Simple Differential Detection of *Entamoeba histolytica* and *Entamoeba dispar* in Fresh Stool Specimens by Sodium Acetate-Acid/Formalin Concentration and PCR

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Amoebiasis is caused by two distinct species, a pathogenic form (*Entamoeba histolytica*) and a nonpathogenic form (*Entamoeba dispar*), which are morphologically identical. Although the distinction between these two species is of great clinical importance, the methods developed for this purpose either are very time-consuming or involve laborious procedures for isolation of the DNA. We report here a simple PCR method starting with fresh stool specimen that allows for the sensitive and reliable distinction between *E. histolytica* and *E. dispar*. After initial concentration by the sodium acetate-acid-formalin (SAF) method and digestion with proteinase K, a 0.88-kb sequence of the multicopy 16S rRNA gene served as a target for PCR amplification. The method starting with unpreserved specimens proved to be very sensitive and was not influenced by the quick exposure to SAF fixative during the initial concentration step. However, storage in SAF fixative prior to testing resulted in a decreased sensitivity within 2 days. The detection limit of the method was as low as one copy of the 16S rRNA gene. No cross-reactivity was observed with other common intestinal protozoa. Mixed infections involving both *E. histolytica* and *E. dispar* could easily be detected at a ratio of 1:10,000 by agarose gel electrophoresis or a DNA hybridization immunoassay.

**MATERIALS AND METHODS**

**Parasites.** Identification of parasite species was performed by microscopic examination of stool specimens after concentration in sodium acetate-acid-formalin (SAF) fixative (31). *E. histolytica*, *E. dispar*, *Entamoeba hartmanni*, *Entamoeba coli*, *Dientamoeba fragilis*, and *Blastocystis hominis* were isolated from stool samples by xenic cultivation in Robinson’s medium (20) in bijou bottles on an agar slope to a density of 2.0×10^6 to 2.0×10^7 cells/vial. Differentiation of isolates as *E. histolytica* or *E. dispar* was done by isoenzyme electrophoresis by the protocol of Sargeaunt and Williams (25). *E. histolytica* HK-9 was grown axenically in TYI-S-33 medium (10) in 12.0-ml glass tubes to a density of 2×10^5 to 5×10^6 cells. *Entamoeba invadens*, a species affecting reptiles, was cultivated on slopes of coagulated horse serum and horse serum diluted 1:4 with Ringer’s solution (103 mM NaCl, 1.34 mM KCl, 0.7 mM CaCl_2 · 2H_2O [pH 7.4]).

**Stool specimens.** The stool specimens used either were unpreserved (fresh specimen stored in the refrigerator and in contact with SAF fixative only during concentration procedure) or were stored in SAF fixative for a period of up to 30 days. DNA extraction. Trophozoites of strain HK-9 were harvested by centrifugation at 300×g for 5 min after chilling on ice for 5 min. The pellet was washed twice in cold 0.02 M phosphate-buffered saline (pH 7.2). Parasites cultivated in Robinson’s medium and *E. invadens* were harvested by centrifugation at 350×g for 10 min in a 2.0-ml Eppendorf tube and were subsequently rinsed twice with phosphate-buffered saline. The final pellet was resuspended in 50 μl of lysis buffer consisting of 1 mM EDTA, 1 mM dithiothreitol, and 1 mM ε-amino caprylic acid, and the mixture was centrifuged at 13,000×g for 20 s. The supernatant was diluted 1:10 in distilled water for PCR. For experiments with a defined number of cells, the parasites were counted in a counting chamber and were lysed in the appropriate volume of lysis buffer.

Stool samples were prepared for PCR as described by Acuna-Soto et al. (2), with minor modifications. In brief, the amoebae were concentrated by the SAF method (31). The target sequences of the 16S rRNA gene were amplified in a 50-μl reaction mixture, which consisted of 1.25 U of Taq polymerase (Life Technologies, Basel, Switzerland), 0.6 mM dUTP (Pharmacia, Du¨bendorf, Switzerland), 18 pmol of each primer (Ed-5′ and Ed-3′) for *E. histolytica* or 16 pmol of each primer (Ed-5′ and Ed-3′) for *E. dispar*, 1.25 U of Taq DNA polymerase, and 0.5 U of uracil DNA glycosylase (UDG; Life Technologies). UDG and dUTP (instead of dTTP) were used to remove eventual (dUTP-containing) carryover contaminants from previous PCR amplifications (17). Samples were overlaid with 50 μl of mineral oil to prevent evaporation. To degrade potential dUTP-containing contaminants.

