Thermophilic potentially pathogenic amoebae isolated from natural water bodies in Poland and their molecular characterization

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Abstract
The free-living amoebae (FLA) may live in the environment and also within other organisms as parasites and then they are called amphizoic. They are potentially pathogenic for humans and animals and are found in water that is a source of infection. The aim of this study was molecular detection and identification of these FLA in natural water bodies in North-Western Poland to evaluate the risk of the pathogenic amoebae infections. We examined surface water samples collected from 50 sites and first, the tolerance thermic test was performed in order to select thermophilic, potentially pathogenic strains. For molecular identification of FLA, regions of 18S rDNA, 16S rDNA and intergenic spacers were amplified. Acanthamoeba T4 and T16 genotypes of 18S rDNA gene and 18S rDNA of H. vermiformis were detected. We identified two variants of Acanthamoeba T4 genotype, two variants of Acanthamoeba T16 genotype and one variant of H. vermiformis. Identification of the T16 genotype and H. vermiformis in water was for the first time in Poland. Additionally, we made attempts to adapt the RLB method for detection and differentiation of FLA species and strains. PCR seems to be more sensitive than RLB hybridization, though.

Keywords
Acanthamoeba T4 and T16 genotypes, free-living amoebae, genotyping, Hartmannella vermiformis, natural water bodies

Introduction
Free-living amoebae (FLA) are unicellular organisms living in the environment. They are isolated from water, soil, dust and air samples throughout the world. These of them, which may live also within other organisms as parasites are called amphizoic (Nagyoá et al. 2010) and they include Acanthamoeba spp., Balamuthia mardrillaris, Naegleria fowlerii, Sappinia pedata and probably Hartmannella vermiformis (Kuiper et al. 2006; da Rocha-Azevedo et al. 2009; Gianinazzi et al. 2010). The source of infection is mainly water or soil containing amoebae that enter host’s organism through corneal surface, nasal mucosa, and breaks in the skin or via an oral route (Schuster and Visvesvara 2004).

Acanthamoeba is an opportunistic protist that has two stages in the life cycle, an active trophozoite stage that reveals vegetative growth and a resistant cyst stage. Acanthamoeba is widely present in the environment; has been isolated from diverse natural and artificial water bodies, from the air and from soil. It is a causative agent of cutaneous lesions, can cause amoebic keratitis (AK) – an acute sight-threatening infection of the cornea, granulomatous amoebic encephalitis (GAE) – a chronic, progressive infection of the central nervous system with unspecific clinical symptoms that may involve the lungs, and disseminated tissue infections in animals and humans (da Rocha-Azevedo et al. 2009; Corsaro and Venditti 2010). There is no recommended treatment and in the most cases of GAE, diagnostics has been done at the postmortem phase (Siddiqui and Khan 2012).

According to the recent analysis of the gene encoding 18S rRNA, eighteen genotypes of Acanthamoeba genus have been identified and only some of them are pathogenic (Lanocha et al. 2009; Corsaro and Venditti 2010; Nuprasert et al. 2010; Qvarnstrom et al. 2013). Genotypes T2–T6, T10 and T11 are causative agents of AK and T4 genotype is dominant. Genotypes T1, T2, T4, T5, T10, T12 and T18 are responsible for GAE and other non-AK infections (Nagyoá et al. 2010; Nuprasert et al. 2010; Qvarnstrom et al. 2013). A new geno-
type, found in bronchoaspirate fluid of a man with chronic marrow leukaemia and bronchoalveolar lavage of newborn with symptoms of atypical pneumonia and named ‘T16’ (Lanocha et al. 2009) may be involved in those clinical symptoms in humans. Since 2010, other authors have described different new strains and described them as ‘T16’ using the 18S rRNA sequences of various length (Corsaro and Venditti 2010; Landell et al. 2013; Tanveer et al. 2013). The classification of described until now ‘T16’ strains is not clear and needs to be rearranged.

Other amoebae causing pathologies regarding central nervous system are Balamuthia marrillaris, that causes Balamuthia amoebic encephalitis (BAE) – a subacute and chronic granulomatous amoebic encephalitis with nonspecific syndroms and Naegleria fowleri, an etiological agent of primary amoebic meningoencephalitis (PAM), an acute and potentially fatal illness (Schuster and Visvesvara 2004; da Rocha-Azevedo et al. 2009; Qvarnstrom et al. 2009). There are also two other species that may be involved in causing human infections: Sappinia pedata, which caused a single case of Sappinia amoebic encephalitis (SAE) and Hartmannella vermiformis isolated from cerebrospinal fluid of a young patient with meningoencephalitis and bronchopneumonia (Kuiper et al. 2006; da Rocha-Azevedo et al. 2009; Qvarnstrom et al. 2009; Gianinazzi et al. 2010).

