CONVENTIONAL AND MOLECULAR STUDY OF *Entamoeba* spp. IN DOMESTIC DOG'S FECAL AT BAGHDAD CITY, IRAQ.

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ABSTRACT

This study was aimed to detect *Entamoeba* spp. in domestic dogs by using the microscopic method and PCR. The study includes collection of 100 fecal samples by visiting a small animal private clinics in Baghdad, at the period from January 2019 to February 2020. The microscopical results showed the rate of infection at *Entamoeba* spp. was (15%). Show a higher infection rate in females (15.55%)) than in males (14.54%)without significant difference (P.0>0.05). The Prevalence of *Entamoeba* related to age was (22%), (6%) in young and adult age respectively. Molecular examination results, the total infection rate of *Entamoeba* in dogs fecal showed 15% . Ten positive PCR products were sequenced and deposited in the Genebank database, the first time in Iraq, the phylogenic analysis demonstrated that 10sequences belong to *Entamoeba histolytica* (MT296770, MT296771, MT296772, MT296773, MT296774, MT296775, MT296776, MT296777, MT296778, and MT296779).

Key word: Microscopic examination, molecular techniques, *Entamoeba* , dogs, Iraq.

الدراسة التقليدي والجزيئي لطفيلي.*Entamoeba* spp في براز الكلاب المنزلية في مدينة بغداد ،العراق

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المستخلص

تم التخطيط للدراسة الحالية للكشف عن *Entamoeba spp.* في الكلاب المنزلية باستخدام الطريقة المجهرية والجزيئية، اشتملت الدراسة على جمع 100 عينة براز من خلال زيارة العيادات الخاصه بالحيوانات الصغيرة في ، الفترة من كانون الثاني 2019 إلى شباط 2020. وأظهرت النتائج المجهرية معدل الإصابة الكلية لل *Entamoeba spp.* كان (15٪). أظهرت الإناث معدل إصابة أعلى (15.55%) مقارنة بالذكور (14.54٪) بدون وجود فرق معنوي . انتشار *Entamoeba spp.* المرتبطة بالعمر كانت (22٪) ، (6٪) في الاعمار الصغيرة والكبيرة على التوالي. بينت نتائج الفحص الجزيئي معدل الإصابة الكلي لـ *Entamoeba spp.* في عينات براز الكلاب(15٪) . تم تسلسل عشرة منتجات إيجابية من PCR و إيداعها في قاعدة بيانات بنك الجينات العالمي لأول مرة في العراق ، اظهرت نتائج شجرة التطور الوراثي أن 10 عينات متسلسلة تنتمي الى*Entamoeba spp* (MT296770, MT296771, MT296772, MT296773, MT296774, MT296775, MT296776, MT296777, MT296778, and MT296779).

مفتاحية كلمات:الفحص المجهري، سلسلة البلمرة ،انتميبابا ، كلاب، العراق

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INTRODUCTION

Amoebiasis, an infection by intestinal protozoa belongs to the genus *Entamoeba* consist of several species (e.g. E. *histolytica, E. dispar, E. moshkovskii, E. coli, E. poleki and E. hartmanni*) (2). *E. histolytica* considered the third most important parasitic cause of human death rate after malaria and schistosomiasis. Genus *Entamoeba* has two forms, the trophozoite which has a short life span and is mobile that can foray into the different organ systems, while the cysts are a long-surviving form of *Entamoeba* that colonize the patient(1). The cyst is composed of resistant walls which keep these species from drought in the environment, also, the cysts can persist for several days, weeks or month mostly in moist conditions, and are accountable for transmission of infection (11). The clinical signs of amoebiasis comprise diarrhea, amoebic dysentery, and liver abscess (5). The routes of infection of cyst occur with the ingestion of contaminated food or water and trophozoite proliferate in the lumen of the large intestine that may cause disease (17). Light microscope is used to detection of *Entamoeba* by wet smear but the results mostly difficult to distinguish the trophozoite and cyst of pathogenic *E. histolytica* from the cyst and trophozoite of non-pathogenic (16). Accurate molecular methods available PCR that can distinguish *E. histolytica, E. moshkovskii,* and *E. dispar* (9,3,20). A few is known about the molecular diagnosis of the species of this parasite in dogs in Iraq. This study determined the prevalence of *Entamoeba* species in domestic dogs in Baghdad city using molecular diagnosis and determined the genetic identity of these *Entamoeba* species by phylogenetic analysis.

