



## Potentially human pathogenic *Acanthamoeba* isolated from a heated indoor swimming pool in Switzerland

Christian Gianinazzi <sup>a</sup>, Marc Schild <sup>a</sup>, Fritz Wüthrich <sup>b</sup>, Norbert Müller <sup>a</sup>, Nadia Schürch <sup>b</sup>, Bruno Gottstein <sup>a,\*</sup>

<sup>a</sup> Institute of Parasitology, University of Bern, Faculty of Veterinary and Medicine, Länggass-Strasse 122, CH-3012 Bern, Switzerland

<sup>b</sup> Labor Spiez, CH-3700 Spiez, Switzerland

### ARTICLE INFO

#### Article history:

Received 18 September 2008

Received in revised form 31 October 2008

Accepted 5 November 2008

Available online 17 November 2008

#### Keywords:

Free-living amoebae

*Acanthamoeba*

Granulomatous amebic encephalitis

Immunocompromised

Central nervous system

Lungs

*Acanthamoeba lenticulata*

Thermophilic *in vitro* cultivation

Rag2-mice

PCR

*Naegleria fowleri*

*Balamuthia mandrillaris*

*Sappinia diploidea*

### ABSTRACT

Some free-living amoebae, including some species of the genus *Acanthamoeba*, can cause infections in humans and animals. These organisms are known to cause granulomatous amebic encephalitis (GAE) in predominantly immune-deficient persons. In the present study, we isolated a potentially human pathogenic *Acanthamoeba* isolate originating from a public heated indoor swimming pool in Switzerland. The amoebae, thermophilically preselected by culture at 37 °C, subsequently displayed a high thermotolerance, being able to grow at 42 °C, and a marked cytotoxicity, based on a co-culture system using the murine cell line L929. Intranasal infection of Rag2-immunodeficient mice resulted in the death of all animals within 24 days. Histopathology of brains and lungs revealed marked tissue necrosis and hemorrhagic lesions going along with massive proliferation of amoebae. PCR and sequence analysis, based on 18S rDNA, identified the agent as *Acanthamoeba lenticulata*. In summary, the present study reports on an *Acanthamoeba* isolate from a heated swimming pool suggestive of being potentially pathogenic to immunocompromised persons.

© 2008 Elsevier Inc. All rights reserved.

### 1. Introduction

Free-living amoebae (FLA)<sup>1</sup> basically represent a large and important part of the fauna of practically any environmental biotope. However, some FLA including some species of the genus *Acanthamoeba*, are furthermore able to invade and multiply within a mammalian host. The exact reasons and mechanisms, why and how a switch from a free-living status to a colonizing and invading pathogenic status occur, are still poorly understood. These organisms have been documented to be responsible for opportunistic and non-opportunistic infections in humans and animals (Visvesvara et al., 2007). A weakened immune system of the host is known to predispose for infection with FLA.

*Acanthamoeba* spp. infect different organs in humans including skin, eyes, lungs, and brain, with pneumonitis and subacute

granulomatous encephalitis and dermatitis being less common. Skin, brain and lung infections have exclusively been documented in immune-deficient or immune-weakened persons including AIDS patients and pregnant women, respectively (Marciano-Cabral and Cabral, 2003; Bloch and Schuster, 2005; Walia et al., 2007). Keratitis as a result of infection of the cornea has been associated with contamination of water or wearing contact lenses.

With regard to the central nervous system (CNS), some *Acanthamoeba* spp. have been shown to be the etiologic agents of granulomatous amebic encephalitis (GAE), a multifocal chronic encephalitis (Bouton et al., 2005). Infection occurs in the lower respiratory tract, and spread to the CNS is hematogenous. Therapeutic treatment for GAE is still unsatisfactory, and the outcomes of most reported cases of GAE have been uniformly fatal despite various treatment regimens with few exceptions (Schuster and Visvesvara, 2004).

Worldwide, the interest in pathogenic FLA, especially in *Acanthamoeba* spp., and pathologies associated with FLA is increasing as shown by the rapidly rising number of studies during the last years (Khan, 2007), which documents the growing importance of FLA in human health. Habitats of pathogenic FLA include soil, dust,

\* Corresponding author. Fax: +41 31 631 24 77.

E-mail address: [bruno.gottstein@ipa.unibe.ch](mailto:bruno.gottstein@ipa.unibe.ch) (B. Gottstein).

<sup>1</sup> Abbreviations used: GAE, granulomatous amebic encephalitis; PCR, polymerase chain reaction; FLA, free living amoebae; CNS, central nervous system; rDNA, ribosomal deoxyribonucleic acid; PBS, phosphate-buffered saline; RT, room temperature.

air, and water where they provide a potential source of infection. *Acanthamoeba* spp. are the most common amoebae, and probably the most common protozoa, to be found in soil and water samples (Page, 1988), and have been isolated from e.g. air-conditioning units, bottled mineral water, and eyewash stations (Khan, 2006). The ubiquity of *Acanthamoeba* spp., the lack of therapies for infections and the severity of injury caused by amoebae of this genus justify further efforts in investigating the pathogenic potential of *Acanthamoeba* isolates, pathogenesis and improving therapeutic treatment of acanthamebiasis.

