

# Detection and Molecular Characterization of Potentially Pathogenic Free-living Amoebae from Water Sources in Kish Island, Southern Iran



## Supplement Issue: Microbial Diversity

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**ABSTRACT:** Amoebic keratitis, a sight-threatening corneal infection, mainly occurs in contact lens wearers who wash their eyes with tap water. The present research was conducted to identify the occurrence of potentially pathogenic free-living amoebae (FLA) in tap water sources on Kish Island, a tourist region in Iran. Amoebae were detected using a culture-enriched method and by polymerase chain reaction (PCR)/sequencing of the diagnostic fragment 3 region of the 18S rRNA gene of *Acanthamoeba*. In the case of other free-living amoebae species, PCR/sequencing analysis of the 18S rDNA was conducted. Results of this study showed the presence of *Acanthamoeba* belonging to T3, T4, T5, and T11 genotypes in tap water sources. Additionally, *Vermamoeba vermiformis* was detected in three water samples. This is the first report of the *Acanthamoeba* genotypes T3, T4, T5, and T11 and *V. vermiformis* species in tap water sources in a tourist region in Iran.

**KEYWORDS:** free-living amoebae, Kish Island, sequencing analysis, *Acanthamoeba*, *Vermamoeba vermiformis*

**SUPPLEMENT:** Microbial Diversity

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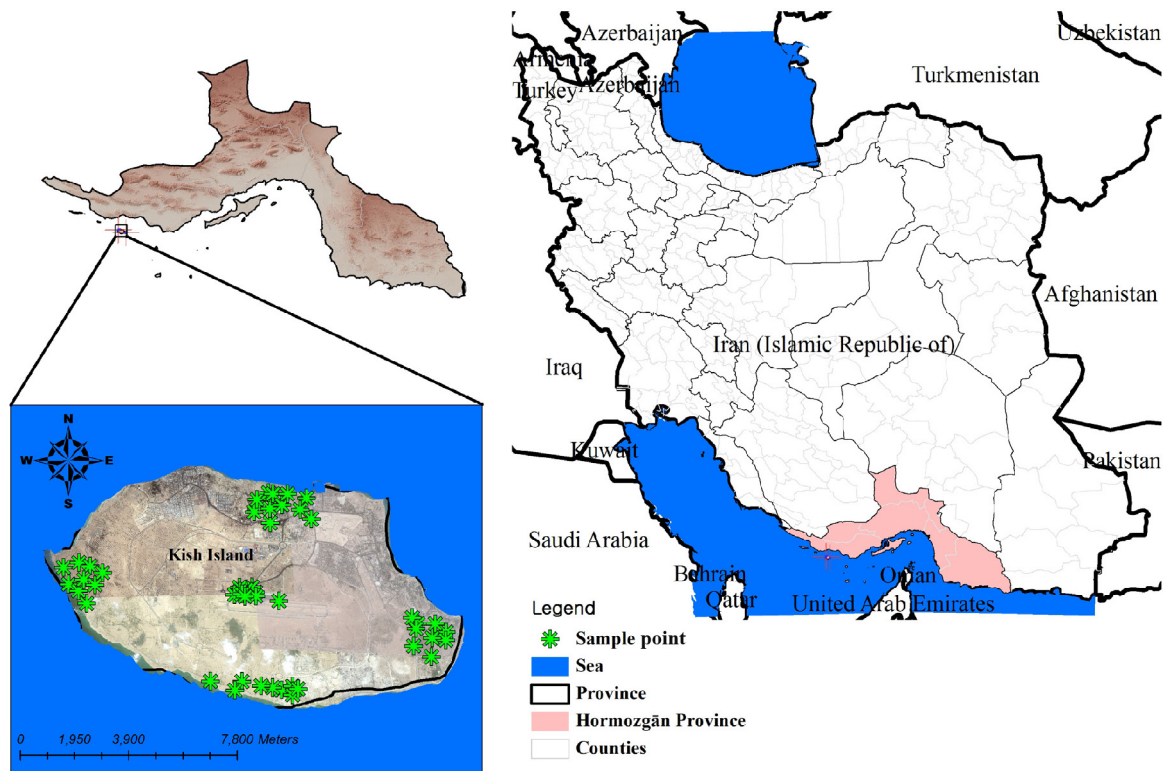
## Introduction

