J. Hyg., Camb. (1975), **75**, 7
Printed in Great Britain

The effect of thermal pollution on the distribution of *Naegleria fowleri*

BY J. DE JONCKHEERE, P. VAN DIJCK AND H. VAN DE VOORDE
Laboratory of Hygiene, School of Public Health,
Vital Decosterstraat 102, *Katholieke Universiteit Leuven, Belgium*

(*Received* 28 *November* 1974)

SUMMARY

The distribution in the environment of *Naegleria fowleri*, the causal agent of primary amoebic meningoencephalitis has been investigated in this study. *N. fowleri* was isolated only from a thermally polluted canal. These amoebo-flagellates were not isolated from another thermally polluted canal in the neighbourhood indicating that, apart from high temperature, other factors are involved in the selective proliferation of *N. fowleri*. This species was absent in all other samples originating from two canals, a stream, two lakes, several reservoirs and slow sandfilters of a water supply service and also a water distribution network. Many other amoebae able to grow at 42° C. were found in different places. Most of the *N. fowleri* strains isolated were not virulent for mice, although they showed all the characteristics of the pathogenic strains.

INTRODUCTION

Ever since cases of primary amoebic meningoencephalitis (PAME) were recognized as being caused by *Naegleria fowleri*, research has been undertaken pertaining to the occurrence of this amoebo-flagellate in the environment. Many of the PAME cases were in persons who had been swimming in warm waters (Carter, 1972). In the U.S.A. the disease was prevalent during the summer months after swimming in very warm lakes (Duma *et al.* 1971). The water temperature during these months averages over 28° C. Cases in Australia showed a positive relation with drinking-water piped overland where the water reached average temperatures of 31° C. during the summer months (Jamieson, 1973). In New Zealand (Mandal *et al.* 1970) water from hot springs was involved, and in Czechoslovakia (Červa & Novák, 1968) and Belgium (Jadin *et al.* 1971) many PAME infections were contracted in warm indoor swimming-pools.

In 1973 our attention was drawn in Belgium to a very specific case, which occurred after swimming in a thermally polluted stream (Van Den Driessche *et al.* 1973) in the province of Antwerp. It is worth noting that the thermally polluted canal which is at the origin of the stream is in connexion with the canals where a water supply service takes its water. This water is delivered after treatment to the swimming-pools where other Belgian cases occurred in 1970–72.

We have now tried to isolate *N. fowleri* from this thermally polluted water in order to make a positive contribution to the ecology of *Naegleria* and to the epidemiology of PAME. Only on a few occasions have mouse-pathogenic strains of *N. fowleri* been isolated from the environment (Singh & Das, 1972; Anderson & Jamieson, 1972; Gordeeva, 1973; Kasprzak & Mazur, 1974) owing to inadequate isolation techniques. In this work a more selective screening method was used.

MATERIAL AND METHODS

Sampling sites

A canal receiving warm effluent from a lead and zinc factory and the connected stream where a boy had contracted PAME last year (Van Den Driessche *et al.* 1973) were the main objects of our investigation (Figure 1). The water temperature ranged on two occasions from 29 to 30° C. at the effluent of the factory, the thermal plume, and dropped to 22–23° C. at the farthest sampled point in the stream some 2 km. away.

Another canal in the neighbourhood with a temperature ranging from 21 to 23° C. was also sampled. This canal receives cooling waters of an electricity power plant. At the time of sampling the thermal discharge had a temperature of ± 29° C. Samples could not be taken from the warmest part of the canal because at that time this area was inaccessible.

Control samples were taken in the surrounding area from canals with water temperatures of 15° C.

Two lakes were sampled, one of which receives sewage drained from small villages and had a temperature of 13° C.; the other is a fishpond where a temperature of 11° C. was recorded. A canal and a stream (the Dijle), crossing the city of Leuven, receiving domestic and industrial sewage, in both of which a temperature of 11 to 12° C. was recorded, were also sampled.

