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# Morphological and Molecular Detection of Some Opportunistic Free-Living Amoebae Isolated from Environmental and Clinical Sources in Thi-Qar Province / Iraq

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***Abstract*— Predatory heterotrophic protozoa known as free-living amoebae (FLA) are found in various environmental sources. They have been linked to deadly cases of encephalitis, blinding keratitis, and pneumonia in humans. This study was performed in Thi-Qar province, south of Iraq, to identify the opportunistic FLA and detect it using morphological characteristics in the culture and Polymers Chain Reaction (PCR) amplification – targeting specific genes for each genus. One hundred and two (102) samples were collected between February and September 2020; these samples were collected from several environmental sources, soil, water (rivers, tap water, tank water, stagnant water, the marshes water ,and drops of water from the air conditioner units ) and animals wastes(lizard, birds ,and wild mice), and 27 clinical samples ( eye, skin, ear, and Cerebral Spinal Fluid) gathered from private laboratories in the province of Thi-Qar as well as the Al-Hussain, Bint AL-Huda, and Al-Hboobi teaching hospitals.**
**All samples were grown in Non Nutrient agar medium (NN-agar medium), which was subsequently studied under a light microscope to identify the trophozoites and cysts of opportunistic amoebas from the following genera: *Sappinia spp. (S. pedata, S. diploidea), Acanthamoeba spp. (A. triangularis, A. astronyxis, A. castellini, A. polyphaga), and Naegleria fowleri* and *Balamuthia mandrillaris*, the study found 62 (60.78%) samples were positive for total opportunistic FLA, and 40(39.21%) samples were negative, the incidence of FLA in environmental samples was 76% and in clinical samples were18.51 %. *Sappinia* spp. was the most common amoeba in both environmental and clinical samples while *Naegleria fowleri* was less present. Among the 62 environmental and clinical isolates of common FLA that were positive microscopically, only 48(47.05%) of them were positive after polymerase chain reaction, the incidence of FLA in environmental samples was 58.66% and in clinical samples was 14.81%.**

***Keywords*— Free-living amoebas, Environmental samples, Clinical samples,Thi-Qar province**

## I. INTRODUCTION

Free-living amoebae (FLA) are heterotrophic predatory protozoa that attach to surfaces and feed as trophozoites on bacteria, cyanobacteria, fungi, and algae through phagocytosis [1]. Normally found living freely in freshwater environments and soil, these amoebae are extremely pathogenic and capable of facultative parasitism in humans. When deadly cases of primary amoebic meningoencephalitis (PAM) were reported concurrently in Florida and Australasia in 1965, their toxicity in humans was first recognized. since then, reports of more than 150 cases - from other parts of the United States have been made globally. The majority of victims report having recently been in fresh, brackish, warm, stagnant water like those found in lakes, ponds, and swimming pools [2].

Of the many species of FLA found in nature, only four genera or species are recognized as helpful agents of human infections in their natural environments. *Acanthamoeba spp., Naegleria fowleri, Balamuthia mandrillaris and Sappina pedata***.** Due to their poor adaptation to parasitism, these amoebae may survive in the human environment without a host. Even though these amoebae infections have minimal morbidity, they are characterized by a very high mortality rate and present significant clinical issues [3].

The most pathogenic of FLA, *Naegleria fowleri*, is recognized as being the cause of Primary Amoebic Meningoencephalitis (PAM). This acute and quickly lethal disease that strikes healthy people who drink polluted water [4]. .*Acanthamoeba* spp. , *Balamuthia mandrillaris* and *sappina pedata* are associated with Granulomatous Amoebic Encephalitis (GAE) a subacute or chronic disease of individuals with a compromised immune system. Additionally, infections of the lung, liver, kidneys, spleen, heart, sinuses, adrenal glands, and skin are brought on by *Acanthamoeba* spp.. Additionally, certain strains of *Acanthamoeba* are responsible for a potentially blinding corneal infection known as Acanthamoeba keratitis (AK), primarily linked to healthy contact lens wearers [4,5].


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Because of these threats and their potential impact on human , this study aims — to assess the prevalence of pathogenic free- living amoebae in Iraq in environmental and clinical sources.

