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Development of a rapid DNA extraction method and one-step nested PCR for the detection of *Naegleria fowleri* from the environment

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**Abstract**

*Naegleria fowleri* is a small free-living amoeboid-flagellate found in natural and manmade thermal aquatic habitats worldwide. The organism is pathogenic to man causing fatal primary amoebic meningoencephalitis (PAM). Infection typically results from bathing in contaminated water and is usually fatal. It is, therefore, important to identify sites containing *N. fowleri* in the interests of preventive public health microbiology. Culture of environmental material is the conventional method for the isolation of *N. fowleri* but requires several days incubation and subsequent biochemical or molecular tests to confirm identification. Here, a nested one-step PCR test, in conjunction with a direct DNA extraction from water or sediment material, was developed for the rapid and reliable detection of *N. fowleri* from the environment. Here, the assay detected *N. fowleri* in 18/109 river water samples associated with a nuclear power plant in South West France and 0/10 from a similar site in the UK. Although culture of samples yielded numerous thermophilic free-living amoebae, none were *N. fowleri* or other thermophilic *Naegleria* spp. The availability of a rapid, reliable and sensitive one-step nested PCR method for the direct detection of *N. fowleri* from the environment may aid ecological studies and enable intervention to prevent PAM cases.

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**1. Introduction**

*Naegleria fowleri* is a thermophilic, free-living amoeboid-flagellate characterised by a life-cycle of trophozoite, flagellate and cyst stage (Ma et al., 1990; Carter, 1972; Marciano-Cabral, 1988; Schuster and Visvesvara, 2004; De Jonckheere, 2002; John, 1982). The organism is pathogenic to humans, causing primary amoebic meningoencephalitis (PAM) (Carter, 1972; Marciano-Cabral, 1988; De Jonckheere, 2002; John, 1982; Martinez, 1993). Infection results from the instillation of the organism into the anterior nares usually whilst bathing. From here the amoebae penetrate the nasal epithelium and olfactory nerves and migrate through the cribriform plate to invade the brain and meninges (Carter, 1978; Cain et al., 1981). PAM is almost invariably fatal with death occurring in 3–7 days following exposure (John, 1982; Cain et al., 1981).

It is, therefore, important to identify sites containing *N. fowleri* in the interests 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 tolerating growth up to 45 °C, cytopathogenicity for tissue culture cells and antigenicity (*Marciano-Cabral, 1988; Stevens et al., 1980*). Although mouse pathogenicity was originally used to differentiate the species the recognition of *Naegleria australiensis* that was shown to be pathogenic for mice, albeit less so than *N. fowleri*, rendered the test nonspecific (*John, 1982; De Jonckheere, 1981*).

The isolation of *N. fowleri* and *N. lovaniensis* involves the culture at 44 °C of sediment and water concentrates on non-nutrient agar plates (NNA) seeded with a living suspension of *Escherichia coli* (NNA-E. coli) (*Anon, 1990; Page, 1988; John and Howard, 1996*). Presumptive *Naegleria* of are then picked from the plates and incubated at 37 °C in deionised water or ¼ strength Ringer’s solution and observed for trophozoite transformation into the temporary flagellate stage (*Anon, 1990; Page, 1988*). Flagellate positive isolates must then be further characterised to differentiate the species. In natural thermal environments, *N. lovaniensis* tends to predominate and often necessitates the screening of large numbers of isolates when attempting to identify *N. fowleri*. Furthermore, it has been reported that the enflagellation test may not always be positive with *Naegleria* (*Kilvington et al., 1991; De Jonckheere et al., 2001; Behets et al., 2003*).

