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MOLECULAR IDENTIFICATION OF SOME SPECIES OF *ENTAMOEBA* ISOLATED FROM PATIENTS WITH DIARRHEA IN AFAK CITY/ AL-QADISIYAH GOVERNORATE USING REAL-TIME PCR TECHNIQUE

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ABSTRACT

Objective: The study aimed to identify *Entamoeba histolytica*, *E. dispar,* and *E. moshkoviskii* isolated from patients with disturbance gastrointestinal and to compare that to Real-Time PCR technique and microscopic examination results.
Methods: 142 stool samples were obtained from patients suffering from gastrointestinal disorders, abdominal pain, and diarrhea and visiting Afak General Hospital and some civil diagnostic laboratories in the city during the period June to October 2015. The patients age ranged from 1-73 years and for both sexes.
Results: Microscopic examination results revealed that 66 samples contained the *Entamoeba* parasite with a percentage of (46.48%) while it is isolated with a percentage of (51.41%) 73 samples when examining the same samples using the RT-PCR technique. Molecular examination results proved the presence of three species of the genus *Entamoeba*, which are *E. Histolytica*, *E. dispar*, and *E. moshkoviskii* in single and mixed infections. The highest infection percentage of *E. dispar* reached (72.6%), distributed to (56.16%) of single-type infections and (16.44%) of mixed-type infections (*E. dispar* + *E. histolytica* and *E. dispar*, + *E. moshkoviskii*), followed by *E. histolytica* with a percentage of (41.1%), distributed to (26.03%) of single-type infections and (15.07%) of mixed-type infections, then of species *E. moshkoviskii* with a percentage of (2.74%) in single and mixed infections.
Conclusion: Real-Time PCR technique revealed high sensitivity in the examination of samples positive for microscopy at a percentage of (92.42%), which confirms that the identification of Amoebiasis utilizing molecular techniques is useful not only in terms of diagnosis of *Entamoeba* spp but also in epidemiological studies of Amoebiasis by avoiding possible microscopic examination mistakes.

INTRODUCTION

Amoebiasis is one of the leading causes of disease and death around the world, and it is responsible for about 40,000 to 100,000 deaths a year; this disease is a real health problem, especially in developing countries (1). Infectious Amoebiasis happens as a consequence of infection of the gastrointestinal tract by *Entamoeba histolytica*, a protozoan parasite able to invade the intestinal mucosa layer and spread to other organs, especially the liver (2). This species coexists with other species of the genus *Entamoeba*, such as *E. dispar* and *E. moshkoviskii* (3).

It is difficult to differentiate between these species due to the lack of remarkable phenotypic differences between the Trophozoite and cyst stage (4). Several research has established that *E. histolytica* is the main cause of Amoebiasis while the ability of the other two species to cause the disease is still not clear yet (5).

Because of the morphological similarities between the three species in human stool, there is an urgent need to identify them; for example, an individual may be infected with *E. moshkoviskii* or *E. dispar* and diagnosed as infected with *E. histolytica*, decreasing the importance of addressing the chemical drugs for Amoebiasis (6). Amoebiasis identification using microscopic examination is considered by the researchers qualitatively 100% for the diagnosis (7). In spite of this, the bloody diarrhea cases are few somewhat. The absence of Trophozoite stage omnivorous red blood cells limits the sensitivity of the microscope due to its insufficiency to recognize samples containing *E. histolytica* from those

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containing non-pathogenic species belonging to the genus *Entamoeba* spp. In addition, the incidence of suspicion between amoeba and leucocytes in the samples often results in a wrong diagnosis of Amoebiasis (8).

Recently, molecular biological techniques have developed to be the main way for the diagnosis and investigation of *E. histolytica*. One of these techniques is Polymerase Chain Reaction test (PCR), which is characterized by its sensitivity to diagnose the presence of a parasite amoebic dysentery even if the there is only one parasitic cell in the stool sample. PCR has also a high ability to distinguish between the non-pathogenic members belonging to the genus *Entamoeba* and the *E. histolytica*. One of the reasons that prevent the use of these tests is the exorbitant cost required for these tests as well as a high level of expertise needed (1).

The remarkable similarity between the species of the genus *Entamoeba* has raised a lot of questions about the diagnostic and epidemiological studies accuracy of Amoebiasis, making the estimate the infection rate based on traditional diagnostic methods are very misleading. If assert in its results that the pathogen is the only species *E. histolytica*. So the aim of this study is the molecular diagnosis of the three *Entamoeba* spp. (*E. histolytica*, *E. dispar*, and *E. moshkoviskii*) using Real-time PCR technique, which is considered the most modern, accurate, and sensitive method at the recent time.