All described FLA amoebae are potentially pathogenic for humans and animals and are found in different kinds of water reservoirs throughout the world, including human-related aquatic habitats (da Rocha-Azevedo et al. 2009; Gianinazzi et al. 2010; Nagyová et al. 2010). Therefore, the aim of this study was molecular detection, identification and characterization of these amphizoic FLA strains in human-exposed natural water bodies in order to evaluate the risk of the pathogenic amoebae infections in North-Western Poland.

**Materials and Methods**

**Water bodies selection**

Surface water samples were collected from 50 sites of 36 water tanks in four year period (from 2009 to 2012). We collected 200 water samples from 30 sites of 27 lakes, 12 sites of 8 rivers and 8 sites of Baltic Sea. 49 sites chosen for this study are used as bathing places (all lake and sea sites and 11 river sites) and 3 sites – as drinking water sources for people (2 lake sites and 1 river site).

**Samples collection and preparation**

All water samples were examined earlier for detecting the presence of Cryptosporidium and Giardia (oo)cysts (Adamska et al., unpublished yet). In order to improve monitoring of these pathogens (oo)cysts and amoebae cysts in the samples, we performed modified 1623 Method introduced and recommended by United States Environmental Protection Agency (USEPA) for concentration and detection of Cryptosporidium and Giardia (oo)cysts in water (Krometis et al. 2009; Skotarczak et al. 2009). In accordance to this method the 50 liter water samples were collected from each site by passing through separate compressed-foam depth filters with pores of 1 μm diameter (Idexx Laboratories, USA) with the use of a pump (Grundfoss, Denmark) with a flow rate of 4 l/min. The high water samples volume increases the (oo)cysts recovery and amounts over 50 liters caused the clogging of foam filter pores. After filtration, elution procedures were carried out in accordance to the 1623 Method with the use of a Manual Filta Max® Wash Station (Idexx Laboratories, USA) and auxiliary equipment. Concentration of eluted (oo)cysts was performed using membrane filters with pores of 3 μm diameter (Idexx Laboratories, USA).

**Thermic tolerance test and DNA extraction**

The thermic tolerance test was performed to select thermophilic, potentially pathogenic species and strains of amoebae. Received eluates with the residue were placed on agar medium (NN Agar) covered with inactivated bacteria Escherichia coli and incubated at 37°C for 72h. In order to isolate thermophilic strains, proliferated amoebae were passaged at 42°C, observing the intensity of growth every day. The thermophilic strains from cultures were scrapped and resuspended in 1 ml of PBS buffer. DNA was extracted from pelletted trophozoites using QIAamp® DNA Mini Kit (Qiagen, Germany), according to the manufacturer’s instructions.

**PCR amplification, sequencing and genotyping**

For molecular identification of FLA, regions of 18S rDNA, 16S rDNA and intergenic spacers were amplified according to the descriptions provided by the authors (Table I). All analyses were carried out in two replicates. PCR products were visualized in 1.5% agarose gels stained with ethidium bromide. The positive controls were DNA isolates obtained earlier from infected patients and environmental samples, where the presence of amoebae DNA was revealed by PCR and sequencing.

Both strands of all amplicons were sequenced with the amplification primers (Macrogen, Korea) and their sequences were initially compared with homology sequences deposited in GenBank database using the Basic Local Alignment Search Tool (BLAST) at the National Centre for Biotechnology Information. Our sequences were aligned with each other using ClustalW (Mega 5.10 software) and separate alignment were performed for T4 and T16 strains of Acanthamoeba. The ends of the alignment were trimmed in order to form blunt ends on all the sequences in the alignment. The final alignment covered nucleotides corresponding to nucleotide positions 1 to 742 of Acanthamoeba strains with GenBank accession numbers JQ408986 – 88, 1 to 708 of Acanthamoeba strains with GenBank accession numbers JQ408993 – 96 and 10 to 508 of H. vermiformis with GenBank accession number HQ632780.
Free-living amoebae in natural water bodies

Trimmed sequences were aligned with other sequences from GenBank with ClustalW using Mega 5.10. The STATISTICA 7.0 was used to perform all statistical analysis. The obtained results were compared and significance was assessed by a χ² test (especially χ² test with Yates’ correction for small groups). The level of statistical significance was set at p < 0.05.

