DOI: 10.1515/ap-2017-0022
© W. Stefański Institute of Parasitology, PAS
Acta Parasitologica, 2017, 62(1), 188–191; ISSN 1230-2821

DE GRUYTER

# Molecular diagnosis of *Entamoeba* spp. versus microscopy in the Great Cairo

**Mohamed H. Roshdy[1], Nour M. Abd El-Kader[1], Marwa Ali-Tammam[2,3], Isabel Fuentes[4], Magdy M. Mohamed[1], Nabila A. El-Sheikh[2] and Jose Miguel Rubio[4]***

[1]Biochemistry Department, Faculty of Science, Ain Shams University, Cairo, Egypt;
[2]Molecular Immunology Unit for Infectious Diseases, Al-Azhar University, Cairo, Egypt;
[3]Faculty of Pharmaceutical Sciences & Pharmaceutical Industries.Future University in Egypt (FUE), New Cairo, Egypt
[4]Malaria & Emerging Parasitic Diseases Laboratory. National Microbiology Center. Instituto de Salud Carlos III, Madrid, Spain

## Abstract

Amoebiasis is a human disease produced by *Entamoeba histolytica* which causes widespread mortality and morbidity worldwide through diarrheal disease and abscess establishment in parenchymal tissues such as liver, lung, and brain. The true prevalence of infection is unknown for most areas of the world due to the difficulty to characterise E*ntamoeba histolytica* versus other non-pathogenic amoebas with identical morphology, as E*ntamoeba dispar*, and E*ntamoeba moshkovskii*. To overcome microscopy misidentification issues, we tested a nested multiplex polymerase chain reaction (PCR) and a real-time PCR on 194 stool samples collected from incoming dysentery patients in Cairo hospitals diagnosed with *E. histolytica* by microscopy. Nested PCR showed only 20 (10.3%) samples positive to *E. histolytica* and 17 (8.7%) to *E. dispar*. The real-time PCR detected only 19 and 11 samples positive to *E. histolytica* and *E. dispar* respectively, showing less sensitivity than the nested PCR. The data show that prevalence of *E. histolytica* in Cairo is lower when specific diagnosis methods are used instead of traditional microscopy, allowing to differentiate between morphologically identical human amoebas species.

## Keywords

Amoebiasis**,** molecular diagnosis, *Entamoeba histolytica*, *Entamoeba dispar*, Cairo, Egypt

## Introduction

Amoebiasis is still mentioned as one of the main health problems in tropical and subtropical regions. The true prevalence of infection caused by *Entamoeba histolytica* is unknown for most areas of the world (Ali 2015). *Entamoeba histolytica* causes widespread mortality and morbidity worldwide through diarrheal disease and abscess establishment in parenchymal tissues such as liver, lung, and brain (Skappak *et al.* 2014). In contrast, other amoebae that infect humans; including *Entamoeba dispar*, *Entamoeba moshkovskii*, *Entamoeba coli*, *Entamoeba hartmanni*, and *Endolimax nana*; have been considered non-pathogenic (Blessmann *et al.* 2002; Calegar *et al.* 2016). *Entamoeba histolytica*, *E. dispar*, and *E. moshkovskii* are morphologically undistinguishable (under microscope their cysts have the same shape and diameter and contain 4 nuclei), but are biochemically and genetically different. Although *E. histolytica* is recognised as a pathogen, the ability of the other two species to cause disease is unclear (Fotedar *et al.* 2007; Oliveira *et al.* 2015). It is also worthy to note that until recently, the differentiation of *E. histolytica* from the non-pathogenic amoebic species was not possible (Ali *et al.* 2008; Calegar *et al.* 2016; Fotedar *et al.* 2007; Khairmar *et al.* 2007; Zebardast *et al.* 2016).

In our study, we used two molecular diagnostic techniques to determine the real prevalence of *E. histolytica* in microscopically diagnosed positive samples in the Great Cairo, Egypt. The molecular tests were a nested multiplex PCR (Nested PCR), to examine the prevalence of *E. histolytica*, *E. dispar* and *E. moshkovski* infections, and a TaqMan Real Time PCR (RT-PCR), using specific probes for *E. histolytica*.

