Epidemiol. Infect. (2016), **144**, 2456–2461. © Cambridge University Press 2016
doi:10.1017/S095026881600073X

Presence of *Balamuthia mandrillaris* in hot springs from Mazandaran province, northern Iran

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Received 26 December 2015; Final revision 27 February 2016; Accepted 26 March 2016; first published online 18 April 2016

SUMMARY

Balamuthia mandrillaris is an opportunistic free-living amoeba that has been reported to cause cutaneous lesions and *Balamuthia* amoebic encephalitis. The biology and environmental distribution of *B. mandrillaris* is still poorly understood and isolation of this pathogen from the environment is a rare event. Previous studies have reported that the presence of *B. mandrillaris* in the environment in Iran may be common. However, no clinical cases have been reported so far in this country. In the present study, a survey was conducted in order to evaluate the presence of *B. mandrillaris* in hot-spring samples of northern Iran. A total of 66 water samples were analysed using morphological and molecular tools. Positive samples by microscopy were confirmed by performing PCR amplification of the 16S rRNA gene of *B. mandrillaris*. Sequencing of the positive amplicons was also performed to confirm morphological data. Two of the 66 collected water samples were positive for *B. mandrillaris* after morphological and molecular identification. Interestingly, both positive hot springs had low pH values and temperatures ranging from 32 °C to 42 °C. Many locals and tourists use both hot springs due to their medicinal properties and thus contact with water bodies containing the organism increases the likelihood of infection. To the best of our knowledge, this is the first report on the isolation of *B. mandrillaris* from hot-spring sources related to human activity. Therefore, *B. mandrillaris* should be considered as a possible causative agent if cases of encephalitis are suspected following immersion in hot springs in addition to *Acanthamoeba and Naegleria.*

Key words: *Balamuthia mandrillaris*, hot spring, Iran.

INTRODUCTION

Free-living amoebae of medical relevance include the genera/species *Acanthamoeba* spp., *Balamuthia mandrillaris* and *Naegleria fowleri* which are causative agents of lethal encephalitis and other diseases in humans and animals [1–4]. Regarding *B. mandrillaris*, this amoebae is the causative agent of *Balamuthia* amoebic encephalitis (BAE), which has been reported in more than 200 cases worldwide [4, 5]. The United States and Latin America have reported the highest number of cases, with the southwest United States

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and Peru being the most affected regions [3, 4, 6, 7–10].

The pathogenicity mechanisms of this amoeba have not been established so far; however, it is suspected that the amoeba is able to enter the host through the olfactory neuroepithelium, the respiratory tract or a skin lesion. The amoeba is then able to reach the central nervous system by haematogenous spread [4, 11, 12].

There is an urgent need for the development of reliable diagnostic and therapeutic measures for BAE. In the case of diagnosis, it is often carried out post-mortem since the disease is lethal in more than 98% of cases [4, 13, 14].

Although the environmental niches of *B. mandrillaris* seem to be associated with soil-related habitats, the pathogen has only been isolated from the environment on nine previous occasions: dust and soil sources in the United States [15, 16] and Iran [17, 18], mud in Jamaica [14], soil and water in Mexico [10], soil in Peru [5], dust in Costa Rica [19] and water in Guinea–Bissau [20].

In Iran, previous studies have reported the isolation of *B. mandrillaris* from dust collected in the city of Tehran [17] and it was also recently isolated from soil samples in northwestern Iran [18]. Nevertheless, even though the pathogen appears to be present in the environment, no clinical cases have been reported so far in Iran [18].

A recent study by Todd *et al.* [14], reported the presence of *B. mandrillaris* in a local mud spa in Jamaica. Therefore, since we have previously detected *B. mandrillaris* in dust and soil in Iran, in this study we checked water samples for the presence of this amoebic pathogen from hot springs widely visited by locals and tourists in Iran.

To the best of our knowledge, this is the first report on the isolation of *B. mandrillaris* from water sources in Iran related to human activity and the third time that this pathogen has been isolated from the environment in this country. Moreover, this is the first time that *B. mandrillaris* has been isolated from hot springs worldwide.

MATERIALS AND METHODS

Geographical study area

This study was conducted in Mazandaran province, northern Iran. Mazandaran province is located on the southern coast of the Caspian Sea. The province enjoys a moderate, subtropical climate with an average temperature of 25 °C in summer and about 8 °C in winter. The region has many therapeutic and recreational mineral springs and geothermal streams, thus many locals and tourists travel to this region to use the facilities (mostly those located in the capital of Mazandran province, Ramsar), which are used to treat skin diseases, rheumatism, neuralgia and muscular pains (Fig. 1).