MATERIALS AND METHODS

One hundred fecal samples (10 g) were collected from domestic dogs(different sexes and ages) by visiting small animal private clinics in Baghdad, Iraq. During the period from January 2019 to February 2020. For the detection of *Entamoeba* species, the samples were kept in 5 ml tubes containing 2% potassium dichromate and 70% ethanol as a preservative. The samples were transported to the Parasitological laboratory - College Veterinary Medicine -University of Baghdad. . Each fecal sample was divided into two parts the first one for the direct microscopic examination using iodine staining, the second part the positive fecal samples with *Entamoeba* species cysts/trophozoite were kept at -20 ^{o}C for molecular diagnosis (PCR).

Laboratory analysis of samples

Iodine wet mount: A total of 2 mg fecal dog samples were mixed with Lughole's iodine. a drop of the sample was placed on a glass slide and the coverslip and microscopic observation were performed at 10× and 40× magnification(18).

DNA extraction:

Total genomic DNA was extracted by using a commercial DNA extraction kit (ADDBIO, South Korea) from 100 fecal specimens according to the manufacturer's recommendation. Briefly, about 50 mg of fecal sample was lysed by the provided lysis buffer (200 µl) with 20 µl of proteinase k (20 mg/ ml). The obtained lysate was then purified via spin column and numerous steps of washing were then eluted using the provided elution buffer.

Genomic DNA estimation

The extracted genomic DNA was measured by utilizing a Nanodrop spectrophotometer (THERMO. South Korea), in which the purity was measured at the absorbance 260 /280 nm. The extracted DNA was kept in a deep freezer until further PCR analysis.=

Polymerase chain reaction (PCR)

100 fecal samples were randomly selected from domestic dogs for PCR screening. PCR technique was performed for direct detection of *Entamoeba* spp. targeting the *18SrRNA*gene (Table 1).

Table 1. The primer with their sequence and product size

Primers		Oligos 5-------3	Reference
Amplicon size≤900 bp	forward primer) (reverse primer)	E-1 5' TAAGATGCACGAGAGCGAAA 3' E-2 5' GTACAAAGGGCAGGGACGTA 3'	(8)

PCR master mix preparation: PCR master mix was implemented by (ADDBIO, SOUTH KOREA kit), according to manufacturer's instructions as in (Table2).

Table2. Protocol of PCR reaction mixture volume

Components	Volume
Master mix	10 µl
DNA template 5-50 ng/µ	5 µl
Forward primer (0.5 pm	1µl
Reveres primer (0.5 pmc	1µl
High pure water	3µl
Total volume	20µl

These PCR components were transported into T100 thermal cycler (BIO-RAD, USA) to that amplify the targeted gene with the following thermal conditions (Table3).

Table 3. The optimum condition of detection *Entamoeba spp.*

PCR step	Tem	Time	Repeat
Initial Denaturation	95 °(	5min	1
Denaturation	95 °(	30 sec.	
Annealing	58 °(	30 sec	35 cycl
Extension	72 °(	40 sec	
Final extensio	72 °(	5 min	1
Hold	4 °C	Foreve	-

PCR product analysis

The PCR products were analyzed by electrophoresis in a 1% agarose gel utilizing 1X TBE buffer. Firstly, the agarose was dissolved in 1x TBE by heating in a microwave for 2 minutes then cooled down to 60 C followed by adding 7 µl of Safe gel stain (ADDBIO, South Korea); this was poured into the electrophoresis cassette and the PCR products were loaded into the set wells and electrophoresed at 100 volts and 80 AM for 1 hour in parallel of DNA marker (INTRON). Finally, the amplicon was demonstrated under UV Transilluminator (Cleaver Scientific, UK).

Sequencing and phylogenetic analysis

PCR products were purified by using (INTRON kit) and analyzer (Macrogen) using terminator cycle sequencing and BLAST analysis database http://blast.ncbi.nlm.nih.gov), edited with (Mega 6) then analyses by (Neighbour Joining Method).

Statistical analysis

The Statistical Analysis System- SAS (2012) program was used to detect the effect of different factors in study percentage. Chi-square test was used to significantly compare between percentage in this study(19).