In the present study, we report on and discuss a potentially human pathogenic *Acanthamoeba* isolate originating from a public heated indoor swimming pool in Switzerland. To our knowledge, this is the first time a potentially human pathogenic FLA isolate has been found and assessed in view of potential CNS and lung infection in Switzerland.

## 2. Material and methods

### 2.1. Sampling

Sampling was carried out in a heated public indoor swimming pool with a water temperature of 29.1 °C. Macroscopically, the water was clear. Water samples were collected by immersion of a 50 ml–plastic Falcon tube into the upper 2 cm of the water body. The tubes were subsequently centrifuged for 15 min at 1000g using a Heraeus Varifuge 3.0R centrifuge (Kendro Laboratory Products, Zurich, Switzerland). The pellet was re-suspended in 50 µl of supernatant, and used as described in the following sections.

### 2.2. Isolation and culturing of trophozoites

The suspension was gently pipetted onto an agar plate (1.5% agar in Page's saline) which was sealed with Parafilm®, and incubated upside down at 37 °C in order to select for thermophilic amoebae. A daily inspection was done by light microscopy until morphological structures compatible with amoeba trophozoites were detected. Upon detection of trophozoites, a single colony was selected and transferred to a fresh agar plate in order to obtain pure cultures without contaminating organisms. The cultures were kept at 37 °C by a biweekly transfer onto fresh agar plates coated with heat-inactivated (1 h at 60 °C) *Escherichia coli* (for initial isolation, the agar plates were lacking heat-inactivated *E. coli*). Further plates were cultured at 18 and 42 °C, respectively, in order to assess temperature tolerance by comparing growth and performance of the trophozoites at the temperatures mentioned. No antibiotics were used for isolation and further culturing of the isolates.

### 2.3. DNA extraction, PCR and sequencing

The agar plate was covered with 1 ml of PBS, and the trophozoites were gently scraped from the plate by using a bent Pasteur pipette. The liquid containing the trophozoites was collected and transferred to an Eppendorf tube, which was centrifuged for 10 min at 1000g. The supernatant was removed, and the pellet used for DNA extraction using the DNAeasy kit (Qiagen, Basel, Switzerland) following the manufacturer's instructions. The DNA was subjected to PCR aimed at the specific recognition of 18S rDNA from amoeba of the genus *Acanthamoeba* (Schroeder et al., 2001). The following primer pairs and protocols were applied: forward primer (JDP1; 5'-GGCCAGATCGTTACCGTGAA), reverse primer (JDP2; 5'-TCTCACAGCTGCTAGGGAGTCA). Incubation protocol: 7 min at 95 °C, followed by 45 cycles of 1 min at 95 °C, 1 min at 60 °C, and 2 min at 72 °C.

The sequencing was carried out on the PCR product obtained. 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 compared with sequences of different *Acanthamoeba* spp. published (Genbank).

PCR analysis of tissue samples was carried out using exactly the same protocol as described above (lysis of brain, lung as well as nasal and olfactory mucosa was performed overnight in order to guarantee proper tissue lysis).

### 2.4. Morphological analysis of the amoeba isolate

In order to characterize and document the characteristic morphology of the isolated amoebae, trophozoites and cysts were analyzed by light microscopy and photographed under different conditions and at different magnifications (Fig. 1).

### 2.5. Cytotoxicity in vitro

To investigate the cytotoxic potential of the isolate, we used an approach based on a co-culture system using the murine 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 (total volume per well: 0.5 ml) at 37 °C in a 5% CO<sub>2</sub>-enriched atmosphere. The amoebae were grown on agar plates and subsequently harvested when the density was adequate to allow for co-culture. The trophozoites were carefully removed from the plates by scraping with a bent Pasteur pipette, pelleted in PBS, and re-suspended in 50 µl of PBS. The volume corresponding to 2.5 × 10<sup>4</sup>, 2.5 × 10<sup>3</sup>, and 2.5 × 10<sup>2</sup> trophozoites, respectively, was added to the wells (containing 10 µg/ml of penicillin and streptomycin). Co-culture was performed at 37 °C in a 5% CO<sub>2</sub>-enriched atmosphere. The L929 cultures were investigated by light microscopy, and the cytotoxicity was documented as the loss of confluence and upon intracellular morphological changes such as e.g. picnosis and granulation of nuclear chromatin.

