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Isolation of Pathogenic Naegleria from Artificially Heated Waters*

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ABSTRACT

Investigations were undertaken to determine whether heated waters facilitate the proliferation of free-living amoeba that cause primary amoebic meningoencephalitis. Water samples were taken close to the discharges of power plants situated on lakes or rivers in Florida and Texas and from cooling towers in Tennessee. The water temperatures ranged from 29 to 42°C. Water samples were also taken from several lakes in Florida and Texas without associated power plants. The water temperatures of these ranged from 30° to 34°C. Twenty-five-250-ml samples were filtered through membranes. Samples taken from the control lakes and cooling towers showed no growth of pathogenic amoeba, whereas growth was obtained from 2 of the 8 lakes and rivers in Florida and from 1 of the 7 man-made lakes in Texas that were artificially heated. The amoebae were identified as belonging to the genus Naegleria from their trophozoite and cyst structure, ability to grow at 45°C, to transform into flagellates, and to produce primary amebic meningoencephalitis (PAME) in mice after intranasal instillation. Their identification as N. fowleri was confirmed by indirect immunofluorescent analysis with antiserum produced against N. fowleri. These findings indicate that artificial heating of waters may facilitate the growth of pathogenic free living amoeba.

INTRODUCTION

The ability of pathogenic Naegleria and Acanthamoeba to outgrow their nonpathogenic counterparts at elevated temperatures was suggested in laboratory tests by Griffin (1972). This has also been suggested from various studies of naturally or artificially heated waters in Australia and Europe that have shown the presence of pathogenic Naegleria fowleri and its association with fatal cases of meningoencephalitis (Anderson and Jamieson, 1972; Van den Driessche et al., 1973). Similarly, epidemiologic studies in the United States have shown an association between Naegleria and Acanthamoeba infections and warm waters (Duma et al., 1971). Relatively few studies, however, have been undertaken in the United States regarding the presence of pathogenic-free-living amoeba in artificially heated waters. Consequently, waters receiving thermal discharges from electric power plants were studied to determine whether there is increased proliferation of pathogenic free-living amoeba.

MATERIALS AND METHODS

Isolation

Water samples ranging in volume from 25-250 ml were taken either from surface waters containing little or no sediment or from along the shore, in which case they generally contained some underlying sediment. The water was filtered through cellulose acetate membrane filters with a porosity of 1.2 μM. The membranes were inverted and placed on agar plates spread with a lawn of heat killed (60°C-30°) Aerobacter aerogenes. Mud, water plants or detritus also were placed directly on the agar plates. Most samples were plated the same day as collected; the remainder within 48 hours of sampling. After sealing with parafilm, duplicate

plates for each sample were incubated at 45°C and 37°C. The 45°C incubation temperature was used for the selective outgrowth of pathogenic Naegleria. The plates were monitored daily with an inverted phase microscope for assessment of growth. When sufficient growth occured, the trophozoites were flushed from the surface and aliquots sub-cultured onto fresh agar plates. The heterologous outgrowth from these sub-cultures was used for characterizing the amoebic isolates.

Flagellation, a characteristic of Naegleria species, was tested for by harvesting trophozoites in sterile distilled water and microscopically examining the suspension in a hanging drop slide for evidence of flagellar locomotion. These preparations were examined and observed at 30-minute intervals for three hours.

Weanling ICR or BALB/c mice were used for determining pathogenicity of various isolates. The heterologous amoeba populations that grew at either 37°C or 45°C were suspended in distilled water at concentrations ranging from 2.4×10^4 - 1.2×10^6/ml. Each of five mice per test group was inoculated intranasally with 0.05 ml of amoeba suspension and observed for 2-3 weeks for acute symptoms of primary amoebic meningoencephalitis (PAME). Brain tissue from dead or moribund mice was examined unfixed or histologically for presence of amoeba. In addition, suspensions of brain tissue were plated on agar plates seeded with A. aerogenes or inoculated in axenic casitone-based media C.G.V.S. (Willaert, 1971) for culture of the amoeba. Only sick or dead mice whose brain tissue showed microscopic or cultured evidence of amoeba were considered as positive for PAME.