Sample collection and processing

Water samples were collected from the 22 most popular hot springs of Mazandaran province, northern Iran using 1-l sterile bottles in triplicate, thus a total of 66 samples were processed in this study. Each sample was filtered using nitrocellulose membrane (0·22 µm pore size, 45 mm diameter) and cultured using the enrichment cultivation method. Briefly, 1·5% non-nutrient agar were obtained using Bacto agar (Difco, USA), filters were then placed on the surface of the medium and incubated at room temperature. Plates were monitored for outgrowth of *Balamuthia*-like amoebae every 24 h [19, 20].

DNA extraction, PCR analysis, and sequencing

A modified phenol–chloroform method for DNA extraction from cysts and trophozoites on positive plates was used based on our previous work [17]. For the molecular identification of *B. mandrillaris*, the 16S rDNA gene was targeted and sequenced as described previously [17, 21], using the Balspec16S primer pair (5′-Balspec16S: 5′-CGCATGTATGAAGAAGACCA-3′ and 3′-Balspec16S: 5′-TTACCTATATAATTGTCGATACCA-3′).

PCR was performed using the Ampliqone kit (Taq DNA Polymerase Master Mix RED, Denmark) which is a pre-made mixture. According to the manufacturer's instructions, 15 µl Ampliqone was mixed with 3 µl suspicious DNA, 1·3 µl primers and 10·7 µl distilled water. The thermal cycling conditions were an initial denaturing step at 94 °C for 1 min and 35 repetitions at 94 °C for 35 s, annealing steps were at 56 °C for 1 min, and 72 °C for 1 min.

PCR products were purified and sequenced in both directions. Obtained sequences were edited using Chromas software (Informer Technologies Inc.) and aligned using the Mega 5.0 program (http://mega.software.informer.com/5.0/). Homology analysis was then performed against all available genes in the Genbank

https://doi.org/10.1017/S095026881600073X Published online by Cambridge University Press

