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ORIGINAL PAPER

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The identification of free-living environmental isolates of amoebae from Bulgaria

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Abstract A survey was carried out in Bulgaria to determine the presence of free-living amoebae (FLA) from environmental sources. In 171 (61.1%) of 280 samples, isolates of *Acanthamoeba* with group II or III morphology, as well as *Hartmannella* spp. were recovered. Five isolates named "6" (artificial lake), Ep (lake), G2 (soil), R4* (river) and PK (spring water)—all exhibiting a highly efficient proliferation in axenic cultures—were subsequently cloned and subjected to molecular analyses for identification and genotyping In accordance with morphological findings, PCR-based analyses identified four isolates (6, Ep, G2, R4*) belonging to the genus *Acanthamoeba*. Confirmation of these findings was obtained by phylogenetic analysis using partial sequencing of the 18S rDNA (ASA.S1) *Acanthamoeba*-gene. Comparison of these sequences with corresponding regions from other *Acanthamoeba* strains available from GenBank sorted all four isolates into the sequence type group T4 that contains most of the pathogenic *Acanthamoeba* strains already identified. The fifth isolate (PK) exhibited morphological characteristics matching those of *Hartmannella,* and scored negative in the *Naegleria fowleri* and *Acanthamoeba* PCRs.

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Introduction

Free-living amoebae (FLA) group within the genera of *Naegleria*, *Acanthamoeba* and *Balamuthia*. FLA are amphizoic protozoa that are ubiquitous in nature. They have been found in soil, fresh water lakes (Johan and De Jonckheere 1985), swimming pools (Janitschke et al. 1980; Kadlec 1981), therapeutic pools, tap water (Visvesvara and Stehr-Green 1990), natural thermal water (Rivera et al. 1990), and air samples (Rodriguez-Zaragoza and Magana-Becerrs 1997) from all over the world. These amoebae can survive severe conditions by forming resistant cysts. Some are of medical importance as causative agents of infections and disease in humans (Cerva 1980; Martinez 1985; Ma et al. 1990) and animals (Kadlek 1978). *Naegleria fowleri* produces primary amoebic meningoencephalitis (PAM). Several species of *Acanthamoeba* as well as *Balamuthia mandrillaris* are pathogenic "opportunistic" FLA that cause granulomatous amoebic encephalitis (GAE), mainly in immunocompromised humans and in animals (Martinez 1985; Gonzalez et al. 1986; Clayton and Wiley 1987; Ma et al. 1990; Friedland et al. 1992; Gregorio et al. 1992; Slater et al. 1994). Some *Acanthamoeba* spp. can produce a severe chronic infection of the cornea that can potentially lead to blindness (Jones et al. 1975; Ledee et al. 1996; Mathers et al. 2000). Other possibly pathogenic amoebae have been isolated from the nose and throat of apparently healthy individuals (Kurdova-Mintcheva 1979). *Hartmannella* sp., also ubiquitous in nature, has recently also been associated with human disease (Fields et al. 1990; Kennedy et al. 1995; Aitken et al. 1996; Inoue et al. 1998).

The genus *Acanthamoeba* consists of 18 different species, 15 of which have been described as potential pathogens. Pussard and Pons (1977) divided the members of the genus into three groups based on cyst size and shape. Although this classification scheme has been extensively used by investigators, the differentiation of *Acanthamoeba* at the species level is still problematic. Moreover, cyst morphological variation within a clone depends on culture conditions (Page 1988). Therefore, the reliability of morphological characters alone in species identification is of limited value (Visvesvara 1991).

Acanthamoeba spp. of group II and III are among those most often isolated from human infections. It is also well known that these two groups are morphologically and genetically closely related. In recent years, ribosomal RNA gene sequences (rDNA) have been increasingly studied and used for the investigation of the phylogeny, systematics and pathogenicity of *Acanthamoeba* organisms (Ledee et al. 1996; Walochnik et al. 2000b, 2001; Schroeder et al. 2001; Booton et al 2002). Thus, Stothard et al. (1998) identified 12 lineages referred to as sequence types. These 1–12 DNA sequence variations within the nuclear small subunit ribosomal RNA gene (*Rns*; 18S rDNA) were obtained by analysing sequences from 53 isolates of all three morphological groups and 16 species. The results showed that sequence type T4 included most of the species involved in eye pathology, as well as three sequence types which are representatives of species belonging to morphological group I. Rns sequence variation was found insufficient for the full taxonomic validity of many *Acanthamoeba* strains at the species level.

Walochnik et al. (2000b) demonstrated a correlation between phylogenetic relationship and pathogenicity. They determined that clinically relevant isolates exhibited *Acanthamoeba* group II morphology and were sequence type T4, which is also the sequence type of most of the non-pathogenic *Acanthamoeba* strains. However, they found that the strains which are of no clinical relevance clustered together within T4 (Walochnik et al. 2000b).

In Bulgaria, many natural freshwater bodies (springs, rivers, lakes, etc.) and the Black Sea represent ideal environments for FLA. High summer temperatures may favor the dispersion of FLA in outdoor swimming pools and natural water bodies. Previous investigations on FLA (Kurdova-Mintcheva 1979, 1984) documented the presence of *Acanthamoeba* spp. in fresh water, soil, and Black Sea salty water, including swimming pools and the nasopharyngeal cavities of healthy individuals. Some parasites displayed potential pathogenicity in cell cultures (Kurdova-Mintcheva et al. 1979; Tsvetkova and Kurdova 1998) and in experimentally infected mice (Kurdova-Mintcheva 1979).