Biochemical and molecular techniques have been developed for the differentiation of *Naegleria* spp. and associated identification of *N. fowleri* (*De Jonckheere, 2002*). These include monoclonal antibodies (*Visvesvara et al., 1987; Flores et al., 1990; Reveiller et al., 2000*), isoenzyme electrophoretic profiles (*Kilvington, 1995; Pernin and Grelaud, 1989; De Jonckheere, 1982; Adams et al., 1989*) and analysis of restriction fragment length polymorphisms from either whole-cell or PCR amplified DNA (*De Jonckheere, 1987a; Kilvington and Beeching, 1995a; Pelandakis et al., 1998, van Belkum et al., 1992*). More recently the rapid identification of *N. fowleri* by PCR methods have been developed (*Kilvington and Beeching, 1995b; Pelandakis and Pernin, 2002; Qvarnstrom et al., 2006; Reveiller et al., 2002; Sparagano, 1993; Maclean et al., 2004*). Whilst these methods enable the reliable and specific identification of *N. fowleri* and *Naegleria* spp., they typically require the primary culture and subculture of isolates from environmental samples. This is a time consuming process and can result in lack of detection sensitivity due to overgrowth from the more rapidly growing but non-pathogenic species such as *N. lovaniensis* and other FLA (*De Jonckheere, 2002; Kilvington et al., 1991; Kilvington and Beeching, 1995a*).

Developments in the PCR, combined with improved DNA extraction methods, enable the direct detection of microorganisms in environmental samples without the need for primary culture isolation (*Yeates et al., 1997; Fitzpatrick et al., 2010; Fierer et al., 2005; Tzeneva et al., 2009*). This has been applied to the rapid detection of *N. fowleri* from the environment through application of conventional, nested and real-time PCR methods (*Pelandakis and Pernin, 2002; Maclean et al., 2004; Puzon et al., 2009; Sheehan et al., 2003; Jamerson et al., 2009*). Although PAM is a rare infection (111 cases confirmed in the USA since 1962 (*Yoder et al., 2010*) reports have suggested that the incidence is increasing (*Yoder et al., 2010; Heggie, 2010*). Accordingly, the rapid and reliable identification of environmental habitats containing *N. fowleri* may enable the implementation of preventative public health measures to reduce the risk of PAM cases. Here we describe a DNA extraction and one-step nested PCR for the reliable detection of *N. fowleri* from thermally enriched environmental samples.

2. **Materials and methods**

2.1. *N. fowleri* nested PCR development

The nested PCR was developed from a cloned fragment of *Naegleria fowleri* (MCM) DNA (pUC PB2.3), shown from hybridization and PCR analysis to be specific for the organism (*Kilvington and Beeching, 1995a, b*). The outer primers were: OP4F 5′-gaccttccccgctcgctag-3′ and OP4R 5′-cttgagtcgcc-agcactatg-3′ while the internal primers were: IP4F 5′-cag-gaattctcaac-c-3′ and IP4R 5′-gaattgactgtgctgc-3′. Testing of these primer sets alone and in combination against purified DNA from several strains of *N. fowleri*, *Naegleria* spp. and other organisms showed them to be specific only for the species. The Tm values for the outer and inner primer sets were calculated as 65 °C and 54 °C respectively, enabling the nested PCR to be conducted as a one-step procedure in a single tube to minimise carry over and cross-contamination. The PCR produce sizes were 767 bp and 506 bp for the outer and inner primer sets, respectively. PCR was performed in 40 μl volumes consisting 2X ReddyMix PCR Master Mix (ABgene, Surrey, UK), 0.1 μM of each outer primer, 0.5 μM of each internal primer and 4 μl of environmental DNA sample. The PCR conditions comprised: 94 °C for 4 min, followed by 20 cycles of 94 °C for 1 min, 60 °C for 1 min, 72 °C for 1 min, and then 35 cycles of 94 °C for 1 min, 48 °C for 1 min, 72 °C for 1 min. A final elongation step of 72 °C for 10 min was used.