MATERIAL AND METHODS

Samples collection

142 stool samples were obtained from patients suffering from gastrointestinal disorders, abdominal pain, and diarrhea and visiting Afak General Hospital and some civil diagnostic laboratories in the city during the period June to October 2015. The patients age ranged from 1-73 years and for both sexes. Stool specimens were gained in a sterile plastic box with a tight lid to keep samples moisturized. Samples were examined using optical microscopy by the direct wet swab method for half an hour after collection. All samples were stored without adding any preservatives in a freezer at a -20 C° until extracting the DNA samples and subjecting them to the molecular examination processes.

Laboratory Diagnosis

Two tests were utilized: **1. Macroscopic examination** by checking stool samples in terms of color, textures, and the existence of blood or mucus. **2. Microscopic examination** by direct wet swab method the use of physiological saline visits to investigate Trophozoites first and the Lugol's iodine solution second to stain the cyst stage, especially the nucleus, as stated in (**9**).

Diagnostics using Real-Time PCR technique

The complete sequence of the 18S rRNA gene for each species of parasite Entamoeba was obtained by using NCBI-Genbank website. The primers were designed by using IDT online website.

Genomic DNA was extracted from stool samples by using AccuPrep® Genomic DNA Extraction Kit (Bioneer , Korea) and performed as described in the manufacturer protocol.

Real-Time PCR Technique was conducted as stated in (8) by using (AccuPower® Dualstar qPCR Master Mix, kit, Bioneer. Korea) following the manufacturer instructions mentioned in the protocol.

Table 1 primers used in this research with the sequencing nucleotide.

Gene	Primer	Sequence (5'→ 3')
Entamoeba histolytica isolate EH_IQ1 18S ribosomal RNA gene, partial sequence. GenBank: **KP233836.1**	**F**	**GAATTGACGGAAGGGCACAC**
	R	**AACTAAGAACGGCCATGCAC**
Entamoeba dispar isolate ED_IQ1 18S ribosomal RNA gene, partial sequence. GenBank: **KP722596.1**	**F**	**ACCAAGACCGAACAGTAGAA**
	R	**GTTTCAGTCTCGTTCGTTAC**
Entamoeba moshkovskii isolate EM_IQ1 18S ribosomal RNA gene, partial sequence. GenBank: **KP722601.1**	**F**	**GCGGACGGCTCATTATAACA**
	R	**TCAGAATGGCATTCGCACTC**

Statistical Analysis

The results were analyzed statistically by Chi-squared test, significant results were attributed to probability values $P \leq 0.01$ by using SPSS program.

RESULTS

The microscopic examination results of showed for 142 stool samples showed that only 66 samples contained the Trophozoite or cystic stage of *Entamoeba* in the percentage (46.48%) while (53.52%) of the samples were negative for the parasite.

The molecular examination of the same samples revealed that percentage of samples positive for the parasite *Entamoeba* was (51.41%) while the percentage of samples negative was (48.59%). (Table:2).

Table 2 Infection percentage of parasitic *Entamoeba* by microscopic examination and RT - PCR results.

Methods	Positive (%)	Negative (%)
Microscopic examination	**66** (46.48%)	**76** (53.52%)
RT- PCR	**73** (51.41%)	**69** (48.59%)

The current study recorded high sensitivity using Real-Time PCR Technique in the examination of samples positive for microscopy in the percentage (92.42%) (61 samples out of 66 positive samples for microscopic examination). It is worth mentioning that there are (12 samples) showed a positive result through molecular examination although they gave negative results in the microscopic examination, and (5 samples) were positive for the microscopic examination gave a negative result in the molecular examination. Statistics shows significant differences between the two methods at α=0.01. (Table: 3).

Molecular identification using the Real Time – PCR technique

The Results proved the presence of three species of the genus *Entamoeba*, which are *E. Histolytica*, *E. dispar*, and *E.*

moshkoviskii in single and mixed infections. Statistics shows significant differences between the three species in the single and mixed infections percentage at α =0.01. (Table: 4).

