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Differentiation of *Entamoeba histolytica* and *E. dispar* DNA from cysts present in stool specimens by polymerase chain reaction: its field application in the Philippines

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Abstract It has been established that two distinct species exist within what was originally known as *Entamoeba histolytica*. These are *E. dispar* and *E. histolytica*, for the nonpathogenic and pathogenic forms, respectively. Differentiation of these two organisms is of great clinical importance since they are morphologically indistinguishable and both forms can infect the human intestinal cavity to different degrees. A simple and rapid DNA-extraction method that can be used directly on formalin-fixed stool specimens has been developed. The extracted DNA was used for the identification of the species existing in the stools by polymerase chain reaction (PCR). A total of 72 randomly collected stool samples from the Philippines were analyzed. In all, 19 samples reacted with *E. dispar* primers, resulting in the expected 101-bp PCR products; however, none reacted with *E. histolytica* primers. Furthermore, sensitivity assay suggests that genomic DNA from as few as five cysts can be used as a template for PCR. These observations imply that the use of genomic DNA directly extracted from formalin-fixed stool specimens for PCR amplification is a useful tool for obtaining a sensitive and accurate diagnosis that can be applied even in epidemiology studies.

Introduction

The existence of pathogenic and nonpathogenic species of *Entamoeba* has recently been established. On the bases of biochemical, genetic, and immunological evidence, *E. histolytica* Schaudinn 1903 is now recognized as the pathogenic species, distinct from *E. dispar* Brumpt 1925, the nonpathogenic species (Diamond and Clark 1993). The invasive form usually penetrates the mucosa, resulting in massive destruction of host tissue, and causes diseases such as hemorrhagic colitis and extraintestinal abscess, whereas the noninvasive form passively inhabits the cavity of the lower intestine in a commensal manner (Leippe et al. 1993). In recent years, isoenzyme patterns of the carbohydrate metabolism have been shown to be useful in distinguishing pathogenic from nonpathogenic isolates (Sargeaunt 1987). Specific DNA sequences have subsequently been identified and used in several laboratories as probes for the detection of pathogenic and nonpathogenic species (Garfinkel et al. 1989; Tannich et al. 1989; Tachibana et al. 1991a, b). However, the use of these methods depends on the culture of amebae, which usually takes several days. Moreover, in vitro cultivation of amebae sometimes results in failure. Thus, the use of template DNA from cultivated trophozoites of *E. histolytica* and *E. dispar* for polymerase chain reaction (PCR) can defeat the purpose of a rapid diagnostic procedure. Also, at present, diagnosis of amebiasis in many endemic countries such as the Philippines relies mainly on microscopic examination of stool samples for the presence or absence of amebae. Since pathogenic and nonpathogenic species are morphologically indistinguishable, the sole use of this method frequently leads to indiscriminate use of antiamebic drugs. Hence, development of a simple and reliable method to distinguish *E. histolytica* from *E. dispar* through the use of DNA for diagnosis would be of utmost importance.

In view of these conditions, this paper reports on the use of PCR for field diagnosis of *Entamoeba* using DNA directly extracted from ameba cysts present in stool samples. Furthermore, the sensitivity of the PCR using DNA from cysts is also evaluated.

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Materials and methods

Cultivation of the *Entamoeba* reference strain

Trophozoites of the pathogenic strain of *E. histolytica*, SAW 1453, were xenically grown in Inoki medium (Inoki et al. 1953). Ame-

bae were harvested after completion of log-phase growth at 72 h using Percoll-gradient centrifugation as described previously (Tachibana et al. 1990). Trophozoites were isolated from the medium components by centrifugation at 400 *g* for 5 min, and were washed three times with phosphate-buffered saline (PBS, pH 7.4) at 4°C. Also, DNA extracts of *E. dispar*, SH-5, isolated from Shanghai, China (Cheng et al. 1993), were obtained and used as the nonpathogenic control strain.

