The Occurrence *Acanthamoeba* (Free Living Amoeba) in Environmental and Respiratory Samples in Poland

Natalia ŁANOCHA¹, Danuta KOSIK-BOGACKA¹, Agnieszka MACIEJEWSKA², Marek SAWCZUK², Aleksandra WILK¹, Wanda KUŹNA-GRYGIEL†

¹ Chair and Department of Biology and Medical Parasitology, Pomeranian Medical University, Szczecin, Poland; ² Department of Genetics, University of Szczecin, Szczecin, Poland

Summary. Free-living amoebae are amphizoic organisms living in soil, air, fresh and sea water. Species belonging to the genera *Acanthamoeba*, *Naegleria* and *Balamuthia* are human parasites, which may invade the central nervous system, lungs, adrenal glands, skin, eyes and lower jaw. In this paper, we examine the occurrence of potentially virulent strains of amoebae in water bodies of the West Pomeranian and Lubuskie voivodeships (north-western Poland) and in biological material from patients with symptoms of pneumonia. We examined 45 natural water bodies (lakes, ponds, rivers, seas and lagoons), 39 artificial water bodies (swimming pools, firefighting and reservoirs fountains) and the water supply system in the city of Szczecin, and collected altogether 216 samples. We also examined biological material from 130 immunocompromised patients with atypical symptoms of pneumonia. From the collected environmental samples we isolated 110 strains of amoebae from the amphizoic group, 24 of which were thermophilic. Based on the morphological attributes of trophozoites and cysts, all the isolates were classified to the genus *Acanthamoeba*. Amoebae from the genus *Acanthamoeba* were also isolated from biological samples from patients with symptoms of pneumonia with secondary immune deficiencies. The presence of amoebae was determined in 2 samples of bronchoaspirate fluid taken from patients after chemotherapy and in 2 samples of bronchoalveolar lavage from patients with respiratory insufficiency. Molecular identification of the amoebae isolated from biological samples confirmed their affinity to *Acanthamoeba* genus, two of them classified as T4 genotypes. The two other sequences could not be classified to any known *Acanthamoeba* genotype. Thus there is a need for further investigation to establish *Acanthamoeba* genotype in biological material from patients with immunosuppression and pneumonia symptoms.

Key words: Free-living amoebae (FLA), *Acanthamoeba*, patients with immunosuppression.

INTRODUCTION

Free-living amoebae (FLA) are amphizoic organisms that can also be endozoic, occurring as facultative pathogens (Page 1974). Strains of free-living amoebae have been isolated from lake water (Kasprzak et al. 1974, Befinger et al. 1986), rivers (Ettinger et al. 2003, Lorenzo-Morales et al. 2005), moist soil, desert sand (Rezaeian et al. 2008) and air (Kingston and Warhurst 1969). Trophozoites and cyst of these protozoa have also been detected in bottled mineral water (Marciano-Cabral et al. 2003) and chlorinated water from swimming pools (Cerva 1969, Cerva and Huldt 1974).
Amoebae have also been detected in gastric and intestinal lavages, in the nasopharynx of healthy individuals, in purulent discharges from the ear and in liquid faeces (Lengy et al. 1971, Jadin 1974, Khan 2006).

A potential threat for humans is posed by species from four genera: *Acanthamoeba*, *Naegleria*, *Balamuthia* and *Sappinia* (Visvesvara et al. 2007). They affect mainly the central nervous system, causing primary amoebic meningocencephalitis (PAM) and granulomatosus amoebic encephalitis (GAE). They also may invade lungs, adrenal glands, the skin, eyes, and lower jaw (Khan 2006).

This paper examined the occurrence of potentially virulent strains of amoebae in water bodies of the West Pomeranian and Lubuskie voivodeships (north-western Poland) and in biological material from patients with pneumonia symptoms.

