Experimental Parasitology 126 (2010) 45–53

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Screening of Swiss hot spring resorts for potentially pathogenic free-living amoebae

Christian Gianinazzi [a], Marc Schild [a], Beatrice Zumkehr [a], Fritz Wüthrich [b], Irina Nüesch [c], Regula Ryter [c], Nadia Schürch [b], Bruno Gottstein [a], Norbert Müller [a,*]

[a] Institute of Parasitology, University of Berne, Berne, Switzerland
[b] Spiez Laboratory, Federal Office for Civil Protection, Spiez, Switzerland
[c] Kantonales Laboratorium des Kt. Aargau, Aarau, Switzerland

ARTICLE INFO

Article history:
Received 6 October 2009
Received in revised form 11 December 2009
Accepted 17 December 2009
Available online 29 December 2009

Keywords:
Free-living amoebae
Acanthamoeba spp.
CNS infection

ABSTRACT

Free-living amoebae (FLA) belonging to *Acanthamoeba* spp., *Naegleria fowleri*, *Balamuthia mandrillaris*, and *Sappinia pedata* are known to cause infections in humans and animals leading to severe brain pathologies. Worldwide, warm aquatic environments have been found to be suitable habitats for pathogenic FLA. The present study reports on screening for potentially pathogenic FLA in four hot spring resorts in Switzerland. Water samples were taken from water filtration units and from the pools, respectively. Amoebae isolated from samples taken during, or before, the filtration process were demonstrated to be morphologically and phylogenetically related to *Stenoamoeba* sp., *Hartmannella vermiformis*, *Echinamoeba exundans*, and *Acanthamoeba healyi*. With regard to the swimming pools, FLA were isolated only in one resort, and the isolate was identified as non-pathogenic and as related to *E. exundans*. Further investigations showed that the isolates morphologically and phylogenetically related to *A. healyi* displayed a pronounced thermotolerance, and exhibited a marked *in vitro* cytotoxicity upon 5-day exposure to murine L929 fibroblasts. Experimental intranasal infection of Rag2-immunodeficient mice with these isolates led to severe brain pathologies, and viable trophozoites were isolated from the nasal mucosa, brain tissue, and lungs *post mortem*. In summary, isolates related to *A. healyi* were suggestive of being potentially pathogenic to immunocompromised persons. However, the presence of these isolates was limited to the filtration units, and an effective threat for health can therefore be excluded.

1. Introduction

Free-living amoebae (FLA) have a worldwide distribution in soil and water, and do generally not represent threats to human health. However, FLA belonging to the genus *Acanthamoeba*, *Balamuthia mandrillaris*, *Naegleria fowleri*, and *Sappinia pedata* have been documented to invade and multiply within a mammalian host leading to opportunistic and non-opportunistic infections in humans and animals (Martinez and Visvesvara, 1997; Khan, 2007). The reasons for switching from the free-living status to the pathogenic status including invasion of the host still remain poorly understood. Interestingly, infections due to *Acanthamoeba* spp. have exclusively been, with the exception of eye infections, documented in immune-deficient or immune-weakened persons, e.g. in AIDS patients and pregnant women (Marciano-Cabral and Cabral, 2003; Bloch and Schuster, 2005; Walia et al., 2007). For the other pathogenic FLA, infection seems to occur independently of the immune status.

With regard to the central nervous system (CNS), two pathologies are known. Primary amoebic meningoencephalitis (PAM) with *N. fowleri* as infecting agent, and granulomatous amoebic encephalitis (GAE) caused by *Acanthamoeba* spp. and *B. mandrillaris*. *Acanthamoeba* spp. may also affect other organs including skin, eyes, and lung. Treatments for these infections are usually empirical and still unsatisfactory, with the vast majority of reported cases ending up fatally (Schuster and Visvesvara, 2004a).

Habitats of pathogenic FLA include soil, dust, air, and water which provide a potential source of infection. With regard to aquatic environments, pathogenic FLA have been isolated from, e.g. cooling waters from power plants, tap water but also from bottled mineral water, and eyewash stations (Behets et al., 2006; Khan, 2006; Pelandakis and Pernin, 2002; Schuster and Visvesvara, 2004a; Visvesvara et al., 2007). Additionally, warm-water swimming pools, hot tubs, and hot water resorts are worldwide known to be suitable aquatic environments for pathogenic FLA, and infections due to the presence of pathogenic FLA in these environments have been largely documented (Lekkla et al., 2005; Gornik and Kuzna-Grygiel, 2004; Rivera et al., 1993; De Jonckheere, 1982; Kadlec et al., 1980; Scaglia et al., 1983).

In Switzerland, only two studies have addressed so far the presence of potentially pathogenic FLA in aquatic environments

* Corresponding author. Address: Institute of Parasitology, University of Berne, Länggass-Strasse 122, CH-3012 Berne, Switzerland. Fax: +41 31 631 24 77.
E-mail addresses: norbert.mueller@ipa.unibe.ch, nmueller@ipa.unibe.ch (N. Müller).

0014-4894/$ - see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.exppara.2009.12.008

(Gianinazzi et al., 2009a,b). Potentially pathogenic FLA were isolated from a constantly warm habitat, but not from colder aquatic habitats. In the present study, we report on screening for and identifying potentially pathogenic FLA in four hot spring resorts in Switzerland, and on investigating their potential pathogenicity *in vitro* and *in vivo*.

2. Materials and methods

2.1. Sampling procedure

In total, 31 water samples were collected in four hot spring resorts (A, B, C, and D) at the sites indicated in Table 1 by immersion of a 50-ml plastic Falcon tube into the upper 2 cm of the respective water body. The tubes were subsequently cooled to 4 °C for 30 min, and the water sample was pelleted for 15 min at 1200 rpm using a Heraeus Varifuge 3.0R centrifuge (Kendro Laboratory Products, Zurich, Switzerland). The pellets were re-suspended in 100 µl of supernatant, and used as described in the following sections.

2.2. Isolation of trophozoites and establishment of cultures

Re-suspended pellets were gently pipetted onto a non-nutrient agar plate (1.5% agar in Page's saline) and were allowed to adsorb and dry. Subsequently, the plates were sealed with Parafilm®, and incubated upside down at 37 °C in order to select for thermotolerant FLA. Since all water samples lacked fungal contaminations, migration of FLA from the pellet to the edge of the agar plate was fast and resulted in a low density distribution of, and presumably non-competitive growth among, the few amoebic cells present in the individual cultures. Thus, we did not incubate the initial samples at 45 °C, a temperature generally used to select for pathogenic *Naegleria* spp., although these parasites can essentially be isolated at 37 °C (Scaglia et al., 1983). A daily inspection of plates was done by light microscopy until morphological structures suggestive of amoeba trophozoites were detected. Cultures lacking morphological features of amoebae within 2 weeks were considered as negative and discarded. Upon detection of trophozoites, clones were established by means of the migration technique (Gianinazzi et al., 2009a). Briefly, a block of agar containing small numbers of amoebae was transferred to a fresh plate. From amoebae migrating onto the plate, a single trophozoite was then selected and transferred to a fresh plate. The clones were then kept at 37 °C, and at 42 °C, in order to investigate isolates for increased thermotolerance (Table 2), and biweekly transferred onto fresh agar plates coated with heat-inactivated (1 h at 60 °C) *Escherichia coli*. For documentation, trophozoites, and, where applicable, cysts were photographed (Fig. 1 and 2).

