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Evaluation of the Indirect Fluorescent-Antibody Technique for Identification of *Naegleria* Species

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The indirect fluorescent-antibody technique was used to assess a rapid method for identification of amoebae belonging to the genus *Naegleria*. Thirty-eight *Naegleria* and eight other limax amoeba strains were examined by using one *N. gruberi* and two *N. fowleri* antisera. All pathogenic *Naegleriae*, most of which originated from fatal cases of primary amoebic meningo-encephalitis, were identified as belonging to the *fowleri* species. Most of the *N. gruberi* strains showed irregular fluorescence. Other limax amoebae, such as *Vahlkampfia*, *Acanthamoeba*, *Hartmannella*, and *Schizopyrenus* sp. gave negative responses with the prepared antisera. The indirect fluorescent-antibody technique allows the identification of *N. fowleri* in a mixed culture of both *N. fowleri* and *N. gruberi* strains. Twenty-two *Naegleria* isolated from a suspected stream, other surface waters, and muddy soil could be excluded from the *fowleri* species with the indirect fluorescent-antibody technique. The results obtained demonstrate that this immunological technique is a valid method for the rapid identification of *N. fowleri* trophozoites.

Serological techniques have been used extensively for the identification and classification of parasitic as well as free-living amoebae (1, 5, 11, 14).

Since *Naegleria fowleri* was recognized as the causal agent of primary amoebic meningo-encephalitis (PAME) (4) many of these techniques were also applied to *Naegleria*.

Saygi (12) observed the lack of specificity of the *N. gruberi* antiserum, which gives rise to a cross-reaction with *N. fowleri* HB-1 antigen. In 1970 Singh and Das (15) proved that *N. fowleri* could be separated from *N. gruberi* with the immobilization reaction.

The agglutination test for identifying *Naegleria* species was introduced by Anderson and Jamieson (2) thus permitting the identification of 19 wild isolates as *N. fowleri*.

Later it appeared that some of these differed from *N. fowleri* and *N. gruberi*. Willaert et al. (19) investigated the antigenic differences between *Naegleria* species by immunoelectrophoretic analysis. These authors found 27 hydrosoluble antigenic components in *N. fowleri* and 19 in *N. gruberi*. *N. gruberi* and *N. fowleri* were shown to have eight antigenic components in common.

Willaert identified 35 antigenic components in another *Naegleria* isolate (0.400) from which 12 and 14 components were in common with *N. gruberi* and *N. fowleri*, respectively.

A new species was thus named *N. jadini* on this basis. The antigenic uniformity of four pathogenic *Naegleria* was established by Červa (7) by means of the indirect fluorescent-antibody test. Preliminary studies (18) showed that *N. gruberi* 1518/le can be discerned from four pathogenic *N. fowleri* with the indirect fluorescent-antibody technique (IFAT). Like Červa we obtained a nonspecific reaction with cysts. For further evaluation of the IFAT for differentiation of limax amoebae, the reaction was applied to 46 strains obtained from various laboratories throughout the world and to several freshly isolated strains.

MATERIALS AND METHODS

Amoebae. The *Naegleria* and other limax amoebae used in this study are described in Table 1.

Culture methods. Amoebae were cultivated in liquid MYAS medium in association with living *Escherichia coli*. MYAS is composed of: malt extract (Difco B 186), 0.1 g; yeast extract (Difco B 127), 0.1 g; and 1,000 ml of amoebae saline according to the instructions of the Culture Centre of Algae and Protozoa. Composition of amoebae saline: NaCl, 0.12 g; $MgSO_4 \cdot 7aq$, 0.004 g; $CaCl_2 \cdot 2aq$, 0.004 g; Na_2HPO_4, 0.142 g; KH_2PO_4, 0.136 g; distilled water, 1,000 ml.

The amoebae were cultivated in 1-liter Roux bottles containing 70 ml of MYAS medium. All strains of *N. fowleri* and *Acanthamoeba* sp. were incubated at 37 C, whereas *N. gruberi*, *Vahlkampfia*, *Schizopyrenus*, and *Hartmannella* sp. were cultivated at 28 C.

