STUDIES OF AMOEBAE

OF THE GENUS *NAEGLERIA*

by

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SUMMARY

Primary amoebic meningoencephalitis (PAME) is a disease of man caused by the free-living amoebo-flagellate, Naegleria fowleri. This thesis is a study of the genus with particular reference to this species.

The growth requirements of the genus were studied, and it was found that the presence of other living cells was not essential. This was an area of controversy even though Cerva in 1969 and Fulton in 1970 had already achieved axenic culture of these organisms. The ability of Naegleria to grow on commonly isolated gram negative bacteria was not able to be used to distinguish between species.

A method for isolating Naegleria (Jamieson & Anderson, 1973) from water, soil and dust samples which is suitable for routine laboratory surveillance of swimming pools and water supplies in "risk" areas (i.e. areas in which cases of PAME have been reported) was developed. This method is now being used by two other public health laboratories. Nineteen strains of Naegleria and seven strains of Acanthamoeba pathogenic to mice were isolated using this technique. The pathogenic isolates of Naegleria were the first ever made from a probable source of infection of a case of PAME; in fact, they were the first pathogenic Naegleria to be isolated from the environment.

Ether vapour was shown to lyse trophozoites of Naegleria and is therefore considered to be unsuitable as an anesthetic for pathogenicity testing in mice. Efforts to infect mice with N. fowleri by the oral and ocular routes were unsuccessful.

An agglutination test (Anderson & Jamieson, 1972b) was developed as an aid in the classification of Naegleria. The use of this test revealed the existence of a diversity of serotypes within the genus.

The anti-fungal antibiotic clotrimazole was found to have significant activity against N. fowleri in vitro (Jamieson & Anderson, 1974) but it was unable to protect mice from infection, even though adequate serum levels were achieved (Jamieson, 1975).

As a result of the work involved in this study, the public water supplies to "risk" areas of South Australia have been more heavily chlorinated and no N. fowleri have been isolated since adequate levels of chlorine were established.

Three cases of PAME occurred during this study. In 1971 a 14 year old Aboriginal boy from Mount Morgan, Queensland was successfully treated with amphotericin B (Anderson & Jamieson, 1972a). In1972 a five year old boy and a seven year old girl, both from Port Augusta, died from PAME despite treatment with this drug. The diagnoses were confirmed here by isolation of N. fowleri from cerebrospinal fluid. Laboratory studies of the isolates were conducted by myself. These three cases are not reported in the body of the thesis because the course of the disease has already been adequately documented by Dr. R.F. Carter (1968).

DECLARATION

This thesis does not contain any material previously accepted for the award of any degree or diploma at any University. Nor to the best of my knowledge does it contain any material previously published or written by any other person without due acknowledgement.

ACKNOWLEDGEMENTS

I am deeply indebted to the late Dr. Kevin Anderson who was Director of Clinical Microbiology at the Institute of Medical and Veterinary Science, for his advice and help in the earlier parts of this work.

My thanks also go to my Supervisor, Dr. Rodney Carter, Director of Pathology, Adelaide Children's Hospital, for his tuition in the techniques of handling pathogenic amoebae, for his gift of cultures and for his continued interest and advice.

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Dr. Z. Seglenieks of the Department of Public Health helped in co-ordinating environmental investigations with Local Boards of Health, and the Engineering and Water Supply Department.
The Division of Histopathology of the Institute of Medical and Veterinary Science processed all histological specimens.

Dr. E. Willaert and Professor J.B. Jadin of the Prince Leopold Institute of Tropical Medicine, Antwerp, Belgium supplied many strains of *Naegleria* used in this study.

The Pharmacy of the Royal Adelaide Hospital prepared drugs for chemotherapy experiments. The Division of Animal Science of the Institute of Medical and Veterinary Science supplied and cared for mice used in pathogenicity testing. Antisera were prepared by Mr. E.F. Crowder of the Serum Production Unit.

I wish to thank Mrs. M. Priede for typing the manuscript and Miss J. Rust and Mrs. M. Newman for their valuable technical assistance throughout this study. Grants for equipment were received from the National Health and Medical Research Council, E.R. Squibb & Sons, Beecham, Roche, Upjohn and Lilly Industries.

I am grateful to Dr. J. Bonnin for allowing me to undertake this study in the Division of Clinical Microbiology, Institute of Medical and Veterinary Science, Adelaide.

INTRODUCTION

This thesis is about Naegleria fowleri (Carter 1970) (Figures 1 and 2) the free-living amoeba-flagellate which causes the disease primary amoebic meningo-encephalitis (PAME) in man. When the work on which this thesis is based, was begun in February 1971, many of the problems surrounding this recently recognised disease (it was first described in the literature by Fowler and Carter in 1965) had been solved. Both Carter, in South Australia (Carter, 1968) and Butt, in Florida, U.S.A. (Butt, et al., 1968) had isolated the causative organisms from cases of PAME and had shown that it belonged to the genus Naegleria and not to the genera Acanthamoeba (Figure 3) or Hartmanella as had previously been thought (Fowler & Carter, 1965, Culbertson et al., 1965). This misapprehension arose because in 1958 Culbertson, Smith and Minner showed that free-living amoebae of the Hartmanella-Acanthamoeba group could be pathogenic in animals. Subsequent cases of meningoencephalitis caused by free-living amoebae were thought to be due to this group, but the identification of the amoebae relied solely on morphology in tissue sections - the organisms were never isolated from patients and studied. When Carter isolated the amoeba from a patient in 1968 he found that it belonged to the genus Naegleria. In 1970 he described his isolate as a new species, Naegleria fowleri. Singh and Das described a similar isolate as a new species, Naegleria aerobia, also in 1970 but after Carter's description had been published and the priority of the name N. fowleri had been established. Chang (1971) proposed the name Naegleria invades but it has not been generally accepted.

In 1968, Carter described the clinical, pathological and epidemiological features of PAME as seen in six cases. Subsequently he described (1970) the experimental pathological changes produced by N. fowleri in mice.

Figure 1 *Naegleria fowleri* (Strain NHI) trophozoites.

Note limax (slug-like) shape and anterior speudopod.

Wet mount. Bright field (B.F.) X 1000.

Figure 2. *Naegleria fowleri* (Strain NHI) trophozoites.

Note nucleus with large nucleolus.

Haematoxylin and Eosin (HE) X 1000.

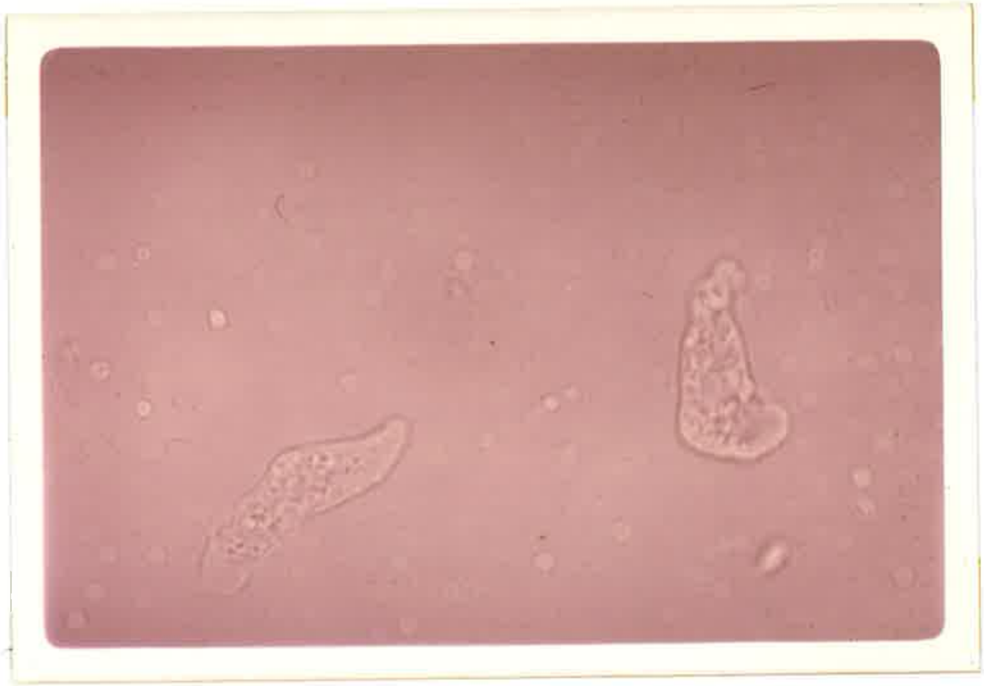

FIG 1.

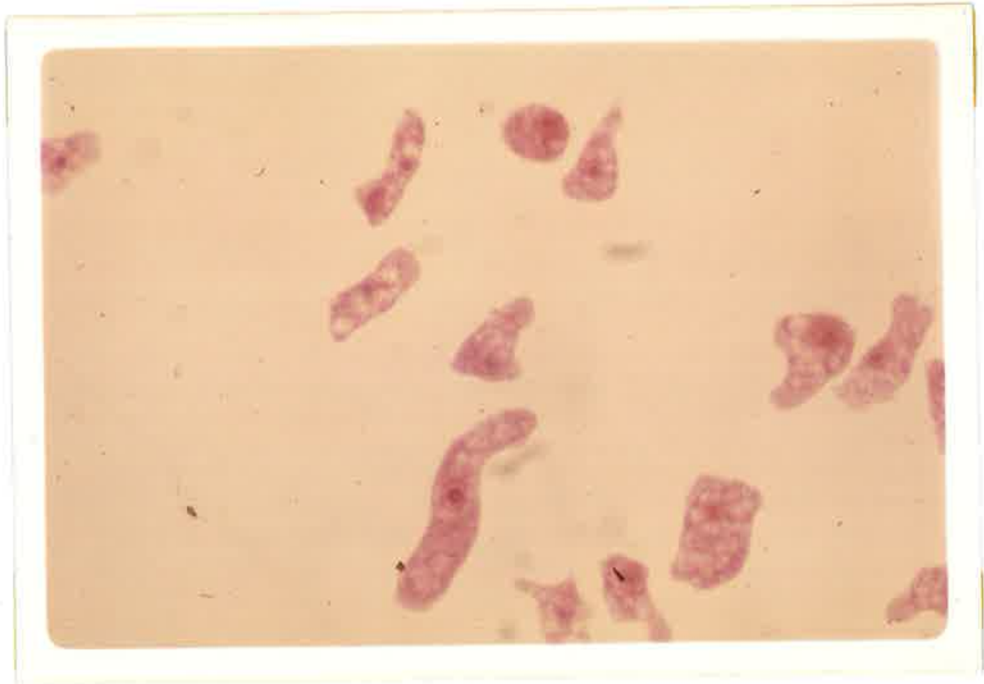

FIG. 2.

Figure 3. *Acanthamoeba* sp. (Strain A-1).

Note spiny pseudopodia and vacuolated cytoplasm.

B.F. X 2,500.

Figure 4. *N. gruberi*

Note darkly staining, large nucleolus.

H.E. X 1,000.

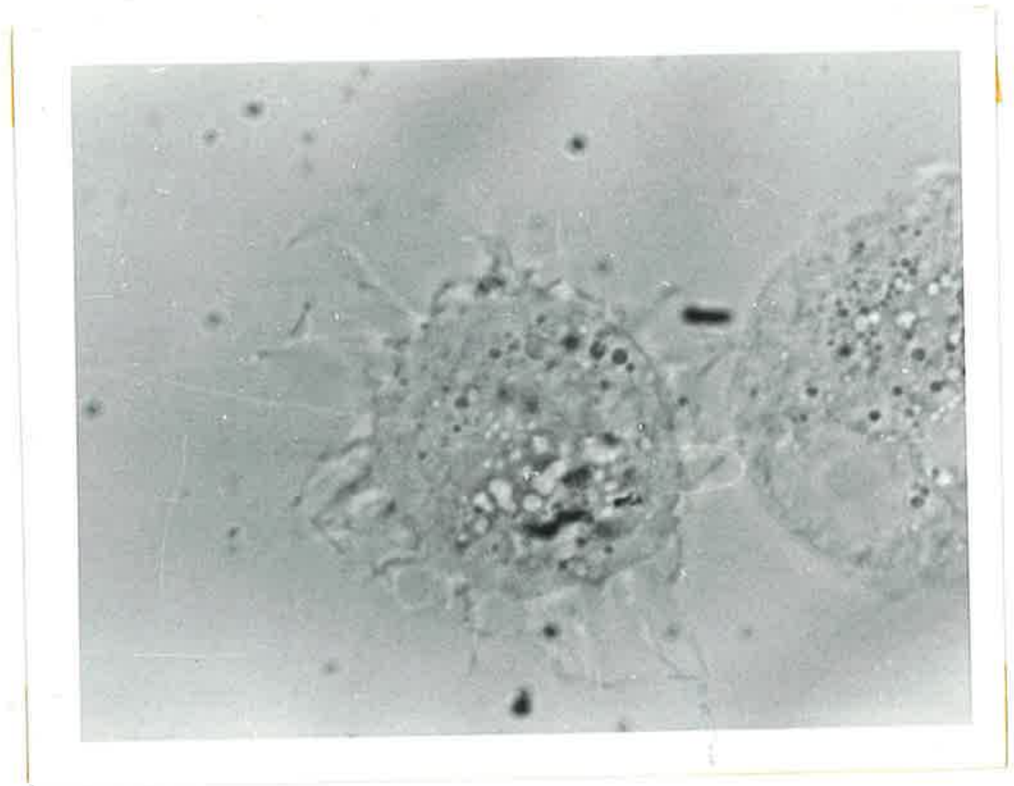

Fig 3.

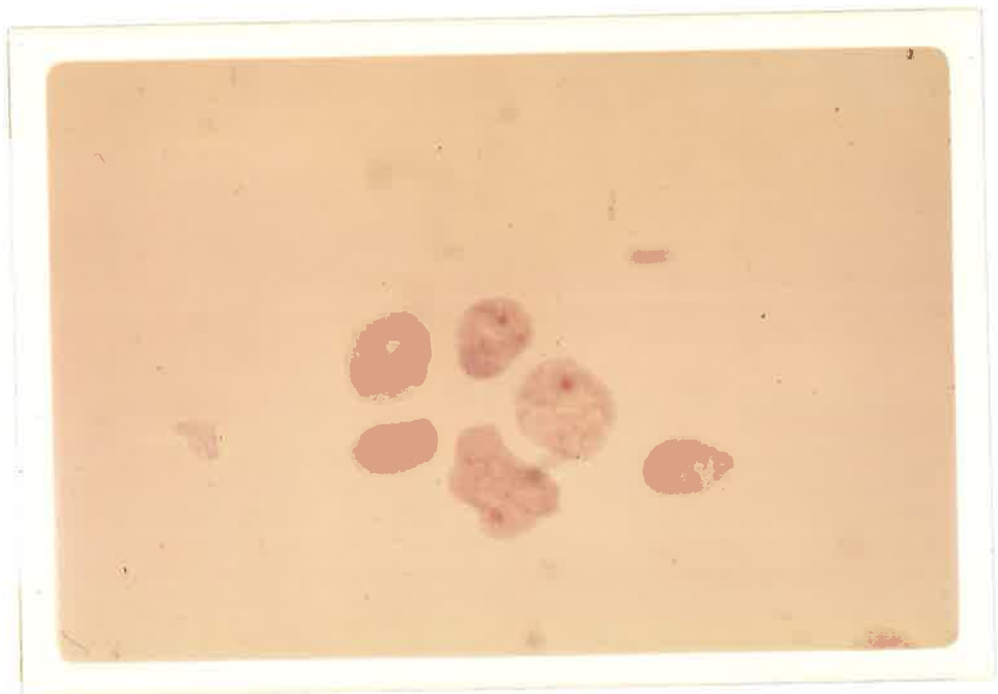

Fig 4

In 1969 Carter showed that N. fowleri was sensitive to the anti-fungal antibiotic amphotericin B.