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In addition, cell lysates of *E. invadens* DNA of resulting in two fragments of 0.55 and 0.35 kb, as predicted which distinguishes both species from other protozoan para-

Dra tide in length but they differ in a fragment, which is revealed as a single band by agarose gel and *E. coli* Eco\textsuperscript{I}; S, Sau96I. The sizes of the restriction fragments are indicated to the right and left (in kilobases).

by UDG, the reaction mixture was incubated for 2.5 min at 50°C prior to PCR. Furthermore, to ensure double-stranded DNA denaturation, the samples were incubated at 94°C for 3 min. Subsequently, 40 amplification cycles were performed in a thermal cycler (Perkin-Elmer Cetus, Rotkreuz, Switzerland) by using the following cycle: 1 min of denaturation at 94°C, 1 min of annealing at 60°C, and 2 min of primer extension at 72°C. After the last cycle, primer extension was continued for 10 min at 72°C before 50 μl of chloroform was added for inacti-

**Primer**

The following pairs of primers were used for the study: Eh5' (5'-GTAATTTACTTAAACCGGTTAACAATG-3'), Eh3' (5'-TTCTCTGTAAACAAGATCTCAGGCT-3'), Ed5' (5'-TTGATGTTATTTAACCGGTGAAACATG-3'), and Ed3' (5'-TTCTTTGTAACAAAGATATTAGGTCA-3').

Analysis of PCR products was by agarose gel electrophoresis (1% agarose) in ethidium bromide solution (10 mg/ml). Furthermore, the amplification products were detected by an enzyme-linked immunosorben assay (ELISA; Gen-etiK-DEIA; Sorin Biomedica, Saluggia, Italy) according to the manufactur-

**RESULTS**

The chosen target for the PCR in the coding region of the 16S rRNA gene allowed for the amplification of a 0.88-kb gene fragment, which is revealed as a single band by agarose gel electrophoresis. Both PCR products vary by only one nucleo-

**SPECIFICITY OF THE PRIMERS**

The specificity of the primers for *E. histolytica* and *E. dispar* were verified by using cell lysates of different *Entamoeba* strains with known zymodeme patterns. In addition, cell lysates of *E. invadens*, a species infecting reptiles, and several human protozoan parasites were tested. PCR was performed with both primer sets in independent reactions with each cell lysate. In all 14 cases tested, the result of the PCR corresponded to the one obtained by zymodeme analysis (Table 1). Additionally, no unspecific priming was seen with DNAs from *E. coli*, *E. invadens*, *E. hartmanni*, *D. fragilis*, or *B. hominis*. Results for inhibition controls, carried out to exclude the possibility that a negative PCR result was due to the failure of amplification, were negative for all reac-

**Sensitivity of the assay**

The sensitivity of the PCR was assessed by using serial dilutions of lysates of 100 to 0.001 trophozoites of *E. histolytica* or *E. dispar*. After 40 PCR cycles, as little as one copy of the target gene was detected by both primer sets, as determined by agarose gel electrophoresis (Fig. 2). If amplification products were analyzed with the Gen-etiK-DEIA system, the same sensitivity was obtained (Fig. 2). In order to determine the effect of feces on the assay sensitivity, we performed the SAF concentration procedure with a stool sample negative for parasites, resuspended the pellets in digestion buffer containing proteinase K, and added cell lysates corresponding to decreasing numbers of parasites. It was found that the PCR was completely inhibited by undiluted feces and was partially inhibited at a dilution of 1:10, resulting in decreased sensitivity. However, at a dilution of 1:100, the detection limit for the 16S rRNA gene is similar to that obtained with cell lysates in the absence of stool (data not shown). In order to estimate the sensitivity of the reaction for mixed infections, various numbers of *E. histolytica* trophozoites were mixed with a constant number of *E. dispar* trophozoites before lysis, and vice versa. One hundred cells of one species as the background still allowed for the detection of one copy of the target gene of the other species, i.e., a ratio of 1:10\(^4\) (Fig. 3).

**Diagnosis of infections with *E. histolytica* and *E. dispar* in stool specimens**

Target DNA in 19 stool samples stored in SAF for between 3 and 30 days and 18 unpreserved stool specimens, which all contained *E. histolytica* or *E. dispar*, as verified by microscopy, were amplified starting from a 1:100 dilution of the proteinase K-digested DNA extract. The target DNA was amplified in 25 cases, identifying 23 *E. histolytica* infections. The PCR was falsely negative for 12 specimens. As it turned out, the PCR was highly influenced by the starting material. By using unpreserved stool specimens, all 18 samples examined were positive by PCR, whereas by using samples stored in SAF for more than 2 days, only 7 of 19 samples (36.8%) were positive by PCR, accounting for all 12 false-negative results. Time course experiments with aliquots of SAF-fixed samples taken every 2 days over 2 weeks for PCR analysis revealed that the sensitivity of the PCR decreased strongly within 2 days, as reflected by the fact that the specific DNA band became more faint. In contrast, if unpreserved stool specimens stored in the refrigerator were used, the sensi-