Free-living amoebae (FLA) include genera with pathogenic potential, such as *Acanthamoeba*, *Balamuthia*, *Naegleria*, and *Sappinia*. These ubiquitous FLA are widespread in the environment, including different types of water sources.<sup>1–3</sup> Contamination of water sources, such as pools, sea, and recreational waters, particularly those associated with human activity, is an important risk factor for amoebic infections.<sup>2,4,5</sup> One of the important issues in water contamination is the presence of pathogenic amoebae in tap or drinking water supply sources. Tap water goes through filtration and chlorination processes before human use; thus, this may reflect in the lower microbial contamination of such waters. However, this depends on many factors, such as effective filtration and disinfection systems of the mentioned water sources.<sup>5–9</sup> Amoebae belonging to *Acanthamoeba*, *Vermamoeba* (previously known as *Hartmannella* spp.), and *Vahlkampfiids* may be the cause of amoebic keratitis (AK) in high-risk populations, such as contact lens wearers and individuals who have undergone eye surgery.<sup>10</sup> Prognosis of AK is very poor, and most patients show episodes of recurrence even after effective therapy with a combination of drugs, such as propamidine isethionate and polyhexamethylene biguanide (0.02% PHMB). Moreover, most of the reported cases of AK in

cosmetic soft contact lens wearers in Iran as well as worldwide are caused by *Acanthamoeba* and *Vermamoeba* genera.<sup>8,11–13</sup> Interestingly, these patients report a history of contact with tap water before wearing their lenses. Improper maintenance of lenses, including cleaning of contact lenses with tap or distilled water, is still one of the main causes of AK.<sup>8</sup>

Unfortunately, FLA-related infections continue to increase in Iran.<sup>14–16</sup> The first reported blind cases in Iran were approximately 20 years ago; since then, several studies indicating an increased incidence of the disease in Iran have been published. However, there are still many unreported cases because of the lack of knowledge regarding the diagnosis of AK.<sup>14,16</sup> Other diseases related to FLA have been reported as cases of mixed infections caused by *Acanthamoeba* and *Hartmannella* or *Acanthamoeba* and *Vahlkampfiids*.<sup>12,13</sup> There are no reports of granulomatous amoebic encephalitis (GAE) in the country yet; however, there has been a single report of primary amoebic meningoencephalitis (PAM) in Qhum city (central Iran) isolated from the cerebrospinal fluid (CSF) of a 6-month-old-boy with a history of washing his nasal passages with contaminated tap water.<sup>17</sup>

Kish Island is located in Hormozgan Province in southern Iran (Fig. 1) and attracts many people annually due to its climate and tourist interest. Because of the warm climate and



**Figure 1.** Kish Island location in Hormozghan province, Southern Iran (sampling points from five different districts of the region are shown by stars).

the manner in which tap water is stored in the region, it is suspected that the presence of amoebae may be high in water storage tanks. In this connection, it should be mentioned that there are also reported cases of AK infections caused by washing contact lenses in tap water sources in this region.

Therefore, tap water sources were surveyed to address the occurrence of FLA in the water sources of Kish Island, Iran, using both morphologically and molecular-based methods. To the best of our knowledge, this is the first study reporting the presence of potentially pathogenic FLA in this region.

## Methods

**Sampling geographical area.** This study was conducted in Kish Island, southern Iran. Kish Island is part of the Hormozgan province and is located in the Persian Gulf 19 km (12 mi) from mainland Iran and has an area of approximately 91 km<sup>2</sup> (Fig. 1). The island is positioned along the 1,359-km (844-mi)-long Iranian north coastline in the Persian Gulf at the first quarter from the Hormuz entrance to the Persian Gulf (Fig. 1). The island is 15.45 km long from west to east coast. The average temperature in summer is 40°C. Tap water supplies in Kish Island are mainly maintained using reservoir tanks located in houses or resort yards. All tap water is chlorinated and filtered by the city officials in a drinking water plant, according to World Health Organization–defined criteria (0.2–0.7 ppm).<sup>9</sup>

**Processing samples and morphological identification.** Fifty-five water samples were collected randomly from the tap water of five districts of Kish Island (north, south, east, west,

and central). All tested tap water had been stored in water tanks in the yard of houses, resorts, or malls, and was in direct contact with sunlight all day. Notably, water storage tanks are drained and cleaned approximately every 6 months. Processing of the samples was performed within eight hours of collection. Each sample was filtered using nitrocellulose membranes (0.22 μm pore size and 45 mm diameter) and cultured using an enrichment cultivation method. Briefly, culturing was performed using 1.5% non-nutrient bactoagar (Difco, USA) along with heat-inactivated *Escherichia coli* as food sources of amoebae.<sup>3,14</sup> Because the medium was non-nutrient, unwanted organisms could not grow.