Reverse line blotting

Macroarray method (reverse line blotting) was also used in order to detect different FLA species and strains. Appropriate 5’ biotinylated starters and probes with 5’ terminal aminogroup, complementary to 18S rDNA gene of amoebae, Table I. Primers used in the study in order to detect DNA of amoebae

<table>
<thead>
<tr>
<th>FLA genus or species</th>
<th>Molecular marker</th>
<th>Primer</th>
<th>Size of amplification product (bp)</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acanthamoeba spp.</td>
<td>18S rDNA</td>
<td>Ami6F1</td>
<td>~830</td>
<td>Thomas et al. 2006</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ami9R</td>
<td>~600</td>
<td></td>
</tr>
<tr>
<td>H. vermiformis</td>
<td></td>
<td>NFFW</td>
<td>~300</td>
<td>Lares-Villa and Hernández-Peña 2010</td>
</tr>
<tr>
<td>N. fowleri</td>
<td>ITS</td>
<td>NFRW</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B. mandrillaris</td>
<td>16S rDNA</td>
<td>Balspec16S</td>
<td>~230</td>
<td>Yagi et al. 2008</td>
</tr>
<tr>
<td>S. pedata</td>
<td>ITS</td>
<td>Bal16Sr610</td>
<td>~150</td>
<td>Brown et al. 2007 – modified</td>
</tr>
</tbody>
</table>

Table II. Primers and probes designed for detecting of amoebae DNA with the use of RLB method

<table>
<thead>
<tr>
<th>FLA species or strain</th>
<th>PRIMERS</th>
<th>PROBES</th>
</tr>
</thead>
</table>
| Acanthamoeba          | Forward | 5’–CTCATCGTCTGTCAGCAAT–3’  
|                       | Reverse | 5’–ATTTCAGTACATGCGCGTACAAAT–3’ |
| Acanthamoeba          | Forward | 5’–TTGAGTCTGACCTCTCATTAT–3’  
|                       | Reverse | 5’–TCCATCGTCTGTCAGCAAT–3’  
| Acanthamoeba          | Forward | 5’–CTCATCGTCTGTCAGCAAT–3’  
|                       | Reverse | 5’–ATTTCAGTACATGCGCGTACAAAT–3’ |
| Acanthamoeba          | Forward | 5’–CTCATCGTCTGTCAGCAAT–3’  
|                       | Reverse | 5’–ATTTCAGTACATGCGCGTACAAAT–3’ |
| Hartmannella          | Forward | 5’–TAAATAGTCACGCGAACC–3’  
| vermiformis           | Reverse | 5’–AACTTTAGCTGGACAGGA–3’  
| Naegleria             | Forward | 5’–CCTGACCCAACCGGATATATT–3’  
| fowleri               | Reverse | 5’–ACTGACCCAACCGGATATATT–3’  
| Balamuthia            | Forward | 5’–CCTGACCCAACCGGATATATT–3’  
| mandrillaris          | Reverse | 5’–ACTGACCCAACCGGATATATT–3’  

*The genotype described as T16 by Lanocha et al. (2009)
were designed (Table II). Due to the high level of genetical variability of *Acanthamoeba*, two probes were designed for T3 and T11 strains. The positive control was DNA extracted earlier from infected patients and environmental samples, where the presence of amoebae DNA was revealed by PCR and sequencing. All reactions were performed using Miniblotter® MN45 System (Immunetics, Netherlands) and Mini-Hybridization Oven OV3 (Whatman Biometra, Germany). In order to detect the reaction products, we applied incubation in BM Blue POD substrate (Roche Applied Science, USA). The positive controls were DNA isolates obtained earlier from infected patients and environmental samples, where the presence of amoebae DNA was revealed by PCR and sequencing.

**Results**

Among all 200 water samples, 20 of them, collected from 7 sites, showed ability to proliferate at 42°C. *Acanthamoeba* sp. DNA was detected in 13 samples collected from 6 water bodies. DNA of *H. vermiformis* was noticed in 11 samples collected from 4 water bodies (Table III). The presence of *B. mandrillaris*, *N. fowleri* and *S. pedata* DNA was not detected in any examined water body.

