Microscopic and Molecular Detection of Entamoeba species in Human Al-Diwaniyah Province

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Abstract

The aim of this study which conducted in Al-Diwaniyah province during the period from septemper (2022) to march (2023) was to determine the prevalence of Entamoba infection in human and study the effect of some epidemiological factor such as sex ,age and months on the infection rates addition to that and molecular identification of Entamoba. 105 stool sample were collected, from goat in four different regions in Al-Diwaniyah province included (AlShamiya, Al-Sunyih, Ghamas and Al-Mohanawih) at one visit per a week for each regions. The results of microscopic examination showed that 105(65%) of human were infected. according to the sex The results showed that the rate of infection with Entamoeba spp was 40(66.6%) in male and in female 25(55.5)% With significant difference at P<0.05 , according to the ages The higher infection rate was recorded in (1<6) months (87.5%) while the lower infection rate (26.66%) was recorded in age(20-30) years Statistical analysis of the data showed significant variation (P<0.05) on the overall prevalence of Entamoba species between both age groups, according to the Month The study found significant differences (p<0.05) in the Infection rates of Entamoeba spp in human during the months of the study, The highest infection rate was 93.33% occurred in December, while the lowest was 33.33% was recorded in march, results of PCR technique showed that, out of 100 human stool samples (78%) were positive for (18S rRNA) gene.

INTRODUCTION

Amoebiasis is still considered to be a global health problem that spreads in tropical and subtropical regions [1]. The transmission of amoebiasis in developing countries is usually due to poor sanitation, poor hygiene, and crowded living conditions, whereas it is mostly transmitted in developed countries by people who travel from endemic countries [2,3]. About 500 million people are believed to be with amoebiasis worldwide [4]. Previous studies have estimated that 50 million people contract amoebiasis annually and that 100,000 people die from amoebiasis every year [5,6]. However, amoebiasis is considered the third-highest cause of death among human parasitic infections [7,8].

Although the genus Entamoeba contains six species (E. coli, E. histolytica, E. dispar. E. moshkovskii, E. bangladeshi, E. hartmanni, and E. poleki) that colonize the human large intestine, only E. histolytica is considered a pathogen that invades the intestinal tract [9]. E. histolytica is an enteric extracellular protozoan that can attach and then destroy epithelial tissue. The most common manifestations accompanying this disease are bloody diarrhea, fever, abdominal pain, colitis,

malaise, fatigue, flatulence, and weight loss [10]. It has 2 forms (trophozoites and cysts) in its life cycle. Infection occurs by the ingestion of water or food contaminated with cysts. In the small intestine, trophozoites excyst to develop and colonize the colonic region, and then adhere to the mucosal layer of the large intestines. E. histolytica causes intestinal and extra-intestinal infections [11]. In addition, some trophozoites may be excreted in stool outside the human host, but are not able to survive. The signaling pathways that lead to excystation or encystation are not clearly understood [12]. In extra-intestinal infections, E. histolytica parasites may penetrate the intestine wall to reach the liver through the portal circulation to form hepatic abscesses, which can be fatal if untreated. Abscesses may infect other organs, including the lungs and brain [8].

Previously, microscopic examinations were the only technique used in routine diagnostic laboratories to determine the presence of gastrointestinal parasites in stool specimens [13]. However, traditional diagnostic methods do not discriminate among the causative species of disease [14]. Since E. dispar, E. bangladeshi, and E. moshkovskii are morphologically identical to E. histolytica, molecular techniques have been used to facilitate the identification of E. histolytica at the genotype level [8]. Generally, polymerase chain reaction (PCR)-based techniques have higher sensitivity and specificity than microscopic tests [15]. Many studies have widely targeted unique regions of the small subunit ribosomal RNA fragment to diagnose the parasite, as a high copy number provides increased sensitivity [16]. of the species of this parasite in dogs in Iraq. This study determined the prevalence of Entamoeba species in domestic dogs in AlDewineya city using molecular diagnosis and determined the genetic identity of these Entamoeba species by phylogenetic analysis.

Materials and Methods

Fecal Samples Collection

stool samples (10-15) grams were collected from 105 human at different ages, and of both sexes (males and females) during the period from the first of septambe (2022) to end of march (2023) from four different regions in Al-Diwaniyah province included four districts (Al-Shamiya, Al-Sunyih, Ghamas and Al-Mohanawih) at one visit per a week for each regions.

