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Original papers

Amoebicidal or amoebostatic influence of disinfectants used in health facilities and laboratories on corneal strains of *Acanthamoeba*

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ABSTRACT. Different *Acanthamoeba* species are amphizoic organisms distributed in wide range of habitats in natural and man-made environments; they are also detected on surfaces of equipment and accessories in health facilities. Some strains of the amoebae are causative agents of the vision-threatening human disease *Acanthamoeba* keratitis, mainly reported in contact lens wearers. An exceptional high resistance of *Acanthamoeba* trophozoites and particularly cysts to chemicals, disinfectants and drugs is believed as influencing difficulty resulting in unsuccessful therapeutic management. As *Acanthamoeba* keratitis is the serious medical problem worldwide, different chemicals with possible activity against environmental and clinical *Acanthamoeba* strains are tested. In our study, selected disinfectants used in health care settings and laboratories were tested and their efficacy against the corneal strains *Acanthamoeba castellanii* and *A. polyphaga* , and environmental *A. castellanii* Neff strain was assessed. Comparative assessment of results of the assays show that, apart from amoebistatic effects, the disinfectants indicated expected cysticidal efficacy.

Key words: *Acanthamoeba* strains, *in vitro* dynamics, influence of disinfectants

Introduction

The amphizoic *Acanthamoeba* strains belong to organisms that complete their life cycles as free-living protists in the outer environment, and in predisposing circumstances may enter and colonize human body and exist as parasites. The amoebae are ubiquitous in natural and man-made environments in various parts of the world, including Poland [1–6]. It has been revealed by serological and genotypic studies that different human populations are exposed to both, the non-pathogenic and pathogenic *Acanthamoeba* strains [5–9]; the latter strains have been recognized from skin surface, oral cavity, paranasal sinuses, lungs and brain as causative agents of the rhino-sinusitis, skin inflammation, skin ulceration and pneumonia. The amoebae have been detected also in health care settings and the hospital environment on surfaces of different equipment and accessories [10–16].

Particularly serious threats for human health present trophozoites and/or cysts of some *Acanthamoeba* species as causative agents of fatal granulomatous amoebic encephalitis (GAE) developing in immune-compromised individuals [1,10–12] and also *Acanthamoeba* keratitis (AK). The vision-threatening disease occurs in immune-competent individuals and is mainly reported as non-opportunistic disease in contact lens wearers; more than 90% of cases is linked with the T4 genotype [7,17–23].

Recently, the use of contact lenses has risen and incidents of an acute AK are constantly increasingly recognized. The therapy applied in this devastating eye disease, including loss of the visual acuity, is often unsuccessful and therapeutic results disappointing. Diagnostic mistakes that cause delay of an appropriate treatment may result in a prolonged, severe course of AK and vision deterioration.

An exceptional high resistance of *Acanthamoeba* trophozoites and particularly cysts to chemicals, disinfectants and drugs is believed as mainly influencing difficulty resulting in unsuccessful therapeutic management. The emerging threats for the public health generated by these amoebae is the serious medical problem worldwide. For this reason, different chemicals with possible activity against various environmental and clinical *Acanthamoeba* strains were tested and are still examined [17,22–31]; standards to test a susceptibility of the facultative parasitic amoebae to chemical agents are yet expected.

In the present work, selected disinfectants used in health care settings and laboratories were tested and compared in terms of their *in vitro* efficacy against several *Acanthamoeba* strains, the causative agents of the vision-threatening *Acanthamoeba* keratitis.

Materials and Methods

***Acanthamoeba* strains examined.** Corneal isolates originating from patients with severe course of *Acanthamoeba* keratitis (AK), diagnosed initially by noninvasive methods: the slit-lamp and *in vivo* confocal microscopy, and next by corneal scrapings examinations in contrast phase light microscope to visualize cysts or/and trophozoites of the amoebae were *in vitro* cultivated in the laboratory of the Department of Medical Biology, Medical University of Warsaw, Poland. The isolates were also assessed for the specific identification at cytological and molecular level based on genotype identification of the 18S rRNA gene sequencing, according to procedure described previously [5,31].