Table 3 Comparison of microscopic examination and RT - PCR results

Method		Microscopic examination		
		Positive	Negative	Total
RT- PCR	Positive	61	12	73
	Negative	5	64	69
	Total	66	76	142

Table 4 The percentage of single and mixed infections of three species of the genus *Entamoeba*

The type of parasite	number of samples	The percentage
E. dispar	41	56.16 %
E. histolytica	19	26.03 %
E. histolytica+ *E. dispar*	11	15.07 %
E. moshkoviskii	1	1.37 %
E. dispar + *E. moshkoviskii*	1	1.37 %
Total	73	100 %

The results showed the presence of species *E. dispar* in 53 samples (72.6%), including 41 samples (56.16%) of single infection and 12 samples (16.44%) of mixed infection. The results in (Figure: 1) display the Amplification plot of *Entamoeba dispar* positive samples by 18S rRNA gene.

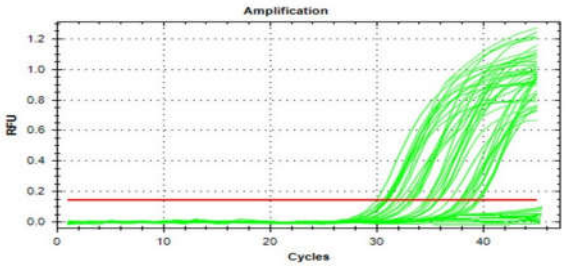

Figure 1 The Real -Time PCR Amplification plot of *Entamoeba dispar* positive samples.

The results also showed the presence of species *E. histolytica* in 30 samples (41.1%), including 19 samples (26.03%) of single infection and 11 samples (15.07%) of mixed infection. (Figure: 2).

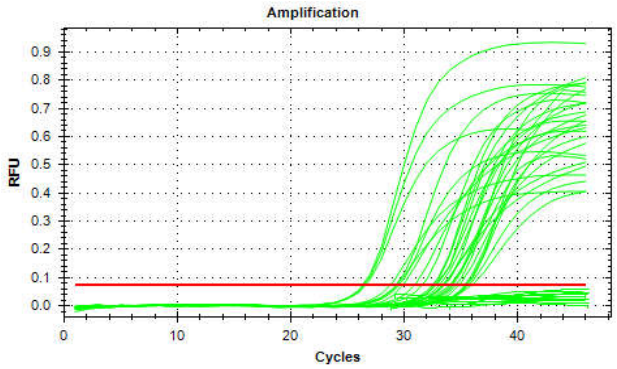

Figure: 2 The Real -Time PCR Amplification plot of *Entamoeba histolytica* positive samples.

while this study showed the presence of species *E. moshkoviskii* in two samples (2.74%) including one sample (1.37%) of both single and mixed infection. (Figure: 3).

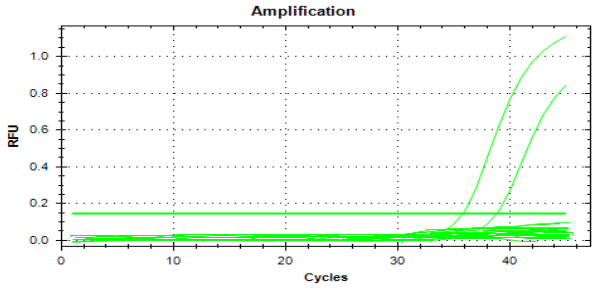

Figure 3 The Real -Time PCR Amplification plot of *Entamoeba moshkoviskii* positive samples.

DISCUSSION

Infection percentage of parasite Entamoeba using microscopic examination

The current study found the infection percentage of parasitic *Entamoeba* amounted to 46.48% (Table: 2). This percentage is similar to (10), where it was 53.18% in her study to diagnose Amebiasis in patients with gastrointestinal symptoms in an endemic region in Turkey. Our results were higher than what both (11) (2.33%) in Erbil and (12) (17.4%) in Babylon. The infection percentage in the current study was less than (13) (68%) and (8) (61.26%) in the city of Diwaniyah. The high prevalence parasite *Entamoeba* may be due to direct transmission by contaminated food and water, insufficient attention to hygiene (14), and unhealthy practices of street vendors and that cause exposure of food to contaminated dust and insects. The difference of the results in the present study compared to other studies may be probably to the difference in the sewage level, personal hygiene, population density, geographical location, climatic conditions, the total number of samples tested, the test methods (15), as well as the different duration of the study, months of the year, different age groups, and the living style.

