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Research Article
Identification of *Entamoeba histolytica* in Patients with Suspected Amebiasis in Jordan Using PCR-based Assays

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Abstract

Background and Objective: Identification of *Entamoeba histolytica* (*E. histolytica*) by microscopy alone can be problematic because *E. dispar* and *E. moshkovskii* are morphologically similar to *E. histolytica*. Therefore, this study aimed to assess the performance of microscopy in the detection of *E. histolytica* in stool specimens with the help of PCR-based assays and enzyme-linked immunosorbent assay (ELISA). **Materials and Methods:** Between September, 2017 and September, 2018, 200 stool specimens were obtained from Jordanian patients with suspected amebiasis. All specimens were subjected to microscopic analysis. DNA was extracted from the microscopy-positive stool samples. A conventional PCR and a duplex real-time PCR were performed to detect *E. histolytica* and *E. dispar*. **Results:** By microscopy, 35% (70/200) of specimens were tested positive for *Entamoeba* complex. All 70 microscopic-positive *Entamoeba* complex samples were negative for the presence of *E. histolytica* by the NOVITEC® *E. histolytica* ELISA assay. All 70 samples positive by microscopy were negative for the presence of *E. histolytica* and *E. dispar* by PCR-based assays. **Conclusion:** We suspect some of these microscopy-positive stool specimens might contain a potentially novel species of *Entamoeba* that could not be detected by ELISA or PCR-based assays specific for *E. histolytica* and *E. dispar*. Diagnosis of amebiasis remains challenging here in Jordan and hence highlighting the need for improved diagnostic method.

Key words: *Entamoeba histolytica*, infectious disease, microscopic identification, molecular diagnosis, protozoan parasite

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Infection with the protozoan parasite *Entamoeba histolytica* (*E. histolytica*) is known as amebiasis. About 40-50 million cases of amebiasis occur worldwide yearly, resulting in 100,000 deaths annually. Amebiasis is ranked as the 3rd leading cause of death among parasitic infections[1,2]. *E. histolytica* can thrive in the damaged and inflamed tissues of the gut mucosa of the infected host. Infection by *E. histolytica* may spread from the intestine to the liver via portal circulation and may result in hepatic abscess formation, which can be fatal if left untreated. The abscess may burst and spread the infection to other organs such as the lungs and brain[3-5]. The global burden of amebiasis is directly ascribed to its association with severe and life-threatening symptoms, its low infectious dose, chlorine resistance and environmental stability[6-8].

Amebiasis occurs through the ingestion of *E. histolytica*-contaminated food and water which can affect people of every age and gender[6,9]. This infection is more common in developing countries, particularly in tropical areas with crowded living conditions and poor sanitation. In developed countries amebiasis occurs mostly in returning travelers or immigrants from endemic countries. An increase in the number of patients who are chronically immunosuppressed or taking chronic immunosuppressive drugs has been noted[10-12]. Accordingly, amebiasis will likely persist in most parts of the world and possibly increase.

The true epidemiology of amebiasis is unknown in many developing countries due to lack of accurate diagnostic methods[11,13,14]. Detection of *E. histolytica* in many developing countries still relies on microscopic examination of fresh stool specimens, which is not ideal due to presence of morphologically identical non-pathogenic species such as *E. dispar*, *E. moshkovskii* or *E. bangladeshi*. On the other hand, diagnostic confirmation of *E. histolytica* based on clinical manifestations alone can be difficult or impossible because of the nonspecific nature of gastrointestinal symptoms of amebiasis[9]. Amebiasis-like symptoms are often occur in other infectious diseases. For example, the diarrheal illness caused by *Shigella dysenteriae* and *Shigella flexneri*, *Salmonella*, *Campylobacter*, enterohemorrhagic *Escherichia coli* and enteroinvasive *Escherichia coli*, may present clinical manifestations similar to amebiasis[15,16]. These bacterial species are widely distributed over large portions of tropical and subtropical regions. Therefore, it may not be possible to distinguish amebiasis from these infectious diseases.