Collection and processing of field samples

Human stool samples were collected from San Antonio, Sasmoan, Pampanga, the Philippines. The samples were taken from persons who had given their informed consent prior to the collection. Each stool sample was put in a covered glass vial with 10% phosphate-buffered formalin solution and transported to the laboratory for microscopic examination. Each sample was then macroscopically inspected for its consistency and for the presence of soil contamination, blood, or mucus. Diagnosis of parasites was performed as previously described by Acuna-Soto et al. (1993). *Entamoeba* cysts were extracted from microscopically positive stools using a formalin-ether sedimentation procedure based on that described by Beaver et al. (1984).

Genomic DNA extraction from stool samples

Genomic DNA was extracted from cysts present in the stool samples according to a modified version of the method of Acuna-Soto et al. (1993). Briefly, about a 70-µl volume of pellet resulting from the formalin-ether sedimentation procedure was placed in a 1.5-ml plastic Eppendorf tube and was then resuspended in 1 ml distilled water and centrifuged for 30 s at 2,000 *g* in a microfuge. This washing step was repeated three times. The supernatant of the last wash was decanted and the pellet was resuspended in a small volume (50–100 µl) of a solution containing 100 m*M* TRIS (pH 8) and 25 m*M* ethylenediaminetetraacetic acid (EDTA). The tubes were immersed in a mixture of dry ice and ethanol for 3 min and were placed in water at room temperature for 2 min. This process was repeated three times to rupture the cysts.

After the last treatment, the solution was mixed with 200 µl of a solution containing a 200-µg/ml concentration of proteinase K, 100 m*M* TRIS (pH 8), 1% sodium dodecyl sulfate (SDS), and 25 m*M* EDTA. The mixture was incubated at 50°C for 24 h, and boiled for 10 min. The DNA was extracted twice with phenol-chloroform-isoamyl alcohol (25:24:1, by vol.) and then precipitated with 3 *M* sodium acetate and absolute ethanol. The resulting DNA was resuspended in 10 m*M* TRIS (pH 7.4)/1 m*M* EDTA (TE buffer) and stored at –20°C until use. To increase the purity of the DNA sample, 50 µl of 25% polyethylene glycol (PEG 6000) in 2.5 *M* NaCl was added to the 50 µl of DNA suspended in TE buffer and stored at 0°C overnight. Thereafter, the suspension was centrifuged at 14,000 rpm for 10 min at 4°C and the DNA was reextracted using the usual phenol-chloroform extraction procedure as described by Sambrook et al. (1989).

PCR of DNA extracted from stool samples

Genomic DNA segments were amplified by PCR. The reaction mixture contained (final concentration) 10 m*M* TRIS-HCl (pH 8.9), 1.5 m*M* $MgCl_2$, 80 m*M* KCl, 500 µg bovine serum albumin (BSA)/ml, 0.1% sodium cholate, 0.1% Triton X-100, 0.2 m*M* each of the four deoxynucleoside triphosphates, 25 p*M* each of the two primers, 2.0 U of *Tth* DNA polymerase (Toyobo Co., Ltd.), and 0.5 µg genomic DNA as the template in a final volume of 50 µl. The mixture was overlaid with 100 µl of light mineral oil (Sigma Chemical Co.). The reactions were amplified using the "hot-start" technique for 30 cycles in an automated PCR machine (ASTEC Program Temp Control System p-700). PCR was carried out using two sets of primers, p11 plus p12 and p13 plus p14, as described elsewhere (Tachibana et al. 1991b). Cycling conditions for these primers were denaturation (94°C) for 1 min (3 min in cycle 1), annealing (59°C) for 1.5 min, and polymerization (72°C) for 1.5 min (8.5 min in cycle 30). Aliquots (7.5 µl) of the amplified products were subjected to electrophoresis in 2% LO3 (Takara Biomedicals) agarose gels, and the presence of specific bands was visualized with UV light after ethidium bromide staining.

Southern-blot hybridization

DNAs extracted from formalin-fixed stool samples containing *E. histolytica* were amplified through PCR using p13 plus p14 primers. They were size-fractionated on agarose gels and subsequently transferred to nylon Immobilon-N (Millipore Corp.) membranes using the capillary transfer technique (Sambrook et al. 1989). Membranes were air-dried, baked at 80°C for 1 h, prehybridized at 65°C overnight, and then hybridized with the p13 DNA probe labeled with digoxigenin (DIG; Boehringer Mannheim 1993).

