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SHORT COMMUNICATION

Genotypic characterization of amoeba isolated from *Acanthamoeba* keratitis in Poland

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Abstract Free-living amoebae belonging to the genus *Acanthamoeba* are the causative factor of many diseases. Among others, they cause *Acanthamoeba* keratitis (AK), a condition that usually occurs in contact lens wearers, though it is also observed in non-wearers. The number of diagnosed cases of AK increased more than eightfold during 8 years in the USA, and a proportional increase in frequency also occurred in Poland and Europe. Cases of AK are usually diagnosed late, and their therapy is difficult and rarely successful. AK is an uncommon diagnosis in Poland. The increased number of positive cases observed in our laboratory may reflect the growing at-risk population of contact lens wearers. *Acanthamoeba* as a genus of facultative human parasites is currently classified into 17 genotypes. Isolates belonging to seven genotypes were found to be associated with AK. One genotype in particular, T4, was found to be overrepresented in human disease. The main finding of our study is that in Poland, AK is almost always associated with the T4 genotype.

Keywords *Acanthamoeba* · *Acanthamoeba* keratitis · Cornea · Genotyping

Introduction

Amoebae belonging to the genus *Acanthamoeba* are free-living organisms, widespread in the environment, and excellently adjusted to various environmental conditions (Stockman et al. 2011). They occur in two developmental stages: trophozoites and cysts, resistant to environmental conditions (Marciano-Cabral and Cabral 2003). The invasive forms for the human are cysts as well as trophozoites of amoebae. Infection occurs most often in the warm seasons and is associated with contact with water. In particular cases, the infection may take place through damaged skin or accidently injured cornea, e.g., during earth works or playing with sand or water, or through contact lenses.

The most significant and most common disease caused by the free-living amoebae is *Acanthamoeba* keratitis (AK).

The first cases of the amoeba's invasion of the cornea were described in 1974 (Naginton et al. 1974). In subsequent years, only a few cases of AK were described. After 1981, a rapid increase in the number of cases of keratitis was observed, which reached a peak in 1985. Most cases were described in the USA.

Within the last two decades, amoebae of the *Acanthamoeba* genus have become a well-known and significant risk factor for the keratitis. Contributory factors include global climate warming and the increase in the population of patients with immunological deficiencies. The number of diagnosed cases of AK in the USA increased from 22 in 1999 to 170 in 2007 (Yoder et al. 2012). A proportional increase of the number of diagnosed cases of AK also occurred in Poland.

So far, apart from the USA, AK cases have been described in Europe, Australia, Asia, and Africa. The approximate number of infections is counted in thousands. The majority of cases, as many as 85–88 %, are connected with the wearing of contact lenses (Dart et al. 2009). Among this group, 88 % of cases are connected with wearing hydrogel contact lenses and 12 % with wearing rigid lenses. Seal (2003) stated that the probability of contracting AK each year is 1:30,000 in persons wearing contact lenses.

In persons not wearing contact lenses, AK is usually diagnosed late, and in these cases, the disease is usually already at an advanced stage.

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AK usually affects one eye. The first symptoms are blurred vision, photophobia, and severe pain in the eye not explained by corneal injury. Conjunctival swelling and eyelid swelling also appear. Patients have bleary and teary eyes. In the front layers of the corneal stroma, there are diffuse, ring-shaped, or crescent infiltrates and less specific satellite infiltrates. The ring-shaped infiltrates are single or numerous. The epithelium covering the corneal stroma can be unharmed or may have punctate erosions. In the late stage of the disease, pus appears in the eye. Together with the development of the disease, the corneal edema increases (Kosik-Bogacka et al. 2010).

Material and methods

Samples of amoebae

Eight *Acanthamoeba* strains were isolated from corneal scrapings from patients suspected of having keratitis. Scrapings were placed on 2 % non-nutrient (NN) agar plates, covered with *Enterobacter aerogenes* bacteria, and the plates were incubated at a temperature of 28 °C. After 3–5 days, the increase in the number of amoebae was observed and examined with an inverted microscope at×200. The plates were monitored microscopically for up to 2 weeks for growth of *Acanthamoeba* trophozoites or for the presence of cysts.

Pathogenicity test

The amoebae 2–3 days old obtained from the NN agar culture were washed down with sterile distilled water. The suspension thus obtained was thickened and used to infect 2-week-old mice, strain BALB/c, by intranasal inoculation of five mice for each isolate. The mice were kept for 2 weeks, and thereafter anaesthetized and killed, unless they died within the first few days of being infected. The brains and lungs of the mice, irrespective of the method of death, were collected in order to isolate the amoebae. This study was conducted to verify the pathogenicity and virulence of isolated amoeba.

