Thermotolerant *Acanthamoeba* spp. isolated from therapeutic hot springs in northwestern Iran

Rahmat Solgi, Maryam Niyyati, Ali Haghighi, Niloofar Taghipour, Seyyed Javad Seyyed Tabaei, Mohamad Eftekhar and Ehsan Nazemalhosseini Mojarad

ABSTRACT

This study was conducted to address the distribution of *Acanthamoeba* genotypes in therapeutic hot springs in Iran. Sixty water and sediment samples were collected from bicarbonate, sulphur, and sodium chloride thermal springs in the northwest. All hot springs examined are used mainly for health purposes in Iran. *Acanthamoeba* were identified by both morphology and PCR (polymerase chain reaction). Genotype identification was based on the sequencing of a highly variable and informative region of Diagnostic Fragment 3 (stem 29–1 of 18S rRNA gene) within *Acanthamoeba*-specific amplimer (ASA.S1). Twenty percent of hot springs were contaminated with thermotolerant *Acanthamoeba* belonging to the potentially pathogenic T4 and T3 genotypes. A high number (91.7%) of strains showed growth at 37 °C, and eight isolates showed growth at 42 °C. A single isolate (HSNW2) was detected in waters at 70 °C. The presence of thermotolerant *Acanthamoeba* highlights a risk factor for susceptible individuals, as *Acanthamoeba*-related keratitis continues to rise in Iran. Periodic surveillance of thermal waters as well as improved filtration and disinfection is recommended to prevent disease related to pathogenic *Acanthamoeba*. This is the first comprehensive molecular study of *Acanthamoeba* genotypes in hot springs in Iran and the first to report the occurrence of the T3 genotype (corresponding to *Acanthamoeba griffini*) in thermal water sources in this country.

Key words | *Acanthamoeba* spp., hot springs, Iran, sequencing

Rahmat Solgi
Maryam Niyyati (corresponding author)
Ali Haghighi
Niloofar Taghipour
Seyyed Javad Seyyed Tabaei
Mohamad Eftekhar
Department of Medical Parasitology and Mycology,
School of Medicine,
Shahid Beheshti University of Medical Sciences,
Tehran,
Iran
E-mail: *maryamniyati@yahoo.com*

Ehsan Nazemalhosseini Mojarad
Research Center for Gastroenterology and Liver Diseases,
Shahid Beheshti University of Medical Sciences,
Tehran,
Iran

INTRODUCTION

Free-living amoebae (FLA) include opportunistic protozoans that can cause severe disease, including fatal encephalitis, meningoencephalitis, and painful keratitis (Marciano-Cabral & Cabral 2003; Visvesvara *et al.* 2007). Among many taxa of FLA, *Acanthamoeba*, with 17 identified genotypes (T1-T17), is ubiquitous in aquatic habitats (Khan 2006; Nuprasert *et al.* 2010). *Acanthamoeba* has widespread distribution in lakes, ponds, streams, hydrotherapy pools, thermally polluted waters, ocean sediments, and coastal waters (Kilvington *et al.* 2004; Khan 2009; Marciano-Cabral *et al.* 2010). These amoebae, especially those with more pathogenic potential, can tolerate extremes of pH, osmolarity, and temperature (Khan 2009). Therefore thermal waters including jacuzzis, hot tubs, and hot springs may be favourable habitats for pathogenic *Acanthamoeba* strains (Lekkla *et al.* 2005; Gianinazzi *et al.* 2010; Huang & Hsu 2010). *Acanthamoeba* may also be a suitable carrier for pathogenic microorganisms such as *Legionella* (Thomas *et al.* 2008).

Reports of FLA-related keratitis have become more frequent in Iran during recent decades due mainly to increasing number of contact lens wearers and insufficient education regarding lens maintenance (Maghsood *et al.* 2005; Niyyati *et al.* 2009). Summer produces higher

doi: 10.2166/wh.2012.032

numbers of reported amoebic keratitis (AK) cases among contact lenses users associated with water-related activities.

Acanthamoeba belonging to various genotypes, including T2, T3, T4 and T11, have been found to be the leading cause of AK in Iran (Maghsood *et al.* 2005; Niyyati *et al.* 2009). Other FLA, such as *Vahlkampfia*, have also been found to be an agent of keratitis in this region. However, since the latter reported case exhibited both the *Acanthamoeba* T3 genotype and *Vahlkampfia*, the causality is not clear (Niyyati *et al.* 2010). There have been no reports of other human disease associated with FLA in Iran. Thus, there is an urgent need for more comprehensive research regarding these potentially pathogenic FLA.