**MATERIALS AND METHODS**

**Sampling**

Between 2006 and 2008 we collected 185 water samples from natural and artificial water bodies in the West Pomeranian and Lubuskie voivodeships, and 31 samples from the water supply system in Szczecin, Poland.

Water samples from lakes, ponds, lagoons, seashore and rivers were collected together with mud (at ratio 1:1) from the sites on the border of water and soil. Individually, 53 samples were taken from 23 lakes, 10 samples from 2 ponds, 16 samples from 5 rivers, 2 samples from a lagoon, and 32 samples from the seashore.

In swimming pools and fountains, samples were collected from places close to the walls of the pool. In this way, 13 samples were taken from 12 swimming pools, 20 samples from 10 firefighting reservoirs, and 39 samples from 17 fountains. 31 samples were taken from the water supply system from 15 households in the city of Szczecin.

We also examined biological material from 130 patients with immune deficiencies, showing atypical pneumonia symptoms. This included 6 spits, 46 bronchoaspirates fluid, 64 bronchoalveolar lavage (BAL) and 4 epiglottal swabs.

**Sample preparation**

The collected water samples were filtered using a vacuum pump, Seitz filter and GF 50 Schleicher & Schuell membranes. Membranes with the residue were placed on NN Agar plates and incubated at 37°C. After 72 h amoebae were observed on the surface of the agar plates.

In order to isolate the strains of *Acanthamoeba* amoebae from the biological material, we began cultivation using standard methods (Culberston et al. 1965, Marciano-Cabral et al. 2003). We used agar plates (NN Agar) covered with a suspension of deactivated bacteria *Escherichia coli* (deactivated at 70°C for 2 h) and incubated at 37°C for 72 h.

**Morphological classification**

The morphological identification was based on morphological criteria for cysts and trophozoites using Page’s key. From the actively growing in vitro cultures, we made preparations stained with eosine and Gomori-Wheatley stain, after fixing them in the Schaudin’s liquid.

In order to identify genus *Naegleria*, we performed the flagellar test to identify flagellar stages. The suspension of amoebae was rinsed from the agar surface and placed in distilled water at 42°C, and after 2 h the amoebae were observed under microscope.

In order to isolate thermophilic strains, the proliferated amoebae were passaged at 45°C, observing the intensity of growth each day. The growth was evaluated on a scale between 0 and 3. The cultures showing moderate and intense growth at 42°C were classified as thermophilic.

**Molecular analysis**

The amoebae from cultures obtained from biological material after were harvested after three or four subcultures by scraping and then resuspended in 1 ml of PBS buffer. DNA was extracted from pelleted trophozoites using a GenElute DNA Extraction Kit (Sigma, Germany), then an 18S rRNA PCR adapted from the method of Thomas et al. (2006) was performed with primers Ami6F1 (5’ CCAGCTCCAATAGCGTATAAT 3’) and Ami9R (5’ GTTGAGTCCAATTTAAGCGGC 3’). PCR amplicon spanned a region of 828 bp between 640 and 1467 bp of *A. castellanii* (U07400) sequence deposited in GenBank and contained a large piece of the GTSA.B1 amplimer described by Schroeder et al. (2001). Each 10 μl reaction tube contained 1× concentration of PCR buffer, 4 pM of each primer, 1.5 mM MgCl2, 0.5 U of Taq DNA polymerase (Sigma, Germany), 0.75 nM of each deoxynucleoside triphosphate and 1 μl of template DNA. After a first step consisting of 94°C for 2 min, 30 s, 40 cycles of amplification were performed by using denaturation at 94°C for 45 s, annealing at 55°C for 30 s, and elongation at 72°C for 1 min. 30 s and a final cycle at 72°C for 5 min. The PCR products were sequenced (both strands) with the amplification primers, without cloning.

Sequencing reactions were carried out using the ABI PRISM BigDye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, California), and sequenced on an ABI 3100 (Applied Biosystems, Foster City, California).

