Prevalence and species distribution of *E.histolytica* and *E.dispar* in symptomatic and asymptomatic patients

Buthenia Abdul Hamza, Ekhlas Mushref, Majeed Arsheed Sabbah

Abstract

The objectives of this study were shed light to determine the prevalence and differential detection of two species *Entamoeba histolytica* (pathogenic) and *Entamoeba dispar* (non-pathogenic) that were morphologically identical as both cysts and trophozoite in two different groups, the first groups includes stools of fifty patient have diarrhea and abdominal pain (symptomatic) and ninety five patient have no diarrhea and abdominal pain (asymptomatic), who attending the AL-alwyia childhood Teaching Hospital and AL-zafaranyia General Hospital in Baghdad were collected during the period from the beginning of July 2011 to the end of May 2011. The polymerase chain Reaction (PCR) was used to identify the *Entamoeba* species, *E.histolytica* (pathogenic) and *E.dispar* (non-pathogenic) by amplification DNA sequences of two genes, cystein proteinase (EhCP) gene, present only in *E. histolytica* and Actin gene (Act) which is present in both *E. histolytica* and *E. dispar*. The Results showed that both parasites were presents in both groups of patients and the percentage of *E. dispar* was higher than *E.histolytica* in two groups symptomatic and asymptomatic. In conclusion, it should not depend on direct wet mount technique identification of *Entamoeba* and it should be used PCR for exact identification of both species *E. histolytica* and *E. dispar* in the diagnosis of amoebic dysentery.

Key word: Prevalence, distribution, *E.histolytica*, *E.dispar*, symptomatic, asymptomatic.

Introduction:

Amoebiasis is an important public health problem in developing countries and it’s the third cause of death among parasite disease. Approximately people die yearly due to this parasite infection worldwide [1]. The percentage of the world population infected by *Entamoeba histolytica* is calculated to be 10% and 90% of these individuals are not
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Symptomatic [\(^7\)]. It has been known that many people who are apparently infected with *E. histolytica* never develop symptoms and spontaneously clear their infection. Differential diagnosis of *E. histolytica* and *E.dispar* in stool samples is not easy on the basis of microscopy alone. Currently some expensive methods such as amoebic antigen and DNA detection, isoenzyme electrophoretic pattern, PCR-basis methods, are available to differentiate both non pathogenic *E. dispar* from pathogenic *E. histolytica* [\(^74,45423\)]. In Iraq, there have been several studies on the prevalence of *E.histolytical/E.dispar* complex which used morphological or ELISA Technique [\(^3,7\)] the aim of this study, however, was to address the prevalence of *E.histolytica* and *E.dispar* in stool samples of patients with symptomatic and asymptomatic diarrhea using PCR.

**Materials and Methods**

**Collection sample & Microscopy**

A cross-sectional study was conducted on 160 stool samples collected from fifty patients with symptoms diarrheas, Ninety five patients with a symptom diarrheas and twenty healthy individuals as a control. The samples collected from the childhood Teaching Hospital and AL-Zafaranyia General Hospital in Baghdad Governorate, during July 2011 to the end May 2014. Stool was collected with a clean and labeled container and patients were asked and instructed on how to bring approximately 7 g of stool, which was enough for direct saline [\(^1\)].

**DNA Isolation**

All DNA isolation procedures were carried out in a biological safety cabinet in a room physically separated from that used to set up nucleic acid amplification and also from the "post-PCR" room, in order to minimize contamination and hence the possibility of false positive results. Parasites genomic DNA was extracted from stool samples by using Genomic DNA Purification kit Accuprep® stool DNA Extraction kit (Bioneer, Korea) according manufacture instructions.

**PCR assays**

Specific primers were used for PCR analysis of the two genes sequences, the internal segment of the cystein proteinase \(^5\) (EhCP\(^5\)) gene, present only in *E. histolytica* and Actin gene which is present in both *E. Histolytica* and *E. dispar* is shown in Table 2. These primers synthesized by Alpha DNA Company, Canada.

PCR reaction was conducted in 25 μl of a reaction mixture containing 25 μl Go Taq® Green Master (Promega, USA), 1 μl of 100 pmol of each of primer (Ehcp\(^5\)+Act ) ,7 μl of DNA template, 1 μl MgCl\(_2\) and 17 μl of Deionized water. Negative control (containing Deionized water instead of DNA template)
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amplification was included in every set of PCR reactions. Amplification was conducted using a Master cycler (Eppendorf) programmed with 1 cycle at 95°C for 5 min; 3 cycles of 93°C for 1 min, 99°C for 1 min, 72°C for 2 sec; 72°C for 1 min. The amplified products were subjected to 2% Agarose gel electrophoresis, and visualized under UV (Image master VDS, Pharmacia Biotech, USA) after Ethedium bromide staining.

