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Screening Swiss water bodies for potentially pathogenic free-living amoebae

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Abstract

Free-ling amoebae (FLA) including *Acanthamoeba* spp., *Naegleria fowleri*, *Balamuthia mandrillaris* and *Sappinia pedata*, can cause opportunistic infections leading to severe brain pathologies. Human infections with pathogenic FLA have been increasingly documented in many countries. In Switzerland, thus far, the occurrence and distribution of potentially pathogenic FLA has not been investigated. Swiss water biotopes, including swimming pools, lakes, rivers and ponds, have now been screened for the presence of FLA, and assessment of their pathogenicity potential for a mammalian host has been undertaken. Thus, a total of 17 isolates were recovered by in vitro cultivation from these different aquatic sources. Characterization by sequence analysis of *Acanthamoeba* spp.-specific and 'FLA-specific PCR products amplified from 18s rDNA based on morphological traits, thermotolerance, and cytotoxicity towards murine fibroblasts yielded the following findings: *Echinamoeba* cf. *exundans* (3 isolates), *Hartmannella* spp. (3), *Vannella* spp. (4), *Protacanthamoebica* cf. *bohemica* (1), *Acanthamoeba* cf. *castellanii* (1) and *Naegleria* spp. (5). *B. mandrillaris* and *N. fowleri* did not range amongst these isolates. None of the isolates exhibited pronounced cytotoxicity and all failed to grow at 42 °C; therefore, they do not present any potential for CNS pathogenicity for humans.

Keywords: Free-living amoebae; *Acanthamoeba* spp.; Central nervous system (CNS) infection

1. Introduction

Free-living amoebae (FLA) have worldwide distribution in soil and water. Generally, FLA, in contrast to the medically well-known parasitic amoeba *Entamoeba histolytica*, which causes colitis and liver abscesses, do not represent threats to human health. However, FLA of some genera or species, such as *Acanthamoeba* spp., *Naegleria fowleri*, *Balamauthia mandrillaris* and *Sappinia pedata,* can act as opportunistic parasites, leading to severe brain pathologies [24,34]. In addition, amoebae of the genus *Acanthamoeba* and *B. mandrillaris* are known to cause skin infections [26,27]. In eye infections, *Acanthamoeba* spp. are known to cause keratitis [21].

Central nervous system (CNS) infections caused by FLA include primary amoebic meningoencephalitis (PAM) with *N. fowleri* as the infecting agent, and granulomatous amoebic encephalitis (GAE) which is due to infection with *Acanthamoeba* spp. and *B. mandrillaris*. Pathogenesis is not yet fully understood, and chemotherapy and other treatments for these infections are usually empirical and remain unsatisfactory. Patient recovery is frequently problematic in that the vast majority of reported cases infections are fatal [31]. In recent years, numerous countries and areas, e.g. Portugal [32], Italy [6], Chile [23], Madagascar [17], and southeast Asia [16],

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have recorded their first cases of CNS infections due to FLA. Moreover, a recent report has emphasized the growing number of keratitis cases due to FLA [1].

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 few years [20] documenting the importance of FLA in human health. Potentially pathogenic FLA have been found in environments such as lakes, pools, thermal waters, cooling waters from power plants and tap water, but also in air-conditioning units, bottled mineral water and eyewash stations [2,14,20,24,31,34]. Due to this cosmopolitan distribution, multiple opportunities for contact with humans are provided, as evidenced by antibody titers in human populations [31].

The aim of the present study was to investigate selected water bodies in Switzerland, related to human activities and recreation, for the presence of FLA potentially pathogenic to humans. Potential pathogenicity was assessed by the extent of cytotoxicity revealed in mammalian cell line L292 cells, the capacities of the isolates to grow at 37 °C and 42 °C, respectively, and by PCR protocols specific to FLA, *N. fowleri*, *Acanthamoeba* spp. and *B. mandrillaris*, followed by respective sequence analyses.

2. Materials and methods

2.1. Sampling

Water samples were collected in summer 2005 at selected sites by immersion of a 50 ml plastic Falcon tube into the upper 2 cm of the respective water body (Table 1). The tubes were subsequently cooled to 4 °C for 30 min (in cases in which the samples could not be processed immediately, tubes were stored at room temperature for a period not exceeding 48 h before cooling), coarse debris was removed by filtration on a sieve 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 resuspended in 100 µl of supernatant and were used as described below.