TABLE 1. *Origin of the limax amoeba type strains studied by the IFAT*

Amoeba	Strain	Source	Date of isolation	Author	Received from[a]
N. fowleri	HB-1	CSF, U.S.A.	1966	C. Butt	2
	HB-2	CSF, U.S.A.		C. Butt	1
	Nf 66	CSF, Australia	1966	R. F. Carter	6
	Nf 69	CSF, Australia	1969	M. Fowler	6
	0.359	CSF, Belgium	1970	J. B. Jadin	8
	0.360	CSF, Belgium	1970	J. B. Jadin	8
	Vitek	CSF, Czechoslovakia	1969	L. Červa	8
	KUL	CSF, Belgium	1973	J. Vandepitte	7
	CJ	CSF, U.S.A.	1967	R. J. Duma	6
	LEE	CSF, U.S.A.	1968	R. J. Duma	6
	TY	CSF, U.S.A.	1969	R. J. Duma	6
	Wm	CSF, U.S.A.	1969	R. J. Duma	6
	GJ	CSF, U.S.A.	1973		1
	Morgan	CSF, Australia	1971	K. Anderson	10
	PA 90	Drinking water, Australia	1972	A. Jamieson	10
	LEE-2	Mousebrain infected with LEE			6
N. aerobia	Na1	Sewage sludge, India	1972	B. N. Singh	3
	Na2	Sewage sludge, India	1972	B. N. Singh	3
	Na3	Sewage sludge, India	1972	B. N. Singh	3
	Na4	Sewage sludge, India	1972	B. N. Singh	3
	Na5	Sewage sludge, India	1972	B. N. Singh	3
Naegleria sp.	TS-1	Vero-cell culture, U.S.A.	1972	W. O'Dell	6
	L1-L	CSF, England; reisolated by Chang from L1	1969	G. Saygi	4
	161 A	Human nasal swab		J. Shumaker	1
N. gruberi	1518/1e	Millpond, U.S.A.	1964	F. C. Page	2
	EGS	Eucalyptus grove, U.S.A.	1961	F. L. Schuster	10
	27	Riverwater, Australia		A. Jamieson	10
	BG-6	Riverwater, Australia		A. Jamieson	10
	Ng7	Outdoor swimming pool, U.S.A.	1969		1
	Lake 4	Lake, U.S.A.	1971		1
	1518/1a			E. G. Pringsheim	2
	1518/1b			E. G. Pringsheim	2
	1518/1c			E. G. Pringsheim	2
	1518/1d			W. Balamuth	2
	1518/1f			F. C. Page	2
	1518/1g			F. C. Page	2
	1518/1s			E. G. Pringsheim	2
N. jadini	0.400	Swimming pool, Belgium	1971	J. B. Jadin	10
V. avara	1588/1a			F. C. Page	2
V. inornata	1588/2			F. C. Page	2
V. jugosa	P1	Dried out puddle, England	1969	G. Saygi	9
	FP 1	Fishpond, England	1969	G. Saygi	9
Schizopyrenus sp.	L1-S	Brain tissue, England	1969	G. Saygi	4
H. vermiformis	1534/7			F. C. Page	2
A. culbertsoni	A-1	Tissue culture, U.S.A.	1959	C. G. Cultbertson	5
Acanthamoeba sp.	m.c.v.-180	U.S.A.		E. C. Nelson	6

[a] 1, Chang, S. L., U.S. Environmental Protection Agency, Cincinnati; 2, Culture Centre of Algae and Protozoa, Cambridge, England; 3, Das, S. R., Central Drug Research Institute, Lucknow, India; 4, Griffin, J. L., Armed Forces Institute of Pathology, Washington; 5, Lumsden, W. H. R., London School of Hygiene and Tropical Medicine, London, England; 6, Stevens, A. R., Veterans Administration Hospital, Gainesville; 7, Vandepitte, J., Academisch Ziekenhuis St. Raphaël, Leuven, Belgium; 8, Verstraeten, J., Provinciaal Instituut voor Hygiëne, Antwerpen, Belgium; 9, Warhurst, D. C., Liverpool School of Tropical Medicine, Liverpool, England; 10, Jadin, J. B., Instituut voor Tropische Geneeskunde, Antwerpen, Belgium. Abbreviations: CSF, cerebrospinal fluid; *N.*, *Naegleria*; *V.*, *Vahlkampfia*; *H.*, *Hartmannella*; *A.*, *Acanthamoeba*.

Isolation of amoebae from water samples and muddy soil. Water samples ranging from 50 to 500 ml were filtered through 5-μm cellulose acetate membranes. The membranes were inverted on nonnutrient (NN) agar inoculated with living *E. coli*.

For sampling from muddy soil, a piece of mud was deposited in the middle of an NN agar plate spread with living *E. coli*. Plates were incubated at 28 and 37 C.

