

Cloning of the Gene Encoding the 44-Kilodalton Antigen of the Agent of Human Granulocytic Ehrlichiosis and Characterization of the Humoral Response

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Antibodies in the sera of patients with human granulocytic ehrlichiosis (HGE) commonly recognize a 44-kDa antigen. We cloned the gene encoding the 44-kDa protein of the agent of HGE (aoHGE) by probing an aoHGE lambda ZAP II genomic DNA expression library with sera from aoHGE-infected mice. The gene, *hge-44*, is part of a multigene family, with sequence similarity to the *Anaplasma marginale msp-2* genes. RNA-PCR studies confirmed that *hge-44* is expressed by aoHGE cultured in HL-60 cells and by aoHGE during murine infection. Recombinant HGE-44, expressed and purified as a glutathione transferase fusion protein, was used as the substrate in immunoblots to help diagnose HGE. Antibodies in eight sera from eight patients with HGE and in two sera from two aoHGE-infected mice bound recombinant HGE-44. Antibodies in the sera of healthy individuals or patients with *Ehrlichia chaffeensis* or *Borrelia burgdorferi* infection did not recognize HGE-44. We conclude that *hge-44* is a member of a multigene family and that *hge-44* is expressed and elicits specific antibodies during infection.

Human granulocytic ehrlichiosis (HGE) is a recently recognized tick-borne infectious disease in the United States and Europe (4, 5, 10, 24, 26). *Ixodes scapularis* is a vector of the agent of HGE (aoHGE) (21), an organism that is closely related to *Ehrlichia equi* and *Ehrlichia phagocytophila* (6). Clinical infection produces acute symptoms including fever, leukopenia, thrombocytopenia, and myalgias; severe secondary complications can occasionally result in death (1, 13). Diagnosis may be aided by the identification of the characteristic aoHGE morulae in neutrophils in a peripheral blood smear (4) or by DNA detection methods such as PCR (6). Serologic tests such as immunofluorescence (IFA) (1, 17, 18) and immunoblotting with *E. equi*-infected equine neutrophils or aoHGE cultured in HL-60 cells have also proven helpful for diagnosis (15, 28).

The aoHGE 44-kDa protein is commonly recognized by immunoglobulin M (IgM) and IgG antibodies in the sera of patients with HGE (8, 15, 19, 28). Examination of aoHGE isolated from different patients reveals that although this antigen usually migrates at about 44 kDa, the molecular size may vary from 42 to 49 kDa, suggesting molecular structural differences which may account for the observed antigenic diversity (3, 30). Fractionation studies indicate that the 44-kDa protein may be located in the aoHGE outer membrane (30). It has recently been shown that sera from mice immunized with aoHGE lysates are sufficient to partially protect mice from aoHGE infection (25). This murine aoHGE antiserum has high concentrations of antibodies that bind the 44-kDa protein, suggesting that 44-kDa-protein-specific antibodies may play a role in immunity against infection. This immunogenic antigen may therefore be important in pathogenesis and laboratory

diagnosis and serve as a candidate for an HGE vaccine. The gene encoding the aoHGE 44-kDa antigen has now been cloned, and the humoral response to the recombinant protein has been characterized.

MATERIALS AND METHODS

Isolation of HGE organisms. The promyelocytic cell line HL-60 (ATCC CCL 240) was cultured in Iscove's modified Dulbecco's medium supplemented with 20% fetal bovine serum and was maintained at 37°C with 5% carbon dioxide (11). HL-60 cells were infected with the NCH-1 strain of aoHGE and cultured (15). The cell density was maintained at between 5×10^5 and 1.5×10^6 cells per ml by feeding the cells twice a week. If the cell count was below 5×10^5 cells per ml, fresh HL-60 cells were added to a final concentration of 1×10^6 cells per ml. Light microscopy slides of cultured HL-60 cells were air dried, stained with Diff-Quik (Baxter Healthcare Corp., Miami, Fla.), and examined for aoHGE infection.

