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Molecular Identification And Genetic Diversity Of *Entamoeba* Species From Diarrheic Patients In Baqubah / Iraq.

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ABSTRACT

*Entamoeba histolytica*protozoan is a most public health problem worldwide**.**Microscopical and molecular identification of different human species of *Entamoeba*.The populations of this study included 326 patients with different age groups who came to the parasitology laboratory in BaqubahTeaching Hospital and AL-Batool Maternity Teaching Hospital/Diyala province, suffering from gastrointestinal complaints with acute diarrhea. All stool samples were laboratory diagnosed by microscopy, andconventional PCR was performed to amplify the small subunit ribosomalRNAgene, and the sequencing was done after successful amplification of *Entamoeba* species.Among 150 fecal samples from patients with amebiasis diagnosed by microscope, the PCR products with approximately 166, 580 and 752 bp were generated from 56 *E. histolytica*, 22*E.moscovskii* and 8 *E. dispar*.Sequenced of 14isolates 8 for (*E. histolytica*), 3 for each *E. moshkovskii* and *E. dispar*. Two of 14 sequence data, presented by 3 and 10 were not included in the analysis because of its short band size obtained after sequencing.Multiplex conventional PCR method was a rapid and effective in differentiating *E. histolytica* from *E. dispar* and *E. moshkovskii*. This method is an optional tool in the diagnosis and epidemiological studies of amoebiasis.

Keywords: *Entamoeba*, Molecular identification, genetic diversity

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INTRODUCTION

Amoebiasis is a common intestinal protozoan infection of the human gastrointestinal tract produced by *Entamoeba histolytica* which causes widespread mortality and morbidityworldwide through diarrheal disease and abscess establishment in parenchymal tissues suchas liver, lung, and brain. *E. histolytica* is a protozoon of humans. It movies by rapidly produce thick and hyaline pseudopodis (1). The prevalence of infection is unknown for most areas of theworld due to the difficulty to characterize *E. histolytica* versus other amoebas with identical morphology, as *Entamoeba dispar*, and *Entamoeba moshkovskii* (2). For many years *E. dispar* was considered a non-pathogenic, noninvasive parasite that did not cause disease. However, *E. dispar* has been associated with a few cases of amebic colitis and amebic liver abscesses, putting in question its status as a virulent (3,4). Both species occur in two forms: the hardy, infective cyst and the fragile, potentially pathogenic trophozoite (5). Another four-nucleated morphologically identical organism, *E. moshkovskii*, has been observed in sewage as a free-living amoeba, but is also capable of colonizing the human intestine (6). Although, dysentery and extraintestinal disease have been proposed to be potentially associated with *E. dispar* and *E. moshkovskii* (7). These findings complicated our understanding of the pathogenic behavior and public health importance of indistinguishable *E. histolytica/ dispar/ moshkovskii* complex (8).

Symptoms, when present, range from mild abdominal discomfort with diarrhea containing blood or mucous to acute or fulminating dysentery with fever, chills and bloody or mucoid diarrhea. Complications of prolonged infection include extraintestinal disease such as ameboma or abscesses in the liver, lungs, heart, brain, skin or other organ (9). Transmission is through the ingestion of fecally contaminated food or drinks, sexual exposure (usually anal sex) or through the unwashed hands of an infected food handler. The Prevalence of *E. histolytica/dispar* in Iraq is 48% and 3.7% respectively. Najaf, Wasit, Basra, Diwaniya and Miasan provinces showed the highest prevalence rates, while the lowest prevalence was reported in Anbar, Diyala, Thiqar, and Erbil (10).

Microscopic examination of stool for cysts and trophozoites remains the most common test available for amoebiasis diagnosis.However, it lacks specificity for *E. histolytica*, easy diagnosis of amoebiasis now depends on the use of immune-chromatography and/or PCR is extremely sensitive and useful in differentiating *E. histolytica* from *E. dispar* and *E. moshkovskii* (11). The phylogenetic analysis of the*Entamoeba* species showed a variety of genotypes which can explain the big variation in pathogenicity in humans (1).

The present study aimed to microscopical and molecular identification of different human species of *Entamoeba*.

