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Isolation of *Balamuthia mandrillaris* (Free Living Amoeba) from Shatt Al-Arab River in Basrah, South of Iraq

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Abstract: *Balamuthia mandrillaris* is a free living, opportunistic amoeba was first discovered in 1986 in a mandrill baboon at the Wild Animal Park in California suffered from a neurological disease, later it was associated with many human CNS fatal infection and skin infection all over the world, and it considered to be ubiquitous. We investigate the presence of *B. mandrillaris* in Shatt Al-Arab, the main river in Basrah south of Iraq, the amoeba was identified morphologically and genetically by PCR. Trophozoite and cyst were observed in culture, the trophozoite with finger like pseudopodia that subdivided into small arms. Rounded cyst of about 13-30 µm surrounded by outer thin wrinkled layer gave the shape of a rose flower. Our finding was the first in Iraq, *Balamuthia mandrillaris* represent a health hazard in such main river in Basrah.

Keywords: Balamuthia mandrillaris, Opportunistic Amoeba, Shatt Al-Arab River, Basrah, Iraq

Introduction

Balamuthia mandrillaris is an opportunistic free-living amoebae that causes serious cutaneous infections and fatal encephalitis in human (Siddiqui & Khan, 2015) and may invade the skin causing extensive skin lesions (Martinez and Visvesvara, 1997). *B. mandrillaris* infection has been identified in other mammalian species, such as sheep, horses and dogs (Schuster *et al.*, 2009).

B. mandrillaris was first isolated in 1986, from the brain tissue of a mandrill baboon (*Papio sphinx*) at the San Diego Zoo Wild Animal Park in California that died after a neurological disease (Visvesvara *et al.*, 1990). Later it was associated with fatal human infections involving the CNS (Anzil *et al.*, 1991). The organism was suggested to enter the body through wounds in the skin that contaminated by soil or by inhalation of cysts carried by wind from soil to the lower respiratory tract (Martinez & Visvesvara, 1997).

The trophozoite is pleomorphic, uninucleated and binucleated forms are occasionally seen (Lokhande *et al.*, 2015). It characterized by the irregular branching pattern (Visvesvara *et al.*, 1993). *Balamuthia* trophozoite is similar to *Acanthamoeba* but larger with a specific morphology (Booton *et al.*, 2003b). *Balamuthia* cyst possessing three walls that seems to be proteinaceous containing mostly cysteine-rich proteins, no polysaccharides or carbohydrate moieties were detected in the cyst wall; mesocyst seems to contain cellular debris such as lipid granules (Klieščiková, 2013).

B. mandrillaris isolation and cultivation is difficult (Schuster, 2002), but thought to be ubiquitous in the environment (MMWR, 2010). *Balamuthia* and *Acanthamoeba* supposed to occupy the same ecological habitats and found naturally in soil (Booton *et al.*, 2003b).The first environmental isolation of this amoeba was from soil by Schuster *et al.* (2003), and recently was isolated from aquatic environment by Lokhande *et al.*, (2015), it was also recorded from infection of two dogs who swam in pond water previously (Finnine *et al.*, 2007).

Material and Methods:

Water samples were collected in 100 ml sterile cups from Shatt Al-Arab River. The date and site details were fixed for each sample. Within the next 24 hours of collection, 2-3 ml of each water sample was cultured on non-nutrient agar medium (2%) in petri-dishes; three replicates were done; incubated at 25C° for three days at least before examination under light microscope.

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Purification was done by using a corck-borer like device, designed by Dr. Muslim A. Rahman, that allow to cut a piece of wet agar medium, of about 0.5 mm diameter under light microscope, Fig.(1), to ensure the presence of targeting amoeba and minimizes the chance for getting other amoeba spp. Then the piece was transported to a fresh wet NN-agar medium usually, incubated at 25 Cº for 3 days at least before growth can be observed. This technique was repeated till obtained a pure isolates.

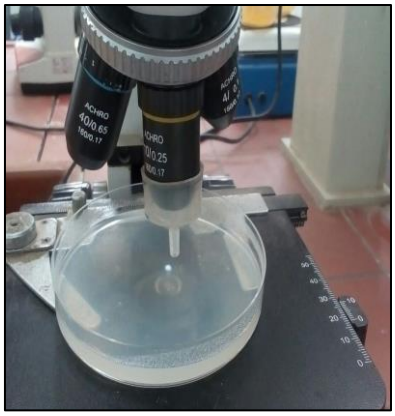

Fig. (1):The crock borer-like devise used in purification.