Mineral waters and hot springs are popular for recreation and therapeutic purposes worldwide (Brown *et al.* 1983; Rivera *et al.* 1989; Kuroki *et al.* 1998; Sukthana *et al.* 2005; Hsu *et al.* 2009; Huang & Hsu 2010). Hot springs and mineral waters are frequently accessed as supportive treatment of arthritis, skeletal disorders, and gastrointestinal disorders. Chemical composition and mineral concentration define the specific properties of various hot springs (Chou 2004).

Ardebil Province in the northwest of the country is a region of natural and man-made hot springs and spas, attracting seven million tourists annually (Mahdizadeh & Panjaliasl 2003). Most hot springs are natural hot water systems without filtration; therefore the occurrence of thermotolerant *Acanthamoeba* spp. is likely. Indeed, cases of keratitis after using thermal water have increased in this region (Mahdizadeh & Panjaliasl 2003).

The extent to which *Acanthamoeba* spp. are present in water including waterfalls, ponds, and cold streams in Iran and worldwide has been previously reported (Tsvetkova *et al.* 2004; Maghsood *et al.* 2005; Niyyati *et al.* 2009; Marciano Cabral *et al.* 2010). In Iran, a single study has shown the presence of FLA in hot springs identified mainly from a morphological study (Badirzadeh *et al.* 2011). However, no comprehensive information on the presence of *Acanthamoeba* genotypes in hot springs of Iran is available. The major aim of the present study was to determine the presence of *Acanthamoeba* genotypes in hot springs, using a culture enrichment method and *Acanthamoeba*-specific amplimer (ASA.S1) sequencing.

MATERIAL AND METHODS

Geographical information of Ardebil Province

Ardebil Province is located in northwestern Iran (Figure 1). The area has a cold climate throughout the year and the average temperature is 15 °C. However, it attracts many tourists due to the presence of as many as 30 natural and man-made hot springs and spas serving recreational and health purposes, depending on chemical composition (bicarbonate, sodium chloride, and sulphur springs) and mineral concentration. Nearly all hot springs are used for health purposes for various diseases including skeletal and skin disorders. The water of some of these hot springs is ingested as a treatment for gastrointestinal disorders.

Sample collection and water analysis

Sixty samples were collected from surface water (<10 cm below) (30) and sediments (30) of natural and man-made hot springs in cities in Ardebil Province (Figure 1). Thirteen bicarbonate springs, 6 sodium chloride, and 11 sulphur springs were examined for the presence of *Acanthamoeba* spp. All hot springs included are used for both recreation and for therapeutic purposes. Briefly, 500–1,000 ml of water was placed into sterile bottles and transported to the Department of Parasitology and Mycology, School of Medicine, Shahid Beheshti University of Medical Sciences, Iran. Sediments were also obtained from the bottom of each spring. Water temperature and pH were measured *in situ* using a portable pH meter.

Processing of the samples and culture method

Samples were first thoroughly stirred and filtered using cellulose nitrate membranes with a 1.2 μm pore size. Three cultures of each sample were performed. The filters were transferred onto 1% non-nutrient agar plates overlaid with autoclaved *Escherichia coli*. All plates were sealed and the three plates per sample were incubated at 30, 37, or 42 °C for 30 days. Microscopic detection of amoebae was conducted according to Page's key using inverted microscopy (Page 1988). Cloning of positive plates was done by single cell dilution in order to eliminate bacterial and fungal