Plates were then monitored daily by inverted microscopy.<sup>18</sup> Positive plates were then cloned to eliminate bacterial and fungal contamination. Axenic culture of the isolated strains was performed according to previous work.<sup>4,16</sup>

**DNA extraction, PCR analysis, and sequencing.** DNA extraction was performed on positive plates using the Insta-gene matrix (Chelex; Biorad)<sup>19</sup> for trophozoites or phenol–chloroform method for cysts. Based on the morphological identification of the amoebae in cultures, primer sets were used for further molecular analysis. Morphological identification of *Acanthamoeba* genus was based on the presence of flat trophozoites with acantopodia and double-walled cysts with various shapes of endocysts, such as stars and triangles. *Vermamoeba* were detected morphologically by their elongated, thin trophozoites, their length exceeding their girth by approximately sixfold, and cysts were detected by their smooth, spherical, or slightly oval appearance with a single wall.

Polymerase chain reactions (PCRs) were performed using genus-specific primers targeting the diagnostic fragment 3 (DF3) of 18s rRNA gene for *Acanthamoeba*<sup>20,21</sup> and NA primers targeting various genera of amoebae such as *Vermamoebae*.<sup>22</sup> The sequences of primer sets used in the present study were as follows: JDP1 5'-GGCCCAGATCGTTTACCGTGAA-3' and JDP2 5'-TCTCACAAGCTGCTAGGGAGTCA-3' and NA1 5'-GCT CCA ATA GCG TAT ATT AA-3' and NA2 5'-AGA AAG AGC TAT CAATCT GT-3'. PCR was performed in 30  $\mu$ L Ampliqone (Taq DNA Polymerase Master Mix RED, Denmark) as a readymade mixture. Twenty-five microliters of Taq Master mix was used with 5 ng template DNA, 0.1  $\mu$ M of each primer, and distilled water. The thermal cycling conditions were an initial denaturing step of 94°C for 1 minute and 35 repetitions at 94°C for 35 seconds; annealing steps were carried out at 56°C and 50°C for 45 seconds for *Acanthamoeba* and *Vermamoeba*, respectively, and 72°C for 1 minute. The extension time was prolonged for five minutes at 72°C. PCR products were electrophoresed on 2% agarose gel stained with a solution of ethidium bromide and visualized under ultraviolet light.

**Genus and genotype/species identification.** Identification of *Acanthamoeba* genotypes and other FLA genera was based on alignment analysis of the obtained sequences with the genes available in the basic alignment search tool of the U.S. National Center for Biotechnology Information. All sequences were then submitted to the Genbank database, and are under the following accession numbers: KP337292–KP337308.

