1997

Serologic and Molecular Detection of Granulocytic Ethrlichiosis in Rhode Island

Min-Tsung Yeh
University of Rhode Island

Thomas N. Mather
University of Rhode Island

Richard T. Coughlin

Cindy Gingrich-Baker

John W. Sumner

See next page for additional authors

Citation/Publisher Attribution

Yeh, M-T., Mather, T. N., Coughlin, R. T., Gingrich-Baker, C., Sumner, J. W., & Massung, R. F. (1997). Serologic and Molecular Detection of Granulocytic Ethrlichiosis in Rhode Island. *Journal of Clinical Microbiology, 35*(4), 944-947. Retrieved from https://jcm.asm.org/content/35/4/944
Available at: https://jcm.asm.org/content/35/4/944

Serologic and Molecular Detection of Granulocytic Ethrlichiosis in Rhode Island

Authors

Min-Tsung Yeh, Thomas N. Mather, Richard T. Coughlin, Cindy Gingrich-Baker, John W. Sumner, and Robert F. Massung

JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1997, p. 944–947 Vol. 35, No. 4
0095-1137/97/$04.00+0

Serologic and Molecular Detection of Granulocytic Ehrlichiosis in Rhode Island†

MIN-TSUNG YEH,[1] THOMAS N. MATHER,[1]* RICHARD T. COUGHLIN,[2] CINDY GINGRICH-BAKER,[2] JOHN W. SUMNER,[3] AND ROBERT F. MASSUNG[3]

Center for Vector-Borne Disease, University of Rhode Island, Kingston, Rhode Island 02881[1]; Aquila Biopharmaceuticals, Inc., Worcester, Massachusetts 01605[2]; and Division of Viral and Rickettsial Diseases, National Center for Infectious Diseases, Centers for Disease Control and Prevention, Atlanta, Georgia 30333[3]

Received 7 October 1996/Returned for modification 21 November 1996/Accepted 14 January 1997

A new indirect fluorescent-antibody (IFA) assay with antigen produced in vitro in the human promyelocytic leukemia cell line HL60 was used to identify the first recognized case of human granulocytic ehrlichiosis in Rhode Island. This IFA assay was used to detect granulocytic ehrlichiae in white-footed mice and in a dog inhabiting the area surrounding the patient's residence. Host-seeking *Ixodes scapularis* ticks found in the same habitat also were infected. *I. scapularis* ticks collected from other locations were fed on dogs and New Zealand White rabbits to assess the competency of these species as hosts of granulocytotropic *Ehrlichia*. Tick-induced infections of dogs were confirmed by serologic testing, tissue culture isolation, and PCR amplification, whereas several rabbits seroconverted but were PCR and culture negative. PCR amplification of the 16S rRNA gene and DNA sequencing of the PCR products or culture isolation was used to confirm granulocytic *Ehrlichia* infections in humans, dogs, white-footed mice, and ticks.

The agent of human granulocytic ehrlichiosis (HGE), a newly emerged tick-transmitted infection, is closely related to two veterinary pathogens, *Ehrlichia equi* and *Ehrlichia phagocytophila*, the agents of equine granulocytic ehrlichiosis (2, 15–17) and caprine ehrlichiosis (10), respectively. Both HGE and equine granulocytic ehrlichiosis appear to be tick transmitted (1, 3, 4, 15, 22, 26). Patients with HGE often exhibit relatively nonspecific clinical symptoms, including fever, chills, severe headache, and myalgia. Hematologic findings often include anemia, thrombocytopenia, and leukopenia, and morulae may be seen in the cytoplasms of neutrophils (1, 4, 12, 26, 27). HGE recently has emerged in several localities in the United States, including California, Massachusetts, Minnesota, New York, and Wisconsin, is occasionally fatal, and poses a serious threat to public health (1, 3, 4, 11, 12, 26, 27).

