

## The Emergence of Another Tickborne Infection in the 12-Town Area around Lyme, Connecticut: Human Granulocytic Ehrlichiosis

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Human granulocytic ehrlichiosis (HGE) is an emerging tickborne infection, increasingly recognized in areas in which Lyme disease is endemic, but there are few data on the incidence of HGE. Prospective population-based surveillance was conducted in the 12-town area around Lyme, Connecticut, by means of both active and passive methods, from April through November of 1997, 1998, and 1999. Five hundred thirty-seven residents presenting to their primary care provider with an acute febrile illness suggestive of HGE were identified. Of these, 137 (26%) had laboratory evidence (by indirect fluorescent antibody staining or polymerase chain reaction) of HGE; 89 were confirmed cases, and 48 were probable cases. The incidence of confirmed HGE was 31 cases/100,000 in 1997, 51 cases/100,000 in 1998, and 24 cases/100,000 in 1999. A subset of sera was tested by use of immunoblot assays, and results were in agreement with indirect fluorescent antibody methods for 86% of samples analyzed. Thus, HGE is an important cause of morbidity and is now the second most common tickborne infection in southeastern Connecticut.

The agent of human granulocytic ehrlichiosis (HGE) is increasingly recognized as an important cause of acute febrile illness. The organism, closely related or identical to *Ehrlichia equi* or *Ehrlichia phagocytophila*, is transmitted by the same tick (*Ixodes scapularis*) that transmits the agents of Lyme disease and human babesiosis [1–3]. Patients diagnosed with one tickborne infection are at increased risk for other tick-associated diseases [4–7]. The incidence of emerging infections such as HGE often remains poorly defined because of a time lag between the first recognition of the infections and the development of sensitive and widely available laboratory tests. With the advent of sensitive tests, including the use of polymerase chain reaction (PCR) to detect ehrlichial DNA and the use of antibody detection assays, the true incidence of HGE can be more accurately assessed [4, 8, 9]. To date, no prospective studies

have sought to determine the incidence of HGE in a population at risk of frequent exposure to *I. scapularis* ticks.

In Connecticut, which has the highest incidence of Lyme disease in the United States [10], HGE has been a reportable disease since 1995. The first 3 years of passive surveillance determined annual HGE incidence to be 1.1–2.9 cases/100,000 [11]. Previous experience with Lyme disease reporting suggests that passive surveillance may severely underestimate disease incidence [12]. We hypothesize that the same enabling factors that have driven the emergence of Lyme disease in Connecticut, such as changes in environment, demographics, and residential preference [13, pp. 72–6], are likely to have promoted a similar increase in HGE. To more accurately assess the incidence of HGE, we supplemented the ongoing passive surveillance of the Connecticut Department of Public Health with an active surveillance system. We used PCR and antibody testing to confirm ehrlichial infection in patients with an acute febrile illness in the same 12-town area around Lyme, Connecticut, where Lyme disease was first identified and remains highly endemic [14, 15]. In this same area, *I. scapularis* ticks have been found to contain the agents of both diseases [16, 17].

### Methods

**Active surveillance.** In the early spring of 1997, active surveillance for HGE was established in the 12-town area surrounding Lyme, Connecticut (Chester, Clinton, Deep River, East Haddam, Essex, Haddam, Killingworth, Lyme, Madison, Old Lyme, Old Saybrook, and Westbrook; total population, 83,600). The high incidence of Lyme disease in this area has resulted in a greater awareness of tickborne illnesses among physicians and residents. All pri-

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Informed consent was obtained from all patients or their parents or guardians according to a protocol approved by the Yale University Human Investigations Committee.

