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Article

Morphological and molecular investigation of free-living amoeba *Sappinia* spp. isolated from environmental and clinical samples in Thi-Qar province-southern Iraq

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ABSTRACT

Sappinia is a free-living amoeba, a single-celled living organism found in the environment. It has recently been attributed to being an opportunistic human pathogen. There are two known species of *Sappinia*: *S. diploidea* and *S. pedata*. The current study detected *Sappinia* species using morphological and genetic approaches in various environmental and clinical samples in the Thi-Qar province of southern Iraq. The study was conducted from February - September 2020; one hundred and two samples for this study were obtained from various environmental and clinical sources. PCR was carried out with a positive culture after the samples were cultivated on an NN-agar medium. Overall, the study showed that 53(51.96%) samples were positive on morphological characters as well as PCR analysis showed that only 47 (46.07%)) of *Sappinia* morphologically positive samples were positive by using a specific primer. *Sappinia* spp. were observed in all types of samples of environmental and clinical sources except CSF, and clinical ear samples were negative. The current study showed two species of *Sappinia* that have similar morphology but differ in the diameter of trophozoite and cyst, number of nuclei, and cyst morphology. The current study will pave the way for additional epidemiological research by considering the presence of potential Sappinia species in various samples, whether from environmental or clinical sources, to better understand the function of *Sappinia* as a potential health danger to people.

Keyword: *Sappinia* spp. Free-living amoeba, opportunistic amoeba, Thi-Qar, Iraq

INTRODUCTION

Free-living amoebae (FLA) are heterotrophic protozoa that feed as trophozoites on bacteria, fungi, cyanobacteria, and algae by phagocytosis while attaching to their surface [1]. These amoebae, usually free living in fresh water and soil, are capable of facultative parasitism in humans and are highly pathogenic [2]. Among FLA distributed naturally, four genera/species are known as pathogenic factors of human in their natural habitat: *Acanthamoeba spp., Naegleria fowleri, Balamuthia mandrillaris and Sappina pedata*. [3] . *Sappinia* is a free-living amoeba, a single-celled living organism found in the environment. There are two known species of *Sappinia*: *S. diploidea* and *S. pedata* [4,5]. It includes three species: *S. pedata, S. diploidea and S. platani* [6]. *Sappinia* are usually found in soil in natural environment [7]; they widely found in mammalian feces, soil, freshwater, forest liter, elk, bison, cattle and lizard rectum [8,9], and are usually exist in buffalo and elk feces, farm animals, soil consisting rotting plants, and freshwater [10,9]

The first and only case of amoebic encephalitis caused by *Sappinia pedata* (identified initially as diploid) was described in 2001 by Gelman *et al*. [11] . The life cycle of *Sappinia* spp. Includes two stages: trophozoites and cysts [5]. Infection is thought to occur either through nasopharyngeal inhalation or hematogenous dissemination to the brain, while the incubation period and exact transmission mode are uncertain[11]. The study aimed to detect free-living amoeba in the environmental and clinical samples.

MATERIALS AND METHODS

Samples from environmental sources

Samples were taken from various 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 (February - September 2020).

A-It was collected the water samples 100 ml in sterile cups, and each cup was tagged with the date and the location. Each sample was immediately cultivated in two replicates on a non-nutrient agar (NN-agar) medium and incubated at 26 C^0. For four weeks, amoebic growth was observed daily 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. After collecting each sample for 24 hours, 3 grams were dissolved in 5 ml of sterile distilled water, and the supernatant was then grown in two replicates on a non-nutrient agar (NN-agar) medium and incubated at 26 C^0. 3 ml of sterile distilled water was added twice a week to keep cultures moist. Amoebae growth was checked daily for four weeks under a microscope on a wet mount slide**.**

Clinical samples

Samples were collected from different clinical sources, including eyes, skin, ear, and CSF collected from Al-Hussain teaching hospital, Bint AL-Huda teaching hospital, Al-Hboobi teaching hospital and private laboratories in Thi-Qar Province during the period from February to September 2020. The clinical samples were cultured on a non-nutrient agar medium incubated at 26C^0 and examined weekly.