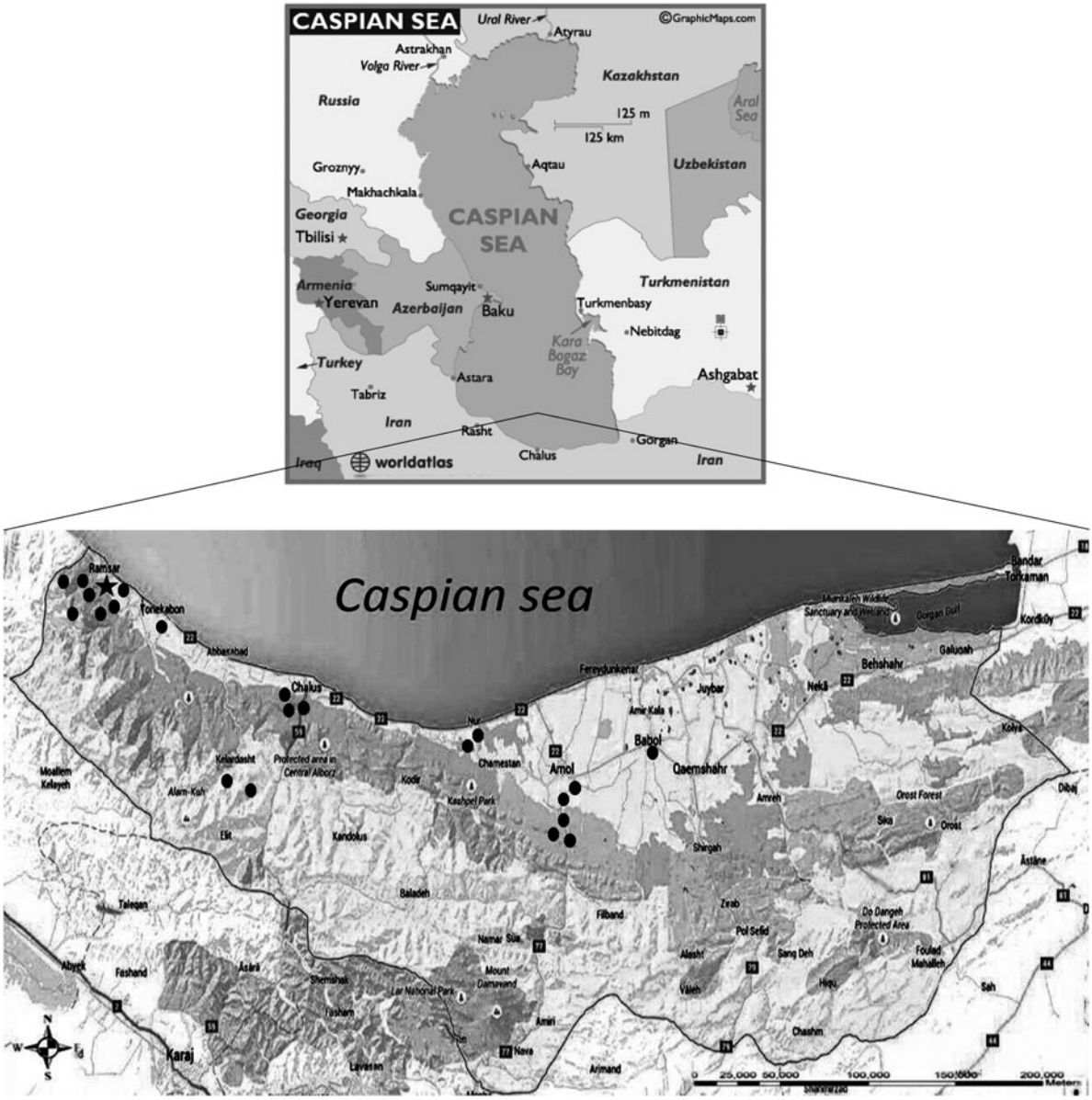

Fig. 1. Map of the Caspian Sea showing outline of Caspian Sea coast and country boundaries, Mazandaran province, northern Iran. The 22 sampling points are indicated by solid circles (●). Ramsar city, where *Balamuthia mandrillaris* isolates were detected, is indicated by a star (★)

database. The obtained sequences for the new isolates have been deposited in Genbank (accession nos. KU184268–KU184269).

RESULTS AND DISCUSSION

From the 66 water samples collected from the 22 mineral and hot springs of Mazandaran province included in this study, two plates showed suspected *Balamuthia* growth in the non-nutrient agar plates. *Balamuthia* trophozoites were approximately 30–120 µm in diameter with dendritic pseudopodia and most of the time their trophozoites appeared stretched out and branched (Fig. 2). Subsequent cultures revealed the elimination of unwanted amoebae, bacteria and fungi and the suspected amoebae were cloned successfully. Both positive plates belonged to popular hot springs in the city of Ramsar (Tables 1 and 2). One of the isolated strains, designated LN-HSR5-*Balamuthia*, exhibited very slow growth rates and was not fully grown until 20 days. This strain was isolated from an acidic and sulphurous hot spring with a temperature of 32 °C. The other strain, designated LN-HSR1-*Balamuthia*, was isolated from a hot spring

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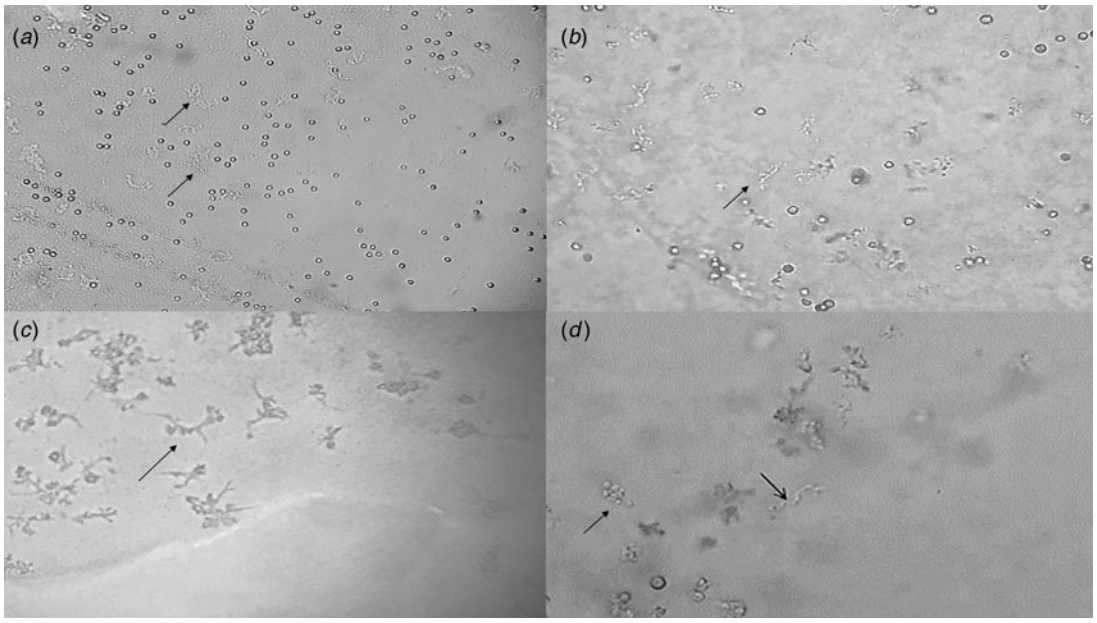

Fig. 2. *Balamuthia mandrillaris* trophozoites and cysts (*a, b*, magnification ×100). *B. mandrillaris* trophozoites (*c, d*, magnification ×400) in non-nutrient agar plates, during the early stages of isolation.

