Molecular detection of Entamoeba histolytica in diarrheic fecal samples from patients attending National Hepatology and Tropical Medicine Research Institute

Azza Hasan Abbas
Medical Parasitology, Immunology, Microbiology and Molecular Biology Department, National Hepatology and Tropical Medicine Research Institute (NHTMRI), Cairo, Egypt, azzahas17@gmail.com

El Shahat Ahmed ELShahat
Medical Parasitology, Immunology, Microbiology and Molecular Biology Department, National Hepatology and Tropical Medicine Research Institute (NHTMRI), Cairo, Egypt

Heba Mohamed Abdelglil
Community and Occupational Medicine Department, Faculty of Medicine, Al Azhar University, Cairo, Egypt

Follow this and additional works at: https://jmisr.researchcommons.org/home

Part of the Diagnosis Commons, Investigative Techniques Commons, Laboratory Medicine Commons, and the Medical Sciences Commons

Recommended Citation
Abbas, Azza Hasan; ELShahat, El Shahat Ahmed; and Abdelglil, Heba Mohamed (2023) "Molecular detection of Entamoeba histolytica in diarrheic fecal samples from patients attending National Hepatology and Tropical Medicine Research Institute," Journal of Medicine in Scientific Research: Vol. 6: Iss. 1, Article 11.
DOI: https://doi.org/10.59299/2537-0928.1033

This Original Study is brought to you for free and open access by Journal of Medicine in Scientific Research. It has been accepted for inclusion in Journal of Medicine in Scientific Research by an authorized editor of Journal of Medicine in Scientific Research. For more information, please contact m_a_b200481@hotmail.com.
**ORIGINAL STUDY**

**Molecular detection of *Entamoeba histolytica* in diarrheic fecal samples from patients attending National Hepatology and Tropical Medicine Research Institute**

Azza H. Abbas\(^a\)\(^*\), ElShahat A. ElShahat\(^a\), Heba M. Abdelgilil\(^b\)

\(^a\) Medical Parasitology, Immunology, Microbiology and Molecular Biology Department, National Hepatology and Tropical Medicine Research Institute (NHTMRI), Cairo, Egypt

\(^b\) Community and Occupational Medicine Department, Faculty of Medicine, Al Azhar University, Cairo, Egypt

**Abstract**

**Background:** Amoebiasis is a worldwide disease caused by *Entamoeba histolytica* infection. The true prevalence is unknown due to the difficulty in distinguishing *E. histolytica* from nonpathogenic amoebas especially when microscopic diagnosis is performed, to overcome microscopy misidentification. Polymerase Chain Reaction (PCR) assay is necessary as a highly sensitive and specific technique.

**Objective:** To determine the value of Real-Time PCR (RT.PCR) in the diagnosis of *E. histolytica* in the stool samples of diarrheic patients attending NHTMI versus microscopy, copro-antigen, and serum antibodies detection techniques.

**Subjects and methods:** A cross-sectional study was conducted on 150 diarrheic patients. Their stool samples were examined macroscopically and microscopically (by direct smear, parasep concentration, and formol-ether methods), by coproantigen, and by RT.PCR and their blood samples were examined for serum *E. histolytica* antibodies by Indirect Hemagglutination (IHA).

**Results:** Through RT. PCR (42%) were positive samples for *Entamoeba histolytica*, microscopy detected 64.7% positive samples with a sensitivity of 100% and specificity of 61.0%, coproantigen detected 73.3% positive samples with a sensitivity of 100% and specificity of 85.1%, while serology detected 38.7% positive samples with sensitivity 92.1% and specificity 100%.

**Conclusion:** The incidence of the true *E. histolytica* is lower when specific and sensitive diagnostic methods (RT.PCR/coproantigen) are used, allowing to differentiate between pathogenic and other nonpathogenic amoebas that help to avoid the unnecessary treatment that relies on misdiagnosis as well as to reduce medical costs and hazards.