<table>
<thead>
<tr>
<th>The name and location of examined site</th>
<th>The year of sampling and detected FLA species or strain</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2009</td>
</tr>
<tr>
<td>Glebokie Lake (Szczecin)</td>
<td><em>Acanthamoeba</em> T4 genotype</td>
</tr>
<tr>
<td>Dabie Duze Lake (Lubczyna)</td>
<td><em>Acanthamoeba</em> T16 genotype *, <em>H. vermiformis</em></td>
</tr>
<tr>
<td>Miejskie Lake (Trzcinisko Zdroj)</td>
<td>none</td>
</tr>
<tr>
<td>Nowogardzkie Lake (Nowogard)</td>
<td>none</td>
</tr>
<tr>
<td>Odra River – (Dziewoklicz, Szczecin)</td>
<td><em>Acanthamoeba</em> T16 genotype *</td>
</tr>
<tr>
<td>Odra River – (warm water channel, Gryfino)</td>
<td><em>H. vermiformis</em></td>
</tr>
<tr>
<td>Ina River (Goleniow)</td>
<td><em>Acanthamoeba</em> T16 genotype *</td>
</tr>
</tbody>
</table>

*Strains with the maximum similarity to the genotype described as T16 by Lanocha et al. (2009)*

We obtained weak positive signals of RLB reaction – for T4 genotype of *Acanthamoeba* in water samples obtained from Miejskie Lake (Trzcinsko Zdroj) in 2011, for *Acanthamoeba* T16 genotype (described by Lanocha et al., 2009) in water samples obtained from Dabie Duze Lake (Lubczyna) in 2009 and 2010 and Odra River – Dziewokicz bathing place (Szczecin) in 2010 and 2011, and for *H. vermiformis* in water samples obtained from Dabie Duze Lake (Lubczyna) in 2009.

Table III. The presence of *Acanthamoeba* spp. and *H. vermiformis* DNA in examined water basins

<table>
<thead>
<tr>
<th>FLA genus or species</th>
<th><em>Acanthamoeba</em> sp.</th>
<th><em>H. vermiformis</em></th>
</tr>
</thead>
<tbody>
<tr>
<td>Year of sampling</td>
<td>2009</td>
<td>2010</td>
</tr>
<tr>
<td>Number of positive samples</td>
<td>4/50</td>
<td>4/50</td>
</tr>
<tr>
<td>Percentage</td>
<td>8%</td>
<td>8%</td>
</tr>
</tbody>
</table>

Five of 7 sites in which amoebae DNA was detected (all lakes and Odra River – Dziewokicz) are used as bathing places during summer months. Odra River – warm water channel is a place where water temperature is at least 10°C and reaches up to 30°C in summer months.

The presence of *Acanthamoeba* sp. DNA was detected in 6.5% of all collected samples, whereas *H. vermiformis* DNA was found in 3.5% of examined isolates. The prevalence of amoebae DNA in each year is presented in Table IV. The statistical analysis showed that the difference in the prevalence of *Acanthamoeba* sp. DNA in all years was not significant, as well as the prevalence of *H. vermiformis* DNA. The difference in *Acanthamoeba* sp. and *H. vermiformis* DNA prevalence in each year and in general was not statistically significant, either.

We obtained weak positive signals of RLB reaction – for T4 genotype of *Acanthamoeba* in water samples obtained from Miejskie Lake (Trzcinsko Zdroj) in 2011, for *Acanthamoeba* T16 genotype (described by Lanocha et al., 2009) in water samples obtained from Dabie Duze Lake (Lubczyna) in 2009 and 2010 and Odra River – Dziewokicz bathing place (Szczecin) in 2010 and 2011, and for *H. vermiformis* in water samples obtained from Dabie Duze Lake (Lubczyna) in 2009.
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Genotyping

Acanthamoeba 18S rDNA PCR products were sequenced. According to this gene sequence analysis, two variants of T4 strain were detected in four samples, and in nine – two variants of T16 strain (described by Lanocha et al. 2009). Sequence analysis of eleven H. vermiformis 18S SSU rDNA PCR products revealed the presence of one variant in all samples. The most of obtained sequences were deposited in GenBank where separate accession numbers were assigned to them (Table V). Four of obtained sequences were not deposited in GenBank because of their poor quality.

