APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Dec. 1977, p. 661-667
Vol. 34, No. 6

Printed in U.S.A.

Isolation and Identification of Pathogenic *Naegleria* from Florida Lakes

F. M. WELLINGS,[1] * P. T. AMUSO,[1] S. L. CHANG,[2] AND A. L. LEWIS[1]

Epidemiology Research Center, Department of Health and Rehabilitative Services, Tampa, Florida 33614[1], and U. S. Environmental Protection Agency, Health Effects Research Laboratory, Cincinnati, Ohio 45268[2]

Received for publication 5 July 1977

Five cases of primary amoebic meningoencephalitis associated with swimming in freshwater lakes have been recorded in Florida over the past 14 years. The present study demonstrated that pathogenic *Naegleria*, the causative agent, is relatively widespread. Twelve of 26 lakes sampled only once yielded the amoeba. Populations in three of five lakes sampled routinely reached levels of one amoeba per 25 ml of water tested during the hot summer months. Overwintering in freshwater lake bottom sediments was demonstrated, showing that thermal-discharge pollution of waters plays a miniscule, if any, role in the maintenance of pathogenic *Naegleria* in nature in this semitropical area.

The presence of pathogenic *Naegleria* amoeba in four freshwater lakes in Florida was indicated by epidemiological data accrued on five of the seven fatal primary amoebic meningoencephalitis (PAM) cases that occurred in the state since 1962 (2, 3, 5). These five cases associated with freshwater lake swimming had all occurred in the Orlando area.

Butt (2) reported isolating pathogenic amoebae from a lake in the Orlando area that was considered the source of PAM infection in two cases. Although intracerebral inoculation of these amoebae into mature white Swiss mice resulted in death, overt infections after intranasal instillation failed to occur. Thus, it is unclear whether these isolates were pathogenic *Naegleria* or strains of *Acanthamoeba*.

In an attempt to study the role of lake waterborne pathogenic *Naegleria* in PAM infections in Florida, a study was initiated in January 1975. The first priority was to determine the distribution of the agent in Florida lakes. Results of this facet of the study are reported here.

MATERIALS AND METHODS

Sampling sites. A thermally polluted lake receiving cooling waters from an electric power generating plant and two freshwater lakes in the Orlando area were sampled initially. The former was selected since isolations of pathogenic *Naegleria* from thermally polluted waters had been reported initially from Belgium (6). The latter were chosen because they were suspected as sites of exposure for PAM cases. As the study progressed, additional lakes throughout Florida were randomly selected for study.

Sample collection. Large quantities, 50 to 100 gallons (189.3 to 378.5 liters), of lake water were filtered on site through a sand column 1.5 inches (38.1 mm) in diameter and 2 feet (60 cm) long containing approximately 600 ml of Ottawa silica sand. The flow rate was approximately 1 gallon/min. One- to two-liter samples were also obtained in sterile glass jars from several sites. These were returned to the laboratory within 4 h and processed by passage through 5-μm membrane filters (Millipore Corp., Bedford, Mass.). Water samples were obtained approximately 5 feet (1.5 m) from the shoreline in relatively shallow water, i.e., 3 to 5 feet (0.9 to 1.5 m).

The water depth in which lake bottom samples were taken dictated the collection technique used. In shallow waters, from 0.2 to 1 foot (63.5 to 304.8 mm), a small can was used to scoop up bottom sand. Water was decanted, and the remaining sediments were placed into a resealable plastic bag for transport to the laboratory. In water depths from 4 to 8 feet (1.2 to 2.4 m), a core sample was obtained. A polyvinyl-chloride tube, approximately 10 feet (3 m) long with a 2.5-inch (63 mm) internal diameter, was forced approximately 10 to 12 inches (254 to 305 mm) into the lake bottom; the top was sealed with a cork; and the pipe was removed from the water. There was always some loss of the core on withdrawal, but the upper 6 to 7 inches (152.4 to 177.8 mm) of the core was retained in the pipe. The core was allowed to drain from the tube into a resealable plastic bag, and the water column was discarded.

