Morphological and molecular identification of a free-living Acanthamoeba spp. Isolated from the environmental sources in the central province / Iraq

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Abstract

Acanthamoebaas a free-living protozoan which is one of the most commonly isolated amoebae in environmental samples.It is ubiquitous and found in a variety of habitats including domestic water supplies , hospital water , dental water units , air and soil . The current study under taken investigation and detection of Acanthamoeba spp. throughout morphological and molecular methods in different environmental samples at province central Iraq. This study included fifty eight sample were collected from different environmental source during the period from August to September 2022.. The samples were cultured on NN-agar medium after that PCR was conducted on the culture positive samples .Over all 25/58 (43.301%) samples were positive on morphological characters , PCR –analysis showed that only 20/25(80%) of Acanthamoeba morphologically positive samples were positive by specific primer .

The Acanthamoeba spp. It was isolated from soil, potato soil and water and confirmed by molecular examination. In this study, four species of Acanthamoeba can be identified morphologically, namely, A. triangleis, A. astronyxis, A. castellini, and A. polyphaga. study considering the possible presence of Acanthamoeba species in different samples, whether in the environmental source, which will be opened for further epidemiological studies in order to better understand the role of Acanthamoeba as a potential threat to the spread of pollution in different environments in Iraq.

INTRODUCTION

The term "amoebae" refers to a range of unicellular creatures that differ from other protozoa in that they move by the use of pseudopodia, which are transient extensions of the plasma membrane (Adiba et al., 2010). Protozoa called free-living amoeba (FLA) can be found all over the natural world. They clump together as a relatively diverse group of facultative parasitic amoebae among free-living protozoa, lacking any unified phylogenetic, systematic, or taxonomic origin (Walochni & Aspöck, 2007) . Free – living amoebae can be found both in natural aquatic environments and in artificial, man-made aquatic environments, for a long time FLA were considered to be harmless protozoa of soil and water, anyway research since the 1960s has demonstrated that FLA can be pathogenic to humans and animals (Scheid, 2018).

Acanthamoeba belongs to the family Acanthamoebidae (Reveiller et al., 2003). It

was first isolated in 1913 and named Acanthamoeba polyphagus (Marciano-Cabral and Cabral, 2003).. The life cycle of this amoeba contains two forms that are vegetable phase known as trophozoite and doormat phase known as cyst. The cyst can be found in environments for many years and when the environmental condition become suitable the cyst transfers to trophozoite and leads its vital function ,Acanthamoeba is opportunistic amoeba that's mean its survives as free living in environmental but can be cause disease when enter the human and animals body and live as parasite. The genus of Acanthamoeba consist of 20 species some of them are very important because they are causing infection such as A. castellanii, A. polyphaga, A. triangularis, A. culbertsoni and other .These species can be causing infection in skin, lung , eye and center nerve system (Gardner et al., 1991).

Contaminations by free-living amoebae, mainly of the genera Acanthamoeba, and Balamuthia are emergent diseases in human and animals, They can cause several different diseases (Daft etal.,2005; Morales etal.,2006), Acanthamoeba and Balamuthia cause focal sensory system (CNS) as well as disseminated diseases, and Acanthamoeba in addition to CNS infections.

Materials & Methods:

Environmental samples collection:

Samples were collected from different environmental sources in Karbala, Qadisiyah, Amarah, Kut and Babel province of iraq , including, soil(different kind of soil and potato soil), water samples (rivers, tap water , tank water ,stagnant water and water from the air conditioner units, Filtered water ,Water for the animals to drink, for my markets in the farms, Turtle puncture, Large ponds for breeding fish and Water for washing the owner's hands).these samples were collected in the period from August to September 2022.tabl (1)

Sample Cultivation :

A-Water samples were collected in 60 ml sterile cups,the date and site details were fixed for each sample. In the lab 3-5 ml of each sample was cultured on non-nutrient agar (NN-agar) medium in one replicates within 24 hours of collection and incubated in 26 C0 and 37C0 and amoebic growth was examined daily by light microscope on slide and followed for 4 weeks.(Moker,2017).

B-Soil samples was collected in sterile containers, the date and site details were fixed for each sample, within the next 24 hours of collection two grams of each sample were suspended in 5 ml of sterile distilled water and supernatant was cultured on non-nutrient agar (NN-agar) medium in one replicates and incubated in 26 C0 and 37 C0 with 3 ml of sterile distilled water were added twice a week to keep cultures wet and amoebic growth was observed daily by microscope examination for a wet mount slide for 4 week .(Moker,2017).