Fecal samples were collected directly from the intestines, in a clean plastic container and were tightly closed, given sequential numbers, with taking off protective measure such as wearing disposable gloves. All information included age, sex, and date of sampling. The samples were transported in refrigerated bags to a Parasitology laboratory in the College of Veterinary Medicine-University of AlQadisiyah.

Microscopic examination

Flotation technique is most commonly used in veterinary medicine for examination of stool it is based on differences in specific gravity of parasite eggs (Dryden et al., 2005). Floatation solutions include zinc sulfate, NaCl and Sheather's (Eckert et al., 1995). Flotation method produce clear material than sedimentation for lighter egg amount, it is easy and inexpensive to perform (Christie et al., 2011).

Molecular detection of the Entamoba spp

The primers were provided as lyophilized form and were dissolved in a high pure water to give a final concentration 100 Pico mole/µl as primer stocks. These were kept at – 20oC until further use in a concentration (0.5 Pico mole/ 20 µl in total PCR reaction). These primers were supplied from Macrogen /Korea (Albanse et al., 2019). Preparation of master mix was done by (AccuPower® PCR

PreMix). Composition of PCR premix tube is formed from (one U of Taq polymerase enzyme 1U, 250µM of dNTPs, 10mM of Tris-HCl ,30mM of 1.5mM of KCl, stabilizer, MgCl2 and dye). Preparation of master mix is done according to companys directions in total volume 20µl after adding extracted DNA 2µl , master mix 10µl ,forward primer and reverse primer at 1µl respectively .Completing the remaining size by deionizer water into 6µl then exposed for mixing by vortex (Bioneer company. Korea). The final reaction was done by using a thermocycler device(Mygenecompany. Made in Korea) depending on company instructions as following:

1. The first stage (denaturing stage) was done at 94°C for a 5 minute

2. 2.The second stage consists of thirty-five cyclesat 94°C for 35 seconds.

3. The third stage (annealing stage) was done at 60°C for 30 seconds.

4. The fourth stage was done at 72°C for 45 seconds

5. The fifth stage (final extension stage) was done for 5minute at 72°C .

The electrophoresis was carried out using agarose gel 1% ;ethidium bromide dye was used for staining and watching under UV light device(table 1) .

Table 1. The primer with their sequence and product size

F:ATTGGAGGGCAAGTCTGGTG 600BP

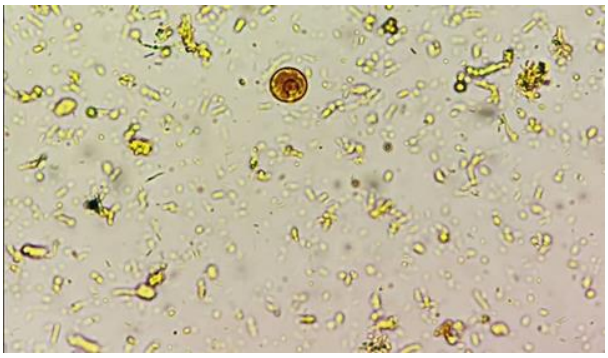
R: CATACTCCCCCTGAAGTCCA

Results

Prevalence according microscopic examination

Out of 105 stool samples 65(61.90%) were found positive for Entamoba spp in microscopic examinationin Figure(1).

Figure 1: The cysts of Entamoeba spp by arrow direct wet mount at (40X)



Infection rate of Entamoba species according to age

The higher infection rate was recorded in human in age (94.44%) and in the age range between (6-12) months in rate (80%), while the lower infection rate (50%) was recorded in animal age more than 12 months.(Table 2) .

Table (2): The infection rate of Entamoeba spp according to age

Age/Years	No. of Examined	No. of infected	Percentage (%)	X^2/ P value
1<6	40	35	87.5	21.71/ 0(S)
6-10	20	10	50	
10-15	15	9	60	
15-20	15	7	46.66	
20-30	15	4	26.66	
Total	105	65	61.90	
S: Significant difference at P<0.05				

Infection rate of Entamoeba spp according to the Month

The study found significant differences (p<0.05) in the Infection rates of Entamoeba spp in human during the months of the study,

The highest infection rate was 93.33% occurred in December, while the lowest was 33.33% was recorded in march. Table 3

Table 3: The infection rate of Entamoeba spp according to month

Months	**No. Exam**	**Positive cases**	%	X^2/ P value
September	**15**	10	66.66	20.19/ 0.003(S)
October	**15**	7	46.66	
November	**15**	14	86.66	
December /2022	15	13	93.33	
January /2023	15	10	66.66	
February	15	6	40	
March	15	5	33.33	
Totale	105	65	61.90	
S: Significant difference at P<0.05				

Infection rate of Entamoeba spp according to the sex

The results showed that the rate of infection with Entamoeba spp was 40(66.6%) in male and in female 25(55.5)% With significant difference at P<0.05.

