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Development of a PCR for Identification of *Naegleria fowleri* from the Environment

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A species-specific PCR for the identification of *Naegleria fowleri* was developed. In sensitivity studies, 10 trophozoites or cysts and 1 trophozoite or cyst could be detected after 35 and 45 cycles, respectively. In conjunction with a rapid DNA isolation method, this PCR was used to identify *N. fowleri* directly from primary cultures of environmental samples.

Naegleria fowleri is a small free-living amoeba (FLA) found in warm freshwater habitats worldwide. This organism is pathogenic to humans, causing fatal primary amoebic meningoencephalitis (PAM) (3, 11). During monitoring of the environment for the presence of *N. fowleri*, it is important to reliably differentiate this species from other closely related thermophilic *Naegleria* spp. (5, 12, 13, 22).

Several techniques have been developed for the identification of *N. fowleri*. These include species-specific monoclonal antibodies (25), isoenzyme electrophoretic profiles (5, 17), characterization of DNA restriction fragment length polymorphisms (RFLPs) (6, 12, 15), and PCR. Amplification of repetitive DNA has been used for the identification of *N. fowleri* from purified nucleic acids, in crude preparations of infected mouse brains (16), and from environmental cultures (19, 20). Randomly amplified polymorphic DNA typing has been used to differentiate *Naegleria* spp. and has also been found to detect minor variations in *N. fowleri* strains (24).

Potentially, PCR is a highly specific, sensitive, and rapid method for the identification of microbes. Here, we describe the development of a PCR-based protocol for the identification of *N. fowleri* soon after primary culture isolation from the environment.

The organisms used in this study are listed in Table 1. The *Naegleria* sp. designations reflect recently proposed changes to this genus (8). The culture and extraction of DNAs from these strains were carried out as described previously (12). The *N. fowleri*-specific chromosomal DNA probe pB2.3 (12) was partially sequenced by using the Sequenase version 2.0 kit (United States Biochemical Corp., Cleveland, Ohio) according to the manufacturer's protocol. Forward and reverse PCR primers were designed from the sequence data of the clone. The pB2.3 forward primer (p3f) used in PCR was GCTATCGAATGGA TTCAAGC, and the reverse primer (p3r) was CACTACTCG TGGAAGGCTTA.

PCR was performed in a 100-μl volume consisting of 1× *Taq* DNA polymerase buffer (10 mM Tris-HCl [pH 9.0], 50 mM KCl, 1.5 mM $MgCl_2$, and 0.1% Triton X-100), 0.2 mM deoxynucleoside triphosphates (dNTPs), 1 μM (each) each primer, 2.5 U of *Taq* DNA polymerase (Promega Corp., Southampton, England), and approximately 100 ng of DNA that had previously been heated at 96°C for 5 min. The reaction mixture was overlaid with 100 μl of mineral oil. The standard temperature program was 1 min at 95°C, 1 min at 55°C, and 1.5 min at 72°C for 35 cycles, with a final 7 min at 72°C.

A rapid extraction method for the isolation of *N. fowleri* DNA from trophozoites or cysts taken directly from nonnutrient agar plates seeded with the bacterium *Klebsiella edwardsii* (NNA-*K. edwardsii* plates) was developed. A 1-cm area of amoeba growth was swept from the plate with a disposable bacteriological loop. Amoebae were resuspended in 70 μl of ice-cold PCR lysis solution (10 μl of 10× *Taq* DNA polymerase buffer, 1 μl of 10-mg/ml proteinase K, and distilled water to 70 μl), overlaid with 100 μl of sterile mineral oil, and incubated at 60°C for 1 h. The tubes were placed in a boiling water bath for 10 min to inactivate the proteinase K and were chilled on ice. Following the addition of dNTPs, primers, and 2.5 U of *Taq* DNA polymerase to a final volume of 100 μl, PCR was performed as described above. Care was taken during sampling, as the carryover of agar was found to be inhibitory to PCR.

Amplification products were detected by analyzing 20 μl of PCR mixture on 1.2% agarose–Tris–borate–EDTA gels in the presence of 0.5 μg of ethidium bromide per ml (18). DNAs from gels were transferred to Hybond N nylon membranes (Amersham International, Buckinghamshire, England) for hybridization with the pB2.3 plasmid clone labelled with 5′-[α-^{32}P]dCTP (18). PCR and hybridization studies were also performed with 10-fold dilutions of *N. fowleri* MCM DNA from 10 ng to 100 fg.

