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Studies on the Molecular Biology of Naegleru Fowleri and Identification of N. Fowleri in the Environment

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STUDIES ON THE MOLECULAR BIOLOGY OF *NAEGLERIA FOWLERI* AND IDENTIFICATION OF *N. FOWLERI* IN THE ENVIRONMENT

A Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

by

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ABSTRACT

STUDIES ON THE MOLECULAR BIOLOGY OF *NAEGLERIA FOWLERI* AND IDENTIFICATION OF *N. FOWLERI* IN THE ENVIRONMENT

By Rebecca Carmean MacLean, Ph.D.

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Naegleria fowleri, a free-living ameboflagellate, is the causative agent of primary amebic meningoencephalitis. Healthy humans sporadically become infected with *N. fowleri* and develop fatal PAM after recreational or work exposure to freshwater; accordingly, there is a need for monitoring the presence of pathogenic amebeflagellates in public freshwater. The present study was conducted to determine whether a nested PCR assay could be used for detection of *N. fowleri* in freshwater habitats. PCR analysis was

used to test samples from Virginia, Connecticut, Arizona, and Oklahoma for the presence of *N. fowleri* in lakes, ponds, soil, and domestic water supplies. The amebae were identified in all 4 states from soil and water sources, including domestic water supplies. In addition to identification in the environment, it is also important to determine virulence factors of the ameba. Although virulence factors have not been defined, resistance to complement lysis and production of phospholipases may account for pathogenicity of this ameba. Studies were performed to determine the gene encoding a complement regulatory protein, CD59, found in membrane fractions of *N. fowleri*. The genome of this organism has not been sequenced, therefore, we have constructed a genomic DNA library to search for putative virulence factors or drug targets. We have performed partial sequencing of 155 plasmids and have identified putative genes for cell motility, chromosome segregation, gene regulation, protein synthesis and degradation, protein regulation, cell signaling, respiration and energy production, membrane synthesis and metabolism, amino acid synthesis, as well as genes with unknown functions. Also, we have identified a putative virulence factor, a patatin-like protein. Patatin has been shown to exhibit phospholipase A_2 activity in other organisms and has been shown to be involved in invasion into human tissue in certain pathogens. Northern analysis demonstrated hybridization with *N. fowleri* RNA at 3kb, but not with RNA from other free-living amebae tested. RT-PCR analysis was positive for pathogenic *N. fowleri* and negative for nonpathogenic *Naegleria* spp. Further studies are needed to determine whether the patatin-like protein in *N. fowleri* serves as a virulence factor and plays a role in invasion in human tissue.

INTRODUCTION

The genus *Naegleria* consists of free-living amoeboflagellates that are ubiquitous in nature and are capable of causing a human disease called primary amebic meningoencephalitis (PAM). *Naegleria* amebae are unique from other free-living amebae in the environment because of their flagellate stage. There are three morphological stages, including a trophozoite, flagellate, and cyst stage. The vegetative trophozoites are the diving stage of the ameba and can feed on bacteria in the environment. *Naegleria* trophozoites display a predominantly monopodial "limax" pattern of locomotion, which is a slug-like movement (Marciano-Cabral 1988). It has been suggested that the ameboid trophozoite is the infectious form (Maricano-Cabral 1988). The trophozoite displays a prominent sucker-like structure called a food-cup that is used to ingest bacteria and yeast in the environment as well as brain tissue during infection. Under nutrient limiting conditions, the trophozoite can transform into a non-feeding flagellated stage, which involves a change in cell shape and a change in synthesis of all organelles of the flagellar apparatus (Griffin 1978; Marciano-Cabral 1988). The flagellar apparatus consists of two terminal flagella, two basal bodies, microtubules, and a single rootlet (Parija and Jayakeerthee 1999). The flagellated form found in water swims to the surface, docks, and transforms into the ameboid stage to feed on bacteria (Preston and King 2003). The trophozoites can also transform into a cyst stage, which provides protection from desiccation and food deprivation. The encysting *Naegleria* contains a nucleus and a variety

of cytoplasmic vacuoles, including food vacuoles and contractile vacuoles (Marciano-Cabral 1988). The cyst stage is resistant to chlorination (Rubin et al. 1983) and heat, but not freezing temperatures (Biddick et al. 1984).

Naegleria spp. are widely distributed in soil (Brown et al. 1983; Wellings et al. 1979) and freshwater (Duma 1980; Griffin 1983; Marciano-Cabral 1988) habitats throughout the world. *N. fowleri* has been isolated from recreational water facilities including chlorinated swimming pools, (Cerva 1971), natural hot springs (Brown et al. 1983; Scaglia et al. 1983), and hot tubs (Rivera et al. 1993). The ameba has been isolated, also, from domestic water supplies (Anderson and Jamieson 1972a, 1972b; Anderson et al. 1973; Marciano-Cabral et al. 2003), and from artificially heated industrial water sources (De Jonckheere et al. 1975; Fliermans et al. 1979; Huizinga and McLaughlin 1990; Kasprazak et al. 1982; Sykora et al. 1983; Tyndall et al. 1989; Wellings et al. 1979).

Although many species of *Naegleria* have been described (De Jonckheere 2004), only *N. fowleri* has been associated with human disease. The earliest known case of a human infection with *N. fowleri* was 1937 and occurred in a patient from Virginia. This case was not reported until 1968 when Dos Santos identified the patient during a retrospective review of autopsies. The first published report of *N. fowleri* causing CNS disease was reported in Australia by Malcolm Fowler and Rodney F. Carter in 1965 (Marciano-Cabral 1988). *N. fowleri* is the causative agent of primary amebic meningoencephalitis (PAM), a rapidly fatal disease of the central nervous system (CNS). The disease is generally acquired while swimming and diving in freshwater lakes and ponds (Martinez and Visvesvara 1997). Although widespread in nature, the true incidence

and prevalence of *N. fowleri* remains unknown. Surveillance studies for water borne-disease outbreaks in the United States indicate that the occurrence of PAM attributed to *N. fowleri* has increased in recent years (Lee et al. 2002; Taylor et al. 1996).

PAM occurs most often in immune competent children and young adults with a history of swimming or diving in freshwater (Marciano-Cabral 1988). Infection results when water containing the amebae is introduced into the nasal passages. After adhering to the nasal mucosa, the amebae migrate up the olfactory nerves, traverse the cribriform plate, and enter the CNS where they activate complement, induce proinflammatory cytokines, and feed on brain tissue. Swelling and hemorrhagic necrosis of brain tissue results and death ensues 10-14 days post-infection. *N. fowleri* has a predilection for the CNS. In experimental animals and humans, *N. fowleri* are found primarily in the brain. Previous studies in our laboratory have demonstrated that pathogenic *N. fowleri* move chemotactically to soluble components of nerve cells (Cline et al. 1986). *N. fowleri* attach to nerve cells and lyse them by producing a pore-forming peptide and ingest brain tissue using a food-cup structure on the surface (Herbst et al. 2002).

It is not known why infection occurs in one individual while not in other individuals swimming in the same water source. Contact with *N. fowleri* without progression to amebic encephalitis is evidenced by the presence of antibodies in screened human populations (Schuster and Visvesvara 2004). It has been established that animal passage enhances pathogenicity. In fact, virulence of *N. fowleri* is maintained in laboratories by mouse intranasal inoculation and reisolation of the amoebae, or by serial passage of amoebae in tissue cultures (Martinez 1985). Recently, *N. fowleri*-associated

PAM was diagnosed in a herd of Holstein cattle from California, and in a captive South American tapir in a zoo in Arizona and in a cow from Costa Rica (Morales et al. 2006). Pathogenicity is related to challenge level, so exposure to exceptionally high numbers of *N. fowleri* may be the determinative factor. The ability to survive and grow at temperatures of 37°C and above does not appear to determine pathogenicity since thermophilic nonpathogenic species such as *N. lovaniensis* have been described. It has also been suggested that a deficiency in secretory IgA may contribute to the ability of *N. fowleri* to establish an infection in human hosts (Marciano-Cabral 1988).

It is thought that the higher incidence of infection in young children is due to the duration of time in the water, a higher level of activity, and are more likely to engage in diving in water when compared to other age groups (Schuster and Visvesvara 2004). Studies have been performed to determine the risk of acquiring an infection with *N. fowleri* amebae. These studies were performed in France using mathematical modeling and the risk was calculated to be 8.5 x 10-8 in water containing 10 *N. fowleri* amebae/liter, with the risk rising as the number of amebae increase (Cabanes et al. 2001)

The high level of mortality associated with PAM indicates the need for a rapid and sensitive test to identify *N. fowleri* in recreational, industrial and domestic water supplies. The correct identification of *N. fowleri* is often difficult because several genera of amebae are morphologically similar (Page 1988) and pathogenic *N. fowleri* and nonpathogenic *N. lovaniensis* are antigenically related (Stevens et al. 1980). Furthermore, polyclonal antiserum to *N. fowleri* cannot distinguish this species from nonpathogenic *N. lovaniensis* by immunologic assays. A sensitive and specific nested PCR assay was developed to

identify *N. fowleri* in water and soil (Reveiller et al. 2002), however, the assay was developed using laboratory strains of *N. fowleri* in sterile water. The application of this nested PCR assay for environmental monitoring of soil and water was reported (MacLean et al. 2004).