### 2.6. Pathogenicity in vivo

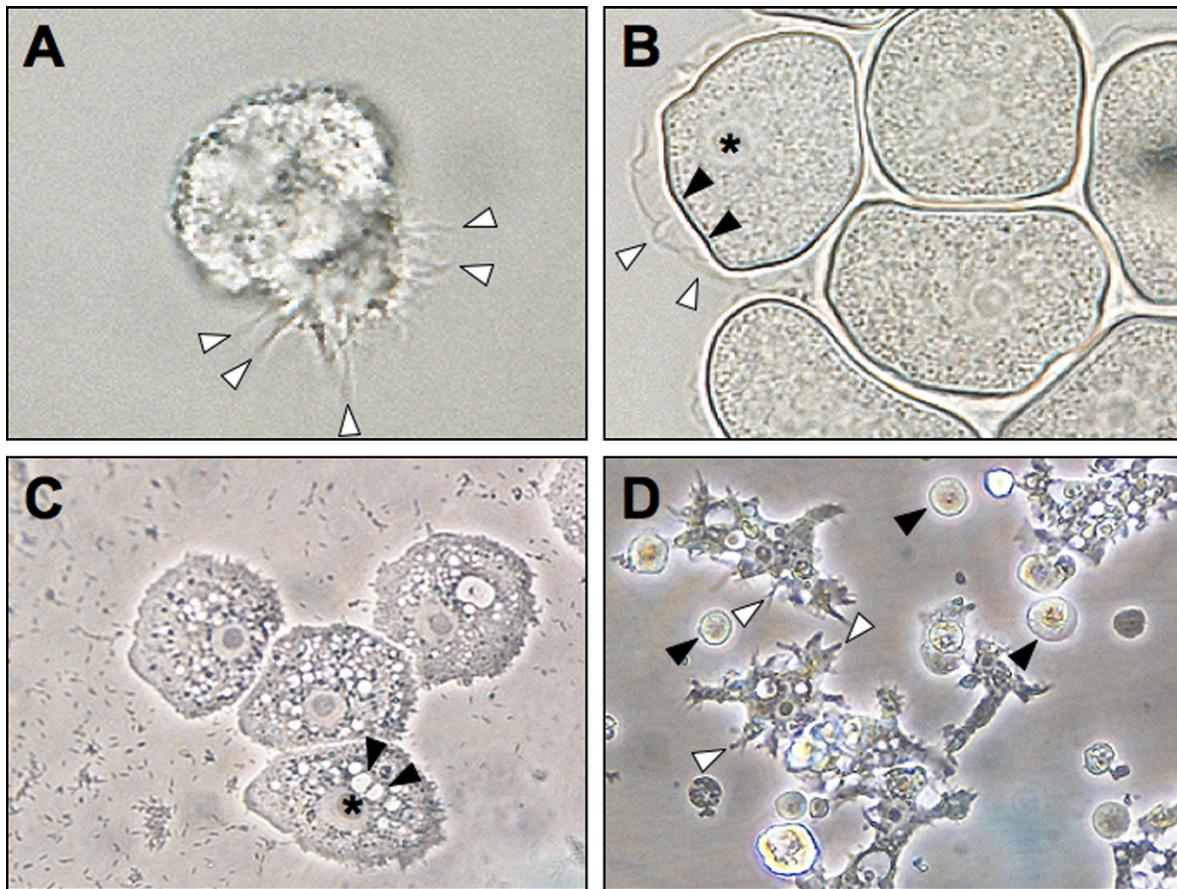
Three six-week-old Balb/c (wild-type) and three six-week-old Rag2(–/–)gamma(c)(–/–) mice of Balb/c back-ground (lacking T-cell, B-cell, and natural killer cell function) (Goldman et al., 1998; Baenziger et al., 2006) were intranasally infected with 1 × 10<sup>6</sup> trophozoites/animal as previously 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, muzzle, and blood were collected and used for (i) histopathological investigations; for (ii) PCR studies; and (iii) for isolation of viable amoebae.

### 2.7. Histopathology

The tissues of interest were fixed in a 4% PBS-buffered formalin solution, embedded in paraffin, sectioned to 8 µm-thick slices, and stained with hematoxylin-eosin, a technique employed universally for routine tissue examination.

### 2.8. Isolation of trophozoites from tissue samples

Tissue samples of primary interest (i.e. brain, nasal and olfactory mucosa, and lungs), in addition to the liver, were gently



**Fig. 1.** Morphological documentation of the *Acanthamoeba* isolate investigated in the present study. (A) A trophozoite re-suspended in water displaying acanthopodia (arrowheads), spine like surface projections characteristic for this genus (original magnification 1000PCR-). (B) Double-walled cysts consisting of the endocyst (black arrowheads) and the ectocyst (white arrowheads). The nucleus is highlighted by an asterisk (original magnification 1000 $\times$ ). Cyst formation occurs under adverse environmental conditions but is also commonly observed in cultured *Acanthamoeba* spp. (C) Trophozoites grown on agar and covered with a cover slip. This method allows to clearly visualize the contractile vacuoles (black arrowheads), and nucleus consisting of dense central nucleolus (asterisk) surrounded by a clear nuclear halo (original magnification 400 $\times$ ). (D) *Acanthamoeba* trophozoites (white arrowheads pointing at acanthopodia) in co-culture with the mammal cell line L929. After four days of co-culture, the L929 monolayer was completely degraded with the majority of the cells being lysed or rounded and detached (black arrowheads) from the surface of the culture flask. This was paralleled by considerable growth of trophozoites (original magnification 400 $\times$ ).

dipped on agar plates (1.5% agar in Page's saline). The plates were sealed, kept at RT, and daily investigated for the presence of amoebae (i.e. trophozoites and/or cysts).

