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Molecular Comparison of Free -Living Amoeba Isolated From Iraqi, Iranian and Turkish Waters

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Abstract

Amoebas live freely in different climates and parts of the world. Several species of Free Living Amoeba (FLA) are capable of causing serious as well as fatal infections in human beings. The aim of this study was to identify and compare genotypes of water-polluting FLA in major rivers and lakes of Iraq and compare them with FLA isolates from Iran and Turkey. For this purpose, the study included 20 water samples from the Tigris River, Euphrates River , Najaf Sea and Dukan lake in Iraq, 20 water samples from Marivan, Velasht, and Soleimanshah lakes and Caspian sea in Iran, and 20 water samples from Sabanca, Seyfi , Hazar and Yay lakes in Turkey. The samples were studied by culture methods, invert microscope, and molecular methods.

After inoculation and microscopic examination, cysts and trophozoites were detected in 18 cultured specimens. Overall, out of 60 water samples, 30 cases (30%) were found to be contiminated in the three countries. The highest pollution was in Turkish waters (40%), while the rate in the Iraqi and Iranian water samples was the same (25%). Because of the various species of FLA , it may be difficult to distinguish pathogenic from non-pathogenic species by culture on non-nutrient agar. Therefore, the molecular technology was applied in this study. Only a specific band of *Acanthamoeba* Rns genes which ranged from 423 to 551 nucleotides was observed. The isolates belonged to the T3 genotype . In addition, it was a new isolate that differs from what exists in other neighboring countries , registered in the GenBank under accession number MN462973 as the *Acanthamoeba* genotype T3 isolate T3 Iraq . This is the first study to detect pathogenic FLA in Iraq by PCR and Sequencing techniques. Given the high prevalence of *Acanthamoeba* potential pathogenic genotypes in various environmental sources and the evidence of T3 genotype in Iraqi specimens , more studies about *Acanthamoeba* and other pathogenic FLA for various environmental sources in Iraq are required.

Keywords: Acanthamoeba, FLA, genotyping, Iraq, water resource .

مقارنة جزيئية للأميبا الحرة المعيشة المعزولة من المياه العراقية والإيرانية والتركية

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الخلاصة

يوجد الاميبا الحرة المعيشة Free Living Amoeba في مناخات مختلفة وفي اجزاء مختلفة من العالم . هناك عدة انواع من FLA قادرة على التسبب في اصابات خطيرة ومميتة في البشر. كان الهدف من هذه الدراسة هو تحديد ومقارنة الانماط الوراثية للاميبات الملوثة للمياه في الانهار والبحيرات الكبرى في العراق ومقارنتها بايران وتركيا . لهذا الغرض , تم جمع 20 عينة مياه من انهار دجلة والفرات وبحر النجف وبحيرة دوكان و20 عينة مياه من بحيرات مريوان , ولشت , سليمانشاه وبحر قزوين الايرانية و 20 عينة مياه من بحيرات صبنجه , سيفي , هازار وياي بتركيا. وقد تمت دراسة العينات بواسطة طرق الاستنبات والفحص المجهري باستخدام المجهر المقلوب والطرق الجزيئية .

بعد الفحص المجهري وزراعة العينات , تم الكشف عن الاكياس والتروفوزويت في 18 عينة . اجمالا , من بين 60 عينة مياه , تم العثور على 30 حالة (30 %) في ثلاثة بلدان . كان اعلى تلوث في المياه التركية (40 %) , ولكن النسبة في عينات المياه العراقية والايرانية كانت مطابقة (20 %) . وبسبب تنوع اجناس FLA , فقد يكون من الصعب التمييز بين الاجناس المرضية عن غير المرضية من خلال تنميتها على non– nutrient agar . لذا تم استخدام التقنية الجزيئية , اذ تراوحت فقط مجموعة محددة من جينات Rns الاكانثاميبا من 423 الى 551 نيوكليوتيدات . انتمت العزلات الى النمط الوراثي T3 , بالاضافة الى كونها عزلة جديدة مختلفة عن ماهو موجود في الدول المجاورة ايران وتركيا. وقد تم تسجيلها في بنك الجينات تحت رقم التسلسل MN462973 كعزلة عراقية من النمط الوراثي T3 (Acanthamoeba genotype T3 Iraq) . هذه الدراسة هي الاولى في العراق للكشف عن FLA المرضية بتقنية PCR و Sequencing . نظرا لارتفاع انتشار الطرز الوراثية المحتملة المسببة للامراض في الاكانثاميبا في مختلف المصادر البيئية والادلة على التركيب الوراثي T3 في العينات العراقية , يتطلب المزيد من الدراسات حول الاكانثاميبا وباقي FLA لمختلف المصادرالبيئية في العراق .

