Original
ISSN 2521-8492

Detection of *Entamoeba* Spp. in Jordanian Clinical Samples by Traditional and Molecular Methods

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(Submitted: 19 July 2024 – Revised version received: 14 August 2024 – Accepted: 29 September 2024 – Published online: 26 December 2024)

Abstract

Objective: To evaluate the efficacy of polymerase chain reaction (PCR), enzyme-linked immune-sorbent assay (ELISA), and immunochromatography-based methods compared to conventional microscopy for detecting *Entamoeba histolytica* in diarrheal stool samples, and to identify a reliable diagnostic tool for amoebiasis.

Methods: A total of 68 fresh diarrheal stool samples were collected from three hospitals in Amman. Samples were tested using microscopy, TechLab *E. histolytica* II ELISA, and nested-PCR. Additionally, Operon Simple immunochromatography test was performed on 29 randomly selected samples. Detection rates of the methods were compared, and the concordance between techniques was analyzed.

Results: Microscopy detected *E. histolytica* cysts and/or trophozoites in 76.5% (52/68) of the samples, while ELISA and PCR detected the parasite in 10.3% (7/68) and 29.4% (20/68), respectively. Among the 29 samples tested by immunochromatography, 79.3% (23/29) were positive by microscopy, and 48.3% (14/29) were positive for *E. histolytica* antigen. Discrepancies were noted, as 3 PCR-positive samples were negative by both ELISA and microscopy, and 7 of the microscopically negative samples were PCR-positive. Immunochromatography detected antigens in 2 of the 16 microscopically negative samples.

Conclusion: None of the methods was found superior to microscopy, though microscopy's limitations, such as its inability to distinguish pathogenic from non-pathogenic Entamoeba species, compromise its reliability. Antigen- and DNA-based methods demonstrated higher specificity, with immunochromatography outperforming ELISA and PCR in simplicity and usability. Further studies with larger sample sizes and a standardized reference method are needed to establish the most effective diagnostic approach for *E. histolytica*.

Keywords: Amoebiasis, diarrhea, *Entamoeba*, antigen detection, PCR, Jordan

Introduction

Entamoeba histolytica, an intestinal protozoan parasite that causes amoebiasis was first discovered by Fedor A. Lösch in 1875.[1–5] The first difference between the pathogenic *E. histolytica* and the non-pathogenic *E. dispar* was suspected in 1925.[5] Practically, microscopic detection of *E. histolytica* parasite in the stool sample could be difficult and technical expertise is needed since artifacts and cells similar to amoebae could be misdiagnosed as *E. dispar-E. histolytica* complex.[5–8] Nevertheless, so far, the diagnosis of amoebic infection depends mainly on the microscopic identification of the cyst morphology.[9,10]

In addition to the intestinal problems, *E. histolytica* can result in serious complications, which makes proper diagnosis of amoebiasis more essential. The parasite can disseminate to extraintestinal sites such as liver causing hepatic amoebiasis, lungs resulting in pulmonary amoebiasis, and brain leading to amoebic encephalitis.[11–13]

Various fecal antigen enzyme linked-immunosorbent assays (ELISAs) and polymerase chain reaction (PCR)-based assays have been used worldwide for the identification of *Entamoeba* species and the detection of *E. histolytica* infections[9,13] However, diagnosis and epidemiology of amoebiasis have become more complicated with the appearance of *Entamoeba* complex including pathogenic *E. histolytica* with non-pathogenic *E. dispar* and *E. moshkovskii*.[7,13,14]

The use of molecular approaches for detection of *E. histolytica* has re-evaluated the epidemiology in considering morbidity and prevalence of amoebiasis, especially in regions with high endemic rates.[15–17]

Search for new methods able to differentiate the morphologically similar *Entamoeba* species is crucial for understanding the epidemiological causes and clinical management of patients with amoebiasis, and to avoid unnecessary treatment.[13,18–22] Therefore, molecular approaches based on DNA and antigen detection may be necessary for confirmation of amoebiasis.[22–24] Also, antigen detection methods were set up to differentiate between pathogenic and non-pathogenic species of *Entamoeba* (TechLab, *E. histolytica* II). However, investigation of different diagnostic methods showed a degree of controversy in results. For example, Khairnar and Parija found that the *E. histolytica* antigen detection with ELISA assay was more sensitive than the standard diagnostic methods (microscopy and/or culture) but comparable to PCR[25] others found PCR more sensitive than the antigen detection method, since the latter method failed to detect *Entamoeba* in PCR positive stool samples.[26] Some studies found PCR accurate in detection of strains, more sensitive, and more specific than microscopy and culture techniques.[27–30]

