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Acanthamoeba polyphaga strain – causative agent of vision-threatening keratitis uncommonly detected in Poland, susceptible *in vitro* to toyocamycin

Acanthamoeba polyphaga – rzadko wykrywany w Polsce czynnik zagrażającego utratą wzroku zapalenia rogówki, podatny in vitro na przeciwpasożytnicze działanie tojokamycyny

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Abstract:

Introduction: *Acanthamoebic keratitis*, detected with increasing frequency worldwide in the last decades, mainly is reported in contact lens wearers.

Other risk factors include damage to corneal cells, eye surgery and ocular exposure to water containing developmental forms of the amphizoic amoebae. In this study we analyzed *Acanthamoeba* isolates from complicated keratitis and evaluated some corneal strains in terms of their *in vitro* susceptibility to toyocamycin.

Material and methods: Six strains of *Acanthamoebic keratitis* in contact lens wearers, one with a history of swimming in a lake, previously improperly diagnosed and treated were analyzed. *Acanthamoeba* corneal isolates of T4 genotype cultured *in vitro* were monitored.

A. polyphaga corneal strain and environmental *A. castellanii* Neff exposed to toyocamycin, the agent with an established anti-trichomonal and anti-toxoplasmal activity, were evaluated.

Results: *A. polyphaga* strain, which is not commonly diagnosed as an etiological agent of *Acanthamoeba* keratitis in Poland, was the most virulent *in vitro*. Amoebostatic effect of toyocamycin, without stimulation of encystation, was stronger for the corneal strain than for the environmental *A. castellanii* Neff.

Conclusions: There is no sufficient knowledge of the epidemiology of *Acanthamoeba* keratitis in Poland. Extremely high antimicrobial resistance of *Acanthamoeba* cysts leads to treatment failure, thus chemicals of possible anti-amoebic activity are still tested. Promising results of our study of adenosine analogue as the agent against *Acanthamoeba* pathogenic strains justifies further tests with this drug and various amphizoic amoebic strains. This was the first study of *in vitro* susceptibility of corneal *Acanthamoeba* strain to toyocamycin.

Key words: *Acanthamoeba* keratitis, *A. polyphaga* corneal strain, *in vitro* monitoring, amoebostatic activity of toyocamycin.

Abstrakt:

Cel: Pierwotniakowe zapalenia rogówki są coraz częściej wykrywane w różnych rejonach świata, głównie u osób stosujących soczewki kontaktowe. Inne czynniki ryzyka zapalenia rogówki o etiologii amebowej to uszkodzenie nabłonka rogówki, zabiegi chirurgiczne i kontakt oka z wodą zawierającą formy rozwojowe ameb amfizoicznych. W pracy analizowano izolaty rogówkowe ze skomplikowanych przypadków pierwotniakowego zapalenia rogówki, oceniono podatność wybranych szczepów na tojokamycynę.

Materiał i metody: materiał do analizy stanowiło sześć szczepów przypadków pierwotniakowego zapalenia rogówkizwiązanych z noszeniem soczewek kontaktowych oraz jeden szczep pochodzący z przypadku zakażenia podczas pływania w jeziorze, poprzednio błędnie rozpoznanych i leczonych w innym ośrodku. Monitorowano dynamikę populacji rogówkowych izolatów *Acanthamoeba* o genotypie T4 w aksenicznych hodowlach *in vitro*. Szczep rogówkowy zidentyfikowany jako *A. polyphaga* i środowiskowy *A. castellanii* Neff poddano działaniu substancji o znanej skuteczności przeciwrzęsistkowej oraz przeciwtoksoplazmowej – tojokamycyny.

Wyniki: najbardziej wirulentny *in vitro* okazał się szczep z gatunku *A. polyphaga* rzadko rozpoznawanego w Polsce jako czynnik etiologiczny pierwotniakowego zapalenia rogówki. Stwierdzono wyraźny amoebostatyczny wpływ tojokamycyny *in vitro* na obydwa szczepy, bez stymulacji encystacji, z silniejszą redukcją liczby ameb w populacjach szczepu rogówkowego.