Amplicons were detected by electrophoresis, through 1.5% (w/v) agarose gels containing 0.5 μg/ml ethidium bromide in Tris-Acetate-EDTA buffer (*Sambrook et al., 1989*). *N. fowleri* positive PCR products were excised from the agarose gel, purified using NucleoSpin Extract II (*Macherey–Nagel, Germany*) according to the suppliers instructions and sequenced by MWG-Biotech (Ebersberg, Germany) using the primer IP4R. DNA sequences were compared to the cloned *N. fowleri* DNA sequence using BLAST software (*Altschul et al., 1990*).

2.2. **Samples collection and DNA extraction**

A total of 119 water samples (1 L) were collected from the River Tarn, South West France (109 samples) and the River Trent, United Kingdom (10 samples) between June 2008 and August 2009. Samples were collected into sterile polypropylene containers and processed within 5 days of collection. Depending on the turbidity of the water samples, processing was done either by filtration (clear samples) or direct centrifugation. Briefly, 750 ml of water was filtered slowly (approximately 5 min) through a 0.45 μm pore size cellulose nitrate membrane (*Sartorius, Surrey, UK*). Filtration was stopped when approximately 20 ml of water remained above the membrane. The residual water above the membrane and the membrane were added to a 50 ml sterile centrifuge tube so
that the sample surface faced inwards from the walls of the container. The tube was vortexed for 10 s and the surface debris removed from the membrane with sterile cotton-tipped swab and expressed into the tube. The membrane was then removed and the tube centrifuged at 1000× g for 10 min. The supernatant was discarded, leaving approximately 1 ml and the pellet resuspended by vortexed for 5 s. Turbid samples were centrifuged at 1000× g for 10 min and the pellets pooled and resuspended as described above.

The resuspended deposits were inoculated over a series of NNA-E. coli as 5 small drops per plate. After allowing the drops to dry, the plates were sealed in polythene bags and incubated at 44 °C. Plates were examined microscopically for up to 7 days for FLA trophozoites migrating away from the inocula. A part of each isolate growth was scraped from the plate with a disposable 1 μl bacteriological loop and inoculated into the well of a flat bottomed 96 well microtitre plate containing 100 μl of ¼ strength Ringer’s solution. The plate was sealed and incubated at 37 °C and observed after 1–3 h for trophozoite morphological characteristics of the genus Naegleria and transformation into the flagellate stage (Page, 1988). Culture positive, enflagellating isolates are then identified as either N. fowleri or N. lovaniensis using a duplex species specific PCR (S. Kilvington, unpublished methodology). In addition, FLA that exhibited limax type trophozoite movement were also tested using this PCR to exclude the possibility that they were N. fowleri or N. lovaniensis but failed to flagellate.

2.3. DNA extraction

The remainder of the pellet was mixed with 10 ml of UNSET lysis solution (urea 8 M, sarcosyl 2%, NaCl 0.15 M, EDTA 0.001 M, Tris pH 7.5 0.1 M) and vortexed for 1 min with 4 g of 0.25 mm glass beads (Jencons, Sussex, United Kingdom) (Hugo et al., 1992). The suspension was left at room temperature for 30 min, centrifuged at 3900× g for 10 min. The supernatant was then incubated at RT for 2 h or overnight at 4 °C in a half-volume of 30% (v/v) polyethylene glycol in 1.6 M NaCl (Yeates et al., 1997). Following centrifugation at 3900× g for 40 min, the resulting pellet was resuspended in 5 ml TE buffer (10 mM Tris–HCl, 1 mM sodium EDTA, pH 8.0) and potassium acetate (7.5 M) added to 0.5 M and placed on ice for 5 min prior to centrifugation at 3900× g for 30 min at 4 °C (Yeates et al., 1997). The supernatant was extracted twice with phenol:chloroform (1:1) and once with chloroform by centrifugation at 3900× g for 3 min at RT. The nucleic acids were precipitated with 0.8 volume of ice-cold isopropanol, pelletted by centrifugation at 3900× g for 30 min and washed twice with 70% (v/v) ethanol. The final pellet was dried and dissolved in 50–100 μl 10 mM Tris-0.1 mM EDTA (pH 8.0).

