Real-Time PCR for Detection and Differentiation of *Entamoeba histolytica* and *Entamoeba dispar* in Fecal Samples

Joerg Blessmann,1 Heidrun Buss,1 Phuong A. Ton Nu,2 Binh T. Dinh,2 Quynh T. Viet Ngo,2 An Le Van,2 Mohamed D. Abd Alla,3 Terry F. H. G. Jackson,4 Jonathan I. Ravdin,3 and Egbert Tannich1*

Department of Molecular Parasitology, Bernhard Nocht Institute for Tropical Medicine, Hamburg, Germany1; Medical College, Hanoi, Vietnam2; Department of Medicine, University of Minnesota, Minneapolis, Minnesota3; and Medical Research Council (Natal), Congella, South Africa4

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A closed-tube, real-time PCR assay was developed for sensitive and specific detection and differentiation of the two closely related intestinal protozoan parasites *Entamoeba histolytica* and *Entamoeba dispar* directly from human feces. The assay is performed with the LightCycler system using fluorescence-labeled detection probes and primers amplifying a 310-bp fragment from the high-copy-number, ribosomal DNA-containing ameba episome. The assay was able to detect as little as 0.1 parasite per g of feces. The two pairs of primers used were specific for the respective ameba species, and results were not influenced by the presence of other *Entamoeba* species even when present in exceeding amounts. PCR was evaluated using several hundred stool samples from areas of amebiasis endemicity in Vietnam and South Africa, and results were compared with those of microscopy and ameba culture. PCR was found to be significantly more sensitive than microscopy or culture, as all samples positive by microscopy and 22 out of 25 (88%) samples positive by culture were also positive by PCR, but PCR revealed a considerable number of additional *E. histolytica*- or *E. dispar*-positive samples. Compared to culture and subsequent ameba differentiation by isoenzyme analysis, PCR was 100% specific for each of the two *Entamoeba* species. Interestingly, the comparison with PCR revealed that culture, in particular, underestimates *E. histolytica* infections. Given the high sensitivity and specificity of the developed PCR assay, the inability of microscopy to distinguish between the two ameba species, and the time it takes to culture and subsequently differentiate entamoebae by isoenzyme analysis, this assay is more suitable than microscopy or culture to correctly diagnose intestinal *E. histolytica* or *E. dispar* infection.

The intestinal protozoan parasite *Entamoeba histolytica* is endemic in large parts of the world and is considered responsible for millions of cases of dysentery and liver abscesses each year (32). Prior to invasive disease, *E. histolytica* may reside in the human gut for months or even years as asymptomatic infection (15, 37). To interrupt transmission of the parasite and to avoid progression of infected individuals to invasive disease, treatment of *E. histolytica* carriers has been recommended (36).

Traditionally, the laboratory detection of *E. histolytica* in human feces has relied upon the microscopic examination of fresh or fixed stool samples. However, the recent identification of *Entamoeba dispar* as a separate but nonpathogenic species which is morphologically indistinguishable from *E. histolytica* and does not require treatment has indicated the need of numerical rather than qualitative, allowing appropriate diagnostic statistics to be applied.

Here we report the application of closed-tube, real-time PCR for the detection and differentiation of *E. histolytica* and *E. dispar* directly from fecal samples.

**MATERIALS AND METHODS**

*Entamoeba* strains and culture conditions. The two *E. histolytica* isolates HK-9 and HM-1:IMSS (obtained from the American Type Culture Collection) and the two *E. dispar* isolates SAW142 and SAW760 were used to spike fecal samples. All isolates were cultured in TY-S-33 medium (9). The *E. histolytica* isolates were grown monoxenically in the presence of *Crithidia fasciculata* (6).

