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Viability of Pathogenic and Nonpathogenic Free-Living Amoebae in Long-Term Storage at a Range of Temperatures

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The long-term storage of pathogenic and nonpathogenic strains of both *Naegleria* and *Acanthamoeba* spp. were tested on Page amoeba saline agar slopes for 24 months at room temperature and for 8 months at −10, 4, 10, and 15°C. *Acanthamoeba* strains showed better survival potential than *Naegleria* strains, particularly when they were stored at temperatures equal to or lower than room temperature.

Free-living amoebae of the genera *Naegleria* and *Acanthamoeba* are found very widely in nature and include pathogenic species which cause primary amoebic meningoencephalitis (4) and granulomatous amoebic encephalitis (6), respectively, in humans. Their ubiquity is largely due to an ability to form resistant cysts, enabling them to overcome unfavorable conditions or take advantage of intermittently favorable factors such as thermal enrichment (8).

Acanthamoeba spp. have been isolated from soil in the harsh Antarctic environment (2) and their cysts have been shown to be more resistant than those of *Naegleria* spp. (5). In a study of isolates from frozen swimming areas in Norway, all 50 incubated plates yielded amoebae, including the nonpathogen *Naegleria gruberi* and pathogenic *Acanthamoebae castellanii* (1). The effects of desiccation and high- and low-temperature incubation on *Naegleria* cyst survival over a short-term period have been studied previously (3). Cysts of pathogenic *Naegleria* spp. quickly become nonviable when dried owing to a loss of cytoplasmic water that leads to protein denaturation. The cysts of the nonpathogen *N. gruberi* retain their normal appearance over the same time and, indeed, are viable after 23 months of drying (3).

Low temperatures have a detrimental effect on pathogenic *Naegleria* spp., with shortened survival of cysts exposed to temperatures below freezing. *N. gruberi* cysts fared considerably better, surviving 3.5 to 4 months when stored at −25 to −30°C compared with 1 to 1.5 h at −10 to −30°C for *Naegleria fowleri*. Conversely, cysts of pathogenic *Naegleria* spp. were more resistant to heat than were those of *N. gruberi* (3).

The current study tests the long-term viability of pathogenic and nonpathogenic strains of *Naegleria* and *Acanthamoeba* spp. The amoebae were incubated on Page amoeba saline (PAS) agar (7) at room temperature for 24 months and at −10, 4, 10, and 15°C for 8 months.

Viable 24-h-old axenic cultures grown in CYM medium (4) were spun in a bench centrifuge. The pellet was washed three times and plated on PAS agar (7), with the addition of 4 ml of PAS solution and 0.1 ml of an overnight broth culture of *Enterobacter cloacae*. After 24 to 48 h at the appropriate incubation temperature (30 or 37°C), the cultures were subcultured onto duplicate plates, and a pool of viable amoebae was grown up for inoculation of a series of PAS agar slopes in screw-capped bottles. Inoculation of the slopes involved transferring 0.5 ml of washed amoebic suspension containing ca. 10^3 cysts ml^{-1} plus 0.1 ml of a solution of *E. cloacae* to the surface of the slope. The slopes were incubated at the appropriate temperature and were angled so that they were covered by the bacteria-amoeba suspension. After incubation, the slopes were stored at the temperatures stated above. Viability testing was carried out every month with duplicate agar slopes for each strain at the designated temperature. Extra slopes were prepared to replace any which dried out owing to faulty seals. To test viability, cysts were washed off the agar slopes with PAS solution. The amoebic cyst suspension was then transferred to a PAS agar plate with the addition of 4 ml of PAS solution and 0.1 ml of a solution of *E. cloacae* and initially incubated for 48 h. The amoebae were said to be viable if excystation was initiated and trophozoites were seen on microscopic examination. Excystment could, however, take longer than 48 h, and amoebae were kept for up to 96 h after being transferred from slopes to plates before results were finally recorded.

At room temperature all strains were viable for 6 months and the nonpathogens *A. castellani* 1501 and *N. gruberi* P1200f and the pathogen *Acanthamoeba culbertsoni* A1 were viable for 24 months. The pathogenic *N. fowleri* strains MsM, $MsMbr_4$, NHI, Nth, and MsT showed fluctuating viability at room temperature, as did the nonpathogen *Naegleria lovaniensis* (Table 1). Storage for longer than 6 months in an encysted state cannot be recommended for reliable regrowth; however, some slopes of *N. fowleri* were shown to be viable late in the 2-year period. Strains MsM, $MsMbr_4$, NHI, MsT, and Nth were viable at 17 months, strain MsM was viable at 20 months, and strains Nth and MsT were viable at 22 months. Factors such as moisture retention in the slope or cyst concentration may have affected viability in long-term storage.