Microscopic identification of *E. histolytica* in stool specimens often leads to uncertain identification or even misidentification, especially in cases of presence of morphologically identical species such as *E. dispar*, *E. moshkovskii* or *E. bangladeshi*[11]. However, there is no compelling evidence that these *Entamoeba* species are responsible for diarrhea or dysentery or extra intestinal diseases in humans. Therefore, use of molecular tools is required to facilitate species-specific identification of *E. histolytica*.

Recently, several antigen-based ELISA kits have been developed for specific diagnosis of *E. histolytica* distinguishing it from *E. dispar*[17-19]. Moreover, molecular analysis by PCR-based assays are currently highly recommended for species-specific identification of *E. histolytica* in stool and liver aspirate specimens[16,20-24]. This is because PCR-based assays have been found to be more specific and sensitive than the conventional microscopic and immunological approaches by allowing definitive detection of *E. histolytica*. In addition, PCR-based assays have high reproducibility and stool specimens or the extracted DNA can be stored for long periods of time for re-assay, if needed or for performing molecular-based epidemiological surveys.

Despite medical advances and improvements in sanitation, water supply, nutrition and the availability of medications in Jordan, amebiasis still exists and has been reported in various districts but little is known about the true prevalence of amebiasis in Jordan. More importantly, detection of the parasite in stool specimens in Jordan is mainly accomplished by microscopic examination of stool specimens, which is neither specific nor sensitive. Therefore, this study aimed to assess the performance of microscopy in the identification of *Entamoeba* species in fresh stool specimens in comparison with the ELISA and PCR-based assays obtained from patients with gastrointestinal complications that were microscopy-positive for *Entamoeba* complex.

MATERIALS AND METHODS

Study design: This study was conducted at 3 different governmental hospital in Jordan including King Talal Military Hospital at Al-Mafraq city, Al-Zarqa Governmental Hospital at Al-Zarqa city and King Abdullah the First University Hospital at Al-Ramtha city. The study was carried out during a 1 year period from September, 2017 to September, 2018. Two hundred patients of different age groups with suspected amebiasis were recruited during this study. A patient was considered as having amebiasis when there was a clinical history suggestive of the disease such as diarrhea, stomach pain, bloody stools and fever. The mean age of the 200 patients in diagnosis was 32.5 years old and age range

was 2-65 years. These patients lived in urban neighborhoods and rural communities. All patients were treated at these local governmental hospitals by metronidazole (Flagyl).

Specimen collection: Stool specimens were collected from all patients with suspected amebiasis into a sterile plastic stool container. Stool specimens were placed in an ice box and brought to the Laboratories within one and half h of collection, at the Department of Biological Science, Al al-Bayt University, Al-Mafraq, for further examination and analysis. Microscopy-positive (for *Entamoeba* complex) stool samples were kept frozen at -20°C until they were processed for DNA purification or ELISA testing. This study was carried out after ethical clearance was obtained from human ethics committee of Ministry of Health, Jordan. Human ethical guidelines were followed strictly before engaging individuals for specimen collection.

Microscopic examination: Fresh specimens were used for the detection of *Entamoeba* by microscopy. Two rounds of microscopic examinations were performed. The stool specimens were first subjected to microscopic examination by a trained microbiology laboratory technician at the Al-Mafraq Hospital. This was followed by a second microscopic analysis by a medical technologist at the Al al-Bayt University (Table 2). Briefly, few drops of normal saline (0.9%) were added to a clean microscopic slide. About 2 mg stool specimen were added over a slide. The stool specimen was evenly spread over the clean slide and covered with cover-slip. All slides were examined for the presence of trophozoites and cysts forms of *Entamoeba* species at low (10x) and high (40x) magnifications. All stool specimens were examined within one and half hour of the time of passage.

Identification of *E. histolytica* by enzyme-linked immunosorbent assay (ELISA): NOVITEC® *E. histolytica* microplate assay was used to detect *E. histolytica* by following the manufacturer's recommendations (Diagnostics GmbH, Germany).

DNA extraction and PCR amplification: DNA was extracted from the stool specimens using the (QIAamp) DNA Stool Mini Kit according to the manufacturer's instructions (Qiagen Inc., Hilden, Germany). All PCR assays were performed at the Centers for Disease Control and Prevention (CDC, GA, USA). About 1 µL of template DNA was added to each PCR reaction mixture. The total reaction volume was 20 µL in all assays. A previously characterized *E. histolytica*-positive stool sample available at the laboratory was used as positive extraction control, while a previously characterized *Entamoeba*-negative stool sample was used as negative extraction control.