**Keywords:** Copro-antigen, *Entamoeba histolytica*, Microscopy, Real-time PCR, Serology

1. **Introduction**

*Entamoeba histolytica* (*E. histolytica*) is an anaerobic protozoan intestinal parasite belonging to the genus *Entamoeba*, class Archamoeba. It is the only amoeba that is associated with pathological injuries, while other amoebic species, such as *Entamoeba dispar*, *Entamoeba moshkovskii*, *Entamoeba coli*, *Entamoeba hartmanni*, and *Entamoeba polecki* are included in *Entamoeba* genus are nonpathogenic [1]. Transmission occurs by ingestion of fecally contaminated water, food or from contaminated hands with *E. histolytica* cysts that can withstand viable in the external environment for several weeks due to its protective wall, the cysts transform to trophozoites and multiply in the small intestine,
followed by colonization, cell death, and ulcer formation in the large intestine subsequently, a new cysts formation excreted once more time in human feces [2].

E. histolytica is responsible for amoebiasis and is listed among the top 15 of diarrheal causes in developing countries, and still a challenge especially where poverty and low income are prevalent [3,4]. Annually, amoebiasis affect up to 50 million people causing death up to 100,000 worldwide [5]. The trophozoites harboring the colon as a nonpathogenic parasite, sometimes, trophozoites transferred into invasive and virulent, causing amoebic dysentery, that may migrate to the liver resulting in hepatocellular damage [6]. About 90% of the patients are asymptomatic carriers and 10% presented with clinical symptoms [5]. Amoebic Liver Abscess (ALA) is the most common clinical extraintestinal amoebiasis, delayed diagnosis or treatment may be fatal [7]. No vaccine is available to prevent this infection [8].

Diagnosis of intestinal amoebiasis is based on microscopy, immunological and molecular diagnostic methods. The gold standard in diagnosis in developing countries is the microscopic stool examination. The major drawback of microscopy is the challenge for an inexperienced technician to differentiate between pathogenic and nonpathogenic species [9]. Molecular diagnosis is highly sensitive and specific; the financial cost is an obstacle in most of countries. Patients with abdominal pain and watery or bloody diarrhea should be suspected as amoebiasis, there is a great difference in the laboratory diagnosis in patients with extraintestinal from those with intestinal amoebiasis in two major points; first, most extraintestinal amoebic patients, especially ALA have no concurrent amoebic colitis, so, stool analysis is not recommended for ALA patients, unless there are intestinal symptoms. Second, most of intestinal amoebic patients have developed IgG antibodies that persist for years, so, diagnosis depending on IgG antibody detection shows a challenge due to the difficulty in distinguishing past from current infections, [10,11].

3. Subjects and methods

The current study is a cross-sectional study, was conducted on randomly selected Egyptian diarrheic patients attending NHTMRI from the period between December 2018 and November 2019. After the application of inclusion criteria, the number of studied subjects was 150 patients. Ethical approval was obtained from the Ethics Committee of the General Organization for Teaching Hospitals and Institutes. Written consent was taken from each patient.

3.1. Samples collections

(1) Fresh stool samples were collected in labeled, dry, clean, and sterile containers. Each sample was divided into three subsamples: 1st, was immediately examined macroscopically/microscopically, the 2nd, for copro-antigen, and the 3rd, for Real-Time PCR.

(2) Blood samples: Five ml of peripheral blood was collected from each patient, then centrifuged to separate serum that was kept in cryotubes and preserved at −20°C until examined for E. histolytica antibodies.

3.2. Inclusion criteria

Patients’ stool and blood samples for those who had positive stool samples for E. histolytica/dispar with no co-infection, or negative for any parasitic infections were included in the study.

3.3. Procedures and examinations

3.3.1. Macroscopic examination

The visible fecal blood, mucus, pus, worms, larvae, and consistency were detected.

3.3.2. Microscopic examination

All stool samples were examined microscopically (immediately without delay for diagnosis of E. histolytica/dispar cysts or trophozoites, otherwise, trophozoites identification may become impossible as the amoebae lose its motility, round up, and extrude food vacuoles containing red cells, and round up) by using the following methods:
(1) Direct smear: Two slides for each sample, 1st slide was stained with iodine, and the 2nd slide was stained by Eosin (for easy detection of amoebic motility by providing pink background; as Eosin does not stain living amoeba), and by Formol-Ether concentration methods, Garcia et al. [12]. Microscopic examination using the 10× objective with sufficient closure for condenser iris to obtain good contrast, then examination with the 40× objective to identify *E. histolytica* trophozoites motility.

