Molecular Typing of the Etiologic Agent of Human Granulocytic Ehrlichiosis

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The p44 gene of the agent of human granulocytic ehrlichiosis (aoHGE) encodes a 44-kDa major outer surface protein. A technique was developed for the typing of the aoHGE based on the PCR amplification of the p44 gene followed by a multiple restriction digest with HindIII, EcoRV, and AspI to generate restriction fragment length polymorphism patterns. Twenty-four samples of the aoHGE were collected from geographically dispersed sites in the United States and included isolates from humans, equines, canines, small mammals, and ticks. Six granulocytic ehrlichiosis (GE) types were identified. The GE typing method is relatively simple to perform, is reproducible, and is able to differentiate among the various isolates of granulocytic ehrlichiae in the United States. These characteristics suggest that this GE typing method may be an important epizootiological and epidemiological tool.

Human granulocytic ehrlichiosis (HGE) is an emerging infectious disease first reported in the United States in 1994 (7). The disease is caused by a as-yet unnamed Ehrlichia species similar or identical to Ehrlichia equi and Ehrlichia phagocytophila, which are known veterinary pathogens. Clinical symptoms of HGE include fever, myalgias, leukopenia, and thrombocytopenia (1, 3). HGE can be treated readily with tetracyclines; however, the disease has proven fatal for several patients with serious underlying medical conditions (3, 13, 18).

Most cases of HGE are reported from the northeast and upper midwestern United States. The four states reporting the highest overall incidence of HGE are New York, Connecticut, Wisconsin, and Minnesota (21). The geographic locations of reported cases correspond to the natural habitat of the implicated tick vectors. In the northeast and upper midwestern United States, the agent of HGE (aoHGE) is transmitted to humans by the black-legged tick, Ixodes scapularis (8, 14, 24, 25, 31), and in the western United States it is transmitted to humans by Ixodes pacificus (4, 5, 9, 26, 27). The HGE agent can also infect a variety of small mammals. HGE agent has been detected by PCR or culture in white-footed mice (Peromyscus leucopus) (25, 30), deer mice (Peromyscus maniculatus) (23, 33), meadow voles (Microtus pennsylvaticus) (32), eastern chipmunks (Tamias striatus) (32), and dusky-footed woodrats (Neotoma fuscipes) (20, 23). One drawback to studying the epidemiology and epizootiology of HGE is that, at present, there is no standard typing method to distinguish between unique strains of the HGE agent. Attempts to compare 16S sequences and the ank gene sequences of isolates of the HGE agent have shown little or no variability (6, 19).

We investigated the possibility of using the gene that encodes the P44 protein for the typing of the aoHGE. P44 is the designated name of a family of HGE agent outer membrane surface proteins. P44 is capable of eliciting immunogenic responses in infected patients (2, 16, 34). The gene that encodes the outer membrane protein P44 is present in multiple copies in the genome and has shown significant sequence diversity. The p44 gene was first cloned and sequenced by Ijdo et al. (17) and is homologous to the multigene family msp-2 genes in Anaplasma marginale (22). Zhi et al. (35) have estimated the copy number of the p44 gene at 18 to 22. They are of different sizes and are random dispersed throughout the HGE agent genome. Zhi and coworkers have reported the transcription of at least 5 copies of the p44 gene by reverse transcription-PCR (35). This indication of genetic diversity makes p44 an attractive target for restriction fragment length polymorphism (RFLP) analysis. PCR amplification of specific gene sequences followed by RFLP analysis has been used successfully to type other organisms, such as Helicobacter pylori (29), Staphylococcus aureus (15), Borrelia spp. (10), and Rickettsia spp. (11).

Bacterial isolates were supplied as viable cultures or DNA extracts as described in Table 1. The HGE agents were cultivated in the HL60 cell line (CCL240; American Type Culture Collection), grown in RPMI 1640 (Gibco, Grand Island, N.Y.) containing 10% fetal bovine serum (Gibco) at 37°C with 5% CO2 as described by Goodman et al. (12). The HL60 cells were harvested by centrifugation at 500 × g for 5 min when greater than 70% of the HL60 cells had visible morulae upon microscopic examination of Giemsa-stained cytopsin preparations. The cellular DNA was extracted from the HGE-infected HL60 cells using the guanidium isothiocyanate method (IsoQuick; ORCA Research Industries, Inc., Bothell, Wash.). The purified HGE agent DNA was used as template DNA in the PCR. The 50 µl of PCR contained 25 ml of Taq PCR Master Mix (Qiagen Inc., Valencia, Calif.), 1 to 5 µl of DNA template (about 0.5 µg of DNA), 5 µl of each primer (10 pmol/µl), and distilled water to bring the reaction mixture to a volume of 50 µl. The primers used were previously published by Ijdo et al.: 5'AGCGTAATGATGTCTATGGC-3' and 5'-ACCCTAAC-3', which amplify a 1,279-bp portion of the p44 gene (17). The PCR was carried out by denaturation at 94°C for 2 min and then 40 cycles at 94°C for 1 min, 58°C for 2 min, and 72°C for 3 min (1 s was automatically added to each