### 3. Results

#### 3.1. Isolation and culturing of trophozoites

After 2 days of incubation at 37 °C, the pellet obtained from the water sample yielded organisms compatible with the morphology of amoeba trophozoites (i.e. pseudopodia (Fig. 1A) and contractile vacuoles (Fig. 1C)). After 5 days formation of cysts was observed on the plates. Within 10 days of culture (37 °C), about 80% of the amoebae were present in the cystic stage (Fig. 1B). At 18 °C, growth was reduced, while increasing the temperature to 42 °C resulted in faster growth and higher density of the trophozoites, which suggests the isolate as being highly thermophilic. Microscopical examination of cysts and trophozoites revealed that only one species was present on the culture dish. Detailed microscopical analyses of the isolate revealed acanthopodia, characteristic spine-like structures on the surface of trophozoites belonging to the genus *Acanthamoeba* (Fig. 1A), which changed into a characteristic shape after co-culture with the mammal cell line L929 (Fig. 1D). Moreover, double-walled cysts consisting of an inner endocyst and an outer ectocyst were identified, a characteristic of amoeba belong-

ing to the genus *Acanthamoeba* (Fig. 1B), among some others. Covering the culture plate by a glass slide allowed to clearly visualize significant morphological traits including contractile vacuoles and nucleus consisting of dense central nucleolus (asterisk) surrounded by a clear nuclear halo (Fig. 1C).

#### 3.2. PCR and sequencing

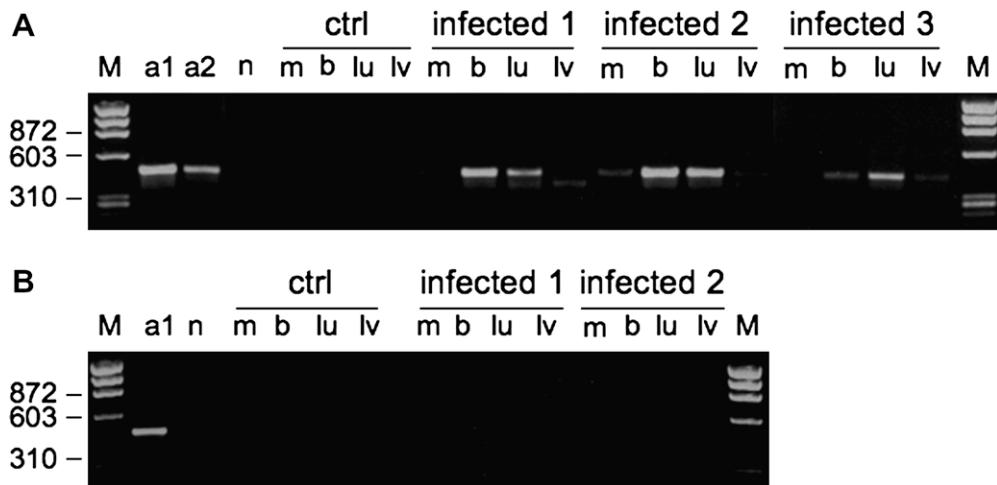
Analysis of the PCR product on an agarose gel showed an amplification product of approximately 450 bp (Fig. 2). The nucleotide sequence of the PCR product yielded a 99% homology with that of *Acanthamoeba lenticulata* (Fig. 3).

#### 3.3. In vitro cytotoxicity

Co-cultures of the isolate with L929 cells resulted in complete damage of the cell monolayer within 4 days, even when challenged with the lowest inoculum of trophozoites ( $2.5 \times 10^2$ ). Host cell damage went in line with an increasing density of the parasite population (Fig. 1D).

