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High occurrence of *Acanthamoeba* spp. in the water samples of public swimming pools from Kerman Province, Iran

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ABSTRACT

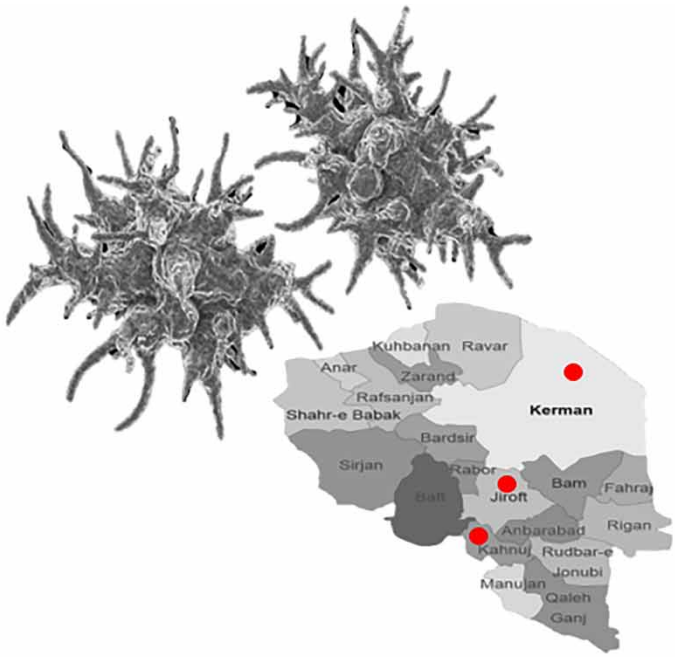
Acanthamoeba spp. is a free-living amoeba that can cause major infections in humans, including keratitis and granulomatous encephalitis. Thus, water resources play an important role in transmitting *Acanthamoeba* spp. infection to humans. The purpose of this study was to investigate the presence of *Acanthamoeba* spp. in public swimming pools from three cities of Kerman Province, southeastern Iran. Eighty water samples of 20 public indoor swimming pools were taken from Kerman, Jiroft, and Kahnauj cities. Water temperature (°C), pH, and free chlorine concentration (ppm) were measured. Filtration and cultivation were applied on non-nutrient agar medium. The polymerase chain reaction was applied by using the genus-specific primers (JDP1 and JDP2) on positive samples; these primers can amplify the 423–551 bp fragment. Eighteen of the 20 swimming pools (including 32/80; 40% samples) were contaminated with *Acanthamoeba* spp. All swimming pools of Jiroft and Kahnauj and 88.2% of swimming pools in Kerman were contaminated. As such, all 32 *Acanthamoeba* isolates were amplified using the JDP primer pairs. Two genotypes, T3 and T4, were also identified. The present research is the first to report *Acanthamoeba* spp. in public swimming pools from Kerman Province. Due to high occurrence of this protozoan, it is recommended to use warning signs around swimming pools to create awareness of this infection.

Key words: *Acanthamoeba*, Iran, swimming pool

HIGHLIGHTS

- This is the first study on the occurrence of *Acanthamoeba* spp. in public swimming pools from Kerman Province.
- Eighteen of the 20 swimming pools (including 32/80; 40% samples) were contaminated with *Acanthamoeba* spp.
- It is recommended to use warning signs around swimming pools to raise awareness of this infection.

GRAPHICAL ABSTRACT



***Acanthamoeba* shadow on public swimming pools from three cites of Kerman province (Kerman, Jiroft, and Kahnuj).**

1. INTRODUCTION

Free-living amoebae from the genus *Acanthamoeba* have a broad global distribution and can be found in a wide range of environmental sources such as water, soil, and air (Trabelsi *et al.* 2012; Niyyati & Rezaeian 2015). *Acanthamoeba* spp. has a biphasic life cycle that exhibits two distinct forms: a vegetative form (trophozoite) and a resistant form (cyst) (Paknejad *et al.* 2020; de Lacerda & Lira 2021). In favorable conditions, trophozoite forms are identified by having spine-like structures (known as acanthopodia) (Khan 2001). In this stage, trophozoites are able to replicate, move, feed, or adhere to the host cell surface through acanthopodia (Khan 2003; de Lacerda & Lira 2021). During the unfavorable condition, *Acanthamoeba* spp. can be surrounded by a resistant double-walled cyst structure (Gabriel & Panaligan 2020). These cysts withstand adverse environmental conditions such as very high or low temperatures, severe dryness, ultraviolet (UV) radiation, the presence of chemical compounds (i.e., accumulation of metabolic products and toxins), disinfectant solutions, changes in osmolarity, and pH (Aksozek *et al.* 2002; Dudley *et al.* 2005; Lonnen *et al.* 2010; Taher *et al.* 2018).

