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Survival of *Acanthamoeba* Cysts after Desiccation for More than 20 Years[▽]

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***Acanthamoeba* is a free-living ameba that is found throughout the world and that causes encephalitis, keratitis, and cutaneous infections in humans. It has two stages in its life cycle: a trophic stage and a resistant cyst stage. We describe here the ability of *Acanthamoeba* cysts to survive desiccation for more than 20 years.**

Acanthamoeba, a free-living ameba, is an opportunistic pathogen of humans and other animals, including gorillas, monkeys, dogs, ovines, bovines, horses, and kangaroos, as well as birds, reptiles, amphibians, and fishes. In humans, *Acanthamoeba* causes a spectrum of diseases, including infections of the central nervous system, namely, granulomatous amebic encephalitis (GAE); infection of the skin; and *Acanthamoeba* keratitis (AK), an infection of the eye. GAE and cutaneous infections have often occurred in patients with human immunodeficiency virus infection and AIDS, as well as immunodeficient patients, including transplant recipients. *Acanthamoeba* keratitis, however, has occurred in immunocompetent persons wearing soft contact lenses and those with trauma to the eye. *Acanthamoeba* feeds on bacteria and occurs worldwide. It has been isolated from a number of habitats, including soil; freshwater ponds; pools; lakes; brackish water; seawater; heating, ventilating, and air-conditioning filters; and medical equipment, such as gastric wash tubing, dental irrigation units, contact lens paraphernalia, as well as vegetables, cell cultures, and even human and other animal tissues (13, 21, 26).

Acanthamoeba has two stages in its life cycle: a trophozoite stage and a cyst stage. Both the trophozoite and the cyst are uninucleate, although binucleate trophozoites are occasionally seen. The nucleus is characterized by a large densely staining nucleolus. The trophozoite feeds on bacteria and reproduces by binary fission. The cyst stage is a dormant and resistant stage. The cyst has double walls. The outer ectocyst is wrinkled and is proteinaceous, whereas the inner cyst wall, the endocyst, is either stellate, polygonal, round, or oval and contains cellulose (15, 16). According to a few previous studies the cyst stage of *Acanthamoeba* spp. is resistant to extreme physical and chemical conditions, including pH 2.0, freezing, γ irradiation (250 rads), and UV irradiation (800 mJ/cm^2) (2, 8); moist heat (60°C) with a contact time of 60 min (11); prolonged storage at room temperature for 24 months (4) or 24 years at 4°C in water (14); and heavy metals and polychlorinated biphenyls (PCBs) (18).

Over the past 30 years, we have established in culture 45 isolates obtained from diverse human specimens, including cerebrospinal fluid (CSF), brain, skin, and nasal and corneal tissues, as well as contact lens paraphernalia and water. The isolates were grown on nonnutrient agar plates coated with live *Escherichia coli* cells. After the amebae differentiated into cysts, the agar plates were tightly wrapped with Parafilm and stored at room temperature in laboratory cabinets (Table 1). The agar plates that were retrieved from storage were dry and parched, and either the entire agar layer or part of the agar layer had detached from the surface of the petri dish. The Parafilm wrappings were removed and 10 ml William Balamuth saline (24), a modified ameba saline, was added to each plate. The plates were allowed to rehydrate overnight, the agar surface was scraped with a cell scraper, and the scraped materials were transferred to 50-ml centrifuge tubes. The tubes were centrifuged at 500 × *g* for 10 min at 4°C. The supernatant was aspirated, the sediment was inoculated into fresh agar plates coated with a layer of live *E. coli* cells, and the plates were incubated at 30°C. The plates were observed daily with an inverted microscope equipped with differential interference contrast optics, and in some cases, amebae were visualized within 24 h. The presence of amebae in the plates could be easily identified on the basis of the characteristic track marks that the amebae left behind on the agar plates coated with bacteria (Fig. 1). If trophozoites were seen in the plates, the area was marked and a small piece of agar was cut out and transferred face down onto a fresh agar plate coated with bacteria, and the plates were sealed with Parafilm and incubated as described above. The amebae consumed the bacteria, colonized the fresh agar plates, and subsequently produced double-walled cysts (15, 16). Microscopic examination of the cysts revealed that they all belonged to *Acanthamoeba* group II (17). Of the 45 plates processed, 32 (71%) were positive for amebae. Of these 32 positive plates, 17 (53%) contained samples from keratitis patients, 6 (19%) contained samples from patients with GAE, 4 (13%) contained samples from patients with skin infections, 3 (9%) contained samples from patients with nasal sinus infections, and 1 each (3%) contained CSF and water samples. The geographic origin, the sources of isolation, and the genotypic information for the recovered isolates are given in Table 1. Three-day-old agar plates containing large numbers of trophozoites were scraped and washed by centrifugation; the sediment was inoculated into a 25-cm^2 Corning tissue culture flask containing 10 ml proteose pep-

