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Identification and Epidemiological Typing of *Naegleria fowleri* with DNA Probes

SIMON KILVINGTON[1]* AND JOHN BEECHING[2]

Public Health Laboratory, Royal United Hospital, Combe Park, Bath BA1 3NG,[1] and School of Biology and Biochemistry, University of Bath, Claverton Down, Bath BA2 7AY,[2] England

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***Naegleria fowleri* is a small free-living amoeboflagellate found in warm water habitats worldwide. The organism is pathogenic to humans, causing fatal primary amoebic meningoencephalitis. When monitoring the environment for the presence of *N. fowleri*, it is important to reliably differentiate the organism from other closely related but nonpathogenic species. To this end, we have developed species-specific DNA probes for use in the rapid identification of *N. fowleri* from the environment. Samples were taken from the thermal springs in Bath, England, and cultured for amoebae. Of 84 isolates of thermophilic *Naegleria* spp., 10 were identified as *N. fowleri* by probe hybridization. The identity of these isolates was subsequently confirmed by their specific whole-cell DNA restriction fragment length polymorphisms (RFLPs). One DNA clone was found to contain a repeated element that detected chromosomal RFLPs that were not directly visible on agarose gels. This enabled the further differentiation of strains within geographically defined whole-cell DNA RFLP groups. *N. fowleri* DNA probes represent a specific and potentially rapid method for the identification of the organism soon after primary isolation from the environment.**

Naegleria fowleri is a small free-living amoeba found in warm freshwater habitats worldwide. The organism is pathogenic to humans, causing primary amoebic meningoencephalitis (PAM) (4). Infection results from the inoculation of the organism into the nasal passages, usually while bathing, and is almost invariably fatal (17, 21). It is therefore important to identify sites containing *N. fowleri* in the interest of preventive public health microbiology. This requires methods that are both accurate and reliable for the differentiation of *N. fowleri* from other closely related thermophilic *Naegleria* spp. Although not pathogenic, *Naegleria lovaniensis* resembles *N. fowleri* in growth at temperatures of up to 45°C, cytopathogenicity for tissue culture cells, and antigenicity (35). Although mouse pathogenicity was originally used to differentiate the species (3, 16), the recognition that *Naegleria australiensis* was pathogenic for mice, albeit less so than *N. fowleri*, rendered the test nonspecific (9).

A variety of techniques have been investigated for the identification of *N. fowleri*. These include serological tests with species-specific monoclonal antibodies (38), isoenzyme electrophoretic profiles (10, 19, 27), characterization of DNA restriction fragment length polymorphisms (RFLPs) (11, 23, 26), and the amplification of unique regions of *N. fowleri* DNA by PCR (25, 34). The advantage of isoenzyme and whole-cell RFLP analyses is that they allow the simultaneous identification of all currently recognized *Naegleria* species. However, such methods are expensive and time-consuming and require large numbers of amoebae that usually have to be adapted to axenic (bacteria-free) culture for testing.

Genomic DNA is a highly complex structure in which specific sequences that uniquely characterize an organism occur. Advances in recombinant DNA technology enable these regions to be isolated, cloned in to a suitable vector, and labelled as probes in the detection of homologous sequences in the rapid identification of organisms (1, 36). The key step in these procedures is to identify probes that will detect DNA sequences in a given organism and not cross-react with others. Such an approach has been used successfully to develop specific and sensitive DNA probes for a variety of microbes, including pathogenic protozoa such as *Entamoeba histolytica* (2, 33), *Giardia intestinalis* (*duodenalis*) (13, 22), *Trichomonas vaginalis* (31), *Babesia bovis* (24), and *Plasmodium falciparum* (1).

Although DNA clones from *Naegleria gruberi* and *N. fowleri* have been described, these cross-hybridize with other species and have been used only for taxonomic studies (7, 8). The object of this study was to isolate clones of *N. fowleri* DNA for use as probes in the development of a method to rapidly and specifically identify the organism soon after primary isolation from the environment.

MATERIALS AND METHODS

Organisms studied. The organisms used in this study and their sources are given in Table 1. Strains of *Naegleria* spp., *Acanthamoeba* spp., and *Willaertia magna* were maintained at 32°C in serum-casein-glucose-yeast extract medium (ATCC 1021), modified by the inclusion of 0.1% liver digest, or Proteose Peptone-yeast extract-nucleic acid-folic acid-hemin medium supplemented with 10% heat-inactivated fetal calf serum (ATCC 1034 medium). Other free-living amoebae (FLA), *Hartmannella vermiformis*, *Vannella* spp., and a *Vahlkampfia* sp., were grown at 32°C on nonnutrient agar plates (1.5% plain agar in 0.25× strength Ringer's solution) seeded with the bacterium *Klebsiella edwardsii* K10896 (NNA-*K. edwardsii* medium). *Tetrahymena pyriformis* was grown in brain heart infusion broth at room temperature. *Trichomonas vaginalis* was grown in brain heart infusion broth supplemented with 0.1% cysteine hydrochloride, 0.02% ascorbic acid, and 10% heat-inactivated horse serum at 37°C. *Pseudomonas aeruginosa*, *Escherichia coli*, and *K. edwardsii* were grown on nutrient agar in air at 37°C.

DNA isolation. For axenic cultures of FLA, *Tetrahymena pyriformis*, *Trichomonas vaginalis*, human leukocytes, tissue culture cells, and algae, approximately 10^8 late-log-phase cell cultures were harvested by centrifugation at 500 × *g* for 10 min at room temperature, washed once with phosphate-buffered saline (PBS), and resuspended in cell lysis buffer (10 mM Tris-HCl [pH 8.0], 0.1 M NaCl, 0.1 M EDTA). For FLA growing on NNA-*K. edwardsii*, approximately 10^7 trophozoites were washed from the plates with ice-cold PBS, centrifuged at 500 × *g* for 10 min, washed once with PBS, and resuspended in cell lysis buffer. For the bacterial strains, 10 colonies were picked directly into cell lysis buffer.

* Corresponding author. Phone: 0225-424624/824743. Fax: 0225 448262.