Storage at the controlled temperatures (−10, 4, 10, and 15°C) showed that *A. culbertsoni* A1 and *A. castellanii* 1501 could survive for 8 months at −10, 4, 10, and 15°C and that *N. fowleri* strains $MsMbr_2$ and NHI and *N. gruberi* P1200f could survive for 8 months at 10 and 15°C. *N. fowleri* NHI survived for 6 months at 4°C and for 4 months at −10°C, whereas *N. fowleri* $MsMbr_2$ survived for 4 months at 4°C and for only 1 month at −10°C (Table 2).

These results further confirm that *Acanthamoeba* spp. are capable of surviving under conditions that are lethal for *Naegleria* spp. Although nonpathogenic *N. gruberi* appears to survive under normal temperatures far better than pathogenic *Naegleria* spp., results at lower temperatures do not confirm those from earlier work (3). This may be because Chang (3) used amoebae that had already completed encyst-

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TABLE 1. Viability of amoeba strains stored at room temperatures for 24 months on PAS agar slopes

Amoeba	Viability at month(s)[a]:																		
	1–6	7	8	9	10	11	12	13	14	15	16	17	18	19	21	21	22	23	24
N. fowleri MsM (pathogen)	+	+	−	+	−	−	−	−	−	−	−	+	−	+	+	−	−	−	−
N. fowleri $MsMbr_4$ (pathogen, Baquacil-resistant)	+	+	+	−	+	−	−	−	−	−	−	+	−	−	−	−	−	−	−
N. lovaniensis TS-1 (nonpathogen)	+	+	+	+	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−
N. fowleri NHI (pathogen)	+	−	+	−	−	−	−	−	−	−	−	−	+	−	−	−	−	−	−
N. fowleri Nth (pathogen)	+	+	−	−	−	−	−	−	−	−	−	−	+	−	−	−	+	−	−
N. fowleri MsT (pathogen)	+	−	+	−	−	−	−	−	−	−	−	−	+	−	−	−	+	−	−

[a] +, Viable; −, nonviable.

TABLE 2. Viability of amoebae stored at −10 and 4°C for 8 months on PAS agar slopes

Species and strain	Temp (°C)	Viability at month[a]:							
		1	2	3	4	5	6	7	8
N. fowleri $MsMbr_2$	−10	+	−	−	−	−	−	−	−
	4	+	+	+	+	−	−	−	−
N. fowleri NHI	−10	+	+	+	+	−	−	−	−
	4	+	+	+	+	+	+	−	−
N. gruberi P1200f	−10	+	−	−	−	−	−	−	−
	4	+	+	−	−	−	−	−	−

[a] +, Viable; −, nonviable.

ment before exposure to the storage temperatures; in the present study most of the amoebae were trophozoites before exposure to the storage temperatures, and viable trophozoites that were present were thus exposed to a temperature stimulus to initiate encystment. This situation closely emulates that found in nature. The lowered number of viable *N. gruberi* cysts could then be explained by a reduced efficiency of encystment at the lower temperatures or by premature trophozoite death. Different strains of amoeba also differ in their encystment potential.

Laboratory storage of amoebae at room temperature in an encysted state is reliable for 6 to 7 months for *N. fowleri* strains and for up to 24 months for *Acanthamoeba* and *N. gruberi* strains. *N. fowleri* strains $MsMbr_2$ and NHI can be stored for up to 8 months at 10 or 15°C.

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LITERATURE CITED

1. **Brown, T. J., and R. T. M. Cursons.** 1977. Pathogenic free-living amoebae (PFLA) from frozen swimming areas in Oslo, Norway. Scand. J. Infect. Dis. **9**:237–240.
2. **Brown, T. J., R. T. M. Cursons, and E. A. Keys.** 1982. Amoebae from Antarctic soil and water. Appl. Environ. Microbiol. **44**:491–493.
3. **Chang, S.-L.** 1978. Resistance of pathogenic *Naegleria* to some common physical and chemical agents. Appl. Environ. Microbiol. **35**:368–375.
4. **Cursons, R. T. M., T. J. Brown, and E. A. Keys.** 1978. Diagnosis and identification of the aetiological agents of primary amoebic meningo-encephalitis (PAM). N.Z. J. Med. Lab. Tech. **32**:11–14.
5. **De Jonckheere, J., and H. Van de Voorde.** 1976. Differences in destruction of cysts of pathogenic and nonpathogenic *Naegleria* and *Acanthamoeba* by chlorine. Appl. Environ. Microbiol. **31**:294–297.
6. **Martinez, A. J.** 1980. Is *Acanthamoeba* encephalitis an opportunistic infection? Neurology **30**:567–574.
7. **Page, F. C.** 1976. An illustrated key to freshwater and soil amoebae. Scientific publication no. 34. Freshwater Biological Association, Kendal, Cumberland, United Kingdom.
8. **Stevens, A. R., R. L. Tyndall, C. C. Coutant, and E. Willaert.** 1977. Isolation of the etiological agent of primary amoebic meningo-encephalitis from artificially heated waters. Appl. Environ. Microbiol. **34**:701–705.