Several known species of Acanthamoeba were tested for pathogenicity in mice or for cytopathogenicity in cultured human tumor cells (HeLa) or mouse cells transformed by the Kirsten sarcoma virus (KBALB). These assays were carried out at 37°C or 28°C using Plus I tissue

culture media (IBL) supplemented with 10% fetal calf sera. Amoeba were added to semi-confluent cell cultures at a ratio of 1 to 5. The cultures were observed twice daily for signs of cytopathogenicity.

The pathogenic Naegleria isolated from mouse brains and grown in C.G.V.S. media were used as a source of antigen for the indirect immunofluorescent antibody test (IFAT) (Willaert, 1976) using antisera produced against various Naegleria species.

Samples

All samples were taken during the summer of 1976. Samples were generally collected from sites close to power plant discharges. Water temperatures of sampling sites ranged from 25°C-41°C.

Sampling sites in Florida that received thermal effluents from power plants included three fresh water lakes (A,C and E), a river (D), a river estuary (F,G, and H), and a canal (B). The canal was contiguous with the Atlantic Ocean and contained brackish water. Samples were also taken from lakes with no associated power plants (I,J,K, and L).

Sampling sites in Texas included seven man-made lakes (M,N,O,P,Q,R,S) and one river (T), which received heated effluent from power plants. Two lakes (V,W) and a creek (U) not contaminated with thermal effluents were also tested.

The Tennessee samples were obtained from three cooling towers (X,Y,Z) at three sites in Oak Ridge. In contrast to other sampling sites the cooling towers were closed circulation systems which had been treated with standard biocidal chemicals.

RESULTS

Of a total of 30 samples taken in Florida waters receiving power plant effluent, 15 showed growth of amoebae at 45°C (Table 1). Amoebae were identified as amoeboflagellates in 4 of these samples. Three were isolated from lake E with an average temperature of 38°C and the fourth was obtained from river D with a temperature of 35°C. The positive lake samples were all obtained from water samples taken along the shoreline and containined some sediment. The amoebae grew out within a few days after plating. In contrast, amoebae isolated from the river only grew out from plant material.

Subsequently, five mice per group were inoculated with these four isolates. Sixty to eighty per cent of the mice inoculated with the amoebic flagellates isolated from lake E died within 4-10 days after inoculation. Some deaths occurred in mice inoculated with all three isolates (Table 3). All five mice inoculated with the isolate from river D died within 7 days after exposure.

Phase microscopic examination of the brain tissue from the dead or moribund mice showed numerous motile amoeba that grew luxuirantly when inoculated into CGVS media. Histologic and electron microscopic examination confirmed the presence of amoeba in the brain tissue. Seven other non-flagellating heterologous amoeba populations isolated from canal and river water were also tested for pathogenicity in mice. No obvious effects resulted from intranasal injection of these amoeba (Table 1).

Indirect fluorescence tests were performed on two of the three Naegleria isolates from lake (E) and the isolate from river D. The amoebae fluoresced to positive titers of 1/512 (lake) and 1/1024 (river)

when reacted with anti-N. fowleri serum, whereas they reacted minimally with anti-N. jadrini and anti-N. gruberi sera (Table 4).

In addition to the growth of amoebae at 45°C, amoebae grew from all Florida water samples at the 37°C incubation temperature, but none were found to be flagellated. Three of the isolates taken at one site at lake C were inoculated into mice, but produced no overt signs of pathogenicity during the observation period. By morphological characteristics some of these strains were identified as belonging to the genus Acanthamoeba. Other amoebae were identified as Vahlkamphilidae, Hartmannellidae and Mayorellidae.

