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High occurrence of potentially-pathogenic free-living amoebae in tap water and recreational water sources in South-West Iran

Niyyati, M.[1*], Saberi, R.[1], Lorenzo-Morales, J.[2] and Salehi, R.[1]
[1]Department of Parasitology and Mycology, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran
[2]University Institute of Tropical Diseases and Public Health of the Canary Islands, University of La Laguna, Avda Astrofísico Francisco Sanchez s/n, Campus de Anchieta, 38271 La Laguna Tenerife, Canary Islands, Spain
[*]Corresponding author e-mail: maryamniyati@yahoo.com; maryamniyati@sbmu.ac.ir
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Abstract. The present study was conducted to evaluate the presence of potentially pathogenic free living amoebae in drinking and recreational water sources in south-western Iran. From 75 collected water samples, 40 samples (53.3%) were positive for free living amoebae identified using morphological tools. Interestingly, all recreational waters in Ilam city included in the present study were positive for *Acanthamoeba*, Vahlkampfidae and *Vermamoeba*. Thirty percent of tap water samples in Ahvaz city were also positive for potentially pathogenic Free Living Amoebae. Moreover, the three genera identified in the present study have been previously reported as keratitis causative agents in Iran. The present research highlights the need to improve filtration methods for tap waters and to establish awareness in recreational water sources in Iran, in order to prevent Free Living Amoebae related infections. To the best of our knowledge, this is the first report of the isolation of potentially pathogenic free living amoebae including *Acanthamoebae*, *Vermamoebae* and Vahlkampfiids in the South-West of Iran.

Free-living amoebae (FLA) include potentially pathogenic genera such as *Acanthamoeba*, *Vahlkampfia* and *Balamuthia* (Khan *et al.*, 2006; Visvesvara *et al.*, 2007; Niyyati & Rezaeian, 2015). These genera are widespread protozoa and they occur in many niches such as soil, water and dust (Khan, 2009). The outcome of FLA-related diseases mainly depends on early diagnosis and treatment (Marciano-Cabral & Cabral, 2003; Marciano Cabral *et al.*, 2010; Niyyati *et al.*, 2010). Diseases related to free living amoebae include keratitis, cutaneous nodules and amoebic encephalitis among others. In Iran, the increased in the reported cases of amoebic keratitis was described in 2007 in cosmetic soft contact lens wearers (Rezaeian *et al.*, 2007). Later reports regarding AK in Iran were mainly due to improper maintenance of contact lenses. Accordingly, poor hygienic practice such as rinsing of contact lenses in domestic tap water leads to lens contamination (Khan, 2006; Visvesvara *et al.*, 2007; Rezaeian *et al.*, 2007). It should be mention that most AK patients in Iran reported a history of contact with suspected contaminated water bodies (Rezaeian *et al.*, 2007; Niyyati *et al.*, 2010). Moreover, amoebic keratitis cases continue to increase in Iran as it has been recently reported (Niyyati *et al.*, 2009; Abedkhojaste *et al.*, 2013). Regarding amoebic encephalitis, a recent case of encephalitis due to *Naegleria fowleri* was reported in Iran in a six month child. Interestingly, the patient recovered after intensive medical care (Movahedi *et al.*, 2012).

Overall, the main aim of the present study was to address the occurrence of potentially-pathogenic free living amoeba in tap and recreational water sources of South West Iran. This is the first report of the presence of potentially pathogenic amoebae in tap water sources South West, Iran.

Sampling sites in the present study were limited to two cities located in South-West Iran, Ilam and Ahvaz (Fig. 1). Ilam city was chosen due to the presence of very popular recreational water bodies in the area and Ahvaz was selected since this region has reported the use of filtration methods to treat their tap water supplies and also due to the *Acanthamoeba* keratitis cases previously reported in the area.

A total of 75 water samples were collected in this study, 25 recreational water samples in the city of Ilam and 50 tap water samples in Ahvaz. The collected samples were taken to the laboratory to be processed for the identification of Free-Living Amoebae. Briefly, 250 ml of each water sample were filtered through a nitrocellulose membrane with 0.45 µm pore size which allows the isolation of free living amoebae trophozoites and/or cysts. Filter were then cultured in 2% Non-nutrient agar plates (NNA) containing a layer of heat killed *E. coli* which is used as a food source for the outgrowth of amoebae (Memari *et al.*, 2015). All plates were monitored for up to two months. Plates that were positive for amoebic growth were cloned in order to isolate amoebae, this process was carried out as previously described (Lorenzo-Morales *et al.*, 2006). Briefly, blocks of agar containing a few amoebae were transferred to fresh clean NNA plates and replicated until a clean plate only containing one type of amoebae was obtained. Isolated amoebae were identified to the genus level using morphological characteristics as previously described (Page, 1988) such as cell size, contractile vacuoles, acanthopodia and nucleus criteria. Vahlkampfiids members were identified by the presence of rounded endo and ectocysts and wormy shaped trophozoites.

