Annals of R.S.C.B., ISSN: 1583-6258, Vol. 25, Issue 1, 2021, Pages. 6704 - 6714
Received 15 December 2020; Accepted 05 January 2021.

Morphological and Molecular Identification of Free- living Amoebae *Acanthamoeba* spp. Isolated From Environmental and clinical Sources in Thi- Qar province / Iraq

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Abstract :

*Acanthamoeba*as a free-living protozoan which 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 and soil .Several species of *Acanthamoeba* can cause Granulomatous Amoebic Encephalitis (GAE) ,cutaneous acanthamoebiasis or Amoebic Keratitis (AK) . The current study undertaken investigation and detection of *Acanthamoeba* spp. throughout morphological and molecular methods in different environmental and clinical samples at Thi-Qar province south Iraq. This study included one hundred and two sample were collected from different environmental and clinical source during February to September 2020. The samples were cultured on NN-agar medium after that PCR was conducted on the culture positive samples .Overall 17 (16.66%) samples were positive on morphological characters , PCR –analysis showed that only 13 (12.74%) of *Acanthamoeba* morphologically positive samples were positive by specific primer . For the first time in Iraq, *Acanthamoeba* spp. was isolated from CSF, potato soil , lizard and wild mice wastesamples this was confirmed by molecular examination .In the present study four species of *Acanthamoeba* can be morphologically recognized namely *A. triangularis , A. astronyxis , A. castellini* and *A. polyphaga* .Eventually,the present study considering the presence of potentially *Acanthamoeba* species in different samples whether in environmental or clinical source that will be opened gate to further epidemiological studies for a better understanding of the role of *Acanthamoeba* as a potential health threat to humans .

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

Introduction

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

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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 fresh-water 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 O157 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 the 18Sribosomal 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).

Material and Methods :

Sample collection &cultivation:

Environmental samples

Samples were collected from different environmental source , including ,soil , water from (rivers , tapwater , the marshes ,ponds and drops of water from the air conditioning equipment outside the building) as well as animals wastes. Furthermore, 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 then incubated in

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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- Soilsamples :soil samples and animal wastes were collected in sterile containers the date and site details were labeled for each sample after 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 and incubated in 26 C^0. 3 ml of sterile distilled water were added twice a week to keep cultures wet and then amoebic growth was observed daily by microscope examination for a wet mount slide for 4 week.

Clinical samples :

Samples were collected from different clinical source including eyes, skin , ear ,and CSF collected from Al-Hussan teaching hospital , Bint AL-Huda teaching hospital , Al-Hboobi teaching hospital and private laboratories in Thi-Qar province during the period from March to Septemper 2020. the clinical samples were cultured on non-nutrient agar medium and incubated in 26$C^{0.}$. that was weekly examination .

The identity of *Acanthamoeba spp.* was confirmed ,after morpholgical characterization , genetically by conventional PCR using a set of *Acanthamoeba* spp specific two primers designed by Schroeder *et al*. (2001) : Forward JPD1 (5'GGCCAGATCGTTTACCGTG 3') Reverse JPD2 (5' TCTCACAAGCTGCTAGGGAGTCA 3') (manufactured by Alpha DNA) .

Genomic DNA from cell culture of *Acanthamoeba* spp. were extracted by using gSYNC ^{TM} DNA Extraction kit , Geneaid . Korea , and done according to company instructions . The PCR product yield was a 450b- 500 bp from 18S -rRNA genes in *Acanthamoeba* spp. according to the following protocol :

Initial denaturation 95C^0 for 10 min and 35 cycle of 35 sec at 95C^0 , 35 sec at 56 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 :

The occurrence of *Acanthamoeba* spp. in environmental and clinical samples

Acantamoeba spp. cysts and trophozoite were observed in 17 (16.66%) sample out of 102 samples from different environmental and clinical source of Thi-Qar province.Among the 17 environmental and clinical isolates of *Acanthamoeba* spp. that were positive microscopically , only 13(12.74%) of them were positive after polymerase chain reaction for *Acanthamoeba* spp.using thespecific *Acanthamoeba spp.* primer JPD1 / JPD2Fig (1) .