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TABLE 1. Source of HGE specimens

| Sample | Source | State | Provided by |
|--------|--------|-------|-------------|
| 1003   | Meadow vole (M. pennsylvanica) | Minn. | Johnson* |
| 1007   | Eastern chipmunk (T. striatus) | Minn. | Johnson |
| 1008   | Eastern chipmunk (T. striatus) | Minn. | Johnson |
| 1011   | White-footed mouse (P. leucopus) | Minn. | Johnson |
| 1014   | Eastern chipmunk (T. striatus) | Minn. | Johnson |
| 1023   | Meadow vole (M. pennsylvanica) | Minn. | Johnson |
| 1026   | Eastern chipmunk (T. striatus) | Minn. | Johnson |
| 1029   | White-footed mouse (P. leucopus) | Minn. | Johnson |
| 1033   | Southern red-backed vole (Clethrionomys gapperi) | Minn. | Johnson |
| PL59   | White-footed mouse (P. leucopus) | Minn. | Johnson |
| HGE-1  | Human | Minn. | Goodmanb |
| HGE-2  | Human | Minn. | Goodman |
| HGE-3  | Human | Minn. | Goodman |
| HGE-4  | Human | N.Y. | Goodman |
| HGE-5  | Human | N.Y. | Goodman |
| HGE-6  | Human | N.Y. | Goodman |
| Marim | Canine | Minn. | Munderloh |
| Equi   | Equine (E. equi MRK strain) | Calif. | Munderloh |
| GOM2   | Tick (I. spinipalpis) | Colo. | Piesmand |
| GOM4   | Tick (I. spinipalpis) | Colo. | Piesman |
| TB41   | Mexican wood rat (Neotoma mexicana) | Colo. | Piesman |
| TB64   | Deer mouse (P. maniculatus) | Colo. | Piesman |
| TB80   | Deer mouse (P. maniculatus) | Colo. | Piesman |
| TB108  | Mexican wood rat (N. mexicana) | Colo. | Piesman |

* Cultures from mammals trapped in Morrison and Washington counties, Minn.  
* Cultures supplied by Jesse Goodman, University of Minnesota, Minneapolis.  
* Cultures supplied by Ulrike Munderloh, University of Minnesota, St. Paul.  
* DNA extracts supplied by Joseph Piesman and William Nicholson, Centers for Disease Control and Prevention, Fort Collins, Colo.

TABLE 2. GE types characterized by restriction fragment sizes

| GE type | Fragment sizes (bp) |
|---------|---------------------|
| A       | ..................1,100, 610, 470, 225 |
| B       | ..................1,100, 490, 480, 225 |
| C       | ..................1,100, 570, 430, 310, 225 |
| D       | ..................1,100, 610, 560, 490, 470, 225 |
| E       | ..................1,100, 610, 570, 490, 470, 225 |
| F       | ..................1,100, 890, 600, 530, 490, 470, 225 |

a May also have a faint band at 750 bp.  
b May also have a faint band at 750 bp.

g eoHGE from the various hosts analyzed are shown in Table 3. Although the number of samples from the northeast and the western regions were limited, the results suggest that this typing method has strong discriminatory potential. GE types B and F were present in Colorado, and types C, D, and E were found in the north-central states (Minnesota and Wisconsin). In contrast, GE type A was present in specimens from California, Minnesota, and New York.

Three GE types were identified among the six human specimens. They were type A for the two New York patients, type C for the three Minnesota patients, and type E for the Wisconsin isolate. The Minnesota canine isolate was GE type C, which also contained molecular size markers (Kb DNA Ladder; Stratagene, La Jolla, Calif.)
the same GE type as that of the three Minnesota human isolates. The Minnesota human isolate, type C, was also present in a chipmunk and a southern red-backed vole from Minnesota. The Wisconsin human isolate, type E, was also present in eastern chipmunks captured in Minnesota. The GE type A, present in the two New York human isolates, was also present in white-footed mice and meadow voles from Minnesota as well as a horse isolate from California (Table 3). Four different GE types were represented among the 14 isolates of the aoHGE from Minnesota. GE type F, present in the Mexican wood rat and the deer mouse from Colorado, was also identified in the tick vector, *Ixodes spinipalpis*.

We investigated the stability of the GE type in two isolates, one from a human and one from a white-footed mouse. The human isolate was determined to be GE type C at tissue culture passage number 15, and after 164 passages in HL60 cells it remained type C. A white-footed mouse isolate was identified as GE type A at its second passage in HL60 cells. Its GE type remained type A after isolation from an experimentally infected mouse, 1 week postinoculation, and following 48 passages in HL60 cells. Based on the above typing results of these two aoHGE isolates, we conclude that the GE type is a stable characteristic.

The GE typing method we described is relatively simple to perform, is very reproducible, and appears to be a sensitive means to differentiate among the various isolates of granulocytic ehrlichiae. Another advantage of this technique is that it can be performed on animal and human specimens without the need to cultivate the organism. These characteristics suggest that the GE typing method has the potential of being an important epizootiological and epidemiological tool.

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