Introduction

Free-living amoebae (FLA) are ubiquitous and widely distributed in the environment [1]. They have been isolated from soil, fresh water lakes, swimming pools, therapeutic pools, domestic tap water, natural thermal water, and air all over the world [1, 2, 3, 4]. Among the free-living amoebae, only members of the genera *Acanthamoeba, Neglaria, Balamothia mandillaris, and Sapinia diploididae* are responsible for opportunistic and non-opportunistic infections in humans and other animals [5].

During the last two decades, *Acanthamoeba* species have become increasingly recognized as important microbes. They are now well recognized as human pathogens causing serious as well as life-threatening infections, having a potential role in ecosystems, and acting as carriers and reservoirs for prokaryotes. A detailed current understanding of these microbes was reviewed elsewhere [5]. Although several studies reported the presence of FLA in various environmental and clinical samples in neighboring countries. including Turkey and Iran, but so far there has been no study of the FLA prevalence and other related issues in Iraq. That is why the objectives of the present study involved the isolation and molecular diagnosis of some pathogenic FLA species isolated from Iraqi waters along with the comparison of these FLA isolates with those existing in Turkey and Iran.

Site and period of the study

The study was carried out from the beginning of May 2018 to the end of April 2019 in the Environmental Research laboratory, University of kirkuk – Iraq . Water samples were collected from different regions (Figure-1) in sterile plastic containers (250 ml) and kept in a special refrigerated box prepared for the purpose of conservation and transport of biological and medical specimens, as follows:

From Iraq: Five isolates from the Euphrates River, 5 from Najaf Sea, 5 from Dukan Lake and 5 from Tigris River .

From Iran: Five isolates from Mariwan Lake, 5 isolates from Velasht Lake, 5 isolates from Suleymanshah Lake and 5 isolates from Caspian Sea.

From Turkey: Five isolates from Sabanca Lake, 5 isolates from Seyfi Lake , 5 isolates from Hazar Lake and 5 isolates from Yay Lake.
All samples were transported to the laboratory on the same day or on the second day as a maximum.

Figure 1-The map of Iraq which is bordered by Turkey to the north and Iran to the east [www.google maps].

Materials and Methods

Filtration and Cultivation

The water samples were filtered by a Wattman filter then spinned using a centrifuge. The sediments were cultured in a non-nutrient agar (NNA) medium supplemented with killed *E.coli* bacteria.

The study excluded cultures that were negative for *Acanthamoeba* cysts and trophozoites after one month. Cultures containing *Acanthamoeba*-specific cysts and trophozoites were maintained for further work.

Preparation of solutions and culture medium

The Page's Saline solution (PAS) was preparing by dissolving the following salts in 1000 ml of distilled water; NaCl (2.5 mM) 120 mg, $MgSO_4$. $7H_2O$ (20 µM) 4 mg, $CaCl_2$. $2H_2O$ (40 µM) 4 mg, Na_2HPO_4 (0.5 mM) 142 mg, KH_2PO_4 (1 mM) 136 mg. The pH of the solution was adjusted to 6.9 [6 , 7]. For the preparation of 1.5% NNA medium, 15 g of DIFCO agar was dissolved in one liter of saline page solution [8]. Approximately 10 ml of each plate was poured from the culture medium to a thickness of about 4-5 mm. After cooling and closing of the culture medium, the plates were completely sealed using paraffin and stored in a plastic bag in the refrigerator until used for culture. To prepare the suspension of *E.coli*, a culture on EMB medium was used. Bacterial colonies were collected from EMB using fildoplatin and dissolved in saline. In order to inactivate the bacteria by heat, the suspension was autoclaved at 121 ° C. This suspension (Heat-killed *E.coli*) was stored in the refrigerator and used for *Acanthamoeba* cultivation in the NNA medium [9].