Spiking of fecal samples. Cultured ameba trophozoites were sedimented by centrifugation and resuspended in phosphate-buffered saline, pH 7.4. Cell den-
sity was measured by counting the amebae in a hemocytometer (Neubauer chamber) under the microscope. Serial dilutions of cells were mixed with 10 g of human feces prior to DNA extraction. Human feces microscopically free of any parasite were obtained from German residents who had never been in an area where amebiasis is endemic.

Real-time PCR. DNA was extracted from human feces using the QIAamp DNA stool mini kit (Qiagen, Hilden, Germany), according to the manufacturer’s protocol. Real-time PCR was performed using the LightCycler (34). Basic reagents for the LightCycler PCR were purchased from Roche Diagnostics, Mannheim, Germany. Oligonucleotide primers and probes (ThermoScientific, Waltham, Mass.) to minimize primer dimer and other primer secondary structures. The 10-μl reaction mixture volume in a glass capillary tube contained 1 μl of FastStart reaction mix hybridization probes (a component of the FastStart DNA master hybridization probes kit; Roche Diagnostics), 1.2 μl of MgCl₂ (25 mM), 3.8 μl of H₂O, 1 μl each of sense and antisense primer (10 pmol/μl), 0.5 μl each of LC-Red 640- and fluorescein-labeled probe (4 pmol/μl), and 1 μl of DNA extract. Primers and probes for E. histolytica-specific PCR were Eh-S26C (5' GATA CAA AAT GCC CAA TTC ATT CAA CG), Eh/Ed-AS25 (5' /H9262 AA TTG GCC ATC TGT AAG TTA TAA GCA), and Eh/Ed-25-fluorescein (fluorescein-GCC ATC TGT AAA GCT CCC TCT CGA X). For E. dispar-specific PCR the same primers and probes were used except that Eh-S26C was replaced by Ed-27C, 5' GATA CAA AGT GGC CAA TTT ATG TAA GCA. Reaction conditions were chosen according to a standard LightCycler protocol in our laboratory and were 5 min at 95°C, followed by 50 cycles of 10 s at 98°C and 20 s at 72°C. Temperature change rates were 20°C/s for the first two steps and 3°C/s for the last step. In addition, a touch-down PCR mode was incorporated in that the annealing temperature was stepwise decreased from 62 to 58°C by steps of 0.5°C within the first 8 of the 50 cycles. Readout was performed in channel F2/Back-F1. A sample was regarded as positive when the LightCycler software, version 3.5, determined a crossing point in the quantification analysis screen.

Stool microscopy, culture, and serology. Stool samples were examined microscopically for the presence of protozoan parasites using the Formol-ether concentration technique and subsequent staining with Lugol’s iodine solution (19, 20). Amebae were cultured from fecal samples and differentiated by isoenzyme analysis essentially as described by Sargeaunt and coworkers (24). Serum samples were investigated for the presence of antiameba antibodies using an enzyme-linked immunosorbent assay based on a recombinant E. histolytica surface antigen as previously described (17). This assay has been proven to be highly specific even when applied in countries where amebiasis is endemic, and the test is able to detect anti-E. histolytica antibodies for about 6 to 12 months after successful antiamebic treatment (16).

Statistical analysis. All data collected were computer coded and analyzed by use of Sigma-Stat (SAS Software; Jandel Scientific, Erkrath, Germany). Binomial or χ² tests were used for comparisons between two groups. A P value of <0.05 was considered to be significant.

RESULTS

Sensitivity and specificity of PCR for the detection of E. histolytica or E. dispar in fecal samples. Sensitivity of the developed real-time PCR assay was evaluated using Entamoeba-negative human feces, which were spiked with various numbers of cultured E. histolytica or E. dispar trophozoites prior to DNA extraction and subjected to LightCycler PCR. Depending on the number of cells introduced, positive results were obtained after 20 to 45 cycles. For both ameba species, detection limits were 0.1 cell per g of feces (Fig. 1). Each of the two sets of primers used for PCR was specific and did not amplify DNA from the opposite ameba species. In addition, PCR was negative with both sets of primers when DNA was introduced
TABLE 1. Comparison of fecal PCR with microscopy and serology*