The resources of drinking-water are of special interest; for this reason the water of reservoirs and slow sandfilters of the Antwerp Water Works (A.W.W.) was also examined. Samples from a water distribution network, where the water was very turbid with a high organic content, were also taken. Sampling was undertaken during the months of March, April and May 1974.

In August of the same year, samples were again taken from the first canal when the discharge from the factory showed a temperature of 34° C. In October 1974 the two thermally polluted canals were sampled once more. At this time the temperature of the discharge was 24° C. for both factories.

Sampling

Sterile 500 ml. containers were filled with surface water and processed the same day in the laboratory. On some occasions waterplants or detritus were taken in sterile plastic bags. Samples of 50 ml. and 250 ml. were filtered with slight suction through sterile 5·0 μm. cellulose acetate membranes. The suction procedure was interrupted before the membranes were allowed to dry and the membranes were inverted on nutrient-agar (NN) inoculated with living *E. coli*. Pieces of waterplants or detritus were placed directly on the medium. On some occasions soil samples taken near the water were placed directly on the culture medium. In August and October 1 ml. samples poured immediately on NN agar with living *E. coli* were also included.

The composition of NN agar was 15 g. Difco Bacto-Agar, and amoeba saline (Page, 1967) to make 1 litre. The sealed plates were incubated at 42° C. for selective cultivation of *N. fowleri* (Griffin, 1972). Daily observation of clearing zones around the membranes caused by migrating amoebae was made with the naked eye until the 10th day.

Identification of amoebae

The following criteria were used for this purpose.

Ability to grow at 42° C. According to the criterion of Griffin (1972), only pathogenic *Naegleria* strains are able to grow at 42° C. From a large series of naeglerias tested, only *N. fowleri* and a few non-pathogenic naeglerias could develop at such a temperature (unpublished results).

Morphological examination. The morphology of amoebae and cysts were examined in the living state using a phase contrast microscope.

All amoebae without the eruptive-like pseudopode formation and the typical *Naegleria* cyst morphology (Page, 1967) were discarded. No attempt was made to identify the other genera.

Flagellation test at 42° C. A piece of NN agar cut from the dense migrating amoeba ring was placed in 1 ml. sterile distilled water and incubated at 42° C. After 30 min., 1 hr., 2 hr. and 3 hr. the tubes were examined for the presence of flagellates using an inverted phase contrast microscope.

Pore structure of the cysts. After prolonged incubation on NN agar at 37° C. empty cysts were easily obtained. Special attention was then given to the structure and number of pores in the cyst wall, observed at a magnification of × 625. *N. gruberi* shows many crater-like pores with elevated walls, whilst *N. fowleri* shows only a small number of flat-edged pores (Jadin, Eschbach, Verheyen & Willaert, 1974).

Indirect Immunofluorescence (IFAT). Cultures on NN agar or in liquid medium showing heavy development were processed for indirect immunofluorescence according to the methods published earlier (De Jonckheere, Van Dijck & van de Voorde, 1974).

Mouse inoculation. Naegleria strains with the characteristics of the *fowleri* species were inoculated intranasally to C3H mice.

For each strain 5 mice were inoculated with 20,000–50,000 amoebae, washed off with sterile distilled water from the dense ring of proliferating amoebae on the medium.

RESULTS

N. fowleri were isolated only in the thermally polluted canal receiving the cooling water from the lead and zinc factory (Table 1). In this thermally polluted water one case of PAME had occurred in 1973. More *N. fowleri* were isolated from the thermal plume and its immediate surroundings than further away from the