TABLE 1. Organisms examined in the development of *N. fowleri*-specific DNA probes

Species	Strain[a]	Origin	Source[b]
N. fowleri	MCM	PAM, Bath, England	
	NF-3	Thermal spring water, Bath, England	
	158-44-3	Power station, Nottingham, England	
	168-44-5	River water, Nottingham, England	
	KUL	PAM, Belgium	a
	NF-59	Czech Republic	k
	HB-1	PAM, USA	a
	6088	PAM, USA	a
	CDC:0487:1	PAM, USA	g
	NF-124	Thermal water, USA	a
	Carter 69	PAM, Australia	a
	CCAP 1518/3 (Morgan)	PAM, Australia	b
	CCAP 1518/4 (PA-90)	Domestic water supply, Australia	b
	Ng 060	Domestic water supply, Australia	d
	MSM	PAM, New Zealand	b
	NHI	PAM, New Zealand	a
N. lovaniensis	Aq/9/1/45D^T	Aquarium, Belgium	a
	C-0490	Thermal spring water, Bath, England	
	EX5D/5	Hospital cooling tower, England	
	HSP 154	Hot springs, USA	c
	Ng 045	Water supply, Australia	d
N. australiensis	PP397^T	Flood water, Australia	b
	4684.11	Thermal spring water, Bath, England	
	5858.3	Thermal spring water, Bath, England	
	5858.5	Thermal spring water, Bath, England	
	LSR34a	Thermal water, France	b
	PV2891	Thermal spring water, Italy	b
	NJ	Pond, India	b
N. australiensis italica	AB-T-F_3^T	Thermal spa water, Italy	a
N. andersoni	PPMFB-6^T	Aquarium, Australia	a
N. andersoni jamiesoni	T56E^T	Tropical fish import, Singapore	a
N. jadini	CCAP 1518/2^T	Swimming pool, Belgium	f
N. gruberi	CCAP 1518/1e	Freshwater, USA	f
Willaertia magna	Z503^T	Bovine feces, France	a
	NI 13	Pond, India	e
	PAOB CL_4	Pond, Spain	e
Acanthamoeba castellanii	CCAP 1501/1a (Neff)	Soil, USA	f
Acanthamoeba culbertsoni	ATCC 30171 (A-1)	Tissue culture, USA	h
Acanthamoeba lenticulata	PD_2S	Swimming pool, France	a
Acanthamoeba rhysodes	ATCC 30973	Soil, England	h
Acanthamoeba polyphaga	CCAP 1501/16	Freshwater, USA	f
	Dav	Keratitis, England	
	SHI	Keratitis, England	
Hartmannella vermiformis	9115.1	Bottled mineral water, England	
	RB-1	Thermal spring water, Bath, England	
Vannella sp.	BF 1690	Tap water, Northern Ireland	
Vahlkampfia sp.	B-1270	Bottled mineral water, England	
Tetrahymena pyriformis	CCAP 1630/1w	Unknown, USA	f
Trichomonas vaginalis	Q442	Vaginal infection, Bath, England	
	Q7828	Vaginal infection, Bath, England	
HeLa cell line			
Hep2 cell line			
Rhesus monkey kidney cell line			
Human DNA		Leukocytes	
Scenedesmus quadricande			j
Ulothrix fimbriata			j
Anabaena variabilis			j
E. coli	JM101		
K. edwardsii	K10896	National Collection of Type Cultures, Colindale, England	
Pseudomonas aeruginosa	Rb-1760	Thermal spa water, Bath, England	
	W5789	Contact lens storage case, England	
Legionella pneumophila sg 1	Philadelphia	Legionnaires' disease, USA	i
Legionella micdadei			i

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

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

Whole-cell DNA was isolated by adding proteinase K (10 mg/ml) and sarcosyl (20%) to a final concentration of 150 μg/ml and 2%, respectively, and incubating at 56°C for 4 h or overnight. The lysate was extracted once with phenol-chloroform-isoamyl alcohol (25:24:21) saturated with 10 mM Tris-HCl (pH 8.0)–1 M EDTA and once with chloroform-isoamyl alcohol (24:1). These steps were facilitated by the use of SST blood collection tubes (Becton Dickinson, Oxford, England), which contain a silicone gel material that forms a barrier between the organic and aqueous layers after centrifugation (37). The denatured protein is also trapped with the organic layer below the gel barrier. The upper aqueous phase containing the nucleic acids can then be simply poured off into a fresh tube. Nucleic acids were precipitated with an equal volume of isopropanol at −20°C overnight, pelleted by centrifugation at 3,000 × *g* for 15 min, and washed twice with 70% ethanol. After drying briefly, the pellet was dissolved in TE buffer (pH 8.0) containing 20 μg of RNase A per ml and stored at −20°C. DNA concentrations were estimated by comparison with standards spotted onto agarose plates containing the dye Hoechst H33258 with UV illumination (30).

Construction and analysis of λ phage library. Standard protocols were used to construct a genomic library of *N. fowleri* MCM DNA in the λ phage vector EMBL3 (32). Briefly, purified *N. fowleri* MCM DNA was partially digested with limiting amounts of the restriction endonuclease *Sau*3AI (Northumbria Biologicals Ltd., Northumberland, England) to produce fragments ranging from 15 to 22 kbp, as determined by electrophoresis on a 0.5% agarose gel in 0.5× Tris-borate-EDTA (TBE) buffer (32). The fractionated DNA was then extracted with phenol-chloroform-isoamyl alcohol, precipitated with isopropanol, washed with 70% ethanol, and dissolved in distilled H_2O. Following dephosphorylation with calf intestinal alkaline phosphatase (32), 300 ng of DNA was ligated with 1 μg of the λ phage vector EMBL3 according to the supplier's recommendations (Stratagene, Cambridge, England). Ligation was performed for 1 h at 22°C followed by 48 h at 4°C with 4 U of T4 DNA ligase in buffer supplied by the manufacturer (Northumbria Biologicals Ltd.). Recombinant phages were packaged by using Gigapack II Plus packaging extract (Stratagene) and used to infect *E. coli* P2392. Dilutions of the cells were made in phage dilution buffer and plated onto NZY agar for incubation at 37°C overnight (32).

Duplicate plaque lifts from NZY agar plates to Hybond N nylon membranes (Amersham, Buckinghamshire, England) were made, and the membranes were screened with whole-cell DNA from either *N. fowleri* MCM or *N. lovaniensis* C-0490 labelled with deoxycytidine 5′-[α-^{32}P]triphosphate ([^{32}P]dCTP) by the random primer method (15). Prehybridization and hybridization of membranes were performed at 65°C in Westneat buffer (39). Membranes were washed twice at room temperature for 15 min each in 2× SSC–0.1% sodium dodecyl sulfate (SDS) (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate), for 15 min in 2× SSC–0.1% SDS at 65°C, for 30 min in 1× SSC at 65°C, and finally for 30 min in 0.1× SSC at 65°C. Autoradiography was performed with Fuji RX medical X-ray film in autoradiography cassettes with intensifying screens at −70°C.

