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# Isolation of the Etiological Agent of Primary Amoebic Meningoencephalitis from Artificially Heated Waters†

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To determine whether artificial heating of water by power plant discharges facilitates proliferation of the pathogenic free-living amoebae that cause primary amoebic meningoencephalitis, water samples (250 ml) were taken from discharges within 3,000 feet (ca. 914.4 m) of power plants and were processed for amoeba culture. Pathogenic *Naegleria fowleri* grew out of water samples from two of five lakes and rivers in Florida and from one of eight man-made lakes in Texas. Pathogenic *N. fowleri* did not grow from water samples taken from cooling towers and control lakes, the latter of which had no associated power plants. The identification of *N. fowleri* was confirmed by pathogenicity in mice and by indirect immunofluorescence analyses, by using a specific antiserum.

Free-living amoebae belonging to the genera *Naegleria* and *Acanthamoeba* can be pathogenic to man (13). *Naegleria* is recognized as the etiological agent of fulminant primary amoebic meningoencephalitis (PAME) (1, 2), whereas *Acanthamoeba* can produce both chronic and acute encephalitis in man (7, 15). Both pathogenic and nonpathogenic species of *Naegleria* and *Acanthamoeba* are found in water and soil. However, since their potential danger to humans was recognized, reports of the isolation of pathogenic species from the environment have been infrequent.

Isolations have been reported from pipeline and drinking water and from soil in Australia (A. Jamieson, *in* Progress in protozoology, Fourth International Congress of Protozoology, Clermont-Ferrand, France, 1973, p. 159), from sewage sludge in India (9) and the Union of Soviet Socialist Republics (L. M. Gordeeva, *in* Progress in protozoology, Fourth International Congress of Protozoology, Clermont-Ferrand, France, 1973, p. 159), and from a thermally polluted canal that received cooling water from a factory in Belgium (4). Recently, we reported preliminary results on the isolation of pathogenic *Naegleria* from Florida lakes (16). Subsequently, Wellings et al. (11) confirmed our findings. Potentially pathogenic *Acanthamoeba* have been isolated only from sewage sludge in India (9) and from hot water springs in New Zealand (A. Jamieson, personal communication).

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As evidenced in the preceding reports, one important factor selecting for pathogenic amoebae in water is temperature. According to Griffin (6), only pathogenic species of free-living amoebae are able to grow at temperatures as high as 42°C. However, more recent studies (4) showed that not only pathogenic *Naegleria*, but also other nonpathogenic free-living amoebae were able to proliferate at 42°C. Consequently, in the present study of discharge cooling waters from electric power plants, we screened for pathogenic amoebae at 45°C.

## MATERIALS AND METHODS

**Sampling sites.** To obtain a spectrum of independent environments, in 1976, warm-water samples were taken from Florida (June), Texas (July), and Tennessee (August). The sampling was done as close as possible to the discharges of the power plants in Florida (usually within 100 feet [ca. 30.48 m]). Samples were obtained from most Texas lakes at greater distances from the discharge point (up to 3,000 feet [ca. 914.4 m] away) because of limited access to the outlets.

Table 1 lists the names and respective temperatures of bodies of water sampled in Florida and Texas. The bodies of water without associated power plants were sampled as controls. All of the Texas lakes sampled are man-made lakes. Power plant cooling towers in Oak Ridge, Tenn., were also screened for amoebae to determine whether closed-cycle cooling tower systems provide favorable conditions for pathogenic strains to develop. The temperatures ranged between 25 and 30°C.

