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A Prevalence study of *Entamoeba* spp. in Basrah Province using Different Detection Methods

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Abstract:

This study aims to determine the prevalence of *Entamoeba histolytica*, *Entamoeba dispar* and *Entamoeba moshkovskii* by three methods of diagnosis (microscopic examination, cultivation and PCR) that were compared to obtain an accurate diagnosis of *Entamoeba* spp. during amoebiasis. Total (n=150) stool samples related to patients were (n = 100) and healthy controls (n= 50). Clinically diagnosed stool samples (n=100) were collected from patients attending the consultant clinics of different hospitals in Basrah during the period from January 2018 to January 2019. The results showed that 60% of collected samples were positive in a direct microscopic examination. All samples were cultivated on different media; the Brain heart infusion agar showed high efficiency and was the most suitable in cultivating the parasite. Data and results of molecular study were indicated by DNA extraction from stool samples and used in PCR technique with specific primers. This study identifies different infection percentage for the three species. The highest infection in Basrah patients was *Entamoeba moshkovskii* 15% followed by *Entamoeba dispar* 10% and *Entamoeba histolytica*, which was 5%.

Keywords: *Entamoeba* spp, Cultivation, PCR.

Introduction:

In many countries, amoebiasis has been considered one of the major public health problems. It is caused by the genus *Entamoeba*, which includes several species, such as *E. histolytica*, *E. dispar* and *E. moshkovskii*. One of the main reasons that this disease is widely spread in developing countries is the lack of hygienic measurements and the supply of clean drinking water. In addition, the disregarding of parasitic infections in such countries increases the number of these infections (1).

Many studies worldwide investigate asymptomatic amoebiasis. There is an idea of some researchers that the pathogenesis of the parasite differs according to the species and strains, while others believe that the severity of the parasite pathogenicity and the condition of host may increase the clinical symptoms. The prevalence of *E. histolytica* is normally over estimated, this is because of the overlap of the epidemiology of *E. dispar* and *E. moshkovskii* which are similar in morphology to *E. histolytica* (2).

Generally, the prevalence of *Entamoeba* spp. is not well described in different parts of the world. The only well-known side is in human that *E. histolytica* can cause invasive diseases, while the other two species (*E. dispar* and *E. moshkovskii*) are considered as non-pathogenic and free-living amoeba until now. Even though, there are many studies that have suggested the pathogenicity of these two species in human. The diagnosis of amoebiasis requires more sensitive method than the ones used now as trophozoites that have low viability in feces sample and the cysts, which are irregularly excreted in the sample in asymptomatic (3)

Although *E. histolytica*, *E. dispar*, and *E. moshkovskii* have identical appearance, the pathogenicity of the *E. dispar* and *E. moshkovskii* have not been well understood. The main laboratory diagnosis of *Entamoeba* spp. in human stool is microscopic examination but it is not possible to distinguish between these types depending on it (4).

The morphological similarity between *Entamoeba* spp. is a major concern, which may lead to mistreating the patients. Therefore, the need of accurate diagnostic method is important, this method is represented by the molecular diagnosis using PCR technique in different regions around the world (5).

Molecular diagnosis has showed that the distribution of *E. dispar* was 10 times higher than that of *E. histolytica* worldwide; many studies reported the relationship between *E. dispar* and the clinical symptoms (6). Most molecular studies used polymerase chain reaction (PCR) techniques in differentiating *E. histolytica* and *E. dispar*. Whereas the diagnosis of *E. moshkovskii* was ignored, although recent studies have shown that *E. moshkovskii* cause gastrointestinal disorders (GIDs) in human, which is an appropriate host for this species (7). In Basrah province many studies were conducted regarding *Entamoeba* spp. i.e., Al-Yaqub (8) who used the PCR technique for the detection and differentiation of *Entamoeba* spp. and compared it with microscopically examination. Moreover, Al-Abodi (9) studied phylogenetic sequencing for species *E. histolytica*, *E. dispar*, and *E. moshkovskii* in Al-Qadisiya province. Therefore, this study aims to clarify an accurate method for evaluating the prevalence of *E. histolytica*, *E. dispar* and *E. moshkovskii* in Basrah, to the south of Iraq, using three methods of diagnosis (microscopic examination, cultivation and PCR).

Materials and Methods:

Samples collection

The study was subjected a 150 stool samples related to a 100 patients and a 50 for healthy controls. Clinically diagnosed stool samples were collected from outpatients suffering from gastrointestinal disorders, abdominal pain and diarrhea, those patients were attending the consultant clinics of different hospitals in Basrah. The collection took place during the period from January 2018 to January 2019. All patients ranged from 1 – 70 years old with both genders. The samples were collected in a labeled sterile plastic container and brought directly to the laboratory of parasitology in the Department of Biology/ College of Science/ University of Basrah. Each sample was divided into two parts, the first one for direct microscopic observation and culturing, while the second part kept at -20C° for DNA extraction.