Large volumes of infected HL-60 cells were grown for purification of bacteria by Renografin density gradient centrifugation as described previously (7, 12) with some modifications. Briefly, cultures with at least 70% aoHGE-infected HL-60 cells were centrifuged and resuspended in phosphate-buffered saline (PBS)–0.1% glucose. HL-60 cells were lysed by shearing with a 21-gauge needle, and the cellular debris was pelleted by centrifugation at $1,500 \times g$ (Sorvall RT600B; Sorvall, Newtown, Conn.) for 10 min. The supernatant was collected and incubated with RNase and DNase (Boehringer, Mannheim, Germany) (final concentration, 50 µg/ml). By using Renografin with a noncontinuous gradient of 42 and 30% (Hypaque 76; Nycomed Inc., New York, N.Y.), ultracentrifugation was performed at $87,000 \times g$ for 75 min at 4°C in an SW-28 swing bucket rotor (Beckman, Fullerton, Calif.). The interface band was collected in a sterile pipette, dissolved in SPGN (7.5% sucrose, 3.7 mM KH_2PO_4 , 7 mM K_2HPO_4 and 5 mM L-glutamine), and pelleted at $12,000 \times g$ (Sorvall rotor SS-34), and the HGE bacteria were resuspended in SPGN at a concentration of 2 µg/µl and stored at -70°C .

HGE library construction, screening, and sequencing. For construction of the lambda ZAP II aoHGE genomic DNA expression library, purified aoHGE was used to extract DNA as described previously (22). After random shearing of 100 µg of aoHGE DNA, *EcoRI* adapters were ligated to the ends and subsequently size fractionated (from 1 to 9 kb), and DNA was inserted into the lambda ZAP II vector (Stratagene, La Jolla, Calif.). The lambda ZAP II phages were plated on a lawn of *Escherichia coli* XL-1 Blue (Stratagene), and protein expression was induced with 10 mM isopropyl-β-D-thiogalactoside (IPTG).

In order to identify immunogenic aoHGE proteins, nitrocellulose filters containing the expressed proteins were incubated with hyperimmune murine antiserum (1:1,000 dilution). Hyperimmune antiserum was produced by immunizing 10 C3H/HeJ mice with a lysate of purified aoHGE in complete Freund's adjuvant and boosting the animals twice with the same preparation in incomplete Freund's

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1 GAATTCCTGAAAAGTATGAGAAAAGGAAAGATAAATCTTAGGAAGCGTAATGATGTCTATGGCTATAGTCATGGCTGGGAATGATGTTAGGCGTCATGATG
  M R K G K I I L G S V M M S M A I V M A G N D V R A H D
101 ACGTTAGCGCTTTGGAAACTGGTGGTGCGGGATATTTCTATGTTGGTTGGATTACAGTCCAGCGTTTAGCAAGATAAGAGATTTTACTATAAGGGAGAG
  D V S A L E T G G A G Y F Y V G L D Y S P A F S K I R D F S I R E S
201 TAACGGAGAGACTAAGCGAGTATCCATACTTAAAGGATGGAAAGAGTGTAAAGCTAGAGTCACACAAGTTTGGACTGGAACACACCTGATCCCTCGGATT
  N G E T K A V Y P Y L K D G K S V K L E S H K F D W N T P D P R I
301 GGGTTAAGGACACATGCTTGTAGCTATGGAAGGCAGTGTGGTTATGGTATGTTGGTGGCCAGGTTGAGCTTGAGATTGGTTACGAGCGCTTCAAGA
  G F K D N M L V A M E G S V G Y G I G G A R V E L E I G Y E R F K
401 CCAAGGGTATTAGAGATAGTGGTAGTAAGGAAGATGAAGCTGATACAGTATATCTACTAGCTAAGGAGTTAGCTTATGATGTTGTTACTGGACAGACTGA
  T K G I R D S G S K E D E A D T V Y L L A K E L A Y D V V T G Q T D
501 TAAGTCTACCGCTGCTCTTCCCAAGACCTCCGGTAAAGATATCGTTCAGTTTGTCTAAGGCCGTGGAGATTTCCTCCCCTAATATCGAAAAGAAGGTTTTC
  K L T A A L A K T S G K D I V Q F A K A V E I S S P N I E K K V C
601 AGGACCAAGAAGTATGGGGTTCTCGTTATAGTAAGTATGCTTCGGAAACTGCTAATAGCTCGGATGCGAGCGAAGCGGATGAGCTGTGTGTAGTGCAG
  R T K K N G G S R Y S K Y A S E T A N S S D A A K A D V A V C S A
701 CCTCTACGCCAGCAATAGTTCTCATGGGGCAGTGGTGGAGAGACTTTAAAGAACTTTGTGAGTGCACCGCTAAGTGGTGTGAGTGCAGTGTGAAGTGGCC
  A S Y A S N S S H G G T G E E T L K N F V S A T L S G D G S V N W P
801 CACGTCGAAAAAGCGGAAAGCAATGCAGGCACTCCGGAACCCGTTCAAAAACGATAACCGCGCAGCTGTAGCGAAGGACCTAGTCAAGGAATTAACCCCC
  T S K K A E S N A G T P E P V Q N D N A A A V A K D L V K E L T P
901 GAAGAAAAAACCATAGTGGCAGGGTTACTAGCTAAAACATTTGAAGGGGGCAGGTTGTTGAGATCAGGGCGGTTTCTTCTACTTCTGTGATGGTCAATG
  E E K T I V A G L L A K T I E G G E V V E I R A V S S T S V M V N
1001 CTTGTTATGATCTTCTTAGTGAAGGTTTGGCGGTTGTTCTTATGCTTGCCTTGGTCTCGGTGTAACCTTCTGGGGTTGTTGATGGCCATATCACTCC
  A C Y D L L S E G L G V V P Y A C V G L G G N F V G V V D G H I T P
1101 TAAGCTTGCTTATAGATTAAGCGCTGGGTTGAGTTATCAGCTCTCTCTGAAATATCTGCTTTTGCAGGTGGTTTCTACCATCGTGTGTGGGAGATGGT
  K L A Y R L K A G L S Y Q L S P E I S A F A G G F Y H R V V G D G
1201 GTTATGATGATCTCCGGCTCAACGCTTGTAGATGATACAGTCCGGCGGGTCTGACTAAGGATACTGCTATTGCTTACTTCCATGGCTTATGTGC
  V Y D D L P A Q R L V D D T S P A G R T K D T A I A N F S M A Y V
1301 GTGGGAATTTGGTGTAGGTTTGTCTTTTAAAG
  G G E F G V R F A F *

