RESEARCH ARTICLE

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# Morphological and Molecular Identification of *AcanthamoebaSpp* from Surface Waters in Birjand, Iran, During 2014-2015

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**A-R-T-I-C-L-EI-N-F-O**

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**A-B-S-T-R-A-C-T**

**Background & Aims of the Study:** Free-living amoebae (FLA) are opportunistic and ubiquitous protozoa that are widely found in various environmental sources. They are known to cause serious human infections including a fatal encephalitis, a blinding keratitis, and pneumonia. So, due to their medical importance, the identification of free living amoeba in water resources, as a source of human infection, is necessary. The objective of this study was to isolate the *Acanthamoebaspp* from the surface waters of Birjand, Iran, during 2014-2015 by Morphological and molecular method.

**Materials and Methods:**
In a cross-sectional study, 50 samples were collected from different localities of Birjand city including the surface waters, pools and fountains in parks,squares and water stations from the October 2014 to the January 2015.Each sample was filtered through a nitrocellulose membrane filters and cultured on non-nutrient agar (NNA) with *Escherichia coli* suspension and incubated for 1 week to 2 months at room temperature.The plates were examined by the microscopy to morphologically identify *Acanthamoeba* species. Following DNA extraction, PCR specific primers was used to confirm the identification morphologically.

**Results:**
Out of 50 water samples, 19 (38%) were positive for *Acanthamoebatrophozoites* and cysts according to the morphological criteria. In addition, *Acanthamoebaspp* was identified by PCR method, using genus specific primers pairs in 15 (78.9%) cases of positive cultures, showing anearly 500bp band.

**Conclusion:** According to the prevalent of *Acanthamoebaspp* in the surface stagnant waters of Birjand, more attention to the potential role of such waters in transmission of infection by the regional clinicians and health practitioners is necessary.

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## Background

*Acanthamoeba* is an opportunist amphizoic protozoan which is found in the environmental sources. Researchers showed that *Acanthamoeba* can be found in quite different media including sea water, treated water, swimming pool, aquarium, bottled water, soil, air dust, sewage water, contact lenses washing solution, food stuff, air conditioners, digest organs and dialysis machines (1). This

protozoan has two stages in its life cycle, active trophozoite and the resistant cyst. The double-layered coat of the cyst enables it to survive in the unfavorable conditions including presence of disinfectants such as chlorine compounds and antibiotics. It also well tolerates rang of temperature as wide as -2 °C to +45 °C. A variety of microorganism, such as *Legionella sp* and *Burkholderiapicketti*, which nest in the form of endosymbiont in this amoeba as amoeba-associated bacteria, can also survive after chlorination and applying other disinfectants (2,3,4,5). These amoebae are the causative agents of multifocal encephalitis called granulomatous amebic encephalitis, a chronic central nervous system disease that usually occurs in immuno compromised hosts, amoebic keratitis (AK) and pneumonitis. AK is a corneal infection mainly associated with the contact lenses use (6). An increase in the number of intra cerebral infections caused by worldwide has been reported (7). The presence of *Acanthamoeba* and other contaminants in water, soil, dust, cow feces and swimming pool have been shown in Iran (8,9).

Detection of *Acanthamoeba* can be improved by means of a molecular detection of the organisms by polymerase chain reaction (PCR). This technique can detect the presence of DNA of *Acanthamoeba* even in small amounts which can be missed by culture techniques (10).

**Aims of the study:**

Since there was no information regarding the distribution of *Acanthamoeba* in recreational water sources, the main aim of the present study was the isolation of *Acanthamoebaspp* from the surface waters in Birjand, South Khorasan province of Iran, during 2014-2015 by morphological and molecular methods.

## Materials & Methods

**Sampling:**

In a cross-sectional study, 50 samples were collected from different localities in Birjandcity, South Khorasan province including surface waters, pools and fountains in parks and squares and water stations from October 2014 to January 2015 (Fig 1). From each sampling point, one to three water samples were collected in 500 ml sterile bottles and In order to morphological isolation were transferred immediately to the Microbiology Laboratory of Birjand University of Medical Sciences, Birjand, Iran, within 24 hours and stored at room temperature.