Microscopic examination of *Entamoeba* spp.

The microscopic examination of 150 stool samples (100 patients and a 50 for healthy controls) were performed by direct wet smear method (which

included a diagnostic examination for trophozoite by normal saline solution and cyst stages by using Lugols iodine solution of *Entamoeba* spp.) and concentration methods using simple sedimentation and formalin-ether technique. In order to get a precise identification for *Entamoeba* spp., the parasites were stained by trichrome stain including Basic and Modified by using a test tube instead of slide (10).

Cultivation of *Entamoeba* spp.

In order to obtain a clear growth of *Entamoeba* spp. a 150 stool samples (100 patients and a 50 for healthy controls) were cultivated in different media, which were xenic and diphasic (11).

A. Basic culture media: Locke's egg medium, Robinson's medium, Trypticase yeast extract iron serum (TYI-S-33) medium (11).

B. New culture media for *Entamoeba* spp. including

1. Brain heart infusion agar (Hi media, India)

2. Sheep testes agar

Sheep test extract: five gm. of sheep testes was taken, added to 150 ml of distilled water and then put in a mixture for 15 min to be filtrated. This medium was prepared from mixing 1.5g agar with 100 ml sheep testes extract.

3. Buffalo milk agar

This medium was prepared from mixing 1.5g agar with 100ml buffalo milk.

4. Sheep liver agar

Sheep liver extract: five gm. of sheep liver was taken, added to 150 ml of distilled water and then put in a mixture for 15 minute to be filtrated. This medium was prepared from mixing 1.5g agar with 100 ml sheep liver extract.

5. Chicken liver agar

Chicken liver extract: five gm. of chicken liver was taken, added to 150ml of distilled water and then put in a mixture for 15 min to be filtrated it. This medium was prepared from mixing 1.5g agar, protein 1 g with 100 ml chicken liver extract.

Then 15 ml from each media was added to tubes. Two type of antibiotics were added to all the media above, Erythromycin 0.5g /100 ml and Nystatin 0.5g/100ml, both antibiotics were sterilized by Millipore filtration and stored at 4°C.

Inoculation of *Entamoeba* spp. in culture media

Samples (n=150) were cultivated by using the different culture media. Each media was supplied with the inoculation of Nystatin 0.25mg/ml, Erythromycin 0.25mg/ml, phosphate buffer saline (pH 7.4) 2 ml, Human serum 7 ml and suspension of parasite 1 ml (which contains 750

trophozoite). The tubes were incubated at 35.5-36 °C for 48 hour. The presence of the parasite was confirmed by light microscope after 48 hour. The numbers of the parasite were calculated by using Neubauer chamber (HIRSCHMANN E M TechcoloR) (12, 13).

Molecular diagnosis of *Entamoeba* spp.

At first, molecular methods were used to diagnose all samples (n=150, a 100 for patients and a 50 for healthy as controls). After that, the molecular analysis was repeated for samples that were succeeded by cultivated methods (n=10).

DNA extraction

DNA was extracted from stool samples according to the procedure of Presto™ Stool DNA Extraction Kit (Geneaid, Taiwan).

Diagnosis of *Entamoeba* spp. by PCR

The PCR amplification reaction was performed to the final volume of 25 µl. The reaction was optimized to combine the specific primers, for *E. histolytica* (SPEH): forward primer 5'-ATGCACGAGAGCGAAAGCAT-3' and reverse primer 5'-GATCTAGAAACAATGCTT CTCT-3', for *E. dispar* (SPED): forward primer 5'-ATGCACGAGAGCGAAAGCAT-3' and reverse primer 5'-CACCACTTACTATCCCTACC-3', for *E. Moshkovskii* (SPEM): forward primer 5'-ATGCACGAGAGCGAAAGCAT-3' and reverse primer 5'-TGACCGGAGCCAGAGACAT-3'. The volumes for a single reaction were 1.5 µl of each forward and reverse primer, Go Tag Green master mix 12 µl, Nuclease free water 7 µl and 3 µl of extracted DNA samples. The thermal cycler (Thermo cycler, Applied Bio system, USA) program used to amplify each species-specific DNA fragment was started with an initial denaturation at 95 °C for 4 min., followed by 35 cycles of 95 °C for 35 sec., 55 °C for *E. histolytica*, 53 °C for *E. dispar* and 52.7 °C for *E. moshkovskii* for 35 sec. and 72 °C for 35 sec. with a final extension at 72 °C for 7 min. PCR products were detected by 2 % agarose gel electrophoresis, stained with (0.2 µl) ethidium bromide and visualized by UV transilluminator (14).