Up to now, using molecular techniques based on the 18S rDNA gene region, *Acanthamoeba* spp. is classified into 20 genotypes (T1–T20) (Taher *et al.* 2018; Chelkha *et al.* 2020). It is suggested that some genotypes play a potential role in human pathogenesis, especially in immunocompromised individuals (Memari *et al.* 2017; Megha *et al.* 2018), and are capable of causing granulomatous amoebic encephalitis (GAE) (Megha *et al.* 2018), *Acanthamoeba* keratitis (AK) (Risler *et al.* 2013; Khosravinia *et al.* 2021), and cutaneous lesions (Morrison *et al.* 2016). Among the genotypes, T4 is the most common genotype isolated from clinical (AK, GAE) and environmental specimens (Marciano-Cabral & Cabral 2003; Mirjalali *et al.* 2013; Megha *et al.* 2018); nevertheless, some studies have reported other genotypes such as T2, T3, T5, T6, T11, and T12 for AK (Seal 2003; Risler *et al.* 2013) and some GAE-related genotypes including T1, T3, T10, and T12 (Khan 2006; Megha *et al.* 2018).

According to published studies, most reports of AK occur after water exposure or a history of swimming in lakes and pools while wearing soft contact lenses (Ibrahim *et al.* 2007; Lindsay *et al.* 2007). Therefore, these environmental sources can be considered as major potential risks for human infection. Since there was no information regarding the status of *Acanthamoeba* spp. in Kerman Province in the southeastern region of Iran, the main aim of the present study was to investigate the presence of *Acanthamoeba* using morphological and molecular tools in public swimming pools from three cities of Kerman Province (Kerman, Jiroft, and Kahnauj).

2. MATERIALS AND METHODS

2.1. Sampling

During June–August 2018, a total of 80 water samples (~500 mL for each sample) were collected into sterile bottles from 20 public indoor swimming pools of three cities of Kerman Province in the southeastern region of Iran (Figure 1). These cities were Kerman (17 swimming pools including 68 samples), Jiroft (two swimming pools including eight samples), and Kahnuj (one swimming pool including four samples) (Table 1). Two sampling stations, 1 m from the pool wall and the middle of the pool, were selected, as listed in Table 1. Moreover, the water temperature (°C), pH, and free chlorine concentration (ppm) were measured during sampling. Then, all of the samples were immediately transported to the Laboratory of Parasitology and Mycology, Department of Kerman University of Medical Sciences, Iran.

2.2. Filtration, cultivation, and microscopy detection

At first, the samples were thoroughly stirred to mix the contents. After shaking, each water sample was filtered through an autoclaved 2.5 μm pore filter size (Whatman, Grade 42, UK) by mild suction using a vacuum or pressure pump (a new sterile membrane was used for each water sample) (Solgi *et al.* 2012). Then, the filters were transferred onto 1.2% non-nutrient agar (NNA) medium covered with autoclaved *Escherichia coli* K12 (HB101), a noninvasive laboratory strain (Tanveer *et al.* 2013). All plates were sealed with parafilm and the three plates per sample were incubated at 30, 37, or 42 °C for up to 30 days (Solgi *et al.* 2012). The subculture was performed for all the positive plates on fresh NNA plates to obtain pure and noncontaminated isolates (Solgi *et al.* 2012). Finally, the microscopic detection of *Acanthamoeba* was conducted based on Page keys (Page 1988) using inverted microscopy.