Table 1. *Isolation of amoebae* at 42° C. from the environment*

Sampling site	Water temperature (°C.)	Number of samples	Number of amoeba-positive samples	Number of *N. fowleri*-positive samples	Number of *N. gruberi*-positive samples
A. *Thermally polluted water*					
1. Lead and zinc factory					
April–May					
(*a*) factory outlet	29–30	4	4	2	0
(*b*) immediate surroundings of the factory outlet	25–27	6	6	4	0
(*c*) canal and connected stream below factory outlet	20–23	12	9	3	3
August					
(*a*) factory outlet	34	4	4	4	0
(*b*) immediate surroundings of the factory outlet	32	4	4	4	0
(*c*) canal and connected stream below factory outlet	25–29	24	14	6	0
October					
(*a*) factory outlet	24	3	2	1	0
(*b*) immediate surroundings of the factory outlet	21·5	3	3	0	1
(*c*) canal and connected stream below factory outlet	16	6	3	0	1
Total		66	49	24	5
2. Electricity power plant					
May	21–23	11	4	0	0
October					
(*a*) outlet	22–24	12	6	0	3
(*b*) cooling tower	20–26	6	4	0	3
Total		29	14	0	6
B. *Various waters*					
Canals in surroundings	15–15·5	9	3	0	0
Polluted canal in a city (Leuven)	11–12	7	7	0	1
Polluted stream in a city (Leuven)	11–11·5	6	5	0	2
Lake polluted by sewage	13	6	0	0	0
Fishpond	11	4	1	0	0
Spare-basins and slow sandfilters	17–18	24	2	0	0
Soil samples	—	3	1	0	0
Water distribution network	10	5	0	0	0

* Apart from *N. fowleri*, these are *N. gruberi* and other amoebae of the families *Valhkampfiidae*, *Hartmannellidae*, *Mayorellidae* and others able to grow at 42° C.

cooling water discharge point. This observation suggests that a raised temperature favours the growth of *N. fowleri* in the environment of the factory. Downstream their number is diluted out as they are no longer multiplying and even overgrown by other amoebae. In the month of August samples were again taken from this thermally polluted canal (temperature up to 34° C.) and *N. fowleri* were again identified.

In the warm waters of the electricity power plant no *N. fowleri* could be isolated. In May the water samples taken near the cooling water outlet did not show

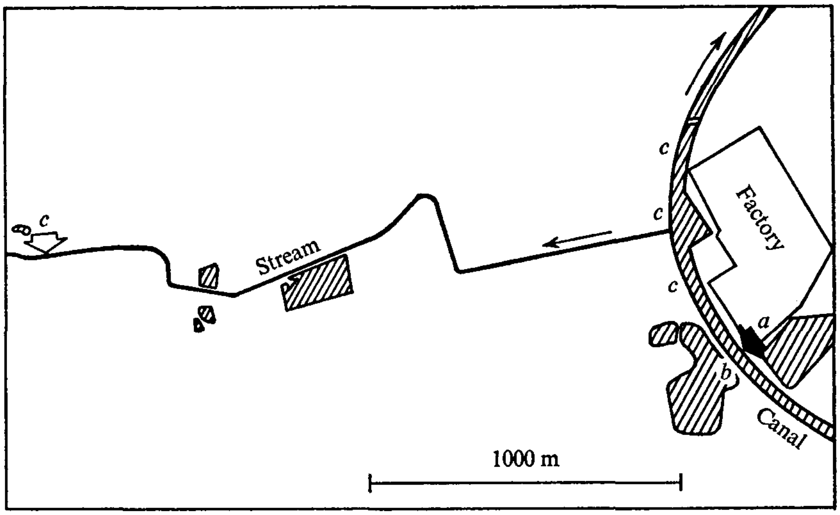

Fig. 1. Map of the thermal polluting lead and zinc factory with canal and connected stream where *N. fowleri* was isolated. The black arrow at point *a* indicates the cooling water discharge of the factory. The white arrow at point *c* indicates the farthest sampled point in the connected stream. The sampling sites are marked with letters referring to Table 1: (*a*) factory outlet; (*b*) immediate surroundings of the factory outlet; (*c*) points in the canal and connected stream, below factory outlet.

temperatures above 21–23° C., and we failed to isolate *N. fowleri* from the samples, though we had isolated it from samples at a similar temperature from the lead and zinc factory. The cooling discharge temperature at that time was the same for both factories (29° C.).

