

Original papers

In vivo confocal microscopy and *in vitro* culture techniques as tools for evaluation of severe *Acanthamoeba* keratitis incidents

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ABSTRACT. Amphizotic amoebae belonging to the genus *Acanthamoeba* are known as etiological agents of sight-threatening *Acanthamoeba* keratitis. The leading risk factor for the development of this serious human disease is contact lens wearing which popularity increases worldwide, also in Poland. The disease with active epithelial inflammations, corneal ulcers, including loss of the visual acuity is a serious medical problem as an emerging threat for the public health related to improper contact lens hygiene. The treatment of the amoebic keratitis is difficult, often unsuccessful due to delayed proper diagnosis. The clinical picture of the disease, often with severe course is nonspecific, similar to that occurring in viral, fungal or bacterial keratitis, thus clinical symptoms alone are not sufficient to identify the causative agent of the amoebic infection. Early diagnosis is decisive for the suitable therapeutic management and the treatment efficacy. In our studies, several complicated, difficult to treat *Acanthamoeba* keratitis incidences pertaining Polish patients using contact lenses have been retrospectively analyzed in terms of the usefulness of non-invasive methods of *in vivo* confocal microscopy and *in vitro* culture techniques applied for diagnosis. Hyper-reflective double-walled spherical *Acanthamoeba* cysts, with a more reflective outer wall were detected in the epithelium and anterior layers of the corneal stroma. *In vivo* confocal microscopy, if available, may be a valuable, sensitive tool for diagnosis in late identified severe infections mainly with strong viability strains, however confoscan may offer limited value at low-intensity amoebic infections. The microscopic visualization of amoebae in slides prepared directly from corneal scraping and laboratory examinations of specimens from *in vitro* cultivated corneal isolates allow to confirm or verify results of *in vivo* examinations, furthermore to identify directly the pathogens and to clarify previous misdiagnoses.

Key words: *Acanthamoeba* keratitis, *in vivo* confocal microscopy, *in vitro* cultivation

Introduction

Different amoebic species belonging to the genus *Acanthamoeba* are known in many parts of the world as free-living organisms. The amoebae occur in a wide range of aquatic and soil habitats, in air and also common in natural environments. The protists were also found in a hospital environment, on surfaces of equipment and surgical instrument; they were isolated from air-conditioning systems, from

dental irrigation units, dialysis units, contact lenses and contact lens solutions [1–4]. The amoebae are believed to be amphizotic organisms. Although they exist in the different outer environments, under predisposing circumstances are able to enter the human body, colonize certain organs and tissues and multiply. Thus, several *Acanthamoeba* strains can exist as free-living protists and as facultative parasites. The amoebae may be causative agents of serious human diseases affecting skin, brain, lungs

[4–10]. Trophozoites and cysts of *Acanthamoeba* species were also found among oral cavity microbiota associated with gingivitis and periodontitis [11,12]. Moreover, *Acanthamoeba* keratitis (AK), the eyesight-threatening disease caused by the pathogenic strains of the amphizoic amoebae may develop in immune-competent persons.

The leading predisposing factor for AK is contact lens wearing. The popularity of the contact lens use is rising in Poland; for this reason, severe, vision-threatening AK cases are reported with increasing frequency every year. [4,13–16]. The clinical pattern of the disease may include redness, photophobia, excessive tearing, severe eye pain, eyelid edema and progressive visual impairment; epithelial inflammations and hyper reflective tissue of corneal ulcers appearing in affected eyes may be detected by slit-lamp. A visualization of hyper reflective objects, *Acanthamoeba* cysts, is also possible using other non-invasive methods, *in vivo* confocal microscopy [17–23]. The symptoms are nonspecific, similar to those occurring in the course of other eye diseases, thus diagnosis based on clinical symptoms alone is not sufficient to indicate a causative agent of human keratitis. A classification of *Acanthamoeba* spp. has changed following the recognition of the amoebae in the human environment. For years, the *Acanthamoeba* species were classified using morphological criteria, however, advances in molecular methods influenced the classification of *Acanthamoeba* strains. So, 18–19 genotypes are recently distinguished based on genotype associations, the 18S rRNA gene sequence. It is considered that T2 and T11 genotypes, and mainly T4 genotype are detected of human organs and tissues [24–27].

The disease may also develop as etiologically mixed keratitis caused by concomitant bacterial, fungal, viral infections, which is the reason of misdiagnosed cases. However, literature data, and also our experience indicate that amoebic etiology of keratitis should be taken into consideration in persons previously unsuccessfully treated with antiviral, antibacterial and/or antifungal medications.

In the present study, we analyzed retrospectively selected data pertained several complicated, difficult to treat *Acanthamoeba* keratitis incidences in terms of an usefulness of *in vivo* and *in vitro* techniques applied in the diagnostics of the eyesight-threatening disease.