Table 1
Overview of geographic sites sampled for FLA.

Sampling site (Kt.)	Sample	Water temperature	Description of site	Isolation
Egelsee (BE)	3i	23.0 °C	L, art, turb, org	+
Rosengarten (BE)	4i	15.0 °C	Pd, art, cl, org	+
Aare (BE)	5i	17.5 °C	R, nat, cl, −	−
Brissago (TI)	6i	20.3 °C	Po, art, cl, −	+
Lago Maggiore (TI)	7i	23.0 °C	L, nat, cl, org	+
Lago della Piazza (TI)	8i	9.2 °C	L, nat, cl, −	−
Wohlensee (BE)	9i	14.1 °C	L, nat, turb, org	+
Murtensee (FR)	10i	18.0 °C	L, nat, turb, org	+
Bielersee (BE)	11i	19.6 °C	L, nat, turb, org	+
Moossee (BE)	12i	21.1 °C	L, nat, turb, org	+
Gürbe (BE)	13i	13.0 °C	R, nat, cl, org	−
Weyermannshaus (BE)	14i	15.7 °C	Po, art, cl, −	−
BoGa Bern (BE)	15i	20.5 °C	Pd, art, cl, org	+
BoGa Bern (BE)	16i	29.3 °C	Pd, art, cl, org	+
Binningen (BL)	S001	24.0 °C	Po, art, cl, −	+
Laufen (BL)	S002	−	Po, art, cl, −	+
Greifensse (ZH)	1e	26.7 °C	L, nat, cl, org	+
ARA Neugut (ZH)	2e	24.4 °C	R, nat, cl, org	+
Zürichsee (ZH)	3e	25.5 °C	L, nat, cl, −	+
Brugg (ZH)	4e	25.5 °C	Pd, nat, turb, org	+
ARA Brugg (ZH)	5e	22.5 °C	R, nat, cl, −	+
Lerchenfeld (BE)	LS001	−	Pd, nat, cl, −	−
Übeschisee (BE)	LS002	−	L, nat, cl, org	+
Thunersee (BE)	LS003	−	L, nat, cl, −	−

Kt.: Kanton; BE: Bern; FR: Fribourg; TI: Ticino; ZH: Zürich; L: lake; Pd: pond; Po: pool; R: river; art: artificial water body; nat: natural water body; cl: clear water; turb: turbid water; org: decaying organic material on the ground. "−" indicates a 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 did not grow at 37 °C.

2.2. Isolation and culturing of trophozoites

Resuspended pellets were gently pipetted onto a non-nutrient agar plate (1.5% agar in Page's saline) and allowed to adsorb and dry. The plates were then sealed with Parafilm®, and incubated upside down at 37 °C to carry out pre-selection based on broader-spectrum thermotolerance. In a subsequent step, thermotolerance of pre-selected isolates was further investigated by incubation of plates at 42 °C (see below). Daily inspection was carried out by light microscopy until morphological structures suggestive of amoeba trophozoites were detected. Cultures lacking morphological features of amoebae within two weeks were considered negative and discarded. Upon detection of trophozoites, clones were established by means of the migration technique [12]. 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. In cases of fungal contamination, this procedure was repeated until amoebae cultures were free of contamination. The clones were then kept at 37 °C (and at 42 °C in order to investigate isolates for increased thermotolerance) and transferred onto fresh agar plates coated with heat-inactivated *Escherichia coli* (1 h at 60 °C) every two weeks. For documentation, cysts and trophozoites were photographed (Fig. 3).

2.3. Cytotoxicity in vitro

In order to screen for clones that exhibit cytotoxic potential, we employed an approach based on a coculture 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% fetal 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. Amoebae to be investigated were harvested from agar plates when the density reached a level sufficient to allow subsequent infection of mice. For this, trophozoites were carefully removed from the plates by scraping with a curved Pasteur pipette, pelleted in PBS and resuspended 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 L 929 cultures were investigated by light microscopy 1 and

4 days after infection 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. 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.

Data obtained on cytotoxicity, growth of trophozoites in co-cultures, assessment of viability of amoebae and thermotolerance at 42 °C were compared to data obtained with control isolates of pathogenic FLA (*N. fowleri* [ATCC 30894], *B. mandrillaris* [ATCC 50209], *Acanthamoeba lenticulata* [12]) and non-pathogenic FLA (*Vannella* sp. [ATCC 30947], *Hartmannella vermiformis* [ATCC 50802]) obtained from ATCC (Fig. 1, Table 2).