2.3. DNA extraction and polymerase chain reaction amplification

Trophozoites and cysts were harvested from plates and washed using sterile phosphate-buffered saline (PBS, pH 7.4). Next, the extraction of DNA was performed using the DNAeasy kit (Qiagen, Basel, Switzerland) according to the manufacturer's instructions.

DNA was amplified by using the genus-specific primers (JDP1 and JDP2) for 18 s rRNA of *Acanthamoeba* spp. (Schroeder *et al.* 2001; Behniafar *et al.* 2015), including the primer pair JDP1 (5′-GGCCCAGATCGTTTACCGTGAA-3′) as forward primer and JDP2 (5′-TCTCACAAGCTGCTAGGGAGTCA-3′) as reverse primer (Schroeder *et al.* 2001). These primers can amplify the 423–551 bp fragment. Then, a total of 20 μL volume of polymerase chain reaction (PCR) were fixed by using 10 μL (2×) of Taq Master Mix (Cinnagen, Iran), 25 pmol of each primer, distilled water, and 2 μL of DNA template. The PCR was carried out under the following conditions: 45 cycles of denaturation at 95 °C for 1 min, followed by 45 repetition cycles at 95 °C for 1 min, annealing at 60 °C for 1 min and 72 °C for 2 min, and final extension at 72 °C for 5 min. Finally, PCR

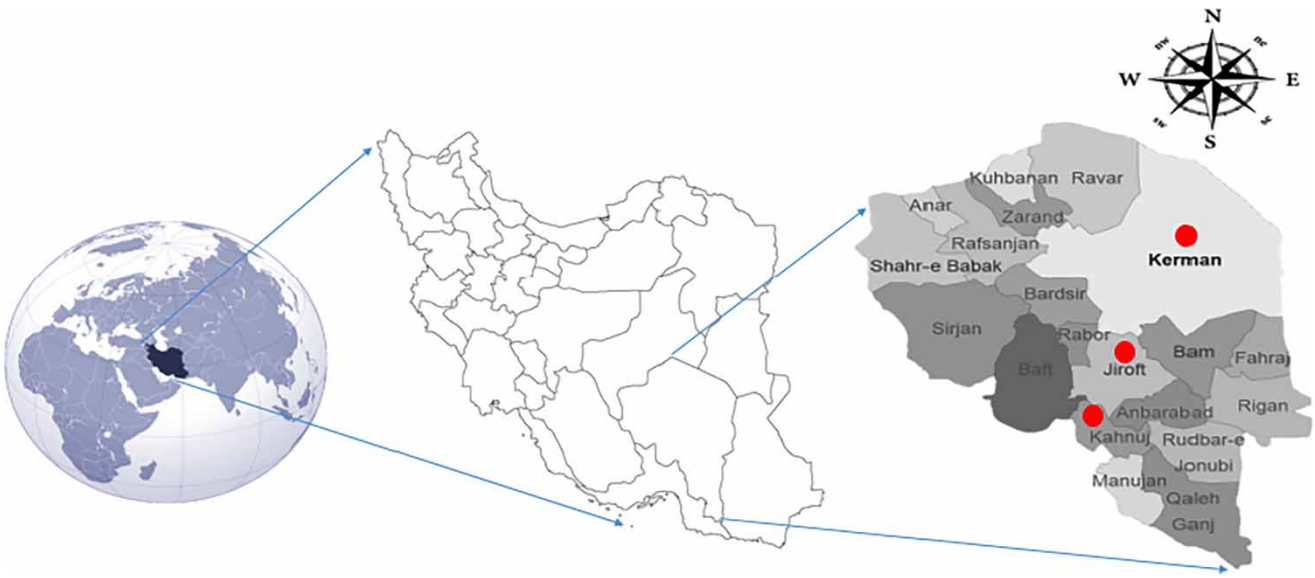


Figure 1 | Geographic map of three cities (Kerman, Jiroft, and Kahnauj) of Kerman Province in the southeastern region of Iran.