The aim of the present study was to isolate and characterize—by morphological and molecular means—FLA from various environmental foci of Bulgaria.

Materials and methods

Sources of amoebic isolates and sample collection

Samples were collected from environmental sources, including natural (rivers, lakes, springs and mineral springs) and artificial (outdoor or indoor swimming pools and lakes) freshwater reservoirs, as well as wastewater treatment plants (WTP, at nine sampling points: untreated wastewater, and water and sludge at all steps of its purification), the Black Sea, and various soils. In addition, bottled mineral water and tap water were analyzed (Table 1).

Table 1 The sources sampled and isolates obtained

Sources (number of sampling points)	Samples examined	Positive samples		Isolates					Amoebae growing in axenic cultures	
				Total number of primary isolates	Including isolates growing at					
					37°C		45°C			
	n	*n*	%	*n*	*n*	%	*n*	%	*n*	%
National freshwater reservoirs (41)	75	52	69.33	69	51	73.91	18	26.09	16	23.18
Rivers (13)	33	31	93.93	45	31	68.88	14	31.11	14	31.11
Lakes (7)	18	14	77.77	15	13	86.66	2	13.33	1	6.66
Springs (10)	10	4	40.0	4	4	100.00	0	0	1	25.0
Mineral springs (11)	14	3	21.42	5	3	60.0	2	40.0	0	0
Artificial freshwater reservoirs (32)	46	28	60.86	32	28	87.50	4	12.50	2	6.25
Outdoor swimming pools (6)	7	6	85.71	7	6	85.71	1	14.28	0	0
Indoor swimming pools (19)	24	9	37.5	9	9	100.0	0	0	1	11.11
Lakes artificial (7)	15	13	86.66	16	13	81.25	3	18.75	1	6.25
Black Sea (12)	14	4	28.57	4	4	100.0	0	0	0	0
Soil (7)	11	11	100.0	39	11	28.20	5	12.82	1	2.56
Sand (24)	24	22	91.66	24	22	91.66	2	8.33	0	0
Wastewater treatment plants (9)	45	42	93.33	72	42	58.33	30	41.66	1	1.38
Tapwater sources (34)	60	11	18.33	12	11	91.66	1	8.33	0	0
Bottled mineral water (4)	5	1	20.0	1	1	100.0	0	0	0	0
Total (163)	280	171	61.07	230	170	73.91	60	26.08	20	8.69

A total of 280 samples were collected, consisting of 245 water, sand-containing water, or mud-containing water (the samples were taken near the shore), 11 clay and 24 sand samples. Liquid samples consisted of approximately 500 ml, and wet but solid ones 100 g each. Excluding swimming pool samples, all were collected during each of the four seasons of 1995 and 1996, in order to determine the influence of environmental temperature on the presence and detectability of FLA. Some of the source points were sampled more than once per season. The samples were transported under normal conditions to the laboratory as soon as possible, usually no more than 1 day after sampling.

Reference isolates

All isolates, their sources and GenBank accession numbers for 18S rDNA sequences are listed in Table 2. *Acanthamoeba castellanii* strain Douglas isolated from soil in the United States and *A. astronyxis* strain Ray and Hayes, isolated from fresh water in the United States were obtained from the Culture Collection of Algae and Protozoa (Ambleside, England: CCAP 1501/1a and 1534/1, respectively), and were cultured axenically in PPG (CCAP medium). *A. polyphaga* strain "eye" (ATCC 30461), and *N. fowleri* (ATTCC 30863) were obtained from the American Type Culture Collection and were grown axenically in proteose peptone glucose (PPG) and 1034 modified PYNFH media, respectively. *A. castellanii* strain 1BU and *A. hatchetti* strain 2HH were isolated from the clinical specimens of patients who had developed a severe keratitis (Walochnik et al. 2000b). *A. lenticulata* strain 72/2 was originally isolated from a healthy individual (Michel et al. 1982) but in this study we used the re-isolate from the brain of an experimentally infected mouse (De Jonckheere and Michel 1988). Strains Pb40, De610 and Rhodos were isolated by Dr. Rolf Michel from the Central Institute of the Federal Armed Forces Medical Services, Koblenz, Germany; strain Pb40 from a physiotherapeutic pool in Germany, strain De610 from a dental unit in Germany, and strain Rhodos from an environmental sample in Greece. *Acanthamoeba* strains Douglas, 1BU, "eye", 72/2, Ray and Hayes and 2HH were grown as axenic cultures in PPG medium, while amoebae strains Pb40 (*A. comandoni*), C3/8, De610, and Rhodos, as monoxenic cultures, on non-nutrient agar (NNA) plates. *Hartmannella vermiformis* strain C3/8 originated from the sediments of water reservoirs in Germany (Smirnov and Michel 1999; Walochnik et al. 2002).