2.4. Characterization of the isolates

Amoebic trophozoites were gently scraped from one agar plate using a bent Pasteur pipette, resuspended in PBS and subsequently pelleted by sedimentation at 6000 × *g*. Genomic DNA (gDNA) was extracted from the native pellet using the DNAeasy kit (Qiagen, Basle, Switzerland) following the manufacturer's instructions. The gDNA was subjected either to PCRs yielding the specific recognition of *N. fowleri* (Nf-PCR), *Acanthamoeba* spp. (ACA-PCR) and *B. mandrillaris* (Bm-PCR) (refs. see below), or to a pan-PCR recognizing FLA in general (FLA-PCR) (for refs. see below). The following primer pairs were used: Nf-PCR, *N. fowleri*: Nae3-For (forward primer): 5′-CAAACACCGTTATGACAGGG-3′; Nae3-Rev (5′-CTGGTTTCCCTCACCTTACG-3′; target: 18S rDNA [29]. ACA-PCR, *Acanthamoeba* spp.: JDP1 (forward primer): 5′-GGCCCAGATCGTTTACCGTGAA-3′; JDP2 (reverse primer): 5′-TCTCACAAGCTGCTAGGGAGTCA-3′; target: 18S rDNA [30]. Bm-PCR, *B. mandrillaris*: 5′ Balspec

