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Molecular and Morphological Identification of Balamuthia mandrillaris (Free- living Amoeba) Isolated From Environmental Sources in Thi- Qar province / Iraq

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Abstract
Balamuthia mandrillaris is a free- living amoeba that lives in soil and water near human settlements . It was supposed to occupy the same ecological habitats of Acantamoeba . B. mandrillaris has been reported as the causative agent of fatal Balamuthia Amoebic Encephalitis (BAE)essentially, there is a little information about this genus. So that, this study attempted to determent characterization of B. mandrillaris at molecular level and morphological features. Seventy five of environmental samples were collected from different sources in Thi-Qar province south Iraq during the period February to September 2020. Samples were screened for B. mandrillaris and identified by morphologically and genetically by PCR method. Results stated that B. mandrillaris cyst and trophozoite were observed in some environmental sample were 12 (16%). There was only 9 (12%)were positive out of 12 suspected environmental isolates that confirmed by the presence of distinct amoeba cyst or both trophozoites and cyst by using PCR method including Balamuthia mandrillaris specific primer Balspec 16S forward and reverse that amplified a portion of mitochondrial rRNA gene yield a 1075 bp product . Trophozoite and cyst were observed in culture ,the trophozoite stage approximately 35-50 micron in diameter. it has finger like pseudopodia the trophozoite showed an ability to produce pseudopodia from any part of the amoebic body , the cysts of B. mandrillaris were spherical and approximately 12-25 micron surrounded by a double layer membrane , an inner wall that was endocyst and an outer wrinkled wall that was ectocyst. the ectocyst had no pores like Acanthamoeba and endocyst without arms . The presence of B. mandrillaris can be considered a health hazard , as B. mandrillaris is an opportunistic amoeba and also may harbor some pathogenic bacteria .

Keywords : Balamuthia mandrillaris , free-living amoeba , Thi-Qar province , Iraq , opportunistic amoeba

INTRODUCTION

Balamuthia mandrillaris is a protista 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 of 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).

MATERIAL AND METHODS :

Sample collection &cultivation:

Samples were collected from different environmental source , including ,soil , water from (rivers , tapwater , the marshes ,ponds, drops of water from the air conditioning equipment outside the building ,) , and animals wastes, these samples collected from different region in Thi- Qar province during the period from February to September 2020 .

A- Water samples : 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 incubated in 26 C^0 and amoebic growth was examined daily by light microscope on slide or inverted microscope on agar and followed for 4 weeks . **B- Soil samples** : soil samples and animal wastes were collected in sterile containers the date and site details were fixed for each sample , within the next 24 hours of collection from each sample 3 grams were suspended in 5 ml of sterile distilled water and supernatant was cultured on non-nutrient agar (NN-agar) medium in two replicates within 24 hours of collection incubated in 26 C^0 with 3 ml of sterile distilled water 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. The identity of *Balamuthia mandrillaris* was confirmed ,after morpholgical characterization , genetically by conventional PCR using a set of *B. mandrillaris* specific two primers designed by Booton *et al*. (2003 a) .: 5Balspec 16S (5- CGCATGTATGAAGAAGACCA-3)and 3Balspec 16S (5-TTACCTATATAATTGTCGATACCA-3) . (manufactured by Alpha DNA) .

Genomic DNA from cell culture of *Balamuthia mandrillaris* were extracted by using gSYNC ™ DNA Extraction kit , Geneaid . Korea , and done according to company instructions . The PCR product yield was a 1075 bp from mitochondrial small- subunit-rRNA genes in *B. mandrillaris* according to the following protocol :

Initial denaturation 10 min at 95C^0 , 35 cycle of 35 sec at 95C^0 , 50 sec at 49 C^0 and 40 sec at 72 C^0 followed by 10 min final extension at 72 C^0 ,PCR product was electrophoresed on 1.5% agarose gel and visualized by UV.

RESULTS

B. mandrillaris cyst and trophozoite were observed in 12(16%) out of 75 samples from different environmental sources , the highest occurrence was in tanky water was 2 (50%) and no occurrence was in both tap water ,marshes water , potato soil and wild mice waste . Among the 12 suspected environmental isolates that confirmed by the presence of distinct amoeba cyst or both trophozoites and cyst , only 9 (12%)were positive after the PCR method using *Balamuthia mandrillaris* specific primer Balspec 16S forward and reverse that amplified a portion of mitochondrial rRNA gene yield a 1075 bp product . Table (1) Fig (1) .

Table (1) : Occurrence of *Balamuthia mandrillaris* in environmental samples obtained by microscopic and molecular examination .