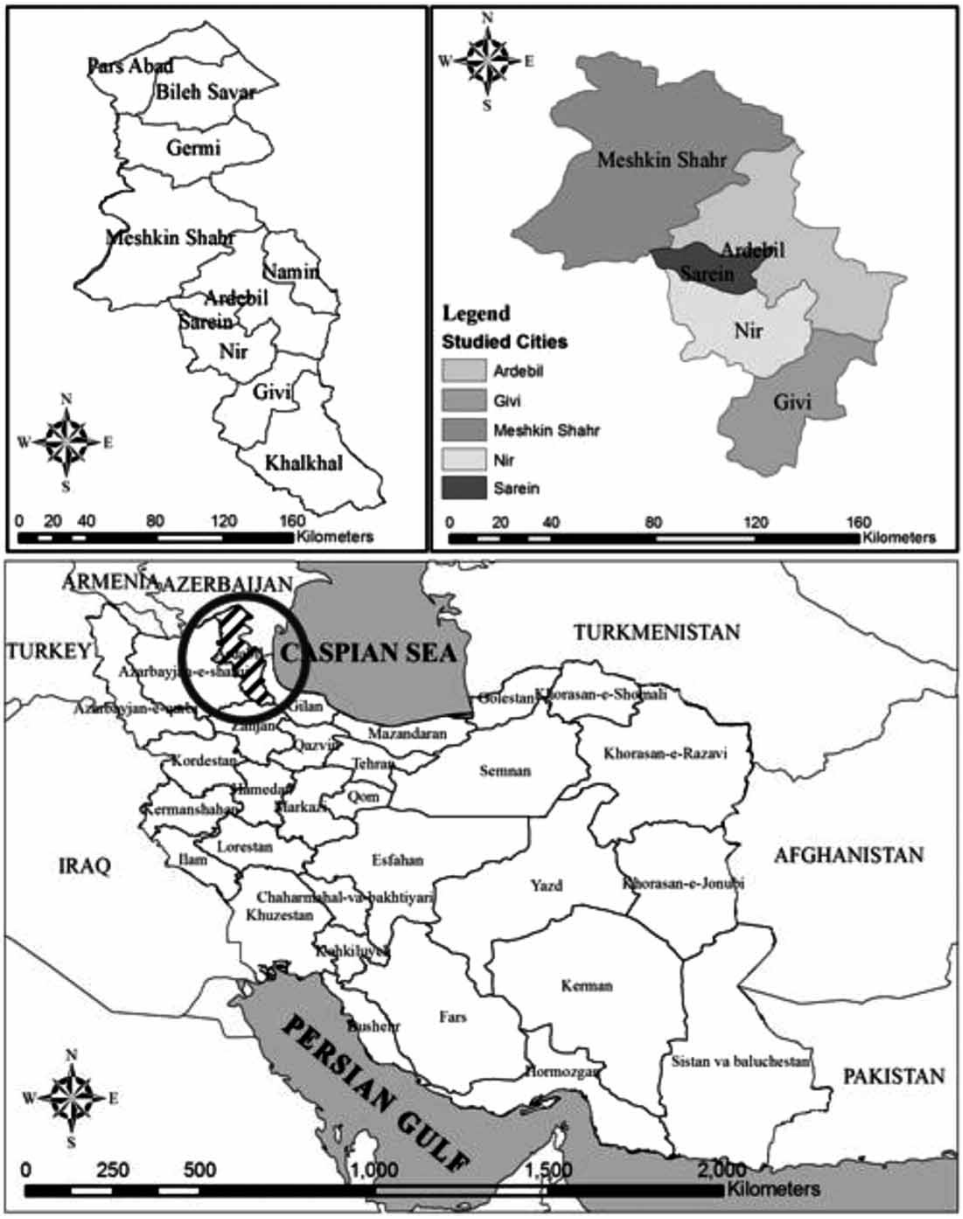

Figure 1 | Map showing Ardebil Province in northwestern Iran, the investigated cities are shown (right) (Created by Arc GIS version 9.3).

contamination according to previous studies (Lorenzo-Morales *et al.* 2005).

Diagnostic Fragment 3 amplification (stem 29–1 of 18S rRNA gene) and gel electrophoresis

Amoebae were harvested from plates and washed using phosphate-buffered saline (PBS pH 7). Extraction of DNA was performed using the Instagene matrix (Chelex; Biorad) according to manufacturer's instruction with minimal modifications. Briefly, approximately 10^3 cells were incubated with 50 µl Chelex. Incubation was at 56 °C for 20 min, followed by 10 min incubation in boiling water. A DNA pellet was obtained by centrifuging the samples at 10,000 g for 5 min and the supernatant was used as the DNA template for the polymerase chain reaction (PCR). DNA extractions for some isolates with higher numbers of cysts were performed using a modified phenol-chloroform

method according to our previous study (Niyyati *et al.* 2009; Lasjerdi *et al.* 2011).