In water depths greater than 8 feet (2.4 m), drag samples were collected. Lead weights were attached to one side of a commercial food can (approximately 2-liter volume), and draglines were attached to the same and opposing sides of the container. The weight permitted the can to settle onto the lake bottom when dropped over the back of the boat. With the boat's forward motion, tension on the top tow line (opposite the weight) forced the lip of the can to cut into the soft lake bottom and permitted collection of the lake bottom drag sample. As the can filled, tension on the dragline increased, and, when maximum, the filled can was withdrawn. Sediments were placed in resealable

plastic bags for transport to the laboratory. On arrival at the laboratory, all samples not processed immediately were placed in the 43°C incubator for overnight storage. Processing was done the following morning.

Isolation. Each of the large mud and sand samples from the columns was placed in separate beakers and mixed with 400 to 600 ml of 1% beef extract as an eluate. After vigorous stirring, the sand was permitted to settle out, and the supernatant was quickly decanted into 200-ml centrifuge bottles. After centrifugation at 500 × *g* for 30 min, all but 80 ml of the supernatant was aspirated and discarded. The pellet was resuspended, transferred into 40-ml conical tubes, and recentrifuged at 500 × *g* for 15 min. The supernatant was discarded.

Sufficient inorganic agar plates (IA) (grams per liter of distilled water: $Na_2HPO_4 \cdot 7H_2O$, 2.0; KH_2PO_4, 0.4; $MgCl_2 \cdot 6H_2O$, 0.213; $CaCl_2$, 0.1; $FeSO_4$, 0.025; agar, 15.0) bearing a heavy suspension of living *Enterobacter aerogenes* were used to permit inoculation of all the sediments, using approximately 20 plates per sample. Inoculum was placed in the center of each and the lid was applied. These were incubated in lidded, petri dish cans at 43 to 44°C. Early studies showed that pathogenic *Naegleria* proliferated at this temperature, whereas growth of other amoebae, saprophytes, and bacteria was depressed.

Preparation of immune sera. Antisera were prepared in rabbits against one pathogenic and one nonpathogenic strain of *Naegleria*. The former, GJ strain, was originally isolated from the cerebral spinal fluid of a Florida PAM case, and the latter was the 43-9 strain isolated in February 1976 from a thermally polluted Florida lake. Antigen was prepared by growing the pathogenic strain axenically in Chang calf serum–yeast extract–casein (CYSEC) medium (4). The amoebae were pelleted by centrifugation, washed four times in phosphate-buffered saline (PBS), pH 7.2, and suspended in 20 ml of Formalin-treated PBS (1:1,000) to a concentration of 8.0×10^6 trophozoites per ml. The antigen was combined with an equal amount of Freund complete adjuvant and injected intramuscularly in 1.0-ml amounts per rabbit at weekly intervals. Titers were determined by the slide agglutination test (1), and animals were sacrificed at 8 weeks when optimal titers were obtained.

The 43-9 strain was grown axenically in inorganic solution supplemented with heat-killed *E. aerogenes* as described below. Antigen for immunization was prepared and inoculated as described for the pathogenic strain, but animals were bled out at 6 weeks. These sera were absorbed with heat-killed *E. aerogenes* to remove antibodies produced against bacterial antigens present in the vaccine before being used in the indirect fluorescent-antibody test.

Preparation of absorbed sera. Equal volumes of axenically grown GJ strain amoebae, heat-killed *E. aerogenes*, and 43-9 hyperimmune sera were mixed and permitted to react at 37°C for 2 h. Particulates were removed by centrifugation at 1,100 × *g* for 10 min. The absorbed serum was ampouled and stored at −20°C until used.

Absorbed GJ antiserum was prepared by mixing equal volumes of axenically cultured 43-9 strain amoeba and GJ hyperimmune rabbit serum. Treatment and storage were the same as described above.

Axenic cultivation of 43-9 strain. One-liter cultures of *E. aerogenes* were prepared in tryptic soy broth (Difco Laboratories, Detroit, Mich.) and incubated at 35°C for 4 days. Bacteria were concentrated by centrifugation at 10,000 × *g* for 5 min and washed three times in distilled water. Washed cells were suspended in sterile distilled water at a dilution of 1:16. Bacteria in suspensions were disrupted sonically at 200 W for 20 min (Sonifier Cell Disruptor, model 350, Branson Sonic Power Co., Stamford, Conn.), using a ¾-inch (1.9-cm)-diameter tapped disruptor horn.

