**Diagnosis and distinguishing of *Entamoeba* spp. By PCR technique**

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## Abstract

This study conducted between October 2016 to September 2017, with the aims of distinguishing between amoeba species by molecular methods and determination of the prevalence of pathogenic and non-pathogenic species, In Imam Hussein Teaching Hospital, in the province of Thi Qar southern Iraq. 96 stool samples were positive in microscopic examination. After the PCR test, positive samples were found in 86 samples and 10 negative samples *E.histolytica* were the most common 82 (85.4%)Followed by *E.dispar* 14 (14.5%) These two parasites were double *E.histolytica* + *E.dispar* 10 (10.4%) cases, and *E.histolytica* + *E. hartmanni* 8 (8.3%) cases.

Kewords:Entamoeba spp.,PCR technique,DNA Sequance

## Introduction

Amoebiasis caused by the infection of *E. histolytica*, the world's third parasite, which is responsible for the deaths of 40,000 to 100,000 people annually[1]. *E. histolytica* is the most widespread in the world [2] Dangerous infection of amoebiasis is caused by intestinal protozoa [3]. Intestinal protozoa infections are higher in developing countries because inappropriate sanitation and environmental Countries with fecal wastes[4] Rural areas have poor sanitation systems and waste is often disposed inappropriate ways[5].Trophozoite of *E. histolytica*, inhabit in human colon, doubles and distributes cysts, after released these cysts to environment. After the cycts are delivered to the environment, the infection is transmitted to another host when eating food and water contaminated with these cycts, and causes many diseases when parasite invasion of intestinal mucosa, such as dysentery[6]. Also causes rare liver abscesses at transmitted parasites by blood [7]. When the virulence factors of trophozoite break down the mucosal barrier, the invasive disease occurs after the passage through mucous layer and the damage of the intestinal cells, which causes inflammation leading to dysentery[8]. It has most of the infection was asymmetrical[9]. The presence of nonpathogenic cannot be distinguishable among *E.histolytica*, *E.dispar*, *E.hartmanni* and *E.moshkovskii* ,Where they live in the human intestines as commensals It was known for decades, for example, in 1926 that Brumpt proposed the presence of *E.dispar*, which cannot be distinguished from the *E.histolitica* by optical microscopy However, the *E.dispar* exhibits physiological characteristics, biochemical properties and distinct synthetic properties which have been described recently by[10,11,12]. *E.hortmanni* cysts are smaller than the cysts of the *E.histolytica* and thus can be distinguished as the cysts size is (5-10) microns in diameter while the cysts of the *E.histolytica* range between( 12-14) microns[13]. It has recently been suggested that *E.displars* and *E.moshkovskii* are linked to amoebic dysentery and extraintestinal diseases [14]. These results were held to understand the causative manner and public health importance of *E. histolytica*, which cannot be distinguished from *E.displar* , *E.moshkovskii* and *E.hartmanni*. [15]. To determine the incidence of *Entamoeba* species has always been controversial and microscopy examination is usually used to diagnose parasites in stool samples However, this method is unable to differentiate *E. histolytica* from non-pathogenic and similar species wherefore WHO recommends the development and application of new diagnostic methods [16]. Molecular technique one of the methods used in the diagnosis of parasites they are highly sensitive and accurate. They can distinguish between strains of the same type, identify pathogens from non-pathogens, study the genetic structure of the parasite and the relationship of virulence to pathogens. Identification and selection

of appropriate treatment. [17]. The PCR technique also helps to better understand epidemics by knowing the prevalence of parasites with high accuracy [18].

## Material and method

After the collection of 1393 stool samples during the period from October 2016 to July 2017 from the auditors to hospitals in the province of Thi Qar in southern Iraq .The sample examined by direct smear and flotation method using sodium chloride and sedimentation using formalin ether. 96 samples of containing *Entamoeba* spp. were isolated for examination PCR for the purpose of distinguishing *E.histolyica,E.dispar* and *E.hartmanni*.

