ACTA
PROTOZOOLOGICA

Acta Protozool. (2015) 54: 77–84
www.ejournals.eu/Acta-Protozoologica
doi: 10.4467/16890027AP.15.006.2193

Molecular Identification of Free-living Amoebae Isolated from Artificial Water Bodies Located in Poland

Agata LEOŃSKA-DUNIEC[1,2], Małgorzata ADAMSKA[1] and Bogumiła SKOTARCZAK[1]

[1]Department of Genetics, Szczecin University, Szczecin, Poland; [2]Faculty of Physical Culture and Health Promotion, University of Szczecin, Poland

Abstract. Free living amoebae (FLA) are amphizoic protozoa that are widely found in various environmental sources. They are known to cause serious human infections, including a fatal encephalitis, a blinding keratitis, and pneumonia. The main aim of the study was detection and molecular identification of *Acanthamoeba* spp., *Naegleria fowleri, Balamuthia mandrillaris*, *Sappinia pedata*, and *Vermoamoeba vermiformis* (formerly *Hartmannella vermiformis*) in artificial water bodies in North-Western Poland. We examined 86 water samples collected during 2-year period from 43 water bodies, including outdoor and indoor swimming pools, firefighting reservoirs, fountains, as well as water network. The samples were filtrated using Filta-Max® membrane filters (IDEXX Laboratories, USA) and, in order to select potentially pathogenic, thermophilic strains and to limit the number of PCR examined samples, the thermal tolerance test was carried out. Obtained filtrates were transferred to non-nutrient agar plates with *E. coli*. The agar plates were incubated at 37°C and then proliferated amoebae were passaged at 42°C. DNA was extracted from the thermophilic trophozoites and then polymerase chain reactions and sequence analysis were performed for molecular identification of FLA. From the 86 collected water samples 57 strains of FLA were able to proliferate at 37°C and 7 of them showed ability to proliferate at 42°C. For molecular identification of *Acanthamoeba* spp. and *V. vermiformis*, regions of 18S rDNA were amplified. In order to detect *B. mandrillaris* DNA, we used mitochondrial 16S rDNA as a marker, and for detection of *N. fowleri* and *S. pedata* – ITS regions. Based on molecular analysis, isolates were classified to the genus *Acanthamoeba* (T4 and T11 genotypes, as well as the new genotypes detected earlier in clinical samples and named T16) and *V. vermiformis* species. Detected strains were highly similar or identical to pathogenic strains detected earlier in patients. Our results show a wide distribution of potential pathogenic FLA, as *Acanthamoeba* T4, T11, T16 genotypes, and *V. vermiformis* species in various artificial water bodies located in North-Western Poland and suggest a potential threat to health of humans in this part of the country.

Key words: Artificial water samples, free living amoebae, thermal tolerance test, PCR, DNA sequencing, *Acanthamoeba*, *Vermoamoeba.*

INTRODUCTION

Free-living amoebae (FLA) are amphizoic protozoa commonly found in various environmental sources throughout the world. They have been isolated from fresh water and seawater, soil, dust, and air (Visvesvara *et al.* 2007). Mainly the strains belonging to *Acanthamoeba* spp. (T3, T4, and T11 genotypes) (Culbertson *et al.* 1958, 1959; Jager and Stamm 1972; Naginton *et al.* 1974; Booton *et al.* 2005), *Naegleria fowleri* (Fowler and Carter 1965, Carter 1970)*, Balamuthia mandrillaris* (Visvesvara *et al.* 1990, 1993) and *Sappinia pedata*

Address for correspondence: Bogumiła Skotarczak, Department of Genetics, Szczecin University, Felczaka 3c, 71-412 Szczecin, Poland; E-mail: boskot@univ.szczecin.pl, genetyka@univ.szczecin.pl

(Gelman *et al.* 2001, 2003; Qvarnstrom *et al.* 2009) have been documented to invade and multiply within a host leading to life-threatening infections in humans and animals. According to some reports, *Vermoamoeba vermiformis* (formerly *Hartmannella vermiformis*, Smirnov *et al*. 2011) has recently also been associated with human disease (Kennedy *et al.* 1995, Centeno *et al.* 1996, Lorenzo-Morales *et al.* 2007), however, the pathogenicity of this species is discussed (De Jonckheere and Brown 1998).

