DETECTION AND IDENTIFICATION OF NAEGLERIA FOWLERI

REPORT TO THE DEPARTMENT OF THE ENVIRONMENT

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Title of Project:

Detection and identification of *Naegleria fowleri*

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Staffing: Mrs Gillian Harkness (nee Young) BSc has been employed full time to carry out the laboratory development and validation of the techniques. Mr. Kalim Laloo, MSc, has carried out some development work at the Hospital for Tropical Diseases.

Dr. D.J. Carucci and Dr. S. Wilson acted as advisers on this project.

Review: As part of this study a review has been prepared of current knowledge of the distribution of *Naegleria fowleri* in the environment, its medical implications and public health significance. This has already been passed on to the Department of the Environment (see appendix 12).
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SUMMARY

The contract, from March 1993 to March 1995 was funded by the Department of the Environment (reference PECD 77/7/412) and was carried out under the supervision of the Drinking Water Inspectorate.

OBJECTIVES

To develop and test Polymerase Chain Reaction (PCR) and other techniques for the specific identification of *Naegleria fowleri* from environmental sources. Adaptation of the techniques to enable safe, non-radioactive probes to be used. Development of colorimetric methods for PCR which are semi-quantitative and applicable to large numbers of samples. Studies on optimal isolation of the organism from water and a limited programme of environmental sampling were also envisaged following the development of identification techniques, in order to develop a suitable detection protocol for further testing.

BACKGROUND

*N. fowleri* is a free-living amoeba found in warm fresh water. It causes a rare and usually fatal meningitis following infection while swimming. A fatality has occurred, associated with contamination of the hot mineral springs in Bath in 1978 and the organism has recently been detected in power station effluent in the UK.

We can expect *N. fowleri* colonisation of heated fresh water habitats, such as warm swimming pools and fish farms, with consequent risk of deaths.

Reliable methods for detection and identification of the organism are required. Non-pathogenic species of *Naegleria* inhabit many waters and may not only be mistaken for the pathogen but also interfere with its isolation.

The knowledge exists to detect microorganisms such as *N. fowleri* using molecular biological techniques. DNA probes have potential to detect a particular microorganism specifically by virtue of unique sequences of bases in its DNA. PCR enables detection of minute numbers of organisms in water samples or early cultures using DNA amplification and a DNA probing process. The integration of DNA methods into water sampling procedures offers the potential for more specific and sensitive techniques.

STRATEGY

The studies were carried out in the Applied Molecular Biology Unit in the Department of Medical Parasitology, London School of Hygiene and Tropical Medicine. DNA sequences for testing were obtained from the literature and from GenBank. The plan was to design primers and probes based on analysis of available sequences, then to test them for their specificity and sensitivity. Having obtained suitable results, the method would be adapted to use non-radioactive safe probes. Then the development of a colorimetric method capable of high throughput in the detection of specific
PCR product would begin. Having optimised this technique, the application to detection of organisms from water samples would be developed, and finally tests on samples from the environment would be carried out. An optimal protocol would then be drawn up for further testing by other groups.

**PCR FOR DETECTION OF *N. FOWLERI***

PCR optimisation:

Primers and DNA probes were developed from 2 available gene sequences. Ability of specific PCR methods to detect one organism in laboratory conditions was confirmed. Safe methods of probe labelling using digoxigenin were optimised.

Enumeration:

Currently, cultivation is the only technique able reliably to measure concentrations of the viable organisms. A microtitre plate method of cultivation is better than the present method on Petri plates. Most probable number method can be applied to cultures from water samples after serial dilution.

Identification:

Apart from culturing at temperatures higher than 37°C to encourage growth of the thermophilic amoebae *N. fowleri*, *N. lovaniensis* and *N. australiensis*, and avoiding the use of animals, it is best to confirm the identity of *N. fowleri* using isoenzyme, DNA probe or Polymerase Chain Reaction (PCR) methods. We have found that *N. fowleri* has a lower mobility of glucose phosphate isomerase than other *Naegleria* spp. A lysate suitable for electrophoretic analysis can be prepared from as few as 5000 organisms.

Using PCR primers for the amoebic mitochondrial ATPase gene we were able to detect 400 organisms but not 40 by fluorescence and size of the PCR product in agarose gel electrophoresis. When a [32P]radiolabelled specific probe was used to detect *N. fowleri* PCR product, the sensitivity was greatly increased, and DNA from 1 or fewer organisms was detected. The technique was adapted using the safe label digoxigenin for the probe, and specificity was confirmed in a simple blot-hybridisation protocol.

Using primers for amoebic serine carboxypeptidase we confirmed the specificity of a PCR reported by Sparagano using a safe, digoxigenin-labelled probe.

Development work was carried out on a PCR-Solution Hybridisation Enzyme Linked Assay (PCR-SHELA) which we have used successfully for the dysentery amoeba. PCR-SHELA allows a rapid, highly sensitive semi-quantitative visualisation of PCR product from a large number of reactions without use of hazardous or expensive materials.
Conventional PCR techniques depend on carcinogenic ethidium bromide for staining electropherograms which are examined under ultra violet light, and probing the product with radioactive probes. The method is hazardous, tedious and not easily applicable to large numbers of samples. A colorimetric technique carried out in microtitre 96-well plates, the PCR-Solution Hybridisation Enzyme Linked Assay (PCR-SHELA) would allow a rapid, highly sensitive visualisation of specific PCR product, without use of hazardous or expensive materials. The amplified specific DNA sequence, labelled with biotin, is identified using a digoxigenin-labelled specific internal probe. On addition to a microtitre plate well which has been coated with avidin, DNA strands which are biotinylated are bound and the digoxigenin label can be detected with enzyme-labelled antibody. A reaction linked to the enzyme gives a colour which can be detected visually or using an ELISA reader. The technique is semi-quantitative and susceptible to automation for large numbers of samples. A modification of this technique applicable to *N. fowleri* has been developed by us which not only has the potential for a large throughput and automated reading of the colour reaction, but also has the high sensitivity of a nested PCR technique. In the modified 'demi-nested PCR-SHELA', the two consecutive PCRs of the nested technique are carried out in the same tube which is kept sealed throughout, avoiding cross-contamination which is a problem for other nested reactions. The tube is opened only for the calorimetric detection stage in the microtitre plate, which is not susceptible to contamination.

The technique is specific for the pathogen *N. fowleri* and will currently detect 10 organisms. Used together with the modified initial concentration and culture protocol we have developed, it will enable *N. fowleri* to be detected in water supplies even at low concentration and in the presence of other organisms.

After optimisation of the technique 5 samples kindly supplied by Dr. Simon Kilvington were tested blind, and the 3 *N. fowleri* samples (from Hong Kong, Bath Spa and an English power station), were detected accurately. No detectable colour was seen in the tests on the two other samples, *N. australiensis* and *N. lovaniensis*.

**PRACTICAL IMPLICATIONS**

The PCR protocols we have developed are applicable to specific identification of *Naegleria fowleri* among amoebae isolated and enumerated in microtitre plate cultures from water samples. Protocols for conventional PCR and for probing the product on membranes with digoxigenin-labelled probes are tedious and are unlikely to be adopted by the water industry. Direct DNA probing without PCR (not studied here), similarly, is unlikely to be practicable on a large scale except in specialist laboratories. The relatively simple technique of isoenzyme electrophoresis can be applied for identification, but demands large numbers of organisms. The demi-nested PCR-SHELA method, will, we believe, be usable in the water-industry because of its relative simplicity, high throughput and capacity for automation.
1. INTRODUCTION

Facultatively parasitic free living amoebae, normally found in soil and water, cause three important diseases in man: primary amoebic meningoencephalitis (PAM), granulomatous amoebic encephalitis (GAE), with invasion of other tissues and chronic amoebic keratitis (CAK). Both PAM and CAK occur in healthy individuals while GAE and related diseases are associated with immunodeficient states\(^1\).

The causative agent of PAM belongs to the amoeboflagellate group, and is a species of the genus *Naegleria*, *N. fowleri*. The organism is found worldwide in warm fresh water, normally feeding on bacteria. Its life cycle has three stages, the feeding, growing, multiplying form or trophozoite found on surfaces of vegetation and mud, the rapidly motile biflagellate form found in the surface layers of water and the dormant cyst form found in the same locations as the trophozoite. Infection takes place through the nose when the organisms are inhaled in contaminated water, usually during swimming. The symptoms and features of the disease are characteristic of a purulent bacterial meningitis, but there is no response to antibacterials. Coma rapidly sets in and culminates in death. Treatment has been successful in only four of more than one hundred and forty four\(^2\) recorded cases. Although relatively few cases of PAM are recorded, the disease is significant among those associated with recreational water use, because of its almost invariably fatal outcome. In the 2 year period 1989 and 1990, three cases of PAM were reported among 1062 cases of illness associated with recreational water use in the USA\(^3\).

*N. fowleri* will grow well at temperatures up to 45° C and has been isolated from thermally elevated aquatic environments worldwide. Other species of *Naegleria*\(^4\) are regularly found in association with *N. fowleri* and this causes identification problems.

In a study of a newly created cooling reservoir (Clinton Lake, Illinois) before and after thermal additions from a nuclear power plant, *N. fowleri* was isolated from the thermally elevated arm but not from the ambient-temperature arm of the reservoir. It has also been isolated from hot springs of Bath and electricity generator cooling towers in the UK. Even in temperate areas we can expect *N. fowleri* colonisation of any newly introduced heated fresh water habitats, such as warm pools and possibly, CenterParc-like facilities.

It is therefore vital that reliable methods for detection and identification of the organism are developed.

1.1 Glossary:

DNA: Deoxyribonucleic acid which comprises the genetic material (chromosomes) of bacteria, animal and plant cells.

RNA: Ribonucleic acid plays important roles in functioning of bacterial, animal and plant cells.

Genetic Probe: A DNA molecule of defined base sequence which binds by hybridisation to a specific region (target site) of DNA from an organism, and which may be labelled to enable its detection.
PCR Primer: Short DNA molecule serving to initiate the cycles of DNA replication in PCR.

Hybridisation: Binding of DNA probe to its specific target site on the DNA of the organism of interest.

Polymerase chain reaction (PCR): Method to amplify (many times) a specific sequence of DNA as defined by two PCR primers.

Oligonucleotide: Short artificially synthesised DNA sequence. These are usually used in pairs to carry out PCR reactions, and singly when labelled, to detect specific target sites on the PCR product.

Template: The target sequence of DNA present in the prepared sample which will be replicated in PCR.

Trophozoite: Actively moving, feeding and multiplying form of a protozoan.

Cyst: Dormant stage of a protozoan which is resistant to environmental stress.

1.2 Safety:

*N. fowleri* is a pathogenic free living amoeba which is infective to man by the intranasal route. There is a treatment available (amphotericin B), but unless the diagnosis is made early the outcome is usually fatal. According to ACDP the organism falls into hazard group 3 and cultures should be handled in a class 1 (exhaust protective) or class 3 (fully enclosed) cabinet. The laboratory used should have a vented lockable door with an air inlet vent, and entry should be restricted to authorised persons whilst *Naegleria* is being handled. (see appendix, 8.1.).
2. OBJECTIVES AND PROGRAMME OF WORK

To develop and test PCR and other techniques for the specific identification of *Naegleria fowleri* from environmental sources.

Proposed work programme:

1. Validation of a polymerase chain reaction technique (PCR) for the detection of small amounts of *Naegleria fowleri* DNA, and to adapt this system for use with environmentally-friendly specific detection systems to avoid use of ethidium bromide and radiophosphorus.