## Results and Discussion

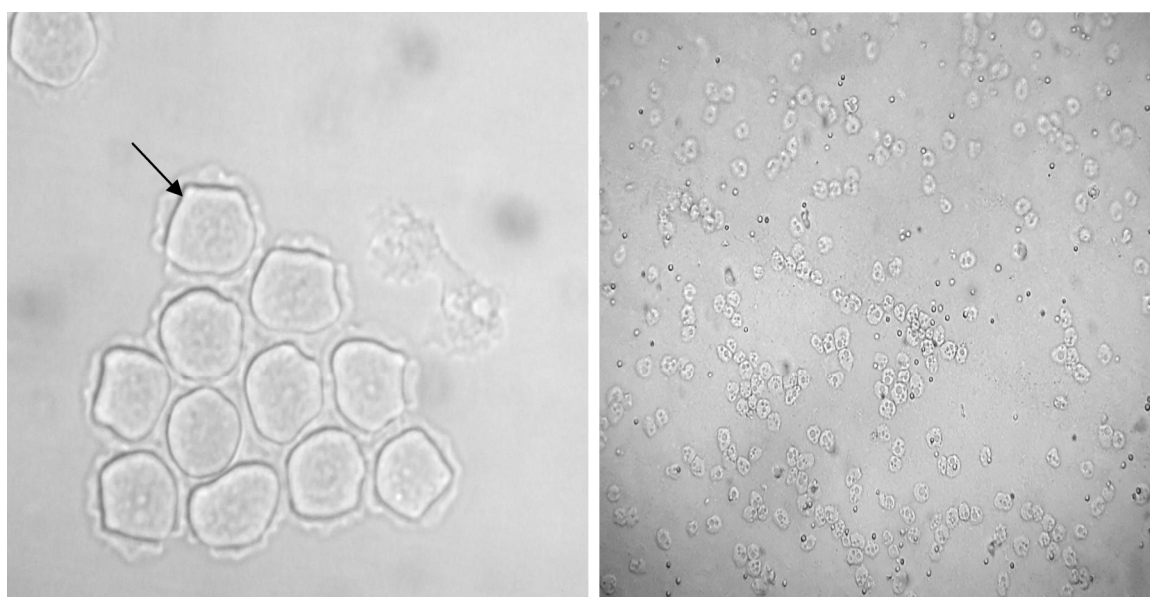
Overall, out of 55 tap water samples collected from five different part of Kish Island, 21 (38.2%) isolates were recovered for FLA after 3 days to 1 month of incubation. The most

**Table 1.** Percent contamination of tap water sources in different district of Kish Island, Iran.

DISTRICT OF THE ISLAND	TOTAL	POSITIVITY n (%)
North	11	8 (72.7)
South	11	4 (36.3)
West	11	3 (27.2)
East	11	4 (36.3)
Center	11	2 (18.1)
<b>Total</b>	<b>55</b>	<b>21 (38.2)</b>

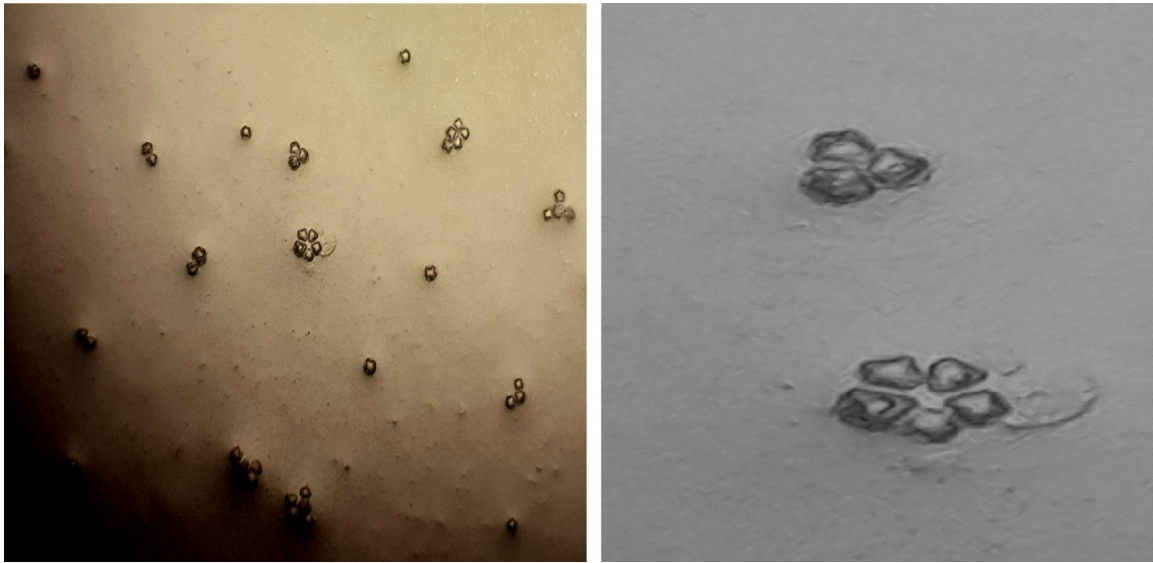
contaminated water sources were in the northern district of Kish Island, which the endemic population inhabits. However, water contamination in the central part of the island was less in comparison with other districts (Table 1). Fourteen samples (66.7%) contained *Acanthamoeba* spp. with double wall cysts and flat trophozoites with acanthopodia. Axenic cultivation was successful in all isolated strains (Fig. 2). The cysts were different regarding the size and morphology of the endocysts and included star-, triangle-, and round-shaped cysts (Figs. 3 and 4). Three plates (14.2%) contained elongated amoebae and small round cysts measuring between 10 and 15  $\mu$ m. These amoebae were identified as members of the Hartmannellidae family (*V. vermiformis* species) by morphological (Fig. 5) and molecular tools. Moreover, two samples positive for *Acanthamoeba*, T3 and T4 (NL19 and NL27), also contained *V. vermiformis*. Furthermore, other FLA, such as *Miniamoebae* and *Thecamoebae* genera, were detected in four samples (19%) but were excluded from our research.