Probe labeling

The p13 probe was labeled using the Boehringer Mannheim DIG oligonucleotide 3′ end-labeling kit and was detected by an anti-DIG antibody conjugated with alkaline phosphatase and a subsequent enzyme-catalyzed color reaction with 5-bromo-4-chloro-3-indolyl phosphate (BCIP/x-phosphate) and nitroblue tetrazolium salt (NBT).

Results

Macroscopic and microscopic examination of collected stool samples

Of the 72 samples collected, 10 were loose and contained liquid and mucus, whereas none contained blood.

Table 1 Comparison of microscopy, macroscopic examination, and PCR in the identification of *Entamoeba histolytica* and *E. dispar* in the study population

Microscopic examination		Macroscopic examination				PCR	
Species	Number of samples	Formed	Formed with liquid/mucus	Loose	Loose with liquid/mucus	*E. histolytica* (p11 plus p12)	*E.dispar* (p13 plus p14)
Protozoa							
E. histolytica	13	9	0	2	2	0	13
E. coli	11	4	2	2	3	0	6
Endolimax nana	6	1	2	2	1	0	0
Blastocystis	2	2	0	0	0	–	–
Helminth:							
Ascaris lumbricoides	2	1	0	0	1	–	–
Negative samples:							
No parasite	38	28	0	7	3	–	–

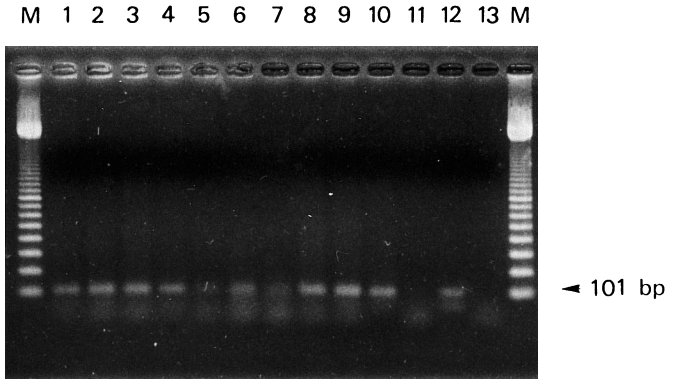

Fig. 1 Agarose-gel electrophoresis of PCR products amplified by *Entamoeba dispar* primers p13 plus p14. Template DNAs were extracted from cysts collected after a formalin-ether concentration technique from positive stool samples. Amplified products were subjected to electrophoresis in 2% LO3 (Takara Biomedicals) agarose gels and visualized under UV after ethidium bromide staining (*Lanes 1–10* Test samples, *lane 11* SAW 1453 – *E. histolytica* control, *lane 12* SH-5 – *E. dispar* control, *lane 13* without template, *M* DNA size marker – 100-bp ladder). The *arrowhead* indicates the position and size of PCR products

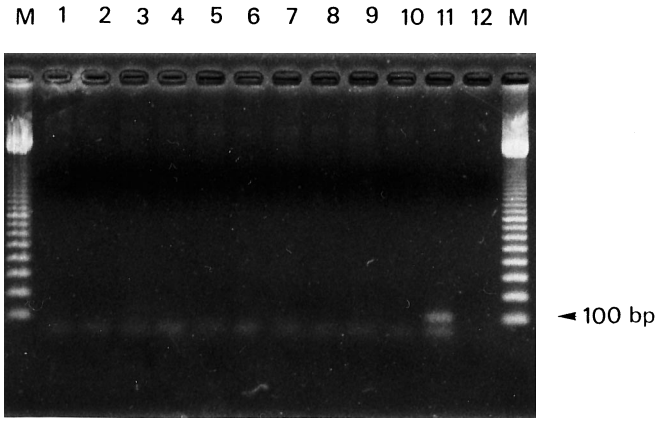

Fig. 2 Agarose-gel electrophoresis of PCR products amplified by *E. histolytica* primers p11 plus p12. Template DNA were extracted from cysts collected after a formalin-ether concentration technique from positive stool samples. Amplified products were subjected to electrophoresis in 2% LO3 (Takara Biomedicals) agarose gels and visualized under UV after ethidium bromide staining (*Lanes 1–10* Test samples, *lane 11* SAW 1453 – *E. histolytica* control, *lane 12* without template, *M* DNA size marker – 100-bp ladder). The *arrowhead* indicates the position and size of PCR products