## II. MATERIAL AND METHODS

### A. Collection of Sample and Cultivation:

#### 1) Samples from environmental sources

Samples were taken from a variety of environmental sources, including animal feces, soil, and water from (rivers, tap water, ponds, marshes, and air conditioning units outside buildings). Additionally, these samples were gathered from various locations in the province of Thi-Qar between February and September 2020.

**A-**For collecting the water samples 100 ml sterile cups were used, and each cup was tagged with the date and the location. Each sample was immediately cultivated in two replicates on non-nutrient agar (NN-agar) medium and incubated at 26 $C^0$. For four weeks, amoebic growth was observed every day using a light microscope on a slide and an inverted microscope on agar.

**B-** In sterile containers, soil samples and animal waste were gathered. Each sample was tagged with the date and the location. Following the collection of each sample during 24 hours, three grams were dissolved in 5 ml of sterile distilled water, and the supernatant was then grown in two replicates on non-nutrient agar (NN-agar) medium and incubated at 26 $C^0$. To keep cultures moist, 3 ml of sterile distilled water was added twice a week. Growth of Amoebae was then checked–each day for four weeks under a microscope on a wet mount slide**.**

### B. Clinical Samples

During the period from February to September 2020, samples were taken from several clinical sources, including - the eyes, skin, ears, and CSF, from Al-Hussan Teaching Hospital, Bint AL-Huda Teaching Hospital, Al-Hboobi Teaching Hospital, and private laboratories in Thi-Qar Province. The non-nutrient agar medium was used to cultivate the clinical samples, and they were incubated at 26C0 for weekly evaluation.

### C. Amoebae Isolate Dagnosis

When amoebic growth was observed on the culture media, the samples were mounted in sterilely conditions on a microscope slide made with a cotton swab. Next, trophozoites and cyst phases were detected by imaging the samples at 100x and 400x magnification. Next, each step's measurements were taken with the microscopic stage ruler. and the condition was then diagnosed [6]. After morphological identification of free- living amoeba. was confirmed genetically by conventional PCR using targeting specific genes for each genus shown in Table (1). The genomic DNA of free- living amoeba spp. wasisolated from cell culture using the gSYNC TM DNA Extraction kit from Geneaid Korea, following the manufacturer's instructions, the PCR product from 18S-rDNA genes in accordance with the procedure shown in Table (2). The PCR product was electrophoresed on a 1.5 % agarose gel and visualized with UV.

Table (1 ) PCR primers used in this study ( 5'-3')

| Species | Forward primer | Reveres primer | Reference |
|---|---|---|---|
| *Acanthamoeba* spp. | GGCCCAGATCGTTTACCGTG | TCTCACAAGCTGCTAGGGAGTCA | (7) |
| *Naegleria fowleri* | CAAACACCGTTATGACAGGG | CTGGTTTCCCTCACCTACG | (8) |
| *Balamuthia mandrillaris* | CGCATGTATGAAGAAGACCA | TTACCTATATAATTGTCGATACCA | (9) |
| *Sappinia* spp. | TCTGGTCGCAAGGCTGAAAC | GCACCACCACCCTTGAAATC | (10) |

Table (2 ) PCR Steps

| Primers | PCR Step | | | | | |
|---|---|---|---|---|---|---|
| | Initial denaturation | | Denaturation | Annealing | Extension | Final Extension |
| *Acanthamoeba* | Tem. | 95 $C^0$ | 95$C^0$ | 56$C^0$ | 72$C^0$ | 72$C^0$ |
| | Tim. | 10 min. | 35 sec | 35 sec. | 40sec. | 10min. |
| | Rep. | 1 | 35cycle | | | 1 |
| *N. fowleri* | Tem. | 95 $C^0$ | 95$C^0$ | 58 $C^0$ | 72$C^0$ | 72$C^0$ |
| | Tim | 10 min. | 35 sec | 35 sec. | 40sec. | 10min. |
| | Rep. | 1 | 35cycle | | | 1 |
| *B. mandrillaris* | Tem. | 95$C^0$ | 95$C^0$ | 49 $C^0$ | 72 $C^0$ | 72 $C^0$ |
| | Tim. | 10 min. | 35 sec. | 50 sec. | 1 min. | 10 min. |
| | Rep. | 1 | 35 cycle | | | 1 |
| *Sappinia* | Tem. | 95$C^0$ | 95$C^0$ | 56$C^0$ | 72$C^0$ | 72$C^0$ |
| | Tim. | 10 min | 35 sec | 35 sec. | 40sec | 10min. |
| | Rep. | 1 | 35cycle | | | 1 |