I began this study of Naegleria in 1971 after Dr. R.F. Carter of the Adelaide Children's Hospital had taught me the techniques of handling and culturing these organisms. He supplied the first culture of N. fowleri, the Northcott strain isolated from a patient with fatal PAME in Port Pirie, South Australia in February 1971.

My objectives were to achieve axenic growth of N. fowleri, to devise a serological means of identification, to isolate N. fowleri from the environment and to investigate chemotherapeutic agents for anti-amoebic activity. In the earlier parts of the work I collaborated with Dr. K.F. Anderson until his death in July 1973.

Axenic culture of N. fowleri was desirable for studies involving the preparation of antisera and antigens. A liquid medium was more convenient for routine maintenance of strains and for studying the effects of various agents on the amoebae.

A serological means of identification offered the possibility of more rapid differentiation between the pathogenic N. fowleri and the non-pathogenic N. gruberi (Figure 4), which are morphologically identical, than could be achieved using animal inoculation. It also represented another tool for gaining more information about the amoebae.

Although over forty cases of PAME had been reported by early 1971, the causative amoeba had not yet been isolated from the environment. Location of sources of infection and subsequent control measures depended on proof of the existence of N. fowleri in these sources.

The control measures could then consist of either limiting access to the source or treating the source with an agent lethal to N. fowleri.

Amphotericin B was successful in the treatment of one patient and unsuccessful with two in the early part of this study. The main disadvantage with amphotericin B was its nephrotoxicity. Because of this, treatment of a suspected case was not started until a fairly firm diagnosis of PAME was made. This caution was necessary because, in the normal anti-fungal use of the drug, doses are gradually increased from 0.25 mg/kg body weight/day up to 1 mg/kg/day. The daily increment is usually 0.25 mg/kg/day and this practice is necessary to minimise toxic effects and is quite acceptable in the treatment of fungal infections. PAME, however, is fatal usually within 5 - 7 days of infection and since the diagnosis is usually made at least 48 hours after injection there is not sufficient time for this gradual increase in dose. The maximum dose with its attendant risks is therefore recommended. A less toxic drug, with equivalent or better activity against N. fowleri and good absorption into serum and cerebrospinal fluid was, and still is, required.

MEDIA

INTRODUCTION

Living cells were long considered necessary for the growth of Naegleria spp. in artificial culture media. The cells were usually live bacteria or tissue culture cells (Culbertson et al., 1968, Carter, 1970 and Mandal et al., 1970). Butt et al. (1968) had used dead bacteria successfully. Dead bacteria, or preferably, an axenic culture medium, are more convenient than live bacteria for the maintenance of large numbers of Naegleria strains over a long period during which their properties are studied. Axenic cultures are desirable for the preparation of antisera and antigens. Growth on agar spread with dead bacteria and axenic methods of culture were therefore investigated.

METHODS

Growth on dead bacteria

Live gram negative bacilli had been used successfully as a food source for Naegleria and so Escherichia coli A.T.C.C.11129 was chosen for study. The bacteria were grown on the surface of 20 ml of plate-count agar (Oxoid) dispensed in flat bottles. After 18 hours' incubation at 37°C, growth was washed off with 5 ml of distilled water. The bacterial suspension was distributed in 3 ml aliquots and immersed in a 60°C water bath for periods ranging from 30 - 120 minutes. One sample was steamed at 100°C for 10 minutes. Each volume was subcultured to test sterility and then stored at 5°C. Thirty minutes' exposure at 60°C proved insufficient to kill this organism and these suspensions were consequently excluded from further experiments.

Initially, an infected mouse brain, removed from an animal inoculated by the intranasal route with N. fowleri, was immersed in distilled water containing gentamicin in a final concentration of 10 ug/ml.

This amoebic suspension was incubated at 37°C for six days and cultured on blood agar medium to exclude the presence of viable bacteria. The material was then inoculated onto 1.25% agar plates spread with the heated bacterial suspensions. Growth of the amoebae was observed by stereoscopic microscopy after two days on all the plates, including controls spread with live bacteria. To exclude the serial transfer of viable intracellular bacteria, N. fowleri propagated on dead cells were subcultured to the surface of MacConkey's medium containing 0.5% bile salts. (Carter (1970) showed that Naegleria trophozoites are lysed by bile salt concentrations of 0.3% or less in 15 minutes.) Lysis of amoebae was observed by stereomicroscopy and the plates were then incubated at 37°C for 36 hours.

Growth in Axenic Media

Cerva (1969) medium

Twenty grammes of Bacto-Casitone were dissolved in one litre of distilled water. The solution was distributed in 100 ml amounts and autoclaved at 15 lbs/15 minutes. Uninactivated horse, bovine and rabbit sera, sterilised by Seitz filtration, were added in 10 ml amounts to separate bottles. To each bottle was added 500 units/ml penicillin and 50 ug/ml streptomycin. The media were dispensed in 10 ml volumes in polycarbonate containers and inoculated with N. fowleri from an agar plate spread with dead E. coli. The cultures were incubated at 37°C without agitation or aeration. The growth of wild unidentified strains of Naegleria was tested in Cerva's medium with bovine serum. The cultures were inoculated as described above and incubated at 28°C.

Fulton (1970) medium

Fulton's medium "A" was tested. It is composed of:

PO4	KH2PO4	3.4 gm
	Na2HPO4.7H2O	6.7 gm
	Dissolved in 100 ml distilled water and autoclaved (15 lbs/15 minutes).	
DEX	Distilled water	50 ml
	Glucose	13.5 gm
	Dissolved and autoclaved (15 lbs/15 minutes).	
HL-5	Distilled water	650 ml
	Proteose peptone	10 gm
	Yeast extract	5 gm
	Dissolved and distributed in 65 ml volumes.	
	Autoclaved (15 lbs/15 minutes).	
	Add 2 ml PO4 and 2 ml DEX = HL-5.	

Complete medium:

HL-5	7 ml
Distilled water	2 ml
Foetal calf serum	1 ml

Penicillin and streptomycin, in final concentrations of 500 units/ml and 50 ug/ml respectively, were added. Later the concentrations were increased to 2,000 units/ml penicillin and 100 ug/ml streptomycin without any adverse effect on the amoebae, and with the beneficial effect of further discouraging bacterial growth. An axenic culture of N. fowleri in Cerva's medium with bovine serum wasused to inoculate 5 ml volumes of Chandler Fulton's medium "A" (CFA). The cultures were incubated at 37°C without agitation or aeration.

Growth in CFA with various peptones

Proteose peptone is used in the HL-5 component of CFA. Experiments were conducted to find the peptone which best supported the growth of

N. fowleri, *N. gruberi* and wild, unidentified isolates of *Naegleria*. The following peptones were tested in the concentrations used by Fulton:

- Proteose peptone No. 3 (Difco)
- Tryptone (Difco)
- Tryptose (Difco)
- Casitone (Difco)
- Soya Peptone (Oxoid)
- Proteose peptone (Control) (Difco)

N. fowleri and a wild strain, presumed to be *N. gruberi* because of its lack of pathogenicity and its agglutination pattern, were inoculated into 10 ml volumes of the various media and incubated.

Reduction of the Amount of Foetal Calf Serum in CFA

The supply of foetal calf serum is always uncertain because present methods of collection depend on the slaughter of pregnant cows in abattoirs; the Commonwealth Serum Laboratories obtain blood from the foetuses. Therefore, the amount of serum in CFA was reduced to determine whether this had an adverse effect on the growth of amoebae.

1. Reduction of foetal calf serum - all the ingredients of CFA except the serum constituted the Base. A series of 10 ml volumes was prepared by increasing the amount of Base by 0.1 ml and reducing the amount of serum correspondingly. The series ranged from the control of 9 ml Base + 1 ml serum to 10 ml Base + 0 ml serum. 0.5 ml of axenically growing *N. fowleri* was added to each 10 ml volume, so that each received approximately the same inoculum. The cultures were incubated at 37°C for 7 days.
2. Dilution with distilled water - the amount of CFA was reduced by 1 ml and the amount of distilled water was increased correspondingly in 10 ml volumes. The cultures were inoculated and incubated as described above.

Bacterial Suspensions for the Growth of *Naegleria* spp.

(Anderson and Jamieson, 1974)

When the growth of wild strains and *N. fowleri* was well established on both dead and live *E. coli*, investigations were carried out to determine whether *N. fowleri* and other strains of *Naegleria* could be distinguished by their ability to grow on different bacteria. Suspensions were prepared in distilled water by washing growth from the surface of agar slants, followed by storage at 5°C. Heat-killed suspensions were made by immersing 3 ml volumes in a 60°C water bath for one hour. Sterility was confirmed by subculture to the surface of blood agar plates, followed by overnight incubation at 37°C. Turbidity was adjusted by dilution with distilled water and comparison with standard opacity tubes (Wellcome). The following gram negative bacilli were selected for testing:

Proteus vulgaris
Pr. mirabilis
Pseudomonas aeruginosa
Escherichia coli A.T.C.C.11129
Enterobacter aerogenes
Ent. cloacae
Ent. hafniae
Shigella sonnei
Salmonella typhi-murium
Providencia sp.

Plates containing 10 ml of 1.25% agar (Difco) in distilled water were spread with suspensions using a cotton wool swab. A central area, some 6 cm in diameter, was covered so as to appear visibly moist. Prepared plates were not dried, but were used immediately, or stored in plastic bags at 5°C. Plates were inoculated in the centre of the spread area with 0.02 ml of axenic culture containing approximately 700 trophozoites/cmm. Control plates, without bacteria, were prepared in parallel.

Cultures were incubated at 28°C or 37°C depending on the temperature requirements of the amoebae. Plates were examined by stereomicroscopy at 18 and 36 hours. Growth was considered satisfactory if, at 18 hours, amoebae were seen migrating from the point of inoculation to form, at 36 hours, an expanding ring of dense, syncytium-like growth. (Figures 5 and 6).

The Effect of Agar on Axenic Growth

Considerable difficulty has been experienced in establishing axenic growth of some strains of *Naegleria*. These strains grow well on plain agar in association with dead bacteria and so supplementation of the axenic medium with agar was investigated. Agar consists of:

Water	7.5 - 12.0%
Ash	1.5 - 2.0%
PO4	0.3 - 0.6%
Total N	0.3%
Cu	0.0002 - 0.001%
Fe	0.015 - 0.03%
Ca	0.25 - 0.5%
Mg	0.2 - 0.4%

Amounts ranging from 0.1 ml - 1.0 ml of molten 1.25% agar were added to 10 ml volumes of CFA. The supplemented media were inoculated with equal amounts of *N. fowleri* and incubated at 37°C for 7 days.

RESULTS

Growth on Dead Bacteria

All suspensions of heat-killed bacteria supported growth of *Naegleria*. Heating at 60°C for 1 hour was chosen for routine use because it was the shortest time tested which killed the bacteria. The pathogenicity of *N. fowleri* maintained on plates spread with heat-killed organisms was confirmed by mouse inoculation.

Figure 5. Growing edge of a culture of *N. fowleri* on plain agar with live *E. coli*.
B.F. X 100.

Figure 6. Growing edge of a culture of *N. gruberi* on plain agar with heat-killed *E. coli*.
B.F. X 100.

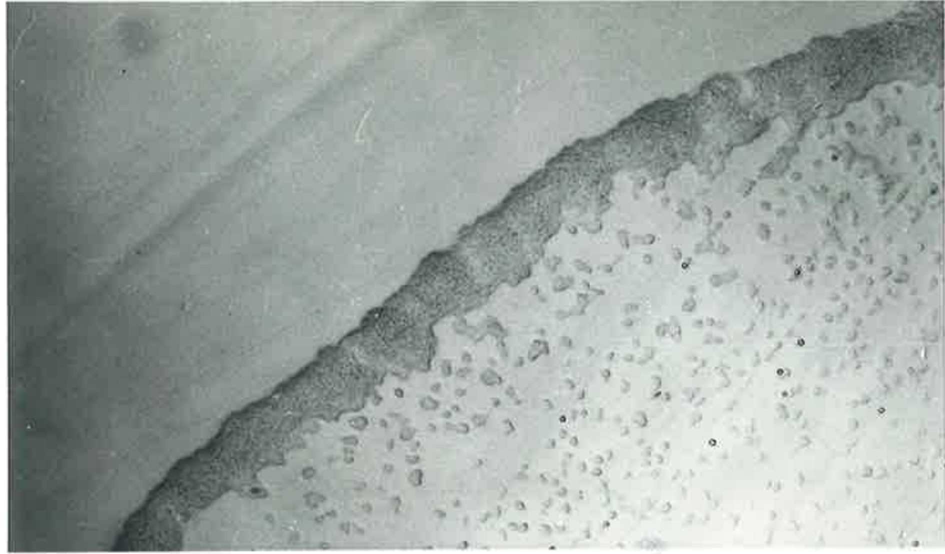

FIG 5.

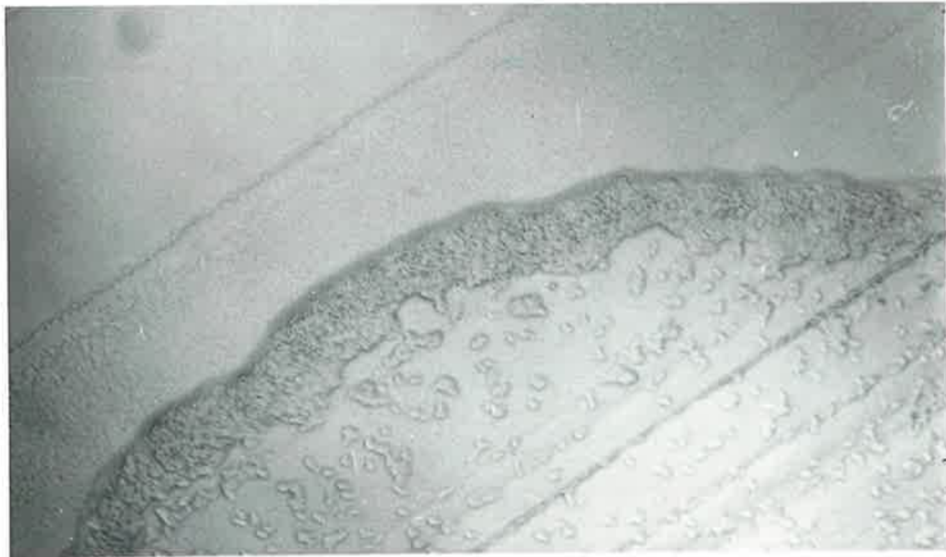

FIG 6.

