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Original papers

Comparison of chlorhexidine disinfectant in vitro effect on environmental and ocular *Acanthamoeba* strains, the amoebic agents of human keratitis − an emerging sight-threatening corneal disease in Poland

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ABSTRACT. Small amoebae belonging to the *Acanthamoeba* genus complete their life cycles in different environmental niches as free-living protists however some of them are facultative parasites that can cause severe disease in humans. The sight-threatening *Acanthamoeba* keratitis develops in immune-competent persons, mainly in contact lens wearers; it is detected with increasing frequency along with the spread of contact lens use. The high abundance of the amoebae in the environment is important for dispersion and transmission of the infections among humans. Emerging threats for the public health generated by these amoebae is the serious medical problem worldwide. Nonspecific symptoms, similar to those occurring in the other eye diseases, diagnostic mistakes, the delay of an appropriate treatment, an exceptional high resistance of the amoebae to chemicals and drugs result in a prolonged course of the disease and often unsuccessful therapeutic management. Thus, different chemicals are still examined for their potential activity *in vitro* against various species, strains/isolates of *Acanthamoeba*. As the prolonged therapy often induces encystation subsequently leading to excystment and recurrences of amoebic keratitis, apart from anti- amoebic activity, cysticidal effect of examined agents is desirable. In the present study, results of our comparative investigations showed that cationic antiseptic chlorhexidine digluconate indicated *in vitro* anti-amoebic effect on environmental *Acanthamoeba castellanii* Neff strain and pathogenic corneal *Acanthamoeba polyphaga* T4 genotype. Amoebostatic effect of the disinfectant was expressed in reduced number of surviving amoebae in comparison to the respective control cultures; simultaneously, despite prolonged incubation with the agent no stimulation of encystation was noted. The corneal strain was more resistant to the tested compound than the Neff strain. The cysticidal efficacy of chemicals is very expected, thus further *in vitro* studies on pathogenic *Acanthamoeba* strains with different application chemicals pattern are needed.

Key words: environmental and corneal pathogenic *Acanthamoeba* strains, chlorhexidine digluconate, *in vitro* effect, *Acanthamoeba* keratitis

Introduction

In the life cycle of free-living amoebae (FLA) of *Acanthamoeba* genus, active vegetative trophozoite forming acanthopodia with characteristic spine-like protrusions develops that can transform into double-walled cyst under adverse conditions. The amoeboid protists inhabit different environmental niches and are widely distributed in many parts of the world, also in Poland [1–7].

Some strains of the free-living-exozoic amoebae are believed to be amphizoic because they are able to exist as endozoic organisms and infect and parasitize humans. Serological and molecular

studies revealed that different human populations are exposed to the non-pathogenic and pathogenic strains of *Acanthamoeba* [8–13]. Some FLA strains are facultative parasites causing *Acanthamoeba* keratitis (AK), a sight-threatening disease mainly reported as non-opportunistic disease in contact lens wearers and related to improper contact lens hygiene; the disease is detected with increasing frequency along with the spread of contact lens use [1,4,6,11–14]. A corneal epithelial injury and exposure of eye to water containing amoebic trofozoites or cysts are, apart from contact lens wear, other factors predisposing to AK. The high abundance of *Acanthamoeba* strains in the environment is important for dispersion and transmission of the infections among humans. Emerging threats for the public health generated by *Acanthamoeba* keratitis is the serious medical problem worldwide [4–7,15–18].

The proper diagnosis of the keratitis is difficult due to nonspecific symptoms, similar to those occurring in other eye diseases – in viral, fungal or bacterial keratitis. Treatment of AK is often unsuccessful because diagnostic mistakes delay an appropriate therapy [4,11,13–15]. Exceptional high resistance of the *Acanthamoeba* cysts to chemicals and drugs is mentioned as the key contributors of treatment failure and a prolonged course of the disease, thus, different chemical agents were tested, also by us, and are still examined for their potential anti-amoebic *in vitro* activity against various species, strains/isolates of *Acanthamoeba* [4,19–24].

In the present study, subsequent pathogenic *Acanthamoeba* isolate acquired and identified from complicated case of amoebic keratitis and environmental strain were investigated to assess the chlorhexidine cationic antiseptic in term of its anti-amoebic *in vitro* efficacy.

Materials and Methods

The material of corneal isolate originating from case with severe course of *Acanthamoeba* keratitis and environmental *Acanthamoeba* strain included in this study was *in vitro* cultivated in the laboratory of the Department of Medical Biology, Medical University of Warsaw, Poland.

The eye disease was initially diagnosed clinically by noninvasive methods: the slit-lamp and *in vivo* confocal microscopy. The study was performed in accordance with the tenets of the Declaration of Helsinki. Laboratory microbiology and parasitology investigations were applied to identify causative agents of the ocular disease. Molecular techniques based on associations the 18S rRNA gene sequence, analysis of PCR products, cycle sequencing were performed as described previously [19]; sequences obtained were compared with data available in the GenBank using GeneStudio Pro Software (GeneStudio, Inc., Suwanee, Georgia) to determine genotype of the individual isolate.