Wnioski: w Polsce wiedza z zakresu epidemiologii zapalenia rogówki o etiologii amebowej jest niewystarczająca. Ekstremalnie wysoka oporność cyst *Acanthamoeba* na leki to jeden z powodów trudności terapeutycznych, nadal testuje się substancje o potencjalnej aktywności przeciwamebowej. Obiecujące wyniki, wskazujące na amebostatyczny wpływ tojokamycyny na patogeniczny szczep *Acanthamoeba*, zachęcają do podejmowania dalszych prób z tą substancją i z innymi szczepami ameb amfizoicznych. Według naszej wiedzy są to pierwsze badania nad podatnością *in vitro* rogówkowego szczepu *Acanthamoeba* na tojokamycynę.

Słowa kluczowe: pierwotniakowe zapalenia rogówki, szczep rogówkowy *A. polyphaga*, *in vitro* monitoring, amoebostatyczna aktywność tojokamycyny.

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Introduction

Infectious eye diseases in humans are the problem of the public health worldwide. These are often difficult to diagnose, due to their diverse etiology, which causes therapeutic difficulties. Among microorganisms, various bacterial, fungal, viral and protozoan species may be causative factors of severe visual impairment. Particularly, some *Acanthamoeba* species may be etiological agents of vision-threatening keratitis reported with increasing frequency in different regions of the world (1–5).

Amoebae of the *Acanthamoeba* genus are free-living organisms, ubiquitous in natural and man-made environments and widely distributed in many parts of the world (1–4). These protists occur in various soil and aquatic habitats: sea water, fresh water, as well as chlorinated and tap water; they have been found in drinking water systems, bottled mineral water, thermal recreational waters, swimming pools, air, air-conditioning systems, humidifiers, dust and sewage; they were also isolated from vegetables, fruits and animals. Likewise, the amoebae have been detected in hospital environment: on equipment surfaces, accessories, surgical instruments, in dialyzers, dental irrigation units, in contact lenses and their boxes (4–7).

Acanthamoeba spp. exist in two morphological forms: as active vegetative trophozoites with pseudopodia that create characteristic protrusions (spine-like acanthopodia), and as dormant forms, double-walled cysts, highly resistant to chemicals. They feed on bacteria, algae, yeasts and protozoans and develop in various environments as free-living protists, without entering human or animal organisms. However, some *Acanthamoeba* strains exist in two different modes: as free-living-exozoic amoebae and as endozoic organisms – facultative parasites able to enter and colonize human organs and multiply within them causing pathogenic effects. The amphizoic amoebae may be etiological agents of rare but fatal granulomatous *Acanthamoeba* encephalitis, an opportunistic disease developing in immune-compromised individuals; they may also be causative factors of other system disease including pneumonia and keratitis (4, 8–10).

Acanthamoebic keratitis (AK) is non-opportunistic, progressive, vision-threatening corneal disease, detected with increasing frequency in various parts of the world, including Poland; up to 90% cases have been described in contact lens wearers. The risk factors predisposing to AK include: damage to the corneal epithelial cells, eye surgery and exposure of human eye to water containing trophozoites and/or cysts of the amoebae (2, 8, 11).

The proper diagnosis of the keratitis is difficult due to non-specific clinical symptoms. For this reason, it is impossible to determine the causative agent based on the symptoms alone. Literature data and results of our studies showed that optimal material for AK diagnosis should come from corneal scrapings; laboratory analysis is needed for specific pathogen detection: by microscopic visualization of amoebae corneal scraping specimens, and *in vitro* cultures of isolates derived from these specimens (1–4). Treatment of AK is difficult and often unsuccessful, with diagnostic errors delaying appropriate treatment and extremely high antimicrobial and anti-parasitic resistance of *Acanthamoeba* cysts mentioned as the key contributors of treatment failure. *In vitro* susceptibility of various species, strains and isolates of *Acanthamoeba* to different chemical agents has been tested (12–15). Still, the optimum treatment strategy is yet to come.

Currently in Poland, there is no sufficient knowledge about epidemiology of AK and the distribution of *Acanthamoeba* strains and their genotypes. As emphasized in the literature and confirmed by our studies (1, 2, 6, 16–22), AK with photophobia, epithelial defects, severe pain, corneal ulcerations, visual impairment is diagnosed increasingly more often in Poland, especially as contact lenses become more popular. We analyzed serious cases of AK posing diagnostic and therapeutic challenge, with varied response to topical therapy, including non-contact lens wearers. The detected pathogenic *Acanthamoeba* strains represented T4 genotype believed to be the most common cause of the vision-threatening corneal disease in humans.