Sequences obtained in this study were compared with the GenBank database using the Basic Local Alignment Search Tool (BLAST) at the National Center for Biotechnology Information (NCBI). The selected sequences of the 18S rRNA gene retrieved from the GenBank database were aligned using the program Clustal W (Thompson et al. 1994) (Table 1). Phylogenetic analysis was performed with the DNAMAN program (Lynon BioSoft, Canada) and the phylogenetic tree was constructed by the neighbor-joining method (Saitou and Nei 1987). The reliability of internal branches was assessed via bootstrap analysis (Felsenstein 1988) of 1000 replicates. Sequence data reported in this paper were deposited in GenBank and are available under the following reference numbers: strain AM101, GQ342609; strain AM19, GQ342606; strain AM22, GQ342607; strain AM38, GQ342608.
RESULTS

Isolation of amoebae from the natural environment

The examination covered 45 natural water bodies, 39 artificial water bodies, and the water supply system in Szczecin, altogether 216 water samples (Table 2). From the collected environmental samples, at 37°C, 110 strains of free-living amoebae were isolated, 24 of which showed thermophilic attributes. Based on the morphological traits of trophozoites and cysts, all the isolates were classified to the Acanthamoeba. Flagellation tests, that enabled the identification of the genus Naegleria, had negative results. Detailed data on the occurrence of Acanthamoebae in the examined water bodies and in drinking water are presented in Table 2.

Table 1. List of Acanthamoeba sequences included in the phylogenetic tree constructed from SSU rRNA gene sequence alignment, obtained from GenBank.

<table>
<thead>
<tr>
<th>Sequence name</th>
<th>Accession number</th>
<th>Source</th>
<th>Place of origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acanthamoeba royreba</td>
<td>U07417</td>
<td>Human</td>
<td>USA</td>
</tr>
<tr>
<td>Acanthamoeba rhysodes</td>
<td>AY351644</td>
<td>Soil</td>
<td>Great Britain</td>
</tr>
<tr>
<td>Acanthamoeba mauritaniensis</td>
<td>AY351647</td>
<td>Soil</td>
<td>Great Britain</td>
</tr>
<tr>
<td>Acanthamoeba polyphaga</td>
<td>AY237735</td>
<td>Water from pond</td>
<td>Great Britain</td>
</tr>
<tr>
<td>Acanthamoeba lugdunensis</td>
<td>AF005995</td>
<td>Swimming pool</td>
<td>France</td>
</tr>
<tr>
<td>Acanthamoeba sp. KAE26</td>
<td>EF140627</td>
<td>Human</td>
<td>Korea</td>
</tr>
<tr>
<td>Acanthamoeba sp. KAE12</td>
<td>AF316545</td>
<td>Human</td>
<td>Korea</td>
</tr>
<tr>
<td>Acanthamoeba sp. CDC V388</td>
<td>AY703003</td>
<td>Human</td>
<td>USA</td>
</tr>
<tr>
<td>Acanthamoeba pearsei</td>
<td>AF019053</td>
<td>Ocean sediment</td>
<td>Atlantic Ocean</td>
</tr>
<tr>
<td>Acanthamoeba stevensoni</td>
<td>AF019069</td>
<td>Ocean sediment</td>
<td>USA</td>
</tr>
<tr>
<td>Acanthamoeba hatchetti</td>
<td>AF251937</td>
<td>Human</td>
<td>Austria</td>
</tr>
<tr>
<td>Acanthamoeba castellanii</td>
<td>U07400</td>
<td>Human</td>
<td>USA</td>
</tr>
</tbody>
</table>

Table 2. The occurrence of Acanthamoeba in water bodies in West Pomeranian and Lubuskie voivodeships, north-western Poland.