Table (1): No. of Environmental samples collected from different sources.

Type of samples	No. of samples collected
Soil	20
Potato soil	1
Water for washing the owner's hands	6
River water	2
Tank water	3
Tap water	3

Stagnant water	**4**
Filtered water	**3**
Puncture water	**1**
Airconditioner water	**5**
Animal drinking water	**10**
Total	**58**

Preparation of media

Non-nutrient agar medum (NNA):

Twelve grams of non-nutrient agar powder were added to 400 ml of distal water, then autoclaved at 121C0 for 15 minute, the medium was left to cool (45C0) after autoclaving then poured in Petri-dishes (8.5 cm diameter) and left till become solid at room temperature . five ml of sterile distal water was added on the agar surface as a liquid phase for cultivation of amoeba, this media was used in routine primary culture.(Page ,1988)

When amoebic growth was identified on culture media, a microscopic slide was made using a cotton swab to mount the samples under sterile condition then examined under 10X and 40X to detect trophozoites, cyst and /or floating stages of various amoebae, dimension of each stage were recorded using the microscopic stage ruler, then diagnosed according to Page (1988).

Molecular study:

The identity of Acanthamoeba spp. was confirmed ,after morpholgical characterization , genetically by conventional PCR using a set of Acanthamoeba spp specific two primers designed by Schroeder et al. (2001) : Forward JPD1 (5'GGCCAGATCGTTTACCGTG 3') Reverse JPD2 (5' TCTCACAAGCTGCTAGGGAGTCA 3') (manufactured by Alpha DNA) . Genomic DNA from cell culture of Acanthamoeba spp. were extracted by using AddPrep Genomic DNA Extraction kit , Addbio. Korea, and done according to company instructions . according to the following protocol : Initial denaturation 95C0 for 10 min and 35 cycle of 35 sec at 95C0 , 35 sec at 56 C0 and 40 sec at 72 C0 followed by 10 min final extension at 72 C0 ,PCR product was electrophoresed on 1.5% agarose gel and visualized by UV.

Sequencing :

The PCR products of positive samples were sent to Macrogen Company(Korea) for sequencing .The tube of each sample was labeled with a number identical to the number of Excel sheet that sent by the company . The sequences were processed and analyzed using Basic Local Alignment Search Tool (BLAST) to search for homologous sequences in the National Center for Biotechnology Information database (NCBI) . All the analyzed sequences were submitted to NCBI to obtain accession numbers which then analysed by using MEGA X software.

Results:

the results showed that the Acanthamoeba spp. was the most common amoeba in environmental samples.

The occurrence of Acanthamoeba spp. in environmental samples

Acanthamoeba spp. cysts and trophozoites were observed in 25/58 (43.301%) of samples (12 water ,12 soil, 1 potato soil samples) out of 58 samples that were positive microscopically were collected from different environmental source of province middle of Iraq. The highest occurrence of Acanthamoeba spp. were in the Environmental soil samples was 12(6.629%) and water samples was

12(6.629%) followed by Potato soil samples was 1(1%) .Found three species (A. castellanii, A. triangularis, A. astronyxi) ,out of 20 postive samples were confirmed by molecular examination. Acanthamoeba spp. showed occurrence in 20/25(80%) samples.by using the specific Acanthamoeba spp. primer JPD1 / JPD2 Table (2) Fig (1),(2).

Table (2): Occurrence of Acanthamoeba spp. in environmental samples by microscopic examination and molecular examination

Type of sample	No. sample EX. By microscope	Positive sample		PCR positive samples	
		No.	%	No.	%
Water for washing the owner's hands	6	3	5.172	2	10
River water	2	0	------	0	------
Stagnant water	4	2	3.448	0	------
Tank water	3	1	1.724	1	5
Tap water	3	0		0	------
Filtered water	3	1	1.724	1	5
Puncture water	1	0	------	0	------
Airconditioner water	5	1	1.724	2	10
Animal drinking water	10	4	6.896	3	15
Soil	20	12	20.689	10	50
Potato soil	1	1	1.724	1	5
Totel	58	25	43.103	20	100

Fig. (1): Agarose gel electrophoresis image that show the PCR product analysis of spicific gene from genomic DNA of Acanthamoeba spp. from environmental samples : Where M: Marker (2000- 100 bp) all lance positive samples at 460 -500 bp.