The PCR mixture was performed in 30 μl Ampliqone (Taq DNA Polymerase Master Mix Red, Denmark) as a ready-made solution. Briefly, 25 μl of master mix with 5 ng DNA templates and 20 pmol primers were combined to achieve a volume of 30 μl. Genus-specific primer pairs (JDP1 and JDP2) were used to amplify a fragment of 18S rRNA gene (*Acanthamoeba*-specific amplimer, ASA1) that contains a subset of nuclear small subunit ribosomal RNA gene (Diagnostic Fragment 3) (Schroeder *et al.* 2001; Booton *et al.* 2002). Each PCR cycling condition included 35 cycles of denaturation at 94 °C for 1 min, followed by 35 repetition cycles at 94 °C for 35 s, annealing at 56 °C for 45 s, and extension at 72 °C for 1 min.

Gel electrophoresis was conducted to fractionate PCR products using 1.5% agarose gel stained with a solution of ethidium bromide (25 mg ml^{-1}) and examined under ultra violet (UV) illumination.

Sequencing, genotype identification, and multi-alignment

PCR-products were subjected to sequencing using an ABI 3130X automatic sequencer at the Research Center for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran. BLAST software from the National Center for Biotechnology Information (NCBI) webpage was used for the alignment of obtained sequences with genes archived in the gene data bank. The highest homology and query coverage was the base of genotype identification. Nucleotide sequence accession numbers were deposited in the GenBank database (accession number: JN585806-JN585817). Comparison of a primary sequence alignment of a variable region of DF3 (stem 29–1 of 18S rRNA gene) of *Acanthamoeba* T4 and T3 strains isolated from hot springs with two published subtypes of T4 (Accession number: GQ342609.1) and T3 (Accession number: HM036185.1) were performed using MultAlin software (http://multalin.toulouse.inra.fr/multalin/multalin.html) (Figure 2).

RESULTS

The temperature and pH of hot springs were recorded as 32–70 °C and 3.88–7.9, respectively (Table 1). Of 60 water and sediment samples, 12 (20%) were positive for *Acanthamoeba* spp. (Table 2) according to the morphological criteria of double-walled cysts and the characteristic trophozoites. Positivity for outgrowth of *Acanthamoeba* spp. was observed in 33.3% of 30 water samples and in 6.6% of 30 sediments samples. All *Acanthamoeba* isolates were successfully cloned. Sequencing of the DF3 region (stem 29–1 of 18S rRNA gene) of PCR products revealed 10 (83.3%) isolates belonging to the T4 genotype with a homology of 98–100%.

```
                        1       10        20        30        40        50        59
                        |--------+---------+---------+---------+---------+---------|
      SN-HSSW7-IR-T4    GGTGCGGTCGTCCTTGGCGTTGGTCTTCAAAAGCCAGCGCGGGGGTGGCTTAGCCCGG
      SN-HSNW2-IR-T4    GGTGCGGTCGTCCTTGGCGTCGGTCTTTCGGGGCCGGCGCGGGGACGGCTTAGCCCGG
      SN-HSGW2-IR-T4    GGTGCGGTCGTCCTTGGCGTCGGTCTTTCGGGGCCGGCGCGGGGACGGCTTAGCCCGG
      SN-HSMW5-IR-T4    GGTGCGGTCGTCCTTGGCGTCGGTCTTTCGGGGCCGGCGCGGGGACGGCTTAGCCCGG
      SN-HSAW6-IR-T4    GGTGCGGTCGTCCTTGGCGTCGGTCTTTCGGGGCCGGCGCGGGGACGGCTTAGCCCGG
      SN-HSAS7-IR-T4    GGTGCGGTCGTCCTTGGCGTCGGTCTTTCGGGGCCGGCGCGGGGACGGCTTAGCCCGG
      SN-HSAW7-IR-T4    GGTGCGGTCGTCCTTGGCGTCGGTCTTTCGGGGCCGGCGCGGGGACGGCTTAGCCCGG
      SN-HSSW8-IR-T4    GGTGCGGTCGTCCTTGGCGTCGGTCTTTCGGGGCCGGCGCGGGGACGGCTTAGCCCGG
      SN-HSSS3-IR-T4    GGTGCGGTCGTCCTTGGCGTCGGTCTTTCGGGGCCGGCGCGGGGACGGCTTAGCCCGG
T4GenBank(GQ342609.1    GGTGCGGTCGTCCTTGGCGTCGGTCTTTCGGGGCCGGCGCGGGGACGGTTTAGCCCGG
      SN-HSAS9-IR-T4    GGCGCGGTCGCCCTTGGCGTTCGTGTTCACGCACGGGCGCGAGGGCGGCTTAGCCCGA
      SN-HSAW2-IR-T3    ATTGCGGTCGTCTTTGGTGTCG---CTCACAAGGCGGCACCGGGGCGGCTTAGCTCGCA
      SN-HSNW5-IR-T3    ATTGCGGTCGTCTTTGGTGTCG---CTCACAAGGCGGCACCGGGGCGGCTTAGCTCGCA
T3GenBank(HM036185.1    ATTGCGGTCGTCTTTGGTGTCG---CTCACAAGGCGGCATCGGGACGGCTTAGCTCGCA
            Consensus   ggtGCGGTCGtCcTTGGcGTcggt.tTcacaagccgGCgcggGGgcGGCTTAGCcCG..
```

