Molecular characterization of Acanthamoeba isolated from Kayseri well water

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Aim: Potentially pathogenic free-living amoebae have a cosmopolitan distribution in soil, dust, air, and water. Generally, environmental free-living amoebae do not threaten human health. This study aimed to investigate the presence of Acanthamoeba in well waters drawn from different locations in Kayseri, Turkey, which are mainly used for drinking or irrigation by the residents in the region.

Materials and methods: Twenty-nine samples, including 26 well water sediment samples and 3 tap water samples as the control, were collected from 3 different locations. Melting snow feeds 5, rain water 18, and tap water 3 of these wells.

Results: Five of 26 well water samples were 19.23% positive for Acanthamoeba by both PCR and agar culture. All of these Acanthamoeba were characterized as the T4 genotype group.

Conclusion: This is the first report from Turkey on the isolation and identification of Acanthamoeba. Further studies with a wide series are warranted, focusing on in vitro cytotoxicity and in vivo pathogenicity of isolates.

Key words: Acanthamoeba, well water, genotype, Kayseri, Turkey

1. Introduction

Parasitic diseases are widespread and dangerous in humans and animals worldwide (1–3). Potentially pathogenic free-living amoebae (FLA) have worldwide distribution in water, soil, dust, and air. They have been isolated from lakes, pools, swimming and therapeutic pools, tap water, thermal water, cooling water, bottled mineral waters, hot tubs, air-conditioning units, and eyewash solutions (4–8). Infections due to pathogenic FLA in these environments and items have been largely documented. People may inevitably come into contact with potentially pathogenic FLA due to this worldwide distribution as has already been evidenced by antibody titers in human populations (9,10).

In general, environmental FLA do not threaten humans. However, FLA such as Acanthamoeba spp., Naegleria fowleri, Balamuthia mandrillaris, and Sappinia pedata are known to be opportunistic parasites that can lead to severe pathologies (11–13). Central nervous system (CNS) infections caused by FLA include primary amoebic meningoencephalitis with N. fowleri and granulomatous amoebic encephalitis, which is due to infections with several Acanthamoeba as well as B. mandrillaris, mainly in immunocompromised humans and in animals. In addition, Acanthamoeba and B. mandrillaris are known to cause skin infections, but it is also important to emphasize that Acanthamoeba spp. also cause keratitis, which may further result in blindness (12,14).

Therefore, the interest in pathogenic FLA, epidemiology of FLA, and infections associated with FLA is gradually increasing. To the best of our knowledge, this study will be the first report from Kayseri investigating Acanthamoeba in well water.

2. Materials and methods

2.1. Sampling

Samples were obtained from well water sediment in the town of Hacılar in the foothills of Erciyes Mountain between March and May 2011. The wells are also known as snow wells and are 2 and 3 m deep and square-shaped. This well water is currently used for drinking and irrigation. The altitudes of Erciyes Mountain and Hacılar are 3917 m and 1350 m from sea level, respectively. Twenty-nine water samples, including 26 well and 3 tap waters, were collected from 3 different locations as presented in the Table. Plastic tubes of 50 mL were used to collect water and were pelleted for 10 min at 1000 rpm. The pellets were resuspended in 100 μL of supernatant and used.

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2.2. Isolation and culturing of trophozoites

Resuspended pellets were gently pipetted onto a nonnutrient agar plate (1.5% agar in Page's saline) and were allowed to adsorb and dry. Subsequently, the plates were sealed with Parafilm and incubated upside down at 37 °C and additionally at 42 °C. A daily inspection of plates was done by light microscopy until morphological structures suggestive of amoeba trophozoites were detected. Cultures lacking morphological features of amoeba within 15 days were considered as negative and discarded.