2.3. In vitro cytotoxic activity of isolates

In order to screen for clones that exhibit a cytotoxic potential, we employed an approach based on microscopical inspection of a co-culture system using the murine fibroblast cell line L929. Briefly, L929 cells were grown to confluence in 24-well plates in MEM Earle medium supplemented with 1% L-glutamine, 5% foetal calf serum, 1% non-essential amino acids, and 10 µg/ml penicillin and streptomycin (total volume per well: 0.5 ml) at 37 °C in a 5% CO_2-enriched atmosphere. Isolates to be investigated were harvested from agar plates by carefully scraping with a curved Pasteur

Table 1
Overview of water samples investigated for free-living amoebae.

Thermal bath	Sampling site	ID of sample	Temperature[a] (°C)	Isolation[b]
A	Basin reservoir	A1	40.0	–
	Fresh water reservoir	A2	37.0	+
	After sand filtration	A3	34.0	+
	After ozone treatment	A4	34.0	–
	After degassing	A5	34.5	–
	Swimming pool 1	*A6*	*34.3*	+
	Swimming pool 2	*A7*	*35.0*	–
	Swimming pool 3	*A8*	*36.0*	–
	Swimming pool 4	*A9*	*35.1*	–
B	*Swimming pool*	*B1*	*35.3*	–
	Before sand filtration/ozone treatment	B2	35.3	+
	After sand filtration/ozone treatment	B3	35.3	+
	After ozone pre-treatment	B4	35.3	–
	Waste water from pool	B5	35.3	–
	Whirl pool	*B6*	*37.0*	–
	Indoor swimming pool	*B7*	*35.2*	–
	Outdoor swimming pool	*B8*	*35.2*	–
	Outdoor river bath	*B9*	*35.2*	–
C	Before filter 1 (sand)	C1	33.0	–
	Backflush filter 1	C2	33.0	–
	Before filter 2 (sand), standing water	C4	34.0	+
	After filter 2	C5	34.0	–
	Sink basin filter 2	C6	34.0	+
	Backflush filter 2	C7	34.0	+
	Before filter 2, after pipe flushing	C8	34.0	–
	Indoor swimming pool, after backflush	*C9*	*34.0*	–
	Outdoor swimming pool, after backflush	*C10*	*34.0*	–
D	After sand filtration, for indoor pool	D1	35.2	+
	After sand filtration, for outdoor pool	D2	36.0	–
	Indoor swimming pool	*D3*	*35.2*	–
	Outdoor swimming pool	*D4*	*36.0*	–

Sampling sites marked in italic indicate sites of potential contact with FLA and people.
[a] Water temperature at sampling.
[b] "–" indicates a water sample where no trophozoites were detected within 14 days of incubation, either due to the absence of trophozoites in the sample or due to the fact that the trophozoites were not able to grow at 37 °C. In case of trophozoite isolation (+), the isolate was assigned the same ID as the water sample of origin.

Table 2
Cytotoxicity profile and thermotolerance of control and study isolates.

	Cytotoxicity[a] (25,000/2500/250)[c]		Growth[b] (25,000/2500/250)[c]		Viability	Thermotolerance		
	Day 2	Day 5	Day 2	Day 5		18 °C	37 °C	42 °C
Pathogenic species (positive control)								
N. fowleri	1/4/4	1/1/1	0/0/0	3/3/3	+	+	+	+
B. mandrillaris	4/4/4	1/1/1	3/2/1	3/3/3	+	+	+	+
A. lenticulata	4/4/4	1/1/1	0/0/0	3/3/3	+	+	+	+
Non-pathogenic species (negative control)								
Vannella sp.	4/4/4	2/4/4	0/0/0	0/0/0	–	+	+	–
H. vermiformis	3/3/3	2/2/2	0/0/0	0/0/0	–	+	+	–
Isolates from hot spring resorts								
A2	4/4/4	3/3/3	0/0/0	0/0/0	–	+	+	+
A3	1/3/4	1/3/4	0/0/0	0/0/0	–	+	+	–
A6	n.a.[d]	n.a.	n.a.	n.a.	n.a.	+	+	–
B2	4/4/4	1/1/1	0/0/0	3/2/1	+	+	+	+
B3	4/4/4	1/3/4	0/0/0	3/2/1	+	+	+	+
C4	4/4/4	1/3/4	0/0/0	3/2/2	+	+	+	+
C6	4/4/4	1/1/1	0/0/0	2/2/1	+	+	+	+
C7	1/4/4	1/1/1	3/1/0	3/3/2	+	+	+	+
D1	4/4/4	4/4/4	0/0/0	0/0/0	–	+	+	+

Viability: viability of trophozoites on day 5 of co-culture.
[a] Cytotoxicity measured via confluence of L929 cell layer, i.e. confluent to 90–100% and preserved morphology (score 4); confluent to 50–90% and preserved morphology (score 3); loss of confluence (20–50%) and alteration of morphology (score 2); complete loss of confluence (0–20%) and consistent alteration of morphology (score 1).
[b] Growth of trophozoites characterized as follows: high (score 3), medium (score 2), and low (score 1) density; no trophozoites visible (score 0).
[c] No. of trophozoites used as inoculum for cytotoxicity assay.
[d] Not applicable.

pipette, pelleted in PBS, and re-suspended in 50 µl of PBS. A volume corresponding to 2.5×10^4, 2.5×10^3, and 2.5×10^2 trophozoites, respectively, was added to the wells. Co-culture was then performed at 37 °C in a 5% CO_2-enriched atmosphere. The L929 cultures were investigated by light microscopy on day 2 and 5 of co-culture, and cytotoxic effects evaluated according to the following scale: 4 = confluent to 90–100%, preserved morphology; 3 = confluence between 50% and 90%, preserved morphology; 2 = loss of confluence (20–50%) and alteration of morphology; 1 = complete loss of confluence (0–20%), and consistent alteration of morphology. Growth of trophozoites co-cultured with L929 cells was assessed as follows: 3 = high density; 2 = medium density; 1 = low density; 0 = no trophozoites visible (Gianinazzi et al., 2009b). At the end of the co-culture, remaining cells were removed from the well and assessed for viable amoebae by plating on non-nutrient agar plates.

The cytotoxicity profile (cytotoxicity), the ability to multiply in presence of L929 cells (growth), the viability of isolates on day 5, and the thermotolerance at 42 °C were compared to data obtained for control isolates of pathogenic FLA (*N. fowleri* [ATCC 30894], *B. mandrillaris* [ATCC 50209], *Acanthamoeba lenticulata* [Gianinazzi et al., 2009a]), and non-pathogenic FLA (*Vannella* sp. [ATCC 30947], *Hartmannella vermiformis* [ATCC 50802]) (Table 2 and Fig. 1B and C).

