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Detection and Differentiation of *Entamoeba histolytica* and *Entamoeba dispar* Isolates in Clinical Samples by PCR and Enzyme-Linked Immunosorbent Assay

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Differential diagnosis of *Entamoeba histolytica* (pathogenic) and *Entamoeba dispar* (nonpathogenic), which are two morphologically identical species of amebae, is essential both for treatment decision and public health knowledge. The study reported here was designed to choose a reference differentiation technique. Stool samples (*n* = 95) were tested by microscopy, TechLab enzyme-linked immunosorbent assays (ELISAs), and an in-house PCR. The target for the PCR amplification was a small region (135 bp) of the SSU rRNA selected to increase the sensitivity of the test. Sixty-eight specimens tested positive by PCR: 2 for *E. histolytica* and 66 for *E. dispar*. For detection of *E. dispar*, ELISA performance was lower than that of microscopy in this reference context, while PCR was much more sensitive than microscopy. Given the low proportion of *E. histolytica* cases, test performance for this species is difficult to assess. However, for differentiation, PCR performed well on simulated samples, while ELISA gave a discordant result for one of the two samples PCR positive for *E. histolytica* during the study. This report also confirms that *E. dispar* infection is significantly higher among travelers and underlines the possibility of acquiring *E. histolytica* infection in regions that are not areas of endemicity. Because of its lower sensitivity, the interest of ELISA for *Entamoeba* detection and differentiation in stools seems questionable in nontropical regions. On the other hand, results suggest that PCR should be useful as a reference test for sensitive differentiation of both species and to contribute to physicians' decision in treatment of *E. histolytica*- or *E. dispar*-infected patients.

Amebiasis is an important parasitic disease in humans (18). *Entamoeba histolytica* and *Entamoeba dispar* parasitize approximately 10% of the world population, of which 90% are asymptomatic infections. It is estimated, however, that amebiasis causes up to 110,000 deaths a year (15). While the infectious agent was discovered in 1875 by Fedor A. Lösch and the distinction between *E. dispar* and *E. histolytica* was first suspected in 1925 (3), the evidence for the dichotomy in two different species, pathogenic (*E. histolytica*) and nonpathogenic (*E. dispar*), is relatively recent (5). However, *E. dispar* and *E. histolytica* are morphologically indistinguishable from one another. Isoenzyme analysis is considered the "gold standard" for differentiating *E. histolytica* and *E. dispar*, but this method is not currently available and not readily usable for routine diagnosis (16). More recently, several studies have been devoted to the development of new techniques either based on monoclonal antibodies (8, 9, 10, 20, 21) or molecular biology methods (1, 2, 4, 6, 19, 22, 23) to successfully distinguish the two species in human feces. Reliable distinction would have a medical impact as until now, both infections are usually treated, whereas only approximately 10% (pathogenic infections) need to be treated. This proportion drops to much lower levels in developed countries, where *E. histolytica* infection is not endemic and occurs mostly after traveling to areas of endemicity. Technically, however, differentiation is still a challenge. Microscopic examination of intestinal parasites is usually performed on fixed stools. Numerous groups have tried with variable success molecular methods on this type of sample, and recently, the effect of formalin fixation on PCR was further investigated (17). It was shown that even if its effect on DNA is indirect, concentrations of formalin higher than 1% seemed to inhibit PCR amplification from 4 days of fixation. This is corroborated by the work of Troll et al. (23), who showed that sensitivity of PCR usually decreased within 2 days in feces stored in sodium acetate-acetic acid-formalin (SAF) fixative. Both teams concluded that the effects of formalin are time dependent. In the context of a reference center, it is difficult to be sure of swiftly obtaining all specimens. Moreover, fixed stools are not suitable for enzyme-linked immunosorbent assay (ELISA). This study was thus undertaken with frozen unfixed samples. To circumvent the potentially inhibiting nature of stool samples on PCR, an efficient technique amplifying the multicopy small-subunit (SSU) rRNA gene and including an internal amplification control was developed. The purpose of this study was to establish the comparative performances of the TechLab (Blacksburg, Va.) ELISAs and an in-house PCR for detection and differentiation of *E. dispar* and *E. histolytica*. It also allowed us to estimate the relative proportions of each of these species in our area, which is not an area of endemicity.