A total of 13 individuals had loose stool samples, 45 had formed stool samples, and 4 had formed stools containing liquid and mucus. At the time of collection of samples, two individuals complained of diarrhea. The prevalence of all parasites found in the samples collected from individuals was also identified. *Endolimax nana*, *Entamoeba coli*, *Blastocystis*, and the helminth *Ascaris lumbricoides* were found in low numbers. However, no correlation was observed between the prevalence of parasites and the consistency of the stool samples (Table 1).

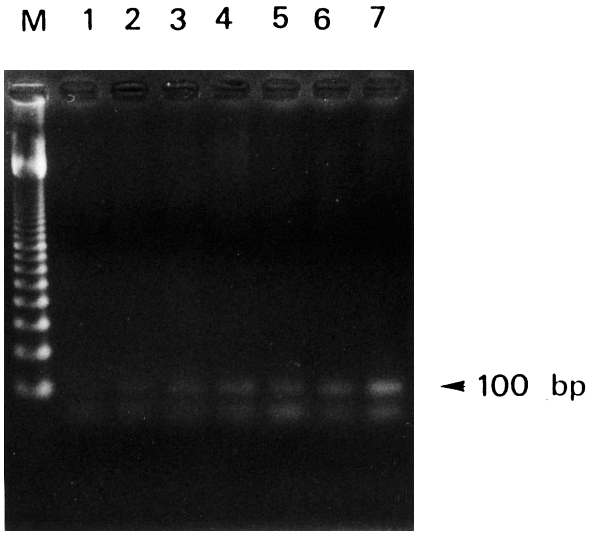

Fig. 3 Sensitivity of PCR amplification of genomic DNA from *E. histolytica* cysts. The DNA extracted from cysts isolated from an asymptomatic cyst passer in Japan was serially diluted and amplified for 30 cycles using p11 plus p12 primers. Amplified products were subjected to electrophoresis in 2% LO3 (Takara Biomedicals) agarose gels and visualized under UV after ethidium bromide staining. The number of cysts equivalent to the template DNA used as shown in *lanes 1–7*: 0, 5, 15, 35, 50, 75, and 100, respectively. *M* Size marker – 100-bp ladder). The *arrowhead* indicates the position and size of PCR products

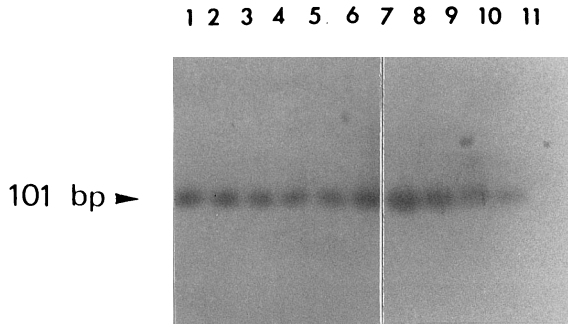

Fig. 4 Southern-blot hybridization analysis of PCR products amplified by primers p13 plus p14. The p13 probe labeled with digoxigenin (DIG)-ddUTP was used and detected by an anti-DIG antibody conjugated with alkaline phosphatase. After agarose-gel electrophoresis of PCR products, DNA was transferred to Immobilon-N membranes and hybridized with the labeled p13 probe (*Lanes 1–10* Test samples, *lane 11* SAW 1453 – *E. histolytica* control). The *arrowhead* indicates the position of the hybridization bands

Amplification of DNA extracted from stool samples

When the DNAs extracted from the samples were used as templates for PCR amplification using *E. dispar* primers p13 plus p14, the expected 101-bp PCR product resulted from 19 collected samples (Fig. 1). On the other hand, the *E. histolytica*-specific primers p11 plus p12 did not affect the PCR reactions; hence, no band was detected (Fig. 2). These results indicate that all of the 13 samples microscopically diagnosed as *E. histolytica* were *E.*

dispar. Furthermore, we detected *E. dispar*-positive bands in 6 of the 11 samples microscopically observed as *E. coli.*

Determination of the minimal number of cysts detected by PCR

The cyst content of *E. histolytica* isolated from an asymptomatic cyst passer in Japan was determined using a hemocytometer. DNA was extracted from this known amount of cyst sample and was serially diluted and assayed by PCR. The set of primers for pathogenic isolates, p11 plus p12, was tested. As shown in Fig. 3, template DNA equivalent to 5 cysts gave a faint band after PCR amplification; on the other hand, a distinct band was observed after ethidium bromide staining of the amplified DNA extracted from the serially diluted sample equivalent to 15 cysts.