Assessment of cytotoxicity of amoebae based on measurement of release of lactate dehydrogenase (LDH) by disrupted L929 cell monolayers in co-cultures was performed as previously described (Sissons et al., 2006). Briefly, L929 cells were grown to confluent monolayers using 24-well plates. To individual wells, 5×10^5 cells of *N. fowleri, Vannella* sp. and different *Acanthamoeba healyi*-related amoeba isolates were added and incubated at 37 °C in 5% CO_2 with L929 monolayer in RPMI 1640 medium containing 2 mM glutamine, 1 mM pyruvate, and non-essential amino acids. At different time-points of incubation, the supernatants from co-cultures were collected and the LDH activity in these samples as well as negative (supernatant from L929 cells alone) and positive (supernatant from L929 cells lysed with solution 3 from the kit; used for determination of maximal LDH activity release in cultures) control samples was analysed using the LDH Assay Cytotoxicity Detection Kit^{Plus} (Roche Applied Science, Basle, Switzerland). The percentage of cytotoxicity corresponding to LDH activity release in the individual cultures was determined according to the instructions of the manufacturer of the kit (Fig. 3).

2.4. Identification of isolates by PCR

The trophozoites were gently scraped from one agar plate by using a bent Pasteur pipette, re-suspended in PBS and subsequently pelleted by sedimentation at 1000*g* for 10 min. DNA was extracted from the pellet using the DNAeasy kit (Qiagen, Basle, Switzerland) following the manufacturer's instructions. DNA was subjected to PCRs either yielding the specific recognition of a pan-PCR recognizing FLA in general (FLA-PCR) (references see below), *Acanthamoeba* spp. (ACA-PCR), or *N. fowleri* (Nf-PCR). The following primer pairs were applied: FLA-PCR, free-living amoeba: P-FLA-F (forward primer): 5′-CGCGGTAATTCCAGCTCCAATAGC-3′; P-FLA-R (reverse primer): 5′-CAGGTTAAGGTCTCGTTCGTTAAC-3′; target: 18S rDNA (Tsvetkova et al., 2004); ACA-PCR, *Acanthamoeba* spp.: JDP1 (forward primer): 5′-GGCCCAGATCGTTTACCGTGAA-3′; JDP2 (reverse primer): 5′-TCTCACAAGCTGCTAGGGAGTCA-3′; target: 18S rDNA (Schroeder et al., 2001); Nf-PCR, *N. fowleri*: Nae3-For (forward primer): 5′-CAAACACCGTTATGACAGGG-3′; Nae3-Rev: 5′-CTGGTTTCC CTCACCTTACG-3′; target: 18S rDNA (Schild et al., 2007). The PCR products were visualized on a 2% agarose gel containing ethidium bromide. For sequence analyses, 50 µl of PCR product were purified using the High Pure PCR Product Purification Kit (Roche, Applied Science, Basle, Switzerland) according to the manufacturer's protocol, and sequenced using a 310 Genetic Analyzer (Applied Biosystems). The nucleotide sequences were then compared to sequences published in GenBank using the BLAST (Basic Local Alignment Search Tool) (Table 3). For comparative BLAST analyses, only sequence segments exhibiting complete identity in at least two independent sequencing runs were accepted.

2.5. In vivo pathogenicity of isolates

Cultures of isolates B2, C4, C6, and C7 were used for *in vivo* infection of two 6-week-old Balb/c (wild-type) and three 6-

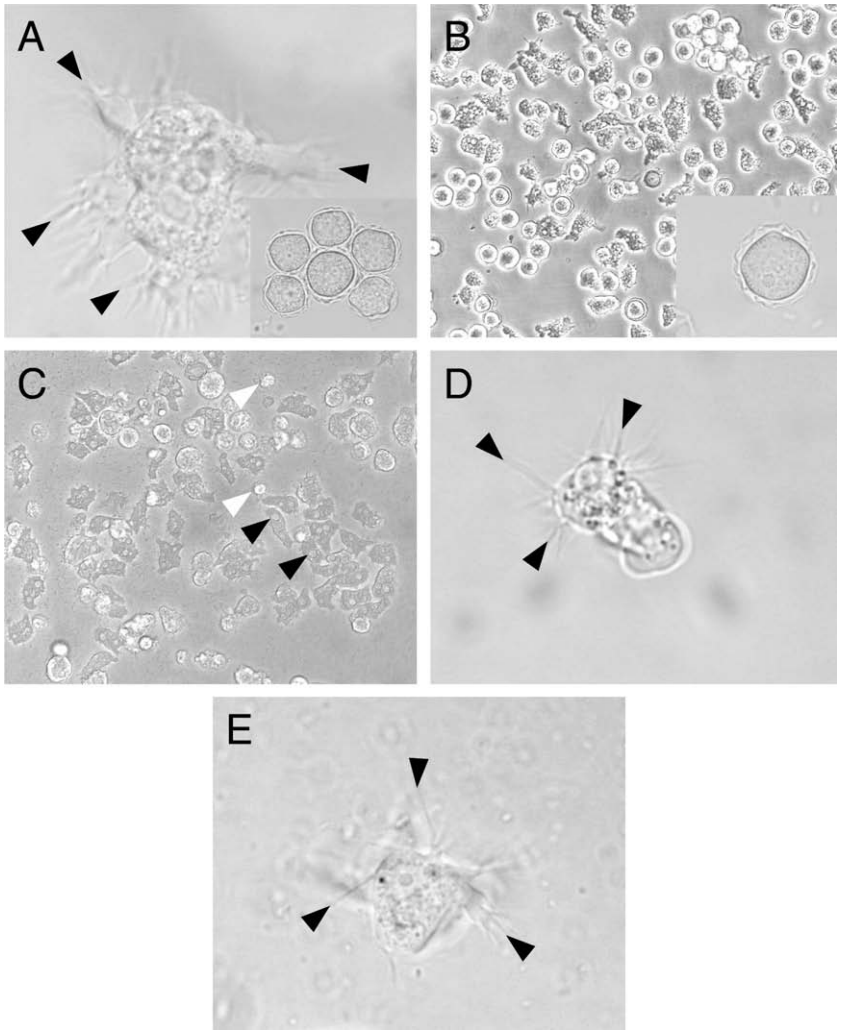

Fig. 1. Morphological documentation of *Acanthamoeaba* isolated from hot spring resorts. (A, D and E) A trophozoite of isolate B2 (A), C6 (D), and C7 (E), respectively, re-suspended in water displaying acanthapodia (arrowheads), spine-like surface projections characteristic for this genus (original magnification: 1000×). (B and C) Isolate B3 (B) and C4 (C) multiplying in the presence of L929 cells and displaying contractile vacuoles (black arrowheads). Cytotoxicity manifests by detachment and alteration of morphology of L929 (white arrowheads) (original magnification: 200×). Insets in (A) and (B) show *Acanthamoeba*-characteristic double-walled cysts of the respective isolates.

week-old Rag2(−/−)gamma(c)(−/−) mice of Balb/c back-ground (lacking T-cell, B-cell, and natural killer cell function) (Goldman et al., 1998; Baenziger et al., 2006). The animals were intranasally infected according to Table 4 with 1×10^6 trophozoites/animal as previously described (Gianinazzi et al., 2005), with a mock-infected negative control animal per group. The mice were daily checked for clinical signs indicating a possible CNS infection such as loss of weight, obtundation, and ataxy. Upon onset of these symptoms, the animals were sacrificed, and tissue samples from brain, lungs, and nasal mucosa were collected, and used to investigate the presence (recovery) of viable trophozoites (Table 4 and Fig. 4C), and for histopathological investigations of tissues (Fig. 4A and B). The experiments were stopped on day 21 (sacrifice of all remaining animals).

