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Molecular Study of *Enamoeba dispar* and *Entamoeba moshkovskii* isolated from amoeboid dysentery in comparison with *Entamoeba histolytica* infections

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Abstract

Background: *Entamoeba moshkovskii* and *E. dispar* are impossible to differentiate microscopically from the pathogenic species *E. histolytica.* polymerase chain reaction (PCR) is a widespread molecular biology technique for amplification of multiple targets in a single PCR experiment.

Methods: For detection and differentiation of the three-microscopy indistinguishable *Entamoeba* species in human, PCR assay using different DNA extraction methods was studied. A conserved forward primer was derived from the middle of the small-subunit rRNA gene, and reverse primers were designed from signature sequences specific to each of these three *Entamoeba* species.

Results : The current study included the examination of 634 stool samples of patients attended Baghdad Teaching hospital, Al-sadir hospital and Al Zahraa hospital Hospital in Baghdad city, during the period between 1/2016 to 10/2016. The current study revealed the out of 634 stool samples examined by wet mount and formalin-ether concentration 17.03%, 108 were infected with *E.dispar* trophozoite and /or cyst ,as found in stool samples varying from solid to semi-solid to diarrhea (watery or bloody and mucus together). The epidemiological survey based on the results of microscopic examination showed the highest infection rate were among males 19.44%(70 of 360) than females 13.86% (38 of 274), with the highest infection rate of 4.1% in the age groups (≤ 1 – 10year) and (11-20year), the highest percentage of infection between residents of rural areas 9.77% compared to residents of urban areas 7.26%. Extraction method used in the current study proved to be a good way to get the suitable concentration and purity for the use of DNA as a template output in the PCR technique through direct extraction of DNA from 108 feces samples. The present study recorded a single parasite infection *E.dispar* in 90(90 or 108 as mention above??)or you mean 90 detected by PCR out of 108) samples (83.33%), while the mixed infection by *E.dispar* + *E.moshkovskii* in 2 samples (1.85%), out of 108 positive samples were examined under microscope and were subjected to PCR technique, where the overall proportion recorded of infection in 92 samples (85.18%) of the total 108, the out of 634 (14.5%), the current study did not register of *E. histolytica* infection. As well as the *E.moshkovskii* infection did not register individually and recorded 16 negative samples (14.8%).

Conclusion : Study the epidiomoloigcal prevalence of *Entaembia* spp ,study the route of cut off life cycle of the parasite and make further study for vaccine production . We recommend this PCR assay as an accurate, rapid, and effective diagnostic method for the detection and discrimination of these three *Entaembia* species in both routine diagnosis of amoebiasis and epidemiological surveys

