Original Article

Evaluation of multiplex PCR assay against microscopy in diagnosing *Entamoeba histolytica* in stool samples at Assiut Governorate Hospitals, Egypt

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ABSTRACT

Background: Cysts of *E. histolytica*, *E. dispar*, *E. moshkovskii*, and others are not microscopically distinguishable. Since not all are pathogenic to human, unnecessary prescription of anti-amoebic therapy may lead to drug resistance.

Objective: To evaluate usefulness of the molecular assays to differentiate between pathogenic and non-pathogenic *Entamoeba* species.

Patients and Methods: Fecal samples were collected and examined from 187 patients suffering from diarrhea and/or dysentery, and attending the outpatient clinics of the Assiut Governorate Hospitals. Stool samples were examined by saline wet mount preparations, iodine, methylene blue and trichrome stains. Positive stool samples for *Entamoeba* cysts were preserved for multiplex PCR (mPCR) assay.

Results: Among 187 samples, 24 (12.8%) were positive for *Entamoeba* complex by microscopic examination. Multiplex PCR detected *E. histolytica*, *E. moshkovskii* and *E. dispar* in 5 samples (2.7%), 2 samples (1.1%), and one sample (0.5%), respectively.

Conclusion: Multiplex PCR is more suitable for differentiating between *Entamoeba* spp. for better treatment strategies and successful control.

Keywords: Assiut; differentiation; Egypt; *Entamoeba* spp.; microscopy; mPCR.

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INTRODUCTION

Entamoeba histolytica is an anaerobic parasite, of genus *Entamoeba* that infects about 35-50 million people around the world[1]. It is considered a great public health problem, especially in tropical regions with low socioeconomic areas and poor sanitary conditions[2]. Amoebiasis is the world's third mortality leading parasitic cause after malaria and schistosomiasis[3]. Amoebiasis annually kills more than 55,000 patients[4]. There are six species of the genus *Entamoeba* inhabiting the human intestinal lumen including, *E. histolytica*, *E. dispar*, *E. moshkovskii*, *E. poleki, E. coli*, and *E. hartmanni*. Among them, *E. histolytica* is the only pathogenic species[5]. Notably, *E. histolytica*, *E. dispar* and *E. moshkovskii* are conjointly called *E. histolytica* complex. Although morphologically indistinguishable, they are different biochemically and genetically[2]. Accordingly, several studies were carried out using molecular assays for detection and differentiation between *Entamoeba* spp.[6-8].

In fact, accurate assessment of amoebiasis is a challenge. Other than stool examination, several methods were evaluated such as immunochromatographic tests (ICTs)[3], culture[9], and serological methods including latex agglutination[10], and ELISA[11]. Microscopy is the less reliable tool in diagnosis of amoebiasis because of delayed delivery to the laboratory that deteriorates trophozoites' motility and viability. Notably, microscopy had poor sensitivity (60%) yielding false-positive results[9].

Entamoeba cultivation is technically difficult, and less sensitive than light microscopy with a success rate of 50–70% and not undertaken in a routine clinical laboratory[9]. In their report[10], the researchers discussed several commercial and homemade dipstick assays such as indirect hemagglutination, bentonite flocculation, cellulose acetate membrane precipitation, counter immune electrophoresis, fluorescent immunoassay[10]. Besides, although commercial serological methods are more sensitive than traditional microscopic and ICTs are practical and easy to perform, both are unable to differentiate pathogenic from non-pathogenic *Entamoeba*[12]. Therefore, identification of the pathogenic *Entamoeba* is essential for accurate diagnosis to obtain valid epidemiological studies, and successful treatment strategies without drug resistance[13]. Since the results revealed high specificity of molecular diagnosis, a recent study conducted in Egypt recommended its use whenever possible[14] .

Notably, patients infected by *E. moshkovskii*, E. *hartmanni*, and *E. dispar* could be unnecessarily

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treated with anti-amoebic drugs[15]. For proper treatment regimen, and control of amoebiasis, The WHO approved PCR as the most reliable diagnostic tool for differentiation of *Entamoeba* spp.[14]. That inspired us to utilize mPCR technique for identification and molecular differentiation of *E. histolytica*, *E. dispar* and *E. moshkovskii* spp. in stool samples of Egyptian patients complaining of diarrhea and/or dysentery from Assuit Governorate, Egypt.