Diagnosis of amoebae isolates

When amoebic growth was seen on the culture media, a microscopic slide was created using a cotton swab to mount the samples in sterile conditions. The samples were then imaged under 10x and 40x to find trophozoites and cyst stages. The dimensions of each stage were then recorded using the microscopic stage ruler, and the condition was diagnosed according to Page (1988)[4].

After the morphological identification of Sappinia spp., it was confirmed genetically by conventional PCR using a set of *Sappinia spp.* specific two primers designed by Qvarnstrom *et al*.(2009): Sapp-F1576 (5' TCTGGTCGCAAGGCTGAAAC3') Sapp-R1736(5' GCACCACCACCCTTGAAATC3').The genomic DNA of *Sappinia spp.* was isolated from cell culture using the gSYNC DNA Extraction kit from Geneaid Korea; following the manufacturer's instructions, the PCR product yield was a160 bp from 18S-rDNA genes following the following procedure. Initial denaturation at 95^{0}c for 10 minutes was followed by a 35-cycle of 35 seconds at 95^{0}c, 35 seconds at 56^{0}c, and 40 seconds at 72^{0}c, followed by a 10-minute final extension at 72^{0}c. The PCR product was electrophoresed on a 1.5 % agarose gel and visualized with UV.

RESULTS

The occurrence of Sappinia spp. in environmental and clinical samples

Sappinia spp. Trophozoites and cysts were observed in 53(51.96%) samples out of 102 samples collected from different environmental and clinical sources in Thi-Qar province. *Sappinia* spp. were observed in all environmental and clinical sources except CSF, and clinical ear samples were negative. Among the 53 environmental and clinical isolates of *Sappinia* spp. that were microscopically positive, only 47(46.07%) were positive after polymerase chain reaction for *Sappinia* spp. (Figure 1). The incidence of *Sappinia* spp. in environmental samples was 58.66% and 11.11 % in clinical samples, as shown in Table (1).

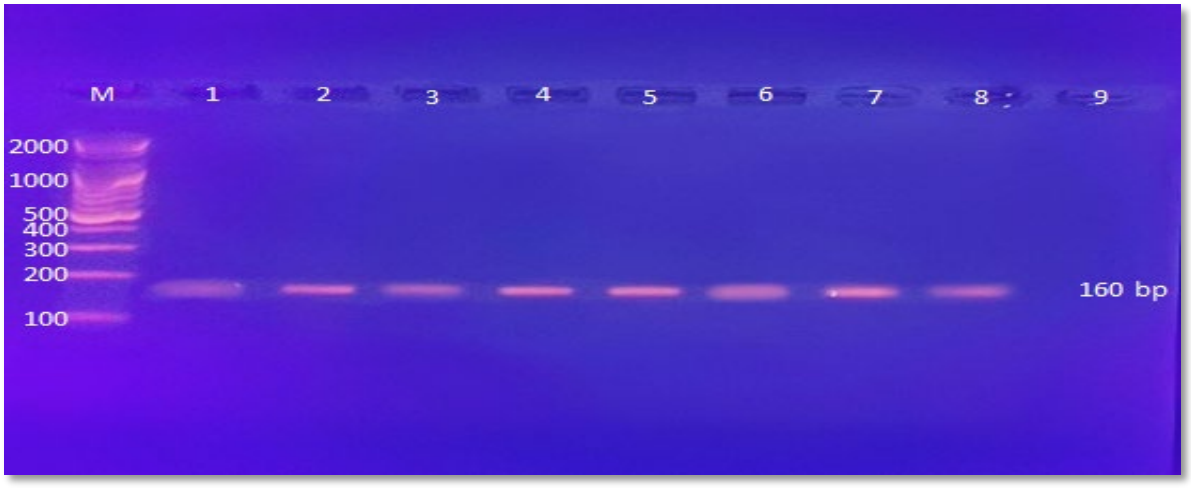

Figure 1. Agarose gel electrophoresis image that shows the PCR product (160 bp) analysis of *18S rDNA* gene from genomic DNA of *Sappinia* spp. from environmental and clinical samples: Where M: DNA Marker (100-2000 bp) lance (1-8) positive samples and lance (9) negative sample