<table>
<thead>
<tr>
<th>Water body</th>
<th>No. of sites</th>
<th>No. of samples</th>
<th>No. of strains in samples</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>total</td>
<td>%</td>
</tr>
<tr>
<td>Lake</td>
<td>23</td>
<td>53</td>
<td>29</td>
<td>54.72</td>
<td>5</td>
</tr>
<tr>
<td>Pond</td>
<td>2</td>
<td>10</td>
<td>4</td>
<td>40</td>
<td>–</td>
</tr>
<tr>
<td>River</td>
<td>5</td>
<td>16</td>
<td>6</td>
<td>37.5</td>
<td>–</td>
</tr>
<tr>
<td>Lagoon</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>50</td>
<td>–</td>
</tr>
<tr>
<td>Sea</td>
<td>14</td>
<td>32</td>
<td>9</td>
<td>28.12</td>
<td>1</td>
</tr>
<tr>
<td>Swimming pool</td>
<td>12</td>
<td>13</td>
<td>10</td>
<td>76.92</td>
<td>5</td>
</tr>
<tr>
<td>Firefighting reservoir</td>
<td>10</td>
<td>20</td>
<td>12</td>
<td>60</td>
<td>6</td>
</tr>
<tr>
<td>Fountains</td>
<td>17</td>
<td>39</td>
<td>21</td>
<td>53.85</td>
<td>4</td>
</tr>
<tr>
<td>Tap water</td>
<td>15</td>
<td>31</td>
<td>18</td>
<td>58.06</td>
<td>3</td>
</tr>
<tr>
<td>Total</td>
<td>99</td>
<td>216</td>
<td>110</td>
<td>50.92</td>
<td>24</td>
</tr>
</tbody>
</table>
Isolation of amoebae from biological material

Free-living amoebae were also isolated from the biological material from patients with pneumonia symptoms, with secondary immune deficiencies. We examined 6 spits, 46 bronchoaspirates fluid, 64 bronchoalveolar lavage (BAL) and 4 epiglottal swabs (Table 3).

Table 3. The occurrence of Acanthamoeba strains in biological material from patients with pneumonia symptoms.

<table>
<thead>
<tr>
<th>Biological material</th>
<th>No. of samples</th>
<th>No. and % of isolated Acanthamoeba strains</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spit</td>
<td>6</td>
<td>0</td>
</tr>
<tr>
<td>Bronchoaspirate fluid</td>
<td>46</td>
<td>2</td>
</tr>
<tr>
<td>Bronchoalveolar lavage (BAL)</td>
<td>64</td>
<td>2</td>
</tr>
<tr>
<td>Epiglottal swab</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>130</td>
<td>4</td>
</tr>
</tbody>
</table>

The presence of free-living amoebae was detected in 2 bronchoaspirate fluid samples taken from patients after chemotherapy, and in 2 bronchoalveolar lavage samples taken from patient with respiratory deficiency. The biological samples were microbiologically sterile and additional microscopic examination excluded the possibility of infection with Pneumocystis jiroveci.

We detected the AM 19 strain in the bronchoaspirate fluid of a 15 year old boy after the allogenic transplantation of bone marrow in an acute myeloblastic leukemia. In the bronchoaspirate fluid of a man with chronic marrow leukemia, we isolated the AM 22 strain. Strains AM38 and AM 101 were detected in the BALs of two newborns with symptoms of atypical pneumonia. All the aforementioned strains were thermophilic, and proliferated at 42°C. Based on morphological and molecular studies, we identified the isolated amoebae as Acanthamoeba.

We observed a loss of weight and respiratory efficiency in the patients. In respiratory tracts we observed a discharge. In the radiological examination we observed interstitial changes with a visible pulmonary swelling.

Molecular studies of amoebae from biological material

PCR and sequencing of the fragment of the SSU rRNA gene were proceeded for amoebae isolated from 2 bronchoaspirate fluid samples and 2 bronchoalveolar lavage samples and four types of sequences were subsequently obtained. Sequences from sample AM19 and AM101 of approximately size 830 bp were almost identical with 3 polymorphic points and were considerably different from AM22 and AM38 (both sequences 840 bp long). The latter two sequences were very similar to each other and differs by only one substitution and one indel.