N. fowleri is the most pathogenic of FLA and it is known as a causative agent of an acute and rapidly fatal disease of healthy humans following exposure to contaminated water, called primary amoebic meningoencephalitis (PAM) (Visvesvara *et al.* 2007). *Acanthamoeba* spp., *B. mandrillaris*, and *S. pedata* are associated with granulomatous amoebic encephalitis (GAE), a subacute or chronic disease of individuals with a compromised immune system. *Acanthamoeba* spp. also causes infections of lungs, liver, kidneys, spleen, heart, sinuses, adrenal glands, and skin. Furthermore, *Acanthamoeba* strains are causative agents of a sight-threatening corneal infection mainly associated with healthy contact-lens wearers, called *Acanthamoeba keratitis* (AK) (Khan 2006, Visvesvara *et al.* 2007). *V. vermiformis* has been isolated from the cerebrospinal fluid of a patient with meningoencephalitis and bronchopneumonia (Centeno *et al.* 1996). Moreover, the pathogenic potential of *V. vermiformis* isolated from patient with AK has been also proven (Kennedy *et al.* 1995, Lorenzo-Morales *et al.* 2007). In addition to their own pathogenic abilities, FLA are known to be a reservoir of many microorganisms, including bacteria, viruses, fungi and also other protozoa (Greub and Raoult 2004).

Because of all mentioned threats and their possible impact on human and animal health, it is significant to detect and also identify these potentially pathogenic FLA in human-exposed aquatic environments. In Poland, *Acanthamoeba* strains which have showed pathogenicity for mice (Górnik and Kuźna-Grygiel 2004) and *N. fowleri* (Kasprzak *et al.* 1982) have been isolated from various natural and man-made water bodies. It shows that there is a real risk of the pathogenic parasite infections throughout the country. In our previous studies, we revealed the occurrence of potential human and animals pathogenic FLA (*Acanthamoeba* T4 strain and new, potentially pathogenic strain, as well as *V. vermiformis*) in human-exposed natural water bodies (Adamska *et al.* 2014). The main aim of this study was detection and molecular identification of potentially pathogenic FLA strains (*Acanthamoeba* spp., *N. fowleri, B. mandrillaris*, *S. pedata*, and *V. vermiformis*) in artificial water bodies located in North-Western Poland.

MATERIALS AND METHODS

During two years (2010 and 2011) 86 water samples were collected from 43 artificial water bodies, including outdoor and indoor swimming pools, firefighting reservoirs, fountains, as well as water network (Tab. 1). All examined water bodies are localized in North-Western Poland and used for recreational purposes or located in urban areas.

Each time, the 5-litre water samples were collected into sterile containers and filtered using a vacuum pump and separated in Filta-Max® membrane filters (IDEXX Laboratories, USA). Agar plates (NN Agar) were prepared and covered with inactivated *Escherichia coli* bacteria. 100 µl of each eluate with the residue, received after filtration, were placed on the agar plates and then they were incubated at 37°C for 72 h. After the incubation, proliferated amoebae were passaged at 42°C and the intensity of growth were observed each day in order to isolate thermophilic strains that were scrapped and resuspended in 1 ml of PBS buffer after 72 h incubation.

QIAamp® DNA Mini Kit (Qiagen, Germany) was used in order to isolate DNA from pelleted trophozoites of thermophilic strains, according to the manufacturer's instructions. Different species of FLA were detected in obtained isolates using PCR protocols previously published by the authors (Tab. 2). All reactions were carried out in two replicates. Amplification products from all PCR reactions were separated by electrophoresis in 1.5% agarose gel stained with ethidium bromide and viewed in UV light. DNA isolates obtained earlier from clinical and environmental samples, in which the presence of amoebae DNA was revealed by PCR and sequencing, were the positive controls.

Both strands of all obtained PCR products were sequenced using the amplification primers (Macrogen, Korea) and the sequences were initially compared with other homology sequences from GenBank database using the Basic Local Alignment Search Tool (BLAST) at the National Centre for Biotechnology Information. Separate alignments were performed for our each strain of *Acanthamoeba* and for *V. vermiformis* using ClustalW (Mega 5.10 software) to compare them to each other and to other homologous sequences from GenBank.

The obtained results were compared and significance was assessed by a χ^2 test with Yates' correction for small groups using the STATISTICA 7.0 statistical package. The level of statistical significance was set at $p < 0.05$.

RESULTS

Among the 86 collected water samples, 57 strains of FLA able to proliferate at 37°C were isolated and 7 of them showed ability to proliferate at 42°C (Tab. 1).

Table 1. Isolated strains of FLA and their thermal properties.

Water source	No. of sites	No. of samples	Thermal tolerance			
			37°C		42°C	
			n	%	n	%
Indoor swimming pool	10	20	15	75	2	10.00
Outdoor swimming pool	3	6	6	100	2	33.33
Firefighting reservoir	5	10	9	90	1	10.00
Fountain	7	14	11	78.57	1	7.14
Tap water	18	36	16	44.44	1	2.78
Total	43	86	57	66.28	7	8.14

Table 2. Primers used in the study in order to detect amoebae DNA.