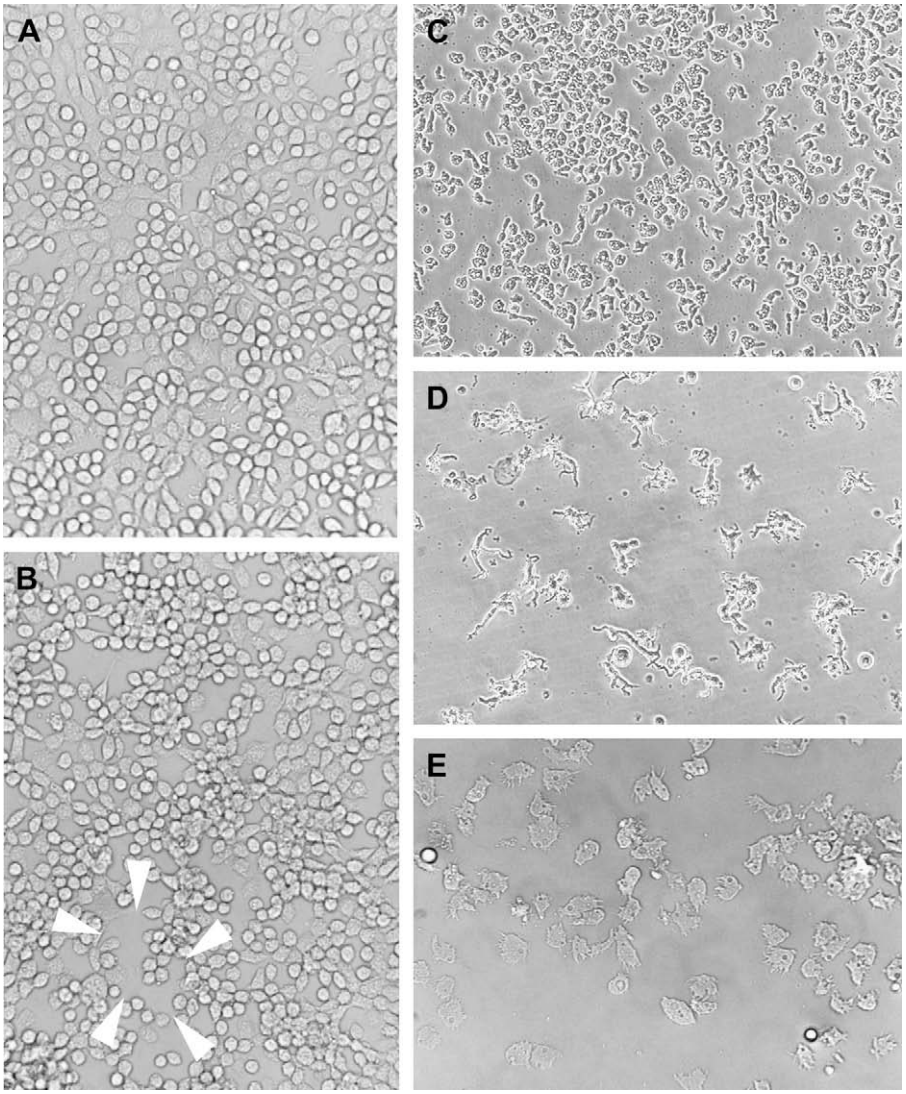

Fig. 1. Effect of pathogenic amoebae on fibroblast cells. A: Confluent monolayer (control). B: Developing damage as shown by loss of integrity of the cell layer (arrowheads). C–E: Complete loss of the fibroblast monolayer paralleled by massive proliferation of trophozoites of *N. fowleri* (C), *B. mandrillaris* (D), and *A. lenticulata* (E). Original magnification × 200.

Table 2
Cytotoxicity profile and thermotolerance of control and FLA field isolates.

Pathogenic species (positive controls)	Cytotoxicity* (25,000/2500/250)***		Growth** (25,000/2500/250)***		Viability	Thermotolerance 18 °C/37 °C/42 °C		
	day 2	day 5	day 2	day 5				
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 controls)								
Vannella sp.	4/4/4	2/4/4	0/0/0	0/0/0	−	+	+	−
Hartmannella vermiformis.	3/3/3	2/2/2	0/0/0	0/0/0	−	+	+	−
Study isolates								
S001	4/4/4	4/4/4	0/0/0	0/0/0	+	+	+	−
S002	4/4/4	4/4/4	0/0/0	0/0/0	−	+	+	−
4i	4/4/4	4/4/4	0/0/0	0/0/0	−	+	+	−
6i	1/4/4	1/4/4	0/0/0	0/0/0	−	+	+	−
7i	1/1/3	1/1/3	1/1/0	0/0/0	+	+	+	−
9i	4/4/4	4/4/4	0/0/0	0/0/0	−	+	+	−
10i	4/4/4	4/4/4	1/1/0	1/1/0	−	+	+	(+)
11i	4/4/4	4/4/4	0/0/0	0/0/0	−	+	+	−
12i	−/4/4	−/4/4	−/0/0	−/0/0	+	+	+	−
15i	4/4/4	4/4/4	0/0/0	0/0/0	−	+	+	−
16i	4/4/4	4/4/4	0/0/0	0/0/0	+	+	+	−
2e	4/4/4	1/3/4	0/0/0	0/0/0	+	+	+	−
4e	4/4/4	1/3/4	0/0/0	0/0/0	−	+	+	−
LS002b	4/4/4	4/4/4	0/0/0	0/0/0	−	+	+	−

*Cytotoxicity measured via confluency 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 confluency (20–50%) and alteration of morphology (score 2); complete loss of confluency (0–20%) and consistent alteration of morphology (score 1).
**Growth of trophozoites characterized as follows: high (score 3), medium (score 2), and low (score 1) density; no trophozoites visible (score 0).
***No. of trophozoites used as inoculum.

(forward primer): 5′-CGCATGTATGAAGAAGACCA-3′; 3′ Balspec 16S (reverse primer): 5′-TTACCTATA TAATTGTCGATACCA-3′; target: 16S rDNA [3]. FLA-PCR, free living amoeba: P-FLA-F (forward primer): 5′-CGCGGTAATTCCAGCTCCAATAGC-3′; P-FLA-R (reverse primer) 5′-CAGGTTAAGGTCTCGTTCGTTAAC-3′; target: 18S rDNA [33]. The PCR products were visualized on 2% agarose gels containing ethidium bromide. For sequence analyses, 50 µl of PCR product were purified using the High Pure PCR product purification kit (Roche, Diagnostics GmbH, Mannheim, Germany) 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 BLAST (basic local alignment search tool) (see Table 3). For comparative BLAST analyses, only sequence segments exhibiting complete identity in at least two independent sequencing runs were accepted.

3. Results

3.1. Isolation and culture of trophozoites

After in vitro culture at 37 °C for two weeks, 18 water samples (75%) from 24 different geographic sites were positive for free-living amoebae (75%) (Table 1). Samples that scored negative were either from river habitats (5i, 13i), an alpine lake at 2000 m a.s.l. (8i), a swimming pool (14i), a natural pond (LS001) or a lake (LS002). The 18 "positive" cultures led to the obtaining of 18 different clones (one clone per positive sample), which were able to grow at 37 °C for a minimum time period of four months and were thus considered as relatively thermotolerant. Four of the 18 clones (1e, 3e, 5e and 3i) were lost due to fungal contamination and could not be used for further investigation.