<table>
<thead>
<tr>
<th>Specimen group (n)</th>
<th>No. (%) of specimens with result(s)</th>
<th>E. histolytica</th>
<th>E. dispar</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive positive for E. histolytica and E. dispar</td>
<td>Positive serology for E. histolytica</td>
<td></td>
</tr>
<tr>
<td>All samples (491)</td>
<td>45 (9.2)</td>
<td>120 (24.4)</td>
<td></td>
</tr>
<tr>
<td>E. histolytica-positive PCR (46)</td>
<td>29 (63.0)*</td>
<td>38 (82.6)*</td>
<td></td>
</tr>
<tr>
<td>E. dispar-positive PCR (24)</td>
<td>16 (66.7)*</td>
<td>5 (20.8)**</td>
<td></td>
</tr>
<tr>
<td>E. histolytica- and E. dispar-negative PCR (421)</td>
<td>0*</td>
<td>77 (18.3)***</td>
<td></td>
</tr>
</tbody>
</table>

* Symbols for significant difference compared to all samples: *, P < 0.001; ***, P < 0.03; **, not significantly different (P = 0.63).

which had been isolated from stool samples containing other Entamoeba species such as Entamoeba coli, Entamoeba hartmanni, or Entamoeba chattoni. Moreover, double infections with E. histolytica and E. dispar did not influence PCR results, as cross-contamination of E. histolytica-spiked fecal samples with E. dispar trophozoites or vice versa in a ratio between 1:1 and 1:10,000 produced virtually identical results as did cases without contamination (Fig. 1).

Comparison of PCR with microscopy and serology. A total of 491 fecal samples collected from residents of an area with a high incidence of amebic liver abscess (ALA) in Hue, Vietnam (4), were subjected to PCR analysis. Outcome was compared with the results of microscopy and antiameba antibody status (Table 1). Prevalence of E. histolytica and E. dispar was found to be 9.2% (45 of 491) based on microscopy versus 14.3% (70 of 491) according to the results of PCR (P = 0.02). Remarkably, all samples positive by microscopy were also positive by PCR. There was a good correlation between the number of PCR cycles required in order to reach the crossing point and the relative parasite burden. In general, PCR became positive within 20 to 30 cycles when relatively high numbers of amebae were seen under the microscope, whereas more than 40 cycles were required with samples containing very low parasite numbers. There was a strong association between PCR and microscopy, as none of the samples negative by PCR were positive by microscopy and 64.3% (45 of 70) of PCR-positive samples were also positive by microscopy (P < 0.001). Differentiation into E. histolytica and E. dispar by PCR revealed 9.4% (46 of 491) of samples to be positive for E. histolytica versus 4.9% (24 of 491) positive for E. dispar (P < 0.01). The association between positive PCR and microscopy was slightly but not significantly higher for E. dispar than for E. histolytica (66.7 versus 63.0%; P = 0.93). Interestingly, PCR revealed no double infection with E. histolytica and E. dispar.

In addition to microscopy, a strong association was found between E. histolytica-positive PCR and serology. A significant antiameba antibody titer was present in 24.4% (120 of 491) of individuals tested but in 82.6% (38 of 46) of individuals with a positive E. histolytica fecal PCR result (P < 0.001). In contrast, individuals with fecal PCR results positive for E. dispar or negative for both organisms revealed positive serology in only 20.8% (5 of 24) and 18.3% (77 of 421) of cases, respectively (P = 0.79).