MATERIALS AND METHODS

Stool samples. Seventy-nine stool samples originating from 69 patients for whom an *Entamoeba histolytica-E. dispar* diagnosis had been established from May 1999 to May 2001 on the basis of a previous SAF-fixed sample using conventional techniques (formalin-ethyl acetate concentration and iron hematoxylin staining) were tested at the Laboratoire de santé publique du Québec (LSPQ), the reference center for stool parasitology in the province of Quebec,

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Canada. Stools samples were tested for differential diagnosis of digestive tract symptoms of unknown etiology. No patient was treated prior to specimen collection. The mean delay between testing at the hospital and referral was 3 weeks. Sixteen additional samples, from 15 patients, containing other species of *Entamoeba* ($n = 4$), coming from contacts of infected patients ($n = 4$), or sent for routine screening ($n = 8$) were also included to rule out *E. histolytica* and/or *E. dispar*. Fecal samples for ELISA and PCR were quickly frozen, shipped on dry ice, and kept at −20°C prior to analysis. Most stools were formed. A portion of each stool specimen was fixed in SAF for microscopy and shipped at room temperature.

Microscopic examination and ELISA. Microscopic examination was carried out on sediments obtained after stool concentration and on iron hematoxylin-stained smears. ELISA tests were run according to the instructions of the manufacturer (TechLab), using the *Entamoeba* Test and *E. histolytica* II Test kits. The *E. histolytica* II kit is a second-generation test that uses an improved capture antibody (12). Briefly, specimens were first tested using the *Entamoeba* Test for *E. histolytica*-*E. dispar* complex detection. Positive samples were subsequently tested using the *E. histolytica* II kit, which specifically detects *E. histolytica*. Specimens negative by the latter test were interpreted as positive for *E. dispar*, according to the manufacturer's instructions.

DNA extraction and PCR. All extraction and amplification steps were performed in duplicate. DNA was extracted using the QIAamp DNA mini kit (catalog no. 51304; Qiagen Inc., Mississauga, Ontario, Canada) according to manufacturer's recommendations using approximately 10 mg of partly thawed stools for the first ATL buffer step, without concentration of the starting material. DNA was frozen at −20°C till analyzed.

PCR amplifications were carried out using a 50 μM concentration of each deoxynucleoside triphosphate, 2 mM $MgCl_2$, 20 pmol of primers, 1× HotStar *Taq* 10× buffer, and 5 U of HotStar *Taq* DNA polymerase (Qiagen Inc.). The target for PCR amplification was the SSU rRNA (4). Forward primers ED1 (5′-TACAAAGTGGCCAATTTATGTAAGTA-3′) and EH1 (5′-GTACAAAATGGCCAATTCATTCAATG-3′) were used for *E. dispar* and *E. histolytica* detection, respectively, with the unique reverse primer EHD2 (5′-ACTACCAACTGATTGATAGATCAG-3′). Forward primers were derived from those of Clark and Diamond (4), while the reverse primer was designed to yield a small 135-bp amplicon. Cycling conditions were as follows: 15-min incubation at 94°C followed by 40 cycles consisting of 30 s at 94°C, 60 s at 51°C, and 40 s at 72°C, with a final 5-min elongation at 72°C. PCR performance evaluation was carried out on 50 simulated samples, each at three dilutions of *E. dispar* and *E. histolytica* SSU RNA plasmid (from C. G. Clark). In case of discrepancy between ELISA and PCR, tests were repeated at least twice.