**TABLE 1. Comparison of PCR results with the results of zymodeme analysis**

<table>
<thead>
<tr>
<th>Species</th>
<th>Zymodeme</th>
<th>No. of isolates tested</th>
<th>PCR result</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Entamoeba dispar</em></td>
<td>I, IV</td>
<td>10</td>
<td><em>E. dispar</em></td>
</tr>
<tr>
<td><em>Entamoeba histolytica</em></td>
<td>II, XIX</td>
<td>4</td>
<td><em>E. histolytica</em></td>
</tr>
<tr>
<td><em>E. coli, E. hartmanni, E. invadens, B. hominis, D. fragilis</em></td>
<td>8</td>
<td>Negative for all organisms</td>
<td></td>
</tr>
</tbody>
</table>
Amoebiasis affects about 500 million people worldwide, yet only about 10% of the infections are caused by *E. histolytica*, which leads to severe disease like dysentery or amoebic liver abscess (30). Considering the fact that most infections are due to the noninvasive species *E. dispar*, a rapid and sensitive diagnostic procedure for differentiating the two species would be of great medical importance. This report describes a very sensitive method which allows for the reliable distinction between *E. histolytica* and *E. dispar* by a procedure that starts with unpreserved fresh fecal samples.

It is well established that fresh fecal material has a strong inhibitory effect on the PCR, resulting in a considerable loss of sensitivity (16). Concentration with SAF fixative as a first preparatory step is an elegant way to concentrate parasites and at the same time eliminate the inhibitory effect of the feces. The method does not require any further DNA purification or a time-consuming in vitro cultivation, which often ends up with a negative result. Furthermore, our PCR was very sensitive at detecting one copy of the target gene of one species in a background of $10^4$ copies of the other species when mimicking mixed infections.

For optimal results, starting with unpreserved fecal material turned out to be crucial. By using unpreserved stool specimens, no false-negative results occurred when microscopy was used as a “gold standard,” even after storage of the fecal specimen in a refrigerator for 2 weeks. The very short time period during which the sample is in contact with SAF does not have an adverse effect on the PCR, because the preservative is removed immediately by the washing steps. However, by using feces stored in SAF fixative, the sensitivity of the PCR usually decreased within 2 days due to degradation of the target DNA, although exceptionally, a few samples performed well for up to 14 days. Since SAF itself showed no effect on DNA degradation, as verified by the addition of the amoeae DNA to the SAF fixative and subsequent PCR, we conclude that the DNA in SAF-preserved specimens is degraded enzymatically. This is supported by the finding that the addition of 100 mM EDTA to λ DNA incubated in SAF-stool specimens for 16 h prevents degradation. SAF does not inhibit
the DNA-degrading enzymes present in stool. After fixation, the passage of DNases through the wall of the cyst may be facilitated, while this process would not occur in unpreserved specimens. The first method described for the differentiation of *E. histolytica* and *E. dispar* was zymodeme analysis (26). However, this method requires cultivation of the amoebae starting with a fresh fecal sample, a lengthy procedure which is hampered by frequent failures. In recent years several investigators reported the use of monoclonal antibodies directed against the 170-kDa subunit of the galactose-specific adherence lectin for the differentiation of *E. histolytica* and *E. dispar* (1, 13, 14). However, since those investigators did not dispose of an antibody specific for *E. dispar*, the assays require two consecutive ELISAs. Furthermore, the sensitivity as well as specificity were not satisfactory. Alternatively, molecular DNA-based detection methods were developed. Several investigators have described the use of DNA probes, e.g., by relying on intergenic repeated sequences of the rRNA gene circles (6, 12, 21) or on a probe named IE-gen1, which is related to genomic sequences present on intergenic repeated sequences of the rRNA gene circles. Investigators have described the use of DNA probes, e.g., by relying on intergenic repeated sequences of the rRNA gene circles. Several investigators have described the use of DNA probes, e.g., by relying on intergenic repeated sequences of the rRNA gene circles. Several investigators have described the use of DNA probes, e.g., by relying on intergenic repeated sequences of the rRNA gene circles. Several investigators have described the use of DNA probes, e.g., by relying on intergenic repeated sequences of the rRNA gene circles.
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Volume 35, no. 7, p. 1701–1705, 1997. Page 1702, column 1, line 9 should read “Eh-5’ (5’-GTACAAAATGCCAATTCAT TCAATG-3’), Eh-3’ (5’-CTCAGATCTAGAAACAATGCTTCTCT-3’), Ed-5’ (5’-GTACAAAGTGGCCAATTTATGTAAGT-3’), and Ed-3’ (5’-ACTTGGATTTAGAAACAATGTTTCTTC-3’).”

Genotyping of Rotaviruses in Environmental Water and Stool Samples in Southern Switzerland by Nucleotide Sequence Analysis of 189 Base Pairs at the 5’ End of the VP7 Gene

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Volume 38, no. 10, p. 3681–3685, 2000. Page 3683, Fig. 2 legend: The last sentence, “Numbers indicate percent similarity,” should be deleted.