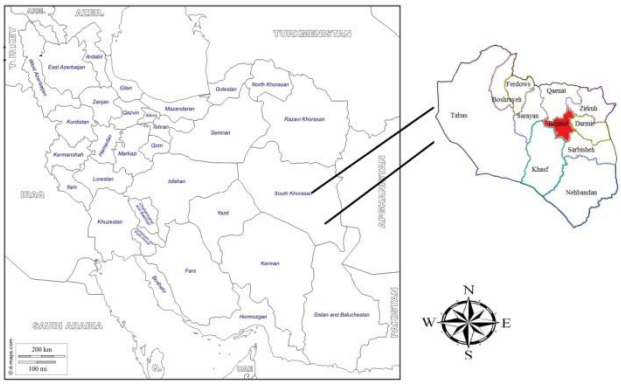



**Figure 1) map of the South Khorasan Province and Birjand city**

**Isolation of *Acanthamoeba* species and culture:**

For the isolation of *Acanthamoeba* species, 250 ml of the collected water samples were filtered through a cellulose nitrate membrane with pore size 0.45 µ approximately. Filter was transferred on non-nutrient agar plates enriched with the Gram-negative bacteria (*Escherichia coli*) as a food source. Plates were incubated at room temperature, and 7 days later, they were examined for the presence of *Acanthamoebatrophozoites* microscopically. However, in the absence of amoebae, plates were monitored for up to 2 months. *Acanthamoeba* were identified at the genus level, based on the morphological characteristics of trophozoites and cysts, using a light microscope.

**DNA extraction and PCR (Polymerase Chain Reaction):**

Amoeba cells were harvested from the surface of culture plates by sterile PBS, pH 7.2, concentrated by centrifugation at 500g for five minutes; then DNA extraction was performed

by DNA extraction Kit (Cinnagen, Iran), according to the manufacturer's instructions.

**PCR assay and Gel electrophoresis:**

The *Acanthamoeba* specific primer pairs JDP1 (5′GGCCCAGATCGTTTACCGTGAA-3′) and JDP2 (5′-TCTCACAAGCTGCTAGGGGAGTCA-3') as described by Schroeder et al (11) were used for the amplification of the 500 bp of 18S rDNA gene approximately (12).Thermal cycling conditions were 94°C for 3 min; 35 cycles of 94°C for 35 s, 56°C for 45 s, 72°C for 45 s; followed by a final extension at 72°C for 5 min.

The PCR-products electrophoresis was done on 1.5 % gel agarose stained with ethidium bromide solution and visualized under the UV light (13).

## Results

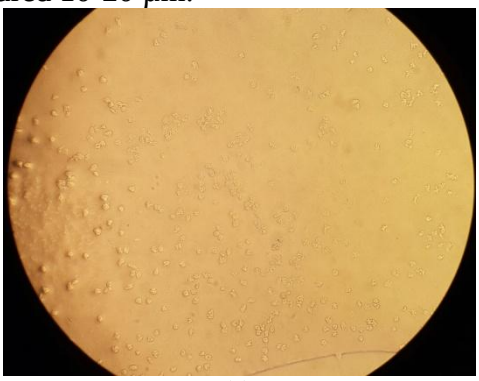
**Morphological identification:**

Overall, 19 out of 50 (38%) surface waters samplesof different parts of the Birjand city were found positive *Acanthamoeba* spp by microscopic method.