## III. Results

### A. *Total Prevalence of Opportunistic FLA by Microscopic Study:*

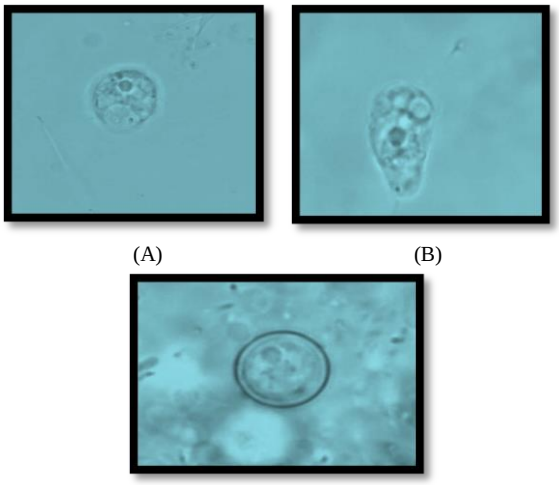
Out of 102 samples obtained from various clinical and environmental sources, 40 (39.21%) samples tested negative for total opportunistic FLA- based morphological observation in NN-Agar culture, while 62 (60.78%) samples showed positive results. Some samples showed more than one type of FLA. In clinical samples, the incidence of FLA was 18.51%, while it was 76% in environmental samples. Soil samples exhibited the largest incidence in environmental sources, accounting for 95.6%, whereas skin samples showed the highest frequency in clinical sources, 40 percent. Table (3).

Table (3 ) : Total opportunistic FLA spp. were found by microscopic analysis in environmental and clinical samples. .

| Type of Sample | No. sample Examed. | Positive samples | | Negative samples | |
|---|---|---|---|---|---|
| | | No. | % | No. | % |
| **River water** | 8 | 7 | 87.5 | 1 | 12.5 |
| **Tap water** | 5 | 3 | 60 | 2 | 40 |
| **Tank water** | 4 | 3 | 75 | 1 | 25 |
| **Stagnant water** | 4 | 3 | 75 | 1 | 25 |
| **Marshes water** | 5 | 4 | 80 | 1 | 20 |
| **Air conditioner water** | 4 | 3 | 75 | 1 | 25 |
| **Soil** | 23 | 22 | 95.6 | 1 | 4.3 |
| **Potato soil** | 4 | 3 | 75 | 1 | 25 |
| **Lizard waste** | 7 | 5 | 71.42 | 2 | 28.51 |
| **Birds waste** | 5 | 3 | 60 | 2 | 40 |
| **Mice waste** | 6 | 1 | 16.66 | 5 | 83.33 |
| **Total** | 75 | 57 | 76 | 18 | 24 |
| **Clinical eye samples** | 10 | 1 | 10 | 9 | 90 |
| **Clinical skin samples** | 5 | 2 | 40 | 3 | 60 |
| **Clinical ear samples** | 4 | 0 | 0 | 4 | 100 |
| **Clinical CSF samples** | 8 | 2 | 25 | 6 | 75 |
| **Total** | 27 | 5 | 18.51 | 22 | 81.48 |
| **Total** | 102 | 62 | 60.78 | 40 | 39.21 |