The amoebae maintained in 1.5 ml Eppendorf tubes, each containing 1 ml BSC culture medium [10] enriched with 10% calf serum were grown under bacteria-free condition – in the absence of external live food organisms. Simultaneously, all strains were also grown in parallel cultures containing liquid PYG medium [17,25] with an addition of Antibiotic Antimycotic Solution (Sigma-Aldrich). All *in vitro Acanthamoeba* cultures were carried out in 26°C and subcultured twice a month. Seven days after current subculturing, the assays were undertaken for assessment of anti-amoebic influence of selected disinfectants used in health facilities and laboratories. In this study, three strains of T4 genotype: two isolated of AK patients – *Acanthamoeba castellanii* and *A. polyphaga* – and one environmental – *A. castellanii* Neff strain were tested and compared in terms of their *in vitro* susceptibility to selected chemicals.

Assays for testing in vitro influence of selected disinfectants on *Acanthamoeba* strains. For these assays, in exponential growth phase of the amoeba cultivation cycle, 8th day following subculturing all cultures maintained in 26°C were vigorously vortexed for 20 sec. and 10 µl samples of each strain taken for the assessment at baseline density of the amoebae. A range of protozoan number of two or three counts, with the use of the counting chamber, Burker's hemocytometer and calculation for 1 ml of culture medium, was determined. Next ~1ml strain cultures were exposed to selected chemicals; simultaneously, the respective control amoebic assays without disinfectants were assessed.

Two types of disinfectants frequently used in health facilities and laboratories were examined *in vitro*. Aerodesin®2000 (distributed by MEDILAB) containing propan-1-ol, ethanol (denatured) and glutaral is alcohol-based agent for the rapid disinfection of medical devices. Medicarine (distributed by ECOLAB GMBH GERMANY) containing sodium dichloroisocyanurate is active chlorine-releasing agent used for cleaning and disinfection of objects and surfaces.

The viability and dynamics of each particular strain populations were microscopically examined after 24 h and 48 h from exposure. Then, second portion of the same disinfectant was applied and the changes in population dynamics of the investigated *Acanthamoeba* strains after 24 h and 48 h from the second application (72 h and 92 h from the first application) were determined. The overall number of surviving amoebae in different growth media was counted, a percentage content of cysts, the ability of amoebae to multiply *in vitro* after exposition to selected disinfectants, as well as differences in comparison to respective control cultures, were assessed. All assays were performed at 26°C,

Table 1. Overall number of amoebae and percentage of cysts before exposure *in vitro* cultured *Acanthamoeba* strains to disinfectants

Acanthamoeba strain	Culture medium	Range of amoebae number ($\times 10^3$)*	Range of cysts (%)
A. castellanii	BSC	85.55–103.3	1.30–1.35
	PYG	102.22–111.1	4.35–5.00
A. polyphaga	BSC	94.40–104.4	1.20–3.2
	PYG	65.55–72.22	1.69–4.62
A. castellanii Neff	BSC	75.55–83.33	1.1–2.5
	PYG	83.33–94.4	3.8–6.1

* calculated for 1 ml of culture medium

repeated twice and results were analyzed statistically (ANOVA, Student-Newman-Keuls method). The level of statistical significance was set at $p < 0.05$.

Results

The evaluation of *Acanthamoeba* strains of T4 genotype examined in the study: the corneal isolates of *Acanthamoeba castellanii* and *A. polyphaga*, and environmental *A. castellanii* Neff strain showed that the number of live amoebae was low in the early adaptive growth phase and successively has increased in the exponential phase while the amoebae multiplied and increased density of strain populations. Although this general dynamics was independent from kind of culture medium, in the control assays, differences in a density of amoebae strains maintained on BSC and PYG medium were visible in overall number of amoebae and the percentage content of cyst forms.

Overall number of amoebae and percentage of cysts before exposure the *in vitro* cultured *Acanthamoeba* strains to disinfectants is presented in Table 1.