2.3. Characterization of the isolates

The trophozoites were gently scraped from an agar plate using a sterile pipette, resuspended in phosphate buffered saline, and pelleted at 1000 rpm for 15 min. DNA was extracted from the pellet using the QIAamp DNA Mini Kit (QIAGEN, USA) according to the manufacturer's instructions. The genomic DNA was subjected either to polymerase chain reaction (PCR) yielding the specific recognition of *Acanthamoeba* spp. (Nelson-PCR, JDP-PCR), or to a pan-PCR recognizing FLA in general (FLA-PCR). The following primer pairs were used for PCRs: FLA-PCR: P-FLA-F (5'-CGCCGTAATTCGCTCCAATGC-3'); P-FLA-R (5'-CAGGTTAAGGTCCTCGTTAAC-3'); target: 18S rDNA; Nelson PCR: NelsonF (5'-GTGTTGGGCAATAACAGGT-3'); NelsonR (5'-GAATTCTCGTTGGAAGAT-3'); target: 18S rDNA; JDP PCR: JDP1 (5'-GCGCCGATCTGTTACCCTGAA-3'); JDP2 (5'-CTTCACAAAGCTGCTAGGGATCA-3'); target: 18S rDNA (8,15,16). The PCRs were carried out in a 25-µL volume containing 12.5 µL of 2X PCR Master Mix (Vivantis, Malaysia), 2 µL of 20 pmol of each primer, and 1 µL of genomic template DNA, and they were performed in a PCR labcycler (SENSQUEST, Germany). Products from all PCRs were visualized on 2% agarose gel containing ethidium bromide.

2.4. Phylogenetic analysis

For sequence analyses, PCR products were purified using the Wizard SV Gel and PCR Clean-Up System (Promega, USA) according to the manufacturer's instructions. The purified products of JDP-PCR of *Acanthamoeba* were subjected to cycle sequencing by the dideoxynucleotide chain termination method using the DYEnamic ET Terminator Cycle Sequencing Kit (Amersham), with JDPF used as a sequencing primer. The products were separated in an automated DNA genetic analyzer (MegaBACE 500 Genetic Analyzer). The GenBank BLAST program was used for comparisons. A sequence of 423–551 bp was read for each isolate. These were aligned with the corresponding sequences from 18 reference *Acanthamoeba* isolates, including genotypes T1–T15 under the following accession numbers: T1: *A. castellanii* CDC:0981:V006 (U07400); T2: *A. palestinensis* Reich; ATCC 30870 (U07411); T3: *A. griffini* S-7 ATCC 30731 (U07412); T4: *A. castellanii* Ma ATCC 50370 (U07414); T4: *Acanthamoeba* sp. S27 (DQ087323); T4: *Acanthamoeba* sp. ACA1 (EF654665); T4: *Acanthamoeba* sp. ACA7 (EU741257); T4: *Acanthamoeba* sp. ACA9 (EU741252); T5: *A. lenticulata* PD2S (U94741); T6: *A. palestinensis* 2802 (AF019063); T7: *A. astronyxis* CCAP 1534/1 (AF239293); T8: *A. tubiashi* OC-15C (AF019065); T9: *A. comandoni* Comandon & de Fonbrune (AF019066); T10: *A. calberstoni* Lilly A-1 (AF019067); T11: *Acanthamoeba* sp. PN14 (AF333608); T12: *A. healyi* CDC 1283:V013 (AF019070); T13: *Acanthamoeba* sp. UWC9 (AF132134); and T15: *A. jacobi* AC305 (AY262365); T15: *Acanthamoeba* sp. ACA12 (FJ195367).

Three sequences of our *Acanthamoeba* isolates were used in phylogenetic tree construction. JDP sequences were entered in MEGA5 for construction of the phylogenetic trees using maximum parsimony (MP) and distance methods, namely neighbor-joining, an unweighted pair-group method with arithmetic averages (UPGMA), and minimum evolution (17). Branch support was given using 1000 bootstrap replicates in MEGA5 (17).

3. Results

3.1. Isolation and culture of trophozoites

Sources, sample ID, purpose and temperature of water, and positive samples are shown in the Table. Five water samples (OV1, HM3, HM4, SF2, and SF3) from 26 different samples were positive for *Acanthamoeba* (19.2%) after in vitro culture at 37 °C for 15 days (Table). One sample from a well fed by snow water and 4 samples from wells fed by rain water were positive for FLA. Samples originating from 3 different tap waters were negative (OV10, HM10, and SF9) (Table). The amoebae have characteristic spine-like pseudopodia called acanthapodia on the surface of trophozoites, and double-walled cysts. Therefore, isolates OV1, HM3, HM4, SF2, and SF3 were identified as belonging to the genus *Acanthamoeba*.

