Folia Parasitologica 2015, 62: 064
doi: 10.14411/fp.2015.064

Folia Parasitologica
http://folia.paru.cas.cz

Research Article OPEN ACCESS

Presence of potential pathogenic genotypes of free-living amoebae isolated from sandboxes in children's playgrounds

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Abstract: Some free-living amoebae are a potential threat to human health. The best known species are those of the genus *Acanthamoeba* Volkonsky, 1931, which cause *Acanthamoeba* keratitis, granulomatous amoebic encephalitis and other forms of tissue inflammation. The aim of the present study was to search for potential pathogenic genotypes of free-living amoeba in the sand in children's playgrounds. Our results confirmed that free-living amoebae were present in all examined playgrounds. Sequences of the 18S rDNA have shown that all isolated potentially pathogenic strains of amoebae belong to genotype T4 of *Acanthamoeba*. The potential pathogenicity of isolates was confirmed on mice. The presence of pathogenic amoebae in the examined sand may be a potential source of human infection.

Keywords: genotyping, *Acanthamoeba*, parasites, molecular study

Free-living amoebae of the genera *Acanthamoeba* Volkonsky, 1931, *Balamuthia* Visvesvara, 1986, *Echinamoeba* Page, 1975, *Hartmannella* Page, 1975, *Mastigina* Frenzel, 1897, *Naegleria* Alexeieff, 1912, *Saccamoeba* Bovee, 1972, *Vannella* Bovee, 1965, *Vexillifera* Schaeffer, 1926 and others are organisms commonly occurring in the natural environment (Michel and Schneider 1980, Bolivar et al. 2001, Łanocha et al. 2009, Corsaro et al. 2010). Some feed on bacteria, fungi and other solid particles, and are well adapted to the natural environment (Gupta and Das 1999, Schuster et al. 2003). Trophozoites and cysts of amoebae are found in oceanic deposits, waste water, in bottled mineral water, swimming pools, air conditioners, on vegetables and mushrooms, as well as on nasal cavity swabs, pharyngeal swabs and in purulent secretions from the ear.

Several genera of amoebae are also known as amphizoic organisms, which means that as free-living organisms they are able to penetrate and multiply in host organisms – including the human body – and function as parasites (Geisen et al. 2015). Free-living amoebae are the subject of research conducted not only by biologists, but also by geneticists, microbiologists, cytologists and pathologists (Walochnik 2014). The human and animal pathogenicity of amoebae was first reported in 1958. Research performed by Culbertson et al. (1959) and Fowler and Carter (1965) described the first fatal instances of human encephalitis caused by species of *Acanthamoeba*. A few thousand cases of diseases caused by free-living amoebae have been described in the literature (Trabelsi et al. 2012).

Potentially, the most virulent and dangerous to humans are species and strains of the genera *Acanthamoeba* and *Naegleria*, as well as species and strains of the genera *Balamuthia* (see Siddiqui and Khan 2012, Walochnik 2014).

Amoebae of the genus *Acanthamoeba* may represent a potential etiological agent in granulomatous amoebic encephalitis (GAE), *Acanthamoeba* keratitis (AK), pneumonia (AP) and amoebic inflammation of the skin, as well as in other diseases of organs and tissue in both humans and animals. Amoebae of the genus *Naegleria* cause primary amoebic meningoencephalitis (PAME), usually fatal, whereas amoebae of the genus *Balamuthia* bring about symptoms and pathological changes similar to those occurring during an infection with *Acanthamoeba* spp. (Sriram et al. 2008, LaFleur et al. 2013, Mirjalali et al. 2013, van der Beek et al. 2015).

The objective of the present study was to search for potential pathogenic genotypes of free-living amoebae in the city's sandboxes on children's playgrounds. These amoebae can be a potential threat to human health, especially for children (Lorenzo-Morales et al. 2005) because inhalation of sand or invasion of eyes by sand or dust on playgrounds constitutes a potential risk of amoeba infection (Shamsuzzaman and Hashiguchi 2002, Conza et al. 2013, Todd et al. 2015).

MATERIALS AND METHODS

The samples of amoebae were isolated from wet sand in thirteen sandboxes on children's playgrounds in Poznań, Poland (Table 1). All sandboxes are not covered and exchange of sand takes

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Table 1. Pathogenicity of isolates of *Acanthamoeba* spp. from sandboxes on children's playgrounds in Poznań, Poland.