## Materials and Methods

A total of 194 stool samples reported to be *Entamoeba* positive by microscopy examination were included in the study from March 2012, till December 2014.The samples were collected from different hospitals in Cairo and surroundings including the Cairo University pediatric Hospital, Ain Shams paediatric

*Corresponding author: jmrubio@isciii.es

Author's copy

hospital and some private laboratories in Ain Shams and El-Salam city. Stool specimens were screened microscopically using formol / ether concentrated slide smear for the presence of *Entamoeba* spp. (Nazemalhosseini *et al.* 2010). Genomic DNA was extracted from fresh or frozen unpreserved stool using QIAamp DNA Stool Kit (QIAGEN, Venlo, Netherlands). Nested PCR amplification was carried out according to Khairnar and Parija (2007). Primary PCR amplification was performed targetting the 16S-like rRNA (SSU rRNA) gene to produce an *Entamoeba* spp.-specific amplicon of about 900 bp in length, which was then used as a template for the species specific secondary PCR amplification giving either 553, 439 or 174 bp long PCR product for *E. moshkovskii*, *E. histolytica* and *E. dispar* respectively. Briefly, both PCR amplification were performed using 1x Biotools Tth PCR buffer (Biotools, Madrid, Spain) with 2 mM $MgCl_2$, 200 µM of dNTPs, 0.7 U of *Tth* DNA polymerase (Biotools, Madrid, Spain) and 3 µM of each primer (Sigma, San Luis, MI, USA) in a final volume of 25 µl. Five µl of extracted DNA were included in the primary PCR and two µl of the amplification product of this was used as template for the second amplification. The PCR amplification conditions for the primary amplification were an initial denaturation at 95°C for 5 minutes, followed by 40 cycles consisting of 94°C for 20 seconds, 56°C for 20 seconds and 72°C for 30 seconds followed by a final extension step at 72°C for 10 minutes. For the secondary PCR, the amplification conditions were an initial denaturation at 95°C for 5 minutes, followed by 35 cycles consisting of 94°C for 15 seconds, 48°C for 15 seconds and 72°C for 20 seconds, and a final extension step at 72°C for 10 minutes. Non-template and positive controls were included in each run. Amplified PCR products were visualised with 1x Pronasafe Nucleic Acid Staining Solution (Pronadisa, Madrid, Spain) after electrophoresis on 1.8% agarose gels.

In order to test the presence of PCR inhibitors in the isolated DNA, twenty randomly selected negative samples were tested by adding an equal volume of *E. histolytica* positive control to each sample aliquot. Both the seeded as well as the unseeded aliquots of the same sample were tested in the same nested multiplex amplification protocol as stated earlier.

The real time amplification targeting 16S-like rRNA (SSU-rRNA) gene was performed according to Verweij *et al*. (2004). The specific primers amplified a 172 bp fragment inside the16S-like rRNA gene and the MGB TaqMan probes were used to detect *E. histolytica*-specific amplification. The amplification was performed using a Rotor-Gene (Corbett, Sydney, Australia). The RT-PCR protocol used TaqMan gene expression Master Mix (Applied Biosystems, Foster City, CA, USA), 0.5 µM of each forward and reverse primer (Sigma, San Luis, MI, USA), 0.1 µM specific probe for *E. histolytica* and 3 µl of template DNA was added for 25 µl final reaction volume with the following conditions, an initial hold at 95°C for 10 minutes, followed by 45 cycles, each consisting of 95°C for 15 seconds and 60°C for 60 seconds. Positive and negative control samples were included in each reaction.

## Results

Thirty seven cases out of 194 (19 %), of the samples characterised as *E. histolytica* positive by microscopy, were confirmed as *E. histolytica / E.dispar / E. moshkovskii* by the nested multiplex PCR. The genotyping discrimination revealed the presence of 17 out of 37 cases of *E. dispar* (8.7%) and 20 cases (10.3%) of *E. histolytica* without detection of *E. moshkovskii* (Fig. 1).