### B. *Observations of Opportunistic Amoebas in Clinical and Environmental Samples:*

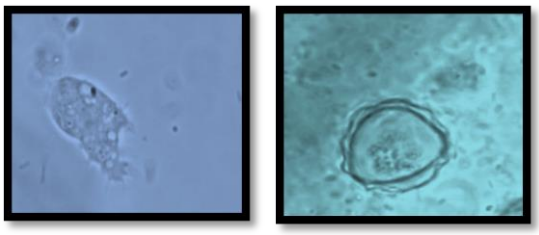
The findings indicated that *Sappinia* spp. was the most often found amoeba in environmental and clinical samples. 53 samples (51.96%) had amoeba recovered from them, including 21 soil samples, 2 potato soil samples, 7 animal waste samples, and 3 clinical samples. In comparison to *Sappinia, Acanthamoeba* spp. were found in 17 (16.66%) cases (3 water, 6 soil, 1 potato soil, 3 animal waste, and 4 clinical samples)., *Balamuthia mandrillaris* was, less common than *Sappinia* and *Acanthamoeba* was observed in 12(11.76 %) cases ( 6water, 4 soil, 0 potato soil, 2 animals waste, and 0 clinical samples ) , while *Naegleria fowleri.* was less present in environmental and clinical samples which were observed in 8(7.84%) (6 water and 2 soil ). Table (4).



(A) (B)

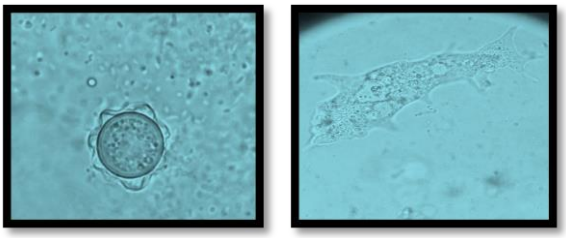
(c )

Fig (1 ) *Naegleria fowleri.*(A) amoeboid trophozoite (B) flagellate trophozoite , (c) cyst stage (unstained ) (1000X).



(A) (B)

Fig (2) *Acanthamoeba triangularis* (A) Trophozoite (B) Cyst (unstained ) (1000X).



(A) (B)

Fig (3) *Balamuthia mandrillaris* (A) Cyst (B) trophozoite (unstained ) (1000X).

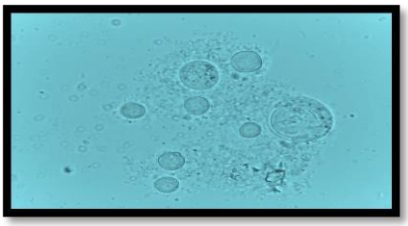


Fig.( 4 ) *Sappinia* spp. showed stage of trophozoite emerged from a cyst shell (unstained) (1000X)

Table (4) : Microscopic analysis of environmental and clinical samples revealed the presence of opportunistic amoebas.

| Type of sample | No. samples examination | *Acanthamoeba* spp. ve+ | | *Sappinia* spp. ve+ | | *Balamuthia mandrillaris* ve+ | | *Naegleria fowleri* ve+ | |
|---|---|---|---|---|---|---|---|---|---|
| | | No. | % | No. | % | No. | % | No. | % |
| Water | 30 | 3 | 10 | 20 | 66.66 | 6 | 20 | 6 | 20 |
| Soil | 23 | 6 | 26.08 | 21 | 91.30 | 4 | 17.39 | 2 | 8.69 |
| Potato soil | 4 | 1 | 25 | 2 | 50 | 0 | 0 | 0 | 0 |
| Animals waste | 18 | 3 | 16.6 | 7 | 38.88 | 2 | 11.11 | 0 | 0 |
| Total | 75 | 13 | 17.33 | 50 | 66.66 | 12 | 16 | 8 | 10.66 |
| Clinical samples | 27 | 4 | 14.81 | 3 | 11.11 | 0 | 0 | 0 | 0 |
| Total | 102 | 17 | 16.66 | 53 | 51.96 | 12 | 11.76 | 8 | 7.84 |

## IV. Molecular study

Only 48 (47.05%) of the 62 ambient and clinical isolates of common FLA that were microscopically positive were positive following polymerase chain reaction. Incidences of FLA were 14.81 % in clinical samples and 58.66 % in environmental samples. The frequency of total opportunistic FLA spp. in clinical and environmental samples examined under a microscope and through DNA analysis is displayed in Table 5 .