Performed alignment showed 1 indel and 7 transversions among our T4 sequences (Table VI) and revealed that one of our Acanthamoeba sp. T4 sequence (HQ632779) is identical to our three sequences obtained earlier from Polish fountain (JQ408989) and lake (JQ408989 and HQ632778) water and eight sequences from GenBank: Acanthamoeba T4 GQ342606 sequence obtained from bronchoalveolar fluid (BAL) of 15-year boy after the allogenic transplantation of bone marrow in Poland (Lanocha et al. 2009), Acanthamoeba T4 GQ397463, GQ397464, GQ397467 and GQ397471 sequences obtained from Slovenian swimming pool water, swimming pool scrape and salt cave salt water (Nagyová et al. 2010), Acanthamoeba T4 GQ342606 sequence obtained from hospital water network in France and Korean Acanthamoeba lugdunensis AF005995 sequence. The rest of our Acanthamoeba sp. T4 sequences showed similarity of 99.63% to HQ632779 and mentioned above eight sequences obtained by others.

Among our Acanthamoeba T16 sequences, two indels and 3 substitutions (1 transition and 2 transversions) were observed (Table VII). Our T16 sequences with GenBank accession numbers HQ632776, JQ408993 and JQ408994 showed 100% similarity to Acanthamoeba T16 GQ342606 sequence obtained from bronchoalveolar lavage (BAL) of Polish newborn with symptoms of atypical pneumonia (Lanocha et al. 2009), in opposite to our remaining T16 sequences (JQ408995, JQ408996, HQ632775 and HQ632777) which similarity to GQ342606 was 99.68%. All our H. vermiformis strains were identical to each other and they showed the highest similarity (99%) to the sequences obtained earlier from different kinds of water bodies, isolated by us (HQ632781 – lake water, JQ409010 – fountain water, JQ409009 – fire water reservoir and JQ409008 – hospital tap water) and other au-

Table V. The accession numbers of amoebae 18S rDNA gene sequences obtained from examined water samples

<table>
<thead>
<tr>
<th>The name and location of examined site</th>
<th>Accession numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Acanthamoeba</td>
</tr>
<tr>
<td></td>
<td>H. vermiformis</td>
</tr>
<tr>
<td>Glebokie Lake (Szczecin)</td>
<td>HQ632779</td>
</tr>
<tr>
<td>Dabie Duze Lake (Lubczyna)</td>
<td>HQ632776, JQ408993, JQ408994</td>
</tr>
<tr>
<td>Miejskie Lake (Trzcinski Zdroj)</td>
<td>JQ408987, JQ408988</td>
</tr>
<tr>
<td>Nowogardzkie Lake (Nowogard)</td>
<td>JQ408986</td>
</tr>
<tr>
<td>Odra River – Dzwoklicz bathing place (Szczecin)</td>
<td>–</td>
</tr>
<tr>
<td>Odra River – warm water channel (Gryfino)</td>
<td>–</td>
</tr>
<tr>
<td>Ina River (Goleniow)</td>
<td>HQ632775</td>
</tr>
</tbody>
</table>

Table VI. Nucleotide polymorphism among aligned Acanthamoeba T4 sequences

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Nucleotide position</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>36 262 497 569 611 627 667 670</td>
</tr>
<tr>
<td>HQ632779</td>
<td>G G A A C C G T</td>
</tr>
<tr>
<td>JQ408986, JQ408987, JQ408988</td>
<td>– T T T A A T A</td>
</tr>
</tbody>
</table>

Table VII. Nucleotide polymorphism among aligned Acanthamoeba T16 sequences*

<table>
<thead>
<tr>
<th>Accession number</th>
<th>Nucleotide position</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>28 29 33 578 595</td>
</tr>
<tr>
<td>HQ632776, JQ408993, JQ408994</td>
<td>C A – C T</td>
</tr>
<tr>
<td>HQ632777, JQ408995, JQ408996, HQ632775</td>
<td>T T A – A</td>
</tr>
</tbody>
</table>

*The sequences with the maximum similarity to the genotype described as T16 by Lanocha et al. (2009)
thors (e.g. AY230090, AY230093 – hospital drinking water network, JQ678665 – swamp water), as well as obtained from compost (KC164244), sludge (FJ628004) and gill tissue of rainbow trout (Oncorhynchus mykiss) (Dyková et al. 2010).