Key words : DNA extraction, *E.dispar*, *E. histolytica* , *E.moshkovskii*., PCR

INTRODUCTION

Intestinal parasitic infections have long been recognized as an important health problem worldwide(1). Infection with pathogenic parasites has been associated with significant morbidity and mortality especially in the young, malnourished and immunosuppressed(1). Parasitic infections are regarded as a serious public-health problem, as they cause malnutrition, iron-deficiency anemia, growth retardation in children, and other physical and mental health problems (2). The prevalence of intestinal parasitic infections depends on the socio- economic level of the society, social practice and traditions, poor sanitary and environmental conditions, inadequate personal hygiene, absence of safe drinking water supplies and climatic factors(3). In developing countries, prevalence rates rang from 30-60%, as compared to < 2% in the developed countries(4,5). Diarrheal diseases are a major cause of mortality in developing countries; their control and prevention are one of the main objectives of the World Health Organization (WHO) within the Division of Diarrheal and Acute Respiratory Disease Control programs in developing countries (5) It is impossible to give an accurate estimate of the economic importance of parasitic disease because it varies so greatly between countries and between regions (5). Despite the great development that has occurred on the quality of medical services in terms of diagnosis of parasitic diseases, treatment and control, which in turn led to a decrease evident in the spread of these diseases in many industrialized countries and developing countries, most of the parasitic diseases still consider a major challenge for health staff in many developing countries and poor (6). The importance of intestinal parasites in the capacity of spreading and infecting a large segment of the population, especially children in most developing countries and poor countries. , For ease of infection in most types example *Entamoeba histolytica* and *Giardia lamblia*, and some worms example *Hymenolepis nana*, which is contagion by eating food and drinks contaminated with one of the roles of her life and be on the likely contamination of vegetables and water sanitation, excreta, as is the case in many parts of the world (6). *Entamoeba moshkovskii*, *Entamoebadispar* and *Entamoeba histolytica* are morphologically identical but biochemically and genetically are different and microscopic examination is unable to detect and differentiate these three *Enatamoeba* spp. Although *E. histolytica* is known to be pathogen, the other two species are non-pathogen or the ability of them to cause disease is unclear (7). Before redescription of *E. histolytica* and *E. dispar* in 1997 (7), several epidemiological studies in Iran have shown *Entamoeba* spp. infection rate of about 2.2 to 30 percent (7). In the past decade, these three *Entamoeba* have been differentiated and reported by molecular methods in some areas of Iraq.(7).

Extraction of DNA is often an early and important step in many diagnostic processes used to detect bacteria, viruses and parasites in the environment as well as diagnosing disease and genetic disorders .(8).

In the presence study, we aimed the detection the presence of *E. histolytica*, *E. dispar* and *E. mosh kovskii kovskii* in stool samples by molecular techniques and study the prevalences of Entamoeba spp according to the age ,sex,geographical distribution .

MATERIALS AND METHODS

The current study included the examination of 634 stool samples of patients attended Baghdad Teaching hospital, Al-sadir hospital

and Al Zahraa hospital Hospital in Baghdad city, during the period between 1/2016 to 10/2016.

The current study revealed the out of 634 stool samples examined by wet mount and formalin-ether concentration 17.03%, 108 were infected with *E.dispar* trophozoite and /or cyst ,as found in stool samples varying from solid to semi-solid to diarrhea (watery or bloody and mucus together).The age was ranged from (1-60 years), from various socio- economic status had been examined for intestinal parasites in their stool by using both direct smear and concentration methods. A detailed questionnaire was filled with all necessary information's for each

Stool samples: Samples were collected for each patient in clean universal screw cap battles, one gram of the feces was kept to be ready examined by direct smear method, and a drop of Lugol's iodine solution was mixed with a fleck of the feces (approximately 0.2 gm) and spread by means of an applicator stick on a clean microscopic slide to suitable thickness. A cover slip was put on the smear and examined under low and high objectives lens. Finally, the samples were concentrated using the procedure of zinc-sulphate flotation technique and iodine stained slides were prepared and examined microscopically(8). The results were presented in numbers, percentages and by using chi-square as a test of significance.

DNA Extraction :

For DNA extraction, 200 mg of stool sample was taken to extract DNA using QIAamp DNA stool mini kit (Qiagen) according to the manufacturer's protocol with few modifications: all the centrifugations steps were carried out at 800 g except the final step of purification in which centrifugation was done at 1300 g. The pellet was washed with PBS buffer pH 6.8 and resuspended in the same buffer. A 200 µl volume of this suspension was used to extract DNA from cysts and trophozoites of *Entamoeba*. The extracted DNA was used as positive control for the amplification reactions. The purity of the extracted DNA was estimated from the absorbance ratio 260/280 and its concentration in all the samples was estimated from the 280 nm readings.