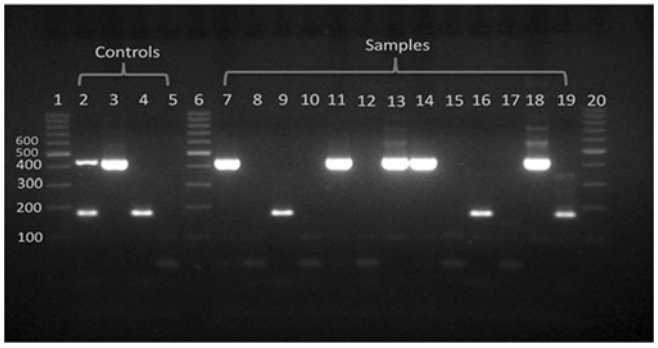


**Fig. 1.** Agarose gel electrophoresis (1.8 % TAE) of 16S-like rRNA (SSU rRNA ) amplified PCR product staining with 1x Pronasafe Nucleic Acid Staining Solution (Pronadisa, Madrid, Spain) [9] Lanes 1, 6 and 20: 100 bp molecular marker (Biotools, Madrid, Spain); lane 2: positive mixed control of *E. histolytica* (439bp) and *E. dispar* (174bp); lane 3: *E. histolytica* positive control; lane 4: *E. dispar* positive control; lane 5: non-template (non-DNA) control, lanes 7, 11, 13, 14 and 18: *E. histolytica* positive patient's samples; lanes 9, 16 and 19: *E. dispar* positive patients' samples and lanes 8, 10, 12, 15 and 17: Negative patients' samples

RT-PCR amplification detected 11 and 19 out of 194 samples positives for *E. dispar* and *E. histolytica* respectively. Threshold cycle (Ct) values for *E. dispar* cases were between 37 and 44 (median threshold of 41 cycles) and for *E. histolytica* cases were between 30 and 38 (median threshold of 32 cycles).

Presence of inhibitory contaminants was not detected in the twenty randomly selected PCR negative samples mixed with *E. histolytica* positive control.

## Discussion

An accurate diagnosis of intestinal amoebiasisis allowing to distinguish *E. histolytica* from *E. dispar* and *E. moshkovskii* is essential for determining the true prevalence of pathogenic *E. histolytica* in the community. Most physicians in Egypt prescribe systematic treatment for amoebiasis upon microscopic examination. Efficient differentiation techniques are required in order to avoid unnecessary treatment when *E. dispar*, a non-pathogenic species, is present. The present study used two molecular techniques for the discrimination of the pathogenic amoeba species, a nested multiplex PCR for the differentiation of *E. histolytica*, *E. dispar* and *E. moshkovski* in stool

Author's copy

samples (Khairnar *et al.* 2007) and a quantitative real time-PCR with specific probe to differentiate *E. histolytica* (Verweij *et al.* 2004).

In our study the nested multiplex PCR detected 8.7% (17/194) of *E. dispar* and 10.3% (20/194) of *E. histolytica* positive samples, while the Real Time PCR only detected 5.7% (11/194) of *E. dispar* and 9.8% (19/194) of *E. histolytica* positive samples. This result shows that the RT-PCR method missed seven positive samples which were detected by the nested PCR. The seven missed samples were six *E. dispar* and one *E. histolytica* by nested-PCR. The main reason of this discrepancy could be the low parasite concentration present in the samples, especially in *E. dispar* as it was shown in the higher Ct threshold average, and a lower sensitivity of the Real Time PCR method used. This lower sensitivity of the Real Time PCR versus nested PCR also happens in other molecular diagnosis (*Cryptosporidium spp*, *Giardia lamplia*, *Leishmania donovani*, *Toxoplasma gondii*) and it is not exclusive of *Entamoeba* (Bastien *et al.* 2008; Zebardast *et al.* 2016).

Clearly, the number of positive samples is much lower than by microscopy, i.e. 37 *versus* 194 (19.0%) and only 20 (10.3%) of them are characterised as *E. histolytica*, the pathogenic amoebae. A possible explanation for such high discrepancies could be the presence of potential PCR inhibitory materials in stool samples (Forsell *et al.* 2015), but inhibitory test performed in the twenty randomly selected PCR negative samples mixed with *E. histolytica* positive control did not show any inhibition of the PCR. In any case, the inclusion of an internal amplification control could eliminate this risk (Verweij *et al.* 2004). Another possible explanation may be the microscopists' limited experience (Hamzah *et al.* 2010; Leiva *et al.* 2006; Sharbatkhori *et al.* 2014; Tanyuksel and Petri 2003), and the use of suboptimal microscopical equipment. In our case, no micrometer was available to properly measure the amoebas forms, which can produce a misdiagnosis with other similar *Entamoeba* species such as *E. coli*, *E. hartmanni* or others, and not only with species being identical morphologically, such as *E. dispar*, and *E. moshkovskii* (Calegar *et al.* 2016; Tanyuksel and Petri 2003).