2.6. Isolation of trophozoites from tissue samples

Tissue samples obtained from nasal mucosa, brain, and lungs were gently dipped on agar plates (1.5% agar in Page's saline). The plates were sealed, kept at 37 °C, and daily investigated for the presence of viable trophozoites (Fig. 4C).

2.7. Histopathology

Tissue samples obtained from brain, and lungs were fixed in a 4% PBS-buffered formalin solution, embedded in paraffin, sectioned to 8-μm-thick slices, and stained with haematoxylin–eosin, a technique employed universally for routine tissue examination and used as diagnostic tool to visualize FLA in tissue samples (Fig. 4A and B) (Walochnik and Aspöck, 2005).

3. Results

3.1. Isolation of trophozoites and establishment of cultures

Out of a total of 31 water samples, nine samples scored positive for free-living amoebae (29%) after culturing at 37 °C for 2 weeks (Table 1). Each of the nine samples displayed morphologically identical trophozoites within the same sample. For this reason, one single trophozoite was selected per sample, and used to obtain clones. The 9 "positive" clones (one clone per positive sample) isolated were able to grow at 37 °C for a minimum time period of 4 months, and were thus considered as thermotolerant (A2, A3, A6, B2, B3, C4, C6, C7, D1). Seven out of the nine clones were able

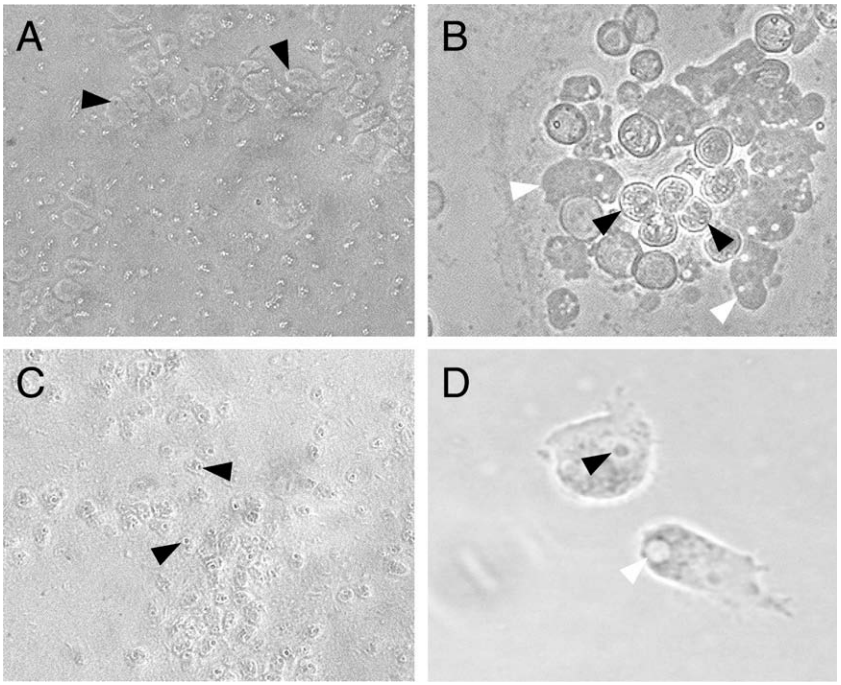

Fig. 2. Morphological documentation of non-*Acanthamoeba* isolates from hot spring resorts. (A) *Stenamoeba* sp.-related isolate A2 on agar with black arrowheads pointing at contractile vacuoles (original magnification: 400×). (B) Trophozoites (white arrowheads) and cysts (black arrowheads) of *H. vermiformis*-related isolate A3 cultured on agar (original magnification: 400×). (C) *E. exundans*-related isolate A6 cultured on agar showing contractile vacuoles (arrowheads) (original magnification: 200×). (D) Trophozoites of *E. exundans*-related isolate D1 re-suspended in water showing nucleus (black arrowhead) and a contractile vacuole (white arrowhead) (original magnification: 1000×).

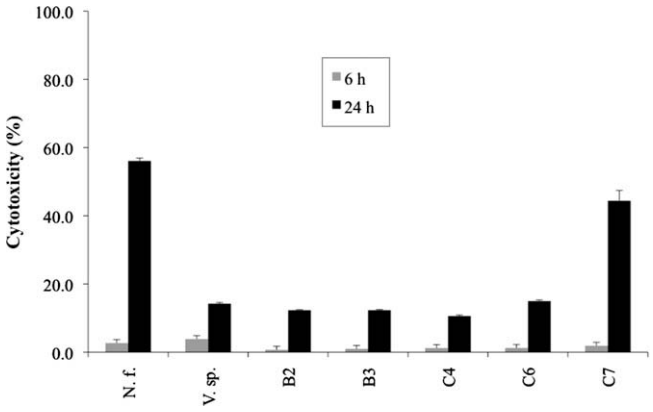

Fig. 3. Cytotoxicity of *Naegleria fowleri* (N. f.; pathogenic FLA reference strain [ATCC 30894]), *Vannella* sp. (V. sp.; non-pathogenic FLA reference strain [ATCC 30947]), and *Acanthamoeba healyi*-related isolates B2, B3, C4, C6, and C7 against L929 murine fibroblasts. Samples taken after 6 h and 24 h of cultivation were tested in triplicates and results are given as mean values and error bars represent standard deviations. Note that all results were confirmed in a second assay that was based on an independent co-cultivation experiment.

to grow at 42 °C, while two lost their viability within 3–5 days (A3, A6) as evidenced by the loss of characteristic morphology and absence of growth when the respective cultures were re-incubated at 37 °C after exposure to 42 °C (Table 2). Isolates A6 and B3 were lost during culture, and could therefore not be further investigated for cytotoxic activity (A6), and in *in vivo* infection (B3), respectively. Eight out of nine isolates were obtained from samples taken during, or before, filtration process, while only one sample (A6) from the pools scored positive for FLA (Table 1).

All FLA isolates characteristically showed contractile vacuoles (Figs. 1 and 2). Morphologically, isolates B2, B3, C4, C6, and C7, displayed acanthapodia, characteristic spine-like structures on the surface of trophozoites and the presence of double-walled cysts identified the isolates as belonging to the genus *Acanthamoeba* (Fig. 1).