Half of the suspension was placed in a water bath at 85°C for 20 min to heat kill any surviving bacteria and, thus, avoid contamination of the growth medium used for culturing the amoebae. This preparation was designated bacterial homogenate (BH). The remainder of the initial suspension was centrifuged at 17,000 × *g* for 20 min, followed by filtration through a Swinny filter (0.45-μm membrane), and was designated cell-free lysate (CFL). Increments of these preparations were added to Chang CSYEC medium, Page malt–yeast extract–amoeba saline medium (8), and inorganic solution. Comparable growth rates were based on visual observation and scored on a scale of 0 to 4.

Indirect fluorescent-antibody technique. The indirect fluorescent-antibody (IFA) technique described by Van Dijck et al. (9) was used with slight modifications. A drop of a trophozoite suspension in PBS was evenly distributed to predesignated sections on Clay Adams fluorescent-antibody slides, air dried, and acetone fixed for 10 min at room temperature. A drop of serially diluted antiserum (1/20 to 1/2,560) was added to the correspondingly marked, fixed amoebae on the slides. After a 30-min incubation period at 37°C in a humidified chamber, slides were washed twice in fresh PBS, remaining in the second wash for 15 min at room temperature. They were removed and air dried, and fluorescein-labeled anti-rabbit globulin (Baltimore Biological Laboratory, Cockeysville, Md., no. 40602; Scientific Products, Ocala, Fla.), diluted to 1:20 with PBS, was added to each antigen-antibody preparation. These were incubated for 30 min at 37°C in a humidified chamber and then washed and air dried as described above. Cover slips were mounted with buffered fluorescent-antibody mounting fluid (Difco), and the slides were examined under an Olympus microscope with an HBO-200 light source, dark-field condenser, and filters (BG 38, OG1, and a Wrattin 2 eyepiece filter). Normal rabbit serum was used as a control. Absorbed antisera were also included in the test.

Identification. Morphology of isolates was determined under ×100 magnification. All amoeba isolates showing limax form with the eruptive-like pseudopod formation typical of the family *Schizopyrenidae* were tested for formation of flagellates. For this, a piece of agar approximately 1 cm^2 was cut from the migrating ring of amoebae on the plate and placed in a culture tube containing 0.5 ml of sterile distilled water. These were incubated at 35°C and examined for flagellate forms at 1, 2, and 3 h under the inverted microscope.

Cysts from plaques giving rise to flagellate forms were carefully examined under the phase microscope.

Trophozoites from plaques yielding single-walled cysts with one or no pores were then subjected to the IFA test and/or inoculated directly into Chang medium.

Mouse inoculation. Many isolates that were tentatively identified as pathogenic *Naegleria*, based on IFA tests and growth in CSYEC medium, were made from a single-field sample. Not all isolates were tested for mouse pathogenicity by intranasal instillation, but at least one isolate from each positive sample was tested. These strains were either grown out on IA plates in the presence of *E. aerogenes* or in CSYEC medium. Amoebae growing on the former were removed from the plate by washing with PBS, and a suspension was prepared containing approximately 0.5 $\times 10^6$ to 1.0 $\times 10^6$ amoebae. Those grown in the CSYEC medium were pelleted by centrifugation, and a comparable suspension was made. Three- to 4-week-old white Swiss mice were inoculated by intranasal instillation of 0.02 ml of the amoeba suspension. Mice were examined daily for evidence of infection. At death, brains were harvested, and wet-mount smears were examined under the microscope for confirmation of fatal amoebic infections. In some instances, internal organs were also harvested for isolation attempts.

RESULTS

The initial pathogenic isolate obtained in this study was from a 100-ml sample of bottom sediments from a thermally polluted lake at a water depth of approximately 15 cm on 19 February 1976 when the water temperature was 26°C. The same day, a 50-gallon (189.3 liters) water sample yielded multiple seropositive nonpathogenic *Naegleria*, i.e., amoeba with single-walled cysts, which reacted in the IFA test but were not pathogenic for mice by intranasal instillation. The following week, when the water temperature reached 32°C, pathogenic *Naegleria* were isolated from a 600-ml water sample collected near the shoreline.