Place of samples collection	Isolate name	Pathogenicity*	Invaded tissue	
			brain	lungs
Zbigniew Zakrzewski Green Garden Square	AcP1	3/1/1	-	+
Zbigniew Zakrzewski Green Garden Square	AcP15	3/1/1	+	-
Solacki Park	AcP2	4/1/0	+	-
Izabela i Jarogniew Drweski Park	AcP7	4/3/0	+	-
Izabela i Jarogniew Drweski Park	AcP8	3/2/0	+	-
Izabela i Jarogniew Drweski Park	AcP17	4/3/0	+	-
Izabela i Jarogniew Drweski Park	AcP14	3/1/0	-	+
Rusalka Lake Park	AcP12	3/1/0	-	+
Henryk Wieniawski Park	AcP16	3/2/0	+	-
John Paul II Park	AcP13	3/1/0	+	-
John Paul II Park	AcP20	4/1/0	-	+
John Paul II Park	AcP21	3/1/1	-	+
John Paul II Park	AcP23	3/1/1	+	-
Adam Asnyk Square	AcP49	4/2/0	-	+
Adam Wodziczko Park	AcP54	4/1/0	+	-
Thomas Wilson Park	AcN4	4/0/0	-	-
Jurij Gagarin Park	AcN5	4/0/0	-	-
Grunwaldzka Street	AcN6	4/0/0	-	-
Malta Lake Park	AcN9	4/0/0	-	-
Gustaw Manitius Park	AcN10	4/0/0	-	-

* pathogenicity of the strains is expressed as the ratio of the number of animals inoculated to the number of infected and dead animals.

place two times in a year. For the study approximately 50 g of sand were collected in sterile disposable containers.

The amoebae were isolated by placement of about 1 g samples of sand on agar plates (diameter of 80 mm). They were cultured at temperature of 28 °C on 2% non-nutrient agar (Difco Laboratories, Detroit, USA) covered by bacteria (*Enterobacter aerogenes* strain 535i). After 3–5 days, an increase in the number of amoebae was observed and examined with a microscope at 200×. The plates were monitored microscopically for up to two weeks for growth of trophozoites or for the presence of cysts. For monitoring, a Nicon (Precoptic Co., Warsaw, Poland) microscope was used.

The amoebae 2–3 days old obtained from NN agar culture were washed down with sterile distilled water. The suspension thus obtained was used to infect two-week-old mice, strain BALB/c, by intranasal inoculation (Mazur 1984). Autopsy was performed immediately after death of mice and pieces of the brain and the lungs were placed on NNE medium for culturing of amoebae. Mice that survived for over four weeks were anesthetised and their organs were examined. The brains and lungs of the mice, irrespective of the cause of death, were collected in order to isolate the amoebae. The amoebae isolated from tissues were identified according to morphological criteria, measurements of the size of cysts, and tests for flagellation (Page 1988, Smirnov et al. 2011).

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'GGCCCAGATCGTTTACCGTGAA'3), and the reverse primer JDP2 were used (5'TCTCACAAGCTGCTAGGGAGTCA'3) for genetic characterisation targeting an ~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 MgCl2, 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* Douglas, 1930 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 analysed 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, Waltham, Massachusetts, 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). Sequences were analysed using the program Chromas. Next, the gene sequence fragments of the isolates of *Acanthamoeva* were compared with the reference sequences deposited in GenBank (National Center for Biotechnology Information).

RESULTS

Free-living amoebae were found in all sandboxes, with pathogenic isolates detected in eight sandboxes (Table 1). Based on morphological characteristics of trophozoites or cysts and PCR, all isolates of pathogenic amoebae belonged to the genus *Acanthamoeba*. Pathogenic strains were obtained from the brain and the lungs of experimentally infected mice (Table 1).

DNA was isolated from 15 pathogenic samples and partial 18S rDNA sequences were obtained (see Table 2 for sequence accession numbers). Amplicons of the desired fragment were obtained from the amoeba isolates from two tissues of experimentally infected mice. Six strains of *Acanthamoeba* sp. were from the lungs: AcP1, AcP12, AcP14, AcP20, AcP21 and AcP49. Nine isolates of *Acan-*

Table 2. Comparison of isolates of *Acanthamoeba* spp. from children's playgrounds in relation to reference strains deposited in GenBank (all isolates belonged to genotype T4).