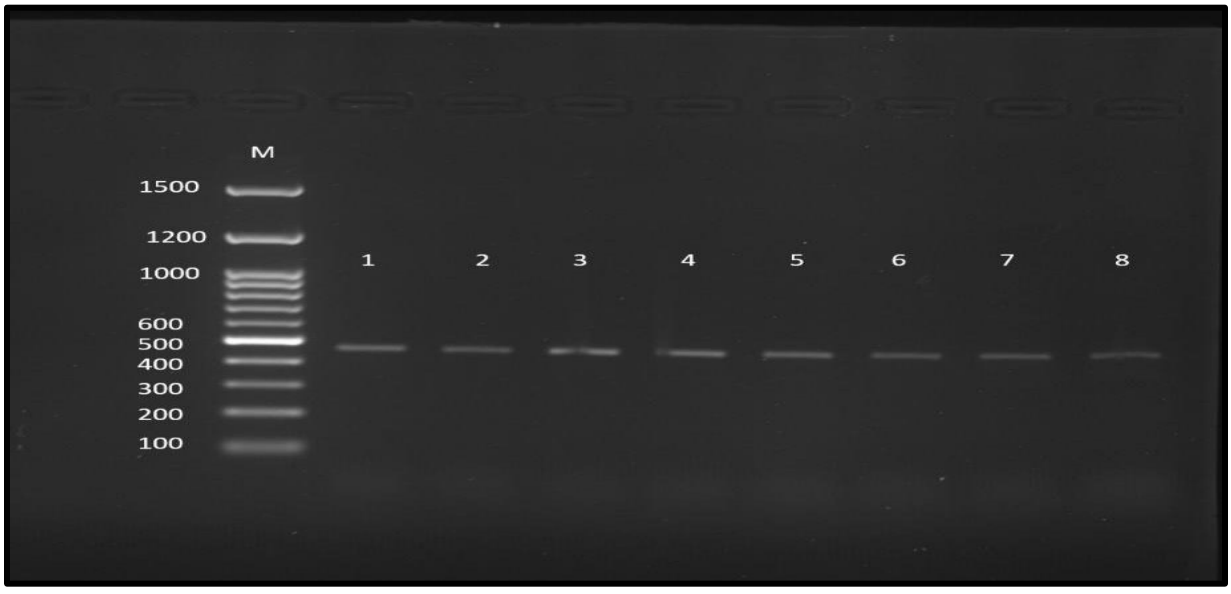
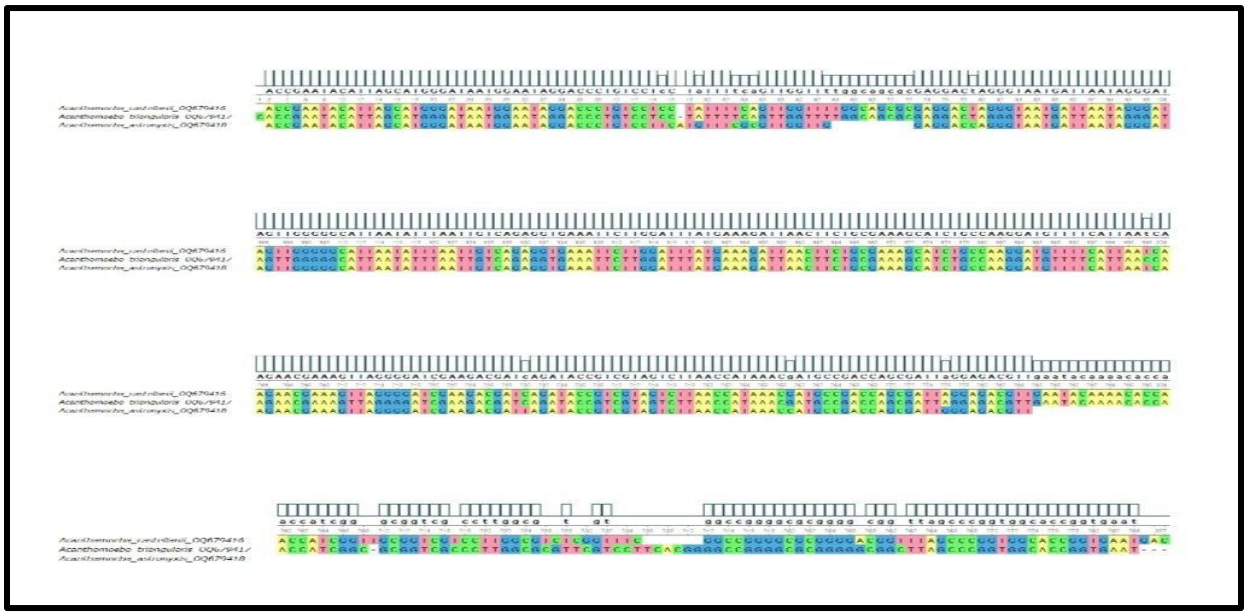


Table (3): the NCBI-BLAST Homology Sequence identity (%) in local T. annulata in cattle. These sequences were deposited in gene bank under the following accession numbers and these were being compared with other global sequences.