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TABLE 1. Geographic origin, source of isolation, and genotypic information for recovered isolates[a]

Date recv'd (mo/day/yr)	CDC no.	Origin	Sex	Original source	Strain no.	Species	Genotype, GenBank accession no.	Date reproc'd (mo/day/yr)	OSU identifier
04/02/1984	84023461	MA	F	Corneal scraping	CDC:V014	*Acanthamoeba* sp.	No growth		
04/05/1984	84037022	MA	F	Corneal scraping	CDC:V016	*Acanthamoeba* sp.	No growth		
05/30/1985	85033424	LA	M	CL	CDC:V025	*Acanthamoeba* sp.	T4, AY702985	11/08/2006	03-010
06/06/1985	No number	TX	F	CL	CDC:V026	*Acanthamoeba* sp.	T4, AY702986	11/08/2006	03-011
07/08/1985	85037352	CA	F	Brain tissue	CDC:V028	*Acanthamoeba* sp.	T4, AY702987	11/08/2006	03-012
08/07/1985	85041057	MA	M	Corneal scraping	CDC:V029	*Acanthamoeba* sp.	T4, U07402	11/08/2006	
04/10/1986	86027199	WI	F	CL solution	CDC:V036	*A. castellanii*	T4, FJ196654	11/08/2006	07-027
04/23/1986	86027491	OK	F	corneal scraping	CDC:V037	*A. culbertsoni*	No growth		
07/17/1986	86038248	IL	F	Corneal button	CDC:V042	*A. castellanii*	T4, U07403	11/08/2006	07-026
08/13/1986	86038744	CA	M	Corneal scraping	CDC:V043	*A. polyphaga*	T4, AY702988	11/08/2006	03-013
02/07/1986	86017860	FL	M	Corneal biopsy specimen	CDC:V045	*A. culbertsoni*	T1, FJ196645	11/08/2006	07-025
09/17/1986	86043531	PA	F	Lens case	CDC:V048	*Acanthamoeba* sp.	No growth		
01/08/1987	87014900	MA	M	Corneal scraping	CDC:V062	*A.polyphaga*	T4, AY702989	03/22/2002	03-014
04/14/1987	87026622	CA	F	Corneal scraping	CDC:V077	*A.polyphaga*	T4, FJ196652	08/05/2002	07-024
04/27/1987	87026823	WI	M	Lens case	CDC:V078	*A.castellanii*	T4, FJ196651	08/09/2002	07-023
04/24/1987	87026834	MS	M	CL solution	CDC:V079	*A. polyphaga*	T4, FJ196650	08/09/2002	07-022
04/24/1987	87026833	MS	F	Corneal scraping	CDC:V080	*A. polyphaga*	T4, FJ196655	08/05/2002	07-028
06/09/1987	87032192	IL	M	Corneal biopsy specimen	CDC:V084	*A. polyphaga*	No growth		
09/08/1987	87038557	TX	M	Corneal biopsy specimen	CDC:V093	*A. polyphaga*	No growth		
09/11/1987	87038692	India	M	Corneal biopsy specimen	CDC:V095	*A. castellanii*	No growth		
03/23/1988	88020754	TX		Tap water	CDC:V118	*A. castellanii*	No growth		
06/17/1988	88031108	India	F	Brain tissue	CDC:V124	*A. polyphaga*	T4, FJ196656	08/05/2002	07-030
01/24/1989	89013839	CA	NA	Hot spring	CDC:V155	*Acanthamoeba* sp.	T4, AY702991	04/22/2002	03-016
03/15/1989	89017685	India	M	Corneal scraping	CDC:V160	*Acanthamoeba* sp.	T4, FJ196657	08/09/2002	07-031
11/24/1989	90000616	CO	F	Corneal scraping	CDC:V185	*Acanthamoeba* sp.	No growth		
11/02/1990	91000597	GA	M	Brain tissue	CDC:V210	*Acanthamoeba* sp.	No growth		
04/23/1991	91020919	PA	M	Skin tissue	CDC:V221	*Acanthamoeba* sp.	T4, AY702993	04/22/2002	03-017
10/25/1991	92001385	Italy	M	Corneal biopsy specimen	CDC:V235	*Acanthamoeba* sp.	T4, AY702994	03/04/2002	03-018
12/16/1991	92016015	OR	F	Skin biopsy specimen	CDC:V240	*Acanthamoeba* sp.	T4, AY702995	03/29/2002	03-019
01/14/1992	92016053	NZ	F	Corneal biopsy specimen	CDC:V241	*Acanthamoeba* sp.	No growth		
02/19/1992	92016854	VA	M	Skin biopsy specimen	CDC:V245	*Acanthamoeba* sp.	T4, AY702996	05/22/2002	03-020
12/30/1992	93016008	CA	M	Sinus swab	CDC:V280	*A. castellanii*	T1, FJ196642	02/28/2002	07-008
02/12/1993	93000045	Chile	NA	CL in case	CDC:V524	*A. polyphaga*	No growth		
04/01/1993	93016016	GA	F	Corneal scraping	CDC:V286	*Acanthamoeba* sp.	No growth		
06/03/1993	93019480	Spain	M	Corneal scraping	CDC:V285	*Acanthamoeba* sp.	T4, FJ196647	02/27/2002	07-009
08/17/1993	93025166	Canada	F	Corneal scraping	CDC:V291	*Acanthamoeba* sp.	T4, FJ196648	05/22/2002	07-010
06/07/1994	94001218	GA	M	Nasal tissue	CDC:V313	*Acanthamoeba* sp.	T4, AY702997	04/12/2002	03-021
11/04/1994	95000122	OH	M	Brain tissue	CDC:V329	*Acanthamoeba* sp.	T1, AY703000	06/27/2002	03-022
01/30/1995	95000134	GA	M	Brain tissue	CDC:V333	*Acanthamoeba* sp.	T1, FJ196644	06/27/2002	07-012
01/09/1996	96000312	NE	M	Brain tissue	CDC:V369	*Acanthamoeba* sp.	T10, AY703001	03/29/2002	03-023
01/18/1996	96000313	AZ	M	Sinus swabs	CDC:V370	*Acanthamoeba* sp.	T4, FJ196649	06/27/2002	07-013
02/04/1997	97001904	MO	M	Corneal tissue	CDC:V391	*Acanthamoeba* sp.	T4, AY703005	07/10/2002	03-024
08/18/1998	98003046	Spain	M	Brain tissue	CDC:V411	*Acanthamoeba* sp.	T4, AY703007	07/10/2002	03-026
02/12/1999	99010812	CT	M	Skin biopsy	CDC:V425	*A. culbertsonii*	T4, AY703008	04/12/2002	03-027
01/25/2000	2000020285	India	M	CSF	CDC:V501	*Acanthamoeba* sp.	T74, AY703010	05/24/2002	03-029