2. Development of a suitable PCR method, determinations to be carried out of the sensitivity and specificity of the system on artificially contaminated water and mud samples in the laboratory.

Use of organism-specific polyclonal rabbit antibody attached to magnetic Dynabeads for capture of the organism from water samples, and PCR on the dynabeads themselves to detect *N. fowleri*.

The programme was expected to be complete within the 2 years available, and towards the end it was proposed that sampling would be carried out at Bath Spa to test the protocols in a practical situation.

A protocol would be developed to allow others to carry out confirmatory studies.
3. THE POLYMERASE CHAIN REACTION (PCR) AND THE USE OF DNA PROBES TO ENHANCE SENSITIVITY.

A DNA probe is a sequence of deoxyribonucleic acid which has been made so that it is complementary to a known sequence in a microorganism. Complementarity means that the sequence will bind to one of the strands making up the double helix in the target DNA. In PCR two short sequences each complementary to alternate strands of the DNA double helix are made. These are termed primers, and, in the presence of DNA polymerase will direct the synthesis of a double-stranded DNA sequence between the bound primers. This copying process can be repeated using cycles of denaturation (strand separation), annealing (binding of new primer pairs) and extension (polymerisation of nucleotides by DNA polymerase) so that many (up to a million) copies of the original microorganism DNA sequence are produced. Electrophoresis on agarose allows the amplified DNA molecule (of known predicted size) to be detected after DNA-staining with a fluorescent dye. Apart from measuring the size of the PCR product on a gel, it is more informative and also generally more sensitive to detect specific sequences in the product using hybridisation to a DNA probe labelled with radioactivity ($^{32}$P) or, more safely and economically, with a marker which can be detected using a colour-generating process.

Conventional PCR tests tend to take 2 days to complete, and the specimens cannot be bulked together in large numbers. Modifications of the test using probe technique and colour reaction in 96 well microtitre plates enable the process to be speeded up, carried out in larger batches, and completed in one day or less. The PCR-Solution Hybridization Enzyme-linked Assay (PCR-SHELA) is one of these techniques, and there are others which are being developed by commercial firms for widespread use.
4. THE DEVELOPMENT OF PCR TESTS AND DNA PROBES FOR

DETECTION OF *Naegleria fowleri*.

There are several stages in the development of a suitable PCR test and DNA probe PCR product identification technique.

1. Identification of a suitable nucleotide sequence.

2. Design of primer sequences to allow amplification of that sequence.

3. Design of a suitable internal probes to detect the PCR product specifically.

4. Optimization of the PCR conditions such as annealing temperature, timing, number of cycles and reagent concentration.

5. Optimization of the hybridisation conditions for the probe.

6. Optimization of the label used for the probe to give sensitivity with safety and economy.

In addition, when a suitable conventional PCR has been developed, a technique, such as PCR-SHELA, for increasing throughput and simplicity needs to be optimised.

4.1 Identification of suitable nucleotide sequences.

In a literature and GenBank search two sequences were found to have been reported as usable for *N. fowleri* identification.

4.11 *N. fowleri* mitochondrial ATP-ase gene\(^5\) (GenBank M55009).

This sequence has the advantage of being present in 10 or more copies in each *N. fowleri* cell since it is found on the mitochondrial DNA. This means that there should be an enhanced probability of detection of even a single organism over a single copy nuclear gene. The primers described by McLaughlin et al. were reported to be specific for *N. fowleri* under stringent annealing conditions. A PCR product was however obtained with less-stringent (lower temperature) annealing conditions for the other species.
Naegleria fowleri mitochondrial ATPase gene sequence, showing positions of forward (--->) and reverse (<=--) primers Nf1 and Nf2, and internal reverse probe sequence MP.

```
1 AAGCTTTTT TATTCCTTAT TAAAGGTAC GTATAATT TTATAGTTA
51 CTATATATTC GTATCTAGTA GATAGAACAA AAATGTTTTT TCTTTTTTT T------------------- Nf1 --------------->|
101 TTTATTTAT TCTTTATTTAT CTGTTAAGT AATTTAGTCG GTATAGTTCC
151 ATTAGTTTTC ACAATAACAA GCCATTTAAA TATAACATTT AGTCTATCTT
201 TTTGCTAGT GTGGGCTACT TGTTTATTAG GTTTTATGA AAGTGGTCTT |
251 GCTTTTATG CTATATTCTA TGTAAAAGGT ATTCTTTTG TATTAGTTCC
301 ATTTTGGGCA TTAATAGAAG TTATAAGTTT TATTATTAGA TCTGTAGGTT |<=-------Nf2--
351 TGTCGTAGCG
```

Forward primer Nf1: 5' CGTATCTAGTAGATAGAACA 20 bases

Reverse primer Nf2: 5' CGTACGACAAACCCCTACAGA 20 bases

Reverse probe MP: 5' AACAAAG TAGCCCAACCCTACAGA 19 bases

Sparagano⁷ claimed that the PCR (above) described by McLaughlin and colleagues was non-specific in his hands (even to the extent of reacting with Acanthamoeba DNA).

A second PCR system for identification of *N. fowleri* was reported in 1993 by Sparagano and colleagues. A gene, described originally as virulence-related⁶, and believed to code for a serine carboxypeptidase was the basis for Sparaganos's new test. Sparagano reported the amplification of a 678 bp fragment from *N. fowleri* DNA only and no reaction with other species.
Naegleria fowleri virulence-related protein cDNA sequence: GB M88397

Showing forward primer (----> and probe (-----) sequences and reverse primers (<-----) used in polymerase chain reaction and specific identification of product by nucleic acid hybridisation.

201  CATCTCAGGA TCCACTTGTG ATGTGGACCA ATGGTGGAAC TGGGATGCAGT

251  AGTTTGGGCG GTGAGGCGCA TGAACATGGT TTATTCCTTG TCAATGCAAG
|--Nf5------------>
|--Nf3------------>|
|--NF5A------------|

301  TGGTGCAACC ATTACAAGAA ATCCCTATTC TIGGAA1CGT GTTTCTAAATA

351  TTCTCTATAT TGAACAAACCA GTGGGTGTTG GATTITICATA TTCCAATTCG

401  ACCGATGATT ATCAAATCT CAATGATGTA CAAGCTGTTT CTGATATGAA

451  TAATGCATTG AGAGATTCTT TGAATCGATT TCCTCAATTT ATGGAAGAG

501  AAACCTATTG GCCAGGTTGA TCGTATGTTG GAGTTTATGT TCCAAACACG

551  GCTTACAATA TTGGTGAAAG AAATGGAAAG GGACAAACAC CCTATGTGAA

601  TTTAGTGGGT ATTTTAGTTG GTAATGTTGT GACTGATGCT GAAGCGGATA
|--Nf7-------------------------------|
|--Nf7a------------------------------|
|--Nf7b------------------------------|

651  GTAATAGTAT TCCACAAATG ATGAAATATC ACAGTTGGAT TTCTATCAAG

701  TATATGAAAG AGGGATATAA GCCATGTCAA GTGTTTTTTT ATGCAGATCA

751  GAATTTGCCA GCTTGCTAAA AATTTTGAC AGATAGTAGT ATGCGCATTG

801  GAAATATTAA TCCCTATTAT ATTTATGATT CATGTCCATG GTTAGGAATC

851  AACTTGCAAC AAAAATCTAA AAACACACAA GAAATGACAT TCCAGGTGT
|--Nf4------------------|
|--Nf6------------------|
|--Nf6a------------------|

901  GGATCCAAAG ACTCAAAAC CTGTCAAAT TCATCCACTC TTCAAATGT
We prepared generic primers Nf3 and Nf4 based on the same sequence, which gave a product of similar relative molecular mass (750-800 bp) for all the species of *Naegleria* tested. These should be useful in detecting the genus *Naegleria* in comparison with specific detection systems.

These primers are as follows:

Forward: Nf3 \(5'\) CCAGTGAAACATGGTTATTCCCTTG \(24\) bases  
Reverse: Nf4 \(5'\) TTGAGTCTTTGGATCCAACACTTG \(24\) bases  

product: predicted 649bp from c-DNA sequence, actually 750 bp.

Primers used by Sparagano:

Forward primer: Nf5: \(5'\) ATGCAGTAGTTGGGCCG \(17\) bases  
Reverse primer: Nf6: \(5'\) AGTTTGTTAGTCTTTGG \(18\) bases  
Internal probe: Nf7: \(5'\) GTTGGTAATGGGTGACTGCTGAAGCGGAT \(33\) bases  

The internal probe, marked Nf7a above, was designed by us for experiments upon the shortened PCR-SHELA technique (see later).

**4.2 LABELLING AND DETECTION OF PROBES WITH RADIOACTIVE AND NON-RADIOACTIVE MARKERS.**

For conventional PCR DNA probes we used 2 types of labelling techniques (see appendix 8: Hybridisation and probes):

4.21 Radioactive marker method using \(^{32}\)P. This method adds a chain of radionlabelled bases to the end of the probe oligonucleotide. Radiophosphorus is dangerous and has a short half-life, and so the labelling of the probe has to be carried out under strictly controlled conditions. When labelled its useful life is short. The label was detected using exposure of X-ray film to the probed membrane, development and examination of the intensity of darkening.

4.22. Digoxigenin labelling method (Boehringer Mannheim). This technique poses no hazard and when labelled the probe can be stored for several months. The label was detected by incubation of the probed membrane with a chromogenic substrate and linker mixture which gave a blue deposit in situ.

For the PCR-SHELA we used a capture system with microtitre plates coated with avidin to which PCR product labelled with biotin would bind. The specific product was also labelled with digoxigenin which could then be detected colorimetrically using alkaline-phosphatase conjugated anti digoxigenin antibody. The yellow-brown colour intensity in wells after development could then be judged by eye or using a plate reader at 405 nm.
5. IDENTIFICATION OF *Naegleria fowleri* IN WATER SAMPLES.

5.1 PCR IDENTIFICATION OF CULTURED MATERIAL.

5.11 Using the mitochondrial ATP-ase sequence.

In our hands the specificity of the technique used under stringent conditions appears satisfactorily high. No product was produced from the other amoebae: *N. lovaniensis* 1479 and 6604.2 (isolates from Bath) *Acanthamoeba polyphaga* or *Entamoeba histolytica*, flagellates: *Leishmania donovani*, *L. enrietti*, *Trypanosoma cruzi*, *Cryptosporidium parvum*, *C. muris*, bacteria: *Escherichia coli*, or an environmental isolate of yeast. At the same time, product of the correct size was produced from *N. fowleri* from USA (HB1), Colombia (Colombia 1) and Australia (Morgan).

We have carried out sensitivity tests on this protocol which involve amplifying DNA extracted from different quantities of amoebae in the PCR.

We could detect and positively identify, by the agarose gel technique, from 10-fold dilutions of amoebae, 400 organisms but not 40. This suggests that the limit of detection for visual evaluation of ethidium bromide fluorescence is between these figures.

When a Southern transfer was carried out from such a run, and a radiolabelled PCR product (validated by hybridisation to the specific internal probe) was used to detect the *N. fowleri* PCR product, the sensitivity was greatly increased, and DNA from fewer than 1 organism was detected.

The sensitivity is what would be expected from a target molecule, mitochondrial DNA, where there are more than 10 examples per cell. However, under conditions of water examination, there may be materials present which may interfere with the PCR reaction itself. Additionally, the production of a product from *N. lovaniensis* under less stringent conditions may not be an encouraging sign, and may suggest that the PCR test could be unduly influenced by laboratory variables.

