Detection of some opportunistic free-living amoeba in some water samples in Thi-Qar province Southern of Iraq

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ABSTRACT

Free living amoeba (FLA) are widely distributed in various environmental sources and they have been found world-wide in aquatic habitats, soil and air.This study was performed in Thi –Qar province south of Iraq in order to detection of the opportunistic FLA in water samples and identified by morphological characters in the culture .During the period from November 2021 to February 2022 forty samples of water were collected ,All samples were cultured in the NN-agar medium and then examined by light microscope and we recognized morphologically the trophozoites and cysts of opportunistic amoebas that belong to the genera ; Naegleria fowleri. ,Acanthamoeba spp , Sappinia spp and Balamuthia mandrillaris , the study found 27(67.5%) samples were positive for total opportunistic FLA , and 13(32.5%) samples were negative , Where Sappinia spp. was the most common amoeba in water samples while Naegleria fowleri was less present.

Keywords: opportunistic free living, Southern of Iraq, water

Introduction: 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 . However research since the 1960s has demonstrated that FLA can be pathogenic to humans and animals (Scheid , 2018) . Strains of FLA have been isolated from lake water , river , moist soil

, desert soil and air , trophozoites and cysts of these protozoa also been detected in bottled mineral water and chlorinated water from swimming pools , amoeba have also been detected in gastric and intestinal lavages , in the nasopharynx of healthy individual in purulent discharges from the ear and liquid face (Lanocha et al . , 2009) . The presence of FLA in tap water may represent a healthy risk to both immunocompromised and / immunocompelent individuals and they are resistant to extreme condition of temperature , PH and exposure to various chmical (Kubra et al . , 2013) .

Among FLA that are widely distributed in nature only four genera / species are known as agents of human infections beneficial in their natural habitat, *Acanthamoeba* spp., *Naegleria fowleri, Balamuthia mandrillaris* and *Sappina pedata*. These amoebae are not well adapted to parasitism and could exist in the human environment without the need for a host. Infections due to these amoebae, despite low morbidity, are characterized by relatively high mortality rate and pose serious clinical problems (

Krol -Turminska and Olender, 2017). Hartmannella sp. (resp. Vermamoeba), Vahlkampfiae, Allovahlkampfia spelae and Dictyostelium these other free living amoebae with pathogenic capability polycephalum discovered to date (Scheid, 2018).

In view of the potential health consequences due to infection with these amoebae, rapid diagnosis is critical for early treatment. Microscopic examination and culture of biopsy specimens, cerebral spinal fluid (CSF), and corneal scrapings have been used in the clinical laboratory. For amoebic keratitis, confocal microscopy has been used to successfully identify amoeba in corneal tissue. More recently conventional and real time PCR assays have been developed that are sensitive and specific for the amoebae. In adition multiplex PCR assays are available for the rapid identification of these pathogens in biopsy tissue, CSF and, corneal specimens (Bruno et al., 2009).

1.1. Naegleria fowleri :

Naegleria is a genus that comprises single- celled, heterotrophic protists that are widely distributed in natural environments (Bellini et al., 2020). Naegleria belongs to the family Vahlkampfidae (Sawyer and Griffin, 1975;Ma et al.,1990). There have been over 40 species of *Naegleria* described to date, but only N. fowleri is pathogenic to humans (De Jonckheere, 2004).Two other species of Naegleria, N. australeinsis and N. italic can cause infection in mice but have never been identified from human infection (Martinez and Visvesvara, 1997). N.

fowleri a thermophilic flagellate amoeba known as a "brain – eating " amoeba is the a etiological agent of a as primary amoebic perilous and devastating waterborne disease known meningoencephalits (PAM). The first case of PAM was reported in Florida, USA, in 1962, the name Naegleria fowleri was given to it after the discovery of disease primary amoebic meningoencephalitis (PAM) in Australia in 1965 by Malcom Fowler of the Adelaide Childern's Hospital, Australia and

R. F. Carter, who first describe this disease.The name "primary amoebic meningoencephalitis " was given 11 to it in 1966 by Butt and then in 1968 by Carter to differentiate it from the rare brain invasion caused by *Entamoeba histolytica* (Jahangeer *et al*., 2019) . N. fowleri with stands higher temperatures, as found in geothermal sources and heated recreational aquatic systems, the species has been classified as thermophilic with the ability to

