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Rapid identification of thermophilic *Naegleria*, including *Naegleria fowleri* using API ZYM system

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SUMMARY The suitability of the API ZYM system for identifying thermophilic *Naegleria* species, based on enzyme presence and activity, was investigated. Replicate testing on strains of *N fowleri, N lovaniensis,* and *N australiensis* cultured in a monoxenic and an axenic medium showed that the system could provide a rapid and reproducible means of identifying the species soon after primary isolation. No single enzyme was found specific for any one species, but considerable differences were found in the patterns of activity of acid phosphatase and leucine arylamidase. When these were compared the species could be differentiated. Use of the system in conjunction with a simple culture method is proposed as a readily available means of monitoring environmental and public bathing sites to prevent primary amoebic meningoencephalitis.

Naegleria fowleri is a pathogenic free living amoeba that causes fatal primary amoebic meningoencephalitis in man.[1] It is widely distributed in nature and can be recovered from thermally polluted water, mud, and soil. Cases of primary amoebic meningoencephalitis have been recognised world wide, and infection is usually attributed to swimming in an infected public bath or environmental water. Found in association with *N fowleri* are two other thermophilic species, *N lovaniensis*[2] and *N australiensis*.[3] When the environment is monitored for the presence of such amoebae isolates should be identified clearly, accurately, and quickly.

N lovaniensis is closely related to *N fowleri* but is non-pathogenic. Both species can grow on bacteria at 44°C, produce a cytopathic effect in tissue culture, and cross react serologically.[2] *N lovaniensis* has been isolated from hydrotherapy pools and indoor aquaria[4 5] and is considered to be an indicator of environmental conditions favourable to the growth of *N fowleri*. *N australiensis* is pathogenic for mice on intranasal inoculation but its virulence is generally less than that of *N fowleri,* and it has not yet been shown to infect man. Recent isolates from a thermal spa in Italy, however, were as virulent as *N fowleri* for mice, increasing speculation about the pathogenic potential of the species in man.[6]

The close ecological, biological, and pathogenic relation between the thermophilic *Naegleria* makes accuracy and rapid identification soon after primary isolation imperative. Inability to grow above 42°C and the serological specificity of the indirect fluorescence antibody test distinguish *N australiensis,* but more complex techniques are required to differentiate *N fowleri* from *N lovaniensis*. Isoenzyme profiles,[7] electron microscopy, and lectin agglutination[2] are all discriminatory. De Jonckheere and Dierickx showed that measurement of specific enzyme activity of acid phosphatase and leucine aminopeptidase, using a colorimetric assay method, distinguished *N fowleri* from non-pathogenic *Naegleria* spp.[8] Such techniques are expensive and time consuming, rely on the axenic culture of isolates, and are unavailable to many laboratories.

The API ZYM system is a commercially available assay strip for the semiquantitative measurement of specific enzyme activity in various biological specimens (Table 1). It permits the rapid identification of enzyme presence and activity using very small sam-

Table 1 *Enzymes detected by API ZYM system*

Test	*Enzyme detected*	*Test*	*Enzyme detected*
1	Control	11	Acid phosphatase
2	Alkaline phosphatase	12	Phosphoamidase
3	Esterase (C4)	13	α Galactosidase
4	Esterase lipase (C8)	14	β Galactosidase
5	Lipase (C14)	15	β Glucuronidase
6	Leucine arylamidase	16	α Glucosidase
7	Valine arylamidase	17	β Glucosidase
8	Cystine arylamidase	18	N acetyl B glucosamidase
9	Trypsin	19	α Mannosidase
10	Chymotrypsin	20	α Fucosidase

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Table 2 *Naegleria strains used in this study*

Species	*Strain*	*Origin*	*Reference*
N fowleri	HB	Cerebrospinal fluid, Florida, United States	11
	MCM	Cerebrospinal fluid, Bath, England	12
	MsM	Cerebrospinal fluid, New Zealand	13
	NHI	Cerebrospinal fluid, Wellington, New Zealand	14
	C-0503	Spa water, Bath, England	
	C-0504	Spa water, Bath, England	
N lovaniensis:	C-0490	Spa water, Bath, England	
	4786.1	Spa water, Bath, England	
	4788.4	Spa water, Bath, England	
	4978.6	Spa water, Bath, England	
	5857/D.5	Spa water, Bath, England	
N australiensis:	LSR 34a	Thermal water, France	15
	NJ	Pond water, India	16
	PP 397	Flood drainage water, Australia	3
	PV 2891	Mud, thermal Spa, Italy	17
	4684.11	Spa water, Bath, England	
	5858/D.3	Spa water, Bath, England	
	5858/D.5	Spa water, Bath, England	
	5858/D.12	Spa water, Bath, England	

ples, giving a result in four hours. The substrates are incorporated into support strip wells that permit contact with the enzyme test suspension. Enzyme activity is shown by adding developing reagents and recording the intensity of colour development. This paper describes an investigation into the suitability of the API ZYM system for specifically identifying thermophilic *Naegleria,* using axenic and simple monoxenic culture.

