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Original papers

Comparative examination on selected amphizoic amoebae in terms of their *in vitro* temperature tolerance – a possible indirect marker of potential pathogenicity of *Acanthamoeba* strains

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ABSTRACT. *Acanthamoeba* species are ubiquitous in natural and man-made environments worldwide; some strains are able to colonize human eyes as facultative parasites. It has been shown that environmental and clinical isolates/species of *Acanthamoeba* vary in their pathogenicity. In this study we examine and compare the *in vitro* effects of the changing temperature on the population dynamics of subsequent amoebic strains. Identification of *Acanthamoeba* strain by morphological and molecular methods and temperature assays were performed. Monitoring of the corneal and environmental strains showed changes in population densities and a termo-tolerance correlating with pathogenicity of amoebae. Comparative assessment of results indicated differences in viability of amoebic populations in exponential growth phase *in vitro* cultivation. The increased awareness of the threat is needed for better understanding of impact of factors examined on pathogenesis in human infected with *Acanthamoeba* strains.

Key words: environmental and corneal *Acanthamoeba* strains, *in vitro* termo-tolerance, pathogenicity

Introduction

Different species of free-living amoebae of the genus *Acanthamoeba* ubiquitous in natural and man-made environments in various parts of the world may also exist as endozoic organisms [1–5]. Potentially pathogenic amphizoic amoebae have been detected in environmental samples worldwide; the protists have been isolated in different regions of Poland, from natural water bodies including lakes, ponds, rivers, and lagoons, in tap water of the water supply system at the area of the cities, in swimming pools and fountains [6–10]. The *Acanthamoeba* strains have been also recognized in the hospital environment as contaminants of surgical instruments and dental irrigation units, in various human cavities and tissues, on skin surfaces, in oral cavities, paranasal sinuses, in the brain and lungs [11–18].

Some of the amoebae have been recognized to be able to colonize human eyes, exist as parasites and cause a severe sight-threatening disease *Acanthamoeba* keratitis [19–27]. Symptoms of the eye infection are unspecific, the redness, photophobia, excessive tearing, severe eye pain, and significant deterioration of the visual acuity may occur; without consistent therapy the amoebic infection may lead to blindness. The vision-threatening disease is mainly related to contact lens wearers, and poor

contact lens hygiene is indicated as the most predisposing risk factor [14–16].

The treatment of the keratitis is difficult and the therapy applied is often unsuccessful due to, among others, nonspecific symptoms and high resistance, especially of amoebic cyst forms to disinfectants and drugs. Moreover, it has been shown that environmental and clinical isolates/species of *Acanthamoeba* vary in their pathogenicity, and the virulent strains indicate different intensity of pathogenic effects [21–23].

The number of reported cases of the eye infection caused by pathogenic *Acanthamoeba* strains is increasing every year worldwide due to more common contact lens use, however, the awareness and knowledge about the risk for human health that generate the amoebae is still insufficient. It is considered that *in vitro* temperature tolerance of *Acanthamoeba* strains, particularly an ability to growth at higher temperature, is possible indirect marker of potential pathogenicity of the amoebae [12,16–18,23].

The aim of this study was to examine and compare the *in vitro* effects of a physical factor – the changing temperature conditions – on the population dynamics of subsequent amphizoic amoebae strains.

Materials and Methods

Two *Acanthamoeba* strains: the environmental and the originating from eye of patient with severe course of *Acanthamoeba* keratitis (AK) were included in this study.

Identification of *Acanthamoeba* strains. The environmental strain classified earlier based on morphological criteria within the species belonging to *Acanthamoeba* group II was *in vitro* cultivated for years under bacteria-free conditions in BSC medium [24] in the Department of Medical Biology Laboratory, Medical University of Warsaw, Poland. This type *Acanthamoeba* strain has been also assessed using molecular techniques based on genotype associations the 18S rRNA gene sequence; it has been defined according to procedure described by Schroeder et al. [20] for ATCC 30010 *A. castellanii* Neff T4 genotype.

The pathogenic isolate was acquired from contact lens wearer with a complicated *Acanthamoeba* keratitis; the duration of symptoms was one month, after unsuccessful treatment in other units the patient was admitted to our hospital and then the correct diagnosis was made. Initially, the keratitis was diagnosed by non-invasive methods: slit lamp and the *in vivo* confocal microscopy. Laboratory microbiological and parasitological investigations were performed to identify causative agents of the eye disease; corneal scrapings collected from this eye were directly examined under the light microscope to confirm AK.

Simultaneously, the material of the isolate was *in vitro* cultured under bacteria-free condition in the absence of external live food organisms. Samples of the scrapings were maintained, like *A. castellanii* Neff strain, in the sterile 1.5 ml tubes, each containing BSC medium enriched with calf serum, incubated at 26°C and sub-cultured into this growth medium twice a month.