PATIENTS AND METHODS

The present cross-sectional study was conducted at the Medical Parasitology Department, Faculty of Medicine, Assuit University during the period from February 2021 to May 2022.

Study design: Stool samples were collected from patients complaining from diarrhea and/or dysentery, and attending the outpatient clinics of the Assiut Governorate Hospitals. They were examined microscopically, and the positive samples for *Entamoeba* spp. were subjected to mPCR.

Study patients: Included in the study were 187 male and female patients of all ages, presenting with diarrhea and/or dysentery.

Samples collection and processing: Three stool samples were provided by each patient on three successive days in dry, clean, leak-proof plastic disposable cups labeled with name, age, date and sex of each patient. A questionnaire was conducted and related demographic data were recorded. All fecal samples were transported within half an hour to our laboratory.

Stool examination: Stool samples were examined macroscopically and microscopically by direct wet mount, and slides stained with iodine, methylene blue and trichrome stains for detection of *Entamoeba* cysts and trophozoites. Positive, and negative stool samples with blood and/or mucous (suspected samples) were stored frozen at −20°C for mPCR assays.

Molecular identification

- **Extraction and preparation of DNA:** The DNA purification kit (Qiagen, Hilden, Germany) was used for DNA extraction from fresh frozen fecal samples as per instructions of the manufacturer[16]. The extracted DNA concentration was measured by nanodrop.
- **Amplification of DNA[17]:** We applied mPCR targeting the ssu-rRNA gene sequence. The forward primer sequence (*Enta*F, 5'-ATGCACGAGAGCGAAAGCAT-3') was conserved in all three *Entamoeba* spp., whereas the specific reverse primers, *Eh*R (5'-GATCTAGAAACAATGCTTCTCT-3' X64142), *Ed*R (5'-ACCACTTACTATCCCTACC-3' Z49256), and *Em*R (5'-TGACCGGAGCCAGAGACAT-3' AF149906), were specific for *E. histolytica*, *E. dispar*, *E. moshkovskii*, respectively.
- **Reagents and reaction conditions[6]:** The mPCR was performed using amplicon (TaqDNA Polymerase Master Mix Red, Denmark) as a ready-made solution. The reaction was carried out in 25 µl final volume (per sample) containing: 12.5 µl My Taq TM Red Mix, 75 µl forward primer, 75 µl of each reverse primer, 4.5 µl DNA free water, and 5 µl template DNA extract. Amplification of each species-specific DNA fragment started with an initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 7 min.
- **Gel electrophoresis:** Amplified DNA fragments were analyzed by gel electrophoresis and visualized under UV illumination. The analysis of the images was performed by Gel Imager and Documentation System (Compact M, Biometra, Germany). The size of each fragment was based on comparison with 100-bp ladder.

Statistical analysis: It was performed using the IBM SPSS 26.0 software. Categorical variables were described by number and percent, where continuous variables were described by mean and standard deviation. The Chi-square test and fisher exact test were used to compare between categorical variables, where MannWhitney test was used to compare between continuous variables. A two-tailed $P<0.05$ was considered statistically significant.

Ethical consideration: The present study protocol gained the approval of the Ethics Review Committee of Assiut Faculty of Medicine. The study was registered at Clinical Trials.gov with registry No. (NCT04466449). The patients and children's parents were informed about the study objective, and their consent was obtained to collect the stool samples. All patients were informed with the study results, and received the appropriate treatment and care. The research policy is in agreement with the 1964 Helsinki declaration.

RESULTS

Microscopic examination: Collected stool samples were loose, offensive and few of them were mixed with blood and mucous. Out of the total 187 examined total stool samples, 24 (12.8%) were microscopically positive for *Entamoeba* complex (cysts and/or trophozoites), and the remaining 163 (87.2%) were negative. Among them, 30/163 (18.4%) were mixed with blood and mucous and considered suspected cases.