**Sampling and processing.** At each site several water samples were taken in sterile 250-ml containers from surface water either before or after stirring the

TABLE 1. *Isolation of amoebae at 45°C from Florida and Texas waters*

| Sampling site | No. of samples | Water temp (°C) | Samples positive for amoebae | Samples positive for pathogenic *N. fowleri* |
|---|---|---|---|---|
| Florida | | | | |
| Lake Lotello[a] | 4 | 36–37 | 0 | — |
| Dania Land Cutoff[a,b] | 4 | 35 | 1 | 0 |
| Lake Worth[a,b] | 3 | 29–31 | 0 | — |
| Lake Montoe[a] | 6 | 38 | 5 | 3 |
| St. Johns River (Sanford)[a] | 2 | 35 | 1 | 1 |
| St. Johns River (Jacksonville, Southside)[a,b] | 4 | 34–35 | 3 | 0 |
| St. Johns River (Jacksonville, Kennedy)[a,b] | 2 | 32 | 2 | 0 |
| St. Johns River (Jacksonville, Northside)[a,b] | 5 | 41 | 3 | 0 |
| Lake Conway | 4 | 33–34 | 0 | — |
| Little Lake Conway | 3 | 33–34 | 0 | — |
| Lake Eloise | 3 | 32–33 | 0 | — |
| Lake Alfred | 3 | 32 | 0 | — |
| Texas | | | | |
| Eagle Mountain Lake[a] | 5 | 31–33 | 0 | — |
| Lake Weatherford[a] | 3 | 29 | 2 | 0 |
| Lake Graham[a] | 4 | 35 | 0 | — |
| Lake Palo Pinto[a] | 4 | 36–37 | 0 | — |
| Lake Arlington[a] | 9 | 39–41 | 7 | 1 |
| Mountain Creek Lake[a] | 4 | 32–34 | 1 | 0 |
| North Lake[a] | 8 | 39–41 | 1 | 0 |
| Trinity River[a] | 3 | 35 | 3 | 0 |
| Possum Kingdom Creek | 2 | 33–34 | 1 | 0 |
| Benbrook Lake | 4 | 32 | 3 | 0 |
| Lake Worth | 3 | 30 | 2 | 0 |

[a] Waters receiving discharges from power plants.
[b] Brackish water.

underlying soil. These samples were processed in the laboratory often on the same day or at least within 48 h. Samples (250 ml) were filtered through acetate membranes (1.2-μm porosity), which were then inverted on nonnutrient agar seeded with heat-killed *Aerobacter aerogenes*. Pieces of water plants, mud, or detritus were placed on the medium, either at the sampling site or in the laboratory. During the sampling in Texas, between 25 and 50 ml of water also was filtered through sterile membrane filter holders (Swinnex, Millipore Corp., Bedford, Mass.) fitted with 1.2-μm cellulose acetate membranes, which were then planted directly onto nonnutrient agar plates seeded with heat-killed bacteria.

The parafilm-sealed plates were incubated at 45°C for selective cultivation of *N. fowleri* (6). The plates were observed for growth daily under an inverted microscope. As soon as good growth was obtained on the original plates, the heterogenous population was harvested and seeded onto several new plates. Populations from the secondary cultures were used to identify the amoebae by established criteria.

Cultures were also incubated at 37°C for isolation of other thermophilic species. Results of the studies of these cultures will not be reported here, but it should be noted that amoebae grew abundantly from all samples incubated at 37°C.

**Identification of the amoebae.** Amoebae that grew from samples incubated at 45°C were identified as belonging to the genus *Naegleria* from their morphological characteristics and their ability to flagellate. Page's (8) criteria for the structure of *Naegleria* trophozoites and cysts were used. The flagellation tests were performed as described previously (14), except that the samples were incubated at 45°C.

Each sample growing at 45°C was tested for mouse pathogenicity by intranasal instillation of weanling ICR or BALB/c mice. For each heterogenous population of amoebae, five mice were inoculated with trophozoites that had been harvested from the plates and suspended in 0.05 ml of sterile distilled water. The number of trophozoites inoculated from each sample is shown in Table 2. The mice were observed for up to 2 weeks for acute symptoms of PAME. Brains from dead or moribund mice were examined fresh or after fixation for presence of amoebae. Mice were considered to have died from other causes when amoebae were not identified upon microscopic examination or after culturing of brain tissue.