FLA	Molecular marker	Primer	Size of amplification product (bp)	References
Acanthamoeba spp. *V. vermiformis*	18S rDNA	Ami6F1 5'-CCAGCTCCAATAGCGTATATT-3' Ami9R 5'-GTTGAGTCGAATTAAGCCGC-3'	~ 830 ~ 600	Thomas *et al.* 2006
N. fowleri	ITS	NFFW 5'-TGAAAACCTTTTTTCCATTTACA-3' NFRW 5'-AATAAAAGATTGACCATTTGAAA-3'	~ 300	Lares-Villa and Hernández-Peña 2010
B. mandrillaris	mitochondrial 16S rDNA	5'Balspec16S 5'-CGCATGTATGAAGAAGACCA-3' Bal16Sr610 5'-CCCCTTTTTAACTCTAGTCATATAGT-3'	~ 230	Yagi *et al.* 2008
S. pedata	ITS	Sap-ITS-542F 5'-ATGCCTGCGATTTCTCTATC-3' Sap-ITS-675R 5'-AACCCACCTTTGCATTTCG-3'	~ 150	Brown *et al.* 2007 – modified

Thermophilic strains were collected in 2 indoor and 2 outdoor swimming pools, 2 fountains, 1 firefighting reservoir, as well as 1 sample of tap water from hospital (Tab. 3). Statistically significant differences in the FLA strains distribution in particular water bodies were not observed and the strains distribution in 2010 and 2011 was also insignificant.

PCR and sequencing of the 18S rDNA gene fragment, performed for thermophilic strains, revealed the presence of DNA of *Acanthamoeba* spp. (T4 and T11 genotypes, as well as the new genotype described in 2009 as T16 by Lanocha *et al*.) and *V. vermiformis*. In 5 samples, DNA of *Acanthamoeba* spp. has been identified, in 2 – *V. vermiformis* and in 1 sample – both of them (Tab. 3). Three other investigated species: *N. fowleri*, *B. mandrillaris* and *S. pedata* have not been identified.

Statistically significant differences in the distribution of *Acanthamoeba* spp. and *V. vermiformis* DNA in particular water bodies in 2010 and 2011 were not observed ($p = 0.233$). However, it should be noted that the low number requires great caution in interpreting the results.

The sequence comparison has showed that two detected T11 sequences are identical, as well as two detected sequences representing the new strain described earlier as T16 (Lanocha *et al*. 2009). We detected two variants of *V. vermiformis* 18S rDNA sequence: JQ409008 sequence has T in 88 position and C in 89 position, while JQ409009 and JQ409010 sequences are identical and have C in 88 position and T in 89 position. The two detected variants of *V. vermiformis* sequence showed similarity of 99.87%.

Table 3. Accession numbers of the 18S rDNA gene sequences of *Acanthamoeba* spp. and *H. vermiformis* deposited in the Gene Bank database.

Accession numbers in the Gene Bank	Genus or species	Sequence type	Water body type
JQ408990	*Acanthamoeba* sp.	T4	Fountain in shopping centre
JQ408991	*Acanthamoeba* sp.	T11	Indoor swimming pool, river
JQ408992	*Acanthamoeba* sp.	T11	Indoor swimming pool, jacuzzi
JQ408997	*Acanthamoeba* sp.	T16*	Outdoor swimming pool for children
JQ408998	*Acanthamoeba* sp.	T16*	Outdoor swimming pool, salt water
JQ409008	*V. vermiformis*	"TC"	Tap water, hospital
JQ409009	*V. vermiformis*	"CT"	Firefighting reservoir
JQ409010	*V. vermiformis*	"CT"	Fountain in a shopping centre

*the genotype described as T16 by Lanocha *et al.* (2009)