Calculate sensitivity to diagnostic methods for clinical samples (n=100)

For the three diagnosis methods (direct microscopic examination, cultivation and molecular diagnosis), the sensitivity of detecting *Entamoeba* spp. were calculated according to the equation below (15).

$$\text{Sensitivity} = \frac{\text{No. of true positives}}{\text{No. of true positives} + \text{No. of false negative}} \times 100$$
$$= \frac{\text{No. of true positives}}{\text{Total number of sick individuals in population}} \times 100$$

Results:

Prevalence of *Entamoeba* spp. infection according to microscopic examination

In general, the present study showed that 60% was positive samples in direct microscopic examination, regarding sex. The infection was found at 56.6% for male patients and 43.4% for female patients.

The results showed that percent infection of *Entamoeba* spp. was different according to age groups. The highest percentage infection of *Entamoeba* spp. was in group 1 (1-10 years) and group 7 (61-70 years) with 31.6% and 23.3%. While, the lowest percentage was associated with group 3 (21-30 years) and group 4 (31-40 years) with 1.6 % for each.

The results also showed a difference in the percentage of infections between months of the year, the highest infection was in July 18.3%, while the lowest infection was in December 1.6%. Moreover, there were differences in the stool appearance, which differ from bloody, mucous, fatty, and semisolid. The mucus samples were the most frequent with 31.6% followed by semi-solid samples 23.3%.

Direct microscopic examination showed positive results, the trophozoite was clearly shown as a static colorless or moving using normal saline method and the cysts were diagnosed by Lugol's Iodine stain (Fig. 1 A and B), the diagnostic method by both basic and modified trichrome stain showed the *Entamoeba* spp. trophozoite as in (Fig. 2 A and B).

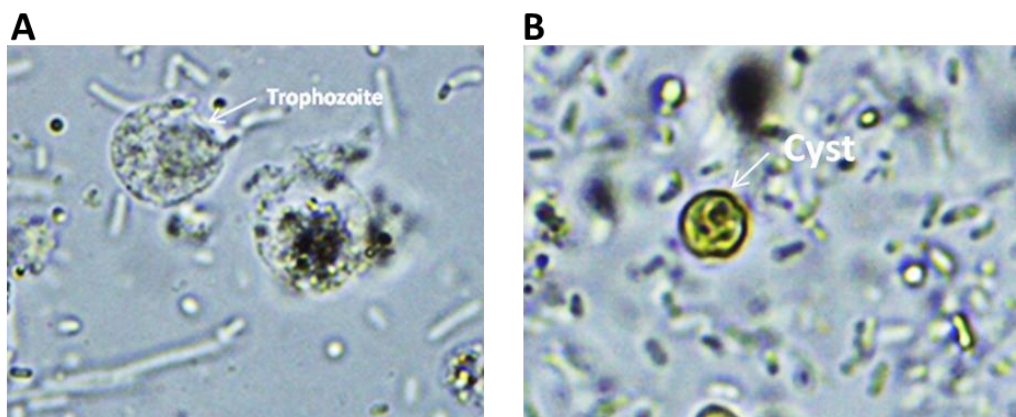


Figure 1. Direct examination of *Entamoeba* spp. trophozoite and cyst. A. trophozoite with irregular chromatin in the nucleus original magnification 100x by normal saline method. B. Cysts have round shape with one nucleus original magnification 100x. By Lugol's Iodine method.

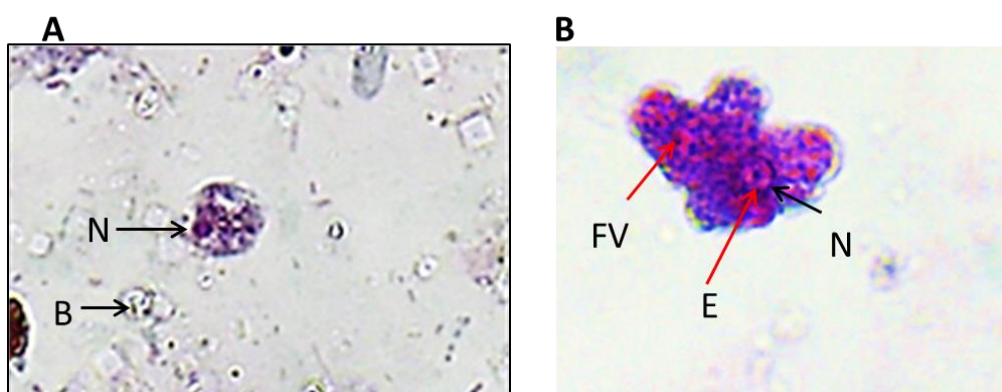


Figure 2. Direct examination of *Entamoeba* spp. trophozoite. A. by basic trichrome stain. Characteristic features of trophozoite, the nucleus (N). Note the bacteria in the samples original magnification 100x. B. by modified trichrome stain. Characteristic features of trophozoite, the nucleus (N), endoplasm (E) and the food vacuoles (FV). Note reduction of bacteria the samples original magnification 100x.