Sample number	Obtained accession number	Identical to	GenBank accession number	Country	Identity
1	OQ679416	Acanthamoeba castellanii	KU872061	Iran	100
2	OQ679417	Acanthamoeba triangularis	MZ310461	South Korea	98.69
3	OQ679418	Acanthamoeba astronyxis	AF239293	USA	100

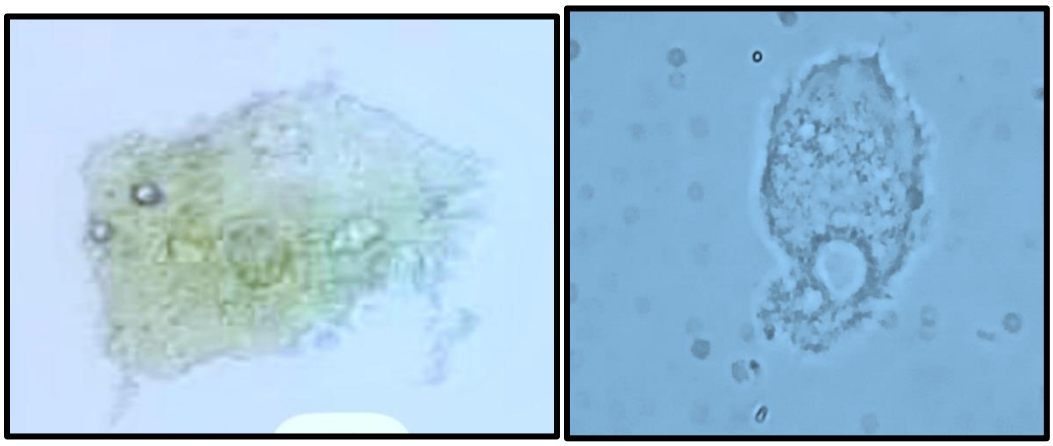
Fig (2): multiple alignment of the A. triangularis,A. astronyxis,A. castellanii strain .analyzed by MEGA



Morphological characteristics Trophozoites

Acanthamoeba spp. showed trophozoite stage after five day of cultivation which characteristic by non uniform , trophozoite of all isolates were irregular in form that were measuring 27- 32 µm µm. .Acanthamoeba trophozoite showed a prominent contractile vacuole and acanthopoda , the typical morphology of Acanthamoeba trophozoite moved freely and presence of lobopoda and needle like fine projections of pseudopodia called acanthopoda. Fig. (3).

Fig. (3) Acanthamoeba trophozoites show contractile vacuole (red arrow) and acanthopodia (blue arrow). (unstained) (A) isolated from clinical source . (B) isolated from environmental source.

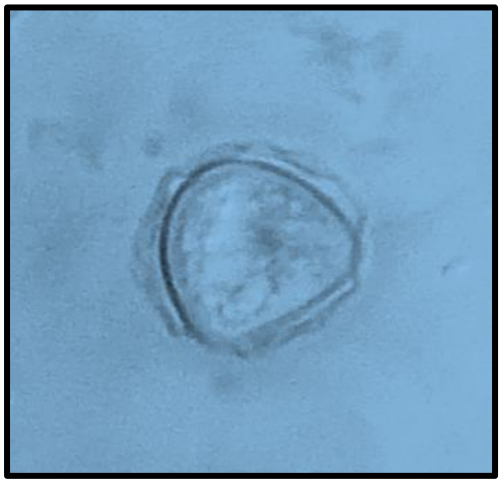


Cysts

Different species of Acanthamoeba genus were recognized according to the shape and size of cysts in addition to the number ,size , shape and arrangement of the cyst pores . In our current study four species of Acanthamoeba was morphologically recognized namely A. triangularis , A. astronyxis , A. castellini .

A. triangularis The mean diameter of cyst 12.5 µm, endocyst which could be stellate , polygonal and triangular. ectocyst was thick wrinkled and corrugated but not spherical however ray of endocyst was broad and slightly curved , the average number of pores were 3 or 4 .Fig (4).