Recent reports have shown that dogs may serve as hosts for granulocytotropic *Ehrlichia* (GE) that is indistinguishable on the basis of 16S ribosomal DNA sequences from the agent that causes HGE (13, 14). Likewise, recent experimental-transmission studies have shown that rodents are competent host species for such GE (25). Evidence implicating *Ixodes scapularis* ticks as a vector has been provided by several groups (18, 22, 25). Although those studies indicate the potential of those species as hosts, broader studies using reliable serologic and molecular methods will be needed to identify the natural reservoirs and vectors of GE and to determine the extent of human infection risk.

E. equi-infected horse leukocytes have been successfully used to detect antibodies against HGE organisms by indirect fluorescent-antibody assay (IFA) for humans clinically diagnosed with HGE (7, 12), but preparation of *E. equi* antigen is inconvenient. Not only do horses need to be inoculated with *E. equi* but also *Ehrlichia*-infected leukocytes need to be separated from the erythrocytes during the peak of bacteremia (7, 16, 21). We report here on the first use of an IFA with GE antigen derived from HL60 cell cultures for serologic studies of GE in different animals. This method also was used to identify the first reported case of HGE in Rhode Island.

MATERIALS AND METHODS

Isolation of HGE in cell culture. The human promyelocytic leukemia cell line HL60 (ATCC CCL 240) was used to isolate GE from blood samples. Cells were cultured in RPMI 1640 supplemented with 10 to 20% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, and 0.1 mM minimal essential medium nonessential amino acids. Cells were maintained at 37°C and 6% CO_2 in a humidified chamber. The HGE agent (strain USG3) was originally isolated in 1994 from a purpose-bred beagle challenged experimentally by adult *I. scapularis* ticks collected from both Westchester County, N.Y., and Montgomery County, Pa. Confirmation of the newly isolated *Ehrlichia* was made by PCR assay and DNA sequence analysis.

Preparation of antigen. GE antigen was prepared from stock cultures of the USG3 strain grown in HL60 cells. Cultures grown for 2 to 3 days were harvested by centrifugation at 2,000 × *g* for 10 min. Cell pellets were washed three times by suspension in 500 μl of phosphate-buffered saline (PBS) (0.01 M, pH 7.38), recentrifugation at 12,000 × *g* for 25 s, and removal of the supernatant. After being washed, the cell pellet was resuspended in PBS to a final concentration of 5×10^7 cells/ml. Ten microliters of this cell suspension was applied to each of eight wells on a Teflon-coated glass slide (Cell-Line, Newfield, N.J.). Slides were air dried, fixed in acetone for 10 min, and stored in airtight containers at −70°C until use. Uninfected HL60 cell suspensions were prepared by the same procedure to make antigen-free control slides.

Blood and serum collections. Serum samples from patients having a history of tick bites from adult *I. scapularis* and of fever, headaches, muscle aches, and fatigue were obtained from attending physicians in South Kingstown and Charlestown, R.I. Twelve human serum samples were collected from September to October 1995. Whole blood and serum were also collected from six purpose-bred beagles exposed to the bites of numerous (>20 pairs) adult *I. scapularis* ticks collected from sites in Connecticut, Indiana, New York, North Carolina, Pennsylvania, and Rhode Island (5). Blood was collected from these six dogs by venipuncture prior to, and at 2 and 4 weeks following, tick feeding. In addition, blood and serum were taken from a dog residing in the area surrounding the home of a patient with confirmed HGE (23). The dog reportedly was nearly continually infested with nymphal and adult *I. scapularis* ticks.

White-footed mice (*Peromyscus leucopus*) also were livetrapped at the residence of this HGE patient in coastal Charlestown, R.I., during a 4-week period between October and November 1995. After their capture, mice were transported to biosafety level 2/3 facilities at the University of Rhode Island. All mice were tagged and anesthetized with 2% methoxyflurane (Metofane; Pitman-Moore Inc., Mundelein, Ill.), and whole-blood samples were obtained by cardiac puncture and either mixed with EDTA or left untreated. Serum was collected

* Corresponding author. Phone: (401) 874-5616. Fax: (401) 874-4017.

† Contribution 3408 from the Rhode Island Agricultural Experiment Station.

