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Original papers

Morphological, physiological and genetic characteristics of protozoa of genus *Acanthamoeba*, isolated from different deposit of bentonite in Ukraine

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ABSTRACT. The representatives of genus *Acanthamoeba* are widespread in the environment. The presence of free-living *Acanthamoeba* sp. in such mineral deposits as bentonite was shown for the first time. Identification of isolated amoeba was conducted according to morphological features of trophozoites and cysts, as well as using sequencing of gene 18S RNA (amplifier GTSA.B1). The obtained data showed that isolated amoebae belong to the genotype T4 and II morphological group (cyst size <18 μm). For its growth, "bentonite" amoebae are intensively used bacteria of the genus *Cellulosimicrobium sp*. as a food substrate.

Keywords: bentonite, *Cellulosimicrobium* sp., *Acanthamoeba* sp., genotype

Introduction

The protozoa of the genus *Acanthamoeba* are free-living amoebae that inhabit ecological niches of the environment (water, soil, air) [1]. They can cause eye infections – amoebic keratitis and granulomatous amebic encephalitis under certain conditions [2]. Acanthamoebae have two stages in their life cycle – active vegetative trophozoites, which intensively grow and divide, and the stage of rest with minimal metabolic activity – cysts. Amoebae can use different bacterial agents, yeast, organic substances, etc. as a food substrate, when they are in the environment.

The basic methods of amoebae isolation are based on principle of cultivation on nutrient media with previously inoculated bacteria and axenic cultivation without using of microorganisms [3].

The first representative of *Acanthamoeba* was described by Castellani in 1930 [4]. By this time, the classification of amoeba within the limits of this genus is not completed. More often, for identify different species of *Acanthamoeba* used morphological criteria and sequencing of the genome's various sections. Immunological, biochemical and physiological criteria may also be used in addition [5,6]. In 1977, Pussard and Pons [20] distributed representatives of the genus *Acanthamoeba* into 3 groups to morphological features. The basis of this classification is peculiarities of the trophozoites structure, size, shape and structure of the cyst. According to the nucleotide sequence of 18S rRNA, the genus *Acanthamoeba* is divided into 20 (T1–T20) different genotypes [7]. The difference between genotypes is from 5% or more nucleotide sequences [8].

Materials and Methods

Collection of samples and isolation of cultures.