Type of Sample	No. sample Examined By microscope	Microscopic Positive samples		No. samples Examined By PCR		PCR positive samples
		No.	%		No.	%
River water	8	6	75	6	5	**62.5**
Tap water	5	2	40	2	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 water	4	2	50	2	1	**25**
Soil	23	21	91.3	21	18	**78 .2**
Potato soil	4	2	50	2	2	**50**

Lizard waste	7	4	57.14	4	4	**57.14**
Birds waste	5	2	40	2	2	**40**
Mice waste	6	1	16.66	1	1	**16.66**
Total	75	50	66.66	50	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	0	0	0	0	**0**
Total	27	3	11.11	3	3	**11.11**
Total	102	53	51.96	53	47	**46.07**

The current study showed that two species of Sappinia have similar morphology but differ in the diameter of trophozoite and cyst, as well as the number of nuclei and cyst morphology.

Table 1. Occurrence of *Sappinia* spp. in environmental and clinical samples obtained by microscopic and molecular examination

A-*Sappinia diploidea*

The trophozoite of *Sappinia diploid* vary in measurement from 15- 41 µm long by 12- 32 µm, with transparent cytoplasm, two neighboring nuclei of about surrounded by nuclear envelop were noticed they moved together as a single unit, within the granular endoplasm, during cytoplasm streaming the pseudopodium is anterior transparent, large took about third the cell size (Figure 2). *Sappinia diploidea* showed a trophozoite stage that emerged from a cyst shell (Figure 3). Resting rounded trophozoites were also observed in the same culture, with a single nucleus within the granular endoplasm surrounded by a thin layer of clear ectoplasm, and no contractile vacuole was seen (Figure 4). Young cysts with two amoebae still separated by the visible border were also observed in the same culture (Figure 5).

Mature cyst rounded 17- 20 µm in diameter present distinctive double nuclei in which the two nuclei closely apposed with a central flattening (Figure)