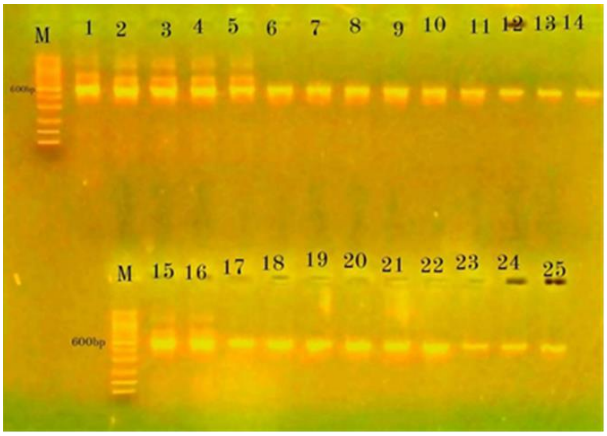
Table (4.1)

Sex	No.of examined patients	No.of infected patients	(%)	X^2/ P value
Male	60	40	66.66	1.34/ 0.246(NS)
female	45	25	55.55	
Total	105	65	61.90	
NS: No significant difference at P<0.05				

Infection rates depending on PCR

Out 105 stool samples 65(78)% were found positive by PCR technique . (Fig. 2).

Figure (2) Agarose gel electrophoresis image(1.5% agarose) that show the PCR product analysis of 18S rRNA gene in Entamoba species of DNA extracted from stool samples human, where ladder (3000-100bp), lanes (1, 2, 3, 4, 5 , 6 7,8, 9, 10, 11,12, 13,14,16,17,18,19,20,21,22,23,24 and 25) shown positive Entamoba species at 600bp PCR product size



Result of DNA sequencing and phylogenetic tree construction

The result of nucleotide sets of local Entamoba species in the present study checked and confirmed by using National Center for Biotechnology Information (NCBI). The local Entamoba species isolates were submitted in NCBI-Genbank data base and Genbank accession numbers were obtained. Based on Clustal W alignment tool of (MEGA 6.0), Sequences of local strains alignment with references strains for Entamoba species which previously recorded in GenBank. Five local E. histolytica isolates (OP522452, OP522453, OP522454, OP522455 and OP522456) were recorded were homology sequence (100%) E. histolytica and identity with three NCBI-Blast of E. histolytica in Iraqi (OQ504207) and iran(DQ899179) and Egypt(MK332025).

Five local E. disper isolates (Op874688, Op874689, Op874690, Op874691 and Op874692) were recorded. were homology sequence (100%) identity with three NCBI-

Blast E.disper of Iraq (OM268859), iran(OM190405) and india (Z97871).

Five local E.moshkovskii (OP529844, OP529845, OP529846, OP529847 andOP529848)identity with NCBI-Blast E. moshkoviskii of Iraq(MT250838) and iran (AB520687) and india.(ON965450) identity (100%). Five local E. poleki isolates (OP564991, OP564992, OP564993, OP564994 and OP564995) were showed closed related to NCBI-Blast to E. poleki of Iraq identity (100%), (FR686399). five local E. hartmanni. While No.(OP529844, OP529845, OP529846, OP529847 and OP529848) were homology to two NCBI-Blast E. hartmanni in Iraq and Russia MW026789 , MF421531 identity (100%).

Phylogenetic tree construction was performed by using MEGA 6.0 version. Phylogenetic tree construction was performed by using MEGA 6.0 version. Most Entamoba species isolates were close related to NCBI-Blast Entamoba of some countries as shown in (Table 5), Figures (3)

Fig (3) Multiple sequence alignment of the identified Entamoa spp. in comparison with homologues global strains. Highlighting with black color to indicate the similarity