Growth was observed by using an inverted microscope (magnification $\times$250). Subcultures of growth were obtained by cutting out a piece of agar and placing it on a fresh plate. This procedure eliminated most of the bacteria and fungi. After 1 or 2 days 1 ml of sterile distilled water was poured into the plates for flagellate transformation and the morphology of amoebae was studied under a microscope. Amoebae with typical *Vahlkampfiidae* morphology and flagellates were inoculated into Roux bottles containing 70 ml of MYAS medium. Some of the *Naegleria* strains isolated from water have been cloned. One amoeba selected by microscope examination was taken from the medium with a micro-pipette and transferred to a fresh plate. After growth it was cultivated in liquid MYAS medium for the IFAT.

Antigen preparation. Antigen was prepared from *N. gruberi* 1518/1e and *N. fowleri* HB-1 harvested from monoxenic growth in MYAS medium and *N. fowleri* 0.359 from axenic growth in Cerva (6) medium, supplemented with 20 μg of biotin per liter. The medium was centrifuged for 5 min at 750 $\times$ *g* and washed four times with distilled water, and the amoebae were counted in a Bürker cell and standardized to 10^6 amoebae per ml. The amoebae were disintegrated by sonic treatment in a small volume and filtered through 5-μm membranes to eliminate the cysts. Formalin was added for disinfection to a final concentration of 0.1%.

Immunization. Rabbits were injected intramuscularly with 0.5 ml of antigen mixed with 0.5 ml of complete Freund adjuvant (Difco 0638-60-7) at weekly intervals for a period of 3 to 7 weeks. Three rabbits were immunized with each antigen and the two sera showing the highest titer were used for the IFAT.

Indirect fluorescence technique. All reactions were performed with amoebae obtained from monoxenic growth, as preliminary studies showed that the fluorescence was more specific than with axenic cultures. The amoebae were concentrated from the liquid medium by centrifugation and washed twice with distilled water. Slides coated with teflon (PTFE/A Aerosol Spray, Fisons Scientific Apparatus, England) were prepared according to Goldman (8). One drop of washed amoebae was placed on each uncoated circular area. The smears were dried on a hot plate and fixed for 10 min in acetone. The antisera serially diluted with phosphate buffer (pH 7.3) 1/20 to 1/640 were added to each smear and the slides were incubated for 30 min at 37 C in a moist chamber. The slides were then rinsed and left for 15 min in phosphate buffer on a reciprocating shaker with slow motion. After drying, the smears were covered with fluorescent anti-rabbit serum. After 30 min of incubation at 37 C in a moist chamber, rinsing with phosphate buffer was repeated for 30 min as before.

When dry, the slides were mounted with buffered glycerin (pH 8.5). Fluorescence of the stained amoebae was observed in bright field by using a Wild microscope equipped with a pressure mercury tube HB0.20, a BG 12 excitation filter and a FITC barrier filter. Fluorescence of cysts was not taken into consideration because of the nonspecific reaction. Sera from two different rabbits were used for each reaction. The brightness of fluorescence was rated on a subjective scale ranging from 1+ to 4+. A reaction was still noted as positive when the intensity was rated as 1+.

RESULTS

Thirty-eight *Naegleria*, two *Acanthamoeba*, four *Vahlkampfia*, one *Schizopyrenus*, and one *Hartmannella* strain have been investigated. Most strains were typified by the authors (Table 1) and their reaction with the IFAT is summarized in Table 2. Because two different sera for each antigen were used two values were sometimes recorded, when the highest titers did not correspond.

A strong positive reaction was observed up to 1/640 with *N. fowleri* HB-1 and 0.359 antisera for *Naegleriae* causative of PAME isolated from all over the world.

In this case, the fluorescence was very clear (2 to 4+) even with the last dilution (1 to 2+). *N. gruberi* 1518/1e antiserum, on the other hand, gave a weak positive reaction to a titer of only 1/40 to 1/80 with these strains. Wild-living pathogenic strains such as *N. aerobia* 1 to 5 identified by Singh and Das (16) and strain PA 90 identified by Anderson et al. (2, 3) also gave positive fluorescence to a titer of 1/640 with antisera prepared with *N. fowleri* isolated from cerebrospinal fluid (CSF). According to the IFAT result, strain TS-1 must be regarded as belonging to the pathogenic species *N. fowleri*.