The 14 *Acanthamoeba* isolates amplified a 500-bp product using the DF3 region primers, and the sequencing results



**Figure 2.** Light photomicrograph of *Acanthamoeba* cysts (left:  $\times 400$ ) and trophozoites (right:  $\times 100$ ).



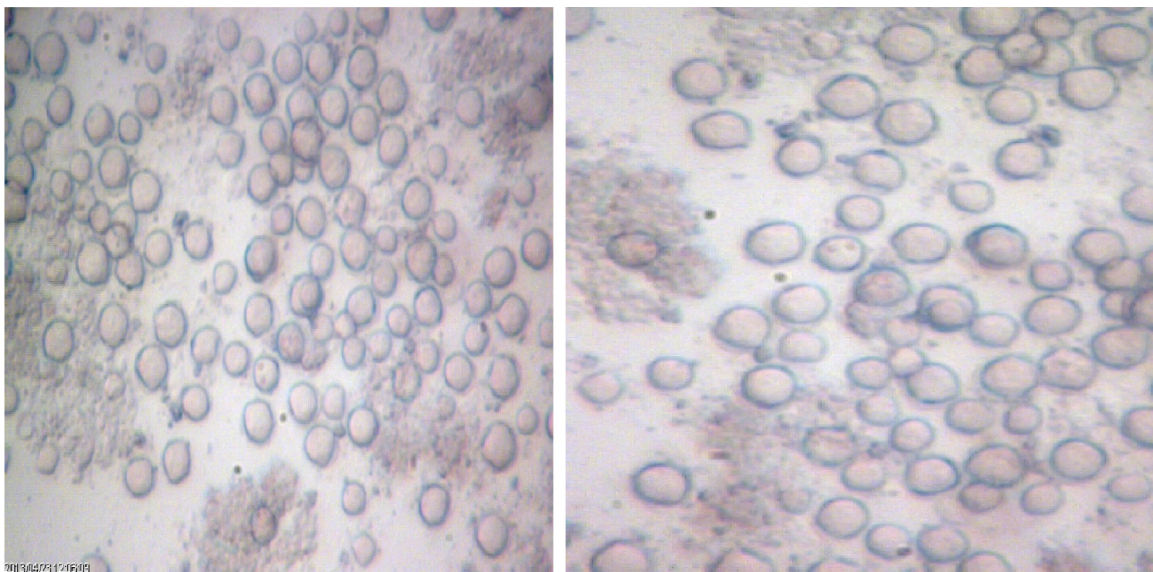


**Figure 3.** *Acanthamoeba* belonging to T4 genotype with star-shaped endocysts (left:  $\times 100$ ; right:  $\times 400$ ) isolated from tap water in the north of Kish Island, Iran.