Our results are similar to other studies performed in Egypt where the presence of the *E. histolytica* / *E. dispar* complex ranges between 22.2 to 10.8%, depending on the method used, microscopy, immunochromatographic assay or ELISA (Banisch *et al.* 2015; Ibrahim *et al.* 2015; Leiva *et al.* 2006; Nazeer *et al.* 2013; Uslu *et al.* 2016; Van den Bossche *et al.* 2015). However, they showed a higher proportion of *E. histolytica* than others. Ibrahim *et al*. (2015), using a specific ELISA test, detected only a 3.6% of *E. histolytica* and Nazeer *et al*. (2013) detected only a 2% of *E. histolytica* using a multiplex real-time PCR. It is very difficult to assess the accuracy of our data and our method because any meaningful evaluation inevitably involves comparison with other methods of diagnosis, which may themselves be wrong. In any case, all controls and known samples gave the expected results.

**Conclusions**

In the absence of an easy reproducible test for the detection of *E. histolytica* other than the microscopic examination, most of the reported cases would have been over estimated infection and treated unnecessarily with anti-amoebic drugs. We therefore, recommend the use of molecular assays based on PCR amplification to confirm the diagnosis of *E. histolytica* infection. Methods must incorporate an internal amplification control to discard cases of PCR inhibition.

**Acknowledgement.** This work was supported by the Spanish Agency of International Cooperation and Development (AECID) grant number A1/0355539/11 and by the AESI-ISCIII grant number PI14CIII/00014.

## References

Ali I.K. 2015. Intestinal *amebae*. *Clinics in Laboratory Medicine*, 35, 393–422. DOI: 10.1016/j.cll.2015.02.009

Ali I.K., Clark C.G., Petri W.A. 2008. Molecular epidemiology of amebiasis. *Infection Genetics and Evolution*, 8, 698–707

Banisch D.M., El-Badry A., Klinnert J.V., Ignatius R., El-Dib N. 2015. Simultaneous detection of *Entamoeba histolytica*/*dispar*, *Giardia duodenalis* and cryptosporidia by immunochromatographic assay in stool samples from patients living in the Greater Cairo Region, Egypt. *World Journal of Microbiology and Biotechnology*, 31, 1251–1258. DOI: 10.1007/s11274-015-1875-5

Bastien P., Procop G.W., Reischl U. 2008. Quantitative Real-Time PCR is not more sensitive than "conventional" PCR. *Journal of Clinical Microbiology*, 46, 1897–900. DOI: 10.1128/JCM.02258-07

Blessmann J., Buss H., Nu P.A., Dinh B.T., Ngo Q.T., Van A.L., Alla M.D., Jackson T.F., Ravdin J.I., Tannich E. 2002. Real-time PCR for detection and differentiation of *Entamoeba histolytica* and *Entamoeba dispar* in fecal samples. *Journal of Clinical Microbiology*, 40, 4413–4417

Calegar D.A., Nunes B.C., Monteiro K.J., Santos J.P., Toma H.K., Gomes T.F., Lima M.M., Bóia M.N., Carvalho-Costa F.A. 2016. Frequency and molecular characterisation of *Entamoeba histolytica*, *Entamoeba dispar*, *Entamoeba moshkovskii*, and *Entamoeba hartmanni* in the context of water scarcity in northeastern Brazil. *Memórias do Instituto Oswaldo Cruz*, 111, 114–119. DOI: 10.1590/0074-02760150383

Forsell J., Koskiniemi S., Hedberg I., Edebro H., Evengård B., Granlund M. 2015. Evaluation of factors affecting real-time PCR performance for diagnosis of *Entamoeba histolytica* and *Entamoeba dispar* in clinical stool samples. *Journal of Medical Microbiology*, 64, 1053–1062. DOI: 10.1099/jmm.0.000129

Fotedar R., Stark D., Beebe N., Marriott D., Ellis J., Harkness J. 2007. PCR detection of *Entamoeba histolytica*, *Entamoeba dispar*, and *Entamoeba moshkovskii* in stool samples from Sydney, Australia. *Journal of Clinical Microbiology*, 45, 1035–1037

Hamzah Z., Petmitr S., Mungthin M., Leelayoova S., Chavalitshewinkoon-Petmitr P. 2010. Development of multiplex real-time polymerase chain reaction for detection of *Entamoeba histolytica*, *Entamoeba dispar*, and *Entamoeba moshkovskii* in clinical specimens. *The American Journal of Tropical Medicine and Hygiene*, 83, 909–913. DOI: 10.4269/ajtmh.2010.10-0050