A TaqMan-based duplex real-time PCR assay was performed using the commercially available Platinum Quantitative PCR SuperMix-UDG w/ROX (Invitrogen, USA). A common forward primer (Ehd-239F:ATTGTCGT GGCATCCTAACTCA), a common reverse primer (Ehd-88R:GCGGACGGCTCATTATAACA), an *E. histolytica* specific probe (Histolytica-96T: 5'-FAM-TCA TTG AAT GAA TTG GCC ATT T-MGBNFQ-3') and an *E. dispar* specific probe (Díspar-96T: 5'-HEX-TTA CTT ACA TAA ATT GGC CAC TTT G-BHQ1-3') were used according to the Qvarnstrom *et al.*[20].

Two conventional PCRs to amplify tRNA gene-linked loci, SD (S^{TGA}-D5: CTCTGGATGCGTAGGTTCAA and S^{TGA}-D3: GTATC-TTCGCCTGTCACGTG) and SQ (S-Q5: GTGGTCTA-AGGCGTGTGACT and S-Q3: GAGATTCTGGTTCTTAGGACCC) were also used to amplify *E. histolytica* and/or *E. dispar* DNAs according to Ali *et al.*[25].

Another broad specificity conventional PCR for *Entamoeba* species was employed using a forward primer (Entagen-F: ACTTCAGGGGGAGTATGGTCAC) and a reverse primer (Entagen-R: CAAGATGTCTAAGGGCATCACAG) as described previously[26].

Agarose gel electrophoresis: The amplified DNA fragments were subjected to separation by agarose gel electrophoresis. The DNA bands were visualized under UV light. The size of the PCR product was obtained by comparing relative mobility on agarose gel with the standard molecular size marker (100-bp DNA ladders).

DNA sequencing: All resulting PCR products were directly sequenced using the forward and reverse primers used in the original amplification as sequencing primers. An ABI 3730XL sequencer was used for this Sanger sequencing according to the manufacturer's instructions. The resulting Sanger sequences were compared to the sequences available at the NCBI database by performing a BLAST search.

RESULTS

Baseline characteristics for the enrolled suspected patients with amebiasis are tabulated in Table 1. During this study, a total of 200 stool specimens were collected from suspected patients with amebiasis from September, 2017 to September, 2018. Of these 200 patients, 50.0% were children

Table 1: Baseline characteristics for the enrolled patients with suspected amebiasis

		Patients	
Demographic data	Age	Number	Percentage
Groups			
Children	2-12	35	50.0
Adolescents	13-17	18	25.7
Adults	18-59	14	20.0
Older Adults	≥60	3	4.3
Gender			
Male		45	64.3
Female		25	35.7
Residence area			
Rural area		45	64.0
Urban area		25	36.0

Table 2: Results of microscopic analysis for detection of *Entamoeba* complex in stool specimens collected from suspected patients with amebiasis

	Stool specimens (n = 200)			
	Positive		Negative	
Location of test	Number	Percentage	Number	Percentage
Hospital*	100	50	100	50
Al al-Bayt University**	70	35	130	65

*Microscopic examination was performed at 3 different hospitals: King Talal Military Hospital at Al-Mafraq city, Al-Zarqa Governmental Hospital at Al-Zarqa city and King Abdullah the First University Hospital at Al-Ramtha city, **70 microscopy-positive results obtained during the second round of examination by the medical technicians at the Al al-Bayt University were all included in the 100 microscopy-positive results obtained by the medical technicians at those 3 hospitals in the first round of examination

Table 3: Results of ELISA test and PCR-based assays for identification of *Entamoeba* complex in stools specimens collected from microscopy-positive suspected patients with amebiasis

	Positive		Negative	
Test	Number	Percentage	Number	Percentage
Microscopy	70	35.0	0	0.0
ELISA	0	0.0	70	35.0
Duplex real time PCR	0	0.0	70	35.0
tRNA gene-linked PCRs	0	0.0	70	35.0
Entagen PCR#	3	1.5	67	33.5

ELISA: enzyme-linked immunosorbent assay, PCR: Polymerase chain reaction, #Although the Entagen PCR was positive for 3 specimens but the sequencing of the amplicons revealed that they were of human origin (non-specifically amplified during PCR) and not related to *Entamoeba* species

(aged 2-12 years old) and 4.3% were older adults (≥60 years old), a greater proportion were males (64.3%). Based on residence area, about 64% of suspected patients with amebiasis were from rural areas compared with around 35-36% were from urban areas.