1.2. Acanthamoeba spp.;

Acanthamoeba belongs to family Acanthamoebaebidae (Reveiller *et al.,* 2003). It was first isolated in 1913 and named Acanthamoeba polyphagus (Marciano- Cabral and Cabral,2003).Acanthamoeba 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, soil and water(Bruno et al ., 2009). High number of *Acanthamoeba* spp. are found in surface layers of freshwater lakes and sediments, corresponding to high density of bacterial populations, members of genus Acanthamoeba colonize chemical showers, hot tubs, drinking water fountains, eyewash fountains, dialysis units, dental units, air conditioning systems, swimming pools, hot-water systems and humidifiers (Hsu *et al*.,2009; Al- Herrawy *et al.,* 2015). They are able to tolerate a wide range of environmental conditions, as some Acathamoeba strains are thermotolerant they are capable of infection humans (Scheid,2018). A.griffin, A. rhysodes, A. lugdunensis, A. culbertsoni, A. quina, A. hatchetti, A. polyphaga and

A. castellanii are the most common species infecting humans (Polat et al., 2007; Rivera and Adao, 2009). Acanthamoeba spp. are natural hosts of many bacteria (Legionella spp., Burkholderia cepacia, Vibrio cholera, Escherichia coli 0157 and Listeria monocytogenes) and viral pathogens (coxsackie viruse and adenoviruses)(Lorenzo - Morales et al., 2007 ; Pagnier et al., 2009) . To date, molecular classification of Acanthamoeba genus based on the18S ribosomal RNA sequence has described 21 genotypes (T1-T21) (Corsaro et al., 2017),among Acanthamoeba genotypes the most prevalent type is T4 that cause disease in human (Shokri et al ., 2016). Several species of Acanthamoeba can cause Granulomatous Amoebic Encephalitis (GAE),cutaneous acanthamoebiasis or Amoebic Keratitis (AK).AK is a sight -threatening infection of the cornea that occurs in immune competent individual, mainly contact lens user (Khan,2006).

1.3. *Balamuthia mandrillaris*

Balamuthia mandrillaris is aportist pathogen that can cause encephalitis with a fatality rate of > 95% (Siddiqui and Khan, 2015). *B. mandrillaris* is a free – living amoeba found in the environment causes rare cases human disease including cutaneous and central nervous system (CNS disease called granulomatous amebic encephalitis(GAE) (Jennifer et al., 2019). The disease it causes are similar to those of *Acanthamoeba* spp., including granulomatous amebic encephalitis and cutaneous lesions (Visvsevara et al., 2007) *B. mandrillaris* is the only species of Balamuthia known to cause infection in humans or animals, Balamuthia was first isolated from the brain of a mandrill baboon that died at the San Diego Zoo from meningoencephalitis. The amoebae was first described as a leptomyxid amoebae but later identified and named Balamuthia mandrillaris (Schuster and Visvsevara,2008). B. mandrillaris had ability to host bacteria, enhances their infective capacity for host cells or support transmission to a new susceptible hosts, thus amoeba may serve as a biological host as well as a transmission vector (Matin et al., 2008). B. mandrillaris isolation and to cultivation is difficult (Schuster,2002), but thought to be ubiquitous in the environment (MMWR, 2010). The first environment isolation of the amoeba was from soil by Lokhande *et al*.(2015), it was also recorded from two dogs who swam in pond water previously (Finnine *et al*., 2007).

fibrillar middle layer the mesocyst (Martinez *et al*., 2001). The cysts are mE resistant to physical and chemical conditions, the trophozoites emerge from cysts under favorable conditions and actively reproduce, thus completing the cycle (Siddiqui and Khan, 2009)Fig (1-3). *Balamuthia* is thought to enter the human body via the lung by inhalation of cy direct contamination of a break in the skin (Schuster and Visvesvara,

2004a). CDC

1.4. *Sappinia* spp:

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 (Page, 1988; Walochnik *et al*., 2010). The first and only case of amoebic encephalitis caused by Sappinia pedata (originally identified as diploidea) was described in 2001 by Gelman et al. (Gelman *et al*., 2001). Sappinia with the single species,

S. pedata was established by Pierre Augustin Dangeardin 1896 for a free-living,mostly binucleate amoeba with a dense glycocalyx. The name *Sappinia* was chosen as a tribute to Mister Sappin Trouffy, who had performed remarkable work in mycology. The second species of *Sappinia, S. diploidea*, was originally described as Amoeba diploidea by Max Hartmann and Kurt Nagler in 1908.They had isolated it from intestinal material of the colon of a lizard (Lacerta agilis) and soon found great interest in this peculiar amoeba (Walochnik et al.,2010) It includes at present three species, S. pedata, *S. diploidea* and *S. platani* (Corsaro *et al.,* 2017b). *Sappinia* can be found around the world (Visvesvara *et al.,* 2007a; Walochnik et al. 2010).Sappiniae are usually found in soil in natural environment (Scheid, 2008), they widely found of mammalian feces, soil, freshwater, forest liter , elk, bison, cattle and lizard rectum(Gelman et al., 2003 ; Visvesvara et al., 2007a), and usually found in elk and buffalo feces, places where farm animals are known to eat, soil containing rotting plants and freshwater sources (Gelman et al., 2001; Visvesvara et al., 2007a).

1-5 Method :

Sample Collection & Cultivation: Water samples:

were collected in 100 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 two replicates within 24 hours of collection and incubated in 26 C and 37C" and amoebic growth was examined daily by light microscope on slide or inverted microscope on agar and followed for 4 weeks

Non-nutrient agar medium (NNA):

Twelve grams of non-nutrient agar powder were added to 500 ml of distal water then autoclaved at 121C for 15 minute, the medium was left to cool (45C) after autoclaving then poured in Petri-dishes (8.5 cm diameter) and lett till become solid at room temperature. Three milliliter of sterile distal water was added on the agar surface as a liquid phase for cult

Non- nutrient agar medium covered with killed Escherichia coli (NNE medium):

Non-nutrient agar medium was prepared as described by Al-Malıky (2014), a suspension of autoclaved E coli was used as a liquid phase medium added on the agar surface An E coli isolate was obtained of science, activated overnight in 100 ml of nutrient broth at 37 C. The culture then autoclaved at 121 C" for 15 minutes, the killed bacteria broth was transported to sterile tubes, centrifuged at 10000 rpm/ min for 10 min. the broth was discarded and the bacteria was washed twice again with sterile distal water and finally was suspended in 10 ml of sterile distal water to

be used as a nutrient source for amoeba (Zeybek et al., 2010). It was used in maintenance of isolation of amoeba, this media was used in routine primary culture.

Page amoeba saline - agar medium :

Page saline was prepared according to page(1998):

Sodium chloride	0.12g/l
Mg .7	0.004g/l
	0.004g/l
	0.142g/l
	0.136g/l

Two grams of agar were dissolved in 100 ml of Page saline, autoclaved at 121C" for 15 minutes, then left to cool at room temperature to 45C before pouring in Petri- dish (8.5cm). This medium was used in routine primary culture and routine maintenance of isolates.

1-6 Occurrence of total opportunistic free living amoeba (FLA)

Out 40 samples of water from different source 27(67.5%) samples were positive for total opportunistic FLA . based morphological observation in NN-agar culture (were some samples recorded one type of FLA and other samples recorded more than one type of (FLA) and 13(32.5%)samples were negative .

The incidence of FLA in tap water samples was 60% and in tank water was 75%

.Table (1)

Table (1) occurrence of total opportunistic (FLA) in water samples by microscopic examination.

Type of sample	No. sample Examined	Positive samples		Negative samples	
		NO.	%	NO.	%
Tap water	20	12	60	8	40
Tank water	20	15	75	5	25
Total	40	27	67.5	13	32.5

1-7 Opportunistic amoebas observed in water samples

The results showed that *Sappinia* spp. was the most common amoeba in both tap and tank water were observed in 27(67.5%) of samples . *Acanthamoeba* spp. wre observed in 10 (25%) .