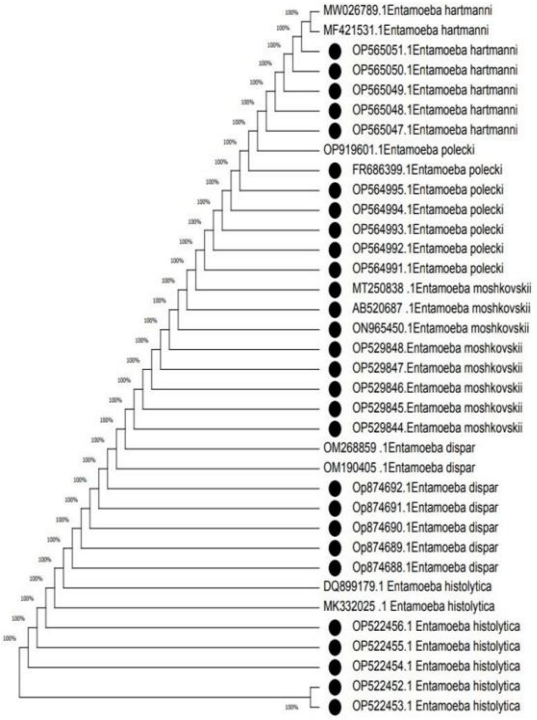


Table (5) the NCBI-BLAST Homology Sequence identity (%) between local Entamoba species and NCBI-BLAST submitted Entamoba species isolates

Nolocal E.histolytica isolate	**Local accession number**	**Country related NCBI**	**Accession no.**	**Identity (%)**
E histolytica GY28460.1 *E.histolytica* KGY284622 *E. histolytica* KG8459 *E. histolytica* KG28458 *E. histolytica* KT8457	OP522452 OP522453 OP522454 OP522455 OP522456	Egypt Iran Iraqi	MK332025 DQ89917 OQ504207	100 100 100
E. disper rmb38 *E. disper* rmb9 *E. disper* rbm3 *E. disper* rbm2 *E. disper* rbm1	Op874688 Op874689 Op874690 Op874691 OP874692	Iran Iraq India	OM190405 OM268859 Z97871	100 100 100

E. moshkovskii K0228 *E. moshkovskii* KT229 *E. moshkovskii KG0230* *E. moshkovskii* KF0231 *E. moshkovskii* KH0232	OP529844 OP529845 OP529846 OP529847 OP529848	India Iran Iraq	ON965450 AB520687 MT250838	100 100 100
E. polecki F7668 *E. polecki* MT7667 *E. polecki MF666* *E. polecki* MF665 E. *polecki* MF764	OP564991 OP564992 OP564993 OP564994 OP564995	FR686399 OP919601	DENMARK iraq	100 100
E. hartmanni F69 *E. hartmanni* OH233 *E. hartmanni* ON6232 *E. hartmanni O231* *E. hartmanni OH27*	OP565047 OP565048 OP565049 OP565050 OP565051	MW026789 MF421531	Russia iraq	100 100

Discussion

Diagnosis of Endameba spp by microscope in 105 Human samples revealed high infection rate65(61.90) The high prevalence of Entamoba species of Human in Al-Diwaniyah province could be attributed to many risk factors such as, the ignorance, overcrowding, inadequate and contaminated water supplies (quality of water consumed), poor sanitation, toilet habit, low socioeconomic status, absence of adequate urban services, place of residence, age, ingestion of raw vegetables (Hamad and Ramzy,2012; Ahmed et al., 2012 Karaman et al., 2006). proved that Intestinal parasitic infections are widely prevalent in developing countries due to poor sanitation, and inadequate personal hygiene. The incidence is also closely related to climate and environmental conditions. Additionaly the prevalence of Entamoeba spp is high among families who eat together from the same plate, among those who eat with their hands, among those who eat away from home and sanitary workers.

The result of study agreed with Alsadi (2022) in Al-Diwaniyah province recorded the prevalence of Entamoba species were 66 % from 200 samples in Al-Diwaniyah province also Alsharaa (2022) recorded the prevalence of Entamoba species (60%), Al- Ammash et al. (2015) in Saladin which recorded (63.64 %), in Thi-Qar Province Al-Yasari et al. , (2019) recorded in rural areas (68.6 %) , But, it was lowest than the results recorded by (lina,2021) in Baghdad recorded (33.33%) ,alsoThe result of study disgreed with Nasser,(2014) who recorded 32% in Basra province, and Al-Azawi (2009) who reported 32.5%, in Baghdad Amidou et al.(2006) recorded (42.2%), in Basra Al-Azawi (2009) found rate infection (32.5%.) .