Molecular study

Preparation of samples for DNA extraction

For DNA extraction, cysts and trophozoites of FLA in NNA medium were used. One ml of *Acanthamoeba* cysts and trophozoites were added on NNA culture medium inside the hood and in sterile conditions. The culture medium was scraped using a sterile Pasteur pipette to remove the cysts. The fluid was also removed several times from the culture medium and poured again on it to completely remove the cysts. The suspension containing the isolated cysts was poured into a 1.5 ml sterile microtube. The microtubes containing *Acanthamoeba* were centrifuged at 2000 g for 5 minutes.

The supernatant was then discarded and added to the PBS precipitate and washed three times. Finally, the supernatant was discarded and the precipitate was stored at -20 ° C for free extraction of DNA from the parasite [6].

Primers

The primers were provided by Macrogen, Korea to amplify the ASA.S1 fragment of the 18S rRNA gene of Acanthamoeba (JDP1), ITS1 and ITS2 of Naegleria (Naegleria), and 18S rRNA gene of Vermamoeba vermiformis (NA). The sequences of primers are shown in Table- 1 [10, 11].

Table 1-Primers

Primer Name	Seq.	Annealing Temp. (°C)	For detection of
NA1	GCTCCAATAGCGTATATTAA	48	*Vermamoeba vermiformis*
NA2	AGAAAGAGCTATCAATCTGT		
JDP1-F	GGCCCAGATCGTTTACCGTGAA	56	*Acanthamoeba*
JDP1 -R	TCTCACAAGCTGCTAGGGAGTCA		
Naegleria-F	CAAACACCGTTATGACAGGG	58	*Naegleria*
Naegleria -R	CTGGTTTCCCTCACCTTACG		

DNA extraction

Wizard genomic DNA purification kit (Promega, USA) was used for extracting DNA from samples containing trophozoite and cysts of *Acanthamoeba*. The samples were first subjected to freezing / thawing using liquid nitrogen for three cycles for DNA extraction. The extraction procedure was performed according to the kit instructions.

Quantitation of DNA

Quantus Florometer was used to detect the concentration of the extracted DNA in order to detect the quality of samples for downstream applications. For 1 µl of DNA, 199 µl of diluted QuantyFlour Dye was mixed. After 5 min of incubation at room temperature, DNA concentration values were detected.

Reaction Setup and Thermal Cycling Protocol

For molecular confirmation of AFL isolates, NA1, JDP and *Naegleria* Genes were used for amplification Tables -(2 , 3) [10,11].

Table 2-Master mix components

Master mix components	Volume (1 sample)
Master Mix	12.5
Forward primer	1
Reverse primer	1
Nuclease Free Water	6.5
DNA	4
Total volume	25
Aliquot per single rxn	21µl of Master mix per tube and add 4µl of Template

Table 3-PCR Program

Steps	°C	Time	Cycle
Initial Denaturation	95	5 min	1
Denaturation	95	40 sec	30
Annealing	48 or 56 or 58	45 sec	
Extension	72	55 sec	
Final extension	72	7 min	1
Hold	10		

Agarose Gel Electrophoresis

After PCR amplification, agarose gel electrophoresis was adopted to confirm the occurrence of amplification. PCR was completely dependent on the extracted DNA criteria. 1 gm (for 1%) agarose was added to 1 X TAE buffer, loading dye, DNA ladder marker and Ethidium bromide (10mg / ml).

Standard Sequencing

PCR products were sent for Sanger sequencing using ABI3730XL automated DNA sequencer by Macrogen Corporation – Korea. The results were received by email then analyzed using Geneious software.

Phylogenetic analysis

In order to investigate the phylogenetics of the ASA.S1 fragment, sequences of both strands were compared using Sequencher software (version 5.4.6). The sequences obtained were compared with the sequences recorded in the gene bank using the BLAST program.

Genotypes were identified based on the highest homology and query cover. Sequences obtained from environmental isolates were also recorded in the gene bank .

MEGA software (version 7) was used to perform multiple alignment, score determination and sequence similarity. Phylogenetic analysis was performed using maximum likelihood method and the phylogenetic tree was plotted [NCBI] .