Isolates			Nucleotide sequence similarity in relation to reference strains			
Isolate name	Re-isolated from tissue	Accession no.	Origin		Accession no.	Reference
AcP1	L	KR259807	100% identity to *Acanthamoeba* sp., NI134 ex-arable fields,	Netherlands	KF928942	Geisen et al. 2014
AcP2	B	KR259808	100% identity to *Acanthamoeba* sp., AcaVN10 swimming pool scrape,	Slovakia	GQ397472	Nagyová et al. 2010a
			100% identity to *Acanthamoeba* sp., Mbc_3E, a rice field of the Istituto Sperimentale della Risicoltura,	Italy	AB425952	Murase and Frenzel 2008
			100% similarity to *A. castellanii* Neff, 1957, 1BU keratitis patient, cornea	Austria	AF260721	Walochnik et al. 2000
AcP7 AcP13 AcP16 AcP49 AcP54	B B B L B	KR259809 KR259812 KR259815 KR259820 KR259821	100% identity to *Acanthamoeba* sp., DRB1 from Danube river bank soil, (strain most closely related to *A. castellanii*)	Austria	KF924599	Lagkouvardos et al. 2014
AcP8	B	KR259810	99% similarity to *Acanthamoeba* sp., 116MAF corneal scrape	France	DQ087324	Yera et al. 2007
AcP12	L	KR259811	99% similarity to *Acanthamoeba* sp., CDC#V390 from non-AK	USA	AY703004	Booton et al. 2005
AcP14	L	KR259813	99% similarity to *Acanthamoeba* sp., JPH13 from patient with ocular infection	Japan	AB741047	Rahman et al. 2013
AcP15	B	KR259814	99% similarity to *Acanthamoeba* sp., AG-2012 swamp water	Spain	JQ678625	Garcia et al. 2013
			99% similarity to *Acanthamoeba* sp., AcaVNAK03 from corneal scrape	Slovakia	GQ905497	Nagyová et al. 2010b
AcP17	B	KR259816	100% identity to *Acanthamoeba* sp., DRS3 from the River Danube sediment,	Austria	KF924603	Lagkouvardos et al. 2014
			100% identity to *Acanthamoeba* sp., AcaVN11 from air conditioner scrape	Slovakia	GQ397473	Nagyová et al. 2010b
AcP20	L	KR259817	100% identity to *Acanthamoeba* sp., 116MAF from corneal scrape	France	DQ087324	Yera et al. 2007
AcP21	L	KR259818	100% identity to *Acanthamoeba* sp. SM6_6A from a rice field of the Istituto Sperimentale della Risicoltura,	Italy	AB425948	Murase and Frenzel 2008
AcP23	B	KR259819	100% identity to *A. hatchetti* Sawyer, 1977, 2HH	Austria	AF260722	Walochnik et al. 2000
			100% identity to *Acanthamoeba* sp., BP:P8:LCS/T4 from corneal scrape	Italy	FJ422512	Ledee et al. 2009

L – lung; B – brain; AK – *Acanthamoeba* keratitis.

thamoeba sp. were from the brains: AcP2, AcP7, AcP8, AcP13, AcP15, AcP16, AcP17, AcP23 and AcP54. No double peaks in the chromatograms occurred in the tested locus.

The results showed (Table 2) that the obtained sequence from the isolate AcP1 shared 100% identity with the sequence from the isolate NI134 of *Acanthamoeba* sp. obtained from an ex-arable field (GenBank accession No. KF928942; see Geisen et al. 2014). It was found that the DNA sequences of the fragment of the gene 18S rRNA of AcP2 was 100% identical to the reference sequences of the *Acanthamoeba* strains AcaVN10 (GQ397472) from a swimming pool scrape (Nagyová et al. 2010a) and Mbc_3E (AB425952) from a rice field and 1BU (AF260721) from a cornea (Walochnik et al. 2000).