Molecular identification: Out of the 24 microscopy-positive samples; 6 samples were successfully amplified and characterized as *Entamoeba* spp. Based on amplicon size; three samples (12.5%), two samples (8.3%), and one sample (4.2%) were identified as

E. histolytica (at 166 bp), *E. moshkovskii* (579 bp), and *E. dispar* (753) bp, respectively (Figs. 1, and 2). Application of mPCR on the 30 suspected samples revealed that only two samples (6.6 %) were positive for *E. histolytica* (Fig. 3). The remaining samples were negative by mPCR (Fig. 4).

The overall molecular detection of *Entamoeba* spp. infections among the collected samples was 5 samples (2.7%) for *E. histolytica* (166 bp), 2 samples (1.1%) for *E. moshkovskii* (579 bp) and 1 sample (0.5%) for *E. dispar* (753 bp). The difference between mPCR and microscopic examination for positive and suspected samples of *Entamoeba* is shown in table (1). According to ROC curve, microscopy is of poor sensitivity (71.43) and specificity (59.57) in the diagnosis of *Entamoeba* spp. infection in comparison to mPCR (Table 2, and Fig. 5).

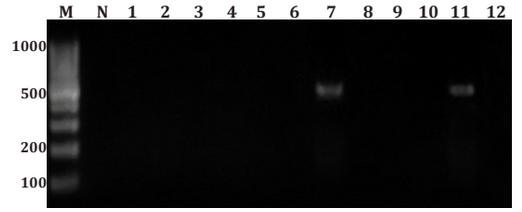

Fig. 1. Agarose gel electrophoresis with ethidium bromide showing mPCR of *ssu-rRNA* gene products of *Entamoeba*. **M:** 100 bp DNA ladder marker; **N:** Negative control. **Lanes 7, 11:** Positive for *E. moshkovskii* (~579 bp).

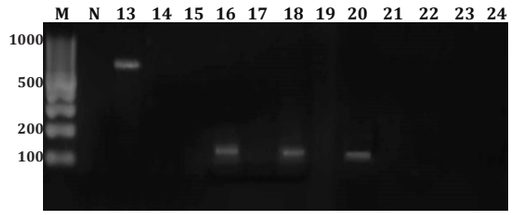

Fig. 2. Agarose gel electrophoresis with ethidium bromide showing mPCR of *ssu-rRNA* gene products of *Entamoeba* spp. **M:** 100 bp DNA ladder marker; **N:** Negative control. **Lanes 16, 18, 20:** Positive for *E. histolytica* (~166 bp); **Lane 13:** Positive for *E. dispar* (~753bp).

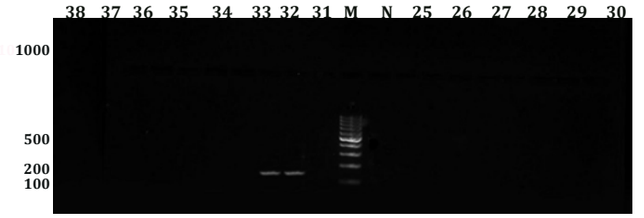

Fig. 3. Agarose gel electrophoresis with ethidium bromide showing mPCR of *ssu-rRNA* gene products of suspected samples of *Entamoeba* spp. **M:** 100 bp DNA ladder marker; **N:** Negative control. **Lanes 32, 33:** Positive for *E. histolytica* (~166 bp).

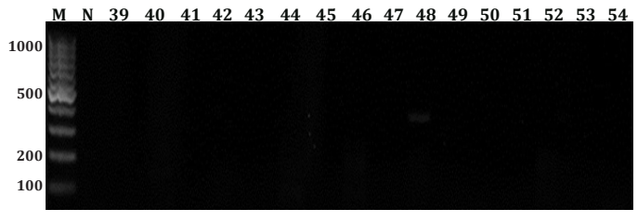

Fig. 4. Agarose gel electrophoresis with ethidium bromide showing mPCR of *ssu-rRNA* gene products of suspected samples of *Entamoeba* spp. **M:** 100 bp DNA ladder marker; **N:** Negative control. No specific bands were detected.

