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Diagnostic and molecular study of *Entamoeba dispar* in Nineveh Province, Iraq

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Abstract
Amoebiasis is one of the important parasitic intestinal diseases that is widespread in mosul and all Iraq, responsible for many deaths, especially in children, caused by an elementary parasite *Entamoeba histolytica*, and there are other species of the genus Amoeba, which are not pathological, such as *Entamoeba dispar*., and because of the similarity between the two species in morphology, it has become necessary to use the accurate diagnosis of amoebiasis, this study was conducted for the period between October 2021 and until April 2022, included the examination of 96 stool samples from the patients in Nineveh Governorate hospitals and some private medical laboratories whose ages ranged from less than one year to 50 years and for both genders, during this study, microscopic and molecular diagnostic methods were used for two species of the genus Amoeba, as the microscopic examination was carried out using a direct wet smear to detect the stages of the trophozoite parasite, the cyst, or both. The study showed that there was an infection rate of 33.3%, with a rate of (32) of the stool samples diagnosed microscopically out of a total of 96 samples among the examined persons, while the results of using molecular diagnosis using polymerase chain reaction technology showed for 50 stool samples out of 96 samples (32 positive samples and 18 negative samples), the presence of amoeba infection was observed by 38%. In addition, two species of amoeba were diagnosed in the examined samples, the highest rate of infection was for *Entamoeba histolytica*, followed by *Entamoeba dispar*, at a rate of 52.63% and 47.36%, respectively. In addition, the Gene sequencer for the species *E.dispar* was performed. Which was isolated for the first time in Nineveh Governorate and registered in the National Center for Genetic Information with serial number 692812.

Keywords: *Entamoeba dispar*, diagnostic and molecular, Nineveh Province

Introduction
Entamoeba histolytica is a widespread parasite in many countries of the world, including Iraq. The infection rate is estimated at 500 million parasite infections worldwide, as well as 100,000 deaths recorded annually according to the World Health Organization. (Saidin *et al*., 2019) [21], which makes it the third leading cause of death among parasitic diseases, and it spreads in hot tropical and subtropical countries that suffer from poor sanitation (Roshdy *et al*., 2017; Shirley *et al*., 2018) [19, 23]. Including Southeast Asia, Africa, South America, Mexico and many Arab countries.

This parasite belongs to the genus *Entamoeba*, which includes many species that infect humans, including: *Entamoeba histolytica, Entamoeba coli, Entamoeba dispar, Entamoeba polecki, Entamoeba moshkovski, Entamoeba hartmani*. Although all *Entamoeba* species live in the lumen of the intestine, they are coexisting parasites and do not cause disease effects in humans, except for *Entamoeba histolytica*, which is one of the pathogenic species that invades the intestines, causing severe diarrhea cases, especially in children (Parija *et al*., 2014).

The importance of amebiasis lies in causing diarrhea of different ages, as the symptoms of the disease range from mild diarrhea to severe bloody diarrhea with mucus as the parasite invades the intestinal mucosa, in advanced stages of infection, cysts of the parasite can travel through the bloodstream to tissues outside the intestine and reach the liver through the portal circulation to form liver abscesses, which can be fatal if left untreated. (Parija., *et al* 2014; Rure *et al*., 2019) [22]. The infection may develop, causing abscesses in sensitive organs such as the lungs and brain, which would cause serious complications when infected with the parasite *Entamoeba histolytica* in the case that the correct diagnosis is delayed and appropriate treatment is given (Maldonado-Barrera *et al*., 2012) [16].

Therefore, it has become important to correct and accurate diagnosis of the parasite, as the diagnosis is most often made by examining the samples in the laboratory under a light microscope through the presence of tetranuclear cysts or by using immunological techniques such as antigen and antibody detection, but these methods are unable to distinguish between pathogenic and non-pathogenic amoebae due to the phenotypic affinity between these species. (Chacin-Bonilla, 2013; Flaih, 2020) [7].