No growth was obtained on the MacConkey plates, thus excluding the presence of intracellular bacteria.

Growth in Axenic Media

Cerva (1969) medium

N. fowleri grew in Cerva medium containing horse and bovine serum, but not with rabbit serum (Figures 7 and 8). None of the wild, unidentified strains of *Naegleria* grew in Cerva's medium with bovine or horse serum.

Fulton (1970) medium

Five mls of CFA were inoculated with *N. fowleri* growing axenically in Cerva's (bovine) medium, and incubated at 37°C. The numbers of trophozoites and cysts produced over 14 days are given in Figure 9.

Growth in CFA with Various Peptones

Tables 1 and 2 show the numbers of cells (both trophozoites and cysts) produced when *N. fowleri* and *N. gruberi* were inoculated into CFA with various peptones. Proteose peptone No. 3 was selected for routine use.

Reduction of Foetal Calf Serum

When the amount of foetal calf serum in CFA was reduced and replaced by extra base, adequate growth for the maintenance of strains was achieved in tube 6 which contained 9.6 ml base and 0.4 ml serum. (Table 3).

CFA could be diluted by adding 6 ml distilled water to 4 ml CFA (tube D) and still provide an adequate medium for the maintenance of strains (Table 4).

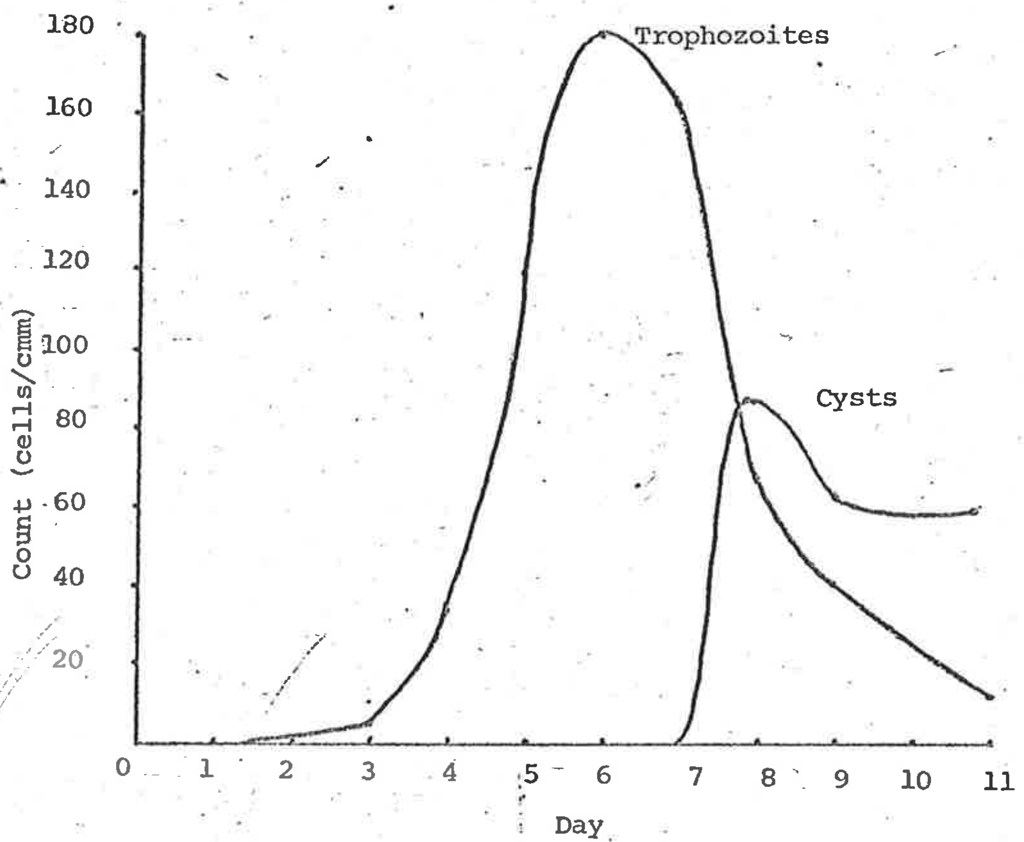

Figure 7. Growth of N. fowleri on Cerva (1969) Horse Medium.

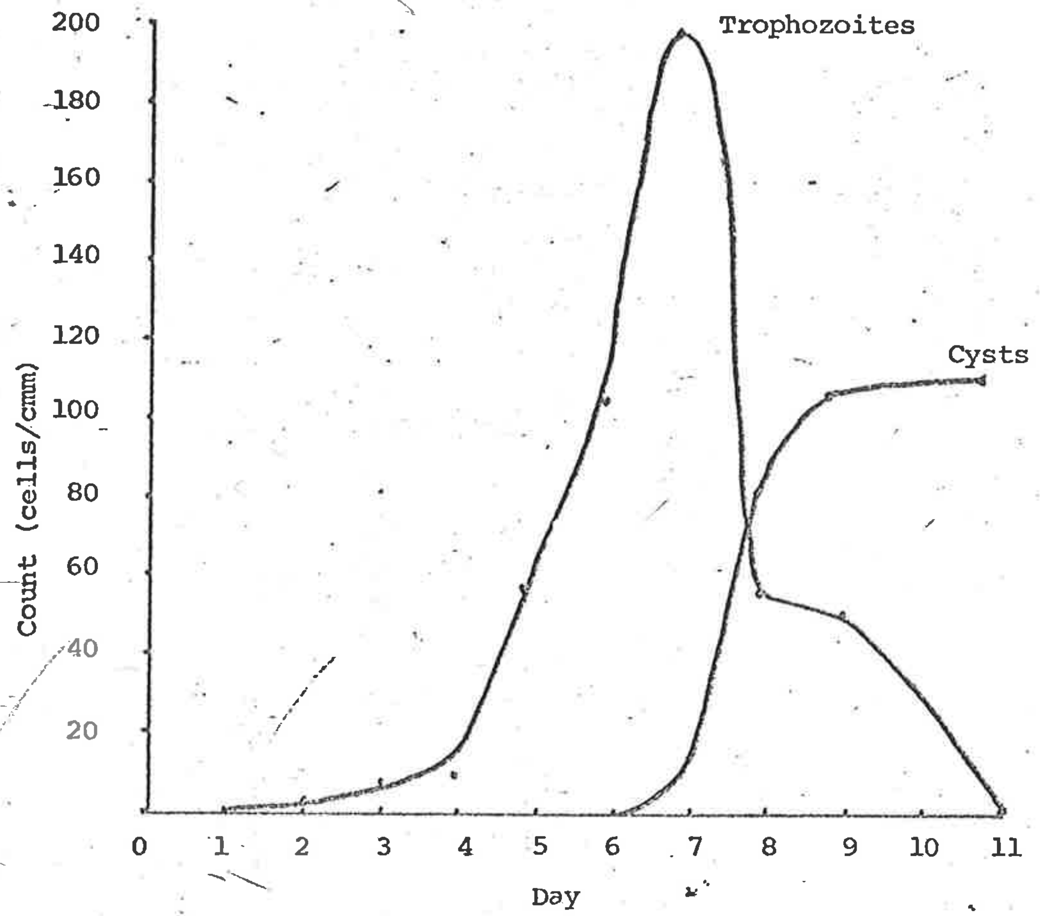

Figure 8. Growth of _N. fowleri_ in Cerva (1969) Bovine Medium

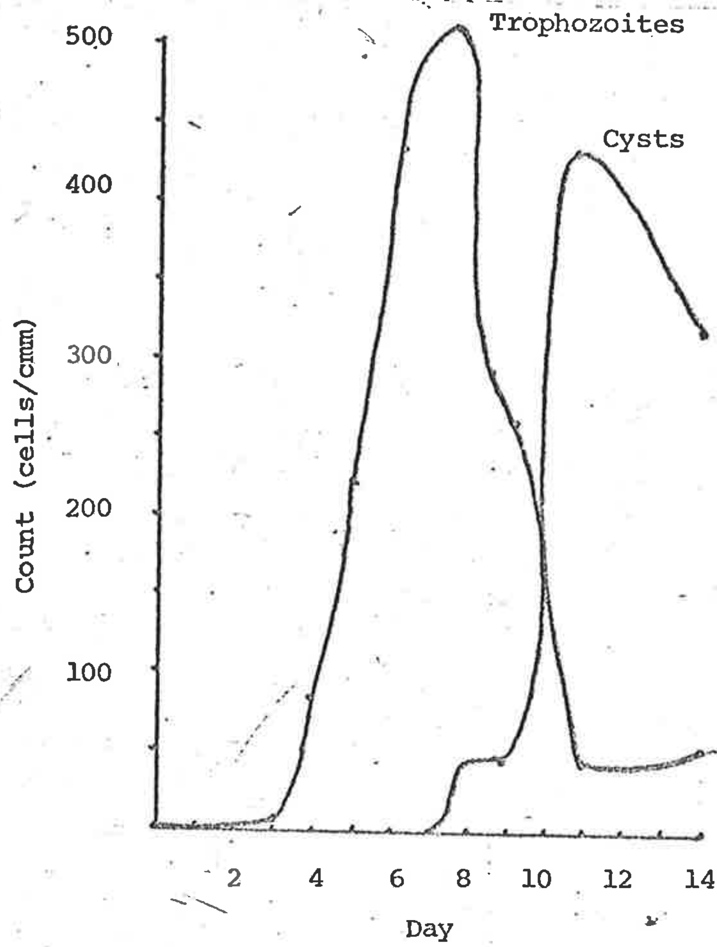

Figure 9. Growth of N. fowleri in Fulton (1970) Medium

Table 1. Growth of *N. fowleri* in CFA with various peptones.

Peptone	Days of incubation 3	4	5	6	7
Proteose peptone No. 3	334	568	786	896	1,030
Tryptone	434	668	882	928	854
Tryptose	270	476	670	828	964
Casitone	248	420	664	734	808
Soya peptone	0	0	0	0	0
Proteose Peptone (Control)	316	442	610	824	1,126

All coutns cells/cmm.

Table 2. Growth of *N. gruberi* in CFA with Various Peptones

Peptone	Days of incubation 2	3	4	5	6	7	8
Proteose peptone No. 3	12	30	140	432	1,000	1,144	1,296
Tryptone	40	98	218	332	454	638	652
Tryptose	24	62	114	322	382	604	436
Casitone	22	68	126	234	364	546	426
Soya peptone	0	0	0	0	0	0	0
Proteose peptone (Control)	24	54	114	260	400	638	688

All counts cells/cmm.

Table 3. Reduction of Foetal Calf Serum in CFA.

		Count (cells/cmm)									
Day	Cont	1	2	3	4	5	6	7	8	9	10
1	158	142	96	152	84	88	84	90	54	64	10
2	488	572	452	212	392	364	290	398	494	340	106
3	880	796	686	574	626	774	514	434	600	466	112
4	1152	1216	1066	1132	1080	1330	1000	820	1070	460	130
5	1900	1850	1220	1370	1120	1420	1360	910	600	700	86
6	1310	1220	1080	1010	1140	970	780	690	920	690	48
7	1160	810	850	820	720	710	870	482	454	760	12

Cont = 9 ml Base + 1 ml serum,

1 = 9.1 ml Base + 0.9 ml serum, etc.

Table 4. Dilution of CFA with distilled water.

Day	Cont	A	B	C	D	E	F	G	H	I	J
	Count (cells/cmm)										
1	184	290	282	174	110	120	40	28	12	8	6
2	662	640	706	838	362	340	228	104	16	2	8
3	814	960	1074	634	528	450	278	98	56	0	0
4	1100	1190	1180	1000	720	290	170	78	46	0	0
5	1580	1130	840	738	424	194	64	46	30	0	0
6	990	1080	760	650	580	202	74	48	24	0	0
7	780	786	518	360	416	176	28	20	32	0	0

Cont = 10 ml CFA + 0 ml Distilled water

A = 9 ml CFA + 1 ml Distilled water, etc.

Bacterial Suspensions for the growth of *Naegleria* spp.

Live suspensions

With the exception of *Pseudomonas aeruginosa*, all the bacterial suspensions tested gave satisfactory growth, regardless of variations in optical densities within the range provided by the standard tubes. It became apparent, on inspecting the plates after incubation, that bacterial multiplication had occurred due to the small amounts of nutrients washed from the agar slant with the suspending fluid. *Ps. aeruginosa*, because of its capacity for rapid growth in the presence of a minimum of nutrients, produced a dense lawn on which the amoebae clearly found it difficult to move and multiply. The bacterial lawn became "moth-eaten" by small groups of amoebae, forming interrupted circles of growth with little or no syncytium formation (Figure 10). In some areas, tracks left by amoebae migrating through the dense growth could be seen.

Heat-killed suspensions

Live suspensions, matching an opacity tube No. 1 which gave an abundant amoebic growth, were found to be unsatisfactory when killed by heat. At best, the expanding ring of growth showed scanty groups of trophozoites (Figure 11) while on less evenly spread areas of the plate, only single amoebae could be detected. The optical density of the suspensions was therefore increased until it was found that samples, requiring a 1:50 dilution to match an opacity tube No. 2, gave the best results. Thereafter, all bacteria tested were found to be satisfactory, including *Ps. aeruginosa*. These standardised suspensions gave consistently good growth. Both *N. fowleri* and wild *Naegleria* grew with equal abundance.

The Effect of Agar on Axenic Growth

The addition of a minute amount of agar (0.0001%) produced a doubling of the total cell count after 7 days' incubation (Table 5).

Figure 10. *N. fowleri*.

Growth at 36 hours on live suspension of *Pseudomonas aeruginosa*. B.F. X 100.

Figure 11. *N. fowleri* sub-optimal growth at 36 hours on heat-killed suspension of *E. coli*. B.F. X 100.