A homogeneous population of the pathogenic isolate was obtained by inoculating brain tissue from dead or moribund mice into an axenic Casitone-based medium (CGVS) (12). The strain designations of the amoebae reisolated from the brain tissues are listed in Table 3. After axenization, species identification was accomplished by indirect immunofluorescence analyses. Antisera that were produced against nonpathogenic species of *Naegleria* and against *N. fowleri* were

TABLE 2. *Mouse pathogenicity tests with amoeba populations growing at 45°C*

| Location | Population designation | Total no. of amoebae inoculated | Day of death | % Mortality | Amoebae in brains |
|---|---|---|---|---|---|
| Florida | | | | | |
| Dania Land Cutoff | La-1 | $1.6 \times 10^4$ | | 0 | |
| Lake Monroe | Ent-1 | $7 \times 10^3$ | 5, 7, 7, 10 | 80 | Yes |
| | Ent-3 | $6 \times 10^3$ | 4, 5, 6, 6 | 80 | Yes |
| | Ent-5 | $1.9 \times 10^4$ | 7, 7, 8 | 60 | Yes |
| St. Johns River (Sanford) | Sa-3 | $1.6 \times 10^4$ | 5, 5, 5, 6, 7 | 100 | Yes |
| St. Johns River (Jacksonville, Southside) | S1 | $4 \times 10^3$ | 3, 7 | 40 | No |
| | S6 | $2 \times 10^3$ | 8 | 20 | No |
| St. Johns River (Jacksonville, Kennedy) | Ke-1 | $3.4 \times 10^3$ | | 0 | |
| | Ke-3 | $3.4 \times 10^3$ | 11 | 20 | No |
| St. Johns River (Jacksonville, Northside) | N-1 | $7.9 \times 10^3$ | 8 | 20 | No |
| | N-5 | $8 \times 10^3$ | | 0 | |
| Texas | | | | | |
| Lake Weatherford | LW-1 | $9.6 \times 10^3$ | | 0 | |
| Lake Arlington | Ar-1 | $1.6 \times 10^4$ | | 0 | |
| | Ar-3 | $8 \times 10^4$ | | 0 | |
| | Ar-5 | $3.3 \times 10^4$ | | 0 | |
| | Ar-7M$_1$ | $4 \times 10^3$ | | 0 | |
| | Ar-9M$_1$ | $8 \times 10^3$ | 5 | 20 | No |
| | Ar-12 | $8 \times 10^3$ | 5, 5, 6, 7, 7 | 100 | Yes |
| Mountain Creek Lake | Mc-6M$_1$ | $5 \times 10^4$ | | 0 | |
| Trinity River | NM-3M$_1$ | $6 \times 10^3$ | 3 | 20 | No |
| | NM-4M$_1$ | $1 \times 10^4$ | | 0 | |
| Lake Benbrook | Be-4 | $1.2 \times 10^3$ | | 0 | |
| Lake Worth | LWo-3M$_1$ | $2.4 \times 10^3$ | | 0 | |

used for the immunofluorescence analyses (14). The strain of *N. fowleri* was an isolate from a patient who died from PAME (14).

## RESULTS

**Florida.** Several strains of *N. fowleri* were isolated from bodies of water that received thermal discharges from electric power plants in Florida (Table 1). Amoebae grew after a few days at 45°C from five of six samples obtained from Lake Monroe, which had a temperature of 38°C at the sampling site. Amoebae from three of these five samples exhibited morphological characteristics of *Naegleria* and were positive when tested for their ability to flagellate. The three positive samples were all obtained from water samples.

*Naegleria* also grew from one of the two samples obtained from the St. Johns River (Sanford). In contrast to the lake samples, however, the positive sample was a piece of water plant incubated at 45°C. The water sample from the St. Johns River (Sanford) remained negative for amoeba growth.