Cultivation of *Entamoeba* spp. for clinical samples

Generally, clinical samples (n=100) were cultivated, however, only ten samples (10%) were positive in culture. These 10 samples were also positive in direct microscopic examination.

Cultivation of *Entamoeba* spp. for control samples

Control isolates (50) were free from 3 species infection

Parasite cultivation in basic media

Three culture media were used in order to obtain the best medium for *Entamoeba* spp. The best growth period for each culture medium after incubation for 48h. was determined by confirming that the parasite stags alive for the longest period. Locke's egg medium showed that this period reaches a maximum to 4 days with parasite alive, while Robinson's and TYI-S-33 media recorded a

maximum to 2 days that the parasite is still alive (Table 1). From these results it is clear that Robinson's and TYI-S-33 media are less efficient than Locke's egg medium in maintaining the parasites growth.

Parasites cultivation in new culture media

The new five culture media were compared to Locke's egg medium, which have the best results. The results showed that the growth of the parasites varied according to the culture media. Brain heart infusion agar was the best in maintaining the growth parasite for 10-11 days .Secondly, the Sheep testes agar that have a maintaining period of 7-8 days. Thirdly, the Buffalo milk agar with 4-5 days maintaining period while both Sheep liver agar and Chicken liver agar culture media showed the least efficiency in maintaining the parasite growth (Table 1).

Table 1. Maintenance periods of *Entamoeba* spp. trophozoite on variable culture media

Samples	Culture media	Maintenance period (days) after incubation for 48h.											
		1	2	3	4	5	6	7	8	9	10	11	12
S1	Locke's egg medium	+	+	+	+	-	-	-	-	-	-	-	-
	Robinson medium	+	+	-	-	-	-	-	-	-	-	-	-
	TYI-S-33 medium	+	-	-	-	-	-	-	-	-	-	-	-
	Brain heart infusion agar	+	+	+	+	+	+	+	+	+	+	+	-
	Sheep testes agar	+	+	+	+	+	+	+	+	-	-	-	-
	Buffalo milk agar	+	+	+	+	+	-	-	-	-	-	-	-
	Sheep liver agar	+	+	+	-	-	-	-	-	-	-	-	-
	Chicken liver agar	+	+	+	-	-	-	-	-	-	-	-	-
S2	Locke's egg medium	+	+	+	+	-	-	-	-	-	-	-	-
	Robinson medium	+	+	-	-	-	-	-	-	-	-	-	-
	TYI-S-33 medium	+	+	-	-	-	-	-	-	-	-	-	-
	Brain heart infusion agar	+	+	+	+	+	+	+	+	+	+	-	-
	Sheep testes agar	+	+	+	+	+	+	+	-	-	-	-	-
	Buffalo milk agar	+	+	+	+	+	-	-	-	-	-	-	-
	Sheep liver agar	+	+	-	-	-	-	-	-	-	-	-	-
	Chicken liver agar	+	+	+	-	-	-	-	-	-	-	-	-
S3	Locke's egg medium	+	+	+	+	-	-	-	-	-	-	-	-
	Robinson medium	+	+	-	-	-	-	-	-	-	-	-	-
	TYI-S-33 medium	+	+	-	-	-	-	-	-	-	-	-	-
	Brain heart infusion agar	+	+	+	+	+	+	+	+	+	+	-	-
	Sheep testes agar	+	+	+	+	+	+	+	+	-	-	-	-
	Buffalo milk agar	+	+	+	+	+	-	-	-	-	-	-	-
	Sheep liver agar	+	+	+	-	-	-	-	-	-	-	-	-
	Chicken liver agar	+	+	+	-	-	-	-	-	-	-	-	-
S4	Locke's egg medium	+	+	+	+	-	-	-	-	-	-	-	-
	Robinson medium	+	-	-	-	-	-	-	-	-	-	-	-
	TYI-S-33 medium	+	+	-	-	-	-	-	-	-	-	-	-
	Brain heart infusion agar	+	+	+	+	+	+	+	+	+	+	+	-
	Sheep testes agar	+	+	+	+	+	+	+	+	-	-	-	-
	Buffalo milk agar	+	+	+	+	+	-	-	-	-	-	-	-
	Sheep liver agar	+	+	+	-	-	-	-	-	-	-	-	-
	Chicken liver agar	+	+	+	-	-	-	-	-	-	-	-	-
S5	Locke's egg medium	+	+	+	+	-	-	-	-	-	-	-	-
	Robinson medium	+	+	-	-	-	-	-	-	-	-	-	-
	TYI-S-33 medium	+	+	-	-	-	-	-	-	-	-	-	-
	Brain heart infusion agar	+	+	+	+	+	+	+	+	+	+	-	-
	Sheep testes agar	+	+	+	+	+	+	+	+	-	-	-	-
	Buffalo milk agar	+	+	+	+	+	-	-	-	-	-	-	-
	Sheep liver agar	+	+	+	-	-	-	-	-	-	-	-	-
	Chicken liver agar	+	+	+	-	-	-	-	-	-	-	-	-
S6	Locke's egg medium	+	+	+	-	-	-	-	-	-	-	-	-
	Robinson medium	+	-	-	-	-	-	-	-	-	-	-	-
	TYI-S-33 medium	+	+	-	-	-	-	-	-	-	-	-	-
	Brain heart infusion agar	+	+	+	+	+	+	+	+	+	+	+	-
	Sheep testes agar	+	+	+	+	+	+	+	+	-	-	-	-
	Buffalo milk agar	+	+	+	+	+	-	-	-	-	-	-	-
	Sheep liver agar	+	+	-	-	-	-	-	-	-	-	-	-
	Chicken liver agar	+	+	+	-	-	-	-	-	-	-	-	-
S7	Locke's egg medium	+	+	+	+	-	-	-	-	-	-	-	-
	Robinson medium	+	-	-	-	-	-	-	-	-	-	-	-
	TYI-S-33 medium	+	+	-	-	-	-	-	-	-	-	-	-
	Brain heart infusion agar	+	+	+	+	+	+	+	+	+	+	+	-
	Sheep testes agar	+	+	+	+	+	+	+	+	-	-	-	-
	Buffalo milk agar	+	+	+	+	-	-	-	-	-	-	-	-
	Sheep liver agar	+	+	+	-	-	-	-	-	-	-	-	-
	Chicken liver agar	+	+	-	-	-	-	-	-	-	-	-	-
S8	Locke's egg medium	+	+	+	-	-	-	-	-	-	-	-	-
	Robinson medium	+	+	-	-	-	-	-	-	-	-	-	-
	TYI-S-33 medium	+	+	-	-	-	-	-	-	-	-	-	-