Table (5 ): Total opportunistic FLA spp. presence in clinical and environmental samples examined under a microscope and by molecular analysis

| Type of Sample | No. sample Examed By microscope | Microscopic Positive samples | | No. samples Examed By PCR | PCR positive samples | |
|---|---|---|---|---|---|---|
| | | No. | % | | No. | % |
| River water | 8 | 7 | 87.5 | 7 | 5 | 62.5 |
| Tap water | 5 | 3 | 60 | 3 | 2 | 40 |
| Tank water | 4 | 3 | 75 | 3 | 3 | 75 |
| Stagnant water | 4 | 3 | 75 | 3 | 3 | 75 |
| Marshes water | 5 | 4 | 80 | 4 | 3 | 60 |
| Air conditioner | 4 | 3 | 75 | 3 | 1 | 25 |
| Soil | 23 | 22 | 95.6 | 22 | 18 | 78.2 |
| Potato soil | 4 | 3 | 75 | 3 | 2 | 50 |
| Lizard waste | 7 | 5 | 71.42 | 5 | 4 | 57.14 |
| Birds waste | 5 | 3 | 60 | 3 | 2 | 40 |
| Mice waste | 6 | 1 | 16.66 | 1 | 1 | 16.66 |
| Total | 75 | 57 | 76 | 57 | 44 | 58.66 |
| Clinical eye samples | 10 | 1 | 10 | 1 | 1 | 10 |
| Clinical skin samples | 5 | 2 | 40 | 2 | 2 | 40 |
| Clinical ear samples | 4 | 0 | 0 | -------- | --------- | 0 |
| Clinical CSF samples | 8 | 2 | 25 | 2 | 1 | 12.5 |
| Total | 27 | 5 | 18.51 | 5 | 4 | 14.81 |
| Total | 102 | 62 | 60.78 | 62 | 48 | 47.05 |

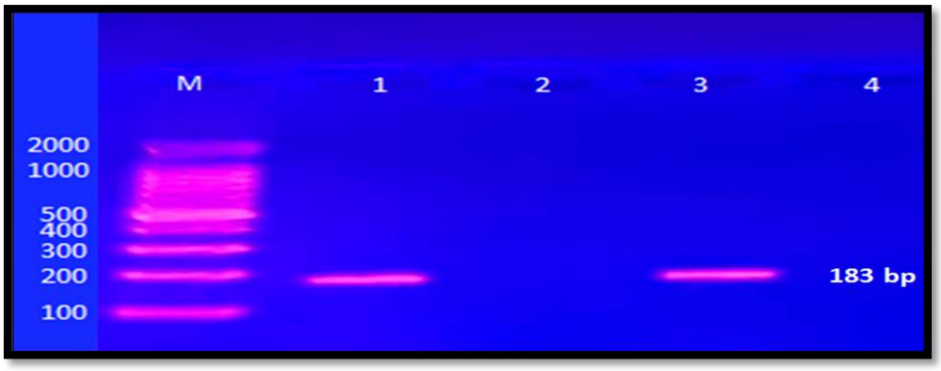



Fig. (5 ) : The PCR result (183 bp) of the 18S r RNA gene analysis from the environmental samples of *Naegleria fowleri's* genomic DNA is shown in this Agarose gel electrophoresis image: Where M: DNA marker (100–2000 bp) positive samples and negative samples are lance (1,3) and lance (2,4).