Clones reacting strongly with the *N. fowleri* whole-cell DNA probe and weakly or not at all with that of *N. lovaniensis* were picked, replated, and rescreened under the same conditions. Large-scale λ phage preparations of the selected recombinant clones were made from NZY plate cultures (32).

Subcloning into plasmid pUC18 vector. Purified λ phage clones were digested with the restriction endonuclease *Sal*I and separated by electrophoresis in 0.7% agarose–Tris–acetate–EDTA (TAE) gels (32). Clones showing internal *Sal*I restriction sites were selected, and fragments were recovered from the gels by absorption to silica particles (Geneclean II; Stratech Scientific, Bedfordshire, England). The purified DNA inserts were then subcloned into the plasmid vector pUC18 that had been linearized with *Sal*I and dephosphorylated with calf intestinal alkaline phosphatase (32). Ligation, transformation of *E. coli* JM101, and selection of recombinant cells on ampicillin–5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal)–isopropylthiogalactopyranoside (IPTG) agar were done by standard methods (32). Plasmid subclones from small- and large-scale cultures of transformed *E. coli* JM101 were isolated by the alkaline lysis method (32).

Southern hybridization analysis of DNA. Whole-cell *Naegleria* DNA (3 μg) was digested with 10 to 20 U of restriction endonucleases for 18 h at 37°C with the appropriate reaction buffers provided with the enzymes (Northumbria Biologicals). Samples were loaded onto horizontal 0.7% agarose–TBE gels for electrophoresis in 0.5× TBE buffer at 1.5 V/cm overnight (32). DNA standards of *Hin*dIII-digested λ plus *Hae*III-digested ϕX174 RF or *Hin*dIII-digested λ plus *Hin*cII-digested ϕX174 RF (Pharmacia LKB, Milton Keynes, England) were included as size markers. Gels were stained with 1 μg of ethidium bromide per ml in distilled H_2O for 1 h, destained in distilled H_2O for 20 min, and photographed under shortwave UV transillumination with Polaroid 665 film and a Kodak Wratten 23A orange filter.

The gels were soaked in 0.25 M HCl for 15 min, rinsed in distilled H_2O, and gently agitated in 0.4 M NaOH–1.5 M NaCl twice for 15 min each, and the DNA was transferred overnight by capillary blotting onto Hybond N nylon membranes with 0.4 M NaOH–1.5 M NaCl. The membranes were then air dried and exposed, DNA side down, on a UV transilluminator for 5 min. pUC18 recombinant clones were digested with *Sal*I and separated on 0.7% agarose–TAE gels. The desired fragments were excised from the gels, purified by silica absorption, and labelled with [^{32}P]dCTP and used in hybridization studies under the conditions described above.

Dot-blot hybridization. Purified DNA from the organisms listed in Table 1 was adjusted to approximately 500 ng in 200 μl of 0.4 M NaOH–10 mM EDTA, heated at 70°C for 15 min, and chilled on ice. The denatured DNA was then immobilized onto Hybond N nylon membranes with a commercial dot-blotting apparatus (Bio-Rad, Hemel Hempsted, England), air dried, and exposed, DNA side down, on a UV transilluminator for 5 min. Membranes were hybridized with *N. fowleri* pUC18 subclones labelled with [^{32}P]dCTP as described above. *N. fowleri* MCM DNA dilutions ranging from 1.6 ng to 1.6 pg were also immobilized onto membranes and used to test the sensitivity of detection by the probes. *N. fowleri* MCM DNA was also extracted by the alkaline lysis method used for the isolation of *E. coli* plasmid DNA and tested in the dot-blot studies.

Identification of *N. fowleri* from the environment with DNA probes. The natural hot springs in Bath, England, have previously been shown to contain *N. fowleri* and were linked to a fatal case of PAM in 1978 (20). Mud and algal samples were collected in sterile polycarbonate containers and transported to the laboratory without delay for processing the same day. The contents were vigorously shaken, and the samples were spotted onto the surface of square NNA-*K. edwardsii* plates (100 by 100 mm). For each sample, two lots of 16 mud or algal spots were distributed evenly over an NNA-*K. edwardsii* plate and allowed to dry into the agar. Plates were incubated at 44°C in sealed polythene bags and examined daily for up to 5 days for the presence of *Naegleria* trophozoites migrating away from the inocula.

For each *Naegleria* isolate on NNA-*K. edwardsii* medium, a 2- to 3-cm region of dense trophozoite growth was gently scraped with a disposable 1-μl bacteriological loop (Nunc, Gibco, Middlesex, England) and inoculated into a well of a 96-well microtiter plate containing 200 μl of 0.4 M NaOH–10 mM EDTA. Previous studies had shown that approximately 10^4 trophozoites were recovered on the loop, which is equivalent to 2 ng of *N. fowleri* chromosomal DNA. The use of 0.4 M NaOH–10 mM EDTA was found to cause immediate lysis of the trophozoites and inactivation of amoebal nuclease activity. Inoculated plates were sealed with clear adhesive film and stored at 4°C until required for testing.

With a fresh loop, the primary sampling area was again scraped and inoculated into a corresponding well of a second microtiter plate containing 100 μl of NNA-*K. edwardsii*. Sufficient trophozoites remain at the primary sampling site to establish growth in the well of the microtiter plate. The plate was then sealed with clear adhesive film and incubated at 37°C for 2 days. Encystment also occurs during incubation, and the culture plates may then be stored at 4°C for at least several weeks for subsequent recovery of the strains.

The primary microtiter plate containing the alkali-lysed trophozoites was incubated at 70°C for 15 min on the surface of a water bath and chilled on ice. The contents of the wells were then transferred to a Hybond N nylon membrane with a dot-blotting apparatus. This process was simplified by use of a 12-place multichannel pipette. Controls of *N. fowleri*, *N. lovaniensis*, and *K. edwardsii* DNA were also included. After drying at room temperature, the membrane was placed DNA side down onto a transilluminator and exposed for 5 min to fix the DNA. The DNA dot-blots were then hybridized with *N. fowleri* probe pB2.2.4 labelled with [^{32}P]dCTP. Following hybridization and washing, the membranes were autoradiographed overnight.