The PCR assay was used to identify *N. fowleri* in water collected from the James River, Richmond, VA, water and soil samples from Connecticut, and domestic and environmental samples from Arizona and Oklahoma (Marciano-Cabral et al. 2003; MacLean et al. 2004). The James River is a body of water previously reported to contain *Naegleria* amebae (Ettinger et al. 2003) and an area from which cases of PAM have occurred (Callicott et al. 1968; dos Santos 1970; Duma et al. 1969; Martinez 1985). The PCR assay and Western immunoblot analysis were positive for *N. fowleri* from water samples taken from the James River. Additionally, a survey of soil and water samples from Connecticut was conducted to determine whether *N. fowleri* is present in Connecticut, since neither *N. fowleri* nor PAM have been reported previously from this state. Of eighty-six samples from Connecticut examined by PCR, fifteen tested positive for *N. fowleri* (MacLean et al. 2003). Samples collected in Arizona and Oklahoma were tested for the presence of *Naegleria* because of the recent deaths of children from those areas. *N. fowleri* was identified by PCR in domestic water samples from both Arizona and Oklahoma.

The complement system

In addition to identifying *N. fowleri* in the environment to prevent disease, it is also important to determine virulence factors of the ameba. *N. fowleri* appear to have developed

strategies to avoid destruction by both the innate and acquired immune systems. The complement system is a biochemical cascade that is the first line of defense against invading organisms. The complement system consists of soluble and membrane bound proteins that interact in a sequential cascade that results in irreversible damage to target cell membranes (Makrides 1998). Three biochemical pathways activate the complement system: the classical complement pathway, the alternate complement pathway, and the mannose-binding lectin pathway (Matsushita and Fujita 1992). Activation of any of the three pathways results in the generation of homologous variants of the protease C3 convertase. The C3 convertase cleaves and activates component C3, creating C3a and C3b. The association of C3b with the C3 convertase of any pathway results in the production of a C5 convertase, which cleaves C5 in C5a and C5b. Complement component C5b binds the membrane of the invading organism and serves as a binding site for C6, C7, and C8. Multiple molecules of C9 polymerize and form a pore in the membrane, which results in osmotic lysis of the cell (Makrides 1998). It has been demonstrated that pathogenic and nonpathogenic *Naegleria* activate the alternative pathway of complement (Holbrook et al. 1980; Whiteman and Marciano-Cabral 1987). Pathogenic *N. fowleri* are able to resist complement-mediated lysis and damage host brain tissue, but nonpathogenic *N. gruberi* are sensitive to lysis by complement (Whiteman and Marcicano-Cabral 1987). Complement resistance is an important factor in establishing disease in the infected host since it has been shown that mice treated with cobra venom factor to deplete complement activity and complement deficient A/HeCr mice are more susceptible to *N. fowleri* infections (Haggerty and John 1978; Reilly et al. 1983). *N. fowleri* evade the humoral

immune system by capping, internalizing, and degrading anti-*Naegleria* antibodies attached to the ameba surface (Ferrante and Thong 1979). Extensive damage to the brain may be the result of the direct action of the amebae and indirect action of complement activation and production of proinflammatory cytokines (Marciano-Cabral et al. 2001).

Previous studies in our laboratory have shown that *N. fowleri* have at least two mechanisms of resisting lysis by complement. The first process occurs by membrane vesiculation. The amebae extrude the lytic pore-forming membrane attack complex (MAC) of complement from the cell surface on membrane vesicles, leaving behind an intact ameba (Toney and Marciano-Cabral 1994). Immunofluorescent staining of pathogenic amebae using an antibody to the MAC demonstrated that complement components, C5b-C9, were concentrated on ameba membrane blebs on the surface of the ameba (Toney and Marciano-Cabral 1994).

Another mechanism of resisting lysis by complement may involve a membrane protein on the surface of *N. fowleri* that reacts with an antibody to human CD59. CD59 is an 18 to 20 kDa glycosyl-phosphatidylinositol inositol (GPI)-anchored glycoprotein found on the surface of a variety of cell types which functions to inhibit complete formation of the MAC of complement. CD59 inhibition of complement lysis occurs by binding complement components C8 and C9, ultimately preventing C9 insertion and polymerization into the cell membrane (Davies and Lachmann 1993).

A number of pathogens reportedly express or acquire complement regulatory proteins. *Schistosoma mansoni*, a blood fluke, has a protein that is cross-reactive with antibodies to CD59 and protects the cells against complement mediated lysis (Parizade et

al. 1991). *Borrelia burgdorferi*, the Lyme disease bacterium, has been shown to evade complement-mediated lysis by a molecule on the surface that is cross-reactive with human CD59 (Pausa et al. 2003).

Characterization of the CD59 molecule has been undertaken. Studies by other investigators have shown that treatment of mammalian K562 erythroleukemic cells with phospholipase C (PIPLC) resulted in removal of approximately one-half of surface CD59 (Marchbank et al. 1995). Similarly, treatment of *N. fowleri* with PIPLC to cleave GPI-linked proteins or endo-β-N-acetylglucosaminidase H (Endo H) to cleave N-linked high mannose residues of glycoproteins resulted in an increased susceptibility of the amebae to complement-mediated lysis. Similar treatment of nonpathogenic, complement-sensitive *N. gruberi* did not result in a significant increase in lysis. In addition, treatment of the amebae with tunicamycin, an antibiotic that inhibits the formation of N-linked protein-carbohydrate linkages, results in an increased susceptibility of pathogenic *N. fowleri* to complement lysis. Again, treatment of nonpathogenic, complement-sensitive *N. gruberi* did not result in a significant increase in lysis (Toney and Marciano-Cabral 1992).

In addition, Northern blot analyses demonstrated hybridization of a radiolabeled cDNA probe for CD59 to RNA from pathogenic *N. fowleri* (Fritzinger et al. 2006). An 18 kDa immunoreactive protein was detected on the membrane of *N. fowleri* by Western immunoblot and immunofluorescence analyses using monoclonal antibodies for human CD59. Complement component C9 immunoprecipitated with the *N. fowleri* "CD59-like" protein from amebae incubated with normal human serum. In contrast, a gene or protein

similar to CD59 was not detected in nonpathogenic, complement-sensitive *N. gruberi* amebae (Fritzinger et al. 2006).

Our studies suggest that a protein reactive with antibodies to human CD59 is present on the surface of *N. fowleri* amebae and may play a role in resistance to lysis by cytolytic proteins. These data suggest that a GPI-linked protein on the surface of *N. fowleri* contributes to the ameba's ability to evade complement lysis and that the protein is similar to mammalian CD59.

Therefore, studies were performed to determine the sequence of the gene encoding the CD59 protein. A genomic DNA library was constructed in order to screen with a cDNA probe encoding CD59. Immunoprecipitations were also performed using an anti-human CD59 antibody.

Patatin-like phospholipase

Random colonies were selected from the *N. fowleri* genomic DNA library for sequencing. We were able to identify genes that may be important in treatment or determination of virulence of *N. fowleri*. In particular, we obtained a DNA sequence encoding a patatin-like phospholipase protein. Patatin proteins are a group of plant storage proteins initially discovered in *Solanum tuberosum*, the potato (Park et al. 1983). The first indication that patatin exhibits enzymatic activity was by Galliard (1971) who purified an enzyme from potato tubers that catalyzed the deacylation of a range of lipid substrates. Later studies demonstrated that this acyl hydrolase activity was due to patatin (Racusen 1984). Further characterization of this protein has demonstrated that patatin is a lipid acyl

hydrolase (Andrews et al. 1988). More recently, it has been suggested that patatin has phospholipase A_2 activity, which was confirmed by solving the crystal structure of patatin and mutagenesis of the putative active sites (Senda et al. 1996; Rydel et al. 2003).

It has been demonstrated that patatin may be a virulence factor in *Pseudomonas aeruginosa*, *Rickettsia* species, as well as other pathogenic bacteria (Sato et al. 2003; Baberji and Flieger 2004; Blanc et al. 2005). In this study, a putative patatin-like phospholipase A_2 protein was found in *N. fowleri* and studies were performed to determine the complete gene sequence, similarity to other patatin-like proteins and its presence in other pathogenic and nonpathogenic amebae.

MATERIALS AND METHODS

Amebae

Naegleria fowleri LEE (ATCC 30894) obtained from the American Type Culture Collection (ATCC) was originally isolated from a patient with a fatal case of PAM at Virginia Commonwealth University. A highly virulent mouse-passaged strain of *N. fowleri* (LEEmp) was obtained by continuous passage of *N. fowleri* LEE through 8-week old, female $B_6C_3F_1$ mice at monthly intervals. The LEE and LEEmp strains of *N. fowleri* were maintained in Cline growth medium supplemented with heat-inactivated donor calf serum and hemin at 37 °C (Cline et al. 1983). Other amebae obtained from the American Type Culture Collection (ATCC) included *N. lovaniensis* (Aq/9/1/45D), *Acanthamoeba culbertsoni* (ATCC 30171), *Acanthamoeba castellanii* (ATCC 30010), *and Acanthamoeba astronyxis* (ATCC 30137). All were maintained in Cline growth medium supplemented with heat-inactivated donor calf serum and hemin at 37 °C. *N. gruberi* (EG_B) was maintained in Cline medium at 30 °C or 37 °C. Other free-living amebae used in select studies were *Naegleria galeacystis* (ATCC 30294), *Vahlkampfia avara* (ATCC 30964), *Willaertia magna* (ATCC 50036), *Hartmannella vermiformis* (ATCC 50599), *Vannella aberdonica* (ATCC 50815) and *Balamuthia mandrillaris* (ATCC 50209). *Naegleria galeacystis* (ATCC 30294) and *Vahlkampfia avara* (ATCC 30964) were maintained at

room temperature in ATCC 997 medium. *Willaertia magna* (ATCC 50036) was maintained at room temperature in ATCC 1034 medium. Also maintained at room temperature was *Hartmannella vermiformis* (ATCC 50599) in ATCC 711 medium, and *Vannella aberdonica* (ATCC 50815) in ATCC 944 medium. *Balamuthia mandrillaris* (ATCC 50209) maintained at 37 °C in BM3 medium were used in select experiments (Schuster and Visvesvara, 1996).

