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THE DISTRIBUTION OF *NAEGLERIA FOWLERI* IN SELECTED NORTHEAST ARKANSAS LAKES

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ABSTRACT

Seven northeast Arkansas recreational lakes were examined for the presence of pathogenic and nonpathogenic *Naegleria fowleri*. Cultural differentiation and microscopic morphology were used as species determining tests, while mouse pathogenicity tests were conducted to determine virulence. Only one isolate met all criteria utilized for definite identification of *Naegleria fowleri*, although *Naegleria* type organisms were found in all of the lakes. None of the isolates were pathogenic in mice.

INTRODUCTION

Increasing attention has been directed to the recently discovered pathogenic freshwater amoeba, *Naegleria fowleri*, implicated as the causative agent of primary amoebic meningoencephalitis in humans (Carter, 1972). De Jonckheere and De Voorde (1977a) and Wellings et al. (1977) found that environmental isolates of *Naegleria spp.* can be readily cultured by collecting sediment samples from thermally polluted lakes and streams. By the use of varied culture conditions established by Griffin (1972) and Weik and John (1977), species can be separated. Both smaller size and cultural fastidiousness distinguish *N. fowleri* from *N. gruberi*, a nonpathogenic species (Carter, 1972).

De Jonckheere (1977) reported that both pathogenic and nonpathogenic strains of *N. fowleri* exist and that pathogenic strains were almost invariably associated with nonpathogenic variants. Most distribution studies with pathogenic strains have involved water reservoirs that received thermal effluent which raised the water temperature, creating conditions favorable for the growth of *N. fowleri*. Relatively little is known about its distribution in temperate climate reservoirs which only seasonally reach favorable temperatures. However, Wellings et al. (1977) isolated *N. fowleri* from several Florida lakes which were subject to seasonal temperature fluctuations. They demonstrated that the organisms can overwinter in lake bottom sediments at temperatures as low as 12°C. Bone and Becker (1975) reported negative results from isolation attempts employing surface water samples from recreational lakes in western Arkansas.

The purpose of the current study was to determine if *N. fowleri* was present in several northeast Arkansas lakes. Sediment samples were collected during seasonal warm temperatures and processed for use in differential cultivation, microscopic examination and mouse infectivity.

MATERIALS AND METHODS

Two strains of *N. fowleri*, LEE and HB-4, were obtained from D.T. John *(per. comm.)*. These were used for comparison purposes and as cultivation controls.

Seven lakes in northeast Arkansas were selected as sampling sites because of frequent use by the public, and the variety of ages, depths, sizes and sediments. The seven lakes were Lake Charles, Lake Frierson, Lake Hogue, Craighead Lake, Lake Poinsett, Big Lake and Mallard Lake. Five of the lakes have restricted use and do not allow swimming. Both Lake Charles and Craighead Lake permit swimming in specified areas, while water skiing is also allowed on the remainder of the latter lake. All the lakes were man-made, with the exception of Big Lake, and none receive significant thermal effluent.

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Sediment samples were taken at the mud-water interface. Samples from depths less than 62 mm were simply scooped up in 400 ml biological specimen jars, while deeper samples were collected by the use of an Eckman dredge, 21 X 21 cm (Wildlife Supply Co., Saginaw, Michigan). Subsurface water and sediment were separated and placed in 400 ml biological specimen jars. The water temperature was immediately taken, and some of the water was mixed with the sediment to form a muddy slurry. All samples were processed in the laboratory on the day of collection. The pH of the mud slurry was determined with an electronic pH meter immediately upon opening the jars.

The procedure described by Wellings et al. (1977) was followed, with modifications, for the processing of samples. The samples were washed with 100 ml of 1% beef extract. After vigorous shaking, heavy material was allowed to settle, and the supernatant fluid was centrifuged at 500 X G for 30 minutes. The resulting supernatant was discarded, and the pellets were resuspended in 25 ml of 1% beef extract and centrifuged at 500 X G for 15 minutes. The supernatant was again discarded, and the pellets were placed on 15 ml non-nutrient agar bearing a heavy suspension of *Escherichia coli* (NNE) (De Jonckheere and De Voorde, 1977b). These were incubated in petri dish cans with lids at 30 and 45°C, the latter temperature reportedly being inhibitory to *N. gruberi* and some nonpathogenic *N. fowleri* (De Jonckheere and De Voorde, 1977b). Plaque-forming cultures were transferred and maintained on NNE by removing a 1 X 1 cm piece of agar from the dense ring of migrating amoebae and placing it on a fresh NNE plate. Transfers were also made to a modified Nelson's medium containing 0.1% (w/v) liver concentrate (Sigma Chemical Co.), 0.1% (w/v) glucose and 2% (v/v) fresh rabbit serum in Page's saline (Page, 1967), pH 6.5 in screw-cap culture tubes and incubated at 30°C (Weik and John, 1977).