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mary care physicians in the area were provided with detailed information about HGE and were encouraged to participate in the prospective study. Seven medical practices, representing 46 physicians, and an emergency medicine–outpatient clinic (total of 70,000 patient visits per year) agreed to participate. Patients were enrolled from April through November of 1997, 1998, and 1999. A clinically suspected case of HGE was defined as an acute febrile illness associated with headache and malaise in a resident of the 12-town area. Providers collected an acute-phase blood specimen and completed a brief case report form detailing clinical symptom(s) on presentation, date of disease onset, and choice of antibiotic treatment, if any. Decisions about antimicrobial therapy were made by each patient's physician. Convalescent-phase blood was drawn 4–10 weeks after the acute-phase sample, except for children, from whom a convalescent sample was not solicited. Study personnel traveled to the providers' offices at least once a week to retrieve specimens and enrollment packets (case report forms and consent forms). These visits also served to encourage continued cooperation in the study and to ensure the collection of convalescent samples. Laboratory test results were reported to the medical care providers once a week.

**Passive surveillance.** HGE has been a reportable disease in Connecticut since 1995. Serum samples submitted by physicians from patients with suspected ehrlichiosis were tested at the Connecticut Department of Public Health or the Connecticut Agricultural Experiment Station [11]. Cases of HGE among residents of the 12-town area identified through passive surveillance were combined with cases detected through active surveillance to yield the total numbers of cases for 1997, 1998, and 1999.

**Case definition.** Case definitions used in active and passive surveillance were previously defined by the Centers for Disease Control and Prevention [18]. A confirmed case of HGE was defined by a positive result on PCR of the acute-phase blood specimen, by seroconversion determined with the use of indirect fluorescent antibody (IFA) staining methods, or by a 4-fold change in titer of antibody between acute- and convalescent-phase specimens. A probable case was defined by a single acute titer  $\geq 80$ , without the submission of a convalescent sample. For incidence calculations, only confirmed cases were used. Immunoblot analysis was done on specimens submitted to active surveillance in 1998 to validate IFA and PCR results but was not used as part of the case definition.

**PCR assays.** DNA for PCR analyses was extracted from EDTA-anticoagulated blood specimens collected during the acute phase of disease, as described elsewhere [19]. PCR was done for all acute-phase blood specimens collected through active surveillance. In 1998 and 1999, PCR assays also were done on convalescent blood samples, for laboratory test comparisons. All DNA extraction procedures and PCR assays included positive control blood samples from a patient with HGE (confirmed by the presence of morulae on peripheral smear and by positive results on PCR) and negative control samples from a healthy person (without evidence of ehrlichial DNA). Primers used for the PCR assay (ehr-521 and ehr-747), which amplify a portion of the 16S ribosomal sequence of the HGE agent, have been described elsewhere [2]. During the second and third year of active surveillance, an additional primer set was used in parallel PCR analyses. This primer set, hge-396 (5'-TCAAGACCAAGGGTATTAGAGATAG-3') and hge-921 (5'-GCCACTATGGTTTTTCTTCGGG-3'), is based on

the sequence of *hge-44*, the gene that encodes the immunodominant 44-kDa protein of the HGE agent [20]. Compared with the 16S primers, these primers have shown superior performance [21]. PCR testing was considered positive when ehrlichial DNA was detected by either primer set.

**Serologic assays.** Antibodies to the HGE agent were detected by IFA methods and by immunoblot [7, 9]. The slides used for the IFA assay were examined for distinct fluorescence of inclusion bodies (morulae) in infected HL-60 cells (NCH-1 strain), and antibody titers  $\geq 1 : 80$  were considered positive. Tests included positive control sera from persons with proven HGE (confirmed by the presence of ehrlichial DNA), negative control sera from healthy subjects (without reactivity to HGE antigen), and PBS. Serial dilutions of all positive sera were retested to determine titration end points. Sera collected in 1998 (acute and convalescent) were tested by immunoblot with use of lysed HGE bacteria (NCH-1 strain) cultivated in HL-60 cells as antigen. Specimens were tested at a dilution of 1 : 100 and were considered positive if there was reactivity to the 44-kDa peptide, which is specific for the agent of HGE [9]. Positive and negative control sera were included with all immunoblot testing.