RESULTS AND DISCUSSION

icroscopic examination results

This study showed that the overall rate of *Entamoeba spp.* infection among domestic dogs was 15%, in which 14.54% male and 15.55% female, with no significant difference ($P \geq 0.01$) by using microscopy examination as in (Table4)(figure1).

Table 4. *Entamoeba* spp. infection rate according to sexes in the domestic dog

Sex	No. of animal examined	No. of an animal infected	Percentage (%)
Male	55	8	14.54
Female	45	7	15.55
Total	100	15	15
Chi-Square -χ^2	---	---	0.328 NS
(P-value)			(0.607)

NS: Non-Significant

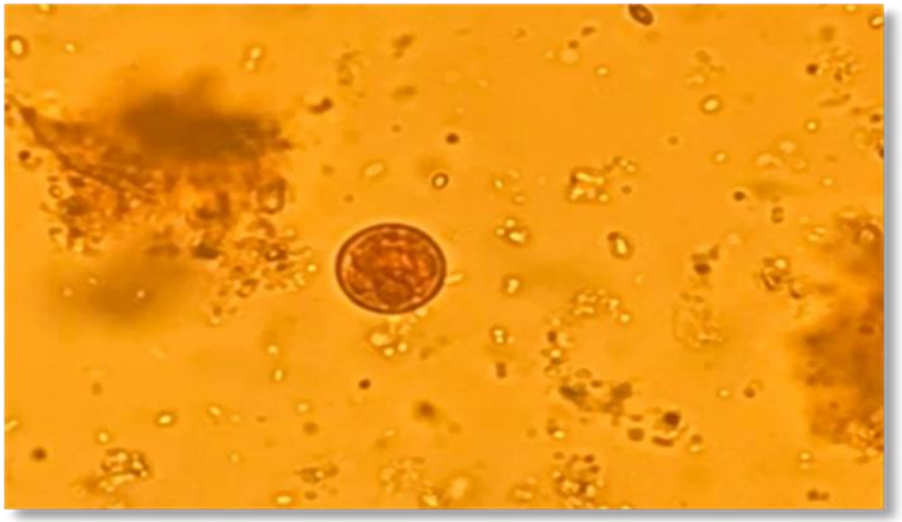

Figure 1. *Entamoeba* cysts stained by Lughole's iodine stain (X100).

A significant difference (P≤0.01) was shown among different age groups. The high infection rate was recorded in young dogs (22%) and the lowest infection rate in adult dogs 6% (Table 5).

Table 5. *Entamoeba* spp. infection rate according to age in domestic dogs

Age	No. of animal examined	No. of animal infected	Percentage (%)
Young	50	11	22.00
Adult	50	3	6.00
Total	100	15	15
Chi-Square -χ^2	---	---	6.019 **
(P-value)			(0.0086)

** (P≤0.01).

The study showed that the rate of infection of *Entamoeba* spp. in domestic dogs in Baghdad city was 15%. The results indicate that dogs possibly serve as reservoirs to many types of protozoa and may be a potential source of infection to human, the parasite has been reported in dogs, including in Malaysia (14), Spain (12), Pakistan (2), and Nigeria (8). In this study, no differences were observed between dog sex with the rate of infection, which is consistent with (14). This could be due to both sexes exposed to the same environmental condition. This study showed that *Entamoeba* infection is higher among young dogs compared to adults dogs with statistically significant. This result is in agreement with studies (12,2), which also found a significant association of *Entamoeba* infection with age. The high prevalence of *Entamoeba* infection among young dogs may be due to low immunity at young ages or probably as a consequence of single or repeated exposures(6).

Polymerase Chain Reaction (PCR) results

Traditional microscopic examination is the most commonly used diagnostic tool for examining the presence of *Entamoeba* in fecal samples (8). However, several species of *Entamoeba* with the same morphological characteristics, for instance, the *E. dispar*, a nonpathogenic species, is morphologically identical to *E. histolytica* cannot be distinguished by microscopic examination. Therefore, accurate identification of species of *Entamoeba* was performed with molecular assay (2, 4, 7, 15, 13). The PCR assay was used for specific indirect confirmative detection of *Entamoeba* spp. in 100 samples were randomly collected from the domestic dogs by visiting small animal private clinics in Baghdad city. The PCR results showed that 15 positive samples that among 100 examined the fecal samples appeared at ≤900 *for18s rRNA* gene on 1% agarose gel (figure2).