Materials and Methods

The present retrospective study includes data pertaining ten patients, 26–41 years old, contact lens wearers, previously treated without success in other ophthalmic units with antibacterial and antifungal medications. The patients were reported to our hospital in 2009–2014 at different periods – from 4 to 35 days after first symptoms of keratitis appeared. As most of the patients were probably misdiagnosed, the slit-lamp and *in vivo* confocal microscopy were applied for the clinical assessment of the eye deteriorations; parasitological examinations and microbiological tests for specific identification of bacteria and fungi were also performed. Samples of corneal scrapings from affected eyes were directly examined in wet-mount slides with the aid of a contrast phase light microscope to identify *Acanthamoeba* cysts or/and trophozoites. Simultaneously, isolates deriving from corneas were investigated by PCR technique and *in vitro* grown in BSC medium in sterile 15 ml tubes under bacteria-free condition in BSC medium [28] enriched with calf serum, incubated at 26°C and sub-cultured into this growth medium twice a month.

The amoebae were maintained in these axenic cultures as long as particular corneal strains survived. Overall amoeba number and percentage of particular stages: trophozoites and cysts were assessed according to previously described [10]. The morphophysiological status of amoebic developmental forms was monitored; particularly, *in vitro* viability of *Acanthamoeba* from different strain populations expressed, among other as an ability of amoebae to multiply, was examined and compared.

Results

Comparative evaluation of the analyzed data showed that among ten keratitis cases, finally five were undoubtedly the *Acanthamoeba* keratitis incidents. Clinical symptoms of this keratitis with severe eye pain, photophobia, excessive tearing, inflammation and serious visual impairment were observed with different intensity in affected eyes of contact lens wearers from which assessed corneal material was acquired. The duration of the symptoms until proper diagnosis, after patients were admitted to our hospital was 4, 12, 21, 30 and 35 days, thus a time when a verification of diagnosis and topical anti-*Acanthamoeba* therapy start was also different.

In the slit-lamp, epithelial inflammations and corneal ulcerations with hyper reflective tissue have been detected in all eyes affected, and characteristic ring-like stromal infiltration were revealed in three of them.

Simultaneously, there were differences in evaluation of results received with the use of *in vivo* confocal microscopy. Presumable *Acanthamoeba* cysts revealed as numerous hyper-reflective double-walled polygonal or round objects, with their outer wall more reflective than the internal one were detected by this *in vivo* technique in three AK cases. The cysts were detected in the epithelium and anterior layers of the corneal stroma. However, amoebic cysts were *in vivo* visualized only in the incidents in which AK diagnosis was performed late, three or more weeks after the first keratitis symptoms appeared. The affected eyes were treated earlier in other ophthalmic units with antibacterial and antifungal medications. In remaining cases, no cysts were visualized by *in vivo* confocal microscopy.

Amoebic etiology of analyzed keratitis cases has been confirmed by laboratory examinations. *Acanthamoeba* cysts or also trophozoites were found in microscopically examined wet-mount slides prepared from corneal scrapings deriving from several cases. Live trophozoites with characteristic acanthopodia and polygonal or round cysts were detected in the material deriving from all corneal isolates during *in vitro* cultivation in axenic growth medium. The pathogenic corneal amoeba isolates/strains detected directly in corneal scrapings and cultivated *in vitro* were identified as belonging to *Acanthamoeba* morphological group II. The results of molecular examinations of the

corneal isolates, based on molecular genotype associations – the 18S rRNA gene sequence and a comparison with those available in GenBank showed that they belong to T4 genotype.

There was a variability in the dynamics of particular cultivated strain populations. Amoeba numbers increased with a progress of population development and next decreased at the end of cultivation cycle. Successive monitoring of the pathogenic *Acanthamoeba* corneal strains cultured *in vitro* showed changes in their density with varying intensity in different protozoan populations. The highest number of the amoebozoans was revealed in all assessed strains during the log /exponential growth phase, at 6–9 days of cultivation cycle. *Acanthamoeba* strains deriving from corneas in which initially no cysts were detected *in vivo* by confocal microscopy, *in vitro* indicated lower value of overall amoeba number in comparison with three other strains analyzed. However, no regular increase/decrease in cyst percentage was observed in monitored strains. At the same time, clear differences occurred in the viability of particular *Acanthamoeba* populations that were expressed, among other, in various ability of trophozoites to multiply and in different *in vitro* survival time of amoebae in cultures.

The amoebic strains deriving of corneas in which cysts were visualized already *in vivo* by confocal microscopy indicated stronger *in vitro* viability in comparison with those of shorter *in vitro* survival time in which no cysts were *in vivo* detected.

Comparative characteristics of several AK incidences in terms of usefulness of *in vivo* confocal microscopy and *in vitro* culture techniques applied in diagnostics are presented in Table 1.

