

Isolation of Thermotolerant Freelifving Amebae from Lake Tenkiller, Oklahoma

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Received: 1995 Dec 06; Revised: 1996 Mar 12

Pathogenic freelifving amebae cause serious human disease including infection of the eye and central nervous system. Disease-producing freelifving amebae are able to grow at 37 °C or higher, and are considered to be thermotolerant. Nonpathogenic species are often, although not always, thermointolerant. We sampled the water at various depths in Lake Tenkiller to isolate thermotolerant and, therefore, potentially pathogenic freelifving amebae. Pathogenicity was tested by intranasal inoculation of amebae into mice. The prevalence of thermotolerant amebae in the water sampled was 1 ameba/171 ml; however, no pathogenic amebae were isolated.

INTRODUCTION

Pathogenic freelifving amebae can cause serious illness in humans (1). *Naegleria fowleri* causes a rapidly fatal infection involving the central nervous system (CNS) known as primary amebic meningoencephalitis. Another species, *N. australiensis*, is pathogenic to mice and should be considered a potential human pathogen (2). A number of *Acanthamoeba* species may produce a chronic CNS infection called granulomatous amebic encephalitis, or an eye infection referred to as *Acanthamoeba* keratitis. A newly described leptomyxid ameba, *Balamuthia mandrillaris*, causes a chronic CNS infection similar to that produced by *Acanthamoeba* (3).

All pathogenic freelifving amebae are thermotolerant; that is, they are able to grow at 37 °C or higher. However, not all thermotolerant freelifving amebae are pathogens. The ability to grow at 37 °C or higher is the criterion used in environmental studies to initially isolate amebae potentially pathogenic to human beings. Pathogenicity is determined by intranasal inoculation of mice. The purpose of this study was to collect water samples at various depths in Lake Tenkiller and to isolate therefrom thermotolerant, and possibly pathogenic, freelifving amebae. Our sampling efforts yielded thermotolerant amebae at all depths examined, but none were pathogenic to mice.

MATERIALS and METHODS

Sampling site: All water samples were collected at Lake Tenkiller, Oklahoma, at weekly intervals during July 1992. Water samples were obtained by SCUBA at the surface, and at 10, 20 and 30 m below the surface. Three collecting trips were made to Lake Tenkiller, and 12 water samples were collected at each depth on each trip.

Sample collection and ameba isolation: 50-ml water samples were collected in sterile, 50-ml, screw-cap centrifuge tubes. Tubes were opened at a specific depth, filled with water and tightly recapped to prevent mixing by water of other depths. Water temperature was measured at the site of collection, and pH was determined on the samples after they were brought to the shore using a portable digital meter. All water samples were processed in the laboratory on the day of collection.

Table 1 gives a flow diagram of the procedure used for processing samples and isolating thermotolerant and pathogenic freelifving amebae. Briefly, water samples were centrifuged (1,200 g, 10 min, 20 °C) and the sediment placed on nonnutrient agar (1.5%) spread with a washed suspension of living *Escherichia coli* (1). Agar plates were sealed and incubated at 42 °C for 48 h, and then transferred to 37 °C for 72 h. Amebae that were present in the samples grew on the moist agar surface and used *E. coli* as food, producing plaques as they cleared the bacteria. Amebae were subcultured to other plates as a means to reduce or avoid fungal overgrowth.

After 48 h of growth on the subcultured plates, amebae were transferred to Mix ameba medium (1) supplemented with

TABLE 1. Procedure for the isolation of thermotolerant and pathogenic freeliving amoebae from the environment.

50 ml water sample
↓
Centrifuge (1200×g, 10 min, 20 °C)
↓
Sediment
↓
Place on nonnutrient agar (1.5%) spread with <i>Escherichia coli</i> (42 °C for 48 h; then 37 °C for 72 h)
↓
Subculture to nonnutrient agar with <i>Escherichia coli</i> (37 °C for 48 h)
↓
Subculture to liquid medium ^a with <i>Escherichia coli</i> and antibiotics (37 °C for 48–72 h)
↓
Mouse inoculation (intranasal) (10 ⁴ –10 ⁵ amoebae/mouse; 3 mice/isolate)
↓
Culture brain tissue for isolation of amoebae ^a

^a Mix amoeba medium [(1); see Materials and Methods for composition] or Page amoeba saline for isolates that failed to grow in Mix medium.

E. coli, 200 µg streptomycin/ml and 200 U penicillin/ml. Mix amoeba medium is an equal mixture of Balamuth's (4) and Nelson's (5) media that, is 0.55% liver digest, 0.50% proteose peptone, 0.25% yeast extract and 0.30% glucose in Page's amoeba saline [0.12 g NaCl, 0.004 g MgSO₄ • 7H₂O, 0.004 g, CaCl₂ • 2H₂O, 0.142 g Na₂HPO₄ and 0.136 g KH₂PO₄ per liter of distilled water,(6)] supplemented with 4% bovine calf serum and 1 µg hemin/ml. Some of the amoeba isolates would not grow in Mix medium and were cultivated in Page's saline with *E. coli* (7).

Following 48–72 h of growth in liquid medium, amoebae were harvested by centrifugation (1,200 g, 5 min, 20 °C) and inoculated intranasally into 21-day-old male CD-1 mice (purchased from Charles River Breeding Laboratories, Wilmington, MA) at a dose of 1×10⁴–1×10⁵ amoebae/mouse using 3 mice per isolate (7). While mice were under anesthesia (Metofane; Pitman-Moore, Inc., Washington Crossing, NJ), a 10-µl drop containing amoebae in Page's saline was introduced into a single naris with an Eppendorf pipet (Brinkman Instruments, Inc., Westbury, NY). Mice were observed daily for signs of illness. Had illness or death occurred, brain tissue from all dead or dying mice would have been cultured for amoebae in Mix medium.