S9	Brain heart infusion agar	+	+	+	+	+	+	+	+	+	-	-
	Sheep testes agar	+	+	+	+	+	+	-	-	-	-	-
	Buffalo milk agar	+	+	+	+	-	-	-	-	-	-	-
	Sheep liver agar	+	+	+	-	-	-	-	-	-	-	-
	Chicken liver agar	+	+	-	-	-	-	-	-	-	-	-
	Locke's egg medium	+	+	+	+	-	-	-	-	-	-	-
	Robinson medium	+	+	-	-	-	-	-	-	-	-	-
	TYI-S-33 medium	+	-	-	-	-	-	-	-	-	-	-
	S10	Brain heart infusion agar	+	+	+	+	+	+	+	+	+	-
Sheep testes agar		+	+	+	+	+	+	-	-	-	-	-
Buffalo milk agar		+	+	+	+	-	-	-	-	-	-	-
Sheep liver agar		+	+	+	-	-	-	-	-	-	-	-
Chicken liver agar		+	+	+	-	-	-	-	-	-	-	-
Locke's egg medium		+	+	+	+	-	-	-	-	-	-	-
Robinson medium		+	-	-	-	-	-	-	-	-	-	-
TYI-S-33 medium		+	+	-	-	-	-	-	-	-	-	-
Brain heart infusion agar		+	+	+	+	+	+	+	+	+	-	-
Sheep testes agar	+	+	+	+	+	+	+	-	-	-	-	
Buffalo milk agar	+	+	+	+	-	-	-	-	-	-	-	
Sheep liver agar	+	+	-	-	-	-	-	-	-	-	-	
Chicken liver agar	+	+	-	-	-	-	-	-	-	-	-	

+ Positive growth, -Negative growth

Molecular diagnosis of *Entamoeba* spp.

DNA extraction

The genomic DNA showed in Fig. 3.

