chaffeensis*, the cause of human monocytic ehrlichiosis (24).

Arthropod vectors have been implicated in the transmission of *Ehrlichia* infections (1, 10, 18), and there is substantial evidence that the deer tick, *Ixodes scapularis*, is a vector for HGE. The majority of patients with HGE recall a recent tick bite (4), and *E. equi* DNA has been found in the midgut and salivary glands of an *Ixodes* tick that was removed from a patient with HGE, as well as in unfed ticks from Wisconsin (20, 22). A recent study by Telford et al. (23) confirmed that *I. scapularis* is a competent vector for HGE and that the white-footed mouse (*Peromyscus leucopus*) is a competent reservoir host.

Ehrlichia infections have been recognized in hoofed mammals for many years. *E. phagocytophila* was identified as the cause of tick-borne fever among sheep and cattle in the British Isles and Europe (11, 13). *E. equi* was originally reported as a cause of granulocytic infection in North American horses in the late 1960s (14). The white-tailed deer (*Odocoileus virginianus*) is the primary host for adult *I. scapularis* ticks, but little is known about the distribution and types of granulocytic *Ehrlichia* organisms in this species. White-tailed deer in Oklahoma and Georgia have been shown to be infected with an *Ehrlichia*-like agent that closely resembles *Ehrlichia platys*, *E. phagocytophila*, and *E. equi* (9).

The geographic areas of risk for HGE are not well defined. If deer are reliable sentinels for HGE, then the prevalence of granulocytic *Ehrlichia* in deer may provide useful information regarding areas with the highest risk of human *Ehrlichia* infection. In this study, our objectives were to determine (i) the prevalence of granulocytic *Ehrlichia* infection in Wisconsin white-tailed deer by serologic testing and PCR and (ii) the geographic distribution of infected animals.

MATERIALS AND METHODS

Specimen collection. In the autumn of 1995, deer hunters were recruited in central and northwestern Wisconsin to collect deer blood for this study. Several recruitment strategies were used, including presentations to local hunting groups, local media publicity, posters in retail stores frequented by hunters, and direct mailings to members of hunting organizations. Hunters were also recruited during two special hunts at Fort McCoy (Monroe County, Wis.), an area that has been previously shown to have high prevalence of *Borrelia burgdorferi* in *I. scapularis* ticks and white-footed mice (2). Blood collection kits were distributed at group meetings and through cooperating retail stores. Each kit consisted of two blood tubes (one for serum and one containing EDTA) and a 10-ml plastic syringe (without needle) inside a biohazard bag with instructions and a demographic-data form.

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Hunters were asked to obtain samples of pooled blood from the chest cavities of white-tailed deer when dressing freshly killed deer in the field. They were instructed to place the tubes in the biohazard bag (one bag for each deer) and keep the tubes cool after collection by refrigerating them as soon as possible. Multiple sites for dropping off the blood samples were available in central and northwestern Wisconsin. Most samples were collected during the gun hunting season in late November.

To evaluate deer outside the recognized geographic range of *I. scapularis*, we obtained stored serum samples that had been collected in western and southern Iowa during the 1993 hunting season. Whole blood for PCR testing was not available from these deer.

Serological tests. For detection of *E. equi* antibody, substrate slides with *E. equi* antigen (ProtaTek International, St. Paul, Minn.) were used in a polyvalent indirect immunofluorescence assay (IFA) with fluorescein isothiocyanate-conjugated anti-deer immunoglobulin (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Md.). Endpoint titration of serum samples established the highest dilutions at which fluorescence could be detected within infected cells (intensity, 2+ or greater). The titer was defined as the reciprocal of the highest dilution of serum with homogeneously stained cytoplasmic morulae. Titers of ≥64 were considered positive. All samples that were initially positive were retested with a different lot of *E. equi* antigen.