Isolation, axenization and cloning

Inoculation of about 1 ml of various non-concentrated samples onto duplicate 1.5% NNA plates seeded with heat-killed suspensions of *Enterobacter aerogenes* was carried out for amoeba isolation (Kurdova-Mintcheva 1979). Samples were incubated at 37°C and at 45°C, respectively, and examined daily for 7–14 days post-inoculation under an inverted microscope. The cultures containing fungi were discarded. Subculturing of amoeba isolates was performed every 14 days. Axenic cultures were obtained by harvesting cysts from the plate cultures, washing them three times in sterile phosphate buffered saline (PBS), pH 7.0 centrifuging at 2,000 rpm for 15 min and transferring the pellet to liquid culture medium. Two liquid media were used in the study: PPG: 1.5% (w/v) proteose-peptone (Difco) and 1.8% (w/v) glucose (Merck) in 1,000 ml Page's amoeba saline (PAS) (Page 1988), and yeast extract-PAS medium (YAS): 0.1 g yeast extract (Merck) in 1,000 ml PAS. To eliminate bacteria from the liquid growth media, they were supplemented with penicillin G (Pharmacium, Bulgaria) (500 U/ml) and streptomycin (Sopharma, Bulgaria) (50 μg/ml). All isolates adapted to growth in axenic culture media were maintained either at 37°C or 45°C. Some of the amoeba isolates were cloned in the same media without supplementation with antibiotics, either by transferring a single cyst to a fresh plate using a micromanipulator or by the method of limiting dilutions.

Microscopic examination of amoebae

Wet mounted and permanently stained smears (Heidenhain's alum hematoxylin, or trichrome) of amoeba trophozoites and cysts were examined by light microscopy at 100×, 400× and 1,000× magnification. For classification, the Pussard and Pons (1977) and Page (1988) keys were applied. Measurements of both living and fixed amoebae were performed with a standard ocular micrometer.

Processing of DNA samples and PCR

DNA was extracted from cultured organisms using the DNAeasy kit (Qiagen, Basel, Switzerland) according to the standard protocol. DNA was eluted in 100 μl AE buffer (elution buffer from the kit), and subsequently boiled for 5 min.

For molecular identification of FLA, different previously published PCRs were applied according to the descriptions provided by the authors: two *N. fowleri*-specific PCRs based on the use of either

Table 2 Strains used in this study

Number	Species	Strain	18S rDNA genotype	Morphological group	GenBank ref. no. (collection no.)	Reference/source
1	*A. castellanii*	Douglas	T4	II	CCAP:1501/1a	Soil, California, USA
2	*A. castellanii*	1BU	T4	II	(AF260721)	Walochnik et al. 2000b
3	*A. polyphaga*	"eye"	T4	II	ATCC:30461	Corneal scraping, Houston, Texas
4	*A. lenticulata*	72/2	T5	III	ATCC:50704 (U94732)	Michel et al. 1982; De Jonckheere and Michel 1988; Stothard et al. 1998
5	*A. astronyxis*	Ray and Hayes	T7	I	CCAP 1534/I	Fresh water, USA
6	*A. comandoni*	Pb40	T9	I		Walochnik et al. 2003
7	*A. hatchetti*	2HH	T11	II	(AF260722)	Walochnik et al. 2000b
8	*H. vermiformis*	C3/8			AF426157	Smirnov and Michel 1999; Walochnik et al. 2002
9	*N. fowleri*				ATCC 30863	Human male, Charleston, USA
10	*Vannella*sp.	De610				Walochnik et al. 2003
11	*Vahlkamfhia ovis*	Rhodos				Walochnik et al. 2003
12	*Acanthamoeba* sp.	6	T4	II	AY376160	Artificial lake, Bulgaria
13	*Acanthamoeba* sp.	Ep	T4	II	AY376161	Lake, Bulgaria
14	*Acanthamoeba*sp.	G2	T4	II	AY376159	Soil, Bulgaria
15	*Acanthamoeba*sp.	R4	T4	II	AY376162	River, Bulgaria
16	*Hartmannella* sp.	PK				Spring, Bulgaria

primer pair p3f/p3r (targeted to *N. fowleri*-specific chromosomal DNA sequence represented by DNA probe pB2.3) (Kilvington and Beeching 1995) or NAEGF1/NAEGF2 (targeted to the mitochondrial ATPase 6 subunit, derived from mitochondrial DNA) (McLaughlin et al. 1991), and an *Acanthamoeba* spp.-specific PCR including primer pair JDP1/JDP2 (targeted to 18S rDNA stretch ASA.S1) (Schroeder et al. 2001). In order to confirm the presence of FLA DNA and its quality, an as yet unpublished PCR was introduced. This PCR was carried out with forward primer P-FLA-F (5′CGCGGTAATTCCAGCTCCAATAGC3′) and reverse primer P-FLA-R (5′CAGGTTAAGGTCTCGTTCGTTAAC3′) that were targeted at conserved stretches of *Acanthamoeba* 18S rDNA. For all PCRs, amplification reactions were performed in a 25 µl mixture containing 5 µl 10×Gene Amp PCR buffer (Applied Biosystems, Basel, Switzerland), 0.2 mM each of dATP, dGTP, dCTP, and dTTP (Applied Biosystems), and 20 pmol each of primers, 1.25 units of AmpliTaq DNA polymerase (Applied Biosystems). The P-FLA-F/P-FLA-R PCR was done in 40 cycles with denaturation (94°C; 1 min), annealing (63°C; 1 min) and primer extension (74°C; 3.5 min). After the last cycle, a primer extension was continued for 10 min at 72°C. Amplification products from all PCRs were analyzed by electrophoresis through a 2% agarose gel.