## Microscopic examination

The samples examined by direct smear methods where a drop of normal saline placed on one end of the slide and a drop of Lugol's iodine placed on the other end. The amount of stool was taken by a stick and mixed with each drop, covered with the slide cover and examined under the microscope with 10X and 40X. [19]. In Floatation technique, 4 g of feces mixed with sodium chloride solution (1: 200 standard density) in a 20 ml container and shaken well to make it emulsified. The container has been filled up. A glass slide placed on the top of the container to touching the solution for 20 minutes. The glass slide was taken quickly for examination under the microscope [19]. And sedimentation technique using formalin ether[20]. Concentrate cysts and worm eggs at the bottom of the tube for densities greater than the density of the medium [19].  mixed (1) g of stool with (10) ml distilled water, filtered through two pieces of gauze to a test tube capacity (15) ml and transported to centrifuge 500 cycles/min for 5 minutes then removed the leachate, added 10 mL formalin (10%) and 3 ml of ether, mixed its contents well and return to centrifuge 500 cycles / min for 5 minutes removed the leachate and the remaining waste took by stick a quantity of precipitate put on a glass slide, covered with a slide cover and examined under a microscope [20].For the purpose of the distinction between *Entamoeba histolytica*/*dispar*  and *E.hartmanni* was saved 5 g of feces in particular tubes under the temperature of 20 – for confirm and distinguish between them by PCR technique.

## Stool DNA extraction

The DNA extracted from 96 positive samples in microscopic examination, used ( Stool Genomic DNA extraction Kit ) according to the manufacturer's instructions. In short, the proteinase K  placed in the tubes and 200 mg of feces added with the addition of stool lysis buffer and incubated at 60C  for 10 minutes then binding buffer and  incubator, add ethanol alcohol and transferred to the binding column in the subject of collection tubes capacity 2 ml and centrifuge 8000 rpm for 1 min. after adding a washing  buffer 1 and 8000 rpm then washing buffer 2 and 12000 rpm for 3 min.and forsake the deposit. It is then kept DNA at -20 to confirm the PCR  C.

assay**.**

## Primers diagnostic prefixes

The primers designed  for gene (18S rRNA) diagnosis of *Entamoeba* spp. in human stool samples. The Genbank-NCBI site was used to obtain the full gene sequence of the 18S rRNA gene for each *Entamoeba* species  and with Primer3plus the primers were designed. The primers were equipped by Korean Bioneer. primers Eh-F(5- GGCCGTTCTTAGTTGGTGGA-3)and Eh-R(5- GTGTGTACAAAGGGCAGGGA-3) for *E.histolytica* specific at product 501bp,Ed-F(5-TCGAAGACGATCAGATACCGT-3) at product 353bp [21]

and *E.hartmanni* protocol described by [22]. E.hartF(5- GGGGAGTATGGTCACAAGGC-3) and E.hartR(5- CGTGCAGCCCAAGATGTCTA-3) by product 429bp.

## Method of diagnosis using PCR assay

PCR technique was performed using 18S rRNA genes responsible for the diagnosis of *Entamoeba* species from human faecal samples according to [21, 22] as in several steps:

## Preparation of PCR master mix

A PCR reaction mix prepared used the AccuPower® PCR Master Mix kit, manufactured by the Korean company Bioneer, as per the company's instructions,mix of 5µL DNA tample and 1.5 µL Forward primer 10 pmol and 1.5 µL Reverse primer 10 pmol and 12 µL PCR water. These components were put in tubes 0.2ml were transferred to vortex centerfuge 3000 rpm for 3 min then PCR thermocycler condation intial denaturation at 95C for 5 min,deaturation 95C at 5 sec,annealing 58C for 30 sec ,extension 72C for 3 min and final extension 72C for 10 min

## DNA sequencer  method

The DNA sequencing method was carried out to perform the definitive diagnosis of the *Entamoeba* parasite species diagnosed by PCR examination by conducting the phylogenetic tree analysis of a small subunit rRNA gene. After the PCR reaction, the PCR reaction was sent to Macrogen in South Korea for a procedure Sequence of DNA using the AB DNA sequencing system.

## RESULT

DNA extracted from 96 positive stool samples by microscopical examination. After PCR testing, show up 86 positive samples (89.6%) and 10 negative samples (10.4%) were found. *E. histolytica* recorded the highest percentage 82 (85.4%) fig.(1) followed by *E.dispar* parasite 14 (14.5%) fig.(2) *E.hartmanni* parasites (10.3%), *E. histolytica + E.dispar* were 10 (10.4%) and *E. histolytica + E.hartmanni* by 8 (8.3%) fig.(3).