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FIG. 1. Nucleotide sequence of *hge-44* (from clone EM3C) and the deduced protein sequence. The putative cleavage site is indicated by a triangle. Underlined are the two peptide sequences that were obtained from the native aoHGE 44-kDa antigen and show a perfect match with the predicted amino acid sequence. The stop codon is indicated by an asterisk.

adjuvant at 2-week intervals. This serum has been shown to contain high concentrations of antibodies that bind the 44-kDa antigen in aoHGE lysates (25). After being washed, the nitrocellulose filters were incubated with a 1:5,000 dilution of alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Sigma, St. Louis, Mo.), washed again, and then immersed in 5-bromo-4-chloro-3-indolylphosphate-nitroblue tetrazolium (BCIP-NBT) (KPL, Gaithersburg, Md.) for color visualization.

After secondary screening, reactive clones were subjected to *in vivo* excision by simultaneous infection of *E. coli* XL-1 Blue cells with R408 helper phage (Stratagene), resulting in replication and recircularization of a single-stranded DNA molecule of the cloned insert and the pBluescript vector. This single-stranded plasmid was then packaged, secreted, and made double stranded by reinfection with fresh *E. coli* XL-1 Blue cells. Plasmid DNA was purified and sequenced by using the T3 and T7 primers and additional internal primers at approximate distances of 250 bp, so that both strands of the clone were sequenced internally.

Partial internal protein sequencing. aoHGE bacteria, purified from infected HL-60 cultures, were lysed, dissolved in sample buffer (5% 2-mercaptoethanol, 10% glycerol, 2% sodium dodecyl sulfate [SDS], and 0.8% bromophenol blue in 6.25 mM Tris buffer, pH 6.8), and heated for 10 min at 100°C. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) was performed with 20 µg of aoHGE protein. The 44-kDa band was excised from the gel and used for peptide sequencing at the Yale Protein Purification and Analysis Facility. Because amino-terminal sequencing was not successful, the protein was subjected to in-gel trypsin digestion, and two aliquots were selected for sequencing by matrix-assisted laser desorption-ionization mass spectrometry, yielding two peptide sequences from the 44-kDa protein.

Protein expression and purification. By using 5'-AAACCCGAATTCATGTCATGGCTATAGTCATGGCTGGG-3' and 5'-ATATATCTCGAGTCATTAAAAAGCAACCTAACACC-3' primers, a PCR-derived subclone of EM3C (a phage clone that contained the *hge-44* gene [see Results]) containing the full-length gene sequence was then constructed in frame with the glutathione *S*-transferase (GT) gene by using the pMX vector (23). This clone, designated *hge-44*-pMX, was used to transform *E. coli* XL-1 Blue. After lysis of the cells and

pelleting of the cell membranes, the soluble fraction (supernatant) and the cell pellet were subjected to SDS-PAGE and stained with Coomassie blue. Part of the GT-HGE-44 fusion protein appeared to be in the soluble fraction. GT-HGE-44 was then purified from the whole-cell lysate by using a glutathione-Sepharose-4B column.