Figure 2 | Primary sequence alignment of a variable region of DF3 (stem 29–1 of 18S rRNA) of *Acanthamoeba* T4 and T3 strains isolated from hot springs and two published subtypes of T4 (Accession number: GQ342609.1) and T3 (Accession number: HM036185.1) using MultAlin software. The alignment shown is a subset of DF3 of 18S rRNA gene that contains the highly variable and informative section of the gene. Gaps are represented as dashes.

Table 1 | Location and distribution of *Acanthamoeba* genotypes in hot springs of northwestern Iran

Accession number	Growth at 42 °C	Growth at 37 °C	Genotype	Max Identity/ query coverage (%)	pH	Temp (°C)	Sample type	Spring type	Locality	Code
JN585806	+	+	T4	99/100	6.8	43	Water	Bicarbonate	Sarein	HS[a]SW7
JN585813	+	+	T4	100/100	6.7	42	Water	Bicarbonate	Sarein	HSSW8
JN585814	+	+	T4	100/100	6.7	43	Sediment	Bicarbonate	Sarein	HSSS3
JN585810	+	+	T4	100/100	7.9	58	Water	Bicarbonate	Givi	HSGW2
JN585807	_	+	T3 (*A. griffini*)	100/99	4.8	32	Water	Sulphur	Ardabil	HSAW2
JN585815	_	+	T4	98/99	4.3	34	Sediment	Sulphur	Ardabil	HSAS9
JN585816	_	_	T4	99/100	4.8	34	Water	Sulphur	Ardabil	HSAW7
JN585817	+	+	T4	100/100	4.8	34	Sediment	Sulphur	Ardabil	HSAS7
JN585812	_	+	T4	100/100	3.88	34	Water	Sulphur	Ardabil	HSAW6
JN585808	+	+	T3 (*A. griffinii*)	100/100	6.37	40	Water	Sodium chloride	Nir	HSNW5
JN585809	+	+	T4	100/100	6.52	70	Water	Sodium chloride	Nir	HSNW2
JN585811	+	+	T4	100/99	6.22	36	Water	Sodium chloride	Meshkin shahr	HSMW5

[a]HS: Hot springs and their code.

Two (16.7%) isolates (HSNW5 and HSAW2) belonged to the T3 genotype (Table 1). The latter corresponded to *Acanthamoeba griffini* with identity of 99–100% (Accession number: GQ905499.1 and AB594480.1). The percent identity and query coverage between the obtained sequences and reference sequences in gene data banks are shown in Table 1.

A large proportion (91.7%) of strains showed growth at 37 °C, and eight isolates showed growth at 42 °C. A single isolate (HSNW2) was detected in waters at 70 °C and was classified as the T4 genotype. As expected, results of DF3 multi-alignment showed considerable difference (5% or more) between T4 and T3 genotypes. Sequences of the highly variable and informative region of DF3 are shown in Figure 2.