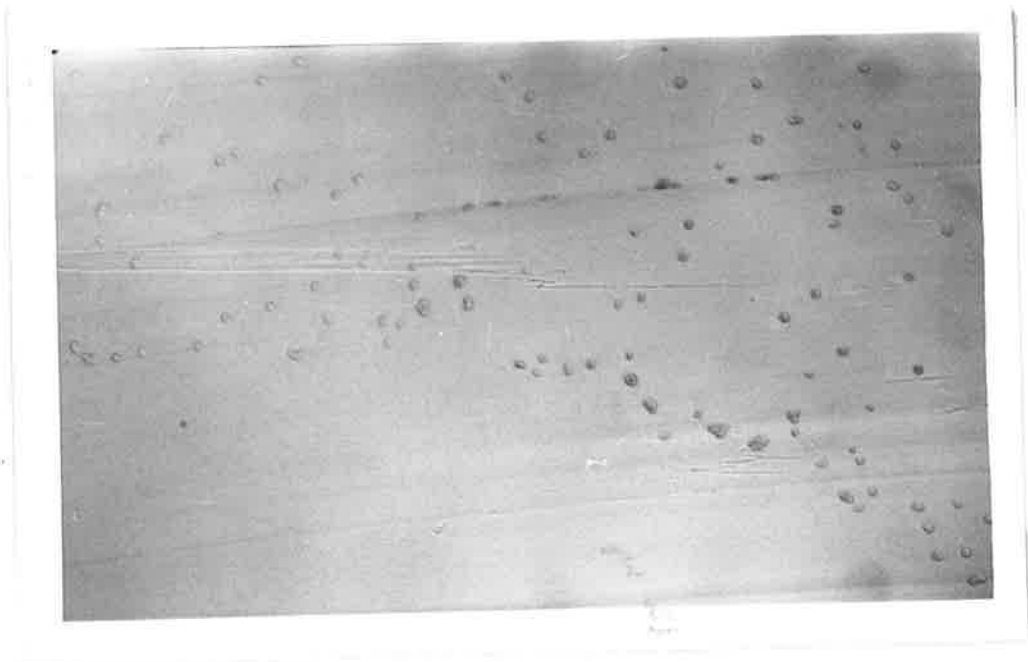

FIG 10.

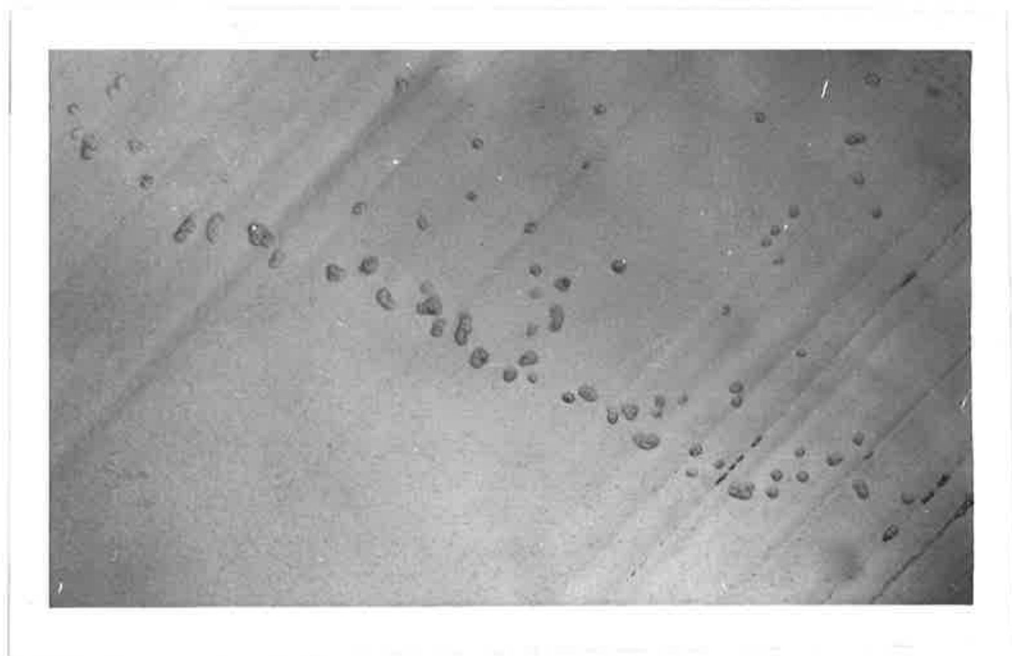

FIG 11.

Table 5. The Effect of Agar on Axenic Growth.

Concentration of Agar (%)	Count (cells/cmm) Day 5	Day 6	Day 7
0	750	330	660
0.0001	1,360	940	1,270
0.0005	990	1,200	1,330
0.001	2,300	3,050	3,000

MEDIA

DISCUSSION

Growth on dead bacteria

Heat-killed E. coli have been used routinely in this laboratory for three years. They adequately support the growth of Naegleria and many other free-living soil amoebae. Live bacteria probably allow more luxuriant growth of Naegleria, but the convenience of using dead bacteria, both for plate culture, and when transferring cultures from agar plates into axenic medium, makes their use preferable. Large batches of bacteria can be prepared at one time and stored at 5°C until required. Axenic growth is achieved more quickly because the dead bacteria transferred with the amoebae to the liquid medium are either eaten or left behind on future subcultures. The growth of Naegleria, both on dead bacteria and in axenic medium, shows that this amoeba does not require the presence of other live cells for its growth.

Growth in axenic media

Cerva's medium with horse or bovine serum will support the growth of N. fowleri but not of wild strains of Naegleria. Hence it is unsuitable for use with unidentified isolates of Naegleria. N. fowleri was unable to grow in the presence of rabbit serum. Fulton's medium allowed all strains tested to grow. This difference between the growth requirements of N. fowleri and other types of Naegleria led eventually to the development of an agglutination test to distinguish between them. The bovine serum used in Cerva's medium caused agglutination and lysis of the wild Naegleria at a dilution of 1:16; its concentration in the medium was 1:10. No agglutination and lysis was seen with N. fowleri at 1:10. Foetal calf serum had no agglutinating effect on any Naegleria.

Axenic culture is convenient and desirable; weekly subculturing only is required; antigens for animal immunisation and agglutination tests (as well as for more sophisticated immunological techniques) can be

prepared with minimal inclusion of unwanted antigens.

Bacterial suspensions for the growth of *Naegleria* spp.

All amoebae of the genus *Naegleria* which were tested grew profusely on a number of commonly isolated gram-negative bacilli. Thus growth, or the lack of it, on one particular bacterial species of those tested cannot be used to distinguish between *N. fowleri* and other types of *Naegleria*.

Live suspensions of bacteria, prepared by the addition of a few ml of distilled water to bacteria on the surface of an agar slant may not, initially, contain sufficient cells to support growth. Subsequent multiplication on prepared plates does, however, make good this deficiency. The failure of such suspensions, when killed by heat, to give comparable results has given rise to the suggestion that viable bacterial cells are essential for the multiplication of amoebae.

Of the bacteria examined, *E. coli* and *Proteus* spp. are the most frequently isolated in routine diagnostic laboratories. Either can be used for the isolation of *N. fowleri* from cerebrospinal fluid or brain tissue taken at autopsy.

The effect of agar on axenic growth.

Supplementation of CFA with small quantities of agar produced a marked increase in the concentration of amoebae after 7 days' growth, compared with un-supplemented CFA. The technique, however, proved to be of little use because when cultures were centrifuged for inoculation into mice or for agglutination tests, the amoebae were entangled in the small amount of agar present. It was not possible to remove this agar by washing the culture. Supplementation of CFA with the trace elements found in agar may produce better growth without this disadvantage. The agar may be providing a substrate which enables the amoebae to divide more easily; in pure CFA in polycarbonate containers, there is no material to which the amoebae can attach to facilitate the act of division.

The absence from the supplemented CFA of the giant multinucleate forms, typically seen in axenic cultures, supports this contention.

ISOLATION OF NAEGLERIA SPP. FROM THE ENVIRONMENT

INTRODUCTION

A major part of the work of the last three years has been the screening of water, soil and dust samples for the presence of *Naegleria*. All *Naegleria* isolated were subjected to mouse pathogenicity tests, which are described separately. In some instances the water and soil were taken from places believed to be the possible source of infection of cases of primary amoebic meningoencephalitis (PAME). The remainder of the samples were taken in an effort to determine whether *N. fowleri* is widespread in the environment, even in places where no cases of the disease have occurred. Many of these samples were collected by the Engineering and Water Supply Department and the Department of Public Health of South Australia.

When a case of PAME occurs it is important to try to locate the source of infection as rapidly as possible so that no more people are exposed to the risk of infection. A dependable method of isolation of amoebae, which could be presumed to be *Naegleria* until proven otherwise, needed to be developed. Such a method had to be as rapid as possible and able to be used with large numbers of samples. This section describes the method developed by the author and the results obtained with its use.

Cases of PAME have occurred in three areas of South Australia approximately 80 kilometers apart (Figure 12). These areas are supplied with water for domestic use by a concrete pipeline which travels overland from the River Murray (Figure 13). When this survey was begun, in August 1971, the unfiltered water was chlorinated at levels of up to 10 mg/l as it left the river at Morgan. The water received no further treatment.

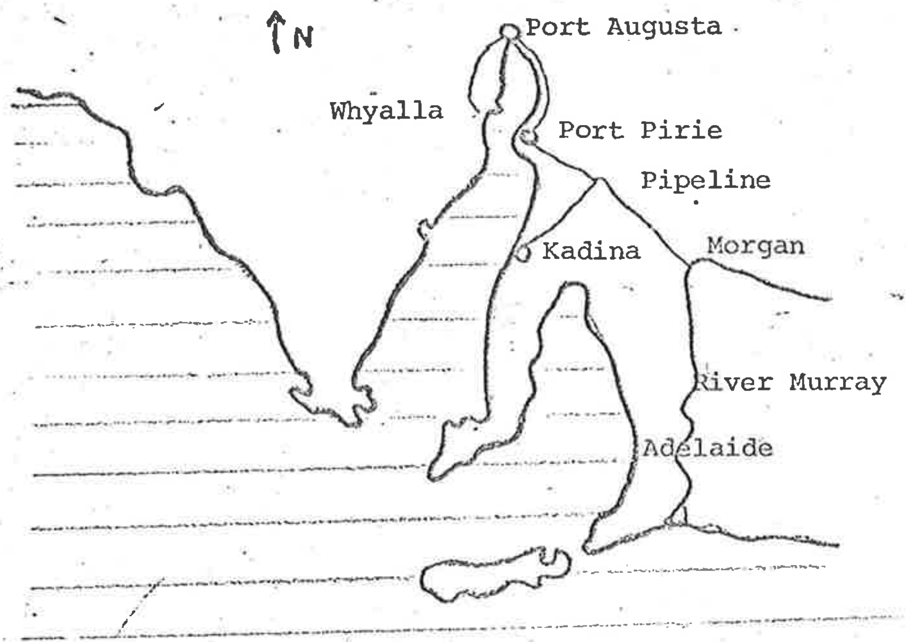

Figure 12. Morgan-Whyalla Pipeline, South Australia.

Figure 13. Overland pipeline from River Murray, South Australia.

FIG 13.

The presence of organic material in the water, the heating of the above-ground pipeline in summer, and the consequently diminishing chlorine levels, directed attention to this water supply which the three areas shared. Therefore a program of sampling at 38 points of the public water supply was undertaken in conjunction with the Engineering and Water Supply Department.

METHOD

Sampling

Water; 250 ml were taken by stirring up the water and including any floating debris or slime found adhering to the sides of swimming pools, scum gutters, the inner surface of taps, and puddles. In the domestic water supply survey, one sample was taken as soon as the tap was turned on, and a second was taken after water had been flushed through the tap for some minutes so that it could be assumed to be coming from the street main outside the house. This was done so that the first sample represented the water the consumer was likely to receive, and the second represented water which still retained an active ihhibitory chlorine level which would be unlikely to be present in the water in the house's reticulation system, particularly if the water had been standing for some time. An air space was left above the samples, and they were kept at ambient temperature - never refrigerated because this was believed to be damaging to the viability of Naegleria (Carter, 1970).

Soil: dry soil (100-150 gm) was placed in plastic bags and moistened with sterile distilled water on arrival in the laboratory. Wet soil was collected in a similar manner but was not further moistened. Soil was examined because it is often carried into swimming pools on the feet of swimmers. If it contains N. fowleri it could lead to contamination of the pool by these organisms. The source of the organisms in the pool needed to be determined to eliminate unnecessary incrimination

of the public water supply which is used to fill these pools.

Dust: slit samplers were left in position for approximately one month. The dust collected was moistened with sterile distilled water; often, however, rain fell during the sampling period and entered the sample - no more water was added to these samples. Dust was examined because it can contaminate swimming pools in the same way as soil.

Culture: culture plates consisted of 10 ml volumes of 1.25% agar (Difco) in distilled water, spread with a suspension of heat-killed *E. coli* to within 2 cm of the edge of the plate. The plates were not dried at any stage of preparation, and were stored at 5°C in sealed plastic bags.

Water: samples were filtered through a 47 mm cellulose acetate membrane with a pore diameter of 8µ (Sartorius). Light suction was applied until the membrane became blocked with sediment. After filtration, membranes were placed in sterile 6.5 cm petri dishes and a few drops of steriledistilled water were added to the surface of each. Sediment was scraped from the surface of each membrane using a bacteriological wire loop, and a loopful was deposited on the centre of a prepared plate. Six cultures were made from each specimen. Two plates were incubated at 21°C, two at 28°C and two at 37°C. The remaining sediment was transferred to sterile Bijou bottles and stored at room temperature in case re-isolation of the strain was necessary.

Soil and dust: the samples were treated in the same way as water except that sedimentation was not required.

Presumptive Identification of *Naegleria* spp.

The plates were examined daily, with the lids removed, using a stereoscopic zoom microscope (Olympus) at a magnification of x60.

Amoebae were clearly seen by slightly oblique, sub-stage illumination, and growth could often be detected after 18-24 hours' incubation. *Naegleria* have a characteristic appearance (Figure 14) and migrate rapidly outwards from the central inoculum which usually contains debris, bacteria, fungi and various protozoa and crustacea.

Micro-pipettes were made for the manipulation of amoebae under direct stereo-microscopic control. Glass tubing, 20 cm long and 3 mm in diameter, was heated and drawn out to a length of approximately 30 cm. The resulting capillary was divided in the middle by flaming. Using the pilot flame of a Bunsen burner, the terminal portion of each capillary was again drawn out to a needle point, which was snapped off by tapping with the detached portion of the capillary held in the right hand. During this operation, protective goggles were worn to prevent fragments of glass entering the eye.

The transformation of *Naegleria* trophozoites to flagellates was demonstrated by a hanging drop technique. Brass curtain rings, two cm in diameter, were dipped in a beaker of molten petroleum jelly and then placed on the surface of microscope slides. Additional petroleum jelly was deposited on the upper surface of each ring from a collapsible tube with a fine nozzle, of the type used for ophthalmic ointments. A small quantity of sterile distilled water was drawn into the capillary of the micro-pipette, using a length of rubber tubing with an attached mouth-piece. Under microscopic control, the water was deposited on an area of a culture plate seen to contain numerous amoebae with the morphology characteristic of *Naegleria*. The drop of water with suspended amoebae was re-aspirated into the capillary and then gently expelled onto the surface of a coverslip. A prepared slide was inverted over the coverslip and pressed into place with the drop located in the centre of the ring. The preparation was then turned over rapidly and incubated at 37°C for two hours. Flagellates were observed using a binocular microscope at a magnification of x100.

Figure 14. *Naegleria* sp. growing edge migrating outwards from central inoculum. B.F. X 100.