Molecular identification using the Real Time – PCR technique

The current study revealed the infection percentage of parasitic *Entamoeba* amounted to 51.41% (Table: 2) when examining all study samples (positive and negative for microscopy). The sensitivity RT-PCR technique was very high in the examination of the positive samples for microscopic examination at a rate of (92.42%) (Table: 2), this percentage asymptotic to his record (16) (94%) when using nested multiplex PCR technique and asymptotic to (8) (88%) when using RT-PCR technique while it was higher than of (5) (81%) and (17) (69.3%) when using the conventional PCR technique.

The study also showed the presence of 5 negative samples for molecular examination despite being positive for microscopic examination (Table: 3). The study agreed with (10) who found (20 samples) negative for molecular examination out of (79 samples) positive for microscopic examination; The mentioned study (8) also found 24 negative sample for molecular examination out of 200 positive samples for microscopic examination. Furthermore, the current study did not agree at all with (18) and (19), who did not reveal any negative result when examined positive samples for microscopy examination. The emergence of some samples the negative result of samples examined by PCR technology although they showed a positive results using microscopic examination may be due to an error

in the laboratory diagnosis of some samples on suspicion white blood cells multiple nuclei or phagocyte with Trophozoite, cystic stage for parasite *E. Histolytica* and *E. dispar*, or with cystic stage of other species of genus *Entamoeba*, such as *E. hartmanni* (4). In addition, the presence of inhibitory substances in some stool samples may be associated with the enzyme DNA-Polymerase turning off the spigot for DNA amplification process (20).

While the current study showed that there were 12 negative samples for microscopic examination, they gave positive results for the molecular examination (Table: 3). The study agreed with (10) that found 24 positive samples for molecular examination despite being negative for microscopic examination. The presence of positive samples for microscopic examination despite being negative for microscopic examination may be due to the time of sampling and analysis and the presence of small numbers of parasites in the sample as well as microscopic examination mistakes.

The current study showed a different infection percentage of three species of the genus *Entamoeba*. The highest percentage of the species *E. dispar* has reached (72.6%), distributed to (56.16%) of single infections and (16.44%) of mixed infections, followed by *E. histolytica* in the percentage (41.1%), distributed to (26.03%) of single infections and (15.07%) of mixed infections, then *E. moshkoviskii* in two samples (2.74%) including one sample (1.37%) of both single and mixed infections. The current study reinforced by (12) in the province of Babylon, which found that the highest percentage of the species *E. dispar* has amounted (87.9%) then *E. histolytica* (22%) but he did not reveal the presence of the species *E. moshkoviskii*. Also, the study agreed with (16) where he revealed the highest percentage of species *E. dispar* (49%) followed by *E. histolytica* (7.4%), while the current study did not agree with (8) that *E. histolytica* had the highest percentage of infection followed by *E. disbar* then *E. moshkoviskii*, which were 74%, 26%, and 7% respectively. It also does not agree with the study (17) in Malaysia, which found that the highest percentage of the species *E. histolytica* (63.5%) then the species *E. dispar* (19.2%) than *E. moshkoviskii* (5.8%). The difference in the results of PCR technique may be, due to a difference in the methods of DNA extraction from stool samples and PCR methods. The parasite amount found in the stool sample in full may lead to different results, as well as, the study time, the geographical location of the study area, population density, the cultural level, the age group targeted by the study (8), as well as the factors that affect the parasite propagation such as the existence of animals and human contact with them, environmental factors, water resources, traffic, and transport.

The study also agreed in terms of the proportion of mixed infection of two species (*E. dispar* + *E. histolytica*) with (8) who scored in his study of infection percentage (14%), while it was higher than (21) (10.2%) and it was lower than (16) (18.8%), the current results also did not agree with (19) who did not reveal any mixed infection of two species (E. dispar + E. histolytica). The study also agreed in terms of the proportion of mixed infection of two species (*E. dispar* + *E. moshkovisikii*) with (19) (14%) in Iran, while it was higher than (22) (0.5%) in Diwaniya, and it was lower than (**5**) (35%). The current study did not reveal a mixed infection of three species (*E. dispar*, E. histolytica, and *E. moshkovisikii*), and therefore agreed with (22) and (8**)**, while it did not agree with (23) who reveal mixed infection of three species in the percentage (19.2%). Mixed infection in a single sample reflects the similarity of the ways that lead to infect a host by the pathogens, which is *Entamoeba* (24). Morphological similarities, mixed infection, and similar life cycles are the main reasons of the late distinguish difficulties in distinguishing the species, the high-resolution molecular techniques are available.

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