Table 2 | Percent contamination of hot springs with *Acanthamoeba* spp. in northwestern Iran

Number Spring type	Total of examined waters	Positivity *N* (%)
Sulphur	11	5 (45.4)
Bicarbonate	13	4 (30.8)
Sodium chloride	6	3 (50)
Total	60	12 (20)

DISCUSSION

This is the first comprehensive molecular study investigating the presence of *Acanthamoeba* spp. in water and sediments of hot springs accessed mainly for health purposes in Iran. The results revealed the presence of potentially pathogenic T3 and T4 genotypes chiefly in surface waters in comparison to sediments (33.3% vs. 6.6%). This could be due to the higher temperature of the bottom of springs or flow and circulation of water which may make the situation unfavourable for a high presence of *Acanthamoeba* in sediments. Both genotypes obtained have been reported as causal agents of *Acanthamoeba*-related infections in Iran and worldwide (Ledee *et al.* 1996; Booton *et al.* 2002; Maghsood *et al.* 2005; Niyyati *et al.* 2009). Previous research revealed the presence of *Acanthamoeba* T2, T4, and T6 in aquatic sources such as streams, ponds, waterfalls, and stagnant waters (Maghsood *et al.* 2005; Niyyati *et al.* 2009). Prior to the present report, only one study showed the presence of FLA in hot springs in one city of Ardebil province (Badirzadeh *et al.* 2011), and no comprehensive molecular research regarding the distribution of *Acanthamoeba* genotypes in hot springs had been done. This study reports, for the first

time, the T3 genotype (corresponding to *A. griffini*) isolated from water sources in Iran. In previous research on AK, T3 was the second most frequent type identified as an agent of keratitis in this region (Niyyati *et al.* 2009). T3 corresponding to *A. griffini* have also been reported as a cause of keratitis worldwide (Booton *et al.* 2002). Recently, many cases of AK report use of hot springs prior to onset of disease in this region. Thus education of people, especially those in the high risk category, is of the utmost importance. Our study provided further evidence of the T4 type as the predominant environmental genotype in contrast to Huang & Hsu (2010) who reported the T15 genotype as the most frequently isolated strain in Taiwan hot springs. Hsu *et al.* (2009) also detected *Acanthamoeba* in 8.8% of mud spring recreation areas. Classification of these amoebae revealed that they belonged to *A. castellanii* and *A. polyphaga* (Hsu *et al.* 2009). Lekkla *et al.* (2005) surveyed natural hot springs of Thailand and observed 13% occurrence of *Acanthamoeba* spp. in a study using only morphological criteria.

Acanthamoeba T4 genotype (HSNW2) was isolated from hot spring water at very high temperatures (70 °C), and another strain (HSAW6) was present in acidic water with a pH of 3.88 (Table 1). Previous research has demonstrated that *Acanthamoeba* strains that tolerate high temperatures and extremes of pH and osmolarity present greater pathogenic potential for humans and animals (Visvesvara *et al.* 2007; Khan 2009; Wannasan *et al.* 2009). It is believed that this strain (HSNW2) is a potential pathogen for humans, since it could tolerate very high water temperature. Growth of this strain at both 37 and 42 °C is another character of the pathogenic potential of the isolated *Acanthamoeba*. It is worth mentioning that the genotype of the mentioned strain (HSNW2) belonged to the T4 type which is the predominant pathogenic *Acanthamoeba*. This may be due to greater virulence of pathogenic strains under environmental stress, possibly related to mechanisms such as secretions of heat shock protein (HSP70) (Khan 2009). Further studies are needed regarding other virulence factors. A large proportion (91.7%) of isolates showed growth at 37 °C. Eight isolates also demonstrated growth at 42 °C. Therefore we consider that the isolates obtained could be pathogenic in humans, and lead to human infections.

In conclusion, the present study highlights that hot springs must be monitored by health authorities. As the use of hot springs for health purposes is increasing, both in this region and worldwide, contamination could lead to increasing numbers of *Acanthamoeba* infections. Periodic surveillance of recreational and therapeutic hot springs and spas along with improved filtration and disinfection is recommended to prevent human disease related to pathogenic *Acanthamoeba*.

ACKNOWLEDGEMENTS

Dr Maryam Niyyati was supported by a grant from the National Elites Foundation for Distinguished Young Assistant Professors. The research was funded by Shahid Beheshti University of Medical Sciences, Tehran. The authors thank Mr Alireza Badirzadeh and Mr Amini for their comments and help. The authors also acknowledge Lucidus Consultancy for language editing.