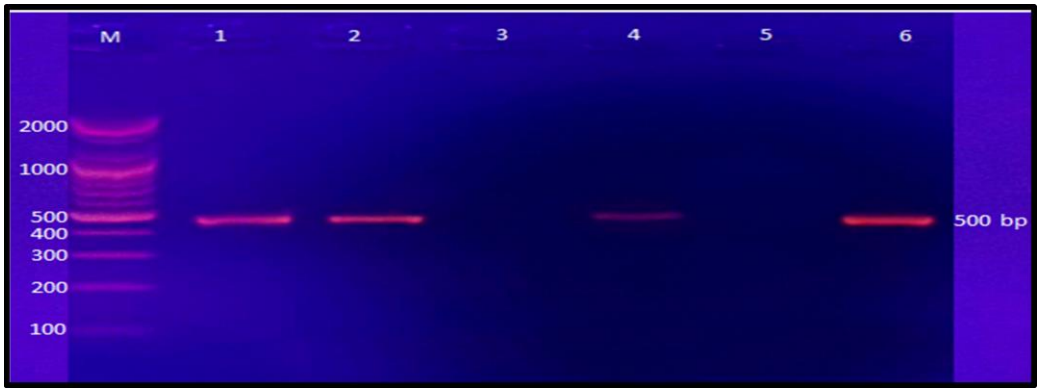



Fig. ( 6 ) : The PCR product (500 bp) of the 18S rRNA gene from the genomic DNA of *Acanthamoeba* spp. from environmental and clinical samples is shown in this Agarose gel electrophoresis image: Where M is a 100–2000 kb DNA marker positive samples from Lance (1, 2, 4, 6) and negative samples from Lance (3, 5).

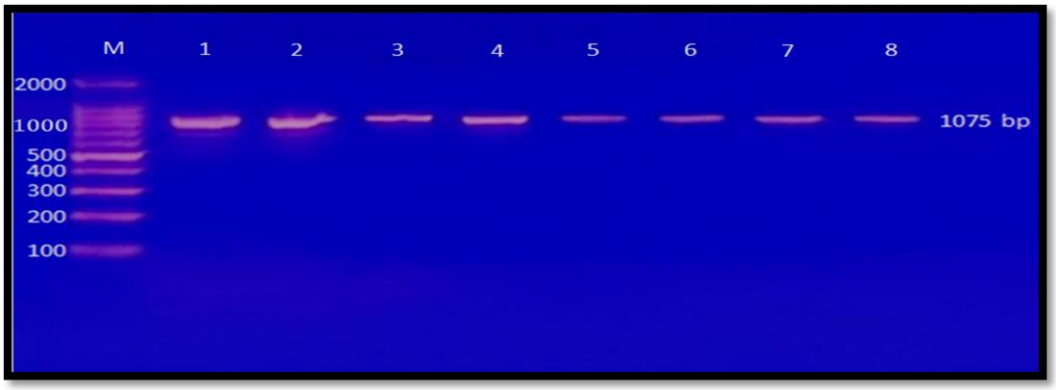



Fig. (7 ) : PCR product (1075 bp) of the 16S ribosomal RNA gene from *Balamuthia mandrillaris* genomic DNA shown on an agarose gel electrophoresis picture. from samples of the surroundings: M:DNA Marker (100-2000 bp) is located where sample counts for lance (1–8) positive and lance (9) negative samples

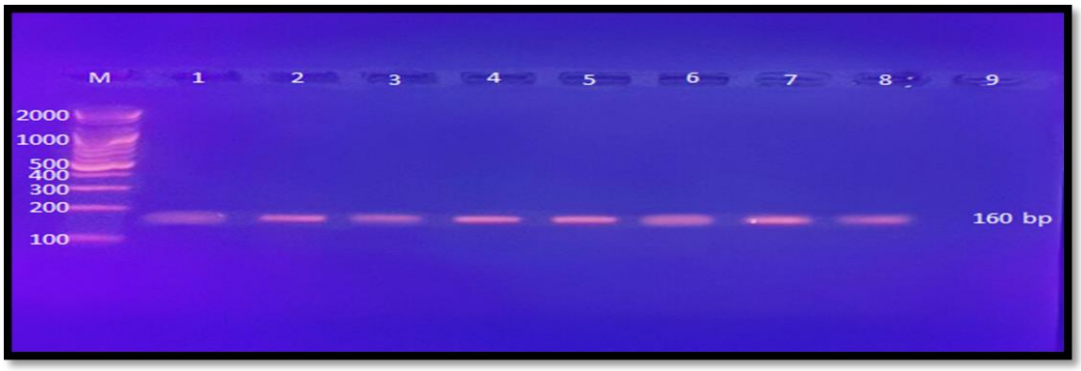



Fig. ( 8 ) : The PCR product (160 bp) of the 18S rRNA gene from the genomic DNA of *Sappinia* spp. from environmental and clinical samples is displayed in this Agarose gel electrophoresis image: Where M is a 100–2000 kb DNA marker positive samples from Lance (1–8) and negative samples from Lance (9)

## V. Discussion

Free-living amoebas (FLAs) are found worldwide in aquatic environments, soil, and air, and they are extensively distributed in a variety of environmental sources [4]. They have the potential to cause deadly encephalitis, blinding keratitis, and pneumonia in humans. [11]. Because free-living amoebae play a significant role in ecosystems and can cause dangerous diseases in humans, the number of investigators studying these organisms has expanded considerably recently [12].