When grown at 42 °C, 17 of the 18 clones lost their viability within three to five days, as evidenced by the loss of characteristic morphology and absence of growth when put back at 37 °C. Field isolate 10i preserved morphology and viability, but failed to grow (Table 2). An increase in temperature from 37 °C to 42 °C resulted in faster growth for pathogenic controls *N. fowleri*, *A. lenticulata* and *B. mandrillaris*, while rapid loss of viability was observed in the non-pathogenic control amoebae *Vannella* sp. and *H. vermiformis*.

3.2. Cytotoxicity in vitro

In general, most of the field isolates showed no cytotoxic effects towards L929 cells, nor did they grow in co-culture. In addition, loss of viability in co-culture was observed in 9 out of 14 isolates. Cytotoxicity was noted for four isolates (6i, 7i, 2e, 4e). Only isolate 7i combined cytotoxicity and growth, although growth was at a low level and occurred exclusively on day 2. Cytotoxicity and growth patterns of the field isolates,

Table 3
Taxonomic identification (genetics and morphology) of field isolates obtained from Swiss water bodies.

Isolate	Water temp. (at origin)	Accession no. (sequence from present isolate)	Taxonomic status (respective to genetically closest organism)	Query coverage/max. identity (to sequence from genetically closest organism)	Accession no. (representing genetically closest organism)
3i	23.0 °C	FJ940706	*Echinamoeba* cf.[a] *exundans*	100/99%	AF293895.1
4i	15.0 °C	n.a.[b]	*Vannella* sp.*	n.a.	n.a.
6i	20.3 °C	FJ948856	*Naegleria* cf. *gruberi***	99/99%	AB298288.1
7i	23.0 °C	FJ940707	*Naegleria* cf. *gruberi***	96/95%	AB298288.1
9i	14.1 °C	FJ948857	*Vannella* cf. *miroides*	99/97%	AY183888.1
10i	18.0 °C	FJ940708	*Echinamoeba* cf. *exundans*	100/99%	AF293895.1
11i	19.6 °C	FJ940709	*Hartmannella* cf. *vermiformis*	100/98%	AF426157.1****
12i	21.1 °C	FJ940710	*Hartmannella* cf. *vermiformis*	95/99%	AF426157.1****
15i	20.5 °C	FJ940711	*Vannella* sp.	95/95%	AY929906.1****
16i	29.3 °C	FJ940712	*Protacanthamoeba* cf. *bohemica****	98/97%	AY960120.1
S001	24.0 °C	FJ940713	*Acanthamoeba* cf. *castellanii****	100/99%	U074131.1****
S002	n.d.[c]	FJ940714	*Vannella* sp.	100/95%	AY929906.1****
1e	26.7 °C	FJ940715	*Naegleria* cf. *australiensis*	99/100%	AY321362.1
2e	24.4 °C	FJ940716	*Hartmannella* cf. *vermiformis*	99/99%	AF426157.1****
3e	25.5 °C	n.a. †*****		n.a.	n.a.
4e	25.5 °C	FJ940717	*Naegleria* sp.**	96/95%	EF378678.1****
5e	22.5 °C	FJ940718	*Echinamoeba* cf. *exundans*	100/98%	AF293895.1
LS002	n.d.	FJ940719	*Naegleria* cf. *australiensis***	96/96%	AY321362.1

*Identification based exclusively on morphological traits.
**Formation of flagellae in water.
***Scoring positive in ACA-PCR.
****No. from only one hit among two, or more, identical BLAST scores indicated.
*****†, Isolate could neither be identified by morphological traits nor yielded the PCR product a significant similarity to known sequences.
[a] cf., taxonomic abbreviation indicating that isolate is similar but not certainly identified as this species.
[b] n.a., not applicable.
[c] n.d., not determined.

however, were not comparable to the pattern of control *N. fowleri*, *B. mandrillaris*, and *A. lenticulata*, where a noticeable cytotoxic effect was matched by marked proliferation of trophozoites (Table 2; Fig. 1).