Type of Sample	No. sample EX. By microscope	Microscopic Positive samples		No. samples Ex. By PCR	PCR positive samples	
		No.	%		No.	%
River water	8	2	25	2	1	12.5
Tap water	5	0	0	0	0	0
Tanky water	4	2	50	2	2	50
Stagnant water	4	1	25	1	1	25
Marshes water	5	0	0	0	0	0
Air conditioner	4	1	25	1	1	25
Soil	23	4	17.39	4	2	8.69
Potato soil	4	0	0	0	0	0
Lizard waste	7	1	14.28	1	1	14.2
Birds waste	5	1	20	1	1	20
Mice waste	6	0	0	0	0	0
Total	**75**	**12**	**16**	**12**	**9**	**12**

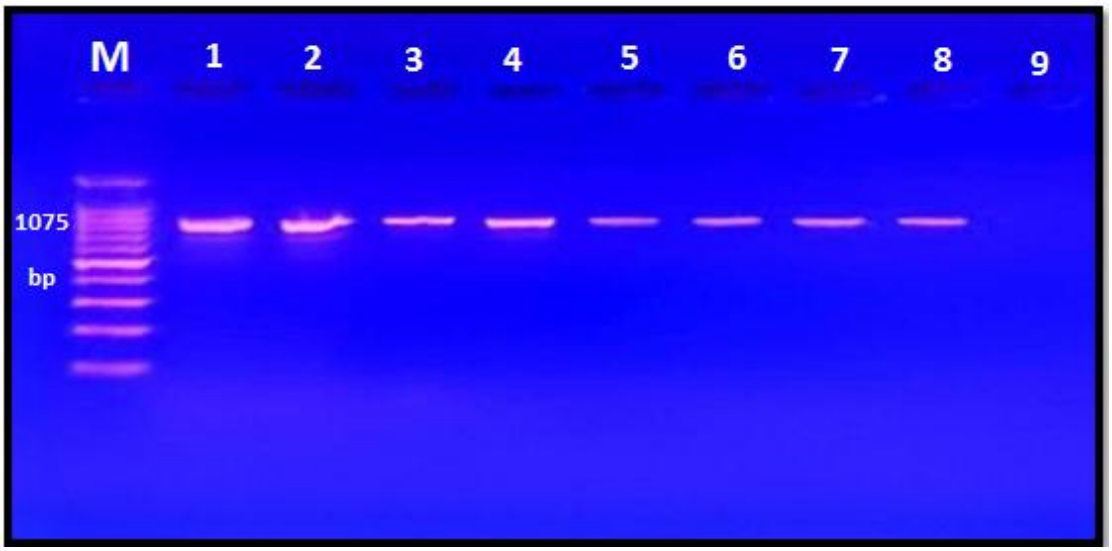

Fig. (1) : Agarose gel electrophoresis image that show the PCR product analysis of 16S ribosomal RNA gene from genomic DNA of *Balamuthia mandrillaris* . from environmental samples : Where M: Marker (2000- 100 bp) lance (1-8) positive samples and lance (9) negative samples at 1075 bp .

Our current study showed *B. mandrillaris* has two stage a vegetative trophozoite stage and dormant cyst stage . The trophozoite stage approximately 35-50 micron in diameter that contain a single nucleus although binucleated form have been observed . It has finger like pseudopodia , transparent ectoplasm and granular endoplasm that contain food and contractile vacuoles . *Balamuthia* trophozoite showed an ability to produce pseudopodia from any part of the amoebic body , trophozoite variable in shape depending on cultivation condition and age of culture Fig. (2)

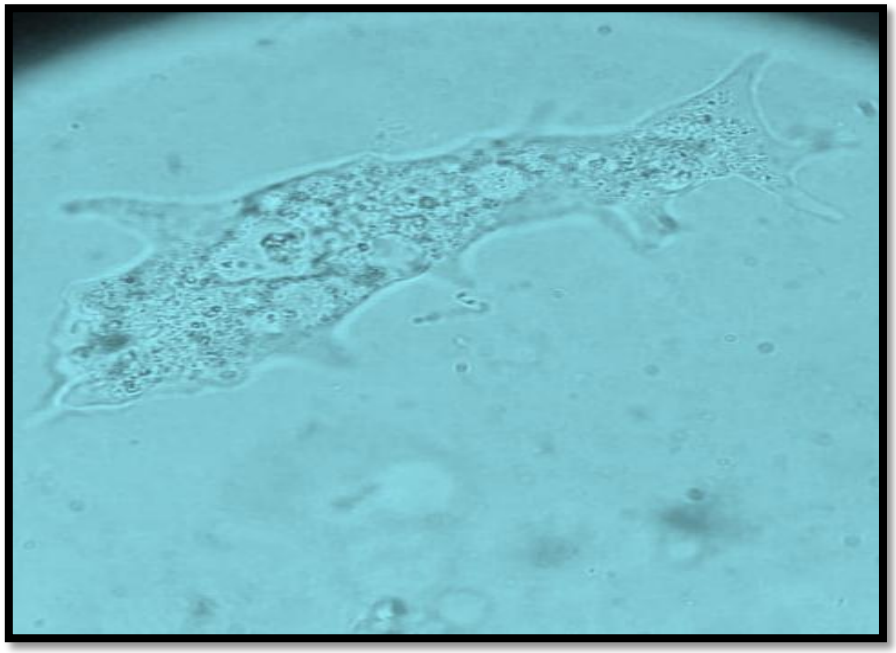

Fig (2) *B. mandrillaris* trophozoite (unstained) 100X

The cysts of *B. mandrillaris* are spherical uninucleus and approximately 12-25 micron surrounded by a double layer membrane , an inner wall that is endocyst and an outer