3.2. Cytotoxic activity of the isolates

As assessed microscopically during long-term co-cultivation of variable numbers of FLA (2.5×10^4, 2.5×10^3, 2.5×10^2 trophozoites per well) on murine L929 fibroblast monolayers in 24-well plates, *A. healyi*-related isolates B2, B3, C4, C6, and C7 displayed a cytotoxic profile comparable to the one of the pathogenic FLA, i.e. *N. fowleri, A. lenticulata,* and *B. mandrillaris*. This profile of cytotoxicity included the complete loss of confluence of the L929 cells (cytotoxicity), increasing density of the co-cultured isolate (growth), and viability of isolates on day 5 of co-culture (Table 2). The other isolates investigated (A2, A3, and D1) failed to grow in co-culture, and no viable trophozoites were present on day 5 of co-culture. For *N. fowleri*, loss of confluence of the L929 monolayer was observed on day 2 without trophozoites being visible (Table 2). Induction of rapid host-cell death by *N. fowleri* could be explained either by a contact-dependent or contact-independent mechanism (Kang et al., 2005; Kim et al., 2008), which is also likely to apply to isolate A3.

In the LDH release assay, the *A. healyi*-related isolates exhibited a differential cytotoxicity pattern when high numbers of amoebae (5×10^5 trophozoites per well) were grown for a relatively short period (24 h) on L929 fibroblast monolayers in 24-well plates (Fig. 3). Here, only isolate C7 exhibited a degree of cytotoxicity that was similar to the one of pathogenic *N. fowleri* reference strain (cytotoxicity: 44.4 + 3.1% versus 56.1 + 0.8%). For long-term (2 and 5 days) cultures, the assay system demonstrated an allover massive reduction of the LDH activity (not shown) indicating a degradation of the released enzyme in the culture medium at these late time-points.

Table 3
Taxonomic identification of isolates.

Isolate	Accession No.[a]		Accession No.[b]	Taxonomic status[c]	Query coverage/max. identity[d]	
	FLA-PCR	ACA-PCR			FLA-PCR (%)	ACA-PCR
B2	GQ996530	GU199433	AF019070.1	*Acanthamoeba healyi*	99/95	100/97%
B3	GQ996531	GU199434	AF019070.1	*Acanthamoeba healyi*	96/78	100/95%
C4	GU016571	GU199435	AF019070.1	*Acanthamoeba healyi*	99/91	100/98%
C6	GQ996532	GU199436	AF019070.1	*Acanthamoeba healyi*	99/95	100/99%
C7	GQ996533	GU199437	AF019070.1	*Acanthamoeba healyi*	90/95	99/95%
A2	GQ996534	n.a.[e]	EU377587.1	*Stenamoeba* sp.	100/98	n.a.
A3	GQ996535	n.a.	AY502959.1	*Hartmannella vermiformis*	100/100	n.a.
A6	GQ996536	n.a.	AF293895.1	*Echinamoeba exundans*	100/99	n.a.
D1	GQ996537	n.a.	AF293895.1	*Echinamoeba exundans*	100/100	n.a.

[a] Sequence from present isolate.
[b] Sequence from genetically closest organism.
[c] Respective to genetically closest organism.
[d] Respective to sequence from genetically closest organism.
[e] Not applicable.

Table 4
In vivo infection, course of infection, and re-isolation of trophozoites.

Isolate	Taxonomic status[a]	Time point of sacrifice in days after infection (isolation of trophozoites)[b]					
		Immunocompetent[c]			Immunodeficient[c]		
		1	2	3	1	2	3
B2	*Acanthamoeba healyi*	a (−/−/−)	a (−/−/−)	n.d.	11 (n/l/b)	a (n/−/−)	n.d.
C4	*Acanthamoeba healyi*	a (−/−/−)	a (−/−/−)	n.d.	8 (n/l/b)	10 (n/l/b)	n.d.
C6	*Acanthamoeba healyi*	8 (−/−/b)	a (−/−/−)	n.d.	5 (n/l/b)	6 (−/−/b)	n.d.
C7	*Acanthamoeba healyi*	a (−/−/b)	a (n/−/b)	n.d.	3 (n/−/−)	9 (n/−/b)	a (n/−/−)

[a] Respective to genetically closest organism.
[b] a: alive; n.d.: not determined; (n/l/b): recovery of viable trophozoites from nasal mucosa (n), lung (l), and brain (b).
[c] 1, 2, 3 indicate individual animals.

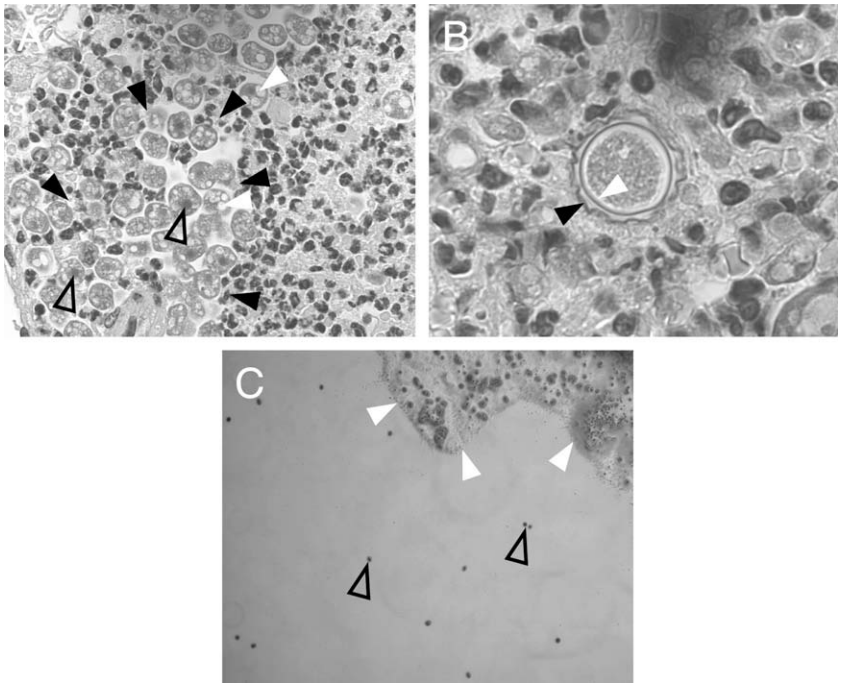

Fig. 4. Histological sections (haematoxylin–eosin staining) from brain and isolation of trophozoites from tissue samples of infected Rag2(−/−)gamma(c)(−/−) mice. (A) A characteristic focus, delineated by black arrowheads, of trophozoites of *Acanthamoeba* (isolate C4) in brain tissue. The trophozoites are readily recognizable by the contractile vacuoles (white arrowheads), and the nucleus (open arrowheads) (original magnification: 400×). (B) Double-walled *Acanthamoeba* cyst in brain tissue consisting of exocyst (white arrowhead) and endocyst (black arrowhead) (original magnification: 1000×). (C) Migration of trophozoites (open arrowheads) from the site where the tissue sample was dipped on the agar (delineated by white arrowheads) towards the edge of the plate (original magnification: 50×).