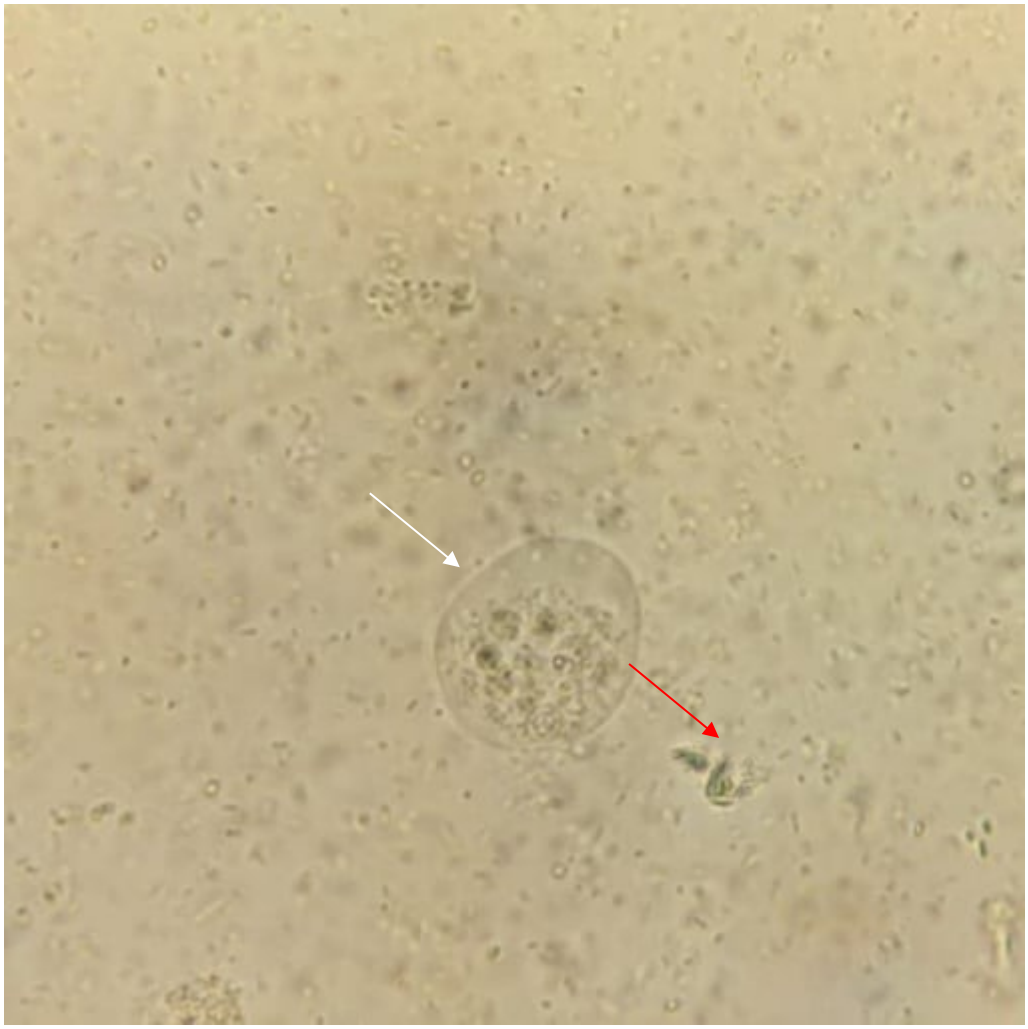

Figure 2. Sappnia *diploidea* trophozoite shows the amoeba moving by pseudopodia (white arrow)and a pair of closely apposed nuclei (red arrow) (unstained). Scale bar 15µm

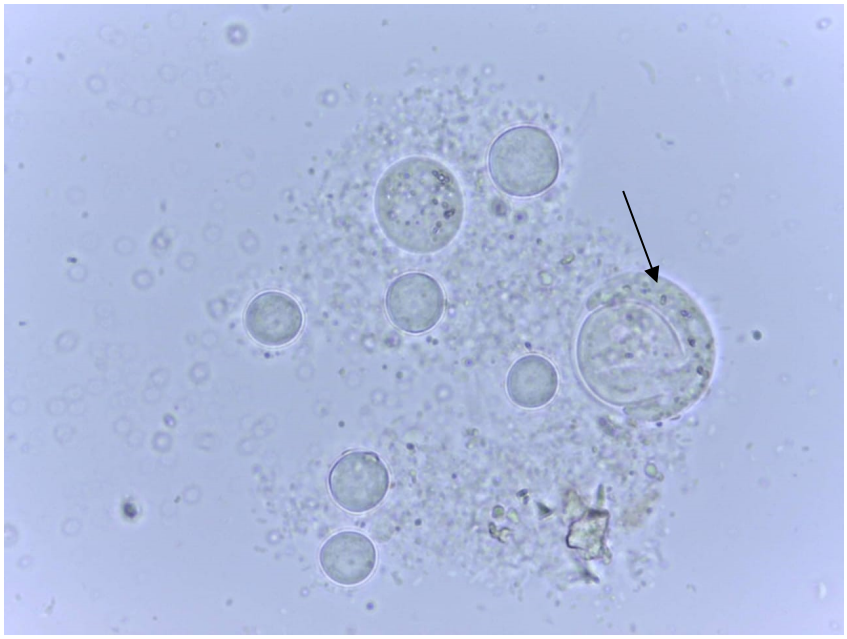

Figure 3. *Sappinia* spp. Showed trophozoite stage emerged from a cyst shell (arrow) (unstained).