Results

Sample culture and microscopic examination

Cysts and trophozoites of *Acanthamoeba* were observed in positive cultured samples after inoculation and microscopic examination of the cultures using invert microscope. No other free living amoeba was detected (Figure-2)

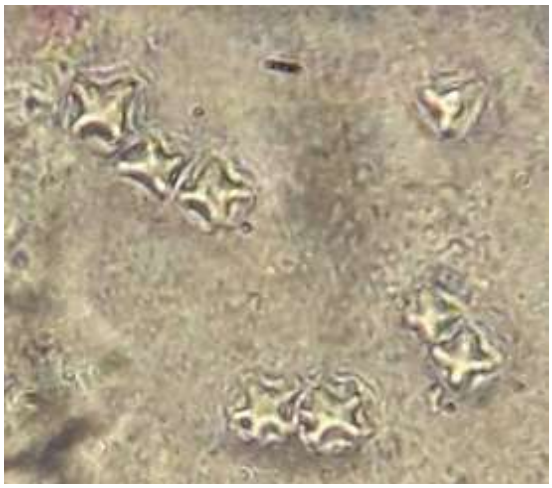

Figure 2-The cyst of *Acanthamoeba* in the NNA. Photographed by inverted microscope with a magnification of 400 x.

Totally, 18 (30%) out of 60 examined water samples were found to be contaminated with FLA in the three countries. Turkey showed the highest (8/20, 40%) while Iraq and Iran showed similar (5/20, 25%) contamination rates. The results of microscopic examination of the cultures are shown in (Table-4).

Table 4-Microscopic diagnosis of FLA after growing in culture medium

Negative		Positive		No.	Location	Country
%	No.	%	No.			
40	2	60	3	5	Euphrates	Iraq
80	4	20	1	5	Najaf Sea	
80	4	20	1	5	Dukan Lake	
100	5	0	0	5	Tigris River	
60	3	40	2	5	Mariwan Lake	Iran
60	3	40	2	5	Velasht Lake	
80	4	20	1	5	Caspian sea	
100	5	0	0	5	Suleymanshah Lake	
20	1	80	4	5	Sabanca Lake	Turkey
60	3	40	2	5	Seyfi Lake	
80	4	20	1	5	Hazar Lake	
80	4	20	1	5	Yay Lake	
70	42	30	18	60	Total	

Molecular study (PCR)

After PCR and electrophoresis of its products, from 18 positive culture samples , only one Iraqi isolate generated a specific band of the Rns gene of *Acanthamoeba*, in the range of 423 to 551 nucleotides (Figure-3).

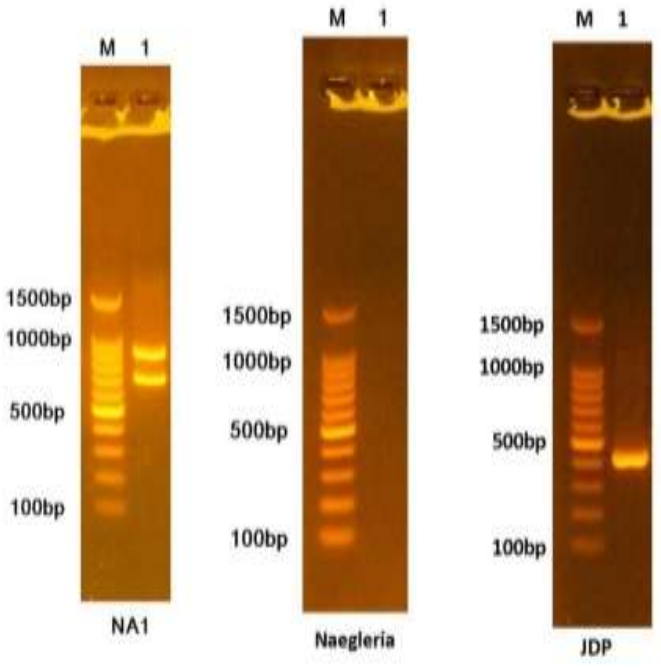

Figure 3-Electrophoresis of PCR product for samples which were only positive for JDP primer .

The PCR product of the samples that produced the expected band was used for cleaning up and sequencing.

Sequencing

Only one sample of the Iraqi isolates was sequenced. After editing the sequences using Sequencher software (version 5.4.6) and comparing them with the sequences in the Genbank, the genotypes of the isolates were identified (Figure-4). The isolates belonged to the T3 genotype which is registered as the *Acanthamoeba* genotype T3 isolate T3 Iraq.