MATERIALS AND METHODS

Patients and samples

The study included 326 patients of different age groups who came to the Parasitology Laboratory in BaqubahTeaching Hospital and AL-Batool Maternity Teaching Hospital/Diyala province, suffering from gastrointestinal complaints with acute diarrhea. Stool samples were collected during the period from 1st August 2017 till 30thApril 2018.Ethical approval(MD8 December 2017 MAI).A questionnaire on personal information was prepared.

Stool samples examinations

1- **Microscopical examination**: Direct method from each stool samples, smears with normal saline and lugols iodine were examinedaccording to WHO (12).
2- **DNA extraction andgene amplification by conventional PCR**

DNA-extraction from stool Samples AccuPrep® Stool DNA Extraction Kit provided by BioNeer/ Korea was adopted by the manufacturer of DNA extraction kit.

A single-round PCR assay and primer sets were used (13). The sequence of the forward primer used (EntaF) was conserved in all 3 *Entamoeba spp*., whereas the specific reverse primers EhR, EdR, and EmR were

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specific for *E. histolytica*, *E. dispar*, and *E. moshkovskii*, respectively. The expected products were 166 bp (*E. histolytica*), 580 bp (*E. moshkovskii*), and 752 bp (*E. dispar*).

The amplified products were analyzed by electrophoresis in 1.5% agarose gel stained with 0.5 mg/mL red stain.

3-The sequencing: The PCR products of *E. histolytica*, *E. dispar* , and *E. moshkovskii* were prepared (35µl for each PCR product with forward and reverser primers) were sent abroad to Macrogen company in South Korea for direct sequencing. Then purified using the purification kit (Macrogen, Korea) according to the manufacturer's instructions. All purified amplicons were used as a template for sequence cycle. The sequencing was done in both directions using the same primer sets (previously used in study for amplification *Entamoeba spp.*) as in the respective PCR assay with a genetic analyzer (Macrogen, Korea).

Alignment was conducted using alignment tool, BLAST for nucleotide sequence. This tool is available online at the National Center Biotechnology Information (NCBI) at (http//www.ncbi.nlm.nih.gov).
The genetic relatedness of the local isolates were analyzed with MEGA 6 software through constriction phylogenetic tree. The accession number of reference sequences were used in the analysis: KP233840.1, KP233838.1, KP233837.1, GQ423748.1, AB608092.1, KP722602.1, KP722603.1, KP722605.1, KT825980.1, and KT825978.1.

Statistical analysis

The Statistical Analysis System- (SAS) was used to find out of different factors in study parameters (14).

RESULTS

Among 150 fecal samples from patients with amoebiasis diagnosed by microscopic, 86/150 were amplified for the gene SSU rRNA (57.33%). However, the amplification of these isolates showed that 56 (65.11%) represented *E. histolytica* and 22 (25.58%) samples contained *E. moshkovskii*, while 8(9.30%) represented *E. dispar*. There were 12 cases of mixed infection between *E. histolytica* and *E. moshkovskii,* and 5 cases of mixed infection between *E. histolytica* and *E. dispar,* as shown in Figure (1). The successful amplification of these species of *Entamoeba* was shown in Figures (2, 3 and 4).

