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Original papers

Evaluation of *in vitro* effects of selected physical and chemical agents on detected in Poland *Acanthamoeba* strains – factors of increasing threats for public health

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ABSTRACT. *Acanthamoeba* keratitis, the vision-threatening corneal disease reported with increasing frequency in Poland is difficult to treat due to extremely high resistance of the amoeba cysts to chemicals. The agents of possible anti-amoebic activity are still tested. Pathogenic *Acanthamoeba* samples/isolates acquired from severe cases of keratitis examined by molecular techniques to determine genotypes, compared to one another as well as to the environmental *Acanthamoeba castellanii* Neff strain were included in the studies. These strains were *in vitro* examined in terms of their sensitivity/resistance to selected chemicals and tolerance to temperature changes. Samples of the strains cultivated *in vitro* under bacteria-free conditions were monitored during different growth phases. Higher amoebic population dynamics was observed in both pathogenic *Acanthamoeba* strains during transfer to 37°C. Agents tested influenced population dynamics in different degree; they showed amoebostatic or amoebicidal effects, however a tendency toward induction of encystment also appeared. Because activation of the dormant cysts can lead to repeated development of amoebae, very important is cysticidal efficacy of chemicals. Further *in vitro* investigations on various *Acanthamoeba* strains with different chemicals are still necessary.

Key words: corneal and environmental *Acanthamoeba* strains, *Acanthamoeba* keratitis *in vitro* dynamics, effect of chemicals, influence of temperature, amoebostatic, cysticidal effects

Introduction

Different species of amphizoic amoebae known from many parts of the world as free-living organisms are detected in a wide range of soil and aquatic habitats and in air. The amoebae are ubiquitous in natural and man-made environments. Trophozoites and cysts of *Acanthamoeba* spp. are detected in all kinds of the water: in the sea, recreational waters, fresh, tap, chlorinated water, in bottled mineral water and also in drinking water systems. They were found as contaminants on vegetables, mushrooms, fruits and in animals: healthy, diseased or dead monkeys, dogs, birds, fishes, reptiles, amphibians; the amoebae have been also isolated in different regions of Poland from natural water bodies including lakes, ponds, rivers, of the water supply system at the area of the cities, in swimming, pools and fountains [1–8]. The protists were recognized in the hospital environment as a pollution of surgical instruments, dental irrigation units, instruments, dialyzers, contact lenses and their boxes [9–11]. Some strains of these amoebae are found in various human cavities and tissues, on skin surfaces, in oral cavities, paranasal sinuses, in the brain and lungs; trophozoites and cysts of *Acanthamoeba* were also found among the microbiota of periodontal biofilms in older persons

and patients with systemic diseases. The amoebae generate a human health threat due to their pathogenic potential as facultative parasites – causative agents of serious human diseases such as fatal granulomatous amoebic encephalitis (GAE) and sight-threatening disease *Acanthamoeba* keratitis. [2,4,12–15]. This severe human eye disease is recently reported with increasing frequency in various parts of the world [16–19]; it is mainly related to contact lens wearers; the poor contact lens hygiene is considered as the most predisposing risk factor. Nonspecific symptoms of the disease, misdiagnostics, different pathogenicity of various *Acanthamoeba* species and extremely high resistance of cysts to anti-microbial and anti-parasitic drugs result in disappointing therapeutic management [20–23]. As the threats for the public health generated by these amoebae is the emerging medical problem worldwide due to increased contact lens use, various chemicals with possible activity against *Acanthamoeba* strains were tested and are still investigated, including the study with nanoparticles as novel therapeutic agents [21–27].

The aim of this study was to examine and evaluate the *Acanthamoeba* corneal strains in terms of their *in vitro* sensitivity/resistance to selected chemicals and to the tolerance to changes of temperature.

Materials and Methods

***Acanthamoeba* isolates/strains – identification and cultivation.** The material included in this qualitative retrospective analysis derived from patients who were proper diagnosed at different times after first symptoms of eye disease appeared. Corneal scrapings were acquired from contact lens wearer and from non-contact-lens wearer with a history of swimming in a lake. The patients were previously misdiagnosed and unsuccessful treated in other ophthalmic units with antibacterial and antifungal medications; they had serious pathogenic changes in their eyes and were under suspicion of *Acanthamoeba* keratitis (AK). In proper diagnosis, corneal ulcer and hyper reflective objects – *Acanthamoeba* cysts were visualized by noninvasive methods: slit lamp and the *in vivo* confocal microscopy [28]. The study was performed in accordance with the tenets of the Declaration of Helsinki.