In October both thermal discharges had a temperature of 24° C. and *N. fowleri* was isolated only in the outfall of the lead and zinc factory. No *N. fowleri* were found this time in the immediate surroundings of the thermal plume. This must be due to the much lower temperatures observed at that time of the year. The highest number of *N. fowleri* isolations were made at the lead and zinc factory in summer when the temperature was highest. It was also at this time of the year that a case of PAME occurred in 1973 in a boy who swam at this site.

Many other amoeba strains able to grow at the selective temperature of 42° C. were isolated. Amongst the samples taken in August and October 1 ml. samples were also included. In October only one sample was positive; it was taken from the cooling discharge of the lead and zinc factory. In August all the 1 ml. samples taken from the cooling discharge and its immediate surroundings were positive. Thus the concentration of amoebae able to grow at 42° C. was greatest in the water with the highest temperature. No *N. fowleri* were isolated from all other sources. The striking incidence of amoebae able to grow at high temperatures in the canal and stream polluted by sewage is worth noting.

N. gruberi strains able to grow at 42° C. were isolated from the two thermally polluted waters examined and from a stream and canal, highly polluted by city sewage. Although many workers reported *N. gruberi* as being widespread the distribution of strains with this high temperature tolerance seems restricted.

Most of the *N. fowleri* isolated in this study possessed all but one of the characteristics for identification of the species. No pathogenicity could be observed in mice, except with one strain which proved to be highly virulent for mice. Controls with *N. fowleri* isolated from human cerebrospinal fluid were highly virulent. A more extended study with two of these non-pathogenic strains of *N. fowleri* is in progress.

DISCUSSION

The epidemiology of PAME described in the literature indicates that this disease is correlated with swimming in warm waters. Our investigation shows that the occurrence of *N. fowleri* is enhanced by thermal pollution, but other factors also appear to have an effect. The reason why *N. fowleri* occurs in one thermally polluted water and not in another will be the subject of further examination. This was not due to the use of a disinfectant in one of the two factories. High temperature seems to be one of the essential requisites for the selective proliferation of *N. fowleri*.

Only one of the *N. fowleri* isolated proved to be virulent for mice. It is possible that the non-pathogenic *N. fowleri* strains we isolated may undergo transformation in a carrier organism. Without this contact with an animal these amoebae would remain harmless. This hypothesis is supported by our observation that strains isolated from CSF which are cultivated for many years in the laboratory, lose some degree of pathogenicity whilst they become again highly virulent after brain passage in mice. Other workers also observed this phenomenon (Visvesvara & Callaway, 1974). *N. fowleri* should not therefore be identified by means of the mouse inoculation test only, more characteristics being needed as mentioned. In this respect IFAT is a very promising technique, as one may assume that these naeglerias which are identified by IFAT as *N. fowleri* but which are not pathogenic for mice, could become virulent under the influence of some unknown factors.

Our technique will not only identify the pathogenic *Naegleria* strains but also the potentially pathogenic ones. These potentially pathogenic strains could be indicator organisms for detection of dangerous environments where PAME may be contracted, as we isolated them only from water where the disease had occurred.

Amoebae isolated at 42° C. belonging to *Vahlkampfiidae*, *Hartmannellidae*, *Mayorellidae* and other families could interest workers studying amphizoic amoebae (Page, 1974) as the ability to grow at high temperatures may be important for their becoming endozoic or parasitic in warm-blooded animals. During the international 'Symposium on the Physical and Biological Effects on the Environment of Cooling Systems and Thermal Discharges at Nuclear Power Stations', held in August 1974 in Oslo, no attention was paid to the proliferation of pathogenic organisms. Yet the possible hazards of inducing growth of pathogens in surface water by discharge from cooling systems of industries must be taken into consideration.