Figure 1 shows all the positive samples obtained during 1976 from thermally polluted and freshwater lakes. All samples obtained from January through mid-October were taken from shallow water, whereas those obtained from late October through December were all from water

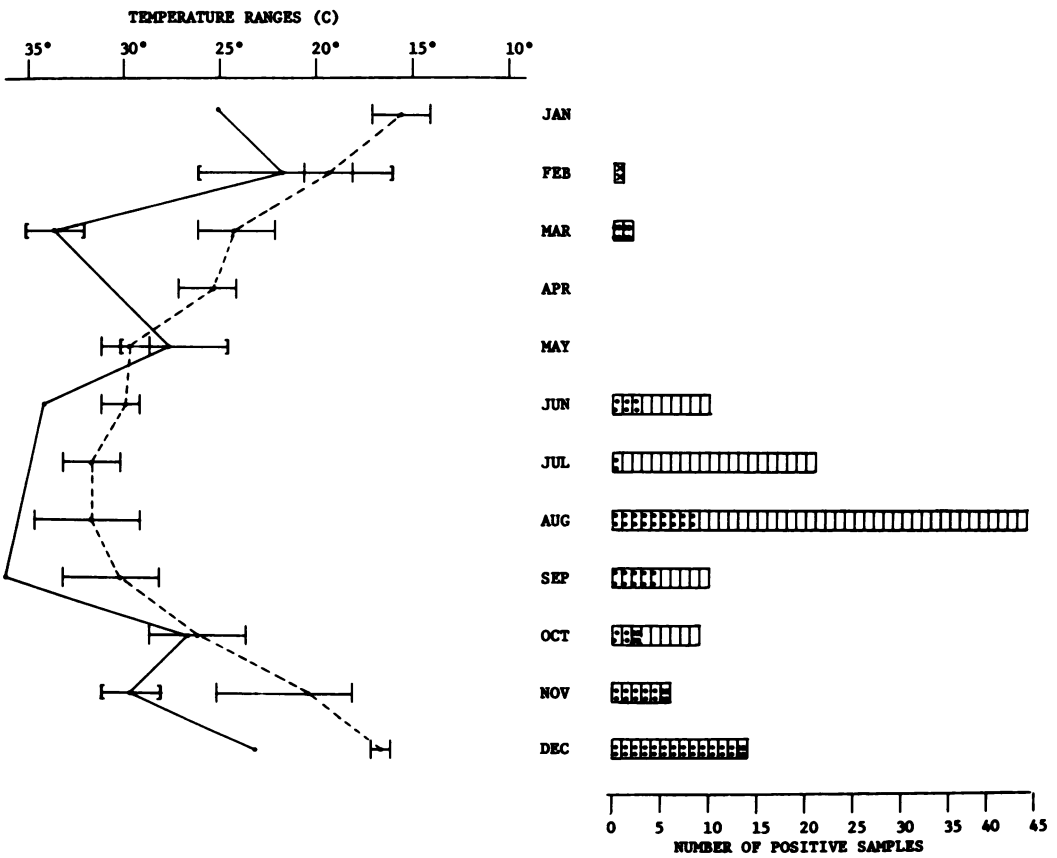

FIG. 1. *Number of positive samples by month and source, and lake water temperatures recorded when samples were obtained. (------), freshwater lakes; (——) thermally polluted lakes; (|——|) freshwater lake temperature range; ([——]) thermally polluted lake temperature range. Freshwater lake: □, water; ⁝, bottom sediments. Thermally polluted lake: ▦, water; ▤, bottom sediments.*

depths varying from 4 to 25 feet (1.2 to 7.6 m). It is readily apparent from Fig. 1 that the number of positive samples reflected the water temperature rise in freshwater lakes. During August, when the largest number of positive samples were obtained, three lakes yielded isolates from 25-ml lake water samples. As the water temperature dropped, isolates could only be obtained from lake bottom samples in water depths of 7 to 25 feet (2.1 to 7.6 m). Only the upper 3 inches (7.7 cm) of core sample yielded isolates. Freshwater lake temperatures during November and December reached a high of 26 and a low of 16°C.