From the foregoing, we decided in this study to use molecular methods that are characterized by a higher sensitivity and specificity than microscopic tests in diagnosing diarrheal pathogenic amoebae to detect the presence of *Entamoeba histolytica* and *Entamoeba dispar* in stool samples isolated from diarrheal cases (Taniuchi *et al*., 2011; Ögren *et al*., 2020)) [26, 18].

Materials and methods

1. Sample collection

Stool samples were collected from patients suffering from intestinal disorders, abdominal pain, and diarrhea cases from Al-Salam Hospital, The General Hospital, Ibn Al-Atheer Hospital for Children and some external medical laboratories in Mosul / Iraq, for the period from 1/10/2021 to 1/4/2022. During this period, 96 samples were collected from different age groups, ranging between less than 1 year - 50 years old. The samples were examined using a light microscope using the direct wet smear method prepared with physiological saline (Tanyuksel *et al*., 2005) [27]. The samples were kept in freezing at -20°C until the start of the DNA extraction process and subjected to molecular examination.

2. DNA extraction

The DNA of the amoeba parasite was extracted from 50 stool samples using the FavorPrep ™ stool DNA Isolation Mini Kit, according to the instructions of the Korean manufacturer Favorgen, the purity of the extracted DNA was measured using a Nanodrop device, and the extracted DNA was stored at -20 °C.

3. PCR Polymerase Chain Reaction

The polymerase chain reaction targeted the gene of 18SrRNA of the amoeba genus by amplifying the DNA fragment using the primer for each species shown in Table (1) for the two species of *Entamoeba histolytica* and *Entamoeba dispar* under study. (Khairnar and Parija, 2007) [14] Using a thermal recycling device Bio Rad/USA, according to the plan shown in Table (2).

Table 1: Represents the primers that were used in this study to detect the parasite in the polymerase chain reaction with their nucleotide sequence and temperature specific to each primer:

No.	Sequence 5-3	Primer	Temperature (°C)
1	EU1-F	TTTGTATTAGTACAAA	35.4
2	EU2-R	GTAAGTATTGATATACT	40.3
3	EU2-F	TAAGATGCACGAGAGCGAAA	56.4
4	EU2-R	GTACAAAGGGAGGGACGTA	60.5
5	EH-F	AAGCATTGTTTCTAGTCTGAG	56.6
6	EH-R	AAGAGGTCTAACCGAAATTAG	55.5
7	ED-F	TCTAATTTCGATTAGAACTCT	51.6
8	ED-R	TCCCTACCTATTAGACATAGC	57.4

Table 2: Shows the components of the main mixture for the polymerase chain reaction

Material	Size
2X Master Mix	12 µl
Forward primer	1 µl
Reverse primer	1 µl
DNA Template	3 µl
PCR water	4 µl
Final size	21 µl

4. Electrophoresis of agarose gel

A 1.5% agarose gel was prepared by dissolving 1.5 g of agarose powder in 100 ml Tris Borat, EDTA buffer with 1X strength using a 250 ml glass beaker. The beaker was shaken gently to ensure that the solution was mixed with the buffer, then the beaker was placed in the microwave for a minute and a half until boiling and then left to cool and before solidification 3 µl of Gel Red dye was added and mixed well and then poured into the casting tray of the gel electrophilic device that contains the comb, and then leave the gel to harden for 15-20 minutes at room temperature, after which the comb is carefully pulled from the hardened agar, leaving empty and ready holes. Then agarose gel was placed in the gel electrophilic device basin, 700 ml of TBE buffer with a strength of 1X was added, until the agarose gel was completely submerged, then 7 µl of PCR products resulting from the polymerase chain reaction were placed in the specified holes, as for the DNA ladder (100 bp), 4 µl was placed in the first hole in the agarose gel. The power supply was equipped with a voltage of 80 volts and 300 mA for a period of 60 minutes, and after completing the electrophoresis process, the gel was extracted and then placed in a special imaging device Gel Documentation (Gel Doc EZ) (Bio Red, USA) that shines UV rays to detect the amplification products and to enable seeing the DNA packages and then saving the images of results to analysis them later.