Table 1. Microscopic versus mPCR in identification of *Entamoeba* spp.

Stool analysis	PCR			
	E. histolytica **(N=5)**	*E. moshkovskii* **(N=2)**	*E. dispar* **(N=1)**	**Negative (N=46)**
Positive (n=24)	3	2	1	18
Negative (n=30)	2	0	0	28

Table 2. The ROC curve analysis of microscopic stool examination for *Entamoeba* spp. infection (sensitivity, specificity, PPV, NPV and accuracy) according to mPCR results.

	Stool analysis for *Entamoeba* spp.
AUC	0.655
Sensitivity	71.43
Specificity	59.57
PPV	20.80
NPV	93.30
Accuracy	65.50

AUC: Area under the curve, **PPV:** Positive predictive value; **NPV:** Negative predictive value.

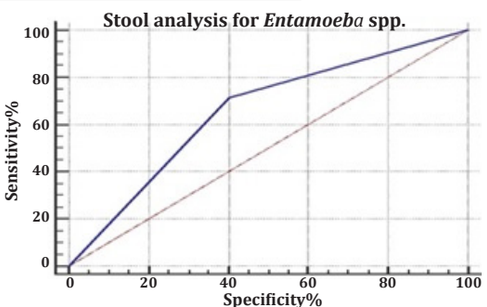

Fig. 5. The ROC curve analysis of microscopic stool examination for detection of *Entamoeba* spp. according to mPCR results.

DISCUSSION

Our direct microscopic examination for the collected samples revealed 24/187 (12.8%) positive for *Entamoeba* complex. This was lower than the rate detected in Sohag-Egypt (43%)[3], in Baghdad, Iraq (35.3%)[17], in South Africa (33%)[18], and in Beni-Suef, Egypt (15.4%)[7]. However, it was higher than 2.3% in Lebanon and 2.5% in Turkey[19], 8.1% among rural school children in Assiut, Egypt[20], and 8.3% reported among school children in Aswan Governorate[21]. The infection rate was nearly like that in Nairobi, Kenya 12.5%[22].

The studies conducted in Egypt[3,7,20,21], were carried out to differentiate between *E. dispar* and *E. histolytica* without including *E. moshkovskii*. According to the current study, 3 out of the 24 positive samples by microscopic examination were detected positive for *E. histolytica* by mPCR; 2 samples were positive for *E. moshkovskii* and only one sample detected positive for *E. dispar*. While the remaining 18 samples were negative by molecular assay. These cases which were positive for *Entamoeba* infection by microscopy but were negative by mPCR, might have been of a different *Entamoeba* species other than *E. histolytica*, *E. dispar* and *E. moshkovskii*, e.g., *E. hartmanii, E. polecki*, and *E. coli*, which are extremely similar. This is in agreement with two previous studies[7,17].

For the suspected 30 samples, 2 samples were reported positive for *E. histolytica* by mPCR while being negative microscopically. This indicates that microscopy was of low specificity and sensitivity in detection of *E. histolytica* stages in fecal samples. This was consistent with previous studies that detected positive cases for *E. histolytica* by PCR while being negative by microscopic examination[7,23]. The remaining dysenteric negative samples for *Entamoeba* may be due to other causes for dysentery rather than amoebic dysentery, *e.g.,* dysentery of bacterial origin[24].

Regarding the results of the present molecular study, the total positive cases for *E. histolytica* by mPCR were 5 out of the 187 included cases. So, the *E. histolytica* detection rate in the studied cohort who presented with diarrhea and/or dysentery at Assuit Governorate hospitals was 2.7%. That was higher than 0.7% reported in Nepal[25], and 1.4 % in Beni-Suef, Egypt[7]. Unlike the present study, a higher infection rate (ranged from 4.1% to 14.7%) was reported in Behira, Egypt[14], Iran[18], and in Nairobi, Kenya[22]. This variation in the detection rate showing wide discrepancy between different world regions according to geographical and climate variations, socioeconomic level and living habits of the studied groups[26].