An attempt was made to suppress nonpathogens to an even greater extent by increasing the incubation temperature as suggested by De Jonckheere and De Voorde (1977b) and Griffin (1972). Mixed isolates exhibiting characteristics of *N. fowleri* were incubated at 40°C on NNE for 7-10 days and examined daily for plague formation.

Wet mount microscopic examinations of trophozoites and cysts were made with a Wild phase-contrast microscope to determine the type of movement and cyst wall morphology. The ability of the organisms to flagellate was also observed microscopically. One drop of amoeba suspension was mixed with two drops of deionized water, incubated at 42°C and viewed at 30 min. intervals for the formation of biflagellates. In order to more critically examine the trophozoites for nuclear detail and the cysts for pore structure, permanent slides were prepared with iron-hematoxylin stain according to the method outlined by Spencer and Monroe (1961).

Isolates which exhibited characteristics of typical *N. fowleri* were utilized in mouse pathogenicity studies. Washings of active plaques on NNE were made with Page's saline, and the number of tropho-

zoites per ml was determined by the use of a Levy hemocytometer. Amoeba suspensions containing 1.0 X 10^6 to 1.1 X 10^7 trophozoites per ml were used to infect mice. Young white mice were anesthetized with ether and inoculated intranasally with 0.02 µl of the suspension. Three weanling mice were injected intracranially with 0.02 µl of amoeba suspension. All mice were observed for 14 days for reactions indicative of meningoencephalitis. Any mice which died or were sacrificed after exhibiting a reaction were autopsied and the brain examined microscopically for amoebae. All other mice were euthanized after 14 days.

Identification of isolates as *N. fowleri* was based on criteria provided by De Jonckheere et al. (1975) and De Jonckheere and De Voorde (1977b). These criteria are summarized as follows:

1. Ability to grow at 45°C.
2. Morphological features: A large karyosome surrounded by clear zone, a fine nuclear rim, a single contractile vacuole and eruptive, finger-like pseudopodia are typical of *N. fowleri*.
3. Flagellation test at 42°C: *N. fowleri* isolates placed in deionized water will form biflagellates at 42°C.
4. Pore structure of the cysts: Cysts of *N. fowleri* show only a small number of flat-edged pores, while *N. gruberi* cysts show many crater-like pores.

RESULTS

Cultivation of *N. fowleri* was considerably better on NNE medium than in the modified Nelson's medium. Growth occurred rather slowly in the latter with sufficient numbers of amoebae being available for transfer only after several weeks of incubation. It was observed, however, that cysts remained viable in this medium for months and produced normal plaques when reinoculated onto NNE.

The varied environmental conditions of the lakes with respect to the differences in age, size, sediment and public use (Table 1) provided a good cross section of thermally non-polluted lakes in Arkansas. The sampling sites generally were protected from disturbance from wind or wave action; however, some sites were located in mid-channel or in public swimming areas. Samples taken from each of the seven lakes produced plaques of *Naegleria*-type organisms on NNE at 45°C (Table 2).

Many of the amoebae exhibited typical *Naegleria spp.* movement. Of the amoebae producing eruptive, finger-like pseudopodia, several were observed to have a spherical nucleolus surrounded by a clear zone and a lack of chromatin granules. These amoebae also transformed to a biflagellate stage when incubated at 42°C in deionized water. Pore structure of the cysts was of two types, those typical of *N. gruberi* and those typical of *N. fowleri*. Incubation at 48°C killed all except two cultures, P3 and P6, both isolated from Lake Poinsett. Subsequent examination indicated that culture P6 was now comprised of organisms fully consistent with *N. fowleri*, including typical cyst pore structure; while P3 still produced some cysts characteristic of *N. gruberi*. None of the isolates were pathogenic to mice when administered intranasally. Amoebae injected intracranially were found living in the brain tissue of the mice which died three days after inoculation.

Table 1. Criteria for the selection of lakes.

Lake (County)	Age (yrs)	Maximum distance length (m)	width (m)	Sediment	Public use
Poinsett (Poinsett)	35	2,414	1,609	Aufwuchs overlying organic mud debris	Fishing
Mallard (Mississippi)	14	2,407	792	Clay mud	Fishing
Big (Mississippi)	108	19,747	2,416	Organic mud debris	Fishing
Hogue (Poinsett)	11	853	836	Mud	Fishing/ hunting
Charles (Lawrence)	16	4,827	1,609	Sand/mud	Fishing/ swimming
Frierson (Greene)	5	2,835	806	Mud	Fishing/ hunting
Craighead (Craighead)	5	1,926	1,628	Gravel	Fishing/ swimming/ water skiing

Table 2. Isolation of amoebae at 45°C.