Results

1. The rate of infection with the amoeba parasite by microscopic examination

By following up on the results of the microscopic examination that was conducted in the laboratory for 96 stool samples collected during the study period, it was noted that 32 samples showed a positive result for the presence of the parasite in its trophic or cystic stage or both, where the percentage of infection with the amoeba parasite was 33.3%, while the percentage of negative samples of the parasite has 66.6% of the total stool samples included in the study, as shown in Table No. (3), which shows the number of samples and the percentage.

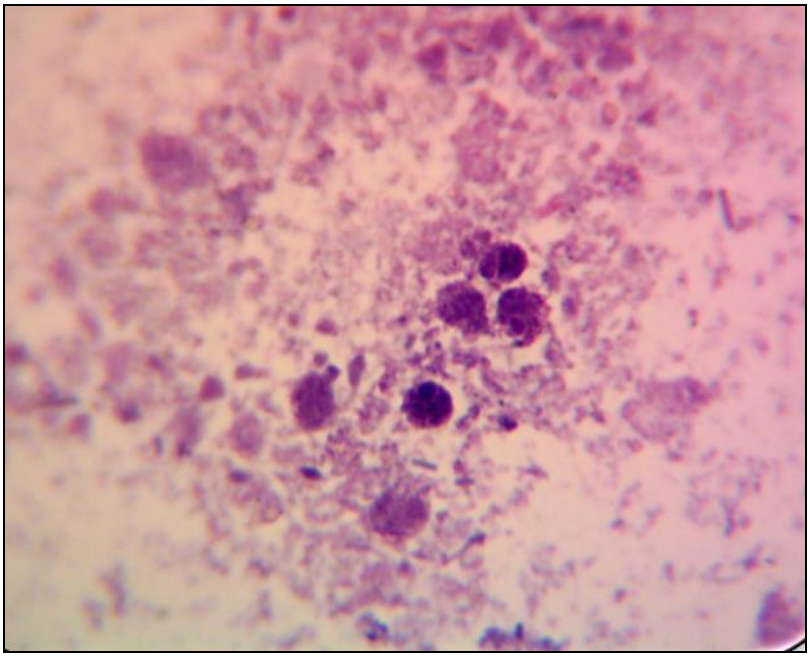

Image 1: Amoeba parasite cysts stained with trichrome (100X)

Table 3: Shows the percentage of infection with amoeba parasite using microscopy.

Number of total samples	Number of positive samples	Percentage	Number of negative samples	Percentage
96	32	33.33%	64	66.66%

2. The results of the molecular examinations

By following up on the results of measuring the concentration and purity of the extracted DNA of the targeted samples, it was noted that all samples appeared with a high purity that ranged between (1.75-2) ng/µl at a wavelength of 260-280. By following up on the results of the molecular examination for 50 random stool samples out of the total samples, the results showed that the number of positive samples was 19 that gave a positive result, while 31 were recorded as negative as shown in Table (3).

Table 3: Shows the number of positive and negative samples of the amoeba genus using PCR technique targeting the 18SrRNA gene.

Molecularly Examined Samples	Positive samples for the amoeba genus	Percentage	Negative samples for the amoeba genus	Percentage
50 (32 positive samples and 18 negative samples)	19	38%	31	62%

The results of the detection of the parasite species belonging to the Amoeba genus diagnosed using the polymerase chain reaction

The study recorded the presence of two species of amoeba genus in Nineveh Governorate, with 10 *Entamoeba histolytica* samples and 9 *Entamoeba dispar* samples using the primers for each species, the results of the molecular examination recorded the presence of the *Entamoeba dispar* for the first time in Nineveh Governorate, with a higher infection rate compared with the *Entamoeba histolytica*. The results were read after the DNA electrophoresis of stool samples on agarose gel with a molecular size of 439bp for the *Entamoeba histolytica* and as shown in Figure (2), and a molecular size of 174bp for the *Entamoeba dispar*, as shown in Figure (3) and Table (4).