When positive probe hybridization was detected on the autoradiographs, the strain was identified in the corresponding well of the replicate microtiter culture plate and subcultured onto fresh NNA-*K. edwardsii*. The trophozoites were then adapted to axenic growth and examined for whole-cell DNA *Eco*RI RFLPs to verify their identity as *N. fowleri* as described above. Random *Naegleria* isolates that were negative by probe hybridization were also examined for their whole-cell DNA *Eco*RI RFLPs.

RESULTS

Characterization of probes. A genomic library of *N. fowleri* MCM DNA was constructed in the λ phage vector EMBL3. The genome size of *N. fowleri* has been estimated as 140,000 kbp (5). To ensure complete cloning of the *N. fowleri* genome in a series of 20-kbp fragments would require 3.2×10^4 PFU/μg of DNA (32). The library constructed in this study yielded 2.5×10^6 PFU and is therefore considered highly representative of the entire *N. fowleri* genome.

Screening of the clones with labelled whole-cell *N. fowleri* or *N. lovaniensis* DNA identified several clones that reacted only with former. Two λ phage clones that had internal *Sal*I restriction sites were selected for subcloning into the pUC18 plasmid vector. This gave rise to the subclones pB2.2, a 6.1-kbp fragment, and pB2.3, a 1.6-kbp fragment. pB2.2 was found to have an internal *Eco*RI restriction site that gave rise to a 0.8-kbp fragment. This also was cloned into the *Eco*RI site in pUC18 and gave rise to the subclone pB2.2.4.

***N. fowleri* and *N. lovaniensis* agarose gel whole-cell DNA RFLPs.** *N. fowleri* and *N. lovaniensis* whole-cell DNA preparations digested with *Eco*RI, *Hin*dIII, or *Eco*RI plus *Hin*dIII

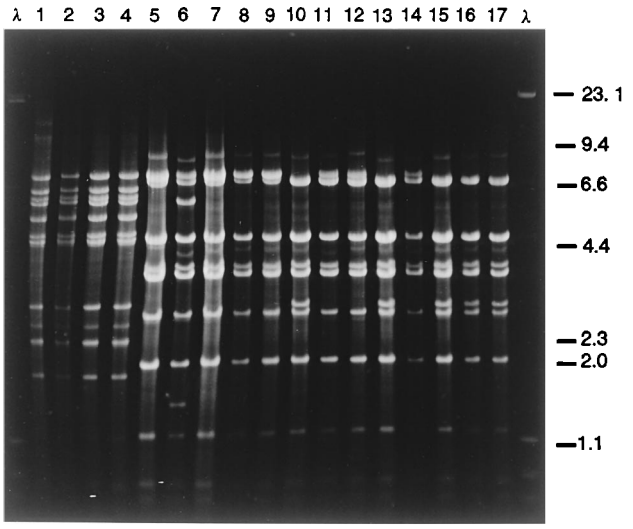

FIG. 1. *N. lovaniensis* and *N. fowleri Eco*RI RFLPs. Lanes λ, lambda *Hin*dIII plus ϕX174 RF *Hin*cII digests as size markers; sizes are shown in kilobases. Lanes 1 to 4, *N. lovaniensis* HSP 154, Aq/9/1/45D, EX5D/25, and C-0490, respectively. Lanes 5 to 17, *N. fowleri* NHI, MSM, 1518/4, 1518/3, Carter 69, CDC:0487:1, 6088, NF-124, HB-1, NF-59, KUL, NF-3, and MCM, respectively.

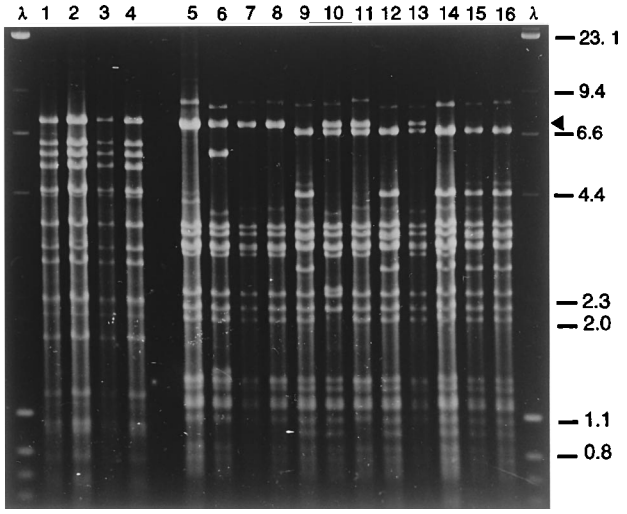

FIG. 3. *N. lovaniensis* and *N. fowleri Eco*RI-*Hin*dIII RFLPs. Lanes λ, lambda *Hin*dIII plus ϕX174 RF *Hin*cII digests. Lanes 1 to 4, *N. lovaniensis* HSP 154, Aq/9/1/45D, EX5D/25, and C-0490, respectively. Lanes 5 to 16, *N. fowleri* NHI, MSM, 1518/4, Carter 69, CDC:0487:1, 6088, NF-124, HB-1, NF-59, KUL, NF-3, and MCM, respectively. The arrowhead indicates a 7.5-kbp band present only in strains NF-59, NF-124, and 6088.

enabled the detection of numerous RFLPs directly by agarose gel electrophoresis (Fig. 1 to 3). All the strains of *N. lovaniensis* gave homologous *Eco*RI, *Hin*dIII, and *Eco*RI-*Hin*dIII RFLPs. These were quite different from those found with *N. fowleri* and readily enabled the differentiation of the two species. In contrast to the findings with *N. lovaniensis*, significant variations in agarose gel RFLPs were detected between *N. fowleri* strains that corresponded to the geographic origin of the strains. With *Eco*RI or *Hin*dIII, strains from Europe were distinguished from those of the Antipodes, while isolates from the United States were of either profile. However, with *Eco*RI-*Hin*dIII digestion, the U.S. strains (6088 and NF-124) that showed RFLPs similar to those of strains from the Antipodes were found to have a unique band of 7.5 kbp, as indicated in Fig. 3. This profile was also found with strain NF59 from Czechoslovakia. Minor interstrain variations were also detected within the geographically defined groups for *N. fowleri*. With *Hin*dIII, strain 6088 from the United States had an additional 2.5-kbp band (Fig. 2). Strain MSM from New Zealand showed a unique 6.2-kbp band with *Eco*RI and *Eco*RI-*Hin*dIII (Fig. 1 and 3).

