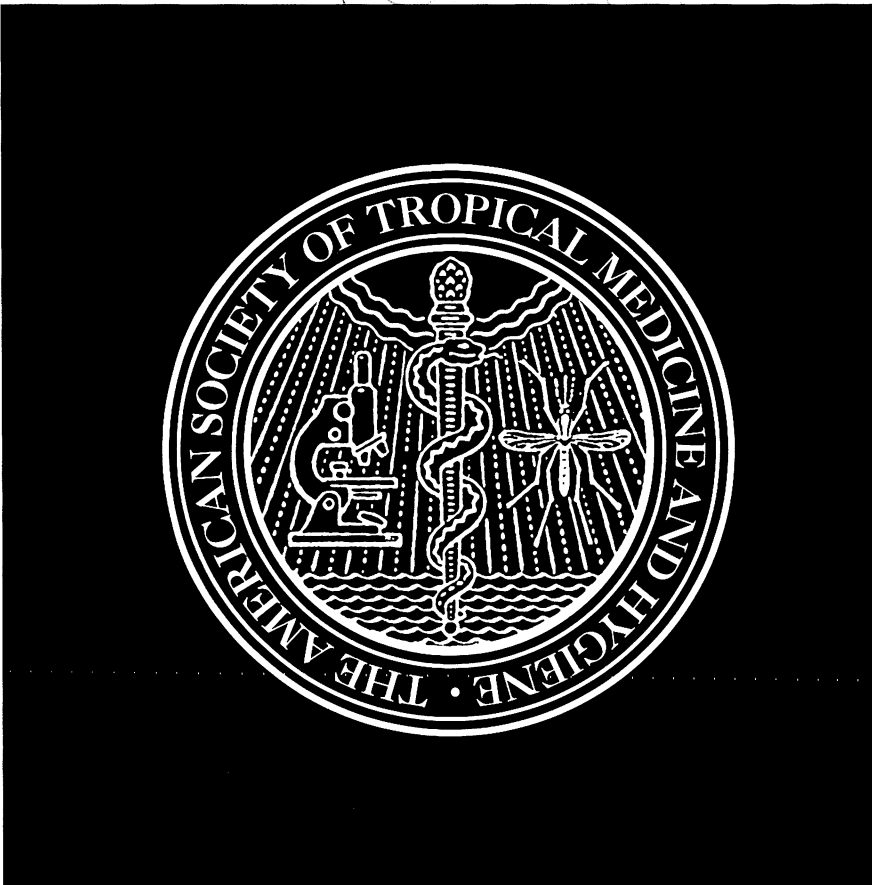


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DIRECT AMPLIFICATION AND DIFFERENTIATION OF PATHOGENIC AND NONPATHOGENIC *ENTAMOEBIA HISTOLYTICA* DNA FROM STOOL SPECIMENS

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Abstract. Discrimination of pathogenic and nonpathogenic *Entamoeba histolytica* is of great clinical importance. A simple and rapid DNA extraction method that can be used directly with stool specimens was developed without the need for prior cultivation of the parasites. The entire protocol can be performed at room temperature in a 1.5-ml microcentrifuge tube format. There is no DNA precipitation step. The subsequent nested polymerase chain reaction consists of an initial *E. histolytica*-specific amplification, followed by two separate amplifications using two primer pairs specific for pathogenic and nonpathogenic *E. histolytica*, respectively. Amplification products can be verified by restriction endonuclease digests. There is no need for hybridizations or the use of radionucleotides. One trophozoite per milligram of stool sample could be detected and differentiated in a 0.1-g specimen.

Amebiasis is an infection of the large intestine caused by the parasitic protozoan *Entamoeba histolytica*. Generally, a nonpathogenic form is discriminated from a pathogenic one that causes invasive amebiasis. Both forms were first differentiated by characteristic isoenzyme patterns.¹ It has been suggested that these forms constitute separate species.² Recently, differences on the genomic level have also been described.³⁻⁵ In this report, those differences in the nucleotide sequences of a small subunit ribosomal RNA gene were used to develop a simple yet sensitive polymerase chain reaction (PCR) technique to detect and differentiate both forms at the same time.

In a first attempt to avoid time-consuming cultivation methods, *E. histolytica* DNA from stool samples was amplified using freezing with dry ice, an ultrasonic generator, and a 24-hr incubation step.⁶ In an effort to find a method that is fast and simple enough to be performed at room temperature without the need for special equipment, a protocol was developed to detect one trophozoite per milligram in a 0.1-g stool sample.

MATERIALS AND METHODS

***Entamoeba histolytica* trophozoites.** Trophozoites of the pathogenic strain HK-9 were grown in axenic culture using TPS-1 medium as described by Diamond and cryopreserved in liquid nitrogen.⁷

Stool specimens. Stool samples (0.1 g) from an asymptomatic person without microscopically detectable parasites were mixed with defined numbers of HK-9 trophozoites. Specimens were also obtained from three patients positive for *E. histolytica* cysts on microscopic examination. Cyst concentrations were not determined.