PCR amplification. DNA was extracted from deer blood anticoagulated with EDTA by using the Isoquick nucleic acid extraction kit (Microprobe Corp., Bothell, Wash.) according to the manufacturer's instructions. Primers Ehr 521 (5′-TGTAGGCGGTTCGGTAAGTTAAAG-3′) and Ehr 747 (5′-GCACTCAT CGTTTACAGCGTG-3′) were used to amplify a variable region of the 16S rRNA gene specific for *E. equi*, *E. phagocytophila*, and the HGE agent (20). PCR amplification with these primers yielded a 247-bp product. A group-specific hybridization probe was constructed within the 247-bp PCR product by PCR amplification with primers Ehr 552 (5′-GCCAGGGCTTAACCCTGGAGC-3′) and Ehr 706 (5′-TCCTGTTTGCTCCCCACGCTTTC-3′) (15). The PCR mixture contained 2.5 μl of extracted genomic DNA in a 50-μl reaction mixture containing 1× PCR buffer (Perkin-Elmer Corp., Norwalk, Conn.), 10% (vol/vol) glycerol, 1.5 mM MgCl$_2$, 0.2 mM (each) deoxyribonucleoside triphosphate (including a 1:1 mixture of dUTP and dTTP), 1.25 U of *Taq* polymerase (Perkin-Elmer), 50 pmol (each) of primers Ehr 521 and Ehr 747, 0.5 U of uracil-DNA glycosylase (Amperase; Perkin-Elmer), and 50 μg of isopsoralen (HRI Associates, Concord, Calif.) per ml. Amplification was performed with a thermal cycler (Barnstead/Thermolyne, Dubuque, Iowa) with a three-step program as follows: 10 min of incubation at room temperature; 10 min of denaturation at 95°C; 40 cycles of 45 s of denaturation at 94°C, 45 s of annealing at 60°C, and 45 s of extension at 72°C; and 5 min of extension at 72°C.

To reduce the risk of contamination, amplifications were done in a building separate from that in which PCR tubes were set up. For photochemical inactivation of amplicons with isopsoralen, PCR tubes were exposed to UV light for 15 min at 4°C prior to electrophoresis. No false-positive reactions were observed in any of the negative control reaction mixtures. Amplification products were visualized on ethidium bromide-stained 3% NuSieve–1% SeaKem agarose gels (FMC Bioproducts, Rockland, Maine). Amplification products were denatured in the gel and transferred onto a nylon membrane (Hybond NT; Amersham, Arlington Heights, Ill.) by Southern blotting. DNA was cross-linked to the membrane by using a Stratalinker UV cross-linker set at 1,200 J.

To generate a chemiluminescent internal probe, 1 to 10 ng of the Ehr 521 and Ehr 747 amplification product from a positive control sample was reamplified with nested primers Ehr 552 and Ehr 706 by using the reaction conditions described above. The 177-bp internal product was then purified by Centricon 100 filtration and labeled directly with the ECL kit (Amersham). Southern blots were hybridized with the chemiluminescence-labeled probe according to the manufacturer's instructions. Autoradiographs (Kodak X-Omat AR film) were obtained with 5 min of exposure; subsequent exposure times were then estimated.

PCR confirmation and DNA sequencing. Four of the initial PCR-positive samples and the two indeterminate samples were retested with a different primer set. Whole-deer-blood samples were extracted with the IsoQuick kit according to the manufacturer's instructions (Orca Research, Bothell, Wash.). Samples were first screened in duplicate for ehrlichial DNA by PCR amplification with a group-specific primer set, Ehr 521 and Ehr 790, as previously described (15). Appropriate positive and negative controls along with unknown samples were included to ensure assay validation.