In an environmental survey, mud and algal samples were collected from various sites at the natural hot springs in Bath, England, the source of a fatal case of PAM in 1978 (12, 13). Thermophilic *Naegleria* spp. were isolated by culture on NNA-*K. edwardsii* plates at 44°C (12). Isolates were tested by PCR by using the rapid DNA extraction method described above. A second scrape was then made of the primary sampling area on the culture plate with a fresh loop and inoculated into a corresponding well of a microtiter plate containing 100 μl of NNA-*K. edwardsii*. When a PCR product was detected by agarose gel electrophoresis, the strain was identified in the well of the microtiter culture plate and subcultured on a fresh NNA-*K. edwardsii* plate. The strain was then adapted to axenic culture and tested for whole-cell DNA RFLPs to confirm the species identity (6, 12).

The results of PCR with purified DNAs of *Naegleria* spp., other FLAs, protozoans, algae, and bacteria are shown in Fig. 1. A single 1.5-kbp product was detected in each of the 19 *N.*

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TABLE 1. Organisms examined in the development of an *N. fowleri* PCR

Species or DNA source	Strain[a]	Origin	Source[b]
N. fowleri	MCM	PAM, Bath, England	a
	NF-3	Hot springs, Bath, England	a
	158-44-3	Power station, Nottingham, England	a
	168-44-5	River water, Nottingham, England	a
	KUL	PAM, Belgium	b
	NF-59	Thermal water, Czech Republic	l
	3e9	Power station, France	m
	NA 420c	Power station, France	m
	HB-1	PAM, United States	b
	6088	PAM, United States	b
	CDC0487:1	PAM, United States	h
	NF-124	Thermal water, United States	b
	Carter 69	PAM, Australia	b
	CCAP 1518/3 (Morgan)	PAM, Australia	c
	CCAP 1518/4 (PA-90)	Domestic water supply, Australia	c
	Ng 060	Domestic water supply, Australia	e
	MSM	PAM, New Zealand	c
	NHI	PAM, New Zealand	b
	HK-1	PAM, Hong Kong	n
N. lovaniensis	Aq/9/1/45D^T	Aquarium, Belgium	b
	C-0490	Hot springs, Bath, England	a
	EX5D/5	Hospital cooling tower, England	a
	HSP 154	Thermal springs, United States	d
	Ng 045	Water supply, Australia	e
N. australiensis	PP397^T	Flood water, Australia	c
	4684.11	Hot springs, Bath, England	a
	5858.3	Hot springs, Bath, England	a
	5858.5	Hot springs, Bath, England	a
	LSR34a	Thermal water, France	c
	PV2891	Thermal spa water, Italy	c
	NJ	Pond, India	c
N. italica	AB-T-F_3^T	Thermal spa water, Italy	b
N. andersoni	PPMFB-6^T	Aquarium, Australia	b
N. jamiesoni	T56E^T	Tropical fish import, Singapore	b
N. jadini	CCAP 1518/2^T	Swimming pool, Belgium	g
N. gruberi (cluster 4)	CCAP 1518/1e	Freshwater, United States	g
Willaertia magna	Z503^T	Bovine feces, France	b
	NI 13	Pond, India	f
	PAOB CL_4	Pond, Spain	f
Acanthamoeba castellanii	CCAP 1501/1a (Neff)	Soil, United States	g
Acanthamoeba culbertsoni	ATCC 30171 (A-1)	Tissue culture, United States	i
Acanthamoeba lenticulata	PD_2S	Swimming pool, France	b
Acanthamoeba rhysodes	ATCC 30973	Soil, England	i
Acanthamoeba polyphaga	CCAP 1501/16	Freshwater, United States	g
Acanthamoeba polyphaga	Dav	Keratitis, England	a
Acanthamoeba polyphaga	SHI	Keratitis, England	a
Hartmannella vermiformis	9115.1	Bottled mineral water, England	a
	RB-1	Hot springs, Bath, England	a
Vannella sp.	BF 1690	Tap water, Northern Ireland	a
Vahlkampfia sp.	B-1270	Bottled mineral water, England	a
Tetrahymena pyriformis	CCAP 1630/1w	Unknown, United States	g
Trichomonas vaginalis	O442	Vaginal infection, Bath, England	a
	Q7828	Vaginal infection, Bath, England	a
HeLa cell line			
HEp-2 cell line			
Rhesus monkey kidney cell line			
Human DNA		Leukocytes	
Scenedesmus quadricande			k
Ulothrix fimbriata			k
Anabaena variabilis			k
Escherichia coli	JM101		
K. edwardsii	K10896	National Collection of Type Cultures, Colindale, England	
Pseudomonas aeruginosa	Rb-1760	Hot springs, Bath, England	a
	W5789	Contact lens storage case, England	a
Legionella pneumophila sg 1	Philadelphia	Legionnaires' disease, United States	j
Legionella micdadei			j

[a] A superscript T denotes the type strain of the species.