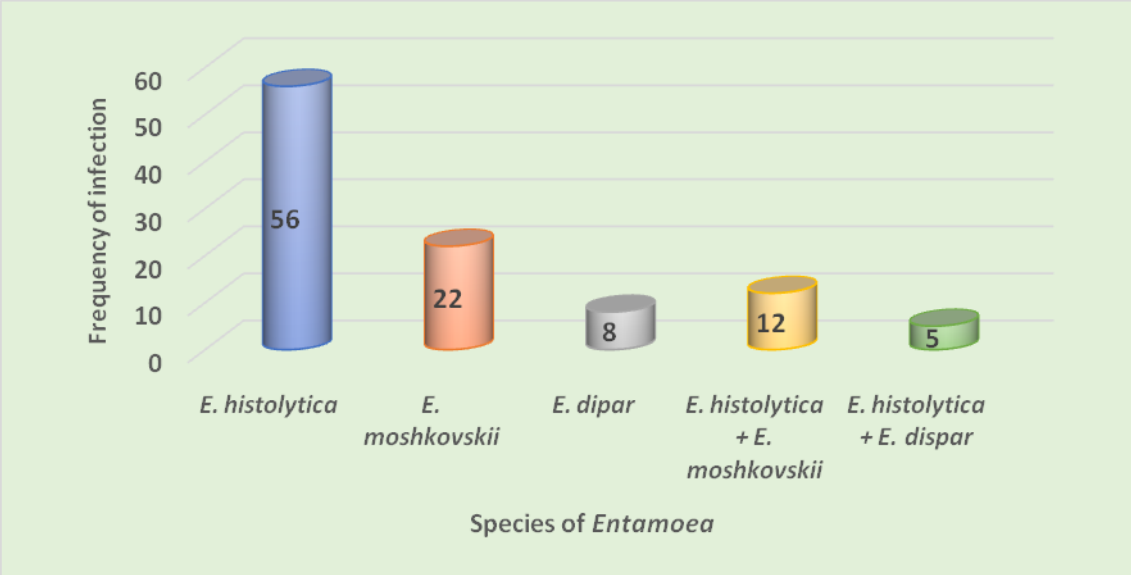

Figure (1): Type and frequency of *Entamoeba* infection.

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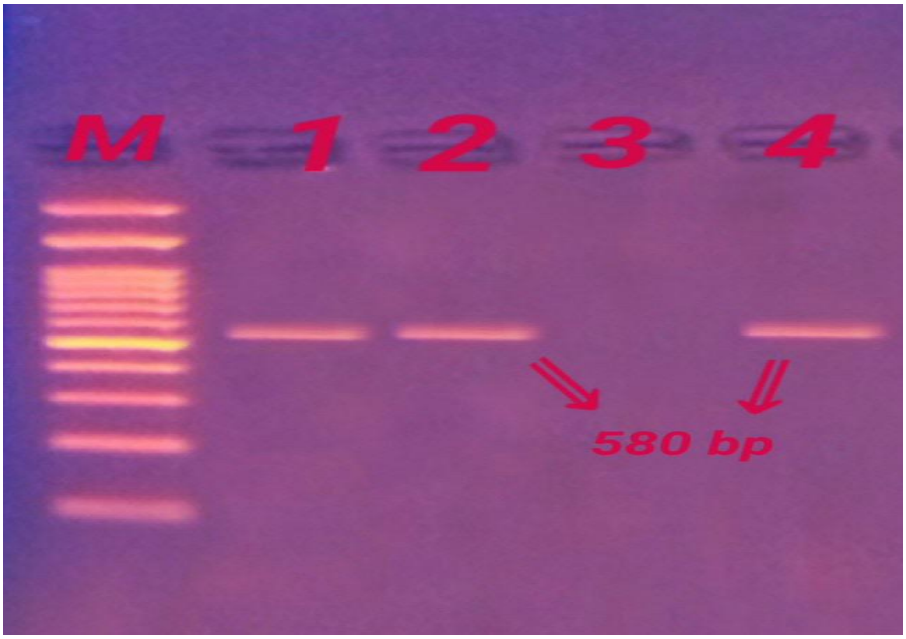

Figure 2: Gel electrophoresis of multiplex PCR products of *Entamoeba spp.* stained with ethidium bromide and visualized under ultra-violate. M: DNA marker (100bp), Lane 1,2,4,5 and 8 are positive result for amplification (580bp) with*E. moscovskii*.