O'Dell and Stevens (10) isolated this strain as a contaminant from a Vero cell culture. They describe it as doubtfully virulent for mice. According to our identification, the pathogenicity of this strain should be reinvestigated. *N. gruberi* 1518/1e reacts with the homologous antisera to a titer of 1/160 to 1/320 and this strain shows a cross-reaction to a titer of 1/40 to 1/80 with *N. fowleri* antisera. However, no clear-cut results could be obtained with other typified *N. gruberi*. Some strains like EGS, 1518/1b, and 1518/1f gave little or no response with both *N. gruberi* and *N. fowleri* antisera.

The brightness of fluorescence with the type strains at a titer of 1/80 was recorded in Table 3. The latter demonstrated that the fluorescence of *N. fowleri* amoebae with the homologous

TABLE 2. *Highest indirect fluorescence titer (reciprocal) of limax-amoebae type strains*

Antigen[a]	Anti-*N. gruberi* 1518/1e	Anti-*N. fowleri* HB-1	Anti-*N. fowleri* 0.359
N. fowleri			
HB-1	40 to 80	640	640
HB-2	40 to 80	640	640
Nf 66	80 to 160	640	640
Nf 69	40	640	640
0.359	40 to 80	320 to 640	640
0.360	80 to 160	640	640
Vitek	40 to 80	640	640
KUL	40 to 80	640	640
CJ	80	320 to 640	640
LEE	20	320 to 640	320 to 640
TY	– to 20	160 to 320	320 to 640
Wm	80	640	640
GJ	80	640	640
Morgan	20 to 40	320	640
PA 90	40 to 80	640	640
LEE-2	40 to 80	320 to 640	640
N. aerobia			640
Na 1	40 to 80	640	640
Na 2	40 to 80	640	640
Na 3	40 to 80	640	640
Na 4	40 to 80	640	640
Na 5	20 to 40	640	640
Naegleria sp.			
TS-1	40	320 to 640	640
L1-L	40 to 80	160 to 320	40 to 80
161 A	640	80	20
N. jadini			
0.400	80 to 160	40 to 80	40 to 80
N. gruberi			
1518/1e	160 to 320	80	20 to 40
EGS	20	– to 20	– to 20
27	160 to 320	40	80 to 160
BG-6	80 to 160	40 to 80	40
7	160	80	160
Lake 4	160 to 320	– to 20	– to 20
1518/1a	640	160	80 to 160
1518/1b	20 to 40	– to 20	– to 20
1518/1c	160 to 320	80 to 160	160 to 320
1518/1d	160 to 320	160	80 to 160
1518/1f	20 to 40	80	20 to 40
1518/1g	160 to 640	160	80 to 160
1518/1s	160 to 320	40 to 80	–
V. avara 1588/1a	20	– to 20	20
V. inornata 1588/2	– to 40	–	–
V. jugosa			
P1	20 to 40	–	–
FP1	20 to 40	20	–
Schizopyrenus sp. L1-S	–	–	–
H. vermiformis 1534/7	–	– to 20	–
A. culbertsoni A-1	–	–	–
Acanthamoeba sp. mcv-180	–	–	–

[a] For abbreviation of the genus names see Table 1. –, Negative fluorescence at 1/20. Two numbers are given when the highest titer of the antisera of the two rabbits differed.

antisera was very bright (2+ to 4+), whereas the fluorescence of *N. gruberi* amoebae with their homologous antisera was much less bright (1+ to 2+). The difference of response of the antisera to the homologous antigen could be explained by the much weaker antigenicity of *N. gruberi* compared with *N. fowleri* in the experimental animals. As a consequence of this lack of uniformity with *N. gruberi*, it was also impossible to assess a difference between *N. gruberi* and *N. jadini* with the IFAT.

Naegleria strain L1 isolated by Saygi (13)

TABLE 3. *Intensity of indirect immunofluorescence at the titer 1/80 obtained with type cultures*