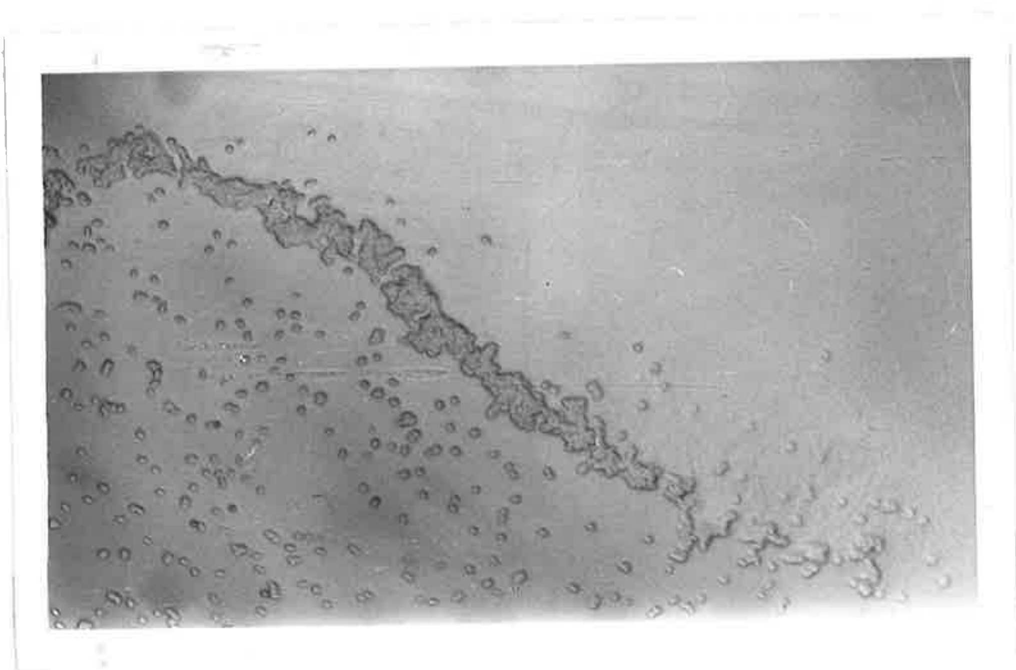

FIG 14.

Specimens showing flagellate transformation were subcultured to fresh plates by removing the coverslip and allowing the drop to enter a micro-pipette by capillary attraction. The suspension was then expelled onto the agar surface and successful transfer was confirmed by stero-microscopy.

Mouse Pathogenicity Tests

Plate cultures from hanging drops frequently showed considerable growth after overnight incubation. This could be scraped off in 0.5 ml sterile distilled water using a sealed Pasteur pipette, bent to a right angle. The suspension, which still contained contaminating bacteria and fungi, could be used for the intranasal inoculation of mice. This method allowed amoebae isolated from samples to be inoculated into mice within 36-72 hours of sampling. The concentrations of amoebae used in these preliminary tests ranged from 100-1000/cmm or more, but the isolates were always tested again when axenic cultivation of the strain had been achieved.

Axenic Culture

Cultures of amoebae were purified by successive transfers to fresh plates. On each occasion, the dense syncytium-like *Naegleria* growth was sampled as it advanced beyond the area of bacterial and fungal contamination. Once freed from contamination amoebae were washed off and inoculated onto six fresh plates, five drops of suspension being added to each plate. After overnight incubation, the washings from these cultures were harvested and pooled. Aprpxoimately 2 ml of suspension was added to 10 ml of CFA.

RESULTS

During one twelve-month period, 400 samples were examined and 130 strains of *Naegleria* were isolated. Of these, two were cultured from tap-water associated with a proven, fatal case of PAME. These strains produced characteristic meningoencephalitis in mice (Carter, 1970) following intranasal inoculation, subsequently confirmed by microscopy, culture and histological examination of brain tissue. Details of *Naegleria* isolated are shown in Table 6.

Table 6. Sources of 130 Isolates of *Naegleria* spp.

Water Source	No. of Isolates	No. of Samples
Swimming pools	50	100
Rainwater tanks	10	50
Piped water supplies (unfiltered, but chlorinated)	20	40
Surface freshwater (rivers, lakes, dams)	46	100
Thermal springs	4	10
	130	400

Details of the sources of all pathogenic amoebae isolated in the three-year period are given in the section on Mouse Pathogenicity Tests.

DISCUSSION

During this large sampling program, N. fowleri was isolated for the first time from a suspected source of infection of a fatal case of PAME. This isolation of pathogens led to further investigations of the reticulated water supply and more pathogens were found throughout the system. Heavy chlorination of the public water supply serving the areas of South Australia where cases of PAME have occurred was begun as a result of these findings. Procedures were altered so that the water was chlorinated at two points after it left the river, and again prior to delivery to these areas. Levels were adjusted to deliver water containing 0.5 ug/l free active chlorine at the household tap. In practice this ideal level, which has been shown to kill N. fowleri after 15 minutes' exposure, is difficult to maintain, often being exceeded or not reached. However, the chlorination program seems to have eliminated N. fowleri from the more recent water samples.

N. fowleri has been isolated from water and soil samples from geothermal springs in New Zealand (where six known case of PAME have occurred), from a metropolitan Adelaide water reservoir, and from the areas in South Australia where cases of PAME have occurred. Pathogenic Acanthamoeba have been isolated from soil taken from the surrounds of Lake Burley Griffin, Canberra, Australian Capital Territory. This is an artificial lake as are three lakes in Virginia, U.S.A. where deaths from PAME following swimming have occurred. A pathogenic Acanthamoeba was detected as an aerial contaminant of a plate culture of Naegleria.

These findings indicate that pathogenic, free-living, soil amoebae are probably widely spread. Disease in humans caused by these organisms probably occurs only when a number of unlikely conditions are satisfied. A large number of pathogenic amoebae (the infective dose for man is, of course, not known) must be introduced to the upper reaches of the nasal mucosa where the olfactory mucosa is situated.

This requires vigorous activity involving water, such as diving, jumping and swimming under water, or it requires unusual activity such as sniffing water up the nose to clear nasal passages, or the complete immersion of the head in water. Unknown factors, such as some undetected form of host susceptibility, or the association of the amoebae with another species (e.g. of bacteria) may be involved. These factors may help to explain the occurrence of only one case of PAME when many people have been exposed to what appear to be the same circumstances.

MOUSE PATHOGENICITY TESTS

INTRODUCTION

At the present time, no rapid, reliable method for differentiating between *N. fowleri* and other morphologically identical *Naegleria*, including *N. gruberi*, has been developed. Both scanning and ordinary electron microscopy have been used to show differences in the ultrastructure of the two recognised species and proposed new species (Carter, 1970, Jadin, 1973, Lastovica, 1974). Immunoelectrophoretic analysis has been used by Willaert, et al. (1972, 1973a & b) and Jadin, (1973). Chang (1971 & 1974) has used differences in behaviour in tissue culture as a guideline to differentiation. In this laboratory we have used an agglutination technique (Anderson & Jamieson, 1972b).

All of these methods have the disadvantage of requiring a monaxenic or axenic culture. This takes varying lengths of time to achieve, depending on the degree of contamination of the isolate. Then the techniques themselves take some time to be performed. At present mouse pathogenicity tests are the quickest reliable method to determine whether an isolate is pathogenic and therefore likely to be *N. fowleri*, or whether it is non-pathogenic and likely to be *N. gruberi*. A result can be expected in five or so days if an adequate inoculum is used. What constitutes an adequate inoculum has yet to be determined, and probably varies for different isolates, according to their virulence. Of necessity, I have arbitrarily defined an adequate inoculum to be a dose of at least 50,000 trophozoites. This large inoculum probably errs on the side of being more than adequate.

METHODS

All mice used in pathogenicity testing were either I.M.V.S. strain Swiss white, or Balb/c weighing from 15-20 gm. Both males and females were used. At first, light ether anaesthesia was used, but it was observed that ether caused lysis of *Naegleria* trophozoites. Nembutal (pentobarbitone sodium, Abbott) given intraperitoneally was then substituted. The veterinary solution of 60 mg/ml was diluted 1:10 with 0.9% saline. Each mouse was given approximately 0.1 ml (0.6 mg) and this was sufficient to produce anaesthesia for at least 10 minutes. The dose was varied slightly for unusually large or small mice. The Nembutal was injected with a one ml tuberculin syringe (Terumo) fitted with a gauge 26, 1.5 cm needle (Terumo).

Initially six mice were inoculated with a strain in each test, but this had to be reduced to four mice per test because of the large numbers of mice being used (approximately 6,580 mice were used in three years). The mice were fed mouse cubes (Charlicks) and tap water *ad libitum*. They were housed in an air-conditioned (21°C) animal house.

After anaesthetisation, the mouse was laid on its back and 50 cmm of a suspension of amoebae in distilled water was dropped onto both nostrils. The inoculum was given with a graduated micro-pipette (Pedersen) using a length of rubber tubing with an attached mouth-piece. Time was allowed for a drop to be breathed in by the mouse before another drop was deposited. When the full dose had been given, the mouse was laid on its back in its cage to allow maximum contact of the amoebae with the upper naso-pharynx. Nembutal, with its longer period of anaesthetisation, allowed longer contact - this was probably an advantage. The mice were inspected daily for 21 days for symptoms of PAME.

The brain of any mouse dying 48 hours or more after inoculation was removed. Mice showing signs of the disease were killed when moribund, and their brains removed. The signs of the disease in the mouse include: incessant circling of the perimeter of the cage, with the circles becoming gradually smaller as the mouse becomes exhausted, a hunched posture, ruffled fur, vertical jumping, and finally, inability to move. A mouse held by its tail at this stage may swing violently in circles for some minutes. The head of a young mouse may be swollen at the top of the skull. This is due, presumably, to cerebral oedema and the absence of fusion of the bony plates öf the skull.

The b rains were removed in the following manner: the dead mouse was pinned by its feet, tail, and nose ventral surface down on a cork board; the head, neck and shoulders were sprayed with a 0.05% solution of chlorhexidene in 70% alcohol; the skin was incised in the midline from the middle of the back to the tip of the nose, the flaps being reflected widely and pinned down (Figure 15); with fine curved scissors and small forceps the vault of the skull was removed (Figure 16). The brain could be removed in two different ways: if the mouse was dead when found, the whole brain was scraped out with a very small spatula and placed in approximately 2 ml of sterile distilled water; the suspension was agitated on a vortex mixer (Scientific Industries); if the mouse was killed, the brain was very carefully removed to preserve the structure; special attention was paid to the extrication of the olfactory lobes; the brain was then bissected longitudinally and one half was placed in 10% buffered formalin for histological processing; the other half was placed in apprpxoimately 1 ml of sterile distilled water and agitated.

The mouse brain suspensions were examined in a haemocytometer (Spencer Bright Line) for the presence of amoebae (Figure 17). One drop of suspension was placed onto a 1.25% agar plate spread with heat killed <u>E. coli</u> and incubated at 37°C. The plate was examined microscopically

Figure 15. Mouse infected with N. fowleri.
Note swelling of brain and haemorrhagic
necrosis of olfactory lobes.

Figure 16. Same mouse as above, top of skull removed.

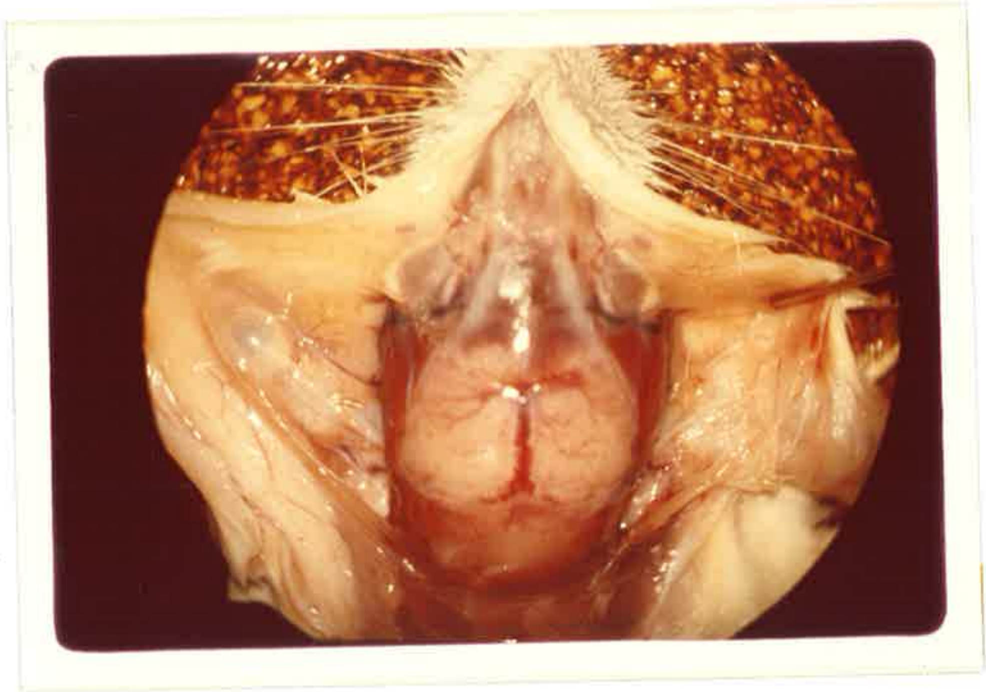

FIG 15

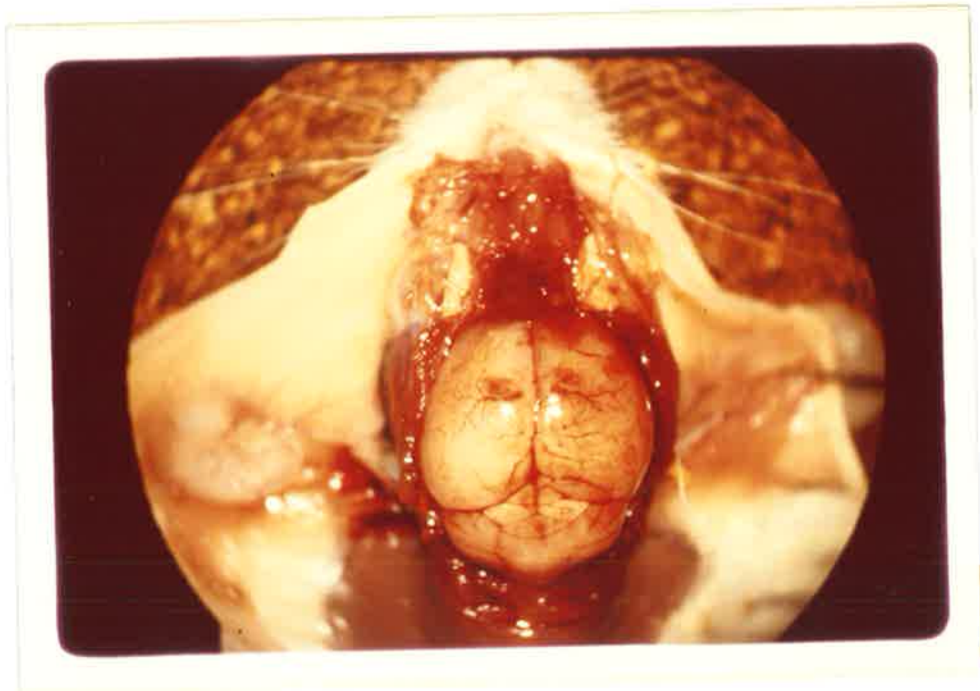

FIG 16

Figure 17. Suspension in water of mouse brain infected with *N. fowleri*.
Amoebae still rounded.
B.F. X 1,000.