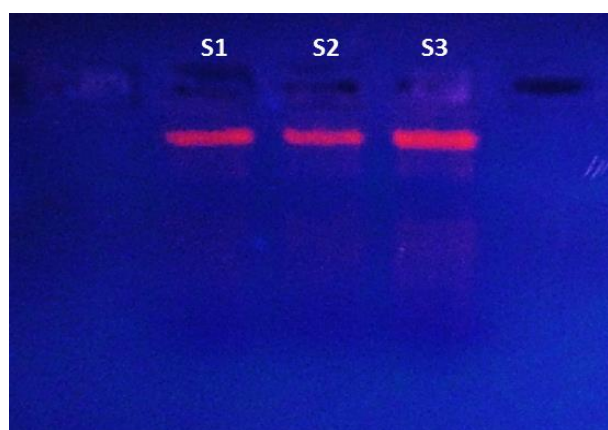


Figure 3. Genomic DNA bands of 3 *Entamoeba* spp. isolates .0.8% agarose gel electrophoresis for DNA samples, in which distinct total DNA bands are observed

Polymerase Chain Reaction of *Entamoeba* spp. from clinical samples by specific primers

A. Diagnosis of *E. histolytica*

Five isolates out of 100 samples (5%) were diagnosed as *E. histolytica* using the specific primer. The gene band size was 166bp as shown in Fig. 4.

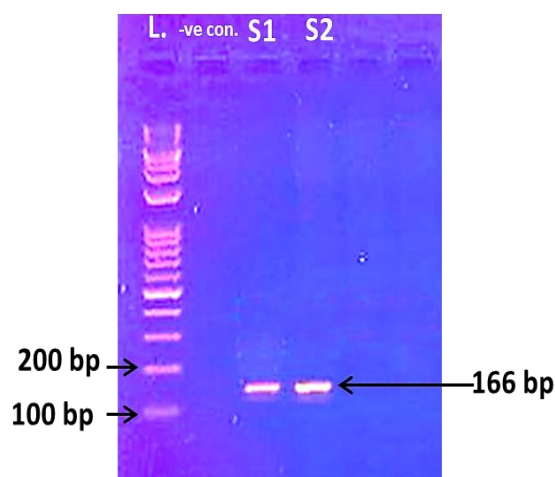


Figure 4. SPEH bands (166bp) of 2 isolates for *E. histolytica* using agarose gel electrophoresis (2%): Lane 1: L (100bp) DNA ladder, Lane 2: negative control (-ve con.), Lane 3: isolate 1(S1), Lane 4: isolate 2(S2).

B. Diagnosis of *E. dispar*

Ten isolates out of the 100 samples (10%) were diagnosed as *E. dispar* using the specific primer. The gene bands size was 752bp as shown in Fig. 5.

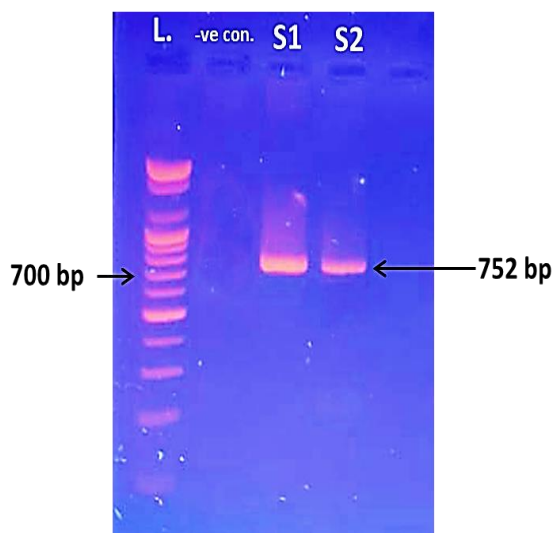


Figure 5. SPED bands (752bp) of 2 isolates for *E. dispar* using agarose gel electrophoresis (2%): Lane 1: L (100bp) DNA ladder, Lane 2: negative control (-ve con.) , Lane 3: isolate 1(S1), Lane 4: isolate 2(S2).

C. Diagnosis of *E. moshkovskii*

Fifteen isolates out of the 100 samples (15%) were diagnosed as *E. moshkovskii* using the specific primer. The gene bands size was 580 bp as shown in Fig. 6.

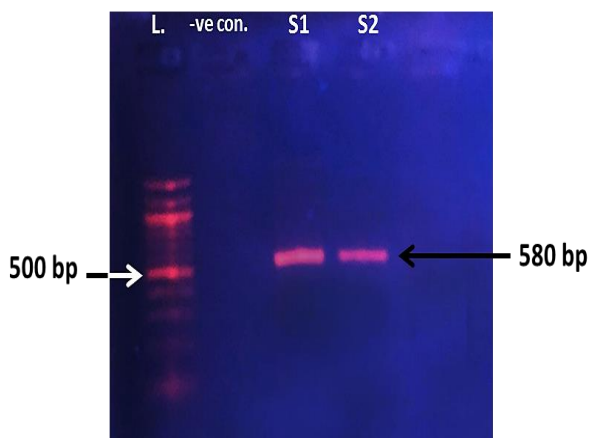


Figure 6. SPEM bands of 2 isolates (580bp) using agarose gel electrophoresis (2%): Lane 1: L (100bp) DNA ladder, Lane 2: negative control (-ve con.), Lane 3: isolate 1(S1), Lane 4: isolate 2(S2).