(2) Parasep concentration tubes, examined by FE5 fecal parasite workstation, preparation, and examination were done following the manufacturers instructions.

NB: - Microscopic examination for the 167 stool samples revealed; 97 stool samples proved to be with *E. histolytica/dispar* with no other co-infection, 17 stool samples with mixed parasitic infection, and 53 stool samples negative for any parasitic infection.

3.3.3. Coproantigen detection

Using RIDA quick *E. histolytica* (N1703), which is an immunochromatographic qualitative rapid test, commercially available by R–Biopharma AG – Germany. Analytic procedures and interpretation were done according to the manufacturers.

3.3.4. Real-time PCR

Test procedures and interpretations were done according to the manufacturers on Qiasymphony – rotor gene system.

The 18S ribosomal RNA (18S) gene Kit, and the Primer for *E. histolytica*, with a broad detection profile kit, specifically, the primers that represent 100% homology and over 95% of the National Center for Biotechnology Information (NCBI) database reference sequences contained in the NCBI Database.

The concentrate was taken from each stool sample and subjected to DNA extraction individually using QIAamp DNA stool mini kit. DNA internal extraction control can be added to the DNA lysis/extraction buffer or to the DNA sample after its resuspension in the lysis buffer.

Mostly, DNA extraction has an exogenous source of DNA template which is added to the lysis buffer, the DNA control is co-purified with the sample DNA and used as a positive control. Successful co-purification and quantitative PCR (qPCR) for the control DNA indicate the absence of PCR inhibitors at a significant concentration.

The specific primer and probe for the *E. histolytica* set included a forward primer: *Entamoeba* (ATGCACGAGACG AAAGCAT) and the reverse primer was *E. histolytica* (GATC-TAGAAACAATGCTTCTCT), Hamza et al. [13].

During the ongoing amplification, forward and reverse primers hybridize to the DNA of *E. histolytica*. A fluorogenic probe is also included in the same reaction mixture that consists of a DNA probe which is labeled with a 5’-dye and a 3’-quencher. The probe is cleaved with the separation of the reporter dye and quencher. Which results in increasing in the fluorescence that can be detected on a range of qPCR platforms.

3.3.5. Serological examination

Using the Indirect Hemagglutination test (IHA), a quantitative test, detect anti – *E. histolytica* antibodies, using commercial kits by FUMOUZE DIAGNOSTICS, France.

Test procedures and interpretations were guided by the manufacturers instructions as the following:

(1) Titer <1: 80 = negative reaction.
(2) Titer ≥160 = significant reaction in favor of visceral amoebiasis.

3.4. Statistical analysis

The chi-squared ($\chi^2$) test was used to determine the statistical significance of the data. A *P*-value < 0.05 was considered statistically significant.

4. Results

*Tables 1 and 2, Figs. 1 and 2.*

**Table 1. Comparison of direct smear, Parasep concentration versus formol-ether methods in diagnosis of intestinal *E. histolytica/dispar.***

<table>
<thead>
<tr>
<th>Method used Results of microscopy</th>
<th>Direct smear</th>
<th>Parasep concentration</th>
<th>Formol-Ether</th>
<th>Test of sig. &amp; <em>P</em> value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. = 150</td>
<td>% 100</td>
<td>No. = 150</td>
<td>% 100</td>
</tr>
<tr>
<td>Cysts only</td>
<td>70</td>
<td>46.7</td>
<td>75</td>
<td>50.0</td>
</tr>
<tr>
<td>Trophozoites only</td>
<td>20</td>
<td>13.3</td>
<td>12</td>
<td>8.0</td>
</tr>
<tr>
<td>Cysts and trophozoites</td>
<td>7</td>
<td>4.7</td>
<td>4</td>
<td>2.6</td>
</tr>
<tr>
<td>Negative</td>
<td>53</td>
<td>35.3</td>
<td>59</td>
<td>39.4</td>
</tr>
</tbody>
</table>

*P value < 0.05 = statistically significant.
5. Discussion

"E. histolytica (E. histolytica) infection is a major diarrhea-causing parasite. Microscopic examination is still the most commonly used diagnostic method in developing countries. Replacing microscopy by more sensitive/specific methods is hampered by the cost, although microscopy lacks sensitivity and specificity. Accurate diagnosis allowing to distinguish pathogenic from nonpathogenic species, which is crucial in determining the true incidence of E. histolytica in the community.