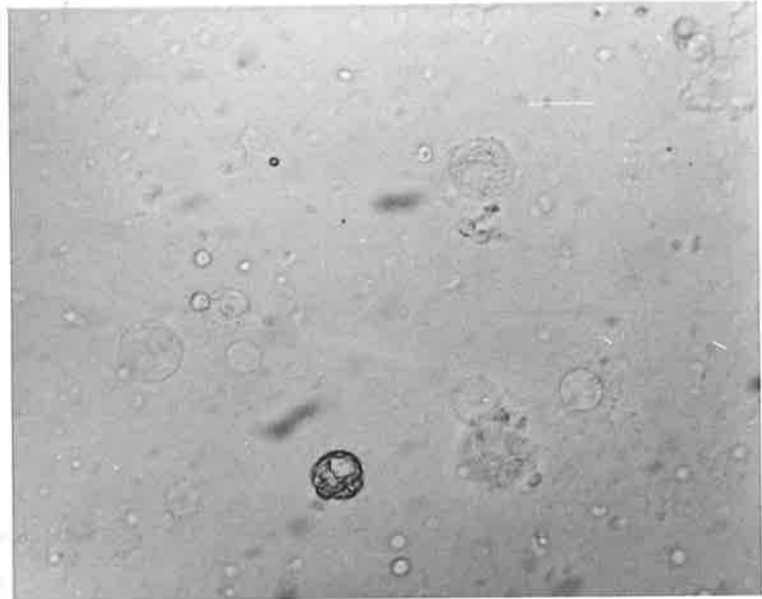

FIG 17

at 18 and 36 hours for the presence of amoebae. The half-brain used for histology was immersed in ascending grades of alcohol after fixation. It was placed in 70% alcohol overnight, then in 95% alcohol for four hours and in absolute alcohol for a further four hours. It was then placed in methyl benzoate overnight. Next day it was subjected to three changes of 56°C melting point paraffin wax, each of an hour's duration. The brain was embedded in paraffin wax and sections were cut. They were stained with haemotoxylin and eosin and examined for the presence of amoebae (Figures 18 and 19).

All mouse remains were disposed of either by autoclaving or incineration. During all procedures in mouse pathogenicity testing the experimenter wore a surgical mask (Bardic). For mouse inoculation, a slit was cut in the mask to allow the rubber tube to be placed in the mouth.

The Effect of Ether on *Naegleria* trophozoites.

(1) A hanging drop of *N. fowleri* in distilled water was exposed to ether vapour for five minutes by cutting segments out of the plasticene mount.

(2) Coverslips spread with a thin film of *N. fowleri* were exposed to ether vapour for 1 - 4 minutes and then transferred to 10 ml of CFA. An unexposed control (four minutes in air) was set up.

The Effect of the Ingestion of *N. fowleri* in Drinking Water.

Because of the finding of *N. fowleri* in public water supplies, an experiment was designed to test whether mice could be infected by ingesting these amoebae. Five groups of four mice and a single mouse all received different doses. A 1 ml suspension of amoebae from the brain of a mouse infected with *N. fowleri* was presented to each group in a rubber tube to which was attached a nozzle of the type normally used in water bottles. When the suspension had been consumed by the group, tap water was made available to the mice in the usual manner.

Figure 18. Mouse brain infected with *N. fowleri*. Note areas of destruction and inflammatory response. Amoebae stained magenta in lytic pockets. H.E. X 250.

Figure 19. As in Figure 18. Amoebae in spaces staining pink with darker nucleus. H.E. X 1,000.

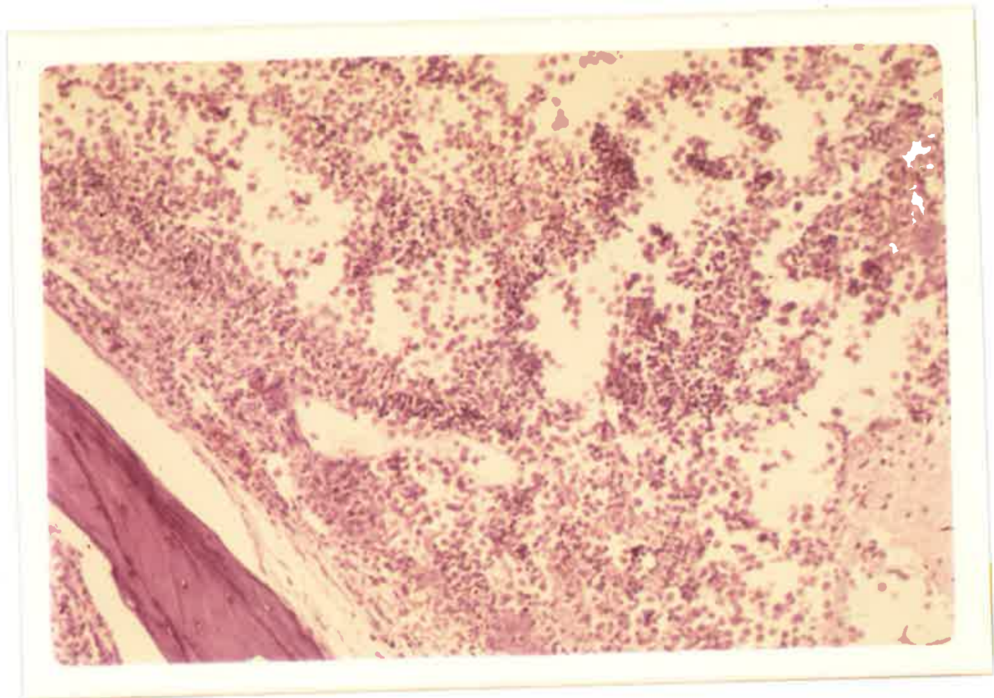

Fig 18

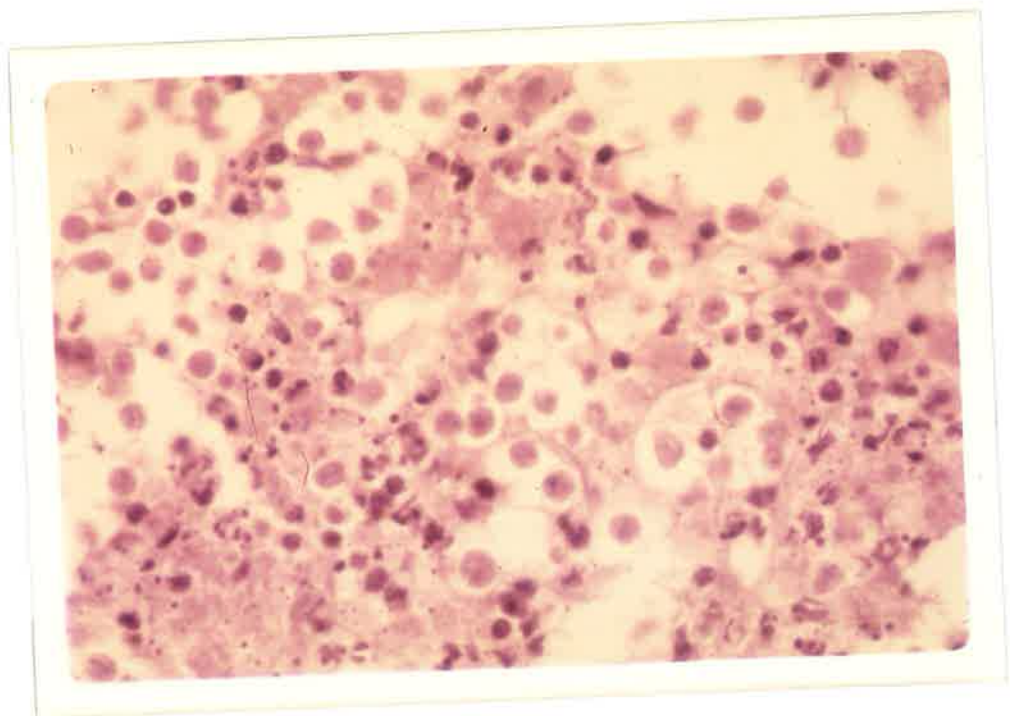

Fig 19

The mice received food ad libitum. They were inspected daily for eight weeks and then killed. Their serum was tested for the presence of agglutinating antibodies. Control mice were inoculated intranasally with the suspensions.

An Attempt to Infect Mice by the Ocular Route.

Most people get water in the eyes, but perhaps less often in the nose, when swimming. The possibility that pathogenic amoebae might gain access to the nose by way of the naso-lachrymal duct was tested in mice. An axenic culture of N. fowleri containing 710 trophozoites/cmm was inoculated into six mice in the following manner: after anaesthesia, two drops were delivered into each conjunctival sac from a tuberculin syringe (Terumo) fitted with a guage 26, 1.5 cm needle (Terumo). The volume of the drops was 0.02ml and so the inoculum in each eye was 14,200 trophozoites. The mice were inspected daily for 14 days. Control mice were inoculated intranasally with the suspension.

An Attempt to Induce Pathogenicity in Non-pathogenic Naegleria.

The possibility was considered that N. fowleri produces an agent which allows the entry of trophozoites from the surface of the olfactory mucosa into the olfactory bulbs via the cribriform plate. Once entry has been achieved, any Naegleria could survive and multiply, causing disease.

An experiment was designed in which a strain of Naegleria considered non-pathogenic after repeated unsuccessful mouse challenges, was resuspended in the supernatant liquid from an axenic N. fowleri culture, and inoculated intranasally into mice. N. fowleri was grown in 20 ml CFA at $37^{\circ}C$ for seven days. The culture was centrifuged at 1,700 rpm for 10 minutes and the supernatant liquid was passed through a 0.45 µ membrane to remove all viable amoebae. The non-pathogenic Naegleria strain was grown in CFA at $28^{\circ}C$ for one week, centrifuged as described above, and the sediment was divided into two equal parts.

One part was resuspended in 1 ml of fresh CFA and the other in 1 ml of the supernatant liquid from the *N. fowleri* culture. In both suspensions the amoebic concentration was 620/cmm. Four mice were inoculated intranasally with each of the suspensions.

RESULTS

In three years, 467 strains of amoebae (mostly Naegleria and Acanthamoeba) isolated from the environment were inoculated intranasally into mice. Each strain was inoculated an average of three times. The average lowest count of the inocula was 320/cmm and the average highest count was 6,150 trophozoites/cmm. Twenty six strains of amoebae pathogenic to mice were recovered from infected mouse brains. Nineteen of these strains were Naegleria (Table 7) and seven were Acanthamoeba (Table 8).

In Tables 7 and 8 the strain designation (indicating source), the number of trophozoites/cmm in the inoculum, the temperature of isolation of the strain, and the number of days taken to kill a mouse, are given. The strain designations representing the source are:

NZ = Water, Crystal Springs, Matamata, New Zealand.

RU = Water, Golden Springs, Rotorua, New Zealand.

RUS = Soil, " " " " "

PP = Water, Port Pirie, South Australia.

HOV = Water, Hope Valley Reservoir, Adelaide, South Australia.

HOV6-2 = Probable aerial contaminant of HOV isolate.

PA = Water and Soil, Port Augusta, South Australia.

MW = Morgan-Whyalla pipeline, South Australia.

CS = Soil, Lake Burley Griffin, Canberra, Australian Capital Territory.

K = Water, Kadina, South Australia.

TABLE 7. Pathogenic *Naegleria*.

Strain	Count	Temp.	Days
NZ-17	660	21°C	6
NZ-17xM	660	37	10
NZ-17xM	1,090	37	17
NZ-27	850	28	10
NZ-27xM	1,140	37	11,14
NZ-27xM	420	37	13
NZ-31	380	28	4
RU-30	1,660	37	6,13
RU-30xM	660	37	8,10,11
RU-x30xM	1,460	37	11
RU-42	1,000	21	4,4
RU-50	1,570	21	7
RU-53	850	28	10,10
PP-291	870	37	16
PP-291xM	420	37	1
PP-291xM	5,030	37	9,15
PP-397	630	37	7,8
PP-397xM	4,370	37	5,5,6,6,7,7
PP-397xM	4,370	37	5,5,6,6,7,7
PP-397xM	1,300	37	8,10,16
PP-397xM	260	37	8
PP-397xM	1,700	37	8,9,9,9
PP-397xM	414	37	21
PP-397xM	707	37	8,9,11,11
PP-397xM	850	37	8
PP-397xM	1,090	37	13,21
HOV-6	1,900	28	14
PA-a	1,300	28	9
PA-axM	4,000	37	4
PA-14	450	28	5,5,5,5
PA-14	464	28	5
PA-34	4,000	37	3,3,3,4,4,4
PA-90	13,000	37	4,4,4,4,4,5
PA-105	6,000	37	5,5,5,5,5,5
PA-117	14,000	28	6,6,6,7,7,8
K-1	2,890	28	5,6
K-71	14,000	28	3,4,4,4,4,4
MW-4U	9,000	37	4,4,4,4,4,4

Count = trophozoites/cmm.

Temp. = C.

xM = isolate from mouse brain.

Table 8. Pathogenic *Acanthamoeba*

Strain	Count	Temp.	Days
CS-21	400	37	13
CS-22	180	37	11,11
HOV-6-2	220	37	3
HOV-6-2xM	210	37	13,13
RUS-19	1,200	37	5
RUS-22	260	37	21
RU-32	390	21	9
RU-32	1,700	21	7,7
RU-32xM	580	37	6,7,9

Count = trophozoites/cmm.

Temp. = C.

xM = isolate from mouse brain.

Three of the *Naegleria* mouse pathogens were isolated at 21°C, eleven at 28°C and six at 37°C.
Two of the *Acanthamoeba* were isolated at 21°C, none at 28°C and five at 37°C.

The Effect of ether on *Naegleria* trophozoites.

(1) Microscopy showed inhibition of motility, swelling and lysis of the trphozoites. Subculture of the hanging drop onto an agar plate showed no growth after incubation at 37°C for 36 hours. The unexposed control grew.

(2) None of the exposed coverslips showed growth after seven days' incubation at 37°C. The control grew. One minute's exposure of a 20 cmm thin film of *N. fowleri* containing 400 trophozoites/cmm killed all cells.

The effect of the Ingestion of *N. fowleri* in Drinking Water.