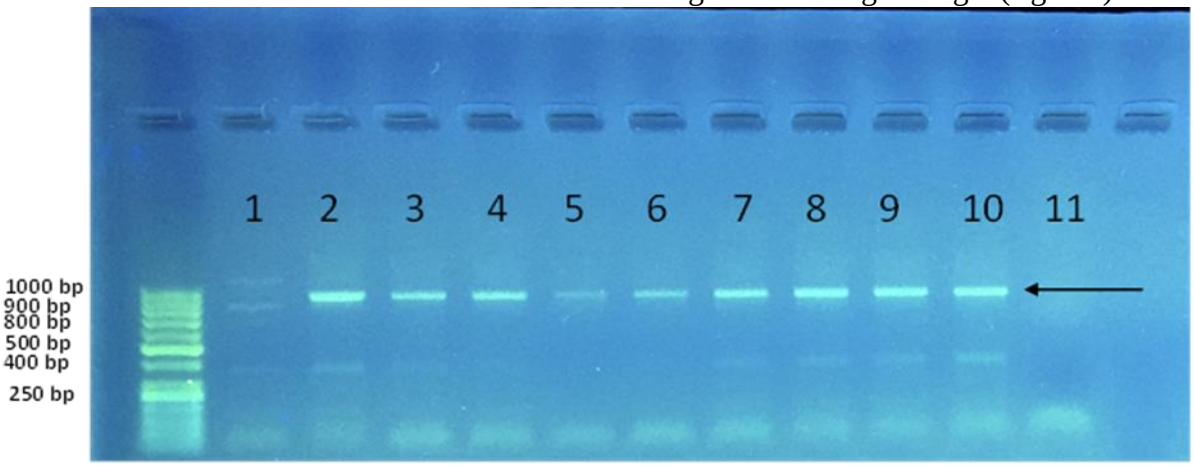

Figure 2. 1% Agarose gel electrophoresis at 100 volt 80 AM for 1 hours ,shows the amplicons of *Entamoeba* (≤ 900 bp in size) targeting *18s rRNA* gene. (molecular marker(100bp); lines 1,2,3,4,5, 6, 7,8,9,10 are positive and 11 negative samples for *Entamoeba*.spp.

Multiple Sequence Alignment Analysis

Ten PCR products from 15 positive PCR samples were collected randomly ,the sequenced PCR products were submitted at NCBI under the following accession numbers (MT296770, MT296771, MT296772, MT296773, MT296774, MT296775, MT296776, MT296777, MT296778,and MT296779) (figure3). That depicts the multiple alignmentoff these sequences.

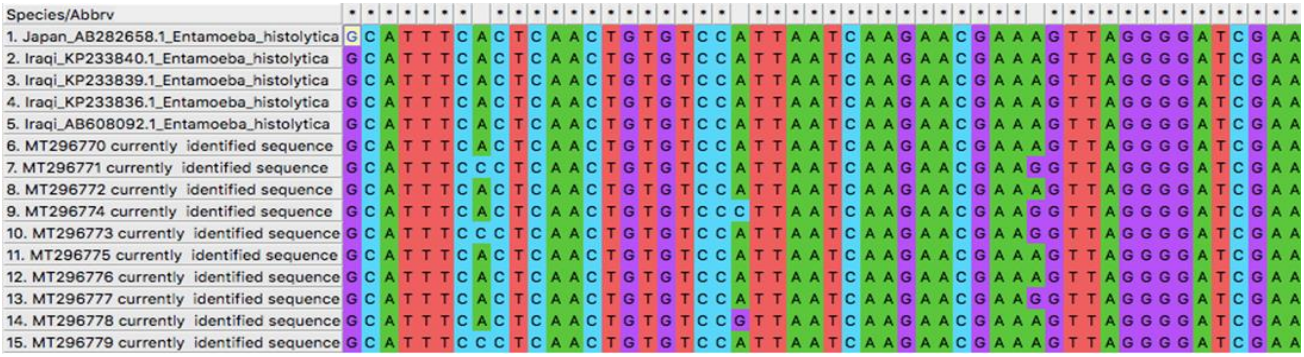

Figure 3. Multiple sequence alignment analysis of 18s rRNA gene of *Entamoeba histolytica* that deposited at NCBI under accession numbers (MT296770, MT296771, MT296772, MT296773, MT296774, MT296775, MT296776, MT296777, MT296778, and MT296779). These were aligned and compared with previously Iraqi sequences (accession numbers: KP233840, KP233839, KP233836, AB608092) as well as Japan's sequence (AB282658). The alignment similarity is depicted as star (*) with some mutations within this gene.