In contrast, Ngui et al. found PCR unable to detect *Entamoeba* in 33% of stool samples that were positive for *Entamoeba* cyst using microscopy.[31]

The contradiction on determining which method is more efficient in detection of *E. histolytica* in clinical samples made the decision in this study to evaluate the efficacy of antigen- and DNA-based methods in detection of *E. histolytica* in Jordanian clinical samples from diarrheal patients who were microscopically diagnosed of having amoebiasis.

Materials and Methods

Collection of Sample

A total of 68 fresh human stool samples were collected from Jordanian patients presenting with diarrhea at three hospitals in Amman, namely, the Jordan University Hospital, Al-Bashir Hospital, and Al-Islami Hospital during the period from May 2017 to August 2018.

Sixty-eight fresh, non-frozen stool samples collected from diarrheal patients attending three hospitals in Amman were examined within a few hours after passage by microscopy and ELISA at the parasitology research lab at the University of Jordan. On the same day, DNA was extracted from the samples, which were then stored at −20C° until use.

Microscopic Examination of Fresh Stool Samples

The fresh stool samples were examined by both saline and iodine wet mounts as described by Parija and Prabhakar.[32] Briefly, smears of stool samples were prepared by adding a small volume of the fresh stool to a drop of saline and another drop of Lugol's solution, which were separately mixed with a wooden stick until a homogenized and transparent emulsion was achieved. The stool suspension was then covered with a transparent glass cover slip. The slides were examined by light microscopy with 40X and oil immersion objectives for the existence of *Entamoeba* spp. cysts and trophozoites. Approximately each slide was examined for about 10–20 min for more accurate detection and to make a difference with the hospital microscopy, which usually is performed for a short time.

Simple Entamoeba Test

The test is a chromatographic immunoassay for *in vitro* detection of *E. histolytica* antigens in human stool samples. It was performed according to the manufacturer's instructions (Operon, Spain).

ELISA Assay

In this study, TechLab *E. histolytica* II test was used to detect *E. histolytica* antigens in stool samples according to the manufacturer instructions (TechLab, USA). The optical densities were measured at 450 nm on Sunrise microplate reader (Tecan, Switzerland). The test sample was considered positive when the absorbance was higher than 0.050, and they were considered as weakly positive when the absorbance was between 0.009 and 0.050. Samples with absorbance less than 0.009 were considered negative for *E. histolytica*.

DNA extraction

DNA was extracted from human stool samples using QIAamp DNA stool mini kit (Qiagen, Germany) according to the manufacturer's instructions. The DNA samples were stored at −20°C until PCR was performed.

Nested-PCR for Detection of E. histolytica

The nested-PCR constituted of outer and inner amplification of PCR. The outer amplification was performed with genus *Entamoeba*-specific primers, whereas the inner amplification involved the species-specific primers for the species *E. histolytica* (Table 1).[25]

The PCR reaction mixture (25 µl) of the outer and inner amplification of the nested PCR was prepared using 0.5 µl of extracted DNA, 0.3 µM of each primer and Platinum high fidelity master mix containing Taq DNA polymerase 22 U/ml, Tris-SO4 66 mM (pH 8.9), 19.8 mM [NH4]2SO4, 2.4 mM MgSO4 and 220 µM dNTPs was added to adjust the total volume of the reaction to 25 µl. Thermal cycling conditions recommended by Platinum Hi-Fi master mix were used for the outer and inner amplification. DNA was denatured at 94°C for 5 min, and the target sequence was amplified for 30 cycles in the outer amplification and 60 cycles in the inner amplification of nested-PCR. Each cycle included denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 68°C for 30 sec. The last cycle of the outer (genus-specific) and inner (species-specific) amplification of nested PCR was followed by a final extension at 72°C for 7 min. The PCR products were stained with ethidium bromide after electrophoresis on a 2% agarose gel. Positive and negative controls were used for each batch of samples analyzed. The size of the PCR product was estimated by loading of 100 bp DNA ladder in the same gel. The gel bands were visualized using the gel documentation system (UVP, USA).