Balamuthia mandrillaris. was less common than *Sappinia* spp. and *Acanthamoeba* spp. was observed in 6 (15%) while *Naegleria fowleri.* was less present in water sample was observed in 5 (12.5%).Table (2)

Table (2) opportunistic amoeba observed in water samples by microscopic examination.

Type of sample	No. sample Examined	Acathamoebspp.		Sappina spp-ve+		Balamuthia		Naegleria	
		N	%	N	%	N	%	N	%
Tap water	20	3	15%	12	60%	2	10%	2	10
Tank water	20	7	35%	15	75	4	20	3	15

1-8 Discussion

Free living amoeba (FLA) are widely distributed in various environmental sources and they have been found world-wide in aquatic habitats, soil and air (Visvesvara et al., 2007). They are known to cause serious human infections including a fatal encephalitis, a blinding keratitis and pneumonia (Leonska- Duniec et al., 2015). Recently investigators in free living amoebae field have been dramatically increased because of their important role in ecosystem and their causing serious infections in human(Rezeaian et al.,2008)

The present study include investigation of opportunistic free amoebae, all the known opportunistic , *Naegleria fowleri Acanthamoeba* spp., *Sappinia* spp. and *Balamuthia mandrillaris* water samples especially with the few previous study in this field in Iraq.

The current study showed that the percentage of occurrence total FLA,was 67.5% based on morphological observation in NN_Agar culture.

Rezaeian et al. (2002) studied 354 samples of soil and water of rivers parishan Lake) in Kazeroon region by culture and microscopic method and reported 13 positive cases of FLA, Kubra et al. (2013) detected FLA in 33 (22%) out of 150 water samples in six Sivas districts / Turkey the results of this studies showed that percent of contamination with FLA were lower than those of the present study.

The current study agreement with another studies done by Eftekhar et al. (2009) studied 22 samples of surface water and water in squares of Tehran city in term of contamination with FLA through polymerase chain reaction method and found that 13 *)%59(* samples were contamination ,Armand *et al*.(2016) they showed of the 82 water samples from different sites of shiraz city 48 (58.53%) samples were positive for FLA based on morphological observation in NN-agar culture and Rezaeian et al.(2007) show nearly high prevalence (46.25%) in Tehran.

The high prevalence of FLA in this study and other studies in worldwide and the composition of these species at certain locations depends on the surrounding Also the spreading of FLA species depends on its tolerance to survive under adverse conditions (Kubra et al. 2013) (The predominance of FLA in various environmental samples are resistant to extreme conditions of temperature, pH and exposure to various chemicals (Thomas et al.,2008) Unlike true parasites, pathogenic free living amoebas can complete their life cycles in the environmental without entering a human or animal host (Martinez and Visvesvara, 1997). The high variability observed in the prevalence values could be attributed to several factors, including the different applied methodologies, as well the features of the water source and of the geographical areas.

Naegleria fowleri:

In current study *Naegleria fowleri* was less present FLA in water samples .The low occurrence of *Naegleria* spp. in Thi-Qar province could be attributed to the features of the water and soil source and their microbial content, and of the geographical areas.

Acanthamoeba spp:

In our study *Acanthamoeba* spp were detected in the examined water samples in Thi- Qar province diagnosed by morphological characteristics. *Acanthamoeba* spp. were isolated from water, the presence of *Acanthamoeba* in samples of water is confirmed by other authors who have observed that water chlorination kills only microorganisms but not FLA. (Khan, 2006)

Sappinia spp:

In current study *Sappinia* spp. were the most common amoebae and probably the most common protozoa detected in water samples in Thi-Qar province .The wide distribution of this genus in water samples in current study attributed to the feeding of *Sappinia* on other free living amoeba like *Naegleria* and yeast beside feeding on bacteria, in Lab. *Sappinia* was grown even on the simplest media, NN-agar and page amoeba saline, that reflected the ability to grow on minimum resources. The feeding on other amoebas beside yeast and bacteria provided a good mean in purification of *Sappinia* isolates from environment (Hanady,2017).