FASTA sequence JDP-Forward

CATTTTCTGCCACCGAATACATTAGCATGGGATAATGGAATAGGACCCTGTC

CTCCTATTTTCAGTTGGTTTGCCGCGAGGACCAGGGTAATGATTAATAGGGA

TAGTTGGGGGCATTAATATTTAATTGTCAGAGGTGAAATTCTTGGATTTATG

AAAGATTAACTTCTGCGAAAGCATCTGCCAAGGATGTTTTCATTAATCA

AGAACGAAAGTTAGGGGATCGAAGACGATCAGATACCGTCGTAGTCTTAAC

CATAAACGATGCCGACCAGCGATTAGGAGACGTTAATACAAAACACCACCA

TGCGCATTGCGGTCGTCTTTGGTGTCGCTCACAAGGCGGCACCGGGGCGGCT

TAGCTCGCATGGCACCGGTGAATGACTCCCTAGCAG

Figure 4-Nucleotide sequences of the ASA.S1 fragment of the 18S rRNA gene of *Acanthamoeba* (partial).

Phylogenetic analysis

Phylogenetic analysis was performed using maximum likelihood and the phylogenetic tree was plotted (Figure-5).

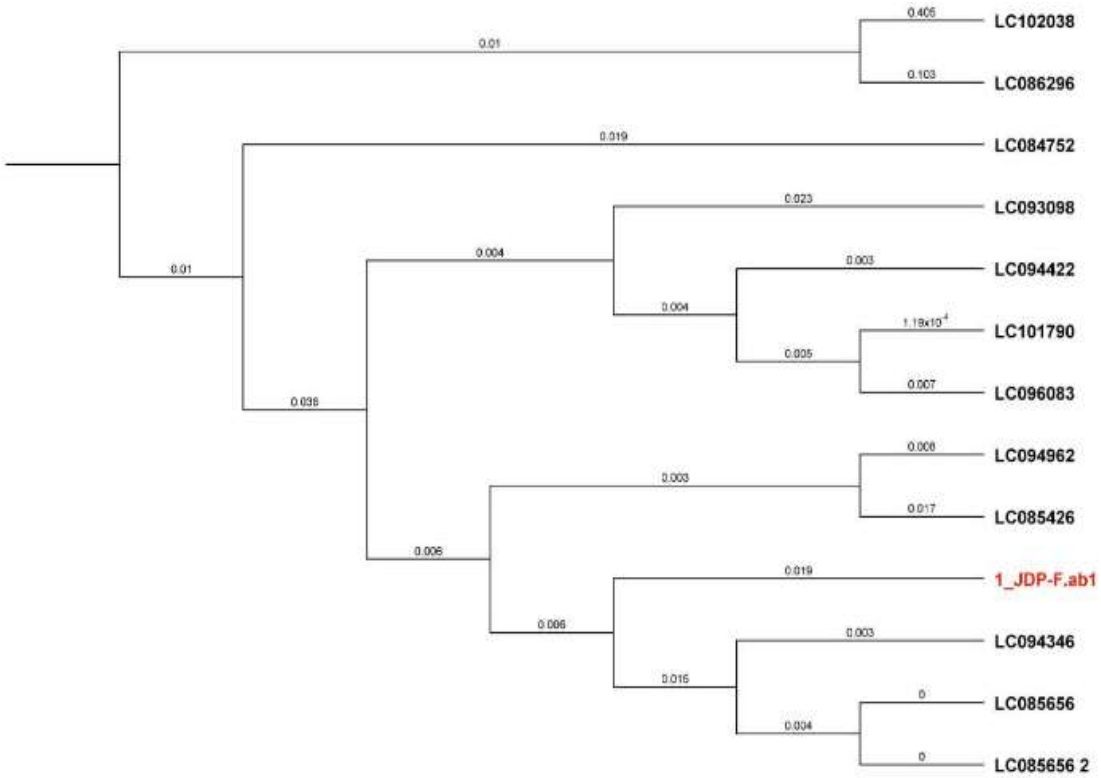

Figure 5 -Phylogenetic tree of the Iraqi isolate 1-JDP-F.ab1 (accession number MN462973) of *Acanthamoeba* compared to other isolates registered in GenBank using maximum likelihood.

Discussion

The wide distribution of FLA in water systems is due to their resistance, especially of the cyst forms, which are resistant against extremes of temperature and pH and against various chemicals used for water disinfection [8, 5]. In their natural habitat, FLA interacts with bacteria in several ways. Although they represent important predators of bacteria and fungi, several of these have evolved to resist digestion by FLA [9]. These amoeba-resistant bacteria (ARB) can survive, multiply within and lyse their host cells, and eventually spread to the environment in large numbers [12, 13,14]. Furthermore, within the amoebae, ARB may develop and maintain virulence traits, including resistance to antibiotics, and may adapt to life within human macrophages [9]. Thus, the presence of FLA in tap water may represent a health risk to both immunocompromised and immunocompetent individuals by their role in spreading pathogenic bacteria in aquatic systems, in addition to their own potential pathogenicity [15, 16].