#### 3.4. In vivo pathogenicity

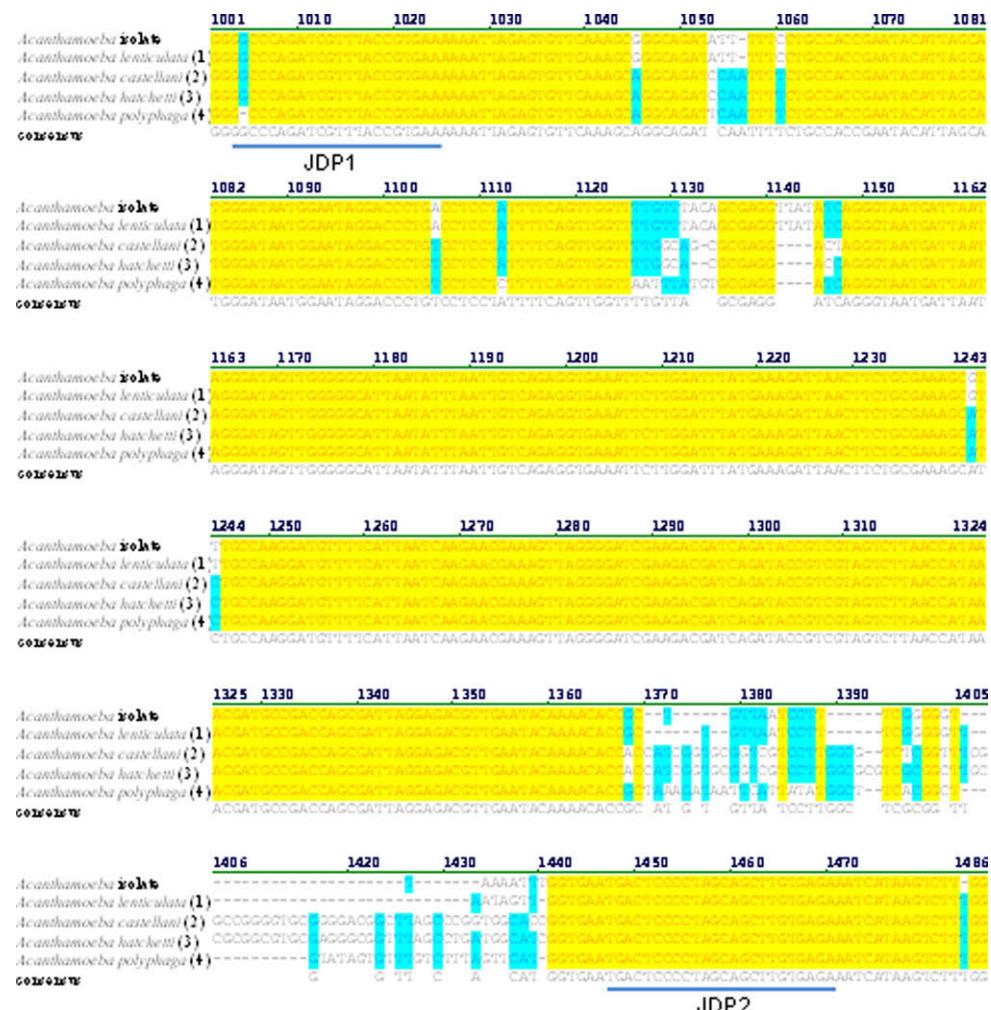
Experimental infection of *Rag2*(-/-)gamma(c)(-/-) mice resulted in the development of clinical symptoms characteristic for



**Fig. 2.** PCR analysis of the amoeba isolate from the swimming pool, and analysis of organs/regions of the animals experimentally infected with this isolate. M, molecular size marker (bp); a1, *Acanthamoeba* isolate; a2, *Acanthamoeba* trophozoites re-isolated and cultured from a brain of an infected animal; n, negative control; m, b, lu, lv: muzzle, brain lungs, and liver, respectively. (A) The PCR product obtained in a1 was purified and used for sequence analysis. *Acanthamoeba*-specific PCR products were found in lungs and brains of all infected immunosuppressed animals as well as in the muzzle (infected 2) and in the liver (infected 2 and 3). (B) No *Acanthamoeba*-specific PCR products were detected in the immunocompetent animals.

amoeba CNS infection: 12, 16, and 23 days after infection, respectively, the animals developed obtundation and ataxy. The animals were sacrificed when the clinical status had seriously deteriorated

(i.e. within 24–48 h after onset of the first symptoms). Gross histopathological examinations of brains and lungs showed marked tissue necrosis, surrounded by inflammatory infiltrates. Additionally,



**Fig. 3.** Comparison of nucleotide sequences obtained on the *Acanthamoeba* isolate using the JDP1 and JDP2 primer, respectively. Alignments with sequences of different *Acanthamoeba* spp., i.e. *A. lenticulata* (U94741.1), *A. castellanii* (AF260724.1), *A. hatchetti* (AF251937.1), *A. polyphaga* (AY026244.1) identified the isolate as *A. lenticulata* (in brackets: GenBank accession numbers).

hemorrhagic lesions were observed on the brain and cerebellum. In all affected organs, the involvement of the vessels was, however, not pronounced. With regard to CNS involvement, two of three animals showed noticeable inflammation and necrosis on the olfactory bulb. Histopathologically, lungs and brains displayed areas marked by a dense presence of trophozoites and cysts (Fig. 4). Within the tissue, trophozoites were easily recognized upon characteristic structures including cell nucleus, contractile and food vacuoles (Fig. 4D). The trophozoites were found to be encircled by neutrophils. Cysts within the tissue were readily identified by the exocyst and endocyst, a characteristic feature of *Acanthamoeba* spp. cysts. Re-isolation of trophozoites was successful for the brain, nasal and olfactory mucosa, lungs and liver, and respective viable amoebae were re-isolated in all infected animals. PCR analyses of all of the tissues where amoeba could be re-isolated confirmed the presence of *Acanthamoeba* (Fig. 2A). In wild-type Balb/c mice, no clinical signs of amoeba infection became apparent, and amoebae could not be re-isolated from tissue samples at any time. Furthermore, PCR analyses and investigation of appropriate histological slices confirmed the absence of amoebae (Fig. 2B).

