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Original papers

Assessment of *in vitro* dynamics of pathogenic *Acanthamoeba* strains originating from contact lens wearers with infectious keratitis

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ABSTRACT. Recently, incidents of *Acanthamoeba* keratitis, the vision-threatening eye disease, are reported with increasing frequency worldwide, particularly in contact lens wearers. In our study, the retrospective assessment of *in vitro* dynamics of subsequent pathogenic *Acanthamoeba* isolates cultured at 24°C, detected in Polish contact lens wearers with keratitis is presented and results compared with those of environmental *A. castellanii* Neff strain. There were delayed the proper diagnosis that influenced prolonged and severe course of this eye disease and treatment difficulties. The corneal material was examined directly to visualize developmental amoeba stages for diagnose verification, microbiologically tested for the specific identification of bacteriae and fungi, and *in vitro* grown in culture medium in temperature 24°C. Among twenty-six keratitis incidents analyzed, eleven were cases of *Acanthamoeba* keratitis; in the six of them, *Acanthamoeba* strains and concomitant bacterial and/or fungal infectious agents were detected. *In vitro* assays showed variability in population density of several clinical strains in the exponential growth phase expressed in various range of overall amoeba number and different proportion between trophozoites and cysts. The clear influence of temperature on the *in vitro* cultivation of the amoebae was observed: statistically significant lower population dynamics was revealed by most of pathogenic clinical isolates in comparison with those showed by environmental strain. The *in vitro* monitoring of dynamics of *Acanthamoeba* strains isolated from infected eyes may be helpful for diagnostics verification, especially in mixed infectious keratitis.

Key words: pathogenic *Acanthamoeba* spp. *Acanthamoeba* keratitis, *in vitro* dynamics

Introduction

Different strains of *Acanthamoeba* spp. are widely distributed in natural and man-made environments of many parts of the world, also in Poland [1–6]. The amoebae are known as free-living organism that complete their life cycles in the outer environment and exist in two morphological stages: as active vegetative trophozoites with characteristic acanthopodia and as double-walled dormant cysts that develop after a growth phase, and under harsh conditions. The protists occur in soil and dust, are found in sea, fresh, tape water, drinking water systems and in swimming pools, air conditioning systems, and humidifiers; they also occur on fruits and vegetables, and in animal bodies. The amoebae have been recognized in the hospital environment as contaminants of surgical instruments and dental irrigation units [6–11].

In predisposing circumstances, some strains of *Acanthamoeba* spp. may enter human bodies, colonize different organs, thus exist as parasites causing pathogenic effects: they are found in various human cavities and tissues, on skin surfaces, in paranasal sinuses, oral cavities, lungs, and brain [9–19]. The protists are called amphizoic amoebae

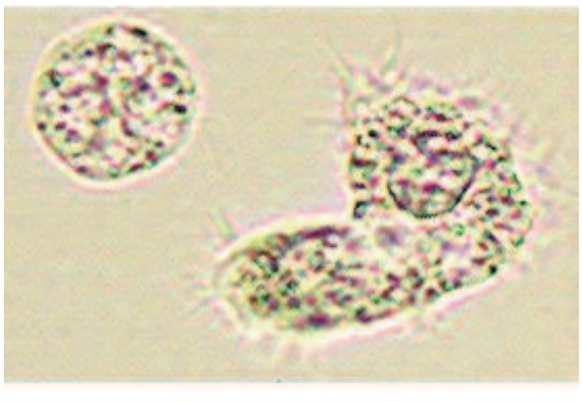

Fig. 1. Light micrograph of the *Acanthamoeba* strain in BSC growth medium. Note: acanthopodia of trophozoite (range 8–40 µm), polygonal wall of cyst (range 6–30µm).

as they are able to exist in two modes: as free-living-exozoic microorganisms and as endozoic parasites.

The pathogenic potential of the amoebae may result in a serious human health threat [12,13,20–25] Some *Acanthamoeba* strains are causative agents of granulomatous amoebic encephalitis (GAE), rare but fatal opportunistic disease developing in immune-compromised persons. Other infection caused by the amoebae is a vision-threatening *Acanthamoeba* keratitis (AK), a non-opportunistic disease that occurs in immune-competent persons, particularly in contact lens wearers [19–28].

During the last years, in different regions of world increases popularity of the contact lens use, and also the frequency of AK incidents. The treatment of AK is often unsuccessful due to, among others, an exceptional high resistance of *Acanthamoeba* cysts to different chemical agents, disinfectants and drugs; also, misdiagnoses of the disease as caused by viral, bacterial, fungal agents result in a delay in the appropriate treatment and deterioration of the vision.