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The incidence of *Acanthamoeba* spp. in environmental was 12% and in clinical samples was 14.81 % . Table (1).

The highest occurrence of *Acanthamoeba* spp. were in the clinical skin samples which attained 40%. .

at the first time, *Acanthamoeba* spp. was isolated from CSF, potato soil samples, lizard and wild mice wastesamples this was confirmed by molecular examination .

Acanthamoeba spp. no occurrence was recorded in the some water samples and clinical ear samples .

Table (1) : Occurrence of *Acanthamoeba* spp. in environmental and clinicalsamples 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	1	12.5	1	0	0
Tap water	5	0	0	0	0	0
Tanky water	4	1	25	1	0	0
Stagnant water	4	0	0	0	0	0
Marshes water	5	0	0	0	0	0
Air conditioner	4	1	25	1	1	25
Soil	23	6	26.08	6	4	17.39
Potato soil	4	1	25	1	1	25
Lizard waste	7	1	14.28	1	1	14.2
Birds waste	5	1	20	1	1	20
Mice waste	6	1	16.66	1	1	16.66
Total	**75**	**13**	**17.33**	**13**	**9**	**12**
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	0	0
clinical ear samples	4	0	0	0	0	0
Clinical CSF samples	8	1	12.5	1	1	12.5
Total	**27**	**4**	**14.81**	**4**	**4**	**14.81**
Total	**102**	**17**	**16.66**	**17**	**13**	**12.74**

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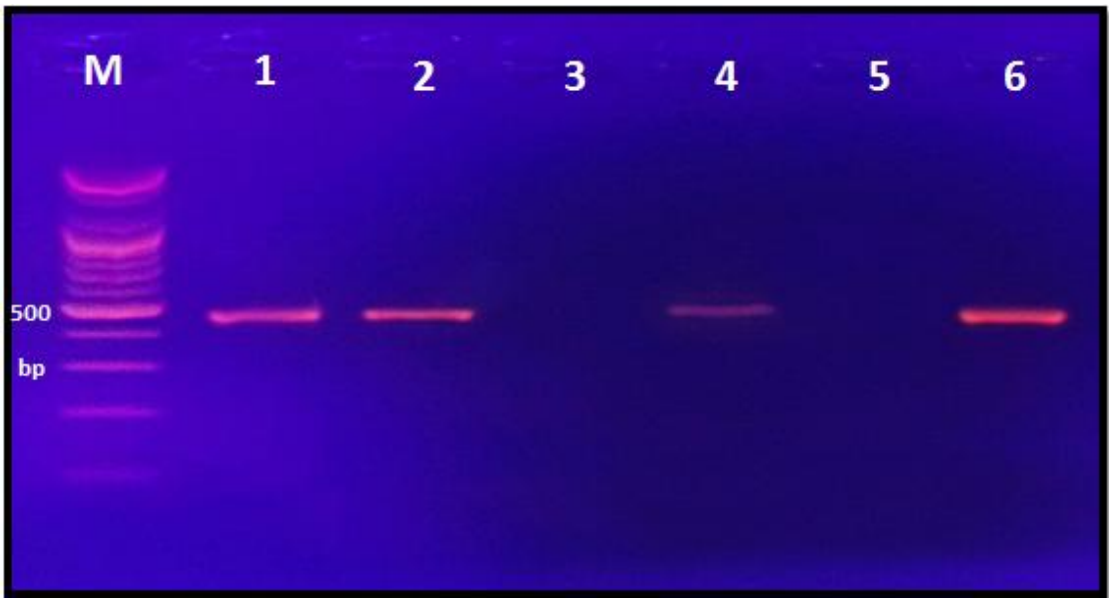
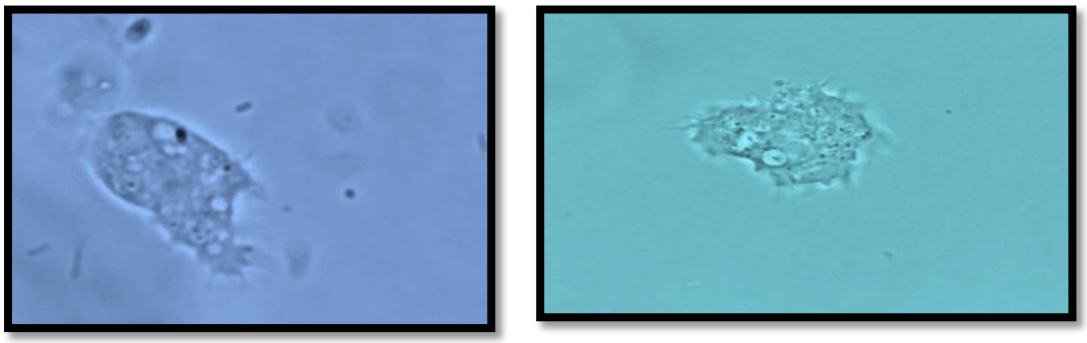