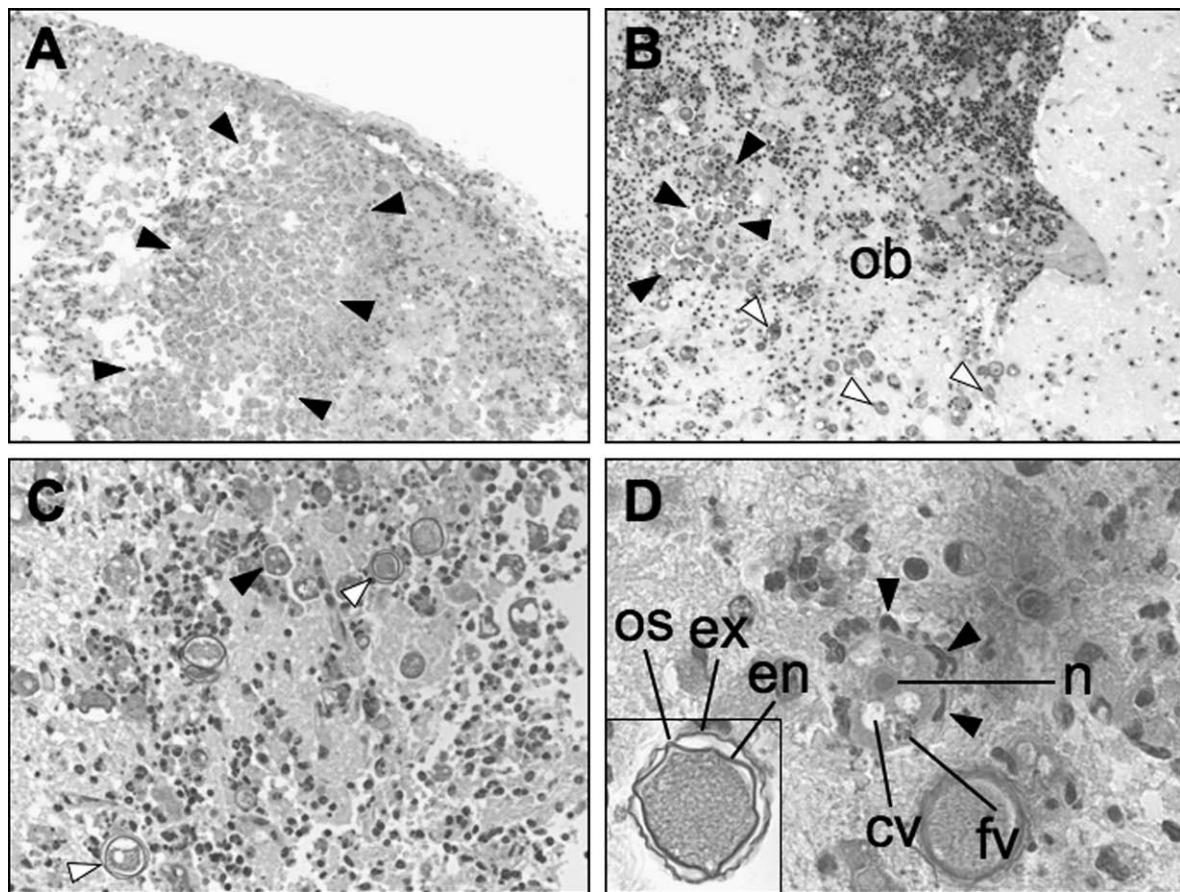
#### 4. Discussion

The present study reports on the isolation of a free-living amoeba from a heated indoor swimming pool during a routine water

control. A high thermotolerance as well as cytotoxic activity during growth of the isolate in co-culture with a mammalian cell line indicated that the isolate exhibits a pathogenic potential, which could be confirmed by subsequent animal experimentations. Morphological criteria and comparative sequence analysis of 18S rDNA identified the isolate as *A. lenticulata* (Mollet and Ermoloeff-Braun, 1976).

*Acanthamoeba* spp. have so far been isolated from different environments (Marciano-Cabral and Cabral, 2003). The presence of *Acanthamoeba* spp. with potential pathogenicity in swimming pools has been documented by several studies (De Jonckheere, 1980; 1993; Kuhlencord et al., 1989; Rivera et al., 1993; Gornik and Kuzna-Grygiel, 2004). However, in contrast to *Naegleria fowleri*, where clinical cases of primary fatal amebic meningoencephalitis 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 neither in immune-competent nor in immune-deficient persons due to the presence of pathogenic *Acanthamoeba* spp. in swimming pools. The involvement of *A. lenticulata* in human disease is limited to one keratitis case (Spanakos et al., 2006), and one case of disseminated acanthamebiasis in an immunosuppressant-treated heart transplant recipient (Barete et al., 2007).