The nucleic acid was further purified using a commercial kit (ZR soil microbe DNA kit, Zymo Research, CA, USA) following the manufacturer’s instructions, with the exception that the bead extraction stage was omitted. The presence of DNA was confirmed by electrophoresis through 1.5% (w/v) agarose gels containing 0.5 μg/ml ethidium bromide in Tris-acetate-EDTA buffer (Sambrook et al., 1989). The purified DNA was stored at −20 °C until required (1–2 weeks).

To assess the suitability of the environmental DNA extracts for PCR, samples were tested using the modified “Universal” 16S prokaryote primers 338F-16S (5’-ACTCC-TAGGGNNGGCNGCA-3’), (Fierer et al., 2005) and 797R–16S (5’-GGACTACAGGTTATCTTAATCTGTT-3’) (Nadkarni et al., 2002). These primers exhibit broad specificity against known prokaryote bacteria for this gene region (personal communication, Dr R. Free, University of Leicester, UK) and have been used previously to assess the quality of environmental DNA extracts for PCR amplification (Ahmad et al., 2011). The sensitivity of the N. fowleri nested PCR was determined using 100, 10 and 1 pg of purified genomic DNA in the assay.

Samples that were negative by this PCR were reamplified using the same primer sets and conditions along with 1 μl from the initial reaction tube. The total volume was 20 μl consisting of 2X ReddyMix PCR Master Mix, 0.5 μM of each primer and 4 μl of environmental DNA sample. The PCR comprised: 95 °C for 5 min, followed by 30 cycles of 94 °C for 30 s, 60 °C for 30 s, 72 °C for 40 s and a final elongation step at 72 °C for 10 min. The expected PCR product is 500 bp. Based on the standard operating procedure for testing the samples stated that samples with negative results during the primary PCR should be reamplified (1 μl) using the same primers. However, if the results were still negative, the samples should be excluded from analysis using the N. fowleri nested PCR.

3. Results

Of the 119 samples extracted for DNA, 108 (91%) gave a positive reaction with the 16S bacterial PCR primers, resulting a product of expected 500 bp size (results not shown). Of the 11 negative samples, all were positive on reamplification of the original PCR tubes using the 16S primers and included in the study.

All 119 water samples were culture negative for N. fowleri although other thermophilic FLA were isolated at 44 °C incubation (Vahikampia sp., Hartmannella sp., Cashia sp., Vannella sp., Platyamoeba sp., Acanthamoeba sp. and an unidentified amoeba) from both the French (54/109) and UK samples (6/10). However, 18/119 (15%) of samples were positive for N. fowleri by the one-step nested PCR, giving the expected nested product of 506 bp (Fig. 1). All N. fowleri nested PCR positive samples were from samples collected from the River Tarn, South West France (18/109). For 4 of the positive reactions, sequencing of the DNA product showed a 99% homology to the DNA region used to design the PCR primers and further confirms that the nested PCR is specific in the amplification of N. fowleri DNA only.

In the sensitivity studies, the nested PCR was able to detect 10 pg of purified genomic DNA of N. fowleri (results not shown). No reaction was obtained with 1 pg of DNA and, therefore, the sensitivity of the assay lies between 10 and >1 pg (equivalent to approximately 50 - >5 cells).

4. Discussion

N. fowleri is found in thermal aquatic environments and can tolerate temperatures up to 46 °C (John, 1982). Although N. fowleri is most likely to be isolated from sites where the
Fig. 1 – Agarose gels showing PCR products from water samples from the River Tarn, France, after extraction by the rapid DNA extraction method followed by one-step nested N. fowleri PCR. Lanes M: 100 bp size marker ladder. Lanes 1–2: positive N. fowleri nested PCR samples from French river water samples (arrow indicates expected 506 bp nested PCR product). Lane 3: positive control of N. fowleri DNA plasmid clone PB2.3 (upper arrow indicates outer primer set PCR product of 767 bp and lower arrow the 506 bp nested PCR product). Lane N: negative control (nanopure water).