The five groups of four mice received the following numbers of amoebae in their one ml inoculum: 1-47,800, 2-104,000, 3-60,000, 4-148,000, 5-78,000 and 6-238,000. 5 was the single mouse - it received the whole inoculum. A mouse died in group 3 on the third day after the inoculum had been consumed. No amoebae were grown from the brain, spleen, liver, kidney or gastro-intestinal tract. No agglutinating antibodies were detected in the serum collected after eight weeks from the remaining 20 mice.

An Attempt to Infect Mice by the Ocular Route

No deaths occurred within the 14 days. The control mice died.

An Attempt to Induce Pathogenicity in Non-pathogenic *Naegleria*.

No deaths occurred within the 21 days. The control mice died.

DISCUSSION

Pathogenic amoebae, either *Naegleria* or *Acanthamoeba*, have been isolated from water and soil from four regions of South Australia, from the Australian Capital Territory, and from two regions of New Zealand. This indicates that these amoebae are widespread, but the detection of only 26 pathogenic strains out of 467 strains tested shows that the numbers of pathogenic strains are relatively few when compared with the populations of non-pathogenic amoebae.

It is interesting to note the temperatures of isolation of the pathogens. They are evenly distributed and no significant difference is evident. They show that a pathogen can be isolated as readily at 21°C as at 37°C, but if all six plates had been incubated at 37°C it is possible that the same number of pathogens would have been isolated. The isolation of a pathogen at 21°C may be due only to the chance occurrence of this amoeba in the inoculum on a plate destined for incubation at 21°C. I have used the three temperatures of isolation so that I could become familiar with all the types of amoebae present in a sample. It was reasoned that some amoebae might be inhibited by the higher temperatures, whereas it was known that free-living pathogens would have to be able to survive temperatures much lower than 21°C. Using the three temperatures therefore probably did not reduce the isolation rate of pathogens at all.

The inclusion of pathogenic *Acanthamoeba* needs to be explained. When isolated, these amoebae appeared to be *Naegleria*. For some time we were processing twelve water samples a day, and it was not always possible to do hanging drop tests for the ability to form flagellates. In these cases, the morphology of the isolate on the agar plate was relied upon, i.e. amoebae with a plate morphology closely resembling that of known *Naegleria* were selected. Some errors were made using this expedient technique, but it had the unexpected advantage of yielding six pathogenic *Acanthamoeba*. Since *Acanthamoeba* spp. are now being implicated in human infections of a more chronic and opportunistic nature (Robert V.B. & Rorke, L.B., 1973, Jager, B.V., & Stamm, W.P., 1972) it is useful to have information about the occurrence of these amoebae in the environment. No human cases of disease caused by these organisms have yet been reported from the areas where pathogenic *Acanthamoeba* were isolated.

The finding that ether vapour lysed Naegleria trophozoites made the use of this anaesthetic inadvisable. Nembutal is actually quicker to use because all of the mice can be injected before the inoculation procedure is begun. The mice take two or three minutes to succumb and so by the time all of the mice have been injected, the first ones are ready to be inoculated. The duration of anaesthesia is long enough to cover the whole period of inoculation which can be easily performed by one person using this technique.

The inability of N. fowleri to infect when ingested is comforting in the light of the finding of this organism in the drinking water supplies of South Australia. This experiment does not rule out the possibility of infection occurring when a person chokes while drinking water and so forces water into the upper naso-pharynx. The inability of N. fowleri to infect when introduced into the eye disproved an early hypothesis we entertained about the mechanics of infection.

Pathogenicity was not able to be induced in non-pathogenic Naegleria when they were suspended in the medium in which N. fowleri had been grown. The ability to invade could not be passively conferred onto these amoebae. At the time of writing similar experiments using concentrated supernatant fluid from cultures and cell extracts are being conducted.

AGGLUTINATION TESTS

INTRODUCTION

Differentiating between *N. fowleri*, *N. gruberi* and other possible species of *Naegleria* is, as yet, impossible using morphological characteristics seen with the light microscope. Members of the genus are morphologically indistinguishable by this means. More sophisticated techniques (viz. normal and scanning electron microscopy, immuno-electrophoresis, and indirect fluorescent antibody staining) have been developed. These appear to enable accurate differentiation but the time involved is a serious disadvantage for their use in screening programs. The indirect fluorescent antibody staining technique (Willaert *et al.*, 1974) may provide a rapid, reliable method, but it is still in the early stages of development.

An agglutinationtest which is easy to perform and can be applied to the residue of axenic culture left after mice have been inoculated for pathogenicity tests has been developed for the genus *Naegleria*. It is helpful in determining whether a strain is likely to be *N. fowleri*, *N. gruberi*, or some other *Naegleria*, but it is not definitive. For instance, we have isolated a number of strains which, by the agglutination test, are *N. fowleri*, but are not pathogenic in mice.

This test can only be used with axenic cultures; amoebae washed from agar plates are held together with the agar skin from the plate and do not form an antigen of free cells. Therefore there is delay with this method also - axenic culture of a strain may be achieved within a few days of isolation, or it may take months.

METHODS

Preparation of Antigen for Rabbit Immunisation

The strains were grown in 10 x 10ml volumes of CFA at 28° or 37°C for five days. Cultures were centrifuged at 1,700 rpm for ten minutes, after which the supernatant fluid was removed and the sedimented amoebae were re-suspended in 10 ml of sterile distilled water. Suspensions were agitated on a vortex mixer (Scientific Industries) and then centrifuged for a further 10 minutes. The deposited amoebae were mixed with 0.25% formol saline to give an approximate concentration of 500 trophozoites/cmm, determined by counting in a Spencer Bright Line chamber.

Immunisation Schedule

Rabbits (I.M.V.S. antibody strain) were selected for immunisation by both intramuscular and intravenous routes. For intramuscular injection, a calcium alginate depot was used. Formalinised antigen was mixed with an equal volume of 4% (w/v) sodium alginate and, following the injection of this material, a 0.4% (w/v) solution of calcium chloride was given through the same needle. The complete schedule is shown in Table 9.

Animals were exsanguinated into agar-lined tubes from which the serum was removed and cleared of red cells by centrifugation. After the addition of merthiolate to a final concentration of 1:10,000 the serum was dispensed in 1 ml volumes in glass ampoules and stored at 5°C. Antisera against N. fowleri (strain NHI), _N. jadini_ (Strain 0400) and _N. gruberi_ (Strain 1518) were prepared.

Preparation of Antigen for serological Examination

Naegleria isolates were grown in 10 ml volumes of CFA for five days at their temperature of isolation. Cultures were centrifuged and washed as for the preparation of antigen for rabbit immunisation.

Table 9. Immunisation Schedule.

Day	Procedure
1	0.5 ml alginate + antigen suspension IM
	0.5 ml calcium chloride IM
	0.25 ml antigen IV
3	0.5 ml antigen IV
7	1.0 ml antigen IV
11	2.0 ml antigen IV
15	Test bleeding
18	1.0 ml antigen IV
21	Rabbit bled out

IM = intramuscular

IV = intravenous

Sedimented trophozoites were re-suspended in 0.25% formol saline. Microscopic examination showed that the morphology was well preserved and that the suspensions consisted of single trophozoites with no tendency to spontaneous clumping.

Agglutination test

Glass slides measuring 7.5 x 5.0 cm were marked into six squares using an ink marking pen (Texta Parcelmate). One drop (0.02 ml) of serum dilution was placed in each square followed by one drop of antigen. A saline control, and, when applicable, positive and negative serum controls were situated in adjacent squares. After mixing all the drops with wooden applicators, the slides were rocked gently until no more macroscopic agglutination was seen. They were examined by stereo-microscopy. Fifty per cent agglutination, shown by clumps of ten or more amoebae, was recorded as the end-point of a titration.

Absorption of Cross-agglutinating Antibodies

Axenic cultures of the antigen were grown for five days. After washing, cells from 10 ml volumes were mixed with two ml of the antiserum and incubated at 37°C for one hour. Then the serum was separated by centrifugation and added to a fresh sediment of trophozoites. The mixture was incubated at 37°C for three hours. After further centrifugation, the serum was removed, tested to show that absorption was complete, and stored in one ml volumes in glass ampoules at 5°C.

RESULTS

The titres of the antisera with their homologous and heterologous antigens are shown in Table 10.

Any agglutination pattern occurring three or more times was regarded as significant. Strains which did not agglutinate at a dilution of at least 1:32 of any of the antisera have been excluded from the results presented here. Their significance has not yet been determined.

The relative frequencies of all significant agglutination patterns are shown in Table 11. The two most common serotypes (40 strains) agglutinated most strongly with N. jadini antiserum - but there is a difference of only one between the dilutions of N. jadini and N. gruberi antisera at which they agglutinated. The two next most common serotypes (31 strains) agglutinated at the same dilutions of these two antisera. ALtogether, 39 of the 296 strains tested had similarly equivocal serotypes (Table 12). Sixty five strains showed a difference of only one between the dilutions of N. jadini and N. gruberi antisera at which they agglutinated (Table 13). Thus 104 strains were not convincingly either N. jadini or N. gruberi, but they did agglutinate at dilutions of at least 1:32 ofantisera to these species. It is interesting that the 81 strains which agglutinated most strongly with N. fowleri antiserum (Table 14) had a difference of at least two between that dilution and the dilution of N. jadini antiserum with which they agglutinated.

Tables 14, 15 and 16 show the strains grouped according to the antiserum with which they agglutinated most strongly. These tables include the 65 strains already shown in Table 13. The range of dilutions for each species-type is shown in Table 17. The ranges for N. jadini and N. gruberi are not mutually exclusive. For example, a strain which does not agglutinate with N. fowleri antiserum, and agglutinates with N. jadini and N. gruberi antisera at dilutions of 1:64 and 1:32

Table 10. Titres of Antisera with Homologous and Non-homologous Antigens

Antiserum / Antigen	NHI	0400	1518
NHI	1:1024	1:16	-
0400	-	1:256	1:128
1518	1:2	1:4	1:1024

- = no agglutination

TABLE 11. Relative Frequencies of Serotypes

Number of Strains	Dilution of Antiserum		
	N. fowleri	N. jadini	N. gruberi
22	-	1:64	1:32
18	-	1:128	1:64
16	-	1:128	1:128
15	-	1:64	1:64
14	1:512	1:16	-
12	-	1:128	1:32
11	-	1:4	1:512
10	1:256	1:32	-
10	-	1:128	1:256
9	-	1:64	1:256
9	1:1024	1:32	-
9	-	1:64	1:128
8	1:256	1:8	-
8	-	1:4	1:256
7	1:512	1:32	-
7	1:128	1:4	-
7	-	-	1:128
7	-	1:4	1:128
6	1:2	1:128	1:32
6	-	1:128	1:16
6	-	1:64	1:16
6	-	1:8	1:512
6	-	1:256	1:128
5	-	1:32	1:32
5	-	1:256	1:32
5	-	1:32	1:128
5	1:512	1:8	-
4	1:128	1:16	-
4	-	-	1:32
4	1:1024	1:64	-
4	-	1:2	1:256
4	1:1024	1:16	-
3	-	1:128	-
3	-	1:64	1:8
3	1:512	1:4	-
3	1:256	1:16	-
3	-	1:256	1:256

TABLE 11. Relative Frequencies of Serotypes (continued)

Number of Strains	Dilution of Antiserum		
	N. fowleri	*N. jadini*	*N. gruberi*
3	-	1:16	1:64
3	1:256	1:4	-
3	-	1:16	1:1024
3	-	1:4	1:1024

- = no agglutination

Table 12. Strains with Equivocal Serotypes

Number of Strains	Dilution of Antiserum		
	N. fowleri	N. jadini	N. gruberi
16	-	1:128	1:128
15	-	1:64	1:64
5	-	1:32	1:32
3	-	1:256	1:256
39			

- = no agglutination

TABLE 13. Strains with One Dilution Difference

Number of Strains	Dilution of Antiserum		
	N. fowleri	N. jadini	N. gruberi
22	-	1:64	1:32
18	-	1:128	1:64
10	-	1:128	1:256
9	-	1:64	1:128
6	-	1:256	1:128
65			

- = no agglutination

TABLE 14. N. fowleri-type strains

Number of Strains	Dilution of Antiserum		
	N. fowleri	N. jadini	N. gruberi
14	1:512	1:16	-
10	1:256	1:32	-
9	1:1024	1:32	-
8	1:256	1:8	-
7	1:512	1:32	-
7	1:128	1:4	-
5	1:512	1:8	-
4	1:128	1:16	-
4	1:1024	1:64	-
4	1:1024	1:16	-
3	1:512	1:4	-
3	1:256	1:16	-
3	1:256	1:4	-
81			

- = no agglutination

Table 15. *N. jadini*-type Strains

Number of Strains	Dilution of Antiserum		
	N. fowleri	*N. jadini*	*N. gruberi*
22	-	1:64	1:32
18	-	1:128	1:64
12	-	1:128	1:32
6	1:2	1:128	1:32
6	-	1:128	1:16
6	-	1:64	1:16
6	-	1:256	1:128
5	-	1:256	1:32
3	-	1:128	-
3	-	1:64	1:8
87			

- = no agglutination.

TABLE 16. *N. gruberi*-type Strains

Number of Strains	Dilutin of Antiserum		
	N. fowleri	*N. jadini*	*N. gruberi*
11	-	1:4	1:512
10	-	1:128	1:256
9	-	1:64	1:256
9	-	1:64	1:128
8	-	1:4	1:256
7	-	-	1:128
7	-	1:4	1:128
6	-	1:8	1:512
5	-	1:32	1:128
4	-	-	1:32
4	-	1:2	1:256
3	-	1:16	1:64
3	-	1:16	1:1024
3	-	1:4	1:1024
89			

- = no agglutination

TABLE 17. Ranges of Dilutions for Species-types

	Dilution of Antiserum		
	N. fowleri	*N. jadini*	*N. gruberi*
N. fowleri-type	1:128-1:1024	1:4-1:64	-
N. jadini-type	- -1:2	1:64-1:256	- -1:128
N. gruberi-type	-	- -1:128	1:32-1:1024

- = no agglutination

respectively, fits both ranges, but has been designated an *N. jadini* type because it agglutinates more strongly with *N. jadini* antiserum.

DISCUSSION

The results show 41 different serotypes which have been seen at least three times. This diversity within the genus could be explained by variations in the "quality" of the antigens and antisera, or it could be due to the genus actually consisting of many different species not yet described.

During all the agglutination tests, the "quality" of the antigens was strictly controlled. Antigens containing more than about 5% cysts, or containing less than approximately 500 trophozoites/cmm were discarded. Antigens were tested within 24 hours of preparation. The antisera used belonged to the same batches. When not in use, antisera and replacement one ml ampoules were stored at 5°C. Often, strains were re-tested some weeks after the initial test, using antisera which had been stored in this manner. These showed a gratifying reproducibility of agglutination pattern.

Approximately 80% of strains showing an N. fowleri type pattern were not pathogenic in mice. Therefore the agglutination test provides little indication of potential pathogenicity, and its usefulness in quickly screening environmental isolates has been disappointing. However, since it has been applied to so many strains isolated in surveys, it has provided much interesting information about the genus.