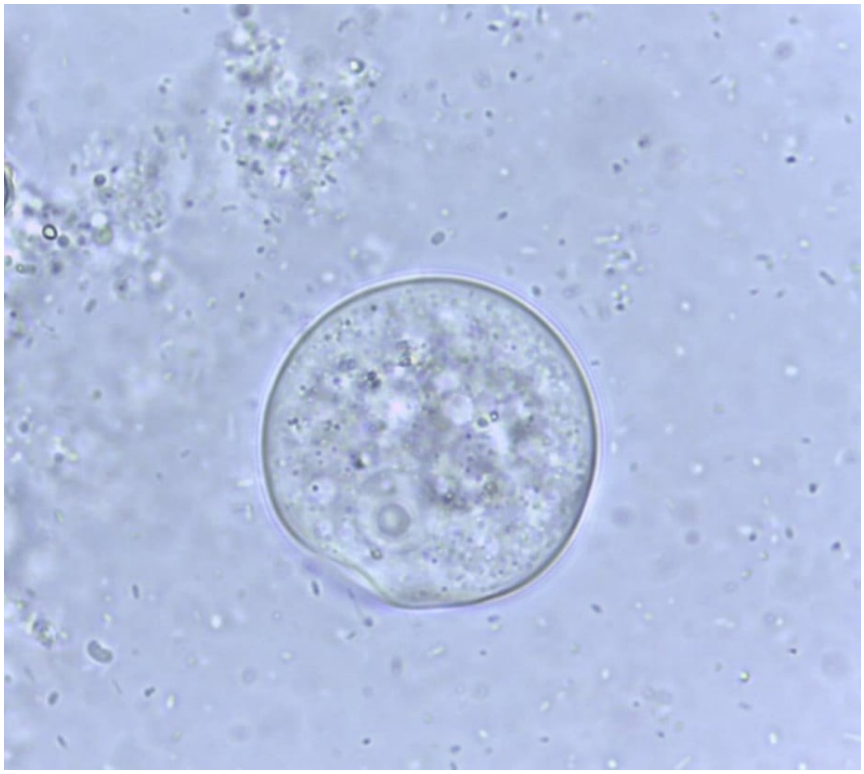

Figure 4. Resting rounded trophozoite of *Sappinia diploidea*, the pseudopodia surround amoeba no contractile vacuole

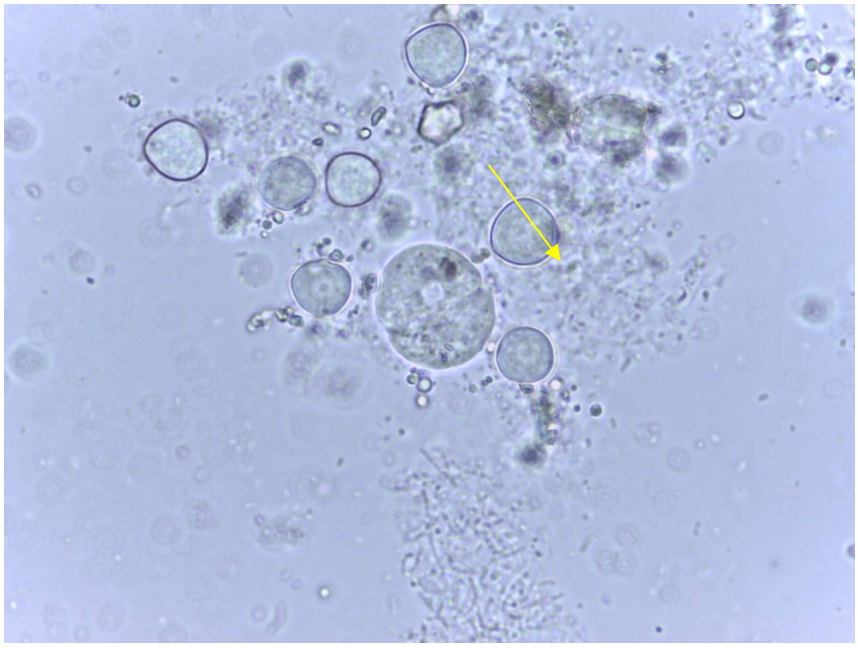

Figure 5. *Sappinia diploidea* show an early cyst stage with two amoebae that are still separate (arrow) (unstained).