Table 1 | Location and distribution of *Acanthamoeba* spp. in public swimming pools from three cities of Kerman Province

Cities	Number of swimming pools	Sampling location (number of samples)	pH	Chlorine concentration (ppm)	Temperature (°C)	*Acanthamoeba* positive
Kerman	1	In the middle of the pool (2)	8	1.5	31	1
		One meter away from the pool wall (2)	7.4	0.5	28	0
	2	In the middle of the pool (2)	7.8	1.5	32	0
		One meter away from the pool wall (2)	7.7	2.5	31	1
	3	In the middle of the pool (2)	7.8	0.5	32	1
		One meter away from the pool wall (2)	7.4	0.5	35	0
	4	In the middle of the pool (2)	7.8	1	32	0
		One meter away from the pool wall (2)	7.6	1.5	32	0
	5	In the middle of the pool (2)	7.6	2.5	31	1
		One meter away from the pool wall (2)	7.8	1.5	30	1
	6	In the middle of the pool (2)	7.4	2	31	1
		One meter away from the pool wall (2)	7.2	1	33	0
	7	In the middle of the pool (2)	7.8	1.5	27.5	1
		One meter away from the pool wall (2)	7.8	2	32	1
	8	In the middle of the pool (2)	7.8	1	34	0
		One meter away from the pool wall (2)	7.5	1.5	31	1
	9	In the middle of the pool (2)	8.2	2.5	31	1
		One meter away from the pool wall (2)	7.6	1	28	1
	10	In the middle of the pool (2)	7.8	2.5	27	0
		One meter away from the pool wall (2)	7.5	1.5	33	2
	11	In the middle of the pool (2)	8.2	2.5	32	1
		One meter away from the pool wall (2)	7.8	3	30	1
	12	In the middle of the pool (2)	8	1	30	1
		One meter away from the pool wall (2)	7.8	2	30	0
	13	In the middle of the pool (2)	7.8	2	33	0
		One meter away from the pool wall (2)	7.5	1	32	2
	14	In the middle of the pool (2)	7.8	2	30	1
		One meter away from the pool wall (2)	7.8	2.5	30	0
	15	In the middle of the pool (2)	7.6	1.5	32	2
		One meter away from the pool wall (2)	7.8	0.8	31.5	1
	16	In the middle of the pool (2)	8	3	30	1
		One meter away from the pool wall (2)	7.8	1	32	0
	17	In the middle of the pool (2)	7.6	1.5	32	0
		One meter away from the pool wall (2)	7.5	1.5	28	0
Jiroft	18	In the middle of the pool (2)	7.8	2	22	2
		One meter away from the pool wall (2)	7.6	2	27	2
	19	In the middle of the pool (2)	7.6	1	26	2
		One meter away from the pool wall (2)	7.4	1	27	1
Kahnuj	20	In the middle of the pool (2)	7.6	0.7	27	1
		One meter away from the pool wall (2)	7.8	1	27	1

products were electrophoresed by using 1.5% agarose gel stained with an ethidium bromide solution and visualized under UV light.

2.4. Sequencing and genotype identification

In this stage, PCR products (randomly) were prepared for sequencing using a sequencer machine (Applied Biosystems® 3130X Automatic Genetic Analyzer) followed by performing homology analysis using the Basic Local Alignment Search Tool (BLAST) program to compare the identified sequence with those available in the GenBank sequence database.

3. RESULTS

As shown in Table 1, a high abundance of potentially pathogenic *Acanthamoeba* spp. was detected in public swimming pools included in this research. Accordingly, 18 of the 20 swimming pools (including 32/80; 40% positive samples) were

contaminated with *Acanthamoeba* spp.; among them, all swimming pools of Jiroft and Kahnauj cities and 88.2% of swimming pools in Kerman city were contaminated (Table 1). Considering Page keys (Page 1988), *Acanthamoeba* genus was identified by the presence of double-walled cysts with various shapes of endocysts, such as stars and triangles (inner wall often polygonal or stellate and outer wall often rippled or wrinkled with an approximate diameter of 15–20 μm), and flat trophozoites with acanthopodia (finger-like tapering pseudopodia) (Figure 2). According to the sampling station, 42.5% (17/40 samples) and 37.5% (15/40 samples) of infections were detected in the middle and within 1 m away from the pool wall, respectively (Table 1). Moreover, the frequency of *Acanthamoeba* spp. is listed in Table 1 based on temperature, pH, and chlorine concentration.