In the control assays, environmental *A. castellanii* Neff strain presented statistically significant ($p<0.05$) higher amoebic density in comparison to *A. castellanii* corneal strain cultured in the same BSC medium (in range calculated for 1 ml of the control culture $1111.1–1222.8\times10^2$ vs. $822.2–991.4\times10^2$, respectively)**.** Simultaneously the strains differed in the control assays also in percent of cysts (in range 1.7–9% vs. 2.6–11.7%, respectively).

With the sensitivity to disinfectant tested, a variability in strain reactivity was observed.

Proportion of *Acanthamoeba* developmental forms changed already after 24 h from the first application of the disinfectant. After an exposure of *A. castellanii* Neff to Medicarine, this strain showed statistically significant decrease in overall number of amoebae (to $222.2–300.0\times10^2$ in BSC cultures and up to $55.6–77.8\times10^2$ in PYG cultures). Most of the amoebic populations constituted cysts (77.8–80% in BSC cultures and 83.3–100% in PYG cultures).

In the *A. castellanii* corneal strain cultures, no trophozoite forms were revealed already after the first exposition to Medicarine, only cysts were detected. The number of the dormant forms decreased during successive days of exposure to the chemical agent.

Comparative assessment of the amoebae after exposure to Aerodesin®2000 indicated high sensitivity to the disinfectant of both, environmental and corneal *Acanthamoeba* strains examined *in vitro*. *A. polyphaga* indicated somewhat higher resistance to the chemical than *A. castellanii* corneal strain that showed particularly low population activity already after the first application of Aerodesin®2000. It was expressed in decreasing amoebic numbers constituted only with cyst forms; moreover, no cyst were revealed independent on the culture medium after 48h from exposure to this disinfectant.

Among the two types of disinfectants, Aerodesin®2000, the alcohol-based agent used for the rapid disinfection of medical devices, influenced *in vitro* examined *Acanthamoeba* strains faster and more powerfully than Medicarine used for cleaning and disinfection of objects and surfaces containing active chlorine-releasing agent.

Overall number of surviving amoebae and percentage of cysts after exposure of the *in vitro* cultured *Acanthamoeba* strains to selected disinfectants are presented in Table 2.

Discussion

Preventive measures undertaken to control the

Table 2. Overall number of surviving amoebae and percentage of cysts after exposure *in vitro* cultured *Acanthamoeba* strains to disinfectants

Acanthamoeba strain	Disinfectant	Culture medium		Range of amoebae number ($\times 10^2$) *24h/*48h **24h/**48h	Range of cysts (%) *24h/*48h **24h/**48h
A. castellanii	MEDICARINE	BSC	*	55.6–88.9/89.0–111.1	100/100
			**	22.2–33.3/11.1–22.2	100/100
		PYG	*	100.0–133.3/77.8–88.9	80.0–90.9/88.9–100
			**	22.2–44.4/11.1–22.2	100/100
	AERODESIN 2000	BSC	*	11.1–22.2/22.2–44.4	100/100
			**	0/0	0/0
		PYG	*	11.1/0	100/0
			**	0/0	0/0
	Control assays	BSC	*	822.2–888.9/766.7–877.8	8.1–10/2.6–6.9
			**	855.6–944.4/900.0–991.4	10.4–11.7/8.7–11.4
		PYG	*	911.1–977.8/855.5–922.2	4.6–7.1/2.6–3.6
			**	922.2–1044.4/1255.5–1311.1	6.0–8.4/9.7–10.2
A. polyphaga	MEDICARINE	BSC	*	166.7–222.2/177.8–211.1	65–100/89.7–93.7
			**	122.2–144.4/111.2–122.2	84.6–90.9/85.7–90.9
		PYG	*	55.6–77.8/33.3–55.6	100/100
			**	11.1–33.3/22.2–44.4	100/100
	AERODESIN 2000	BSC	*	177.8–199.9/77.8–99.9	100/100
			**	44.4–55.6/33.3–44.4	100/100
		PYG	*	22.2–44.4 /11.1–22.2	100/100
			**	0/0	0/0
	Control assays	BSC	*	755.5–911.1/766.7–955.6	10.3–10.9/4.3–5.8
			**	855.5–977.7/1099.9–1166.6	19.5–20.5/17–18.2
		PYG	*	466.7– 677.7/388.8–455.5	9.10–12.5/22.8–24.4
			**	1077.8–1211.0/1322.2–1400.0	7.20–8.2/6.7–7.9
A. castellanii Neff	MEDICARINE	BSC	*	222.2–300.0/244.4–311.1	77.8–80/89.3–95.4
			**	133.3–166.6/111.1–155.6	80.0–83.3/83.3–88.8
		PYG	*	111.1–122.2/177.8–199.9	83.3–100/100
			**	166.7–188.9/133.3–166.6	100/80 -83.3
	AERODESIN 2000	BSC	*	100.0–155.6/66.7–88.9	88.9–100/71.4–85.7
			**	33.3–44.4/22.2–33.3	100/100
		PYG	*	22.2–44.4/66.7–77.8	100/100
			**	66.7–88.9/22.2–33.3	100/100
	Control assays	BSC	#	755.5–833.3	1.7–2.7
			#	1111.1–1222.8	8–9
		PYG	#	733.3–811.1	12–13.7
			#	611.1–800	9–11