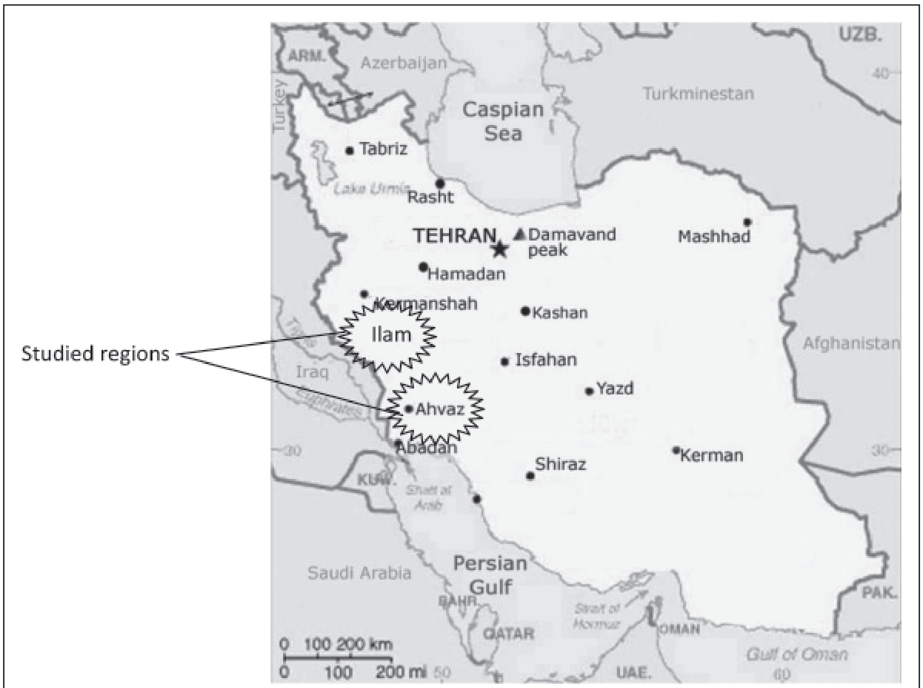

Figure 1. The cities included in this study (recreational water samples were collected in Ilam and tap waters were collected in Ahvaz city)

A high occurrence of potentially pathogenic free living amoebae was observed in both recreational and tap water sources included in this study. Overall, 53.3% of samples were contaminated with free living amoebae. Regarding the recreational water samples collected in Ilam city, 100% of them were positive for Free-Living Amoebae. Interestingly, 16 of the 25 samples were positive for members of the Vahlkampfidae family. Other genera identified in the recreational water sources were *Acanthamoeba*, *Thecamoeba* and some unidentified *Miniamoebae* (Table 1).

Regarding the tap water samples (50), 30% were positive for Free-Living Amoebae belonging to *Acanthamoeba* (6), Vahlkampfiids (4) and *Vermamoeba* (5) genera/family. *Acanthamoeba* genus was identified by the presence of double-walled cysts with star, polygonal or stellate shape endocysts and the two wall layers meet at the corner rather than an arm of endocyst. The cyst diameter was also variable ranging from 15 to 20 µm. All of the isolated cysts were classified as morphological group 2 members according to the morphogical key (Page, 1988). Trophozoites revealed as flat shape with large karyosome inside nucleus and needle like pseudopodia (Fig. 2). Vahklampfiids were identified due to the presence of round single wall cysts and wormy shape trophozoites (Fig. 3). *Vermamoebae* cysts showed spherical or slightly ovoid shape walls with smooth out layer and elongated shaped trophozoites (Fig. 4).