In our earlier multidisciplinary studies, we analyzed, among others, cases of AK difficult to diagnose and treat, variable in the corneal symptom intensity and in response to topical therapy. Diagnosed pathogenic *Acanthamoeba* strains were evaluated in terms of their susceptibility to selected chemical and physical agents, e.g. a viability in changed temperature conditions [23,26,28].

In the present study, we assessed *in vitro* dynamics of subsequent *Acanthamoeba* isolates detected in contact lens wearers with AK complicated by concomitant bacterial and/or fungal infectious agents.

Materials and Methods

Data from twenty-six patients, reported to our hospital in 2013–2016 at different time after first symptoms of keratitis appeared, most of them previously unsuccessfully treated in other units with antibacterial and/or antifungal medications were retrospectively analyzed in this study. In the clinical assessment/verification of diagnosis, noninvasive methods: the slit-lamp and *in vivo* confocal microscopy were applied. The corneal material deriving from the cases was directly examined in wet-mount slides with the aid of a contrast phase light microscope, 100× and 400× magnifications to visualize *Acanthamoeba* cysts or/and trophozoites. Samples of corneal scrapings collected from affected eyes were also routinely tested with microbiological techniques for the specific identification of bacteriae and fungi, and by PCR technique. Simultaneously, isolates deriving from corneas were *in vitro* grown in sterile 15 ml tubes under bacteria-free condition in BSC medium [12] enriched with calf serum, incubated at 24°C and sub-cultured into this growth medium twice a month. Finally, among twenty-six keratitis incidents, eleven were cases of AK; data from the six, in which *Acanthamoeba* strains and concomitant bacterial and/or fungal infectious agents were detected in the corneal material, have been included in this assessment.

The dynamics of particular pathogenic *Acantha - moeba* strains *in vitro* parallel cultivated was monitored and compared to one another, as well as to the environmental *Acanthamoeba castellanii* Neff strain maintained by years in the same growth medium in the laboratory of Department of Medical Biology. Changes in amoeba number and in morpho-physiological status of particular stages were assessed. A range of amoeba overall number and cysts of two or three counts, with the use of counting chamber, Burker's hemocytometer and calculation for 1ml of culture medium was determined for the log growth phase of *Acanthamoeba* pathogenic strain populations, 5-7th day following sub-culturing; results were analyzed statistically (ANOVA, Student-Newman-Keuls method, $p < 0.05$).

Results

Clinical symptoms of *Acanthamoeba* keratitis with redness, photophobia, excessive tearing, pain, a visual acuity deterioration occurred with different

Table 1. Compilation of data of pathogenic *Acanthamoeba* strains detected in material of affected eyes of contact lens wearers with infectious keratitis

Strain	Detected amoebae*		Range of overall amoeba number** (x10^3)	Range of cysts**(%)	Concomitant infectious agent
	stages	material			
S2	cysts, trophozoites	corneal scrapings	12.1–27.0	0.9–1.0	*Candida* spp. *Citrobacter* spp.
S16	trophozoites	*in vitro* culture	36.0–82.0	0.8–1.0	*P. aeruginosa*
S19	cysts	corneal scrapings	55.5–105.5	0–0.3	*E. faecalis*
S21	cysts	corneal scrapings	20.2–34.1	0–0.2	*Candida* spp.
S24	cysts	corneal scrapings	7.5–17.0	0.3–0.5	*E. cloacae, P. aeruginosa*, *K. oxytoca*
S25	cysts	corneal scrapings	2.0–4.0	0–0.2	*Fusarium* spp.
SNeff	–	*in vitro* culture	37.8–42.5	0–0.3	–

*the stage and material in which this *Acanthamoeba* strain the first time was detected. **data from log growth phase of *Acanthamoeba* population. The level of statistical significance was set at $p < 0.05$

intensity in eleven affected eyes of contact lens wearers from which the assessed material was acquired. Active epithelial inflammations, corneal ulcers, characteristic ring-like stromal infiltration detected by slit-lamp, and hyper reflective objects, presumably cysts were revealed in some cases by *in vivo* confocal microscopy.

Results of direct corneal scrapings examinations and *in vitro* cultivations of material deriving of these scrapings confirmed *Acanthamoeba* infections in the eleven cases.

In six of the AK incidents, the microbiological examinations of the corneal material revealed concomitant infections with bacteriae and/or fungi: Gram positive bacteria strains *Enterococcus faecalis*, Gram-negative bacteriae *Klebsiella oxytoca*, *Pseudomonas aeruginosa*, *Enterobacter agglomerans*, *E. cloacae*, fungi of *Candida* spp., *Fusarium* sp.