**Discussion**

In our earlier studies of samples collected from different water sources and various areas in north-western Poland, we investigated the occurrence of Cryptosporidium and Giardia species and analyzed the genotypes (Adamska et al., unpublished yet). Basing on the overall prevalence of C. parvum (0.5%) and G. intestinalis (0.6%) in the examined samples of water, it indicates that the risk of Cryptosporidium and Giardia to humans in the north-western Poland is minimal. Nevertheless these FLA amoebae are widely distributed in various environmental sources, especially in water, which temperature, salinity and chlorination, as well as food availability and ability to form cysts are the factors affecting their distribution (Tsvektova et al. 2004; Visvesvara et al. 2007). Only the most common amoebae, Acanthamoeba spp. and H. vermiciformis were present in the examined natural water bodies and N. fowleri, B. mandrillaris or S. pedata have not been identified, what may be associated with their greater sensitivity to environmental conditions as well as much shorter life of their cysts (De Jonckheere 1980; Schuster and Visvesvara 2004). Acanthamoeba spp. have been reported most frequently in other water bodies throughout the world, also in Poland (Gornik and Kuzna-Grygiel 2004; Tsvetkova et al. 2004; Lorenzo-Morales et al. 2005; Thomas et al. 2006; Lanocha et al. 2009; Gianinazzi et al. 2010; Nuprasert et al. 2010) and our investigations confirm the higher prevalence of this pathogen in water environment. The prevalence of H. vermiciformis in the water bodies in other countries was also high (Tsvetkova et al. 2004; Thomas et al. 2006) nevertheless we detected this amoeba first time in Poland. FLA strains were present in lakes and rivers, in opposite to sea water which salinity is too high for some FLA species, especially for N. fowleri (Schuster and Visvesvara 2004; Tsvetkova et al. 2004). However, Acanthamoeba strains were detected in salt cave salt water (Nagyová et al. 2010) which salinity is higher in comparison to Baltic Sea water. We examined only few number of sea water samples and this may be a reason of lack of amoebae in this kind of water body.

Only thermophilic FLA may be pathogenic for humans and animals, what is correlated with their adaptation to be capable of surviving in 37°C and higher body temperatures, so it is very important to select strains showing ability to proliferate at 42°C. However, not only pathogenic strains are thermophilic and some of the avirulent amoebae can proliferate at the temperature 42°C and higher (Schuster and Visvesvara 2004; Khan 2006; Nagyová et al. 2010). For this reason, the thermal tolerance test is not sufficient to determine the pathogenicity of detected strain and it is important to determine more precise information about its ability to cause disease in humans and animals. Several other methods have been described to find out the pathogenicity of amoebae, such as microscopic and biochemical methods, biological assays and techniques based on polymerase chain reaction (Khan et al. 2002; Khan 2006). Molecular methods, especially sequencing of fragments of 18S rDNA gene, are actually the most informative and allow to obtain the data of FLA pathogenic abilities (Stothard et al. 1998; Schroeder et al. 2001; Booton et al. 2002; Khan et al. 2002; Booton et al. 2005; Khan 2006; Visvesvara et al. 2007). In order to select pathogenic strains and limit the number of PCR examined samples, we performed the thermal tolerance test and obtained 20 thermophilic strains of all 200 collected samples. The only strain which pathogenic abilities are not questionable (Acanthamoeba T4) was detected in 4 of 20 samples.

In 1997, Pussard and Pons clustered Acanthamoeba spp. into three groups based on morphological features of their cysts, however, further biochemical and molecular studies revealed many incoherences in this clustering, such as polyphyletic character of some species, e.g. Acanthamoeba polyphaga (Corsaro and Venditti 2010). Moreover, culture conditions can affect cyst morphology making species identifications based on morphology alone unreliable (Stratford and Griffiths 1978), e.g. cysts have greater diameter in liquid medium (Nagyová et al. 2010), and it is difficult to associate pathogenicity with species morphological classification difficult (De Jonckheere 1980), e.g. A. castellanii can be virulent, weakly virulent or avirulent (Khan 2006). Analysis of the gene encoding 18S rRNA sequence similarity of over 50 strains from the three morphological groups allowed on identification of twelve genotypes (T1 – T12), corresponding to previous species or species complex. Each genotype exhibits at least 5% sequence divergence between different genotypes and genotype number do 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 (Gast et al. 1996; Stothard et al. 1998). From 1998, new genotypes have been classified and named T13 to T18 (Horn et al. 1999; Gast 2001; Hewett et al. 2003; Lanocha et al. 2009; Corsaro and Venditti 2010; Nuprasert et al. 2010, Qvarnstrom et al. 2013).