Molecular identification

The DNA amplification was performed using genus-specific primers previously described by Schroeder et al. (2001). A set of primers that included the forward JDPI (5′GGCCCAGATC GTTTACCGTGAA′3), and the reverse primer JDP2 were used (5′TCTCACAAGCTGCTAGGGAGTCA′3) for genetic characterization targeting the ~450 bp fragment of the *Acanthamoeba* 18S ribosomal rRNA (rRNA) gene. Amplification involved use of a 25 μl suspension of the following reagents: 2.5 mM $MgCl_2$, 0.6–1 μM of each primer, 0.2 mM of each deoxynucleotide triphosphate, and 0.5 U of AmpliTaq Gold DNA polymerase. A clinical isolate of *Acanthamoeba castellanii* belonging to the T4 genotype isolated from a keratitis patient (ATCC 50374) was used as a positive control. A negative control consisting of a reaction mixture without a DNA template was included. Polymerase chain reaction (PCR) was carried out using a GeneAmp 2400 thermocycler. PCR products were analyzed on 1 % agarose gel stained with ethidium bromide. Gel images were illuminated using UV light and captured using a gel documentation system. PCR products were cleaned and sequenced in both directions with the same set of primers. Sequencing was performed with BigDye Terminator v3.1 on an ABI Prism 3130XL Analyzer (Applied Biosystems, USA). Trace files were checked and edited using FinchTV 1.3.1 (Geospiza Inc., Seattle, USA). Contigs were aligned and manually assembled in GeneDoc v. 2.7.000 (Nicholas and Nicholas Jr 1997; Nicholas et al. 1997). Sequencing was conducted in both directions with the same set of primers. Sequences were analyzed using the program Chromas. Next, the gene sequence fragments of the *Acanthamoeba* isolates were compared with the reference sequences deposited in GenBank (National Center for Biotechnology Information).

Results

Pathogenicity test

All studied isolates of amoebae were highly pathogenic and virulent. Among five infected mice, five died within 7–10 days, and the amoebae were isolated from them again.

Molecular identification

The study results of genotyping of amoebae isolates from human cases of AK from Poland are presented in Table 1. The obtained nucleotide sequences were compared with the sequences previously determined and deposited in GenBank.

It was found that all obtained sequences of amoebae isolates from the cases of AK belong to the genotype T4 *Acanthamoeba* sp. and were 100 % identical to the sequences of the same marker obtained from environmental samples but also from people. It was also found that there are small deviations from the typical model, revealing a certain polymorphism within genotype T4. It was found that the particular amoebae isolates form three groups of nucleotide sequences are 100 % identical to the model strains deposited in GenBank.

It was found that the DNA sequences of the fragment of the gene *18S rRNA* Ac34, Ac48, and Ac55 are 100 % identical to reference sequences of the isolates of *Acanthamoeba* strain S6 [accession no. in GenBank DQ087296] (Yera et al. 2008), strain CCS-LG [JX441874] (Duarte et al. 2013), strain 1 FRC-2013 [KF318460] (Mafra et al. 2013), strain CDCV600 [GQ889265] (Visvesvara et al. 2010), strain

Table 1 Comparison of strains isolated from human cases of AK compared to reference strains deposited in GenBank

Strains isolated from AK cases in Poland		100 % nucleotide sequence similarity in relation to reference strains			
Strain	Accession no.	Origin	Region of origin	Accession no.	Reference
Ac32	KP184479	*Acanthamoeba* sp., AcaVN15, dialysis unit water	Slovakia	GQ397477	Nagyova et al. 2010a
Ac35	KP184481	*Acanthamoeba* sp., AcaVN14, salt cave scrape	Slovakia	GQ397476	Nagyova et al. 2010a
Ac36	KP184482	*Acanthamoeba* sp., CRIB-23, river water—Seine River	France	EU273825	Thomas et al. 2008
Ac37	KP184483	*Acanthamoeba* sp., CDCV600, liver of a Temminck's tragopan	USA	GQ889265	Visvesvara et al. 2010
Ac34	KP184480	*Acanthamoeba* sp., S6, contact lens and contact lens case	France	DQ087296	Yera et al. 2008
Ac48	KP184485	*Acanthamoeba* sp., CCS-LG, keratitis,	Brazil	JX441874	Duarte et al. 2013
Ac55	KP120880	*Acanthamoeba* sp., 1 FRC-2013, corneal surface tissue	Brazil	KF318460	Mafra et al. 2013
		Acanthamoeba sp., CRIB53, biofilm	France	EU377583	Corsaro et al. 2009
		Acanthamoeba sp. 222BAL corneal scraping	France	DQ087297	Yera et al. 2007
Ac46	KP184484	*Acanthamoeba* sp., AR881, swamp water	Spain	JQ678632	Garcia et al. 2013
		Acanthamoeba sp., AcaVNAK03, corneal scrape	Slovakia	GQ905497	Nagyova et al. 2010b

CRIB53 [EU377583] (Corsaro et al. 2009), and strain 222BAL [DQ087297] (Yera et al. 2007), originating from Brazil, the USA, and France, isolated from human cases of AK (Yera et al. 2008; Duarte et al. 2013; Mafra et al. 2013; Yera et al. 2007) as well as infected liver of Temminck's tragopan (Visvesvara et al. 2010) and the environment (Corsario et al. 2009).