temperature is above 30 °C, the cysts can survive at 4 °C for at least 12 months with retention of virulence by the excysted trophozoites (Warhurst et al., 1980). N. fowleri occurs worldwide and has been isolated from both natural and artificial thermally enriched habitats such as natural hot springs, fresh water lakes, domestic water supplies, chlorinated swimming pools, water cooling towers and effluent from industrial processes (Martínez and Visvesvara, 1997). Since PAM was first recognised in 1965, several hundred cases of PAM have been reported globally. Although PAM is a rare infection, 111 cases have been confirmed in the USA since 1962 (Yoder et al., 2010) and reports have suggested that the incidence is increasing (Yoder et al., 2010; Heggie, 2010). Clustering of cases can occur when a single site is the source of infection. In Usti, Czechoslovakia, 16 cases were associated with a public swimming pool (Cerva and Novak, 1968). Cases of PAM have been reported from Belgium and Czechoslovakia in persons swimming in warm effluent water from industrial processes (De Jonckheere, 1987b). One confirmed case of PAM occurred in Bath Spa, England in 1978 in a child who swam in a public bathing pool fed with water from the historic thermal springs that rise naturally in the City (Cain et al., 1981). Subsequent analysis confirmed the thermal springs to be the source of the infection (Kilvington et al., 1991). In South Western Australia, several cases were associated with the reticulated mains supply water (Dorsch et al., 1983). Cases linked to the domestic water supply in Arizona, USA have also been reported (Marciano-Cabral et al., 2003). More recently, PAM has been associated with swimming in lakes or ponds in the USA and Italy (Yoder et al., 2010; Cogo et al., 2004).

PAM is almost invariably fatal and emphasises the importance of identifying sources harbouring N. fowleri so that remedial action such as disinfection or prevention of bathing can be implemented (Martínez, 1993; Vargas-Zepeda et al., 2005). Typically, detection relies on the culture of environmental material prior to subsequent genus identification and speciation of N. fowleri using biochemical or molecular techniques (Page, 1988; Behets et al., 2003; Kilvington, 1995; Kilvington and Beeching, 1995b; Pelandakis and Pernin, 2002; Jamerson et al., 2009; Lares-Villa and Hernandez-Pena, 2010). This can take several days or weeks to accomplish and the presence of N. fowleri on the primary isolation culture plates can be obscured by other, faster growing, FLA. PCR enables the rapid and specific identification of N. fowleri and has been applied successfully to the identification of the organism from the environment following culture isolation (Behets et al., 2003; Kilvington and Beeching, 1995b; Pelandakis and Pernin, 2002; Jamerson et al., 2009; Lares-Villa and Hernandez-Pena, 2010).

In a nested PCR, the outer primers amplify DNA in the first round of reactions which act as templates for the internal primers in the second round of the PCR. This can significantly enhance the detection sensitivity of the assay and mitigate the effect of compounds inhibitory to the Taq polymerase present in the extracted DNA. Typically, a nested PCR is accomplished in two separate tube reactions, with the products of the first round of reaction being added to a separate second tube. This can increase the occurrence of false positive reactions from cross-contamination. The advantage of the nested PCR developed here is that the annealing temperatures (Tm) of the outer and inner primer sets are such that they react independently in the first and second round of the assay. This enables the reaction to be conducted in a single tube and greatly simplifies the procedure and reduces the risk of cross-contamination. Key to any PCR is the quality of the DNA used in the reaction. Environmental samples can contain inhibitory compounds, such as humic material, which can contaminate extracted DNA and inhibit the activity and efficiency of Taq polymerase (Yeates et al., 1997; Tsai and Olson, 1992). In this study, we report a reliable method for extracting DNA from water samples which is suitable for PCR amplification. Modification of a previously published method for environmental DNA extraction through the additional use of a commercial kit to further purify the DNA showed that 90% (108/119) were suitable for PCR using universal bacterial 16S primers or 100% when reamplification of the reaction was used. We have also used the method to successfully extract DNA from soil and mud samples for the detection of the pathogenic FLA Balamuthia mandrillaris (Visvesvara et al., 2007) by a nested PCR (Ahmad et al., 2011). Accordingly, the method may be suitable for the DNA extraction and PCR analysis for a wide range of both prokaryote and eukaryote organisms from the environment.