In the present study, subsequent pathogenic *Acanthamoeba* strains acquired and identified from complicated AK cases were analyzed. Laboratory investigations were used in order to examine the *in vitro* population dynamics of the strains and to assess their susceptibility to toyocamycin, an anti-protozoan agent.

Material and methods

The study was performed in accordance with the tenets of the Declaration of Helsinki. The material included in this analysis was collected from seven patients aged 26–43 years, with acute AK, who were referred to our hospital from other centres, following previous misdiagnosis and unsuccessful treatment with antibacterial and/or antifungal medications. Seven AK cases were retrospectively analyzed, six of them in contact lens wearers, whilst the seventh one only had a history of swimming in a lake.

Clinically, active epithelial inflammation and hyper reflective tissue in corneal ulcer were detected using a slit-lamp; hyper reflective objects – *Acanthamoeba* cysts were visualized by *in vivo* confocal microscopy (23). Laboratory microbiology and parasitology investigations were requested to identify

causative agents of the eye disease. The isolates obtained from corneal scrapings of the AK patients were initially examined with the a contrast phase light microscope to visualize cysts or/and trophozoites directly on wet-mount slides. Next, the scrapings were cultured *in vitro* in sterile 15.0 ml tubes under bacteria-free conditions in BSC calf serum-enriched culture medium (24), incubated at 26°C and regularly sub-cultured twice a month. At the same time, the environmental strain of *A. castellanii* Neff was cultivated in the same growth medium and monitored for years in the laboratory of Department of Medical Biology. All corneal strains were also assessed using molecular techniques based on genotype associations the 18S rRNA gene sequence, as described previously (22, 25). The specific detection of *Acanthamoeba* DNA by PCR techniques, analysis of PCR products, cycle sequencing were performed and sequences obtained were compared with data available in the GenBank using GeneStudio Pro Software (GeneStudio, Inc., Suwanee, Georgia) to determine genotypes of the individual isolates. The growth, viability and dynamics of strain populations cultivated *in vitro* were assessed and compared.

In the experimental part of our studies, samples of *A. polyphaga* and *Acanthamoeba* Neff *in vitro* cultures were exposed to anti-protozoan agent, adenosine analogue, toyocamycin [4-amino-5-cyano-7-(β-D-ribofuranosyl)-7H-pyrrolo(2,3-d)pyrimidine] (26–28), kindly provided by Prof. Zygmunt Kazimierczuk from the Division of Organic and Food Chemistry, Warsaw University of Life Sciences. After seven days of the cultivation cycle (log growth phase), the overall amoebae count was 2–5 x 10^4/1.0 mL. Working stock solution at the 1000.0 μM/1.0 mL concentration was prepared by dissolving the compound powder in 9.6% ethanol. Final concentrations of 10.0 μM and 50.0 μM were obtained by diluting the working stock with purified water. The concentration of ethyl alcohol in all assays did not exceed 0.48% and had no significant influence on amoebae. The effect of different compound concentrations on *Acanthamoeba* strains was assessed following 48, 96, and 144 h exposure. The cell morpho-physiology and encystation process was quantified; the resistant amoeba count was determined for each strain following the exposure to the tested compound, as detected in the exponential growth phase, compared to the control culture count considered 100%, and analysed statistically (ANOVA, Student-Newman-Keuls test, $p < .05$).

Results

Analysis of data on pathogenic isolates from acute AK cases indicated that cysts and trophozoites *Acanthamoeba* strains were found in different materials: some of them immediately in wet-mount microscope slides prepared directly of corneal scrapings, whereas others were detected after 2–7 days in cultures of material derived from the isolates. Finally, all strains were successfully cultivated under bacteria-free condition in BSC culture medium, although they differed in growth, viability and population dynamics; the live amoebae count was low *in vitro* in the early adaptive phase and successively increased in the log, exponential growth phase. There were statistically significant differences between the density of *Acanthamoeba* Neff strain and pathogenic strains in the log growth phase: viable amoebae count range of the environmental strain was distinctly higher than of clinical isolates, incubated at 26°C (10–18 x 10^4 and 1–5 x 10^4, respectively).

The comparison of cultivated strain populations *in vitro* showed differences in their viability and dynamics. All sequenced strains analyzed belonged to T4 genotype, although they showed various percentage of homology with the genotype (22); the complete results of these analyses are currently summarized and will be reported in detail in a separate publication.