Sample site and number	Depth (m)	Temperature (C)	pH	Plaque formers
Lake Poinsett 9-19-1979				
1	0.91	27.5	5.17	+
2	0.61	26.5	5.25	+
3	0.91	27.0	5.40	+
4	1.82	24.0	5.13	+
5	0.30	27.0	5.26	-
6	0.61	27.0	5.33	+
7	0.91	26.0	5.42	-
8	0.30	26.5	5.41	+
9	0.76	27.0	4.73	-
10	0.30	27.0	5.51	+
11	4.87	22.0	5.11	-
Mallard Lake 9-22-1979				
1	0.30	18.5	5.87	+
2	1.82	20.0	6.16	+
3	0.61	20.0	6.44	-
4	0.30	20.0	6.47	-
5	2.74	20.0	6.26	+
6	0.30	19.0	6.75	-
Big Lake 9-22-1979				
1	0.30	20.0	6.71	-
2	0.91	20.0	6.63	+
3	0.30	21.0	6.79	+
4	0.30	20.0	6.79	+
5	2.74	20.0	5.88	-
6	1.82	20.0	6.35	-
7	0.61	21.0	6.40	-
Lake Hogue 9-22-1979				
1	0.15	24.0	6.25	-
2	4.57	21.0	5.90	-
3	0.91	21.0	6.25	+
4	0.61	23.0	6.40	-
5	0.76	22.0	6.50	-
6	0.91	22.0	6.20	+
7	0.61	22.0	6.50	+
Lake Charles 9-25-1979				
1	0.76	24.5	6.90	-
2	0.91	24.0	6.60	-
3	0.61	25.5	6.55	+
4	1.82	22.0	6.65	+
5	4.57	20.5	6.76	-
6	0.76	21.5	6.80	+
7	0.76	20.0	6.60	+
8	0.76	24.5	6.37	+
9	3.04	24.0	6.43	+
Lake Frierson 10-2-1979				
1	0.30	25.0	6.50	-
2	0.91	27.0	6.65	+
3	1.21	27.0	6.30	-
4	0.61	28.0	6.49	+
5	0.61	24.0	6.35	+
6	5.18	19.0	6.60	-
Craighead Lake 10-2-1979				
1	0.61	23.0	6.40	-
2	0.30	25.0	6.20	-
3	0.30	24.0	6.50	+

DISCUSSION

The poor growth in modified Nelson's medium suggests that some vital nutritional requirement is not present in the substituted liver concentrate or rabbit serum. The requirement is probably not a carbohydrate, as work by Weik and John (1977) revealed that other substrates are preferred as carbon and energy sources. This deficiency might be a protein, a vitamin or a mineral which is not available in a readily accessible form. Considering the variety of proteins that are present in the serum of any mammal, it seems unlikely that the missing substance is protein.

Earlier work in western Arkansas indicated that *N. fowleri* was not present (Bone and Becker, 1975). Sampling techniques and culture methods may provide the explanation for differing results. Cerva (1978) studied the distribution of *N. fowleri* from industrial effluent and found that all cultivation attempts from water samples were negative. However, he stated that positive cultures were collected from biological material scraped from the walls of the channel and from bottom sediment. In conditions where a small or moderate population of amoebae exists, it is likely that they are more difficult to isolate by sampling only water. Considering the cultural fastidiousness of *N. fowleri*, it is also possible that the BST agar with *Pseudomonas aeruginosa* used by Bone and Becker (1975) may not have supported adequate growth of the organisms to permit isolation. The NNE medium described by De Jonckheere et al. (1975) promotes rapid, luxuriant growth of *N. fowleri* and has been used in other recent studies (De Jonckheere and De Voorde, 1977b; Wellings et al., 1977).

The possibility that pathogenic amoebae exist in water which only seasonally reaches favorable temperatures is clearly indicated by the present investigation. De Jonckheere (1977) found that pathogenic *N. fowleri* are almost invariably associated with nonpathogenic variants. Although none of the isolates in the present study were proven to be pathogenic, the presence of nonpathogens demonstrates that environmental conditions suitable for *N. fowleri* occur in northeast Arkansas, and pathogens may also be present. It has been suggested that nonpathogenic *N. fowleri* might be considered as indicator organisms for environments where primary amoebic meningoencephalitis may be contracted (De Jonckheere et al., 1977).

Wellings et al. (1977) found that pathogenic and nonpathogenic strains show slight antigenic differences when compared by indirect immunofluorescence. Many of the amoebae isolated in this study met some of the basic criteria used for identification of *N. fowleri;* however, the IFA technique was not utilized, and an antigenic comparison between local isolates and reference strains was not made.

The present investigation indicates that *N. fowleri* exists in some northeast Arkansas lakes. Since pathogenicity was not demonstrated, future studies should include other parameters such as antigenic differences by the use of IFA in addition to mouse pathogenicity. No definite relationship was observed between the isolation of the organisms and the pH, temperature, depth, sediment type, size or public use of the lakes.

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