TABLE 1. Reciprocal titers of IgG antibody to culture-derived HGE agent in an IFA with sera from various naturally and experimentally infected host species

Host	No. tested	No. seropositive	Positive cutoff	IgG titer or range	PCR or culture confirmation (no. positive/no. tested)
Human	12	1	<32	512	1/1
Dog[a]	6	2	<64	256–512	2/6
Dog[b]	1	1	<64	1,024	1/1
White-footed mouse	26	4	<32	64–256	1/4
New Zealand White rabbit	7	2	<64	256	0/2

[a] Purpose-bred beagles challenged with field-collected ticks from various locations.
[b] Naturally infected dog from Charlestown, R.I.

from a portion of the sample, and the EDTA-containing blood was frozen and stored for PCR analysis.

New Zealand White rabbits were exposed to the bites of numerous (>45 pairs) adult *I. scapularis* ticks collected from sites where HGE had previously been detected (28). Blood was collected from seven rabbits by venipuncture of the ear prior to and 4 weeks following tick feeding. Sera were prepared and stored at −70°C.

Immunofluorescence assay. Sera from human patients, dogs, feral white-footed mice, and tick-challenged rabbits were initially diluted with PBS in two-fold serial dilutions. Control sera from healthy dogs ($n = 6$), rabbits ($n = 7$), mice ($n = 6$), and symptom-free humans ($n = 3$) were processed in the same way. Each diluted serum sample was applied to IFA slides coated with HGE agent (USG3)-infected HL60 cells and to slides treated with uninfected HL60 cells. Slides were incubated in a humid chamber for 30 min at 37°C. Any unbound antibody was removed by soaking the slides in PBS for 20 min. Slides were air dried. Fluorescein isothiocyanate (FITC)-labeled goat anti-human immunoglobulin G (IgG) conjugate (Southern Biotechnology Associates Inc., Birmingham, Ala.), FITC-labeled anti-dog IgG, FITC-labeled anti-white-footed-mouse IgG (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.), and FITC-labeled goat anti-rabbit IgG (Sigma Chemical Co., St. Louis, Mo.) were diluted in PBS (optimum working dilutions were 1:50, 1:20, 1:10, and 1:80, respectively), and 20 μl of each dilution was applied to each well. Slides were incubated again for 30 min and then soaked in PBS for 20 min. Slides were mounted with PBS-glycerol (1:9) and examined at magnifications of ×40 and ×100. Cutoff values for positives were chosen as the next doubling dilution above the value at which nonspecific autofluorescence was observed in the negative control sera.

DNA purification. Total DNA was purified from 200 μl of EDTA-containing whole blood by using the QIAamp blood purification kit (Qiagen Inc., Chatsworth, Calif.). The protocol used was as suggested by the manufacturer. Briefly, cells were lysed during a 10-min incubation at 70°C and then applied to a QIAamp spin column containing a silica gel-based membrane. Cellular debris was removed by centrifugation and subsequent washes. Purified DNA was eluted from the columns in 200 μl of Tris (10 mM, pH 8.0) and stored at 4 or −20°C until used as a template for PCR amplification.

PCR amplification. PCR amplification was performed with primers designed to amplify the 16S rRNA genes of two species of GE, *E. equi* and *E. phagocytophila*, and the HGE agent. The GenBank/EMBL accession numbers for 16S rRNA DNA sequences that were used for primer design were M73223 (*E. equi*), M73220 (*E. phagocytophila*), and U02521 (HGE agent). Primer GE3a is the forward primer and corresponds to bases 36 to 60 of the HGE agent 16S sequence. Primers EC102 and GE2 are reverse primers and correspond to the complement of HGE agent 16S nucleotides 734 to 758 and 571 to 594, respectively.

Primary PCR amplification was performed with 10 μl of the purified DNA as the template. Reagents were from the GeneAmp PCR kit with AmpliTaq DNA polymerase (Perkin-Elmer), and reactions were performed in a volume of 100 μl in a Perkin-Elmer 9600 thermal cycler. Each primary PCR amplification mixture contained 200 μM each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), 2.5 U of *Taq* polymerase, and 0.5 μM (each) primers GE3a (5′ CACA TGCAAGTCGAACGGATTATTC) and EC102 (5′ GCGTGGACTACAAGG GTATCTAAT). An initial 2-min denaturation at 95°C was followed by 40 cycles, each consisting of a 30-s denaturation at 94°C, a 30-s annealing at 55°C, and a 1-min extension at 72°C. After completion of the 40 cycles, a single 5-min extension at 72°C was done.