Assessment of pathogenic *Acanthamoeba* strains cultured *in vitro* at 24°C showed the occurrence of different amoeba stages developed during cultivation: live trophozoites with characteristic acanthopodia and polygonal or round cysts with their two cyst walls (Fig. 1).

Variability in population density of several strains that was revealed in the exponential growth phase was expressed in various overall amoeba number and in different proportion between trophozoites and cysts. Moreover, statistically significant differences occurred between the clinical strains of *Acanthamoeba* and the *A. castellanii* Neff strain in the population dynamics: the range of overall amoeba number of the environmental strain were significantly higher in comparison to that found in four strains originating from AK incidents.

Molecular examinations of the pathogenic strains and a comparison of the obtained sequences with those available in GenBank showed 98–100% homology with isolates belonging to the T4 genotype; details of these examinations will be reported in a separate publication.

The compilation of data of pathogenic *Acanthamoeba* strains detected in material of affected eyes of contact lens wearers with infectious keratitis and of *A. castellanii* Neff strain is presented in Table 1.

Discussion

Different free-living amoebae of *Acanthamoeba* sp. are ubiquitous and widely distributed in natural and man-made environments worldwide. Potentially pathogenic amoeba strains that have been detected in natural water bodies including

lakes, ponds, rivers, in swimming pools, fountains, in northern and western regions of Poland represented mainly T4 genotype considered to be the most common cause of the vision-threatening eye disease [1,7,8,21,25–36].

Human eye infection with facultative parasitic strains of *Acanthamoeba* sp., mainly related to improper contact lens hygiene, is a serious medical problem as an emerging threat for the public health.

The clinical picture of *Acanthamoeba* keratitis, the sight-threatening, usually progressive corneal disease, is similar to that occurring in viral, fungal or bacterial keratitis. In addition, the amoebae may act as carriers for more than 20 bacterial species and other microorganisms pathogenic for humans; many of them are able not only to survive but even proliferate within the amoebae, thus may be causative agents of secondary/concomitant or mixed infections [27–29]. It is why clinical manifestations alone are not sufficient to identify a causative agent of the keratitis. In our earlier study, etiologically complex keratitis were revealed in more than 50% cases [26,36].

Data of subsequent infections with amoebae regarding persons in other units treated unsuccessfully with antibacterial and antifungal medications were included in our present study. Results of this retrospective assessment showed that AK was identified in all eleven patients; however, there were mistakes in the initial diagnosis. Finally, in some of six cases in which mixed AK bacterial/fungal infections were revealed, the proper diagnosis performed two weeks after the first clinical symptoms appeared, in others – even more than four weeks; thus in these cases, delay in the appropriate treatment occurred.

It should be underlined that co-infections with other microorganisms may complicate the diagnosis, course and treatment in the AK. Literature data and results of our investigations indicated that microscopic visualization of amoebae in slides prepared directly from corneal scraping is usefulness for AK diagnostics, however, it has limited value at low intensity of amoebic infections.

The temperature 24°C, in which *Acanthamoeba* strains were *in vitro* cultivated, influenced in different degree on dynamics of several amoeba populations assessed. It was observed that more intensive growth/multiplication occurred in cultivated environmental *A. castellanii* Neff strain than in most of clinical strains of *Acanthamoeba* originating from human corneas. It is believed that the ability of amfizoic amoebae to accept higher temperatures is considered as an indirect marker of potential pathogenicity of *Acanthamoeba* strains [23,29].

Laboratory examinations of *in vitro* dynamics and population density of specimens from corneal isolates allow to identify directly the facultative pathogens and to verify previous misdiagnoses; it confirms the consideration that culture methods are the gold standard in AK diagnosis. In the cases analyzed in the current study, the diagnostics difficulties were due to unspecific clinical symptoms, misdiagnosis, resulting in delay of proper treatment; in addition, a resistance to antimicrobial and anti-parasitic therapy may occur.

Conclusions

It should be taken into consideration that an awareness and knowledge about AK – the serious, an emerging, vision-threatening eye disease are still insufficient.

Despite advances in pharmacotherapy, treatment of AK is often unsuccessful due to diagnostic mistakes delaying appropriate therapy and resulting in serious course of this disease.

An improvement in duration from first symptoms until suitable diagnosis is crucial for efficacy of the therapeutic management.

Early proper diagnosis, confirmed by detection of live trophozoites, involving *in vivo* and *in vitro* techniques is decisive for therapeutic efficacy, particularly in contact lens users; a strict hygiene while cleaning and using contact lenses is very important preventive measures.