Two of the positive samples were subsequently amplified with a different primer set, designated HGE1 (5′-TCCTGGCTCAGAACGAAC-3′) and HGE537 (5′-CATTTCACCTTTAACTTACCGA-3′), which was designed to allow DNA sequencing of the 5′ portion of the ehrlichial 16S rRNA gene. A 5-μl aliquot of extracted sample was added to a PCR reagent mixture containing 1× PCR buffer II (Perkin-Elmer); 10% glycerol; 200 μM (each) dATP, dCTP, and dGTP; 100 μM (each) dUTP and dTTP; 2.0 mM MgCl$_2$; 100 pmol of each primer; and 2.5 U of *Taq* DNA polymerase (Perkin-Elmer). Reaction mixtures were overlaid with mineral oil and incubated for 4 min at 94°C, thermally cycled 40 times at 94°C for 1 min, 60°C for 1 min, and 72°C for 1 min, and then incubated at 72°C for 5 min to allow complete strand extension. Subsequent to thermal cycling, a 10-μl aliquot of each amplification product was electrophoresed in a 2% agarose gel (Seakem GTG; FMC Bioproducts) to ensure ampli-
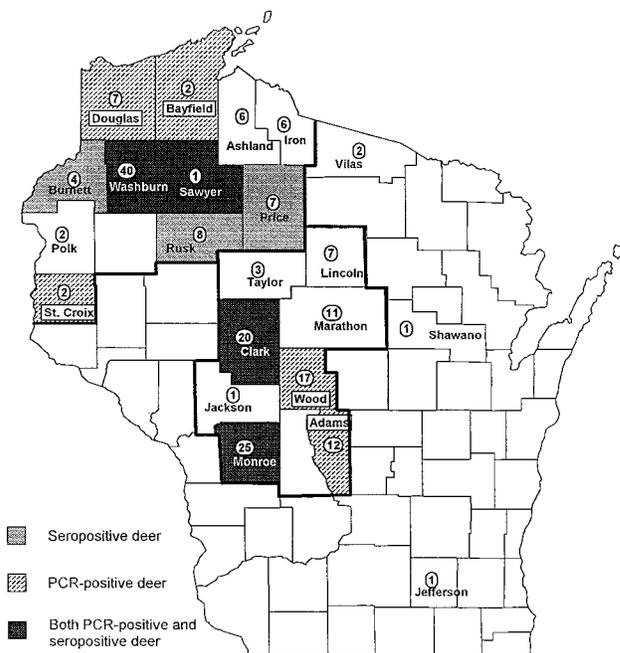

FIG. 1. Geographic distribution of granulocytic *Ehrlichia* organisms among deer as determined by PCR and serologic testing. The circled numbers are the number of serum samples tested from each county. An additional eight samples were received without county identification. Boldface lines show the borders for the central and northwestern regions. (Map provided by Wisconsin Rural Health Research Center).

fication integrity, and the remainder was purified with the QIAquick kit (Qiagen Inc., Chatsworth, Calif.) according to the manufacturer's instructions.

The sense and antisense strands of the purified products were sequenced with an ABI 373A automated sequencing instrument (Perkin Elmer/ABI, Foster City, Calif.) by using PCR primers HGE1 and HGE537 as well as the internal primers HGE20 (5′-GCAAGCT TAAGACATGCAAGTC-3′) and HGE241 (5′-AGAC CAGGTATAGATCATCGCC-3′). Sequence fragments were aligned with the sequence assembly software program Assemblylign (Oxford Molecular), and sequence ambiguities were resolved. A contiguous sequence was established from the overlapping regions of the two sequence fragments, and this was used as a query sequence in the GenBank computer sequence database (The Wisconsin Package, Madison, Wis.).

Statistical analyses. Descriptive statistics, rate ratios, and 95% confidence intervals were generated with standard microcomputer software (Epi-Info 6.0). Two-tailed P values were calculated with the chi-square test or Fisher's exact test, as appropriate. For geographic analyses, we defined three areas where deer specimens were collected. The northwestern region of Wisconsin was defined as encompassing the following 12 counties: Ashland, Barron, Bayfield, Burnett, Douglas, Iron, Polk, Price, Rusk, Sawyer, St. Croix, and Washburn. The central region of Wisconsin was defined as encompassing the following nine counties: Adams, Clark, Jackson, Juneau, Lincoln, Marathon, Monroe, Taylor, and Wood. The Iowa region included the following counties: Appanoose, Buena Vista, Carroll, Cass, Davis, Decatur, Lucas, Madison, Monona, Monroe, Pottawattamie, Ringgold, Wayne, and Woodbury.

RESULTS

A total of 217 serum samples from Wisconsin deer were tested by IFA. Thirty serum samples gave discrepant results on repeated serologic testing, and they were excluded from further analyses. The remaining 187 samples had been collected in 22 different counties (Fig. 1). Ninety-one (49%) of the deer were male and 61 (33%) were female, and the gender was not specified for the remainder.