Despite reports regarding the presence of free-living amoebae in recreational water sources as stated by various researchers worldwide (Garcia, 2007; Khan, 2009; Behniafar *et al.*, 2015), there are limited surveys indicating tap water contamination due to opportunistic *Acanthamoeba* and Vahlkampfiids in Iran. The present study reflected that all of recreational waters of Ilam city Iran and 30% of tap-water supplies in Ahvaz city were contaminated by Free Living Amoebae. Previous studies in order regions regarding the presence of Free-Living Amoebae in water sources are variable. In Korea, from 47% of positive domestic tap water sources only 6% were found to be positive for *Acanthamoeba* genus (Jeong *et al.*, 2007). In contrast, in the Canary Islands *Acanthamoeba* was identified in 60% of the studied tap water samples (Lorenzo Morales *et al.*, 2005a). Another study in Jamaica showed that 30% of tap water supplies were positive for *Acanthamoeba* in which is in accordance to the present study (Lorenzo Morales *et al.*, 2005b). Nevertheless, tap water contamination by Free Living Amoebae may be the result of poor filtration and chlorination of water bodies (Niyyati *et al.*, 2015a, b).

In this study, it is important to highlight that all collected samples in the city of Ilam

Table 1. *Acanthamoeba* Positive isolates in the water sources included in this study

Isolate code	Water source	Morphology
SI 7*	Tap water	*Polygonal endocyst*
SI 7	Tap water	*Star shape endocyst*
WI 8	Tap water	*Star shape endocyst*
SI 9	Pond water	*Star shape endocyst*
SI14	Tap water	*Star shape endocyst*
SI19	Tap water	*Star shape endocyst*
WI30	Pond water	*Polygonal shape endoscysts*
WI32	Pond water	*Star shape endocyst*
SI32	Tap water	*Polygonal endocyst*
SI36	Pond water	*Stellate shape endocyst*
SI39	Pond water	*Polygonal endocyst*

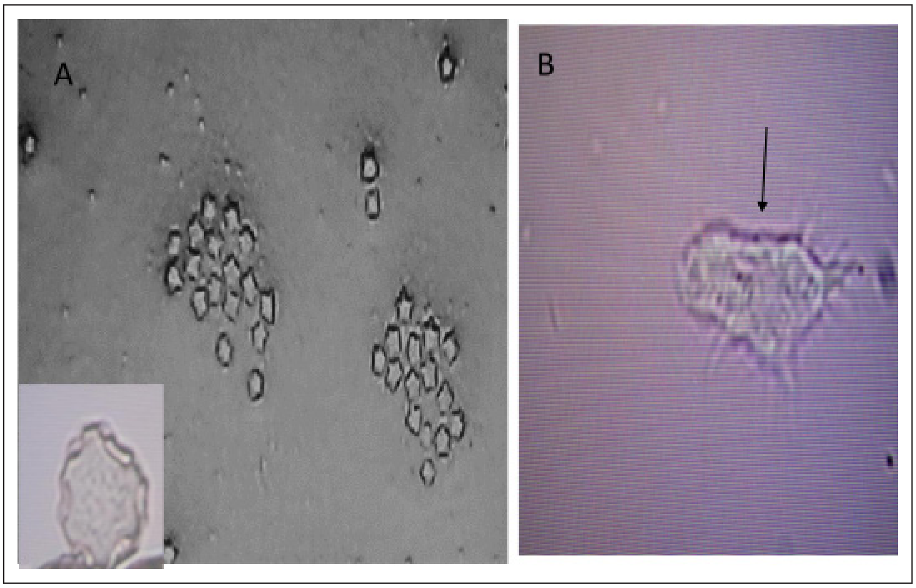

Figure 2. *Acanthamoeba* cysts (A: X100) and trophozoites (B: X400) observed in one of the samples