**Table 1**: Primers used for the amplification of cystein proteinase (CP) & actin genes.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primers</th>
<th>Primer sequence (5’–3’)</th>
<th>Amplicons size bp</th>
<th>GenBank accession number</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>CP</td>
<td>Forward</td>
<td>5’GTTGCTGCTGAAGAAACTGG</td>
<td>242</td>
<td>183223401/183223401/740842/74</td>
<td>[4,14]</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’GTACCATAACCAACTACTGG</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Actin</td>
<td>Forward</td>
<td>5’GGGACGTATGGAAGACTGGTC</td>
<td>300</td>
<td>1608423/167642303/104042381/167642381/167642381</td>
<td>[11,12,13]</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>5’CAAGTCCTAAGGAATAGCATGG</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Statistical Analysis**

Only Chi-square was used for the statistical analysis to analyze the results and comparing between the rates of parasites isolation from stool as well as evaluating the variances between wet mount method and PCR method in identification of *E.histolytica* and *E.dispar* (SAS, 9.6.4) as in the following equation:

\[ \chi^2 = \sum \frac{(o - e)^2}{e} \]

**Result**

**Microscopic examination**

According to microscopic examination, the results showed that 77(13.74) of patient with symptoms and 66(17.3) patients with a symptom were infected with *E.histolytica/E.dispar*. The presence of both amoeba species in both groups (symptom &a symptom) were statically significant (p<0.01), Table 3.

**Table 3**: Distribution of *E.histolytica* / *E.dispar* in the two groups of patients (symptom and a symptom) of selected positive stool samples, according to microscopic examination.

<table>
<thead>
<tr>
<th>Patients groups</th>
<th>NO.</th>
<th>Microscopic examination(Wet mount) for identical <em>E.histolytica/E.dispar</em></th>
<th>Chi-square ((\chi^2))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symptom</td>
<td>30</td>
<td>(%54, 3.5)</td>
<td><strong>(p&lt;0.01)</strong></td>
</tr>
<tr>
<td>A symptom</td>
<td>95</td>
<td>(%32, 9.3)</td>
<td></td>
</tr>
</tbody>
</table>
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**DNA Isolation:**
One hundred and seven isolates, 67 symptom and 61 a symptom patients and twenty fecal samples were collected from healthy individuals as a control, were subjected to DNA isolation. Sharp bands for genomic DNA were obtained for each preparation, the band intensity varied according to concentration of each preparation as shown figure 1.

**Duplex PCR (dPCR)**
Two DNA sequences were amplified, cystein proteinase gene (1,111bp) present only in *E. histolytica* and Actin gene (7,111bp) present both in *E. histolytica* and *E. dispar*, Figure 2.

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**Figure 1:** An Ethedium bromide stained Agaros gel (70˚C) electrophoresis (70 vol for 7 hour) of genomic DNA isolated from stool samples (Lanes: 1-10) collected from symptomatic and asymptomatic patients. M: 100 bp ladder. (Lanes: C1-C4) negative fecal sample by containing distilled water instead of DNA template.

**Figure 2:** An Ethedium bromide stained Agaros gel (70˚C) electrophoresis (70V, 120 min) duplex PCR results showing diagnostic differentiation of *Entamoeba histolytica* from *E. dispar*. The amplicons from the Actin gene, common to both amoebae, and the Ehcp specific to *E. histolytica*, are identified as 711bp and 242bp, respectively. Lane M is

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- bp DNA ladder marker. Other lanes are: (1,2,4,6,8) samples for *E. dispar*; (C) negative fecal sample by (containing distilled water instead of DNA template). *E. histolytica* was identified in direct samples (3,5,7,9).

The dPCR analysis showed that \( \frac{39}{160} \) of stool samples were infected with *E. histolytica/E. dispar*, \( \frac{7}{23} \) (\( \frac{24}{3} \)) infected with *E. dispar* (only Actin gene amplified) and \( \frac{9}{21} \) (\( \frac{11}{3} \)) were infected with *E. histolytica* (both genes Ehcp\(^\circ\) and Actin amplified). Table 7. Which showed a statistically significant?

Table 7: Distribution of *E. histolytica*/*E. dispar* in the two groups of patients (symptom and a symptom) according to dPCR assay which amplified both genes (Ehcp\(^\circ\) and Act).