The *Naegleria* isolates obtained from both the lake and river were highly pathogenic to mice. Sixty to eighty percent of the mice died within 4 to 10 days after infection with amoebae from Lake Monroe (Table 2). All five mice inoculated with the river isolate died within 7 days after infection. Phase-contrast, histological, and electron microscopic examinations of brain tissue of moribund or dead mice that had been infected with either the lake or river isolates confirmed the presence of the amoebae. Additionally, extensive growth of amoebae was obtained within 24 h from brain tissues inoculated

TABLE 3. *Indirect immunofluorescence analyses on pathogenic Naegleria isolates from thermally heated waters*

| Strain | Antiserum[a] | | |
|---|---|---|---|
| | *N. fowleri* (Morgan) | *N. jadini* (ITMAP 400) | *N. gruberi* (CCAP 1518) |
| E-Ent-1 | 1:512 | 1:16 | 1:16 |
| E-Ent-3 | 1:512 | 1:16 | 1:8 |
| D-Sa-3 | 1:1,024 | 1:16 | 1:16 |
| Q-Ar-12 | 1:512 | 1:8 | 1:8 |
| *N. fowleri* (Morgan) | 1:1,024 | 1:32 | 1:8 |

[a] End-point titers are given in this table and compared with *N. fowleri* (Morgan) isolated from a human case of PAME (14).

into CGVS medium. The reisolated strains from the lake were designated as E-Ent-1, E-Ent-3, and E-Ent-5; the river reisolate was designated as D-Sa-3. Indirect immunofluorescence tests were performed on two of the three *Naegleria* isolates from Lake Monroe. The amoebae fluoresced to a positive titer of 1:512 when reacted with anti-*N. fowleri* serum, whereas they reacted minimally with anti-*N. jadini* and anti-*N. gruberi* sera (Table 3). The isolate obtained from the St. Johns River (Sanford) stained to homologous titer (1:1,024) with the *N. fowleri* antiserum and insignificantly with the other antisera (Table 3).

Amoebae also grew at 45°C from one of four samples obtained from the brackish canal water (Dania Land Cutoff) and from 8 of 11 samples taken at three different sites in the brackish estuary of the St. Johns River (Table 1). Because none of the organisms gave a positive flagellation test and none of those tested induced acute pathological signs of PAME in mice (Table 2), no further attempts were made to identify them specifically.

In Florida only two bodies of water that received thermal discharges, Lake Worth and Lake Lotello, showed no growth of amoebae at 45°C. The samples taken from the four freshwater lakes without thermal discharges also showed no growth of amoebae at 45°C (Table 1).

**Texas.** Samples obtained from many of the bodies of water in Texas showed growth of amoebae at 45°C (Table 1). These bodies of water included both those associated and unassociated with power plants. However, *N. fowleri* was positively identified in only one sample that showed amoeba growth at 45°C. This strain of *N. fowleri* grew from a 50-ml water sample taken at Lake Arlington, which received large volumes of thermal effluents of a power plant. This lake also had the highest temperature recorded at the sampling sites, most of which were located over one-half mile (ca. 0.8 km) from the plant outlet.

Mortality in mice was 100% within 7 days after intranasal inoculation with amoebae from the Lake Arlington sample (population designation, Ar-12, Table 2). Amoebae were found in the mouse brains on direct examination and in the histological and electron microscope preparations. Indirect immunofluorescence analyses of the mouse brain reisolate (strain designation, Q-Ar-12) confirmed that this isolate was *N. fowleri* (Table 3).

Many of the populations of amoebae that grew from samples of the remaining bodies of water in Texas were also tested for pathogenicity in mice. None of these, however, produced acute infections in the animals (Table 2).

**Tennessee.** From six water samples obtained at three cooling towers in Oak Ridge, three showed growth at 45°C. None of the amoeba populations were *Naegleria*, since they could not be induced to flagellate. The amoebae were also negative when tested for pathogenicity in mice.