Phylogenetic analysis

The 18S rDNA (ASA.S1) segments from each of our *Acanthamoeba* isolates were subjected to a specific sequencing reaction using primer 892C according to Schroeder et al. (2001). A sequence of 505–513 bp was read for each isolate. These were aligned with the corresponding sequences from 46 reference *Acanthamoeba* isolates, including genotypes T1–T12, plus a single sequence from *B. mandrillaris* (Schroeder et al. 2001, see Fig. 3), using the CLUSTAL X computer program (Thompson et al. 1997). A neighbor-joining tree, rooted with the *B. mandrillaris* sequence, was obtained using MEGA 2.1 (Kumar et al. 2001), with the parameters described in Schroeder et al. (2001).

Statistical methods

Alternative analysis based on the *t*-test was performed according to Sepetliev (1972). $P < 0.05$ were considered statistically significant.

Results

Isolation of amoebae

Sources, sample points and number of positive samples are shown in Table 1. Attempts to isolate FLA from the environmental sources yielded 171 (61.1%) positives out of the 280 samples examined from the 163 sampling points. Altogether, 230 primary amoeba isolates were obtained. In some cases there were multiple isolates from one sampling point. Natural freshwater sources (NFS), artificial freshwater sources (AFS), soil, including clay and sand, and WTP were the sources most abundant with FLA in our survey ($P < 0.05$). In the case of NFS, 31 out of 33 (93.9%) river samples and 14 out of 18 (77.8%) lake samples were positive for amoebae. In the group of AFS, amoebae were cultured from six out of seven (85.7%) outdoor swimming pools (OSP), nine out of 24 (37.5%) indoor swimming pools (ISP) and 13 out of 15 (86.7%) artificial lake (AL) samples. A lower rate of amoeba isolation was obtained from the Black Sea, tap water and bottled mineral water samples. Statistical analysis showed that when grown at 37°C there was a significant difference between the number of isolates recovered from rivers and springs; rivers and mineral springs, NFS and the Black Sea, NFS and soil, NFS and WTP, NFS and bottled mineral water($P < 0.05$). No difference was found among the amoeba isolate numbers obtained from identical sites during different seasons.

Temperature tolerance was applied as a first criterion for potential pathogenicity of the isolated amoebae (37°C versus 45°C) (Table 1). The number of positive cultures obtained at 37°C (73.9%) was greater than that found at 45°C (26.1%) ($P < 0.05$). The highest proportion of amoeba isolates growing at 37°C was detected in the samples from mineral springs, ISP, Black Sea, tap water, sand, lakes and OSP ($P < 0.05$). Incubation at 45°C yielded 30 isolates from WTP and 18 from NFS, of which river isolates were the most frequent. Amoebae tolerating 45°C were also isolated from soil, WTP and AL. None of the isolates from mineral springs, ISP, the Black Sea or bottled mineral water grew at this temperature.

Axenic cultivation and cloning of FLA isolates

Twenty out of the 230 primary isolates (8.69%) grew in axenic culture media. Out of 16 axenic isolates from NFS, 14 originated from rivers (Tables 1, 3).

From all indigenous FLA isolates, only the five strains "6" (from an AL), Ep (from a lake), G2 (from soil), R4* (from a river) and PK (from spring water) were further investigated as they exhibited efficient proliferation in cultures. Strains "6", Ep, G2 and PK tolerated 37°C, while strain R4* even grew well at 45°C (Table 1).

Morphology of cloned amoeba isolates

Among the five indigenous isolates analyzed in this study (Fig. 1), four were morphologically assigned to the genus *Acanthamoeba* ("6", Ep, G2 and R4*) and one (PK) to the genus *Hartmannella*.

Acanthamoeba trophic forms were similar to each other. Two kinds of pseudopods were observed: one broad hyaline lobopodium and spine pseudopods (acanthopodia) (Fig. 1). In the rounded amoebae trophozoites measured approximately 19.1 µm–28.9 µm in diameter. They were uninucleated, and contractile vacuoles were evident in the cytoplasm. The motility was sluggish. Cysts were variable in size and morphology (Fig. 1). The mean diameter was < 18 µm ranging from 11.0 µm to 16.1 µm. Cysts had a wrinkled or wavy ectocyst and a polygonal, stellate or even rounded endocyst. The two walls met at points in which enfoldings of the ectocyst formed pores. Trophozoites of *Hartmannella* isolates were uninucleated. One or more contractile vacuoles were visible within the endoplasm when rounded.