Table 1. *Geographical location of two positive hot springs in northern Iran*

	Geographical location						
	Longitude			Latitude			
Hot spring	Degrees	Minutes	Seconds	Degrees	Minutes	Seconds	Position to Ramsar
Ramsar hotel spa	50	39	26	36	54	9	South
Bridge spa	50	39	41	36	54	6	South East

Table 2. *Positive isolates and contaminated hot-spring data*

Isolate code	Accession number	Hot spring	Temp.	pH	Therapeutic purposes
LN-HSR1-Balamuthia	KU184268	Ramsar hotel spa	42 °C	4·2	Treatment of skin diseases, rheumatism, neuralgia and muscle disorders
LN-HSR5-Balamuthia	KU184269	Bridge spa	32 °C	3·5	Treatment of neurological diseases and joint disorders

with a temperature of 42 °C. Furthermore, PCR of the 16S rDNA mitochondrial gene of *B. mandrillaris* confirmed the morphological observations in both cases. The PCR products of these strains showed sequence homologies between 96% and 99% compared to the *B. mandrillaris* sequences available in GenBank [KT175741·1 and EU934073·1 (Iranian isolate: ID19 strain), respectively]. Furthermore, the isolated strains showed high homology with the previously reported *Balamuthia* strains in Iran (accession nos. KR908788–KR908792). The 96% homology was shown with the mitochondrial genome of *B. mandrillaris* (V039 strain).

This is the first report regarding the isolation of *B. mandrillaris* from water sources in Iran and the first to report regarding the occurrence of this amoeba in hot springs worldwide. Moreover, therapeutic springs in northern Iran are very popular and thus awareness

https://doi.org/10.1017/S095026881600073X Published online by Cambridge University Press

should be raised regarding contact and likelihood of infection with *B. mandrillaris*. A previous study conducted by Todd *et al*. also revealed the presence of *B. mandrillaris* in therapeutic mud used by locals and tourists in Jamaica [14]. In accordance with previous studies, the present study again confirmed that the occurrence of *B. mandrillaris* in environmental sources is very low (2/66 collected water samples) [5, 10, 17, 18]. Moreover, the isolation of the amoebae from various regions of the world also showed low rates, e.g. Iran (5/55, 9% [17]), Costa Rica (1/36, 2·7% [19]), Jamaica (1/72, 1·38% [14]), Guinea–Bissau (1/22, 4·5% [20]) and Peru (4/21, 19% [5]). These data reveal the low rates of occurrence of *Balamuthia* in environmental niches. It should be noted that the low rates of this amoeba can be explained by the fact that it is very rare in nature compared to other free-living amoebae such as *Acanthamoeba*. On the other hand, this could also be due to lack of reliable culture methods for this amoeba and the high contamination of environmental sources with fungi and bacteria that confer the outgrowth of the amoebae [10]. According to a previous study the 'sandwich agar technique' and a new axenic culture could be a way of decreasing fungal contamination [10, 22].

In the present study both positive strains (LN1 and LN5) were isolated from hot springs with acidic pH and relatively high temperature (32–42 °C). The ability of *Balamuthia* to tolerate hostile conditions is not surprising as evidenced by a previous study [12], which showed amoeba viability could not be affected by high temperatures (up to 60 °C for 1 h). Moreover, the cysts could even survive at up to 70 °C for 1 h and they can transfer to proliferating amoebae [12, 23].

The present study is in accord with a report by Lares-Jiménez *et al*. regarding the aquatic environment as a potential source for *B. mandrillaris* [10]. Our study further confirms that the potential risk is not limited to soil and dust samples [17, 18]. Previous studies in Iran have shown the occurrence of *B. mandrillaris* in soil and dust sources of public places including parks and school campuses. It should be also mentioned that most cases of *Balamuthia* infection in Peru, the most affected country, occurred in dusty places and in places with a soil environment [5, 18]. There are studies in the literature demonstrating a direct relationship between the presence of *Balamuthia* in water sources and cases of encephalitis [10, 20, 24].

Overall, the present study reports the contamination of hot springs in Iran due to *B. mandrillaris*, which is also the first time that this pathogen has been isolated from this type of habitat. Therefore, this organism should be considered in cases of encephalitis following immersion in hot springs worldwide.

ACKNOWLEDGEMENTS

J.L.M. was supported by the Ramón y Cajal Subprogramme from the Spanish Ministry of Economy and Competivity RYC-2011–08863. This study was funded by Shahid Beheshti University of Medical Sciences, Tehran, Iran. The authors thank Dr Niloofar Taghipour for her kind comments and Dr Eznolah Azargashb for his kind and helpful comments.

DECLARATION OF INTEREST

None.

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