The heminested PCR was performed by using 1 μl of the primary PCR product as the template. Each nested-amplification mixture contained 200 μM each deoxynucleoside triphosphate (dATP, dCTP, dGTP, and dTTP), 2.5 U of *Taq* polymerase, and 0.2 μM (each) primers GE3a and GE2 (5′ GGCAGTAT TAAAAGCAGCTCCAGG) in a total reaction volume of 100 μl. Nested-cycling conditions were identical to those for the primary reactions, except that 25 cycles were used. Reaction products were maintained at 4 or −20°C until analyzed by agarose gel electrophoresis and DNA sequencing.

DNA sequencing. PCR products were purified with Wizard PCR Preps (Promega Corp.) according to the manufacturer's instructions. DNA sequencing reactions were performed with fluorescence-labeled dideoxynucleotides, with amplification in a Perkin-Elmer 9600 thermal cycler. Sequencing products were analyzed with an ABI 377 automated DNA sequencer and compared with previously published sequences by using the Genetics Computer Group (Madison, Wis.) program package on a SUN workstation (Sun Microsystems, Inc., Mountain View, Calif.).

RESULTS

IFA results for serum samples taken from 12 patients indicated that only 1 patient had been exposed to GE (Table 1). This patient's IgG titer was 512 when tested 5 weeks following acute symptoms and was 1,024 when tested 11 weeks later. PCR confirmation of HGE was obtained in tests conducted on the first but not on the follow-up blood sample from this patient (Fig. 1, lanes 1 and 2). Serum from the dog residing at the property adjacent to that of this patient also exhibited a high IFA IgG titer (1:1,024); the blood sample from the dog was also positive for HGE in the PCR assay (Fig. 1, lane 3). Both the patient and the dog had experienced clinical symptoms consistent with infection by GE, although parasite inclu-

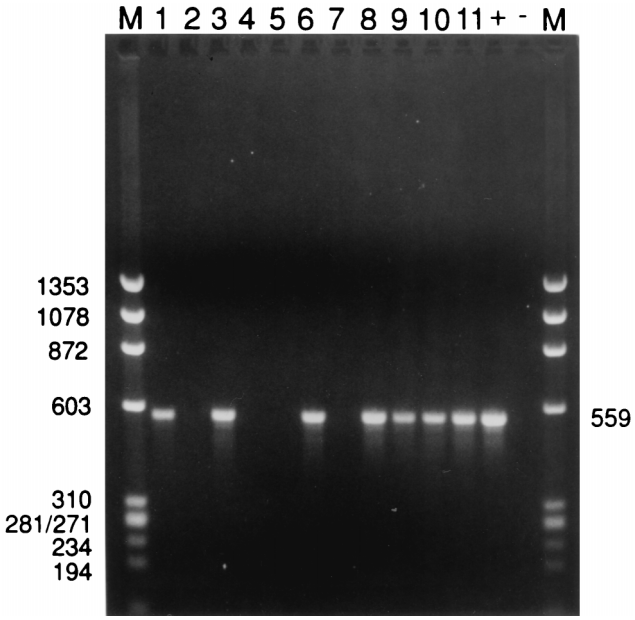

FIG. 1. Agarose gel electrophoresis of PCR products resulting from amplification of 16S ribosomal DNA of GE. Lanes labeled M represent molecular standards; sizes (in base pairs) are indicated on the left. Samples include a sample from a patient from Rhode Island, 5 weeks postinfection (lane 1); a sample from the same patient, 15 weeks postinfection (lane 2); a sample from a dog (lane 3); samples from field-collected *P. leucopus* mice (lanes 4 to 7); and samples from field-collected *I. scapularis* ticks (lanes 8 to 11). Lanes labeled + and − represent positive and negative controls, respectively. The size of the GE 16S ribosomal DNA amplified product is indicated on the right.

sions were not observed in thick or thin Wright- or Giemsa-stained smears. The patient had acute symptoms, including severe myalgia, fever, and lassitude. The dog exhibited mild depression but rather normal hematologic findings, except an elevation of its band neutrophils.