Fig. (1) : Agarose gel electrophoresis image that show the PCR product analysis of 18S ribosomal RNA gene from genomic DNA of *Acanthamoeba* spp. from environmental and clinical samples : Where M: Marker (2000- 100 bp) lance (1,2,4,6) positive samples and lance (3,5) negative samples at 450 -500 bp

Morphological characteristics

Trophozoites

Acanthamoeba spp. showed trophozoite stage after five day of cultivation which characteristic by non uniform , trophozoite of all isolates were irregular in form that were measuring 29- 34 µm. .*Acanthamoeba* trophozoite showed a prominent contractile vacuole and acanthopoda , the typical morphology of *Acanthamoeba* trophozoite moved freely and presence of lobopoda and needle like fine projections of pseudopodia called acanthopoda. Fig. (2).



(A) (B)

Fig. (2) *Acanthamoeba* trophozoite (unstained) show contractile vacuole and acanthopodia 100X:A-isolated from environmental source B- isolated from clinical sources

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Cysts

Different species of *Acanthamoeba* genus were recognized according to the shape and size of cysts in addition to the number ,size , shape and arrangement of the cyst pores . In our current study four species of *Acanthamoeba* was morphologically recognized namely *A. triangularis , A. astronyxis , A. castellini and A. polyphaga* .

A. triangularis

The mean diameter of cyst 13 µm, endocyst which could be stellate , polygonal and triangular. ectocyst was thick wrinkled and corrugated but not spherical however ray of endocyst was broad and slightly curved , the average number of pores were 3 or 4 .Fig (3).

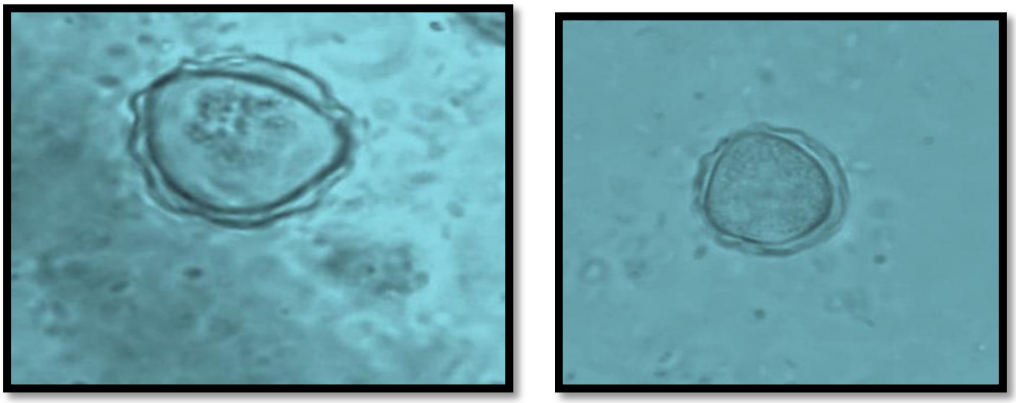

Fig (3) *Acanthamoeba triangularis* cyst stage (unstained) 100X

astronyxis A.