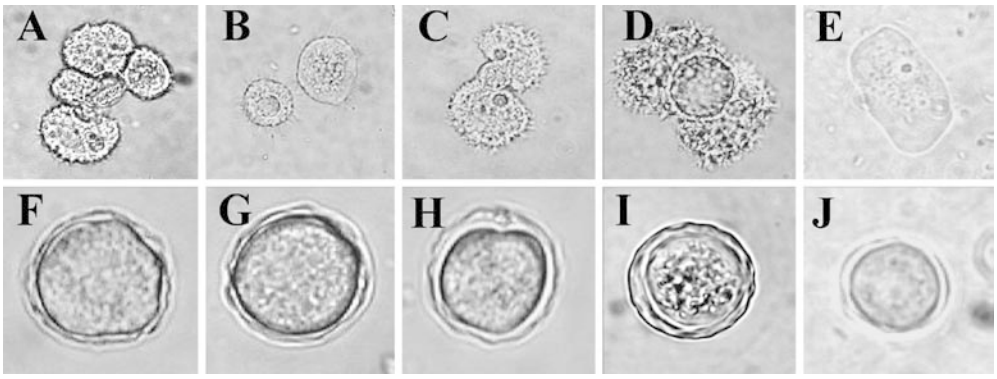

Fig. 1 Photomicrographs of representative trophozoites (**A**–**E**) and cysts (**F**–**J**) of indigenous amoebae clones used in the study: *Acanthamoeba* sp. strains "6" (**A**, **F**), Ep (**B**, **G**), G2 (**C**, **H**), R4* (**D**, **I**) ×400; *Hartmannella* sp. strain PK (**E**, **J**) ×1,000

Their size ranged between approximately 6.2 and 13.2 μm. Cysts exhibited smooth walls, were circular or sometimes ovoid, uninucleated and measured 4.4–9.5 μm.

Further detailed analyses of the morphological features of cysts were done on the basis of criteria listed by Page (1988). *Acanthamoeba* isolates ("6", Ep and G2) were all assigned to *A. castellanii*. The fourth isolate, R4*, could not be unambiguously identified to the species level. In this case, it was only possible to assess its morphology with findings typical for those of the *Acanthamoeba* group II.

Molecular and phylogenetic characterization of FLA isolates

A further objective of the present study was to apply and evaluate molecular methods in parallel to microscopic examinations in tackling the taxonomic identification of the five Bulgarian FLA isolates. As molecular tools, we used a set of known diagnostic PCRs consisting of two specific for *N. fowleri*, one for the genus *Acanthamoeba* and a PCR (based on primer pair P-FLA-F/P-FLA-R) which detects all FLA and was included as a methodical control to confirm the presence of FLA-DNA within the different DNA preparations. In this study, FLA isolates "6", Ep, G2, R4*, and PK were compared with 11 FLA reference strains (Table 2). In the case of the *N. fowleri*-specific PCRs, the amplicons obtained from the reference strain were approximately 1,500 bp (p3f-p3r) and 300 bp (NAEGF1-NAEGF2), respectively (Fig. 2). The sizes of the *Acanthamoeba*-genus-specific ASA.S1 amplicons varied among the different species investigated. These amplicons turned out to have approximate sizes of 420 (*A. lenticulata*), 450 (*A. castellanii*, *A. polyphaga*, *A. hatchetti*; Bulgarian isolates "6", Ep, G2, R4*), 500 (*A. comandoni*) and 550 bp (*A. astronyxis*). The P-FLA-F/P-FLA-R control PCR detected all FLA included in the analysis and provided amplification products with approximate sizes of 800 (*H. vermiformis*; Bulgarian isolate PK), 900 (*N. fowleri*), 950 (*Vannella* sp., *Vahlkampfia ovis*), 1,080 (*A. castellanii*, *A. polyphaga*, *A. lenticulata*, *A. hatchetti*; Bulgarian isolates "6", Ep, G2, PK), 1,350 (*A. comandoni*) and 1,500 bp (*A. astronyxis*).

The Bulgarian isolates "6", Ep, G2 and R4* scored positive in the *Acanthamoeba*-PCR (using primers JDP1/JDP2) but negative in both *N. fowleri*-PCRs (Fig. 2). This result confirmed the above morphological examination, which identified these four isolates as *Acanthamoeba* spp. As expected, isolate PK—morphologically resembling *Hartmannella* spp.)—was negative in these three PCRs.

Acanthamoeba isolates "6", Ep, G2 and R4* were further investigated by phylogenetic analyses. This was done according to Schroeder et al. (2001) by sequencing of ASA.S1 amplimers and subsequent comparison of these sequences with corresponding regions from other known *Acanthamoeba* strains. The comparative investigation included all representative sequences available from GeneBank and had previously been used for the definition of phylogenetic sequence types T1–T12 (Schroeder et al. 2001). Sequences from all four *Acanthamoeba* isolates under study were clustered in sequence type group T4 (Fig. 3). The tree topology was similar to that obtained by other authors either using complete (Stothard et al. 1998) or partial (Schroeder et al. 2001) 18S rDNA (ASA.S1) sequences.

Discussion

This work was prompted by the hypothesis that FLA would be found in various water bodies serving people for various activities in Bulgaria, due to the relatively mild climate of the country and its high summer temperatures. A former, limited survey had detected the presence of FLA in the abundant natural and artificial freshwater reservoirs (Kurdova-Mintcheva 1979). The distribution of FLA in human environments (natural and man-made), and the potential danger that FLA may pose to humans coming into contact with such amoebae, is of considerable medical importance. Of special interest were the isolates capable of proliferating at temperatures of 37°C and above, and the habitats preferred by these FLA. OSP and ISP as well as lakes, rivers and the Black Sea were included in the investigation due to the link between human contact with water and the nasopharyngeal route of infection by FLA.

