Differentiation of Pathogenic from Nonpathogenic *Entamoeba histolytica* by Restriction Fragment Analysis of a Single Gene Amplified In Vitro

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We previously reported the identification of homologous cDNA clones derived from a pathogenic isolate and a nonpathogenic isolate of *Entamoeba histolytica*, which had been designated cEh-P1 and cEh-NP1, respectively. Sequence analysis of both clones had revealed 10% nucleic acid substitutions, which were dispersed over the entire sequence. This genetic difference had been found to be conserved between all four pathogenic and all five nonpathogenic laboratory strains of *E. histolytica* tested. On the basis of nucleic acid substitutions, we have now developed a sensitive assay to distinguish pathogenic from nonpathogenic forms of *E. histolytica* by using fresh clinical isolates. Comparing the sequence of cEh-P1 and cEh-NP1, we identified a 482-bp segment that contained identical 5' and 3' ends but differed in internal cleavage sites for restriction endonucleases. By using oligonucleotide primers corresponding to the 5' and 3' ends of this segment, the corresponding gene was amplified by the polymerase chain reaction. Endonuclease digestion of the amplified DNA yielded restriction fragments that are characteristic for pathogenic and nonpathogenic forms. This assay allows the detection and classification of fewer than 10 amoebae within a few hours. The differentiation of 48 isolates into pathogenic and nonpathogenic strains by using this method corresponded to the clinical status of the infected individuals and to the classification obtained by isoenzyme determination. The results further support the concept that pathogenic and nonpathogenic strains of *E. histolytica* constitute distinct subspecies.

*Entamoeba histolytica* is an enteric protozoan parasite that infests half a billion people worldwide. Ten percent of infected individuals develop disease such as hemorrhagic colitis or extraintestinal abscesses, leading to about 70,000 deaths each year (25). The numerical difference between the occurrence of infection and expression of morbidity is based on the existence of pathogenic and nonpathogenic strains of *E. histolytica*. Although there is disagreement about the nature of pathogenicity, strain-specific differences have been shown by biological assays, isoenzyme analysis, monoclonal antibodies, and DNA probes (9, 13, 20, 21). All current methods of classifying *E. histolytica* have certain disadvantages and are not useful for a rapid and sensitive distinction between pathogenic and nonpathogenic forms. Such a distinction is of considerable medical importance because it aids in the decision of whether to treat an infected individual (15, 19).

We recently reported on genomic DNA differences between pathogenic and nonpathogenic *E. histolytica* (27). On the basis of these differences, we have now developed a rapid and reliable assay to distinguish both forms. By using the polymerase chain reaction (PCR) and subsequent restriction fragment analysis, this assay allows the detection and classification of fewer than 10 amoebae within a few hours.

**MATERIALS AND METHODS**

**E. histolytica strains.** The pathogenic *E. histolytica* strain SAW755 and the nonpathogenic strain SAW 760 were obtained from P. Sargeaunt (London, England) and propagated xenically in TYI-S-33 medium (5). Clinical isolates of *E. histolytica* from microscopically positive stool samples were cultured in Dobell-Laidlaw medium (6) and subsequently transferred to TYSGM-9 monophasic medium (4) containing bacteria.

*E. histolytica* was defined as pathogenic if (i) it originated from patients with symptoms indicative of invasive amebiasis, (ii) the sera of these patients contained antibodies specific for *E. histolytica*, and (iii) the *E. histolytica* isolates had one of the hexokinase isoenzyme patterns which had been shown to be characteristic for pathogenic forms. Isolates were defined as nonpathogenic if (i) they originated from healthy subjects, (ii) the subjects had no detectable antibodies against *E. histolytica*, and (iii) the isolates had one of the hexokinase isoenzyme patterns which were shown to be characteristic for nonpathogenic forms.

**ELISA.** The enzyme-linked immunosorbent assay (ELISA) for the detection of antiamoeba antibodies was performed as previously described (10). In brief, a cell lysate was made from *E. histolytica* isolated from a patient with an amoebic liver abscess. The amoebae (10⁹) were rinsed three times in cold phosphate-buffered saline (PBS). The pellet was resuspended in 1 ml of distilled water, frozen and thawed five times, and subsequently treated by ultrasonation 10 times for 30 s and stored at 4°C overnight. It was then centrifuged at 30,000 × g for 30 min, and the supernatant was stored at −70°C until used as antigen. ELISA was performed in microtiter plates (24). Results were expressed as multiples of normal activity up to a maximum of 100 (8).