Probe hybridization studies. All three pUC18 clones, pB2.2, pB2.3, and pB2.2.4, hybridized specifically to membrane transfers of *N. fowleri* DNA digested with *Eco*RI, *Hin*dIII, or *Eco*RI-*Hin*dIII. No hybridization occurred with digests of *N. lovaniensis*. For probes pB2.3 and pB2.2.4, a single band was detected in all strains of 1.6 kbp for *Eco*RI (Fig. 4), 2.2 kbp for *Hin*dIII (Fig. 5), and 1 or <0.8 kbp for *Eco*RI-*Hin*dIII, respectively.

In contrast, probe pB2.2 detected numerous RFLPs, indi-

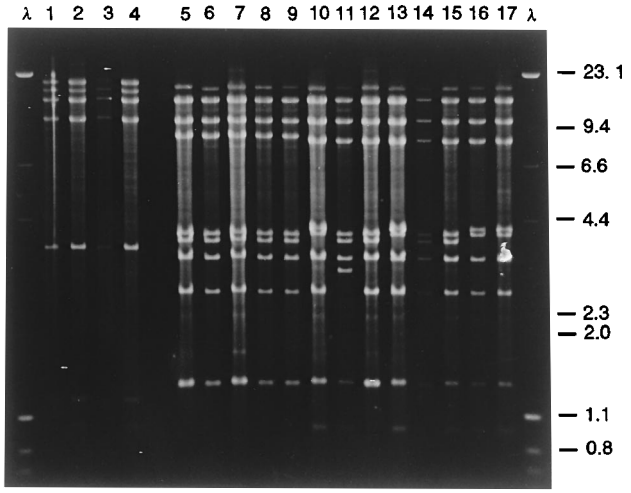

FIG. 2. *N. lovaniensis* and *N. fowleri Hin*dIII RFLPs. Lanes λ, lambda *Hin*dIII plus ϕX174 RF *Hin*cII digests. Lanes 1 to 4, *N. lovaniensis* HSP 154, Aq/9/1/45D, EX5D/25, and C-0490, respectively. Lanes 5 to 17, *N. fowleri* NHI, MSM, 1518/4, 1518/3, Carter 69, CDC:0487:1, 6088, NF-124, HB-1, NF-59, KUL, NF-3, and MCM, respectively.

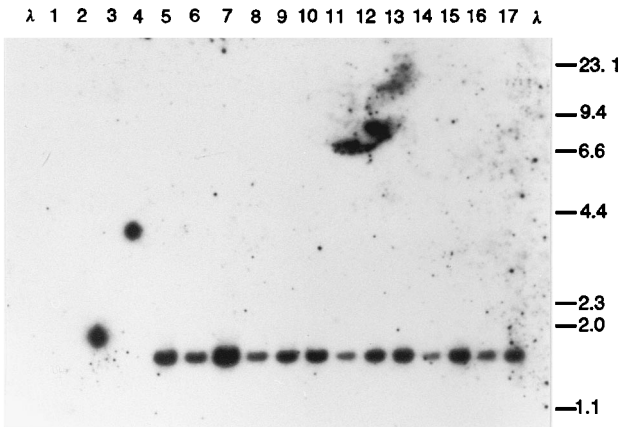

FIG. 4. *N. lovaniensis* and *N. fowleri Eco*RI digests probed with pB2.3. Lanes: λ, lambda *Hin*dIII plus ϕX174 RF *Hin*cII digests. Lanes 1 to 4, *N. lovaniensis* HSP 154, Aq/9/1/45D, EX5D/25, and C-0490, respectively. Lanes 5 to 17, *N. fowleri* NHI, MSM, 1518/4, 1518/3, Carter 69, CDC:0487:1, 6088, NF-124, HB-1, NF-59, KUL, NF-3, and MCM, respectively.

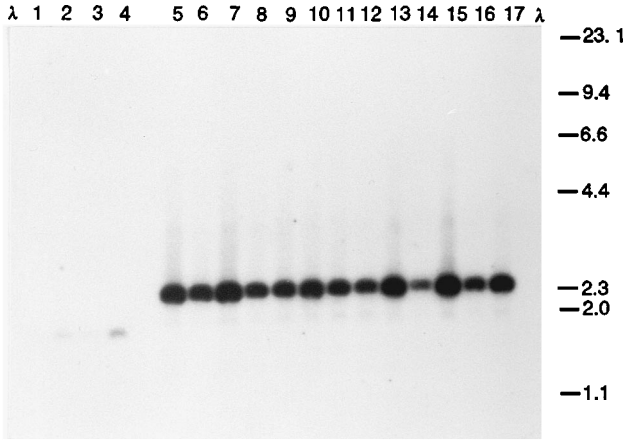

FIG. 5. *N. lovaniensis* and *N. fowleri Hin*dIII digests probed with pB2.2.4. Lanes λ, lambda *Hin*dIII plus ϕX174 RF *Hin*cII digests. Lanes 1 to 4, *N. lovaniensis* HSP 154, Aq/9/1/45D, EX5D/25, and C-0490, respectively. Lanes 5 to 17, *N. fowleri* NHI, MSM, 1518/4, 1518/3, Carter 69, CDC:0487:1, 6088, NF-124, HB-1, NF-59, KUL, NF-3, and MCM.

cating that it was a clone of a repeated element from the *N. fowleri* genome (Fig. 6 to 8). The positions of the variant fragments detected by the probes did not correspond to those detected directly by agarose gel electrophoresis, and they are considered to be of chromosomal origin. Overall, the pB2.2 RFLPs were similar for all the strains, although some minor variations were detected. With *Hin*dIII, all the Antipodean strains had an additional 3-kbp fragment that was absent from the rest, as indicated in Fig. 7. With *Eco*RI-*Hin*dIII, all the Antipodean strains except MSM lacked a 3.4-kbp band that was common to strains from the United States and Europe, as shown in Fig. 8.