3.2. Molecular identification of the isolates and phylogenetic analysis

Five samples scored positive in FLA-PCR while all samples were also positive in both Nelson-PCR and JDP-PCR. We observed an expected amplicon of JDP-PCR in 5 well water sediments (Figure 1). Three different *Acanthamoeba* isolates were obtained from sequence analyzing of the JDP-PCR. While the sequence of JDP DNA of the HM3 isolate was the same as the sequence of HM4, the sequence of JDP DNA of the SF2 isolate was the same as the sequence of SF3. OV1, HM3, and SF2 were named respectively as ERC-B1, ERC-B2, and ERC-B3. Sequences of 3 isolates in the present study were deposited in the GenBank database and are available under accession numbers JN793396 (ERC-B1), JN793397 (ERC-B2), and JN793398 (ERC-B3).

*Acanthamoeba* isolates (OV1, HM3, and SF2) were further investigated by phylogenetic analyses, which were
Table. Water samples investigated for free-living amoebae.

<table>
<thead>
<tr>
<th>Hacilar region</th>
<th>ID of samples</th>
<th>Water content</th>
<th>Temperature(^a)</th>
<th>Isolation(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oren vineyard (OV)</td>
<td>OV1-ww</td>
<td>1</td>
<td>3.8 °C</td>
<td>Acanthamoeba</td>
</tr>
<tr>
<td></td>
<td>OV2-ww</td>
<td>1</td>
<td>8.0 °C</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>OV3-ww</td>
<td>2</td>
<td>6.5 °C</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>OV4-ww</td>
<td>2</td>
<td>7.2 °C</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>OV5-ww</td>
<td>2</td>
<td>0.8 °C</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>OV6-ww</td>
<td>2</td>
<td>9.0 °C</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>OV7-ww</td>
<td>2</td>
<td>9.1 °C</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>OV8-ww</td>
<td>2</td>
<td>12.3 °C</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>OV9-ww</td>
<td>3</td>
<td>7.1 °C</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>OV10-tw</td>
<td>3</td>
<td>19.2 °C</td>
<td>-</td>
</tr>
<tr>
<td>Hasan Mountain (HM)</td>
<td>HM1-ww</td>
<td>1</td>
<td>16.2 °C</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>HM2-ww</td>
<td>1</td>
<td>16.2 °C</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>HM3-ww</td>
<td>2</td>
<td>17.7 °C</td>
<td>Acanthamoeba</td>
</tr>
<tr>
<td></td>
<td>HM4-ww</td>
<td>2</td>
<td>15.1 °C</td>
<td>Acanthamoeba</td>
</tr>
<tr>
<td></td>
<td>HM5-ww</td>
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<td>13.3 °C</td>
<td>-</td>
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<tr>
<td></td>
<td>HM6-ww</td>
<td>2</td>
<td>12.2 °C</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>HM7-ww</td>
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<td>14.4 °C</td>
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<td>HM8-ww</td>
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<td>17.3 °C</td>
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<tr>
<td></td>
<td>HM9-ww</td>
<td>3</td>
<td>11.1 °C</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>HM10-tw</td>
<td>3</td>
<td>19.7 °C</td>
<td>-</td>
</tr>
<tr>
<td>Sakar Farm (SF)</td>
<td>SF1-ww</td>
<td>1</td>
<td>13.7 °C</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SF2-ww</td>
<td>2</td>
<td>14.9 °C</td>
<td>Acanthamoeba</td>
</tr>
<tr>
<td></td>
<td>SF3-ww</td>
<td>2</td>
<td>11.6 °C</td>
<td>Acanthamoeba</td>
</tr>
<tr>
<td></td>
<td>SF4-ww</td>
<td>2</td>
<td>14.7 °C</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SF5-ww</td>
<td>2</td>
<td>11.7 °C</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SF6-ww</td>
<td>2</td>
<td>11.9 °C</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SF7-ww</td>
<td>2</td>
<td>11.8 °C</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SF9-ww</td>
<td>2</td>
<td>11.6 °C</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SF8-ww</td>
<td>3</td>
<td>14.3 °C</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>SF9-tw</td>
<td>3</td>
<td>21.6 °C</td>
<td>-</td>
</tr>
</tbody>
</table>

Samples: ww, well water; tw, tap water. Water content: 1, snow water; 2, rain water; 3, tap water.

\(^a\)Water temperature at sampling.

