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ORIGINAL ARTICLE

Genotyping of *Acantamoeba* spp. from rhisophere in Hungary

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ABSTRACT

The protista *Acanthamoeba* is a free-living amoeba existing in various environments. A number of species among protista are recognized as human pathogens, potentially causing *Acanthamoeba* keratitis (AK), granulomatous amoebic encephalitis (GAE), and chronic granulomatous lesions. In this study, 10 rhizosphere samples were collected from maize and alfalfa plants in experimental station at Institute of Genetics, Microbiology and Biotechnology, Szent István University. We detected *Acanthamoeba* based on the quantitative real-time PCR assay and sequence analysis of the 18S rRNA gene. All studied molecular biological methods are suitable for the detection of *Acanthamoeba* infection in humans. The quantitative real-time PCR-based methods are more sensitive, simple, and easy to perform; moreover, these are opening avenue to detect the effect of number of parasites on human disease. *Acanthamoeba* species were detected in five (5/10; 50%) samples. All *Acanthamoeba* strains belonged to T4 genotype, the main AK-related genotype worldwide. Our result confirmed *Acanthamoeba* strains in rhizosphere that should be considered as a potential health risk associated with human activities in the environment.

KEYWORDS

Acanthamoeba, rhizosphere isolates, genotypes

INTRODUCTION

Acanthamoeba is a genus of free-living amoebae widely distributed in various ecological environments [1–3]. The life cycle of *Acanthamoeba* species (sp.) consists of the active trophozoites and dormant cysts stages. *Acanthamoeba* trophozoites have a size between 20 and 40 μm, although this range can vary significantly among isolates of different species genotypes. Cysts are double-walled and range in size from 10 and 20 μm. This difference in size between the cyst and trophozoite involves a significant loss of cell volume mail due to cellular dehydrations. *Acanthamoeba* spp. are thermotolerant, which are resistant to extreme temperature, pH conditions, UV, as well as to chlorine and other disinfectant media.

Most of the environmental studies are focusing on pathogenic *Acanthamoeba* sp. taxonomic and pathogenic markers, geographic distribution, ecology, and transmission dynamic [4–6]. Unlike obligate parasites, pathogenic *Acanthamoeba* spp. can complete their life cycle, environmental performance without having to enter the human or animal host [7, 8]. The genus *Acanthamoeba* has been currently classified into 21 different genotypes, T1–T21, based on 18S rRNA nucleotide sequence [9, 10]. Some genera of *Acanthamoeba* cause different infections, which produce *Acanthamoeba* keratitis (AK), subacute or chronic granulomatous amoebic encephalitis, and skin infections. Human infections with these amoebae have been reported from all over the world [11]. The first cases that clearly established *Acanthamoeba* as causative agents of disease in humans have been reported in the early 1970s [12]. In many cases, AK infections occur after water exposure or a history of swimming in lakes, following contact with soil or plants, or while wearing contact lenses [13, 14].

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In general, *Acanthamoeba* are metabolically active and use a wide variety of bacteria, fungi, and organic matter as a food source [15].

Therefore, in this study, high microbial activities showing rhizosphere soil used for isolation protozoan organisms to test their occurrences are not in human host. Moreover, the isolated strains morphologically characterized by electron microscopy molecularly characterized based on the 18S rRNA gene sequence and the robust phylogenetic analysis was also measured.

MATERIALS AND METHODS

Samples collection

Rhizosphere samples were collected from experimental station at Institute of Genetics, Microbiology and Biotechnology, Szent István University (longitude: 19°21′39.85″, latitude: 47°35′37′63″) in June 27, 2018. Rhizosphere samples were taken from the depth of 0–20 cm. During the sampling period, altogether 10 samples from rhizosphere of maize and alfalfa plants samples were taken.

The sampling was performed, in which 10 samples were taken from rhizosphere of maize and alfalfa plants (notation: K1_1, K1_2, K1_3; K2_1, K2_2, K2_3; and L1_1, L1_2, L1_3, L2_1).

Culture-confirmed detection method

To concentrate *Acanthamoeba* spp., the samples were filtered, eluted, and centrifuged. Soil samples (1 g) collected from rhizosphere of maize and alfalfa plants were dissolved in 10 ml of sterile physiological saline solution (0.85%) buffer and 500 µl of each sample was inoculated onto PAGE agar 9-cm plates seeded with heat-killed *Escherichia coli* and incubated at 36 °C [16].

Microscopic detection

Samples were examined under a microscope for 72–96 h at 400× with an inverted ZEISS microscope (Figure 1).

Molecular analysis

The *Acanthamoeba* species were isolated by dilution method. For this purpose, the samples of soil (1 g) were suspended in 10 ml of sterile physiological saline solution (0.85%). After preparation, the DNA extraction was treated with High Pure PCR Template Preparation Kit (Germany), according to the instructions of the manufacturer. If further processing was delayed, the isolates were stored at 4 °C for 24 h or at –20 °C for a longer period. The DNA amplification was performed using genus-specific primers and genus-specific fluorescence resonance energy transfer (FRET) hybridization probes, previously described by Orosz et al. [17]. Each experiment included one reaction mixture without DNA as a negative control; positive control and each specimen were run in duplicate for real-time PCR assay in parallel. We have used serial dilutions of *Acanthamoeba* (GenBank accession number: KC434439) strain to determine the calibration curve that the liquid chromatography device could determine the additional samples parasite number in copy numbers.

PCR products were purified with PCR Clean up-M Kit (Viogene, Sunville, CA). The sequence of each amplicon was determined by cycle sequencing with primers for the 5′-NTR region and with primers with BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Germany), according to the manufacturer's instruction. The electrophoresis was carried out on Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems, Budapest, Hungary).