**Isoenzyme determination.** The isoenzymes of hexokinase (EC 2.7.1.1) were examined as previously described (16). In brief, whole-cell lysates of the trophozoites were applied to thin-layer starch gel electrophoresis. The relative mobility of the hexokinase isoenzymes was determined by the localization of the specific enzymatic activity by using formazan development. Slow-migrating hexokinase bands were assigned to nonpathogenic forms, and fast-migrating bands transferred to TYSGM-9 monophasic medium (4) containing bacteria.
were assigned to pathogenic forms of *E. histolytica* (Table 1).

**Preparation of *E. histolytica* genomic DNA.** Cells were harvested in late logarithmic phase by chilling on ice for 10 min and low-speed centrifugation at 4°C for 5 min. Nuclei were obtained from washed cell pellets by lysis in 1% Nonidet P-40 and centrifugation at 500 × g at 4°C for 5 min. The nuclear pellet was resuspended, and DNA was released by treatment with protease K (1 mg/ml) in a buffer containing 100 mM NaCl, 10 mM Tris, 10 mM EDTA, and 0.5% N-lauroylsarcosine at 60°C for 2 h. The DNA was extracted twice with phenol-chloroform, 1:1 (vol/vol), and once with chloroform and was precipitated with ethanol.

**Preparation of *E. histolytica* cell lystate for PCR.** Cells were harvested as described above, washed in a large volume of PBS, and centrifuged at 300 × g at 4°C for 5 min. The cell pellet was resuspended in a small volume of PBS (up to 1 ml), transferred to a 1.5-ml tube, and boiled for 10 min.

**Amplification procedures.** Two oligonucleotide primers, P1-S17 and P1-AS20, were synthesized on an Applied Biosystems DNA synthesizer. The sequences of the primers were derived from two regions of the previously isolated
cDNA clone cEh-P1 (22). These two regions were found to be conserved in all nine _E. histolytica_ isolates tested. P1-S17 (5’-GCAACTAAGGTTAGTGA) is a 17-mer oligonucleotide derived from the sense strand, whereas P1-AS20 (5’-CTCTCC CATGATGTGTTA) is a 20-mer oligonucleotide from the antisense strand of cEh-P1. Both sequences are flanking a 482-bp fragment of cEh-P1.

The amplification reactions were performed with the thermostable DNA polymerase of _Thermus aquaticus_ (Taq; Cetus Corp.) as previously described (18). Briefly, 2.5 U of Taq polymerase was used in a 100-μl reaction volume with 10 mM Tris (pH 8.3), 50 mM KCl, 1.5 mM MgCl2, 200 μM deoxynucleotide triphosphates (Cetus Corp.), each oligonucleotide primer at 1.0 μM, 0.01% gelatin, different amounts of whole-cell lysate or purified genomic DNA as indicated. Samples were overlaid with 100 μl of mineral oil to prevent evaporation in 0.6-ml tubes (Starsted) and amplified for 25 to 35 cycles in an automated thermal cycler (Perkin-Elmer Cetus). Each cycle consisted of 1.5 min of denaturation at 94°C, 2.0 min of annealing at 42°C, and 1.5 to 3.0 min of extension at 72°C. The final extension step continued for an additional 5 min.

**Restriction fragment analysis of amplified DNA.** After PCR, 10 to 20 μl of the amplified DNA was digested with the restriction endonucleases AccI, TaqI, and XmnI under conditions recommended by the supplier (Boehringer Mannheim). Digested DNA was separated on 3% NuSieve–1% SeaKem (FMC Corp.) agarose gels and visualized by ethidium bromide staining.