In previous studies N. fowleri PCR assays have reported a detection sensitivity of 5 pg of purified DNA (which approximates to 25 amoeba) or 5 whole amoebae when spiked into environmental samples. For the nested PCR described in this study a positive reaction was obtained with 10 pg of
purified DNA but not with the next dilution tested of 1 pg. Accordingly, the sensitivity of the assay lies between these values for purified DNA and is likely to be comparable to that reported previously. Although spiking of samples with whole amoebae was not done in this study, it is acknowledged that the sensitivity of the PCR is likely to be reduced due the possible presence of substances inhibitory to the reaction that may co-purify during the extraction procedure. The possibility that the positive results for the nested N. fowleri PCR are derived from the presence of dead organism in the environmental samples cannot be excluded by this technique. However, knowledge that an environment may harbour N. fowleri even in the nonviable state would still be of importance in ecological and preventative public health studies.

Previous studies have shown the sensitivity of nested PCR in the detection of N. fowleri from the environment either with or without primary culture enrichment of the samples (Pelandakis and Pernin, 2002; Maclean et al., 2004; Marciano-Cabral et al., 2003). The sensitivity of the environmental DNA extraction method in conjunction with the N. fowleri nested PCR developed here in the detection of the organism from two river water sites in France and the UK is clearly shown. None of the samples from either site grew N. fowleri on culture analysis but 18/119 (15%) were positive by the direct nested PCR, all from the French waters. Both sites have been shown previously to contain N. fowleri by culture isolation and the absence in the present study is unclear. However, regular samples in the previous year from the French site were also negative on culture for N. fowleri and the UK site had not been examined since 1997, suggesting that presence in the waters have declined or else fluctuate in detectable numbers (Kilvington and Beeching, 1995a, 1997; Pelandakis and Pernin, 2002). Another explanation may lie in the amount of material that can be analysed by both methods. Culture on NNA-E. coli plates permits only a relatively small amount of material to be processed (<1 g) compared with up to 10 g using the direct DNA extraction presented here. In addition, the presence of faster growing FLA can obscure the presence of N. fowleri and reliance on the flagellation test to screen for presumptive Naegleria spp. may be unreliable, resulting in false negative findings (De Jonckheere et al., 2001; Behets et al., 2003).

A reliable and sensitive one-step nested PCR method for the direct detection of N. fowleri from environmental samples has been developed and evaluated. Future developments in the technology may include evaluation of faster DNA extraction from environmental samples and the use of real-time PCR methods that could also quantify the presence of N. fowleri in the processed samples and differentiate viable from dead organisms (Qvarnstrom et al., 2006; Robinson et al., 2006). Although it was shown here that the nested PCR was more sensitive than culture for the detection of the organism, culture isolation should always be attempted in conjunction, as the ability to perform molecular typing assays on isolates is valuable in studying the genetic diversity of N. fowleri and in epidemiological investigations to identify sources of infection (Kilvington and Beeching, 1995a, van Belkum et al., 1992; Pelandakis and Pernin, 2002). The ability to detect the causative agent of PAM in the environment may enable intervention to prevent cases of human infection and aid ecological studies.

5. Conclusion

A reliable and sensitive one-step nested PCR method for the direct detection of N. fowleri from environmental samples has been developed and evaluated. This approach represents a significant advance in the ability to rapidly and reliably detect this human pathogenic free-living amoeba in the environment. This will enable a greater understanding of the ecology of the organism that may, also, help prevent cases of human infection.

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