<table>
<thead>
<tr>
<th>Patients groups</th>
<th>NO. Sample s</th>
<th>Actin gene only</th>
<th>Both genes (Cystein proteinase(^\circ) gene &amp; Actin gene)</th>
<th>Chi-square(( \chi^2 ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Symptom</td>
<td>19</td>
<td>14 (( \frac{24}{7} )) 15 (( \frac{26}{2} ))</td>
<td>** 8,74 **</td>
<td></td>
</tr>
<tr>
<td>A symptom</td>
<td>40</td>
<td>17 (( \frac{15}{8} )) 3 (( \frac{15}{1} ))</td>
<td>** 9,16 **</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>59</td>
<td>31 (( \frac{29}{5} )) 18 (( \frac{20}{1} ))</td>
<td>** 8,89 **</td>
<td></td>
</tr>
</tbody>
</table>

**Microscopic results comparison to the dPCR technique.**

Microscopically result was classified according to the wet preparation. This result was compared with the positive sample result of dPCR. There was statistically significant(\( P<0,01 \)) and negative sample result of dPCR was statistically significant(\( P<0,05 \)) respectively symptom and a symptom group, and control positive result negative for microscopic and dPCR with infected *Entamoeba* sp and was no statistically significant between present *E. histolytica/E. dispar* showed Table 8.

Table 8: The number and percent of positive sample for dPCR for positive and negative of wet mount microscopic examination.
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<table>
<thead>
<tr>
<th>sample</th>
<th>Positve sample</th>
<th>Negative sample</th>
<th>No. 95</th>
<th>No. 77</th>
</tr>
</thead>
<tbody>
<tr>
<td>A symptom</td>
<td>(74.6, 0.0) 33</td>
<td>(67.6, 0.4) 16</td>
<td>(1.9, 0.7) 15</td>
<td>7.86 **</td>
</tr>
</tbody>
</table>

Discussion:

In this report, we used duplex PCR assay for differential diagnosis & prevalence of two species of *Entamoeba*, *E.histolytica* and *E.dispar* which share identical morphology. Recently, differences between *E.histolytica* and *E.dispar* in expression patterns of protein thought to be involved in the virulent behavior of *E.histolytica* [8]. So two primer used for amplification of two genes cystein proteinase (*Ehcp* 5) found only in *E.histolytica* and Actin gene found in both species *E.histolytica* and *E.dispar* [8].

The results of this study showed the result by microscopy indicates that 74% (33/45) and 67% (16/24) of the 265 symptomatic and asymptomatic to be infected with *E.histolytica/E.dispar*. But the species –specific diagnostic with *E.histolytica* found only in 1/11 (9.0%) and *E.dispar* found in 72/117 (3.1%) sample of the 2,537 symptomatic & asymptomatic. So that results indicated that the assay successfully amplified the positive sample with *E.histolytica* & *E.dispar* from each group (symptom & asymptomatic). Also, the result showed that the percentage of *E.dispar* were more than the *E.histolytica* and close to the results of international studies, however, percentages may differ slightly due to time of study, location, sex, age and epidemiology [10,15,16].

Presence of this parasite in asymptomatic patients refers to the need for performing the diagnostic tests in symptomatic in addition to asymptomatic patients.

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non-significant = NS, significant = (P<0.05)*, Higher significant = (P<0.01) **

<table>
<thead>
<tr>
<th>sample</th>
<th>Positve sample</th>
<th>Negative sample</th>
<th>No. 95</th>
<th>No. 77</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>NS</td>
</tr>
</tbody>
</table>

**NS**
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الخلاصة

تهدف الدراسة الحالية لتسليط الضوء على تحديد وتشخيص نسبة الإصابة بنوعين من الاميبا *E.histolytica* والانسجة المتغيرة (الغري مرارية) في المرضى العرضي واللاعرضيين، والذان يحتويان بشكلهما المتماثل في كل الطورين الكسي والخلاقي في مجموعتين مختلفتين. المجموعة الأولى تضمنت 50 عينة لمرضى يعانون من آلام في البطن، والذين تم تجميعهم بشكل متساوي من المسلمين والمسلمين فيобрان من مجموعات الإصابات، وتتضمن 50 عينة لمرضى لا يعانون من آلام البطن في المجموعة الثانية. تم استخدام طفيلي الاميبا بمنطقة الفحص الرطب وتقنية التفاعل التسلسل الضخمي المزدوج dPCR assay من خلال *E.dispar* وفي تشخيص طفيلي الاميبا المرارية *E.histolytica*, والذي يدل وجودة على *gene(Ehcp)* والغير مرارية *Actin gene(Act)*، الذي يتواجد في كل الاميبا الحالة الساطعة للنسل أما الجين الآخر فيمثل الاميبا المتغيرة وتحتاج إلى تأكد العينة من كلا النوعين في كلا المجموعتين. الدراسة آلية لاستخدام تقنية PCR لتشخيص الاميبا الجلية في النسل والاميبا المتغيرة. نستنتج من هذه الدراسة أنه لايمكن الاعتماد على الفحص الرطب في تشخيص الاميبا ولغرض تميز كلا النوعين من خلال *E.dispar*.