Temperature fluctuations of thermally polluted lake water were rapid and extensive during the examination months, depending on the quantity of heated water being discharged. Therefore, readings shown in Fig. 1 may not accurately reflect the average water temperature during the month. However, in freshwater lakes, a more uniform temperature curve rather accurately reflected ambient temperatures. In August, when 43 positive samples were obtained from these lakes, water temperature in one of them reached 34.5°C, only 1.5°C less than the peak temperature recorded for thermally polluted lake water.

Table 1 shows the percentage of samples positive among the water and lake bottom samples tested from five lakes in the Orlando area, one thermally polluted lake and four suspect lakes. Two of the latter, lakes H and B, showed positivity in lake water samples of 75.0 and 88.9%, respectively. However, lakes B and C yielded positive lake bottom specimens of 35.6 and 18.4%, respectively. Bottom samples from lakes M and H yielded approximately the same percentage of positive samples, 7.7 and 7.3%, respectively. The latter lake had a very heavy muck layer on the bottom, whereas the other lake bottoms were sandy and supported limited aquatic growth in shallow areas.

TABLE 1. *Percentage of samples positive for pathogenic Naegleria from five Florida lakes, 1976*

Lake	No. positive/no. tested		Positive (%)	
	Water	Lake bottom	Water	Lake bottom
Thermal				
M	4/45	1/13	8.9	7.7
Other				
H	27/36	3/41	75.0	7.3
B	24/27	16/45	88.9	35.6
C	4/40	9/49	10.0	18.4
S	0/13	0/9	0.0	0.0

Lake S was unique. No isolates were obtained from the lake, even though 13 water and 9 lake bottom samples were tested. It is located approximately 1,000 feet (305 m) away from lake B, which yielded the largest number of positive specimens. Although fewer samples were obtained from lake S than from the others, the quantity of lake water tested was greater than that from lake B, as shown in Table 2. On the average, 24 liters of lake B water yielded a pathogenic *Naegleria* isolate, whereas 5,686.6 liters from the thermally polluted lake was required. Lake bottom samples were much more productive in that an average of 0.4 liter from lake B yielded a pathogenic *Naegleria,* whereas 2.1 liters from lake H was required.

When six additional lakes in Orange County yielded pathogenic *Naegleria* isolates, it became evident that lakes in other contiguous and more distant counties would need to be tested to evaluate the ecological role of thermally polluted water as related to pathogenic *Naegleria.* Twenty-six lakes in 14 counties were sampled only once. A shallow water and lake bottom sample were obtained on the same day. Figure 2 shows the number of lakes positive over the number tested and the counties in which they were located. Three of the lakes yielded pathogenic *Naegleria* isolates from bottom samples only; five yielded isolates from water only; and for four, both water and bottom samples were positive, giving an overall positivity of 41% (12/26). Lakes in the Panhandle counties of Gadsden and Leon were sampled in September when ambient temperatures in that area of the state had begun to drop. Therefore, negative findings were not unexpected.

Axenic culture of 43-9 strain. Chang CSYEC medium supports only meager growth of the nonpathogenic seropositive 43-9 strain *Naegleria.* However, with the addition (per milliliter) of 0.05 ml of supplement of either the

TABLE 2. *Average minimal volume of lake water tested that yielded pathogenic Naegleria isolates in five Florida lakes, 1976*

Lake	No. of samples positive/no. of liters tested		Minimal vol (liters) of water yielding an isolate	
	Water	Lake bottom	Water	Lake bottom
Thermal				
M	5/28,433	1/1.3	5,686.6	1.3
Other				
H	27/1,994.2	3/6.3	73.9	2.1
B	24/575.7	15/6.5	24.0	0.4
C	6/2,933.7	9/6.3	489.0	0.7
S	0/755	0/1.0	0	0