The eye strain was also assessed using molecular techniques based on genotype associations the 18S rRNA gene sequence. An *Acanthamoeba* specific of PCR product following the protocol established by Schroeder et al. [20] was applied; the direct sequencing of the PCR product was performed; sequences obtained was analyzed using GeneStudio Pro Software and compared with data available in the GenBank to determine genotypes of the corneal isolate.

The *in vitro* population density was monitored; particular strains were examined with the contrast phase light microscope to visualize amoebic cysts or/and trophozoites directly on wet-mount slides.

In the early adaptive phase and successively in exponential growth phase, a range of amoebae number of three or four counts, with the use of the Burker's hemocytometer and calculated for 1 ml of culture medium was determined.

The temperature assays. Fourth day following sub-culturing, one ml samples of corneal strain and *A, castellanii* Neff strain cultures were placed in 1.5 ml Eppendorf tubes containing culture medium for the temperature assays. Next, the samples of the respective cultured strains were transferred to another incubator and exposed to 37°C. Simultaneously, the respective amoebic assays were monitored and compared with those left in 26°C. The effects of the different temperature on particular *Acanthamoeba* strains were assessed following 24, 48, 72 and 96 h exposure. Range/average of overall *Acanthamoeba* number were counted and calculated for 1 ml of culture medium The amoebic strain dynamics and a percentage level of cysts were compared for particular assays.

Temperature depending changes in the

Table 1. Effect of *in vitro* exposure of *Acanthamoeba castellanii* Neff strain to the changing temperature in the exponential phase of cultivation

Time of exposure	Range/average of overall *Acanthamoeba* number ($\times 10^3$)* Range/average of cysts (%)*	
	26°C	37°C
before exposure	61.0–80.0/70.4	–
	3.1–5.6/4.7	–
24h	23.3–48.8/36.7	5.55–13.3/95.8
	0	0
	# 4.7–6.8/5.9	–
48h	64.4–76.7/70.7	1.1–2.2/1.65
	0	0
	# 0–3.1/1.02	–
72h	65.5–77.8/70.0	8.9–11.1/11.03
	0	0
	# 0–3.4/1.1	–
96h	87.8–94.4/90.7	8.1–16.7/12.1
	0	0
	–	0–40.1/13.3

*data calculated for 1 ml of culture medium; #the percentage of round forms. The level of statistical significance was set at $p<0.05$; statistically significant differences in relation to data regarding this strain cultivated at 26°C have been underlined.

population density of *A. castellanii* Neff in comparison to the corneal strain at the final phase of *in vitro* cultivation cycle were determined. Results of the investigations were analyzed statistically (ANOVA, Student-Newman-Keuls); the level of statistical significance was set at $p < 0.05$.

Results

This investigation included the specimen deriving from severe infectious keratitis case. In the initial clinical diagnosis, significant deterioration of visual acuity including symptoms of the active epithelial inflammation, corneal ulcers detected by the slit-lamp and hyper-reflective objects identified presumably as *Acanthamoeba* cysts by *in vivo* confocal microscopy were revealed in the contact lens wearer in the course of complicated, severe keratitis. Finally, the amoebic infection was confirmed by laboratory methods: *Acanthamoeba* double-walled cysts and trophozoites forming needle-like protrusions on acanthopodia were found in wet-mount slides prepared from corneal scrapings and, next from *in vitro* cultures.

The results of molecular examinations of the isolate indicated that the causative agent of the severe AK has been determined as *Acanthamoeba castellanii* T4 genotype.

The assessment of the investigated amoebae in successive growth phase of cultivation under bacteria-free condition indicated different population density of the environmental and pathogenic strains before an exposure to changed temperature. The statistically significant lower number of the amoebae was observed in the corneal *A. castelanii* T4 strain in the comparison with the environmental *A. castellanii* Neff strain exposed to the same temperature 26°C, e. g. the overall number of amoebae 4th day following sub-culturing was in range of three counts 44.4–57.8 ($\times 10^3$) and 61.0–80.0 ($\times 10^3$), respectively.

There were temperature depending differences in dynamics and viability of amoebic populations examined microscopically after 24, 48, 72 and 96h of exponential growth phase *in vitro* cultivation.