IC preparation. To assess the presence of inhibitors in extracted fecal DNA, a competitive internal control (IC) was prepared by amplifying a 190-bp fragment of pBR322 with the EHDICF forward (5′-GTACAAAATGGCCAATTCATTCAATGTACAAAGTGGCCAATTTATGTAAGTACCTTGTCTGCCTCCCCGC-3′) and the EHDICR reverse (5′-ACTACCAACTGATTGATAGATCAGTGCTGGAGATGGCGGACG-3′) primers. Amplification of this IC yielded 240- and 266-bp products with the ED1-EHD2 and EH1-EHD2 primer pairs, respectively. To establish the quantity of IC to be added to each fecal DNA, 1:10 serial dilutions of EHDICF-EHDICR product were amplified with ED1-EHD2 and EH1-EHD2 primer pairs in the presence of several positive and negative fecal DNA specimens. To determine the IC working dilution, the lowest dilution consistently detectable was then determined on an ethidium bromide-stained 3% agarose gel. For each test, positive (feces spiked with SSU rDNA plasmid from C. G. Clark [4]) and negative controls were run.

Statistics. To determine the significance of the difference of proportions of *E. dispar*-infected persons having traveled or not to tropical or subtropical regions, an exact binomial test was performed. To quantify agreement between assays (13), PCR was used as a reference. Concordance was determined by McNemar's chi-square test with Yates' continuity correction on contingency tables for comparisons of PCR and ELISA (*E. dispar* and *E. histolytica*) and for comparison of microscopy and PCR results. Agreements between various tests were quantified on the same tables using Cohen kappa indices (κ).

RESULTS

Samples tested were fecal specimens from patients already diagnosed positive for *E. histolytica* or *E. dispar* on the basis of a previous SAF-fixed stool and were referred to the LSPQ. Furthermore, several specimens negative or positive for other species of *Entamoeba* were analyzed to rule out the presence of *E. histolytica* and *E. dispar*. They were also used as negative controls. Among the 95 samples received at LSPQ from 84 patients, 68 specimens from 61 patients were found positive for *E. histolytica* or *E. dispar* by PCR. Of these 61 patients, 34 were symptomatic and 12 were not, and for the remaining 15 no information was available. The main symptoms reported were diarrhea and abdominal pain. Thirteen symptomatic patients were also infected with other parasites such as *Ascaris lumbricoides*, *Dientamoeba fragilis*, *Giardia lamblia*, hookworms, *Schistosoma mansoni*, *Strongyloides stercoralis*, and *Trichuris trichiura*. Among the 48 patients for whom travel history was known, 35 (73%) had traveled recently to or were coming from areas of endemicity such as Mexico, Central or South America, the Caribbean Islands, Asia, or Africa. This proportion is significantly different from what would have happened by chance ($P < 0.0016$ [binomial test]).

Two patients were diagnosed as infected with *E. histolytica*. The first patient was a 4-year-old child who had never traveled. The only known potentially infected contact was an educator, working in the day care center frequented by the child, who had traveled to Africa 10 months before the onset of the child's symptoms. These symptoms were chronic diarrhea (3 weeks), sparse blood in stools, fever (1 day), abdominal cramps, and nausea. The educator was not symptomatic when the child became ill, and her stools were negative for *E. histolytica* and *E. dispar* negative at the time of the diagnosis in the child. The second patient was a 26-year-old man who had stayed for 16 months in Asia, whose symptoms were frequent diarrhea and abdominal pain (onset 2 months after return).

Results of ELISA, PCR, and microscopy are depicted in Tables 1 and 2. Fifty-four samples containing *E. dispar*, 1 sample containing *E. histolytica*, and 40 samples negative for both species were identified by ELISA. Among the 40 negatives, 9

TABLE 1. Comparison of PCR and ELISA results

ELISA result	No. of isolates with PCR result			Total no. of isolates subjected to PCR
	E. histolytica	*E. dispar*	Negative	
E. histolytica	1	0	0	1
E. dispar	1	53	0	54
Negative	0	13	27	40
Total	2	66	27	95

TABLE 2. Comparison of microscopy, PCR, and ELISA results

Result of assay			No. of specimens
ELISA	PCR	Microscopy	
+	+	+	51
+	+	+[a]	3
−	+	+[b]	9
−	+	−	4
+[c]	+	−	1
−	−	−	27

[a] Presence of organisms compatible with *E. histolytica* or *E. dispar* in frozen stools.
[b] Rare or few organisms.
[c] The *E. histolytica* PCR-*E. dispar* ELISA discrepant result.