[b] a, collection of pathogenic FLAs, Bath Public Health Laboratory, Bath, England; b, J. De Jonckheere, Instituut voor Hygiene en Epidemiologie, Brussels, Belgium; c, D. Warhurst, London School of Hygiene and Tropical Medicine, London, England; d, W. O'Dell, Department of Biology, University of Nebraska, Omaha; e, P. Christy, State Water Laboratory, Salisbury, South Australia, Australia; f, R. Michel, Ernst-Rodenwald-Institut Medical Parasitologie, Koblenz, Germany; g, Susan Brown, Culture Collection of Algae and Protozoa, Cumbria, England; h, G. Visvesvara, Centers for Disease Control and Prevention, Atlanta, Ga.; i, T. Nerad, American Type Culture Collection, Rockville, Md.; j, N. Fry, Central Public Health Laboratory, Colindale, England; k, J. Wright, School of Biology and Biochemical Sciences, University of Bath, Bath, England; l, V. Kadlec, Kunz Krajska Hygienicka Stanice, Usti, Czech Republic; m, O. Sparagano, Institut Pasteur De Lyon, Lyon, France; n, K. M. Kam, Hong Kong Government Department of Health, Hong Kong.

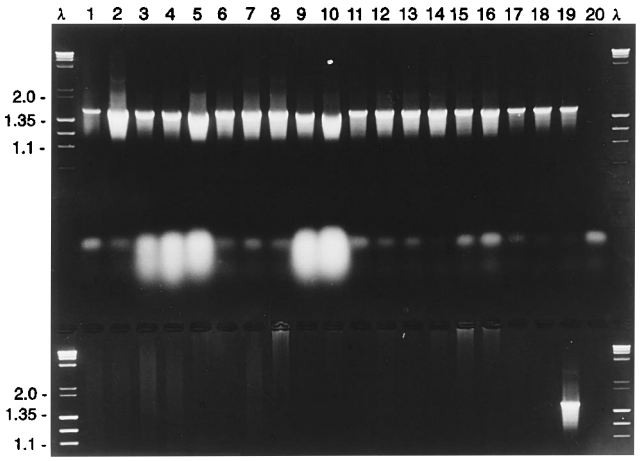

FIG. 1. Agarose gel showing PCR amplification of *N. fowleri* DNA with only primer set p3f and p3r. Top set of lanes: λ, lambda *Hin*dIII/ΦX-174 RF *Hae*III digest; 1 to 19, *N. fowleri* strains; 20, negative control. Bottom set of lanes: 1 to 18, *N. lovaniensis*, *Naegleria australiensis*, *Naegleria italica*, *Naegleria andersoni*, *Naegleria jamiesoni*, *Naegleria jadini*, *Willaertia magna*, *Acanthamoeba* spp., *Tetrahymena pyriformis*, *Trichomonas vaginalis*, mammalian tissue culture cells, algae, *Legionella* spp., *K. edwardsii* K10896, and *Escherichia coli* JM101; 19, positive control of clone pB2.3; 20, negative control. Size standards (in kilobase pairs) are on the left.

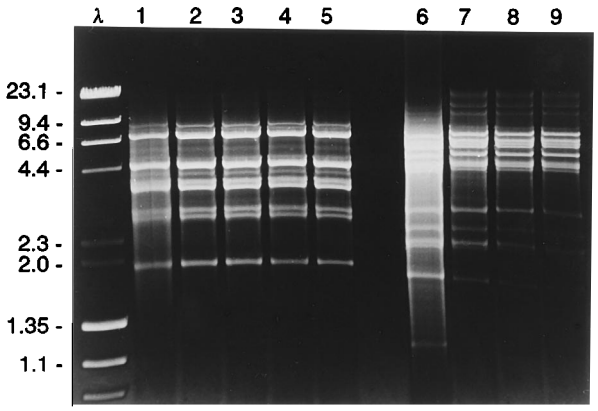

FIG. 2. *Eco*RI whole-cell DNA RFLPs of *N. fowleri* isolates identified by PCR from the thermal springs of Bath, England, and of PCR-negative *Naegleria* strains from that site. Lanes: λ, lambda *Hin*dIII/ΦX-174 RF *Hae*III digest; 1, *N. fowleri* MCM; 2 to 5, *N. fowleri*; 6, *N. lovaniensis* C-0490; 7 to 9, PCR-negative isolates identified as *N. lovaniensis*. Size standards (in kilobase pairs) are on the left.