PCR amplification of the targeted genes:

The primer sequences used were as follows: for EntaF, 5-ATG CAC GAG AGC GAA AGC AT-3; for EhR, 5-GAT CTA GAA ACA ATG CTT CTC T-3; for EdR, 5-CAC CAC TTA CTA TCC CTA CC-3; and for EmR, 5-TGA CCG GAG CCA GAG ACA T-3. All primer sequences were compared to sequences in GenBank. This showed that the forward primer (EntaF) sequence is found only in Entamoeba and that the three reverse primer (EhR, EdR, and EmR) sequences are species specific. They are therefore suitable for species differentiation. The forward primer in combination with the appropriate reverse primer generates a 166-bp PCR product with *E. histolytica* DNA, a 752-bp PCR product with *E. dispar* DNA, and a 580-bp product with *E. moshkovskii* DNA.

The PCR amplification reaction was performed in a final volume of 5l(are u mean 51µl) in 0.1-ml PCR tubes by use of a Px2 thermal cycler (ThermoHybaid, United Kingdom). Reaction conditions were optimized to combine the forward primer (EntaF) with each of the three reverse primers (EhR, EdR, and EmR) in a single reaction mixture and under the same conditions. The reaction mixture contained 200 M of each deoxynucleoside triphosphate, 0.1 M of each forward and reverse primer, 6 mM MgCl2, 0.5 U of Taq polymerase, 1 Taq buffer, and 10 l of extracted DNA samples. Amplification of each species-specific DNA fragment started with an initial denaturation at 94°C for 3 min, followed by 30 cycles of 94°C for 1 min, 58°C for 1 min, and 72°C for 1 min, with a final extension at 72°C for 7 min. Amplified products were visualized with ethidium bromide staining after electrophoresis on 1.5% agarose gels.(How to calculate the final volume 51)???

Determination of the sensitivity and specificity of PCR primers. To determine the sensitivity of the assay, 10 concentrations of DNA from each Entamoeba species were prepared by two fold serial dilution from 5 ng to 0.6 pg of DNA. The sensitivity test was performed using the same protocols described above. A mixture of DNAs from *E. histolytica* , *E. dispar*, and *E. moshkovskii* was tested for any cross-reaction or cross-amplification between primers designed for the three Entamoeba species. The assay was also tested for specificity against a panel of genomic DNAs from different bacterial pathogens and other protozoa. Group I contained eight different genomic DNAs obtained from organisms in culture, including human cell lines and axenic cultures of a variety of pathogens known to caused intestinal infections in humans: Escherichia coli, Salmonella spp., Shigella spp., *Vibrio cholerae* , *Blastocystis hominis*, *Giardia lamblia*, and Cryptosporidium spp. DNA extracted from a parasite-free fecal sample was used as a negative control. Group II consisted of DNA extracted from eight fecal samples, each containing one of the following parasites: *Entamoeba coli*, *Endolimax nana*, *Blastocystis hominis*, *Giardia lamblia*, or *Cryptosporidium parvum*.

RESULTS

The proportion of parasite infection by *E. histolytica* / *E. dispar* were examined by direct microscopic in wet smear of concentration method, examined 634 samples, using local Iodine 1% and brine 0.85% and Eosien 1% and the ether formalin concentration .Our findings shows that 108 samples (17.03%) contained a mutant been Trophozoite or phase cyst parasite as in the were showed in figure (1), and did not score parasite infection in 526 (82.97%) and also in the table (1).

Table (1) showed the prevalence rate of *E .dispar* parasite by direct moist swab and concentration method.

Table (1): Percentage of *E .dispar* in infected samples.

Parasite	No.	Parasite concentration(%)
E. dispar	108	17.3%
Negative samples	526	82.97%
Total	634	100%

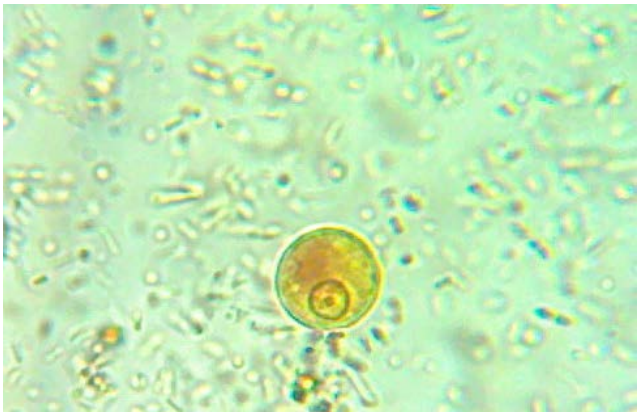

Figure (1): single phase shows the nucleus of the parasite *E.dispar* sample were stained by local iodine dye 1%, under the power of magnification (1000) using oil lens.