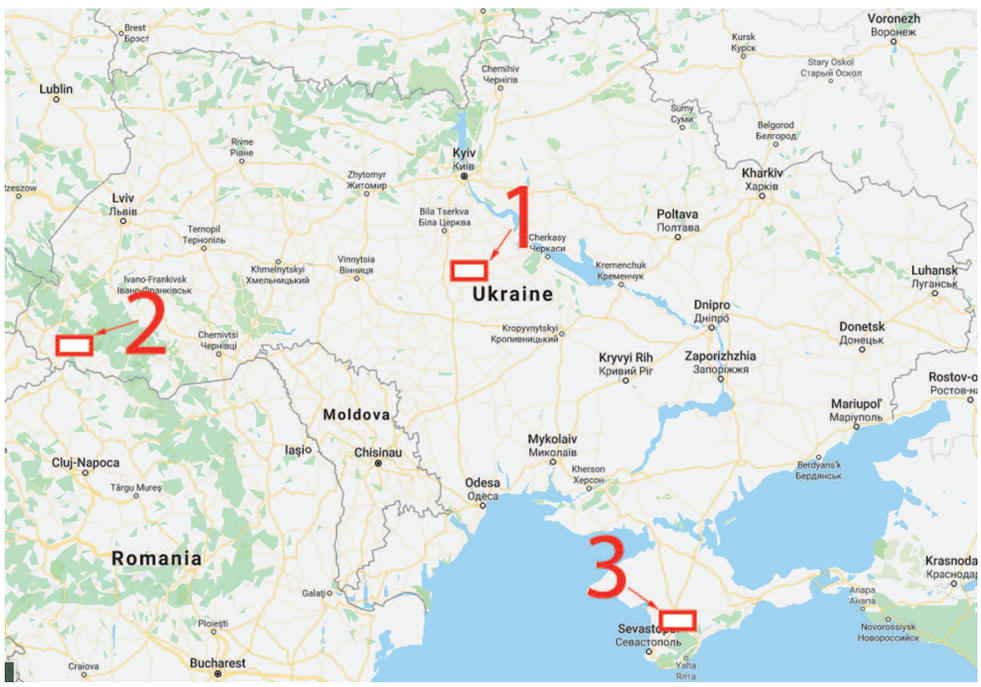

Figure 1. Bentonite samples selection in Ukraine: № 1 – Dashukivske deposit; № 2 – Horbske deposit; № 3 – Kurtsivske deposit

All samples of bentonite were selected on the territory of Ukraine in the following deposit: Cherkasy (Dashukivske) deposit (Lysiansky district of Cherkasy region), Zakarpattia (Horbske) deposit (Vynohradiv district of Zakarpattia region) and Crimean (Kurtsivske) deposit (Simferopol district, ARC) (Fig. 1).

Amoebae were isolated using nutrient media for cultivating microorganisms (peptic digest of animal tissue – 5 g/l, meat extract – 1.5 g/l, yeast extract – 1.5 g/l, sodium chloride – 5.0 g/l; dextrose – 10 g/l) with previous inoculation of bacteria *E. coli (ATCC 25923)*. Later, bacteria of the genus *Cellulosimicrobium* were used to maintain and accumulate amoeba culture. Cultivation was carried out at 35ºC for 5 days. The visible growth of amoebae was seen on 2–3 days.

Bacterial cultures. Bacteria of the genus *Cellulosimicrobium* were isolated by us from samples of bentonite the same as amoebae [9]. There are gram-positive, polymorphic, non-spore forming microorganisms that spread in the soil and sometimes can cause disease in humans [10].

Analysis of morphological features. Morphology of amoebae was studied using electronic (electronic microscope JEM-100CX) and phase-contrast microscopy (microscope Carl Zeiss Axioplan).

DNA extraction, PCR-amplification and gene sequencing. Extraction of DNA was performed from pure cultures that were grown on 1% dextrose MPA for 96 hours. DNA was extracted by adsorption of silica gel by Boom et al. [11].

Genus-specific JDP primers: forward JDP1 (5'>GGCCCAGATCGTTTACCGTGAA<3') and reverse JDP2 (5'>TCTCACAAGCTGCTAGGGGAGTCA<3') were using to confirm the isolated strains belonging to the genus *Acanthamoeba*. This pair of primers amplified ASA.S1 fragment of 18S rDNA gene length 423–551 bp. Amplimer of GTSA.B1 (used to identify individual genotypes) was accumulated using a pair of primers CRN5 (5'>CTGGTTGATCCTGCCAGTAG<3') and 1137 (5'>GTGCCCTTCCGTCAAT<3'). These primers amplify a fragment of 18S rDNA in the range from 1 to 1475 bp. [8]. In addition, primers P2fw (5'>GATCAGATACCGTCGTAGTC<3') and S20R (5'>GACGGGCGGTGTGTACAA<3') were used. Primer P2fw – direct at 1200 bp, primer S20R – reverse ≈ 200 bp from the end of the gene 18S rDNA [12,13].

PCR amplification was performed in volume 25 µl, which contained 2 µl of isolated DNA, 1 unit of Taq DNA Polymerase, 0,2 mM of each dNTPs, 1×PCR buffer with 2,5 mM $MgCl_2$, 10 pm of each primer. PCR program for amoebae included a prolonged denaturation for 10 minutes at 94°C; 35