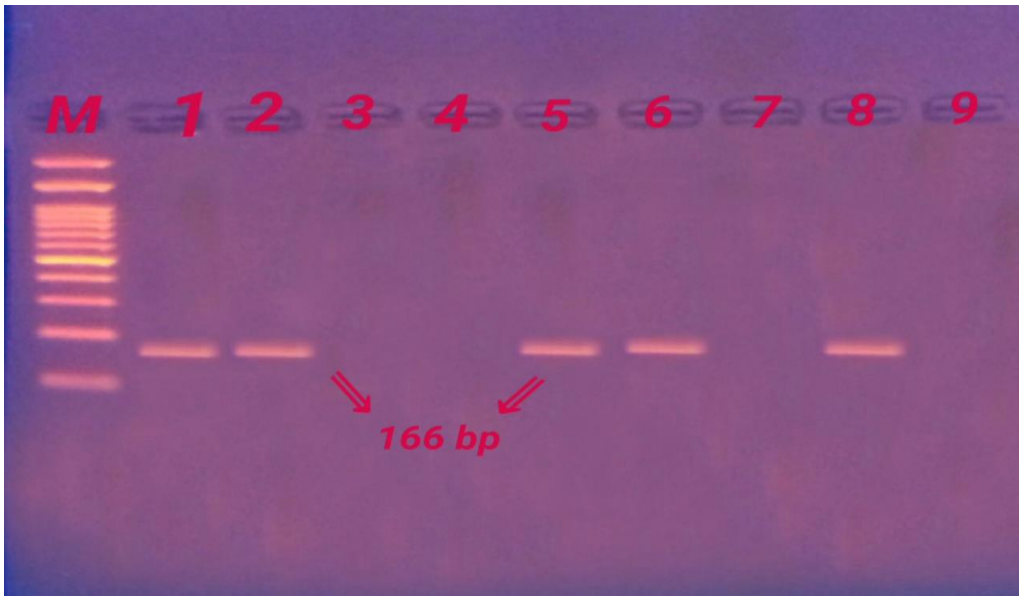

Figure 3: Gel electrophoresis of multiplex PCR products of *Entamoeba spp.* stained with ethidium bromide and visualized under ultra-violate. M: DNA marker (100bp), Lanes 1,25,6, and 8 are positive result for amplification (166bp) with*E. histolytica*.

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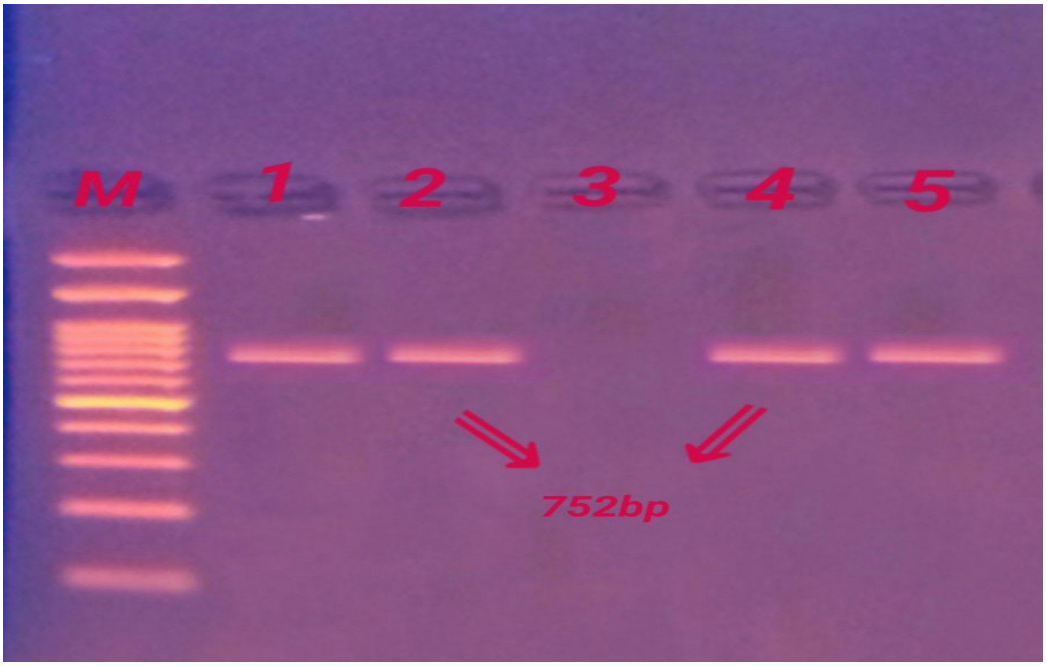

Figure 4: Gel electrophoresis of multiplex PCR products of *Entamoeba spp.* stained with ethidium bromide and visualized under ultra-violate. M: DNA marker (100bp), Lane 1,2,4 and 5 are positive result for amplification (752bp) with*E. dispar*.

