Improved Sensitivity of PCR for Diagnosis of Human Granulocytic Ehrlichiosis Using epank1 Genes of Ehrlichia phagocytophila-Group Ehrlichiae

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The agent of human granulocytic ehrlichiosis (HGE), Ehrlichia phagocytophila, and Ehrlichia equi probably comprise variants of a single Ehrlichia species now called the Ehrlichia phagocytophila genogroup. These variants share a unique 153-kDa protein antigen with ankyrin repeat motifs encoded by the epank1 gene. The epank1 gene was investigated as an improved target for PCR diagnosis of HGE compared with the currently used 16S rRNA gene target. Primers for epank1 flanking a region that spans part of the 5′ ankyrin repeat coding region and part of the unique 3′ region were synthesized. Blood samples from 31 patients with suspected HGE who were previously tested by 16S rRNA gene (16S) PCR and indirect immunofluorescent antibody test (IFA) were retrospectively tested with the epank1 primers. Eleven patients were 16S PCR positive and had a seroconversion detected by IFA (group A), 10 patients were 16S PCR negative but had a seroconversion detected by IFA (group B), and 10 patients were 16S PCR negative and seronegative (group C). Ten of the 11 group A patients were epank1 PCR positive, all 10 of the group B patients were epank1 PCR positive, and all of the PCR-negative and seronegative patients (group C) were epank1 PCR negative. The epank1 primers are more sensitive than the previously used 16S rRNA gene primers and therefore may be more useful in diagnostic testing for HGE.

Ehrlichia species are obligate, intracellular pathogens belonging to the Rickettsiaceae family that are agents of human and veterinary disease in regions throughout the United States and Europe (5, 12). A newly emerging Ehrlichia species, the agent of human granulocytic ehrlichiosis (HGE) has become more frequently diagnosed in regions where Ixodes species tick vectors are abundant (5, 12). Human infection with the HGE agent causes a nonspecific, influenza-like illness that can be difficult to diagnose. Specific etiologic diagnostic tests for HGE include blood smear examination for ehrlichial morulae within neutrophils, PCR with primers based upon the HGE agent 16S rDNA sequence (4, 7), indirect immunofluorescent antibody test (IFA), which detects HGE agent antibodies in patient sera (6), and direct cultivation of the HGE agent from patient blood (8). PCR is a highly useful diagnostic test for HGE since it provides early detection of infection at a time when therapeutic decisions are being made and since there are limitations associated with the other diagnostic tests. Although PCR diagnosis of HGE with the 16S rDNA primers GE9f and GE10r was shown to be highly sensitive (86%) and specific (100%) in a prospective evaluation (7), recent studies have shown a disappointing lack of sensitivity in actual clinical practice (9). Thus, an enhanced PCR method with increased sensitivity for the diagnosis of HGE would be advantageous.

Phylogenetic studies indicate that the HGE agent belongs within a narrow clade including Ehrlichia phagocytophila and Ehrlichia equi (12). The close genetic relationship between these species along with other molecular and biological evidence suggests that they may actually be a single Ehrlichia species that causes granulocytic ehrlichiosis in both humans and animals. Recently, it has been discovered that these Ehrlichia species share a unique gene, epank1, coding for a 153-kDa protein antigen, Epank1, which contains 11 ankyrin repeats of 33 amino acids (11; P. Caturegli, K. M. Asanovich, J. J. Walls, J. S. Bakken, J. E. Madigan, V. L. Popov, and J. S. Dumler, submitted for publication). Recent evidence suggests that the HGE agent contains at least two copies of epank1 in its genome (Caturegli et al., submitted). Considering this evidence, along with the uniqueness of epank1 to the E. phagocytophila genogroup and the fact that the 16S rRNA and groESL genes are highly conserved among prokaryotes, we investigated whether the PCR diagnosis of HGE could be improved by the use of epank1 primers.