Microscopic examination: The results of microscopy analysis are presented in Table 2. Of 200 stool specimens, 100 tested positive for *Entamoeba* complex according to the primary examination. However, by second round of microscopic examination in our microbiology laboratory, 70 out of 200 stool specimens tested positive for the presence of *Entamoeba* complex.

***E. histolytica* ELISA:** All 70 specimens positive by microscopy were negative for the presence of *E. histolytica* antigen by the ELISA assay (Table 3).

Duplex real-time PCR assay: All 70 specimens positive by microscopy were negative for the presence of *E. histolytica* and *E. dispar* by duplex real-time PCR (Table 3).

Conventional PCRs to amplify tRNA gene-linked loci SD and SQ: All 70 specimens positive by microscopy were negative for the presence of *E. histolytica* and *E. dispar* by conventional PCR of the tRNA gene-linked loci SD and SQ (Table 3).

PCR to amplify any *Entamoeba* DNA and sequencing: An *Entamoeba*-general (Entagen) PCR was performed to amplify sequences originated from any *Entamoeba* species. Three out of 70 specimens showed amplifications by the Entagen PCR assay (Table 3). PCR products were separated by agarose gel electrophoresis. The size of these PCR products were approximately 410 bp, which was comparable to those from other *Entamoeba* species (such as *E. histolytica*: 433 bp, *E. dispar*: 434 bp, *E. moshkovskii*: 432 bp). However, sequencing of the resulting PCR products revealed that they were non-specific amplifications with human DNA.

DISCUSSION

In this study, 200 stool specimens were obtained from patients with suspected intestinal amoebic infection. Microscopic examination identified 70 out of 200 stool specimens as being positive for *Entamoeba*. However, microscopic examination cannot distinguish between morphologically similar species such as *E. histolytica, E. dispar*, *E. moshkovskii* and *E. bangladeshi*[9,11,21,27-30]. Therefore, identification of *E. histolytica* solely based on morphologic characteristics of the cysts and trophozoites of *E. histolytica* is likely to produce false positive results. Detection of *Entamoeba* complex by microscopy must be carried out during the first hour of collection of the stool specimens in order to look for motile trophozoites. In case of only *E. histolytica*, trophozoites may contain ingested RBCs. Additionally, a crude stool specimen may also contain

numerous structures such as undigested food particles and human cells such as macrophages resembling cysts or trophozoites of *Entamoeba* species. It is recommended that multiple stool specimens (at least 3) be examined before a negative or positive result is reported[28]. In addition to these limitations, the validity, accuracy and reproducibility of *E. histolytica* identification by microscopy rely on the performance of the microscopist and can be influenced by microscopist's experience[1,31].

The results of this study indicated that all stool specimens tested positive by microscopy for *Entamoeba* complex were negative by *E. histolytica* ELISA test. There are several explanations for the negative result with ELISA. First, none of the specimens was genuinely positive for *E. histolytica*[32]. Second, it is possible that the amount of *E. histolytica* target antigen was too low to be detected by ELISA, which requires about 1,000 trophozoites per well for positive identification[9,28,33]. Third, hypothetically, a new species of *Entamoeba* was present, that is antigenically different from *E. histolytica* but morphologically similar to *E. histolytica* and *E. dispar*.