Sequenced of 14isolates 8 for (*E. histolytica*), 3 for each *E. moshkovskii* and *E. dispar*. Two of 14 sequence data, presented by 3 and 10 were not included in the analysis because of its short band size obtained after sequencing.

Furthermore, the genetic relatedness of the local isolates were analyzed with MEGA 6 software through constriction phylogenetic tree (Figure 5). Isolate 1, 2, 4, 5, 11, 12 and 14 clustered with KP233840.1,KP233838.1, and KP233837.1 which are *E. histolytica* type of Iraqi isolation and also clustered with GQ423748.1 which is *E. histolytica* type of Philippian isolation, as well as clustered with AB608092.1 which is *E. histolytica* type of Japanese isolation. Isolate 8, 9 and 13 clustered withKP722602.1, KP722603.1 and KP722605.1 which are *E. moshkovskii* type of Iraqi isolation. Isolate 6 and 7 clustered with KT825980.1 and KT825978.1 which are *E. dispar* type of Colombia isolation.

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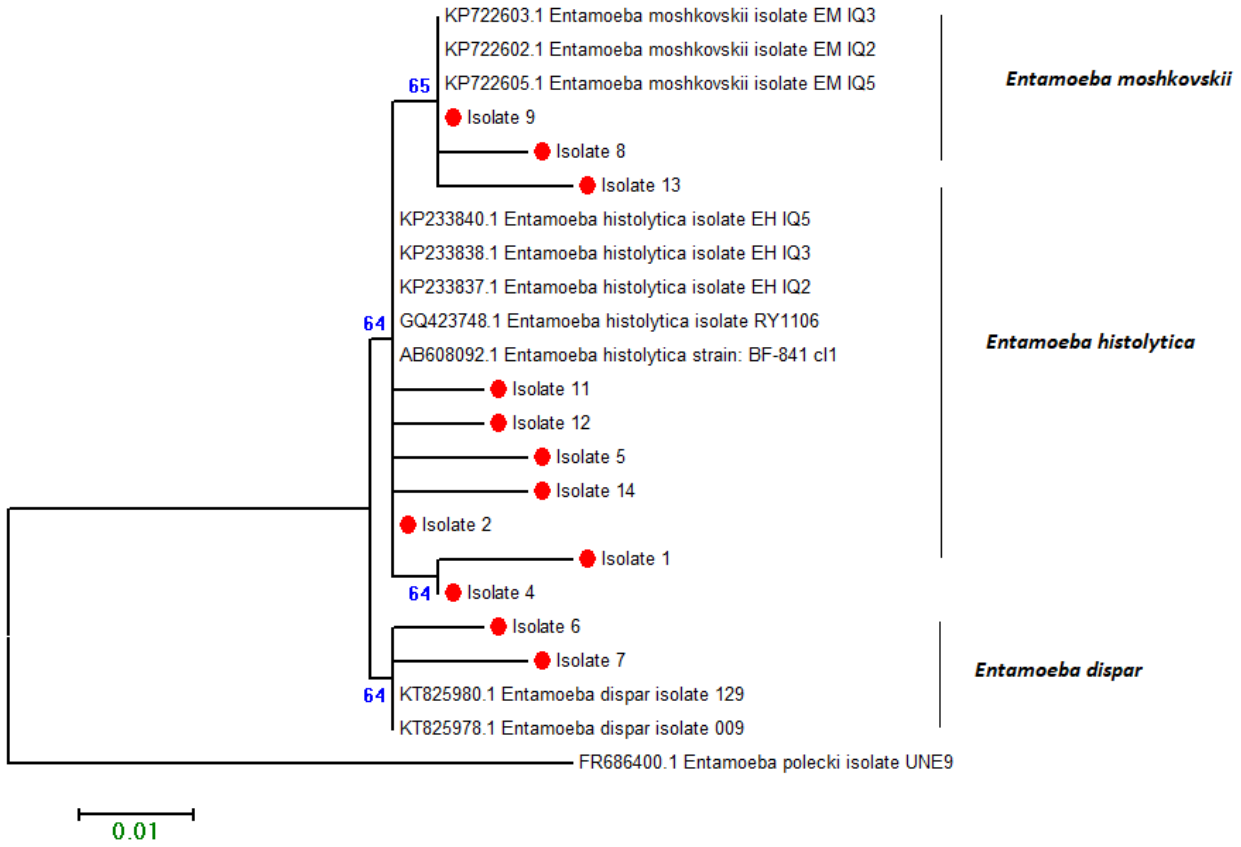