3.3. Identification of field isolates (Table 3; Fig. 2)

All isolates scored positive for the FLA-PCR and, two also scored positive in ACA-PCR. Sequence analysis of PCR products obtained using FLA-PCR identified one isolate (S001) as *Acanthamoeba* cf. *castellanii* (for a comment on "cf." in front of the species name, see the "Discussion" section); sequencing of PCR products obtained using ACA-PCR (Fig. 2) in combination with morphological features characteristic of *Acanthamoeba* sp. confirmed this result (Fig. 3J, K). Three isolates were identified as *Echinamoeba* cf. *exundans* (3i, 10i, 5e) (Fig. 3G, H) or as members of the genus *Hartmannella* (11i, 12i, 2e) (Fig. 3D–F), respectively. Two isolates were classified as *Vannella* spp. (15i, S002) (Fig. 3B and C), and one as *Vannella* cf. *miroides* (9i). Analysis of the PCR products of isolates 4i and 3e yielded no significant match with published sequence data. However, the morphology of isolate 4i (i.e. fan-shaped appearance of the trophozoites) was highly suggestive of *Vannella* sp. (Fig. 3A). Moreover, sequence analysis results placed five isolates within the genus *Naegleria*. Isolates 1e and LS002b (Fig. 3O) were attributed to *Naegleria* cf. *australiensis*, 4e to *Naegleria* sp. (Fig. 3N), and 6i and 7i to *Naegleria* cf *gruberi* (Fig. 3L and M). All *Naegleria* isolates (with the exception of 1e, which was lost during culture and could not be further investigated) formed flagellae when resuspended in water, which is characteristic of the genus *Naegleria*. Isolate 16i scored positive in FLA-PCR and was identified as *Protoacanthamoeba* cf. *bohemica*, a species closely related to *Acanthamoeba* spp.. PCR suitable for detecting *N. fowleri*- (data not shown) and *B. mandrillaris*-DNA (BM) scored negative in all 18 isolates.

4. Discussion

The present work is the first attempt to screen, isolate and identify free living amoebae (FLA) that may exhibit a pathogenic potential from different Swiss water bodies. Some of the water bodies selected were directly related to human activities (i.e. swimming, bathing). These aquatic sites are known from other studies to potentially harbor pathogenic FLA including *Acanthamoeba* spp. and *N. fowleri,* which can lead to severe and lethal CNS infections [7,13,15,18,36].

Our data reported the presence of FLA in 75% of samples investigated. However, it must be taken into account that a temperature of 37 °C may be prohibitive for growth of non-thermotolerant amoebae, and therefore the percentage of positive

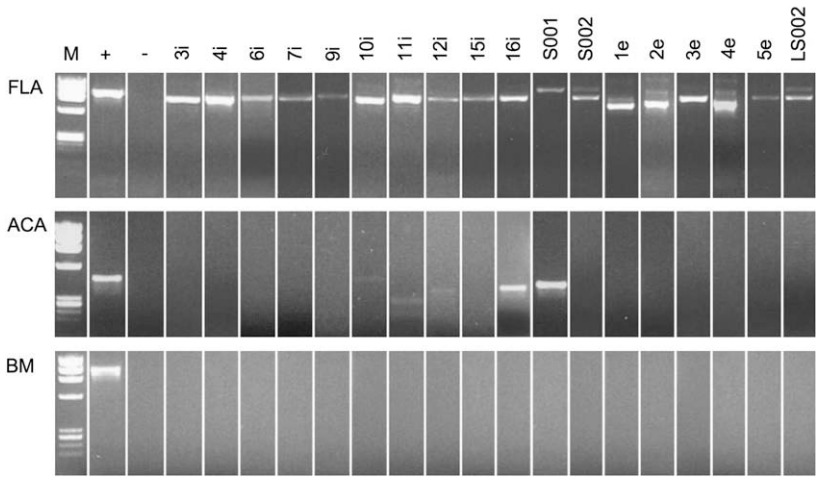

Fig. 2. Visualization of PCR products obtained upon gDNA from amoebic field isolates using specific protocols (FLA: free living amoebae; ACA: *Acanthamoeba* spp., BM: *B. mandrillaris*). Bands obtained with the FLA-PCR were used for sequence analysis. M: molecular markers; +: positive control (*A. polyphaga* for FLA and ACA, *B. mandrillaris* for BM). −: negative control.

samples might be higher if in vitro culture were carried out at lower temperatures. In addition, these data represent the situation at a given moment; seasonal fluctuations as well as fluctuations due to weather conditions are known to influence the number and species collected over time at the same site [11]. Nevertheless, our data confirm the omnipresence of FLA in aquatic environments.