REFERENCES

Badirzadeh, A., Niyyati, M., Babaei, Z., Amini, H., Badirzade, H. & Rezaeian, M. 2011 Isolation of free-living amoebae from Sarein hot springs in Ardebil Province, Iran. *Iranian. J. Parasitol.* **6**, 1–8.

Booton, G. C., Kelly, D. J., Chu, Y. W., Seal, D. V., Houang, E., Lam, D. S. C., Byers, T. J. & Fuerst, P. A. 2002 18S ribosomal DNA typing and tracking of *Acanthamoeba* species isolates from corneal scrape specimens, contact lenses, lens cases, and home water supplies of *Acanthamoeba* keratitis patients in Hong Kong. *J. Clin. Microbiol.* **40**, 1621–1625.

Brown, T. J., Cursons, R. T., Keys, E. A., Marks, M. & Miles, D. M. 1983 The occurrence and distribution of pathogenic free-living amoebae in thermal areas of the North Island of New Zealand. *NZ. J. Mar. Freshw. Res.* **17**, 59–69.

Chou, S. A. 2004 *The Study on the Investigation, Exploitation and Utilization of Hot-Spring Resources in Taiwan (IV)* (In Chinese). Taiwan Water Resource Agency, Ministry of Economic Affairs, Taipei.

Gianinazzi, C., Schild, M., Zumkehr, B., Wüthrich, F., Nüesch, I., Ryter, R., Schürch, N., Gottstein, B. & Müller, N. 2010 Screening of Swiss hot spring resorts for potentially pathogenic free-living amoebae. *Exp. Parasitol.* **126**, 45–53.

Hsu, B. M., Lin, C. L. & Shih, F. C. 2009 Survey of pathogenic free-living amoebae and *Legionella* spp. in mud spring recreation area. *Water Res.* **43** (11), 2817–2828.

Huang, S. W. & Hsu, B. M. 2010 Isolation and identification of *Acanthamoeba* from Taiwan spring recreation areas using

culture enrichment combined with PCR. *Acta. Trop.* **115**, 282–287.

Khan, N. A. 2006 *Acanthamoeba*: biology and increasing importance in human health. *FEMS. Microbiol. Rev.* **30**, 564–595.

Khan, N. A. 2009 *Acanthamoeba, Biology and Pathogenesis*, 1st edition. Caister Academic Press, Norwich, UK.

Kilvington, S., Gray, T., Dart, J., Morlet, N., Beeching, J. R. & Frazer Matheson, M. 2004 *Acanthamoeba* keratitis: the role of domestic tap water contamination in the United Kingdom. *Invest. Ophthalmol. Vis. Sci.* **45**, 165–169.

Kuroki, T., Yagita, K., Yabuuchi, E., Agata, K., Katsube, Y. & Endo, T. 1998 Isolation of *Legionella* and free-living amoebae at hot spring spas in Kanagawa, Japan. *Kansenshogaku. Zassi.* **72**, 1050–1055.

Lasjerdi, Z., Niyyati, M., Haghighi, A., Shahabi, S., Biderouni, F. T., Taghipour, N., Eftekhar, M. & Nazemalhosseini Mojarad, E. 2011 Potentially pathogenic free-living amoebae isolated from hospital wards with immunodeficient patients in Tehran, Iran. *Parasitol. Res.* **109** (3), 575–580.

Ledee, D. R., Hay, J., Byer, T. J., Seal, D. V. & Kirkness, C. M. 1996 *Acanthamoeba griffini*. Molecular characterization of a new corneal pathogen. *Invest. Ophthalmol. Vis. Sci.* **37**, 544–550.

Lekkla, A., Sutthikornchai, C., Bovornkitti, S. & Sukthana, Y. 2005 Free-living *Amoeba* contamination in natural hot springs in Thailand. *Southeast. Asian. J. Trop. Med. Public Health* **36**, 5–9.

Lorenzo-Morales, J., Miranda, C. A., Jimenez, C., Tejedor, M. L., Valladares, B. & Ortega-Rivas, A. 2005 Evaluation of *Acanthamoeba* isolates from environmental sources in Tenerife, Canary Islands, Spain. *Ann. Agric. Environ. Med.* **12**, 233–236.

Maghsood, A. H., Sissons, J., Rezaeia, M., Nolder, D., Warhurst, D. & Khan, N. A. 2005 *Acanthamoeba* genotype T4 from the UK and Iran and isolation of the T2 genotype from clinical isolates. *J. Med. Microbiol.* **54**, 755–759.