Phylogenetic Analysis

The study was the first study on *Entamoeba histolytica* genotypes in dogs in Iraq. In the present study,the sequences have been registered in NCBI under accession numbers ((MT296770, MT296771, MT296772, MT296773, MT296774, MT296775, MT296776, MT296777, MT296778, and MT296779) belong to *Entamoeba histolytica* for analysis and were compared with the NCBI GenBank *Entamoeba histolytica* strain isolates of *18s RNA* gene was highly homology with the *Entamoeba histolytica* sequences reported from Iraq (GenBank Accession Number KP233840.1), from Japan isolate (GenBank Accession Number AB282658.1) from Philippine isolate (GenBank Accession GQ423749.1) at total genetic changes (0.015-0.05%), as shown in (Table 6) (Figure 4). This convergence seems to be taken place because of the continuous importing of animals from other countries meanwhile introducing new strains into Iraq. Actually, this parasite has a wide range of hosts to infect which could lead for the genetic divergence. This genetic variability possibly promotes the infestation strategy of the parasite (13).

Table 6. The NCBI-BLAST Homology Sequence identity (%) between local *Entamoeba histolytica* of dog's feces isolates and NCBI-BLAST deposited strains

Entamoeba histolytica sequence No.1	Accession number	NCBI-BLAST Homology Sequence identity (%)			
		Identical to	Genbank Accession number	Country	Identity (%)
1	MT296770	*Entamoeba histolytica*	KP233840.1	Iraq	98.95
2	MT296779	*Entamoeba histolytica*	KP233840.1	Iraq	98.72
3	MT296777	*Entamoeba histolytica*	KP233840.1	Iraq	99.07
4	MT296776	*Entamoeba histolytica*	KP233840.1	Iraq	99.7
5	MT296773	*Entamoeba histolytica*	KP233840.1	Iraq	98.82
6	MT296772	*Entamoeba histolytica*	KP233840.1	Iraq	99.18
7	MT296775	*Entamoeba histolytica*	KP233840.1	Iraq	99.07
8	MT296774	*Entamoeba histolytica*	KP233840.1	Iraq	98.94
9	MT296771	*Entamoeba histolytica*	KP233840.1	Iraq	98.95
10	MT296770	*Entamoeba histolytica*	KP233840.1	Iraq	98.95
1	MT296770	*Entamoeba histolytica*	AB282658.1	Japan	98.95
2	MT296778	*Entamoeba histolytica*	AB282658.1	Japan	98.95
3	MT296777	*Entamoeba histolytica*	AB282658.1	Japan	99.07
4	MT296776	*Entamoeba histolytica*	AB282658.1	Japan	99.07
5	MT296775	*Entamoeba histolytica*	AB282658.1	Japan	99.07
6	MT296774	*Entamoeba histolytica*	AB282658.1	Japan	98.94
7	MT296773	*Entamoeba histolytica*	AB282658.1	Japan	98.82
8	MT296772	*Entamoeba histolytica*	AB282658.1	Japan	99.18
9	MT296771	*Entamoeba histolytica*	AB282658.1	Japan	98.95
10	MT296779	*Entamoeba histolytica*	AB282658.1	Japan	98.72
1	MT296770	*Entamoeba histolytica*	GQ423749.1	Philippine	98.83
2	MT296779	*Entamoeba histolytica*	GQ423749.1	Philippine	98.60
3	MT296777	*Entamoeba histolytica*	GQ423749.1	Philippine	98.95
4	MT296776	*Entamoeba histolytica*	GQ423749.1	Philippine	98.95
5	MT296773	*Entamoeba histolytica*	GQ423749.1	Philippine	98.70
6	MT296772	*Entamoeba histolytica*	GQ423749.1	Philippine	99.07
7	MT296775	*Entamoeba histolytica*	GQ423749.1	Philippine	98.95
8	MT296774	*Entamoeba histolytica*	GQ423749.1	Philippine	98.82
9	MT296771	*Entamoeba histolytica*	GQ423749.1	Philippine	98.83
10	MT296778	*Entamoeba histolytica*	GQ423749.1	Philippine	98.83