The identity of amoeba was confirmed , after morphological characterization, genetically by conventional PCR using a set of *B. mandrillaris* specific two primers designed by Booton *et al.*, (2003a): 5Balspec16S (5-CGCATGTATGAAGAAGACCA-3) and 3Balspec16S (5-TTACCTATATAATTGTCGATACCA-3) (manufactured by Alpha DNA). DNA was extracted by using chelex resin according to Iovieno *et al.* (2011), lysis buffer preparation according to Mirhendi *et al.* (2006). The PCR product yield was a 1,075 bp from mitochondrial small-subunit-rRNA genes in *B. mandrillaris* according to the following protocol:

40 cycles of 1 min at 94°C, 2 min at 48°C, and 3 min at 72°C followed by a 15min final extension at 72°C . PCR product was electrophoresed on 1.5% agarose gel and visualized by UV.

Results

The trophozoite of *B. mandrillaris* measuring 30-65 µm, it has a finger-like projections, transparent cytoplasm and a single large nucleus, it moved forward very fast on agar surface by the finger like pseudopodia, the most important identification feature of the trophozoites is the subdivided pseudopodia into small arms, *B. mandrillaris* trophozoite showed an ability to produce pseudopodia from any part of the amoebic body, Fig. (2, 3 and 4). It is difficult to diagnose *B. mandrillaris* trophozoites on slide for the first time, because of narrow surrounded space under cover slips, they took the ordinary ovoid appearance of amoeba then the amoebic body elongated and pseudopodium extended and short finger projections appeared, the pseudopodia continued in elongation and sub-branching while the body mass decreased.

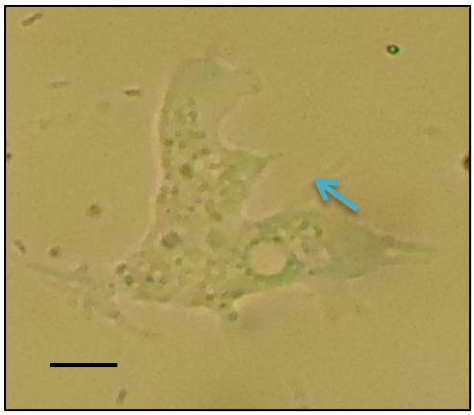

Figure (2): *Balamuthia mandrillaris* trophozoites (arrow: finger like projections). Scale bar 9.69µm.

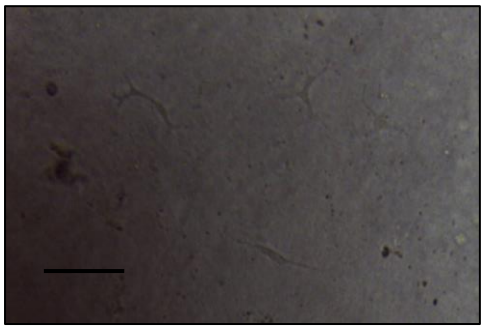

Figure (3): *Balamuthia mandrillaris* trophozoites on agar. Scale bar 50.6µm.

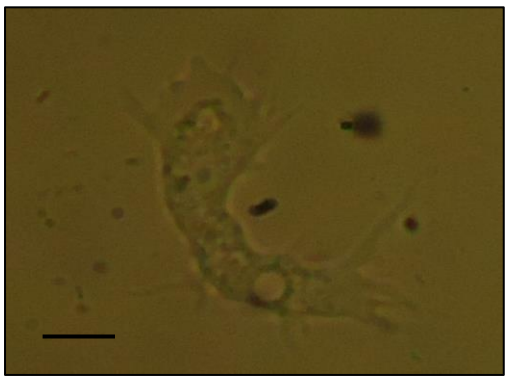

Fig. (4):*Balamuthia* trophozoite with many pseudopodia. Scale bar 13µm.

Cyst of *B. mandrillaris*, Fig. (5), was more easy to observed in cultures and on slide, it was rounded of about 13- 30 µm surrounded by inner thick regular rounded membrane and outer thin wrinkled one gave the shape of a rose flower, only the endocyst and ectocyst could be recognized under light

microscope. The endocyst is rounded without arms and had no pores like *Acanthamoeba*. Some cysts had rounded smooth outer membrane that may cause a confusing during morphological identification, Fig. (6). The cysts were usually dark brown, which gave the surface of culture media a light brown color like dust.