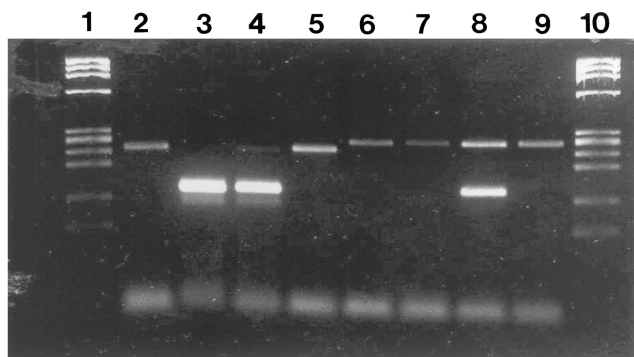

FIG. 1. Sample amplification of stools containing or not containing *E. histolytica* and *E. dispar* with IC. Lanes 1 and 10, marker (ΦX174 RF/*Hae*III fragments [Gibco BRL]: 1,353, 1,078, 872, 603, 310, 281/271, 234, 194, 118, and 72 bp); lanes 2 to 5, *E. dispar* amplification; lanes 2 and 5, negative samples; lanes 3 and 4, positive samples (lane 3, strong positive, no IC amplification); lanes 6 to 9, *E. histolytica* amplification; lanes 6, 7, and 9, negative samples; lane 8, positive sample.

samples displayed rare or few *E. histolytica* or *E. dispar* organisms at microscopic examination (results which were confirmed by PCR). ELISA was thus less sensitive than microscopy or PCR.

A typical PCR amplification is shown in Fig. 1. The PCR target is 135 bp, while the *E. dispar* IC is 240 bp and the *E. histolytica* IC is 266 bp. Results from simulated samples showed that *E. histolytica* and *E. dispar* PCRs performed equally well (not shown). The competitive IC was positive in all samples except for some specimens harboring large quantities of *E. histolytica* or *E. dispar* organisms and for which PCR amplification was strong. Even in negative samples, the IC amplification was minimal due to the deliberate choice of using it at a low copy number. With this technique, 2 samples were determined to contain *E. histolytica*, 66 samples were determined to contain *E. dispar*, and 27 samples were determined to be negative for both organisms. Except for five samples, these results were corroborated by microscopy.

Comparison of both techniques shows that *E. histolytica* was detected in two samples by PCR, of which one was determined by ELISA to be *E. dispar*. This discrepancy was not statistically significant, and agreement was substantial ($\kappa = 0.66$). The specificity of ELISA compared to PCR was 1 for *E. dispar* and 0.98 for *E. histolytica*. Each *E. histolytica*-positive specimen has been tested in duplicate by ELISA and PCR with identical results. No mixed infection was detected by PCR. Among the 66 samples positive for *E. dispar* by PCR, only 53 were detected by ELISA. As expected, the difference between ELISA and PCR results for *E. dispar* was significant ($P < 0.0009$). Agreement was nevertheless substantial ($\kappa = 0.70$).

Among the 68 samples positive for *E. histolytica* or *E. dispar* by PCR, 60 were positive by microscopy. It is noteworthy that microscopy did not give any positive results that could not be corroborated by other techniques. Compared to PCR, the TechLab ELISA *E. histolytica*-*E. dispar* antigen detection in feces was less sensitive than microscopy (0.80 versus 0.92), while microscopy proved to be more in agreement ($\kappa = 0.88$ versus 0.70) with PCR than ELISA. However, it must be kept in mind that no differentiation is possible using microscopy unless ingested erythrocytes are present in trophozoites. The negative predictive value of microscopy was also better than that of ELISA (0.84 versus 0.68), and difference with respect to PCR was not significant as assessed by the McNemar chi-square test with Yates' correction ($P < 0.074$).