3.3. Molecular identification of the isolates

All isolates scored positive for the FLA-PCR, five scored additionally positive in ACA-PCR (not shown). None scored positive in the Nf-PCR (data not shown). Sequence analysis of PCR products obtained using FLA-PCR identified the isolates as phylogenetically closely related to the following organisms: *Stenamoeba* sp. (A2), *H. vermiformis* (A3), *Echinamoeba exundans* (A6, D1), and *A. healyi* (B2, B3, C4, C6, C7) (Table 3). Sequence analysis of the ACA-PCR products confirmed the close relationship of isolates B2, B3, C4, C6, C7 to *A. healyi* (Table 3).

3.4. In vivo pathogenicity

Experimental infection with isolates B2, C4, C6, and C7 in Rag2(−/−)gamma(c)(−/−) mice led to the onset of characteristic symptoms (loss of weight, ataxy, and obtundation) in at least one animal per isolate (Table 4). Clinical symptoms became apparent between 3 and 11 days after infection. Although characteristic symptoms were clearly observed in animal 1 challenged with isolate C7, no viable trophozoites were isolated from brain and lung tissue nor revealed histological examinations of these tissues the presence of trophozoites or cysts. Therefore, it cannot be excluded that factors other than amoebic trophozoites may have contributed to the development of symptoms in this case. Two animals out of nine infected did not develop symptoms by day 21. However, viable trophozoites were isolated from the nasal mucosa after sacrifice.

In immune-competent wild type animals, infection with isolates B2, C4, and C7 failed to develop characteristic symptoms, although viable trophozoites were isolated from the brain on day 21 (isolate C7). One animal developed characteristic symptoms, and viable trophozoites were isolated from brain tissue on after sacrifice on day 8. Altogether, these results clearly indicate that immune-compromised animals were preferentially affected by infection with the isolates related to *A. healyi*. However, the present results may suggest that also immune-competent animal can be affected, but a longer time of infection is needed to develop pathologies. The corresponding mock-infected negative controls did not develop symptoms, nor were viable trophozoites isolated from the tissues on day 21.

3.5. Isolation of trophozoites from tissue samples

The presence of viable amoebae in tissue samples was clearly detectable by characteristic migration of trophozoites from the site of tissue contact on agar towards the edge of the plate (Fig. 4C).

3.6. Histopathology

Lung and brain tissues affected by amoebae displayed areas marked by a dense presence of trophozoites and cysts (Fig. 4A and B). Within the tissue, trophozoites were easily recognized upon characteristic structures including cell nucleus, and contractile vacuoles (Fig. 4A). Cysts within the tissue were readily identified by the exocyst and endocyst, a characteristic feature of *Acanthamoeba* spp. cysts.

4. Discussion

The present study reports on the isolation strains from hot spring resorts in Switzerland. As assessed by co-cultivation of FLA with murine L929 fibroblasts, 5 (B2, B3, C4, C6, and C7) out of the nine isolates identified showed a high thermotolerance paralleled by a high cytotoxic activity, comparable to the cytotoxic profile of pathogenic *N. fowleri*, *B. mandrillaris*, and *A. lenticulata*. It is important to note that this allover high degree of cytotoxicity among isolates B2, B3, C4, C6, and C7 was exclusively detected upon long-term (5-day) co-cultivation and could only be assessed by microscopical inspection of the lytic activity of the five isolates within respective cultures. However, in the LDH release assay suitable for the demonstration of the cytotoxic potential upon short-term (up to 24 h) exposure of the five isolates to L929 cells, high cytolytic activity was restricted to isolate C7. Despite this differential cytotoxicity profile in the LDH release assay, morphological criteria and comparative sequence analysis of 18S rDNA identified all five isolates as organisms closely related to *A. healyi*. The relationship among the different isolates was also – to a certain extent – reflected by the common pathogenicity pattern in experimentally infected immunodeficient mice (assessed by experimental infection of mice with isolates B2; C4, C6, and C7).

Swimming pools have been shown in previous studies to be habitats for potentially pathogenic *Acanthamoeba* spp. (De Jonckheere, 1979; Rivera et al., 1993; Gornik and Kuzna-Grygiel, 2004; Kuhlencord et al., 1989). However, in contrast to *N. fowleri*, where clinical cases of fatal PAM were directly linked to the presence of the parasite in these sites (Cerva et al., 1968; Cursons et al., 1979; Kadlec, 1981), so far no data exist which report clinical cases of CNS infection due to the presence of pathogenic *Acanthamoeba* spp. in swimming pools. It has to be emphasized that the presence of *A. healyi*-related FLA organisms was restricted to the filtration unit of two hot spring resorts (resorts B and C). With regard to the swimming pools, which offer the sole opportunity for a contact between FLA and visitors of the hot spring resorts, FLA were isolated from only one pool (from resort A), and the isolate (A6) was shown to be related to non-pathogenic *E. exundans*. The rigorous cleaning schedule, efficacy of cleaning of the pools, and the filtration unit are likely to be the cause for the weak presence of FLA in the pools. Pathogenic *N. fowleri* were neither found in the filtration unit nor in the swimming pools.

Acanthamoeba spp. are the most common amoebae, and probably the most common protozoa, to be found in soil and water samples (Page, 1988), and evidenced by the presence to a high percentage in samples taken in human-related aquatic habitats (Lorenzo-Morales et al., 2005). Due to the cosmopolitan distribution, multiple opportunities for contacts with humans are provided, as evidenced by antibody titres in more than 80% of surveyed human populations (Cursons et al., 1980; Chappell et al., 2001). Moreover, humans have been shown to be healthy carriers of *Acanthamoeba* spp., as evidenced by the isolation from the naso-pharyngeal and oral cavities (Rivera et al., 1984; De Jonckheere and Michel, 1988). Even so, cases of CNS infections due to *Acanthamoeba* spp. are very rare, but ending in high fatality in almost 100% of the cases. Infection occurs in the lower respiratory tract, and spread to the CNS is haematogenous. Therapeutic treatment for GAE is still unsatisfactory, and the outcomes of most reported cases of GAE have been uniformly fatal (Schuster and Visvesvara, 2004a). *Acanthamoeba* spp. including *A. castellani*, *A. polyphaga*, and *A. culbertsoni* (Castellano-Sanchez et al., 2003; Martinez, 1991; Willaert et al., 1978; Uschuplich et al., 2004), have been shown to be the aetiologic agents of GAE. For *A. healyi*, only one case of GAE has been documented so far (Moura et al., 1992).