Questing adult *I. scapularis* ticks were collected from the yard of the HGE patient and examined for *Ehrlichia* organisms by PCR. Two of 15 female and 2 of 15 male ticks were positive for the organism, indicating that approximately 13% of adult ticks in this location contained GE (Fig. 1, lanes 8 to 11). The DNA sequences determined for the 559-bp PCR products amplified from the human, dog, rodent, and tick samples were identical and corresponded to HGE agent sequences previously described (4). We also tested sera collected from 26 white-footed mice trapped at the residence of the aforementioned patient. In total, 4 of the 26 samples had positive IFA IgG titers ranging between 64 and 256 (Table 1). One of these four mice (titer, 256) was confirmed as being HGE agent positive by the PCR assay (Fig. 1, lanes 4 to 7). Control PCR results for *Ehrlichia chaffeensis*, obtained with DNA purified from the human, rodent, and tick samples, were all negative (data not shown).

Additional ticks, collected either from the yard of the patient or from two other sites located in southern Rhode Island and one site near Philadelphia, Pa., were used to challenge dogs and rabbits in the laboratory. Both dogs and rabbits challenged with field-collected ticks appeared to become infected as determined by our IFA procedure. Two of the six dogs had elevated anti-HGE IgG titers (512 and 256) by the fifth week following tick bite challenge (Table 1). In both cases, HGE organisms were isolated from blood samples and inoculated into fresh HL60 cell cultures. Preinfection IFA and culture samples from all dogs were negative, as were the postinfection samples taken from the other four dogs (IFA IgG titers, <64). Of the seven rabbit sera tested, two exhibited positive IgG titers (256); one of these was from a rabbit challenged with ticks collected from the yard of the HGE patient. The other was from a rabbit challenged with ticks from a cohort that also successfully infected one of the two culture-positive dogs. However, HGE organisms could not be cultivated from any of the rabbit blood samples, nor was any of these samples positive by PCR assay.

Species-specific control sera generally failed to react with culture-derived HGE in the IFA at or above a 1:16 dilution for human and mouse samples and a 1:32 dilution for dogs and rabbits. An exception occurred with one of the experimentally challenged dogs, for which both the pre- and post-tick challenge IgG titers were 128. All positive sera resulted in brightly staining intracellular morulae as well as in extracellular ehrlichial elementary bodies, while only slight background fluorescence was seen in noninfected HL60 cells irrespective of whether the sample was positive or negative (Fig. 2).