Table(2) showed of 214 sample with diarrhea ranging from watery to bloody and mucus together, the parasite was found in 82 samples (38.3%), and 240 semi-formed sample, the parasite were found in 18 samples (7.5%), and 180 formed sample, the parasite were found in 8 samples (4.4%).

Table (2): The prevalence rates of positive stool samples according to the consistancy.

Formed of samples	No.	Positive samples	(%)
Formed	180	8	4.4%
semi-formed	240	18	7.5 %
Diarrhea	214	82	38.3%

The proportion of *E.dispar* infection according the gender

The current study reported the infection in both genuses, were higher in males (19.44%), while the infected females were 36 (13.86%).as showes in table (3).

Table (3): The prevalance of *E.dispar* infection according the gender

Gender	No. of tests	No. of patients	Percentage of patients.	Percentage of total No.
Male	360	70	19.44%	11.04%
Female	274	36	13.86%	5.99%
Total	634	108	33.3 %	17.03%

The current study reported infection in different age groups (≤1 - 60 years), with the highest proportion of infection in age groups (≤1- 10 years old) and (11-20)years, 13 (4.1%) in both, and the lowest percentage of infection in category age (41-50) years 3 (0.94%), while it was in the age group (21-30 years), 11 (3.47%), and the age group (31-40 years), 10 (3.16%) and age (51 - 60 years) 4 (1.26%).as showen in table (4).

Table (4): The proportion of parasite infection *E.dispar* according to age group.

Age group	No. of patients	(%)
1-10	26	4.1%
11-20	26	4.1%
21-30	22	3.47%
31-40	20	3.16%
41-50	6	0.94%
51-60	8	1.26%
Total	108	17.03%

The current study reported infection in the inhabitants of both the rural and the urban where the infection was higher in rural areas and the number of people with 62 (9.77%) and in the city's total number of infected 46 (7.26%) and also in the table (4).

Table (5): The proportion of parasite infection *E.dispar* according the residency.

Residency	Total No.	No. of Patients	The percentage according residency	The percentage according the No. of patients
urban	302	46	15.23	7.26%
Rural	332	62	18.67	9.77%
Total	634	108		17.03%

DNA extraction of *E.dispar* from feces samples:

Extraction method used in the current study proved to be a good way to get the suitable concentration and purity for the use of DNA as a template output in the PCR technique through direct extraction of DNA from 108 feces samples figure (2).

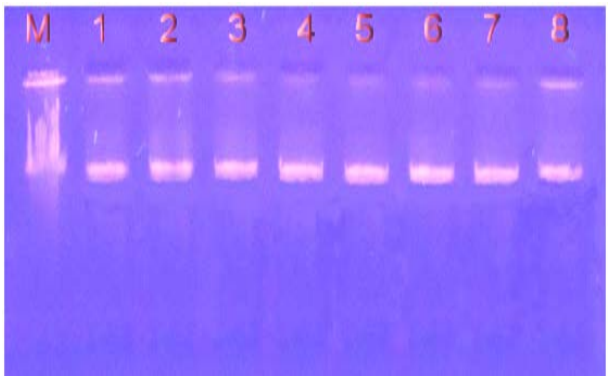

Figure (2): Electrophoresis of DNA extracted from feces samples in agaros gel concentration of 1%, M represents a Genomic marker and samples of DNA extracted.