From the 27 specimens found negative by all techniques, nearly half (11 samples) were collected from patients for whom an *E. histolytica*-*E. dispar* diagnosis had been established on the basis of fixed specimens during the previous weeks. This illustrates one of the problems associated with the necessity of later collecting further samples in order to get unfixed samples for testing, as it is well known that excretion of parasites may be intermittent. As a matter of fact, during our study, we could have missed an interesting case of *E. histolytica* due to intermittent parasite excretion: a patient returning from India presented with a hepatic abscess. Serology for amebiasis was strongly positive, as were the patient's fixed stools collected 1 week before. The remaining negative specimens ($n = 16$) were collected from patients whose infection was not previously proven. In the context of this study (differential diagnose cases of digestive tract symptoms of suspected parasitic origin), no patient was treated prior to specimen collection.

Some samples gave problematic results by ELISA. Two samples diagnosed as *E. dispar* by PCR were initially found to be weakly reactive for *E. histolytica* by ELISA and then were repeatedly found to be *E. dispar* positive on retesting. One sample that tested positive for *E. dispar* by PCR was initially found to be weakly reactive for *E. histolytica* and then was repeatedly found to be negative by ELISA. Another sample negative by PCR was initially ELISA weakly reactive for *E. histolytica* and then was found negative upon retesting. According to the manufacturer of the ELISA and as stated in the ELISA kit *Manual of Instructions*, "some specimens may give weak reactions that are inconclusive. This may be due to a number of factors such as the presence of binding substances or inactivating enzymes in the feces. Under these conditions, the specimen should be retested or a fresh specimen should be tested." This should be kept in mind when doubtful results are obtained and to improve the reliability of these tests for differentiation.

DISCUSSION

Microscopic diagnosis of *E. histolytica*-*E. dispar* complex on stool samples requires technical expertise because of the existence of similar amebae or artifacts that can be misdiagnosed as *E. histolytica*-*E. dispar*. Moreover, *E. histolytica* and *E. dispar* cannot be differentiated by microscopy. More efficient techniques that allow differentiation must be developed in order to avoid unnecessary treatment when *E. dispar* is present, as recommended by the World Health Organization/Pan American Health Organization/UNESCO Expert Consultation on Amoebiasis held in 1997 (24). Till now, however, most physicians have prescribed systematic treatment upon microscopic examination of fixed material at the hospital. The purpose of this work was to develop a reference technique and to estimate the respective proportions of *E. dispar* or *E. histolytica* infections in referred samples from people living in Quebec. The ability to differentiate *E. dispar* infections from *E. histolytica*

infections should lower the number of treatments for *E. dispar*-infected patients. The TechLab ELISA technique (11, 14) was tested and compared to an in-house PCR assay. PCR was designed to target the same region as that targeted by Clark and Diamond (4) for differentiation (forward primer), but with a unique reverse primer nearer to the forward primer to yield a smaller amplicon (135 bp). This technique proved in our hands to be much more sensitive. An amplification using different forward primers but the same reverse primer led to reliable differential identification of both organisms. This was demonstrated with simulated samples spiked with plasmid SSU rDNA and with clinical samples. Indeed, since May 2001, five additional cases of *E. histolytica* infection were determined by PCR, bringing the total to seven. On the whole, PCR was more sensitive than ELISA and microscopy and was more specific than ELISA.

Specificity of PCR could not be assayed by means of a gold standard. However, PCR amplification was always clean, yielding no other product than the expected one. False-positive results can occur in samples like stools that contain DNA from various sources. However, with adequate primers, false-positive results can be expected to occur at a very low rate (7). False-negative results due to residual inhibition can be ruled out thanks to the IC. This IC has been highly diluted so that it does not compete significantly with amebic DNA. It also participated in quality assurance, being of different sizes for *E. histolytica* and *E. dispar*.