Fourteen (8%) of the 187 Wisconsin deer were seropositive (titer, ≥64), compared to 0 of 60 deer from the western and southern regions of Iowa (rate ratio undefined; $P = 0.025$). The titers for all Iowa deer were <16, and the Iowa deer were
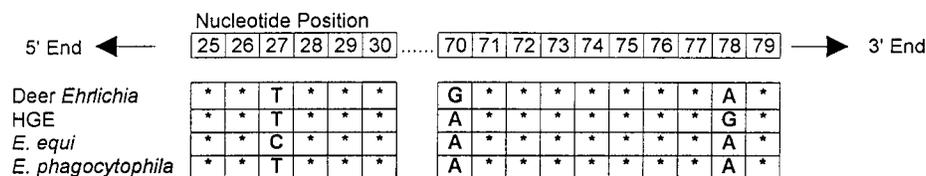
FIG. 2. Nucleotide discrepancies in a 230-bp region of the 16S rDNA sequences of *Ehrlichia* organisms from white-tailed deer and of other granulocytic *Ehrlichia* spp. Asterisks indicate nucleotides that were identical in all four organisms, and all positions not shown were identical. The numbering system is based on the reported sequence for *E. equi*.

excluded from further analyses. The geometric mean titer for positive specimens was 141 (range, 64 to 512). One or more deer were seropositive in 7 of the 22 Wisconsin counties (Fig. 1). Ninety samples (48%) were collected in the northwestern region of Wisconsin, 85 (46%) were collected in the central region, and 12 (6%) were collected in other parts of Wisconsin. The prevalence of *E. equi* antibody was highest among deer in the northwestern region and among male deer, although the differences were not statistically significant. Seven (8.2%) of 85 deer in the northwestern region were seropositive, compared to 4 (4.4%) of 90 deer in the central region (rate ratio, 1.9; 95% confidence interval, 0.6 to 6.1; $P = 0.30$). Six (6.6%) of 91 male deer were seropositive, compared to 1 (1.6%) of 61 female deer (rate ratio, 4.0; 95% confidence interval, 0.5 to 32.6; $P = 0.24$).

PCR testing demonstrated that 25 of 181 deer had *Ehrlichia*-like 16S rDNA, and the PCR result was indeterminate (i.e., discrepant results on two different PCR runs) for 2. Four of the initial PCR-positive samples and both of the indeterminate samples were retested with primer set Ehr 521-Ehr 790, and all were confirmed. Overall, 27 (15%) of the 181 specimens were PCR positive. An additional 12 (46%) of 26 specimens from Fort McCoy (Monroe County) were PCR positive, but these specimens were transported together in a single bag. All other specimens were submitted in individual bags. Due to the possibility of cross contamination, the Fort McCoy PCR results were excluded from the subsequent analyses.

There was no association between the initial PCR result and the IFA result ($P = 1.0$). Of the 27 PCR-positive deer, 21 also had serum samples submitted for serologic testing. Only one (5%) was seropositive. Excluding Fort McCoy, the prevalence of *Ehrlichia*-like 16S rDNA according to PCR was similar in the northwestern region (13%) and the central region (17%), and it was 15% in both male and female deer. Deer in 9 of 22 counties had evidence of HGE genogroup infection according to PCR. Overall, deer in 12 of 22 Wisconsin counties had evidence of HGE genogroup infection according to serology alone (3 counties), PCR alone (5 counties), or both PCR and serology (4 counties). We were not able to exclude *Ehrlichia* genogroup infection in the remaining 10 counties, because fewer than 12 specimens were submitted from each.

DNA sequence analysis of a 300-bp segment from position 521 to 790 of the 16S rRNA genes from two PCR-positive samples indicated that the sequences were identical and that they were 100% homologous with the ehrlichial genogroup that contains the agent of HGE, *E. equi*, and *E. phagocytophila*. They were distinct from the related veterinary pathogen *Anaplasma marginale*, which was 98% homologous.