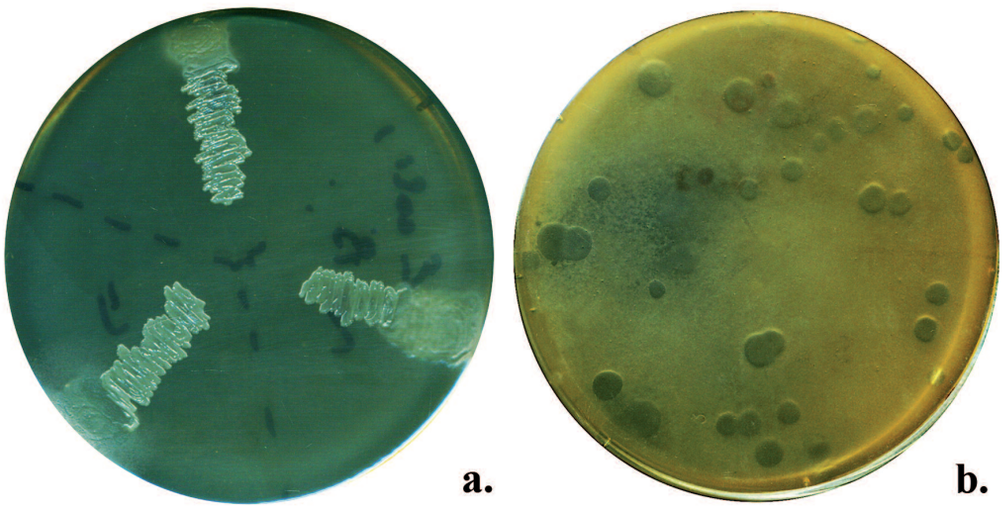

Figure 2. Growth of isolated amoebae on nutrient agar with previously inoculated *Cellulosimicrobium* sp.: a. amoeba growth at the lawn of bacteria; b. the phenomenon of plaque formation

cycles at 94°C during 1 m.,60°C – 1 m., 72°C – 2 m.; final elongation at 72°C during 7 minutes [14].

Gene which code 16S rRNA gene of bacteria, which was used as feeders, was amplified using universal prokaryotic primers: 27F (5'>AGAGTT TGATCMTGGCTCAG<3') and 1492R (5'>GGTT ACCTTGTTACGACTT<3'). PCR program consist of prolonged denaturation for 5 minutes at 95°C; 30 cycles at 95°C during 40s, 50°C during 40s, 72°C during 90s; final elongation at 72°C during 7 minutes [15].

Analysis of amplified DNA fragments was performed by separation of DNA fragments in 1.5% agarose gel, with ethidium bromide as intercalating agent. DNA isolation from agarose gel was carried out using «Gel-Out izolacja DNA z żeli agarozywych» reagent package (© Kucharczyk Techniki Elektroforetyczne, Poland), according to the manufacturer's instructions.

Sequencing of PCR products. PCR products isolated from amoebae and bacteria were sequenced using the apparatus ABI3730 Genetic Analyzer (Institute of Biochemistry and Biophysics, Polish Academy of Sciences).

Phylogenetic analysis. The nucleotide sequences of the homologous gene fragments of *Acanthamoeba* were received from GenBank. Multiple alignment of received sequences and sequences of the 18S rRNA gene from the data bank and construction of a phylogenetic tree were carried out using MrBayes 3.1 [16, 17].

Numbers in GenBank. The resulting nucleotide sequences are deposited in the GenBank under the number: MH620777, MH620776 and MH620775.

Results and Discussion

Amoebae of the genus *Acanthamoeba* are representatives of microbial groups of the environmental objects, directly soil. Their presence in clay's material as bentonite shows an unusual and indescribable phenomenon. For the first time, we isolated free-living amoebae from bentonite samples of various origins [9,18], using the generally accepted method of isolation these microorganisms in nutrient media with previous inoculation of *E. coli* [19]. A differential-diagnostic medium with lactose (Endo agar) was used as a nutrient medium. The test culture was *E. coli B* (ATCC 8739) and *E. coli K12*. But the disadvantage of this method of amoebae cultivation was poor reproducibility of the results. Further investigation of microbial groupings of bentonite clays has made it possible to isolate the bacteria-feeders, which turned out to be the most optimal system for cultivation, and were used as a food substrate by amoebae (Fig. 2a).

A detailed study of morphological, cultural and biochemical features, alongside with sequencing of the 16S RNA gene made it possible to refer isolated microorganisms to gram-positive bacteria of the genus *Cellulosimicrobium*.