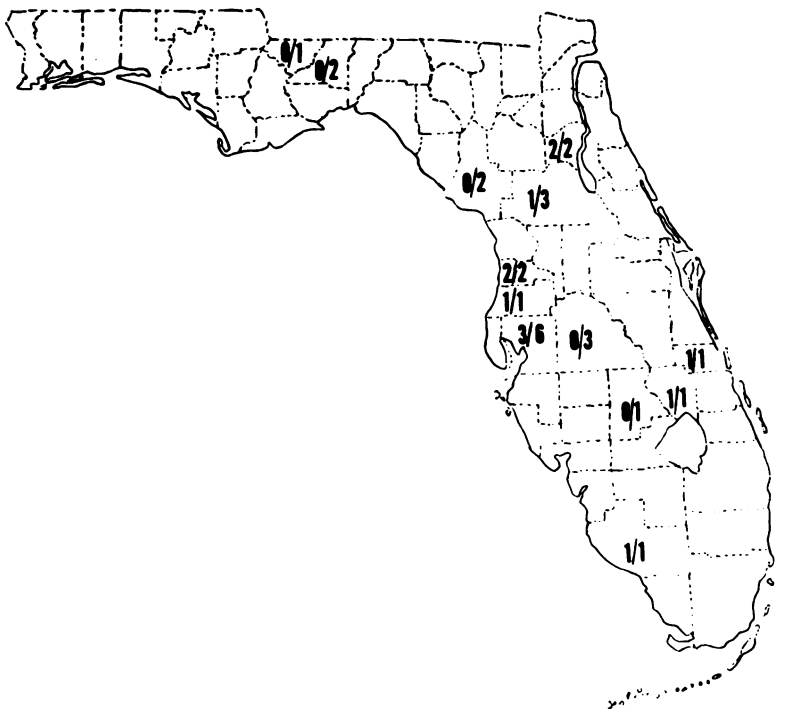

FIG. 2. *Number of lakes yielding pathogenic Naegleria isolates over number of lakes tested in selected Florida counties.*

CFL or BH, lush growth comparable to that of the pathogenic strain in unsupplemented CSYEC medium was obtained. As can be seen in Table 3, there was a direct relationship between growth of the 43-9 strain and the amount of either BH or CFL added, regardless of the medium used. However, there was limited growth in CSYEC medium in the absence of supplements, but no growth occurred in the two unsupplemented media.

IFA studies. The IFA technique readily differentiated between *Naegleria gruberi* and the pathogenic *Naegleria* when antiserum prepared against the latter strain was used. The heterologous titer was 1:20 or 1:40, whereas homologous titers were 1:640 or 1:1,280, depending on the antiserum lot used. Less differentiation was noted between the pathogenic strain and its antigenically related nonpathogenic strain. The latter fluoresced less brightly, and the titer was always one dilution less than the homologous system. Table 4 shows the results of a cross-comparison test, using absorbed and unabsorbed antisera prepared against pathogenic and nonpathogenic strains. Adequate absorption was evidenced by the negative results obtained when homologous antigens were used for absorption. Conversely, heterologous absorption removed antibodies against the common antigens only, resulting in monospecific antisera. Although actual titers in various tests varied by lot of antiserum used, the overall trend did not. One lot (lot 7/8) of GJ antiserum titrated out to 1:1,280 for the homologous system and 1:640 for the heterologous. When this was absorbed with GJ antigen, the homologous titer was unchanged, but the heterologous titer dropped to 1:40, which appears to indicate inadequate absorption.

TABLE 3. *Relative growth rates of nonpathogenic Naegleria in various axenic culture media*

Amount of supplement/ml of media	Growth rating in media					
	CSYEC		MYAS[a]		I.S.[b]	
	BH	CFL	BH	CFL	BH	CFL
0.0125	2	2	1	1	1	1
0.025	2	2	2	3	2	2
0.0375	3	3	2	2	2	2
0.05	4	4	3	2	3	2
0.0	1	1	0	0	0	0

[a] Page malt-yeast extract-amoeba-saline.
[b] Inorganic solution.