* after the first application of disinfectants; ** after the second application of disinfectants;
# control assays performed parallel within several days in the exponential and stationary growth phases.

population of microorganisms that can potentially colonize human organism and cause infections are the most effective way to diminish this threat. Disinfectants are used extensively in laboratories and different health facilities and are also essential for infection control practices in hospitals. Amphizoic amoebae of *Acanthamoeba* genus spreading in natural and man-made environments have been detected also in health care settings and the hospital environment on surfaces of equipment and accessories. The amoebae might play a role in hospital – acquired infections by facilitating transmission of bacteria to the patients [14,17] .

The most frequent infection associated with *Acanthamoeba* spp. is amoebic keratitis AK, first described in 1974. This is a particularly difficult to

treat ocular infection that leads to trauma and corneal necrosis [16]. Despite the growing threat for the public health generated by these amoebae as the causative agents of vision-threatening AK, no international standard exists for efficacy testing of disinfectants against *Acanthamoeba* strains.

Disinfectants are chemical agents, usually broad spectrum, that inactivate microorganisms and are used on objects or surfaces. Various cellular structure, composition and physiology of particular types and developmental stages of microorganisms influence their response to disinfectants [26]. The anti-microbial activity of chemical agents may express in an inhibition of growth that result in bacteriostatic, fungistatic, and sporistatic, and/or sporicidal, virucidal, bactericidal and cysticidal effects.

The both types of disinfectants tested in this study differ in their active agents, however exhibit similar broad-spectrum antimicrobial activity involving against Gram-positive and Gram-negative bacteria, Mycobacteria, fungi, viruses. Among these chemicals, Aerodesin®2000, the alcohol-based agent used for the rapid disinfection of medical devices, influenced *in vitro* examined *Acantha - moeba* strains faster and more powerfully than Medicarine used for cleaning and disinfection of objects and surfaces, containing active chlorine-releasing agent. Comparative assessment of results of the assays show that the tested disinfectants apart from the amoebistatic effect, indicated expected cysticidal efficacy.

It is emphasized that extremely high resistance of *Acanthamoeba* cysts to chemicals, anti-microbial and anti-parasitic drugs is the main factor resulting in disappointing therapeutic management. The encystation process is essential for survival of parasites under unfavorable conditions such as low temperatures, extreme pH changes, starvation, lack of food, high cell density. As the process is associated with resistance to chemicals, blocking the encystation can potentiate the efficacy of chemotherapeutic agents and disinfectants. For this reason, a cysticidal activity is very expected from disinfectants used in different health facilities and laboratories.

Many chemical agents were tested *in vitro* for their anti-amoebic activity and various *Acanthamoeba* strains of different pathogenicity were investigated for their susceptibility to biocides and disinfectants. As contradictory results reported by particular researchers and different views are often presented, further studies on various *Acanthamoeba* isolates/strains are necessary to reduce spreading the pathogenic strains of the amoebae within human environment, especially in health care settings.

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