Cultures of a total of 18 field isolates were established during this study. One of these isolates (4i) was characterized only based on morphological traits, while another isolate (3e) was lost during culture and could not be identified by morphological traits, nor did it yield the FLA-PCR product yield a significant similarity to known sequences. Conversely, the remaining 16 isolates were identified genetically based on PCR product sequence analysis of 18s rDNA. Here it is important to note that BLAST analysis of PCR-amplified 18s rDNA sequences used for identification of the isolates generally did not provide complete identity to the species suggested in our taxonomic classification (see Table 3). Accordingly, we decided to place the ''cf.'' in front of the respective species names. In the system of binomial nomenclature, ''cf.'' stands for ''confer'' (taxonomic meaning: ''compare'') and indicates that a suggested species needs to be viewed in the context of its comparison to another, but by definition is not confirmed as the same (http://en.wikipedia.org/wiki/Cf.).

As concerns PCR methodology, it must be taken into account that positive FLA-PCR amplification also occurs for protozoans others than FLA [2,25]. In fact, amplification of non-amoebic DNA was respectively observed in some FLA cultures, especially during early steps of isolation processes, where some contaminant organisms were still present. However, at later stages of cultivation, at which time cultures were free of contaminating organisms, this was no longer the case. FLA-PCR [33] can therefore be considered a very useful tool for molecular characterization of FLA in cultures, but its use is limited when initial field samples that may contain numerous other organisms are investigated. In addition to molecular biology, morphological traits (e.g. formation of flagellae for *Naegleria* spp., double-walled cysts in *Acanthamoeba* spp.) have been shown to be helpful for confirmation of data obtained using sequence analysis.

With regard to the potential pathogenicity of our field isolates, none of them expressed characteristics of CNS pathogenicity. The identification of one *Naegleria* isolate remained limited to the genus level; however, based on the fact that a cultivation temperature of 42° was prohibitive to proliferation, we could exclude *N. fowleri* as an isolated species. Upon respective sequence analyses, two other isolates were identified as *N.* cf. *australiensis*. Although *N. australiensis* and other *Naegleria* species are known to be cytotoxic for mammalian cells and pathogenic in experimental infection [28], cytotoxicity and pathogenicity do not necessarily correlate and vary considerably within different isolates [9,10]. Although cytotoxicity is not an ultimate criterion for pathogenicity, non-cytotoxic *N.* cf. *australiensis* isolate LS002b (see Tables 1–3) may have to be considered as potentially apathogenic. Unfortunately, we were not able to investigate the second isolate (1e, see Tables 1 and 3) with regard to cytotoxicity. Concerning *Acanthamoeba* spp., the isolate could be identified as *A.* cf. *castellanii*, a species known to cause keratitis and encephalitis in humans [5,19]. However, in experimental infections, *A. castellanii* strains displayed weak pathogenicity and were not able to grow at 42 °C [8]. Within the genus *Acanthamoeba*, it is anticipated that pathogenic strains of clinical relevance generally show an ability to grow at elevated temperatures [35]. The isolate found in this study failed to grow at 42 °C, which is strongly suggestive of a lack of pathogenicity. Although our FLA isolates did not exhibit any potential for being involved in CNS infections, it must be mentioned that other FLA belonging to the genera *Hartmannella*, *Echinamoeba* and *Acanthamoeba* can act as vectors for human pathogenic bacteria including *Legionella pneumophila* and *Pseudomonas aeruginosa* [4,22].

In summary, we have identified and investigated FLA isolated in water bodies in Switzerland and determined their potential pathogenicity. The comparison of temperature tolerance,