Ibrahim S.S., El-Matarawy O.M., Ghieth M.A., Abu Sarea E.Y., El-Badry A.A. 2015. Copro prevalence and estimated risk of *En-*

Author's copy

*tamoeba histolytica* in Diarrheic patients at Beni-Suef, Egypt, *World Journal of Microbiology and Biotechnology*, 31, 385–390. DOI: 10.1007/s11274-014-1791-0

Khairnar K., Parija S.C. 2007. A novel nested multiplex polymerase chain reaction (PCR) assay for differential detection of *Entamoeba histolytica*, *E. moshkovskii* and *E. dispar* DNA in stool samples. *BMC Microbiology*, 7, 47

Leiva B., Lebbad M., Winiecka-Krusnell J., Altamirano I., Tellez A., Linder E. 2006. Overdiagnosis of *Entamoeba histolytica* and *Entamoeba dispar* in Nicaragua: a microscopic, triage parasite panel and PCR study. *Archives of Medical Research*, 37, 529–534

Nazeer J.T., El Sayed K.K., von Thien H., El-Sibaei M.M., Abdel-Hamid M.Y., Tawfik R.A., Tannich E. 2013. Use of multiplex real-time PCR for detection of common diarrhea causing protozoan parasites in Egypt. *Parasitology Research*, 112, 595–601. DOI: 10.1007/s00436-012-3171-8

Nazemalhosseini M., Nochi E., Sahebekhtiari N., Rostami Nejad M., Dabiri, H., Zali M.R., Kazemi B., Haghighi A. 2010. Discrimination of *Entamoeba moshkovskii* in patients with gastrointestinal disorders by single-round PCR. *Japanese Journal of Infectious Diseases*, 63, 136–138

Sharbatkhori M., Nazemalhosseini M., Cheraghali F., Maghsood-loorad F.S., Taherkhani H., Vakili M. 2014. Discrimination of *Entamoeba* spp. in children with dysentery. *Gastroenterology and Hepatology from Bed to Bench*, 7,164–167

Skappak C., Akierman S., Belga S., Novak K., Chadee K., Urbanski S.J., Church D., Beck P.L. 2014. Invasive amoebiasis: a review of *Entamoeba* infections highlighted with case reports. *Canadian Journal of Gastroenterology and Hepatology*, 28, 355–359

Tanyuksel M., Petri W.A. 2003. Laboratory diagnosis of amebiasis. *Clinical Microbiology Reviews*, 16 , 713–729

Uslu H., Aktas O., Uyanik M.H. 2016. Comparison of Various Methods in the Diagnosis of *Entamoeba histolytica* in Stool and Serum Specimens. *The Eurasian Journal of Medicine*, 48, 124–129. DOI: 10.5152/eurasianjmed.2015.0074

Van den Bossche D., Cnops L., Verschueren J., Van Esbroeck M. 2015. Comparison of four rapid diagnostic tests, ELISA, microscopy and PCR for the detection of *Giardia lamblia*, *Cryptosporidium spp.* and *Entamoeba histolytica* in feces. *Journal of Microbiological Methods*, 110, 78–84. DOI: 10.1016/j.mimet.2015.01.016

Verweij J.J., Blangé R.A., Templeton K., Schinkel J., Brienen E.A., van Rooyen M.A., van Lieshout L., Polderman A.M. 2004. Simultaneous detection of *Entamoeba histolytica*, *Giardia lamblia*, and *Cryptosporidium parvum* in fecal samples by using multiplex real-time PCR. *Journal of Clinical Microbiology*, 42, 1220–1223

Zebardast N., Yeganeh F., Gharavi M.J., Abadi A., Seyyed Tabaei S.J., Haghighi A. 2016. Simultaneous detection and differentiation of *Entamoeba histolytica*, *E. dispar*, *E. moshkovskii*, *Giardia lamblia* and *Cryptosporidium spp*. in human fecal samples using multiplex PCR and qPCR-MCA. *Acta Tropica*, 162, 233–238. DOI: 10.1016/j.actatropica.2016.07.004

**Received**: September 14**,** 2016
**Revised**: November 4, 2016
**Accepted for publication**: November 10, 2016

Author's copy