Results

Microscopy of Fresh Diarrheal Stool Samples

The fresh diarrheal stool samples collected from the three hospitals were microscopically examined directly after arrival. Out of the 68 collected stool samples, 52 (76.5%) (95% confidence interval (CI), 0.6494–0.8579) contained trophozoites and/or cysts of *Entamoeba*. Of the 52 positive samples, only cysts were seen in 9 (17.3%) and only trophozoites were observed in 5 (9.6%). Cysts and trophozoites (Figure 1) coexisted in 38 (73%). Neither trophozoites nor cysts were seen in 16 (23.5%) of the 68 samples.

Simple Entamoeba Test

Out of the 68 collected fresh diarrheal stool samples, 29 samples were randomly selected and tested with the Simple *Entamoeba* test, of which 14 (48.3%) (95% CI, 0.2893–0.6762) were positive for *E. histolytica* antigens.

ELISA

The 68 fresh diarrheal stool samples were tested using the TechLab *E. histolytica* II ELISA kit. In this test, only 7 (10.3%) (95% CI, 0.0188–0.1551) of the fresh diarrheal stool were positive or weakly positive for *E. histolytica* antigens. Figure 2 shows representative results for the test with the yellow color indicating positive results.

Nested-PCR Targeting E. histolytica

The nested PCR amplification, inner (species-specific) detection fragment of 439 bp was obtained for *E. histolytica* as

Table 1. **Primer sequences used in the Nested-PCR**

Genus *Entamoeba*-specific primers	E-1 5'TAAGATGCACGAGAGCGAAA 3' (forward primer) E-2 5'GTACAAAGGGCAGGGACGTA 3' (reverse primer)
E. *histolytica* species-specific primers	EH-1 5' AAGCATTGTTTCTAGATCTGAG 3' (forward primer) EH-2 5' AAGAGGTCTAACCGAAATTAG 3' (reverse primer)

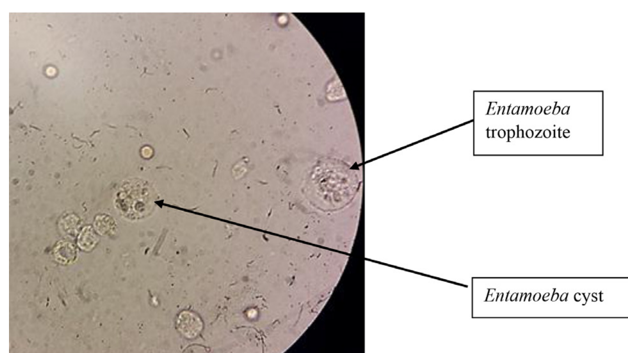

Fig. 1 **Unstained *Entamoeba* trophozoite and cyst examined by the light compound microscopy, 1000X.**

Fig. 2 **Representative ELISA microwells. PC: (positive control); Microwell 1, 2, 3, and 5: (negative samples); Microwell 4: (positive sample).**

shown in Figure 3. Out of 68 clinical diarrheal stool samples. The overall percentage of *E. histolytica*-PCR positive samples were 20 (29.4%) (95% CI, 0.1801–0.3996).

Comparison of the Results of the Microscopy, Antigen-based Methods, and Nested-PCR

Table 2 demonstrates a detailed comparison between microscopy, antigen-based methods (Operon simple *Entamoeba* test, and TechLab ELISA), and nested-PCR. The comparison shows some contradiction in results of the 4 techniques for many of the samples. For example, 39 samples that were positive by microscopy were negative in nested-PCR. On the other hand, 7 samples that were microscopically negative were found to contain *E. histolytica* DNA as detected by nested-PCR. All the 7 sample that where positive by TechLab ELISA were also positive microscopically but only 4 of them were nested-PCR positive. Interestingly, three samples that were negative by microscopically and TechLab ELISA were, on the other hand, positive by nested-PCR (Table 2). Of the 20 nested PCR-positive samples, only 8 were randomly tested by the Operon Simple *Entamoeba* test, of these, 5 were also positive by the immunochromatographic test. On the other hand, of the 48 PCR-negative stool samples 21 were randomly tested by the Operon Simple *Entamoeba* test and nine were found positive (Table 2).