Isolated amoebae showed characteristic signs of growth on nutrient agar at co-cultivating with *Cellulosimicrobium* sp. The best growth was observed when 1% of carbohydrates, in particular glucose, were added to the medium.

A clear dependence of growth „bentonite" amoebae from origin of agar-agar, which was part of

Table 1. Morphological and genetic characteristic isolated from bentonite *Acanthamoeba* sp.

Isolate	Sourse	Size of cysts (μm)	Morph. group*	Genotype
Crimea	bentonite	12.35±0.89	II	T4
Cherkasy	bentonite	10.7±0.97	II	T4
Carpathians	bentonite	11.1±0.74	II	T4

*morphological group to Pussard and Pons [20]

the nutrient media was established during experimental research. Four types of different agar-agar and agarose were used to create the density of media in experiments. The intense growth of amoebae was observed only when using agar B4 (production of SO "Sakhalinmedprom"). This phenomenon indicates the possibility of finding in this type of agar additional components that are necessary for the growth of amoebae. In order to confirm this opinion, further detailed chemical analysis of various agar samples is required.

It was shown that when amoebae and bacteria-feeders were simultaneously inoculated in melted and cooled to 45°C nutrient agar, amoebae form plaques with specific crateroidal depressions in a solid medium (Fig. 2b).

Amoebae showed signs of growth ranging from 20 to 37°C (experimental interval), and in the range of pH from 6.0 to 9.0. Amoebic cysts are fairly stable and maintain their viability in saline at room temperature from several months to several years. The karpaty strain was less stable, which become dead already in two or three months.

"Bentonite" amoebae have sequential stages of development in their life cycle, active form – trophozoit, precyst and resting form – cyst (Fig. 3). Amoebae, from different bentonite deposits, form cysts of different sizes during prolonged cultivation on a solid nutrient medium. The average size of cysts of all isolates which growth in nutrient agar for cultivating microorganisms at a temperature of 37°C was <11.4 μm, directly to the Karpaty strain – 12.35 ± 0.89 μm, Cherkasy strain – 10.7 ± 0.97 μm, Krym strain – 11.1 ± 0.74 μm (by 20 randomly selected cysts of each strain) (Table 1).

According to morphological classification Pussard and Pons [20], all three strains of amoebae belong to the II morphological group. Represen-