Our experimental data suggest that the *Acanthamoeba* isolate might be potentially pathogenic to humans presenting a reduced



**Fig. 4.** Histological sections (hematoxylin-eosin staining) from lung (A) and brain (B–D) of infected *Rag2*( $-/-$ )*gamma(c)*( $-/-$ ) mice. (A) The lung tissue displays major foci (arrowheads) consisting of *Acanthamoeba* trophozoites which are present in a high density (original magnification 100 $\times$ ). (B) Brain section from an animal where the site of CNS invasion is supposedly the olfactory bulb (ob) from where the trophozoites migrate into the cortex (cx). Black arrowheads indicate a focus of trophozoites, white arrowheads single trophozoites migrating into the cortex (original magnification 100 $\times$ ). (C) Trophozoites (black arrowheads) and cysts (white arrowheads) within brain tissue (original magnification 200 $\times$ ). (D) Trophozoites are readily recognized by contractile (cv) and food vacuoles (fv), and nucleus (n), and are usually encircled by neutrophils (black arrowheads). Inset: Double-walled *Acanthamoeba* cysts consisting of exocyst (ex), endocyst (en), and ostiole (os), the site of excystation (original magnification  $\times 600$ ).

or impaired immune status, such as e.g. during pregnancy, diabetes, immunosuppressive therapy, malnutrition, alcoholism, and AIDS, all considered as risk factors for *Acanthamoeba* infections (Gullett et al., 1979; Khan 2006). In agreement with clinical cases, where symptoms include lethargy and deteriorating respiratory conditions (Castellano-Sanchez et al., 2003; McKellar et al., 2006), the experimentally infected immunodeficient mice developed identical symptoms, paralleled by ataxy. However, seizures, which are commonly observed in clinical cases, were not seen during our experiments.

*Acanthamoeba* spp. infections of the CNS in humans resulted in cortical and subcortical necrosis (Martinez, 1982), accompanied by a pronounced perivascular inflammatory infiltrate with amebic trophozoites around and within vessel walls (Castellano-Sanchez et al., 2003; McKellar et al., 2006). Although *A. lenticulata* experimentally showed pathogenic characteristics (De Jonckheere, 1980; De Jonckheere and Michel, 1988), respective histopathology of organs has not been shown so far. Our experimental infection confirmed the development of tissue necrosis, but the involvement of the vessels was not pronounced. Additionally, the lungs of the experimentally infected animals displayed areas of necrotizing amebic pneumonia and showed a marked inflammatory response, which has also been observed in humans suffering from *Acanthamoeba* infections (Castellano-Sanchez et al., 2003).

Generally, *Acanthamoeba* CNS infections in humans occur via the lower respiratory tract leading to amoebae invasion of the intravascular space followed by hematogenous dissemination. Skin lesions may provide direct amoebae entry into the bloodstream, thus bypassing the lower respiratory tract. CNS invasion most likely occurs at the sites of the blood–brain barrier (Martinez and Janitschke, 1985; Martinez, 1991; Khan, 2006). Our experimental data are suggestive for two different routes of infection. Two animals showed noticeable inflammation and necrosis on the olfactory bulb which would indicate CNS invasion via the nasal mucosa and olfactory nerves, as is was found upon experimental *Acanthamoeba* infection (Kuhlencord et al., 1989), *N. fowleri* CNS infection (Jarolim et al., 2000; Rojas-Hernandez et al., 2004) and *Balamuthia mandrillaris* infections (Kiderlen and Laube, 2004). One animal showed no specific involvement of the olfactory bulb, which argues for invasion sites affecting other parts of the CNS, suggesting a hematogenous infection of the CNS, which is supposed to be the usual way of infection in humans.

In addition to the data obtained on the pathogenic potential of the isolate, our study shows the importance of molecular biology (i.e. PCR targeting the 18S rDNA) and sequence analysis) in identifying *Acanthamoeba* spp. Although the genus *Acanthamoeba* has been identified based on distinctive morphological features of trophozoites and cysts, and *Acanthamoeba* spp. have been classified into three distinct morphological groups (I–III) (Pussard and Pons, 1977), identification of the various species based on morphological features alone is problematic e.g. due to inconsistencies and variations in cyst morphology (Sawyer, 1971).

In summary, a potentially human pathogenic *A. lenticulata* isolate has been for the first time isolated from a heated swimming pool of Switzerland. Based upon experimental infection of immunodeficient mice, a potential health threat for humans affected by immunodeficiency needs to be considered and discussed.

## Acknowledgments

The work has been supported by the Swiss Defense Procurement Agency (Project No. 4500303638). We are indebted to Paul Svoboda and Jürg Grimbichler from the Cantonal Laboratory of Basel-Land (Liestal, CH) for their valuable support and logistic contribution to the work.