5.12 Using the virulence-related protein sequence.

In using Nf5 and Nf6 (and the PCR protocol described earlier) we have found *N. fowleri* DNA to give a product of c. 800 base pairs (bp) (678 bp reported). However, as not reported by Sparagano, *N. lovaniensis* gave a band at 500 bp and *N. gruberi* gave a band at 350 bp. The specificity of this PCR technique has further been tested by hybridisation with the "specific" internal sequence Nf7 described by Sparagano. This technique was carried out without using $^{32}$P], by means of a digoxigenin labelled probe, detected by an alkaline phosphatase-labelled antidigoxigenin antibody (Boehringer kit). This is in accordance with our aim of achieving an environmentally and user-friendly system. (See appendix for protocol). The specific probe Nf7 only reacts with the *N. fowleri* product.

Nf5 and Nf6 primers for the virulence-related protein gene also gave, in our study, product from
the 2 other species, *N. lovaniensis* (500bp) and *N. gruberi* (350bp) in addition to a product from *N. fowleri* of about 800bp. However, Nf3 and Nf4 primers close to Nf5 and Nf6 also give a product that is longer than predicted, but it is similar for all the 3 spp. This means that the different length products seen in the other 2 spp are the result of false priming, not that parts of the serine carboxypeptidase sequence are absent from *N. lovaniensis* and *N. gruberi*. This also suggests that the strains of *N. fowleri* we tested contain an intron of c.120 bp in this gene.

According to 2 recent papers of Sparagano and colleagues the primers described have been further tested with success in sampling experiments. No mention is made of bands amplified from the DNA of the other spp., but the specificity is always linked to probing rather than simply the observation of bands.

The aim with PCR detection is to carry out the reaction with as little possibility of contamination from products as possible, so techniques such as nested PCR which usually involve adding reagents to the reaction tube after the process has begun have been avoided in this study. The nested PCR finally developed has been specifically designed to avoid this problem (see later).

**5.2 EXPERIMENTS ON THE PCR-SHELA TECHNIQUE.**

**5.21 Protocol for the PCR-SHELA**

The technique involves conventional PCR using one primer 5'-labelled with biotin, giving rise to double-stranded product which is labelled at one end with biotin. In the usual technique, the product is denatured and a digoxigenin-labelled specific internal probe is allowed to hybridise with the labelled single strands. Then the material is diluted and dispensed into a microtitre plate well which has been coated with avidin. After washing, the biotin and digoxigenin labelled strands bound to the well are detected by use of an alkaline phosphatase-conjugated anti-digoxigenin antibody which is then revealed using alkaline phosphatase substrate linked to a colour-enhancing reaction. The intensity of the colour can be detected visually or using an automated machine. The potential sensitivity of the technique in our other studies is reported to be as high as that achieved using [32P]-labelled probes on a Southern blot from a standard PCR run in agarose. The technique is semi-quantitative and susceptible to automation for large numbers of samples.

In order to speed up the specific identification of the product the specific internal probe can be included in the PCR reaction mixture. Generally this probe has a short 3' poly A tail added (see Nf7a) to prevent it priming for the DNA polymerase. The PCR and hybridization reaction then occur simultaneously, and the product can be diluted and added direct to the avidin-coated wells of the microtitre plate. As a further precaution against priming by the probe, the melting point of the binding portion of the probe is made lower than that of the primers by lengthening the primers or by shortening the probe, or both.

With the avoidance of agarose gel electrophoresis, suitable abbreviation of the PCR protocol, the in-tube hybridization protocol and the ELISA method suitably shortened by optimal choice of reagent concentrations, the limitations on sample numbers are related to the number of PCR machines available and the rapidity in preparing samples for PCR. With one machine, at least 100 tests, including negative and positive controls, should be capable of being performed per day.
Protocol for PCR SHELA: These primers have been lengthened at the 5' end to increase the melting point.

Forward primer: Nf5 : 5' CCTGGATGCAGTAGTGTGGGCG 22mer
Reverse primer: Nf6 : DIG (or BIOTIN) 5' TTTGACAGGTTGTGAGTCTTTGG 24mer
Forward probe: Nf7 : BIOTIN (or DIG) 5' GTTGGTAATGGTGACTGATGCTGAAGCGGAT 33mer
Forward probe: Nf7a: BIOTIN 5' ACTGATGCTGAAGCGGAT (A)10
(Nf7a for shortened protocol with probe added to PCR reaction)

The expected product of the primers, predicted from a cDNA clone, is 684 bp. Previously PCR products from DNA have been ~120 bp longer than reported by Sparagano or predicted from the cDNA and this was observed again on agarose electrophoresis, since the product was about 750 bp.

After the optimal PCR protocol had been found, variation of the conjugate concentration, probe concentration, hybridisation times and other parameters was tested to attempt to produce optimal conditions. Finally the conclusion was reached that owing to the length of the PCR product, bending was taking place, with self-hybridization and inability of the product to react adequately with the avidin-coated wells. A protocol producing a shorter product for binding to the well was required. (See 5.32).

5.22 Protocol for the demi-nested PCR-SHELA.

The probe (modification Nf7b: P18) was used as an internal second stage primer, in a nested PCR. Initially thirty cycles of the normal PCR were performed then 10 pmol of Nf7b were added. Another 5 cycles were performed, with a annealing temperature of 52°C. When the colorimetric reaction was done, only the *N. fowleri* sample gave colour. The *N. lovaniensis* sample and the negative sample were negative. When I tried to add the internal primer at the beginning of the reaction, the result was non-specific colour. However it was possible to do the one-step reaction when the hot-start technique was used.

After optimisation of the technique 5 samples kindly supplied by Dr. Simon Kilvington were tested blind, and the 3 *N.fowleri* samples (from Hong Kong, Rath Spa and an English power station), were detected accurately. No detectable colour was seen in the tests on the two other samples, *N.australiensis* and *N. lovaniensis*. 

20
Forward primerNF5a : 5' CCT GGA TGC AGT AGT TTG GGC G
Reverse primerNF6a : 5' DIG-TTT GAC AGG TTG TTG AGT CTT TGG
Internal primer NF7b : 5' BIOTIN-ACT GAT GCT GAA GCG GAT A

PCR protocol:

The hot-start PCR method was used. In this method all the primers were added to the tube, then a wax pellet placed on top. The Eppendorf tubes were spun down briefly, then placed at 80°C for approximately 5 min until the wax melted. The rest of the PCR reaction mix was then added to the tube. The DNA sample was added to the top layer of reaction mix.

Temperature cycling:

<table>
<thead>
<tr>
<th>Temperature</th>
<th>Duration</th>
</tr>
</thead>
<tbody>
<tr>
<td>94°C</td>
<td>3 min</td>
</tr>
<tr>
<td>94°C</td>
<td>30 s</td>
</tr>
<tr>
<td>65°C</td>
<td>1 min</td>
</tr>
<tr>
<td>72°C</td>
<td>1 min</td>
</tr>
<tr>
<td>94°C</td>
<td>30 s</td>
</tr>
<tr>
<td>54°C</td>
<td>1 min</td>
</tr>
<tr>
<td>72°C</td>
<td>1 min</td>
</tr>
<tr>
<td>72°C</td>
<td>10 min</td>
</tr>
</tbody>
</table>

Colour detection:

1) The PCR tubes were heated to 60°C to melt the wax, the tubes were then flicked and placed on their sides. The wax quickly hardened on the side of the tube, allowing the PCR products to be spun down and removed with a pipette. 20 µl of product was added to 200 µl of diluting solution.

2) The wells of a microtitre plate were coated with avidin. The avidin (1mg/ml) was diluted 1/100 in carbonate buffer, then 100 µl was added to each well to be used. The plate was incubated for at least 1 hr at 37°C.

3) The plate was washed twice with TBS-Tween. Salmon sperm DNA (15mg/ml) was diluted 1/10 000 in TBS-Tween. 100 µl was added to each well, and the plate incubated for 30 min at room temperature.

4) The plate was washed twice with TBS-Tween. 100 µl of each diluted product was added to a well, and the plate incubated for 1 hr at room temperature.

5) The plate was washed twice with TBS-Tween. 1 µl of anti-digoxigenin conjugated to alkaline phosphatase was added to 5 ml TBS-Tween, along with 150 mg milk powder. 100 µl of this solution was added to each well, and the plate incubated for 1 hr at room temperature.

6) The plate was washed four times with TBS. One substrate tablet was dissolved in 5 ml
carbonate buffer containing 5 µl 1M MgCl₂. 100µl of this solution was added to each well, and incubated at room temperature. The optical density of each well, at 410 nm, was read after 1 hr.

SOLUTIONS:

10x stock, Tris buffered saline (TBS) (0.1M Tris, 1.5M NaCl, pH 7.5)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris/HCl</td>
<td>12.5 g</td>
</tr>
<tr>
<td>Tris base</td>
<td>2.36 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>87.66 g</td>
</tr>
</tbody>
</table>

Dissolve in distilled water, pH to 7.5, make to 1 litre.

TBS-Tween (0.05%)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x TBS stock</td>
<td>100 ml</td>
</tr>
<tr>
<td>Tween 20</td>
<td>500 µl</td>
</tr>
</tbody>
</table>

Make to 1 litre with distilled water.

Carbonate buffer (pH 9.6)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂CO₃</td>
<td>0.79 g</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>1.46 g</td>
</tr>
</tbody>
</table>

Dissolve in distilled water, pH to 9.6, make to 500 ml.

Antibody conjugate

Polyclonal sheep anti-digoxygenin Fab fragments, conjugated with alkaline phosphatase, 750U/ml (Boehringer Mannheim, No. 1 093 274). Use diluted 1/5 000 in TBS-Tween, with 3% milk powder (w/v).

Blocking solution

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x SSC</td>
<td>5.0 ml</td>
</tr>
<tr>
<td>10% SDS</td>
<td>100 µl</td>
</tr>
<tr>
<td>1% N-lauryl sarcosine sodium salt</td>
<td>200 µl</td>
</tr>
</tbody>
</table>

Make up to 10 ml, then add 0.3g skimmed milk powder.

Substrate solution

The substrate for the colour reaction is p-Nitrophenyl phosphate, 5 mg tablets.
6. DRAFT PROTOCOL FOR ISOLATION AND IDENTIFICATION OF Naegleria fowleri FROM WATER ETC.

(Numbering of paragraphs is purely internal to this section and has been made comparable to the numbering in the Blue Book: Isolation and Identification of Giardia cysts, Cryptosporidium oocysts, and free living pathogenic amoebae. HMSO, 1989 pp. 15-18).

1. INTRODUCTION

Methods recommended here were developed as result of an evaluation of various protocols for detection of Naegleria fowleri from water samples (Department of the Environment contract number PECD 7/7/412).

2. SAFETY

N. fowleri is potentially pathogenic for man. Samples thought to contain this organism should be processed in a Containment Level 3 facility and all equipment and consumable items autoclaved before being discarded or reused. The culture manipulation of known or suspected N.fowleri should be conducted in an approved biological safety cabinet. Specific information regarding the safety precautions is to be found in “Categorisation of pathogens according to hazard and categories of containment”. Advisory Committee on Dangerous Pathogens, 1995. HMSO London.

3. EQUIPMENT

Filter holders for 47mm diameter membranes, manifold unit, vacuum pump and silicone connecting tubing.

47mm diameter 0.45μm pore size cellulose acetate membranes.

Inverted light microscope with x10 and x20 objectives.

Vortex mixer.

Incubators set at 30°, 37° and 42°C.