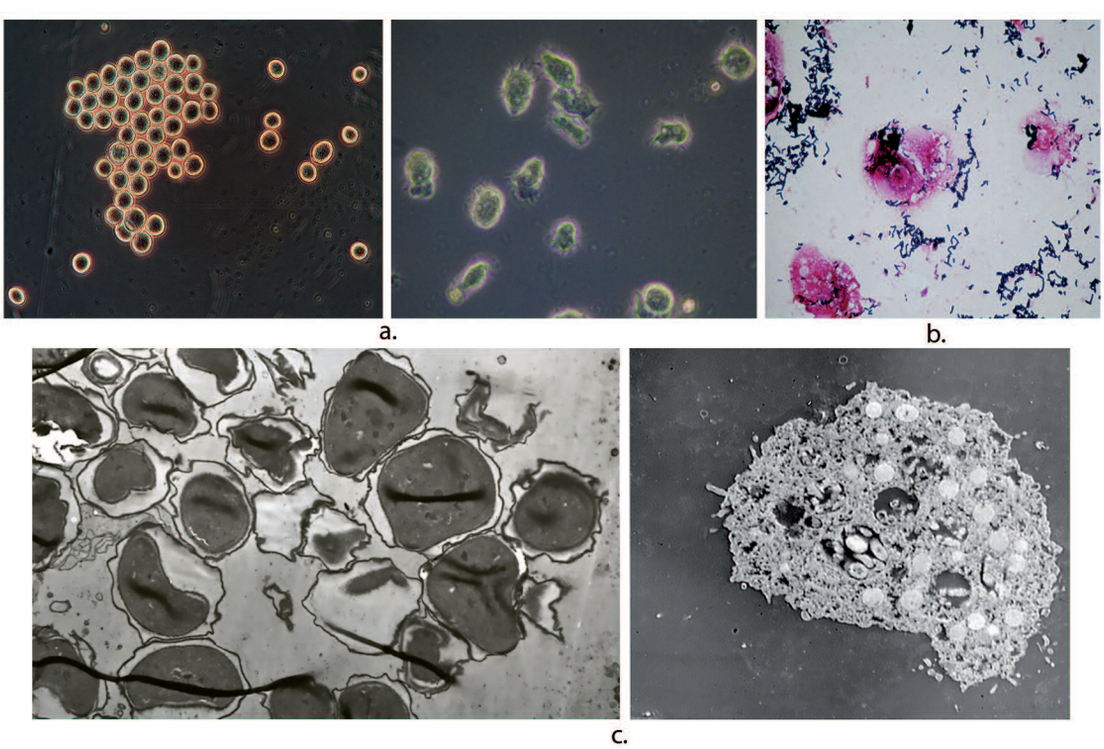

Figure 3. Morphological characteristic of „bentonite" amoebae: a. phase contrast (cysts and trophozoites); b. light microscopy (Gram stain); c. electron microscopy (cysts and trophozoites)

Table 2. Genotyping variants species *Acanthamoeba*, according to literature data [23,24] and GenBank, which used in study

Sequence type	Species affiliation	Number GenBank	Reference (GenBank sequence)
T1	*A. castellanii* CDC:0981:V006	U07400	Gast R.J. (1996)
T2	*A. palestinensis* Reich ATCC 30870	U07411	Gast R.J. (1996)
T3	*A. griffini* S-7 ATCC 30731	U07412	Gast R.J. (1996)
T4A	*A. castellanii Castellani* ATCC 50374	U07413	Gast R.J. (1996)
T4B	A. *castellanii* Ma ATCC 50370	U07414	Gast R.J. (1996)
T4C	*Acanthamoeba* sp. ATCC 50369	U07409	Gast R.J. (1996)
T4D	*Acanthamoeba rhysodes*	AY351644	Chung D. and Kong, H. (2003)
T4E	*A. polyphaga* Page-23	AF019061	Stothard D.R. et al. (1998)
T4F	*A. triangularis*	AF346662	Schroeder J.M. et al. (2001)
T5	*A. lenticulata* strain 118	U94736	Stothard D.R. et al. (1998)
T6	*A. palestinensis* strain 2802	AF019063	Stothard D.R. et al. (1998)
T7	*A. astronyxis* Ray & Hayes	AF019064	Stothard D.R. et al. (1998)
T8	*A. tubiashi* OC-15C	AF019065	Stothard D.R. et al. (1998)
T9	*A. comandoni* Comandon & de Fonbrune	AF019066	Stothard D.R. et al. (1998)
T10	*A. culbertsoni* Lilly A-1	AF019067	Stothard D.R. et al. (1998)
T11	*Acanthamoeba hatchetti* BH-2	AF019068	Stothard D.R. et al. (1998)
T12	*Acanthamoeba healyi*	AF019070	Stothard D.R. et al. (1998)
T13	*Acanthamoeba* sp. UWC9	AF132134	Horn M. et al. (1999)
T14	*Acanthamoeba* sp. PN15	AF333607	Gast R.J. (2001)
T15	*Acanthamoeba jacobsi* AC080	AY262361	Hewett M.K. et al. (2003)
T16	*Acanthamoeba* sp. cvX	GQ380408	Corsaro D. and Venditti D. (2010)
T18	*Acanthamoeba* sp. CDC:V621	KC822470	Qvarnstrom Y. et al. (2013)

tatives of this group most often stand out from clinical material and samples of the environment [21,22].

Low resolution of morphological, physiological and ultrastructural systematics requires the use of molecular sequence data to determine the phylogenetic position of amoebae. Sequencing of gene 18S RNA was used to accomplish the abovementioned task.

Using three pairs of primers made it possible to amplify and sequence the nucleotide sequences of isolated strains of amoeba extending to 1373–1707 bp (the full length of the 18S RNA gene in representatives of the *Acanthamoeba* genus is 2300 to 2700 bp [25]). The resulting fragments encompassed eight variable regions of the gene GTSA.B1 [8], which were allowed differentiate of amoebae and reliably determine their genotype variants. Table 2 lists differential genotype variants of the amoebae genus *Acanthamoeba* from GenBank that were used for comparison.