One strain of N. fowleri was isolated from a Texas lake. This lake had the highest water temperature (39-41°). The isolate was obtained from a filtered 50 ml water sample and showed good outgrowth within three days of plating.

All mice inoculated with this amoeba died within 7 days. Amoeba were observed in unfixed brain tissue and detected histologically and electron microscopically in fixed brain tissue (Table 3). The amoeba was grown in CGVS media and confirmed as N. fowleri by IFAT analysis (Table 4). Of 40 samples collected in Texas, 14 showed outgrowth at 45°C, but only AR-12 was pathogenic for mice.

Six of nine samples taken from lakes not receiving power plant discharge showed amoebic growth at 45°C but the amoeba showed no flagellation. Several of these isolates were tested in mice with no evidence of pathogenicity (Table 2).

Outgrowth of amoeba was seen in all the Texas samples incubated at 37° but no evidence of Naegleria was observed in these samples.

Three of six samples from cooling towers in Tennessee showed outgrowth at 45°C. No flagellation was observed nor were these isolates pathogenic for mice after intranasal inoculation (Table 3). All six cooling tower samples showed growth at 37° but flagellation could not be induced. These amoebae also did not produce PAME in mice.

While the pathogenic potential of most of the Acanthamoeba isolates from the water samples was not tested, the fatal pulmonary and gastro-intestinal infections seen in some mice injected intranasally with axenically cultured A. royreba and A. culbertsoni (strain KA) respectively (Fig. 1 and 2) indicated the importance of further testing to determine what syndromes other than PAME these isolates may produce.

DISCUSSION

The results of this study complement those reported by others (Carter, 1968; Carter, 1970; De Jonckheere et al., 1975) and support the contention that artificially heated waters may facilitate the propagation of pathogenic, free-living amoeba.

Two of the five sampling sites in Florida receiving power plant discharge were positive for N. fowleri whereas N. fowleri was not isolated from control lakes devoid of associated power plants. Considering the minute sample size relative to the total lake volume, however, the absence of N. fowleri in these particular samples does not rule out their presence in these particular bodies of water. In view of our positive results in isolating N. fowleri from heated waters in Florida, it is of interest that all five, recently confirmed cases of PAME in Florida involved individuals who had been swimming in warmed waters (Willaert, 1974).

Recently, Wellings et al. (1977) 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 elevated water temperatures either natural or man-made, enhances the possible proliferation of pathogenic free-living amoebae.

Only one of eleven sampling sites in Texas yielded pathogenic N. fowleri. The positive sample was disconcerting, however, in that it was obtained from only 50 ml of water, indicating a high level of contamination, and from a lake that served as the domestic water supply for the surrounding area. This finding emphasizes the importance of maintaining proper chlorine levels and proper floculation/sedimentation in water purification plants, particularly those obtaining their water supply from lakes or rivers that receive heated discharge. Chlorination alone may not be totally effective in controlling the pathogenic free-living amoeba, particularly Acanthamoeba. Studies have shown that trophozoites of Naegleria and Acanthamoeba are killed if concentrations of 0.5-1.0 µg of free active chlorine per ml of water is maintained; however, the cysts of N. fowleri were destroyed only after 1 hour exposure to 0.5 µg/ml and cysts of pathogenic Acanthamoeba were still viable after 3 hour exposures to 40 µg of free chlorine/ml (De Jonckheere and van de Voorde, 1976; Derreumaux et al., 1974).

Although the samples of the cooling towers in Oak Ridge were negative for N. fowleri, amoebae, tentatively identified as Acanthamoeba, grew from

all samples at 37°C. In addition, an amoeba flagellate, as yet undentified, was isolated from one of the samples.