It is generally accepted that FLA species are widely distributed in nature. Water temperature and salinity, food availability and ability to form cysts are among the factors affecting their distribution (Ma et al. 1990; De Jonckheere 1991). Although amoebae belonging to the genera *Acanthamoeba*, *Naegleria* and *Hartmannella* are free-living organisms, scientists and medical professionals are aware of the potential capability of the first two to cause life-threatening infections of the CNS and of *Acanthamoeba* to cause abscesses in the cornea, skin lesions and other disorders (Jones et al. 1975; Willaert et al. 1978; Martinez 1985; Clayton and Wiley 1987; Ma et al. 1990). This study provides evidence that free-living *Acanthamoeba* and *Hartmannella*, but not *Naegleria*, are found abundantly in waters and soils of Bulgaria.

The production of highly resistant cysts by these protozoa may explain the lack of significant differences between the number of amoeba isolates obtained during the different seasons of the year. FLA of the genera *Acanthamoeba* and/or *Hartmannella* were distributed in the whole range of environmental sources examined. Our observations confirmed the findings of other investigators (De Jonckheere 1991) that *Acanthamoeba* can withstand extremes in temperature, desiccation and disinfection, which correlates well with the high frequency of their isolation compared to that of the other FLA. *Acanthamoeba* spp. were isolated more frequently from natural freshwater reservoirs, artificial freshwater reservoirs, clay and sand, as well as from WTP than *Hartmannella*. *Hartmannella* spp. were the dominant species in spring and tap water sources. On the other hand, *Acanthomoeba* were found at amazingly high frequency in WTP (93% of the examined samples), along with high concentrations of different bacteria. No significant differences were found in the number of amoeba isolates from various stages of the water purification process in the treatment plants. This abundance may indicate that the presence of bacteria in a water source is more important for *Acanthamoeba* multiplication than its oxygen content.

The presence of *Acanthamoeba* and *Hartmannella* in indoor and OSP may be explained by: (1) the resistance of their cyst stages to chlorination of the water, and (2) probably insufficient cleaning and disinfection of the purification installations in the swimming pools. Studies on pathogenic FLA present in swimming pools by De Jonckheere (1979a, 1979b) have shown that the amoebae, especially *Acanthamoeba* spp., are probably introduced into the water from the soil (surrounding grounds) and by humans, and are not permanent residents of the chlorinated water.