This study is the first of its kind in the Thi-Qar province of Iraq. to examined opportunistic free amoebae, as well as all known opportunistic species, including *Naegleria fowleri, Acanthamoeba* spp., *Sappinia* spp., and *Balamuthia mandrillaris*, in environmental and clinical source samples. This is particularly noteworthy given the paucity of prior research in Iraq.

Based on morphological observation in NN-Agar culture, the incidence in the environment was 76% and in clinical samples. It was 18.51%; based on polymerase chain reaction, the incidence in the environment was 58.66% and in clinical samples, it was 14.81 %. These findings indicate that the percentage of total FLA occurrence was 60.78% in the current study.

Rezaeian *et al* [13] used a culture and microscopic approach to examine 354 soil and water samples from the River Parishan Lake in the Kazeroon region. They found 13 positive cases of FLA. Kubra *et al* [14] found FLA in 33 (22%) of 150 water samples from six Sivas districts in Turkey. The findings of this study indicated that the percentage of FLA occurrence was lower than the current study.

This study is in line with previous research conducted by Eftekhar *et al* [15] who examined 22 surface water and water in squares in Tehran, finding that 13 (59%) of the samples were contaminated with FLA through the polymerase chain reaction method. Similarly, Armand *et al*. [16] showed that 48 (58.53%) of the 82 water samples from various sites in Shiraz City were positive for FLA based on morphological observation in NN-agar culture, and Rezaeian *et al*. [12] demonstrated a nearly high prevalence (46.25%) in Tehran.

This study, other studies conducted globally, have found a significant incidence of FLA. The composition of these species varies depending on the surrounding environment. Additionally, the ability of FLA species to endure harsh environments is necessary for their expansion [14]. The prevalence of FLA in diverse environmental samples indicates resistance to harsh temperature, pH, and chemical exposure conditions [17]. Pathogenic free-living amoebas, in contrast to real parasites, can finish their life cycles in the environment without attaching themselves to a human or animal host [18]. Numerous factors, such as the various applied procedures, the characteristics of the water supply, and the geographic locations, could be responsible for the great range in the prevalence levels obtained.

The present investigation focuses on identifying free-living amoebae through molecular methods and NN-agar cultivation. Out of the 102 samples analyzed, 62 (60.78%) tested positive for FLA under microscopic inspection, and 48 (47.05%) tested positive using the PCR method. , the results of the current investigation were in agreement with those of Di Filippo *et al* [19]. who established that the culture method is insufficiently precise to detect FLA. In that study, a total of 160 water samples were subjected to PCR analysis to detect FLA, under a microscope, FLA was found in 46 of the cultured water samples; however, only 39 samples tested positive when the culture was subjected to PCR techniques. This might also be caused by the simultaneous existence of other amoebae in cultured samples; however, direct microscopic analysis of the culture-positive samples is unable to distinguish them from one another. The size variation between amoebae's trophozoite and cyst forms makes it challenging to diagnose those using morphological methods. Magnet's study indicates that PCR is a more sensitive methodology than direct microscopy of culture; nonetheless, in order to obtain more comprehensive results of the true presence of FLA in environmental water samples, the employment of both PCR and culture method is recommended [20].

## CONFLICT OF INTEREST

Authors declare that they have no conflict of interest.

## References


[1] A. M. Spagnolo, M. Sartini, D. Di Cave, B. Casini, B. Tuvo, and M. L. Cristina, "Evaluation of microbiological and free-living protozoa contamination in dental unit waterlines," *International Journal of Environmental Research and Public Health*, vol. 16, p. 2648, 2019.

[2] M. Lalle, E. Pozio, G. Capelli, F. Bruschi, D. Crotti, and S. M. Cacciò, "Genetic heterogeneity at the β-giardin locus among human and animal isolates of Giardia duodenalis and identification of potentially zoonotic subgenotypes," *International journal for parasitology*, vol. 35, pp. 207-213, 2005.