Performed alignment revealed that our T4 sequence (JQ408990) is identical with our sequences detected earlier in lake water (JQ632778, JQ632779, JQ408989, Adamska *et al.* 2014), with sequences obtained from swimming pool and salt cave water in Slovenia (GQ397463, GQ397464, GQ397467, GQ397471, Nagyova *et al.* 2010) and hospital water network in France (AY237735, unpublished data) and with sequences detected in the bronchoaspirate fluid of patient after the allogenic transplantation of bone marrow in Poland (GQ342606, Lanocha *et al.* 2009) and in infected cornea of amoebic keratitis patients in Korea (EF140627, unpublished data). Our T11 sequences are unique and showed similarity of 99.7% to *Acanthamoeba hatchetti* sequence detected in cornea of lens-wearing keratitis patient in Austria (AF251937, Walochnik *et al.* 2000) and to other *A. hatchetti* sequences (AF019068, isolated from water by Stothard *et al.* 1998; JF508857, unpublished data). The similarity of our T11 sequences to other T11 strains from GenBank is lower – 95.3% for AY343664 (isolated from water by Booton *et al.* 2004) and 93.3% for AF333608 (Gast *et al.* 2001). The sequences representing the new strain described earlier as T16 (Lanocha *et al*. 2009) are identical to our sequences detected earlier in river water (JQ408995, JQ408996, HQ632775 and HQ632777) and showed the similarity of 99.4% to the sequence obtained from bronchoalveolar lavage of a newborn with atypical pneumonia (GQ342608, Lanocha *et al.* 2009).

Two of our *V. vermiformis* strains (JQ409009 and JQ409010) were identical and one strain (JQ409008) was unique. They showed the highest similarity (99%) to the sequences obtained earlier by us and other authors from different types of water bodies (e.g. JQ409000 – lake water, JQ408999 – river water), as well as obtained from compost (KC164244), sludge (FJ628004) and gill tissue of rainbow trout (*Oncorhynchus mykiss*) (HM363626, Dyková *et al.* 2010).

DISCUSSION

FLA species are widely distributed in various environmental sources and they have been found worldwide in aquatic habitats, soil, and air (Visvesvara *et al.* 2007). Water temperature, salinity and chlorination, food availability and ability to form cysts are among the factors affecting their distribution (Tsvetkova *et al.* 2004). In this paper, FLA including thermophilic strains were detected in all examined types of artificial water bodies as indoor and outdoor swimming pools, firefighting reservoir, fountain, and tap water. It is consistent with reports from Poland, as well as from other countries around the world, where the thermophilic strains have been reported in numerous natural and man-made water sources (Gornik and Kuzna-Grygiel 2004, Tsvetkova *et al.* 2004, Khan 2006, Visvesvara *et al.* 2007, Lanocha *et al.* 2009, Carlesso *et al.* 2010, Adamska *et al.* 2014).

It is important to determine strains which show ability to proliferate at 42°C, because only thermophilic FLA may be pathogenic for humans and animals. They can proliferate at a temperature about 42°C, which may be correlated with their adaptation to conditions in the host. Amoebae isolated posthumously from organs showed the ability to multiply at 42–45°C. However, not all thermophilic strains are pathogenic, as some of the avirulent amoebae show analogous temperature

preferences (Khan 2006). For this reason, it is important to determine more precise information about their ability to cause disease in humans and animals. Except the thermal tolerance test, several methods have been described to find out the pathogenicity of amoebae, including microscopic methods, biological assays, biochemical methods and techniques based on polymerase chain reaction (Khan *et al.* 2002, Khan 2006). Actually, molecular methods especially sequencing the fragments of 18S rDNA gene is most commonly used to obtain information about pathogenic abilities of FLA (Stothard *et al.* 1998; Schroeder *et al.* 2001; Booton *et al.* 2002, 2005; Khan *et al.* 2002; Khan 2006; Visvesvara *et al.* 2007).

In this study, *Acanthamoeba* spp. and *V. vermiformis* strains were detected in the examined artificial water bodies in North-Western Poland. They are the most common amoebae and probably the most common protozoa detected in environment. However, three other investigated species: *N. fowleri*, *B. mandrillaris* and *S. pedata* have not been identified. The detection of only *Acanthamoeba* spp. and *V. vermiformis* strains may be associated with the much shorter life of other amoebae cysts and their greater sensitivity to chlorination and low temperature (De Jonckheere 1979). It is consistent with reports from around the world, where *Acanthamoeba* spp. and *V. vermiformis* have been reported most frequently in different types of water bodies (Tsvetkova *et al.* 2004, Lorenzo-Morales *et al.* 2005, Thomas *et al.* 2006, Gianinazzi *et al.* 2010, Nuprasert *et al.* 2010, Adamska *et al.* 2014). According to previous reports from Poland, only *Acanthamoeba* strains were found in artificial water bodies and *Acanthamoeba* strains and *V. vermiformis* were found in natural water bodies in the West Pomeranian voivodeship (Górnik and Kuźna-Grygiel 2004, Łanocha *et al.* 2009, Adamska *et al.* 2014). Additionally, *N. fowleri* was previously identified near Poznan (Kasprzak *et al.* 1982), while *V. vermiformis* has not been earlier detected in Polish artificial water bodies, which is surprising taking account of its widespread occurrence. However, the previous identification of amoebae species was carried out on the basis of cysts morphology, which is very inaccurate. Several studies have demonstrated inconsistencies and/or variations in cyst morphology of the same strain (Khan 2006). For example, culture conditions can affect cyst morphology making species identifications based on morphology alone unreliable (Stratford and Griffiths 1978). Moreover, attempts to associate pathogenicity with species morphological classification have proven difficult (De Jonckheere 1980). For example, several studies revealed that strains within *A. castellanii* can be virulent, weakly virulent or avirulent (Khan 2006). Whereas, molecular methods used in this study allow us to identify pathogens at the species or strain levels, and to explore the impact of genetic diversity on significant biomedical parameters such as pathogenicity, resistance to drugs, host species, or site of infection. Recent data have shown the applicability of rDNA sequencing as a tool for differentiating pathogenic and nonpathogenic *Acanthamoeba* spp. (Khan *et al.* 2002).