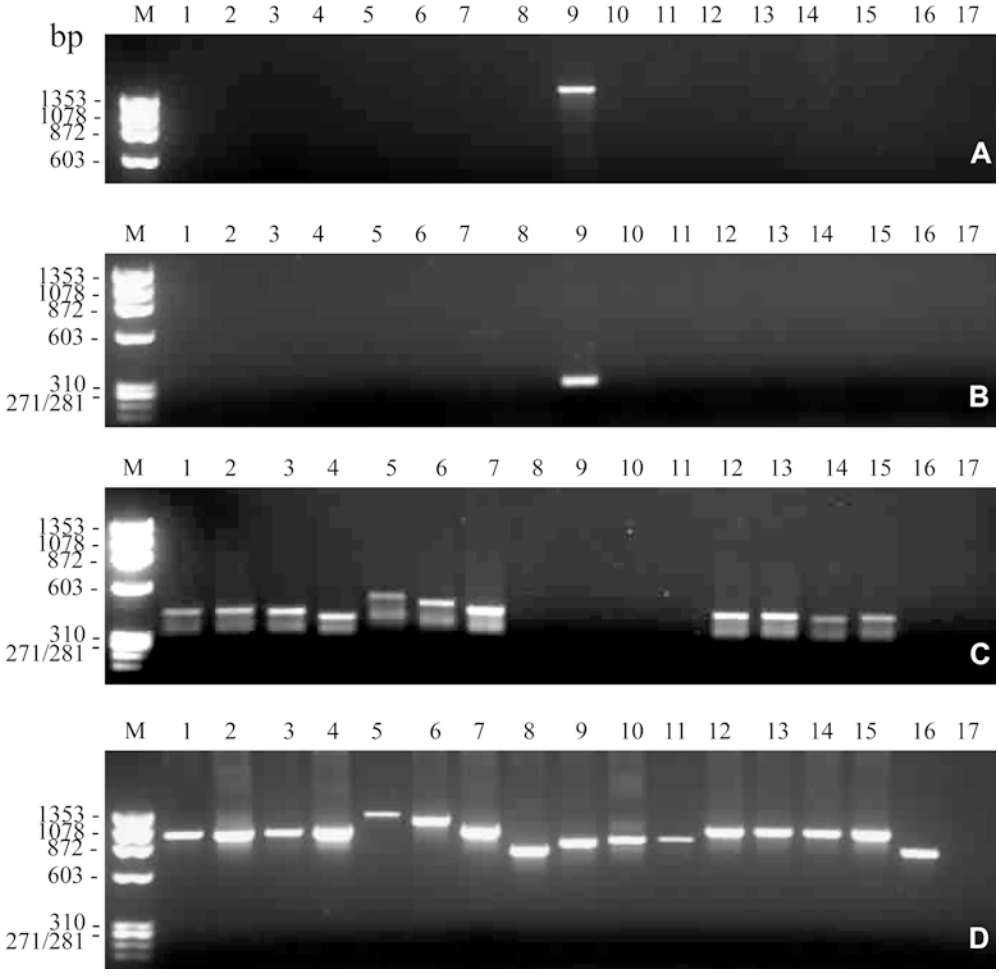

Fig. 2 PCR analysis with DNA from *A. castellanii* CCAP 1501/1a (*lane 1*), *A. castellanii* strain IBU (*lane 2*), *A. polyphaga* ATCC 30461 (*lane 3*), *A. lenticulata* strain 72/2 (*lane 4*), *A. astronyxis* CCAP 1534/1 (*lane 5*), *A. comandoni* strain Pb40 (*lane 6*), *A. hatchetti* strain 2HH (*lane 7*), *H. vermiformis* strain C3/8 (*lane 8*), *N. fowleri* ATCC 30863 (*lane 9*), *Vannella sp.* strain De610 (*lane 10*), *Vahlkampfia ovis* strain Rhodos (*lane 11*), strain "6" (*lane 12*), strain Ep (*lane 13*), strain G2 (*lane 14*), strain R4* (*lane 15*), strain PK (*lane 16*), and without DNA (*lane 17*) using *N. fowleri*-specific primer pairs: **A** NAGF1/NAEGF2 and **B** p3f/p3r, and *Acanthamoeba* genus-specific primer pair **C** JDP1/JDP2, and **D** primer pair P-FLA-F/P-FLA-R () which amplifies DNA from all FLA. Amplification products were analyzed by 2.5% agarose gel electrophoresis. Size markers (*M*) are given in base pairs

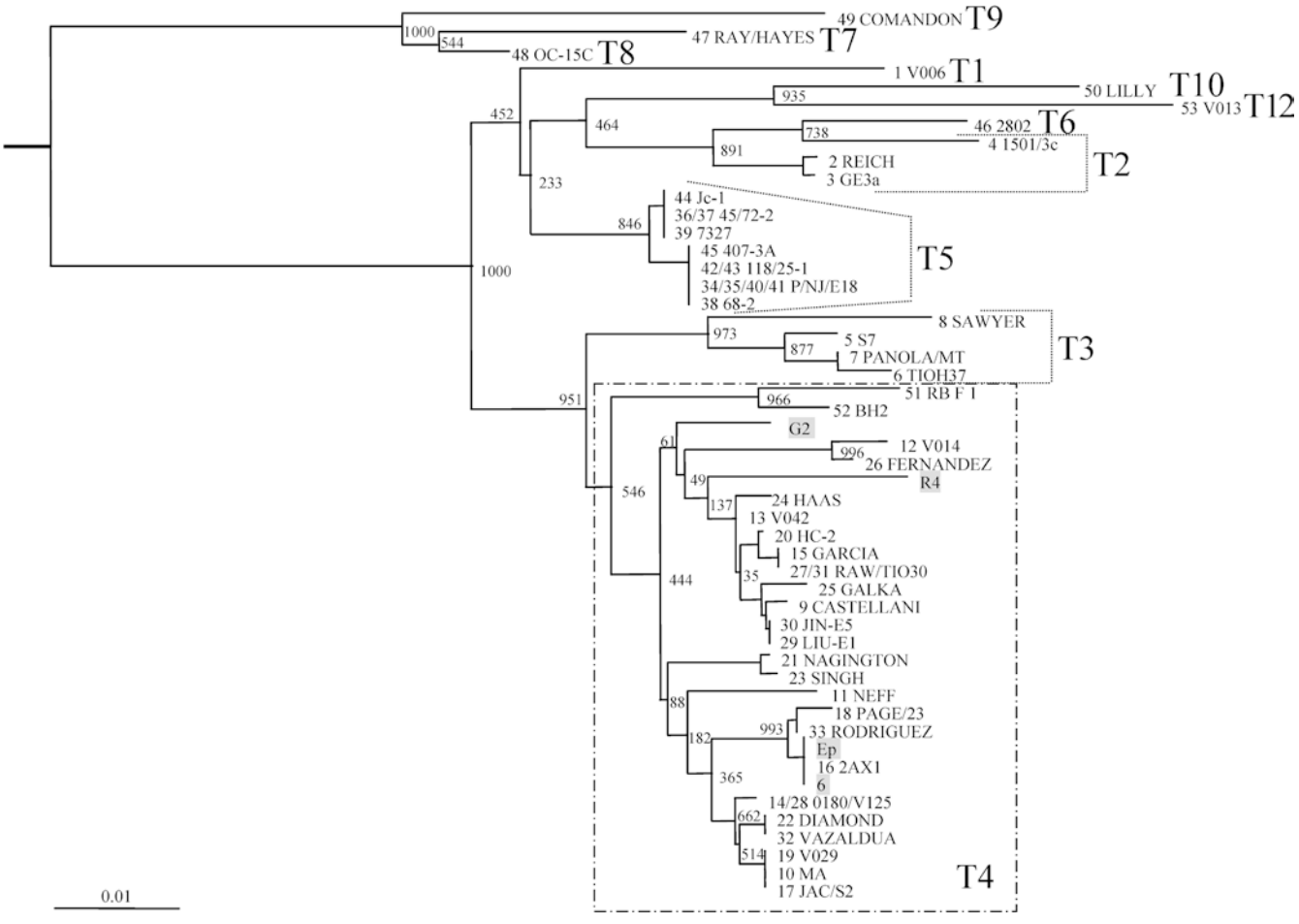

Fig. 3 Neighbor-joining distance tree based on partial 18S rDNA sequences aligning to reference bp 1,271–1,377 within amplimer ASA.S1. The sequences from Bulgarian isolates "6" (Gene Bank, accession no. AY376160), Ep (AY376161), G2 (AY376159) and R4* (AY376162) (indicated by a *grey background*) were aligned with the corresponding sequences from 46 reference *Acanthamoeba* isolates available from GenBank, including genotypes T1–T12 plus a single sequence from *Balamuthia mandrillaris*. *Bar* Index of dissimilarity (0.01) among the different sequences

The low number of amoeba positive samples from the Black Sea suggests that the high salt concentration of the sea water suppresses the re-production of these organisms.