REFERENCES

ANDERSON, K. & JAMIESON, A. (1972). Primary amoebic meningoencephalitis. *Lancet* i, 902–3.

CARTER, R. F. (1972). Primary amoebic meningo-encephalitis. An appraisal of present knowledge. *Transactions of the Royal Society of Tropical Medicine and Hygiene* **66**, 193–213.

ČERVA, L. & NOVÁK, K. (1968). Amoebic meningoencephalitis: sixteen fatalities. *Science, New York* **160**, 192.

DE JONCKHEERE, J., VAN DIJCK, P. & VAN DE VOORDE, H. (1974). Evaluation of the indirect fluorescent-antibody technique for identification of *Naegleria* species. *Applied Microbiology* **28**, 159–64.

DUMA, R. J., ROSENBLUM, W. I., MCGEHEE, R. F., JONES, M. M. & NELSON, E. C. (1971). Primary amoebic meningoencephalitis caused by *Naegleria*. Two new cases, response to Amphotericin B. and a review. *Annals of Internal Medicine* **74**, 923–31.

GORDEEVA, L. M. (1973). Isolation and cultivation of limax amoebae capable to grow at 37° C. *Progress in Protozoology*, p. 159.

GRIFFIN, J. L. (1972). Temperature tolerance of pathogenic and nonpathogenic free-living amoebas. *Science, New York* **178**, 869–70.

JADIN, J. B., HERMANNE, J., ROBYN, G., WILLAERT, E., VAN MAERCKE, Y. & STEVENS, W. (1971). Trois cas de meningo-encephalite amibienne primitive observés à Anvers (Belgique). *Annales de la Societé Belge de Médicine Tropicale* **51**, 255–66.

JADIN, J. M., ESCHBACH, H. L., VERHEYEN, F. & WILLAERT, E. (1974). Etudes comparatives des kystes de *Naegleria* et *d'Acanthamoeba*. *Annales de la Societé Belge de Médicine Tropicale* **54**, 259–64.

JAMIESON, A. (1973). The epidemiology of primary amoebic meningo-encephalitis in South Australia. *Progress in Protozoology*, p. 198.

KASPRZAK, W. & MAZUR, T. (1974). Small free-living amoebae isolated from 'warm' lakes: investigations on epidemiology and virulence of the strains. *Proceedings of the Third International Congress of Parasitology* **1**, 188–9.

MANDAL, B. N., GUDEX, J. D., FITCHET, M. R., PULLON, D. H. H., MALLOCH, J. A., DAVID, C. M. & APTHROP, J. (1970). Acute meningoencephalitis due to amoebae of the order *Myxomycetale* (slime mold). *New Zealand Medical Journal* **71**, 16–23.

PAGE, F. C. (1967). Taxonomic criteria for limax amoebae, with descriptions of 3 new species of *Hartmannella* and 3 of *Vahlkampfia*. *The Journal of Protozoology* **14**, 499–521.

PAGE, F. C. (1974). *Rosculus ithacus* Hawes, 1963 (*Amoebida, Flabelluidae*) and the amphizoic tendency in amoebae. *Acta Protozoologica* XIII (12), 143–54.

SINGH, B. N. & DAS, S. R. (1972). Occurrence of pathogenic *Naegleria aerobia*, *Hartmannella culbertsoni* and *H. rhysodes* in sewage sludge samples of Lucknow. *Current Science* **41**, 277–81.

VAN DEN DRIESSCHE, E., VANDEPITTE, J., VAN DIJCK, P. J., DE JONCKHEERE, J. & VAN DE VOORDE, H. (1973). Primary amoebic meningoencephalitis after swimming in stream water. *Lancet* ii, 971.

VISVESVARA, G. S. & CALLAWAY, C. S. (1974). Light and electron microscopic observations on the pathogenesis of *Naegleria fowleri* in mouse brain and tissue culture. *The Journal of Protozoology* **21**, 239–50.