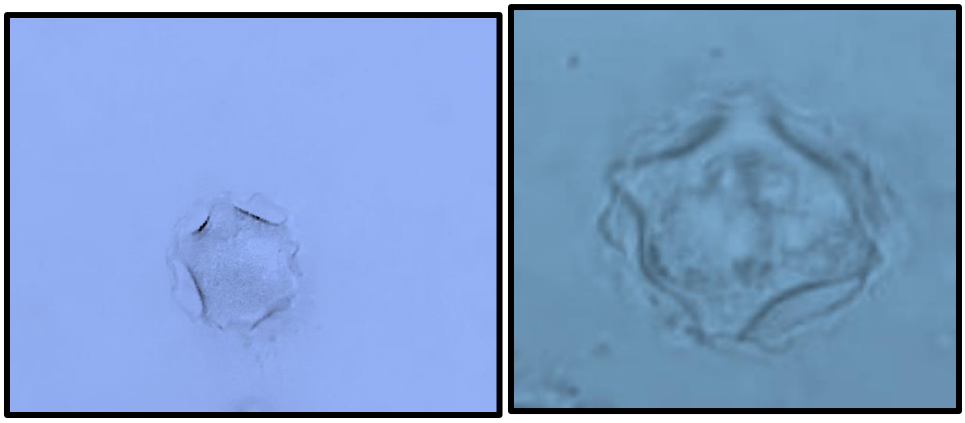
Fig (4) Cysts of Acanthamoeba triangularis (unstained).



B. astronyxis

The cyst diameter was 19 µm,endocyst was usually stellate with mainly 5-6 rays ending with pores. the number of cyst pores reached 4-6 meanwhile ectocyst was smoothing circular or nearly so. all rays of endocyst usually contacted ectocyst in approximately the same plane while the ectocyst was separated from the endocyst by a clear region of changing the width . Fig (5)

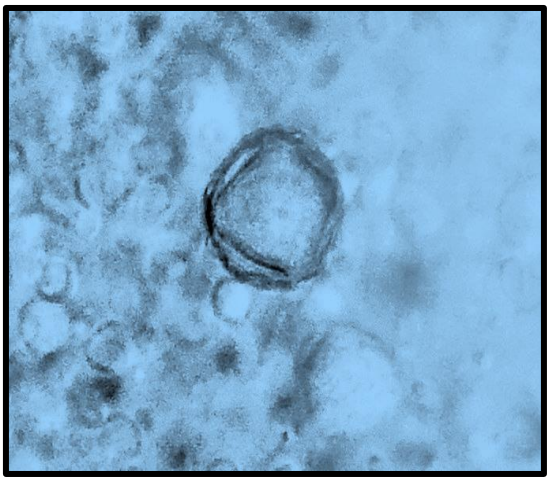
Fig (5) Cysts of Acanthamoeba astronyxis (unstained).



C. Acanthamoeba castellanii :

It,s cyst diameter was 17 µm the ectocyst thick and typically wrinkled the endocyst stellate usually does not have well developed arms or rays , ectocyst and endocyst are both close together . Fig. (6).

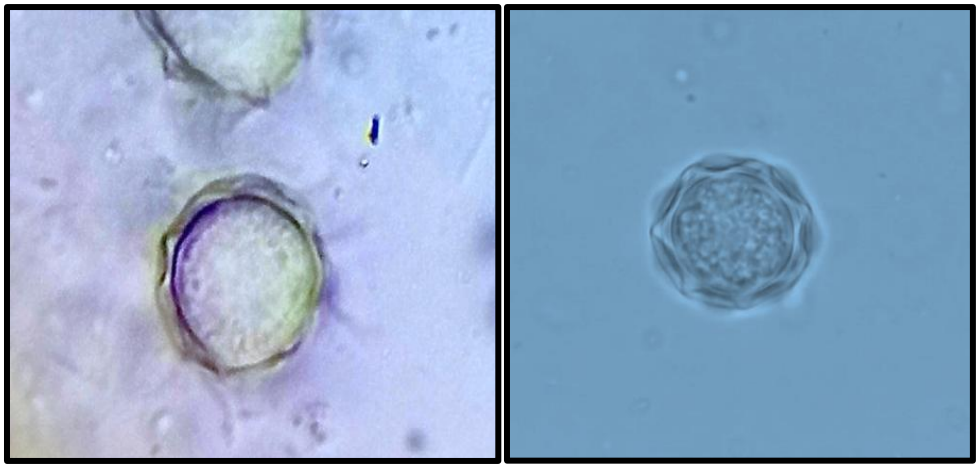
Fig. (6) Cysts of Acanthamoeba castellanii (unstained).