Ibrahim et al., (2019) in Baqubah province which recorded (30%) Jasim(2016)in Al-

Diwaniya province who reported (44%), In Egypt Naguib et al;(2019) Found rate of Entamoba (32%), in United Arab Emirates ali (2009) recorded infection rate (19%) according to the sex The results showed that the rate of infection with Entamoeba spp in the Male40(66.66%) and females25(55.55%) no segnificant difference between males and females $p<0.05$.

The result of study agreed with Rose et al .(2004) from africana showed that there were non-significant difference in the infection rate with Entamoeba spp prevalence between males and females Male (40%) and female (42%),also Nayyef et al. (2011) in Al-Najaf city study showed that there were non-significant difference in the infection rate with Entamoeba spp prevalence between males 51.42% and females 48.58%.In baghdad Harith (2011) Found rate infection of Entamoeba spp In Male (12.28)%and female (15.35)%, Also in Baghdad ali et al. (2020) Show non different between males and females The infective in Male (24)% and female (26)%.

Rasha (2020) from kufa found infection rate in Male (46.80)% and female (45)%. In kirkuk jangeez et al found the infection in Male(36) % and female (37.3)%. Also in the Al-Diwaniyah province alshraa (2022) recorded infection in male (51.5)% and female(48)%. In karbalaa ali et al. (2011) study showed that there were non-significant difference ($P > 0.05$) in the infection rate with Entamoeba spp prevalence between males 51.42% and females 48.58%.

Mari et al . (2008) in america found infection rate in male (13)%and female (10)%. Also mohammed et al from Pakistan recorded the prevalence of Entamoba species (30)% in the male and(33.3) in female. In libya khafaa et al. (2016) shows infection rate in Male (24)% and female (26)%.Also in libya Rugaia (2017) found non different between males and female the male (30) % and female (32)%.

The result of study disagreed with nasser (2014) which recorded infection in male (44.68)% higher than female (15.66)%. Ahmad who recorded in male (20)% and female(4)% and Al-Ammash ,2015 in Saladin who recorded that male (63.64%) and female (36.36%).

In Malaysia Mengistu et al. (2007) showed tinfection of Entamoeba spp was more prevalent in male(31.5%)as compared with Female(19.6%). Also in Malaysia Muna et al(2017) also which recorded infection in male (46.68%) higher than in females (15,2%).

Taswar et al (2010) in pakstan recorded infection in male Found higher infection in male (69.56%) than Female (55%), A previous study of Ejaz et al. (2011) &Mengistu et al. (2007) showed that the infection of Entamoeba spp was more prevalent in male(31.5%)as compared with Female(19.6%).

InAustralia Rodney et al (2015) showed that the infection of Entamoeba spp was more prevalent in male(52,5%)as compared with Female(20.6%). In malysia Muna et al(2017) also which recorded infection in male (46.68%) higher than in females (15,2%)also Taswar et al (2010) , in pakstan recorded infection in male (22,36%) higher than in females (20,9%) ,Haneen et al (2018) in Baghdad Found higher infection in male (69.56%) than Female (55%) obied et al, (2014) in kufa recorded highest infection in female (8%) than male (6%),also in iran nazary et al ,(2011) Found highest infection in female (52%) than male (37.3%), also In Kenya recorded highest infection in female (63.3%) than male (27.8%) by matery et al,(2016) according to the ages The results of the current study showed that the highest infection rate with Entamoeba spp found in age group (1<6) years with the percentage of (87.5%) , while the lowest occurred in the age group ($\geqslant 20$) years with the percentage (26.66%). However the statistical analysis showed Significant difference ($P<0.05$)

between the percentage of infection between age groups, The results of the present study agreed with many previous studies such as Alreequi et al.(2017) in Yemen, in which they recorded high prevalence of infection (45.3%) in age of less than 1 years compared to lower rate (6.1%) in ages over 41 years.They also reported that children at age 1-10 years were more susceptible infection with Entamoeba spp than other ages. Entamoeba spp infection is more prevalent in younger age groups, this could be explained on the basis of that the children have lower resistance as compared to adults and because many of the crucial defense systems that help to protect adults from diseases are not fully developed in children. They are much more sensitive to parasites than adults, other reasons could be that the children are more exposed to overcrowded conditions (schools, nurseries, playgrounds etc)(Al-Kaeebi and AlDifaie,2016). Parasitic infection among school children may be due to poor conditions in schools, they do not take care of their personal hygiene, such as playing in contaminated outdoor environments, in and around disposal sites (which can certainly cause serious health problems), lack of fecal hygiene and lack of washing hands before meals. Kadir and Naki, 2000).