PCR and RNA-PCR. Total aoHGE RNA, isolated from cultured and purified aoHGE, was used as a template for PCR to verify the presence and expression of *hge-44*. The primers used for PCR and RNA-PCR were 5'-AGCGTAATGATGTCATATGGC-3', starting at position 43, and 5'-ACCCTAACACCAAATTCAC-3', starting at position 1322. The denaturing, annealing, and extension temperatures were 94, 58, and 72°C, respectively, for 1 min at each step for 30 cycles, yielding an expected product of 1,279 bp. For RNA-PCR, total RNA was isolated from cultured HGE bacteria and from spleens of experimentally infected mice, by using a Micro RNA isolation kit (Stratagene). First-strand cDNA was synthesized with random primers from 5 µg of total aoHGE RNA and splenic RNA. Control studies were performed without reverse transcriptase to eliminate the possibility of DNA contamination.

Southern blotting. DNA of aoHGE (5 µg per lane) was digested with restriction enzymes *Bam*HI, *Bgl*II, *Kpn*I, and *Pst*I, electrophoresed, blotted onto nylon membranes (Hybond; Amersham, Arlington Heights, Ill.), and probed with insert of EM3C. The probe was labeled by use of a nonradioactive chemiluminescence kit (ECL; Amersham). After hybridization, blots were washed with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), 1× SSC, and 0.5× SSC for 15 min each and then exposed to X-ray film (Kodak, Rochester, N.Y.) for 24 h.

Immunoblotting. SDS-PAGE separation of recombinant protein was performed by using 10% acrylamide gels under reducing conditions (22). Two micrograms of GT-HGE-44 or GT (control) dissolved in sample buffer (5% 2-mercaptoethanol, 10% glycerol, 2% SDS, and 0.8% bromophenol blue in 6.25 mM Tris buffer, pH 6.8) and heated for 10 min at 100°C was loaded onto each lane of the gel. Molecular mass standards (Bio-Rad Laboratories, Hercules, Calif.) were used for each panel. Protein was transferred to nitrocellulose, and the blocking procedure was performed with PBS-5% nonfat dry milk. The

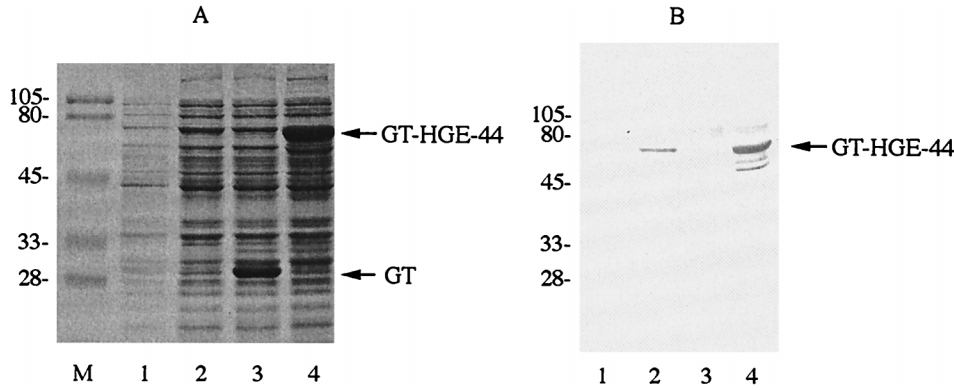


FIG. 5. Expression of GT-HGE-44. (A) Coomassie blue staining of *E. coli* lysates that expressed GT (lanes 1 and 3) and GT-HGE-44 (lanes 2 and 4). Lanes 1 and 2, no IPTG induction; lanes 3 and 4, with IPTG induction. (B) The *E. coli* lysates in panel A were probed with patient serum in an IgG immunoblot, showing reactivity to GT-HGE-44 but not to GT. The numbers on the left of each panel are molecular masses in kilodaltons.

Inc., Campbell, Calif.), followed by the putative cleavage site at six amino acids from the end of the hydrophobic core.