DNA extracted by using  (Stool Genomic DNA extraction kit  ) and in the method [21] where a segment of the 18S rRNA gene was amplified to diagnose *Entamoeba* species using primers Eh-F(5- GGCCGTTCTTAGTTGGTGGA-3)and Eh-R(5- GTGTGTACAAAGGGCAGGGA-3) for *E.histolytica* specific at product 501bp,Ed-F(5-TCGAAGACGATCAGATACCGT-3) at product 353bp.[21] and *E.hartmanni* protocol described by [22]. E.hartF(5- GGGGAGTATGGTCACAAGGC-3) and E.hartR(5- CGTGCAGCCCAAGATGTCTA-3) by product 429bp.
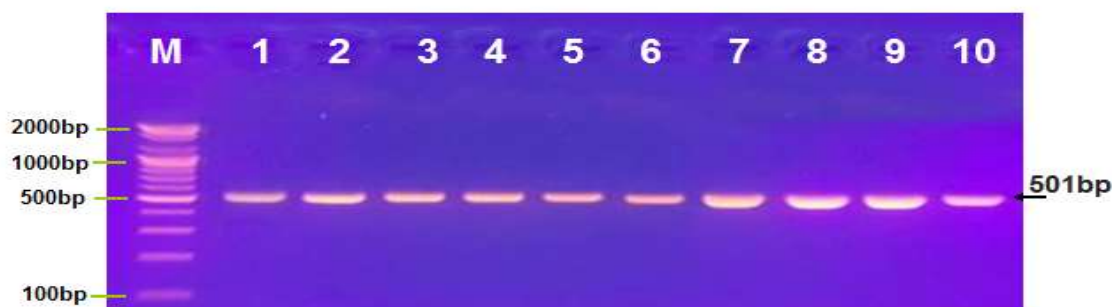
Figure (1):PCR profile , which contains the results of the PCR examination of the 18S rRNA gene for *E.histlytica* diagnosis, for the DNA sequencer analysis where M: Marker ladder 10000-100bp and the numbers from (1-10). Some test positive samples represent the 18S rRNA gene and the 501bp model.
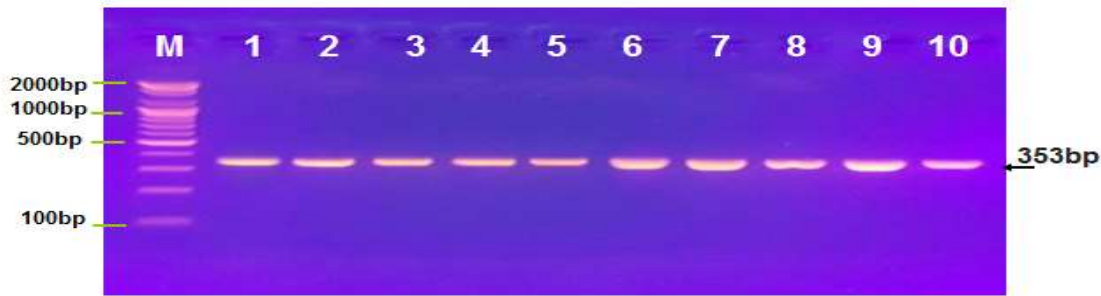


Figure (2): PCR profile, which contains the results of the PCR examination of the 18S rRNA gene of the *E.dispar*, for the DNA sequencer analysis where M: Marker ladder 10000-100bp and the numbers from (1- 10) represent some positive test samples for the 18S rRNA gene and 353bp.



Fig.(3) PCR profile, which contains the results of the PCR examination of the 18S rRNA gene of the *E.hartmanni* genotype for the DNA sequencer analysis where M: Marker ladder 10000-100bp and the numbers from (1- 8) represent  positive test samples for the 18S rRNA gene and 429bp.

## *DNA sequencing*

The results obtained from a PCR assay of isolates of the studied species (one isolate of each species), which carried on the electrophoresis gel of the 18S rRNA gene, which represents the diagnostic gene of these species with a particle size (501bp) of type *E. histolytica* as in Fig. (2-4) and molecular size (353bp) for species *E.dispar* as in Fig. (3-4) and molecular size (429bp) for type *E.hartmanni* and Fig. (4-4) shows this. This proves the popularity of these isolates to globally recognized species. After confirming these isolates by PCR technique, We have registered in the database of the National Center Biotechnology Information (NCBI) in GenBank according to our   accession numbers which we obtained on her. These accession numbers are MF421529 for *E.dispar*, MF421530 for *E.histolytica* and MF421531 for *E.hartmani.*