Discussion

The study aimed to investigate the usefulness of PCR and TechLab *E. histolytica* II ELISA kit in the diagnosis of *E. histolytica* infection. To assess this goal, fresh stool samples that were considered positive (58 samples) for *E. histolytica* using microscopy or those from patients having symptoms of amoebiasis (10 samples) were collected. These 68 stool samples were examined by microscopy, the TechLab *E. histolytica* II ELISA kit, and nested-PCR. The focus in this study was to examine fresh stool to exclude factors related to frozen or old stool samples that may interfere with the results of the tests under investigation. For example, freezing and thawing of stool samples influence the results of the TechLab *E. histolytica* II ELISA as reported in the manufacturer instructions. Moreover, freezing and thawing of stool sample cause DNA degradation.[33]

The TechLab *E. histolytica* II ELISA antigen-based kit could detect *Entamoeba* antigen in only 10.3% (7/68) of the stool samples. The high percentage of negative results for microscopically positive samples obtained by this test may be

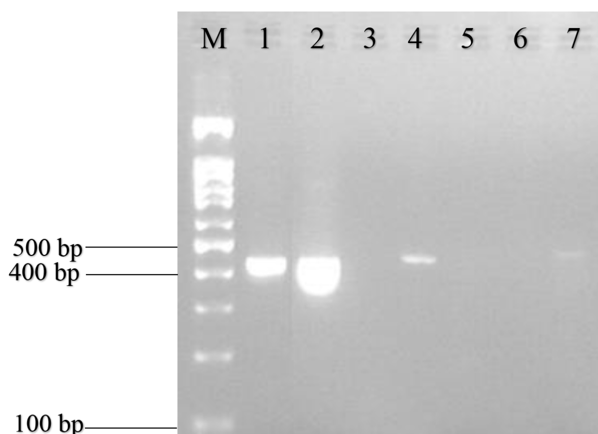

Fig. 3 **A representative ethidium bromide agarose gel of the *E. histolytica* nested-PCR product (439 bp). M: 100 bp DNA marker; Lane 1: positive control; Lane: 2, 4 and 7 positive fresh diarrheal samples; Lane 3 and 5: negative fresh diarrheal samples; Lane 6: blank.**

Table 2. **Combined results of microscopy, antigen-based methods, and nested-PCR for detection of *E. histolytica* in fresh diarrheal stool samples**

Parasitology research lab microscopy	Operon simple *Entamoeba* test	TechLab ELISA	Nested-PCR	No. of sample(s)
+	+	+	+	2
+	+	–	–	8
+	–	–	+	2
–	–	–	+	1
+	–	+	–	1
+	–	–	–	8
–	–	–	–	3
+	N/P	–	–	20
+	N/P	–	+	6
–	N/P	–	–	6
–	N/P	–	+	1
–	N/P	–	+	3
+	N/P	+	+	2
+	N/P	+	–	1
+	+	+	–	1
+	+	–	+	1
–	+	–	+	2

+: positive; –: negative; N/P: not performed.

attributed to several factors. Low concentrations of *E. histolytica* antigen, below the detection threshold of the kit, may exist in many of the stool samples since a high level of *E. histolytica* antigen is required for a positive reaction.[34] Additionally, polymorphism in *E. histolytica* lectin antigen used in TechLab *E. histolytica* II ELISA kit may also contribute to poor ELISA performance.[35] Another factor is that the TechLab *E. histolytica* II ELISA kit specifically detects *E. histolytica* antigens of the trophozoite stage of the parasite only during acute amoebiasis.[36] Therefore, ELISA kit cannot detect *E. histolytica* in stool samples containing only cysts of the parasite. This, however, does not explain the low detection capacity obtained here, since the samples tested in this study are diarrheal stool reflecting potentially acute amoebiasis. In similar studies low sensitivity of TechLab *E. histolytica* II ELISA kit was also reported. This kit did not give any positive results for 456 stool samples that were microscopically positive for *Entamoeba* complex.[37] In a study performed in the United Arab Emirates by El-Bakri, et al.,[26] TechLab *E. histolytica* II ELISA kit failed to detect any of the PCR positive samples. However, Tüzemen and Doğan[38] detected *E. histolytica* antigen in 13 (15.5%) out of 84 microscopically positive stool samples for *Entamoeba*.