Worldwide, the interest in pathogenic FLA and pathologies associated with FLA is increasing, as shown by the rapidly rising number of studies during the last years (Khan, 2006). It is noteworthy that in recent years numerous countries and areas of the world have recorded their first cases of CNS infections due to FLA, e.g. Portugal (Tavares et al., 2006), Italy (Cogo et al., 2004), Chile (Oddo et al., 2006), Madagascar (Jaffar-Bandjee et al., 2005), and south-east Asia (Intalapaporn et al., 2004). A recent work has emphasized on the growing number of keratitis cases due to infection with *Acanthamoeba* spp. (Acharya et al., 2007). So far, successful treatments of *Acanthamoeba* spp. infections are mostly due to multi-

drug therapies but remain an exception despite various treatment regimens. Generally, infections with *Acanthamoeba* spp. are not readily recognized, because many patients present with underlying disease (Marciano-Cabral and Bradley, 2001; Schuster and Visvesvara, 2004b). Increased awareness for the potential of infection with *Acanthamoeba* spp. is an important factor for early detection and treatment.

In summary, we have documented the presence of potentially pathogenic *A. healyi*-related organisms isolated in the water filtration units of hot spring resorts in Switzerland. FLA found in the pools belonged to a non-pathogenic species. Continuative studies will be aimed at screening further habitats of potentially pathogenic FLA in Switzerland. Recently, potentially pathogenic *A. lenticulata* have been isolated from warm aquatic habitat (Gianinazzi et al., 2009a), while screening of cold-water bodies resulted in the isolation of non-pathogenic species (Gianinazzi et al., 2009b). The focus will not be limited to constantly warm-water habitats (heated pools, hot water resorts), but will also be directed to seasonally warm habitats, which may become more and more suitable habitats due to global warming. In the hot summer of 2003, the first European case of CNS due to FLA related to bathing activities in a natural water body was published (Cogo et al., 2004).

Acknowledgment

The work has been supported by the Federal Office for Civil Protection, Spiez, Switzerland (Project No. 4500303638).

References

Acharya, N.R., Lietman, T.M., Margolis, T.P., 2007. Parasites on the rise: a new epidemic of *Acanthamoeba* keratitis. American Journal of Ophthalmology 144, 292–293.

Baenziger, S., Tussiwand, R., Schlaepfer, E., Mazzucchelli, L., Heikenwalder, M., Kurrer, M.O., Behnke, S., Frey, J., Oxenius, A., Joller, H., Aguzzi, A., Manz, M.G., Speck, R.F., 2006. Disseminated and sustained HIV infection in CD34+ cord blood cell-transplanted Rag2(−/−)gamma(c)(−/−) mice. Proceedings of the National Academy of Sciences 103, 15951–15956.

Behets, J., Declerck, P., Delaedt, Y., Verelst, L., Ollevier, F., 2006. Survey for the presence of specific free-living amoebae in cooling waters from Belgian power plants. Parasitology Research 100, 1249–1256.

Bloch, K.C., Schuster, F.L., 2005. Inability to make a premortem diagnosis of *Acanthamoeba* species infection in a patient with fatal granulomatous amebic encephalitis. Journal of Clinical Microbiology 43, 3003–3006.

Castellano-Sanchez, A., Popp, A.C., Nolte, F.S., Visvesvara, G.S., Thigpen, M., Redei, I., Somani, J., 2003. *Acanthamoeba castellani* encephalitis following partially mismatched related donor peripheral stem cell transplantation. Transplant Infectious Disease 5, 191–194.

Cerva, L., Novak, K., Culbertson, C.G., 1968. An outbreak of acute, fatal amebic meningoencephalitis. American Journal of Epidemiology 88, 436–444.

Chappell, C.L., Wright, J.A., Coletta, M., Newsome, A.L., 2001. Standardized method of measuring *Acanthamoeba* antibodies in sera from healthy human subjects. Clinical and Diagnostic Laboratory Immunology 8, 724–730.

Cogo, P.E., Scagli, M., Gatti, S., Rossetti, F., Alaggio, R., Laverda, A.M., Zhou, L., Xiao, L., Visvesvara, G.S., 2004. Fatal *Naegleria fowleri* meningoencephalitis, Italy. Emerging Infectious Diseases Journal 10, 1835–1837.

Cursons, R.T., Brown, T.J., Keys, E.A., Gordon, E.H., Leng, R.H., Havill, J.H., Hyne, B.E., 1979. Primary amoebic meningo-encephalitis in an indoor heat-exchange swimming pool. NZ Medical Journal 90, 330–331.

Cursons, R.T., Brown, T.J., Keys, E.A., Moriarty, K.M., Till, D., 1980. Immunity to pathogenic free-living amoebae: role of humoral antibody. Infection and Immunity 29, 401–407.

De Jonckheere, J.F., 1979. Pathogenic free-living amoebae in swimming pools: survey in Belgium. Annals of Microbiology 130B, 205–212.

De Jonckheere, J.F., Michel, R., 1988. Species identification and virulence of *Acanthamoeba* strains from human nasal mucosa. Parasitology Research 74, 314–316.

De Jonckheere, J.F., 1982. Hospital hydrotherapy pools treated with ultra violet light: bad bacteriological quality and presence of thermophilic *Naegleria*. Journal of Hygiene 88, 205–214.

Gianinazzi, C., Schild, M., Müller, N., Leib, S.L., Simon, F., Nuñez, S., Joss, P., Gottstein, B., 2005. Organotypic slice cultures from rat brain tissue: a new approach for *Naegleria fowleri* CNS infection in vitro. Parasitology 131, 797–804.

Gianinazzi, C., Schild, M., Wüthrich, F., Ben Nouir, N., Füchslin, H.P., Schürch, N., Gottstein, B., Müller, N., 2009a. Screening Swiss water bodies for potentially pathogenic free-living amoebae. Research in Microbiology 160, 367–374.

Gianinazzi, C., Schild, M., Wüthrich, F., Müller, N., Schürch, N., Gottstein, B., 2009b. A potentially human–pathogenic *Acanthamoeba* isolate from a heated indoor swimming pool in Switzerland. Experimental Parasitology 121, 180–186.

Goldman, J.P., Blundell, M.P., Lopes, L., Kinnon, C., Di Santo, J.P., Thrasher, A.J., 1998. Enhanced human cell engraftment in mice deficient in RAG2 and the common cytokine receptor gamma chain. British Journal of Haematology 103, 335–342.

Gornik, K., Kuzna-Grygiel, W., 2004. Presence of virulent strains of amphizoic amoebae in swimming pools of the city of Szczecin. Annals of Agricultural and Environmental Medicine 11, 233–236.

Intalapaporn, P., Suankratay, C., Shuangshoti, S., Phantumchinda, K., Keelawat, S., Wilde, H., 2004. *Balamuthia mandrillaris* meningoencephalitis: the first case in southeast Asia. American Journal of Tropical Medicine and Hygiene 70, 666–669.

Jaffar-Bandjee, M.C., Alessandri, J.L., Molet, B., Clouzeau, J., Jacquemot, L., Samperiz, S., Saly, J.C., 2005. Primary amebic meningoencephalitis: 1st case observed in Madagascar. Bulletin de la Societe de Pathologie Exotique 98, 11–13.

Kadlec, V., Skvárová, J., Cerva, L., Nebáznivá, D., 1980. Virulent *Naegleria fowleri* in indoor swimming pool. Folia Parasitologica 27, 11–17.