90mm polystyrene petri dishes, glass universal containers, plastic Pasteur pipettes, flat-bottomed 96 well microtitre plates, Eppendorf tubes for PCR, adjustable pipettes and sterile tips.

Sterile swabs, wax crayons or marker pens, scalpel blades.

Programmable thermal cycler for Polymerase Chain Reaction

ELISA plate reader.
Strips of flat bottom, high binding capacity 2x8 microtitrewells, with frames.

Microcentrifuge

4. MATERIALS AND REAGENTS

4.1 Media:

Page's amoeba saline (PAS) at pH 6.8-6.9

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>0.120 g</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.004 g</td>
</tr>
<tr>
<td>CaCl₂·2H₂O</td>
<td>0.004 g</td>
</tr>
<tr>
<td>Na₂HPO₄</td>
<td>0.142 g</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.136 g</td>
</tr>
</tbody>
</table>

Dissolve in distilled water, adjust to pH 6.8 and make up to 1 litre. Autoclave at 121° and store at room temperature.

PAS - *E. coli* medium

Add 1 ml of *E. coli* suspension (OD 650 : 6.0) to 100 ml of PAS and store at 4°C, for two weeks.

4.2 Non-nutrient agar - *E. coli* plates (NNA- *E. coli*) plates; non-nutrient agar seeded with a lawn of *E. coli* on which the amoebae feed. These are prepared as follows:

4.2.1 Non-nutrient agar plates (NNA) . 1.5% w/v bacteriological grade agar in PAS. Autoclave at 121° C for 15 minutes. Distribute approximate 25ml volumes into Petri dishes and dry at 37° C for 24 hours. Plates should not be dried uncovered as cysts of free-living amoebae may be present in the air of the laboratory. Plates are stored in sealed polyethylene bags at room temperature for up to 14 days.

4.2.2 *Escherichia coli* NCTC 10418 is grown on nutrient agar plates at 37° C for 24 hours. A stock culture plate can be stored at 4° C for up to 1 month. This may be used to seed several nutrient agar plates. Following incubation, the plate cultures are stored at room temperature in sealed polythene bags for up to 7 days or at 4° C for two weeks.

4.2.3 A thick portion of *E. coli* is taken from a seeded nutrient agar plate using a sterile swab and spread over the entire surface of one NNA plate. Alternatively, the bacteria may be washed from the surface of the culture plate and dilutions of the suspension made in PAS to give a suspension with an estimated OD 650nm of 6.0. This may be kept as a stock at 4° C for two weeks. This stock is diluted 1/100 before use in PAS to give an estimated OD 650 of 0.06. 50 μl are then added to each well of microtitre plates, to which 50μl aliquots of samples for testing (before or after concentration by filtration) will be added.
SSC. Standard saline-citrate solution. (Stock solution, 20x: 3.0M NaCl, 0.3M trisodium citrate in deionised and distilled water. Filter before use.)
Wax pellets for hot-start technique.
Taq polymerase and manufacturer's concentrated buffer with 1.5mM MgCl₂.
Sterile distilled water, deionised and autoclaved.
Stock solutions of the 3 specific primers, Nf5a, Nf5a and Nf7b.
Forward primer Nf5a: 5' CCT GGA TGC AGT AGT TTG GCC G
Reverse primer Nf5b: 5' DIG-TTT GAC AGG TTG TTG AGT CTT TGG
Internal primer Nf7b: 5' BIOTIN-ACT GAT GCT GAA GCG GAT A
Deoxynucleotide triphosphates (dNTPs) (dATP, dGTP, dCTP and dTTP)s at 10 mM.
$p$-Nitrophenyl phosphate, 5 mg tablets.
Polyclonal sheep anti-digoxigenin Fab fragments, conjugated with alkaline phosphatase, 750U/ml
Proteinase K
phenol-chloroform
chloroform
sodium acetate solution (final conc. 0.3M, pH 5.2)
100% ethanol
70% ethanol
Salmon sperm DNA

4.2.5 Tris - buffered saline (TBS).

TBS (0.1M Tris, 1.5M NaCl, pH 7.5)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris HCl</td>
<td>12.5 g</td>
</tr>
<tr>
<td>Tris base</td>
<td>2.36 g</td>
</tr>
<tr>
<td>NaCl</td>
<td>87.66 g</td>
</tr>
</tbody>
</table>

Dissolve in distilled water, adjust pH to 7.5, make to 1 litre.

4.2.6 TBS-Tween (0.05%)  

10x TBS stock: 100 ml
Tween 20: 500 µL
Make to 1 litre with distilled water.

4.2.7 Carbonate buffer (pH 9.6)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂CO₃</td>
<td>0.79 g</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>1.46 g</td>
</tr>
</tbody>
</table>

Dissolve in distilled water, pH to 9.6, make to 500 ml.

4.2.8 Blocking solution

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x SSC</td>
<td>5.0 mL</td>
</tr>
<tr>
<td>10% Sodium dodecyl sulphate</td>
<td>100 µL</td>
</tr>
<tr>
<td>1% N-laurylsarcosine sodium salt</td>
<td>200 µL</td>
</tr>
</tbody>
</table>
5. SAMPLE MATERIAL AND COLLECTION

Surface water, mud, soil aquatic plants, water treatment system and bathing pool filter deposits, swimming pool water and and potable water samples are all suitable for the isolation of pathogenic free living amoebae. Samples should be collected into sterile glass or polypropylene containers and the environmental temperature recorded. For chlorinated water samples, sodium thiosulphate as an 18g/l stock solution should be added to give a final concentration of 18mg/L. Samples should be transported to the laboratory without refrigeration and processed on arrival. It is generally accepted that refrigeration is harmful to trophozoites of thermophilic free living amoebae like N. fowleri and that delay in processing may lead to overgrowth of pathogenic species by non-pathogenic.

6. SAMPLE VOLUME

The volume of water to be processed for the detection of N. fowleri is determined by the nature of the sample, the sample site and the purpose of the examination. A single 250 ml sample of water may be sufficient for the routine monitoring of domestic drinking water or an adequately maintained chlorinated bathing pool, since if there are no amoebae whatsoever growing at 30°, 37° or 42° C from that volume, there are unlikely to be any pathogenic species even in much larger volumes. Much larger volumes, up to several litres, may have to be examined from a probable source of human infection. In such cases it may be preferable to process the sample in several subsamples rather than as one single specimen. When examining surface waters, filterbed samples, etc, the volumes tested may need to be reduced if initial samples produce very high numbers of free living amoebae which may obscure the presence of the pathogen.

7. ISOLATION METHODS

7.1 Concentration techniques:

Filtration

Thoroughly mix the water sample and filter through a 0.45µm pore size cellulose acetate membrane by suction at a flow rate not exceeding 30mL per minute. Do not allow the membrane to dry; stop filtration when 2-3mL of sample remains above the membrane.

Carefully wash the membrane in situ with the residual sample, using a plastic Pasteur pipette.

Transfer the whole of the residual sample used to wash the membrane into a a sterile glass universal container. Place the membrane in the same universal, so rolled that the upper or sample surface in inward and not in contact with the wall.  
Shake vigorously with a vortex mixer for 10 seconds.
A: Distribute the whole of the fluid from the last step over the surface of 2-3 NNA-E. coli plates and allow to absorb to dryness at room temperature. Where amoebae are scarce, pour the fluid on the surface of a single NNA-E. coli plate and leave at room temperature for 2 hours. Pipette off the excess fluid and allow to dry. Plates should not be dried uncovered as airborne cysts may contaminate them. Include uninoculated NNA-E. coli plates as controls.

B: Distribute 50uL aliquots of the fluid concentrate from the filter into a row of 12 wells of a microtitre plate to each of whose wells has been added 50uL PAS-E. coli medium. Make serial 2-fold dilutions, using a multipipette set at 50uL, from the 12 wells into the remaining 7 rows of the plate. Incubate the plate in a moist box at 30° C.

Repeat this process to set up plates to be incubated at 37° and 42° C.

7.2 Direct plating of samples

Untreated water samples may contain large numbers of free living amoebae. This can result in failure to obtain clonal isolates because of overcrowding on the plates. To avoid this, unconcentrated samples may be subjected to procedures A and/or B above.

Solid material may be inoculated directly on NNA-E. coli plates, or, in the case of mud, for example, a known weight vortexed in a known volume of PAS and then subjected to procedures A and/or B.

Vortex swab samples in 2mL of PAS and culture the liquid as in A and/or B above.

8. DETECTION OF AMOEBAE

Microtitre and Petri dish plates should be examined, using the x 10 objective of the inverted microscope, daily for 7 days.

Amoebae can be seen on Petri plates as feeding trophozoites producing tracks or clearings in the bacterial lawn.

In microtitre plates the organisms can be easily seen on the flat bottom of the well.

Positive plates or wells should be marked with the marker pen or wax crayon.

8. ESTIMATION OF NUMBERS OF AMOEBAE GROWING AT EACH TEMPERATURE.

Estimations of viable counts can be made after serial dilutions of samples in the microtitre plates, using tables of most likely numbers as for bacteria. The estimation of numbers of viable amoebae per litre will be carried out taking into account the total volume of water sampled, the volume after concentration, the volume used to inoculate each well of the microtitre plate.

9. Identification of *Naegleria fowleri* in the cultures.

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After growth for 7 days, the positive 37° and 42° cultures should contain sufficient amoebae for confirmation of the presence of *N. fowleri* by semi-nested polymerase chain reaction solution hybridisation enzyme-linked assay (DN PCR-SHELA).

Harvesting the cultures and DNA preparation.

Amoebic colonies from plates may be cut out using a sterile scalpel, vortexed briefly (10 secs) in a sealed Eppendorf tube with a small volume of PAS and centrifuged for 5 secs in a microfuge at 12,000rpm, after removal of the agar, to give a pellet of amoebae and bacteria.

200 μl of proteinase K buffer [10mM Tris, pH 7.8; 5mM EDTA; 0.5% SDS] was added to the pellet, and the resuspended material transferred to an Eppendorf tube.

1 μl of proteinase K (at 25 mg/ml) was added and the tube was incubated for 2 hours at 55°C.

DNA purification:

1) 200 μl of phenol:chloroform was added to the sample. The tube was vortexed for 5 sec and microfuged (13 000 rpm, 2 min).

2) The aqueous layer (upper layer) was carefully removed to a fresh Eppendorf and the first tube discarded into the phenol waste.

3) 200μl of chloroform was added to the second Eppendorf, the tube was vortexed for 5s and microfuged (13 000 rpm, 2 min).

4) The aqueous layer was removed to a third tube and the second discarded.

5) Steps 3 and 4 were repeated.

6) 20 μl sodium acetate (final conc. 0.3M, pH 5.2) and 400 μl 100% ethanol were added and the sample left at -20°C overnight.

7) The tube was microfuged (13 000 rpm, 20 min), the supernatant carefully removed with a fine-tipped pipette and discarded.

8) The pellet was resuspended in 400 μl 70% ethanol and left at -20°C overnight.

9) The tube was microfuged (13 000 rpm, 20 min), the supernatant carefully removed with a fine-tipped pipette and discarded.

10) The pellet was left to air dry, then from 2 to 50 μl sterile distilled water was added (depending on the sensitivity desired). The DNA solution may be stored at -20°C.

Protocol for the demi-nested PCR-SHELA.
The hot-start PCR method is used.