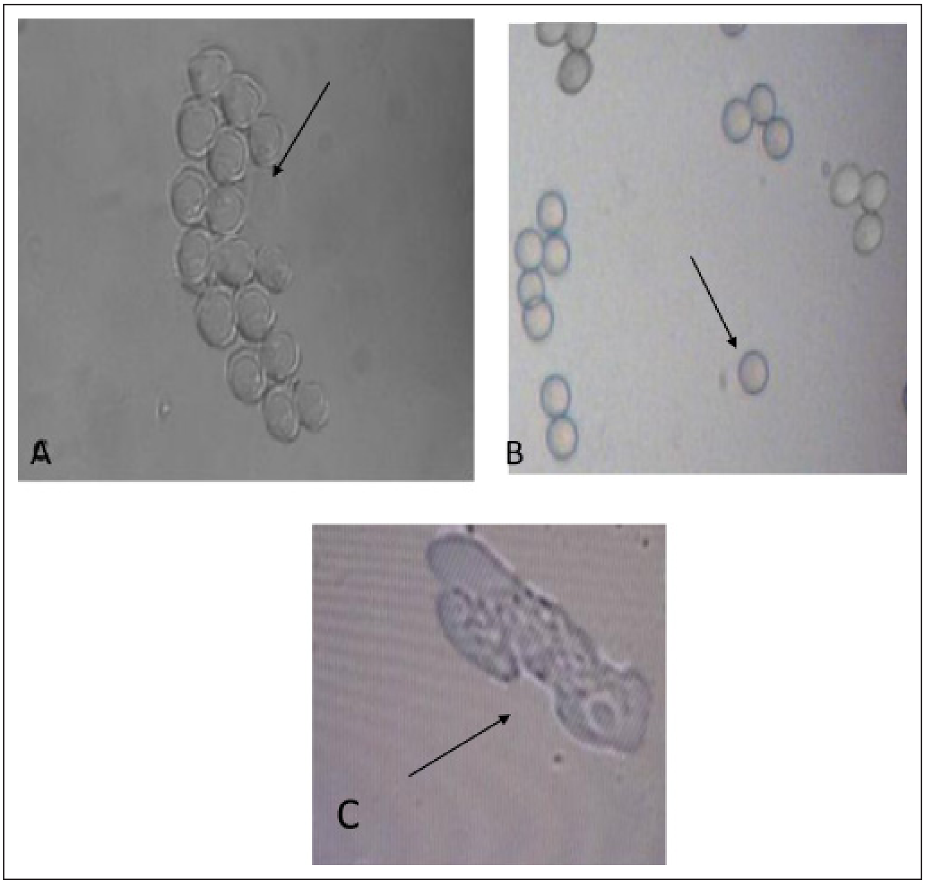

Figure 3. Vahlkampfiid cysts (A, BX400) and trophozoites (C X400)

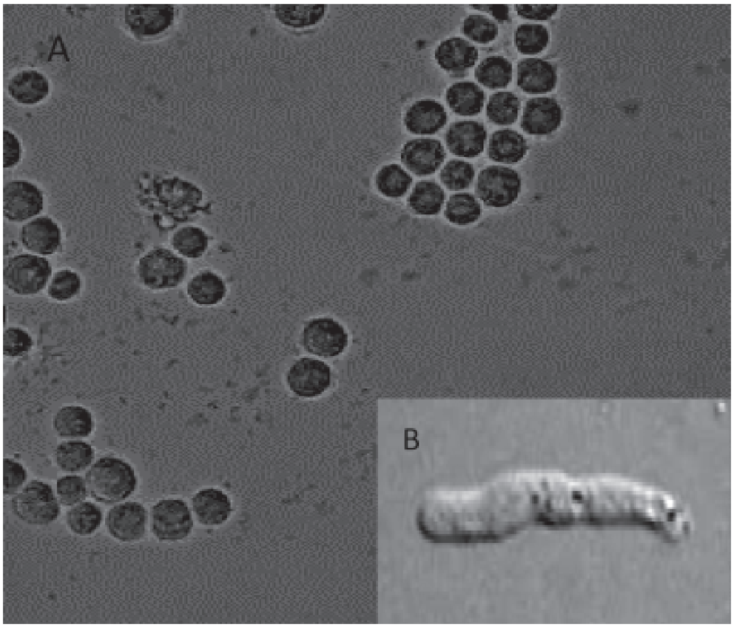

Figure 4. *Vermamoeba* cysts (A) and wormy shape trophozoites (B) (Magnification: X400)

(recreational water bodies) were positive for Free-Living Amoebae. This event could be a serious health hazard in this region. Interestingly, in most of the reported cases of amoebic keratitis in this region patients admitted contact with water sources. Thus health authorities should be aware and implication towards prevention of the establishment of possible infection sites is of utmost importance in this area. Awareness of people at risk regarding avoidance of contact with unfiltered and contaminated water bodies while wearing contact lenses could prevent FLA-related infections (Niyyati & Rezaeian, 2015). Of note, all three genera of the isolated amoebae in the current study were found in amoebic keratitis cases in Iran as it was mentioned in the introduction section. Furthermore, mixed *Vahlkampfia* and *Acanthamoeba* keratitis cases have been previously reported in Iran by Niyyati *et al.* in a soft cosmetic contact lens wearer (Niyyati *et al.*, 2010). Another case of *Vermamoeba* keratitis recently was reported by Abedkhojaste *et al.* in the country (Abedkhojaste *et al.*, 2013).

Overall, the present research highlights the high occurrence of potentially pathogenic Free Living Amoebae in both tap water and recreational waters sources included in the study. Improved filtration methods of tap water supplies using filters with smaller pores could be of utmost importance for decontamination of tap waters in Iran and worldwide. Posting of warning signs near recreational water sources is also critical to prevent amoebae related infections at least in the city of Ilam.

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