\(^b\)Dashes indicate water samples where no trophozoites were detected within 15 days of incubation, either due to the absence of trophozoites in the sample or due to the fact that the trophozoites were not able to grow at 37 °C.
done by sequencing of JDP-PCR amplimers and subsequent comparison of these sequences with corresponding regions from other known Acanthamoeba strains. The comparative investigation included all representative sequences available from GenBank and had previously been used for the definition of phylogenetic sequence types T1–T15 (18). Sequences from all 3 Acanthamoeba isolates obtained in the study were clustered in sequence type group T4 (Figure 2).

4. Discussion
The present study is the first report to investigate, isolate, and identify the Acanthamoeba that may exhibit a potential pathogenicity from selected Turkish well water sediments. We isolated Acanthamoeba in 19.2% of the investigated water samples. FLA are prevalent in soil and water. These environmental sites are known from other studies to potentially harbor pathogenic FLA including Acanthamoeba spp. and N. fowleri, which can lead to severe and lethal CNS infections (9). Therefore, screening of FLA in these sites is very important for human health. The rate of FLA was lower than in partially similar previous studies from the United States (143 of 330, 43.4%), Thailand (43 of 95, 45.2%), Japan (47 of 95, 49.5%), Osaka in Japan (257 of 375, 68.7%), and Bulgaria (31 of 35, 93%). (4,8,19,20).

Water samples were collected at 4 hot spring resorts in the temperature range of 33–40 °C, and 9 of the 31 water samples scored positive for FLA (29%) (7). The data of Gianinazzi et al. reported the presence of FLA in 75% of water samples investigated (6). In that study, temperature of samples ranged from 9.2 to 29.3 °C, but in ours it ranged from 0.8 to 19.7 °C. This discrepancy may be related to the low temperature of the wells in our region. Additionally, we did not investigate Naegleria in the well water of our region since the water temperatures were quite low (from 0.8 to 19.2°C).

Acanthamoeba spp. are the most common amoebae, and probably the most common protozoa, to be found in soil and water samples, as evidenced by their presence at high percentages in samples taken in human-related aquatic habitats (21). The classification of the genus Acanthamoeba is still debated and molecular approaches have been used for precise identification of isolates. More than 24 named species have been described within the genus Acanthamoeba, including both pathogenic and nonpathogenic isolates. Acanthamoeba species were classified into 3 morphological groups due to alternating cyst appearance (22). The analysis of 18S rDNA of Acanthamoeba isolates has also been described, identifying 12 distinct sequence genotypes (T1–T12) and later adding genotypes T13, T14, and T15 (13,22–24). In our study, 4 of 26 well water sediments were positive for Acanthamoeba sp. A phylogenetic analysis of the 5 sequences showed that
2 sequences of the *Acanthamoeba* isolates were the same and 3 isolates were clustered into genotype T4.

In Turkey, a few studies have addressed the presence of potentially pathogenic FLA in environmental samples (25–27). Eighteen *Acanthamoeba* isolates have been isolated from 28 soil and 2 water samples. Ribosomal DNA sequencing revealed that 10 isolates belonged to the T2 genotype, 5 isolates belonged to T3, 2 isolates belonged to T4, and 1 isolate belonged to T7. These water samples were obtained from hot spring water (28).

The first study identifying *Acanthamoeba* keratitis in Turkey and the first isolation of genotype T9 in the country was performed by Ertabaklar et al. (29). The *Acanthamoeba* strain isolated from a corneal scraping was identified as genotype T4. Three more *Acanthamoeba* strains isolated from sites of possible human contact with *Acanthamoeba* in the same geographical region, including a lens storage case, tap water, and soil, were subjected to molecular biological identification. While the strain from tap water exhibited genotype T4, 2 other isolates were identified as genotype T9. Based on PCR-amplified 18S rRNA gene analysis, the first isolate was identified as *Acanthamoeba* genotype T4 and the second as *Paravahlkampfia* sp. from the patient in İzmir, Turkey (30). Despite these studies, a detailed study has not been conducted for screening of FLA in environmental waters in Turkey.

We have identified, for the first time, *Acanthamoeba* isolates from 26 well water sediments in Turkey. Further research is needed in a wide series of subjects focusing on in vitro cytotoxicity and in vivo pathogenicity of isolates and assessing the phylogenetic and pathogenic association in *Acanthamoeba* infections.

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**References**