Based on SSU rRNA gene sequences, the genus *Acanthamoeba* is divided into 18 different genotypes designated T1–T18 (Stothard *et al.* 1998, Qvarnstrom *et al*. 2013). The genotypes exhibit at least 5% 18S rDNA sequence divergence in comparision to each other (Stothard *et al.* 1998) and they are all described on the basis of the full sequence of 16S rRNA gene (~ 2200 bp) except from the T15 genotype described on the basis of the GTSA.B1 fragment of the gene (~ 1450 bp) (Corsaro and Venditti 2011). Genotype number does not always correspond to specific classifications based on morphology, so it is more reasonable to use the designation of sequence type instead of conventional species name (Stothard *et al.* 1998). Based on this classification scheme, the majority of human infections due to *Acanthamoeba* spp. have been associated with the T4 genotype and it is also the most abundant in the environment, e.g. more than 94% of keratitis cases have been linked with this genotype. Similarly, T4 has been the major genotype associated with nonkeratitis infections such as GAE and other infections (Booton *et al.* 2005). It has been recently shown that the genotype exhibits significant higher binding and produce severe cytotoxicity on host cells as compared to other genotypes (Alsam *et al.* 2003). However, T1, T2, T3, T5, T6, T10, T11 and T12 genotypes have been also described in human invasions (Booton *et al.* 2002, 2005; Spanakos *et al.* 2006; Walochnik *et al*. 2000).

In this study, the 830–840 fragments of 18S rDNA gene sequences, containing 5 variable regions, were amplified and sequenced in order to distinguish *Acanthamoeba* genotypes. In examined artificial water bodies, we detected T4 and T11 genotypes, as well as the new genotype described earlier by Lanocha *et al.* (2009) as T16. The similarity of strains representing the same genotype should be over 95% (Stothard *et al*. 1998) and the dissimilarity between our T11 sequences and four T11 sequences from GenBank is below 5%, however, the dissimilarity between our sequences and AY343664

sequence obtained from water is close to 5%, and between our sequences and AF333608 sequence is over 6%. We aligned our T11 sequences only with GenBank sequences of similar length, and shorter sequences were not used. However, the aligned sequences are only fragments of the *Acanthamoeba* 18S rRNA gene, that is about 2200 bp long, and this may increase dissimilarity between the sequences Corsaro and Venditti (2011). What is more, the classification of the strains described as T16 genotype by Lanocha *et al*. (2009) may be false because the short portion of 18S rRNA gene (the 850-bp Ami fragment), was used to describe this type. This fragment contains only 5 of 8 variable regions of the 18S rRNA gene and the use of this marker increases genetic distances between pairs of aligned sequences. Analysis of longer fragments of the 18S rRNA gene (preferably the full sequence) is require to describe new *Acanthamoeba* genotypes (Corsaro and Venditti 2011). Further studies, based on the whole 18S rRNA gene sequence, are needed to classify our strains that showed the highest similarity to the strains described earlier as T16 genotype by Lanocha *et al*. (2009).

It is important that the two genotypes detected by us: T4 and the new genotype described earlier as T16 by Lanocha *et al.* (2009), show 100% and 99.4% similarity, respectively, to *Acanthamoeba* strains isolated from Polish patients with immune deficiencies and atypical pneumonia symptoms. In addition, our T4 strain is identical with the strain detected in infected cornea of amoebic keratitis patients from Korea, and our T11 strain is similar (99.7%) to *A. hatchetti* sequence isolated from eye cornea in Austria. For this reason, we can suppose that there is a potential threat to health of humans in Poland. In our previous studies, we examined 200 surface water samples collected from 50 sites of 36 water bodies between 2009 and 2012 and we detected *Acanthamoeba* T4 genotype and the new genotype described earlier as T16 (Lanocha *et al*. 2009) in 13 water samples collected from 6 water bodies. The overall presence of thermophilic *Acanthamoeba* sp. DNA was detected in 6.5% of all collected samples (13/200, Adamska *et al.* 2014), and in this study the overall presence was 5.8% (5/86). These results are comparable and the threat to health of humans who have contact with artificial and natural water bodies in the West Pomeranian voivodeship seems to be similar.