Additional DNA sequence analysis of a 230-bp phylogenetically informative region in the 5′ end of the gene demonstrated that its sequence differed slightly from the reported sequences for the HGE agent (99.1% homology), *E. equi* (99.1% homology), and *E. phagocytophila* (99.6% homology). There were 2 nucleotide differences between the deer pathogen amplicon sequence and the published sequence for the HGE agent and a single nucleotide difference between the deer pathogen amplicon sequence and the published *E. phagocytophila* sequence (Fig. 2).

DISCUSSION

Our results indicate that *E. equi* genogroup infection is widely distributed among white-tailed deer within the geographic range of *I. scapularis* ticks in Wisconsin and absent from an area of Iowa where these ticks are not found. The areas of Wisconsin with granulocytic-*Ehrlichia*-infected deer include the counties where most recognized cases of human ehrlichiosis have occurred (21), so deer may serve as useful sentinels for *Ehrlichia* infection in areas where transmission to humans can occur. The utility of deer in this capacity was demonstrated by a longitudinal study of *E. chaffeensis* infection in Clarke County, Georgia (17). From 1981 to 1993, the prevalence and intensity of infestations with the tick vector (*Amblyomma americanum*) increased dramatically, with a parallel increase in the seroprevalence of *E. chaffeensis* in deer.

The DNA sequencing results raise questions about the identity of the deer-derived *Ehrlichia* agent and its relationship to the HGE agent. The 16S rDNA sequence of the HGE agent differs from that of *E. equi* by 3 nucleotides (99.8% homology), and it differs from that of *E. phagocytophila* by 2 nucleotides (99.9% homology) (8). In this study, the white-tailed deer-derived *Ehrlichia* agent was most closely related to *E. phagocytophila*, with only a single nucleotide difference, but it differed from the HGE agent by 2 nucleotides. It also differed from the *Ehrlichia*-like agent previously found in southern white-tailed deer (9). The taxonomic significance of these differences is unclear. Polymorphism of *E. equi* has been reported by Goodman et al. (12), who identified a strain that differed from the published sequence by a single nucleotide. Inoculation of horses with the HGE agent causes a disease that is indistinguishable from that caused by *E. equi* infection, and there is subsequent immunity to infection with *E. equi* (6, 19). It is likely that the HGE agent, *E. equi*, *E. phagocytophila*, and the deer-derived *Ehrlichia* agent are all different strains of the same species, but it is also possible that the sequence polymorphism indicates underlying antigenic differences. Since only two deer samples could be sequenced, we were unable to determine if the 16S rDNA sequence matched the reported sequences for the HGE agent or *E. equi* in the other PCR-positive deer.

There are several possible explanations for the apparent absence of an association between the PCR result and the IFA result. False-positive PCR results are unlikely, since all of our negative controls were appropriately negative and detection of two additional nonoverlapping targets was confirmed in a subset of specimens. The specificity of PCR testing for the *E. equi* genogroup should be very high, but PCR cross-reactivity may occur in samples containing related organisms. Since the spec-

ificity of the IFA test in deer is unknown, there is also a potential for false-positive results with this assay. There are also possible biologic explanations for the PCR and serologic results. This was a cross-sectional study, and we may have sampled deer at different stages of *Ehrlichia* infection. The PCR-positive deer could have been more recently infected than the seropositive deer. In addition, the immune response in some animals may have been blunted due to coinfecting pathogens (16). A more complete understanding of the natural history and immunologic effects of *Ehrlichia* infection in deer is necessary to address these possibilities.

Transovarial transmission of *E. phagocytophila* apparently does not occur (18), and it is unlikely to occur with the HGE agent. As a result, infection of successive tick generations would require a competent reservoir host. The white-footed mouse, *P. leucopus*, is the principal host for immature *Ixodes* ticks, and Telford et al. have convincingly demonstrated the importance of this host as a reservoir for HGE (23). Although white-tailed deer appear to be commonly infected with granulocytic *Ehrlichia* organisms, they may be incidental hosts along with humans. Recent case reports of HGE following contact with deer blood suggest that deer may pose a risk for humans with certain types of exposure, although unrecognized tick bites remain a possible explanation for these anecdotal cases (5).

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