Mammalian Cells

The human chronic myelogenous leukemia cell line K562 (ATCC CCL-243) known to express CD59 was used as a source for the complement regulatory protein, CD59 (Philbrick et al. 1990). K562 cells were cultured at 37 °C in an atmosphere of 5 % $C0_2$ and were maintained in RPMI 1640 supplemented with 10 % fetal bovine serum (BioWhittaker, Walkersville, MD), 1.5 % sodium bicarbonate, 25 mM HEPES buffer, 1 % L-glutamine, 1 % non-essential amino acids, 1 % minimal essential medium vitamins, 100 U/ml of penicillin and 100 μg/ml of streptomycin.

Antibodies

Antibodies used in the environmental testing studies include a polyclonal anti-Mp2Cl5 antibody (Reveiller et al. 2001), polyclonal antibodies to whole cell lysates of *Naegleria fowleri* and *Acanthamoeba* prepared in New Zealand white rabbits (Toney and

Marciano-Cabral, 1994), and a monoclonal anti-*N. fowleri* antibody (5D12) was provided by Electricite de France (Paris, France). For the CD59 studies, two monoclonal antibodies were used including a mouse IgG1κ anti-human CD59 antibody (BRA-10G) (Ancell, Bayport, MN), and mouse IgG2b anti-human CD59 antibody (BRIC 229) (International Blood Group Laboratory, Bristol, UK). A monoclonal anti-human phospholipase A_2 antibody (Chemicon, Temecula, CA) was also employed in select studies. HRP-linked goat anti-rabbit IgG (whole molecule) (Sigma-Aldrich Corporation, St. Louis, MO) and HRP-linked rabbit anti-mouse IgG (whole molecule) (Sigma-Aldrich Corporation, St. Louis, MO) were used as secondary antibodies.

Study Areas and Collection Sites for Environmental Studies

To identify *N. fowleri* in the environment, water or soil samples were collected from the following areas in the United States: Virginia, Connecticut, Arizona, and Oklahoma.

Richmond, VA: Water samples were collected from the James River, Richmond, Virginia, by submerging a sterile 100 ml bottle under the surface of the water.

Connecticut: Water and "swab" samples were collected from Clark's Pond, in Hamden, Connecticut, Wharton Brook swimming area in Wallingford, Connecticut, and a small pond on the campus of Quinnipiac University in Hamden, Connecticut during the months of July and August, 2000. Additionally, climatological data were obtained from the area at the Cook Hill Weather Reporting station in Wallingford, Connecticut. This reporting station is within 8 kilometers of all study sites. At each sampling site, water samples were

collected by submerging a sterile 15 ml centrifuge tube beneath the surface of the water. Swab samples were collected by passing sterile gauze over rocks, logs, and soil comprising the substrate of the bodies of water. Bacterial levels were quantified on selective media following procedures outlined by Richardson and Richardson (2003). Samples were diluted and spread on plates containing either mEndo medium, which supports the growth of coliforms, or on EC+MUG medium (Becton Dickinson, Sparks, MD) which supports the growth of *Escherichia coli* (*E. coli*). The plates were incubated at 37 °C for 48 hours. Colony counts were performed.

Arizona: Swipe samples from sink traps and residual water present in pipes were collected from the homes associated with two cases of PAM and from the home of an adjacent neighbor in Arizona. The domestic water was supplied by a private water company in Arizona directly from a well or a holding tank, depending on the demand in the system. Disinfection of the water supply by methods of chlorination, ozone or UV light treatment, or filtration did not occur at the time of the incidents. Swipe samples from the victims' homes were obtained by passing sterile cotton gauze Mirasorb sponges through the kitchen and bathroom sink traps. Sink traps were removed, and the residual water (~150 to 350 ml) was collected into one or two sterile 250 ml containers. The cotton gauze was placed in one of the containers, and both were topped off with Page's ameba saline (Martinez 1985; Page 1988). A soil sample from outside one home with water leakage was collected and placed in ameba saline. Also, a sample from a Micro-Wynd II filter (Cuno, Incorporated, Meriden, CT) was obtained by collecting approximately 60.8 liters (16 gallons) of water into the bathtubs of each individual's home. Using a positive-pressure displacement pump, the

collected water was passed through a 1 μm pore size polypropylene Micro-Wynd II filter (grade Y) and recycled back into the tub. All of the household pump fittings and hoses used were standard. The pump flow rate was approximately 16 gallons per minute at a pressure of 2.2 lb/in^2 and was operated for approximately 3 minutes so that the water was cycled at least three times. The Micro-Wynd II filter was then placed in a container in sterile Page's ameba saline.

Oklahoma: Water and soil samples were collected from a recreational site containing a "splash pad" area, Valley View Creek, and from tap water from two homes to determine if these areas may have been associated with two cases of PAM in Tulsa, Oklahoma. Soil samples were collected from the "splash pad" by cutting a sterile 25 ml pipette with a heated razor and inserting the pipette into the ground. The pipette containing the soil was placed back into a sterile sleeve. Water samples from the "splash pad" and from the creek were obtained by submerging a sterile 50 ml conical tube into the water. Water samples from the victims' homes were obtained by running water from the bathroom sink for 10 seconds and collecting water in a 50 ml conical tube.

Processing of Samples

Richmond, VA: Four water samples (50 ml each) collected from the James River, Richmond, VA were processed directly or enriched by culture. One set of samples was prepared by centrifuging the water and collecting a supernatant and a pellet and testing the pellet directly. Another set of samples was enriched by placing water into 75 cm^2 tissue culture flasks and maintained for 5 days at 37 °C or placed onto a plate of non-nutrient agar

(NNA) spread with killed *E. coli*, a food source (John and Howard 1996).

Connecticut: Samples collected in Connecticut were transported back to the laboratory and processed within 24 hours of collection. Samples were centrifuged for 5 minutes at 5,000 x *g*. The pellet was suspended in 1 ml of sterile water and pipetted on NNA-*E. coli* plates. The plates were incubated at 42 °C for 48 hours to isolate thermotolerant amebae. Plates were observed for the presence of plaques produced by amebae clearing the bacteria. Amebae were subcultured to new plates by cutting a small portion of the agar from each plaque and placing the agar square onto new plates containing NNA with heat killed *E. coli* to avoid overgrowth of fungi. After incubation for 48 hours at 42 °C, the plates were sealed with parafilm and stored in the laboratory at room temperature. Swab samples collected in the field were applied to plates of non-nutrient agar that had been spread with killed *E. coli* and treated as described above. Cultures were observed by light microscopy for the presence of trophozoites, cysts, or flagellates. Cultures were photographed using an Olympus Ck2 microscope with a computer attachment.

Arizona: Nineteen samples were collected, transported to the laboratory, and processed within 1 week of collection. All 19 samples were dispensed into individual 75 cm^2 tissue culture flasks in 10 ml volumes in duplicate and placed either at 44 °C or 37 °C. The samples in tissue culture flasks were observed daily for the presence of amebae by light microscopy and were kept for polymerase chain reaction (PCR) analysis. A third set of samples was prepared by dispensing 10 ml of fluid into centrifuge tubes and subjecting the samples to centrifugation for 10 minutes at 5,000 x *g*. The supernatant was discarded, and the pellet was suspended in 1 ml of Page's ameba saline and placed onto a plate of non-

nutrient agar spread with heat-killed *E. coli*. The plates were incubated at 44°C for 48 hours to isolate thermotolerant amebae. The plates were observed for the presence of plaques produced by amebae clearing the bacteria. Amebae were subcultured to new plates by cutting a small portion of the agar from each plaque and placing the agar square onto new plates containing non-nutrient agar with heat-killed *E. coli* to avoid overgrowth of fungi. After 48 hours of incubation, the plates were sealed with Parafilm and stored at room temperature for later use. The original 19 samples containing swipes or water were stored at 37°C for 3 months to promote growth of the amebae, and portions were prepared for PCR as needed.

Oklahoma: Eighteen samples were collected, transported to the laboratory, and processed within 48 hours of collection. Two sets of water and soil samples were prepared by dispensing water samples into individual 75 cm^2 tissue culture flasks in 10 ml volumes in duplicate and placed at 37°C. Soil samples were obtained by placing some of the sample from the 25 ml pipette into 75-cm^2 tissue culture flasks in ~15 ml of PAGE ameba saline. The samples were observed daily for the presence of amebae by light microscopy and were kept for PCR. After one week in culture, the flasks were scraped and samples were placed into 50ml conical tubes and centrifuged at 5,000 x *g*. The supernatant was discarded, and the pellet was suspended in 0.1 ml of Page's ameba saline and frozen at -20 °C. PCR was performed on these samples approximately one month after freezing. A third set of samples was prepared by placing 1 ml of water sample or 1 ml of soil sample in Page's ameba saline onto plates of non-nutrient agar spread with heat-killed *E. coli*. The plates were incubated at 44°C for 48 hours to isolate thermotolerant amebae. The plates were observed

for the presence of plaques produced by amebae clearing the bacteria. Amebae were subcultured to new plates by cutting a small portion of the agar from each plaque and placing the agar square onto new plates containing non-nutrient agar with heat-killed *E. coli* to avoid overgrowth of fungi. After 48 hours of incubation, the plates were sealed with Parafilm and stored at room temperature for later use. The original 18 samples containing swipes or water were stored at 37°C to promote growth of the amebae, and portions were prepared for PCR as needed.