**RESULTS**

Amplification of a 482-bp genomic DNA fragment of _E. histolytica_ by PCR. We made use of genomic sequence information derived from the previously described cDNA clones cEh-P1 and cEh-NP1, which were shown to be specific for pathogenic and nonpathogenic isolates of _E. histolytica_, respectively. In cEh-P1 and cEh-NP1 we identified similar segments of 482 bp that contained identical sequences at the 5' and 3' ends but differed in internal cleavage sites for restriction endonucleases (Fig. 1). According to the conserved flanking regions of the 482-bp fragments, oligonucleotide primers were constructed and designated P1-S17 and P1-AS20. These primers and purified genomic DNA either from the pathogenic isolate SAW755 or from the nonpathogenic isolate SAW760 were used in the PCR.

Analysis of the amplified material by electrophoresis in ethidium bromide-containing agarose gels revealed that only a single genomic fragment was amplified, that there was no detectable difference in size between the fragments amplified from the DNA of pathogenic and nonpathogenic amoebae, and that the size of the amplification product (482 bp) was consistent with the size expected from the cloned gene (Fig. 2).

There was no loss of specificity when boiled cell lysates were used instead of purified DNA. To determine the sensitivity of the assay, serial dilutions of cells were tested. After 35 PCR cycles, amplified DNA of fewer than 10 amoebae was detectable (Fig. 3). There was no amplification of any fragment when purified human DNA, bacterial DNA, or DNA from _Entamoeba invadens_ (Entamoeba species found in reptiles) was used in this assay.

**Differentiation between pathogenic and nonpathogenic _E. histolytica_ by restriction fragment analysis.** The amplified DNA was subjected to endonuclease digestion. Within the 482-bp DNA fragment, the sequence differences between cEh-P1 and cEh-NP1 were predicted to lead to recognition sites for the restriction endonucleases XmnI, TaqI, and AccI that were specific for pathogenic and nonpathogenic _E. histolytica_ (Fig. 1).

When the amplified DNA of the pathogenic strain SAW755 was digested with XmnI and TaqI, two fragments each were detected (291 and 191 bp for XmnI and 321 and 161 bp for TaqI), whereas the DNA derived from the nonpathogenic strain SAW760 was not cleaved by these enzymes. In contrast, the amplified DNA of SAW760 was cleaved into two fragments by AccI (292 and 190 bp), but there was no fragmentation of the amplified DNA from SAW755 (Fig. 4). Thus, the fragmentation patterns obtained corresponded to those predicted from the sequence analysis of cEh-P1 and cEh-NP1 and are suitable to distinguish the pathogenic strain SAW755 from the nonpathogenic strain SAW760.

**Correlation of restriction fragment analysis with the clinical status of 48 infected individuals.** _E. histolytica_ isolates were derived from 48 individuals who had acquired the infection in various parts of the world. Thirteen had never been outside Europe; they are male homosexuals. Twelve of the 48 infected persons had clinical symptoms compatible with invasive amebiasis, whereas the remaining 36 were apparently healthy, so-called asymptomatic carriers.

For the determination of antiamoeba antibodies, an ELISA was used. Serum samples from 39 persons, among them 8 patients with clinical symptoms, were available. All of these were serologically positive, but none of the asymptomatic carriers tested was positive.
FIG. 2. Detection of a 482-bp genomic DNA fragment from E. histolytica. One nanogram of purified genomic DNA prepared from the pathogenic strain SAW755 (p) and the nonpathogenic strain SAW760 (np) was subjected to PCR. Ten percent of the amplified material was applied to an ethidium bromide-stained agarose gel. Numbers refer to the length of fragments (in base pairs) deduced from size markers (φX174 DNA digested with the restriction enzyme HaeIII).

All 48 E. histolytica isolates were characterized by hexokinase isoenzyme determinations. All isolates from patients with symptoms showed a pathogenic pattern, and all from asymptomatic carriers showed a nonpathogenic one.

FIG. 3. Sensitivity of E. histolytica DNA detection. A serial dilution of E. histolytica cell lysate (10 to 10,000 cells) was used for in vitro DNA amplification, and 10% of the reaction mixtures was subsequently loaded onto an ethidium bromide-stained agarose gel. The number of cells used for each assay and the size markers (in base pairs) are indicated.

FIG. 4. Differentiation of E. histolytica strains by PCR and subsequent restriction fragment analysis. Whole-cell lysates of the pathogenic strain SAW755 (p) and the nonpathogenic strain SAW760 (np) were subjected to the DNA amplification procedure. All lots of the amplified material were digested with the restriction endonuclease XmnI, TaqI, or AccI as indicated. The digested DNAs were separated on an ethidium bromide-stained agarose gel. Size markers (in base pairs) are indicated.