Table 4: The results of the conventional polymerase chain reaction to determine the species of parasite in the positive tested samples for the genus Amoeba.

Total molecularly examined samples	Positive samples for the amoeba genus	Positive samples for *Entamoeba histolytica*	Percentage	Positive samples for *Entamoeba dispar*	Percentage
50	19	10	52.63%	9	47.36%

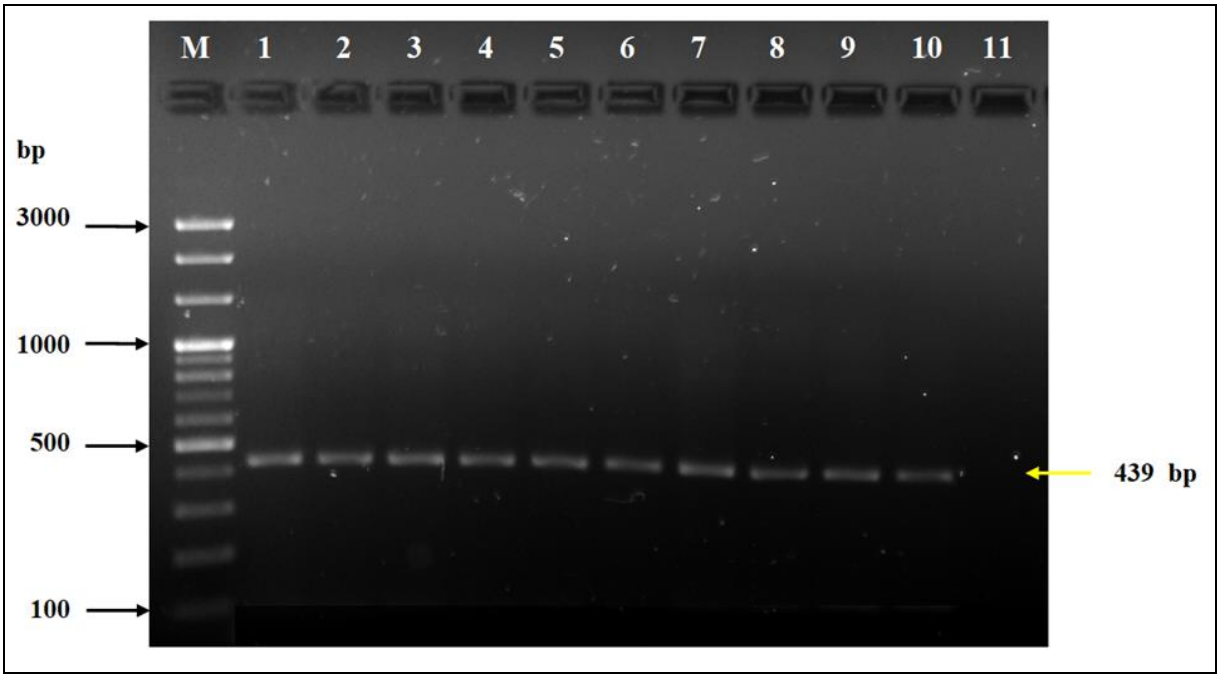

Fig 2: Agarose gel electrophilic of PCR products for detection of *Entamoeba histolytica*. Path M: Marker represents 100 bp size. Path 10-1 represents positive samples with a size of 439 bp, Path 11 represents the negative control.