Preparation of Samples for Nested PCR Analysis

Richmond, VA: Water samples collected in Richmond, VA, were centrifuged directly after collection and the pellet was tested by PCR. Also, flasks containing the samples were harvested by scraping with a sterile cell scraper and centrifuging the contents at 5,000 X g for 5 minutes. The pellets were suspended in 100 µl of PCR grade water and subjected to nested PCR analysis.

Connecticut: Samples from Connecticut, which had been stored at room temperature for 12 months in sealed Petri plates containing NNA-*E. coli* with isolated amebic cysts were processed for PCR. Squares of agar containing cysts were placed in liquid medium (ATCC 802) at 37 °C to stimulate excystment. After 48 hours of maintaining cultures in liquid medium, plates containing excysted amebae were scraped with a sterile cell scraper and samples were centrifuged at 5,000 x *g* for 5 minutes. The supernatant was removed and the pellet was suspended in 100 µl of PCR grade water. Samples were tested for *N. fowleri* by nested PCR analysis. Samples were kept in continuous culture for a further 9

months following the initial excystment process. Samples were cultured in liquid medium by alternating ATCC 802 medium with Page's ameba saline to hinder growth of bacteria and fungi present in the samples. A repeat PCR assay was performed on select samples nine months following continuous culture of the amebae in liquid medium.

Arizona: Samples cultured in flasks containing cysts or trophozoites were maintained in continuous culture in liquid medium by alternating ATCC medium 802 with Page's ameba saline to hinder growth of bacteria and fungi present in the samples. Samples were assayed by PCR beginning 10 days after collection and at intervals for 3 months. Tissue culture flasks maintained at 37°C containing amebae were prepared by scraping the flask with a sterile cell scraper and centrifuging the contents at 5,000 x *g* for 5 minutes. The supernatant was removed, and the pellet was suspended in 100 µl of PCR-grade water and tested for *N. fowleri* by nested PCR. Repeat PCR assays were performed at various intervals during a 3 month period following continuous culture of the amebae in liquid medium kept at 37°C.

Oklahoma: Tissue culture flasks maintained at 37 °C containing amebae were prepared by scraping the flask with a sterile cell scraper and centrifuging the contents at 5,000 x *g* for 5 minutes. The supernatant was removed, and the pellet was suspended in 100 µl of PCR-grade water and tested for *N. fowleri* by nested PCR. Repeat PCR assays were performed at various intervals during a 3-month period following continuous culture of the amebae in liquid medium kept at 37°C.

Nested PCR Analysis

PCR was performed according to the method described by Reveiller et al. (2002) by amplifying a portion of a gene unique to *N. fowleri*. The forward primer, Mp2C15.for (5'-TCTAGAGATCCAACCAATGG-3') and reverse primer, Mp2C15.rev (5'-ATTCTATTCACTCCACAATCC-3'), were used to amplify a 166 bp fragment of the Mp2C15 gene. PCR was performed in a 50 µl volume consisting of 1X *Taq* DNA polymerase buffer (10 mM Tris-HCl, (pH8.3), 50 mM KCl, 2.5 mM $MgCl_2$), 0.2 mM of deoxynucleoside triphosphates (dNTPs), 0.6-µM of primer, and 2.5 U of *AmpliTaq* DNA polymerase (Perkin Elmer, Branchburg, NJ). The positive control consisted of 10 ng plasmid DNA purified from *E. coli* clone Mp2C15. The negative control consisted of PCR-grade water lacking DNA template. Thirty-three µl of the environmental samples were used in the first round of PCR amplification. The standard temperature program was 5 minutes at 95 °C for one cycle and 1 minute at 95 °C, 1 minute at 65 °C, and 2 minutes at 72 °C for 35 cycles. To increase the sensitivity of the assay, nested primers, Mp2C15.for-in (5'-GTACATTGTTTTTATTAATTTCC-3') and Mp2C15.rev-in (5-GTCTTTGTGAAAACATCACC-3'), which amplified a 110 bp fragment of Mp2C15, were used in a second round of PCR. PCR was also performed in 50-µl volume consisting of 1X *Taq* DNA polymerase buffer (10 mM Tris-HCl, pH8.3, 50 mM KCl, 2.5 mM $MgCl_2$), 0.2 mM of deoxynucleoside triphosphates (dNTPs), 0.5 µM of primer, and 2.5 U of *AmpliTaq* DNA polymerase (Perkin Elmer, Branchburg, NJ). Two µl of the PCR product from the first round of PCR were used in the second PCR. The positive control

consisted of 1 μl of the first PCR product of the positive control diluted 50 times in PCR-grade water, as DNA template. The negative control consisted of PCR-grade water lacking DNA template. The standard temperature program was 1 minute at 95 °C, 1 minute at 55 °C, and 1 minute at 72 °C for 35 cycles. Amplified PCR products from the environmental samples were demonstrated on either 1.5 % GenePure (ISC Bioexpress, Kaysville, UT) agarose gels or 3:1 Nuseive agarose gels (Cambrex Bio Science, Inc, Rockland, ME) stained with ethidium bromide.

Specificity of the Nested PCR Assay

Additionally, cultures of amebae observed in environmental samples were purchased from American Type Culture Collection (ATCC) and nested PCR was performed directly on the ameba without prior DNA extraction. The amebae were cultured in their respective media recommended by ATCC and subjected to nested PCR analysis. Amebae tested were *Naegleria galeacystis* (ATCC 30294), *Vahlkampfia avara* (ATCC 30964), *Hartmannella vermiformis* (ATCC 50599), *Willaertia magna* (ATCC 50036), *Vannella aberdonica* (ATCC 50815) and *Balamuthia mandrillaris* (ATCC 50209).

Western Immunoblot Analysis of Environmental Samples

Select samples from Virginia, Connecticut and Arizona cultured in liquid medium were harvested by centrifugation and placed in lysis buffer consisting of 50 mM Tris-HCl (pH 7.4), 1 mM phenylmethylsulfonyl fluoride, 2.1 mM pepstatin A, and 1.5 mM

leupeptin. Samples were subjected to polyacrylamide gel electrophoresis (4 % stacking gel and 12 % separating gel) using a Protean Slab II unit (Bio-Rad Laboratories, Hercules, CA). Samples were transferred to a nitrocellulose membrane overnight using a Trans-Blot cell unit. The nitrocellulose membranes were blocked in Tris-buffered saline containing 0.1 % Tween 20 (TBS/T) and 5 % nonfat milk for 1 hour. Membranes containing the samples were incubated with rabbit polyclonal antiserum to whole-cell lysates of *N. fowleri* (anti-*N. fowleri*), *Acanthamoeba castellanii* (anti-ACN), *N. fowleri* Mp2Cl5 (anti-Mp2Cl5), or with monoclonal antibody 5D12 (Indicia Biotechnology, Oullins, France) to *N. fowleri*. The primary antibodies were preabsorbed three times with heat-killed *E. coli* for 2 hours at 37°C. Membranes were incubated in secondary antibody, which consisted of peroxidase-conjugated goat anti-rabbit immunoglobulin G or rabbit anti-mouse immunoglobulin G (Sigma-Aldrich Corporation, St. Louis, MO) for 1 hour. Blots were washed with TBS/T and then developed by enhanced chemiluminescence (Western blotting detection kit; Amersham Co., Piscataway, NJ).

Cloning and Sequencing of a Positive PCR Sample-Arizona

Cloning of the PCR product from sample 4 (swipe sample from a bathroom sink trap) was performed with a TOPO TA cloning kit (Invitrogen, San Diego, CA) according to the manufacturer's instructions. Briefly, fresh PCR product was ligated into a pCR 2.1 TOPO vector, heat shocked into a competent *E. coli* TOP10 strain, and grown on Luria-Bertani agar plates containing 50 μg of ampicillin/ml. Distinct single colonies were picked

and grown in Luria-Bertani broth containing 50 μg of ampicillin/ml. Plasmids were purified with the Wizard *Plus* Minipreps DNA purification system (Promega Corp., Madison, WI). The sample was sequenced by using M13 reverse primers at the BWH DNA Core Sequencing Facility (Boston, MA) and confirmed by the VCU Massey Cancer Center Nucleic Acids Research Facility (Richmond, VA).

Light Microscopic Examination of Environmental Cultures

Cultures were observed by light microscopy for the presence of trophozoites, cysts, and flagellates. Cultures were photographed with an Olympus Ck2 microscope with a computer attachment.

Studies to Identify Virulence Factors of *N. fowleri*

To examine amebae for the presence of factors that may confer pathogenic potential, we have prepared a genomic DNA library from *N. fowleri*. Additionally, we have examined amebae for expression of proteins in *N. fowleri* that are absent in nonpathogenic *Naegleria*.

cDNA Probe for Southern Analysis

A human CD59 cDNA, from the myelogenous leukemia cell line K562, was provided by Alfred Bothwell, Yale University School of Medicine, New Haven, CT

(Philbrick et al. 1990) and used in Southern blot analysis on various species of *Naegleria* to determine if they possess a gene similar to human CD59.