The monitoring of the corneal strain and the environmental *A. castellanii* Neff strain allowed to evaluate of morpho-physiological features of developmental stages and changes in their population densities. The comparative assessment

Table 2. Effect of *in vitro* exposure of *Acanthamoeba castellanii* T4 corneal strain to the changing temperature in the exponential phase of cultivation

Time of exposure	Range/average of overall *Acanthamoeba* number ($\times 10^3$)* Range/average of cysts (%)*	
	26°C	37°C
before exposure	44.4–57.8/48.8 2.2–12/6.2	– –
24h	25.5–33.3/29.6 0	7.8–12.2/10.4 0
48h	31.1–45.6/35.2 0	51.1–55.6/53.7 0
72h	43.3–55.6/49.3 0	62.3–76.7/70.3 0 # 0–3.3/1.1
96h	76.7–82.2/78.6 0 # 0–2.6/0.8	84.4–89.9/87.2 0

*data calculated for 1 ml of culture medium; #the percentage of round forms. The level of statistical significance was set at $p<0.05$; statistically significant differences in relation to data regarding *A. castellanii* T4 corneal strain cultivated at 26°C have been underlined.

of the both protist's strains cultured *in vitro* showed the significant reduction of the overall amoebic number 24h after exposure to 37°C (Table 1 and 2). However, during next days, the tendency maintained in environmental strain cultures only, while in corneal strain population the number of viable amoebae increased starting from 48h of the exposure to higher temperature.

It was striking that changing temperature conditions have no impact on encystation process of amoebae, although morpho-physiological changes were revealed that were expressed as more frequent appearance of rounded forms, moving slowly or motionless, without forming acanthopodia.

Effects of *in vitro* exposure of type *Acanthamoeba castellanii* Neff strain and *Acanthamoeba castellanii* T4 corneal strain to the changing temperature in the exponential phase of the cultivation are presented in Table 1 and Table 2.

The comparison of changes in the population density of *A. castellanii* Neff and *A. castellanii* T4 corneal strains at the final phase of *in vitro* cultivation cycle has been presented in Table 3.

Discussion

It is considered that *Acanthamoeba* "infecting humans or other mammals must be capable of surviving at 37°C and slightly higher body temperatures" [2]. Currently, the termo- tolerance of *Acanthamoeba* strains is studied and reported as an indirect marker of virulence/pathogenicity of

Table 3. Comparison of *A. castellanii* Neff and *A. castellanii* T4 corneal strains at the final phase of *in vitro* cultivation cycle

Strains	The overall number of amoebae ($\times 10^3$)*: range/average	
	26°C	37°C
A. castellanii Neff environmental strain	197.8–223.3/207	1.1–8.8/4.4
A. castellanii T4 corneal strain	76.7–82.2/78.8	84.4–90.1/87.6

*data calculated for 1 ml of culture medium. The level of statistical significance was set at $p<0.05$; statistically significant differences in relation to data regarding *A. castellanii* strains cultivated at 26°C have been underlined.

environmental samples; simultaneously, it has been shown that an ability of *Acanthamoeba* to grow at higher temperatures correlated with the pathogenicity of *Acanthamoeba* corneal isolates. [15–18,23]

In earlier work [23] an influence of changed temperature conditions on the *in vitro* viability of the several pathogenic *Acanthamoeba* isolates was assessed; the isolates derived from serious AK cases with various corneal symptom intensity and the response to the instituted therapy.

In the presented study, subsequent pathogenic amoebic isolate originating from human AK case poorly responding to topical anti-amoebic therapy was cultured *in vitro*, assessed at the morphological and molecular levels and compared with environmental *A. castellanii* Neff strain in terms of their *in vitro* temperature tolerance. Results of our study indicated that *Acanthamoeba* strains investigated were able to grow at 37°C that is near the temperature normal for eye (34–35°C) and for general human body (~37°C).

Although the amoebae were viable in these conditions, there was various intensity of their *in vitro* temperature tolerance expressed as different population number of particular strains of *Acanthamoeba*. It should be underlined that except of the initial reduction in the population density appearing for short time after the exposure the samples to higher temperature, the overall number of viable amoebae from corneal isolate population significantly increased in next days. The morpho-physiological characteristics of the pathogenic isolate corresponded very well to its temperature tolerance.

The *Acanthamoeba* eye infection is the serious, still poorly known treat for human health and the medical problem emerging worldwide including Poland [25–27].

The termo-tolerance correlating with the pathogenicity of *Acanthamoeba* corneal isolate confirms the opinion that adaptability of pathogenic strains to temperature changes may be one of a complex contributory factors allowing free-living amoebae to exist as facultative parasites [17,23].

The monitoring of various *Acanthamoeba* isolates *in vitro* cultured in higher temperature should be taken into account as important management for evaluation of population dynamics in terms of possible pathogenic effects. As the leading risk factor for the disease is contact lens wear and the vast majority of AK cases occur in person using the lenses, strict hygiene while using the lenses is crucial as preventive measures. In addition, increased awareness of the threat is needed for better understanding of impact of examined factors on the infection of humans with pathogenic *Acanthamoeba* strains.

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