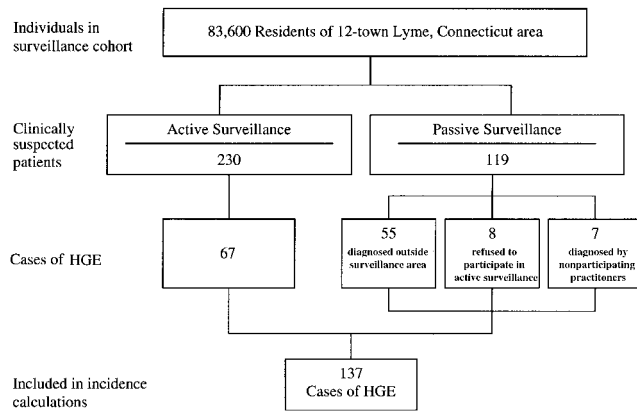
**Laboratory test comparisons and statistical analysis.** Data were entered and analyzed by use of Epi Info 6.04, a database and statistical software package [22]. Comparisons of laboratory test methods were limited to samples submitted through active surveillance. Serologic test methods were compared by contingency table by means of Fisher's exact test. Results were considered significant at the .05 level. The  $\kappa$  coefficient was calculated to assess the agreement between alternative case definitions of HGE.

## Results

A total of 537 residents of the 12-town area, clinically suspected of having HGE, were enrolled in active surveillance (311) or had specimens submitted for passive surveillance (226) from April through November of 1997, 1998, and 1999. Three hundred eight patients (57%) provided a convalescent blood sample, 226 from active surveillance and 82 from passive surveillance. Patients whose serum was submitted for HGE testing were 2–91 years old (mean, 48). During the 3 years of surveillance, 137 patients (26%; 28 in 1997, 66 in 1998, and 43 in 1999) had laboratory evidence (IFA or PCR) of HGE (figure 1); 89 (65%) were confirmed cases, and 48 (35%) were probable cases. The incidence of confirmed HGE was 31 cases/100,000 in 1997, 51 cases/100,000 in 1998, and 24 cases/100,000 in 1999. When both confirmed and probable cases were counted, the incidence of HGE was 33 cases/100,000 in 1997, 79 cases/100,000 in 1998, and 50 cases/100,000 in 1999.

Only 7 patients had HGE antibodies in both acute and convalescent sera without a 4-fold change in titer. These were regarded as having had previous exposure to the HGE agent, rather than having a current acute infection, and were not included in incidence calculations. PCR testing of these acute and convalescent samples failed to detect ehrlichial DNA.

The ages of HGE case-patients were 4–89 years (mean, 51).



**Figure 1.** Cases of human granulocytic ehrlichiosis (HGE; confirmed and probable) identified in 1997, 1998, and 1999 through active and passive surveillance in the 12-town area around Lyme, Connecticut.

Forty-three (45%) were male. Age-specific incidences were elevated in persons >50 years old (figure 2). Evidence of HGE was found in only 8 of 43 enrolled patients who were <18 years old. Of cases reported through active surveillance, 17 (85%) of 20 patients in 1997, 24 (80%) of 30 in 1998, and 11 (65%) of 17 in 1999 received doxycycline at the time of presentation, whereas the majority of the remaining case-patients received antibiotic treatment after laboratory confirmation.

Laboratory testing specific for HGE (PCR and IFA methods) was used in active surveillance. The distribution of cases from active surveillance by test and year is shown in table 1. Ehrlichial DNA was detected in blood samples of 40 (60%) of 67 case-patients, whereas 27 case-patients had antibodies without ehrlichial DNA in the submitted blood samples. To evaluate the performance of IFA procedures, immunoblot assays were done on all available acute and convalescent sera collected in 1998 (180 samples from 114 patients). There was 86% concordance between IFA and immunoblot test results (142 samples negative by both IFA and immunoblot and 12 samples reactive in both IFA and immunoblot). Twenty-six specimens showed discordant results: 19 sera were negative by IFA but positive by immunoblot, and 7 samples were reactive by IFA but not by immunoblot.