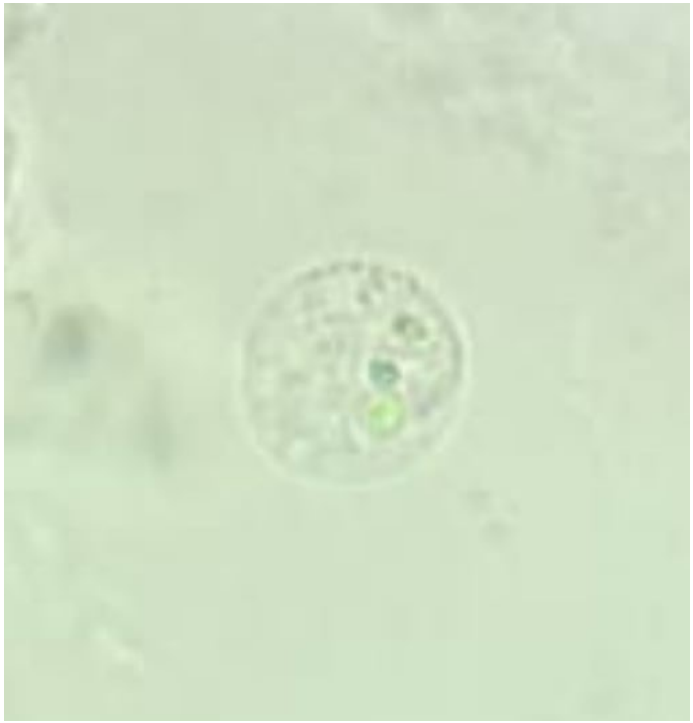

Figure 6. *Sappinia diploidea* mature cyst. (unstained). Scale bar 5.3µm

B- *Sappinia pedata*

The current study showed *S. pedata* trophozoite was irregular shape 30-35 µm by 25- 30 µm highly transparent cytoplasm with obvious clear ectoplasm forming the pseudopodium during motion and granular endoplasm that contains food and contractile vacuoles Figure (7). While the cyst was almost ovoid or rounded about 20-22 µm in diameter, the middle line was hardly observed in most isolates; single, double, triple and tetra nuclei cysts were observed even in the exact culture Figure (8)

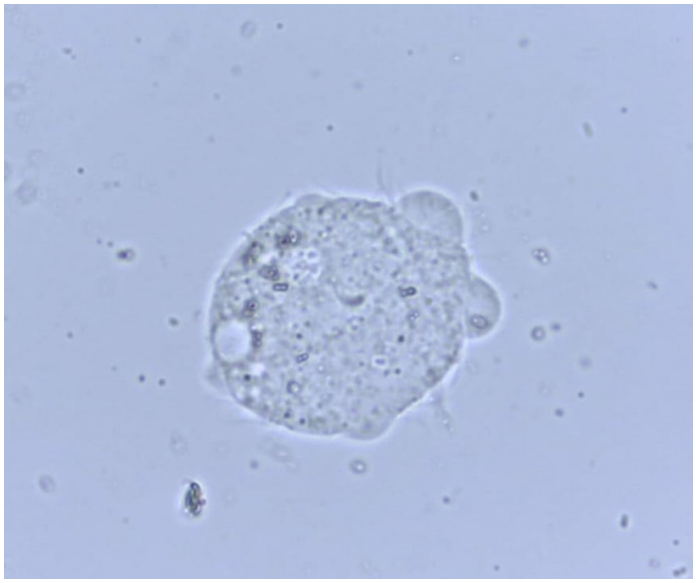

Figure 7. *Sappinia pedata* trophozoite shows the nucleus (red arrow) and contractile vacuole (blue arrow) (unstained). Scale bar 6.5µm