PCR Technique:

The Primers used:

The primers were used showed the sizes were required, where they showed the presence of primers (Edp1F -Edp2R) pairs the size 96 bp base pairs, (Ehp1F-Ehp2R) pairs size of 132 bp base pairs, but were showed the primers (EntaF-EmR) pairs with the size of 580 bp.

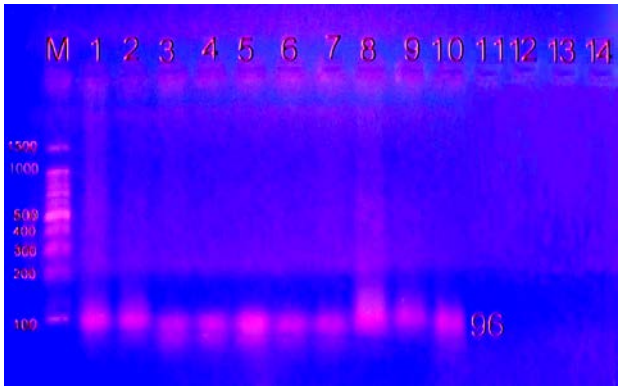

Fig (3): Electrophoresis of PCR products , (bp100) DNA ladder, and samples (1-10) are positive for the parasite *E.dispar*, and samples (11-13) are negative, and the sample (14) as a control.

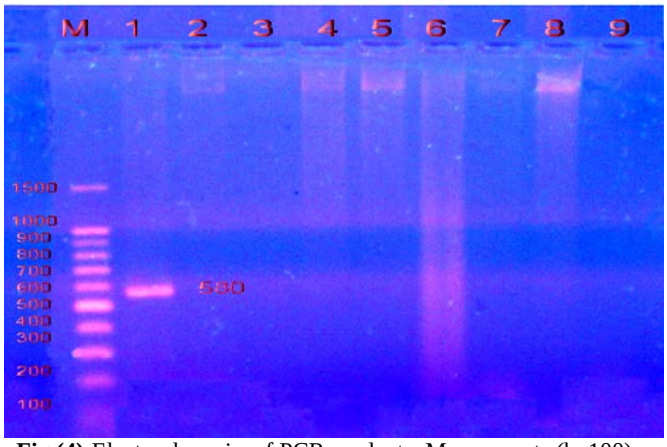

Fig (4): Electrophoresis of PCR products ,M represents (bp100) DNA ladder, the sample (1) is positive for the parasite *E.moshkovskii*, (580 bp) and samples (2-8) are negative, and the sample No. 9 as a control.

Table (6): The proportion of infection parasites *E.histolytica / E.dispar, E. moshkovskii* using PCR technique.

No. of samples	Parasitic Infection	parasites	No (%)	% Total number	negativesamples	% +ve samples
108	90	*E.dispar*	83.33%	14.51%	16	*14.81%*
	2	*E.dispar* + *E.moshkovskii*	1.85%			
Total	92		85.18%			

The proportion of parasites infection with *E.histolytica, E.dispar, E.moshkovskii* using PCR technique.

The present study recorded a single parasite infection *E.dispar* in 90 samples (83.33%), while the mixed infection by *E.dispar* + *E.moshkovskii* in 2 samples (1.85%), out of 108 positive samples were examined under microscope and were subjected to PCR technique, where the overall proportion recorded of infection in 92 samples (85.18%) of the total 108, the out of 634 (14.5%), the current study did not register of *E. histolytica* infection. As well as the *E.moshkovskii* infection did not register individually and recorded 16 negative samples (14.8%). According table(6).

The proportion of infection and parasite *E.dispar E.moshkovskii* according the gender

The current study were reported the infection in both gender with *E.dispar*, but the rate was higher in males where the number of infected males 108 injured male (77.14%), and 104 were infected male single injury (74.28%) parasite with *E.dispar*, and infected male by mixed parasite *E.dispar* + *E.moshkovskii* 2(2.85%), while the 38 infected female (100%) injury with single parasite *E.dispar*, according table(7).