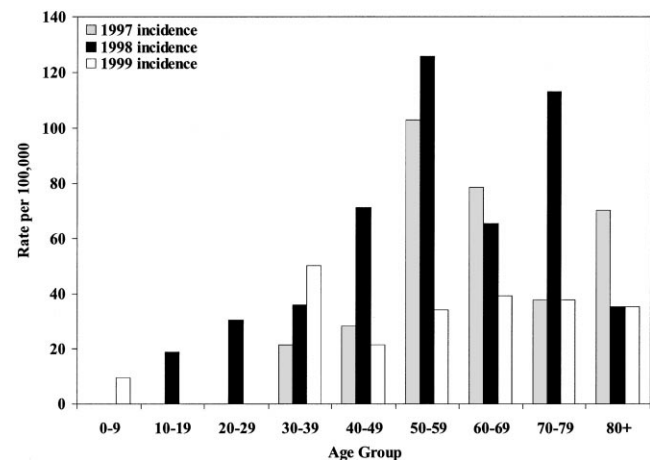
In 1998, a total of 24 HGE cases positive by PCR were detected through active surveillance. The antibody reactivity by IFA was compared with that of immunoblot assays in these cases. Samples from 7 of 24 case-patients were reactive by IFA and immunoblot in either the acute or convalescent sample, whereas 10 did not show any antibody reactivity by either test (table 2). Of the remainder, 5 were reactive by immunoblot but not by IFA, and 2 were reactive by IFA but not by immunoblot, suggesting that immunoblot is more sensitive than IFA ( $P = .045$ , Fisher's exact test).

Given the difference in IFA and immunoblot test results, an alternative case definition incorporating immunoblot proce-

dures was compared with the original definition (PCR combined with IFA methods) to determine whether it would yield substantially different overall results. The alternative case definition was defined as a positive PCR result on the acute blood specimen, seroconversion between acute and convalescent sera by immunoblot, or a single positive result on an acute specimen by immunoblot. Reactivity by immunoblot of both acute and convalescent sera was considered to represent prior exposure and was not included in this definition. With this alternative case definition, 24 cases were identified by PCR (unchanged), and 11 additional cases were detected by immunoblot, resulting in a total of 35 cases in 1998. The original case definition detected 30 cases in 1998. Testing for the  $\kappa$  coefficient assessed the agreement of the 2 case definitions at .71 (0, chance agreement; 1.0, total agreement). This suggests that IFA assays were comparable to immunoblot procedures when used in these case definitions.

Although not part of the case definition, PCR testing also was done on all 128 convalescent blood samples submitted through active surveillance in 1998 and 1999. Ehrlichial DNA was detected in 7 of these samples. One sample was collected from a person whose acute specimen also was positive by PCR 7 weeks earlier, indicating possible persistent ehrlichial infection. Although this patient received a full course of doxycycline and became clinically well, he had detectable HGE antibodies in both acute and convalescent sera by IFA methods (titers of 1 : 1260 and 1 : 160, respectively). Of the 6 other patients with PCR-positive convalescent specimens and corresponding PCR-negative acute-phase blood samples, 1 patient seroconverted by IFA assay, 1 patient had an IFA titer of 1 : 80 on an acute specimen and a negative titer on the convalescent specimen, and the other 4 patients did not show any serologic reactivity by IFA.

During 1998 and 1999, PCR analyses were done with 2 dif-



**Figure 2.** Incidence of confirmed cases of human granulocytic ehrlichiosis, by age group, from active and passive surveillance in the 12-town area around Lyme, Connecticut, for 1997, 1998, and 1999.

**Table 1.** Results of laboratory testing for human granulocytic ehrlichiosis among patients identified through active surveillance (confirmed and probable cases combined).

Patients	1997	1998	1999
No. tested	116	114	81
PCR-positive only	3	15	9
IFA-positive only	16 <sup>a</sup>	6 <sup>b</sup>	5
PCR- and IFA-positive	1	9	3
Total	20	30	17

NOTE. PCR, polymerase chain reaction; IFA, indirect fluorescent antibody.  
<sup>a</sup> One patient with reactive acute- and convalescent-phase sera but <4-fold change in titer was not included  
<sup>b</sup> Two patients with reactive acute- and convalescent-phase sera but <4-fold change in titer were not included.

ferent primer sets on 323 blood specimens from the 195 active surveillance patients (195 acute and 128 convalescent samples). The PCR assay with the 16S gene primers detected ehrlichial DNA in 25 acute blood specimens. The *hge-44* gene primers identified ehrlichial DNA in all but 4 of these specimens, as well as in 15 samples from 13 additional patients (including the 3 patients with convalescent specimens positive by PCR), confirming a previous observation that PCR testing by use of the *hge-44* primers is more sensitive than PCR with 16S primers ( $P < .001$ , Fisher's exact test) [21].