Material and methods

CULTURE OF *Naegleria*

All strains tested (Table 2) other than the Bath isolates of *N australiensis* were cultured monoxenically and axenically using 2 ml volumes in tissue culture tubes incubated at 37°C in a slanted position. The Bath isolates of *N australiensis* have not yet been adapted to axenic culture. Monoxenic culture was achieved in 2mM Tris-hydrochloric acid buffer (pH 7·4) containing a turbid suspension of *Escherichia coli* NCTC 10412 killed by heat.[9] The stock suspension of *E coli* was prepared by centrifuging overnight broth cultures, pooling the deposits, washing twice in distilled water, and adjusting to an optical density of 12 at 546 nm in distilled water. The stock suspension was heated at 70°C for 30 minutes in a water bath and thereafter stored at 4°C; it remained usable for up to one month. Two drops of the bacterial suspension was added per ml of buffer in monoxenic culture. Axenic culture was in Chang's serum-casein-glucose-yeast extract medium.[10]

API ZYM TESTING

Tubes showing semiconfluent growth (usually after 18–36 hours of incubation) were chilled on ice, agitated to free the amoebae, and centrifuged at 600 *g* for 10 minutes. The amoebal pellet was washed once in distilled water and resuspended in about 2 ml distilled water. A cell count was performed in a haemocytometer and adjusted to 1×10^5 amoebae/ml in a final volume of 2 ml distilled water. Only trophozoites (and not cysts) were counted. The API ZYM strips were set up according to the manufacturer's instructions, and 65 μl of the adjusted amoebal suspension was added to each well. The inoculated strips were incubated at 37°C in the dark for four hours. After incubation one drop of the developing reagents ZYM A (Tris (hydroxymethyl) aminomethane 250 g; hydrochloric acid 37%, 110 ml; laurylsulphate 100 g; distilled water to 1000 ml (pH 7·6–7·8)) and ZYM B (fast blue BB 3·5 g; 2 methoxyethanol 1000 ml) were added to each well, and the reaction result was recorded after three minutes by comparison with the API ZYM colour chart. Results were scored from 0 to 5, denoting a negative through to a maximum reaction. Monoxenically cultured strains were tested in triplicate and axenic cultures in duplicate to study the reproducibility and effect of culture medium on the profile results obtained. Each batch of stock suspension of killed *E coli* was similarly tested to confirm inactivation of the bacterial enzymes.

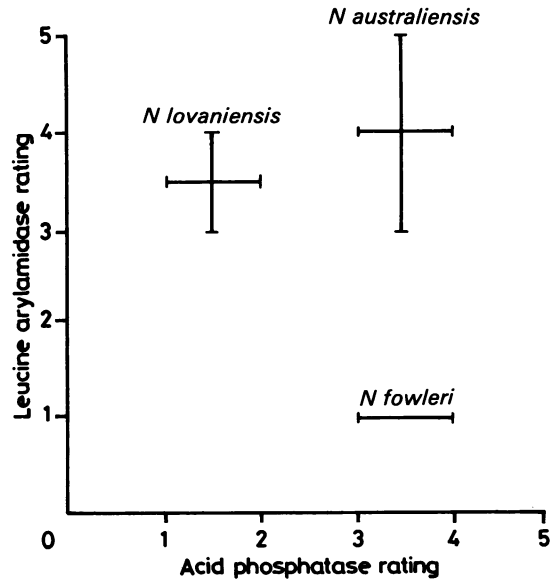
Results

Table 3 shows the profile results of enzyme activity. No enzyme specific to any one species could be shown but important differences in the enzyme activity of leucine arylamidase and acid phosphatase (substrate wells 6 and 11) could be used to differentiate the species. *N fowleri* was characterised by low leucine arylamidase and high acid phosphatase activity, *N lovaniensis* by high leucine arylamidase and low acid phosphatase activity, and *N australiensis* by high leucine arylamidase and acid phosphatase activ-

ity. The results for these two enzymes were consistent for both species and strain on repeated testing. The Figure shows the relation between leucine arylamidase and acid phosphatase activity for the species based on the activity ranges obtained for the strains tested. No differences in overall pattern were observed when monoxenic and axenic culture were compared, and the results in Table 3 include both sets of data. The activity of the other enzymes (substrate wells 2, 3, 4, 12, and 18) remained constant save for minor fluctuations in alkaline phosphatase activity (substrate well 2).