C. Acanthamoeba polyphaga :

It,s cyst diameter about (14-18) µm ,endocyst nearly round with slight angles , ectocyst closely encircling endocyst the ectocyst is thicker than the endocyst , this species didn,t contain pores but contain coroners or ribs , the number of corona are 7.Fig.(7)

Fig. (7) Cyst of Acanthamoeba polyphaga (unstained).



Discussion:

Acanthamoeba spp. is ubiquitous free-living protozoa found in a wide range of environmental niches (Rangsima and Kosol Roongruangchai , 2009). In current study we identification of Acanthamoeba is mainly on cultivation on NN-agar and molecular methods , among 58 samples from environmental sources in central governorates / Iraq included soil samples was 12/25 (6.629%) and water samples was 12/25 (6.629%) followed by Potato soil samples was 1/25 (1%) were examined in this study. The current study showed that Acantamoeba spp. were observed in 25/58(43.301%) were positive for Acanthamoeba spp. using microscopic examination and only 20/25(80%) were positive by using PCR method,this may also attributed due to the concurrent presence of other amoebae in cultured samples , however , they can not be correctly differentiated from each other using the direct microscopic examination of the culture – positive samples . In our study,Acanthamoeba spp. were detected in the examined samples from environmental sources diagnosed by morphological characteristics and PCR method.

Our study agree with other studies done by Lanocha (2009) he was isolated Acanthamoeba from environmental sources in Poland. In same contacts, Azhar (2017) and Hanady (2017) they were isolated Acanthamoeba from different environmental source in Basra province and AL-Aboody (2021) isolated Acanthamoeba from different environmental source in Thi- Qar province .

The current study showed that Acantamoeba spp. were observed in 25/58 (43.103%) sample included soil samples was 12/25 (6.629%) and water samples was 12/25 (6.629%) followed by Potato soil samples was 1/25 (1%) by culture and microscopic method , only 20(100%) of them were positive after polymerase chain reaction included soil samples was 10/20 (50%) and water samples was 8/25 (45%) followed by Potato soil samples was 1/25 (5%). The current study is consistent with the study of AL-Aboody (2021) which showed that Acantamoeba spp. were observed in 11/57 (19.54%) sample included soil samples was 6/11 (10.52%) and water samples was 4/11 (7.017%) followed by Potato soil samples was 1/11 (1.75%) by culture and microscopic method , only 6(10.52%) of them were positive after polymerase chain reaction included soil

samples was 4 (17.39%) and water samples was 1 (25%) followed by Potato soil samples was 1 (5%).

In Egypt detected 56% of Acanthamoeba spp. in of Nile water samples (AL-Herrawy et al., 2013) , other study recorded the percentage of Acanthamoeba spp. was 26.4% in the river water (Lorenzo- Morales et al., 2005) .In Iraq Hanady (2017) showed that Acanthamoeba spp. was found in 20% of collected positive samples in Basra province and Azhar (2017) showed 42 samples (29%) out of 141 were collected from different environmental and clinical sources in same province was positive to Acanthamoeba spp. , these studies recorded a higher incidence of Acanthamoeba than current study . whereas, other studies showed that percentage of Acanthamoeba were lower than the present study , Rezaian et al.(2002) studied water and soil river and Parishan Lake in Kazeroon studied 354 samples by culture and microscopic method reported 10 cases of contamination with Acanthamoeba . In Egypt the prevalence of Acanthamoeba spp. was detected in 8.3% in tap water by real time PCR (Gad and AL-Herrawy , 2016) . The difference in detection rate of Acanthamoeba in different countries and localities may be influenced by geographical conditions and samples sources.

Acanthamoeba spp. were isolated from water, the presence of Acanthamoeba in samples of water is attributed to water chlorination kills only microorganisms but not FLA . (Khan , 2006) . Acanthamoeba spp. were isolated from soil samples at a rate 26.08% , Potato soil at the rate 1.724% the proportion, so that our study suggested that presence of Acanthamoeba with in soil is suitable environments for the growth of Acanthamoeba because they rich with bacteria , which are important food sources for Acanthamoeba .

Based on Magnet, et al .,(2013) study, PCR is more sensitive technique than direct microscopy of culture , but the use of both PCR and culture method is suggested for environmental water samples to gain more complete results of the real presence of Acanthamoeba.

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