The compared sequences of the Ac32, Ac35, Ac36, and Ac37 isolates from AK show 100 % similarity to the sequences of the same marker obtained from environmental samples, identified as *Acanthamoeba* sp. of genotype T4 of such isolates as: strain AcaVN15 from dialysis unit water [GQ397477], strain AcaVN14 from salt cave scrape [GQ397476] (Nagyova et al. 2010a), and strain CRIB-23 from river water [EU273825]. The isolate Ac46 was identical in terms of nucleotide sequences to strain AR881 from swamp water [JQ678632] (Garcia et al. 2013) and strain AcaVNAK03 from corneal scrape [GQ905497] (Nagyova et al. 2010b).

Our *Acanthamoeba* sequences from the Ac32, Ac34, Ac35, Ac36, Ac37, Ac47, and Ac48 isolates obtained from corneal scrapings were deposited in GenBank (NCBI) under accession numbers KP184479 to KP184485.

Discussion

The omnipresence of amoeba in the surrounding environment is undeniable. These cosmopolitan organisms are found in samples of soil, air, water, and animal tissues. *Acanthamoeba* spp. have been isolated and identified in many countries around the world, for example in Bulgaria (Tsetkova et al. 2004), Iran (Mahmoudi et al. 2012), Spain (Magnet et al. 2012), Brazil (Duarte et al. 2013), Japan (Edagawa et al. 2009), the USA (John and Howard 1995), Switzerland (Gianinazzi et al. 2009), and also in Poland (Kasprzak and Mazur 1972).

Recognition of invading amoeba of the genus *Acanthamoeba* in the eye is difficult. Numerous corneal infections caused by viruses, fungi, or bacteria cause symptoms similar to amoeba infection. Very often, an infection of the eye is mistakenly diagnosed as an infection caused by herpes simplex viruses.

In the case of inflammation of the cornea caused by *Acanthamoeba*, there are no uniform standards for diagnostic procedures. The most common diagnostic method is culture of corneal scrapings on NN agar coated with bacteria (Borin et al. 2013). Methods of visualization of the cornea using confocal microscopy and immunofluorescence are also used (Aniśko-Słomińska et al. 2011; Kokot et al. 2012). Additional methods of detection include molecular analysis of nuclear and mitochondrial DNA and methods of testing restriction fragment length polymorphism analysis after digestion of DNA (Kosik-Bogacka et al. 2010).

The taxonomy of the small amoeba species is not well established. Consequently, little is known about the genetic relationships between pathogenic and nonpathogenic strains of *Acanthamoeba*. During the last few years, molecular techniques (such as polymerase chain reaction—PCR) are increasingly being used in genotype identification (Booton et al. 2005; Caumo et al. 2009; Goldschmidt et al. 2012; Howe et al. 1997; Itahashi et al. 2011; Jain and Tilak 2011; Laummaunwai et al. 2012; Le Calvez et al. 2012). Our previous study confirmed the usefulness of PCR for the early, rapid, and sensitive diagnosis of pathogenic *Acanthamoeba* spp. strains (Derda et al. 2014). The best highly specific marker that distinguishes pathogenic *Acanthamoeba* strains in water samples in Poland is a pair of primers of Aca 16. The first results confirming the sensitivity and specificity of a PCR assay for the detection of AK were demonstrated by Lehmann et al. (1998), using two different pairs of primers (P1GP, P2GP). An interesting study showed that a combination of primer pairs (ACARNA, JDP, and NELSON) in the PCR-based method for diagnosing AK is more sensitive than culture and microscopy techniques (Yera et al. 2007).

Acanthamoeba as a genus of facultative human parasites is currently classified into 17 genotypes (T1–T17), each of which arguably represents a species (Stothard et al. 1998; Horn et al. 1999; Gast 2001; Hewett et al. 2003; Nuprasert et al. 2010; Corsaro and Venditti 2010).To date, isolates belonging to seven genotypes (T2, T3, T4, T5, T6, T11, and T15) have been found to be associated with AK (Stothard et al. 1998; Booton et al. 2005; Maghsood et al. 2005; Spanakos et al. 2006; Sharifi et al. 2010; Risler et al. 2013). One genotype in particular, T4, was found to be overrepresented in human disease. The main finding of our study is that in Poland, AK is almost always associated with the T4 genotype.

The correlation between *Acanthamoeba* genotype and clinical presentation of AK or susceptibility of the parasite to treatment has not been well explored. Khan (2003) reported that isolates belonging to AK-related genotypes exhibited significantly higher binding and cytotoxicity to corneal epithelial cells than nonclinical isolates. AK is an uncommon diagnosis in Poland. The increased number of positive cases observed in our laboratory may reflect the growing at-risk population of contact lens wearers. The present study was performed on a low number of isolates and may not reflect the entire spectrum of *Acanthamoeba* genotypes occurring in patients in Poland. However, our results indicate that the distribution of genotypes does not differ from that reported from other countries, with T4 as the predominant finding in AK cases.

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