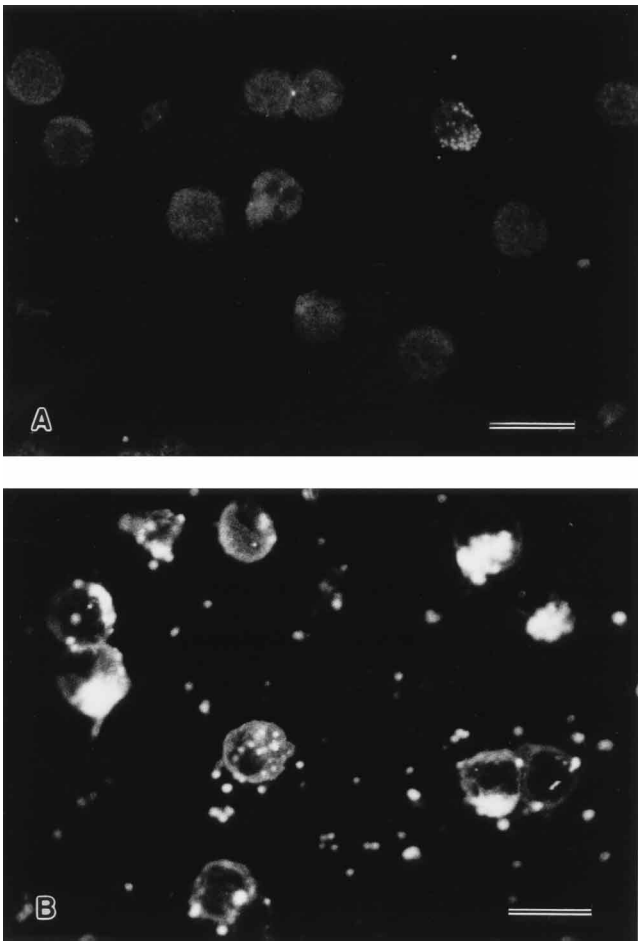

FIG. 2. Immunofluorescence of GE-infected human serum on either noninfected HL60 cells (A) or HL60 cells infected with the USG3 strain of HGE (B). Serum was diluted to 1:32. Bars, 5.0 μm.

DISCUSSION

The human promyelocytic leukemia cell line HL60 has recently been used to isolate GE from human and canine blood (6, 12). This cell line appears to be highly susceptible to infection; on the basis of 16S ribosomal DNA sequences, all GE organisms cultivated in HL60 cells derived from various sources have exhibited similarities to the veterinary pathogens *E. equi* and *E. phagocytophila*. Serologic cross-reactivity between the agent cultivated in HL60 cells and *E. equi* derived from horse blood has also been noted, and it is possible that these agents represent a single species.

An IFA with HL60 cells infected with the USG3 strain of GE as the antigen was used to detect the first case of HGE in Rhode Island. The infection was confirmed by a positive PCR assay performed 5 weeks after the initial onset of acute-phase symptoms. The patient had been treated with amoxicillin for suspected Lyme disease during this interval but remained somewhat symptomatic throughout the period. The only previous report of a protracted HGE infection was for a patient from the upper midwestern United States who was PCR positive 21 days after the onset of symptoms (8), although similar cases of prolonged fever have been described for *E. chaffeensis* infections of humans (9, 24). These data suggest that both the monocytic and the granulocytic forms of human ehrlichiosis have the potential to be prolonged and to include low-grade fever.

Recognition of the first HGE case in Rhode Island caused us to extend this study to investigate potential natural host reservoirs and vectors of the HGE agent in the area surrounding the home of the patient who presented the index case. Within this relatively rural area, a neighbor, who was also symptomatic, remained IFA negative for HGE but was positive for antibodies to *Borrelia burgdorferi*, the agent of Lyme disease. From the same area, the one dog tested was both IFA and PCR positive for HGE, and 13% of adult deer ticks were also PCR positive for HGE. Additionally, 15% of white-footed mice were IFA positive, supporting experimental-transmission studies that

have described *P. leucopus* as a competent host for GE (25). Together, these data suggest that GE is relatively common at this location and presents a significant potential for causing human and veterinary infections. Additional studies will be required to determine if GE is focally restricted to this site or whether this location more broadly represents the situation found throughout regions where *I. scapularis* is endemic in southern Rhode Island (20) and elsewhere. Magnarelli et al. (18) reported a similar proportion of *I. scapularis* ticks positive for GE in two Connecticut towns as determined by reactivity to *E. equi* antibody in an IFA. However, in the same study, other tick samples tested by PCR indicated that GE infection rates for *I. scapularis* populations in Connecticut ranged from 10 to 91%. The reasons for such discrepancies are not yet understood, but primer specificity must always be considered when discussing PCR-based results. In the present study, a novel heminested PCR assay was employed. The specificity of this assay for identifying GE DNA from the samples tested in this study was confirmed by DNA sequencing of all PCR products. The sequences determined for the PCR products from mice, ticks, and a human were identical to the 16S rRNA DNA sequence of the HGE agent previously described (4).

When we challenged laboratory-bred dogs with ticks collected from Rhode Island and other sites, two of six dogs became infected with GE, confirming the competency of canines as hosts and *I. scapularis* as a vector of GE. An analogous experiment with New Zealand White rabbits resulted in seroconversions in two of seven animals. However, DNA samples from seropositive rabbits were consistently PCR negative, suggesting that if an infection was established, it either was very transient or involved a site with a noncirculating cell population.