First time in Poland, *V. vermiformis* strains have been isolated from artificial water bodies: firefighting reservoir, fountain in shopping center, and tap water obtained from maternity ward of hospital. The amoebae, very ubiquitous in environment, has recently also been associated with human disease (Kennedy *et al.* 1995, Centeno *et al.* 1996, Lorenzo-Morales *et al.* 2007). As a result, its presence in human-related aquatic habitats, particularly in hospital tap water seems to be threat to humans, the more since this species may serve as a vector of pathogenic microorganisms (Hsu *et al.* 2009). Previously, we detected 18S rDNA of *V. vermiformis* in 11 water samples collected from 4 water bodies and DNA of this amoeba was found in 5.5% of all examined isolates collected between 2009 and 2012 (11/200, Adamska *et al.* 2014), whereas in this study the overall presence was 3.5% (3/86). Therefore, the risk of human infections is higher during contact with water from natural water bodies in the West Pomeranian voivodeship.

In conclusion, environmental screening of various ecological niches of *Acanthamoeba* spp. is required and may reveal hidden but potentially dangerous habitats (Booton *et al.* 2005). We have identified *Acanthamoeba* genotypes which may cause pneumonia and potential invasion of central nervous system in individuals with a compromised immune system. Additionally, strains which are causative agents of sight-threatening corneal infection mainly associated with healthy contact-lens wearers have been detected. We have also identified *V. vermiformis* species in many artificial water bodies for the first time in North-Western Poland. The presence of strains belonging to T4 and T11 genotypes, as well as to the new genotype detected earlier in clinical samples and described as T16 (Lanocha *et al*. 2009), and probably *V. vermiformis* genotypes, in human-related aquatic habitats may cause health-threatening infections in humans in the country, mainly as these amoebae may serve as vectors of pathogenic microorganisms.

Acknowledgements. This study was supported in part by the Ministry of Science and Higher Education, grant no. N N404 248635.

REFERENCES

Adamska M., Leonska-Duniec A., Lanocha N., Skotarczak B. (2014) Thermophilic potentially pathogenic amoebae isolated from natural water bodies in Poland and their molecular characterization. *Acta Parasitol*. **59:** DOI: 10.2478/s11686-014-0266-7

Alsam S., Kim K. S., Stins M., Rivas A. O., Sissons J., Khan N. A. (2003) *Acanthamoeba* interactions with human brain microvascular endothelial cells. *Microb. Patholog.* **35:** 235–241

Booton G. C., Rogerson G. C., Bonilla T.D., Seal D.V., Kelly D. J., Beattie T. K., Tomlinson A., Lares-Villa F., Fuerst P. A., Byers T. J. (2004) Molecular and physiological evaluation of subtropical environmental isolates of *Acanthamoeba* spp., casual

agent of *Acanthamoeba* keratitis. *J. Eukaryot. Microbiol.* **51:** 192–200

Booton G. C., Kelly D. J., Chu Y. W., Seal D. V., Houang E., Lam D. S., Byers T. J., Fuerst P. A. (2002) 18S ribosomal DNA typing and tracking of *Acanthamoeba* species isolates from corneal scrape specimens, contact lenses, lens cases, and home water supplies of *Acanthamoeba* keratitis patients in Hong Kong. *J. Clin. Microbiol.* **40:** 1621–1625

Booton G. C., Visvesvara G. S., Byers T. J., Kelly D. J., Fuerst P. A. (2005) Identification and distribution of *Acanthamoeba* species genotypes associated with nonkeratitis infections. *J. Clin. Microbiol.* **43:** 1689–1693

Brown M. W., Spiegel F. W., Silberman J. D. (2007) Amoeba at attention: phylogenetic affinity of *Sappinia pedata*. *J. Eukaryot. Microbiol.* **54:** 511–519

Carlesso A. M., Artuso G. L., Caumo K., Rott M. B. (2010) Potentially pathogenic *Acanthamoeba* isolated from a hospital in Brazil. *Curr. Microbiol.* **60:** 185–190

Carter R. F. (1970) Description of a *Naegleria* sp. isolated from two cases of primary amoebic meningoencephalitis, and of the experimental pathological change induced by it. *J. Pathol.* **100:** 212–244