The results of DNA dot-blot hybridization studies with probes pB2.3 and pB2.2.4 against *Naegleria* spp., other FLA,

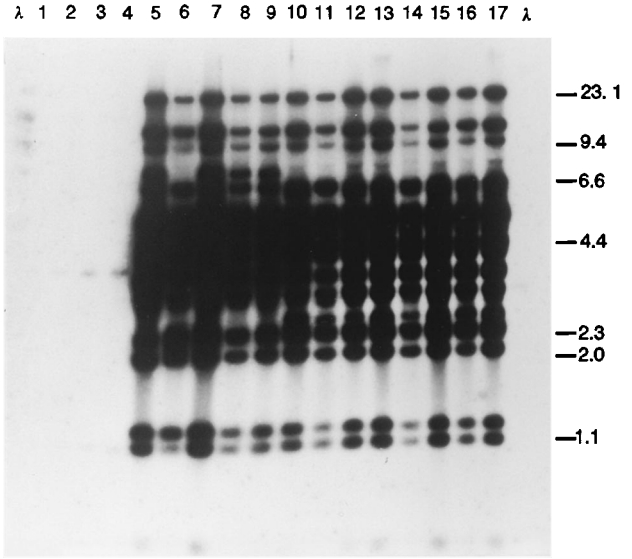

FIG. 6. *N. lovaniensis* and *N. fowleri Eco*RI digests probed with pB2.2. Lanes λ, lambda *Hin*dIII plus ϕX174 RF *Hin*cII digests. Lanes 1 to 4, *N. lovaniensis* HSP 154, Aq/9/1/45D, EX5D/25, and C-0490, respectively. Lanes 5 to 17, *N. fowleri* NHI, MSM, 1518/4, 1518/3, Carter 69, CDC:0487:1, 6088, NF-124, HB-1, NF-59, KUL, NF-3, and MCM.

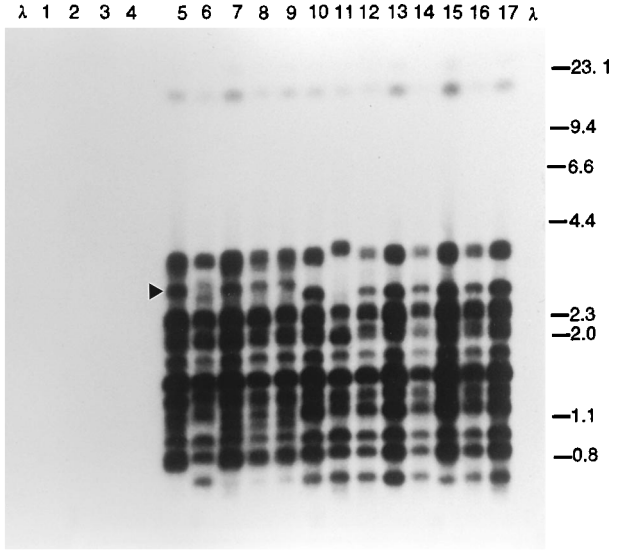

FIG. 7. *N. lovaniensis* and *N. fowleri Hin*dIII digests probed with pB2.2. Lanes λ, lambda *Hin*dIII plus ϕX174 RF *Hin*cII digests. Lanes 1 to 4, *N. lovaniensis* HSP 154, Aq/9/1/45D, EX5D/25, and C-0490, respectively. Lanes 5 to 17, *N. fowleri* NHI, MSM, 1518/4, 1518/3, Carter 69, CDC:0487:1, 6088, NF-124, HB-1, NF-59, KUL, NF-3, and MCM. The arrowhead indicates a 3-kbp band present only in strains from the Antipodes.

Tetrahymena pyriformis, *Trichomonas vaginalis*, mammalian tissue culture cells, human leukocytes, algae, and bacteria are shown in Fig. 9. Only hybridization to *N. fowleri* DNA occurred. A weak hybridization signal was occasionally detected when the probes were tested against *E. coli* JM101 DNA. This

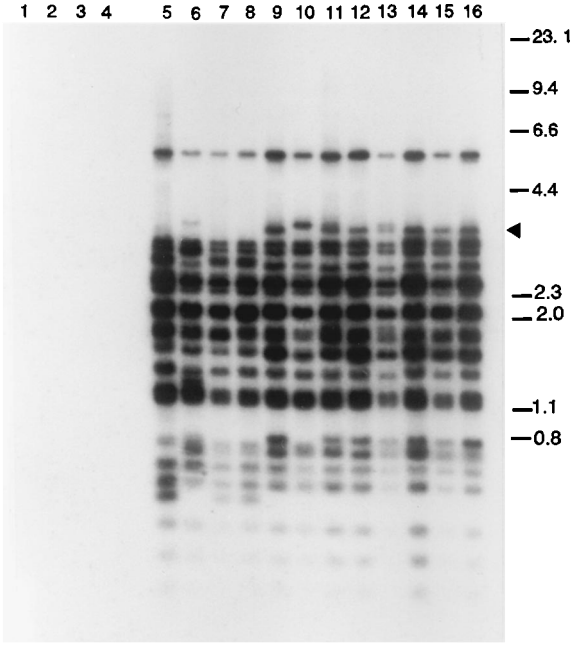

FIG. 8. *N. lovaniensis* and *N. fowleri Eco*RI-*Hin*dIII digests probed with pB2.2. Lanes 1 to 4, *N. lovaniensis* HSP 154, Aq/9/1/45D, EX5D/25, and C-0490, respectively. Lanes 5 to 16, *N. fowleri* NHI, MSM, 1518/4, Carter 69, CDC: 0487:1, 6088, NF-124, HB-1, NF-59, KUL, NF-3, and MCM, respectively. The arrowhead indicates a 3.4-kbp band absent from all Antipodean strains except MSM.