Add 1uL of each stock primer solution at 40pmol/mL to each Eppendorf tube, then place a wax pellet on top. Centrifuge the tubes briefly, then place at 80°C for approximately 5 min until the wax melts. Allow the wax to solidify. Then add the rest of the PCR reaction mixture: To 36.75uL of distilled water add 5uL of the Taq manufacturer’s buffer with MgCl₂, 1uL of each of the 4, 10mM stocks of the dNTPs, 0.25uL of Taq polymerase and finally 1-2uL of the template DNA sample. For positive controls add a sample of N.towleri DNA prepared from a known number of organisms, and for negative, 1-2uL of distilled water from the stock used in the test.

The final composition of the 50-51uL PCR mix was as follows:

40pmol/mL of each primer

200uM of each dNTP

1.5mM MgCl₂

1.25 Units Taq polymerase

Template DNA

The sealed reaction mixtures are then placed in the temperature cycler.

Temperature cycling:

\[
\begin{array}{ccc}
94°C & 3 \text{ min} \\
94°C & 30 \text{ s} \\
65°C & 1 \text{ min} \\
72°C & 1 \text{ min} \\
94°C & 30 \text{ s} \\
54°C & 1 \text{ min} \\
72°C & 1 \text{ min} \\
72°C & 10 \text{ min} \\
\end{array}
\]

(Total 100.5 minutes)

Colour detection protocol:

1) Heat the tubes at 60°C to melt the wax, flick and place on their sides. (The wax quickly hardens on the side of the tube, allowing the PCR products to be centrifuged and then removed with a pipette). Add 20 µL of PCR product to 200 µL of diluting solution.

29
2) Coat the wells of a microtiter plate with avidin. Use 1mg/ml stock avidin diluted 1/100 in carbonate buffer, and add 100 µl to each well. Leave for 1 hr at 37°C.

3) Wash twice with 100µL 1.5 ug/ml salmon sperm DNA in TBS-Tween for 2x30 minutes at room temperature.

4) Briefly wash the plate twice with TBS-Tween and add 100 µl of each diluted product from the PCR, and incubate for 1 hr at room temperature.

5) Briefly wash the plate twice with TBS-Tween and add to each well 100 µl of 1 µl of anti-digoxygenin conjugated to alkaline phosphatase diluted in 5 ml TBS-Tween containing 150 mg milk powder. Incubate the plate for 1 hr at room temperature.

6) Briefly wash the plate four times with TBS.

Dissolve one alkaline phosphatase substrate tablet in 5 ml carbonate buffer containing 5 µl 1M MgCl₂. Add 100µl to each well, and read the optical density of each well, at 410 nm, after 1 hr. at room temperature.

To evaluate the cut off point for identification of N. fowleri, calculate the mean OD of the negative control wells with its standard deviation. OD values over 3 standard deviations above the mean control value are classed as positive.
7. APPENDICES

7.1. SAFETY PROTOCOL

*Naegleria fowleri* is a pathogenic free living amoeba which is infective to man, usually while swimming, by the intranasal route. The organism is found locally in warm fresh water habitats. There is a treatment available (amphotericin B) for infections with this organism, but unless the diagnosis is made early the outcome is usually fatal. There have been no reports of laboratory infection, though it is true that the organism is little studied. There is no risk of spread in the community from an infected person. According to ACDP the organism falls into hazard group 3 and cultures should be handled in a class 1 (exhaust protective) or class 3 (fully enclosed) cabinet. The laboratory used should have a vented lockable door with an air inlet vent, and entry should be restricted to authorised persons whilst *Naegleria* is being handled.

The trophozoite and flagellate stages of *Naegleria* are infective but evidence suggests the cysts are not. Experimental studies indicate that both are killed by desiccation. Nevertheless, spillages must be decontaminated.

Laboratory coat and gloves should be worn, in addition to eye protection, when handling the organism. The gloves should be swabbed at intervals with 70% alcohol and the coats should be laundered (or disposed of) weekly. Cuts and abrasions on the hands should be protected from contamination with waterproof adhesive bandage.

Disinfection:

5% chloros is adequate for the disposal of infected material. Use of sharps should be avoided, and if they are used they must be discarded in sealed plastic bags in a cin bin. Contaminated tissues, agar plates, etc should be bagged and autoclaved.

Plastic pasteur pipettes should be used, not glass, and discarded in 5% chloros. Blunt metal needles are available and must be used in preference to sharp needles wherever possible. The cabinet must be fumigated with formalin vapour on a regular basis (weekly, or following use for this organism, whichever is more frequent).

Working areas must be swabbed with 70% alcohol at the conclusion of any work. Beakers containing 5% chloros and wash bottles containing 70% alcohol respectively must always be available.

Centrifugation:

Centrifugation must be carried out in the Class 3 facility.

Avoid centrifugation in glass containers. Open containers must not be used, and the centrifuge buckets must be sealed. Opening the buckets and tubes after centrifugation should be carried out in the safety cabinet. In case of a breakage while centrifuging, allow the centrifuge to stop, and allow 10 minutes before opening the lid. Handle the contents with gloves and transfer buckets to the cabinet before opening them.

Cryopreservation:

Face mask and gloves and gown must be used for handling material to be added or taken from the cryobank. Thawing of ampoules (plastic only) must be carried out in the safety cabinet, and the ampoules should be kept in the safety cabinet in a sealed metal box for 5 minutes before thawing is attempted. If the cryobank is in a different area from the class 1 or 3 hood, then the unthawed or pre-freezing ampoules may be transported from one room to the other only in a sealed metal box.

Accidents:
Contamination of the hands should be flushed initially with 70% alcohol, then washed with soap and water. Splashes in the eye should be rinsed immediately with tap water. Cuts and needle punctures must be encouraged to bleed and washed well with 70% alcohol. Accidents must be reported to the departmental safety supervisor.
7.2. CULTURE

Routine culture of amoebae:

7.21 Monoxenic culture:

*Escherichia coli* was grown on nutrient agar, then the colonies scraped off and suspended in Page’s Amoebal Saline (PAS). The suspension was centrifuged (600 g, 5 min), and the pellet resuspended in PAS to give a milky suspension. Five drops of suspension were spread on the surface of small agar plates (3% agar fine powder in PAS), and the saline allowed to soak into the agar for 10 min. A square of agar was transferred from an existing culture to the centre of the agar surface. Alternatively, drops of fluid containing amoebae can be spread on the surface of the plate. The plates were incubated at 37°C, in a humid box. This was a self-sealing plastic box with a wet paper towel placed in the bottom. Copper sulphate crystals were spread on the towel to prevent microbial growth in the box.

7.22 Axenic culture:

Axenic culture of *Naegleria* spp. was carried out in Bath Spa Medium. An agar square from a monoxenic culture was placed in a Nuncenon flat sided tissue culture tube in 2 ml of PAS - E. coli medium and incubated at 37°C. The tube was incubated until semi-confluent growth was obtained, then the culture medium was carefully removed to avoid disturbing the amoebae. 2 ml of warm PAS was added, the tube gently rocked and then the PAS removed. Three ml of Bath Spa Medium was added and the tube incubated at 37°C. The medium was replaced when growth became almost confluent. The tubes were then sub-cultured; 1 ml of infected culture fluid was added to 2 ml of Bath Spa Medium.

7.3 MEDIA

7.31 Page’s Amoeba Saline (PAS)

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>1.20 g</td>
</tr>
<tr>
<td>MgSO$_4$.7H$_2$O</td>
<td>0.04 g</td>
</tr>
<tr>
<td>CaCl$_2$.2H$_2$O</td>
<td>0.04 g</td>
</tr>
<tr>
<td>Na$_2$HPO$_4$</td>
<td>1.42 g</td>
</tr>
<tr>
<td>KH$_2$PO$_4$</td>
<td>1.36 g</td>
</tr>
</tbody>
</table>

Dissolve in distilled water, adjust to pH 6.8 and make up to 1 litre. Autoclave. To use, dilute 1:10 with sterile distilled water.

7.32 Bath Spa Medium

Part 1: Casitone 5 g  
Proteose peptone 5 g  
Yeast extract 5 g  
Panmede liver digest 10 g  
Glucose 5 g  
Page’s Amoebal Saline 900 ml

Filter sterilise and store in 90 ml aliquots at -20°C.

Part 2: Hemin 100 mg  
Triethanolamine 4 ml  
Distilled water 96 ml

33
Filter sterilise and store in 1 ml aliquots at -20°C.

To use:

Part 1: 90 ml
Part 2: 1 ml
Donor calf serum (heat inactivated) 10 ml
Gentamycin (40 mg/ml) 0.1 ml

Store at 4°C.
PAS - E. coli medium

Add 1 ml of E. coli suspension to 50 ml of PAS and store at 4°C, for up to 1 month.
7.4. DETECTION OF AMOEBAE BY CULTURE.

For detection of amoebae 2 main approaches are needed, depending on whether the organisms are abundant or scarce. Where the organisms are scarce, concentration of the water is necessary using the filtration technique.

Where amoebae are abundant, direct cultivation of the material is recommended on collection.

Although the current method requires cultivation upon numerous non-nutrient agar plates, with *Esch coli* as food source, it has been found more convenient to make the multiple cultures needed in microtitre plates, using a dilute suspension of the bacteria for growth.

The bacteria are prepared as a suspension washed in sterile Page's saline and a stock with a nominal OD 650 of 6 is prepared. This stock is diluted before use in Page's saline to give an estimated OD 650 of 0.06. 50 µl are then added to wells of microtitre plates, and 50µl aliquots of samples for testing (concentrated or not) are added. Incubation in a moist box at 37°C or as recommended in the blue book protocol (Isolation and identification of Giardia cysts, Cryptosporidium oocysts and free living pathogenic amoebae in water etc 1989) is then carried out, with examination of the plates first after 24hrs, and for up to 7 days. Solid and swab samples should be washed and prepared for plating as described for the filter membranes. Estimations of viable counts can be made after serial dilutions of samples in the microtitre plates, using tables of most likely numbers as for bacteria.

DETECTION OF *Naegleria* spp.

To confirm presence of *Naegleria* spp (i.e. species of *Naegleria* which may include the pathogen *N. fowleri*) in cultured material, the flagellation protocol described in the Blue Book may be carried out. Occurrence of any species of this genus in potable or recreational water is likely to be associated with unacceptable bacterial counts. On identifying the genus in some wells, the most probable numbers in the original sample can be estimated.
DNA EXTRACTION

Standard technique:

A. From monoxenic culture:

Two or three culture plates were used. 1 ml of PAS was added to the first plate and the surface gently scraped with an inoculating loop, suspending the cells in the saline.

The cell suspension was then pipetted onto the surface of the next plate. The surface of this plate was also scraped and the liquid transferred to the third plate.

The surface of this plate was scraped and the cell suspension was added to a centrifuge tube.

1 ml of PAS was then added to each plate and the surface washed by pipetting up and down. All the washings were added to the centrifuge tube.

The tube was centrifuged (450g, 5 min), and the supernatant discarded.

200 µl of proteinase K buffer [10mM Tris, pH 7.8; 5mM EDTA; 0.5% SDS] was added to the pellet, and the resuspended material transferred to an Eppendorf tube.

1 µl of proteinase K (at 25 mg/ml) was added and the tube was incubated for 2 hours at 55°C.

B. From axenic culture: One 25cm² culture flask with a confluent layer of amoebae was put on ice for 1/2 hour, to make the organisms round up and detach from the sides of the flask.

The flask was shaken and the contents pipetted into a centrifuge tube.

The tube was centrifuged and proteinase K added as described above.

DNA purification:

200 µl of phenol:chloroform was added to the sample. The tube was vortexed for 5 sec and microfuged (13 000 rpm, 2 min).

The aqueous layer (upper layer) was carefully removed to a fresh eppendorf and the first tube discarded into the phenol waste.