Centeno M. F., Rivera F., Cerva L., Tsutaumi V., Gallegos E., Calderon A., Ortiz R., Bonilla P., Ramirez E., Suarez G. (1996) *Hartmannella vermiformis* isolated from the cerebrospinal fluid of a young male patient with meningoencephalitis and bronchopneumonia. *Arch. Med. Res.* **27:** 579–586

Corsaro D., Venditti D. (2011) More *Acanthamoeba* genotypes: limits to the use of rDNA fragments to describe new genotypes. *Acta Protozool.* **50:** 49–54

Culbertson C. G., Smith J. W., Miner J. R. (1958) *Acanthamoeba* observation on animal pathogenicity. *Science* **127:** 1506

Culbertson C. G., Smith J. W., Cohen H. K., Miner J. R. (1959) Experimental infection of mice and monkeys by *Acanthamoeba*. *Am. J. Pathol.* **35:** 185–197

De Jonckheere J. F. (1979) Pathogenic free-living amoebae in swimming pools: survey in Belgium. *Ann. Microbiol. (Paris)* **130B:** 205–212

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. *Appl. Environ. Microbiol.* **39:** 681–685

De Jonckheere J. F., Brown S. (1998) There is no evidence that the free-living amoeba *Hartmannella* is a human parasite. *Clin. Infect. Dis.* **26:** 773

Dyková I., Pindová Z., Fiala I., Dvoráková H., Macháčková B. (2005) Fish-isolated strains of *Hartmannella vermiformis* page, 1967: morphology, phylogeny and molecular diagnosis of the species in tissue lesions. *Folia Parasitol. (Praha)* **52:** 295–303

Fowler M., Carter R. F. (1965) Acute pyogenic meningitis probably due to *Acanthamoeba* sp.: a preliminary report. *Br. Med. J.* **2(5464):** 740–742

Gast R. J. (2001) Development of an *Acanthamoeba* – specific reverse dot – blot and the discovery of a new ribotype. *J. Eukaryot. Microbiol.* **48:** 609–615

Gelman B. B., Rauf S. J., Nader R., Popov V., Bokowski J., Chaljub G., Nauta H. W. (2001) Amoebic encephalitis due to *Sappinia diploidea*. *J. Am. Med. Assoc.* **285:** 2450–2451

Gelman B. B., Popov V., Chaljub G., Nader R., Rauf S. J., Nauta H. W., Visvesvara G. S. (2003) Neuopathological and ultrastructural features of amoebic encephalitis caused by *Sappinia diploidea*. *J. Neuropathol. Exp. Neurol.* **62:** 990–998

Gianinazzi C., Schild M., Zumkehr B., Wüthrich F., Nüesch I., Ryter R., Schürch N., Gottstein B., Müller N. (2010) Screening of Swiss hot spring resorts for potentially pathogenic free-living amoebae. *Exp. Parasitol.* **126:** 45–53

Górnik K., Kuźna-Grygiel W. (2004) Presence of virulent strains of amphizoic amoebae in swimming pools of the city of Szczecin. *Ann. Agric. Environ. Med.* **11:** 233–236

Greub G., Raoult D. (2004) Microorganisms resistant to free-living amoebae. *Clin. Microbiol. Rev.* **17:** 413–433

Hsu B. M., Lin C. L., Shih F. C. (2009) Survey of pathogenic free-living amoebae and *Legionella* spp. in mud spring recreation area. *Water Res.* **43:** 2817–2828

Jager B. V., Stamm W. P. (1972) Brain abscesses caused by free-living amoeba probably of the genus *Hartmannella* in a patient with Hodgkin's disease. *Lancet* **2:** 1343–1345

Kasprzak W., Mazur T., Červa L. (1982) *Naegleria fowleri* in thermally polluted waters. *Folia Parasitol. (Praha)* **29:** 211–218

Kennedy S. M., Devine P., Hurley C., Ooi Y. S., Collum L. M. T. (1995) Corneal infection with *Hartmannella vermiformis* in contact-lens wearer. *Lancet* **346:** 637–638

Khan N. A. (2006) *Acanthamoeba*: biology and increasing importance in human health. *FEMS Microbiol. Rev.* **30:** 564–595

Khan N. A., Jarroll E. L., Paget T. A. (2002). Molecular and physiological differentiation between pathogenic and nonpathogenic *Acanthamoeba*. *Curr. Microbiol.* **45:** 197–202

Lares-Villa F., Hernández-Peña C. (2010) Concentration of *Naegleria fowleri* in natural waters used for recreational purposes in Sonora, Mexico (November 2007–October 2008). *Exp. Parasitol.* **126:** 33–36