Table 2. Sensitivity, specificity, positive and negative predictive values of microscopy versus copro-antigen and Real-Time PCR in diagnosis of E. histolytica in diarrheic stool samples.

<table>
<thead>
<tr>
<th>Method</th>
<th>Result</th>
<th>Microscopy E. histolytic/dispar</th>
<th>E. histolytica Copro- Ag</th>
<th>E. histolytica serum Abs</th>
<th>Real-Time PCR for E. histolytica</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No.150</td>
<td>%</td>
<td>No.150</td>
<td>%</td>
<td>No.150</td>
</tr>
<tr>
<td>Positive</td>
<td>97</td>
<td>64.7</td>
<td>110</td>
<td>73.3</td>
<td>58</td>
</tr>
<tr>
<td>Negative</td>
<td>53</td>
<td>35.3</td>
<td>40</td>
<td>26.7</td>
<td>92</td>
</tr>
</tbody>
</table>

E. histolytica Copro antigen detection was performed for 150 samples (97/150) were E. histolytica. /E.dispar positive and (53/150) were negative by microscopy, copro antigen was positive for 110/150, and it was negative for 40/150. RT.PCR. detected 63/150 samples with true E. histolytica infection and 87/150 samples negative for E. histolytica Table 3.

Table 3. Sensitivity, specificity of microscopy versus copro-antigen in detection of E. histolytica in stool samples of diarrheic patients (150 stool samples).

<table>
<thead>
<tr>
<th>Variables</th>
<th>Sensitivity</th>
<th>Specificity</th>
<th>PPV</th>
<th>NPV</th>
</tr>
</thead>
<tbody>
<tr>
<td>Microscopy</td>
<td>100%</td>
<td>61.0%</td>
<td>35.1%</td>
<td>85.6%</td>
</tr>
<tr>
<td>copro antigen</td>
<td>100%</td>
<td>85.1%</td>
<td>42.7%</td>
<td>92.9%</td>
</tr>
<tr>
<td>Serologic examination</td>
<td>92.1%</td>
<td>100%</td>
<td>74.8%</td>
<td>5.4%</td>
</tr>
</tbody>
</table>

Most of the prescriber amoebic treatment depends on microscopic examination, so, specific, sensitive, and efficient differentiating laboratory diagnostic methods are essential to avoid unnecessary treatment [14]. In the current study, 150 stool samples from diarrheic patients were examined by microscopy, coproantigen, and by RT.PCR. blood samples for E. histolytica. The results indicated that among 150 diarrheal stool samples; 64.7% were positive for E. histolytica/dispar microscopically; the Parasep concentration method showed the highest rate of E. histolytica/E. dispar cysts (50%) followed by direct smear (46.7%) and lastly by formol-ether (34.7%) with high statistically significant differences. Direct smear showed the highest rate in detection of both cysts/trophozoites (4.7%) followed by the Parasep method (2.6%). Direct smear showed the highest rate in detection of trophozoites (13.3%)

Fig. 1. Microscopy versus copro antigen, and serology in relation to molecular RT. PCR in detection of E. histolytica.

Fig. 2. Distribution of fecal blood/mucus in positive patient’s samples by RT. PCR and macroscopically/microscopically.
followed by Parasep (8%), then formol-ether (2%), similarly Boucke et al. [15] could detected *trophozites by direct smear*, in contrast, Nazeer et al. [16] found that formol-ether exhibited a higher statistical significant difference in the detection of *E. histolytica/dispar*, while Al-Areequi et al. [17] stated that trichrome staining and formol-ether were the gold standard techniques in fecal examination.