[3] K. Król-Turmińska and A. Olender, "Human infections caused by free-living amoebae," *Annals of Agricultural and Environmental Medicine*, vol. 24, 2017.

[4] G. S. Visvesvara, H. Moura, and F. L. Schuster, "Pathogenic and opportunistic free-living amoebae: Acanthamoeba spp., Balamuthia mandrillaris, Naegleria fowleri, and Sappinia diploidea," *FEMS Immunology & Medical Microbiology*, vol. 50, pp. 1-26, 2007.

[5] N. A. Khan, "Acanthamoeba: biology and increasing importance in human health," *FEMS microbiology reviews*, vol. 30, pp. 564-595, 2006.

[6] F. Page, "A new key to freshwater and soil gymnamoebae Freshwater Biological Association," *Ambleside, Cumbria, United Kingdom ρ,* pp. 14-15, 1988.

[7] J. M. Schroeder, G. C. Booton, J. Hay, I. A. Niszl, D. V. Seal, M. B. Markus, *et al.*, "Use of subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of

acanthamoebae from humans with keratitis and from sewage sludge," *Journal of Clinical Microbiology*, vol. 39, pp. 1903-1911, 2001.
[8] M. Schild, C. Gianinazzi, B. Gottstein, and N. Müller, "PCR-based diagnosis of Naegleria sp. infection in formalin-fixed and paraffin-embedded brain sections," *Journal of Clinical Microbiology*, vol. 45, pp. 564-567, 2007.
[9] G. C. Booton, J. R. Carmichael, G. S. Visvesvara, T. J. Byers, and P. A. Fuerst, "Identification of Balamuthia mandrillaris by PCR assay using the mitochondrial 16S rRNA gene as a target," *Journal of Clinical Microbiology*, vol. 41, pp. 453-455, 2003.
[10] Y. Qvarnstrom, A. J. Da Silva, F. L. Schuster, B. B. Gelman, and G. S. Visvesvara, "Molecular confirmation of Sappinia pedata as a causative agent of amoebic encephalitis," *The Journal of infectious diseases*, vol. 199, pp. 1139-1142, 2009.
[11] A. Leońska-Duniec, B. Skotarczak, and M. Adamska, "Molecular identification of free-living amoebae isolated from artificial water bodies located in Poland," *Acta Protozoologica*, vol. 54, 2015.
[12] M. Rezaeian, M. Niati, S. Farnia, and H. A. MOTEVALI, "Isolation of Acanthamoeba spp. from different environmental sources," 2008.
[13] M. Rezaian, F. Bagheri, S. Farnia, and Z. Babai, "Isolation of pathogenic amoebae (Naegleria and Acanthamoeba) from water sources and margin soils of rivers and lakes in Kazerun," 2002.
[14] K. A. Coşkun, S. Özçelik, L. Tutar, N. Elaldı, and Y. Tutar, "Isolation and identification of free-living amoebae from tap water in Sivas, Turkey," *BioMed research international*, vol. 2013, 2013.
[15] M. Eftekhar, A. M. E. NAZEM, A. HAGHIGHI, K. SHARIFI, Z. Nouchi, and A. Athari, "Detection of Acanthamoeba from fresh water using polymerase chain reaction," 2009.
[16] B. Armand, M. Motazedian, and Q. Asgari, "Isolation and identification of pathogenic free-living amoeba from surface and tap water of Shiraz City using morphological and molecular methods," *Parasitology research*, vol. 115, pp. 63-68, 2016.
[17] V. Thomas, J. F. Loret, M. Jousset, and G. Greub, "Biodiversity of amoebae and amoebae-resisting bacteria in a drinking water treatment plant," *Environmental microbiology*, vol. 10, pp. 2728-2745, 2008.
[18] A. J. Martinez and G. S. Visvesvara, "Free-living, amphizoic and opportunistic amebas," *Brain Pathology*, vol. 7, pp. 583-598, 1997.
[19] A. Magnet, S. Fenoy, A. Galván, F. Izquierdo, C. Rueda, C. F. Vadillo, *et al.*, "A year long study of the presence of free living amoeba in Spain," *Water research*, vol. 47, pp. 6966-6972, 2013.