Al-Qadisiyah J. Vet. Med. Sci. 2025; 24 (3): 10.29079/qjvms.2025.150627.1019
The 1st National and International Scientific Conference November 26 / 2025
ISSN P: 1818-5746
E: 2313-4429
qjvms@qu.edu.iq

Isolation and Molecular Study to Free - Living *Naegleria gruberi* from Different Source Clinical and Environmental in Karbala and Al-Qadisiyah Province, Iraq

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This manuscript is a part of MSc thesis (1st author) and the advisor(2nd author)
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Submitted: June 09, 2025
Revised: June 17, 2025
Accepted: June 27, 2025

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Abstract This study was acted in Karbala and Qadisiyah governarat center of Iraq in perioed from Septemper2021 to October 2022 request to location of the crafty Free living amoebae (FLA) and recognized by morphological characters in culture, Polymeras chain response (PCR) by 18SrRNA general FLA primers. 181 samples were gathered from 123 clinical cases including, eye, skin, nose from animals and human, and CSF from human and 58 samples were gathered from environmental samples were gathered from various sources of water , soil .All samples were refined in the Non Supplement agar medium (NN-agar medina) and then inspected by light microscope to perceived Morphologically the flagellate, trophozoite and cyst of opportunistic amoebas for 12/181 samples included 5/58(2.276%) environmental source (water and soil) and 7/123(3.867%) clinical(eye,skin,nose samples) from only animals were positive to Naegleria spp. Ten from12 tests from environmental and clinical sources were disconnected of common FLA that were positive microscopically, the outcome was positive for three samples for Naegleria gruberi after analyzed sequences. The study of isolation and molecular diagnosis of Naegleria gruberi is the first of its kind in Iraq, and It is the first recorded in Iraq in the Gene Bank database.

Keywords: Naegleria gruberi, Free-living amoebae (FLA), 18S rRNA

Introduction Protozoa called free-living amoeba (FLA) can be found all around the regular world, they bunch all together assorted gathering of facultative parasitic amoebae among free-living protozoa,coming up short on any brought together phylogenetic, systematic, or taxonomic origin (1). The FLA are normal in different conditions, including soil, dust, air, seawater, drinking water, pools, sewage, eyewash arrangements, contact focal points, dialysis units, and dental treatment offices (2). Defilements by free-living amoebae, principally of the genera *Naegleria, Acanthamoeba*, and *Balamuthia* are new illnesses in human and creatures, They can cause several different diseases ; *Naegleria fowleri* causes primary amoebic meningoencephalitis (PAM) in people and creatures (3, 4), *Acanthamoeba and Balamuthia* cause central nervous system (CNS) as well as scattered diseases, and *Acanthamoeba* in addition to CNS infections likewise causes keratitis (5, 6).

Naegleria gruberi is a free-living heterotrophic protist that is commonly found in freshwater and moist soil environments worldwide, in both high-impact and microaerobic conditions (7-9).

The broad, free-living soil and freshwater amoeboflagellate *Naegleria gruberi* The first from an individual from the Heterolobosea, an extraordinary and ecologically relevant basic heredity of amoebae, is addressed in the genome of *N. gruberi*.The unusual three-stage life cycle of many heterolobosean, such as Naegleria, consists of trophozoites, flagellates, and cysts. *Naegleria* is a single-celled organism that divides and grows in culture (8).

Amebae of *N. fowleri* and *N. gruberi* were cytopathic for nine laid out mammalian cell cultures, including mouse and human fibroblasts, rabbit and monkey kidney cells, rodent and mouse neuroblastoma cells, baby hamster kidney cells, and human epithelioma and carcinoma cells, nine strains of *N. fowleri* were similarly cytopathic for rat neuroblastoma cells, as not many as one ameba per million neuroblastoma cells obliterated the mammalian target cells after 9 days, the *N. fowleri* grew and obliterated rodent neuroblastoma cells at 30 to 37C^0 whereas *N. gruberi* grew and obliterated the target cells at 25 to 30 C^0,

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both *N. fowleri* and *N. gruberi* attached efficiently to the target cells at 30 to 37 C^0; *N. gruberi* but not *N. fowleri* attached efficiently at 25 C^0. Electron microscopic perceptions of blended cultures of *N. fowleri* and neuroblastoma cells laid out that the amebae, after 12 hr, had ingested parts of the neuroblastoma target cells without causing cell lysis.On the other hand, *N. gruberi* amebae, subsequent to appending to target cells, upset the plasma film and cytoplasm of the objective cells albeit the objective cell core stayed in one piece, The amebae then ingested the objective cell (10).