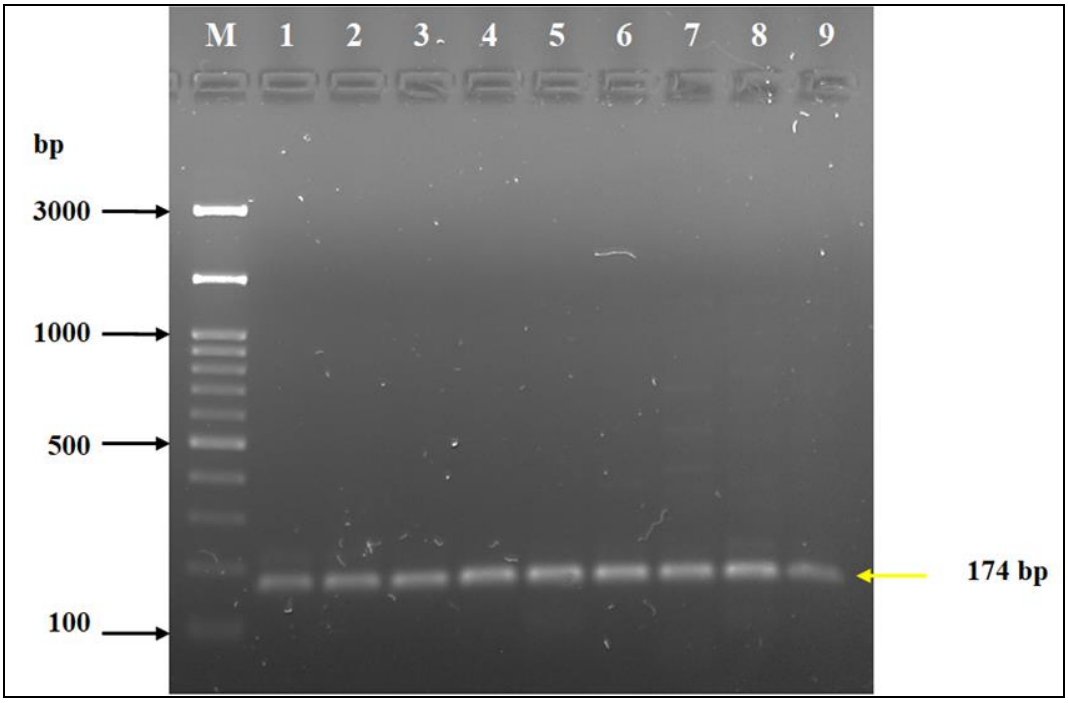

Fig 3: Agarose gel electrophilic of PCR products for the detection of *Entamoeba Dispar*. Path M: Marker represents 100 bp size. Path 9-1 represents positive samples with a size of 174 bp.

Genetic sequence

Results of the genetic sequence of the isolate *E.dispar*

The results of the genetic sequence analysis of the locally isolated *Entamoeba dispar* showed a great congruence of 97% with the isolates recorded in the BLAST database of the gene bank, and the congruence was high for isolates in Iran and Turkey, while the percentage of congruence was lower with the rest of the recorded isolates as shown in Table (6).

It was registered in the gene bank, as it obtained the number identified by the registry ON692812, which is shown in Table (7).

Table 6: Shows the sequence similarity between the local isolate ED-RR-IQ22 (ON692812) and other strains in the gene bank