T4 genotype is the most common Acanthamoeba genotype in the environment and the most widely spread worldwide. It is also the main causative agent of GAE, AK and other infections (Nagyová et al. 2010), e.g. more than 94% of keratitis cases have been linked with this genotype (Booton et al. 2005). Moreover, T4 exhibits significant higher binding and produced severe cytotoxicity on host cells as compared to other genotypes (Alsam et al. 2003). In the present study, we identified two variants of Acanthamoeba T4 genotype in 4 samples obtained from 3 water bodies that are using as bathing places during summer months, however, only in one of them (Miejskie Lake) we detected this pathogen in more than one year of sampling. One sequence variant, isolated...
from Glebokie Lake in 2009, showed 100% similarity to the sequence isolated earlier from respiratory tract of Polish patient after the allogenic transplantation of bone marrow as well as to the sequence obtained from cornea of Korean amoebic keratitis patients. This suggests that bathing in this lake may be associated with the risk of the pathogenic *Acanthamoeba* strain infections. The second variant described in this study, isolated from Miejskie Lake in 2010 and 2011 and from Nowogardzkie Lake in 2010, showed high similarity (over 99%) to the first variant, so these water bodies may also be the sources of human infections.

T16 is a recently described genotype of *Acanthamoeba* and there is an incoherence regarding this type. In 2009, Lanocha et al. detected new *Acanthamoeba* genotype from bronchoaspirate fluid of man with chronic marrow leukaemia and from bronchoalveolar lavage of newborn with symptoms of atypical pneumonia and proposed to name it T16. In 2010, Corsaro and Venditti described another genotype isolated from an environmental sample and also proposed to name this type T16. However, Lanocha et al. (2009) used 830–840 bp Ami fragments of the 18S rRNA gene that contain 5 of 8 variable regions and Corsaro and Venditti (2010) used the full sequence (~2200 bp) of the gene. In their further paper, Corsaro and Venditti (2011) showed that the Ami fragment is not proper to identify new genotypes and longer fragments should be used for this purpose, e.g. the 1450-bp GTSA.B1 fragment or, preferably, the full sequence of the 18S rRNA gene. So, classification of the strains described by Lanocha et al. (2009) requires the use of longer fragments of this gene, as well as the classification of two new genotypes isolated from phylloplane of bromeliad in Brazil (JX683392, Landell et al. 2013) and from water in Pakistan (KC203592, Tanveer et al. 2013). They were both classified as T16 genotype on the basis of 468–bp and 237–bp fragments, respectively. We identified two variants of the genotype described first by Lanocha et al. (2009) in 7 samples obtained from 3 water bodies and this is the first case of detection this type in water. The first variant was detected in 3 samples obtained from Dabie Duże Lake in 2009, 2010 and 2011 and showed 100% similarity to the sequence isolated earlier from BAL of Polish newborn with symptoms of atypical pneumonia. The second variant, identified in Odra River (Dziewoklicz) in 2009, 2010 and 2011 and in Ina River (Goleniów) in 2009, showed over 99% similarity to the first. The genotypes detected recently in Brazil and Pakistan are different from the genotype described earlier by Lanocha et al. (2009) as T16 and our genotype described in this paper. Their similarity to our genotype is 85% for JX683392 and over 93% for KC203592, and to the T16 genotype described in 2010 by Corsaro and Venditti – 90% for JX683392 and 80% for KC203592. To categorize a strain into a genotype, their minimal similarity should be equal over 95% (Stothard et al. 1998). The authors categorized their strains into the T16 genotype on the basis of the phylogenetic analysis which was performed with the use of short fragments of 18S rRNA gene. Such analysis may be biased and longer fragments of the gene are required to identify new genotypes (Corsaro and Venditti 2011). The pathogenicity of *Acanthamoeba* genotype described as T16 by Lanocha et al. (2009) is not confirmed, however, its presence in respiratory tracts of patients with pneumonia and leukaemia suggests that it may be a causative agent of human illnesses, and Odra and Ina Rivers may be the sources of infections because of the presence of this genotype, especially Odra River which is a bathing place during summer months and the genotype was detected in three years.