Comparison of PCR with Entamoeba culture. Ameba culture and subsequent isoenzyme analysis is considered to be the “gold standard” for the differentiation between E. histolytica and E. dispar from fecal samples. Thus, results of ameba culture were compared with those of PCR (Table 2). DNA was extracted from 181 fecal samples, which were collected during a study in Durban, South Africa. Parallel samples were taken into culture, and those cultures positive for E. histolytica and/or E. dispar were subjected to isoenzyme analysis for further differentiation. Of the 181 samples, 81 were obtained from patients with a recent history of ALA. These samples were collected 3 to 9 months after successful ALA treatment with metronidazole. The remaining 100 samples were collected from family members of the various ALA patients living under similar conditions (controls). Culture and isoenzyme analysis revealed a prevalence of 7.2% (13 of 181) for E. histolytica and of 6.6% (12 of 181) for E. dispar. Eleven of the 13 positive E. histolytica cultures were from recent ALA patients, whereas the majority of positive E. dispar cultures (10 out of 12) were from the group of controls. Comparison with PCR revealed a good concordance between culture and fecal PCR, as 12 out of 13 (92.3%) samples with a positive E. histolytica culture and 10 out of 12 (83.3%) samples with a positive E. dispar culture were also positive by the respective PCR. None of the samples with an E. histolytica-positive culture was positive by E. dispar-specific PCR and vice versa. However, PCR identified an additional 21 E. histolytica-positive and two E. dispar-positive samples, which were negative by culture. All of the additional E. dispar-positive samples were from the group of controls, whereas the majority of additional E. histolytica-positive samples (14 of 21, P < 0.02) were from the group of recent ALA patients.

TABLE 2. Comparison of fecal PCR with culture and isoenzyme analysis*

<table>
<thead>
<tr>
<th>Specimen group (n)</th>
<th>No. (%) of specimens with result(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Culture positive</td>
</tr>
<tr>
<td>E. histolytica</td>
<td>13 (7.2)</td>
</tr>
<tr>
<td>E. dispar</td>
<td>12 (6.6)</td>
</tr>
<tr>
<td>All samples (181)</td>
<td>11 (13.6)</td>
</tr>
<tr>
<td>ALA patients (81)</td>
<td>10 (10.0)</td>
</tr>
<tr>
<td>Controls (100)</td>
<td>2 (2.0)</td>
</tr>
</tbody>
</table>

* Symbols for significant difference compared to culture: *, P < 0.001; **, P < 0.03.