No.	*Entamoeba Dispar* isolate	Gene name	GenBank Accession number	Country	Sequence identity
1	*Entamoeba Dispar* isolate Ed_shler20	Small subunit ribosomal RNA	MT250839.1	Iraq	99.22%
2	*Entamoeba Dispar* voucher KEN_ED1	Small subunit ribosomal RNA	MH754938.1	Kenya	99.22%
3	*Entamoeba Dispar* isolate Ed_BS.dc	Small subunit ribosomal RNA	KY823418.1	Iran	99.22%
4	*Entamoeba Dispar* isolate B636-IR	Small subunit ribosomal RNA	OM190405.1	Iran	99.22%
5	*Entamoeba Dispar* voucher KEN_ED2	Small subunit ribosomal RNA	MH754939.1	Kenya	99.22%
6	*Entamoeba Dispar* isolate Ed_BS.dmr	Small subunit ribosomal RNA	KY823423.1	Iran	99.22%
7	*Entamoeba Dispar* isolate ED_IQ5	18S ribosomal RNA	KP722600.1	Iraq	99.22%
8	*Entamoeba Dispar* isolate 42	Small subunit ribosomal RNA	MW165339.1	Iraq	99.22%
9	*Entamoeba Dispar*	18S ribosomal RNA	AB282661.1	Japan	99.22%
10	*Entamoeba Dispar*	Small subunit ribosomal RNA	Z49256.1	Italy	99.22%
11	*Entamoeba Dispar* isolate B14108	Small subunit ribosomal RNA	ON318867.1	Malaysia	98.44%
12	*Entamoeba Dispar* isolate PB041	Small subunit ribosomal RNA	ON318865.1	Malaysia	98.44%
13	*Entamoeba Dispar* strain ED13352	18S ribosomal RNA	HQ153408.1	Pakistan	99.2%
14	*Entamoeba Dispar* isolate PT045	Small subunit ribosomal RNA	ON318866.1	Malaysia	97.66%
15	*Entamoeba Dispar* strain Goat2	Small subunit ribosomal RNA	MW624414.1	Iraq	99.18%
16	*Entamoeba Dispar* strain Sheep4	Small subunit ribosomal RNA	MW624413.1	Iraq	99.18%
17	*Entamoeba Dispar* strain Human5	Small subunit ribosomal RNA	MW624412.1	Iraq	99.18%
18	Uncultured Entamoeba	18S ribosomal RNA	LC259420.1	Japan	97.5%

Table 7: Shows the sequence of nitrogenous bases for the local isolate E.dispar, which is registered in the gene bank

Gene bank ID number	The sequence of nitrogenous bases
ON692812	GGCGGTAAGGAAGGTGAGCTTCAGCAATAACAGGTCTGTGA TGCCCTTAGACATCTTGGGCCGCACGCGCGCTACAATGGAGT TACTAGAGAGCATTTTATCATTTACACCTTATTTATTAGGCTAT GTCTAATAGGTAGGGAAGAGTTCTAATCGAAATTAGAAA

The results of the phylogenetic tree consisting of 18 genetic sequences of the 18SrRNA of *Entamoeba Dispar* that are locally isolated with different isolates from all over the world showed a high similarity ratio ranging between 97.5% - 99.22%. It was noted that the local isolate, which was recorded in the gene bank by sequencing (ON692812), has a high percentage of affinity with the Iranian isolate (KY823423.1) with a percentage of 99.22%. As shown in Figure (4).