Isolation rates in the upper layers of clay (100%) and sand (91.7%) were surprisingly high. This may be explained by the presence of high concentrations of coliform bacteria in these layers, with a secondary importance ascribed to higher concentrations of oxygen, factors enabling the multiplication of *Acanthamoeba* and *Hartmannella* species.

Pathogenic and virulent FLA can withstand temperatures of up to 45°C for *N. fowleri* and 42–43°C for *Acanthamoeba culbertsoni*, and virulent strains multiply well at these temperatures, whereas the non-virulent and the non-pathogenic strains are unable to grow beyond 37° (Visvesvara 1980; De Jonckheere 1991). However, there are also reports on the isolation of many high-temperature tolerant, non-pathogenic strains, which indicates that other factors besides temperature tolerance play an important role in the pathogenicity of these amoebae (De Jonckheere et al. 1977; Stevens et al. 1980). During our investigation, various *Acanthamoeba* species isolated by us from rivers, lakes, soil and WTP were able to grow at 45°C. The growth and isolation of *Acanthamoeba* at these high temperatures were also reported by De Jonckheere (1991).

The growth of FLA in axenic culture medium has been used as an indicator of pathogenicity in the past. It also enables the isolation of pathogenic strains when mixtures of different amoebae are present (De Jonckheere 1977). It was found that *Acanthamoeba* isolates, and especially those from natural freshwater reservoirs (16 out of a total of 20 successfully established axenic cultures, of which 14 were derived from river samples) were better adapted to growth under axenic conditions.

Within the group of amoeba isolates adaptable to in vitro growth, five (strains "6", Ep, G2, R4* and PK) demonstrated highly efficient proliferation in axenic cultures. We decided to clone these isolates, and further characterize them on both the morphological and genetic level. The use of morphology alone as a taxonomic criterion is limited (Visvesvara 1991) by the variation of the cyst shape within a clone (Page 1988). In order to complement this conventional approach for FLA differentiation, amoeba clones "6", Ep, G2 and R4* were

also specified by genus-specific diagnostic PCRs and 18S rDNA (ASA.S1) sequence-based phylogenetic analyses. Several research groups have developed PCR protocols targeting *Acanthamoeba*- (Howe et al. 1997; Schroeder et al. 2001; Booton et al. 2002) and *N. fowleri*-specific (McLaughlin et al. 1991; Kilvington and Beeching 1995; Ledee et al. 1998) genome sequences to detect the organisms in various environmental sources. We applied these PCRs and were able to confirm our morphological findings in that clones "6", Ep, G2, R4* were again identified as *Acanthamoeba* spp. In contrast, clone PK, which exhibited morphological features typical for *H. vermiformis*, did not provide amplification products in any of the genus-specific PCRs and thus genotyping of this clone was not possible.

For phylogenetic analysis of the four clones identified in our study as *Acanthamoeba* spp., we determined the nucleotide sequence of the PCR-amplified ASA.S1 region (Schroeder et al. 2001). At present, ASA.S1 has to be regarded as an ideal target for the specific detection of acanthamoebae because it is highly selective for the genus. As previously demonstrated by Schroeder et al. (2001), ASA.S1 on JDP1/JDP2 amplimers contains substantial inter-strain sequence variation, which allows discrimination between several clusters of 18S rDNA genotypes (genotypes T1–T12). These properties make ASA.S1 particularly useful for species determination of the entire variety of *Acanthamoeba* genotypes to be expected within environment samples (Schroeder et al. 2001). Several research groups have found that nearly all *Acanthamoeba* isolates from AK infections belong to genotype T4 (Stothard et al. 1998; Walochnik et al. 2000a, 2000b; Schroeder et al. 2001) with two exceptions of one T3 isolate (Ledee et al. 1996) and one T6 isolate (Walochnik et al. 2000a, 2000b). Based on this finding, it was assumed that pathogenic *Acanthamoeba* strains would mainly have genotype T4 (Walochnik et al. 2000a, 2000b; Schroeder et al. 2001).

Since our ASA.S1-based phylogenetic analysis clustered the cloned *Acanthamoeba* isolates "6", Ep, G2 and R4* within the T4 sequence group, they may have to be considered as potentially pathogenic strains. However, the pathogenicity of the four strains needs to be confirmed by assessing their infectivity patterns in both mammalian cell lines (Kurdova-Mintcheva 1979) and experimental animal (e.g. murine) models (Kurdova-Mintcheva 1979). Furthermore, the molecular search for pathogenicity factors such as the pore-forming unit found in *N. fowleri* (Herbst et al. 2002) may help considerably to further define the potential for virulence. The results from such future experiments will contribute to the consolidation of the concept of a correlation between the phylogenetic relationship and pathogenicity in association with *Acanthamoeba* infections.

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