Lysates of amoebae, cultured in either medium and prepared by resuspending 2×10^5 amoebae in 2 ml of 1% Triton X-100 in distilled water, did not detect any enzymes in addition to those described for whole amoebae. The enzyme activities were increased with a consequent loss in the differentiating ability of the system.

Several strains of *N gruberi,* which would not grow above 37°C on bacteria, were examined and had a similar profile to *N lovaniensis.*



Relation between leucine arylamidase and acid phosphatase activity for thermophilic Naegleria.

Discussion

The results of this study indicate that the API ZYM system is a simple and reproducible means of identifying thermophilic *Naegleria* spp soon after primary isolation.

Although no enzyme specific for one species was found, differences in the pattern of activity of leucine arylamidase and acid phosphatase effectively differentiated *N fowleri, N lovaniensis,* and *N australiensis.* The observation of De Jonckheere and Dierickx that the activity of acid phosphatase is raised and the activity of leucine aminopeptidase lowered in *N fowleri* compared with other non-pathogenic *Naegleria* spp[8] was confirmed in this study as leucine aminopeptidase reacted with the same substrate used in the API ZYM system (L-

Table 3 *API ZYM profiles of Naegleria species tested*

	API ZYM substrates																			
	1	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*	*13*	*14*	*15*	*16*	*17*	*18*	*19*	*20*
N fowleri:																				
HB_1	0	1–2	1	1	0	1	0	0	0	0	3–4	1	0	0	0	0	0	1	0	0
MCM	0	1	1	1	0	1	0	0	0	0	3	1	0	0	0	0	0	1	0	0
MsM	0	1	1	1	0	1	0	0	0	0	3–4	1	0	0	0	0	0	1	0	0
NHI	0	1	1	1	0	1	0	0	0	0	3–4	1	0	0	0	0	0	1	0	0
C-0503	0	1–2	1	1	0	1	0	0	0	0	3–4	1	0	0	0	0	0	1	0	0
C-0504	0	1	1	1	0	1	0	0	0	0	3–4	1	0	0	0	0	0	1	0	0
N lovaniensis:																				
C-0490	0	1–2	1	1	0	3–4	0	0	0	0	1–2	1	0	0	0	0	0	1	0	0
4786.1	0	1	1	1	0	3–4	0	0	0	0	1–2	1	0	0	0	0	0	1	0	0
4788.4	0	1	1	1	0	3–4	0	0	0	0	1–2	1	0	0	0	0	0	1	0	0
4978.6	0	1	1	1	0	3–4	0	0	0	0	1–2	1	0	0	0	0	0	1	0	0
5857/D.5	0	1	1	1	0	3–4	0	0	0	0	1–2	1	0	0	0	0	0	1	0	0
N australiensis:																				
LSR 34a	0	1	1	1	0	3–4	0	0	0	0	4	1	0	0	0	0	0	1	0	0
NJ	0	1	1	1	0	4–5	0	0	0	0	3–4	1	0	0	0	0	0	1	0	0
PP 397	0	1	1	1	0	4–5	0	0	0	0	3–4	1	0	0	0	0	0	1	0	0
PV 2891	0	1–2	1	1	0	4	0	0	0	0	3–4	1	0	0	0	0	0	1	0	0
4684.11	0	1–2	1	1	0	4	0	0	0	0	3–4	1	0	0	0	0	0	1	0	0
5858/D.3	0	1	1	1	0	3–4	0	0	0	0	3	1	0	0	0	0	0	1	0	0
5858/D.5	0	1	1	1	0	4	0	0	0	0	3–4	1	0	0	0	0	0	1	0	0
5858/D.12	0	1	1	1	0	4	0	0	0	0	3–4	1	0	0	0	0	0	1	0	0
E coli	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

Where a variation in activity occurred the range is given under the respective substrate well for the strain.

leucyl-2-naphthylamide) to detect leucine arylamidase.

The wide distribution of thermophilic *Naegleria* in natural and artificial thermal waters presents a serious threat of infection from *N fowleri* and, possibly, *N australiensis* in man. Most monitoring programmes for bathing sites start only after a case of primary amoebic meningoencephalitis has occurred, and it may well be more prudent to identify potentially hazardous sites and implement preventive measures before such an infection occurs. The API ZYM system, together with simple monoxenic culture techniques, places a reliable and rapid means of identifying the thermophilic *Naegleria* and assessing their distribution in the environment at the disposal of laboratories already committed to the bacteriological monitoring of such areas.

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