It is impressive that free-living amoeba abound in the cooling towers considering the variety of bacteriocidal and algacidal compounds added to these waters. Whether such conditions select for pathogenic amoeba, other than those producing PAME, remains to be determined. Recently, we have found that axenically cultured A. royreba and A. culbertsoni (strain KA), two new isolates from tumor tissue cultures, can produce fatal pulmonary and gastrointestinal disease in mice injected intranasally with these strains. Additionally, several known nonpathogenic Acanthamoeba i.e., A. palestinensis and A. polyphaga were cytopathogenic for cultured HeLa and KBALB when incubated at 28°C. The control, uninfected mammalian cell cultures are not harmed by these lower temperatures (Table 5). Considering the aerosols created by the cooling systems and the capacity of some Acanthamoeba for producing intestinal and pulmonary infections, it is important to define those infections, other than PAME, which the cooling tower isolates may produce in mice.

The observation that nonpathogenic Acanthamoeba that do not normally grow at 37°C are highly cytopathogenic for cultured mammalian cells at 28°C also suggests that adaptation/selection of variants of these species for growth at 37° may also present a public health problem in addition to that already posed by N. fowleri and A. culbertsoni.

The problem of pathogenic free-living amoeba in heated waters is particularly pertinent in lakes or rivers used for recreational purposes such as swimming, boating, water skiing and fishing. In particular the man-made lakes planned expressly for the dual purpose of cooling power plant effluent and recreation are questionable.

In addition to possible human health problems associated with Naegleria and Acanthamoeba they may also pose problems for other aquatic life. That fish may be prone to amoebic infections was shown in recent studies by Taylor (1977). He reported that some "fish kills" in Florida involved several strains of Acanthamoeba.

The results of this study support the observations of De Jonckheere (1975), Griffin (1972) and others (Carter, 1968: Carter, 1970; De Jonckheere et al., 1975; De Jonckheere and van de Voorde, 1976) that artificially or naturally heated waters may be a contributing factor in the propagation of pathogenic free-living amoeba. In view of the increasing demand for energy and the associated warming of bodies of water and expansion of cooling tower systems it is imperative that factors which contribute to the propagation of these thermophilic microbes be determined and the means for controlling their growth and distribution be devised.

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Table 1. Isolation of amoebae at 45 C from Florida waters

Sampling site	Number of samples taken	Water pH	Water temp. C	Samples positive for amoebae	Samples positive for *N. fowleri*	Pathogenic	Other amoebae[a]
		Waters receiving discharges of power plants					
Lake A	4	6.8	36-37	0	--	--	--
Canal B	4	6.9	35	1	0	no	1
Lake C	3	7.65	29-31	0	--	--	--
River D	2	7.65	35	1	1	yes	0
Lake E	6	7.15	38	5	3	yes	2
River F	4	7.5	34-35	3	0	no	3
River G	2	7.55	32	2	0	no	2
River H	5	6.9	41	3	0	no	3
TOTAL	30			15	4		11
		Waters without discharges					
Lake I	4	7.65	33-34	0	--	--	--
Lake J	3	7.5	33-34	0	--	--	--
Lake K	3	9.1	32-33	0	--	--	--
Lake L	3	6.8	32	0	--	--	--
TOTAL	13			0			

[a]These amoebae were identified by their structure as Vahlkamphiidae, Hartmannellidae, Acanthamoebidae and Mayorellidae.

Table 2. Isolation of amoebae at 45 C from Texas and Tennessee water samples

Sampling site	Number of samples taken	Water pH	Water temp. C	Samples positive for amoebae	Samples positive for *N. fowleri*	Pathogenic	Other amoebae[a]
			Waters receiving discharges of power plants				
Lake M	5	7.95	31-33	0	--	--	--
Lake N	3	8.09	29	2	0	no	2
Lake O	4	8.01	35	0	--	--	--
Lake P	4	7.90	36-37	0	--	--	--
Lake Q	9	8.00	39-41	7	1	yes	6
Lake R	4	7.90	32-34	1	0	no	1
Lake S	8	8.10	39-41	1	0	no	1
River T	3	7.55	35	3	0	no	3
TOTAL	40			14	1		13
			Waters without discharges				
Creek U	2	7.50	33-34	1	0	no	1
Lake V	4	8.02	32	3	0	no	3
Lake W	3	8.00	30	2	0	no	2
TOTAL	9			6	0		6
			Cooling Towers (CT)				
CT-X	1	NT[b]	30	1	0	no	1
CT-Y	4	NT	28	2	0	no	2
CT-Z	1	NT	25	0	--	--	--
TOTAL	6			3	0		3

[a]These amoebae were identified by their structure as Vahlkamphiidae, Hartmannellidae, Acanthamoebidae, and Mayorellidae.