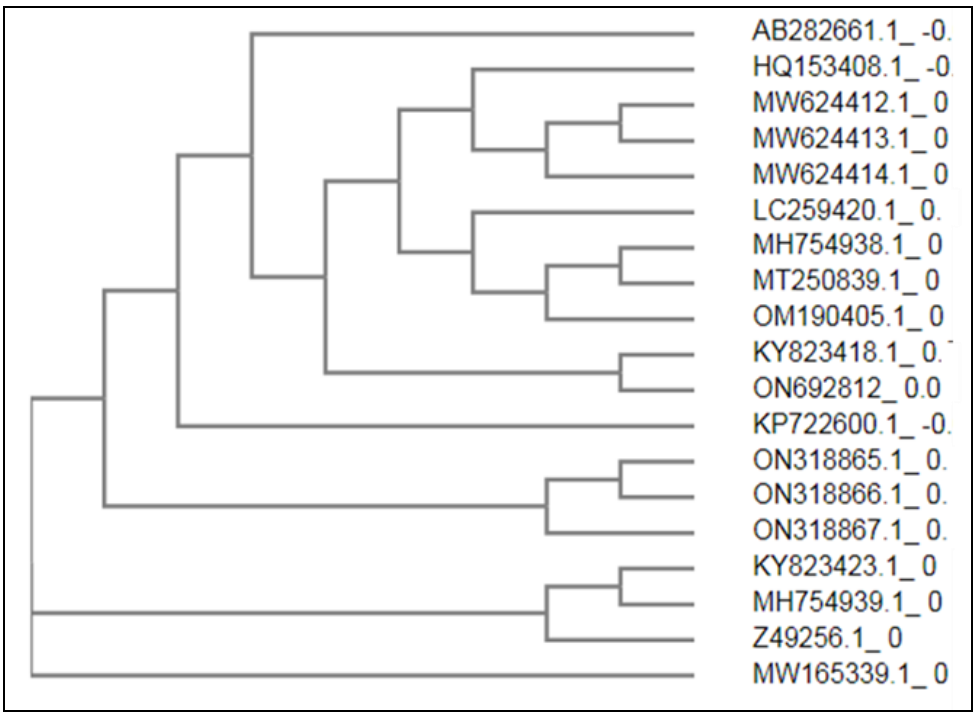

Fig 4: Shows the phylogenetic tree and the relationship between local and global isolates

Discussion

By following up on the results of our current study, it was noted that the number of infections recorded by examining 96 stool samples was (32) positive samples for Entamoeba parasite when using the microscopy method, while the molecular results using PCR showed that only (17) samples were positive for the presence of the parasite. The low infection rate when using molecular diagnosis can be

explained by microscopically incorrect diagnosis, which may be due to the interaction with other elementary parasite cysts diagnosed as a positive result or blood cell residues. (Nugui *et al*., 2012) and (Fallah *et al*., 2014) [9] mentioned that the samples diagnosed positive by microscopic examination and negative by molecular examination may be due to the presence of other species of the genus Amoeba, and this is identical to what happened in our current study, as well as what happened with (Fallah *et al*., 2014) [9].

By following up on the recorded infection rates in Iraq and the extent of the parasite's spread, it shows the importance of studying the presence of the parasite and focusing on the Epidemiology of amoebic dysentery using accurate diagnostic methods to be able to obtain the true percentages of the spread of the disease, as it is noted that the recorded percentages are high in most of the research that adopted microscopic diagnosis, and this is consistent with what we obtained from the results about the spread of the parasite in Nineveh Governorate and the difference in the ratios between it and the molecular methods. Al-Masoudi (2009) [5] recorded 34.3% of *Entamoeba histolytica* parasite in Babylon Governorate. In another study conducted in Baghdad/Rusafa, the prevalence of amoebic dysentery was 31% (Hadi., 2005) [12]. Hamad and Ramzy (2012) [13] also recorded, when studying the epidemiology of *Entamoeba histolytica* among children in Erbil, where the percentage was 30% (Al-Damech and Al-Ebrahimi., 2016) [3], while in Basra, a 60% infection rate was recorded (Al-Hilfi, 2020) [4], and in Salah al-Din, the rate was 9.3% (Kadir *et al*., 2018).

The record of the presence of this parasite in Iraq may be attributed to the low level of sanitation, personal hygiene, population density, climatic conditions and standard of living, therefore, in this study, molecular diagnostic methods were used as an accurate, sensitive and highly efficient method through which we were able to distinguish between two species of the genus Entamoeba and for the first time in Nineveh Governorate, infections with *Entamoeba dispar* parasite were recorded in nine cases diagnosed microscopically as dysentery amoeba, these results are important as they diagnosed the parasite, which could be the cause of a greater pathological effect, and transmission of infection may occur outside the intestine due to error in microscopic diagnosis, which it gave a negative result for the presence of the parasite in some cases, Also, samples that showed a positive result in microscopy and molecularly negative would spare the patient unnecessary treatment and search for the real cause of diarrhea in this case, in addition to the fact that the misuse of medicines would lead to a drug-resistant strain (Santos *et al*., 2007; Dinoop *et al*., 2016; Al-Dalabeeh *et al*., 2020) [22, 8, 2].