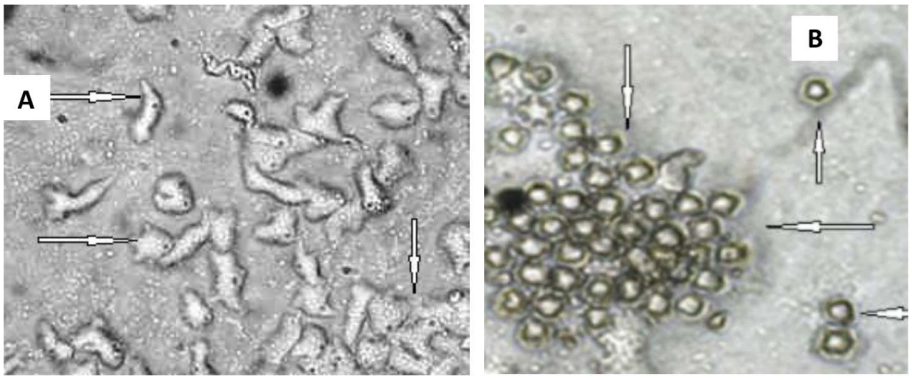
The 5′-NTR and VP1 gene sequences were subject to nucleotide–nucleotide BLAST analysis [18] using the online server at the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast).

The unknown sequences were aligned with known published sequences of the major genotypes using the alignment program MULTALIN (http://multalin.toulouse.inra.fr/multalin) [19]. The genotypes of samples were determined based on this comparison.

The phylogenetic tree was constructed by the neighbor-joining method of genetic distance calculated by the MEGA 6 (http://www.megasoftware.net) [20].

Genotype identification was carried out with a real-time FRET PCR assay based on sequence analysis of the 18S rRNA gene, and sensitivity and specificity were evaluated in comparison with traditional parasitological techniques.

Figure 1. Photomicrograph of *Acanthamoeba* trophozoites (A) and cysts (B) with 400× magnification. Photographer: Erika Orosz



RESULTS

Microscopic detection

All investigated samples revealed *Acanthamoeba* were able to grow at 36 °C, the approximate temperature of the human organism. Microscopically 5 out of the 10 samples were declared as *Acanthamoeba* positive (*Medicago sativa* – L1_2, L1_3 and *Zea mays* – K1_2, K2_1, K2_3). Five rhizosphere samples (*Medicago sativa* – L1_1, L1_3 and *Zea mays* – K1_1, K1_3, K2_2) were microscopically negative. Further examination of the obtained results was conducted by FRET PCR.

Molecular analysis

This study reports successful PCR amplification for 5 (5/10; 50.0%) positive cases. The samples of five *Acanthamoeba* – positive samples, detected by PCR method, were sequenced to identify the species. Sequence analysis using a BLAST search indicated an identity of >98% with *Acanthamoeba* 18r rRNA gene reference sequences. It was found that all obtained sequences of amoebae isolates from the cases belong to the different T4 genotypes *Acanthamoeba* spp. Neighbor-joining analysis inferred relationships between the PCR products isolated from rhizosphere samples reference strains obtained from NCBI GenBank, shown in Figure 2, respectively.

Figure 2. Phylogenetic relations of *Acanthamoeba* species PCR product sample L1_2, sample L1_3, sample K2_1, sample K2_1, sample K2_3, and reference strains from NCBI GenBank inferred by neighbor-joining analysis from pairwise comparisons (180-bp fragments)

DISCUSSION AND CONCLUSIONS

Studies of *Acanthamoeba* have grown exponentially. To the best of our knowledge, this is the second study of occurrence of *Acanthamoeba* similar to T4 genotypes in rhizosphere samples from Hungary. These organisms have gained attention from the broad scientific community studying environmental biology, molecular biology, and biochemistry. Literature describes T4 genotype *Acanthamoeba*, as the most common in the environment. These results are consistent with previous findings indicating that T4 is worldwide predominant [21–24].

However, the correct understanding of the factors influencing the occurrence of the different species appears of great concern, as these amoebae are free-living organisms, and their potential capabilities to cause severe infections of the central nervous system, ocular keratitis, and other disorders are now ascertained worldwide.

All the isolates in this study exhibited morphological features of the genus *Acanthamoeba* confirmed by means of quantitative real-time PCR. Quantitative real-time PCR with FRET hybridization probes method is the most sensitive with a short turnaround time. It is possible even to estimate the parasite number in the samples with method. Therefore, only molecular methods allow reliable differentiation of the *Acanthamoeba* species. Based on rRNA gene sequences, the genus *Acanthamoeba* is divided into 21 different genotypes to date (T1–T21). Each genotype exhibits 5% or more sequence divergence between different genotypes. Five isolates were characterized as similar to genotype T4 due to their strict correspondence to the reference sequences of this genotype (GenBank accession number: KJ786514, KU936114, KJ786526, and MF197424). Sequence date indicate that the vast majority of them causes human infections. Contrary to data on *Acanthamoeba* infections in humans, little is known about infections in rhizosphere. It has been concluded that the rhizosphere isolates are most closely related to strains commonly isolated from human infections, especially AK [25–29].

In conclusion, our results confirm and support previous report on *Acanthamoeba* genotype free-living amoeba in rhizosphere soil. A homologous analysis of the 18S rRNA of five *Acanthamoeba* species isolated from rhizosphere of maize and alfalfa was identified into one genotype, namely T4. These genotypes were associated with AK or encephalitis; therefore, the presence of *Acanthamoeba* should be considered as potential health threat associated with human activity in soil.

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Conflict of Interest: There is no conflict of interest. The corresponding author assures that there are no links with a company whose product is mentioned in the article or a company that distributes a competing product. The presentation of the topic is independent and the presentation of the content is product-neutral.

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