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MATERIALS AND METHODS

Patient specimens. A total of 31 blood samples from patients with suspected HGE agent infection were tested with the epank1 primers. All patient whole-blood samples were previously tested for HGE agent DNA in a PCR assay with the GE9f and GE10r primers (4), and sera were tested for HGE agent antibodies by IFA (2, 6, 10, 13). To exclude false-positive IFA results due to cross-reactivity, patient sera were also tested for Ehrlichia chaffensis antibodies. Patients were initially evaluated for HGE based upon typical history, clinical, and laboratory findings that included at least some of the following: fever, headache, malaise, myalgia, leukopenia, thrombocytopenia, anemia, elevated serum hepatic transaminases, a history of tick bites or exposure to tick-infected areas, and an illness occurring during a period of active tick feeding. Twenty-four of the patients were from southeastern New York, 7 were from either Minnesota or Wisconsin, and 1 was from the Eastern Shore of Maryland. Twelve patients were PCR positive with the HGE agent 16S rRNA gene primers and had a seroconversion detected by IFA (group A). Ten of the patients were previously PCR...
PCR mixture containing (final concentrations) 0.3 mM concentrations of the

CAGTCAGCCATCATTGTGAC-3

Thrombocytopenic purpura, and another had a self-limited influenza-like illness

CGTTCAGCCATCATTGTGAC-3

Perform a 100-μl PCR mixture to assess the analytical sensitivity of the

-AGAGATGCTTATGGTAAGAC-3

444 bp

919 bp

Epank1 DNA was extracted from 300 ml of acute-phase, EDTA-anticoagulated whole blood with the Puregene kit (Genta Systems, Research Triangle Park, N.C.) and quantitated with a GeneQuant UV spectrophotometer (Pharmacia Biotech, Piscataway, N.J.). Two milligrams of DNA was added to a 100-μl PCR mixture containing (final concentrations) 0.3 mM concentrations of the epank1 gene or no plasmid (0).

Comparison of epank1 PCR (top) and 16S rRNA gene PCR (bottom)

A tick bite and persistent (3 months) thrombocytopenia suggestive of idiopathic thrombocytopenic purpura, and another had a self-limited influenza-like illness with no hematologic abnormalities; final etiologic diagnoses were never established for the remaining 3 subjects.

Epank1 PCR. DNA was extracted from 300 ml of acute-phase, EDTA-anticoagulated whole blood with the Puregene kit (Genta Systems, Research Triangle Park, N.C.) and quantitated with a GeneQuant UV spectrophotometer (Pharmacia Biotech, Piscataway, N.J.). Two milligrams of DNA was added to a 100-μl PCR mixture containing (final concentrations) 0.3 mM concentrations of the epank1 primers, 1.5 mM MgCl2, 0.6 mM deoxynucleoside triphosphates, and 2 μl of elongase enzyme mixture (GIBCO/BRL, Gaithersburg, Md.). The forward primer (LA1, 5′-GAGAGATGCTTATGGTAAGAC-3′) and the reverse primer (LA2, 5′-CGGTCAGCCATCATTGTGAC-3′) were designed to flank a region 5′ of

A phagemid that contains the epank1 gene truncated by 90 nucleotides on the 5′ end was obtained by screening an E. equi lambda phage Express (Stratagene) genomic library with polyclonal antisera to E. equi, followed by in vivo excision of the phagemid containing the recombinant insert (Caturegli et al., submitted). The identity of the ehrlichial genes (A) and human blood supplemented with various numbers of HGE agent-infected HL60 cells (B).

The PCR master mixture was directly supplemented with between 106 and 107 plasmids containing the epank1 gene or no plasmid (0).

Determination of analytical sensitivity. A phagemid that contains the epank1 gene was established by sequence analysis (Caturegli et al., submitted).