## References

- 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. *PNAS* 103, 15951–15956.
- Barete, S., Combes, A., de Jonckheere, J., Datry, A., Varnous, S., Martinez, V., Ptacek, S.G., Caumes, E., Capron, F., Frances, C., Gibert, C., Chosidow, O., 2007. Fatal disseminated *Acanthamoeba lenticulata* infection in a heart transplant patient. *Emerging Infectious Diseases* 13, 736–738.
- 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.
- Booton, C.G., Visvesvara, G.S., Byers, T.J., Kelly, D.J., Fuerst, P.A., 2005. Identification and distribution of *Acanthamoeba* species genotypes associated with nonkeratitis infections. *Journal of Clinical Microbiology* 43, 1689–1693.
- Castellano-Sanchez, A., Popp, A.C., Nolte, F.S., Visvesvara, G.S., Thigpen, M., Reedi, 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.
- 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. *New Zealand Medical Journal*, 330–331.
- De Jonckheere, J.F., 1980. Growth characteristics, cytopathic effect in cell culture, and virulence in mice of 36 type strains belonging to 19 different *Acanthamoeba* spp. *Applied Environmental Microbiology* 39, 681–685.
- 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., 1993. Pathogenic free-living amoebae in swimming pools: survey in Belgium. *Annals de Microbiologie (Paris)* 130B, 205–212.
- Gianinazzi, C., Schild, M., Mueller, N., Leib, S.L., Simon, F., Nunez, 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.
- 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 amoebae in swimming pools of the city of Szczecin. *Annals of Agricultural and Environmental Medicine* 11, 233–236.
- Gullett, J., Mills, J., Hadley, K., Podemski, B., Pitts, L., Gelber, R., 1979. Disseminated granulomatous *Acanthamoeba* infection presenting as an unusual skin lesion. *American Journal of Medicine* 67, 891–896.
- Jarolim, K.L., McCosh, J.K., Howard, M.J., John, D.T., 2000. A light microscopy study of the migration of *Naegleria fowleri* from the nasal submucosa to the central nervous system during the early stage of primary amebic meningoencephalitis in mice. *Journal of Parasitology* 86, 50–55.
- Kadlec, V., 1981. Different virulence of *Naegleria fowleri* strains isolated from a swimming pool. *Folia Parasitologica* 28, 97–103.
- Khan, N.A., 2006. *Acanthamoeba*: biology and increasing importance in human health. *FEMS Microbiological Reviews* 30, 564–595.
- Khan, N.A., 2007. *Acanthamoeba* invasion of the central nervous system. *International Journal of Parasitology* 37, 131–138.
- Kiderlen, A.F., Laube, U., 2004. *Balamuthia mandrillaris*, an opportunistic agent of granulomatous amebic encephalitis, infects the brain via the olfactory nerve pathway. *Parasitology Research* 94, 49–52.
- Kuhlencord, A., Mergerian, H., Bommer, W., 1989. Studies on the pathogenesis of *Acanthamoeba*-associated meningoencephalitis. *Zentralblatt für Bakteriologie* 271, 256–260.
- Marciano-Cabral, F., Cabral, G., 2003. *Acanthamoeba* spp. as agents of disease in humans. *Clinical Microbiological Reviews* 16, 273–307.
- Martinez, A.J., 1982. *Acanthamoebiasis* and immunosuppression. Case Report 1: *Journal of Neuropathology and Experimental Neurology* 41, 548–557.
- Martinez, A.J., 1991. Infection of the central nervous system due to *Acanthamoeba*. *Reviews of Infectious Diseases* 13 (Suppl 5), S399–S402.
- Martinez, A.J., Janitschke, K., 1985. *Acanthamoeba*, an opportunistic microorganism: a review. *Infection* 13, 251–256.
- McKellar, M.S., Mehta, L.R., Greenlee, J.E., Hale, D.C., Booton, G.C., Kelly, D.J., Fuerst, P.A., Sriram, R., Visvesvara, G.S., 2006. Fatal granulomatous *Acanthamoeba* encephalitis mimicking a stroke, diagnosed by correlation of results of sequential magnetic resonance imaging, biopsy, in vitro culture, immunofluorescence analysis, and molecular analysis. *Journal of Clinical Microbiology* 44, 4265–4269.
- Molet, B., Ermoliev-Braun, G., 1976. Description d'une amibe d'eau douce: *Acanthamoeba lenticulata*, sp. nov. (Amoebida). *Protistologica* 12, 571–576.
- Page, F.C., 1988. A new key to fresh water and soil amoebae. *Freshwater Biological Association Scientific Publications*, Cumbria, UK.
- Pussard, M., Pons, R., 1977. Morphologie de la paroi kystique et taxonomie du genre *Acanthamoeba*. *Protistologica* 13, 557–598.
- Rivera, F., Ramirez, E., Bonilla, P., Calderon, A., Gallegos, E., Rodriguez, S., Ortiz, R., Zaldivar, B., Ramirez, P., Duran, A., 1993. Pathogenic and free-living amoebae

isolated from swimming pools and physiotherapy tubs in Mexico. *Environmental Research* 62, 43–52.

Rojas-Hernandez, S., Jarillo-Luna, A., Rodriguez-Monroy, M., Moreno-Fierros, L., Campos-Rodriguez, R., 2004. Immunohistochemical characterization of the initial stages of *Naegleria fowleri* meningoencephalitis in mice. *Parasitology Research* 94, 31–36.

Sawyer, T., 1971. *Acanthamoeba griffini* a new species of marine amoeba. *Journal of Protozoology* 18, 650–654.

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., 2004. Free-living amoebae as opportunistic and non-opportunistic pathogens of humans and animals. *International Journal of Parasitology* 34, 1001–1027.

Spanakos, G., Tzanetou, K., Miltsakakis, D., Patsoula, E., Malamou-Lada, E., Vakalis, N.C., 2006. Genotyping of pathogenic *Acanthamoebae* isolated from clinical samples in Greece—report of a clinical isolate presenting T5 genotype. *Parasitology International* 55, 147–149.

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 Immunological and Medical Microbiology* 50, 1–26.

Walia, R., Montoya, J.G., Visvesvara, 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.