When compared with the GenBank database using the BLAST application AM19 sequence showed 100% identity with Acanthamoeba lugdunensis (AF005995), A. polyphaga (AY237735) and Acanthamoeba sp. KAE26 (EF140627) sequences as well as high degree of similarity to Acanthamoeba sp. KAE12 (AF316545) and Acanthamoeba sp. CDC V388 (AY703003). The AM101 sequence showed similarity of 99% to mentioned above sequences. Sequences AM22 and AM38 showed the highest similarity of 92% with A. castellanii sequence (U07400).

In phylogenetic analysis the AM19 and AM101 sequences cluster together with A. lugdunensis, A. polyphaga, Acanthamoeba sp. KAE12, KAE26 and CDC V388. All these sequences form a sister group to a cluster that includes A. rotyreba, A. rhysodes and A. mauritaniensis. The AM22 and AM38 sequences form a separate group with A. castellanii (Fig. 1).

DISCUSSION

Free-living amoebae are common in nature. They live in water, soil and air. In water bodies, one may find strains from the genera Acanthamoeba and Naegleria, and the species Balamuthia mandrillaris, which are human pathogens (Martinez and Visvesvara 2001).

In this study, Acanthamoeba strains were detected in all the examined natural and artificial water bodies in the West Pomeranian and Lubuskie voivodeships in NW Poland. It is consistent with a report by Görnik and Kuźna-Grygiel (2004), who found only Acanthamoeba strains in natural and artificial water bodies in the West Pomeranian voivodeship. The detection of only the Acanthamoeba strains may have been associated with the much shorter life of Neagleria cysts and their greater sensitivity to chlorination (De Jonckheere 1979).
In Poland, genus *Acanthamoeba* and the species *Naegleria fowleri* have been detected in many various areas, for example near Poznań (Kasprzak and Mazur 1972, Kasprzak et al. 1982, Mazur 1984), Lublin (Gieryng and Gieryng 1987) and Gdańsk (Befinger et al. 1986).

In this paper, *Acanthamoeba* were detected in natural water bodies like lakes, ponds, rivers, and lagoons, however thermophilic strains were only found in lakes. Only thermophilic strains may be pathogenic for humans; they can proliferate only at a temperature above 40°C, which may be associated with their adaptation to conditions in the host (Visvesvara and Stehr-Green 1990).

Amoebae isolated posthumously from organs always showed the ability to proliferate at 42°–45°C. However, not all strains that are thermophilic are necessarily pathogenic, as some of the nonpathogenic strains show similar temperature preferences (Mazur 1984).

*Acanthamoeba* strains, including one thermophilic strain, were detected in all the examined samples from the Baltic Sea. It is consistent with results obtained by Górnik (2005) who identified thermophilic *Acanthamoeba* strains in the Baltic Sea waters. *Acanthamoeba* species, including *Acanthamoeba castellanii*, have also been identified in ocean sediments (Sawyer et al. 1977, Liu et al. 2006), which may indicate the *Acanthamoeba* have a resistance to salinity. We also detected *Acanthamoeba* strains in artificial water bodies, and the number of detected strains was greater than in natural ones. It may be associated with continuous pollution by precipitation and organic pollution from the air, dust, and soil deposited by the wind, humans and animals (Schuster and Visvesvara 2004). We also found *Acanthamoeba* strains in swimming pools, firefighting reservoirs and fountains. Potentially virulent attributes were exhibited by half of the strains isolated from swimming pools and firefighting reservoirs and 4 strains of amoebae isolated from fountains. Similarly to our results, *Acanthamoeba* strains have been observed in swimming pools in Belgium (De Jonckheere 1979) and Germany (Kuhlen-
cord et al. 1989). In Swedish and in Czech swimming pools (Cerva and Huldt 1974) Naegleria fowleri strains have been found. In Poland, the presence of Acanthamoeba with a considerable degree of invasiveness and virulence, has been observed in pools in the Lubelskie region (eastern Poland) (Toczołowski et al. 2000) and in Szczecin (Górnik and Kuźna-Grygiel 2004). The presence of amoebae in swimming pools is confirmed by other authors, who have observed that water chlorination kills only microorganisms but not free-living amoebae (Khan 2006) and water heating enhances the proliferation of thermophilic amoebae (Griffin 1972). It has been reported that the permissible concentration of disinfectants in swimming pools does not destroy amoebae (Vesaluoma et al. 1995). The lack of Naegleria strains in the examined swimming pools may be associated with the higher sensitivity of their cysts to chlorination (De Jonckheere 1979).