Corneal scrapings acquired from the infected eyes were directly parasitologically examined in wet-mount slides by the light microscope; cysts and trophozoites of the amoebae have been visualized and AK confirmed. The two corneal isolates were cultured *in vitro* in sterile 15.0 ml tubes under bacteria-free conditions in BSC culture medium [29] (composed of Bacto Casitone, Difco, dissolved in water, enriched with 10% calf serum from Wytwórnia Surowic i Szczepionek, Lublin, with the addition of an aqueous solution of antibiotics: streptomycinum, penicilinum), at 26°C and regularly sub-cultured twice a month.

At the same time, the environmental *A. castellanii* Neff strain classified based on morphological criteria within the species belonging to *Acanthamoeba* group II, cultivated and monitored for years under bacteria-free conditions in the same growth medium in the Department of Medical Biology Laboratory, Medical University of Warsaw was used as the reference strain. At molecular level, based on genotype associations the 18S rRNA it has been defined according to procedure described by Schroeder et al. [30] for ATCC 30010 *A. castellanii* Neff T4 genotype. The corneal strains were also assessed based on genotype associations the 18S rRNA gene sequence. The specific detection of *Acanthamoeba* DNA by PCR techniques, analysis of PCR products, cycle sequencing were performed and sequences obtained compared with data available n the GenBank using GeneStudio Pro Software (GeneStudio, Inc., Suwanee, Georgia) to determine genotypes of the corneal isolates.

Acanthamoeba corneal and environment strains were sub-cultured twice a month and their samples regularly monitored under direct light microscope for *in vitro* population growth. Specific identification of the corneal isolate performed using molecular techniques indicated that the causative agent of severe AK course acquired from contact lens wearer and from non-contact-lens wearer are the *Acanthamoeba castellanii* and *Acanthamoeba polyphaga,* respectively, both belonging – as the *A. castellanii* Neff strain – to T4 genotype.

Dynamics of developmental forms of the amoebic strains and a range of amoeba number of three counts with the use of Bürker's hemocytometer calculated for 1 ml of culture medium was assessed during each sub-culturing and in the exponential growth phase.

After 2-4 days, sub-culturing, the assays were undertaken in terms of *in vitro* susceptibility of corneal and environmental *Acanthamoeba* strains to selected physical and chemical agent.

Effect of the temperature on *in vitro* growth of *Acanthamoeba* populations. The fourth day following sub-culturing,1ml samples of corneal and environmental *Acanthamoeba* isolates/strain were placed in 1.5 ml Eppendorf tubes containing culture medium for the temperature assays. Subsequently, 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 the room temperature, 20°C or in 26°C. The effects of the temperature on examined *Acanthamoeba* strains were assessed following 24, 48, 72 and 96 h exposure. The status of the surviving *Acanthamoeba* was determined microscopically and compared to the control cultures. The overall *Acanthamoeba* number were counted and calculated for 1 ml of culture medium. The dynamics of amoebic strain and a population growth and in vitro viability were compared for particular assays.

***In vitro* effect of selected chemicals on *Acanthamoeba* population dynamics.** In the exponential phase of the cultivation cycle, seven–eight days after current sub-culturing, samples of cultures of *Acanthamoeba* corneal strains and *A. castellanii* Neff strain were exposed to the tested chemicals and compared in terms of their *in vitro* susceptibility to these agents.

Chemicals with possible *in vitro* activity against the *Acanthamoeba* protozoans were applied. All assays were performed in sterile 1.5 ml tubes. 25 µl of the tested compound were added to the 475 µl of calibrated culture. Low to mid concentrations of chemicals non-toxic for human cells were applied; thus, inhibitory drug concentrations were not determined.

Two kind of agents were used and assessed: substances intended to the rapid disinfection and chemicals used as antiseptics and antimicrobial agents.

Among disinfectants, *in vitro* effect of alcohol-based Aerodesin®2000 (by MEDILAB) and active chlorine-releasing Medicarine (by ECOLAB GMBH GERMANY), frequently used in health facilities and laboratories on two *Acanthamoeba* corneal strains and *A. castellanii* Neff strain were examined. Samples of particular strains were microscopically examined for the overall number of surviving amoebae and population dynamics after 24 h and 48 h exposure; the *in vitro* viability of amoebae strains exposed to disinfectants were compared and analyzed.

Among antiseptics antimicrobial agents, *in vitro* effects of Octenisept - octenidine dihydrochloride (Schulke & Mayr GmbH, Germany), dissolved to a final concentration of 50µg/ml and Povidone Iodine (PI) Solutio 10% Teva (PI) (Teva Pharmaceuticals, Poland), a complex of iodine, the bactericidal component with povidone, disinfectant and preoperative antiseptic dissolved to a final concentration of 5 mg/ml on *A. polyphaga* T4 and *A. castellanii* Neff strains were examined and compared. The number of live amoebae after exposure to the agents was assessed after 24 h, 48 h, 96 h, 120 h and 144 h and anti-amoebic effects compared with the control cultures. The morpho-physiological dynamics of amoebic populations and *in vitro* viability of amoebae from both strains were monitored.