RT.PCR. revealed 42% positive and 85% negative results for *E. histolytica*. Microscopy stated that *Entamoeba histolytica/E. dispar* detection was positive for 64.7% with a sensitivity of 100% and specificity of 61%, while *histolytica* copro antigen was positive for 73.3%, with a sensitivity of 100% and specificity of 85.1% and positive predictive value was 42.7%. Serology reported that 38.7% of serum samples were positive (titer<1:180) indicating visceral amoebiasis and 61.3% were negative (titer <1:180), with a sensitivity of 92.1% and specificity of 100%. With a negative predictive value (5.4%), similarly, Beyls et al. [11] stated that *E. histolytica* antibodies had a sensitivity of 95% and specificity of 94%, concluded that *E. histolytica* serum antibodies detection is useful to exclude a potential diagnosis of amoebiasis and could be used as a screening diagnostic method.

Comparing microscopy with RT PCR is the number of positive samples by RT.PCR were much lower than by microscopy, 63 versus 97, similarly Hamza et al. and Sharbat Khori et al. [18,19] detected 37 positive cases versus 194 for *E. histolytica* by PCR and microscopy respectively explaining that the high discrepancies were due to the potential PCR inhibitory materials that may be present in the stool samples or due to the using of suboptimal microscopically equipment. The current results detected that microscopy showed many false positive/negative results that gave lower specificity (61%) as well as positive/negative predictive values, similarly, Stark et al. and Haque et al. [20,21] estimated a lower sensitivity of microscopic examination (10.8%) compared to RT.PCR in the detection of *E. histolytica*; less than one-fifth of these cases (2%) were truly *E. histolytica* by RT.PCR., without detection of *E. histolytica* DNA in diarrheic stool samples, as *E. dispar* is none pathogenic, and the presence of *E. histolytica* DNA in the stool is an indicator for association rather than being an etiologic cause. Also, Hamza et al. [18], reported that many false positive/negative results were obtained by microscopic examination, with a low positive predictive value of 18.6%, explaining that these results are due to misdiagnosis of other *Entamoeba species such as E. coli or Entamoeba hartmani* or due to the morphologically identical as *E. moshkovskii*. Al-Areequi et al., [17], stated that molecular assay could overcome the limitation of the prevalence of *E. species* due to the temporal variation in cyst shedding over hours and day. In Egypt, Ibrahim et al., [22], stated that *E. histolytica/dispar* was 22.2%–10.8% according to the type of laboratory techniques. The current results by RT.PCR revealed that 34 samples were negative for *E. histolytica* were detected positive by microscopy, this may be due to the low parasite concentration in the stool samples or those samples were *E. species* other than *E. histolytica*, similarly Bastien et al. and Rosshdy et al. [23,24] found *E. histolytica* in 19/194 and in 11/194 by RT.PCR respectively with seven cases missed (one case *E. histolytica*, six cases *E. dispar*) were detected by Nested Multiplex PCR, explaining these discrepancies due to the lower sensitivity of the RT.PCR method used or due to low parasitic fecal concentration especially in *E. dispar*.

Among RT.PCR. Positive samples, nearly one-third (30.2%) with fecal blood only, (22.2%) with both blood and mucus, while among positive samples macroscopically/microscopically, there were nearly one-fifth (22.7%) with fecal blood only, 18.6% with mucus, and 39.2% with both blood and mucus, with statistically significant differences. These are clearly significant predictors of true *E. histolytica* infection, also, Boucke et al. [15] estimated that although fecal blood or mucus are not frequent in true amoebic infection, but they are almost never present in *E. dispar* infection, concluded that the presence of fecal blood/mucus in a patients samples, should be treated as amoebic dysentery if *E. histolytica/dispar* cyst/are detected microscopically.

5.1. Conclusion

The current study had shown that; perspectives, proper diagnosis of *E. histolytica* infection is very crucial to avoid misdiagnosis or unnecessary treatment that may lead to disease transmission, morbidity, or even death.

Microscopic examinations using different methods and copro-antigens as well as serum antibodies are needed especially when there is a lack of financial resources, or where highly sensitive and specific laboratory techniques are not available.

With a recommendation for pooling stool samples of diarrheic patients at high risk to a reference molecular laboratory to reduce the running cost of the test.

Conflicts of interest

None declared.