We observed Acanthamoeba strains in tap water in the city of Szczecin. Similarly to our results, Acanthamoeba strains have been observed in 2.8% of samples in South Florida (Shoff et al. 2008), 10% of samples of tap water in Hong Kong (Boost et al. 2008) and in 36.1% of samples from tap water in western India (Lorenzo-Morales et al. 2005). Naegleria fowleri have been detected in the Arizona tap water (Marciano-Cabral et al. 2003).

Due to the common occurrence of amoebae in the environment, frequent human contact with amoebae is unavoidable. It is confirmed by a report by Chapell et al. (2001), who observed anti-Acanthamoeba IgG antibodies in the peripheral blood of 80% of the human population.

Therefore, amoebae are indeed a serious threat to human health, especially in immunocompromised patients, since the pathogenicity of amoebae is a complex process which depends on time and the site of pathogen transmission, on the host, and on the actual overcoming of the host’s barriers and resistances (Martinez 1982).

Pathogenic strains from the genus Acanthamoeba cause brain invasions and also others organs. For example, they may cause granulomatous amoebic encephalitis (GAE). In the described 103 cases of GAE, the invasion of Acanthamoeba developed in patients with immune deficiencies, chronically ill, in the mentally disabled, and in patients receiving immunosuppression drugs after organ transplantation, and in 50 cases in AIDS patients (Khan 2006). Other predispositions for such invasion may be alcoholism, anorexia, cancer, chemotherapy and radiotherapy (Martinez 1980, Schuster et al. 2004, Khan 2006). This is why GAE has been classified as an opportunistic invasion (Khan 2006).

Acanthamoeba species may cause acanthamoeba keratitis in persons wearing contact lenses (Toczołowski et al. 2000, Wesolowska et al. 2006). Acanthamoeba may also invade adrenal glands, the skin and lower jaw (Khan 2006), with symptoms of chronic rhinitis, inflammation of paranasal sinuses (Teknos et al. 2000), inflammation of lymph glands and mucous membranes of genitals and also acute rheumatic arthritis. One proof of the virulent extra-brain invasions is the case of lethal pneumonia caused by Acanthamoeba spp. in a 7 month old newborn with thrombocytopenia, in Korea (Im and Kim 1998).

So far no such cases of Acanthamoeba-induced pneumonia have been observed in Poland. However, amoebae isolated from water bodies in Lubelskie voivodeship in eastern Poland caused pathological changes in the lungs of mice (Gieryn et al. 1993).

In our study, Acanthamoeba amoebae were isolated from patients with immunosupresion. Similarly, amoebae from the genus Acanthamoeba were isolated from the spit from patients with chronic pneumonia and from pulmonary tumours in lungs (Martinez 1982). Studies from recent years have shown that amoebae with non-virulent properties may transmit symbiotic bacteria in vacuolae, including bacteria harmful to humans (Derda et al. 2006). Amoebae may create a specific environment for various pathogenic micro-organisms, e.g., Chlamydia, Pseudomonas aeruginosa, Escherichia coli, Legionella pneumophila, Shigella (Derda et al. 2006). A number of authors showed that Legionella pneumophila may be pathogenic for Acanthamoeba and Naegleria genera in vitro. Therefore, the occurrence of amoebae in patients with atypical pneumonia does not have to indicate amoebic ethiology, and bacterial ethiology must also be taken into consideration. This is confirmed by the observed Acanthamoeba in the epipharynx of healthy subjects (Khan 2006).