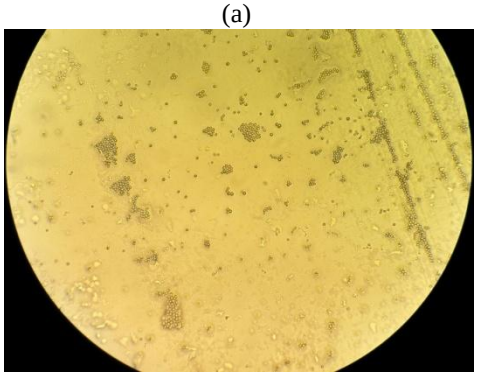
**Table 1) Identification of *Acanthamoebaspp* in surface water samples of Birjand using culture**

| Name of the source | number of samples | number of positive samples | Positive percentage |
|---|---|---|---|
| **Surface waters of squares** | 25 | 12 | 48% |
| **Pools of parks** | 17 | 5 | 29.5% |
| **fountains** | 5 | 2 | 40% |
| **water stations** | 3 | 0 | 00% |
| **Total** | 50 | 19 | 38% |

Some of the samples contain other free-livingorganisms. Acanthamoeba can be identified at the genuslevel based on distinctive features of trophozoites and cysts, especially the double-walled cyst shape that is unique to the genus. Morphological detection revealed flat-shaped trophozoites with a single nucleus, various vacuoles and spine-like pseudopodia called acanthopodia. Trophozoites were between 15-30 µm long. Cysts were detected by their star, triangular or square endocysts and measured 10-20 µm.



(a)



(b)

**Figure 2)(a) *Acanthamoebaspp* flat shape trophozoites after isolation from water samples and cultivation(x100), (b) *Acanthamoebaspp* cysts after isolation from water samples and cultivation(x100)**

Moreover, In 2 samples (4%), a colony of Vahlkampfiidae were observed. Vahlkampfiidae had round cysts measuring 15-20 µm approximately, their trophozoite were long and wormy shape.

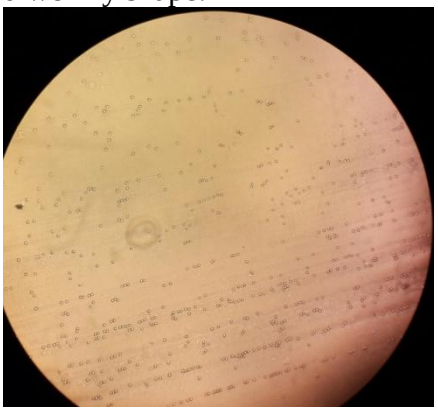


**Figure 3)*Vahlkampfiidaespp* cysts after isolation from water samples and cultivation(x100)**

Most contamination was related to the surface water squares and fountains. Also, in the

samples of Toheed park and Imam Khomeyni fountains, as two crowded and recreational places of Birjand, Acanthamoeba was observed. In three samples of water stations, Acanthamoeba were not found.

molecular identification:

Further detection and identification of *Acanthamoeba* spp from the culture plates has been approved by the PCR.

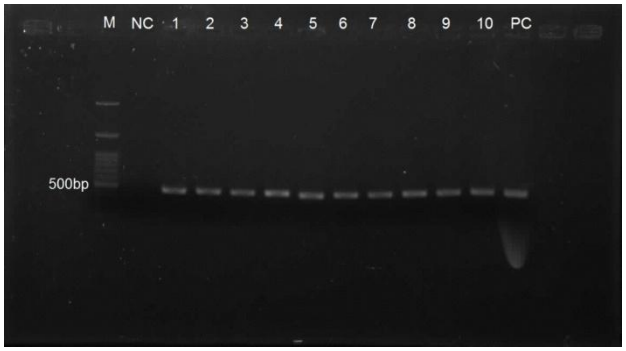



**Figure 4) PCR products on a 1.5% agarosegel.Lanes 1–10 Acanthamoeba positive isolates with JDP primers. Lane NC*Acanthamoeba* negative control, lane PC positive control *Acanthamoeba*, lane M 100bp marker**

The primers which have been used in this study were specific for Acanthamoeba spp.(JDP1 and JDP2). From 19 positive samples microscopically, 15 (78.9%) were also positive by PCR and a specific 500 bp band was detected on gel agarose approximately.