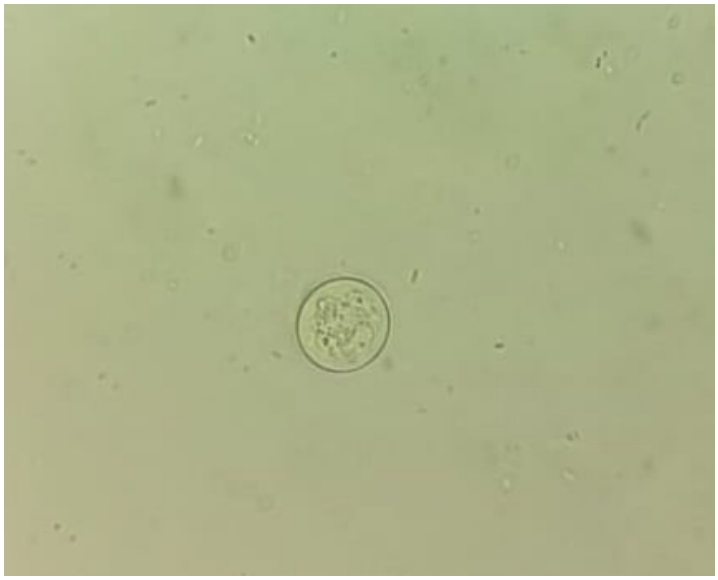

Figure 8. *Sappinia pedata* cyst (unstained). Scale bar 14.5µm

The sequencing and analysis of *Sappinia* culture PCR products from different environmental and clinical sources from Thi–Qar province, Blast results indicate that the sequence of *rDNA* of isolates No.1 and No. 2 showed 94.96% homology identity to *Sappinia pedata 18SrDNA* gene (accession number KR816830.1). Isolates No. 3 and 4 showed 94.31% homology identity to the *Sappinia diploidea 18S rRNA* gene (accession number KP277502.1). These isolates were recorded in the Gene Bank database for the first time in Iraq, with the accession number being NCBI, as shown in Table (2).

No.	Species identified	Accession number	Source of isolate
1	*Sappinia pedata*	LC629637.1	**Tank water**
2	*Sappinia pedata*	*----------	**Air conditioner water**
3	*Sappinia diploidea*	LC629638.1	**Eye**
4	*Sappinia diploidea*	LC629639.1	**Tank water**

*Not registered in NCBI

Table 2. Species, GenBank accession numbers and isolation sources of *Sappinia* sp.

DISCUSSION

The study "Morphological and molecular investigation of free-living amoeba Sappinia spp. isolated from environmental and clinical samples in Thi-Qar province-southern Iraq" provides valuable insights into the distribution and potential pathogenicity of Sappinia species in the region. [4,5]

The study's findings have several implications for the epidemiology and pathogenicity of *Sappinia* spp.:

The high prevalence of *Sappinia* spp. in both environmental and clinical samples (except CSF and ear samples) suggests a widespread distribution of these amoebae in Thi-Qar province. This finding is consistent with previous studies that have reported the presence of *Sappinia* in various environmental sources worldwide, including soil, water, and animal feces. [13]

Although only one case of amoebic encephalitis caused by *Sappinia pedata* has been reported so far, the isolation of *Sappinia* spp. from various clinical samples in this study raises concerns about its potential pathogenicity. The authors suggest that the wide distribution of *Sappinia* spp. in the environment and its ability to feed on other microorganisms could contribute to its opportunistic nature.[11]

The study highlights the importance of using morphological and molecular methods to identify Sappinia species accurately. The authors observed that not all morphologically positive samples were confirmed by PCR, indicating the limitations of relying solely on morphological characteristics for diagnosis.

The study emphasizes the need for further research to investigate the epidemiology, pathogenicity, and transmission routes of *Sappinia* spp. The authors suggest that future studies should focus on identifying the specific environmental factors that contribute to the growth and survival of *Sappinia* spp. and on investigating the potential role of these amoebae in causing human infections. [14]

In conclusion, this study's morphological and molecular findings provide valuable information on the distribution and potential pathogenicity of *Sappinia* spp. in Thi-Qar province. The study's findings highlight the importance of using a combination of morphological and molecular methods for accurate identification and the need for further research to understand the epidemiology and pathogenicity of these amoebae.

CONCLUSIONS

The analysis of PCR product sequencing of *Sappinia* from different environmental and clinical sources showed sequence homologies between 94.31% and 94.96% when compared to the *S. diploidea and S.pedata* strains sequences available in GenBank. *Sappinia* sequencing and its taxonomy is essential because it has recently been referred to as an opportunistic human pathogen.

The present study confirms the presence of Sappinia spp. in both environmental and clinical samples from Thi-Qar province, Iraq, highlighting their wide distribution in the region. This prevalence and the pathogenic potential of these amoebae emphasize the need for further epidemiological surveillance and the development of control measures to prevent possible human infections.

The results of this study underscore the importance of employing a combination of morphological and molecular methods for accurately identifying Sappinia spp. and other free-living amoebae. Accurate identification is crucial for understanding the epidemiology of these amoebae and assessing their potential risk to human health. However, this study also highlights the need for further research. Future studies should urgently investigate the environmental factors that influence the distribution and prevalence of Sappinia spp. and elucidate the molecular mechanisms underlying their pathogenicity.

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