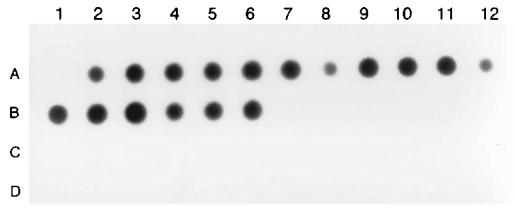

FIG. 9. Dot-blots of DNA from *N. fowleri* and other organisms hybridized with pB2.3. A1, *N. fowleri* MCM mtDNA/rDNA; A2 to B6, *N. fowleri* strains; B7 to B12, *N. lovaniensis* strains; C1 to C12, strains of *N. australiensis*, *N. australiensis italica*, *N. andersoni*, *N. andersoni jamiesoni*, *N. jadini*, and *W. magna*; D1 to D12, strains of *Acanthamoeba* spp., other FLA, *Tetrahymera pyriformis*, mammalian tissue culture cells, *Trichomonas vaginalis*, algae, *Pseudomonas aeruginosa*, *Legionella* spp., *K. edwardsii* K10896, and *E. coli* JM101.

was thought to be due to the presence of small amounts of *E. coli* chromosomal DNA that were not completely removed by the alkaline lysis method used to isolate the plasmid clones from the bacterium. The contaminating DNA then becomes labelled with the probe insert and cross-hybridizes with *E. coli* DNA. No hybridization was found with the probes against *N. fowleri* MCM DNA extracted by the alkaline lysis method. This procedure selects for the recovery of double-stranded circular DNA, such as plasmids, and was found to be suitable for the recovery of mitochondrial DNA (mtDNA) and extrachromosomal ribosomal DNA (rDNA) present in *N. fowleri* (8). This further confirmed that the clones are derived from *N. fowleri* chromosomal DNA.

pB2.3 and pB2.2.4 were of similar sensitivity and could detect as little as 6.25 pg of *N. fowleri* MCM DNA if autoradiographs were exposed for up to 5 days (results not shown). This value is equivalent to approximately 36 trophozoites, assuming a chromosomal DNA content of 0.17 pg per trophozoite (5).

Identification of *N. fowleri* from the environment with DNA probes. A total of 84 isolates of thermophilic *Naegleria* spp. were obtained from the sample sites examined at the hot springs complex in Bath, England. All the isolates were examined by dot-blot analysis with the *N. fowleri* DNA probe pB2.2.4, and a total of 10 positive strains were identified, as shown in Fig. 10. Positions A1, B9, D1, and H1 and A2, B10, D2, and H2 relate to positive and negative controls of known *N. fowleri* and *N. lovaniensis* strains, respectively. Reprobing of the membrane with pB2.3 gave identical hybridization reactions (results not shown).

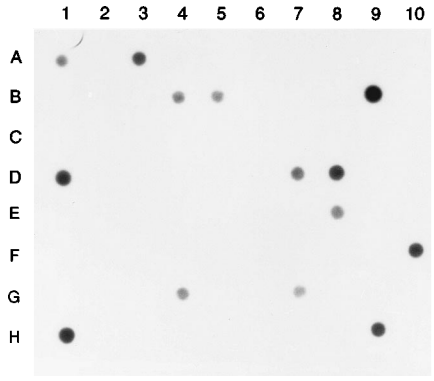

FIG. 10. Detection of *N. fowleri* from the Roman baths by dot-blot hybridization of DNA from environmental isolates of *Naegleria* spp. with pB2.2.4. A1, B9, D1, and H1 are positive controls of *N. fowleri*; A2, B10, D2, and H2 are negative controls of *N. lovaniensis.*

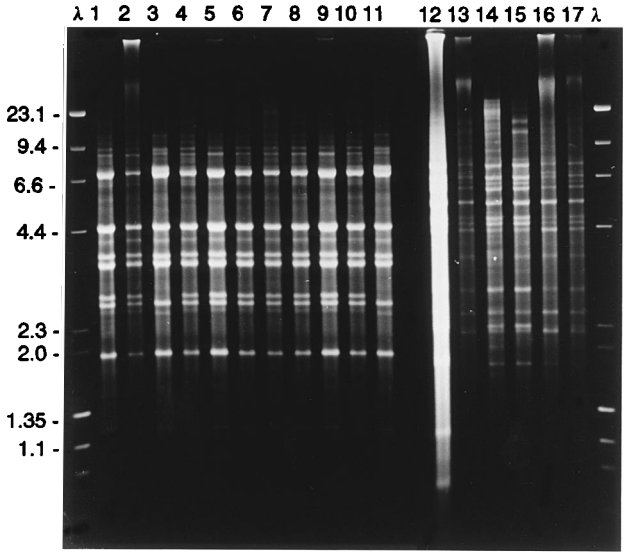

FIG. 11. Whole-cell DNA *Eco*RI RFLP profiles of Roman baths isolates of *N. fowleri* and *N. lovaniensis*. Lanes λ, lambda *Hin*dIII plus ϕX174 RF *Hae*III digests. Lane 1, *N. fowleri* MCM; lanes 2 to 11, *N. fowleri* isolates from the Roman baths identified by probe pB2.2.4; lane 12, *N. lovaniensis* C-0490; lanes 13 to 17, *Naegleria* isolates from the Roman baths which did not hybridize with probe pB2.2.4.

All isolates giving positive hybridization with probe pB2.2.4 were found also to have whole-cell DNA *Eco*RI RFLP profiles typical of *N. fowleri* (Fig. 11). Five isolates which did not hybridize with the probe all had whole-cell DNA *Eco*RI RFLP profiles typical of *N. lovaniensis* (Fig. 11).

DISCUSSION

The genus *Naegleria* and other members of the schizopyrenid amoeba group have been shown to carry their rRNA genes on extrachromosomal plasmids of 14 to 17 kbp (rDNA) (6, 7). As the rDNA of *N. gruberi* has been shown to cross-hybridize with other *Naegleria* species (8), this part of the genome was considered an unsuitable target for the development of *N. fowleri*-specific probes. To minimize the chances of cloning rDNA, the λ phage vector EMBL3, which accepts DNA of 9 to 23 kbp, was chosen to construct a genomic library of *N. fowleri* MCM. The rDNA of *N. fowleri* is cut frequently by the restriction endonuclease *Sau*3AI, used here to generate DNA fragments for cloning, and would therefore be of insufficient size for cloning. This rationale was justified by the isolation of clones that hybridized only to *N. fowleri* chromosomal DNA. Two such clones were subsequently subcloned into the plasmid vector pUC18 to give the DNA probes pB2.2 and pB2.3, representing 6.1- and 1.6-kbp inserts, respectively. A third probe, pB2.2.4, was derived from pB2.2 and represented an internal 0.8-kbp *Eco*RI fragment.