Extractions of DNA. For the comparison of DNA extraction methods, 5000 HK-9 trophozoites were added to 0.1 g of stool. In a modification of an *Echinococcus multilocularis* DNA extraction method used with fox feces,⁷ these 0.1-g stool samples were transferred to 1.5-ml microcentrifuge tubes, 33.3 μ l of 1 M KOH and 9.3 μ l of 1 M DTT were added and mixed thoroughly by stirring with a pipette tip, followed by brief shaking.⁸ After incubation at 65°C for 15 min, the samples were neutralized with 4.3 μ l of 25% HCl, buffered with 80 μ l of 2 M Tris-HCl (pH 8.3), and the suspension was mixed again. The DNA was extracted by shaking with 250 μ l of phenol:chloroform:isoamyl alcohol (PCI, 25:24:1) saturated with 10 mM Tris (pH 8.0), 1 mM EDTA (Sigma, St. Louis, MO). The phases were separated by a 4-min spin in a microcentrifuge. The aqueous phase was transferred to a new tube and the DNA was further purified by adsorption to 5 μ l of a silica gel suspension (Geneclean; Bio 101, La Jolla, CA). The DNA was eluted in 38.2 μ l of water.

For comparison, some 0.1-g stool samples were first washed by suspending them in 700 μ l

of 0.9% NaCl. After centrifugation, the supernatant was discarded. Some samples were PCI-extracted three times and others were not extracted. The proteinase K digestion process used was adopted from a standard protocol.⁹

Nested PCR. Primer construction was based on pathogenic and nonpathogenic sequences from a small subunit ribosomal RNA gene.⁴ For the first PCR, the primer pair EH-1 (TTT GTA TTA GTA CAA A) and EH-2 (GTA (A,G)TA TTG ATA TAC T), which specified a 0.9-kb fragment, was used. For the second (nested) PCR, two different primer pairs that were specific for the pathogenic form (EHP-1: AAT GGC CAA TTC ATT CAA TG and EHP-2: TCT AGA AAC AAT GCT TCT CT) or the nonpathogenic form (EHN-1: AGT GGC CAA TTT ATG TAA GT and EHN-2: TTT AGA AAC AAT GTT TCT TC) were used. All primers were obtained from Genzentrum (Martinsried, Germany). The PCRs were done using a hot start technique in which the 38.2- μ l DNA extracts or 2 μ l of the first PCR in 36.2 μ l water were denatured at 96°C for 2 min after the addition of 1 μ l each of 50 mM solutions of the primers and two drops of mineral oil. After cooling to 80°C, 9.8 μ l of a freshly prepared mixture of 5 μ l of buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl), 4 μ l of 50 mM MgCl₂, 0.5 μ l of dNTP-mix (25 mM each; United States Biochemical Corp., Cleveland, OH), and 0.5 μ l (5 U/ μ l) of *Taq* polymerase (Amersham Buchler, Braunschweig, Germany) was added. Fifty cycles were performed with denaturation at 92°C for 60 sec, annealing at 43°C for 60 sec for EH-1/2 or at 62°C for 60 sec for both EHP-1/2 and EHN-1/2, and extension at 72°C for 90 sec. Products were visualized on a 13% agarose gel containing 0.2 μ g/ml of ethidium bromide (Sigma).

Restriction endonuclease digests. Bands excised from the agarose gel were silica gel-purified as described above, eluted in 9.6 μ l of water, and after the addition of 1.2 μ l of buffer (Nr. 4; New England Biolabs, Beverly, MA), digested with 0.8 μ l (10 U/ μ l) of *Dra* I (Appligene, Illkirch, France) for 60 min at 37°C, followed by the addition of 0.4 μ l (10 U/ μ l) of *Sau* 96 I (New England Biolabs) and further incubation at the same temperature for another 90 min.

RESULTS

DNA extraction methods. After 90 min of digestion with proteinase K, no amplification

products could be detected. To generate a rapid protocol, longer incubation times were not considered. In contrast, the alkaline lysis method yielded a 0.9-kb band. In an attempt to simplify the method, it was found that the initial washing step with 0.9% NaCl could be omitted without a detectable loss of product. However, the PCI extraction proved to be essential, but no change in yield resulted from repeating it two more times.

Volume of specimens and detection limit. Since stool components are known to inhibit PCR amplifications, 0.03 g, 0.08 g, 0.2 g, and 0.5 g of stool at a concentration of 50 HK-9 trophozoites per milligram were extracted and the DNA was amplified as described. The optimal amount of stool was found to be between 0.08 g and 0.2 g. Using a sample size of 0.1 g, the detection limit was found to be one trophozoite per milligram of stool when 1,000, 300, 100, 30, and 10 trophozoites, respectively, were tested in a 0.1-g stool sample.