All 32 *Acanthamoeba* isolates were amplified the 423–551 bp product using the JDP primer pairs. According to the reference (Schroeder *et al.* 2001), *Acanthamoeba* species can be identified based on band size (Table 2). In this regard, 22 out of 32 samples (68.75%) were amplified in the band size of 450 bp, and the rest were amplified in the sizes ranging between 500 bp (18.75%) and 550 bp (12.5%) (Table 2). Some of the amplified samples are shown in Figure 3. Three random samples (one sample from each band size) were sequenced, of which two isolates belonged to T3 (*A. griffini*) and one isolate belonged to T4 (*A. hatchetti*). As such, all three samples were submitted in the GeneBank under the accession numbers MT292605, MT292606, and MT292607.

4. DISCUSSION

Although there are numerous reports of *Acanthamoeba* spp. in swimming pool water samples by various researchers around the world (Magnet *et al.* 2012; Mahmoudi *et al.* 2012), only sporadic studies have been performed on *Acanthamoeba* spp. in different parts of Iran. Recently, a meta-analysis study on the prevalence of *Acanthamoeba* spp. was published in Iran (Motavallihaghi *et al.* 2019), which showed that no studies have been conducted in Kerman Province so far. Therefore, providing results from the status of this free-living amoeba in Kerman Province can be important for policymakers and health officials in order to control and prevent this protozoan. The current study has highlighted that all swimming pools in Jiroft and Kahnauj cities and 88.2% of swimming pool water sources in Kerman city have been contaminated by *Acanthamoeba* spp. This prevalence rate is almost congruent with reports from previous studies in the river water samples of Guilan (88%) (Mahmoudi *et al.* 2015) and reports on surface water samples of Shiraz (99.6%) (Ghalehbin *et al.* 2014) in Iran. Moreover, the present contamination rate was higher than results obtained from river waters of Tonekabon (23%)

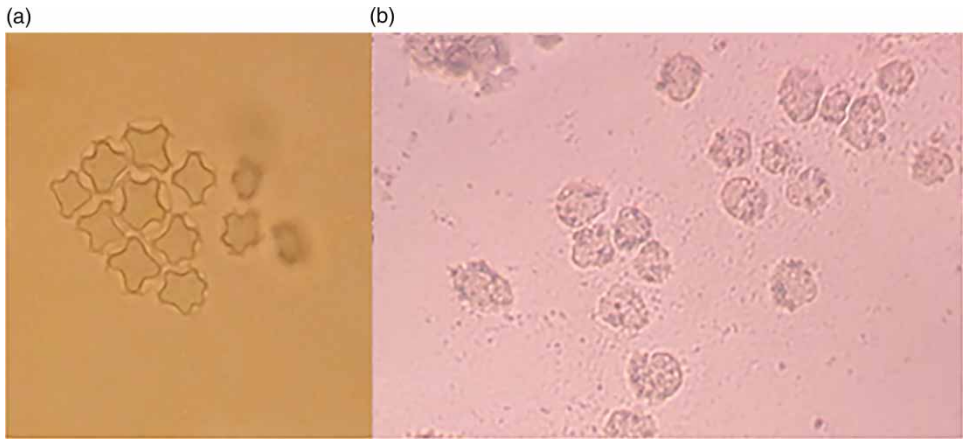


Figure 2 | *Acanthamoeba* cysts (a) ×100 and trophozoites (b) ×100.

Table 2 | Frequency of DNA fragments based on *Acanthamoeba*-specific primer (JDP1 and JDP2) in the present study

Band size	450 bp	500 bp	550 bp
Frequency *n* (%)	22 (68.75)	6 (18.75)	4 (12.5)
Acanthamoeba species	*A. castellani*, *A. polyphaga*, *A. hatchetti*, *A. culbertsoni*	*A. comandoni*, *A. griffni*	*A. astronyxis*