Initially, the probes were tested against membrane transfers of restriction endonuclease digests of *N. lovaniensis* and *N. fowleri* whole-cell DNA. As has been reported previously, digestion of *Naegleria* whole-cell DNA enables the detection of RFLPs directly by agarose gel electrophoresis and is a potent technique for the identification of the species (8). In addition, the RFLPs of *N. fowleri* exhibit interstrain variations that correlate with geographic origin. Strains from the Antipodes are distinct from those of Europe, while those from the United States are of either profile (12, 14, 28). However, here it was found that double digestion with *Eco*RI and *Hin*dIII further

differentiated the U.S. strains with the Antipodean profile and thus defines a fourth geographic subgroup of the species. This profile was also found with strain NF59 from a case of human infection in Czechoslovakia (18) and suggests that the U.S. subgroup also occurs in Europe. Recently, strains of *N. fowleri* from France that showed RFLPs typical of those from the Antipodes have been reported (28). As this profile was only seen in recent isolates of *N. fowleri*, the authors speculated that such strains have only recently emerged in Europe, possibly through importation from the Antipodes. It would be of interest to examine the French strains for their *Eco*RI-*Hin*dIII RFLPs to establish whether they, like NF59, are actually related to the U.S. subgroup defined here.

The RFLPs detected on agarose gel electrophoresis in this study would appear to be derived from both rDNA and mtDNA. The size of the rDNA plasmid has been estimated as 15.0 kbp in *N. lovaniensis* and 16.5 kbp in *N. fowleri* (8). Milligan and Band (26) estimated the size of the mtDNA genome of *N. fowleri* HB-1 to be 54.1 kbp by summation of *Hin*dIII restriction endonuclease fragment sizes. Here it was found that the sum of the *Hin*dIII RFLPs for *N. lovaniensis* C-0490 and *N. fowleri* HB-1 seen directly on agarose gel electrophoresis were 67.2 and 68.9 kbp, respectively. If the reported sizes of the rDNA plasmid and mtDNA in *N. fowleri* are combined, a total of 70.60 kbp is obtained, which approximates the value of 68.9 kbp obtained in this study.

The pUC18 clone pB2.2 was found to contain a repeated element that detected numerous chromosomal RFLPs in *N. fowleri* that showed strain differences also corresponding to their geographic origin. However, this did not allow additional differentiation of strains beyond that of the mtDNA and rDNA RFLPs observed directly on agarose gel electrophoresis. The use of different restriction endonucleases and screening of the EMBL3 λ phage library for the occurrence of other repeated element clones may allow further subtyping of *N. fowleri* strains that would be of value in epidemiological surveys and also help resolve the origins of the geographic dissemination of the organism.

With the *N. fowleri*-specific DNA probes pB2.3 and pB2.2.4, a simple and rapid assay was developed for the identification of the organism directly following primary isolation. It is essential that the target DNA be single stranded when immobilized on nylon membranes for probe hybridization. This is usually achieved by alkali or heat denaturation of the DNA (29, 32). In this study, it was found that inoculation of amoebae into 0.4 M NaOH–10 mM EDTA resulted in immediate lysis of the trophozoites, with inactivation of nuclease activity. This led to the development of a simple sampling strategy that, together with the use of microtiter plates and a commercial DNA dot-blotting apparatus, readily enabled *N. fowleri* to be identified among large numbers of other thermophilic *Naegleria* isolates soon after primary culture isolation. Ten of 84 isolates of thermophilic *Naegleria* species were identified as *N. fowleri* in mud samples taken from the thermal springs complex in Bath. All the strains were subsequently confirmed as *N. fowleri* by their whole-cell DNA *Eco*RI RFLP profiles. This represents a significant increase in the usual number of *N. fowleri* isolates obtained from this site. This increase may be due to several reasons. The thermal springs contain large numbers of thermophilic *Naegleria* spp., of which *N. lovaniensis* is the predominant species (20). In previous surveys, only a small proportion of isolates were examined for species identification because of the practical constraints of the isoenzyme or whole-cell DNA analytical methods available. It was also noted that *N. lovaniensis* grows at a faster rate than *N. fowleri* on NNA-*E. coli* or NNA-*K. edwardsii* medium and may therefore suppress the presence of the latter during primary culture isolation. Accordingly, the DNA probe strategy developed here for the identification of *N. fowleri* enables all isolates to be sampled as soon as they emerge from the inocula on the culture plates and before possible overgrowth by *N. lovaniensis* can occur.

The identification of *N. fowleri* by PCR in conjunction with a DNA probe derived from a region internal to the amplified product has been described (34). In artificially seeded water samples, the presence of environmental sediment was inhibitory to the PCR, and only when amoebae were first isolated by culture on NNA-*E. coli* medium was the test able to detect *N. fowleri*. While PCR is a highly sensitive technique, capable of detecting as little as one *N. fowleri* cell (34), the protocol described is time-consuming. After culture isolation, DNA must be extracted from the amoebae, PCR must be performed, and the amplified product must be separated by agarose gel electrophoresis and then transferred to membranes for probe hybridization. In the DNA probe detection of *N. fowleri* described here, amoebae are tested directly from primary isolation plates, and no separate DNA extraction step is involved. Following the primary isolation of thermophilic *Naegleria* spp., the process of dot-blotting, probe hybridization, and autoradiography can be completed within 2 days. This represents a considerable advantage over conventional identification methods, such as isoenzyme and whole-cell DNA RFLP analysis, which usually require strains to be adapted to axenic culture and do not lend themselves readily to the examination of the large numbers of isolates that are frequently encountered in natural thermal environments (20).

A disadvantage of the method described here for the detection of *N. fowleri* is the use of [^{32}P]dCTP to label the DNA probes. Besides the potential hazards associated with the use of radionucleotides, the half-life of the isotope is 14 days, and probes must be prepared fresh each time. Several nonradioactive methods for the labelling of DNA are now available commercially, most commonly using a biotin or digoxigenin molecule coupled to a nucleotide in the DNA labelling reaction (40). Probe hybridization is detected by incubation with an enzyme-labelled antibody specific to the modified nucleotide and visualization by a colorimetric or chemiluminescent assay (40). Typically, probes labelled by such methods are stable for many months and can be reused several times, and the detection of hybridization is usually complete within hours rather than days. Although not extensively investigated, digoxigenin labelling of probe pB2.3 with chemiluminescent detection has been tested against dot-blots of *N. fowleri* and *N. lovaniensis* DNA. The results were, however, disappointing, with nonspecific hybridization occurring. The use of more stringent hybridization and washing conditions may resolve this problem and lead to the development of nonradioactive DNA probes, with noted advantages, for the detection of *N. fowleri*.

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