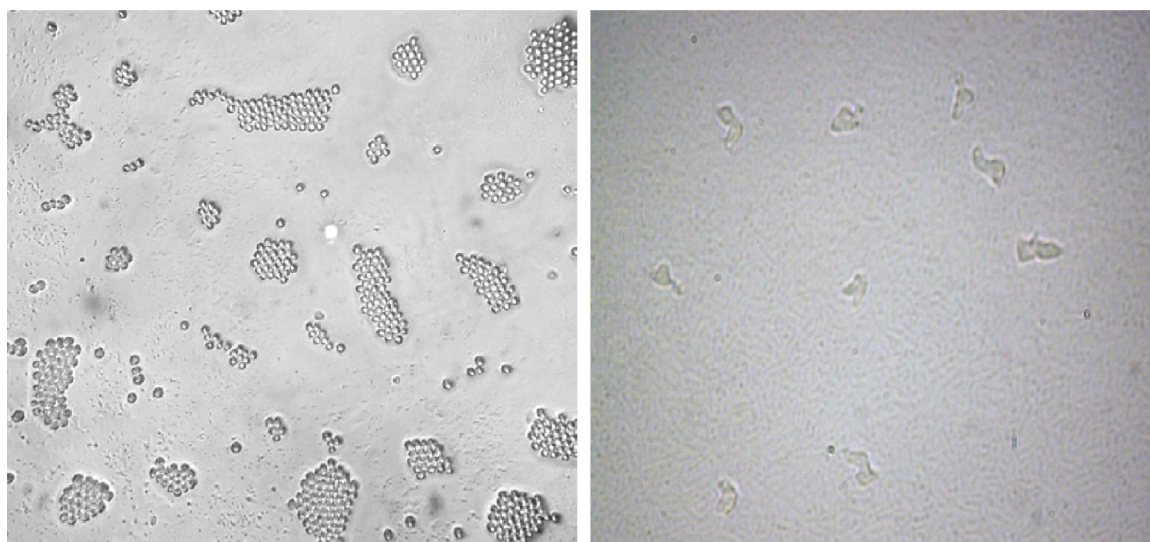
revealed the presence of various genotypes, including T3 (NL15, NL19) (corresponding to *A. griffini*) (14.2%), T4 (57.1%), T5 (NL14, NL18, NL33) (corresponding to *A. lentikulata*) (21.42%), and T11 (NL17) (7.1%) (Table 2). T4 was the most prevalent detected genotype. It should be mentioned that no *Balamuthia*, *Vahlkampfiids*, or *Sappinia* isolates were detected in this survey.

This work was performed to detect the presence of FLA in the tap water supplies in Kish Island, southern Iran. The obtained results revealed that 38.2% of the tested water sources were contaminated with potentially pathogenic amoebae. This may highlight that, despite the filtration, chlorination, and treatment processes, amoebae are able to colonize the water distribution systems in the area probably by biofilm formation,

as previously suggested in other regions by Cabral et al.<sup>23</sup> The most contaminated water was in the northern region, which also presents one of the oldest water distribution systems on the Island. There are several reports regarding contamination of recreational water sources in Iran and around the world, including hot springs, recreational river waters, ponds, and swimming pools.<sup>21,22,24</sup> The range of contamination in different kinds of water sources has been recorded from 23% to even 89%.<sup>4,24,25</sup> Worldwide, a few studies have also shown the presence of potentially pathogenic *Acanthamoeba* spp. in tap water sources.<sup>23,26,27</sup> A study conducted by Hoffmann and Michel in Germany revealed that water sources are widely contaminated with FLA even after filtration processes.<sup>28</sup> In addition, Ozcelic et al showed that FLA were observed in 30% of tap



**Figure 4.** *Acanthamoeba* belonging to T5 genotype with round-shape endocysts ( $\times 400$ ) isolated from tap water in the north of Kish Island, Iran.



**Figure 5.** *Vermamoeba vermiformis* trophozoites (right) and cysts (left) isolated from tap water in the north of Kish Island, Iran.

and recreational water sources in Turkey.<sup>27</sup> However, chlorine may destroy other FLA, such as *Vahlkampfiids*, *Sappinia*, and *Balamuthia*. Water purification in Kish Island consists of both filtration and chlorination procedures. However, according to this study, these processes are not eliminating these amoebae, most likely because of the ability of these organisms to form resistant cysts. Notably, chlorine has been reported not to eliminate *Acanthamoeba* in the doses used for water treatment.<sup>7,10,11</sup> In addition, Thomas et al in 2008 showed that there is a high biodiversity of FLA and associated bacteria in water treatment plants. Their study reflected that potentially pathogenic amoebae were recovered after a treatment chain of sand filtration, ozonation, chlorination, and sand filtration.

Notably, pipe materials and pores of filters are also important in the retention and proliferation of such amoebae.<sup>29</sup> The presence of *Acanthamoeba* and *Vermamoeba* reflects that these FLA can survive the purification process and pass through the filter pores. Indeed, the pore size of the filtration membranes is not capable of preventing amoebae from passing through the pores due to the smaller size of the amoebae.