wrinkled wall that is ectocyst . Both layer are separated with a middle layer composed of amorphous material . Fig. (3) The ectocyst had no pores like *Acanthamoeba* and endocyst without arms ,rounded do not reach the outer edge of outer cyst like *Acanthamoeba* . Those features are helpful in morphological recognition between Balamuthia and some species of *Acanthamoeba* **.**

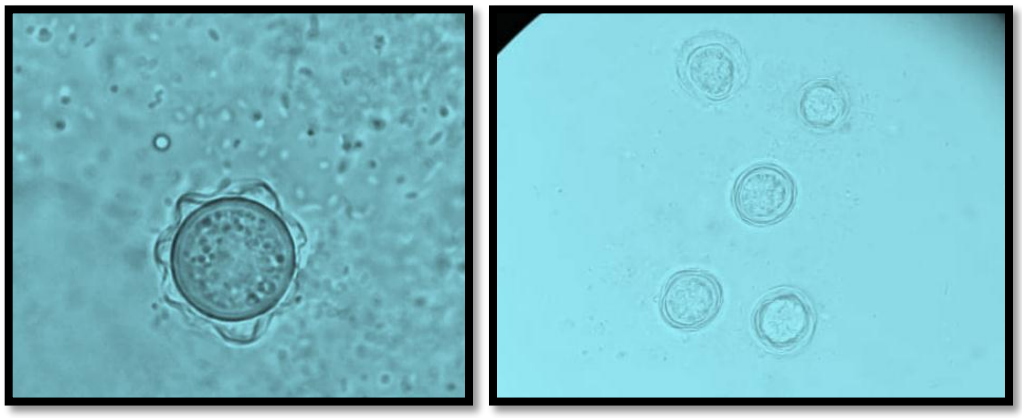

Fig (3) *B. mandrillaris* cyst (unstained)100X

DISSCUSION

B. mandrillaris is a free- living amoeba that lives in soil and water near human settlements (Kanako *et al.*, 2018) . It was supposed to occupy the same ecological habitats of *Acantamoeba* and found naturally in soil (Booton *et al*., 2003b) . *B. mandrillaris* has been reported as the causative agent of fatal Balamuthia amoebic encephalitis (BAE) (Niyyati *et al*., 2015) . Nevertheless environmental isolation of *B. mandrillaris* is rare event and strains of this amoebic species have been isolated from soil and dust sources only in several previous report. The current study is first in Thi- Qar province , that screening environmental detection and isolation of *B. mandrillaris* .In the current study *B. mandrillaris* was observed and diagnosed in 12 (16%) of 75 environmental samples by cultivation and microscopic examination , *B. mandrillaris* was isolated from soil , water and animals waste . PCR using species specific primer was done to confirm the identity ,only 9(12%) samples were positive . There are a number studies that confirmed the presence of *B. mandrillaris* in environmental samples , two cases isolated from the soil in flowerpots in the USA (Schuster *et al*., 2003 ; Yagi *et al*., 2005) , two from Iran (0ne from soil and one from city dust) (Niyyati *et al*., 2009; 2016) ,one from well water in Guinea – Bissau (Baquero *et al*., 2014) ,one from Peruvian soil (Cabello- Vilchez *et al*., 2014) , one from water in Mexico (Lares – Jimenez *et al*., 2014) ,one from a mud bath in Jamaica (Todd *et al*., 2015) , one from soil in the northern region of Japan (Kanako *et al*., 2018) and one from Shatt AL-Arab the main river in Basrah south of Iraq (Moker & Muslim 2018).

The presence of *B. mandrillaris* in soil and animals waste may refer to a need for organic resources this is agree with first isolation of *B. mandrillaris* from plants pots (Schuster *et al*., 2003) , where organic fertilizer usually used . This presence could be in associated with the abundant of bacteria and other organismsin such environment that *Balamuthia* could feed on.

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 .

Morphological identification was confirm by PCR method, study PCR is more sensitive technique than direct microscopy of culture , but the use of both PCR and culture method is suggested for environmental samples to gain more complete results of the real presence of *Balamuthia mandrillaris* .

B. mandrillaris may serve as a biological host as well as a transmission vector for some pathogenic bacteria (Matin *et al*. , 2008) , the presence of *B. mandrillaris* can be considered a health hazard , as *B. mandrillaris* is an opportunistic amoeba and also may harbor some pathogenic bacteria .

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