Balamuthia mandrillaris

The isolation of *B. mandrillaris* from water samples in this study may reveal pollution with organic residues that encouraged their growth and its feeds on other amoebas , the water samples were poured directly into NN-agar medium without filtration or concentration this reflect the high abundance of *B. mandrillaris* in water samples .

References

a. Al-Herrawy, Z.A. ;Heshmat, M.; Abu Kabsha, S.; Gad,M. A. and Lotfy,

b. **W.M. (2015).** Occurrence of *Acanthamoeba* species in the damanhour drinking water treatment plant ,Behera governorate (Egypt). Reports

c. Parasitol.,4: 15 21.

d. Bellini, N. K.;Moreira da Fonseca, A. L. ; Reyes-Batlle, M. ;Lorenzo- Morales

e. **,J.; Rocha, O. and Thiemann, O.H. (2020).** Isolation of *Naegleria* spp. From a Brazilian water source .Pathogens. ,9(2): 90.

f. **Bruno - da Rocha -Azevedo; Herberto T. and Francine, M. (2009)**. Diagnosis of infections caused by pathogenic free-living amoebae .Interdisciplinary perspectives on infectious disease.,2009:251-406.

g. **Corsaro, D.; Kohsler, M. ; Di Filippo, M. W. ;Venditti, D.; Monno, R.; Di Cave, D.; Berrilli, F. and Walochnik, J. (2017)a**. Update on Acanthamoeba jacobsi genotype T15,including full-length 18S r DNA molecular phylogeny Parasitol. Res., 116(4): 1273-1284.

h. Corsaro, D. ; Wylezich, C.; Walochnik, J.; Venditti, D. and Michel, R. (2017)

i. b. Molecular identification of bacterial endosymbionts of Sappinia strains. Parasitol. Res., 116: 549-558.

j. **De Jonckheere, J. F. (2004).** Molecular definition and the ubiquity of species in the genus Naegleria . Protist . 155 (1); 89-103.

k. Finnine, P. Visvsevara, G.; Campbell, B.; Fry,D. and Gasser, R.(2007)

l. .Multifocal *Balamuthia mandrillaris* infection in a dog in Australia. Parasitol. Res., 100(2):423-46

m. .

n. **Gelman, B.B. ; Rauf S.F. ;Nader, R. et al., (2001).** Amoebic encephalitis due to Sappinia diploidea . J. Am. Med. Assoc., 285(19): 2450-2451.

o. **Gelman, B.B.; Popov, V.; Chaljub, G.; Nader, R. ;Rauf, S.J. and Nauta, H.W.(2003).**Neuropathological and ulterastructural features of ainoebic encephalitis caused by *Sappinia diploidea* . J Neuropathol. Exp. Neurol 62(10):990-8.

p. **Hsu, B.M. ;Lin ,C.L. and Shih, F. C. (2009).** Survey of pathogenic free-living amoeba and Legionella spp. in mud spring recreation area. Water Res., 43(11) : 2817-28.

q. **Jahangeer, M.;Mahmood, Z. ; Munir, N.; Waraich, U. ;Tahir, I.M.; Akram,M. ;Shah, S. M. A.; Zulfqar, A. and Zainab, R. (2019).** *Naegleria fowleri* : Sources of infection, pathophysiology, diagnosis and management; a review.Clini. Experi. Pharma. And Physio. ,47(2): 199-212

r. .

s. **Jennifer, R.C.; Janet, L.; Hannah, N. ;Sarah, A. C. ; Carol, G.; Melanie, M.**

t. **;Raghuveer, P. ;Jonathan, S. Y. ; Ibne K.A. and Sharon, I. R. (2019).** The epidemiology and clinical features of *Balamuthia mandrillaris* disease in the United States, 1974-2016 . Clin. Infect. Disease., 68 (11): 1815-1822

u. **Khan, N.A. (2006).** *Acanthamoeba* biology and increasing importance in human health. FEMS Microbio. Rev.,30(4):564-595.