Material and methods

Ethical approval

The current study dose not need an Ethical approval because of the types of samples (Environmental samples).

Clinical Samples Collection & Cultivation

Tests(sampls) were gathered from various clinical cases including eyes, skin, and CSF for human of various ages and sexes from Imam Al-Hassan Emergency clinic skin advisor and eye specialist, Karbala Kids' Showing Emergency clinic Research center Division and Clinical facilities in Karbala territory and Tests were gathered from creatures (sheep , cows and calves) of various ages and sexual orientations, including skin, eyes and nose, from Al-Hussainiya, Al-Awaina, Abu Asid and Abu Tahin in the Karbala governorate and the Taj Al-Nahrain station for reproducing cows and calves in Al-Qadisiyah governorate And the creature house at the College of Al-Qadisiyah, School of Veterinary Medication during the period from Septemper to october 2022 (Table 1).

Eyes samples

Tests were gathered from the eyes of 27 patients between the ages of 8-75 years(10 female, 17 male) from the counseling center at Imam Al-Hassan Clinic and Clinical facilities and gathered 26 tests of creatures between the ages of 1-6 years, including sheep, cows and calves, and these samples were taken from the patient and creatures through a sterile cotton swab, after which the lower conjunctiva of the eye was cleared with a moving development off of the side trench towards the average belt under the management of an expert specialist (11) , eye samples were cultured on non-nutritive agar medium, incubated at 26C^0 and 37C^0 and followed up weekly with examination.

Cerebrospinal Fluid (CSF) tests

One ml of CSF gathered from 8 patients between the ages 2month - 10 years age(3female,5male) they were thought to be contaminated with meningitis which showed a negative bacterial culture in research facility of bacteriology in Karbala Kids' Showing Medical clinic expert specialist, the CSF was refined on non-supplement agar medium,incubated in 26 C^0 and 37 C^0 and followed up week after week by assessment of a wet mount slide for 4 weeks (12) .

Skin samples

Seven skin tests of human and 27skin examples of creatures including sheep, cows and calves were gathered in sterile swabs from patients and creatures with ongoing skin ulcer, after the ulcer was disinfected with iodine for human just, a smear was taken from the profound layer of the ulcer and take 2 skin tests of fish Surface by sterile swabs. then swabs were cultured for every sample on NN- agar medium, incubated in 26 C^0 and 37 C^0 and followed up weekly by examination of a wet mount slide for 4 weeks (12)

Nose samples

One sampl of human, 25 nose tests of creatures including sheep, cows and calves were gathered in sterile swabs from creatures experiencing tingling, runny nose, watery, enlarged eyes, red tone, and somewhat raised temperature, a smear was taken from inside and around the nose and from mucous discharges. then swabs were cultured for every sample on non-nutritious-agar medium and incubated in 26 C^0 and 37 C^0 and followed up weekly by examination of a wet mount slide for 4 weeks (12).

Environmental tests collection

Tests were gathered from different ecological sources in area of iraq (Karbala, Qadisiyah) , including, soil(different sort of soil and potato soil), water tests (rivers, tap water , tank water ,stagnant water and water from the air conditioner units, Water for the animals to drink, for Farmer's puncture, Turtle puncture, large ponds for breeding fish and Water for washing the owner's hands).These samples were gathered by date one year (Table 1).

Samples of water were collected in sanitized 60 ml beakers,and the date and area subtleties were recorder for each test. 3-5 ml of each sample was refined on non-nourishing agar medium (NN-agar) in vitro in one repeat in something lik 24 hours of assortment and brooded at 26C^0 and after two weeks incubated at 37C^0 after which amoebic growth was examined every day by on-slide light microscopy for a period of time 4 weeks year (12) .