As summarized in Table 2, these data correlated well with the results obtained by PCR and subsequent restriction fragment analysis: all 36 isolates from asymptomatic carriers had the same restriction pattern as that seen with the nonpathogenic reference strain SAW760, and 11 of 12 isolates from patients with symptoms reveals the same pattern as that obtained with the pathogenic strain SAW755 (Fig. 4). In one case, DNA analysis did not correlate to clinical data and isoenzyme determinations (case no. 10). The isolate was derived from a European patient returning from Nepal with chronic diarrhea; she was positive for antiamoeba antibodies.

**DISCUSSION**

The results presented here support the view that two genetically distinct subspecies of E. histolytica infect humans. We recently reported on two cDNA clones representing coding genomic sequences that allow the distinction between pathogenic and nonpathogenic forms (22). Initially,

<table>
<thead>
<tr>
<th>Presence of symptoms of invasive amebiasis (n)</th>
<th>No. of E. histolytica isolates as characterized by:</th>
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<tbody>
<tr>
<td></td>
<td>Hexokinase isoenzyme pattern</td>
</tr>
<tr>
<td>Pathogenic</td>
<td>Non-pathogenic</td>
</tr>
<tr>
<td>Present (12)</td>
<td>12</td>
</tr>
<tr>
<td>Absent (36)</td>
<td>0</td>
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Table 2. Genetic analyses compared to standard isoenzyme determinations in the differentiation between pathogenic and nonpathogenic E. histolytica.
these clones were used to test several laboratory strains of *E. histolytica*. We now extend this analysis to a number of fresh clinical isolates and find a nearly complete correlation to the clinical status of the infected individuals and to other markers of pathogenicity. DNA analysis apparently failed in one case. Unfortunately, additional samples from the same patient were not available to repeat the assay and to eventually study the biological properties of the isolate more thoroughly (with regard to a possible mixed infection). We tend to believe that an erroneous switch of samples caused this failure. It seems noteworthy that our study included samples from 13 male homosexuals; all of these isolates exhibited a nonpathogenic isoenzyme pattern and a nonpathogenic DNA restriction fragment pattern. This finding supports previous reports on the prevalence of nonpathogenic *E. histolytica* in American and European homosexual men (1, 11, 14).

Taken together, increasing evidence suggests that *E. histolytica* can be separated into two distinct subspecies, one of which is pathogenic to humans and the other of which is not. A rapid and sensitive diagnostic procedure which allows the distinction between the two forms would obviously be of considerable medical importance. So far, specialized laboratories use isoenzymes of the carbohydrate metabolism to approach the distinction between pathogenic and nonpathogenic isolates. However, this assay needs cell lysates of approximately 10⁶ amoebae, which requires cultivation of the isolate for several days. In addition, it has been questioned whether the isoenzyme pattern of a particular strain is stable over time (15, 19).

As an alternative, DNA probes have already been applied to the classification of *E. histolytica*. Garfinkel et al. isolated probes from tandemly repeated sequences, which are found in highly amplified circular DNA of amoebae. One of the probes selectively hybridized to the DNA of apparently pathogenic *E. histolytica*, whereas the other one was specific for isolates from asymptomatic carriers (9). However, this technique also depends on cultured amoebae and still needs radioactive labeled probes, which limits its use to specially equipped and licensed laboratories.

In recent years, the PCR has been widely used as an in vitro method for detecting specific DNA from minute amounts of starting material (3, 7, 12, 17, 23). Here we present an application of this technique for the distinction between pathogenic and nonpathogenic *E. histolytica*. The assay does not require radioactivity. In principle, it could be performed within 1 day, because its high sensitivity makes cultivation unnecessary. As yet, we have not used a simple extract of feces as starting material for the PCR. Therefore, a short-term culture of the sample is still needed. We and others are currently working on procedures to amplify *E. histolytica* DNA directly out of stool specimens (18a). Combining the two technical approaches, a rapid and sensitive assay for the detection of *E. histolytica* and the identification of pathogenic forms in fecal samples may be developed.

REFERENCES

18a. Samuelson, J. Personal communication.