v. **Krol-Turminska, K. and Olender, A. (2017).** Human infections caused by free living amoebae. Annals of Agricu. And Environ, Medicine., 24 (2), 254- 260 *.)*

w. **Kubra, A.C.; Semra, Q.; Lutfi ,T.; Nazif, E. and Yusuf, T. (2013).** Isolation and identification of free-living amoebac from tap water in Sivas, Turkey, Bio. Med. Research. International., 2013. pp: 8.

x. **Lanocha, N.; Kosik- Bogacka, D.; Maciewska, A.; Sawczuk, M.; Wilk, A. and Kuzna -Grygiel, W. (2009).** The occurrence *Acanthamoeba* (free living amoeba

y.) in environmental and respiratory samples in Poland. Acta Protozool., 48(3): 271-279.

z. **Lokhande, S.; More, B.; Nikam, S.; Sontakke, T. ;Bandra, V. and Bansode V. (2015).** Morphological studies on two rare water amoeba *Balamuthia mandrillaris* and Vannella mirodes from Godavari basin at Gangapur and Vaijapur. Int. J. Recent. Sci. Res., 6(6): 4386-4388

aa. .

bb. **Lorenzo Morales, J.; Coronado - A Ivarez, N.; Martinz-Carretero, E.; Maciver, S. K. and Valladares, B. (2007).** Detection of four adenovirus serotypes within water - isolated strains of *Acanthamoeba* in the Canary Islands, Spain. Am. J. Trop. Med. Hyg., 77(4):753-6.

cc. **Marciano-Carbal ,F. and Carbal, G. A.(2003).** *Acanthamoeba* spp. as agents of disease in human .Clin. Microbiol. Revie.,16(2):127-307.

2. **Martinez, A. J. and Visvsevara, G. S. (1997)."**Free-living amphizoic and opportunistic amoebas "Brain Pathology., 7(1):583-598.
3. **Martinez, A. J.; Schuster, F. L. and Visvsevara, G. S. (2001).** *Balamuthia mandrillaris:* its pathogenic potential .J. Eukaryot Microbiol., Suppl: 6S - 9S

4. **Rivera, W. L. and Adao, D.E. (2009).** 18S ribosomal DNA genotypes of *Acanthamoeba* species isolated from contact lens cases in the Philippines. Parasitol Res., 105(4): 1119-24.

5. **Scheid, P. (2018).** Free-living amoebae as human parasite and hosts for pathogenic microorganisms. Proceedings.,2(11): 692.

6. **Schuster, F. L. (2002).** "Cultivation of pathogenic and opportunistic free- living amoeba Clinical Microbiology Reviews ., 15(3): 342-354.

7. **Schuster, F.L. and Visvesvara, G.S.(2004)a.** Free-living amoebae as opportunistic and non-opportunistic pathogens of humans and animals. Int. J. Parasitol 34(9):10011027.

8. **Schuster, F.L. and Visvesvara, G.S.(2008)."***Balamuthia mandrillaris* in Emerging protozoan Pathogens, N.A.Khan Ed., pp. 71- 118 .Taylor &Francis, New York, NY, USA.

9. **Shokri, A. ;Sarvi, S. ;Daryani, A. and Sharif, M. (2016).** Isolation and genotyping of *Acanthamoeba* spp. as neglected parasites in north of Iran. Kor. J. Parasitol., 54(4): 447- 53.

10. **Siddiqui, R. and Khan,N.A. (2009).** Central nervous system infection due to

11. *Balamuthia* spp. :clinical diagnosis and management. E.J. Biolo. Scie.
12. ,1(1):126-132*.*

13. **Siddiqui, R. and Khan, N.A.(2015).***Balamuthia mandrillaris* :Morpholgy, Biology and virulence. Trop. Parasitol., 5 (1):15-22*.*

14. **Visvesvara, G.S.; Moura, H. and Shuster, F.L. (2007).** Pathogenic and opportunistic free-living amoebae *Acanthamoeba* spp. ,*Balamuthia mandrillaris Naegleria fowleri and Sappina diploidea*. FEMS Immunoi Med. Microbiol .,50 (1): 1-26*.*
15. **Walochnik, J.; Wylezich, C. and Michel, R. (2010)**. The genus *Sappinia* : History , phylogeny and medical relevance. Exp. Parasitol.,126 (1):4-13.