Kadlec, V., 1981. Different virulence of *Naegleria fowleri* strains isolated from a swimming pool. Folia Parasitologica 28, 97–103.

Kang, S.Y., Song, K.J., Jeong, S.R., Kim, J.H., Park, S., Kim, K., Kwon, M.H., Shin, H.J., 2005. Role of the Nfa1 protein in pathogenic *Naegleria fowleri* cocultured with CHO target cells. Clinical and Diagnostic Laboratory Immunology 12, 873–876.

Khan, N.A., 2006. *Acanthamoeba*: biology and increasing importance in human health. EMS Microbiology Reviews 30, 564–595.

Khan, N.A., 2007. *Acanthamoeba* invasion of the central nervous system. International Journal of Parasitology 37, 131–138.

Kim, J.H., Kim, D., Shin, H.J., 2008. Contact-independent cell death of human microglial cells due to pathogenic *Naegleria fowleri* trophozoites. Korean Journal of Parasitology 46, 217–221.

Kuhlencord, A., Mergerian, H., Bommer, W., 1989. Studies on the pathogenesis of *Acanthamoeba*-associated meningoencephalitis. Zentralblatt für Bakteriologie 271, 256–260.

Lekkla, A., Sutthikornchai, C., Bovornkitti, S., Sukthana, Y., 2005. Free-living ameba contamination in natural hot springs in Thailand. Southeast Asian Journal of Tropical Medicine and Public Health 36, 5–9.

Lorenzo-Morales, J., Ortega-Rivas, A., Foronda, P., Martínez, E., Valladares, B., 2005. Isolation and identification of pathogenic *Acanthamoeba* strains in Tenerife, Canary Islands, Spain from water sources. Parasitology Research 95, 273–277.

Marciano-Cabral, F., Bradley, S.G., 2001. Current research on free-living amebae causing granulomatous amebic encephalitis. Journal of Eukaryotic Microbiology (Suppl.), 3S.

Marciano-Cabral, F., Cabral, G., 2003. *Acanthamoeba* spp. as agents of disease in humans. Clinical Microbiology Reviews 16, 273–307.

Martinez, A.J., 1991. Infection of the central nervous system due to *Acanthamoeba*. Reviews of Infectious Diseases 13, 399–402.

Martinez, A.J., Visvesvara, G.S., 1997. Free-living, amphizoic and opportunistic amebas. Brain Pathology 7, 583–598.

Moura, H., Wallace, S., Visvesvara, G.S., 1992. *Acanthamoeba healyi* n. sp. and the isoenzyme and immunoblot profiles of *Acanthamoeba* spp., groups 1 and 3. Journal of Protozoology 39, 573–583.

Oddo, B.D., Ciani, A.S., Vial, C.P., 2006. Granulomatous amebic encephalitis caused by *Balamuthia mandrillaris*. First case diagnosed in Chile. Revista chilena de infectología 23, 232–236.

Page, F.C., 1988. A New Key to Fresh Water and Soil Amoebae. Freshwater Biological Association Scientific Publications, Cumbria, UK.

Pelandakis, M., Pernin, P., 2002. Use of multiplex PCR and PCR restriction enzyme analysis for detection and exploration of the variability in the free-living amoeba *Naegleria* in the environment. Applied and Environmental Microbiology 68, 2061–2065.

Rivera, F., Medina, F., Ramírez, P., Alcocer, J., Vilaclara, G., Robles, E., 1984. Pathogenic and free-living protozoa cultured from the nasopharyngeal and oral regions of dental patients. Environmental Research 33, 428–440.

Rivera, F., Ramírez, E., Bonilla, P., Calderón, A., Gallegos, E., Rodríguez, S., Ortiz, R., Zaldívar, B., Ramírez, P., Durán, A., 1993. Pathogenic and free-living amoebae isolated from swimming pools and physiotherapy tubs in Mexico. Environmental Research 62, 43–52.

Scaglia, M., Strosselli, M., Grazioli, V., Gatti, S., Bernuzzi, A.M., de Jonckheere, J.F., 1983. Isolation and identification of pathogenic *Naegleria australiensis* (*Amoebida*, *Vahlkampfiidae*) from a spa in northern Italy. Applied and Environmental Microbiology 46, 1282–1285.

Schild, M., Gianinazzi, C., Gottstein, B., Müller, N., 2007. PCR-based diagnosis of *Naegleria* sp. infection in formalin-fixed and paraffin-embedded brain sections. Journal of Clinical Microbiology 45, 564–567.

Schroeder, J.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 Acanthamoebae from humans with keratitis and from sewage sludge. Journal of Clinical Microbiology 39, 1903–1911.

Schuster, F.L., Visvesvara, G.S., 2004a. Free-living amoebae as opportunistic and non-opportunistic pathogens of humans and animals. International Journal for Parasitology 34, 1001–1027.

Schuster, F.L., Visvesvara, G.S., 2004b. Opportunistic amoebae: challenges in prophylaxis and treatment. Drug Resistance Updates 7, 41–51.

Sissons, J., Alsam, S., Goldsworthy, G., Lightfoot, M., Jarroll, E.L., Khan, N.A., 2006. Identification and properties of proteases from *Acanthamoeba* isolate capable of producing granulomatous encephalitis. BMC Microbiology 6, 42–49.

Tavares, M., Correia da Costa, J.M., Carpenter, S.S., Santos, L.A., Afonso, C., Aguiar, A., Pereira, J., Cardoso, A.I., Schuster, F.L., Yagi, S., Sriram, R., Visvesvara, G.S., 2006. Diagnosis of first case of *Balamuthia* amoebic encephalitis in Portugal by immunofluorescence and PCR. Journal of Clinical Microbiology 44, 2660–2663.

Tsvetkova, N., Schild, M., Panaiotov, S., Kurdova-Mintcheva, R., Gottstein, B., Walochnik, J., Aspöck, H., Lucas, M.S., Müller, N., 2004. The identification of free-living environmental isolates of amoebae from Bulgaria. Parasitology Research 92, 405–413.

Uschuplich, V., Mileusnic, D., Johnson, M., 2004. Pathologic quiz case. Progressive fatal encephalopathy in an immunosuppressed patient with a history of discoid lupus erythematosus. Subacute granulomatous meningoencephalitis (*Acanthamoeba culbertsoni*). Archives of Pathology and Laboratory Medicine 128, 109–111.

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 Immunology and Medical Microbiology 50, 1–26.

Walia, R., Montoya, J.G., Visvesvera, G.S., Booton, G.C., Doyle, R.L., 2007. A case of successful treatment of cutaneous *Acanthamoeba* infection in a lung transplant recipient. Transplant Infectious Disease 9, 51–54.

Willaert, E., Stevens, A.R., Healy, G.R., 1978. Retrospective identification of *Acanthamoeba culbertsoni* in a case of amoebic meningoencephalitis. Journal of Clinical Pathology 31, 717–720.

Walochnik, J., Aspöck, H., 2005. Diagnostics of infections with free-living amoebae (FLA) 2005. Journal of Laboratory and Clinical Medicine 29, 446–456.