**Table 2) Identification of *Acanthamoeba*spp in surface water samples of Birjand city using PCR**

| Name of the source | number of positive samples using culture | number of positive samples | Positive percentage |
|---|---|---|---|
| **Surface waters of squares** | 12 | 11 | 92% |
| **Pools of parks** | 5 | 3 | 60% |
| **fountains** | 2 | 1 | 50% |
| **water stations** | 0 | 0 | 00% |
| **Total** | 19 | 15 | 78.9% |

Four negative PCR amplifications may include other FLA, like Thecamoebidae and Hartmannellidae family.

## Discussion

Finding *Acanthamoeba* spp in the surface waters is very important, because they are one of the most available sources for people, so it increases the risk of keratitis and the other health problems caused by FLAs in population.

In the present study, we have isolated *Acanthamoeba* species from 19 out of 50 (38%) surface water samples by cultivation and morphologic assessment in different areas of Birjand city; also, 15 samples were reconfirmed by molecular method.

According to contaminated samples like Toheed park and Imam Khomeini Square fountain, the presence of the Acanthamoeba spp is a potential hazard to the public health of the native people and travelling through the villages of Birjand city.

In the present study, more tap water sources have been tested positively for Acanthamoeba than the other sources, as shown in. This result confirms the resistance of the cyst stage of *Acanthamoeba* to chlorine (which is the main and sometimes only material used for cleaning the water). This relatively high occurrence can be caused by the cyst formation and may highlight that, despite filtration, chlorination, and treatment processes, amoebae are able to colonize in the water distribution systems, probably by biofilm formation, as previously suggested in other regions by Cabral et al (14). This relatively high occurrence of *Acanthamoeba* spp in water resources is in accordance with a study by Rezaeian et al, which revealed a significant increase of AK in Iran (15).

In Iran, several studies have reviewed the occurrence of *Acanthamoeba* spp. in water resources of different parts of the country (16,17,18). Some previous studies have reported the presence of FLA in hot springs, particularly two studies that were carried out in Ardebil Province (19,20). *Acanthamoeba* spp.were reported from other water resources of Iran as well; for example, one study reported

their occurrence of 30% in the surface waters of Gilan Province of Iran (21).

Salehi et al in 2014 reported 68% occurrence of *Acanthamoeba* spp. from different localities of Bojnurd including agricultural canals, rivers and swimming pools (22). Niyyati et al in 2015 also reported the presence of 66.8%*Acanthamoeba*spp from tap waters of the tourist attractions on Kish Island in southern part of Iran (23). These results are in agreement with those of the current study.

Karanis et al. were reported *Acanthamoeba* spp as causative agent of an outbreak associated with a contaminated municipal water supply in the USA, and FLAs has been commonly found in various environmental water sources throughout the world (24). Presence of FLAs was reported in rivers and water treatment plants located in Osaka Prefecture, Japan. A total of 257 out of 374 samples (68.7%) were found positive for FLA by microscopy (25). Many Bulgarian environments were examined for FLAs contamination, and 171 (61.1%) out of 280 samples were found contaminated (26). There are also many cases of amoebic keratitis, which occurs after swimming in contaminated pools, usage of homemade saline forwashing contact lenses and exposure to dirt and dust (27).

## Conclusion

In conclusion, the present research showed the high occurrence of *Acanthamoeba*spp in water sources including pools and fountains of parks, squares and water stations in Birjand, South Khorasan province of Iran. Based on the results, it is clear that the pore size of the filtration membranes and the filtration process are not able to eliminate amoebae. To decrease the infections of *Acanthamoeba* spp., water sources must be monitored and disinfected with appropriate disinfectants by health authorities. Based on the results, the potential risk of diseases caused by free-living amoebae should be considered in this area. Further investigations about the various aspects of these important opportunistic protozoa are recommended especially for establishment of appropriate prevention tools.

Moreover, the use of molecular methods to identify free-living amoebae of genus *Acanthamoeba* could provide a more rapid means to diagnose the infections caused by those amoebae.

## Footnotes

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**Conflict of Interest:** The authors declared no conflict of interest.

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