### *phylogenetic tree analysis of* **Entamoeba** *spp***.**

PCR and MEGA 6 used to phylogenetic tree analysis of (*E. histolyica, E.dispar, E.hartmanni*). There was a clear convergence of the nitrogen bases of these isolates with the globally registered isolates carrying serial numbers (Z49256.1, X64142.1, KX618191.1) respectively, as shown in Fig. (5.4), and the sequence of nitrogenic bases of local *E.histlytica* parasites with internationally registered *E.histlytica* showed a (99%) compliance ratio using the  NCBI blast program. Nitrogen bases Following local *E.dispar* with *E.dispar* worldwide registered by match (100%) using NCBI blast program, as well as the rules nitrogenous parasite

*E.hartmanni* local sequence with *E.hartmanni* recorded globally reached match ratio (100%) using NCBI blast program.
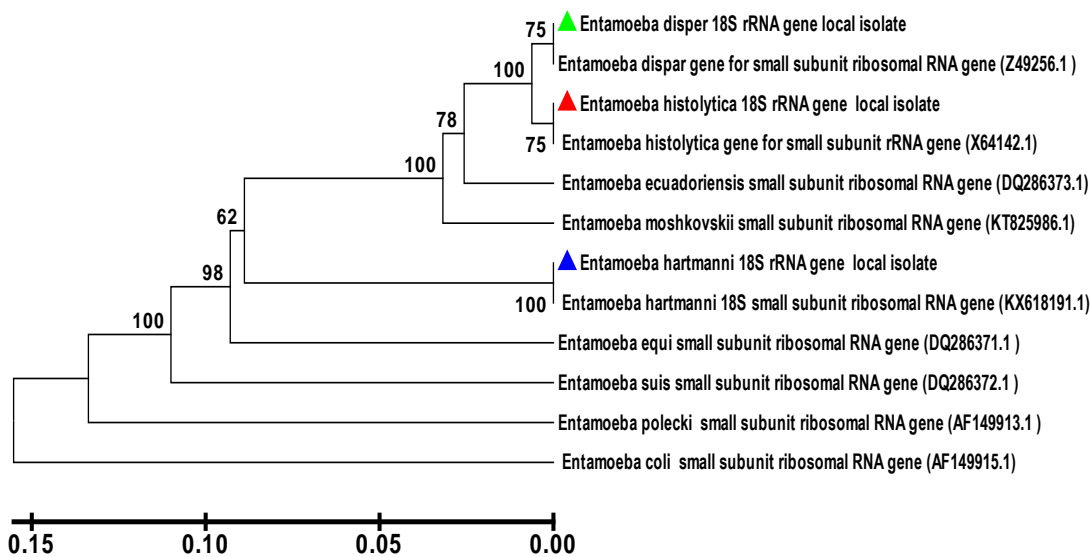


Figure 5-4: The analysis of the phylogenetic tree analysis using the MEGA 6 program, where the UPGMA tree (Unweighted Pair Group Method with Arithmetic Mean) was used in the analysis of the genetic tree, where the results of the analysis showed a clear similarity to the parasite *E.histlytica, E.dispar* and *E.hartmanni* with globally recorded species as opposed to other species of *Entamoeba* which showed a marked difference when compared with local samples.

## *Discussion*

PCR and DNA sequencing used to differentiate between *E. histolytica*, *E.dispar* and *E.hartmanni* species, which cannot be distinguished by microscopy[16]. Microscopy is unable to distinguish pathogens and non pathogens species of *Entamoeba* [23 , 24]. Modern technologies are expensive, so they are not use in many countries, resulting in inaccurate epidemiological estimates [25 , 26].

In order to determine the prevalence of *E. histolytica*, *E.dispar*, *E.hartmanni*, PCR used to target the 18S rRNA gene to confirm the examination of 96 positive stool samples for *Entamoeba* spp. by microscope .But PCR found 86(89.5%) positive stool samples and 10(10.4%) negative, close to that of [27] with 86.2% in Malaysia, The percentage is higher than that recorded by [28] (62.69%). Iran, and [29] ratio (67.7%) in Malaysia.In this study, *E.histlytica* recorded the highest infection rate (85.4%)followed by *E.dispar* (14.4%) and *E.hartmanni* (8.3%). This is consistent with [30]. In addition, the percentage of *E.hartytica* and the lowest proportion of *E.hartmanni* and [31] in Libya also have the highest percentage of *E.histlytica* and the lowest percentage of *E.hartmanni*. However, these results differed with [32] with E. *dispar* (54.8%) followed by *E. histolytica* (25.8%) and [33] in Nigeria, where the incidence of parasitic infection was *E.dispar* (68.8%) and *E. histolytica* (37.5%), and the results recorded by [34] From *E.histolytica / dispar*