Specificity of the PCR primers. The primer pair EH-1 and EH-2 is complementary to both pathogenic and nonpathogenic sequences with the exception of EH-2, in which the pathogenic and the nonpathogenic forms differ at the fourth base. The EH-2 primer was therefore constructed two-fold degenerately, i.e., as a mixture with half corresponding to the pathogenic sequence and the other half corresponding to the nonpathogenic sequence. The first, the preamplification PCR with EH-1 and EH-2, is followed by two additional PCRs, each of which is specific for either the pathogenic or the nonpathogenic sequence. The primers used for these reactions are located downstream of EH-1 and EH-2, making this a nested PCR to increase sensitivity. The two primer pairs used in these second PCRs were constructed in such a way that mutations specifying pathogenic or nonpathogenic forms are placed near the 3' end to increase specificity at the same time. This is in agreement with an earlier observation that an absolute requirement for PCR primers to function is a perfect match of at least two bases at the 3' end.¹⁰ The primer pair EHP-1 and EHP-2, as well as the primer pair EHN-1 and EHN-2, were found to selectively amplify pathogenic or nonpathogenic *E. histolytica* DNA, respectively (Figure 1).

Verification of the PCR products by restriction endonuclease digest. The results of the PCR amplifications were confirmed by a re-

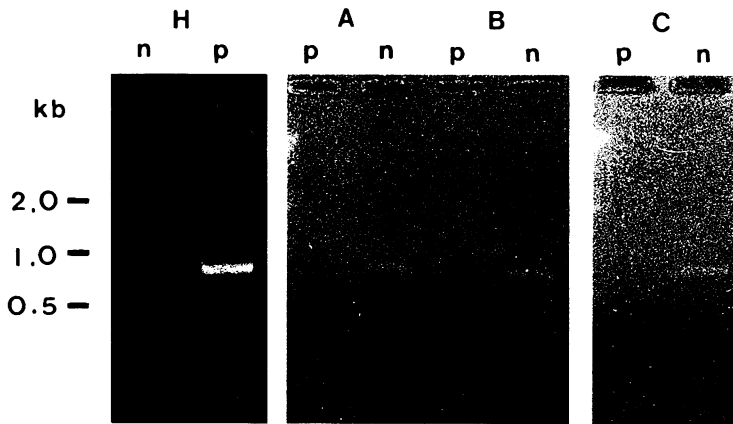


FIGURE 1. Polymerase chain reaction (PCR) amplification products of cultured trophozoites from the pathogenic strain HK-9 of *Entamoeba histolytica* (H) and three nonpathogenic patient specimens (A, B, and C). The second, nested PCR shown was performed with the primer pair EHP-1 and EHP-2 (p) or with the primer pair EHN-1 and EHN-2 (n), respectively. kb = kilobases.

striction endonuclease double digestion with *Dra*I and *Sau* 96 I. The *Dra*I restriction site is common to both forms and confirms the presence of *E. histolytica* DNA by producing a 0.55-kb band. The remaining 0.35-kb fragment con-

tains a *Sau* 96 I restriction site 0.2 kb from the 3' end of the nonpathogenic sequence with a base mutation in the pathogenic form. Therefore, the pathogenic DNA exhibits characteristic bands of 0.55 kb and 0.35 kb, usually with some of the undigested 0.9-kb band remaining. The nonpathogenic DNA yields a band of 0.55 kb and a confluent double band of 0.15–0.2 kb, often with a partial digestion product of 0.7 kb (Figure 2).

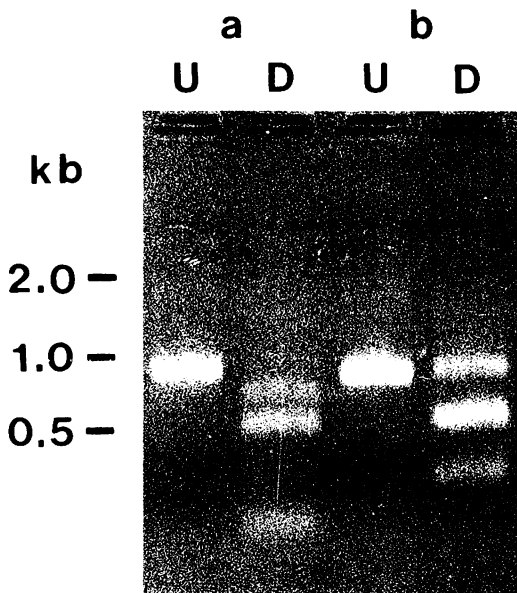


FIGURE 2. Restriction endonuclease digestions of the products from polymerase chain reactions as described in Figure 1. **a**, nonpathogenic *Entamoeba histolytica* DNA. **b**, pathogenic HK-9 DNA. U = undigested; D = digested with *Dra* I and *Sau* 96 I. kb = kilobases.

DISCUSSION

The protocol described provides a method to sensitively and selectively detect and differentiate between pathogenic and nonpathogenic *E. histolytica* DNA directly from stool specimens without the need for prior cultivation. Apart from the often unsuccessful and time-consuming cultivation attempts, possible misdiagnoses by one strain displacing another in mixed infections are being avoided. The method is fast and simple because all extraction steps can be performed at room temperature in a 1.5-ml microcentrifuge tube format. Neither ice nor refrigeration is required. There is no precipitation step, eliminating problems with small amounts of DNA. No laborious hybridizations or the use of radionucleotides are required. The whole procedure can be performed in one day.

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