Molecular identification of the amoebae DNA isolates performed in this study confirmed their affinity to Acanthamoeba genus. On the basis of our analysis there would have been a possibility of Acanthamoeba species recognition (for example AM19 and AM101 as a A. lugdunensis or A. polyphaga), however, it could incur risk of misidentification because genotype do not always correspond to specific classifications based on morphology. On the basis of molecular data it would be more reasonable to use the name of strain or genotype.
number (sequence type) instead of species name (De Jonckheere 2006).

There are 15 genotypes described as T1, T2, T3 etc. in the literature classified on SSU rRNA gene sequences (Walocznik et al. 2000, Hewitt et al. 2003, Booton et al. 2005). The strain of *Acanthamoeba* can be assigned to specific genotype as sequence differ from one another by at least 5% (Stothard et al. 1998). Initially, this classification was performed by sequencing the complete 18S rRNA gene of different *Acanthamoeba* species and strains which resulted in describing 14 sequence types, T1–T14 (Gast et al. 1996, Stothard et al. 1998, Horn et al. 1999). Further analysis revealed that smaller fragments of the gene might contain enough information to differentiate all of the sequence types (Schroeder et al. 2001, Booton et al. 2002). Dykova et al. (1999) demonstrated PCR primer pair that produced an amplimer named ASA.S1, that was reliably specific for the genus *Acanthamoeba* and could be obtained for all *Acanthamoeba* genotypes. However, this amplimer failed to distinguish the most closely related genotypes, therefore, Schroeder et al. (2001) suggested that reliable differentiation such genotypes might be obtained either by sequencing the complete 18S RNA gene or by using the multi locus sequencing of GTSA.B1 fragment of any *Acanthamoeba* species.

Comparative analysis performed to the needs of this paper of 830–840 bp fragment and sequences of GTSA. B1 fragment of all genotypes (T1–T15) retrieved from Genebank revealed similar sequence variation. Therefore, the 830–840 bp fragment is in our opinion sufficient to distinguish *Acanthamoeba* genotypes.

On the ground on the 5% sequence divergence criterion sequences derived from AM19 and AM101 isolates might be classified as T4 genotypes since their similarity to *Acanthamoeba* sequences (AF005995, AF237735, EF140627, AF316545, AY703003), which are the representatives of the T4 genotype, was 99%. AM22 and AM38 sequences were more divergent than 5% from any *Acanthamoeba* sequences deposited in GenBank that was used to establish genotypes (the highest similarity with genotype T1 representative *A. castellanii*-U07400 – 92%). Thus we decided to describe amoebas detected in samples AM22 and AM38 as a new sequence type T16 however their species affinity is not clear. Detailed morphological classification of the specific representatives of genotype T16 needs further investigation.

**CONCLUSIONS**

1. Natural and artificial water bodies in the West Pomeranian and Lubuskie voivodeships in NW Poland are reservoirs of *Acanthamoeba* amoebae.
2. Tap water from the water supply system may be the source of *Acanthamoeba* invasion.
3. 830–840 bp fragment portions of ssu rRNA gene contained 5 variable regions with ASA.S1 within GTSA.B1 amplimer are in our opinion sufficient to distinguish *Acanthamoeba* genotypes. The new sequence type T16 was determined for two clinical samples.
4. Further surveys are needed to identify and examine acanthamebiasis in patients with immunosuppression and pneumonia symptoms.

**REFERENCES**


The Occurrence of *Acanthamoeba* spp. 279


Received on 19th February, 2009; revised version on 22nd June, 2009; accepted on 25th June, 2009