Lorenzo-Morales J., Monteverde-Miranda C. A., Jiménez C., Tejedor M. L., Valladares B., Ortega-Rivas A. (2005) Evaluation of *Acanthamoeba* isolates from environmental sources in Tenerife, Canary Islands, Spain. *Ann. Agric. Environ. Med.* **12:** 233–236

Lorenzo-Morales J., Martínez-Carretero E., Batista N., Álvarez-Martín J., Bahaya Y., Walochnik J., Valladares B. (2007) Early diagnosis of amoebic keratitis due to a mixed infectrion with *Acanthamoeba* and *Hartmannella*. *Parasitol. Res.* **102:** 167–169

Łanocha N., Kosik-Bogacka D., Maciejewska A., Sawczuk M., Wilk A., Kuźna-Grygiel W. (2009) The occurrence *Acanthamoeba* (free living ameba) in environmental and respiratory samples in Poland. *Acta Protozool.* **48:** 271–279

Naginton J., Watson P. G., Playfair T. J., McGill J., Jones B. R., Steele A. D. (1974) Amoebic infection of the eye. *Lancet* **2:** 1537–1540

Nagyová V., Nagy A., Janeček Š., Timko J. (2010) Morphological, physiological, molecular and phylogenetic characterization of new environmental isolates of *Acanthamoeba* spp. from the region of Bratislava, Slovakia. *Biologia* **65:** 81–91

Nuprasert W., Putaporntip C., Pariyakanok L., Jongwutiwes S. (2010) Identification of a novel T17 genotype of *Acanthamoeba* from environmental isolates and T10 genotype causing keratitis in Thailand. *J. Clin. Microbiol.* **48:** 4636–4640

Qvarnstrom Y., da Silva A. J., Schuster F. L., Gelman B. B., Visvesvara G. S. (2009) Molecular confirmation of *Sappinia pedata* as a causative agent of amoebic encephalitis. *J. Infect. Dis.* **199:** 1139–1142

Qvarnstrom Y., Nerad T. A., Visvesvara G. S. (2013) Characterisation of a new pathogenic *Acanthamoeba* species, *A. byersi* n. sp., isolated from a human with fatal amoebic encephalitis. *J. Euk. Microbiol.* **60:** 626–633

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. *J. Clin. Microbiol.* **39:** 1903–1911

Smirnov A. V., Chao E., Nassonova E. S., Cavalier-Smith T. (2011) A revised classification of naked lobose amoebae (Amoebozoa: Lobosa). *Protist* **162:** 545–570

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. *Parasitol. Int.* **55:** 147–149

Stothard D. R., Schroeder-Diedrich J. M., Awwad M. H., Gast R. J., Ledee D. R., Rodriguez-Zaragosa S., Dean C. L., Fuerst P. A., Byers T. J. (1998) The evolutionary history of the genus *Acanthamoeba* and the identification of eight new 18S rRNA gene sequence types. *J. Eukaryot. Microbiol.* **45:** 45–54

Stratford M. P., Griffiths A. J. (1978) Variations in the properties and morphology of cysts of *Acanthamoeba castellanii*. *J. Gen. Microbiol.* **108:** 33

Thomas V., Herrera-Rimann K., Blanc D. S., Greub G. (2006) Biodiversity of amoebae and amoeba-resisting bacteria in a hospital water network. *Appl. Environ. Microbiol.* **72:** 2428–2438

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

Visvesvara G. S., Martinez A. J., Schuster F. L., Leitch G. J., Wallace S. V., Sawyer T. K., Anderson M. (1990) Leptomyxid ameba, a new agent of amebic meningoencephalitis in humans and animals. *J. Clin. Microbiol.* **28:** 2750–2756

Visvesvara G. S., Schuster F. L., Martinez A. J. (1993) *Balamuthia mandrillaris*, n. g., n. sp., agent of amoebic meningoencephalitis in humans and other animals. *J. Eukaryot. Microbiol.* **40:** 504–514

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 Immunol. Med. Microbiol.* **50:** 1–26

Walochnik J., Haller-Schober E., Kölli H., Picher O., Obwaller A., Aspöck H. (2000) Discrimination between clinically relevant and nonrelevant *Acanthamoeba* strains isolated from contact lens-wearing keratitis patients in Austria. *J. Clin. Microbiol.* **38:** 3932–3936

Yagi S., Schuster F. L., Visvesvara G. S. (2008) Demonstration of *Balamuthia* and *Acanthamoeba* mitochondrial DNA in sectioned archival brain and other tissues by the polymerase chain reaction. *Parasitol. Res.* **102:** 491–497

Received on 13th November, 2013; revised on 20th December, 2013; Accepted on 6th March, 2014