[a] recv'd, received; reproc'd, reprocessed; CL, contact lens; F, female; M, male; NA, not available.

tone, yeast extract, and glucose medium (20) with 5% fetal bovine serum and 100 μg/ml gentamicin (PYG medium); and the plates were incubated as described above. After 4 h of incubation, the flask was gently swirled, the supernatant was decanted, and fresh PYG medium was added to the flasks. After 3 days, the flask was shaken and 1 ml of the medium containing the amebae was removed and inoculated into fresh flasks containing the axenic (PYG) medium. An aliquot from the flask was also removed and inoculated into brain heart infusion and sheep blood agar plates for sterility testing. The amebae were next grown in a bacterium-free PYG medium (20) and pelletted by centrifugation, and their DNAs were extracted by use of a DNeasy kit (Qiagen, Valencia, CA). The nuclear 18S ribosomal DNA (rDNA) *Acanthamoeba* genus-specific amplicon ASA.S1 was amplified by PCR with genus-specific primers JDP1 (5′-GGCCCAGATCGTTTACCGTGA A-3′) and JDP2 (5′-TCTCACAAGCTGCTAGGGAGTCA-3′) (5, 19, 22). The amplicon was run on a 1% agarose gel and produced a product of the expected size of ~450 bp. Subsequently, the *Acanthamoeba*-specific PCR product was sequenced with a Terminator 3001 automated fluorescent DNA sequencer system (Applied Biosystems (Foster City, CA), as described previously (5). The nuclear 18S rDNA sequence obtained was compared to other sequences in the *Acanthamoeba* rDNA database and was determined as follows. Of the 32 isolates studied, 27 (84.375%) isolates, including those from keratitis patients, belonged to the most common genotype (genotype T4), 4 (12.5%) belonged to genotype T1, and 1 (3.125%) to genotype T10.