A. polyphaga corneal isolate and environmental *Acanthamoeba* Neff strain were cultured in BSC medium [25] enriched with 10% calf serum under bacteria-free condition at 26°C in 15 mL tubes and sub-cultured twice a month. After 7 days of culturing, during log growth phase, the overall amoebae count was $2–5\times10^4$/1mL. Samples of *A. polyphaga* corneal isolate and environmental *Acanthamoeba* Neff strain from *in vitro* cultures were exposed to the tested compound chlorhexidine digluconate disinfectant; 20% solution of chlorhexidine (Zakłady Farmaceutyczne Polfa, Łódź) was dissolved in purified water and final concentration of 0.02% (0.2mg/mL) was applied. Experiments were performed in sterile 1.5 mL Eppendorf tubes. 25 µL of the tested compound was added to the 475 µL of calibrated culture. In addition, purified water and 0.48% ethanol-solvent were added to the controls containing only the amoebae and submitted to the same procedure used for the experimental cultures. All assays were repeated twice; results were analyzed statistically (ANOVA, Student-Newman-Keuls), the level of statistical significance was set at $p < 0.05$.

Overall amoeba number calculated for 1ml of culture medium, percentage of particular stages: trophozoites and cysts were assessed; morpho-physiological status of amoebic developmental forms, particularly, *in vitro* viability of *Acanthamoeba* from different strain populations was monitored. The effect of the compound on *Acanthamoeba* strains was assessed for each strain following 24, 48, 96, and 120 h exposure and compared to the control culture count considered 100%, the results were statistically analyzed.

Results

The corneal strain included in this study, the causative agent of AK with severe course and recurrences has been identified by molecular techniques as *Acanthamoeba polyphaga* T4

Table 1. Viable amoebae from *in vitro* cultured environmental and corneal strains exposed to chlorhexidine in comparison to specimens count in the control cultures assumed as 100%

Exposure time	Live amoebae of two tested strains after exposure to chlorhexidine	
	environmental strain *Acanthamoeba castellanii* Neff	strain of corneal isolate *Acanthamoeba polyphaga*
24h	84%	74.7%
48h	67.8%	75%
96h	39.6%	68.2%
120h	30.6%	29.9%

genotype (reference strain ATCC 30871).

The comparison of chlorhexidine digluconate *in vitro* effect on environmental *Acanthamoeba* Neff strain and ocular *A. polyphaga* corneal isolate showed that the disinfectant influenced on some changes in amoebic morpho-physiological status and number of trophozoites and cysts in comparison to the respective control cultures. A clear reduction of the amoebae number was observed after 96 and 120 h exposition. Simultaneously, anti-amoebic activity of tested compound was not connected with increased encystations: the percentage number of cysts throughout the duration of the experiment was similar in both amoebic population cultured − around 0.0–3.2% .

The *A. polyphaga* eye strain was somewhat higher resistant to the tested compound than the environmental *Acanthamoeba* Neff strain. The comparison of the percentage of live amoebae from corneal *Acanthamoeba polyphaga* strain and the environmental *A. castellanii* Neff strain in the cultures grown for 7 days and next incubated with chlorhexidine digluconate is presented in Table 1.

Discussion

The sight-threatening disease *Acanthamoeba* keratitis mainly reported as non-opportunistic disease in contact lens wearers is also detected in persons not using contact lenses. The *Acanthamoeba polyphaga* species is rarely identified as an etiological agent of AK in Poland. The strain assessed by us occurred in patient with a history of swimming in a lake, not using contact lenses; a misdiagnosis, improper initial therapy and delayed appropriate treatment influenced the prolonged, severe course of the AK and recurrences.

The high abundance of the amphizoic amoebae in the environment is important for dispersion and transmission of the infections in human environment. Early diagnosis is essential for the successful prognosis. In our previous studies we showed that *in vitro* monitoring of amoebae isolated from the infected cornea may be a useful tool for verification of AK management [7,22].

In our present study, the effect of 0.02% chlorhexidine digluconate on two *Acanthamoeba* strains was tested. Anti-amoebic activity of chlorhexidine against *Acanthamoeba* strains has been already studied and this agent is currently used in the AK therapy [4,7,26–28]. The treatment pattern in this disease has been not fully yet established. An amoebic resistance to the formerly effective drugs that may develop as well as toxicity of used effective drugs concentrations to human corneal cells are very important factors influencing diagnostic difficulties and disappointing therapeutic management in AK. Additionally, contradictory results have been reported by various researchers and different views are often presented.

It is noteworthy that in our present study no encystation was induced by the tested compound; obtained results can be treated as a reference for further investigations.

It should be taken into consideration that prolonged treatment often induces encystation which is very undesirable process and may negatively influence on the recovery prognosis. Moreover, it is also known that isolates within the same genotype may differ in susceptibility to chemical agents or drugs [4,14,20]. Thus, different chemicals should be still examined for their potential activity *in vitro* against various species, strains/ isolates of *Acanthamoeba*, widely distributed in human environment agents of sight-threatening *Acanthamoeba* keratitis.

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