## DISCUSSION

During the last decade, five cases of PAME in humans have been diagnosed in Florida (13). All of these cases were related to swimming in lakes. The epidemiology of PAME, as described in the literature, indicates that this disease has always been correlated with swimming in naturally or artificially heated waters, with infection occurring through the nasal mucosa.

The results of the studies reported in this communication suggest that thermal additions enhance the occurrence of pathogenic *N. fowleri* in the environment. Such a suggestion was made previously by De Jonckheere et al. (4) and by us (16). Nevertheless, other factors must contribute to the distribution of pathogenic *Naegleria* in the environment because in our studies the amoebae were not found in all the bodies of water that received thermal discharges. Negative results, however, are not as meaningful as positive data, in that samples of 50 to 250 ml are minute, relative to the total volume of the site.

An alternative possibility is that nonpathogenic *N. fowleri* were present but not detected in these waters, because we specifically identified only those isolates that produced PAME in mice. De Jonckheere et al. (4) found nonpathogenic *N. fowleri* in a thermally polluted canal in Belgium by indirect immunofluorescence analyses of heterogenous populations that grew at 42°C. These workers suggested that nonpathogenic strains may, under certain conditions, undergo a physiological change and become pathogenic. In our studies, however, the majority of the amoebae that grew at 45°C were not amoeboflagellates and thus were not *Naegleria*. These amoebae were tentatively identified as *Mayorella, Hartmannella,* or *Vahlkamphia*. Considering the growth of these amoebae at 45°C, it is apparent that incubation of samples at 45°C is not completely selective for obtaining pathogenic *N. fowleri* from environmental samples.

The presence of these potentially pathogenic organisms in lakes receiving thermal discharges could indicate the possibly dangerous environment that these lakes may pose to human health. Most of the lakes studied by us are used for recreation—swimming, water skiing, and

boating. Moreover, several of these lakes are currently used for breeding fishes, which might also be infected by the amoebae. For example, Taylor (10) reported that in several epidemics among fish in a Florida lake, he had isolated several strains of free-living amoebae from the organs of the diseased fish.

Another possibly hazardous situation was observed in Lake Arlington, which received thermal discharges and yielded pathogenic *N. fowleri*. This lake is currently used to supply domestic drinking water for the surrounding area. Derreumaux et al. (5) reported that trophozoites of pathogenic *Naegleria* and *Acanthamoeba* are destroyed when concentrations of 0.5 to 1.0 μg of free, active chlorine per ml are maintained in water. However, De Jonckheere and van de Voorde (3) reported that the cysts of pathogenic *Naegleria* and *Acanthamoeba* were more resistant to chlorination than were their trophozoites. Thus, close attention should be given to effective chlorination in water purification stations, especially if they are associated with a body of water that may contain pathogenic amoebae.

Recently, Wellings et al. (11) confirmed our finding of pathogenic *Naegleria* in Florida waters. These workers isolated the organism from some nonartificially heated freshwater lakes in addition to a thermally polluted lake, only one of which was sampled. However, these authors did not obtain pathogenic *Naegleria* from any water with a temperature below 26.5°C, which supports the contention that thermal input, be it solar or man-made, enhances the possible proliferation of pathogenic free-living amoebae.

Nevertheless, quantitative data are still needed to evaluate the relative importance of each heat source. Thus, it is difficult at present to clearly identify man-made thermal additions to lakes in Florida as the only causative factor in maintaining pathogenic *Naegleria* in the environment. The increasing demand for energy production and the concomitant increase in temperature of associated cooling waters, the increasing trend to high-temperature, closed-cycle cooling lakes and towers, and our recent observations that pathogenic *Naegleria* proliferate in such warm waters indicate that further studies of a quantitative nature are needed to evaluate critically the extent and control of this problem.

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