The false-negativity of the TechLab ELISA kit was confirmed in this study since one sample was positive for *Entamoeba* by microscopy, and nested-PCR, but it was negative in TechLab ELISA and only 4 were positive by both the nested-PCR and the TechLab ELISA (Table 2).

In a study similar to the present work conducted by Furrows, et al.[39] that also utilized microscopy, ELISA, and PCR, out of 94 microscopically positive stool samples for *Entamoeba* cyst or trophozoite, 9 samples were positive by TechLab *E. histolytica* II ELISA. When the 9 samples were tested by PCR, a false-positive result was observed in one of the nine stool samples and a false-negative result in another sample of the total 94 stool samples. Moreover, in an Austrian study reported by Stark, et al.[40] TechLab *E. histolytica* II ELISA kit failed to detect any *E. histolytica* antigens in any of PCR positive samples. Additionally, false-positive results by TechLab ELSIA kit were detected in three out of 261 samples (1%) that were negative for *E. histolytica* using PCR. In contrast, other studies utilizing TechLab *E. histolytica* II ELISA kit showed relatively high capacity of this kit in detection of *Entamoeba* antigens. Khairnar and Parija[25] reported that 29 (64.6%) out of 45 microscopically positive stool samples for *Entamoeba*, were positive by TechLab *E. histolytica* II ELISA. These results were confirmed by the nested multiplex PCR. Similarly, Santos, et al.,[41] reported that 30 out of 64 microscopically positive stool samples for *Entamoeba* were positive by TechLab *E. histolytica* II ELISA and 28 of these were positive by PCR as well. The authors, here, stated that the two PCR-negative but TechLab ELISA-positive samples may be due to PCR inhibtiors. Haque et al.[42] reported that all of 16 (100%) culture positive stool samples were also positive by TechLab *E. histolytica* II ELISA.

The other antigen-based method, the Operon Simple *Entamoeba* test, on the other hand, was more sensitive than the TechLab *E. histolytica* II ELISA kit as 14 of 29 (48.2%) sample tested were positive. Out of the 15 samples that were negative in Operon Simple *Entamoeba* test 3 were, in contrast, positive in nested-PCR. To my knowledge, this is the first study that utilizes this kit in a piece of research. The kit is a relatively fast test which gives results within 15 min. The manufacture company of Operon *Entamoeba* tests claimed that the test has a sensitivity above 75%. Further investigation of the sensitivity and specificity of this kit is still needed with a larger number of samples that may reflect a firm conclusion regarding its use as a powerful diagnostic method for amoebiasis.

For epidemiological and clinical studies, PCR-based techniques are considered as the method of choice and have been strongly recommended by the WHO.[15,43–45]

Lin et al.,[46] stated that it is important to establish an accurate detection method for *E. histolytica*. Tanyuksel and Petri Jr[47] claimed that PCR potentially will become the gold standard by which other detection methods (microscopy, antibody detection, etc.) are compared.

López-López, et al.[48] advocated that PCR is useful in detection and differentiation between *E. histolytica* and *E. dispar* in stool samples and it could be a complementary or alternative diagnostic tool. El-Bakri, et al.[26] reported all detection and differentiation of *Entamoeba* species should be carried out by PCR. In the present work, however, *Entamoeba*-specific DNA was detected by nested-PCR in 20 (29%) out of 68 fresh stool samples. Of these, 7 (35%) were microscopically negative for *E. histolytica*, 16 (80%) were negative by TechLab *E. histolytica* II ELISA. Although the nested-PCR was able to detect *E. histolytica* DNA in microscopy-, ELISA-negative stool samples, the test, on the other hand, failed to detect *E. histolytica* DNA in 39 microscopy-, 3 ELISA-positive samples (Table 2).