Accordingly, various *Acanthamoeba* genotypes have been reported in the present study. Moreover, all isolated genotypes have been recorded as agents of AK in Iran and worldwide. In previous research conducted by Niyati et al, genotypes belonging to T3, T4, and T11 were the causative agents of AK cases.<sup>16</sup>

**Table 2.** Location and distribution of free-living amoebae in tap water sources of Kish Island.

CODE	LOCALITY	ORIGIN	GENOTYPE	QUERY COVERAGE/MAX. IDENTITY	ACCESSION NO.
NL7	North	House	T4	94/97	KP337297
NL12	Center	Resort	T4	96/99	KP337304
NL14	North	House	T5	100/100	KP337299
NL15	North	House	T3	100/100	KP337292
NL17	East	House	T11	100/100	KP337301
NL18	Center	Resort	T5	100/100	KP337302
NL19	North	House	T3/ <i>V. vermiformis</i>	100/100	KP337303/ KP337306
NL26	Center	Resort	T4	96/99	KP337298
NL27	North	Hospital	T4/ <i>V. vermiformis</i>	97/96 99/99	KP337293/ KP337307
NL32	East	House	<i>V. vermiformis</i>	96/98	KP337308
NL33	North	House	T5	100/100	KP337300
NL34	North	House	T4	96/99	KP337305
NL36	South	Mall	T4	92/99	KP337294
NL37	West	Mall	T4	98/96	KP337295
NL48	North	House	T4	95/96	KP337296



Overall, the occurrence of 38.2% of potentially pathogenic FLA, including *Acanthamoeba* T3, T4, T5, and T11 genotypes and *Vermamoeba*, in tap water sources in this tourist region in Iran reflects the urgent need to improve water treatment procedures to prevent FLA-related infections.

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### Author Contributions

Conceived and designed the experiments and wrote the first draft of the manuscript: MN. Performed the experiments: ZL. Made critical revisions and approved the final version: JLM. All authors reviewed and approved of the final manuscript.

