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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