RESULTS and DISCUSSION

A total of 42 thermotolerant amoeba isolates were recovered from the 144 water samples collected. Of these, 35 isolates grew in sufficient numbers to be inoculated intranasally into mice as a test for pathogenicity (Table 2). We did not attempt to identify the species of thermotolerant amoebae because our efforts in the overall larger project focused on identifying pathogenic isolates. None of the thermotolerant isolates were determined to be pathogenic; that is, none caused death or produced illness in any of the inoculated animals. Mice are the experimental animal model of choice to determine the pathogenicity of freeliving amoebae because the clinical and histopathologic features of infection in humans and mice are essentially the same (8). Moreover, mice can become infected by swimming in water contaminated with *N. fowleri* (9), a mode of infection identical to that proposed for humans, and a further reason for their use.

The number of thermotolerant isolates that were obtained at various depths was 5 at the surface, 15 at 10 m, 13 at 20 m and 9 at 30 m (Fig. 1). Most environmental studies have isolated thermotolerant and patho-

TABLE 2. Water samples processed for thermotolerant and pathogenic freeliving amoebae.

Depth (m)	Total samples ^a	Positive samples	Number of amoeba isolates	Number of isolates inoculated ^b	Number of pathogens
Surface	36	5	5	5	0
10	36	13	15	9	0
20	36	13	13	13	0
30	36	9	9	8	0
Total	144	40	42	35	0
Percentage	100	28	29	24	0

^a Numbers represent 3 collections of 12 samples each.

^b Inoculated intranasally into mice.

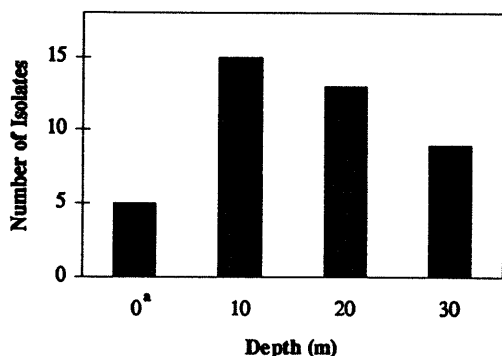


Figure 1. Number of thermotolerant amoebae isolated at various depths in Lake Tenkiller, Oklahoma. Numbers represent amoebae from 3 collections of 12 samples each. Depth = 0 corresponds to surface.

TABLE 3. Average temperature and pH of water samples processed for thermotolerant and pathogenic freeliving amoebae.

Depth (m)	Water temp., °C (range)	Water pH (range)
Surface	28±0.6 (28–29)	8.6±0.5 (8.2–9.2)
10	24±3.5 (20–26)	7.4±0.2 (7.3–7.6)
20	19±1.2 (18–20)	7.3±0.1 (7.3–7.4)
30	16±1.0 (15–17)	7.5±0.3 (7.2–7.7)

genic freeliving amoebae near the shore from surface water, or within 0.5 m of the surface (1). Although the present is the most thorough depth study, there is one report that describes the recovery of thermotolerant amoebae from a South Carolina pond with a depth of 10 m (10). The majority of thermotolerant isolates in that study were obtained from surface waters; very few were isolated from depths of 5–10 m. In our study, the water column had a depth triple that of the South Carolina pond and the majority of the thermotolerant amoebae were isolated from the intermediate depths of 10–20 m. Persons swimming at 10–20 m would likely be using SCUBA equipment and, therefore, would be protected from pathogenic freeliving amoebae, which invade via the nasal mucosa.

Water temperature ranged from an average of 28 °C at the surface to 16 °C at 30 m (Table 3) and thermotolerant amoebae were isolated throughout the range. The majority, however, were recovered from waters between 19 and 24 °C. One would expect thermotolerant amoebae to be most abundant in warmer waters. However, in the present study, the warmest water at the surface yielded the fewest amoebae even though nutrients, presumably, should be greatest at the surface. Obviously there are many yet-to-be-defined factors that prescribe the environmental niche of thermotolerant freeliving amoebae. We reported previously the isolation of pathogenic freeliving amoebae from the shallow waters of Tulsa-area ponds with temperatures of 3 to 34 °C (2,7). The average pH of the water samples from which thermotolerant amoebae were isolated in the present study ranged from 7.3 to 8.6 (Table 3). In previous studies we isolated pathogenic freeliving amoebae from waters in which pH ranged from 5.1 to 8.8 (2,7).

In summary, we obtained 42 thermotolerant, but nonpathogenic, freeliving amoeba isolates from 144 water samples collected in Lake Tenkiller, or approximately 1 thermotolerant amoeba for every 3 samples processed. Thermotolerant amoebae were isolated from all sampled depths: surface to 30 m; however, the majority of isolates were obtained at depths of 10 to 20 m from waters ranging in temperature from 18 to 26 °C. The prevalence of thermotolerant freeliving amoebae in the sampled waters was 1 amoeba/171 ml water. The significance of thermotolerant amoebae in the environment is that they are indicators of the potential presence of pathogenic freeliving amoebae.

ACKNOWLEDGMENTS

We thank Samantha Ahner, Mark Duncan and James Roberson for assistance with sample collection and processing, and Joni Finfrook for typing the manuscript. This investigation was supported by grant R-818106 from the United States Environmental Protection Agency.

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