Investigations on the presence of amoebae in Polish water bodies are not numerous. In 2009, Lanocha et al. examined 113 water samples from 45 natural water bodies and 103 water samples from artificial water bodies from the West Pomerania and Lubuskie voivodship. They identified thermophilic strains of *Acanthamoeba* in 6 natural water bodies samples (5.3%) and in 18 artificial water bodies samples (17.5%). In this study, we detected thermophilic *Acanthamoeba* strains in 6.5% of all samples so our results are similar to these of Lanocha et al. (2009) obtained for natural water bodies. The percentage of artificial water bodies samples in which they detected thermophilic strains was higher in comparison to natural water bodies what may be caused by smaller capacity of artificial water bodies, especially fountains, and in case of swimming pools – the higher temperature of water. The prevalence of thermophilic strains of *Acanthamoeba* was higher also in swimming pools from Szczecin (16 positive samples from 43 – 37.2%; Gornik and Kuznagrygieł 2004), as well as the prevalence of pathogenic *Acanthamoeba* strains in fountains from Poznań (6 positive samples from 9–66.7%; Derda et al. 2013). However, the samples from Poznan were very few what may have an influence on the percentage of positive samples.

*Hartmannella vermiformis* is a species detected earlier in the cerebrospinal fluid of a patient with meningoccephalitis and bronchopneumonia as well as in surface water samples and in fishes. It may also be a causative agent of keratitis, however, the pathogenicity of this species is discussed (Kuiper et al. 2006). We detected one variant of 18S rRNA gene of this amoeba in 11 samples from 4 sites – Dąbie Duże Lake, Miejskie Lake, Odra River and Ina River. The two lakes are using as bathing places and can be the sources of potential infection, however, there were no causes of isolation of *H. vermiformis* from Polish patients. Odra River warm water channel is a place where water temperature is between 10°C and 30°C and the presence of thermophilic strains is expected. Despite this fact, there was no diversity among the strains detected in warm Odra water and in remaining three water bodies. The variant detected by us showed the highest similarity (99%) to the sequences obtained by us and other authors from different water sources and from fishes. These data are not sufficient to confirm or exclude the pathogenicity of detected *H. vermiformis* strain for humans. However, this amoeba may be of great economic importance as a parasite involved in nodular gill disease outbreaks in rainbow trout (*Oncorhynchus mykiss*) in South-Western Germany (Dyková et al. 2010),
more particularly as H. vermiformis is able to produce tissue lesions in experimentally infected fish (Dyková et al. 2005).

The progress in molecular methods allows not only on the precise identification of a parasite at species and/or genotype level but simultaneous detection of several parasites also (Skotarczak 2009). We made attempts to adapt the RLB method for detection and differentiation of FLA species and strains. RLB is not a new way in the diagnostics of parasitic protozoa, because in the end of 90’s of the last century it was improved for the detection and differentiation of Babesia and Theileria species (Gubbels et al. 1999). Hybridization with the RLB technique gets simultaneous detection of many species and strains of inspected organisms. For this purpose, 18S rDNA fragments were amplified with PCR using especially designed primers and appropriate probes were used. Despite the fact that we amplified successfully all 24 positive samples (presented in Table III) with RLB starters, only 7 RLB signals were obtained. RLB reactions (PCR with biotinylated starters and hybridization) were performed in three replicates; however, the positive hybridization results were not repetitive.

Conclusions

To conclude, 7 of 50 examined water bodies are the sources of potential FLA infections. In 3 lakes we identified the pathogenic genotype of Acanthamoeba – T4, associated with GAE, AK and other infections. In 1 lake and 2 rivers, we detected the presence of the new, potentially pathogenic Acanthamoeba genotype, described earlier as T16 by Lanocha et al. (2009) and in two lakes and two rivers – H. vermiformis that may be also involved in human illnesses. T4 is the most prevalent genotype in the environment (Nagyová et al. 2010), however, the new genotype was more frequent in the water bodies in North-Western Poland. The pathogenicity of this Acanthamoeba genotype of is not confirmed, as well as the pathogenicity of H. vermiformis, although these amoebae may have an indirect public health significance as the vectors of pathogenic microorganisms (Hsu et al. 2009). What is more, H. vermiformis may pose a threat for fishes living in Dąbie Duże and Miejskie lakes and in Odra River. Regarding detection methods for FLA, PCR seems to be more sensitive than RLB hybridization.

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

References