Results

The material included in this analysis originated from sever AK cases that were previously unsuccessfully treated because of improper diagnosis. Different amoeba forms were found in wet-mount microscope slides prepared directly from corneal scrapings. The final diagnosis was made/confirmed by corneal scrapings examinations in the light microscope, first directly and next with enrichment during in vitro cultivation; the isolates were assessed at cytological and molecular levels. Examinations of the corneal isolates grown *in vitro* under bacteria-free conditions in BSC culture medium revealed numerous, live trophozoites with characteristic protrusions, acanthopodia, and cysts with wrinkled ectocyst and a round endocyst developed during cultivation. The rounded, but motionless forms were also detected.

Effect of temperature changes on *Acanthamoeba* populations. The temperature influenced the population dynamics of particular *Acanthamoeba* strains. At the beginning, the cultivation *in vitro* at 26°C showed that number of live amoebae were low in the early adaptive phase and successively increased in the exponential growth phase. The evaluation of strains monitored in 20°C indicated that there was a significantly lower population density of pathogenic isolate expressed in lower overall number of amoebae e.g. in *A. polyphaga* cultured *in vitro* than the density of the *Acanthamoeba* Neff strain – 21.1–23.0 (×102) and 378.1–424.6 (×102), respectively.

Monitoring of the *in vitro* population dynamics

Table 1. Comparision of the *in vitro* effect of the temperature changes and chemicals on *Acanthoamoeba* population dynamics

Origin/identified of isolate/strain	Effect on temperature on amoebic cultures		Influence of chemicals used for rapid disinfection		Influence of chemicals applied as antiseptics	
	20°C	37°C	Medicarine	Aerodesin®2000	Octenisept	Povidone Iodine
Corneal scrapings /*A. castellanii* T4	moderate population growth	increased number of viable amoebae after 48h exposure	intense encystations, only cysts, detected after 24h exposure	low cysts number, cysticidal efficacy after 72h exposure	not examined	
Corneal scrapings/ *A. polyphaga* T4	moderate population density	increased population growth	amoebostatic effect, intense encystations	intense encystations, cysticidal effect after 72h exposure	amoebostatic effect, after 24h 63.7% after 120h 40%	amoebostatic effect, after 96h 30% after 120h 18%
Environment type/ *A. castellanii* Neff	high population density	low number of viable amoebae after 24h exposure	amoebostatic effect, intense encystations	intense encystations, only cysts, revealed after 24h exposure	amoebostatic effect, after 24h 42% after 120h 58%	amoebostatic effect, after 48h 38% after 120h 12%

at 37°C showed that the number of viable trophozoites of pathogenic corneal *A. castellanii* was higher, 46.6–78.9 (×102) in comparison to that found in the environmental strain, 3.3–7.8 (×102).

Comparison of the *in vitro* effect of the temperature on *Acanthamoeba* population dynamics is presented in Table 1. Moderate population growth was observed in pathogenic corneal strain cultures at 20°C; contrary to this, increased population growth was revealed in pathogenic corneal strain at 37°C. Simultaneously, high population density was observed in *Acanthamoeba* Neff strain cultures at 20°C. In general, high amoebic population density was observed in both pathogenic *Acanthamoeba* strains during exposure to 37°C, while the number of *Acanthamoeba* Neff was significantly decreased cultivated in the temperature. It is noteworthy that changing temperature influenced an appearance rounded forms more frequent, simultaneously no stimulation the encystation process occurred.

***In vitro* effect of selected chemicals on *Acanthamoeba* strains.** Anti-amoebic effects against *Acanthamoeba* strains were detected after different time exposure to the particular tested agents.

The disinfectants examined showed the clear influence on the *Acanthamoeba* population dynamics, however there were differences in the *in vitro* susceptibility of *Acanthamoeba* corneal strains and *Acanthamoeba castellanii* Neff strain to the chemicals. Medicarine, the active chlorine-releasing agent used for cleaning and disinfection of objects and surfaces indicated weaker anti-amoebic activity than the alcohol-based agent Aerodesin®2000. Among the corneal strains, *A. polyphaga* showed somewhat higher resistance to the chemical than *A. castellanii* strain that showed faster low population activity. It was significant that the *A. castellanii* Neff reference environmental strain indicated *in vitro* lower sensitivity to the tested agents than the corneal *Acanthamoeba* strains It should be underlined that apart from the amoebistatic effect, Aerodesin®2000 indicated expected cysticidal efficacy.