The cyst diameter was 19 µm,endocyst was usually stellate with mainly 5-6 rays ending with pores. the number of cyst pores reached 4-6 meanwhile ectocyst was smoothing circular or nearly so. all rays of endocyst usually contacted ectocyst in approximately the same plane while the ectocyst was separated from the endocyst by a clear region of changing the width . Fig (4)

Fig (4) *Acanthamoeba astronyxis* cyst (unstained) 100X

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A. castellanii

Its cyst diameter was 17 µm. the ectocyst was thick and typically wrinkled. the endocyst stellate usually has no well developed arms or rays. ectocyst and endocyst were both close together . Fig. (5).

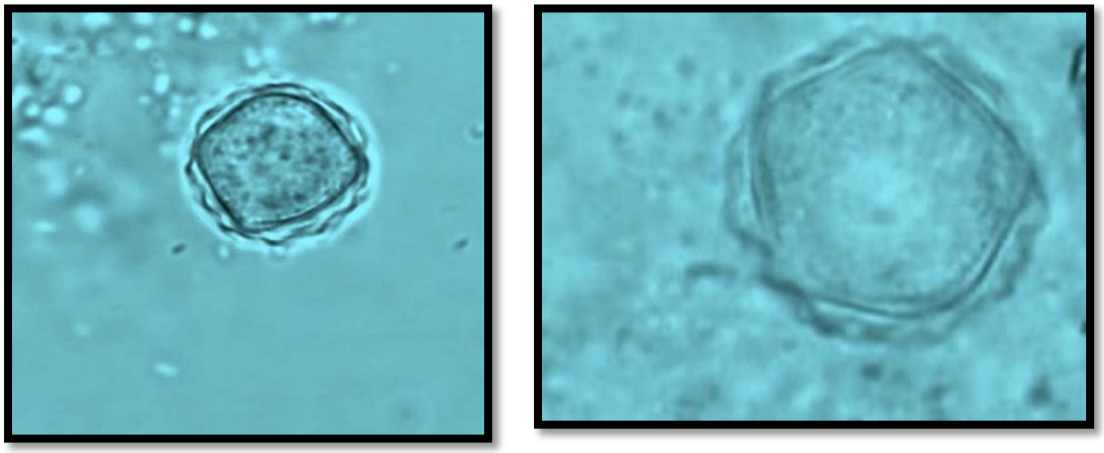

Fig. (5) *Acanthamoeba castellanii* cyst stage (unstained)100X

A. polyphaga

It's cyst diameter about 16.5 µm,endocyst nearly round with slight angles. Ectocyst was closely encircling endocyst . the ectocyst was thicker than the endocyst , this species, there was no pores but contain coroners or ribs , the number of corona were 7 .Fig.(6).

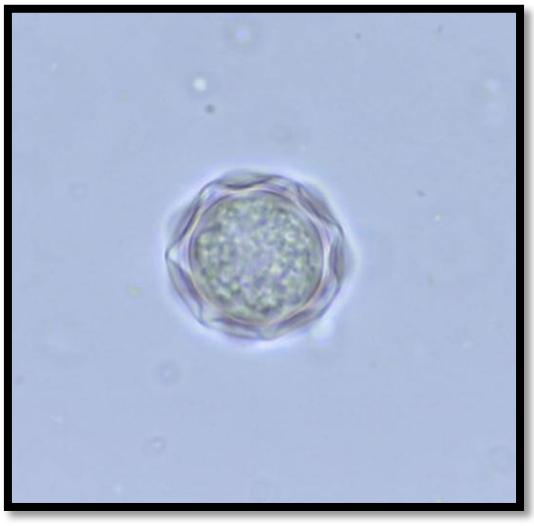

Fig. (6) *Acanthamoeba polyphaga* cyst stage (unstained) 100X

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Discussion

Acanthamoeba spp. is ubiquitous free- living protozoa found in a wide range of environmental niches (Rangsima and Kosol Roongruangchai , 2009) . *Acanthamoeba* strains are said to be responsible for up to 20% of infectious keratitis in CL wearers (McAllum *et al.* , 2009) .