Entamoeba spp was more frequently encountered during childhood since hygienic habits have not been fully developed yet and in hyper endemic regions the disease was seen in young children while with mild or asymptomatic infection in older children (Gunduz et al., 2005). Nasser (2014) in Basra recorded low infection rate of 15% in group of 0-10 months and high infection rate Of 55% in the age group of 30-40years according to the Months The infection with Entamoeba spp according to the months of the study showed variable rates. The highest rate of was (93.33%) was recorded in Decamber, while the lowest was (33.33%) and reported in March with significance difference($P \leqslant 0.05$).(Table 4.3) The results of the present study was compatible with Abd Al-Wahab (2003) who showed that the highest infection rate was in December (100%) whereas lowest infection rate was in march(40.67%) , In Al-Diwaniyah province(Alubadi,2015)highest infection recorded in November (88,8) and december (77,6) and the lowest was February (30,3)and march(22) ,In AlBasra province Ihsan et al (2017) recorded highest infection in December (48%) and lowest was February (20%) and march (11.8%) , In Alnajef Muslim et al(2016) recorded in December (21%) and lowest was February (12%) and march(3%)

And this study disagreed with (Ali,2015) Babylon peovice highest infection recorded in September (41,93) and the lowest was in December (14,06) ,also Haneen et al ,(2021) Found highest rate in February (73%) and lowest rate in November (31%), also in pakistan Found highest rate in july (27.6%) and lowest rate in February (16.9%) by aAurang et al,(2018), in Erbil Narmin et al ,(2012) highest infection recorded in march (34,93) and the lowest was in may (12,06%).

The acceptable environmental conditions like (temperature, humidness and raining), difference between the results of our study and other studies could also be due to the difference in the variety host by pcr based on amplification of DNA that has been used for the diagnosis of Endameba spp parasites in human . A number of approaches have proved to be both specific and highly sensitive for analyses either of parasites grown in vitro or present in tissue samples and clinical materials (Kawahara et al., 2010). PCR based techniques, have been developed and used for accurate identification and diagnosis of Endameba spp cause of their high sensitivity, specificity, rapidity and utility (Yang et al., 2014).

The total results of PCR technique showed that, out of 100 human stool samples

65(78%) were positive for (18S rRNA) gene. result of study agreed with (Ahmed et al:2022) they recorded prevalence of infection(77.8%) and result of study agreed with labeed (2010) they recorded prevalence of infection(73.4%). Also result of study agreed with Alsaddi(2022) they recorded prevalence of infection(77.8%) .the present study agreed with Alabadi (2015) they found prevalence of infection(70%) . The result of study agreed with (Foter,2021) they recorded prevalence of infection(70.8%) . while disagreeed with lina(2021) they recorded prevalence of infection(33.33%) also disagreed with alsharaa(2022) which recorded prevalence of infection (30.8%) and disagreed with Inabo et al.,(2012) .

In molecular phylogenetic studies, one of the attractive genomic DNA targets is the internal transcribed spacer1 (ITS1) region derived from the ribosomal RNA (rRNA) genes, the ITS1 region belonging to a multiple copy gene family provides a large number of targets for PCR assays (Kawahara et al., 2010), 18S rDNA gene sequences have also been used to define the phylogenetic relationship, inter- and intra-species variation existing among some Entamoba spp isolates (Matsubayashi et al., 2005). The 18S rDNA sequences are considered highly species specific and were used widely in differentiating various closely related species of Entamoba (Ruttkowski et al., 2001; Matsubayashi et al., 2005) .Five local E. histolytica isolates (OP522452, OP522453, OP522454, OP522455 and OP522456) were recorded were homology sequence (100%) E. histolytica and identity with three NCBI-Blast of E. histolytica in Iraqi (OQ504207) and iran(DQ899179) and Egypt(MK332025).

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Due to it clustered in the same nodule which explains a close related among each other and that may be due to the presence of these countries near each other and located on a single geographic line also travel between countries, this disease can be transmitted. As a result, the sequencing data generated in this work will aid in understanding the genetic diversity and geographic distribution of Entamoba species that infect human across the world, also the DNA sequences then phylogenetic analysis represent a useful tool to gain information about an organism evolutionary relationship.

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