Polymerase Chain Reaction of *Entamoeba* spp. from samples that were succeeded by cultivated methods

Out of 10 isolates cultivated, 3 isolates were identified as *E. moshkovskii*, while 7 isolates were negative in PCR.

PCR of *Entamoeba* spp. from controlled samples

Control isolates (50) were free from 3 species infection

Sensitivity of diagnostic methods for clinical samples

In terms of the sensitivity, the highest sensitivity was present in the microscopic examination (60%), while the lowest sensitivity value was occurred in cultivation (10%). The molecular diagnosis showed a sensitivity of (30%).

Discussion:

Generally, the most common intestinal parasites in Iraq are *Entamoeba* spp. these species are similar to each other in morphology but they are genetically different (14). The present study showed prevalence infection of *Entamoeba* spp. in Basrah patients (60%) by testing the direct microscopic examination, similar findings were reported by other researchers in Al-Qadisiyah province as they found that the prevalence of *Entamoeba* spp. was 61.26% (9). A variation of amoebiasis prevalence was found among Iraqi provinces, the highest percentage (68%) was reported in a study conducted in Al-Qadisiyah (16), while Kadir *et al.* (17) reported that the lowest percentage in Salah Al-Din 9.3%. This variability may be due to differences in the levels of sanitation, personal hygiene, population density, lifestyle and the climatic conditions suitable for the survival of cysts for a long time, which increase the possibility of causing such infection.

Results regarding sex showed a higher percentage for males than females, this result was confirmed by Al-Kaeabi and Al-Difaie (18) who stated that the infection found in males 58.3% and 41.6% for females in Al-Qadisiyah province. The variations in infection percentages between males and females might be caused by the different social behavior between the two sexes, as males normally are the working sex in the society which made them in contact with the environment (19).

The increased percentages of infection in age group 1 (1-10) yrs. and group 7 (61-70) yrs., could be explained by the influence of different factors as for group1 the behavior of children might be involved less cautious regarding their personal hygiene. In addition, they are more exposed to food from different sources. The high infection percentage in group 7 (61-70) might be resulting from the fact that elderly people exposed to many infectious agents during their life as their immune system is weakened (20).

Our results also reported that summer season associated with increased infection. These results may be attributed to the availability of proper environmental factors for parasites growth, as intestinal parasites were dominant at tropical area. Moreover, the need of fast consumption of

drinking water that contains the infective stage of these parasites (cysts) can be considered as another cause (21). Another reason is represented by our living habit in Basrah city, because of the increased percentages of salt in the tap water and the contamination of drinking water tanks with the infective stages (22).

Direct examination gave a confirmation of *Entamoeba* spp. existence in both trophozoite and cysts stages. However, the use of modified trichrome stain in the test tube was efficient in getting good quality stained samples free of contaminants (Bacteria and debris). The repeated centrifugation in this methods helped the sedimentation of parasites to be precipitated in the bottom of the tube and led to a clear isolation with high purity.

Regarding the parasite cultivation method, in our study stool samples were used, the results showed that it was less sensitive than the microscopic examination. In addition, there is a high risk of contamination with fungi, bacteria and other protozoa, therefore it was not recommended as a routine examination (9). The Locke's egg medium (basic media) was the best among the other media. This is because it contained nutrients that helped parasites to secrete proteases in order to digest proteins, which was essential for rapid growth (23). These nutrients include the eggs that contained the high protein contents necessary for parasite growth and reproduction compared to the presence of peptone found in Robinson medium, which is a simple structured protein. The high protein content allows *Entamoeba* spp. to get nutrients before their consumption by bacteria. In addition to this, it also contained vitamins that stimulated *Entamoeba* spp. for metabolic reactions (24). Regarding Robinson medium which has benzoic acid as one of its components, this compound acts as antibacterial and used to preserve food (25). It has an inhibitory effect on the growth for some microorganisms. For the first time we used 5 new culture media, which were highly efficient in cultivating *Entamoeba* spp. and easy to prepare from different animal sources. The results showed that Brain heart infusion agar was the most efficient medium to give dense growth and increase the number of parasites with a high purity for ten to eleven days period. This efficiency may be due to the high chemical contents of the nutrients necessary for parasite's growth and reproduction because Brain heart infusion agar contains amino acids, glucose, mineral salts, highly lipid complex and cholesterol (23). The Sheep testes agar came in the second place for the efficient medium in parasite isolation and growth, this might be due to its

contents of proteins and carbonate, in addition to the presence of high lipid (26). Moreover, the Buffalo milk agar, which showed a lower growth rate compared with Brain heart infusion agar and Sheep testes agar media may be related to their components which were less suitable for parasite growth (27). The same reason applied for Sheep liver agar and Chicken liver agar media, which have less percentages of fat and proteins, respectively (28, 29). The human serum was added to all media during the culture process, because this serum is normally obtained from media rich with lipoproteins, which had an important role to grow the parasite in pure culture (30).