Figure 5: Phylogenetic tree for *Entamoeba* species genes constructed by the neighbor joining method for 12 local isolates from stool samples and 10 reference isolate from Gene Bank. Current isolates are indicated with redcircle.

DISCUSSION

In the present study, the 150 case of positive microscopical examination with trophozoites or cysts of *Entamoeba* species collected for further study by conventional multiplex single round PCR to differentiate between 3 major *Entamoeba* species *E. histolytica, E.moshkovskii* and *E. dispar*. It can successfully and simultaneously differentiate all the *Entamoebaspp.* from fecal samples in one test (15). Single round PCR is sensitive, specific, easy, and simple and can help in the epidemiological studies concerned with multiple infections in endemic areas (16). The current study revealed 86 out of 150 were amplified for the gene SSU rRNA (57.33%) by this method.

The study showed the presence of 64 negative samples for molecular examination despite being positive for microscopic examination (42.66 %). This agreed with study done Zeyrek *et al*. and Lau *et al*. at 2013 (17,18), who found lower positive rate of PCR than microscopy, this may be due to the unsuccessful in the amplifications of stools samples: low amount of DNA samples, or to their degrading with time, or to the presence of some of PCR inhibitors, handling error, and quick degeneration of trophozoites during time before starting the PCR (19). Although,this may be, due to a difference in the methods of DNA extraction from stool samples and PCR methods (20). On the other hand, the difference in the results of PCR technique may be due to the error in the microscopic 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. coli*, *E. polecki* and *E. hartmanni* (21).

Recently in the developed countries, PCR has been the method of choice for clinical and epidemiological studies and has been strongly recommended to be used as a diagnostic tool by the WHO (22). At the same time, microscopy cannot distinguish the pathogenic from the other strains of amebae (1). For thus the present study was used conventional PCR technique to un ascertain the prevalence of three different

subtypes of amoebic infections. The amplification of these isolates showed that 56 (65.11%) represented *E. histolytica* and 22 (25.58%) samples contained *E. moshkovskii*, while 8(9.30%) represented *E. dispar* among diarrheal patients in Baqubah city.

The results of current study close to the study which was conducted in Yemen that found infection rate of *E. moshkovskii* higher than *E. dispar* with 39.9% and 15.7% respectively but different in *E. histolytica* rate which is less than *E. moshkovskii* with 20.2% (23). *E. moshkovskii* was found associated with gastrointestinal symptoms frequently in regions with a high prevalence of amebiasis (24). The same was found in India (25), in homosexual patients with gastrointestinal symptoms in Australia (26). However, Shimokawa *et al*. (2012) showed that *E. moshkovskii* induced diarrhea, bloody stool and weight loss in susceptible mice and diarrhea in Bangladeshi children(27). This is emphasizing the pathogenicity of this protozoan. For thus, *E. moshkovskii* became relevant because of its ability to infect humans and associated with diarrhea (28).

In contrast, in Tikrit whereas the PCR carried out on the stool samples recorded positive for *Entamoeba*, showed that (83.33%) were *E.dispar* and (1.85%) was mixed infection of *E.dispar* and *E.moshkovskii*, and no infection with *E. histolytica* (29), another study in Diwaniyahthat found *E. histolytica* had the highest percentage of infection followed by *E. dispar* then *E. moshkoviskii*, which were 74%, 26%, and 7% respectively (20). Another study in Babylon, 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* (30).