Genomic DNA Isolation

DNA was isolated from amebae using the cetyltrimethylammonium bromide (CTAB) method (Ausubel et al. 1997). One T75 cm^2 flask was scraped and the medium containing the amebae was placed into a 50 ml conical tube. The tube was centrifuged at 5,000 x g. The supernatant was removed and amebae were washed with 10 ml phosphate buffered saline (PBS) and centrifuged again at 5,000 x g. The supernatant was removed and amebae were resuspended in 100 µl PBS and transferred to a 1.5 ml microcentrifuge tube. Five-hundred µl of CTAB solution (100 mM Tris pH 8, 1.4 M sodium chloride, 10 mM EDTA, 2 % CTAB, 1mg/ml polyvinylpyrrolidone (PVP), and 1mg/ml proteinase K, Sigma-Aldrich Corporation, St. Louis, MO) was added, mixed by inversion, and incubated at 55 °C overnight with gentle shaking. The tubes were allowed to cool to room temperature and 500 µl of chloroform/isoamyl alcohol (24:1) was added and mixed well. The mixture was centrifuged at 16,000 x g for 15 minutes at 4 °C and the resulting supernatant was extracted again with 500 µl of phenol/chloroform/isoamyl alcohol (25:24:1) and centrifuged at 16,000 x g for 15 min at 4 °C. A final extraction was performed using 500 µl of chloroform/isoamyl alcohol (24:1) and the supernatant was decanted into a 1.5 ml tube containing 450 µl of ice cold isopropanol and centrifuged at 16,000 x g for 15 min at 4 °C. The pellet was washed twice with 70 % ethanol and air dried

for 20 minutes. The pellet was resuspended in 100 µl of ultrapure water. RNase (0.05 mg/ml) was added and samples were incubated at 37 °C for 20 minutes and quantified by 260/280 ratio using a Biophotometer (Eppendorf Westbury, NY).

Southern Blot Analysis of the "CD59-like" gene

To determine whether a human CD59 probe hybridizes to genomic DNA (gDNA) from *Naegleria* species, Southern blot analysis was performed. Genomic DNA was isolated using the CTAB method described above. Ten micrograms of gDNA was digested with *Eco*RI, *Bam*HI, or *Hin*dIII (Invitrogen, San Diego, CA) overnight at 37 °C and separated by electrophoresis through a 0.8 % agarose gel. The DNA was transferred to a positively charged nylon membrane (Roche Applied Science, Indianapolis, IN) by downward capillary transfer using 20X SSC. The DNA was cross-linked to the nylon membrane using a UV Stratalinker (Stratagene, La Jolla, CA). A miniprep was prepared from a plasmid containing cDNA encoding human CD59. The miniprep (5 µg) was digested with EcoRI and the fragment containing the CD59 cDNA probe was gel extracted using the Qiagen gel extraction kit (Qiagen Inc., Valencia, CA). Twenty-five nanograms of DNA was labeled with (α-^{32}P) dCTP using the RadPrime DNA labeling system (Invitrogen, San Diego, CA). The membrane was incubated with the labeled CD59 probe at 60 °C in ExpressHyb hybridization solution (BD Biosciences Clontech, Palo Alto, CA) according to the manufacturer's instructions. Briefly, the membrane was incubated in 30 ml ExpressHyb hybridization solution for 30 minutes. The solution was removed and 30 ml fresh ExpressHyb containing 1 x 10^6 cpm/ml radioactively labeled CD59 was added.

The membrane was incubated with the probe for 1 hour. The membrane was washed two times with 2X SSC containing 0.1 % SDS (25 °C for 15 minutes) and two times with 0.5X SSC containing 0.1 % SDS (15 minutes at 50 °C). The membrane was exposed to Kodak Biomax film overnight.

Preparation of Membrane fractions of *N. fowleri* for Two-Dimensional Gel Electrophoresis

N. fowleri LEEmp amebae were maintained for 4 days in Cline growth medium supplemented with heat-inactivated donor calf serum and hemin at 37 °C. Amebae were harvested and cytosolic and membrane fractions were prepared using the 2-D sample prep for membrane proteins kit (Pierce Biotechnology, Inc., Rockford, IL). Membrane proteins were isolated from *N. fowleri* amebae using the protocol for yeast cells. One T75 cm^2 flask was used per preparation. Flasks containing the amebae were scraped and the medium containing the amebae was placed into 50 ml conical tubes and pelleted by centrifugation at 5,000 x g. The supernatants were removed and the pellets were resuspended in 80 µl of Mem-PER Cell Lysis Reagent containing 1 mM phenylmethylsulfonyl fluoride, 2.1 mM pepstatin A, and 1.5 mM leupeptin. Acid-washed glass beads (150 mg) were added to the cell suspension and vortexed continuously for 15 minutes to lyse cells. The beads were pelleted by pulse centrifugation. The supernatants were transferred to new microcentrifuge tubes and incubated on ice. In a separate tube, 2 parts Mem-PER Membrane Protein Solubilization Reagent was added to 1 part Mem-PER Buffer and 720 µl of the prepared solubilization mixture was added to the tubes containing the glass beads and vortexed to

wash. A pulse spin was performed to gather beads and the supernatants were transferred to the tubes containing the cell suspensions. The tubes were incubated on ice and vortexed every 5 minutes. The tubes were centrifuged at 10,000 x *g* for 3 minutes at 4 °C and the supernatants were transferred to new tubes and incubated for 10 minutes at 37 °C. The tubes were centrifuged at 10,000 x *g* for 2 minutes at room temperature to partition the hydrophobic fractions from the hydrophilic fractions. The hydrophilic phases were removed from the hydrophobic protein phases and placed in new tubes on ice. In a separate tube, 1 part Mem-PER Buffer was diluted with 3 parts ultrapure water and 100 ml of the diluted Mem-PER Buffer was added to the hydrophobic fractions to reduce viscosity.

Purification of the Membrane Extract for 2-D gel electrophoresis

The 2-D PAGEprep Resin (Pierce Biotechnology, Inc., Rockford, IL) was vortexed to evenly disperse the resin and 20 µl of dispersed resin into spin cup was inserted in a collection tube. Three-hundred microliters of the membrane extract was added to the spin cup. The spin cup was vortexed for 5 seconds and 300 µl of 100 % DMSO was added to the cup. The spin cup was vortexed for 5 seconds and incubated at room temperature for 5 minutes. The cup was centrifuged at 2,000 x *g* for 2 minutes and 500 µl of 50 % DMSO was added to the resin. The spin cup was vortexed 1 minute and centrifuged at 2,000 x *g* for 2 minutes. The washes were repeated two additional times for a total of three washes. The cup was transferred to a new tube and eluted in 50 µl of elution buffer containing 1 mM phenylmethylsulfonyl fluoride, 2.1 mM pepstatin A, and 1.5 mM leupeptin and incubated at 60 °C for 5 minutes and centrifuged.

Desalting the Membrane Proteins for 2-D gel electrophoresis

A protein desalting spin column (Pierce Biotechnology, Inc., Rockford, IL) was inverted several times to suspend the slurry and centrifuged at 1,500 x g for 1 minute. Three-hundred microliters of 2-D Sample Buffer containing 8 M urea, 2 % CHAPS, 50 mM DTT, 0.2 % Bio-Lyte 3/10 ampholyte , 0.001 % Bromophenol Blue, and 1X ReadyStrip pH 7-10 buffer ampholytes (Bio-Rad Laboratories, Hercules, CA) was added to the top of the column and centrifuged at 1,500 x g for 1 minute. This wash was repeated once. The membrane extract (50 µl) was applied to the column and centrifuged at 1,500 x g for 2 minutes.

Two-Dimensional Gel Electrophoresis and Immunoblotting using Anti-CD59 and Anti-Mp2Cl5 Antibodies

Two-dimensional electrophoresis was performed using precast immobilized pH gradient (IPG) strips (pH 7-10, 11 cm; Bio-Rad, Hercules, CA) in the first dimension. IPG strips were rehydrated overnight in 200 µl 2-D sample buffer containing 8 M urea, 2 % CHAPS, 50 mM DTT, 0.2 % Bio-Lyte 3/10 ampholyte , 0.001 % Bromophenol Blue with the addition of 1X ReadyStrip pH 7-10 buffer ampholytes (Bio-Rad Laboratories, Hercules, CA). Membrane preparations (50 µl) from the 2-D sample prep kit were adjusted to 200 µl 2-D sample buffer containing 1X ReadyStrip pH 7-10 buffer ampholytes and were loaded on each IPG strip and focusing was carried out for 30,000 volt-hours. After IEF separation, the strips were immediately equilibrated for 15 min in equilibration I

buffer (6 M urea, 0.375 M Tris, pH 8.8, 2 % SDS, and 20 % glycerol, 2 % DTT). Strips were equilibrated for 15 min in equilibration II buffer (6 M urea, 0.375 M Tris, pH 8.8, 2 % SDS, and 20 % glycerol, 2.5 % w/v iodoacetamide). The IPG strips were loaded onto Criterion precast Tris-HCl gels 10.5-14 % (Bio-Rad Laboratories, Hercules, CA). The gels were placed into a Criterion cell containing 1x Tris/glycine/SDS buffer (25 mM Tris, pH 8.3, 192 mM glycine, 0.1 % SDS; Bio-Rad Laboratories, Hercules, CA) and proteins were separated at 200 V for 1 hour. Gels were transferred to a nitrocellulose membrane for 1 hour using a Criterion blotter at a constant voltage (100 V). The nitrocellulose membranes were blocked in TBS/T containing 5 % nonfat milk for 1 hour. One membrane was incubated overnight in anti-CD59 monoclonal antibody, BRIC229 (1:25) and then incubated in a peroxidase-conjugated rabbit anti-mouse immunoglobulin G secondary (1:15,000) for one hour. The other membrane was incubated overnight in a polyclonal anti-Mp2Cl5 antibody (1:5000) and then with a peroxidase-conjugated goat anti-rabbit immunoglobulin G secondary (Sigma-Aldrich Co.) for one hour. Blots were washed with TBS/T and then developed by enhanced chemiluminescence (Western blotting detection kit; Amersham Co., Piscataway, NJ).