References

[1] Befinger M., Myjak P., Pietkiewicz H. 1986. Occurrence of amphizoic amoebae in Lake Żarnowieckie. *Bulletin of the Institute of Maritime and Tropical Medicine in Gdynia* 37: 275-286.

[2] Martinez A.J., Visvesvara G.S. 1997. Free-living, amphizoic and opportunistic amebas. *Brain Pathology* 7: 583-598.

[3] Schuster F.L., Visvesvara G.S. 2004. Free-living amoebae as opportunistic and non-opportunistic pathogens of humans and animals. *International Journal for Parasitology* 34: 1001-1027.

[4] Visvesvara G.S., Moura H., Shuster F.L. 2007. Pathogenic and opportunistic free-living amoebae: *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri* and *Sappinia diploidea*. *FEMS*

Immunology and Medical Microbiology 50:1-26.
[5] Aksozek A., McClellan K., Howard K., Niederkorn J.Y., Alizadeh H. 2002. Resistance of *Acanthamoeba castellanii* cysts to physical, chemical and radiological conditions. *Journal of Parasitology* 88: 621-623.
[6] Lorenzo-Morales J., Monteverde-Miranda C.A., Jiménez C., Tejedor M.L., Valladares B.O.R.A. 2005. Evaluation of *Acanthamoeba* isolates from environmental sources in Tenerife, Canary Islands, Spain. *Annals of Agricultural and Environmental Medicine* 12: 233-236.
[7] Dendena F., Sellami H., Jarraya F., Sellami A., Makni F., Cheikhrouhou F. 2008. Free living amoebae: detection, morphological and molecular identification of *Acanthamoeba* genus in the hydraulic system of an hemodialysis unit in Tunisia. *Parasite* 15: 137-142.
[8] Lanocha N., Kosik-Bogacka D., Maciejewska A., Sawczuk M., Wilk A., Kuzna-Grygiel W. 2009. The occurrence *Acanthamoeba* (free living amoeba) in environmental and respiratory samples in Poland. *Acta Protozoologica* 48: 271-279.
[9] Lorenzo-Morales J., Martín-Navarro C.M,. López-Arencibia A., Arnalich-Montiel F., Pińero J.E., Valladares B. 2013. *Acanthamoeba* keratitis: an emerging disease gathering importance worldwide? *Trends in Parasitology* 29: 181-187.
[10] Walochnik J., Obwaller A., Haller-Schober E.M., Aspöck H. 2001. Anti-*Acanthamoeba* IgG, IgM, and IgA immunoreactivities in correlation to strain pathogenicity. *Parasitology Research* 87: 651-656.
[11] Derda M., Hadaś E., Wojtkowiak-Giera A., Wojt W.J., Cholewinski M., Skrzypczak L. 2013. The occurrence of free-living amoebae in fountains. *Problemy Higieny i Epidemiologii* 94: 147-150.
[12] Červa L., Novak K. 1968. Amoebic meningo-encephalitis: sixteen fatalities. *Science* 160: 92.
[13] Nagington P.G., Watson T.J. 1974. Amoebic infection of the eye. *Lancet* 304: 1537-1540.
[14] Teknos T.N., Poulin M.D., Laruentano A.M., Li K.K. 2000. *Acanthamoeba* rhinosinusitis: characterization, diagnosis, and treatment. *American Journal of Rhinology* 14: 387-391.
[15] Van Hamme C., Dumont M., Delos M., Lachapelle J.M. 2001. Cutaneous acanthamoebiasis in a lung transplant patient. *Annales de Dermatologie et de Venereologie* 128: 1237-1240.
[16] Mazur T., Hadas E., Iwanicka I. 1995. The duration of the cyst stage and the viability and virulence of *Acanthamoeba* isolates. *Tropical Medicine and Parasitology* 46: 106-108.
[17] Marciano-Cabral F., Cabral G. 2003. *Acanthamoeba* spp. as agents of disease in humans. *Clinical Microbiology Reviews* 16: 273-307.
[18] Berger P., Papazian L., Drancourt M., Lascolat B., Auffray J.P., Raoult D. 2006. Amoeba associated microorganisms and diagnosis of nosocomial pneumonia. *Emerging Infectious Diseases* 12: 248-255.
[19] da Rocha-Azevedo B., Tanowitz H.B., Marciano-Cabral F. 2009. Diagnosis of infections caused by pathogenic free-living amoebae. In: *Interdisciplinary Perspectives on Infectious Diseases.* Hindawi Publishing Corporation: 1-14.
[20] Wesołowska M., Cisowska A., Myjak P., Marek J., Jurowska-Liput J., Jakubaszko J. 2006. *Acanthamoeba* keratitis in contact lens wearers in Poland. *Advances in Clinical and Experimental Medicine* 15: 553-555.
[21] Booton G.C., Joslin C.E., Shoff M. 2009. Genotypic identification of *Acanthamoeba* sp. isolates associated with an outbreak of *Acanthamoeba* keratitis. *Cornea* 28: 673-676.
[22] Boost M.V., Shi G.S., Lai S., Cho P. 2012. Amoebicidal effects of contact lens disinfecting solutions. *Optometry and Vision Science* 89: 44-51.
[23] Chomicz L., Padzik M., Graczyk Z., Starosciak B., Graczyk T., Naprawska A., Oledzka G., Szostakowska B. 2010. *Acanthamoeba castellanii*: in vitro effects of selected biological, physical and chemical factors. *Experimental Parasitology* 126: 103-105.
[24] Padzik M., Chomicz L., Szaflik J. P., Chrusikowska A., Perkowski K., Szaflik J. 2014. *In vitro* effects of selected contact lens care solutions on *Acanthamoeba castellanii* strains in Poland. *Experimental Parasitology* 145: 98-101.
[25] Adamska M., Leonska-Duniec A., Lanocha N., Skotarczak B. 2014. Thermophilic potentially pathogenic amoebae isolated from natural water bodies in Poland and their molecular characterization. *Acta Parasitologica* 59: 433-441.
[26] Chomicz L., Padzik M., Szaflik J.P., Nahorski W.L., Kryczka T., Szaflik J. 2014. Monitoring of in vitro dynamics of *Acanthamoeba* strains isolated from infected eyes as a useful tool in keratitis management. *Experimental Parasitology* 145: 73-77.
[27] Khan N.A. 2015. *Acanthamoeba*: Biology and Pathogenesis. Caister Academic Press, Norfolk, UK.
[28] Lorenzo-Morales J., Khan N.A., Walochnik J. 2015. An update on *Acanthamoeba* keratitis: diagnosis, pathogenesis and treatment. *Parasite* 22: 1-20.
[29] Kasprzak W., Mazur T. 1972. Free-living amoebae isolated from waters frequented by people in the vicinity of Poznań, Poland. Experimental studies in mice on the pathogenicity of the isolates. *Zeitschrift für Tropenmedizin und Parasitologie* 23: 391-398.
[30] Górnik K., Kużna-Grygiel W. 2004. Presence of virulent strains of amphizoic amoebae in swimming pools of the city of Szczecin. *Annals of Agricultural and Environmental Medicine* 11: 233-236.
[31] Schroeder J.M., Booton G.C., Hay J., Niszl I.A., Seal D.V., Markus M.B., Fuerst P.A., Byers T.J. 2001. Use of subgenic 18S ribosomal DNA PCR and sequencing for genus and genotype identification of