Antigen	Antiserum		
	Anti-*N. gruberi* 1518/1e	Anti-*N. fowleri* HB-1	Anti-*N. fowleri* 0.359
N. fowleri			
HB-1	0 to 1+	2+	2 to 3+
HB-2	1+	2+	2+
Nf 66	0 to 1+	2 to 3+	2+
Nf 69	0	2 to 3+	3 to 4+
0.359	0 to 1+	2+	2+
0.360	1+	3 to 4+	4+
Vitek	0 to 1+	2+	2+
KUL	0	2 to 3+	2 to 3+
CJ	1+	2+	2+
LEE	1+	2 to 3+	3 to 4+
TY	1+	3 to 4+	2 to 4+
Wm	1+	3 to 4+	4+
GJ	1+	2 to 3+	3+
Morgan	0	2+	2+
PA 90	0 to 1+	2+	2+
LEE-2	0 to 1+	2+	2+
N. aerobia			
Na 1	1+	4+	4+
Na 2	1+	2+	2 to 3+
Na 3	0 to 1+	2 to 3+	2+
Na 4	1+	2+	2 to 3+
Na 5	0	2+	2+
Naegleria sp.			
TS-1	0	2 to 3+	2 to 3+
L1-L	0 to 1+	1+	0 to 1+
161 A	3+	0	0
N. jadini			
0.400	1 to 2+	0 to 1+	0 to 1+
N. gruberi			
1518/1e	2+	1+	0
EGS	0	0	0
27	1+	0	1+
BG-6	1 to 2+	0 to 1+	0
7	1+	1+	1+
Lake 4	1 to 2	0	0
1518/1a	2+	1+	2+
1518/1b	0	0	0
1518/1c	2+	1+	1+
1518/1d	1+	1+	0 to 1+
1518/1f	0	1+	0
1518/1g	1 to 2+	1+	1+
1518/1s	1+	0 to 1+	0

[a] For abbreviation of the genera names, see Table 1. The intensity is rated on a scale ranging from 1+ to 4+. 0 means negative fluorescence. Two numbers are given when the intensity of the antisera of the two rabbits differed.

from CSF was nonvirulent for mice and was shown to differ from *N. fowleri*. The IFAT of brain sections, treated with L1 strain antiserum, showed that the same amoebae were found in this tissue (W. P. Stamm, International Colloquium on PAME and free living amoebae, Antwerp, 1973). Thus, the L1 strain was not a contaminant in the CSF culture. This could indicate that besides *N. fowleri* other species may be pathogenic for men.

The reactivity of the *Naegleria* antisera with other common limax amoebae was assessed. *Vahlkampfia jugosa*, *V. avara*, and *V. inornata* did not react with the antisera nor did two *Acanthamoeba* strains, *Hartmannella vermiformis* and a *Schizopyrenus* strain. Apart from morphological characteristics the antigenic structure also seemed to differ from that of *Naegleria*.

Application of the IFAT with mixed cultures. When *N. gruberi* 1518/1e was mixed with *N. fowleri* HB-1 in a medium, overgrowth of the saprophytic *N. gruberi* was obtained. This phenomenon was observed even at a temperature of 37 C. According to the technique of Griffin (9), *N. gruberi* can only be inhibited to the advantage of the pathogenic species at still higher temperatures.

The overgrowth was difficult to prove, because on a morphological similarity the two species are very closely related and a mouse inoculation was necessary to demonstrate the disappearance of pathogenicity. But with the IFAT it was possible to identify both species separately in a mixed culture. The inoculum consisted of a 24-h culture of both strains, with the number of amoebae of *N. fowleri* being twice that of *N. gruberi*. After 24 h at 37 C the mixed growth was processed with the IFAT. Using *N. fowleri* antiserum, the difference in the fluorescence of both strains was obvious up to a titer of 1/640. The fluorescence of *N. fowleri* was very bright, whereas *N. gruberi* amoebae only gave a faint color. This proves that the antisera are rather specific. The number of amoebae of each of the two strains was counted in several microscope fields and it appeared that the proportion had changed in favour of *N. gruberi*.

The saprophytic strain outnumbered *N. fowleri* twice, whereas at inoculation the reverse conditions had prevailed. When using *N. gruberi* antiserum, the identification of both strains in a mixed culture was not possible, since the difference of intensity of the fluorescence was not sufficiently pronounced.

Application of the IFAT with other isolated strains. Attempts were made to isolate *N. fowleri* from a thermally polluted stream where a boy had swum and contracted PAME (17). Several *Naegleriae* were isolated from this water but none reacted positively with *N. fowleri* antiserum. This was also the case with amoebae found on solid matter in the suspected stream. Isolation procedures were extended to canals connected with the stream. Out of 47 strains of amoeba isolated from the surface waters only 15 strains were identified as *Naegleria* species.

The IFAT results of these isolated *Naegleriae* are summarized in Table 4. None of these strains could be identified as *N. fowleri*. The IFAT titer of these isolates were as divergent as the titer of *N. gruberi* type strains.