Sequence analysis of the amplified region of

Epank1 PCR results for suspected HGE patients

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of patients</th>
<th>epank1 PCR positive</th>
<th>Tested</th>
<th>% Positive</th>
<th>IFA geometric mean titer (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>10</td>
<td>11</td>
<td>91</td>
<td>995* (160–2,560)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>10</td>
<td>10</td>
<td>100</td>
<td>1,114* (320–2,560)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0</td>
<td>10</td>
<td>0</td>
<td>&lt;80</td>
<td></td>
</tr>
</tbody>
</table>

* P = 0.80 by Student’s t test.

PCR. As shown in Table 1, 10 of the 11 (91%) group A HGE patients who were previously PCR positive with the HGE agent 16S rRNA gene primers were also PCR positive with the epank1 primers. From group B, all 10 (100%) seropositive patients tested were epank1 PCR positive, and from group C, none of the 10 former PCR-negative and seronegative patients were PCR positive when tested with the epank1 primers. The intervals of illness and the mean geometric antibody titers in convalescence were similar (P = 0.34 and P = 0.80, respectively [Student’s t test]) for both groups of HGE patients regardless of 16S rRNA or epank1 PCR results.

**RESULTS**

Analytical sensitivity. As shown in Fig. 1, epank1 primers detected as few as 10 plasmid copies and also detected chri-

The specificity of PCR with the epank1 primers is additionally high. In a previous study, only antibodies reactive with the E. phagocytophila genogroup reacting with the recombinant Epan1k protein, indicating that this protein antigen is unique to this group (Caturegli et al., submitted); correspondingly, only DNA from isolates of the E. phagocytophila genogroup could be amplified by PCR with the epank1 primers. Sequence analysis of the amplified region of epank1 revealed nucleic acid sequences of E. equi and HGE agent isolates that were nearly identical. Additionally, epank1 primers did not amplify HGE agent DNA from any of the patients who were previously HGE agent 16S PCR negative and seronegative. Therefore, specificity remained 100% when the epank1 primers were used for the PCR diagnosis of HGE, and the sensitivity increased to 95% compared with 48% with the 16S rRNA gene primers in the combined patient cohort.

Although the nucleic acid sequences in the PCR-amplified region of epank1 are nearly identical among the different E. equi and HGE agent isolates, some sequence variation is present among E. phagocytophila isolates (Caturegli et al., submitted). Sequence variation for epank1 between Swedish and
Spanish *E. phagocytophila* strains was noted, and variation between the European *E. phagocytophila* strains and the North American strains is even greater. Amino acid sequence analysis of the Epank1 protein revealed similar variations among isolates. These variations among isolates and strains may explain why HGE agent DNA was amplified from the blood of one HGE patient from group A when alternate epank1 primers were used but not when the LA1 and LA6 epank1 primers were used (data not shown).

We have shown that PCR amplification of HGE agent DNA in infected patient blood with primers based upon epank1, which is unique for the *E. phagocytophila* group ehrlichiae, is both sensitive and specific. However, variation in epank1 sequences among strains may cause occasional false-negative results among North American patients. Regardless, the use of a genogroup-restricted gene with multiple copies in the genome as a target for PCR resulted in an improved clinical sensitivity with these archived blood samples. A pitfall of this strategy is the potentially larger degree of genetic heterogeneity in species- or group-restricted genes that may allow for decreases in sensitivity. It remains to be determined whether epank1 will be an appropriately sensitive target for PCR diagnosis in European patients with HGE. Further characterization of the heterogeneity in epank1 may reveal more-optimal regions that are conserved among the *E. phagocytophila*-group species for the design of primers to further improve the PCR diagnosis of HGE.

Currently, an optimal algorithm for laboratory confirmation of human ehrlichioses is not established. Definitive confirmation during active disease requires the use of PCR or culture. Two approaches for PCR confirmation include assays directed at specific etiologic agent identification and amplification of nucleic acids by using broad-range, “genus-specific” primers (2, 3, 7). Each approach is confounded by the potential for missed diagnoses and increased cost. Until issues of sensitivity, specificity, and cost regarding broad-range PCR for clinical applications are addressed, directed investigation using validated assays that identify specific etiologic agents is a proven approach.

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**REFERENCES**