200µl of chloroform was added to the second eppendorf, the tube was vortexed for 5s and microfuged (13 000 rpm, 2 min).

The aqueous layer was removed to a third tube and the second discarded.

Steps 3 and 4 were repeated.

20 µl sodium acetate (final conc. 0.3M, pH 5.2) and 400 µl 100% ethanol were added and the sample left at -20°C overnight.

The tube was microfuged (13 000 rpm, 20 min), the supernatant carefully removed with a fine-tipped pipette and discarded.

The pellet was resuspended in 400 µl 70% ethanol and left at -20°C overnight.
The tube was microfuged (13 000 rpm, 20 min), the supernatant carefully removed with a fine-tipped pipette and discarded.

The pellet was left to air dry, then 50 µl sterile distilled water was added. The DNA solution was stored at -20°C.

More user-friendly methods of DNA extraction are needed for routine purposes.

Using amoebic trophozoites, boiling a suspension, cooling and adding 1-2 µl of the supernatant after centrifugation to a PCR reaction gives satisfactory results, but this technique did not work for cysts.

Freezing and thawing x3 in liquid nitrogen and water at 37°C was suitable for trophozoites and for cysts.

Sonication was not tested owing to an adverse hazard assessment.

A technique which involves application of 20 µl of a suspension to a glass fibre membrane, drying, washing with distilled water over a sintered filter and using a 1/8 segment of the area where the organisms have been applied for direct addition to the PCR reaction, should be tested because it has proved effective in avoiding the influence of PCR inhibiting substances from blood in malaria PCR tests.

### 7.6 PCR PROTOCOLS

7.6.1 McLaughlin et al: (mitochondrial ATPase, Genbank M55009)

Forward primer Nf1: 5' CGTATCTAGTAGATAGAACA 20 bases

Reverse primer Nf2: 5' CGTAACGACAAACCTACAGA 20 bases

(Reverse primer Nf2: 5' CGTAACGACAAACCTACAGA given in paper, incorrect).

Reverse probe MP: 5' AACAG TAGCCACCATA 19 bases

In our hands the specificity of the technique used under stringent conditions appears satisfactorily high. No product was produced from the other amoebae: N. lovaniensis 1479 and 6664.2 (isolates from Bath) Acanthamoeba polyphaga or Entamoeba histolytica, flagellates: Leishmania donovani, L. enrietti, Trypanosoma cruzi, Crithidia fasciculata, Giardia intestinalis, coccidians: Cryptosporidium parvum, C. muris, bacteria: Escherichia coli, or an environmental isolate of yeast. At the same time, product of the correct size was produced from N. fowleri from USA (HB1), Colombia (Colombia 1) and Australia (Morgan).

We have carried out sensitivity tests on this protocol which involve amplifying DNA extracted from different quantities of amoebae in the PCR.

We could detect and positively identify, by the agarose gel technique, from 10-fold dilutions of amoebae, 400 organisms but not 40. This suggests that the limit of detection for visual evaluation of ethidium bromide fluorescence is between these figures.

When a Southern transfer was carried out from such a run, and a radiolabelled PCR product (validated by hybridisation to the specific internal probe) was used to detect the N. fowleri PCR product, the sensitivity was greatly increased, and DNA from fewer than 1 organism was detected.

The sensitivity is what would be expected from a target molecule, mitochondrial DNA, where there are more than 10 examples per cell. However, under conditions of water examination, there may be materials present which may interfere with the PCR reaction itself. Additionally, the production of a product from N. lovaniensis under less stringent conditions may not be an encouraging sign, and may suggest that the PCR test could be unduly influenced by laboratory variables.
7.62 Hu's virulence related protein: genbank sequence M88397:

Primers NF3 and NF4 were designed.

Forward:  NF3  5' CCAGTGAACATGGTTTATTCTTG  24 bases
Reverse:  NF4  5' TTAGAGTCTTTTGGATCCAACACTTG  24 bases

product: predicted 649bp from c-DNA sequence, actually 750bp.

Sparagano's primers, for Hu's sequence:

Forward primer: NF5:  5' ATGCAGTAGTTGGCCG  17 bases
Reverse primer: NF6:  5' AGTTTTGCAGCTTTGG  18 bases
Forward probe: NF7:  5' GTTGGTAATGTTGACTGATGCAGAACG  33 bases

Reaction mixture: (using Bioline Taq and buffer)

Sterile distilled water 36.75 µl buffer (at x10), incl. MgCl₂ stock ( ): 5 µl
primers, each (40 pmol/ml) 1 µl
dNTPs, each (10 mM) 1 µl
enzyme (5U/ml) 0.25 µl
DNA template (from sample) 2 µl
50 µl of mineral oil was added to the tubes, to prevent
evaporation. The tubes were placed in the PCR machine and the
program set.

The final volume of the aqueous phase in each PCR tube was 50ul, and this contained 40pmol of
each primer, 200µM dNTPs, 1.25 U Taq polymerase and 2ul of template DNA in Bioline buffer containing
1.5mM MgCl₂.

(Calculation of dNTP concentrations:
We assume each base has molecular weight of 330 Da.
OD₂₆₀nm = 1.0 for 20µg/ml solution.
If concentration in µg/ml is x, then molarity is (1000x/330) µM.).

The first protocol used was as follows:

94°C 2.5 min  1 cycle
52°C 1 min
72°C 1 min

94°C 0.45 min 30 cycles
52°C 1 min
72°C 1 min

94°C 0.45 min  1 cycle
52°C 1 min
72°C 10 min

38
The protocol given in McLaughlin et al (1991) was also used:

94°C 1 min  32 cycles  
52°C 1 min  
72°C 3 min  

94°C 1 min  1 cycle  
52°C 1 min  
72°C 6 min  

The PCR products were stored at -20°C. Visualisation of the products was by electrophoresis on a 2% agarose gel which was then stained with 50 µg/ml ethidium bromide in TAE. A 100bp marker ladder was run in the first lane so that the size of the products could be estimated.

8.7 HYBRIDISATION AND LABELLING OF PROBES.

To demonstrate that the desired target sequence had been amplified, a hybridisation was performed.

Dot Blot

Preparation of radiolabelled probe:

For the McLaughlin PCR product, the internal reverse sequence MP (19bp) within the 300bp target sequence was used as a probe:

1) A labelling solution was prepared using a Promega 3' end-labelling system:

sterile distilled water  12.4 ml
buffer (5x)  4 ml
probe (2pmol/ml)  1 µl
terminal transferase  1 µl
\[^{32}P\] dCTP (10mCi/ml)  1.6 µl

The radiolabelled dCTP was added to the labelling solution behind a perspex screen. (use of radiophosphorus necessitates meticulous precautions. The operators must be continuously monitored using film badges, the benches screened using geiger counter, and the HSE regulations adhered to for the disposal of waste materials).

2) The solution was incubated at 37°C for 30 min, then heated to 70°C for 10 min. This is to denatures the enzyme and stops the reaction.

3) The solution was chilled on ice for a few minutes, then spun briefly.

The specific activity of the probe was 2-3 x 10^8 cpm/µg.

Preparation of membrane:

1) 20 µl of the PCR product was denatured by heating for 5 min at 100°C, cooled on ice for 5 min then briefly spun. Herring sperm DNA (0.1 mg/ml) was also denatured and used as a control in case the binding of the probe was non-specific.

2) 10 µl of each solution was dotted onto a piece of nylon membrane (Hybond-N+). The spots were labelled in pencil on the membrane.
3) When dry, the membrane is placed in a UV light oven. The oven emits a spectrum of UV intensities and causes the DNA to covalently bind to the membrane, so it cannot be washed off.

Prehybridisation.

A prehybridisation solution was prepared as follows:

- SSC (20x) 6 ml
- Denhardt's (50x) 2 ml
- SDS (10%) 0.4 ml
- sterile distilled water 11.6 ml
- herring sperm DNA (denatured) 50 μl

5) Approximately 0.1 - 0.2 ml hybridisation solution/ cm² nylon membrane is required. The membrane and solution are placed in a vacuum sealed bag and incubated at 37°C for 4 hours.

Hybridisation:

5 μl of the labelled probe was added to the bag containing prehybridisation buffer, the bag resealed and incubated at 37°C overnight.

Washing:

1) A wash solution was made up as follows:

- SSC (20x) 300 ml
- SDS (sodium dodecyl sulphate) (10%) 10 ml

Make up to 1 litre with distilled water.

2) The membrane was placed in a tray, with some wash solution, and the tray agitated for 5 min at room temperature. The used wash solutions were poured down a radioactive-labelled sink, with large volumes of water. This was repeated.

3) Two washes (5 min) were performed at 37°C in the hybridisation oven with the shaker on.

4) The membrane was then placed in an intensifying screen with a piece of X-ray film and put at -70°C overnight.

5) The film was developed, and an autoradiograph of the membrane obtained.

Southern blot for detection of product:

Preparation of probe:

The 300bp PCR product from a previous reaction, confirmed by a dot blot to have the correct sequence, was used as a probe.

30 μl of PCR product from a reaction tube was run on a 1% agarose gel, then the gel stained with ethidium bromide to visualise the bands. The required band was then cut out of the gel, with a scalpel blade and placed in an Eppendorf.

A Sephaglas Band Preparation was used to extract the DNA from the agarose.

1) 250μl of gel solubiliser was added, the tube vortexed and incubated at 60°C for 5-10 min.

2) 5 μl of Sephaglas BP was added, the tube vortexed and incubated at room temperature for 5
3) The sample was spun for 1 min and the supernatant discarded.

4) 40 µl wash buffer was added and spun. This was repeated twice.

5) The pellet was air-dried, then resuspended in 500 µl water. The sample was spun again and the supernatant removed to another tube.
The probe was then labelled by random hexamer labelling using a Pharmacia Oligolabelling kit

1) A labelling solution was prepared:
   - probe 36 µl
   - (α³²P)dCTP (3000 Ci/mmol) 3 µl (30µCi)
   - reagent mix 10 µl
   - Klenow fragment 1 µl

The radiolabelled dCTP was added to the labelling solution behind a perspex screen. (Safety precautions).

2) The tube was incubated at 37°C for 1 hour, then heated to 70°C for 10 min to inactivate the enzyme.

3) The probe was put through a spun column (Clontech Crome-spin + TE-10) to remove any unincorporated nucleotides.

4) The spun column was mixed gently, then spun for 2 min, then 1 min to remove the buffer.

5) The probe was added dropwise to the top of the column.

6) The column was spun for 5 min, then the cpm of the eluate was checked, with a hand held monitor, to confirm the probe had passed through the column.

Preparation of the membrane

1) The PCR products were run on a 2% agarose gel, and stained with ethidium bromide to visualise the bands as before.

2) A denaturing solution was prepared:
   - NaCl 87.66 g
   - NaOH 20 g
   - Made up to 1 litre with distilled water.

3) The gel was placed in a tray containing 250 ml of the denaturing solution and put on a shaker for 45 min.

4) The tray and gel were rinsed, then the gel placed in 10xTBS (1M Tris, 1.5M NaCl) for 30 min. Then for 15 min.

5) The gel was placed in the electrotransfer cassette, with the nylon membrane cut to the same size. The transfer buffer was TAE (0.2x). The apparatus was switched on and run overnight at 30V.

The gel was removed from the tank and stained with ethidium bromide, to check that all the DNA had been transferred.

The membrane was left to dry and the DNA crosslinked as before.
prehybridisation

A prehybridisation solution was prepared:
- distilled water 12 ml
- Denhardt’s (50x) 2 ml
- SSC (20x) 5 ml
- SDS (10%) 1 ml
- herring sperm DNA (denatured) 200 µl

The herring sperm DNA was denatured by heating to 100°C for 5 min.