The incidence of *E.histlytica* (85.4%) was almost identical with that of [35] with a 75% incidence in Malaysia and was not consistent with other studies where the recorded rate was higher than that recorded by [36]in Mozambique (10%), **[37]** in Ethiopia (1.7%)**,** and [38] with 15.6% in Bangladesh**.** But [39] in Argentina and [40] were unable to record any percentage With this parasite**.**

In this study, *E.dispar* recorded an infection rate of (14.5%). This corresponds to [41] in eastern Brazil, where the parasite was infected (14.3%) and [27] 14.3% in Kampung Bumbun and [29], with (13.4%) in Malaysia but less than [37,41] The results were higher than that recorded by [14] if the infection rate (8.8%) was higher than that recorded by [42] in the United Arab Emirates (2.5%) and so on his [40] in Egypt 2%.

The *E.hartmanni* parasite recorded an infection rate of 8.3%. This is the same as recorded in [36] in Mozambique, where 9% recorded but differed with other studies where it was higher than [41]4.8%, and also higher than [30] in Libya is recorder (4.8%), and [34]) in Iran (0.69%), and [31] recorded a 0.3% rate among African residents in Libya.

## Single and mixed infection rates in stool samples

In this study, *E.histytica* (66.6%) and *E.dispar* (4.1%) were found to be infected with *E.hartmanni* parasites. No single infection were reported.

*Ehistolytica* scored 66.6%, which is almost identical with [35] with (63.5%) The different percentages of our study were results [33] (18.8%) in Nigeria. [42] recorded 10% as the single case of this species, but this percentage is lower than that recorded by [35]In the city of Pos Iskandar (96.6%), in Sungai Layau, the percentage was 75%.

The percentage of parasite *E.dispar* is (4.1%). This result is similar to that of [43] in Iran with a percentage of (3.45%). It differed with [33], (43.8%) and [14] with a percentage of 63.2%, and the results were higher than [40] in Egypt (2%) and [44] by (1.1%) in Malaysia.

*E.hartmanni* did not have a single infection in this study.The mixed infection *E.histolytica* and *E.dispar* wae (10.3%) this Compatible with [35], which was 11.5%, and lower than recorder by [41],14.3%, and [33] (18.8%) and much lower than that recorded by [27] by 39.3% The study of [42] recorded a rate of infection (3.3%).And [44] in Malaysia record rate (1.1%) and these percentages are less than recorded from this study. The proportion of the mixed infection of *E. histolytica* and *E. hartmanni* parasites was 8.3%, which is lower than that recorded by [45], which reached (25%) in Angola, in addition to other species accompanied his studies.

### *DNA sequencer analysis*

It was observed through the analysis of the genetic tree as well as the convergence of the nitrogen bases of the *Entamoeba* spp. In our current study, some samples are genetically similar and different with each other. In our study, *E.dispar* was genetically related to the Z49256.1 serial number recorded by[46] in Milan, (1993),and the serial numberX64142.1 recorded by [47], in India, they express *E.histlytica* parasites, as well as the serial number KX618191.1 recorded by [48] In Singapore are similar to the *E.hartmanni* parasite sample The purpose of DNA sequencer is to ascertain the isolates of the target .recorded locally by our current study. species in our present study and compare them with the global isolates and to know the sequence of the 18S rRNA. The analysis and identification of the DNA sequence are accurate and highly sensitive diagnostic methods and can be used in many aspects of life and practice . It can also be used to carry out many studies Since there is .related to hereditary biologics and compare them with previous studies, both global and local. no previous study on DNA sequencing in *E.hartmanni*, it has been registered in the GenBank database within the NCBI database. The difference in the results of these studies may be due to the conditions surrounding the laboratory such as the conservation of positive samples and the conditions of storage and transfer, as they need

to temperature less than 20 - If kept or delayed at room temperature may work on the decomposition of DNA, which results in the analysis of PCR In the case of preservatives, if they used, they negatively affect DNA [50]. The difference is due to differences in methods of DNA extraction as well as the severity of infection and the number of parasites present in the sample [50].

The cause of positive specimens by microscopy higher than PCR may be due to non-discrimination in appearance between pathogens and nonpathogens species which living  intestinal  the use of sensitive and highly specific diagnostic techniques is therefore required. To overcome the difficulties Encounter by researchers and technicians in distinguishing between these organisms for the purpose of giving accurate indicators and better understanding of epidemiology among individuals [49].

**Conclusion** PCR and DNA sequencer are highly efficient compared with microscopes [23 , 24] and are able to accurately distinguish between species up   to 100% sensitivity (52, 23).

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