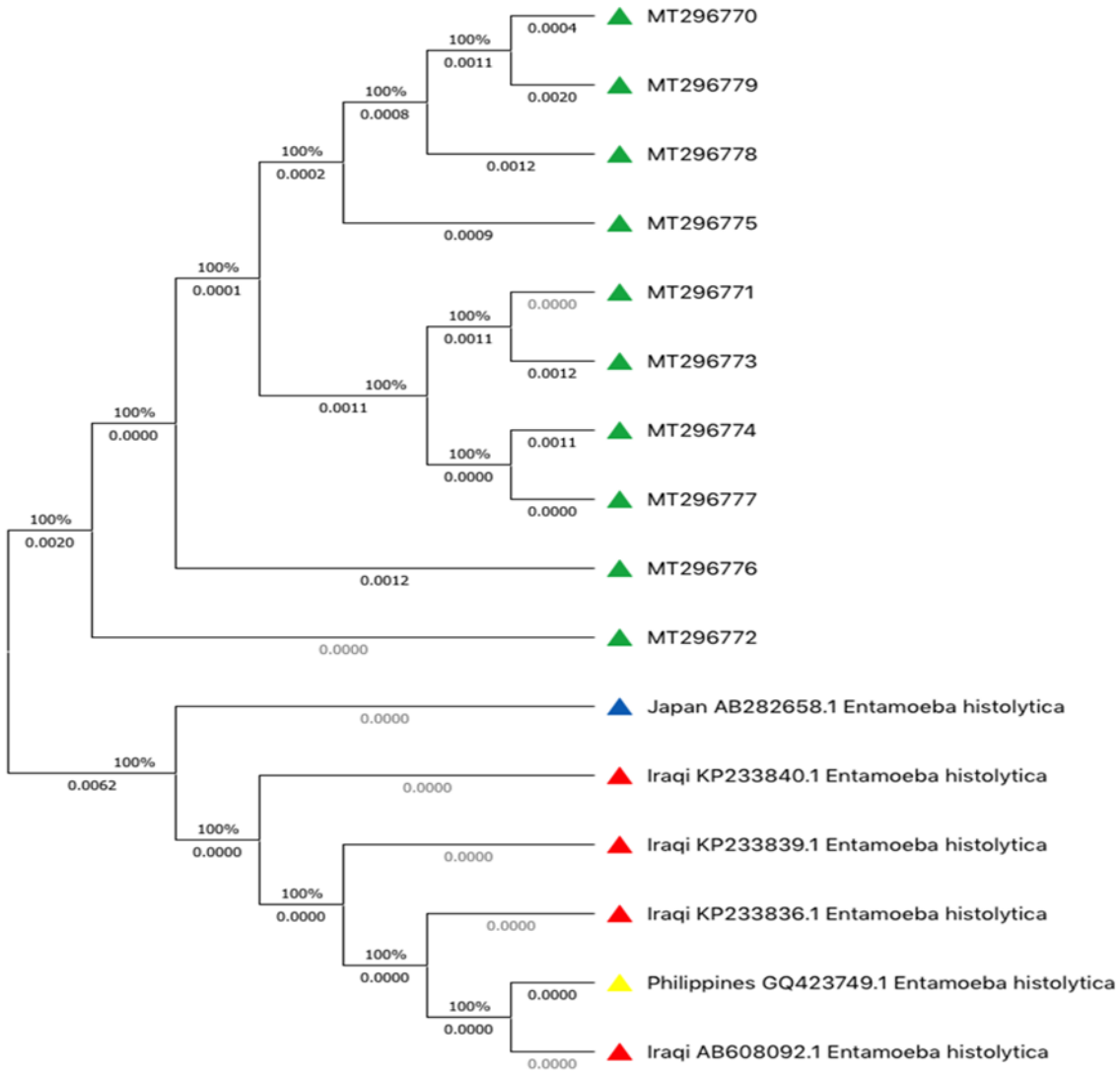

Figure 4. Phylogenetic tree analysis of currently identified sequences of *Entamoeba histolytica*, depicted in green triangle and deposited at gene bank NCBI website under the following accession numbers (MT296770, MT296771, MT296772, MT296773, MT296774, MT296775, MT296776, MT296777, MT296778, and MT296779). These are aligned and compared with previously Iraqi identified sequences, indicated by red triangle and Japan's sequence which is labelled with blue triangle as well as sequence from Philippines indicated with yellow triangle.

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