Marciano-Cabral, F. & Cabral, G. 2003 *Acanthamoeba* spp. as agents of disease in humans. *Clin. Microbiol. Rev.* **16**, 273–307.

Marciano-Cabral, F., Jamerson, M. & Kaneshiro, E. S. 2010 Free-living amoebae, *Legionella* and *Mycobacterium* in tap water supplied by a municipal drinking water utility in the USA. *J. Water Health.* **8**, 71–82.

Mahdizadeh, T. & Panjaliasl, M. 2003 *Treatment with Mineral Water with a Review on Properties of Ardabil Mineral Hot Springs*, 1st edition. Bagh-e-andisheh Press, Iran.

Niyyati, M., Lorenzo-Morales, J., Rezaie, S., Rahimi, F., Mohebali, M., Maghsood, A. H., Motevalli-haghi, A., Martín-Navarro, C. M., Farnia, Sh., Valladares, B. & Rezaeian, M. 2009 Genotyping of *Acanthamoeba* isolates from clinical and environmental specimens in Iran. *Exp. Parasitol.* **121**, 242–245.

Niyyati, M., Lorenzo-Morales, J., Rezaie, S., Rahimi, F., Martín-Navarro, C. M., Mohebali, M., Maghsood, A. H., Farnia, S., Valladares, B. & Rezaeian, M. 2010 First report of a mixed infection due to *Acanthamoeba* genotype T3 and *Vahlkampfia* in a cosmetic soft contact lens wearer in Iran. *Exp. Parasitol.* **126**, 89–90.

Nuprasert, W., Putaporntip, C., Pariyakanok, L. & Jongwutiwes, S. 2010 Identification of a novel T17 genotype of *Acanthamoeba* from environmental isolates and T10 genotype causing keratitis in Thailand. *J. Clin. Microbiol.* **48**, 4636–4640.

Page, F. C. 1988 *A New Key to Freshwater and Soil Gymnamoebae*. Freshwater Biological Association, Ambleside, UK.

Rivera, F., Lares, F., Gallegos, E., Ramirez, E., Bonilla, P., Calderon, A., Martinez, J. J., Rodriguez, S. & Alcocer, J. 1989 Pathogenic amoebae in natural thermal waters of three resorts of Hidalgo. *Mexico. Environ. Res.* **50**, 289–295.

Schroeder, M., Booton, G. C., Hay, J., Niszl, I. A., Seal, D. V., Markus, M. B., Fuerst, P. A. & Byers, T. J. 2001 Use of subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of *Acanthamoeba* from humans with keratitis and from sewage sludge. *J. Clin. Microbiol.* **39**, 1903–1911.

Sukthana, Y., Lekkla, A., Sutthikornchai, C., Wanapongse, P., Vejjajiva, A. & Bovornkitti, S. 2005 Spa, springs and safety. *Southeast. Asian. J. Trop. Med. Public Health* **36**, 10–16.

Tsvetkova, N., Schild, M., Panaiotov, S., Kurdova-Mintcheva, R., Gottstein, B., Walochnik, J., Aspock, H., Lucas, M. S. & Muller, N. 2004 The identification of free-living environmental isolates of amoebae from Bulgaria. *Parasitol. Res.* **92**, 405–413.

Thomas, V., Loret, J. F., Jousset, M. & Greub, G. 2008 Biodiversity of amoebae and amoebae-resisting bacteria in a drinking water treatment plant. *Environ. Microbiol.* **72**, 2428–2438.

Visvesvara, G. S., Moura, H. & Schuster, F. L. 2007 Pathogenic and opportunistic free living amoebae: *Acanthamoeba* spp., *Balamuthia mandrillaris, Naegleria fowleri*, and *Sappinia diploidea*. *FEMS. Immunol. Med. Microbiol.* **50**, 1–26.

Wannasan, A., Chaiwong, P., Manasanant, B. & Morakote, N. 2009 Occurrence of thermotolerant *Naegleria* and *Acanthamoeba* in some natural water sources in Chiang Mai. *Chiang. Mai. Med. J.* **48**, 117–124.

First received 24 January 2012; accepted in revised form 26 July 2012. Available online 27 August 2012