Acanthamoebae from humans with keratitis and from sewage sludge. *Journal of Clinical Microbiology* 2: 1903-1911.

[32] Szaflik J.P., Padzik M., Chomicz L., Oledzka G., Izdebska J. 2012. Usefulness of *in vitro* diagnostics in difficult incidences of *Acanthamoeba* keratitis requiring pharmacotherapy and surgical management. *Okulistyka* 3: 28-32.

[33] Risler A., Coupat-Goutaland B., Pelandakis M. 2013. Genotyping and phylogenetic analysis of *Acanthamoeba* isolates associated with keratitis. *Parasitology Research* 112: 3807-3816.

[34] Derda M., Wojtkowiak-Giera A., Hadaś E. 2014. Comparative analyses of different genetic markers for the detection of *Acanthamoeba* spp. isolates. *Acta Parasitologica* 59: 472-477.

[35] Lass A., Szostakowska B., Idzińska A., Chomicz L. 2014. The first genotype determination of *Acanthamoeba* potential threat to human health, isolated from natural water reservoirs in Poland. *Parasitology Research* 113: 2693-2699.

[36] Chomicz L., Conn D.B., Padzik M., Szaflik J.P., Walochnik J., Zawadzki P.J., Pawłowski W., Dybicz M. 2015. Emerging threats for human health in Poland: pathogenic isolates from drug resistant *Acanthamoeba* keratitis monitored in terms of their *in vitro* dynamics and temperature adaptability. Hindawi Publishing Corporation. *BioMed Research International:* 231285. http://dx.doi.org/10.1155/2015/231285

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