fowleri strains examined. No amplification was obtained with any other eukaryotic or prokaryotic DNA tested. Following membrane transfer and hybridization with DNA probe pB2.3, only the 1.5-kbp *N. fowleri* PCR product was detected (results not shown). In titration studies with purified *N. fowleri* MCM DNA, an amplification product corresponding to an initial DNA concentration of 1 pg was detected by agarose gel electrophoresis; an amplification product corresponding to 100 fg was detected when probe hybridization was used (results not shown). These are equivalent to approximately 6 trophozoites and 1 trophozoite, respectively (4). By the rapid DNA extraction method, 10 *N. fowleri* trophozoites or cysts resulted in a visible amplification product after 35 PCR cycles; a similar result was obtained with 1 organism when PCR was extended to 45 cycles (results not shown). Nonspecific amplification products were not observed with 45 PCR cycles.

A total of 38 thermophilic *Naegleria* strains were isolated from the natural hot springs in Bath, England. Of these, four gave a positive amplification product of the expected size by PCR. These showed whole-cell DNA *Eco*RI RFLPs characteristic of *N. fowleri* (Fig. 2). All three isolates that gave no PCR amplification showed *Eco*RI RFLPs typical of *Naegleria lovaniensis* (Fig. 2). This represents a proportion of *N. fowleri* isolates higher than that usually found at this site (12, 13). *N. lovaniensis* grows at a faster rate on NNA-*K. edwardsii* medium than *N. fowleri* does (12) and may suppress the presence of the latter during culture isolation. The ability to test isolates as soon as they appear on culture plates may prevent the loss of *N. fowleri* due to overgrowth of *N. lovaniensis* and other thermophilic FLAs.

Although a PCR for the identification of *N. fowleri* from the environment has previously been described (20), that protocol required amoebal DNA to be purified by phenol-chloroform extraction and ethanol precipitation. PCR products were then detected by agarose gel electrophoresis and hybridization with an oligonucleotide probe within the amplified region. The protocol developed here uses a rapid DNA extraction method that is applicable to both trophozoites and cysts and can be used directly in PCR. Following primary culture isolation, the process of DNA extraction, PCR, and gel electrophoresis can be completed in about 6 h. This is a significant advantage over other identification methods for *N. fowleri*, such as isoenzyme or whole-cell DNA RFLP analysis, that usually require strains to be adapted to axenic culture, which can be a lengthy process. The *N. fowleri* 1.5-kbp PCR product has two *Alu*I internal restriction sites, producing fragments of 840, 480, and 170 bp, and one site with *Hae*III, giving two fragments of 1,330 and 230 bp (results not shown). This was found for all of the strains tested, indicating that the amplified region is homogeneous within this species and can be used to verify that correct amplification of *N. fowleri* DNA has occurred.

Although environmental samples can contain components inhibitory to *Taq* DNA polymerase, the direct demonstration of microorganisms in water by PCR has previously been reported (1, 2, 10, 14, 21). Methods for the extraction of bacterial DNA from soil samples have also previously been described (9, 23). Although they have not been studied here, evaluations of these methods may lend themselves to the direct PCR detection of *N. fowleri* in environmental samples without the need for prior culture isolation. However, the ability to refer to a culture isolate is important as it enables the PCR finding to be confirmed by other methods, such as whole-cell DNA RFLP analysis. This technique has also been shown to be useful in the epidemiological subtyping of *N. fowleri* (7, 12).

Finally, *N. fowleri* PAM is a rare but usually fatal infection. The clinical symptoms and microscopic appearance of the cerebrospinal fluid are typical of pyogenic meningitis. This can result in a delayed diagnosis or misdiagnosis (3, 11). No amplification of human tissue culture or leukocytes was found with the PCR developed here, indicating that it may have application in the rapid and sensitive diagnosis of PAM.

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