DISCUSSION

The recent identification of E. dispar as a separate but non-pathogenic species which cannot be distinguished by microscopy from pathogenic E. histolytica has prompted the World Health Organization to recommend the development of im-
proved methods for the specific identification of *E. histolytica* from human feces (36). In this respect, we have evaluated the application of closed-tube, real-time PCR technology for the detection and differentiation of *E. histolytica* and *E. dispar* directly from stool samples. The LightCycler PCR method employed here is fast, because it takes only 1 h to process 30 samples, and it is specific, as it requires two primers as well as two fluorescence-labeled probes for DNA detection. DNA was extracted from human feces by a commercially available kit, which has been proven to allow reproducible preparations of PCR-amplifiable DNA, as >99% of all extracted samples do not contain inhibitory activity (30). This was confirmed in this study, as 99.1% of all samples negative for *E. histolytica* or *E. dispar* by PCR were PCR positive with primers amplifying a fragment of the human HLA-DRB gene (27). The rDNA sequence was selected as the target DNA for amebic PCR because it is well conserved between different *E. histolytica* isolates as well as between different *E. dispar* isolates (7) and because it is located on an episomal plasmid, which is present in about 200 copies per cell (3). Compared to chromosomal DNA the episomal plasmid is less sensitive to DNA degradation, and the high copy number should allow sensitive detection even if only a few cells are present. Sensitivity was considered further improved by selecting a relatively small fragment (310 bp only) for PCR amplification. However, rDNA sequences of *E. histolytica* and *E. dispar* share 98.4% nucleotide identity (25). Thus, only very few sites are suitable for the construction of appropriate primers for PCR differentiation. We took advantage of a considerable number of nucleotide polymorphisms around position 190 of ameba rDNA sequences, which represents one of the two sites where, compared to *E. histolytica*, the *E. dispar* sequence contains a nucleotide insertion. In addition, a point mutation was introduced into each of the specific sense primers, in that a thymidine was replaced by a cytidine residue. This created three consecutive mismatches between the 3’ ends of each primer and the respective rDNA sequences of the opposite *Entamoeba* species, which in the case of “cross priming” will strongly prevent DNA elongation by the polymerase (33). Our results with spiked fecal samples indicate that the two sets of primers are indeed species specific and that PCR is not influenced by the presence of considerable amounts of other *Entamoeba* species. In addition, PCR is highly sensitive, as as little as 0.1 cell per g of feces could be detected. The high sensitivity and specificity of PCR was further confirmed by comparison with stool microscopy and ameba culture. All 45 samples positive by microscopy and 22 out of 25 (88%) samples positive by culture were also positive by PCR. According to isoenzyme analysis, PCR classified all samples correctly into *E. histolytica* and *E. dispar*, respectively. Moreover, positive PCR for *E. histolytica* was strongly associated with ameba serology, which is consistent with previous findings indicating that in contrast to *E. dispar*, *E. histolytica* intestinal infection, even if asymptomatic, usually induces a significant systemic antiamebic antibody response (13, 23, 29, 31). However, compared to microscopy or culture, PCR identified a considerably larger number of additional positive samples, suggesting that PCR is more sensitive. Sensitivity of microscopy for the detection of *Entamoeba* by examination of a single fecal sample is considered to be about 70% (11). This value is in good agreement with the results of PCR, as regardless of whether *E. histolytica* or *E. dispar* was identified, about 65% of PCR-positive samples were positive by microscopy. The sensitivity of ameba culture has not been established unambiguously, but is considered to be higher than that of microscopy (19). Interestingly, sensitivity for the detection of *E. dispar* was identical between PCR and culture, but detection of *E. histolytica* was significantly higher by PCR. As for both ameba species the associations of PCR with microscopy and the PCR detection limits were identical, the results strongly suggest that culture in particular underestimates *E. histolytica* infections.

According to PCR about 30% of previous ALA patients were found to be positive for *E. histolytica*. Whether this relatively high number is the result of a high rate of reinfection in these individuals or whether this is due to ineffective treatment remains to be determined. However, the latter is more likely, as all patients were only treated with metronidazole and none of them received a luminal antiamebic agent such as diloxanide furate or paromomycin. A 30% *E. histolytica* prevalence in previous ALA patients 3 to 9 months after metronidazole therapy would be fully consistent with previous findings, which indicated a cure rate of intestinal infection by metronidazole of about 50% (12, 26).

Another interesting result came out of this study was the absence of any double infection with *E. histolytica* and *E. dispar*. This was obviously not due to technical problems, as the two specific sets of primers used for PCR were suitable to detect double infections, even if one of the two species were greatly underrepresented. Thus, it is intriguing to speculate whether intestinal infection with one of the two species is able to prevent colonization with the other one. However, to reach a definite conclusion a larger number of samples has to be analyzed.

Taken together, our results indicate that the PCR protocol presented here is suitable for the detection and differentiation of *E. histolytica* and *E. dispar* directly from human feces. Given the many advantages of closed-tube, real-time PCR technology, the high sensitivity and specificity of the developed PCR assay, the inability of microscopy to distinguish between the two ameba species, and the time it takes to culture and subsequently differentiate *Entamoeba* species by isoenzyme analysis, it is obvious that this protocol or similar protocols are substantially more appropriate than microscopy or culture to correctly diagnose intestinal *E. histolytica* or *E. dispar* infections. On the other hand, although the costs for reagents to extract DNA from human feces and to perform PCR are less than $7 per sample, at present, the application of this method is limited to specifically equipped laboratories running the LightCycler.

**ACKNOWLEDGMENT**

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**REFERENCES**