Table 1. Comparative characteristics of several AK incidences in terms of usefulness of *in vivo* and *in vitro* techniques applied in diagnostics

<i>Acanthamoeba</i> isolate/strain	Duration of symptoms until proper diagnosis	<i>In vivo</i> visualization of cysts by confoscan	<i>In vitro</i> growth amoebae in axenic cultures
2 <i>Acanthamoeba castellanii</i>	12 days	No cyst detected	Weak dynamics of this strain
5 <i>Acanthamoeba</i> sp.	4 days	No cyst detected	Weak dynamics of this strain
12 <i>Acanthamoeba castellanii</i>	35 days	Hyper-reflective cysts detected	Strong viability strain
13 <i>Acanthamoeba castellanii</i>	30 days	Hyper-reflective cysts detected	Strong viability strain
16 <i>Acanthamoeba</i> sp.	21 days	Hyper-reflective cysts detected	Strong viability strain

Discussion

Comparative analysis of corneal strains deriving from several complicated, difficult to diagnose and treat, *Acanthamoeba* keratitis incidences, showed the variability in duration of symptoms until proper diagnosis has been performed and different effects of applied diagnostic techniques. Differences were also expressed in various *in vitro* dynamics /viability of particular *Acanthamoeba* strains.

The amphizoic *Acanthamoeba* species are known as ubiquitous in natural environments; they are also found in different human habitats. Literature data and our investigations indicated that these facultative parasitic amoebae may be detected during infections of various human cavities, tissue and organs: oral cavities, paranasal sinuses, skin surfaces, respiratory tract, brain and corneas [4–9,12,29]. Moreover, these potentially pathogenic protists have been detected in healthy, diseased or dead animals (dogs, beavers), vegetable and fruits, and also in recreational water systems, thus, different environmental sources can not be excluded as factors influencing a development of the vision-threatening disease in contact lens wearing [6–9]. Prevention is very difficult because of this wide distribution of amphizoic amoebae strains in outer and human environments.

Also, it should be taken into consideration that double-walled *Acanthamoeba* cysts are highly resistant to adverse environmental factors, disinfectants and anti-parasitic drugs. Despite advances in pharmacotherapy, the therapeutic management is often unsuccessful and recurrence can appear due to this cyst resistance, strong pathogenicity and viability of amoeba strains [4,16,25,29]. An important role of biofilms, microbial-derived communities, which can be formed on contact lenses, is suggested as providing of “attractive niches for *Acanthamoeba* by fulfilling their nutritional requirements as well as providing resistance to disinfectants” [16]. *Acanthamoeba* keratitis is still considered a rare disease, however generating serious threat for the public health worldwide; incidents of eyesight-threatening human keratitis, the disease caused by pathogenic *Acanthamoeba* strains is reported with constantly increasing frequency in Poland during the last few decades [9,10,25,27].

The nonspecific clinical symptoms of AK as well as mixed microorganism eye infections can result in diagnostic mistakes delaying appropriate

treatment. Initial incorrect diagnosis and a delay in initiating an efficient treatment may influence prolonged and severe course of this human eye disease.

The retrospective analysis showed that *Acanthamoeba* etiology was confirmed in the five among ten eye keratitis incidents. Hyper-reflective objects identified as *Acanthamoeba* cysts have been *in vivo* visualized by confocal microscopy in incidents previously unsuccessfully treated in other ophthalmic units with antifungal and antibacterial pharmaceuticals, and in which proper diagnosis was performed late, three or more weeks after the first keratitis symptoms appeared. Simultaneously, these strains indicated *in vitro* strong population viability and long, many years surviving time in axenic culture medium.

Contrary to this, no cysts were detected by the *in vivo* confocal technique in material from corneal scrapings if infections were early suitable diagnosed, population density was very low and weak *in vitro* strains dynamics were observed.

Hyper-reflective double-walled spherical objects, *Acanthamoeba* cysts, with more reflective outer wall can be detected in the epithelium and anterior layers of the corneal stroma, mainly in severe infections with strong viability strains. *In vivo* confocal microscopy, if available, is a valuable, sensitive tool for the rapid diagnosis and differentiation of AK from other infectious keratitis. However, as trophozoites in confoscan images resemble leukocytes and keratocytes, examiners have to be familiar with amoebic morphology to avoid false results.

It should be taken into consideration that confoscan offers limited value at low-intensity amoebic eye infections. An awareness and knowledge on these serious health threats generated by the facultative parasitic *Acanthamoeba* strains is still insufficient in Poland; particularly, in contact lens wearers, strict hygiene while cleaning and using contact lenses is crucial as preventive measures.

Moreover, the late recognition of corneal amoebic infection is the very important factor influencing diagnostic difficulties and disappointing therapeutic management in AK.

Early proper diagnosis in *Acanthamoeba keratitis*, confirmed by *in vitro* detection of live amoebae in cultures are decisive for the treatment efficacy.

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Received 25 October 2017

Accepted 05 December 2017