Some of the other clones contained only partial ORFs, which were found to have substantial similarity with sequence at the 3' end of *hge-44* but differed significantly in other areas, as shown in Fig. 3. A Southern blot hybridization was then performed, by using the restriction enzymes *Bam*HI, *Bgl*II, *Kpn*I, and *Pst*I, which do not have restriction sites within the full-length EM3C ORF. An *hge-44* probe, generated by PCR with EM3C as a template, would therefore be expected to reveal one hybridizing band if *hge-44* is a single-copy gene. However, Southern blot hybridization revealed multiple bands, suggesting that there are several sequences that hybridize with EM3C (not shown).

PCR and RNA-PCR. To confirm that *hge-44* was present in the genome of aoHGE, the *hge-44* gene was directly amplified from purified aoHGE DNA (Fig. 4, lane 5). Furthermore, to verify that *hge-44* was expressed by aoHGE, total RNA from cultured aoHGE and from spleens of aoHGE-infected mice was prepared for RNA-PCR. The products of RNA-PCR were of the expected size of 1,279 nucleotides (Fig. 4, lanes 1 and 2), and the sequencing of the PCR products confirmed that *hge-44* was transcribed both by aoHGE cultured in HL-60 cells and in vivo in aoHGE-infected mice.

Expression of the *hge-44* gene and immunoblotting. PCR was used to amplify *hge-44* and subclone the gene into the pMX expression vector, such that it was in frame with the GT-coding sequence. Expression in *E. coli* produced a soluble GT-HGE-44 fusion protein of the expected size of 70 kDa on a Coomassie blue-stained gel (Fig. 5A, lane 4), while GT (control) was also expressed and yielded a band at 26 kDa (Fig. 5A, lane 3). *E. coli* lysates that expressed GT-HGE-44 or GT were then probed with serum from a patient with HGE in an IgG immunoblot (Fig. 5B). The patient serum reacted with GT-HGE-44 (Fig. 5B, lanes 2 and 4) but not with GT (Fig. 5B, lanes 1 and 3). The band at 70 kDa in *E. coli* containing the GT-HGE-44 expression plasmid demonstrates that low-level GT-HGE-44 synthesis occurs in the absence of IPTG (Fig. 5B, lane 2). Sera from normal healthy individuals did not recognize either protein (not shown). The GT-HGE-44 was then purified by using a glutathione column and used as a substrate in further immunoblotting studies.

A total of 26 human sera were then tested for IgM and IgG by immunoblotting with purified recombinant GT-HGE-44 as the substrate. Representative immunoblots are shown in Fig. 6. The sera included those from eight patients with HGE (15),

five patients with *E. chaffeensis* infection, six patients with Lyme borreliosis, and seven normal healthy individuals. Sera from patients with *E. chaffeensis* or *Borrelia burgdorferi* infection had high-titer antibodies in *E. chaffeensis* IFA or *B. burgdorferi* enzyme-linked immunosorbent assay, respectively. All sera from HGE patients were obtained between 4 days and 6 weeks after the onset of symptoms, except for patient 2, whose serum was obtained 10 weeks after diagnosis. Six of the eight sera from patients with HGE had detectable IgM to recombinant HGE-44, and sera from all eight patients with HGE had discernible IgG. Faint bands below 70 kDa represent degradation products of the recombinant GT-HGE-44. The two HGE sera that had only IgG were obtained at 3 and 10 weeks, respectively. None of the sera from patients with *E. chaffeensis* or *B. burgdorferi* infection showed reactivity to recombinant HGE-44. In addition, normal healthy individuals did not have antibodies that bound GT-HGE-44. None of the sera that were tested bound recombinant GT (control) in immunoblotting (not shown).