Overall, the concordance of PCR and ELISA was 85%. While the positive predictive value of ELISA was good, the negative predictive value was only 0.68. In our hands, ELISA was less sensitive than microscopy. Indeed, among the 13 ELISA-negative, PCR-positive samples, 9 were positive by microscopy. This differs from the results obtained by Haque et al. (10) in Bangladesh, who found the *Entamoeba* ELISA more sensitive and specific than microscopy compared to culture as the gold standard. However, stools collected in their study came from patients with diarrhea, while here, many of the stools were formed. The latter are more difficult to mix thoroughly than liquid or semiformed stools to ensure adequate sampling. This could be a problem in weakly positive specimens, as organisms might be unevenly distributed in stool. On the other hand, accurate identification of amebae depends on the skill of the microscopist. Results of microscopic examination therefore can vary between studies according to staff experience as well as techniques used for diagnosis.

ELISA might also lack specificity for *E. histolytica*. However, in the case of the sample determined to be *E. histolytica* by PCR and *E. dispar* by ELISA (sample from the patient returning from Asia), no organisms were seen by microscopy, and much macroscopic debris was present in the stools. The problem could be due to a false-negative reaction with the second ELISA test because of the rarity of organisms. However, it could rather be due to a false-positive reaction to the first ELISA test (because of debris or other interfering substances). It should be noted that this sample was the only one that gave a positive ELISA result while giving a negative result by microscopy. This result underlines the lack of a specific ELISA *E. dispar* antibody. Indeed, *E. dispar* diagnosis relies only on the absence of an *E. histolytica* reaction upon the second test.

As expected in our setting, which is not in an area of endemicity, most of the samples were positive for *E. dispar*. These results are similar to those of Pillai and al. (16), in a study also done in a Canadian setting (three cases of *E. histolytica* infection in 73 patients testing positive). Since the end of this study, five new cases of *E. histolytica* have been diagnosed by PCR among 66 cases of *E. dispar*, bringing the proportion of *E. histolytica* to 7%. All the *E. histolytica*-positive patients traveled abroad. One of them had already been treated for a hepatic abscess a few months previously, and another had a positive intestinal biopsy for *E. histolytica*.

The information that could be gathered about patients was not exhaustive but showed, as expected, that most *E. histolytica-E. dispar*-infected persons had traveled to areas of endemicity. Most of them were symptomatic, but some were also infested by other parasites or had a differential diagnosis of chronic bowel disease. Among the two *E. histolytica* cases in this 2-year study, one is intriguing. Indeed, a 4-year-old Canadian who never traveled seems not at all at risk for infection with *E. histolytica*. However, symptoms were in agreement with the laboratory results, and a diagnosis of *E. histolytica* was established. This shows that under special circumstances, the risks of contamination are not exclusively abroad and indicates that even in areas of nonendemicity, transmission of *E. histolytica* can occur from carriers.

This study showed that TechLab ELISAs for differential diagnosis of *E. histolytica-E. dispar* complex lack sensitivity. On the other hand, PCR analysis of frozen stool samples provides reliable results. Moreover, PCR selectively amplifies the different rRNA genes of the two types of amebae, contrary to the TechLab *E. histolytica* II Test, which contains antibodies specific for *E. histolytica* only. This is in accordance with reports by other authors (14, 23), who concluded that PCR is a preferable tool for differentiation. While PCR with fixed samples was attempted with inconsistent results, it would be more convenient to perform PCR with refrigerated stools. Preliminary tests indicate that, in our hands, sensitivity was stable for 3 to 4 days in samples kept at 4°C and then decreased quite rapidly.

On the basis of these results, the in-house PCR with frozen samples was adopted as the reference for differential diagnosis of *E. histolytica* and *E. dispar* in our laboratory, in adjunct to microscopy.

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