In our study,*Acanthamoeba* spp. strains were detected in the examined samples from environmental and clinical sources in Thi –Qar province diagnosed by morphological characteristics and PCR method . In the present study, it was found four species belonging to the genus *Acanthamoeba* spp. these are *A. astronyxis , A. triangularis , A. castellanii and A. polyphaga.*,at the first time in Iraq, *Acanthamoeba* was isolated from CSF ,potato soil samples, lizard and mice waste samples this was confirmed by molecular examination .

Our study agree with other studies done by Lanocha (2009) he was isolated *Acanthamoeba* from environmental and clinical sources in Poland. In same contacts, , Azhar (2017) and Hanady (2017) they were isolated *Acanthamoeba* from different environmental and clinical source in Basra province .

The current study showed that *Acantamoeba* spp. were observed in 17 (16.66%) sample by culture and microscopic method , only 13(12.74%) of them were positive after polymerase chain reaction .

In Egypt detected 56% of *Acanthamoeba* spp. in of Nile water samples (AL-Herrawy *et al*., 2013) , other study recorded the percentage of *Acanthamoeba* spp. was 26.4% in the river water (Lorenzo- Morales *et al.*, 2005) .In Iraq Hanady (2017) showed that *Acanthamoeba* spp. was found in 20% of collected positive samples in Basra province and Azhar (2017) showed 42 samples (29%) out of 141 were collected from different environmental and clinical sources in same province was positive to *Acanthamoeba* spp. , these studies recorded a higher incidence of *Acanthamoeba* than current study . whereas, other studies showed that percentage of *Acanthamoeba* were lower than the present study , Rezaian *et al*.(2002) studied water and soil river and Parishan Lake in Kazeroon studied 354 samples by culture and microscopic method reported 10 cases of contamination with *Acanthamoeba* . In Egypt the prevalence of *Acanthamoeba* spp. was detected in 8.3% in tap water by real time PCR (Gad and AL-Herrawy , 2016) . The difference in detection rate of *Acanthamoeba* in different countries and localities may be influenced by geographical conditions and samples sources.

Acanthamoeba spp. were isolated from water, the presence of *Acanthamoeba* in samples of water is attributed to water chlorination kills only microorganisms but not FLA . (Khan , 2006) .

Acanthamoeba spp. were isolated from soil samples at a rate 26.08% the proportion, so that our study suggested that presence of *Acanthamoeba*within soil is suitable environments for the growth of *Acanthamoeba* because they rich with bacteria , which are important food sources for *Acanthamoeba .*

Acanthamoeba were isolated from clinical samples , this amphizoic protozoan parasite is the etiological agent of several central nervous system infection including *Acanthamoeba* meningitis – meningoencephalitis (AME) and fatal GAE . *Acanthamoeba* has been frequently detected in many environmental sources (Golestani et al., 2018 ; Behera et al., 2016).Consequently , human exposure to *Acanthamoeba* spp. is highly common.

In current study we identification of *Acanthamoeba* is mainly on cultivation on NN-agar and molecular methods , among 102 samples that were examined in this study 17 (16.66%) were positive for *Acanthamoeba* using microscopic examination and 13 (12.74%) were positive by using PCR method , the current study agree with the study done by Di Filippo *et al*. (2015) which that study confirmed that culture method is not precise enough to detect of FLA , also a total of 160 water samples was analyzed for FLA by PCR method . FLA were detected in 46 of the cultured water samples by microscopic examination , but using the PCR methods on the culture only 39 samples were positive . This may also attributed due to the concurrent presence of other amoebae in cultured samples , however , they can not be correctly differentiated from each other using the direct microscopic examination of the culture – positive samples . Based on Magnet's study, PCR is more sensitive technique than direct microscopy of culture , but the use of both PCR and culture method is suggested for environmental water samples to gain more complete results of the real presence of *Acanthamoeba* (Magnet *et al*., 2013) .

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