Sera from two mice that were experimentally infected with

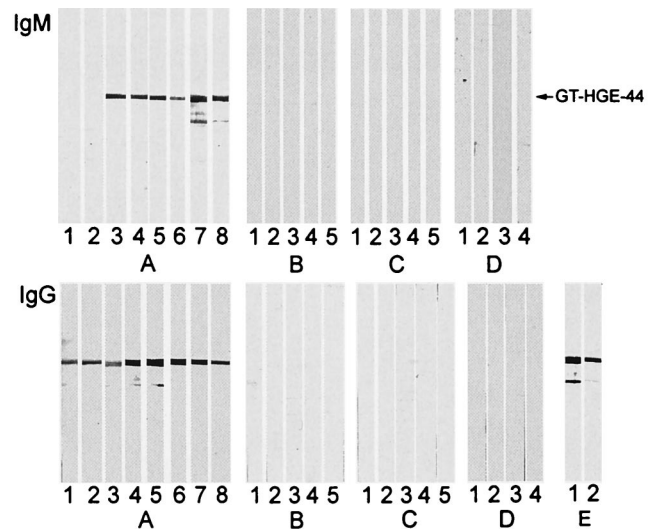


FIG. 6. IgM and IgG immunoblots of purified GT-HGE-44 probed with sera from patients with HGE (A), patients with *E. chaffeensis* infection (B), normal healthy volunteers (C), patients with Lyme disease (D), and aoHGE-infected mice (E) (IgG only).

aoHGE were also examined for antibodies (Fig. 6). Mice were infected by allowing aoHGE-infected ticks to engorge to repletion (25). Infection was documented by the identification of morulae in the murine neutrophils and cultivation of aoHGE in HL-60 cells from murine blood (25). Sera were obtained from the mice after 1 month of infection. Both animals had HGE-44 antibodies. As expected, serum from an uninfected mouse did not have antibodies that bound to GT-HGE-44 (not shown).

DISCUSSION

In this study, we describe the cloning and characterization of *hge-44*, which codes for the immunogenic 44-kDa protein of aoHGE. *hge-44* has similarity with the *A. marginale msp-2* gene family. All sera of patients with HGE that were tested had antibodies recognizing the recombinant HGE-44 on immunoblots. This suggests that a serologic assay with recombinant HGE-44 as a substrate may facilitate laboratory diagnosis of HGE.

A. marginale is an arthropod-borne hemoparasite that induces severe anemia, abortion, and death in cattle (20). The close phylogenetic relationship between *A. marginale* and the *E. phagocytophila* group, including aoHGE, has been elucidated by using the 16S ribosomal DNA sequences (2, 6, 27). It is therefore not surprising that *hge-44* has some similarity with the *msp-2* gene family and that *hge-44* may also be part of a group of closely related aoHGE genes. In *A. marginale*, the surface proteins encoded by the *msp-2* genes are antigenic variants and elicit protective responses, suggesting a possible mechanism to evade the host immune response (9). It is conceivable that different isolates of aoHGE may express homologs of *hge-44* and that aoHGE and *A. marginale* could employ similar strategies for survival within the host. Further studies need to investigate the role of HGE-44 with respect to potential protective immune responses. Already it has been demonstrated that sera from mice immunized with aoHGE-lysates (which contain high concentrations of antibodies that readily recognize HGE-44) afford protection against tick-borne aoHGE infection (25).

We have shown that a recombinant HGE-44 immunoblot can be used for the laboratory diagnosis of HGE. Eight HGE patient sera had either IgM or IgG antibodies that bound recombinant HGE-44, suggesting that an HGE-44-based assay is possible. However, larger numbers of HGE patients need to be tested in order to estimate the sensitivity of this assay. As expected, sera from healthy volunteers did not bind HGE-44. Moreover, none of the sera from patients with Lyme disease or *E. chaffeensis* infection showed reactivity to recombinant HGE-44, suggesting that HGE-44 antibodies are specific for HGE. Serologic IFA assays currently being used in the diagnosis of HGE contain *E. equi*- or aoHGE-infected cells as the substrate and consequently may occasionally yield false-positive results. The false-positive results in assays for aoHGE, *E. chaffeensis*, or *B. burgdorferi* infection are presumably due to cross-reactive antibodies that bind heat shock proteins and other antigens that are present in many bacteria (14, 16, 28, 29). An HGE-44-based assay could help to reduce these difficulties. Furthermore, the use of a recombinant HGE-44 as the substrate, rather than whole aoHGE organisms, eliminates the need to cultivate aoHGE and the possible effects that in vitro culture can cause with respect to pathogen stability, infectivity, and antigenic expression. Finally, recombinant HGE-44 can now be tested as a substrate for an automated enzyme-linked immunosorbent assay, which should reduce the cost and improve the

interlaboratory and intralaboratory reliabilities of serodiagnostic assays for HGE.

HGE is a newly recognized tick-borne infection, and the role of the host immune response to this pathogen in disease remains to be elucidated. The gene, *hge-44*, encoding the major antigen of aoHGE is part of a gene family with similarity to *A. marginale msp-2*. The cloning of this gene represents a first step towards understanding the genetic structure of this pathogen.

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