Figure 4 illustrates the phylogenetic position of the amoebae isolated from bentonite. Analysis of the 18S rDNA gene sequence showed that all three strains of amoebae have 99% identity with the sequences of the *Acanthamoeba* species belong to the T4 genotype. This made it possible to conclude that all investigated amoebae belong to the fourth genotype *Acanthamoeba*.

For determine the pathogenic potential of isolated strains of *Acanthamoeba sp*. were used the thermotolerant and osmotolerant tests [26,27]. During co-culture with *Cellulosimicrobium sp*. on a nutrient agar, presence of moderate osmotic resistance in studied strains (presence of growth at a concentration of mannitol of 0.5 M) and thermosensitivity (presence of growth at 37°C) was showed. This indicates a possible pathogenic potential for humans and animals in all three strains of ”bentonite” amoebae.

Since, the best growth of amoebae in medium with pre-inoculated bacteria *Cellulosimicrobium* sp. (which are representatives of the „normoflore” of the soil), is suggested that amoebae are constantly found in bentonite as autochthonous microflora. This is also confirmed by the fact that amoebae were

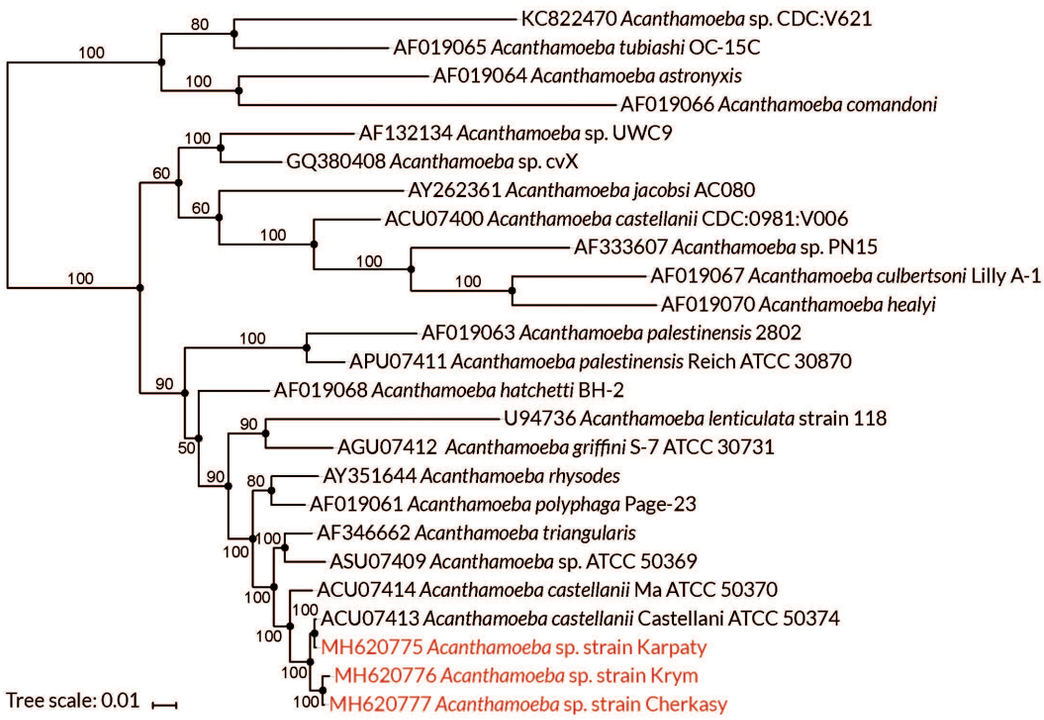

Figure 4. Bayesian inference tree based on fragment of sequences obtained at the small-subunit rRNA gene (SSU rDNA) of *Acanthamoeba* isolates, performed using MrBayes 3.1

isolated from bentonite in all three explored deposits.

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