**Discussion**

We conducted population-based surveillance of HGE in the same 12-town area surrounding Lyme, Connecticut, where Lyme disease was first described and has remained hyperendemic [14]. The combination of active and passive surveillance methods in this area has yielded an incidence of 31, 51, and 24 cases/100,000 in 1997, 1998, and 1999, respectively. These rates are 20–30-fold higher than those previously reported in Connecticut [11]. In southeastern Connecticut, HGE is the second most common tickborne infection.

Few data have been published on HGE incidence in the United States. A retrospective study in Wisconsin estimated the incidence of HGE as 1.1–16.1/100,000, whereas 15% of asymptomatic residents from the same region were found to have antibodies to the HGE agent [23, 24].

There are several possible explanations for the marked difference between our incidence estimates and those previously reported. First, our estimates reflect case finding through the combination of active and passive surveillance, with each system contributing a nearly equal number of cases. Of the HGE case-patients residing in the 12-town area, 55 sought medical care outside the geographic active surveillance area and would not have been identified by active surveillance. Without the concurrent passive surveillance, this phenomenon, inherent in all studies with a circumscribed catchment area, would not have been detected. When defining the incidence of a newly emerging infectious disease, the use of a combination of active and passive surveillance will provide a better estimate of disease inci-

dence. Second, we used various criteria for HGE testing. Patients suspected of having HGE, regardless of whether they had leukopenia or thrombocytopenia, were included in the active study. Of the patients with confirmed HGE cases detected through active surveillance, 21% did not have leukopenia or thrombocytopenia. In passive surveillance, specimens were tested for HGE only if leukopenia or thrombocytopenia was reported. Interestingly, in 1998, this requirement was eliminated, and the number of HGE cases detected through passive surveillance rose from 95 in 1997 to 338 in 1998. Of the 338 cases detected in 1998, 246 (73%) did not have leukopenia or thrombocytopenia reported and, thus, could have been missed had the initial testing criteria remained in place. Third, the use of various laboratory tests affects the overall detection rate of HGE cases. The combination of PCR and IFA testing in active surveillance nearly doubled our detection rate, compared with the use of a single test (PCR or IFA). Case detection in the passive surveillance program relied mainly on IFA methods. On the basis of the data presented here, laboratory confirmation for HGE should include PCR whenever possible, along with an antibody assay (IFA or immunoblot) to optimize the overall detection rate. Fourth, the relatively high number of convalescent samples obtained in the present study enabled us to identify additional seroconversions that would otherwise have been missed. Because 25 HGE patients in the present study did not have a detectable antibody response in their acute-phase serum sample, follow-up with a convalescent blood specimen proved crucial. Furthermore, in our hands, the use of PCR of the *hge-44* gene roughly doubled the number of cases, compared with PCR of the 16S gene. That the additional cases (negative by PCR for 16S but positive by PCR for *hge-44*) are not false-positive results is supported by the fact that roughly half of the corresponding convalescent sera were reactive by IFA or immunoblot procedures. In 1997, when only PCR of the 16S genes was used, several cases could have been missed, which would explain the small number of cases identified by PCR in active surveillance that year.

**Table 2.** Comparison of polymerase chain reaction (PCR), indirect fluorescent antibody (IFA), and immunoblot test results for samples obtained from 114 patients enrolled in active surveillance for human granulocytic ehrlichiosis in 1998.