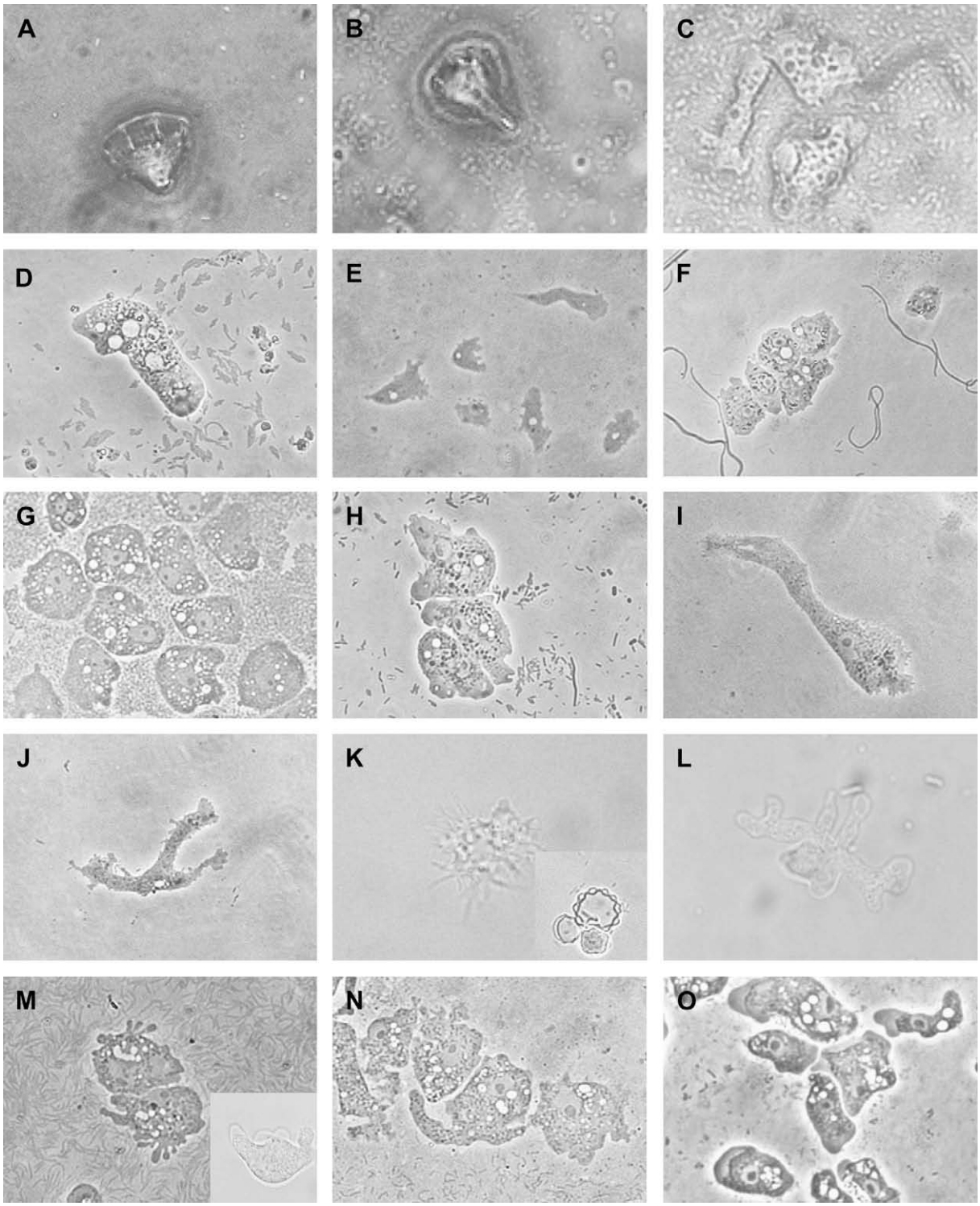

Fig. 3. Documentation of selected isolates (taxonomic status, see Table 3) growing on agar plates. A–C: *Vannella* sp. (4i, 15i, S002). Note the characteristic fan-shaped morphology of the isolates. D–F: *Hartmannella* cf. *vermiformis* (11i, 12i, 2e). G, H: *Echinamoeba* cf. *exundans* (10i, 5e). I: *Protoacanthamoeba* cf. *bohemica* (16i). J, K: *Acanthamoeba* cf. *castellanii* (S001). J: trophozoite on agar plate and resuspended in water (K) displaying characteristic acanthapodia. Inset: double-walled cyst. L–O: *Naegleria* spp.. L: *Naegleria* cf. *gruberi* (6i) resuspended in water. M: *Naegleria* cf. *gruberi* (7i). Inset: trophozoite resuspended in water displaying characteristic pseudopodia, N: *Naegleria* sp. (4e), O: *Naegleria* cf. *australiensis* (LS002). Original magnification × 400, except for E (×200).

cytotoxicity and molecular identification (supported by morphological traits of the isolates) with FLA of clinical relevance, including *N. fowleri*, *A. lenticulata* and *B. mandrillaris,* characterized them as mostly non-pathogenic. Future studies will focus on FLA living in warm waters, sites known to preferentially harbor pathogenic FLA and which are at the origin of a high number of clinical cases. A respective preliminary finding has already been reported [12].

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