In this study we examined the survivability of *Acanthamoeba*

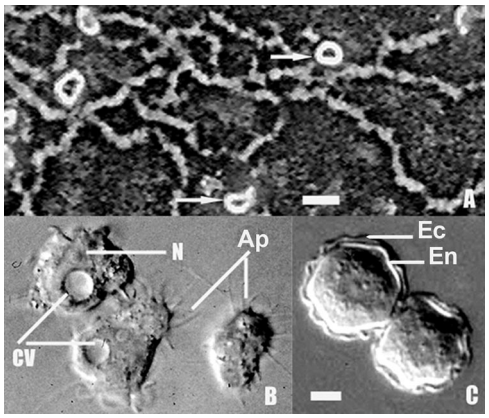

FIG. 1. (A) *Acanthamoeba* trophozoites (arrows) leaving track marks on the agar surface. Bar, 25 μm. (B) Trophozoites exhibiting thorn-like acanthopodia (Ap), nucleus (N), and contractile vacuole (CV). (C) Double-walled cysts. Ec, ectocyst; En, endocyst. Bar for panels B and C, 5 μm.

cysts stored in a state of desiccation for periods of 2 to 21 years. We found that the cysts of 70% of the isolates survived desiccation for 2 to 21 years. Furthermore, among the survivors, cysts of four (12.5%) isolates survived for 21 years even in a completely dry environment. All of the isolates tested here belonged to morphological group II, which is made up of many described species, including *Acanthamoeba castellanii*, *A. polyphaga*, *A. rhysodes*, *A. divionensis*, and *A. hatchetti*, that have commonly been identified from the environment and clinical specimens (13, 21, 26). Additionally, on the basis of sequence analysis, most of the isolates examined here belonged to the T4 clade. It has been well established that group II contains most of the pathogenic genotypes of the 15 recognized clades of *Acanthamoeba* and that the T4 clade contains most of the pathogens that cause AK throughout the world. It is also the most common and dominant genotype, with a universal distribution in the environment throughout the world (6). Booton et al. (6) also found that a majority of *Acanthamoeba* isolates from southern Florida beach sand belonged to the T4 clade. Previous studies have shown that although trophozoites of *A. polyphaga*, a member of the T4 clade, were inactivated after 1 to 2 h of solar photocatalytic (TiO_2) disinfection, cysts of *A. polyphaga* did not show any significant inactivation (9, 12). The ability of amebae to survive in a southern Florida beach, which is constantly exposed to intense sunshine during the daytime, and also to survive in an environment with exposure to seawater may enable them to invade and colonize the corneal surface, where the composition of tears is roughly similar to that of dilute seawater (6). It is also noteworthy that T4 amebae have also been isolated from asymptomatic freshwater fish, from a necrotic lesion in an iguanid lizard, and from the liver of a South American toucan (13, 21, 25–27). These studies, based on sequencing of the small-subunit rRNA gene, have shown that several *Acanthamoeba* isolates from fish, reptiles, and a bird and those associated with human *Acanthamoeba* keratitis infections belong to the same T4 genotype, suggesting that features that enable these amebae to infect animals may also help them to infect humans (25).

Since the infection in humans becomes apparent only after several weeks or even months, the portal of entry is not clearly known, although it is believed that cysts carried by dust in air gain access to the nasal passages, since *Acanthamoeba* has been isolated from the nasal passages of humans. Previous studies conducted with Australian university students and Nigerian children during the Harmattan period, when strong winds carry dust and soil particles, showed that the rates of nasal carriage of *Acanthamoeba* were in the range of 2% in the former population (3) and 24% in the latter one (1). Sinusitis and other nasopharyngeal infections caused by *Acanthamoeba* have also occurred in immunodeficient patients, transplant recipients, and AIDS patients. Amebae may also enter the body through breaks in the skin, resulting in hematogenous dissemination to the lungs and brain (13, 21, 26). The current study highlights that *Acanthamoeba* cysts are able to persist for long periods under adverse conditions, which would facilitate travel over great distances via dust particles in the air.

It has been shown that amebae differentiate into double-walled cysts when the food supply is exhausted and conditions become adverse, especially in the presence of contact lens cleaning and disinfecting solutions (23), and these cysts are resistant to the commonly used contact lens cleaning agents (7, 10). A recent outbreak of *Acanthamoeba* keratitis was associated with the use of AMO complete multipurpose solution (Advanced Medical Optics, INc.) in multiple U.S. states (7), including the Chicago, IL, area (10).

It is clear that acanthamoebae have the ability to tolerate a variety of physical and chemical conditions that occur in their environmental niches and have therefore developed resistance to often used antiseptics, herbicides, pesticides, PCBs, heavy metals, and contact lens disinfectant solutions. Additionally, *Acanthamoeba* cysts, as shown here, can withstand desiccation for more than 20 years. It is therefore necessary to continuously monitor isolates of *Acanthamoeba* for their resistance to environmental pollution, including heavy metals, PCBs, herbicides, pesticides, multipurpose contact lens solutions, and potent pharmaceuticals.

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