The occurrence of *N. fowleri* in the environment was further investigated; out of 20 isolates from muddy soils only two were identified as *Naegleria* sp.

As illustrated in Table 4, these strains differ from *N. fowleri* but the reaction with *N. gruberi* antiserum was irregular as observed with the strains from the above mentioned surface waters. Some strains which were morphologically different from *Naegleria* were also tested with the IFAT but all gave negative reactions.

DISCUSSION

All *Naegleriae* isolated from CSF could be identified by immunofluorescence as *N. fowleri* except one strain, L1, which differs from *N. fowleri* even with other techniques.

Pathogenic *Naegleria* strains isolated from the environment by other workers in two different continents were also identified by the IFAT, and the results indicated that the cultures indeed belong to the *fowleri* species.

The identification of *N. gruberi* seemed more difficult by the IFAT as the reaction was not uniform for the whole series of type strains. *N. jadini* could not be identified as a separate species because of the irregular results obtained with *N. gruberi* antiserum. It is possible that all isolated *N. gruberi* have different antigenic patterns. For Willaert (IV^{ième} Congrés International de Protozoologie, Clermont-Ferrand, 1973) this seems a good reason for designating new species as he already did for *N. jadini*. We observed that the cyst morphology of *N. gruberi* varies greatly amongst the various strains obtained from all parts of the world.

This observation also supports the hypothesis of variation within the *N. gruberi* species. As there was little or no cross-reaction with other *Vahlkampfiidae*, *Acanthamoeba*, and *Hartmannella* sp. and as the cross-reaction was moderate between pathogenic and nonpathogenic *Naegleriae*, the IFAT proves to be a valid method of identification of *N. fowleri*. It became evident that with only a *N. fowleri* antiserum, it was possible to separate *N. fowleri* from *N.*

TABLE 4. *Highest indirect fluorescent antibody titer (reciprocal) of Naeglerina isolated from surface waters and muddy soils*

Naegleria isolates	Anti-*N. gruberi* 1518/1e[a]	Anti-*N. fowleri* HB-1	Anti-*N. fowleri* 0.359
From suspected stream LG			
1	80 to 160	80 to 160	20
2	160	80	20
3	160 to 320	80	40
4	160 to 320	40 to 80	20
4 cloned	160 to 320	80	40
7	40 to 80	20 to 40	–
7 cloned	80	– to 20	40
On solids			
S 1	– to 20	–	–
S 3	– to 20	–	–
S 5	20 to 40	– to 20	–
S 2	160 to 320	80	20 to 40
S 4	80	–	40 to 80
From canals connected to the stream LG			
ATMP2 (28)	160 to 320	20	20
ATMP2 (28) cloned	80 to 160	– to 20	20 to 40
ATMP2 (37)	320 to 640	40	– to 20
ATMP2 (37) cloned	160 to 320	20 to 40	20
ATMP2 (37) cloned	80 to 160	– to 20	20 to 40
ATMP6 (28)	320 to 640	80 to 160	160 to 320
ATMP6 (28) cloned	80	– to 20	– to 20
ATMP6 (37)	80 to 160	80 to 160	20 to 40
ATP 3 (28)	160	40 to 80	20 to 40
ATP 3 (28) cloned	320	80	80
From muddy soil			
BW 3.2	160 to 320	80	20 to 80
ND 3.2	160 to 320	160	80 to 320

[a] –, Negative fluorescence at 1/20. Two numbers are given when the highest titer of the antisera of the two rabbits differed.

gruberi, not only because of a difference in titer but more clearly because of a difference in brightness at a given serum dilution (Table 3). For these reasons, it was not necessary to absorb the antiserum in order to obtain a cross-reaction-free specific antiserum. Although the IFA test does not provide direct information as to the pathogenicity of a given isolate, one may identify a strain as being *fowleri* which is considered pathogenic. For confirmation intranasal instillation of mice can be applied.

The experiment with the IFAT applied to a mixed culture of *N. gruberi* and *N. fowleri* proved the specificity of the *N. fowleri* antisera, without preliminary absorption. The mixing of both strains also showed that the nonpathogenic *Naegleria* overgrew the pathogenic strain even at 37 C. We deduced from this phenomenon that it is difficult to isolate *N. fowleri* from the environment. Only the selective conditions described by Griffin could favour the growth of the pathogenic species (9). Immunofluorescence might probably be an effective and more convenient method for screening *N. fowleri* in the environment.

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