The membrane was placed in a bottle with 10 ml of the
prehybridisation solution and incubated at 65°C for 4 hours, in the rotary incubator.

Hybridisation

100 µl of herring sperm DNA and 50 µl of probe were denatured, then added to the other 10 ml of
prehybridisation solution.

The used prehybridisation solution was poured out of the bottle and replaced with the solution
containing the probe.

The bottle was incubated at 65°C overnight, in the rotary incubator.

Washing:

Two wash solutions were made up as follows:

Wash 1
- SSC (20x) 50 ml
- SDS (10%) 5 ml
Made up to 500 ml with distilled water.

Wash 2
- SSC (20x) 25 ml
- SDS (10%) 5 ml
Made up to 500 ml with distilled water.
Washing was performed as above, but the second set of washes were done at 65°C. If the cpm of the
membrane is still very high, a third more stringent wash could be done.

Wash 3

- SSC (20x) 2.5 ml
- SDS (10%) 5 ml
Made up to 500 ml with distilled water.
This wash would be performed at 65°C also.
The autoradiograph was obtained and developed as before.

Digoxigenin labelling of the internal probe for the virulence related protein product:

A DNA tailing kit (Boehringer Mannheim) was used to label 100pmol quantities of the probe in a
reaction containing 4µl probe, 4µl tailing buffer, 4µl CoCl₂, 1µl digoxigenin-11- dUTP, 1 µl dATP, 5µl
deonised water and 1µl terminal transferase enzyme. Incubation was for 15 min at 37°C. The tailed
product was purified after stopping the reaction using 2μl glycogen. 2.5μl LICl solution and 75μl cold 100% ethanol were added and the tube placed at -20°C for at least 2 hrs. The tube was microfuged for 20 min and the pellet was washed in 50μl cold 70% ethanol, dried and dissolved in distilled water at 2.5pmol/μl.

Hybridization to this product.

The membrane was prehybridised at 68°C for at least 1 hr. The prehybridisation solution contained 10μl/ml poly A solution (Sigma). The membrane was hybridised at 54°C for 6 hrs. The hybridisation solution contained 2pmol/ml of the probe and 10μl/ml of poly A.

Detection

The membrane was incubated in blocking solution for 30 min, then in antibody conjugate solution for 30 min. The colour reaction developed for c. 16hrs overnight and was then stopped and the membrane washed.

The probe was detected on hybridised blots by reaction with anti-digoxigenin /alkaline phosphatase conjugate. A blue product is detected on incubation with the chromogenic substrate/linker mixture, 5-bromo-4-chloro-3-indoly phosphate and nitroblue tetrazolium according to the manufacturer’s instructions.

7.7 POLYMERASE CHAIN REACTION-SOLUTION HYBRIDISATION ENZYME LINKED ASSAY : PCR-SHELA

The PCR-SHELA method allows a rapid visualisation of PCR product with the required internal sequence. The use of expensive agarose gels, carcinogenic ethidium bromide staining and hazardous ultraviolet light examination becomes unnecessary. The technique involves conventional PCR using one primer 5'-labelled with biotin, giving rise to double-stranded product which is labelled at one end with biotin. In the conventional technique, the product is denatured and a digoxigenin-labelled specific internal probe is allowed to hybridise with the labelled single strands. Then the material is diluted and dispensed in to a microtitre plate well which has been coated with avidin (or streptavidin). After washing, the biotin and digoxigenin labelled strands bound to the well are detected by use of an alkaline phosphatase-conjugated anti-digoxigenin antibody which is then revealed using alkaline phosphatase substrate linked to a colour-enhancing reaction. The intensity of the colour can be detected visually or using an automated machine. The potential sensitivity of the technique is reported to be as high as that achieved using [32P] labelled probes on a Southern blot from a standard PCR run in agarose. The technique is semi-quantitative and susceptible to automation for large numbers of samples.

In order to speed up the specific identification of the product the specific internal probe can be included in the PCR reaction mixture. Generally this probe has a short 3'poly A tail added (see N7a) to prevent it priming for the DNA polymerase. The PCR and hybridization reaction then occur simultaneously, and the product can be diluted and added direct to the avidin-coated wells of the microtitre plate. As a further precaution against priming by the probe, the melting point of the binding portion of the probe is made lower than that of the primers.

With the avoidance of agarose gel electrophoresis, suitable abbreviation of the PCR protocol, the in-tube hybridization protocol and the ELISA method suitably shortened by optimal choice of reagent concentrations, the limitations on sample numbers are related to the number of PCR machines available and the rapidity in preparing samples for PCR. With one machine, at least 100 tests, including negative and positive controls, per day should be capable of being performed.

7.71 Protocol for PCR SHELA:

Forward primer: Nf5 : 5' CCTGGATGCAGTAGTTTGCCG 22mer
Reverse primer: Nf6: Biotin or DIG 5' TTTGACAGGTTGAGTCTTTGG 24mer

Forward probe: Nf7:

DIG or Biotin 5' GTTGGAATGTTGACTGATGCTGAAGCGGAT 33mer

Forward probe: Nf7a: Biotin 5'ACTGATGCTGAAGCGGAT (A)ₙ

(for shortened protocol with probe added to PCR reaction)

The expected product of these primers, predicted from a cDNA clone, is 684 bp. Previously PCR products from DNA have been ~120 bp longer than reported by Sparagano or predicted from the cDNA.

Protocol for the PCR:

°C  
94 3 min
94 30 s
65 1 min | 35 cycles
72 1 min
72 10 min

Solution hybridisation (allowing integral or added probe to hybridise)

99 20 min
52 90 min.

Colour reaction:

Wells of a microtitre plate were coated in avidin (0.1 µg/ml in carbonate buffer) at 37°C for 1 hr. the plate was washed twice with TBS-tween (see below) then 100 µl of salmon sperm DNA solution (1.5 mg/ml) was added to each well. After incubation at RT for 30 min the plate was washed twice as before. 20 µl of each PCR mixture after thermal cycling was added to 250 µl of TBS-tween*, and 100 µl of this was added to each well of the microtitre plate. The plate was washed twice after another 30 min incubation. 100 µl of antibody conjugate at 1:5000 in TBS with 1.5% milk powder was added to each well. After 30 min incubation the plate was washed 4 times in TBS and twice in carbonate buffer. Lastly, 100 µl of substrate solution was added and the colour reaction allowed to proceed.

The PCR part of the protocol has been carried out in a variety of modifications, as a two-stage procedure with hybridisation involving addition of probe after PCR and as a one-stage test with integral 3’poly-A substituted probe (poly-A to prevent priming by the probe in the PCR reaction). Results initially were not sensitive or specific, although we obtained excellent results using a similar system for Entamoeba identification*. The major problem with the original protocol appears to be the excessive length of the N.fowleri sequence amplified, which allowed the strands to bend and self-hybridize. The Entamoeba sequences used in our earlier study were just over 100 base pairs in length.

Buffers and Solutions:

44
PCR-SHELA mix (1 reaction)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>36.75 µl</td>
</tr>
<tr>
<td>10x KCl buffer (Biolin)</td>
<td>5.0 µl</td>
</tr>
<tr>
<td>2 Primers (each)</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>Probe (add after PCR for sequential technique)</td>
<td>1.0 µl (1.25pmol)</td>
</tr>
<tr>
<td>4 dNTPs (each)</td>
<td>1.0 µl</td>
</tr>
<tr>
<td>Taq polymerase (Biolin)</td>
<td>0.25 µl</td>
</tr>
<tr>
<td>Target DNA</td>
<td>2.0 µl</td>
</tr>
</tbody>
</table>

Approximately 50 µl mineral oil was added to the tubes, to prevent evaporation of the samples when heated.

Avidin

Stock solution is 10 µg/ml in water. Use diluted 1/100 in carbonate buffer, i.e. 0.1 µg/ml.

Antibody conjugate

Polyclonal sheep anti-digoxigenin Fab fragments, conjugated with alkaline phosphatase, 750U/ml. Use diluted 1/5 000 in TBS-Tween, with 1.5% milk powder (w/v).

Substrate solution

The substrate for the colour reaction is p-Nitrophenyl phosphate. One 5 mg tablet (Sigma) is dissolved in 5ml carbonate buffer with 25 µl 1mM MgCl₂.

TBS (10x)

<table>
<thead>
<tr>
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</tr>
</thead>
<tbody>
<tr>
<td>O.1M Tris, 1.5M NaCl, pH 7.5</td>
<td>12.5 g</td>
</tr>
<tr>
<td>Tris/HCl</td>
<td>2.36 g</td>
</tr>
<tr>
<td>Tris base</td>
<td>87.66 g</td>
</tr>
<tr>
<td>NaCl</td>
<td></td>
</tr>
</tbody>
</table>

Dissolve in distilled water, pH to 7.5, make to 1 litre.

TBS-Tween (0.05%)

1xTBS with 0.05% Tween 20

10x TBS stock 100 ml

Tween 20 500 µl

Make to 1 litre with distilled water and add when required 1.5% fat-free milk powder.

Carbonate buffer (pH 9.6)

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na₂CO₃</td>
<td>0.79 g</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>1.46 g</td>
</tr>
</tbody>
</table>

Dissolve in distilled water, pH to 9.6, make to 500 ml.

Colour detection

The colour reaction could be seen by eye. A more accurate measurement was made by reading the optical density of the samples, at 405nm, in a microplate reader.
7.8 Demi-nested PCR-SHELA system:

Introducing a second amplification stage into the PCR ("nesting"), and using as primers one of the original biotin-labelled primer sequences and a new digoxigenin-labelled primer based on the original probe sequence for the middle of the initial stage PCR product, made three major improvements. It was possible to:

1) shorten the sequence binding to the avidin-coated microtitre plates and avoid self-hybridization.

2) take advantage of the increased sensitivity and specificity of the nested PCR.

3) carry out the 2 PCR reactions consecutively in the same sealed tube to avoid the cross-contamination hazard associated with the nested technique.

Details of this technique are given in section 6 (above).

7.9 IDENTIFICATION OF NAEGLERIA SPP BY ISOENZYME ELECTROPHORESIS

The use of isoenzyme electrophoresis of glucose phosphate isomerase (GPI) for differentiation of Naegleria isolates was introduced in 1978\(^\text{19}\). Fuller details of the technique using cellulose acetate membranes were described by S. Auye (1985) in her London University PhD Thesis, where the enzyme was found to be optimal for differentiation of N. fowleri from N. lovaniensis and N. granulosa.

The technique was designed to be carried out on isolates morphologically identified as Naegleria sp. by their motility and their positive flagellation test (see Blue Book 1989).

Preparation of amoebic extract:

Trophozoites grown in culture axenically or with bacteria were harvested and pelleted by centrifugation. The supernatants were discarded and the pellets were mixed with an equal volume of enzyme stabilising mixture (see below). After standing at room temperature for 15 min the suspensions were frozen and thawed for lysis, and then centrifuged at 6000 g for 30 min. The clear supernatants were removed and could be used for application to the enzyme electrophoresis immediately or stored at -20° C.

**Enzyme stabilising solution:** This is prepared from 3 reagents made up and stored separately in sealed bottles at -20° C as follows:

- **Reagent 1.** 200mM EDTA (0.744g in 10 ml water).
- **Reagent 2.** 0.031g dithiothreitol.
- **Reagent 3.** 0.026g ε amino caproic acid.