Samples of Soil were gathered in sterile cups and the site and date information were fixed for each sample, within the next 24 hours of assortment two grams of each test were suspended in 5 ml of sterile refined water and supernatant was cultured on non-supplement agar (NN-agar) medium in one repeats and incubated in 26 C^0 and and after two week incubated at 37 C^0 with 3 ml of sterile distilled water

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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 (12) .

Preparation of media

Non-nutritious agar medium (NNA)

Twelve grams of non-supplement agar powder were added to 400 ml of distal water, then, at that point, autoclaved at 121C^0 for 15 moment, the medium was passed on to cool (45C^0) subsequent to autoclaving then poured in Petri-dishes (8.5 cm width) and left till become strong at room temperature. five ml of sterile distal water was added on the agar surface as a fluid stage for cultivation of amoeba, this media was used in routine primary culture (13) .

At the point when amoebic development was recognized on culture media, an infinitesimal slide was made utilizing a cotton swab mount the examples under sterile condition then, at that point, inspected under 10X and 40X to identify trophozoites, cyst and/or drifting phases of different amoebae, aspect of each stage were recorded utilizing the microscopic stage ruler, then analyzed by (13). The opportunistic amoebas that have a place with the genera; *Naegleria gruberi* where distinguished and it was recognized morphologically.

Table 1: Number of Environmental & clinical samples concentrate from different sources

Environmental samples		**Clinical samples**	
Type of samples	No. of collected samples	Type of samples	No. of collected samples
Soil	20	human eye	27
		human Skin	7
Potato soil	1	human nose	1
Water for washing the owner's hands	6	CSF	8
River water	2	animal eye (sheep, calves, cow)	8, 8, 10
Tank water	3	animal Skin (sheep, calves, cow,fish)	10, 7, 10, 2
Tap water	3	animal nose (sheep, calves, cow)	8, 6, 11
Stagnant water	4	123	
Filtered water	3		
Puncture water	1		
Airconditioner water	5		
Animal drinking water	10		
Total	58		
Total	181		

Molecular study of samples

Out of 10/12 tests were inspected with common FLA gene. was affirmed, after morpholgical portrayal , hereditarily by traditional PCR utilizing a bunch of general preliminary *18SrRNA gene* common FLA two primers designed by Tsvetkova *et al*., 2004. Forward primer 3'-5' "CGCGGTAATTCCAGCTCCAATAGC" and Reveres primer 5' -3' "CAGGTTAAGGTCTCGTTCGTTAAC".

Genomic DNA from cell culture of normal FLA quality. were extricated by utilizing AddPrep Genomic DNA Extraction pack, Addbio. Korea, and done by organization directions.

Sequencing

The PCR consequences of positive tests were transported off Macrogen Company (Korea) for sequencing. The compartment of every test was set apart with a number indistinct from the amount of Succeed sheet that sent by the association. The National Center for Biotechnology Information database (NCBI) was searched for homologous sequences using the Basic Local Alignment Search Tool (BLAST) after the sequences had been processed and examined. All the analyzed sequences were submitted to NCBI to obtain accession numbers which then analysed by using MEGA X software

Results

The samples *of Naegleria spp*. were diagnosed after morphology according to heat tolerance (26C^0-37C^0) , cysts and trophozites were noticed only total 12 of 181 samples recorder 5 (2.762%) environmental source (water and soil) and 7(3.867%) clinical from animals were positive to *Naegleria spp*. (Table 2).

Morphological characteristics

Morphological attributes of genus *Naegleria spp*. in this study showing three shapes:

Cyst stage: was adjusted , around 11.5 µm in measurement , it had a twofold smooth wall , the nucleolus is huge encircled by pale clear zone. Fig (1 –A).

Amoeboid trophozoite: amorphous, measuring between 13.5 to 22 µm in diameter, with a granular endoplasm and a transparent outer layer, a sizable, dark nucleus encircled by a transparent circle zone, and an easily noticeable vacuole (Figure 1-B).