In this study, *Acanthamoeba* was isolated from water samples of different lakes and rivers from Iraq , Iran and Turkey . Out of 60 water samples from these three countries , totally 18 (30%) were found to be positive for *Acanthamoeba* cyst and trophozoite. Also, the one Iraqi sample that was genotyped belonged to the T3 genotype. No other free living amoeba was detected in the present study. Studies to date have shown that *Acanthamoeba* is present in a variety of environmental sources . In Iran, river water as the most popular surface water has been examined frequently. Rivers in Mazandaran and Gilan provinces (northern Iran), as well as the rivers of Tehran province, have been studied more frequently [17 - 18]. In other regions, more studies are required, even though few studies have been conducted in Bojnurd, Arak, Karaj, Kazeroun, Ahvaz, and East and West Azarbaijan [29- 20]. The infestation rate of FLA was about 47%, among the total samples taken from various rivers. Most of the species were observed, while *Acanthamoeba*, *Naegleria*, *V. vermiformis* and *Saccamoeba* had the highest prevalence, respectively. However, *Acanthamoeba* has still attracted the most attention, which might ignore the impacts of the other FLA [17, 21, 22]. There are limited studies on the prevalence of FLA in the Caspian Sea (North of Iran), since most studies in recreational water have been conducted on springs and pools [23, 19, 24, 25, 26]. However, *Acanthamoeba* was seen in most samples (80%) collected from the Caspian Sea [27, 18]. Moreover, various studies have indicated a prevalence of

27.2% on hot/cold springs in Ardebil, Mazandaran, Gilan, East Azerbaijan and Arak [28, 19, 24, 25, 26]. Importantly, the pathogenic amoebae *Balamuthia menderiallis* were isolated from hot springs [23]. Among the studies that performed sequencing, T4 and T3 are known as the most common genotypes of *Acanthamoeba* [24, 19, 25, 26]. Also, T2, T5 and T15 have been isolated [18, 29, 21]. In Turkey , *Acanthamoebae* have been isolated from various environmental sources in Burdur and Istanbul provinces [30]. Furthermore, both *Acanthamoebae* and *Naegleria* have also been isolated from soil and thermal water specimens in Sivas [31]. *Acanthamoeba* isolates belonging to T2, T3, T4, and T7 genotypes from Ankara [32] and T4 and T9 genotypes from Aydin province [33] have been reported from environmental samples in Turkey.

Studies in other parts of the world have yielded different results. In a study conducted in southern Brazil, 20% of the pools were contaminated with *Acanthamoeba* [34. Another study conducted in Malaysia showed that all pools studied were contaminated with this amoeba [34]. The results of different studies indicate differences in the prevalence of *Acanthamoeba* in the pools of different regions. These differences can be due to differences in sampling method, number of samples, sample size, and sampling time. According to our results, the T3 genotype of *Acanthamoeba* is reported for the first time in Iraq. In Mahdavi *et al.* study, the examination of genotypes of 4 isolates of *Acanthamoeba* isolated from aquatic recreational centers showed that 3 isolates belonged to T4 genotype (75%) and one isolate belonged to T3 genotype (25%) [35]. Previous studies have shown that, in most *Acanthamoeba* infections, the T4 genotype was the causative agent of infection, with more than 90% of amoebic keratitis being the causative agent of the T4 genotype [36]. In previous studies, the T3 genotype has also been isolated from cases of amoebic keratitis [37]. Since the isolates identified in recreational-aquatic centers belong to potentially pathogenic genotypes, this suggests that more attention should be paid to the health of these recreational waters.

Conclusions and recommendations

Given the high prevalence of *Acanthamoeba* potential pathogenic genotypes in various environmental sources and the evidence of T3 genotype in Iraqi specimens, more studies about *Acanthamoeba* and other pathogenic FLA for various environmental sources in Iraq are required. Also, it is recommended to provide the necessary information to those who use contact lenses as well as those with immune defects. On the other hand, since most *Acanthamoeba* isolates carry bacterial, fungal and viral endo-infections, and that their presence in hospital and recreational waters may lead to the transmission of these infections, it is recommended to pay more attention to the health of these sites.

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