Mouse inoculations. In mice inoculated intranasally with pathogenic *Naegleria*, death occurred routinely between days 5 and 7. Onset

TABLE 4. *IFA test results with unabsorbed and absorbed sera*

Antigen	Antiserum				Normal rabbit
	GJ (lot 3)		43-9 (lot 1)		
	Unabsorbed	Absorbed with 43-9	Unabsorbed	Absorbed with GJ	
GJ[a] Pathogenic	640[b]	160	160	0	0
43-9[c] Nonpathogenic	320	0	320	40	0

[a] Isolated by C. Baro, Orlando, Fla., from fatal case of PAM, 1972, and received from S. Chang, 1975.
[b] Reciprocal of serum dilution showing 1+ fluorescence.
[c] Isolated from lake M in February 1976.

of neurological involvement and focal paralysis was noted 24 to 72 h before death. Bacterial suspensions prepared from stock cultures used for seeding the IA plates failed to produce illness or death under the same circumstances. At necropsy, extensive generalized edema and hemorrhage, particularly in the anterior areas of the brain, were noted. Amoebae were always recovered from the brains. In some instances, amoebae were cultured from internal organs such as lung, spleen, liver, and heart. Strains serologically related that failed to grow in CSYEC medium also failed to produce overt infections in mice after intranasal instillation.

DISCUSSION

The extensive distribution of pathogenic *Naegleria* in Florida's freshwater lakes as shown by this study further clouds our understanding of the processes involved in fatal infections. Florida has over 500 freshwater lakes. These are used for water contact sports year round by millions of people. Why then should only seven fatal cases have occurred in 14 years? Based on data obtained from the State of Florida, Department of Natural Resources, Division of Recreation and Parks, more than a billion exposures have occurred over that 14-year period. Many of these have undoubtedly occurred during the winter and spring months when the populations of pathogenic *Naegleria* are depressed. However, thousands of exposures have also occurred during the hot summer and fall months when these populations have reached a level of at least one organism per 25 ml of water tested. Therefore, factors other than the presence of pathogenic *Naegleria* in lake waters must be responsible for infections. Perhaps specific host factors, a critical dose, and/or physical activities at the time of exposure may be the sine qua non of PAM infections.

Lake S, which has remained free of pathogenic *Naegleria* since this study was initiated, may eventually provide the clue to why some freshwater lakes and not others support populations of this amoeba. The constantly negative results from lake S in view of its proximity to lake B, which has been routinely positive, is difficult to explain. Studies are currently under way to define the chemical characteristics and the microflora and fauna of these two geographically, closely related lakes in an effort to elucidate the reasons.

There is little doubt that pathogenic *Naegleria* survive the winter in lake bottom sediments. Early in this study all samples were obtained from shallow areas, which would account for the paucity of positive samples during the first 6 months of the year. However, when negative results began to appear in late October, samples were obtained from lake bottoms in deeper water, which reversed the negative trend. Our ongoing studies have shown that in February 1977, when lake water temperatures reached 12°C, isolates were obtained from lake bottom samples in water depths up to 28 feet (8.5 m).

Plaques developing from the lake bottom samples when water temperatures were below 24°C appeared much later than when temperatures were higher. It is assumed from this that few if any trophozoites were present in the sample and that overwintering is facilitated by encystment. The more stable temperatures afforded by biologically active lake bottom sediments in deeper waters also play a role in overwintering. Preliminary laboratory data indicate that over 50% of cysts survive for 35 days when temperatures are held at 22 or 4°C, whereas only 16% survived when they were held at 22°C for 8 h and then at 4°C for 16 h over the same time period (unpublished data).

Due to the methodology used in collecting bottom samples, the actual site of cyst survival is not known, i.e., at the water/soil interface or buried in the sand. Since all isolations from core samples were made from the upper 3 inches (7.62 cm), the site of survival appears to be in

the surficial bottom sediments. More definitive studies are being pursued.

Belgium investigators (6, 10) have indicated that thermal pollution may play a role in the maintenance and proliferation of pathogenic *Naegleria* in nature. Perhaps in less tropical areas of the world this may be true, but such lakes are of miniscule importance in Florida, as shown by this present study. The types of sampling done in the Belgium studies (7), i.e., small samples taken from shallow sites, may be misleading. Had larger samples been derived from deeper areas of the sampling sites, the apparent absence or disappearance of pathogenic *Naegleria* in winter months may have been shown to be more apparent than real. There is little doubt that increased water temperature, whether from thermal pollution or sunlight, provides the ideal milieu for proliferation of pathogenic *Naegleria.*

The IFA studies have confirmed that the pathogenic *Naegleria* is antigenically closely related to a nonpathogenic strain (6). However, each possesses its own specific antigens. De Jonckheere et al. (6) have suggested that nonpathogenic *Naegleria* strains may undergo transformation in carrier hosts. This hypothesis was based on the observation that pathogenic isolates from human PAM cases serially passed in axenic culture over years showed a decrease in mouse pathogenicity. The original level of pathogenicity was restored after a single mouse passage. This phenomenon has also been noted in this laboratory. However, IFA studies of such strains before and after mouse brain passage showed comparable titers (unpublished data).