However, all deaths could be due to invasive *E.histolytica* infection, data on the prevalence of *E. histolytica* might be an overestimation because the data come from a date in time before the separation of the pathogenic *E. histolytica* from the other species. *E. dispar* and *E. moshkovskii* both might contribute to the prevalence figures (31). *E. dispar* strains were isolated from symptomatic patients in Brazil. These strains were able to produce liver and intestinal lesions that were occasionally indistinguishable from those produced by *E. histolytica*. Parija and Khaimar at 2005 reported gastrointestinal symptoms in Indian patients infected by the association of *E. dispar* with *E. moshkovskii* (25). A similar pathogenic potential was reported by Fotedar *et al*. and Pritt and Clark at 2008(26,32). These and other findings, such as the detection of *E. dispar* DNA sequences in samples from patients with amebic liver abscess, have revived the possibility that this species can produce lesions in humans (8).

However, the present study revealed that 12 cases of mixed infection between *E. histolytica* and *E. moshkovskii,* and 5 cases of mixed infection between *E. histolytica* and *E. dispar.*This may be due to the fact that mixed infection in a single sample reflects the similarity of the ways that lead to infect a host by the pathogens (12).

Molecular tools allow reconstructing a more reliable picture of the true epidemiology of the disease mainly in endemic area and to better understand the role of the parasite and/or host factors that determine the disease outcome. in the present study, all isolates sequences at the SSUrRNA gene which is found to be more sensitive than the best antigen detection method in stool (33). And SSU rRNA is in wide use as it is present in multiple copies on the extra-chromosomal plasmids (34). Furthermore, another study suggested that the SSUrRNA gene is a good phylogenentic developmental marker for analyzing the molecular evolutionary and taxonomic relationshipwith*Entamoeba*species.

The results of the sequencing of the nitrogen bases and analysis of the genetic tree of the species targeted by the present study showed that isolate 8, 9 and 13 they were genetically closer to the sample with the serial number KP722605.1, expressed as *E. moshkovskii*, whose presence was recorded by Al-Mayali and Al-abodi (2017) in Al- Qadisiyah, which is genetically identical to the samples recorded by the same researchers KP722602.1, and KP722603.1 which are *E. moshkovskii* type of Iraqi isolation (35). These isolates from human feces with presence of symptoms. This indicated the anthroponotic prevalence of this strain locally in different city of Iraq.

As well as for the isolate 1, 2, 4, 5, 11, 12 and 14 clustered with serial number AB608092.1, expressed in type *E. histolytica*, which was recorded in Tokyo, Japan by Suzuki *et al*. (2011from human feces (36), and GQ423748.1 recorded in Philippian by Rivera *et al*. (2010) (37), this isolate from captive macaques feces may indicate the zoonotic prevalence of these strain, or may be carried by mechanical vectors such as flies and

contaminate food or water sources (38), which is genetically identical to the samples recorded by Al-Mayali and Al-abodi (2017) in Al- Qadisiyah, with serial number KP233837.1, KP233838.1, and KP233840.1, registered in Iraq (35). These isolates indicated the prevalence of these strain locally in different city of Iraq. While isolate 6 and 7 clustered with KT825980.1 and KT825978.1 which are *E. dispar* type of Colombia isolation recorded by López *et al*. (2015)(39). The existence of these strain may be related to foreign visitors or may be all these isolates are international existence.

The purpose of the DNA sequencer is to confirm our isolates of the three parasite species in addition to knowing who is similar among the global isolates. These results confirm the validity of the isolates currently under study after comparing them with world standard isolates to determine the sequence of the nitrogen bases of the SSUrRNA gene that represents the reference gene to diagnose isolates. The precise diagnosis at the molecular level of similarity of isolating DNA has a diagnostic utility as well as the possibility of using some of these isolates in practical applications as biochemical or pharmacological boosters or in their use for molecular genetics studies, especially since there are no previous studies on the analysis of sequencing of *Entamoeba* in Diyala.

CONCLUSION

Multiplex conventional PCR method was a rapid and effective in differentiating *E. histolytica* from *E. dispar* and *E. moshkovskii*. This method is an optional tool in the diagnosis and epidemiological studies of amoebiasis

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