Flagellate stage: had active movement in fluid buffer with the guide flagellum that emerged from the anterior part of the pear- shaped organism ,the

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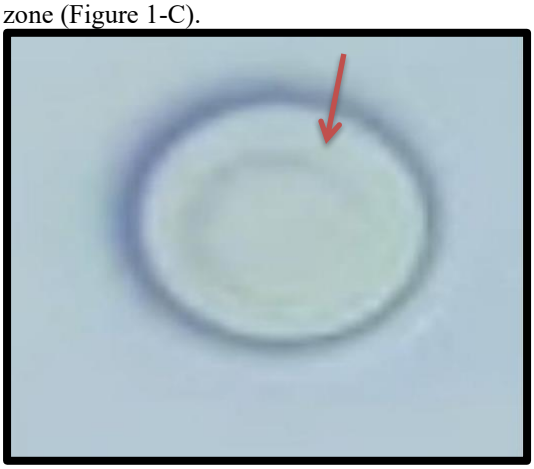
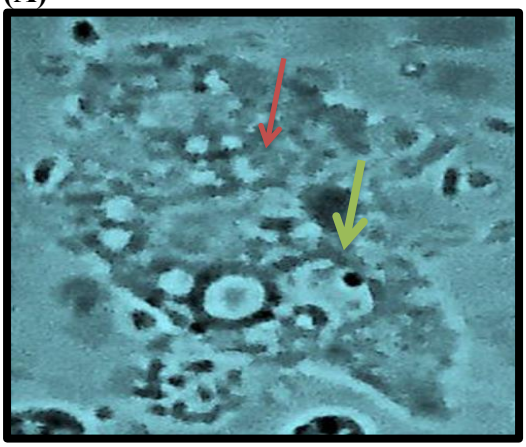
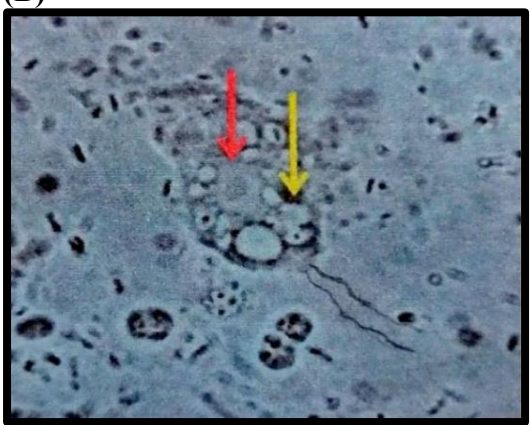
nucleolusexceptionally particular encircled by clear zone (Figure 1-C).



(A)



(B)



(C)

Figure 1: *Naegleria spp.*(A) cyst stage; (B) trophozite stage, (C) Flagellate stage was showd Flagellate (orange arrow). nucleolus (red arrow), vacuole (green arrow): (unstained).from eye cow.

Molecular study.

The current study includes examination 10 from 12 samples (2animal drinking water sample ,2 from soil sample ; 2 Clinical human & animals eye samples,2 Clinical human & animals skin samples, 2Clinical human & animals nose samples) by conventional PCR from microscopically positive for FLA using the general primer for FLA *18S rRNA* gene .The result was positive for three samples for *Naegleria spp.*after using the polymer chain reaction (Table 2 and Figure 2)

The sequences of isolates No. 1, No. 2, and No. 3 showed 98.5% homology identity to *Naegleria gruberi 18S rRNA* gene accession number (OQ678074.1/Iran and OQ678075.1/Iran and OQ678076.1/Iran), according to the sequencing and analysis of *Naegleria spp.* culture PCR products from various environmental and clinical sources central governorates of Iraq.In the Gene Bank database, it is the first instance ever noted in Iraq show in Table 3 and Figure 2.

Table 2: Occurrence of *Naegleria 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	----------	----------	----------	----------
River water	2	----------	-----	-----	------
Stagnant water	4	----------	----------	----------	----------
Tank water	3	----------	------	-----	------
Tap water	3	----------	------	------	------
Filtered water	3	----------	------	-----	------
Puncture water	1	----------	----------	----------	----------
Airconditioner water	5	----------	----------	----------	----------
Animal drinking water	10	2	1.1049	1	1.724
Soil	20	3	1.657	1	1.724
Potato soil	1	----------	----------	----------	----------
Totel	58	5	2.267	2	3.448
Clinical human & animals eye samples	38	3 only animals	1.657	1	1.01
Clinical human & animals skin samples	25	2 only animals	1.1049	----------	----------