### REFERENCES

- Marciano-Cabral F, Cabral G. *Acanthamoeba* spp. as agents of disease in humans. *Clin Microbiol Rev.* 2003;16:273–307.
- Visvesvara GS, Moura H, Schuster FL. Pathogenic and opportunistic free living amoebae: *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*, and *Sappinia diploidea*. *FEMS Immunol Med Microbiol.* 2007;50:1–26.
- Khan NA. *Acanthamoeba*, Biology and Pathogenesis. Caister Academic Press, London; 2009.
- Lorenzo-Morales J, Miranda CA, Jimenez C, Tejedor ML, Valladares B, Ortega-Rivas A. Evaluation of *Acanthamoeba* isolates from environmental sources in Tenerife, Canary Islands, Spain. *Ann Agric Environ Med.* 2005;12:233–236.
- Yousuf FA, Siddiqui R, Subhani F, Khan NA. Status of free-living amoebae (*Acanthamoeba* spp., *Naegleria fowleri*, *Balamuthia mandrillaris*) in drinking water supplies in Karachi, Pakistan. *J Water Health.* 2013;11(2):371–375.
- Winck MA, Caumo K, Rott MB. Prevalence of *acanthamoeba* from tap water in rio grande do Sul, Brazil. *Curr Microbiol.* 2011;63(5):464–469.
- Wang H, Masters S, Hong Y. Effect of disinfectant, water age, and pipe material on occurrence and persistence of Legionella, mycobacteria, *Pseudomonas aeruginosa*, and two amoebas. *Environ Sci Technol.* 2012;46(21):11566–11574.
- Legarreta JE, Nau AC, Dhaliwal DK. *Acanthamoeba* keratitis associated with tap water use during contact lens cleaning: manufacturer guidelines need to change. *Eye Contact Lens.* 2013;39(2):158–161.
- Coşkun KA, Özçelik S, Tutar L, Elaldi N, Tutar Y. Isolation and identification of free-living amoebae from tap water in Sivas, Turkey. *Biomed Res Int.* 2013;2013:675145. doi: 10.1155/2013/675145.
- Khan NA. *Acanthamoeba*: biology and increasing importance in human health. *FEMS Microbiol Rev.* 2006;30:564–595.
- Hassan A, Farouk H, Hassanein F, Abdul-Ghani R, Abdelhady AH. *Acanthamoeba* contamination of hemodialysis and dental units in Alexandria, Egypt: a neglected potential source of infection. *J Infect Public Health.* 2012;5(4):304–310.
- Niyiyati M, Lorenzo-Morales J, Rezaie S, et al. First report of a mixed infection due to *Acanthamoeba* genotype T3 and *Vahlkampfia* in a cosmetic soft contact lens wearer in Iran. *Exp Parasitol.* 2010;126:89–90.
- Abedkhozasteh H, Niyiyati M, Rahimi F, Heidari M, Farnia S, Rezaeian M. First report of *Hartmannella* keratitis in a cosmetic soft contact lens wearer in Iran. *Iran J Parasitol.* 2013;8(3):481–485.
- Rezaeian M, Farnia SH, Niyiyati M, Rahimi F. Amoebic keratitis in Iran (1997–2007). *Iran J Parasitol.* 2007;2:1–6.
- Rezaeian M, Niyiyati M. *Pathogenic Free Living Amoebae*. Tehran: Tehran University of medical Sciences; 2011.
- Niyiyati M, Lorenzo-Morales J, Rezaie S, et al. Genotyping of *Acanthamoeba* isolates from clinical and environmental specimens in Iran. *Exp Parasitol.* 2009;121:242–245.
- Movahedi Z, Shokrollahi MR, Aghaali M, Heydari H. Primary amoebic meningoencephalitis in an Iranian infant. *Case Rep Med.* 2012;2012:782854. doi: 10.1155/2012/782854.
- Page FC. *A New Key to Freshwater and Soil Gymnamoebae*. Ambleside, UK: Freshwater Biological Association; 1988.
- Lasjerdi Z, Niyiyati M, Haghghi A, et al. Potentially pathogenic free-living amoebae isolated from hospital wards with immunodeficient patients in Tehran, Iran. *Parasitol Res.* 2011;109(3):575–580.
- Schroeder JM, Booton GC, Hay J, et al. Use of subgenetic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of *Acanthamoeba* from humans with keratitis and from sewage sludge. *J Clin Microbiol.* 2001;39:1903–1911.
- Nazar M, Haghghi A, Niyiyati M, et al. Genotyping of *Acanthamoeba* isolated from water in recreational areas of Tehran, Iran. *J Water Health.* 2011;9(3):603–608.
- Niyiyati M, Lasjerdi Z, Nazar M, Haghghi A, Nazemalhosseini Mojarad E. Screening of recreational areas of rivers for potentially pathogenic free-living amoebae in the suburbs of Tehran, Iran. *J Water Health.* 2012;10(1):140–146.
- Marciano-Cabral F, Jamerson M, Kaneshiro ES. Free-living amoebae, *Legionella* and *Mycobacterium* in tap water supplied by a municipal drinking water utility in the USA. *J Water Health.* 2010;8:71–82.
- Kilvington S, Gray T, Dart J, Morlet N, Beeching JR, Frazer Matheson M. *Acanthamoeba* keratitis: the role of domestic tap water contamination in the United Kingdom. *Invest Ophthalmol Vis Sci.* 2004;45:165–169.
- Shoff ME, Rogerson A, Kessler K, Schatz S, Seal DV. Prevalence of *Acanthamoeba* and other naked amoebae in south Florida domestic water. *J Water Health.* 2008;6:99–104.
- Thomas V, Loret JF, Jousset M, Greub G. Biodiversity of amoebae and amoebae-resisting bacteria in a drinking water treatment plant. *Environ Microbiol.* 2008;72:2428–2438.
- Ozçelik S, Coşkun KA, Yünlü O, Alim A, Malatyalı E. The prevalence, isolati and morphotyping of potentially pathogenic free-living amoebae from tap water and environmental water sources in Sivas. *Turkiye Parazit Derg.* 2012;36(4):198–203.
- Hoffmann R, Michel R. Distribution of free living amoebae during propagation and supply of drinking water. *Int J Hyg Environ Health.* 2001;203:215–219.
- Loret JF, Jousset M, Robert S, et al. Amoebae-resisting bacteria in drinking water: risk assessment and management. *Water Sci Technol.* 2008;58(3):571–577. doi: 10.2166/wst.2008.423.