The comparison of the sequences at the same molecular marker of the isolates AcP7, AcP13, AcP16, AcP49 and AcP54 with the sequences deposited in GenBank also showed 100% identity to the sequence of this gene of the parasite isolated from the environmental sample DRB1 (KF924599) from bank soil of the River Danube in Austria (Lagkouvardos et al. 2014). The isolate AcP17 was identical in its nucleotide sequence to strain DRS3 (KF924603) from the River Danube (Lagkouvardos et al. 2014) and strain AcaVN11 (GQ397473) from an air conditioner scrape in Slovakia (Nagyová et al. 2010b).

The sequence of the isolate AcP20 was identical to the sequence from strain 116MAF (DQ087324) from a corneal scrape in France (Yera et al. 2007). The sequences from the isolates AcP21 and AcP23 were also identical to the sequences of the same molecular marker from strains SM6_6A (AB425948) from a rice field in Italy (Murase and Frenzel 2008), strain 2HH (AF260722) (Walochnik et al. 2000) and strain BP:P8LCS/T4 (FJ422512) from a corneal scrape in Italy (Ledee et al. 2009).

The 18S DNA sequence from the environmental isolate AcP8 displayed 99% identity with the sequences of the same marker from corneal scraping 116MAF (DQ087324) and had one single-nucleotide polymorphisms (SNP) (Yera et al. 2007). Sequencing of the AcP12 and AcP14 isolate also showed that the molecular marker sequence was 99% identical to the sequence of *Acanthamoeba* strains CDC#V390 (AY703004) from USA (Booton et al. 2005) and JPH13 (AB741047) from patients in Japan (Rahman et al. 2013) and differed in three to four SNP to the compared marker. The genotyping results showed that the sequence from *Acanthamoeba* AcP15 shared 99% identity to the sequence from the swamp water *Acanthamoeba* isolate AG-2012 (JQ678625) (Garcia et al. 2013) and AcaVNAK03 (GQ905497) obtained from a corneal scrape (Nagyová et al. 2010b). Compared sequences differed by one SNP.

DISCUSSION

The research conducted has shown that sand on children's playgrounds in sandboxes is not free of free-living amoebae, including potentially pathogenic strains thereof. It has been determined that amoebae are present in all of the analysed sandboxes in the city of Poznań. The results of the pathogenicity of amoebae have brought to light their potential invasive properties. Experimental infections of mice showed that amoebae were present in the tissues of animals. In the majority of cases, amoebae were isolated from the brain or lung tissue of animals. We may therefore assume that it is probable that granulomatous amoebic encephalitis, *Acanthamoeba* keratitis and pneumonia may be caused in immunocompromised individuals who have come into contact with sand or dust on the children's playgrounds.

Should the presence of amoebae in sandboxes on children's playgrounds be a cause for concern? Can free-living amoebae constitute a real hazard to the participants playing in sandboxes? No clear-cut answer is available. Routine inspections of sand and sandboxes do not serve to determine the presence of pathogenic amoebae. However, when such research was conducted worldwide, it revealed that amoebae are present in 100% of inspected samples. Moreover, free-living amoebae may be isolated not only from moist soil and sand, but also from dust (Booton et al. 2004, Lorenzo-Morales et al. 2005, Geisen et al. 2014).

The lack of screening of municipal children's playgrounds may contribute to an increase in human amoebic infections. Furthermore, cysts of *Acanthamoeba* spp. are not only resistant to the action of numerous physical and chemical agents (considerable temperature fluctuations, drying, radiation) (Sriram et al. 2008, Siddiqui and Khan 2012), but may also function as carriers of pathogenic bacteria (Winniecka-Krusnell and Linder 2001, Derda et al. 2006, Khan and Siddiqui 2014). A special role may be played in this regard by amoebae belonging to non-pathogenic species or genera, for example *Naegleria gruberi* Schardinger, 1899, *Dictiostellium discoideum* Raper, 1935, species of *Hartmannella* and *Vannella* (see Lasjerdi et al. 2011). The fact that amoebae are carriers of other microorganisms (Hadaś et al. 2004) represents an additional challenge. Results of the present study turns our attention to the necessity of broadening routine checks for potentially pathogenic amoebae in sandboxes on children's playgrounds.

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Received 25 June 2015 Accepted 15 October 2015 Published online 9 December 2015

Cite this article as: Cholewiński M., Solarczyk P., Derda M., Wojtkowiak-Giera A., Hadaś E. 2015: Presence of potential pathogenic genotypes of free-living amoebae isolated from sandboxes in children's playgrounds. Folia Parasitol. 62: 064.