Other studies utilizing nested-PCR showed a higher capacity of this test to detect *Entamoeba* DNA in stool sample that were microscopically positive. El-Bakri, et al.[26] reported that out of 36 microscopically positive samples 16 (44.4%) were also positive for *E. histolytica* using nested-PCR. Lau, et al.[49] reported that among 65 microscopically positive samples, 45 (69.2%) were confirmed positive for *E. histolytica* using nested-PCR. Similarly, Ngui, et al.[31] reported that out of 75 microscopically positive samples, 39 (52%) were also positive for *E. histolytica* using nested-PCR. It is also worthy to denote that the nested-PCR utilized in this study and by others showed a higher capacity in detection of *E. histolytica* than other PCR variabilities. For example, in a study conducted in Australia on microscopic positive stool samples, *E. histolytica* was detected in 18 out of 279 (6%) using multiplex PCR.[40]

Similarly, among 162 microscopically positive stool samples, *E. histolytica* DNA was detected in 10 (6.2%) samples using multiplex PCR (Lebbad and Svärd,[50] López-López, et al.[48] reported that out of 62 stool samples collected form patients suffering from gastrointestinal discomfort 10 (16.1%) were positive for *E. histolytica* utilizing PCR-denaturing gradient gel electrophoresis. These results were also confirmed by nested PCR-RFLP. In another study, among 34 symptomatic stool samples, *E. histolytica* was detected in only 2 (5.9%) samples by multiplex PCR.[5]

The relatively high percentage of negative results obtained by nested-PCR reported here and by others with microscopically positive samples raises questions about the use of microscopy as a gold standard by which the sensitivity of other methods is compared. In addition to the presence of artifacts and white blood cells that may interfere in the results of microscopy, the presence of morphologically similar *Entamoeba* species increases the probability of false positive results of microscopy. In this context, Santos, et al.[51] reported that out of 262 microscopically positive stool samples for *E. histolytica*/*E. dispar*, nested multiplex PCR amplification was successful in 227 (86.6%) of the samples and all were positive for *E. dispar*. However, the negativity obtained by nested-PCR can

be significant as trophozoites in stool samples can disintegrate with time which exposes DNA for nucleases.[52] Khairnar and Parija[25] reported that out of 202 positive stool samples for *Entamoeba* complex by microscopy and/or culture, nested multiplex PCR failed to detect 12 samples due to PCR inhibitors. The presence of low number of *E. histolytica* in some of the stool samples, which is lower than the detection limits of PCR may partially explain this high negativity. Khairnar and Parija[25] estimated the detection limit of nested multiplex PCR was approximately 25 *Entamoeba* protozoan cells.

PCR which is known to be a powerful technique to detect DNA in different types of parasitic infections,[53–56] was found to have problems in detecting *Entamoeba*-DNA in stool samples. This experience with stool samples seems to be shared with different researchers. Monteiro, et al.[57] stated that stool samples remain the most difficult samples for DNA extraction and amplification. PCR inhibitors present in the stool, such as complexed carbohydrates, bile salts, heme, and bilirubin negatively affect amplification.[31,58,59]

Conclusions

This piece of research implies that microscopic detection method appeared to be superior to the antigen-based and DNA-based techniques. However, microscopy lacks the advantage of specific detection of *Entamoeba* species. Additionally, TechLab *E. histolytica* II ELISA kit proved to be not useful for routine laboratory diagnosis of amoebiasis. On the other hand, Operon Simple *Entamoeba* test seems to be promising for the routine laboratory diagnosis of amoebiasis. The major difficulty in this study was unavailability of a reference technique by which the sensitivity of the traditional and molecular methods utilized can be measured.

Acknowledgments

We sincerely thank Professor Graham Clark from the London School of Hygiene & Tropical Medicine for his valuable cooperation and for providing an ethanol-fixed culture of *E. histolytica* cells. Thanks also to the lab technician of microbiology labs of Al-Bashir Hospital, the University of Jordan Hospital, and Al-Islami Hospital. Also, thanks to Mr. Ahmed Abdulkarim Alzbeede, the academic instructor in the Department of Medical Laboratory Science at Komar University of Science and Technology, for reviewing this manuscript's drafts and his valuable support. Many thanks to Mrs. Haneen Sarairah, medical technologist at King Hussein Cancer Foundation and Center for sharing her valuable experience and support.

Conflict of Interest

None.

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