Preparation of Membrane Fractions of *N. fowleri* for Immunoprecipitation

N. fowleri were harvested, suspended in 50 mM Tris-HCl, pH 7.4 containing protease inhibitors (1 mM PMSF, 2.1 mM pepstatin A, and 1.5 mM leupeptin), and freeze-thawed three times by alternating cycles in liquid nitrogen and 37 °C water, then subjected to ultracentrifugation at 100,000 X g for 1 hour at 4 °C to generate cytosolic (supernatant)

and membrane (pellet) fractions. Protein determinations were performed using the Bradford assay (Bradford, 1976). Cytosolic and membrane fractions (80 μg) of *N. fowleri* amebae were incubated in a non-reducing sample buffer (400 mM Tris-Cl, pH 6.8, 6 % SDS, 20 % glycerol, 2 mM EDTA, pH 6.8) for 3 minutes at 95 °C. The samples were prepared for immunoprecipitation by using a 2-D clean up kit (Bio-Rad, Valencia, CA). Briefly, samples (400 μg) were incubated in precipitation agent 1 and incubated on ice for 15 minutes. Precipitation agent 2 was added, samples were vortexed and centrifuged at 12,000 x g for 5 minutes. The supernatant was discarded and 40 μl of wash buffer 1 was added and the tubes were centrifuged at 12,000 x g for 5 minutes. Wash buffer 1 was removed at 25 μl of ultrapure water was added. The tubes were vortexed and 1ml of wash buffer 2 containing 5 μl of wash buffer 2 additive was added. The tubes were incubated at -20 °C for 30 minutes with vortexing every 10 minutes. The tubes were centrifuged at 12,000 x g for 5 minutes. The supernatant was removed and the pellets were allowed to air dry. The pellets were resuspended in 300 μl immunoprecipitation buffer (150 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 1 mM EGTA, pH 8.0, 0.2 mM sodium ortho-vanadate, 0.2 mM PMSF, 1 % Triton X-100, 0.5 % NP-40) and stored at -80 °C.

Cross-linking of anti-CD59 Antibody (BRA-10G) to Protein G Magnetic Beads

Protein G coupled magnetic beads (New England Biolabs, Ipswich, MA) were washed in binding buffer (0.1 M sodium phosphate, pH 8.0) and 10 μl of BRA-10G (anti-CD59) antibody was added. The beads were incubated at 4 °C for 30 minutes with agitation. Beads were washed three times in binding buffer. The Protein G immobilized

antibody was washed two times in 1 ml cross-linking buffer (0.2 M triethanolamine, pH 8.2). The beads were resuspended in 1 ml cross-linking Buffer containing 25 mM Dimethyl pimelidate dihydrochloride (DMP) and incubated at room temperature for 45 minutes with agitation. The supernatant was removed and beads were washed one time in 1 ml blocking buffer (0.1 M ethanolamine, pH 8.2). Beads were incubated in 1 ml of Blocking Buffer for 1 hour at room temperature with agitation. Beads were washed three times in PBS. To elute bound antibody that is not cross-linked with DMP, beads were washed in 1 ml Elution Buffer (0.1 M glycine-HCl, pH 2.5). The supernatant was removed and the BRA-10G cross-linked beads were stored in 100 µl PBS, 0.1 % Tween 20, and 0.02 % sodium azide.

Immunoprecipitation of CD59 and Immunoblot Analysis

Membrane preparations (400 µg) were incubated in 25µl of protein G magnetic beads (New England Biolabs, Ipswich, MA) for 1 hour at 4 °C to prevent nonspecific binding to the beads. A magnetic field was applied for 30 seconds to pull the beads to the side of the tube. The supernatant was placed in a clean 1.5 ml microcentrifuge tube. The BRA-10G cross-linked beads (25 µl) were added to the pre-cleared protein extract and incubated overnight at 4 °C. A magnetic field was applied for 30 seconds to pull the beads to the side of the tube and the beads were washed with immunoprecipitation buffer. This wash was repeated three times. The immunoprecipitated proteins were removed from the beads by resuspending the bead pellet in 60 µl non-reducing sample buffer (400 mM Tris-Cl, pH 6.8, 6 % SDS, 20 %, glycerol, 2 mM EDTA, pH 6.8, and 0.01 % bromphenol blue)

and incubating the tubes at 70 °C for 5 minutes. A magnetic field was applied and the supernatant was removed and subjected to polyacrylamide gel electrophoresis (4 % stacking gel and 15 % separating gel) using a Mini-Protean 3 unit (Bio-Rad Laboratories, Hercules, CA). Two gels were run at one time. One gel was prepared for silver staining. The other gel was transferred to a nitrocellulose membrane for 1 hour using a mini Trans-Blot cell unit. The nitrocellulose membrane was blocked in TBS/T containing 5 % nonfat milk for 1 hour. The membrane was incubated with CD59 monoclonal antibody (BRIC 229) diluted 1:25 overnight at 4 °C. The membrane was washed in TBS/T and incubated in a secondary HRP-linked rabbit anti-mouse antibody (Sigma, St. Louis, MO) 1:15,000 for 1 hour at room temperature. The proteins were detected using enhanced chemiluminescence (Amersham Pharmacia Biotech, Buckinghamshire, UK). The membrane was stripped using a method described by Amersham Pharmacia Biotech. Briefly, the membrane was submerged in stripping buffer (100 mM 2-Mercaptoethanol, 2 % SDS, 62.5 mM Tris-HCl, pH 6.7) and incubated at 50 °C for 30 minutes with agitation. The membrane was washed 2 × 10 minutes in TBS/T at room temperature. The membranes were reprobed with an anti-Mp2Cl5 antibody (1:5000).

Silver Staining of Proteins Immunoprecipitated with Anti-CD59

Prior to silver staining, the gel was fixed in 10 % methanol/10 % acetic acid for 30 minutes and washed 5 times in water for 5 minutes each. The fixed gel was incubated in 0.02 % sodium thiocyanate for 90 seconds and then washed in water 3 times, 10 seconds each. The gel was incubated in 0.18 % silver nitrate for 10 minutes, followed by 3 washes

in water, 10 seconds each. The gel was incubated in developer solution (1 % potassium carbonate, 2 ml of 0.02 % sodium thiosulfate solution and 25 µL 37 % formaldehyde, Sigma-Aldrich Corporation) for 5-10 minutes. The reaction was stopped with 10 % MeOH, 5 % acetic acid.

Isolation of Signal Proteins and Immunoblot Analysis using Anti-CD59 and Anti-Mp2Cl5 Antibodies

Signal proteins were isolated from *N. fowleri* using the FOCUS Signal Protein Extraction kit (G Biosciences, St. Louis MO). This kit isolates caveolin-enriched membrane proteins, glycosyl phosphitidylinositol (GPI) linked proteins, glycolipids, and GTP-binding proteins. One T75 cm^2 flask containing *N. fowleri* was scraped and centrifuged at 5,000 x g. The pellet was resuspended in 300 µl of signal protein extraction buffer-I (SPE buffer-I) buffer containing 1 mM phenylmethylsulfonyl fluoride, 2.1 mM pepstatin A, and 1.5 mM leupeptin. Amebae were sonicated in an ice bath with a 10 second bursts and a 10 second chill on ice and was performed 4 times on the amebic lysate. Pre-chilled signal protein extraction buffer-II (SPE buffer-II) (300 µl) buffer was added and the tube was vortexed 4 times for 60 seconds with 30 seconds in an ice bath between vortexing. The tube was incubated on ice for 15 minutes and centrifuged at 20,000 x g at 4 ^{o}C. The supernatant was removed and saved for SDS-PAGE. The pellet was resuspended in 100 µl SPE buffer-I, followed by the addition of 100 µl of SPE buffer-II. The tube was vortexed 4 times for 60 seconds with 30 seconds in an ice bath between vortexing. The tube was incubated on ice for 15 minutes and centrifuged at 20,000 x g at 4 ^{o}C. The

supernatant was removed and saved for SDS-PAGE. The pellet was resuspended in 100 µl of Focus protein solubilization buffer (FPS) to solubilize the insoluble signal protein fraction. The tube was vortexed 4 times for 60 seconds with 30 seconds in an ice bath between vortexing. The tube was incubated at room temperature for 10 minutes and centrifuged at 20,000 x g at 4 °C. The supernatant was removed and any residual pellet was resuspended in 50 µl of FPS buffer. The concentration of protein in each sample was determined by the Bradford Assay (Bradford, 1976). The proteins were separated by SDS-PAGE and immunoblotted using antibodies to CD59 (BRIC229) and Mp2C15 as previously described.