Restoration of pathogenicity to axenically cultured strains has also been noted after continued passage on bacterial-seeded IA plates. It appears that axenic culture medium may lack a necessary nutrient(s) that leads to repression of the cytolytic enzyme, whereas bacteria supply the nutrient(s) necessary for derepression of the enzyme. The fact that nonpathogens have never become pathogenic, regardless of the number of passages afforded the seropositive nonpathogenic *Naegleria* strain, suggests that these are genetically different species. The gross differences between the antigenic composition of pathogenic and nonpathogenic *Naegleria,* as shown by IFA, further support this hypothesis. The two species should be clearly differentiated in the nomenclature. If the pathogenic strain were referred to as *Naegleria invadens* as has been suggested by Chang (4), it would preclude any confusion, since the name for the pathogenic strain would be descriptive of its invasive pathogenicity.

The use of CFL as a supplement in Chang medium provides an excellent medium for culturing large populations of nonpathogenic indirect-fluorescent-positive *Naegleria* free of extraneous matter. This has facilitated absorption of anti-GJ (pathogenic) strain hyperimmune rabbit serum with large numbers of the nonpathogenic *Naegleria* trophozoites, which previously had required much more time and effort. A second advantage has been the preparation of sections free of extraneous matter for electron microscopic studies presently in progress.

ACKNOWLEDGMENTS

We acknowledge with appreciation the excellent technical assistance of Mary Jane Farmelo and Dewey Moody in the laboratory and field aspects of this study.

This study was supported by the State of Florida Department of Health and Rehabilitative Services, Central Operations Services, Office of Laboratory Services and grant no. R-804375-010 from the Environmental Protection Agency.

LITERATURE CITED

1. **Anderson, K., and A. Jamieson.** 1972. Agglutination test for the investigation of the genus *Naegleria.* Pathology **4**:273–278.
2. **Butt, C. G.** 1966. Primary amebic meningoencephalitis. N. Engl. J. Med. **274**:1473–1476.
3. **Butt, C. G., C. Baro, and R. W. Knorr.** 1968. *Naegleria* (sp.) identified in amebic encephalitis Am. J. Clin. Pathol. **50**:568–574.
4. **Chang, S. L.** 1971. Small, free-living amebas: cultivation, quantitation, identification, classification, pathogenesis and resistance. Curr. Top. Comp. Pathobiol. **1**:201–254.
5. **Chang, S. L.** 1974. Cytopathic and pathogenic difference among geographic strains of pathogenic *Naegleria* and their bearing on epidemiology of primary amoebic meningoencephalitis. 3rd Int. Congr. Parasitol. (Munich) ICP III **1**:187–188.
6. **De Jonckheere, J., P. Van Dijck, and H. van de Voorde.** 1975. The effect of thermal pollution on the distribution of *Naegleria fowleri.* J. Hyg. (Cambridge) **75**:7–13.
7. **De Jonckheere, J., and H. van de Voorde.** 1977. The distribution of *Naegleria fowleri* in man-made thermal waters. Am. J. Trop. Med. Hyg. **26**:10–15.
8. **Page, F. C.** 1967. Taxonomic criteria for *Limax amoebae* with descriptions of three new species of *Hartmanella* and three of *Vahlkampfia.* J. Protozool. **14**:499–521.
9. **Van Dijck, P., J. De Jonckheere, G. Reybrouck, and H. van de Voorde.** 1974. Rapid identification of *Naegleria* species by sero-agglutination and fluorescent antibodies—new possibilities for the water supply service. Zentralbl. Bakteriol. Parasitenkd. Infektionskr. Hyg. Abt. 1 Orig. Reihe B **158**:541–551.
10. **Willaert, E., and A. R. Stevens.** 1976. Isolation of pathogenic amoeba from thermal-discharge water. Lancet **ii**:741.