The strain determined as *Acanthamoeba polyphaga* T4 genotype, the causative agent of AK with severe course and recurrences, in which hyper-reflective cysts had already been detected by *in vivo* confocal microscopy, showed the strongest population growth and viability *in vitro.* Microscopic examinations revealed live trophozoites, 10.0 to 34.0 μm in diameter, containing a nucleus with prominent nucleolus, forming pseudopodia and characteristic protrusions, acanthopodia; also,

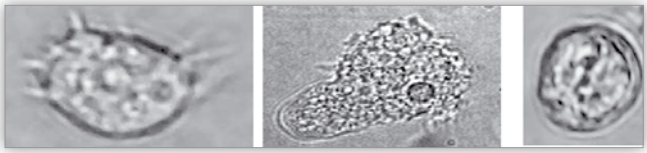

Fig. 2. *Acanthamoeba* polyphaga trophozoites and cyst detected in corneal isolates cultured *in vitro* in BSC growth medium; light micrographs of unstained preparations.

Ryc. 2. Trofozoity i cysta *Acanthamoeba* polyphaga wykryte w kulturach *in vitro* izolatów rogówkowych prowadzonych na podłożu BSC; zdjęcia z mikroskopu świetlnego – preparaty niebarwione.

Toyocamycin concentration/ Stężenie tojokamycyny	Incubation time/ Czas inkubacji	Live amoebae following exposure*/ Liczba żywych ameb po ekspozycji	
		A. castellanii	*A. polyphaga*
10 μM	48 h	100.00%	82.57%
	96 h	73.65%	99.53%
	144 h	99.04%	68.89%
50 μM	48 h	70.84%	74.31%
	96 h	62.52%	24.77%
	144 h	74.06%	24.81%

(* % of the amoebae in comparison to control cultures; amoeba count in control cultures, assessed at identical intervals as in cultures exposed to toyocamycin, was assumed as 100%.)/ (* % ameb w porównaniu do ich liczby w kulturach kontrolnych; za 100% przyjęto liczbę ameb w kulturach kontrolnych, ocenianą w takich samych interwałach czasowych jak w kulturach eksponowanych na tojokamycynę).

Tab. I. Percentage comparison of live amoebae *A. castellanii* Neff and *A. polyphaga* in specimens incubated after exposition to toyocamycin.

Tab. II. Porównanie procentowe żywych ameb *A. castellanii* Neff oraz *A. polyphaga* w próbkach inkubowanych po ekspozycji na tojokamycynę.

cyst forms sized 8.0 to 26.0 μm in diameter with two cyst walls appeared (Fig. 1). The viability of the strain was reflected in the occurrence of numerous trophozoites multiplied intensively in subsequent sub-culturing cycles during long surviving time in the BSC culture medium.

Monitoring the *in vitro* dynamics of the cultivated strains showed different effect of anti-protozoan toyocamycin on *Acanthamoeba* Neff strain and *A. polyphaga* corneal strain, which was reflected by the changed status of surviving amoebae – an appearance of rounded, alive but motionless forms more frequently in populations exposed to the tested agent than in the control cultures. Moreover, a reduction in the total number of amoebae was observed with varying intensity in particular protozoan populations exposed to different toyocamycin concentrations. At the same time, clear differences were noted in the percentage of live amoebae between environmental and corneal *Acanthamoeba* strains. Comparison of the percentage live amoebae *A. castellanii* Neff strain and *A. polyphaga* in the cultures grown for 7 days and next incubated with toyocamycin is presented in Table I.

Discussion

Epidemiology of vision-threatening *Acanthamoebic* keratitis, a distribution of *Acanthamoeba* strains and their genotypes in human environments differ from country to country and between regions. The cases of keratitis caused by the protozoans, relatively rare in populations of several countries, have been constantly increasing during the last few decades. It is underlined (1, 2, 9, 10) that incidence rate of AK does not reflect a geographical distribution of the *Acanthamoeba* species, and is most likely linked to variations in extended contact lens wear. At present, the awareness and knowledge on AK as serious corneal disease is still insufficient (2, 4).