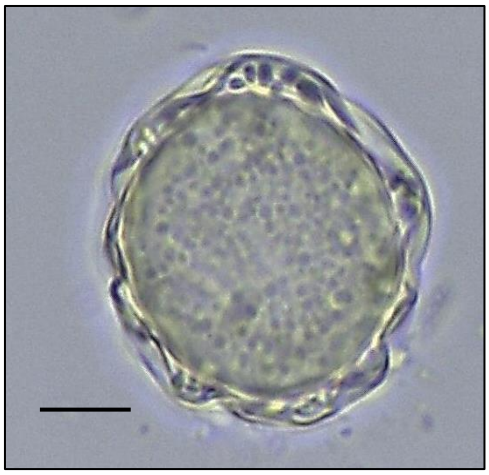

Fig (5): *Balamuthia mandrillaris* cyst. Scale bar 80.6µm

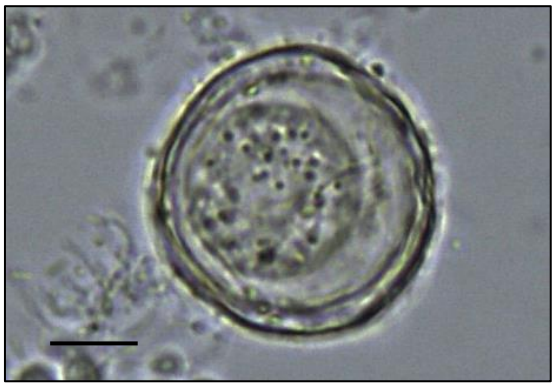

Figure (6): *Balamuthia mandrillaris* cyst with a rounded smooth outer membrane. Scale bar 6.6µm.

Conventional polymerase chain reaction (PCR) using the species specific primer Balspec16S forward and reverse that amplified a portion of mitochondrial rRNA gene yield a 1057 bp product was done to confirm morphological diagnosis, Fig.(7).

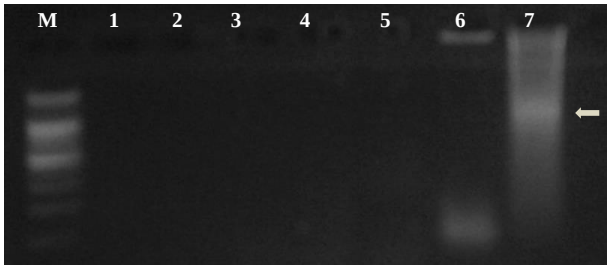

Figure (7): Conventional PCR detecting *Balamuthia mandrillaris*: M: 100 bp ladder, lane 7: positive sample of Shatt Al- Arab isolate, (1075 bp) (arrow), lane 2-6: negative samples.

Discussion

Balamuthia mandrillaris is thought to be ubiquitous in the environment (MMWR, 2010). It was supposed to occupy the same ecological habitats of *Acanthamoeba* and found naturally in soil (Booton *et al.*, 2003b). *Acanthamoeba spp.* were also recovered from many different ecologies all over the world, it found in any moist environment rich in bacterial source including: brackish, fresh water and tap water (Visvesvara *et al.*, 2007 a and b). Yousuf *et al.*, (2013) isolated *Acanthamoeba* and *Naegleria fowleri* from domestic water and recently *B. mandrillaris* was also isolated from aquatic environment by Lokhande *et al.*, (2015).

The present study is the first in Iraq that detect and isolate this opportunistic free living amoeba from the environment, the primary isolation and purification was depended on cyst mainly, in spite of the large trophozoites size of *B. mandrillaris*, it was hard to observe and identify on agar or slide, the trophozoite is transparent and had a polymorphosim but the irregular branching and subdivided pseudopodia with distinct cyst morphology are considered helpful features in morphological recognition between *B. mandrillaris* and some species of *Acanthamoeba.*

First isolation of *B. mandrillaris* from environment was from plants pots (Schuster *et al.*, 2003), where organic fertilizer usually used, this may refer to a need for organic resources or could be in associated with the abundant of bacteria and other organisms in such environment that *Balamuthia* could feed on. The isolation of *B. mandrillaris* from river water in this study may reveal pollution with organic residues that encouraged their growth, the water sample was poured directly into NN agar medium without filtration or concentration this reflect the high abundance of *B. mandrillaris* in river water. Morphological identification was confirm by PCR used genus specific primers designed by Booton *et al.* (2003a), that do not amplify DNA from other free living amoebas even the closest *Acanthamoeba spp*.

B. mandrillaris may serve as a biological host as well as a transmission vector for some pathogenic bacteria (A. Matin *et al.*, 2008). As Shatt Al-Arab river is the main source for domestic water and other usage in Basrah, 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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