Construction of a *N. fowleri* Genomic DNA Library

Construction of the *N. fowleri* gDNA library was performed using the Lambda ZAP® II Predigested EcoRI Kit with Gigapack® III Gold Packaging Extract (Stratagene, La Jolla, CA) kit according to manufacturer's instructions (Fig 1). Genomic DNA was prepared from *N. fowleri* by the cetyltrimethylammonium bromide (CTAB) method described previously. The DNA was digested with *Eco*RI and 0.1 µg of the digested DNA was added to 1 µg of Lambda ZAP II vector (Stratagene, La Jolla, CA) predigested with *Eco*RI. To ligate the DNA, 0.5 µl of 10X T4 ligase buffer, 0.5 µl of 10 mM ATP (pH 7.5) and 0.5 µl of T4 DNA ligase (Stratagene, La Jolla, CA) were added and the tube was incubated overnight at 4°C. The ligation reaction was packaged into phage heads with Gigapack III gold packaging extract. To package the DNA, 1 µl of ligated DNA was added

to the packaging extract and incubated at room temperature for 2 hours. Five hundred µl of SM buffer was added to the tube, followed by the addition of 20 µl of chloroform. The tube was centrifuged at 14,000 x g and the supernatant containing the phage was stored at 4°C overnight. The phagemid stock was titered with the XL1-Blue MRF' *Escherichia coli* strain. The XL1-Blue MRF′ (Minus Restriction) strain is a restriction minus (McrA–, McrCB–, McrF–, Mrr–, HsdR–) derivative of Stratagene's XL1-Blue strain. XL1-Blue MRF′ cells are deficient in all known restriction systems and are tetracycline resistant. XL1-Blue has an F' episome that contains the Δ*M15 lacZ* gene that is required for the β-galactosidase-based nonrecombinant selection strategy. The F′ episome also expresses the genes forming the F′ pili found on the surface of the bacteria. Without pili formation, filamentous phage infection could not occur. Overnight cultures (50 ml) of XL1-Blue MRF′ cells were grown in LB broth at 30 °C. Harvested cells were resuspended in 25 ml of 10 mM $MgSO_4$. The concentration of XL1-Blue MRF' cells was adjusted to an OD600 of 1.0 (8×10^8 cells/ml) in 10 mM $MgSO_4$. One microliter of the packaged phage was mixed with 200 µl of the XL1-Blue cells and incubated at 37 °C for 15 minutes to allow the phage to attach to the cells. NZY (1.0% NZ amine, 0.5% sodium chloride, 0.5% yeast extract, 0.2% magnesium chloride tetrahydrate, 0.7 % agarose) top agar (3 ml) melted and cooled to ~48 °C was added and plated immediately onto dry, prewarmed NZY (1.0% NZ amine, 0.5% sodium chloride, 0.5% yeast extract, 0.2% magnesium chloride tetrahydrate, 1.5% agar) agar plates.

The plates were allowed to set for 10 minutes and incubated at 37 °C overnight. The plaques were counted and the titer in plaque-forming units per milliliter (pfu/ml) was calculated using the following formula:

$$\frac{\text{number of plaques} \times \text{dilution factor}}{\text{volume of extract plated}} = \text{PFU/ml}$$

The packing efficiency was calculated using the following formula:

$$\frac{\text{PFU (clear plaques)/ml}}{\text{concentration of vector DNA packaged}} = \text{recombinants/}\mu\text{g DNA}$$

The primary phage library was 1.6×10^5 PFU/ml. The packaging efficiency was calculated by using the above formulas for only the clear plaques. The packaging efficiency was 1×10^7 recombinants/μg DNA. The primary library was used to create an amplified library.

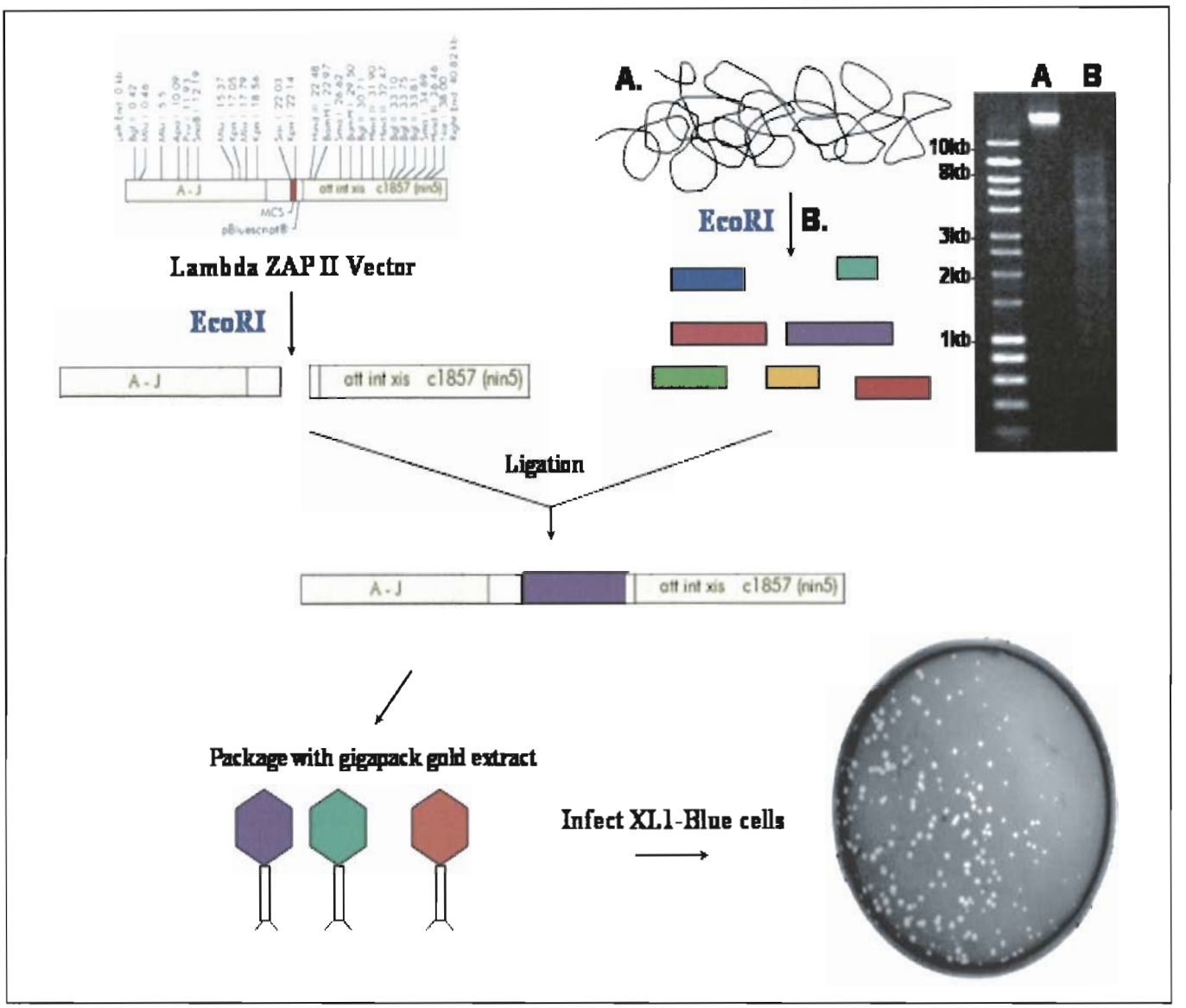

Figure 1. Construction of gDNA library. Genomic DNA was isolated from *N. fowleri* using the CTAB method (lane A) and digested with EcoRI at 37°C overnight (Lane B). Digested DNA was ligated to the Lambda/ZAP II vector arms (Stratagene) and packaged using the Gigapack Gold (Stratagene) packaging extract. The packaged phagemids were plated with XL1-Blue *E. coli* and incubated 16 hours at 37° C. Plaque formation was observed and the titer was calculated to be 1.6 x 10^5 PFU/ml. (Adapted from http://www.biochem.utah.edu/rutter/BC6400/DC2-Libraries.ppt)

Library Amplification

Overnight cultures (50 ml) of XL1-Blue MRF′ cells were grown in LB broth at 30°C. Harvested cells were resuspended in 25 ml of 10 mM $MgSO_4$. The concentration of XL1-Blue MRF' cells was adjusted to an OD600 of 1.0 (8×10^8 cells/ml) in 10 mM $MgSO_4$. Approximately 5×10^4 pfu of bacteriophage was combined with 600 µl of XL1-Blue MRF' cells at an OD600 of 0.5 in 14 ml BD Falcon polypropylene round-bottom tubes incubated for 15 minutes at 37°C. NZY top agar (6.5 ml) melted and cooled to ~48°C was added and plated immediately onto dry, prewarmed NZY agar plates (150 mm). The plates were allowed to set for 10 minutes, then incubated at 37°C for 8 hours. Ten ml of SM buffer was placed on the plates and they were incubated at 4°C overnight on a rocker platform. The suspension was removed from the plates and pooled into a 50 ml conical tube. The plates were rinsed with 2 ml of SM buffer, which was added to the 50 ml conical. Chloroform was added to a 5 % final concentration, the contents were mixed and incubated for 15 minutes at room temperature. The cell debris was removed by centrifugation for 10 minutes at 500 × *g*. The supernatant was removed and transferred to a sterile polypropylene container. Chloroform was added to a 0.3 % (v/v) final concentration and aliquots were stored in 7 % (v/v) DMSO at –80 °C. The titer of the amplified library was 1.2×10^8 pfu/ml.