Mix 1 ml of reagent 1 with reagents 2 and 3 and dilute 1/100 in distilled water.

**Cellulose acetate electrophoresis:**

The Helena technique (Beaumont Texas) is used with Titan III iso-vid cellulose acetate plates, (94 x 96mm: Cat no 3001) and the Super CPK application system for 8 samples (cat No 4092).

**Electrode buffer for GPL:**

0.1 M Na2 EDTA
0.1M MgCl2
0.1M Maleic acid
made up in 0.1M TrischCl in 500ml distilled water adjusted to pH 7.4 with 40% NaOH. This buffer
could be stored at 4°C for up to 4 weeks. Before use 10% w/v of sucrose was added.

Membrane Buffer:
Dilute one part of electrode buffer with 14 parts of distilled water and add 10% sucrose.

Reaction buffer:
0.06M Tris adjusted to pH 8 with 50% HCl.

Developer:
To 60ml reaction buffer containing 20% sucrose, add 25U glucose 6 phosphate dehydrogenase from baker’s yeast, 10mg phenazine methosulphate, 15 mg MTT, 15mg βTPN (Na salt), 120mg MgCl2, 20 mg fructose 6-phosphate and 60mg EDTA (reagents from Sigma).

Electrophoresis protocol

30ml of distilled water was placed in the inner compartment of the electrophoresis tank to ensure a moist atmosphere inside the tank. 100ml electrode buffer was added to each outer electrode compartment of the tank. Filter paper wicks were fitted in the tank to connect the buffer compartments to the sites where the plate would eventually be laid. Before use the tank and buffer were cooled in a refrigerator. A cellulose acetate plate was marked on the back with a waterproof marker to show the position of the samples and the anode and cathode, then it was soaked in membrane buffer for 20 minutes and blotted using filter paper and placed on the applicator template. The enzyme extract samples were transferred to the applicator from the numbered wells of the sample plate and transferred to the cellulose acetate plate in the template. The application procedure was carried out 3 to 4 times to ensure sufficient extract had been applied.

The plate was then placed face downward on the wicks with the application site at the cathode end, and the tank lid was put on. The power supply (Helena supplies a suitable one) was set at constant voltage and switched on, adjusted to give 180V and run for 15 min. On completion of the run a freshly soaked cellulose acetate plate was flooded with enzyme developer and clipped together with the plate which had just been run, face to face between glass plates, with bulldog clips. This was incubated in the dark at 37°C for 20 min. When bands could be clearly seen to have developed the plates were released and immersed in 5% v/v glacial acetic acid to fix the bands and washed in distilled water. The result was photographed and/or the bands were outlined on the back of the plate with waterproof marker.

Controls were carried out at intervals to check the validity of the enzyme reaction using enzyme developer with the substrate (fructose 6 phosphate) omitted.

Results:
The relative mobilities of GPI from species of Naegleria were in the following order:
- fowleri < lovaniensis < gruberi (rough cyst type) < australiensis/gruberi (smooth cyst type).
(See plate). Providing suitable control lysates were run at the same time as test samples, the technique is highly effective in differentiating N. fowleri from the other species of Naegleria not known to be pathogenic to man.

A lysate suitable for electrophoretic analysis can be prepared from as few as 5000 organisms.
8. REFERENCES


temperature factors associated with occurrence of the amoeba remain relatively undefined. It is interesting that although *N. fowleri* will grow well at temperatures up to 45°C, cysts are not readily produced at high temperatures, in contrast to the non-pathogenic species *N. loyvaniensis* 38, which also grows at 45°C. This may perhaps explain persistence of *N. fowleri* in areas of fluctuating temperature or exposed to a temperature gradient. In Bath Spa 39 *N. fowleri* was isolated from water in an area where warm water mixed with cool and only *N. loyvaniensis* in a site where the water was uniformly at a high temperature. In a study of a newly created cooling reservoir (Clinton Lake, Illinois) before and after thermal additions from a nuclear power plant, *N. fowleri* was isolated from the thermally elevated arm but not from the ambient-temperature arm of the reservoir. The probability of isolating thermophilic *Naegleria* and pathogenic *N. fowleri* increased significantly with temperature. Repetitive DNA restriction fragment profiles of the *N. fowleri* Clinton Lake isolates and a known *N. fowleri* strain of human origin were homologous 39. This suggests that even in temperate areas we can expect *N. fowleri* colonisation of any newly introduced heated fresh water habitats, such as warm pools.

**Prevention:**

In the Northern Island of New Zealand the bathing places fed by hot springs are generally lined with earth, and the only preventive measures which are applicable are warnings not to immerse the head. These are presented to the public in graphic notices around the pools. In the UK, the contaminated pools associated with Bath Spa mineral spring have been closed for bathing, and a borehole has been drilled into the aquifer, allowing hot water to reach the surface uncontaminated.

**Water treatment.**

**Filtration**

Treatment of raw water to be used for drinking purposes by coagulation and filtration is generally effective for removal of organisms which do not multiply in the environment, such as bacterial pathogens, *E. histolytica* and *Giardia*. In the case of the potentially pathogenic free living amoebae, even one organism which passes through the filter is significant, since unlimited multiplication is possible in the "purified" water. Chemical or physical disinfection is therefore the only suitable approach.

**Physical treatment**

*N. fowleri* is not usually isolated from waters at temperatures below 25°C. The cysts and trophozoites are killed by temperatures above 60°C. Attractive recreational waters generally exceed 25°C and are at well below amoebicidal temperatures. The amoebae will grow at a wide range of pH in culture, although growth halts below pH 4.6 and above pH 9.5. Ultraviolet radiation appears ineffective in preventing *Naegleria* or bacterial contamination of swimming pools.

**Chemical treatment**

It has been noted that *N. fowleri* will not grow in brackish water. Concentrations of sodium chloride more than 0.75% will inhibit. It has also been shown experimentally that high concentrations of calcium (40-60 mM) are inhibitory.

The cysts of *N. fowleri*, like those of *E. histolytica* and *Giardia*, are relatively insensitive to chlorine. They need a free chlorine residual concentration (mg/L) x time (minutes) factor (C.T factor) in the region

1Depending on the amount of organic material, capable of reacting with chlorine, present in the water, an initial quantity of chlorine added will produce different residual concentrations of chlorine available for microbial inactivation. It is therefore important to determine in any experimental study the residual concentration of chlorine which remains after the experiment.

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of 40 for 99.9% inactivation\textsuperscript{7} i.e. 4mg/L for 10 minutes or 2mg/L for 20 minutes. However, the trophozoites are killed by lower chlorine residuals in the antibacterial region from 0.5 to 1 mg/L. Disinfection efficiency of chlorine is inversely related to pH, and these values are valid up to pH 7.5 but not higher. It is also important to note that chlorination is less efficient at lower temperatures. If bacterial growth is prevented, and this can be confirmed in water masses by a low or nil total plate count, growth of the amoebae should not be possible. It is still possible, however, that bacteria and amoebae may be growing on and in surfaces not adequately in contact with the disinfectant. For example, in the Czechoslovakian series of infections associated with a chlorinated swimming pool, the amoebae were being harboured in unchlorinated water behind a false wall at one end of the pool\textsuperscript{9}. In the Bath Spa episode, there was a channel of communication between contaminated unchlorinated warm spring water flowing under the swimming pool and the chlorinated contents. The South Australian series of PAM cases were apparently infected from the public water supply which was piped over desert after chlorination and thus lost its chlorine content and allowed amoebae to grow. The problem was solved by introducing supplementary chlorination points along the desert pipeline\textsuperscript{9}. In addition, the disinfectant monochloramine has been used because it is more persistent than chlorine itself\textsuperscript{9}. Ozonation has been tested with some success\textsuperscript{7}.

Although the problem of eliminating Naegleria from swimming pools seems immense, this is a much more serious problem for natural waters than for artificial pools, where careful design and proper maintenance should be able to achieve effective control\textsuperscript{7}.

REFERENCES


This level only is relevant in determination of microbial sensitivity. The pH of the water is also relevant, since the active chemical species HOCl, hypochlorous acid, decreases in concentration as pH is raised.

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Agarose gel electrophoresis stained with ethidium bromide and viewed in UV light.

1. PCR primers Nf1 and Nf2

2. PCR primers Nf3 and Nf4

<table>
<thead>
<tr>
<th>Organism</th>
<th>300 bp product</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>N. fowleri</em></td>
<td>+</td>
</tr>
<tr>
<td>Morgan</td>
<td>+</td>
</tr>
<tr>
<td>Colombia</td>
<td>+</td>
</tr>
<tr>
<td>HB 1</td>
<td>+</td>
</tr>
<tr>
<td>C 0504</td>
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</tr>
<tr>
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</tr>
<tr>
<td>1479</td>
<td>-</td>
</tr>
<tr>
<td>6604.2</td>
<td>-</td>
</tr>
<tr>
<td>9240</td>
<td>-</td>
</tr>
<tr>
<td><em>N. gruberi</em></td>
<td>-</td>
</tr>
<tr>
<td>NG</td>
<td>-</td>
</tr>
<tr>
<td><em>A. polyphaga</em></td>
<td>-</td>
</tr>
<tr>
<td>Yeast</td>
<td>-</td>
</tr>
<tr>
<td><em>E. coli</em></td>
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</tbody>
</table>

1 100bp markers
2 1518/3 *fowleri*
3 1518/5 *fowleri*
4 6604.2 *hovaniensis*
5 1479 *hovaniensis*
6 negative control

PCR primers Nf5 and Nf6

1 100bp markers
2 1518/3 *fowleri*
3 1518/5 *fowleri*
4 1479 *hovaniensis*
5 6604.2 *hovaniensis*
6 NG *gruberi*
7 negative control
Probing dot blots:

Primers Nf1 and Nf2, product probed with radiolabelled probe MP.

Dot blots of PCR product from Nf5 and Nf6 primers probed with digoxygenin-labelled probe NF7.

Nylon membrane with dot blots of Naegleria DNA hybridised with the digoxygenin labelled probe SP. From top left: N. fowleri (Morgan); N. fowleri (HB 1); N. lovaniensis (1479); N. lovaniensis (6604,2); N. gruberi (NG); negative control.

Cellulose acetate electrophoresis: GPI profiles of 3 Naegleria spp.
Scheme of PCR-SHELA product detection (digoxigenin labelled primer, biotin-labelled probe).

\[ \text{pNPP} \]
\[ \text{Ab-AP} \]
\[ \text{D} \]
\[ \text{B} \]
\[ \text{A} \]

A: Avidin  
B: Biotin labelled probe  
D: Digoxigenin labelled PCR product  
AB-AP: Anti-digoxigenin Antibody + Alkaline Phosphatase  
pNPP: 4-nitrophenyl phosphate
DEMI-NESTED PCR-SHELA RESULT ON 5 UNKNOWN SAMPLES

Unknown samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Optical density (O.D.)</th>
</tr>
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<tbody>
<tr>
<td>HK-1</td>
<td>2</td>
</tr>
<tr>
<td>A-44-1</td>
<td>1</td>
</tr>
<tr>
<td>CPI</td>
<td>1</td>
</tr>
<tr>
<td>4684.11</td>
<td>0.5</td>
</tr>
<tr>
<td>NF-3</td>
<td>0.5</td>
</tr>
</tbody>
</table>

A. *N. fowleri* HK-1 PAM case Hong Kong
B. *N. fowleri* A-44-1 power station, England
C. *N. lovaniensis* CPI hot springs, USA
D. *N. australiensis* 4684.11 hot springs, Bath, England
E. *N. fowleri* NF-3 hot springs, Bath, England