Southern-blot hybridization

The 101-bp PCR products on agarose gel were transferred to nylon membranes. Figure 4 shows the hybridization pattern obtained after probing of the membrane with p13, a nonpathogenic-specific probe labeled with DIG. Hybridization signals were seen with the 19 nonpathogenic samples but not with the pathogenic control, SAW 1453-amplified DNA, using p11 plus p12 primers. However, on probing with the p11 pathogenic probe labeled with DIG, no hybridization signal was observed on the samples amplified by *E. dispar* primers (data not shown).

Discussion

This report presents an application of PCR for field diagnosis of *Entamoeba histolytica* and *E. dispar* using template DNA directly extracted from stool samples. The DNA-extraction method of Acuna-Soto et al. (1993) was slightly modified to render the protocol feasible to the current situation of clinical laboratories in endemic countries such as the Philippines. The use of a sonicator for the lysis of cysts was omitted. Also, the use of PEG for repurification of the extracted DNA was added to the protocol and proved to be effective in getting rid of residual fecal debris after the phenol-chloroform-isoamyl alcohol extraction, which may affect the PCR. The assay, therefore, does not require cultivation of the organisms and, in principle, is sensitive and can be performed in less than 2 days. Also, the use of formalin-fixed stools for DNA extraction has an advantage in terms of safe handling and storage of samples.

Although the possibility of the presence of *E. histolytica* in the survey area cannot be ruled out, nonpathogenic *E. dispar* predominated in the collected samples. Since the stool samples were collected from asymptomatic carriers, all isolates were thus identified as genotypically nonpathogenic *E. dispar.* Furthermore, 6 of the 11 microscopically observed *E. coli* samples were identified as *E. dispar* through PCR (Table 1). It may have been the single microscopic field observation of each sample and the relatively small numbers of *E. dispar* that made us incapable of observing the prevalence of mixed infections within a sample. Moreover, no mixed infection of *E. histolytica* and *E. dispar* was detected.

To evaluate the sensitivity of this PCR-based analysis, DNA extracted from an asymptomatic cyst passer in Japan was serially diluted and then amplified for 30 cycles. The results of electrophoresis are illustrated in Fig. 3. When template DNA equivalent to 15 cysts was amplified, a distinct band was observed by ethidium bromide staining. A faint band was visible even when genomic DNA equivalent to five cysts was used as a template.

For further detection of the specificity of the primers used in this study, the agarose gels were blotted onto nylon membranes and probed with the p13 probe labeled with DIG. Used as a 3′ primer, this oligomer does not allow amplification of pathogenic *E. histolytica* DNA. A hybridization signal was seen with the 19 nonpathogenic samples but not with the pathogenic SAW 1453 DNA (Fig. 4).

The reliability of PCR for the diagnosis of *E. histolytica* infections has been shown in several studies (Tachibana et al. 1991b, 1992a, b; Tannich and Burchard 1991; Acuna-Soto et al. 1993; Cheng et al. 1993). In the present study the sensitivity and reliability of PCR was also observed through its new application, that is, using template DNA directly extracted from the stool sample. As shown in Table 1, reliance solely on microscopic examination for the diagnosis of *Entamoeba* can be misleading. The small size and variability in life-cycle stages have made it difficult to diagnose accurately *Entamoeba*-related infections. This also makes it difficult to use microscopy as an epidemiologic index of *E. histolytica* infections among different populations (Acuna-Soto et al. 1993). Furthermore, since *E. histolytica* and *E. dispar* are morphologically indistinguishable, the use of DNA probes is of utmost importance.

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