PCR <sup>a</sup>	Test combination		No. of sera
	IFA <sup>b</sup>	Immunoblot <sup>b</sup>	
+	–	–	10
+	–	+	5
+	+	–	2
+	+	+	7
–	–	–	71
–	–	+	11
–	+	–	4
–	+	+	4

NOTE. +, positive test result; –, negative test result.  
<sup>a</sup> PCR results include combination of both 16S and *hge-44* primers.  
<sup>b</sup> Antibody test includes results from both acute- and convalescent-phase sera.

Comparing the incidence of HGE with the temporal trends observed in Lyme disease incidence provides a helpful historical perspective to interpret our findings. Lyme disease incidence figures from the years immediately after the original 1977 investigation of Lyme disease in the 12-town area (43 cases of Lyme disease; incidence, 59/100,000) [14] are similar in magnitude to the HGE incidence reported here. Lyme disease incidence in the 12-town area has increased over the past 20 years (310 and 340/100,000 in 1997 and 1998, respectively) and is currently 5–10-fold greater than our HGE incidence (Connecticut Department of Public Health, unpublished data). However, the Lyme disease rates are the product of a mature surveillance system that has been in place for many years. In addition, patient and physician awareness of Lyme disease is undoubtedly higher than that of HGE. During the few years that HGE cases have been reportable in Connecticut, total numbers of cases have almost doubled each year. This trend may reflect a combination of factors, such as increasing patient and physician awareness, improved laboratory methods, and a maturing HGE surveillance system, rather than a true increase in disease. Continued surveillance over many consecutive years will be needed to accurately assess changes in incidence over time.

Our study is limited with regard to the evaluation of children with HGE because few (8%) patients enrolled were <18 years old, and, of those, only 6% had evidence of HGE. We observed most cases of HGE in older adults. This is in contrast to Lyme disease, in which incidence is elevated in both young children and older adults [15]. It is possible that in children erythema migrans, without the need for a blood specimen, may lead to a correct diagnosis of Lyme disease more readily than does a nonspecific febrile illness to HGE. The reluctance to draw blood, especially convalescent samples, from pediatric patients may have contributed to fewer-than-expected enrolled children and, hence, to an underdiagnosis of HGE in children. Alternatively, the severity of HGE may vary with age. A more asymptomatic course in children also could result in underdiagnosis of ehrlichial infections in children.

Two unexpected results were observed in the present study. First, almost one-third of the HGE cases identified by PCR methods in active surveillance had no detectable antibodies in convalescent sera. Because the majority of these patients were treated with doxycycline, the timely initiation of antibiotic treatment may have curtailed the development of antibodies. Second, ehrlichial DNA was detected in the convalescent blood specimens of 7 patients. One patient had an acute blood specimen that was positive by PCR, suggesting persistence of the HGE organism despite antibiotic treatment and a readily detectable antibody response. Persistent infection with the agent of HGE, albeit rare, has been previously reported [25].

We have observed discordant results from IFA assays and immunoblot procedures. These differences may be due in part to differences in antigen presentation in the 2 techniques. Cur-

rently there is no practical laboratory reference standard for detection of HGE, making interpretation of discordant IFA assay and immunoblot results difficult. Testing for antibodies to the HGE agent is most frequently done by IFA methods, immunoblot, or ELISA [26]. The advent of an ELISA that uses recombinant protein (HGE-44) may simplify serologic testing in the near future [27]. Whether a 2-step testing procedure consisting of ELISA and immunoblot may be necessary for the laboratory diagnosis of HGE, as is the current practice for Lyme disease, needs further evaluation. Continued improvements in laboratory testing for HGE, combined with increased awareness of this disease among physicians and patients living in areas where *I. scapularis* ticks are abundant, may lead to an even greater recognition of the impact of tickborne infections on public health.

The unexpectedly high incidence of HGE and the nonspecific signs and symptoms associated with this disease warrant continuing efforts to educate health care providers about tickborne infections other than Lyme disease. In the absence of readily available, standardized laboratory testing for newly identified pathogens, the methods presented here may serve as a paradigm for conducting surveillance for emerging infections. Our combination of active surveillance with use of multiple laboratory methods, supported by the broad-based case detection provided through statewide passive surveillance, has identified HGE as an important cause of morbidity, particularly among older adults, in southeastern Connecticut.

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