Table (7): the proportion of infection and parasite *E.dispar E.moshkovskii* according the gender

Parasite	Sex	No. of tests	No. of infected patients	%	% total number
E.dispar	male	70	52	74.28%	48.15%
	female	38	38	100%	35.18%
E.dispar+ E.moshkovskii	male	70	2	2.85%	1.85%
	male	70	54	77.14%	
Total			108		85.18%

DISCUSSION

The present study recorded the proportion of infection in males 11.04% higher than females 5.99%, as in the table (1), which is consistent depending on the sex and not value with that recorded where he scored the proportion of infection in males 52.80% and in females 47.20%, Al-Naimi (2001) where he scored the proportion of infection in males and in females 61.26% 37.74% respectively, and where he scored an infection rate of 49.1% in males and 34.6%, in females, Al-Naimi (2001) where he scored the proportion of infection in males and in females 13.33% 12.33%, is also consistent with Al-Harthi and Jamjoom (2007) in Saudi Arabia / Mecca where they scoreded the proportion of infection in males and in females 33.3% and 31.4% respectly . That did not agree with what his record Al-Mashhadani, W.S.H.(2000), where the rate of infection in males scored 19.84% and in females 21.58%. The difference in the rate of infection between males and females may be due to the fact that the male working class in society and this makes them more contact with the environment, and also eat and drink in public places or from street vendors, this increases the chances of exposure to infection.

The present study had the highest proportion of infection of 4.1% in the age groups (≤1- 10 years old) and (11-20) year, also in the table (2), which is consistent to what the record Kadir and Rasheed (2008), in some of the villages of Baghdad where he scored the highest infection rate of 32.5% in the age group (13-18snh), and for his record- (Mohamed *et al* ., 2009) where the highest proportion of infection of 51.7% in the age group (10-19snh), and with the record Kadir and Rasheed (2008), where he scored the highest infection 26.20% in the age group(1-10)years. Fotedar *et al(2007).*, in Australia, where he scored the highest infection rate of 63.6% in the age group (31-50) year. The high rate of infection in the age group may return (≤1- 10 years) under study to the fact that children at this age are more vulnerable to different foods, exercise usually put fingers in the mouth, especially in the children's bags holders, and they are at this age eager for a taste anything this may increase the rate of infection in this age group. The high rate of infection in the age group (11-20)year may have been due to the working conditions and eating foods outside the home and work in agriculture and other businesses, making them more susceptible to infection.

The present study recorded the proportion of infection in rural areas stood at 9.77% higher than the city's 7.26%, and also in the table (4-5), which is consistent with the, where he scored the proportion of infection in the countryside 71.95% 28.05% In the city, however Moran *et al* (2005), where the proportion of record the infection in the rural area were 25.71% male and 16.16% female but in the urban, and did not agree with what his record Al-Janabi(2002), where the proportion of infection scored 21.76% in the countryside and in the city's 26.93%. That the reason for the increase of infection in the countryside due to several factors, including the lack of water availability of clean drinking and rely on river water directly as a source of water or rely on water stored in concrete basins may be exposed often leaving them vulnerable to contamination easily as well as lower health and cultural level of the rural population and the use of animal wastes and sometimes human fertilizer the increase of infection