To further verify the presence or absence of *E. histolytica* and *E. dispar* in 70 microscopy-positive specimens, first a diagnostic duplex real-time PCR was performed. The real-time PCR was negative for all specimens. Secondly, two conventional PCRs to amplify tRNA gene-linked loci were used that are specific for both *E. histolytica* and *E. dispar*. Again, these PCRs were negative for all specimens. Thirdly, we used an *Entamoeba* general primer set to amplify DNA from any known *Entamoeba* species. Although a minority of specimens gave positive amplifications, Sanger sequencing of PCR products showed that they were not of *Entamoeba* origin, instead it is suspected that they were non-specific amplifications of human DNA. It is less likely that our inability to detect *Entamoeba*-specific DNA could be linked to DNA degradation, because the Entagen PCR gave products in three of the 70 DNAs, although the sequencing identified these to be of human origins, ruling out PCR inhibitors affect. The Qiagen procedure used for DNA extraction from fresh stool specimens utilized inhibit tablets which was supposed to adsorb DNA damaging substances and PCR inhibitors. Additionally, *Entamoeba*-positive culture DNA (available at the FLIA Lab) was spiked to the stool DNA of this study to check for PCR inhibition. None of the present study DNA samples inhibited the PCR amplification of the *Entamoeba*-spiked DNA. Overall, the results of these PCR assays suggest either none of the stool specimens were truly positive for *Entamoeba* species or the *Entamoeba* species detected by microscopy was a novel species that has different DNA sequences in the primer-binding regions. At this stage, the presence of *Entamoeba* species in our study remains undetermined. Presence of very low or no *E. histolytica* in microscopy positive specimens by PCR assays is not uncommon. For example, a study in Brazil found that 0 of 59 stool specimens positive for *Entamoeba* complex by microscopy were PCR-positive for *E. histolytica* but 23 were positive for *E. dispar*[32]. Similarly, only 1 out of 246 cases and 1 out of 101 cases that tested positive by microscopic examination also tested positive for *E. histolytica* by PCR in Ghana and in UK, respectively[34,35]. Very recently, Singh *et al.*[24] reported that remarkable genetic polymorphisms exists among *E. histolytica* obtained from a restricted geographic location using a multi-locus genotyping system. These observations might provide a possible explanation as to why the PCR-based test failed to identify the presence of *E. histolytica* in our study populations. Perhaps, metagenomic deep sequencing with the extracted DNA from patients' stool specimens could identify a possible new species of *Entamoeba* in our study population.

We had some limitations in this study: (a) We only collected a single stool specimen from a single patient. Multiple samples from the same patients could have improved the sensitivity of our testing, (b) We were unable to perform culture of *Entamoeba* species, because none of the laboratory facilities used had the culture capability. Otherwise, DNA extracted from the cultured organisms could be used in other researches such as isoenzyme electrophoresis or whole genome sequencing to identify the infecting *Entamoeba* species. The amount of DNA could be extracted from a cultured organism would have been significantly larger than that present in the original stool specimen, (c) All the microscopy-positive patients were treated with metronidazole, which could have negatively impacted the sensitivity of other diagnostic tests used and (d) Stools were not examined by microscopy for the identification of other diarrhea-causing pathogens such as *Giardia lamblia*, *Ascaris lumbricoides*, *Trichuris trichiura*, *Hymenolepsis nana*, *Strongyloides stercoralis* and *Cyclospora cayetanensis*.

CONCLUSION

In conclusion, to our knowledge, this was the first study to utilize PCR-based assays to detect and differentiate *E. histolytica* from other morphologically indistinguishable *Entamoeba* species in stool specimens that were positive for *Entamoeba* complex by microscopy in suspected amebiasis patients. This study suggests that microscopy should not be used as a stand-alone method to detect *Entamoeba* in

stool specimens from suspected amebiasis patients and *Entamoeba* species-specific molecular methods such as PCR should be used instead.

SIGNIFICANCE STATEMENT

The present study utilized PCR-based approaches to evaluate performance of microscopy in the diagnosis of *E. histolytica* in stool samples from suspected patients of amebiasis. Although *Entamoeba* complex could be detected in 35% of stool samples by microscopy, none of these appeared to be belonging to true pathogen *E. histolytica*. In fact, none of the microscopy-positive *Entamoeba* species could be verified by PCR-based assays. This may suggest either a major limitation of microscopy by yielding false-positive results, or, we were unable to identify a novel *Entamoeba* species that was circulating in this geographical region. In summary, while microscopy should not be used in the diagnosis of amebiasis, further studies are needed to understand the true picture of amebiasis in our study populations.

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