Prior to the application of this test, the genus was known to include N. gruberi and N. fowleri was becoming accepted as a new species. Another new species, N. jadini was described by Willaert and Le Ray in 1973. On the basis of agglutination patterns alone, the genus does not appear to consist of distinct species.

Although effors were made to standardise the antigen and antiserum preparations with respect to freshness and cell number and type, there are probably other factors acting which affect the results. For instance, it may be that the age of a culture, whether it is in an exponential phase of growth, or in a stationary phase, is of relevance. Experiments need to be conducted to determine the optimum age of a culture for agglutination testing, the optimum concentration of trophozoites, the effect of the presence of cysts and flagellates and so on.

The antigenicities of the flagellate and the cyst need to be investigated.

In spite of the unknown effects of these variables, the results were reproducible, as mentioned earlier. On the basis of these results, it would appear that either the genus Naegleria consists of many species which have not yet been defined, or that it does not consist of discrete species at all, but rather it contains many strains which differ from each other slightly in the antigenic structure of their surfaces.

In a genus where reproduction is by binary fission, the criterion of the inability of members of different species to interbreed and produce fertile offspring cannot be applied, as it can be to higher organisms which reproduce sexually. Perhaps true speciation is impossible with these amoebae, and is really only a convenience imposed by those studying them.

THE EFFECT OF CLOTRIMAZOLE ON *NAEGLERIA FOWLERI*

INTRODUCTION

Naegleria fowleri is resistant to commonly used antibiotics such as the penicillins, tetracyclines and streptomycin group (Carter 1969). It is sensitive to the antifungal antibiotic amphotericin B. Carter (1969) showed that amphotericin B immobilised *N. fowleri* at a concentration of 0.6 ug/ml, and inhibited growth at 0.075 ug/ml. A concentration of 0.09 ug/ml is attainable in human cerebrospinal fluid (Goodman and Gilman, 1970).

Mice inoculated intranasally with *N. fowleri* were protected from infection by intraperitoneal doses of 7.5 mg/kg/day of amphotericin B. This drug has been used to successfully treat at least one patient with PAME (Anderson and Jamieson, 1972a), but it has the severe disadvantage of being nephrotoxic in 80% of patients (Goodman & Gilman, 1970).

Because amphotericin B is an antifungal antibiotic, attention was directed to other antifungals. One, clotrimazole, showed promising activity *in vitro* against *N. fowleri* and so was extensively tested.

In vitro studies.

A stock 0.1% solution of clotrimazole, which is poorly soluble in water, was prepared with dimethylsulphoxide. This was diluted with water and CFA to give a concentration of 10 ug clotrimazole/ml. All further dilutions were made with CFA. The sources of stains used are shown in Table 18.

Studies of the survival of 18 strains of *N. fowleri* in CFA containing clotrimazole were done as follows: 100 cmm of a culture containing a known concentration of trophozoites was inoculated into tubes with 1 ml of the CFA-clotrimazole in doubling dilutions. The tubes were incubated at 37°C for 5 days. Then motile cells were counted using a Spencer

TABLE 18. Sources of Strains of *Naegleria fowleri*

Strain	Source	Country
NHI	Human	New Zealand
Morgan	"	Australia
Morthcott	"	"
McMahon	"	"
Oram	"	"
Vitek	"	Czechoslovakia
0359	"	Belgium
0360	"	"
Q838	"	"
HB-1	"	U.S.A.
MW4U	Tap Water	Australia
PA14	" "	"
PA34	" "	"
PA90	" "	"
PA105	" "	"
PA117	" "	"
K1	" "	"
K71	" "	"

Bright Line haemocytometer. A control of CFA without clotrimazole, inoculated as above, was used. A control of CFA with dimethylsulphoxide was not included because N. fowleri is able to grow in the presence of 0.7% of this substance (unpublished observation). The highest concentration of clotrimazole in CFA used was 10 ug/ml; this contained only 0.01% dimethylsulphoxide.

In vivo studies

Mouse Protection

Mice were dosed orally with a suspension of clotrimazole in 1% methyl cellulose mucilage. The dose used was 100 mg/kg/day with mice weighing approximately 15 g; 0.75 mg clotrimazole in 0.25 ml methyl cellulose was given morning and evening with an oral dosing needle.

Three groups of mice were used: 10 uninfected and 10 infected control mice were given methyl cellulose, and 10 infected test mice were given clotrimazole in methyl cellulose. Under light ether anaesthesia the mice were infected by intranasal inoculation of 50 cmm. of a culture of strain Q1230 containing 1,600 trophozoites/cmm. Clotrimazole treatment was started immediately after inoculation and continued twice daily for 5 days. The mice were fed and watered ad libitum and observed for 16 days thereafter. After this time the remaining infected mice were killed and their brains were removed and bisected. One half brain and a portion of liver were cultured, and the other examined histologically along with liver, kdiney, spleen and suprarenal tissue.

Bioassay of Clotrimazole Levels in Mouse Serum

A stock 1% solution of clotrimazole in chloroform was prepared and stored in the dark at 4°C. It was diluted with a 0.01 M phosphate buffer (pH 7.2) to give standard solutions containing 0.02, 0.1, 0.5, 2 and 10 ug clotrimazole/ml. Candida pseudotropicalis was used as the test organism because it required a minimal inhibitory concentration

(MIC) of below 0.01 ug/ml (Holt and Newman, 1972). Assay plates were prepared using Sabouraud agar (pH 7.2). A 10 ml base layer in a 9 cmm Petri dish was covered with a 4 ml seed layer containing 0.02 mls of an overnight culture of C. pseudotropicalis in Sabouraud broth without cycloheximide.

Eighty five mice were used: 17 controls received methyl cellulose alone and 68 received clotrimazole in methyl cellulose. The dose was 0.25 ml containing 0.75 mg clotrimazole (as used in the protection study). The first dose was given at 9 am. Four mice receiving clotrimazole and one control mouse were killed hourly for 8 hours, when the next dose was given to the remainder. Five mice were killed at 9 am the following day, the third dose was given to the remainder, and then 5 mice were killed hourly for the next 8 hours.

Blood was obtained by cardiac puncture under ether anaesthesia and the serum was used to determine the degree of inhibition of C. pseudotropicalis in the assay plates.

Antibiotic assay discs (Whatmann AA, 13 mm) were used: one (test) soaked with 0.1 ml serum and one (standard) soaked with 0.1 ml of 10 ug/ml soluton of clotrimazole, were used on each plate. The plates were incubated overnight at 37°C and the zones of inhibition were measured with calipers fitted with a Vernier scale.

Ten sets of standard plates using the same batch of agar as in the test plates were set up with 2 discs/plate. As well as the 5 standard solutions, a control of phosphate buffer alone was used. These plates were incubated and measured as described above.

RESULTS

In vitro studies

The susceptibility of 18 strains of *N. fowleri* to clotrimazole is shown in Table 19. The minimal inhibitory concentrations (MIC) ranged from 0.03 - 0.125 ug/ml and the minimal amoebicidal concentrations (MAC) from 0.125 - 0.25 ug/ml.

The effect of the size of the inocula (which ranged from 185 - 1,700 trophozoites/mm) on the MIC and MAC is shown in Table 20. The number of times each strain was tested at different inoculum sizes is too small to show any reliable trend.

In vivo studies

Mouse protection

All the uninfected controls receiving methyl cellulose survived. The results of the infected mice are shown in Table 21. Twenty-one days after infection there were 3 mice left in the treated, and 2 in the untreated group. These mice were killed and their brains were removed and bisected. One half of the brain and a portion of liver were cultured; the other half was examined histologically. The results of culture are shown in Table 22. Only the mouse with the positive brain culture had amoebae in the histological sections.

Bioassay of Clotrimazole Levels in Mouse Serum

The zone diameters from the 10 sets of standard plates were averaged to give a standard curve from which the serum level corresponding to a given zone diameter could be read. The standard disc on each test plate was used to locate a curve, parallel to the standard, which gave the serum level. The results from the 4 test mice killed each hour were averaged and are presented in Figure 20.

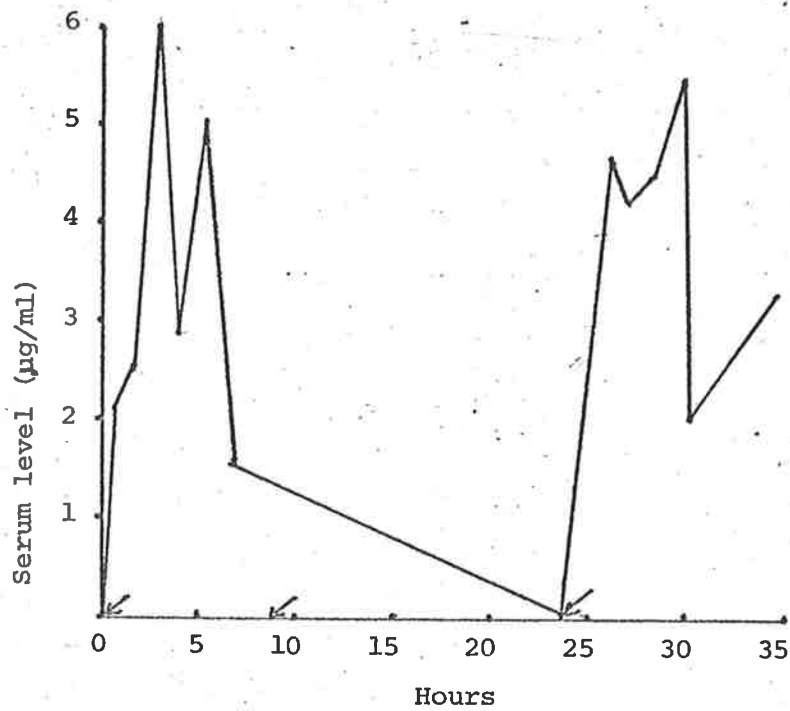

Figure 20. Serum levels of clotrimazole in mice

↙ = dose of 50 mg clotrimazole/kg given.

TABLE 19. *In vitro* Susceptibility of 18 strains of *Naegleria fowleri* to clotrimazole.

Strain	Inoculum	M.I.C.	M.A.C.
NHi	1,700	0.125	0.25
"	1,380	-	0.15
"	260	0.06	0.125
Morgan	870	0.03	0.125
"	390	-	0.15
"	365	-	0.15
Vitek	560	0.06	0.125
"	370	-	0.15
"	300	-	0.15
McMahon	740	0.06	0.125
"	440	-	0.15
0360	310	-	0.15
"	300	0.06	0.25
Northcott	1,000	0.06	0.25
"	375	-	0.15
HB-1	310	0.06	0.125
"	185	-	0.15
Oram	790	0.125	0.25
"	355	-	0.15
0359	600	0.06	0.25
"	410	-	0.15
MW4U	225	-	0.15
PA105	550	0.06	0.25
"	240	-	0.15
PA90	420	0.125	0.25
"	270	-	0.15
K71	1,050	-	0.15
K1	260	-	0.15

TABLE 19 *In vitro* Susceptibility of 18 strains of *Naegleria fowleri* to clotrimazole. (continued)

Strain	Inoculum	M.I.C.	M.A.C.
PA117	720	0.06	0.25
"	300	-	0.15
PA34	650	0.06	0.25
-	350	-	0.15
PA14	420	0.125	0.25
"	410	-	0.15
Q838	830	0.125	0.25

Inoculum = no. of trophozoites/cmm.

M.I.C. = Minimal Inhibitory Concentration.

M.A.C. = Minimal Amoebicidal Concentration - 90% kill of inoculum.

TABLE 20. Effect of inoculum size *in vitro* susceptibility of *Naegleria fowleri* to clotrimazole.

Inoculum	Number of Strains	M.I.C.	M.A.C.
185 - 250	2	-	0.15
251 - 350	9	0.06	0.16
			(0.125 - 0.25)
351 - 450	10	0.125	0.17
			(0.15 - 0.25)
451 - 550	1	0.06	0.25
551 - 650	3	0.06	0.21
			(0.125 - 0.25)
651 - 750	2	0.06	0.19
			(0.125 - 0.25)
751 - 850	2	0.125	0.25
851 - 950	1	0.03	0.125
950 - 1,050	2	0.06	0.2
			(0.15 - 0.25)
1,400	1	-	0.15
1,700	1	0.125	0.25

Inoculum = no. of trophozoites/cmm.

M.I.C. = Minimal Inhibitory Concentration.

M.A.C. = Minimal Amoebicidal Concentration - 90% kill of inoculum.

Values in parentheses represent ranges.

TABLE 21. *In vivo* activity of clotrimazole in mice infected with *Naegleria fowleri*.

Day	Deaths Methyl cellulose	Culture	Deaths Clotrimazole/Methyl cellulose	Culture
5	2	+	2	+
6	5	+	3	+
15	1	+	1	+
17			1	+

Culture + = Amoebae grown from suspension of mouse brain in water.

TABLE 22. Culture of brain and liver of mice infected with *Naegleria fowleri*.

Methyl cellulose		Clotrimazole/Methyl cellulose	
Brain	Liver	Brain	Liver
-	-	-	-
+	-	-	-
-	-		

\+ = Amoebae present.

\- = No amoebae present.

DISCUSSION

The *in vitro* studies showed that the MIC of clotrimazole for various strains of N. fowleri ranged from 0.03 - 0.125 ug/ml and the MAC from 0.125 - 0.25 ug/ml. These concentrations should be attainable therapeutically, although actual cerebrospinal fluid and brain levels in humans are not known.

Plempel *et al.* (1969) successfully treated *Candida albicans* meningitis in mice with doses twice daily of 50 mg/kg clotrimazole for 5 days after infection. Mice were not protected from infection by *N. fowleri* with the same regimen. The serum levels achieved in mice in the first 32 hours after infection should have been sufficiently high to inhibit amoebic multiplication. A level of 6 ug/ml, 3 hours after a single dose of 50 mg/kg, is comparable with a level of 7 ug/ml, 4 hours after a dose of 100 mg/kg (Plempel *et al.*, 1969). In the well-vascularised region of the nasal mucose where the amoebae are found in the first 3 days after inoculation (Carter, 1970), there should be ample exposure of the amoebae to blood containing amoebicidal levels of clotrimazole.

Plempel and Bartmann (1971) showed that an effective level of clotrimazole in the blood from the 4th - 5th day following infection cannot be achieved. This is because the clotrimazole causes the induction or activation of the liver enzymes which catabolise it. The *in vivo* studies showed that adequate serum levels were achieved in mice during the first 32 hours of treatment. As pointed out, this is the period when the amoebae should be especially vulnerable.

Thus, although clotrimazole is very promising in its *in vitro* activity against *N. fowleri*, its efficacy in treating infections (even when given within minutes of inoculation) is negligible.

In the clinical sphere, a patient would be unlikely to receive treatment until at least 72 hours after infection, by which time the amoebae would have begun to invade the meninges and brain (Carter, 1970).

In view of these results, clotrimazole cannot replace amphotericin B as the drug of choice in the treatment of PAME caused by *N. fowleri*.

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