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Clinical human & animals nose samples	22	2 only animals	1.1049	----------	----------
Totel	123	7	3.867	1	1.01
All Totel	181	12	6.629	3	1.657

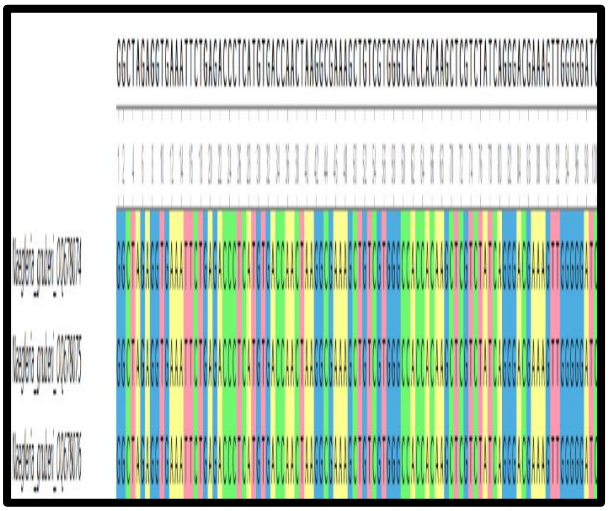

Figure 2: The arrangement investigation of *Naegleria gruberi* strain with reference strain (OQ678074.1, OQ678075.1and OQ678076.1).

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

Sample No.	Obtained accession No.	Identical to	GenBank accession No.	Country	Identity
1	OQ678074	Naegleria gruberi	MT613729	Iran	98.5
2	OQ678075	Naegleria gruberi	MT613729	Iran	98.5
3	OQ678076	Naegleria gruberi	MT613729	Iran	98.5

Discussion

The, current stuedy is the firset in the focal Iraqi territories of Karbala, Qadisiyah, which incorporated the study of free opportunistic amoebae, and known opportunistic species, *Naegleria gruberi* in ecological and clinical source samples. In this study, primary cultures of environmental and clinical samples showed remarkable growth with high diversity of amoebae, and opportunistic amoebae were identified among those free living in the Iraqi environment and clinical, especially with the few previous studies in this field in Iraq.

Our laboratory analysis indicates the importance of the unique morphological features of *Naegleria gruberi*, including complex protrusions, bipolar dumbbell formations, and random movement. As for animal clinical samples, the presence of *Naegleria gruberi* at all stages was evident in our study, as it was detected in the eye of a three-year-old cow for the first time in Karbala, Iraq.

N. gruberi is considered to be the best safe system to study the pathogenic "brain-eating amoeba" *N. fowleri*, which can infect people and cause primary amoebic meningoencephalitis (PAM), a rare but almost always fatal disease (14).

Opportunistic amoebas have been identified among free-living organisms in the Iraqi environment, especially with the lack of previous studies in this field in Iraq. The current study is consistent with the study of Al-Maliky (2014) in Basra, southern Iraq, where *Naegleria spp*. were isolated and *Naegleria fowleri*, which resembles *N*. gruberi in characteristics, was identified. For Moker, the morphology of the turtle pond was consistent (12) where she studied *N. fowleria*, from various sources in Basra Governorate and finally in Dhi Qar Governorate, torophozoites and cysts of opportunistic amoebas belonging to the genus *N. fowleri* were isolated and studied from clinical and environmental sources by AL-Aboody (2021).

The results dosn't agree with the studies by El-Harawi *et al*. (2017) that investigated a *Naegleria*-like organism in 6.7% of the water samples examined from two swimming pools at the University of Alexandria, Egypt, and Ghaddar-Ghadir *et al*. (2012) investigated *Naegleria spp*. In14 cases out of 120 samples of water resources in Shiriaz. While Kubra *et al.,* (2013), FLA in 33, out of 150, water sampeles in six provinces of Sivwas, and there was no representative of any *Naegleria spp*. were present in any of the sameples. But the higher presence of *Naegleria spp*. In this stuedy, this may be attribueted to, feeding *Naegleria spp.* on other genera of FLA or due to its high viciousness (21, 22).

Conflict of interest

There is no conflict of interest in this study as stated by the authors.

Acknowledgment

Not applicable.

Funding source

This research had no specific fund; however, it was self-funded by the authors.

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