[b]NT--not tested.

Table 3. Mouse pathogenicity tests with amoeba populations growing at 45 C

Location	Population designation	Total number of amoebae inoculated	Day of deaths	% mortality	Amoebae in brains
		Florida			
Canal B	La-1	1.6×10^4	--	0	--
River D	Sa-3	1.6×10^4	5,5,5,6,7	100	yes
Lake E	Ent-1	7×10^3	5,7,7,10	80	yes
	Ent-3	6×10^3	4,5,6,6	80	yes
	Ent-5	1.9×10^4	7,7,8	60	yes
River F	S1	4×10^3	3,7	40	no
	S6	2×10^3	8	20	no
River G	Ke-1	3.4×10^3	--	0	--
	Ke-3	3.4×10^3	11	20	no
River H	N-1	7.9×10^3	8	20	no
	N-5	8×10^3	--	0	--
		Texas			
Lake N	LW-1	9.6×10^3	--	0	--
Lake Q	Ar-1	1.6×10^4	--	0	--
	Ar-3	8×10^4	--	0	--
	Ar-5	3.3×10^4	--	0	--
	Ar-7M$_1$	4×10^3	--	0	--
	Ar-9M$_1$	8×10^3	5	20	no
	Ar-12	8×10^3	5,5,6,7,7	100	yes
Lake R	Mc-6M$_1$	5×10^4	--	0	--
River T	NM-3M$_1$	6×10^3	3	20	no
	NM-4M$_1$	1×10^4	--	0	--
Lake V	Be-4	1.2×10^3	--	0	--
Lake W	LWo-3M$_1$	2.4×10^3	--	0	--
		Tennessee			
Cooling towers					
tower X	K-31-1	n.c.[a]	--	0	--
tower Y	HFIR-1	6×10^4	--	0	--
	HFIR-3	n.c.	--	0	--

[a] n.c. = no counts.

Table 4. Indirect immunofluorescent analyses on pathogenic Naegleria isolates from thermally heated waters

Strain	Antiserum[a]		
	N. fowleri (Morgan)	N. jadini (ITAMAP 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	1/1024	1/16	1/16
Q-AR-12	1/512	1/8	1/8
N. fowleri (Morgan)	1/1024	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 primary amoebic meningoencephalitis (12).

Table 5. The relative cytopathogenicity[a] of various Acanthamoeba for cultured mammalian cells at 28°C

	Acanthamoeba			
Target cell	A. culbertsoni (A.)	A. royreba	A. palestinensis	A. polyphaga
HeLa	24-48	24-36	<24	<24
KBALB	24-48	24-36	<24	<24

[a]Time in hours for complete destruction of semi-confluent cell culture by various acanthamoeba. The amoeba were added to the cell cultures at a ratio of 1/5. Cells cultured in plus I tissue culture media with 10% fetal calf serum and 50 units and 50 mcg/ml of penicillin and streptomycin respectively.

FIGURE LEGENDS

Figure 1. Lung tissue of mouse with pneumonia resulting from intranasal inoculation of A. royreba. Mouse was necropsied 28 days after inoculation. Note trophozoites in the diseased tissue. H&E, 500X

Figure 2. Intestinal tissue from mouse injected intranasally with A. culbertsoni strain KA. Note the extensive accumulation of blood in the intestinal lumen and in the crypts. H&E, 200X