The DNA was extracted in order to diagnose the parasites; the technique of PCR was used as a highly sensitive way to differentiate between *Entamoeba* spp. The results showed that the high efficiency of differentiation among the three species was based on the specific primer of each parasite. The highest infection was *E. moshkovskii* 15% followed by *E. dispar* 10% and *E. histolytica*, which was 5%. Al-Abodi (9) also recorded different infection percentages for the *Entamoeba* spp. in the province of Al-Qadisiya, where *E. histolytica* had the highest prevalence (74%) followed by *E. dispar* with (26%), while *E. moshkovskii* was with only 7%. On other part of Asia i.e., in Iran 0.58% was the highest prevalence of *E. dispar*, while the least prevalence was for *E. moshkovskii* 0.07% (5). The specific primers used in this study were derived from the middle of the small-subunit rRNA gene since it was conserved, found in all organism, used to identify and classify organism due to unique characters for each species (15). The differences found in PCR results may be attributed to different reasons, firstly due to the variations in DNA extraction methods from stool samples. Secondly differences in the number of parasites in stool samples. Finally, the fixative and preservative agents used in the study may interfere with cell components resulting in effecting DNA (4).

Regarding the sensitivity of the diagnosis methods, the microscopic examination had the highest sensitivity for detection of parasites trophozoite and cysts in stool samples followed by the molecular diagnosis then cultivation method. The negative results by using PCR or cultivation methods of some positive samples in microscopic examination may be explained by several reasons. Firstly, in microscopic examination there was a chance in misdiagnosing the parasite by way of some times the parasite is misidentified with WBCs as well as with other stages of *Entamoeba* spp. such as *E. moshkovskii*, *E. histolytica* and *E. dispar* (31).

Secondly, the presence of some inhibitory substances in the stool such as urea, bile salt and polysaccharides, which may be bond to DNA polymerase and inhibited its work in replicating the DNA (32).

Conclusion:

In conclusion although microscopic examination is a traditional method for the diagnosis of *Entamoeba* spp. the probability of misdiagnosis of these species is high, the molecular diagnosis method is considered a precise diagnostic method despite the low sensitivity which is due to the presence of both alike forms in the sample

Authors' declaration:

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are mine ours. Besides, the Figures and images, which are not mine ours, have been given the permission for re-publication attached with the manuscript.
- The author has signed an animal welfare statement.
- Ethical Clearance: The project was approved by the local ethical committee in University of Basra.

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دراسة انتشار طفيلي *Entamoeba* وأنواعه في محافظة البصرة باستخدام طرق تشخيصية مختلفة

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الخلاصة:

هدفت الدراسة إلى تحديد مدى انتشار *Entamoeba* spp. من خلال استخدام ثلاث طرق للتشخيص (طريقه الفحص المجهرى المباشر ، الطرق الزرعية و تقنية تفاعل البلمرة المتسلسل PCR) من أجل الحصول على تشخيص دقيق للإصابة. جمعت 150 عينة براز تتضمن 50 عينة للأشخاص الأصحاء و100 عينة مشخصة سريريًا من المرضى في مستشفيات مختلفة من محافظة البصرة خلال الفترة من يناير 2018 إلى يناير 2019. أظهرت النتائج أن 60 ٪ من العينات كانت إيجابية في الفحص المجهرى المباشر. وتمت زراعة جميع العينات على اوساط زرعية مختلفة ، وسجل وسط دماغ القلب Brain heart infusion agar كفاءة عالية وكان أكثر ملاءمة في زراعة الطفيلي. هذا الدراسة تحدد نسبة الإصابة المختلفة للأنواع الثلاثة باستخدام طريقه التشخيص الجزيئي وكانت أعلى نسبة إصابة (15%) للنوع *Entamoeba moshkovskii* ، تليها (10%) للنوع *Entamoeba dispar* و بلغت نسبة الإصابة (5 %) للاميبيا الحالة للنسيج *Entamoeba histolytica* في محافظة البصرة

الكلمات المفتاحية: *Entamoeba* spp، الاستزراع، تقنية تفاعل البلمرة المتسلسل.