To date, serologic diagnosis of HGE has depended on visualizing parasites in granulocytes purified from horses acutely infected with *E. equi* (1). Consequently, serologic testing was not widely practiced because it required obtaining buffy coat smears or purified granulocytes from infected animals. Cultivation of the HGE agent in HL60 cells makes it possible to obtain large quantities of specific antigen. We have shown that the GE antigen produced in cell culture was reactive with sera from an infected human, dogs, and mice, all from a site where a GE infection occurred, as confirmed by either PCR or culture. The availability of the USG3 strain as an antigen should facilitate diagnostic and epidemiologic studies, and the strain can be used for the development of more sensitive diagnostic assays. Additional studies will be necessary to determine the relationship of the USG3 strain to other isolates or clinical samples of HGE and *E. equi*, although preliminary molecular and antigenic studies have found no significant differences among any of the North American GE samples tested to date (2, 7, 13, 17, 19).

ACKNOWLEDGMENTS

We thank the HGE patient in this study for providing access to her yard and Gregory E. Paquette, Clinical Laboratory, University of Rhode Island Health Services, for assistance with hematologic assays. We are grateful to B. Anderson for the original design of primers GE2 and GE3, which were subsequently modified for this study. D. R. Nelson and M. J. Mauel made constructive comments on an earlier draft.

This work was supported, in part, by National Institutes of Health grant AI 30733, CDC Cooperative Agreement U50/CCU106585-06, and a gift from the Island Fund of the New York Community Trust.