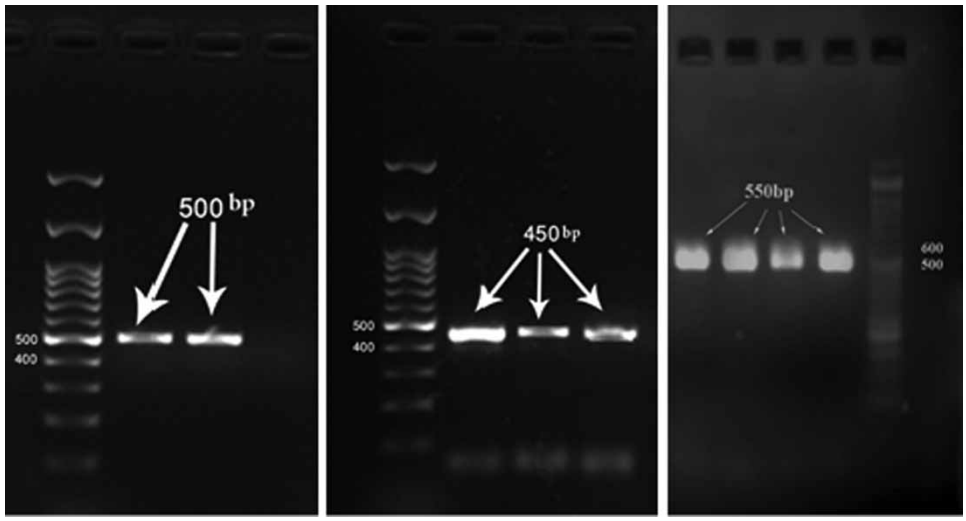


Figure 3 | PCR product of water samples from swimming pools based on the band size.

(Mataji Bandpei & Khataminejad 2016), hot springs of Ardebil (3.6%) (Badirzadeh *et al.* 2011), and surface water sources of Birjand (38%) (Behravan *et al.* 2015) in Iran. In comparison with other countries, the contamination rate of *Acanthamoeba* spp. stated in the present study was higher than tap water from Brazil (9.5%) (Winck *et al.* 2011), river water samples from Turkey (44%) (Koyun *et al.* 2020), and environmental water samples from China (14.68%) (Lass *et al.* 2017). Therefore, all of these studies indicate that *Acanthamoeba* spp. is found in a broad range of water sources.

In the present study, the high prevalence of *Acanthamoeba* spp. could be a serious health risk, especially for high-risk groups (e.g., immunocompromised individuals and those who wear contact lenses) in this area. Therefore, awareness of high-risk individuals regarding the avoidance of contact with untreated and contaminated water, as well as swimming in public pool water while wearing contact lenses, could prevent *Acanthamoeba*-related infections such as AK and GAE (Niyyati *et al.* 2014; Poor *et al.* 2018). In addition, as shown by the results of molecular examinations and sequencing, the presence of major species and genotypes (T3 and T4) greatly increases the importance of attention and awareness of this microorganism. As such, one of the most important reasons for controlling *Acanthamoeba* spp. in different water samples is that this parasite can be used as a substrate for the survival, growth, and transmission of bacteria (i.e., *Legionella*, *Mycobacteria*, and *Vibrio* species) (Denet *et al.* 2017; Muchesa *et al.* 2017); thus, the co-existence of *Acanthamoeba* spp. with opportunistic bacteria may pose a potential health risk to immunocompromised individuals.

Although we used a specific primer to determine the different species of *Acanthamoeba* spp., it is recommended to use the sequencing technique due to the precise determination of genotypes. In this study, due to limited financial resources, only three positive samples were sequenced.

5. CONCLUSION

Overall, the present study reveals a significant occurrence of potentially pathogenic *Acanthamoeba* spp. in the water of the public swimming pools from Kerman Province. The use of improved filtration methods in various water sources by filters with smaller pores could be of greater importance in order to prevent water contamination. Moreover, it is recommended to use warning signs around swimming pools to make people aware of this infection.

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AUTHORS' CONTRIBUTIONS

All authors contributed to study design. K.S. and R.E.-K. contributed to all parts of the study. Z.B. and R.E.-K. contributed to study implementation. H.R. and A.A. collaborated in the analysis and interpretation of data. A.T. and K.S. collaborated in the

manuscript writing and revision. All the authors commented on the drafts of the manuscript and approved the final version of the article.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding the publication of this article.

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ETHICAL APPROVAL

This study was approved by the Jahrom University of Medical Sciences Ethics Committee (ethical approval ID: IR.JUMS.REC.1396.139).

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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