The present study recorded the proportion of single parasite infection *E.dispar* amounted to 83.33% (45 of 54) and also in the table (4-7), which is similar to the record mojarad *et al* (2009)., In Poland, where the proportion of single parasite infection record *E.dispar* 83.78% (31 of 37), and with the record Ahmed *et al* (2006)., in Tunisia, where the rate of infection scored a single parasite *E.dispar* amounted to 81.4% (22 of 27). while the current study recorded the highest rate of infection parasite *E.dispar* Ali *et al*.(2003), in Bangladesh, where the proportion of record infection 35.77% (39 of 109), and Pinheiro *et al*.(2004), in Pernambuco region in the northeast of Brazil, where the proportion of record infection 74.19% (23 of 31), and Hamzah *et al*.(2006), in Bangkok / Thailand, where the proportion of record infection parasite 20% (6 of 30), and Samie *et al* (2006)., in the Venda area, to Mbaboa, Hnob Africa have, where the proportion of record infection 22.13% (54 of 244), and also Santos *et al* (2007)., in Brazil, where the proportion of record infection 33.33% (9 of 27), and Kurt *et al* (2008)., in Izmir province / Turkey, where the rate of infection scored 55.35% (31 of 56). The present study recorded the proportion of infection parasite *E. dispar* less than Hooshyar *et al*.(2003), In Karash and Tehran in central Iran, where the proportion of record infection parasite 93.87% (46 of 49), and Gebertsadik *et al* (2004)., In south-west of Addis Ababa, in Ethiopia, where he scored infection rate of 93.33% (28 of 30), and when his record., in Iran, where the rate of infection scored 91.37% (53 of 58). High rate of parasite infection *E. dispar* recorded in the current study may be due to the fact that is a common parasite of the genus Ontameba, as indicated by a

study in the cities of Karachc and Tehran in Iran that the spread of infection parasite E.dispar represents 11.7 times more common than the parasite infection E.histolytica (Hooshyar *et al.,* 2003), and a study in Kalmnjaro / Tanzania that parasite infection E.dispar were 14.4 times more common than the parasite infection E.histolytica (Moran 2005), is generally considered a parasite E.dispar predominant type and the most common where the prevalence rate of up to a parasite E.histolytica (1*E.dispar*: 10*E.histolytica*) in some areas .In the current study parasite infection *E. dispar* record in cases of bloody diarrhea with symptoms of gastrointestinal Maybe this coincided with the infection by pathogens other diarrhea (Rota virus, Shigella species, Campylobacter species, enterohemorrhagic or enteroinvasive *Esherichia coli*, and Salmonella species), so these cases diarrhea need an alternative explanation.(Santos *et al*., (2010). were registered parasite infection E.dispar in patients suffering from gastrointestinal disorders and bloody diarrhea accompanied Bzhar. Despite the lack of any evidence of the pathogenesis of the parasite *E. dispar* in man, but he recorded localized damage focal lesions in the mucous layer of the intestines of animals infected experimentally parasite itself (Samie A, *et al* ,2010). Also earlier there was some evidence indicated that parasite infection E.dispar, may lead to the occurrence of pathological changes in some people (10). the record later that parasite infection *E. dispar* perhaps may be the causative agent for gastrointestinal symptoms in humans (23), and recently parasite infection *E. dispar* record in patients with hepatic abscesses (amebic or purulent) The present study recorded the proportion of mixed parasite infection *E. dispar* + E.moshkovskii amounted to 1.85% (1 of 54) and also in the table (4-7), which is similar to the record(23). In Tunisia, as the proportion of mixed infection record 7.4% When using PCR technology. The current study did not agree with the (2003) Hooshyar *et al.,* A negative sample did not score when examining (49) is positive microscopic examination of a sample using PCR, however Moran (2005). Did not record a negative sample when examining (58) a positive test sample microscopic using PCR. To the emergence of a negative result when using the PCR technique for some samples positive microscopic examination may be due to several reasons, foremost among which an error in the microscopic diagnosis of some samples on suspicion pellets white blood multiple nuclei or cells phagocyte with eccentric Sarcocystis and feeders for the parasite *E.histolytica* / *E. dispar* or with eccentric Sarcocystis various types of sex Entameba for example, Endolimax (23). Or the presence of inhibitory substances in some feces samples probably linked enzyme polymerase DNA Polymerase, work and prevent get amplified DNA process or it may be because of the time between sampling and analysis Many of the samples are extracted, including DNA and used in the technique PCR is taken from the feces stored for long periods and varying thermal high, that leads to the decomposition of these samples and giving false negative results(23).

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