REFERENCES

1. **Bakken, J. S., J. S. Dumler, S. M. Chen, M. R. Eckman, L. L. Van Etta, and D. H. Walker.** 1994. Human granulocytic ehrlichiosis in the upper Midwest United States: a new species emerging? JAMA **272:**212–218.
2. **Barlough, J. E., J. E. Madigan, E. DeRock, J. S. Dumler, and J. S. Bakken.** 1995. Protection against *Ehrlichia equi* is conferred by prior infection with the human granulocytotropic ehrlichia (HGE agent). J. Clin. Microbiol. **33:**3333–3334.
3. **Centers for Disease Control and Prevention.** 1995. Human granulocytic ehrlichiosis—New York. Morbid. Mortal. Weekly Rep. **44:**593–595.
4. **Chen, S.-M., J. S. Dumler, J. S. Bakken, and D. H. Walker.** 1994. Identification of a granulocytotropic *Ehrlichia* species as the etiologic agent of human disease. J. Clin. Microbiol. **32:**589–595.
5. **Coughlin, R. T., C. Gingrich-Baker, T. N. Mather, M.-T. Yeh, D. Fish, and W. L. Nicholson.** Unpublished data.
6. **Coughlin, R. T., C. Gingrich-Baker, T. N. Mather, C. Murphy, and D. Fish.** Unpublished data.
7. **Dumler, J. S., K. M. Asanovich, J. S. Bakken, P. Richter, R. Kimsey, and J. E. Madigan.** 1995. Serologic cross-reactions among *Ehrlichia equi*, *Ehrlichia phagocytophila*, and human granulocytic ehrlichia. J. Clin. Microbiol. **33:**1098–1103.
8. **Dumler, J. S., and J. S. Bakken.** 1996. Human granulocytic ehrlichiosis in Wisconsin and Minnesota: a frequent infection with the potential for persistence. J. Infect. Dis. **173:**1027–1030.
9. **Dumler, J. S., W. L. Sutker, and D. H. Walker.** 1993. Persistent infection with *Ehrlichia chaffeensis*. Clin. Infect. Dis. **17:**903–905.
10. **Fogge, A.** 1951. Studies on the infectious agent of tick-borne fever and sheep. J. Pathol. Bacteriol. **63:**1–15.
11. **Gerwirtz, A. S., P. J. Cornbleet, D. J. Vugia, C. Traver, J. Niederhuber, C. P. Kolbert, and D. H. Persing.** 1996. Human granulocytic ehrlichiosis: report of a case in northern California. Clin. Infect. Dis. **23:**653–654.
12. **Goodman, J. L., C. Nelson, B. Vitale, J. E. Madigan, J. S. Dumler, T. J. Kurtti, and U. G. Munderloh.** 1996. Direct cultivation of the causative agent of human granulocytic ehrlichiosis. N. Engl. J. Med. **334:**209–215.
13. **Greig, B., K. M. Asanovich, P. J. Armstrong, and J. S. Dumler.** 1996. Geographic, clinical, serologic, and molecular evidence of granulocytic ehrlichiosis, a likely zoonotic disease, in Minnesota and Wisconsin dogs. J. Clin. Microbiol. **34:**44–48.
14. **Johansson, K. E., B. Pettersson, M. Uhlen, A. Gunnarsson, M. Malmqvist, and E. Olsson.** 1995. Identification of the causative agent of granulocytic ehrlichiosis in Swedish dogs and horses by direct solid phase sequencing of PCR products from the 16S rRNA gene. Res. Vet. Sci. **58:**109–112.
15. **Madigan, J. E., J. E. Barlough, J. S. Dumler, N. S. Schankman, and E. DeRock.** 1996. Equine granulocytic ehrlichiosis in Connecticut caused by an agent resembling the human granulocytotropic ehrlichia. J. Clin. Microbiol. **34:**434–435.
16. **Madigan, J. E., and D. Gribble.** 1987. Equine ehrlichiosis in northern California: 49 cases (1968–1981). J. Am. Vet. Med. Assoc. **190:**445–448.
17. **Madigan, J. E., P. J. Richter, Jr., R. B. Kimsey, J. E. Barlough, J. S. Bakken, and J. S. Dumler.** 1995. Transmission and passage in horses of the agent of human granulocytic ehrlichiosis. J. Infect. Dis. **172:**1141–1144.
18. **Magnarelli, L. A., K. C. Stafford III, T. N. Mather, M.-T. Yeh, K. D. Horn, and J. S. Dumler.** 1995. Hemocytic rickettsia-like organisms in ticks: serologic reactivity with antisera to ehrlichiae and detection of DNA of agent of human granulocytic ehrlichiosis by PCR. J. Clin. Microbiol. **33:**2710–2714.
19. **Massung, R. F.** Unpublished data.
20. **Nicholson, M. C., and T. N. Mather.** 1996. Methods for evaluating Lyme disease risks using geographic information systems and geospatial analysis. J. Med. Entomol. **33:**711–720.
21. **Nyindo, M. B. A., M. Ristic, G. E. Lewis, Jr., D. L. Huxsoll, and E. H. Stephenson.** 1978. Immune response of ponies to experimental infection with *Ehrlichia equi*. Am. J. Vet. Res. **39:**15–18.
22. **Pancholi, P., C. P. Kolbert, P. D. Mitchell, K. D. Reed, J. S. Dumler, Jr., and J. S. Bakken.** 1995. *Ixodes dammini* as a potential vector of human granulocytic ehrlichiosis. J. Infect. Dis. **172:**1007–1012.
23. **Rhode Island Department of Health.** Unpublished data.
24. **Roland, W. E., G. McDonald, C. W. Caldwell, and E. D. Everett.** 1995. Ehrlichiosis—a cause of prolonged fever. Clin. Infect. Dis. **20:**821–825.
25. **Telford, S. R., III, J. E. Dawson, P. Katavoloss, C. K. Warner, C. P. Kolbert, and D. H. Persing.** 1996. Perpetuation of the agent of human granulocytic ehrlichiosis in a deer tick-rodent cycle. Proc. Natl. Acad. Sci. USA **93:**6209–6214.
26. **Telford, S. R., III, T. J. Lepore, P. Snow, C. K. Warner, and J. E. Dawson.** 1995. Human granulocytic ehrlichiosis in Massachusetts. Ann. Intern. Med. **123:**277–279.
27. **Walker, D. H., and J. S. Dumler.** 1996. Emergence of the ehrlichioses as human health problems. Emerg. Infect. Dis. **2:**18–29.
28. **Yeh, M.-T., and T. N. Mather.** Unpublished data.