Assessment of the *in vitro* effect of antiseptics octenidine dihydrochloride on cultivated *Acanthamoeba* strains showed differences in dynamics and viability of amoebic populations. The microscopic examination of samples of the amoebic cultures revealed the reduced overall number of the viable trophozoites. The amoebostatic effect occurred both in *A. polyphaga* and *A. castellanii* Neff cultures, however after 120 h exposure, the reduction of the number of live amoebae achieved 40% in *A. polyphaga* culture while the environmental amoebic strain was more resistant; this was reflected as a decrease in the number of live amoebae to 58%.

The significant amoebostatic effect, changes in morpho-physiological status, number of trophozoites and cysts, occurred in assays with Povidone Iodine that has been revealed on both *Acanthamoeba* strains tested. It is noteworthy that the antiseptics reduced

number of viable amoebae by 83% in comparison to the control samples.

Discussion

Acanthamoeba keratitis, the vision-threatening corneal disease is detected with increasing frequency every year worldwide, also in Poland. Comparative assessment of the *in vitro* cultivated *Acanthamoeba* strains after exposure to the chemicals showed changes in the morpho-physiological status of the populations and number of trophozoites and cysts, as well as the proportion between developmental forms in comparison to the respective control cultures. There were differences in the dynamics and viability of corneal and environmental amoebic populations cultivated *in vitro* examined after exposure to particular chemicals.The tested disinfectants are used extensively in laboratories and different health facilities; they are essential for infection control practices in hospitals. *Acanthamoeba* strains spreading in natural and man-made environments are detected also in health care settings and the hospital environment on surfaces of equipment and accessories. Both chemicals used for the rapid disinfection showed some amoebostatic effects after 24 h exposure. Among the two types of disinfectants, Aerodesin®2000, the alcohol-based agent indicated cysticidal efficacy and influenced *in vitro* investigated *Acanthamoeba* strains faster and more powerfully than Medicarine containing active chlorine-releasing agent. It should be taken into consideration that prolonged *in vivo* treatment may induce encystations, an undesirable process: an activation of the dormant cysts can lead to repeated development of amoebae predisposing to disease recurrence. thus, very expected is cysticidal efficacy of chemicals.

Results of the assays with antiseptics revealed time-dependent amoebostatic *in vitro* influence of all agents on the *Acanthamoeba* strains investigated with various degrees of effectiveness. Aerodesin indicated expected cysticidal efficacy: no amoebae after 92 h exposure were detected.

Octenisept, octenidine dihydrochloride, a cationic biguanide agent that has a broad spectrum of activity is known as effective in oral hygiene preventing plaque and gingivitis; it is an antimicrobial effective against most Gram-positive and Gram-negative bacteria at low concentrations, it shows excellent bactericidal and fungicidal activity [31,32]. In present study, the antiseptics reduced overall number of the viable trophozoites by half of the level in control cultures.

Povidone iodine solution, an agent with broad antibacterial and antiviral activity applied in wound care treatment is also recommended as a contact lens care antiseptics, and for use as eye drops in some ophthalmic diseases [33–35]. In the present study, significant amoebostatic effect has been revealed on corneal and type environmental Neff strain. PI after 120 h from application reduced *in vitro* number of viable amoebae to 17% in comparison to the control samples (100%). Results of the study also revealed that povidone iodine may be promising compound against trophozoites of pathogenic *Acanthamoeba* spp.

Comparative assessment of results of the analysis showed the temperature and the strain-dependent differences in population dynamics of *Acanthamoeba* strains *in vitro* cultivated under bacteria-free conditions. Samples of the strains were monitored during different growth phases; higher amoebic population dynamics during exponential growth phase was observed in both pathogenic *Acanthamoeba* strains after transfer to 37°C while the number of the reference environmental *Acanthamoeba* Neff strain was significantly decreased cultivated in this temperature. in comparison to respective control cultures. The ability of these amoebae to grow at higher temperatures correlated with the pathogenicity of *Acanthamoeba* corneal isolates. The termotolerance is considered to be an indirect marker of virulence/pathogenicity of the amoebic strain. Results of the previous and current investigations [11,20,25,37,38] should be taken into consideration as the subsequent evidences that the adaptability of pathogenic strains to changes of temperature may be one of a complex contributory factors allowing free-living amoebae to exist as facultative parasites.

As the serious human eye disease, *Acanthamoeba* keratitis is difficult to treat among other due to extremely high resistance of cysts to antiseptics and drugs, further investigations on various agents of possible anti-amoebic activity should be performed.

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