In this study, 50 stool samples were examined molecularly, 32 samples were determined microscopically as positive, and out of 32 microscopically positive samples, 17 samples appeared positive for the 18srRNA gene, while two samples appeared molecularly positive out of 18 microscopically negative samples, this result is close to the results of a study in Egypt, where a positive molecular sample appeared out of 20 microscopically negative samples and is almost similar to a study in Iran. (Bahrami *et al*., 2019) [6], as two molecularly positive samples appeared out of a total of 55 microscopically negative samples, if through the results a number of negative microscopically positive molecular infections appeared, and the reason for this may be attributed to the delay in the microscopic examination of the stool sample in the laboratory, which leads to the decomposition of the activators and the lack of clarity in the diagnosis in the laboratory, the negative results can also be explained by using the polymerase chain reaction for some positive samples in the microscopic examination for several reasons, including there is an opportunity for incorrect diagnosis of the parasite in the case of microscopic diagnosis, or incorrect identification of polymorphous white blood cells and macrophages, as well as other stages and species of the genus Amoeba in stool samples for diarrhea as being stages of *Entamoeba histolytica* and lead to a false positive result (Schrader *et al*., 2012; Sidstedt *et al*., 2019; Faqe Mahmood *et al*., 2020) [25, 10].

This is the first study in Nineveh Governorate, during which we were able to isolate and diagnose the parasite *Entamoeba Dispar* from the examined diarrhea cases with 9 samples, as they were classified and registered in the gene bank in the name of the authors of the current study with the code (ON692812). A comparison of our current results with studies in Iraq confirms the presence and spread of the parasite *Entamoeba dispar* on a large scale, as (Faqe Mahmood *et al*., 2020) [10] mentioned through the results obtained when examining fecal samples molecularly from diarrhea cases in the city of Erbil, Kurdistan, Iraq, that the infection rate of *Entamoeba histolytica* is 6%, which is the most common, was followed by infection with the non-pathogenic *E.dispar* with a rate of 4.3%, while *E.moshkovskii* was recorded with a lower rate of 0.3% among the population of Erbil, and this is consistent with the percentages recorded in the current study, while the highest percentage was recorded during the study conducted by Al-Abodi (2015) [1]. In Al-Qadisiyah Governorate, the highest infection rate of *E.histolytica* was 74%, followed by the infection rate of *E.dispar* parasite, which reached 26%. The reason may be due to the difference in the methods of extracting DNA from stool samples, as well as the long period of the study, the large number of samples, the time of the study, the geographical location, the population density and the age group targeted by the study.

From the follow-up of the results of the alignment of nitrogenous bases and the analysis of the phylogenetic tree of the species targeted by the current study, *E.dispar*, which is locally isolated and registered in the gene bank, we note that the isolate diagnosed in the current study gave a great match and high affinity with the Iranian isolate by 99.22%, and this indicates a co-evolutionary origin of these isolates, and this is confirmed by the proximity in the geographical location between the isolates of the parasite, it also confirms the correctness of the diagnosis of the isolates under the current study after comparing them with global isolates and knowing the sequence of the nitrogenous bases of the 18srRNA gene, as no previous study recorded the analysis of the sequence of the nitrogenous bases of the parasite *E. dispar* in the city of Mosul.

The purpose of studying the genetic sequence of parasites is to confirm the diagnosis, draw the genetic map of the parasite, and know the origin and development of the infection, as well as the source of infection for that region from which the parasite was isolated, by following the taxonomic tree, it shows us the clear convergence with some of the recorded isolates, especially the Iranian and Turkish, and their divergence with other isolates. This indicates a co-evolutionary origin for these isolates since geographic

location plays an important role in the relationship between organisms, where isolates from similar or nearby regions tend to be more closely related.

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