In Poland, there is still insufficient data on human eye infections caused by *Acanthamoeba*. Recently, it is not exactly known whether the infections are really so infrequent or just misdiagnosed as viral infection caused by *Herpes simplex*, bacterial infection caused by *Pseudomonas aeruginosa*, or fungal infection caused by *Fusarium* spp. due to inadequate awareness of pathogenic role this amoeba plays as an etiological agent in human keratitis. Although an epidemiology of AK remains poorly investigated, the human disease caused by pathogenic *Acanthamoeba* strains has been reported with increasing frequency (4, 6, 16–22); it is emphasized that misdiagnosis delaying appropriate treatment may contribute to a prolonged, severe course of AK and result in serious vision deterioration.

Following the increasing number of *Acanthamoeba* isolates detected in different regions, recently, 18 or 19 genotypes have been distinguished among environmental and clinical *Acanthamoeba* isolates based on genotype associations using the18S rRNA gene sequence method of classification. Over 90% of reported AK cases have been linked to the T4 genotype. At the same time, pathogenic strains described in different studies were diagnosed morphologically as belonging to similar species, e.g. *A. castellanii, A. polyphaga* (12, 17, 29–31).

Monitoring cultivated strains as a part of this analysis revealed the strongest growth and viability *in vitro* of *A. polyphaga* population of the corneal strain obtained from the person with a history of swimming in a lake, not using contact lenses. This amoebic species is rarely identified as an etiological agent of AK in Poland. Moreover, misdiagnosis, improper initial therapy and delayed appropriate treatment all contribute to the prolonged, severe course of the AK and recurrences. Interestingly, there was a correlation between the AK's severity and strong *in vitro* viability of *A. polyphaga*. This confirms that monitoring population dynamics of amoebae isolated from the infected cornea and axenically cultivated *in vitro* is useful for both accurate diagnosis and predicting treatment efficacy in AK (4, 19).

Acanthamoeba keratitis is very difficult to treat due to antimicrobial resistance of trophozoites and cysts. Extremely high resistance of *Acanthamoeba* cysts to disinfectants, chemicals, anti-microbial and anti-parasitic drugs is one of the key contributors of treatment failure (1, 2, 4, 13).

Many chemicals have been examined and are still tested for their potential activity *in vitro* against various species, strains/ isolates of *Acanthamoeba*, however, a common problem is anti-drug resistance. Additionally, new drugs used *in vitro* are often effective in concentrations toxic to human corneal cells (1, 2, 12–15). Furthermore, prolonged treatment often induces encystation, which is very undesirable as it may subsequently lead to excystment, development of trophozoites and recurrences of disease, which adversely affects treatment outcomes. Thus, chemicals examined for their anti-amoebic activity are expected to exert cysticidal effect. Up to now, there have been no established standards of amphizoic amoebae susceptibility testing to chemical agents. In the experimental part of the present study, toyocamycin, the agent with an established anti-trichomonal and anti-toxoplasmal efficacy (26–28) was tested for its possible anti-amoebic activity. The adenosine analogue derived from *Streptomyces toyocaensis* blocks the RNA synthesis and ribosome function.

The dynamics of *A. polyphaga* and *Acanthamoeba* Neff strain was monitored i*n vitro* in term of susceptibility/resistance of the amoebae to the anti-protozoan agent. The comparative evaluation of the dynamics showed that toyocamycin altered the status of surviving amoebae and had a clear amoebostatic effect *in vitro* on populations of both strains. Particularly, trophozoite forms showed high response to toyocamycin. The reduction of viability of amoebae exposed to this agent was expressed by the decrease of their total count; this effect was more pronounced in *A. polyphaga* corneal strain. The intensity of the amoebostatic effect depended on toyocamycin concentration; moreover, despite prolonged monitoring of population dynamics, we did not observe any signs of stimulated encystation *in vitro*.

The results of our study on *in vitro* effect of toyocamycin on the amphizoic amoebae: environmental *A. castellanii* Neff and corneal *A. polyphaga* strain, were preliminarily abstracted and presented during 12th ISOPT Clinical, Berlin, Germany on July 9–12, 2015 (32), pointing to toyocamycin as a promising agent against pathogenic *Acanthamoeba* strains.

To the best of our knowledge, this is the first study of *Acanthamoeba* susceptibility to the anti-protozoan agent, toyocamycin, *in vitro*. Further studies in different clinical strains, with a modified agent application pattern *in vitro* may be useful to determine the actual anti-amoebic activity of the tested com-

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pounds against *Acanthamoeba* strains, etiological agents of vision-threatening keratitis.

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