The detection rate of the molecularly detected *E. moshkovskii* in the current study was 1.1%, that was lower than 3.3% in Beni-Suef, Egypt[7] and 11.8% in El Behira, Egypt[14]. In our study, participants with *E. moshkovskii* detected in their stool samples had symptoms suggesting that it is potentially pathogenic. In confirmation previous studies also detected *E. moshkovskii* among symptomatic patients[7,8]. According to the present molecular study, *E. dispar* detection rate was 0.5% among the studied population, that was lower than 4.6% in Beni-Suef, Egypt[7] and 61.8% in El Behira, Egypt[14].

The results of the present study endorsed the accuracy of PCR more than microscopic stool examination as a diagnostic tool for detection and differentiation of *Entamoeba* spp. It was also more sensitive when it was compared to microscope sensitivity 71.43% and specificity 59.57%, so we can't rely on microscopic examination. It is recommended to apply PCR assay to improve treatment strategies and cut down the unnecessary treatment for nonpathogenic species which may lead to drug resistance[1]. Moreover, by further studies for the dynamics of other species, which may be potentially pathogenic, we shall overcome missing of cases infected by these species. The results agreed with earlier studies demonstrating that the sensitivity of microscopy was only 38% in terms of detection of intestinal parasites compared to the 80% higher sensitivity and 100% specificity of PCR[18,27]. Similarly, Zebardast *et al*.[6], and Fallah *et al*.[28] approved mPCR technology application in recognition and differentiation of *E. histolytica* and *E. dispar* directly from fecal samples in routine diagnosis of *Entamoeba* spp. based on WHO recommendation for better epidemiological information and a better comprehension of amoebic infections.

Aguayo-Patrón *et al*.[23] in Mexico, and Guevara *et al*.[29] in Ecuador confirmed this result as they recorded that detection of *E. histolytica* among the *E. histolytica*/*E. dispar*/*E. moshkovskii* complex will provide accurate diagnosis of amoebiasis, to reduce the morbidity of *E. histolytica* infection, cut down the unneeded treatment of patients infected with nonpathogenic amoeba and understand the actual dynamics of amoebic transmission. Kamidani *et al*.[30] also mentioned that treating amoebiasis detected by microscopic examination or other *E. histolytica* diagnostic tests may be of limited value, and PCR-based epidemiologic studies are required to adjust accurate treatment strategies. That also aligned with the studies conducted in Beni-Suef, Egypt[7], Baghdad, Iraq[17], and Iran[31]. In these studies, it was reported that microscopic diagnosis of amoebic infection in comparison to PCR was of poor sensitivity and limited diagnostic value, resulting in both false-positive and false negative results and mentioned that microscopic examination failed to differentiate between morphologically similar nonpathogenic *Entamoeba* and pathogenic *E. histolytica*. So, it was recommended that PCR assays must be applied to detect the exact

E. histolytica infection rate and to conduct accurate treatment and successful control measures.

In conclusion, the study results proved the importance of applying the advances in molecular techniques in the epidemiology study of amoebiasis. Moreover, *E. moshkovskii*, and *E. dispar* might be related to several gastrointestinal symptoms and so they could be potentially pathogenic.

We recommend PCR technique to be the "gold standard" for diagnosis of *Entamoeba* infection to determine the true infection rate of *E. histolytica* and differente between different species for better epidemiological studies and treatment strategies. Further molecular studies are recommended to be performed using different *Entamoeba* primers to differentiate all the remaining *Entamoeba* species that cause infection. More studies are suggested to understand the transmission dynamics of *E. dispar* and *E. moshkovskii* species and more importantly determine their pathogenic role.

Authors contribution: All authors contributed to the study design and conception. Preparation of materials, data collection and laboratory work up were carried out by Basaly YT. Analysis and assessment of the data and supervision of the work were carried out by Mohamed YM, Mahmoud AE, Elossily NA. All authors revised and accepted the final version before publication.

Conflict of interest: The authors declare that they have no conflict of interest.

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