Mass Excision of pBluescript Phagemids

In vivo excision, involving the ExAssist interference-resistant helper phage along with the SOLR strain of *E. coli,* was used. The ExAssist helper phage contains an amber

mutation that prevents the replication of the phage genome in a nonsuppressing *E. coli* strain like SOLR (Stratagene, La Jolla, CA). This allows efficient excision of the pBluescript phagemid from the Lambda ZAP II vector while eliminating the problems associated with helper phage co-infection. Overnight cultures (50 ml) of XL1 Blue MRF′ or SOLR cells were grown in LB broth at 30°C. Harvested cells were resuspended in 25 ml of 10 mM $MgSO_4$. The concentration of SOLR cells was adjusted to an OD600 of 1.0 (8 × 108 cells/ml) in 10 mM $MgSO_4$. The amplified lambda bacteriophage was diluted to $1x10^5$ phage particles in 250 µl and was mixed with 200 µl XL1-Blue cells. One µl (>1 × 10^6 pfu/µl) of ExAssist helper phage was added and the mixture was allowed to incubate at 37 °C for 15 minutes to allow the phage to attach to the cells. Three ml of LB broth with supplements was added to the mixture and allowed to incubate for 3 hours at 37°C with shaking. To lyse the phage particles and the SOLR cells, the tube was incubated at 70 °C for 20 minutes. The lysed mixture was spun at 1000 x g for 10 minutes to pellet the cell debris and the supernatant containing the excised phagemid was collected and stored at -80 °C. To plate the excised phagemids, 600 µl of freshly grown SOLR cells (OD600 = 1.0) was added to 50 µl of the phage supernatant. The SOLR/phage mixture was incubated at 37°C for 15 minutes and 100 µl of the mixture was plated on a LB-ampicillin agar plate (100 µg/ml) containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) and isopropyl-β-D-thiogalactopyranoside (IPTG) and incubated overnight at 37 °C. A total of six plates were prepared and 155 white colonies were randomly selected for sequencing.

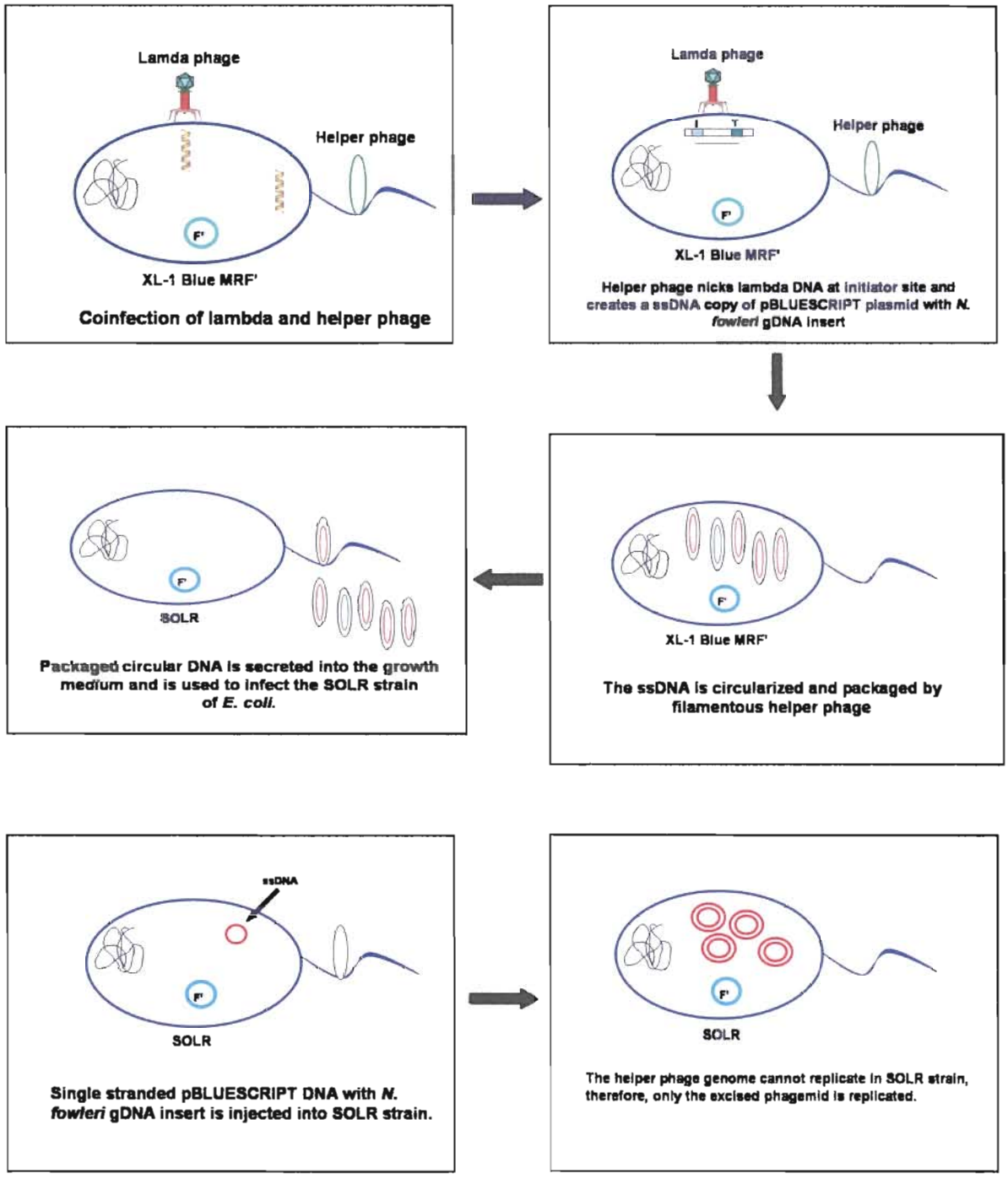

Figure 2. Excision of pBLUESCRIPT phagemids. The amplified phage library was converted to a plasmid library by mass excision to release the phagemid vector. SOLR bacteria containing pBLUESCRIPT KS- phagemids were plated on LB plates containing ampicillin, IPTG and X-gal. White colonies were randomly picked for sequencing.

Plasmid Preparation, Restriction Endonuclease Analysis, and Sequencing of pBLUESCRIPT Phagemids

Plasmids were prepared using the Qiagen miniprep kit (Qiagen Inc., Valencia, CA). Restriction digestions were performed according to the suppliers' directions. One microgram of plasmid DNA was digested with 10 U EcoRI (New England Biolabs, Ipswich, MA) and 0.5 mg of the digest was run on a 1.5 % agarose gel. The minipreps were diluted to 100 ng/µl and sent to VCU Nucleic Acids Research Facilities for sequencing using commercially available KS (5'-CCTCGAGGTCGACGGTATCG-3') and SK (5'-CGGCCGCTCTAGAACTAGTGGATC-3') primers. These are universal primers that flank the insertion site of the pBluescript phagemid. Approximately 600-800 bp of sequencing data was obtained using these primers. A translated BLAST search (blastx) was performed to determine the putative protein encoded by the plasmid sequence. Additional gene and protein analyses were performed using analysis programs available on the Expert Protein Analysis System (ExPASy) website (www.expasy.org) including "Translate" and "ScanProsite" (Gasteiger et al. 2003). Predicted proteins with significant similarities or conserved domains were grouped according to function. I would like to acknowledge the assistance of Matseliso Moloi in the plasmid preparations.

Sequencing of Plasmid 5 (Patatin-like phospholipase)

One gene that was initially sequenced (plasmid 5) was identified as a patatin-like phospholipase. The initial sequencing reaction with KS and SK universal primers only provided 1200 bp of gene sequence data. In order to obtain the whole 2.2 kb sequence of

plasmid 5, primer walking was performed. Sequencing reactions using universal primers, KS and SK, were repeated. In addition, three custom designed primers were used to obtain the sequence in the middle of the plasmid that was not obtained with KS and SK primers. The primers were designed using Vector NTI software and included "5 forward 1" (5'-TGAAACCAAACTCTATGACT -3'), "5 forward 2" (5'TCAATTACCAAATCATGG-3') and "5 reverse 1" (5'- ACGTGATGAAGATCTCTC-3'). Sequencing was performed at the VCU Nucleic Acids Research facility and sequencing data was aligned using Vector NTI software.

DNA Walking of the Patatin Gene

Based on BLAST searches and translation into a protein sequence, it was determined that plasmid 5 did not contain the complete 3' end of the gene encoding the patatin-like protein, therefore, DNA walking was performed using the DNA walking SpeedUp kit (Seegene, Rockville, Maryland) to obtain the 3' end of the gene. Three rounds of PCR were performed using 3 target specific primers (TSP1-3) designed to the patatin gene. The first round of PCR was performed on 100ng *N. fowleri* gDNA with TSP1 (5'-CAGCTCTACAAAGCAGTTCC-3') and each PCR reaction contained 1X SeeAmp ACP (annealing control primer) Master Mix II, 2.5 μM DNA Walking-Annealing Control Primer (reactions contained either DW-ACP 1, 2, 3, or 4), and 10 μM TSP 1, in a total volume of 50 μl. A hot-start PCR program was used and the PCR machine was preheated to 94 °C. The tubes were placed in the machine and one round of cycling was performed at 94 °C for 5 minutes, 42 °C for 1 minute, and 72 °C for 2 minutes. Thirty-five cycles were

performed at 94 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 100 seconds. A final extension was performed for 7 minutes at 72 °C. The products from the first PCR reaction were purified using the Qiaquick PCR purification kit (Qiagen Inc., Valencia, CA). The purified PCR products (1μl) were subjected to a second round of PCR amplification using TSP 2 (5'-TGGCCAAAGATCGAGATGGT-3') in a reaction containing 1X SeeAmp ACP Master Mix II, 10 μM DW-ACPN (DNA Walking-Annealing Control Primer N), and 10 μM TSP 2, in a total volume of 50 μl. A hot-start PCR program was used and the PCR machine was preheated to 94 °C. The PCR reactions were incubated at 94 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 100 seconds for 35 cycles. A final extension was performed for 7 minutes at 72 °C. A third round of PCR was performed using 1μl of the PCR product from the second round and TSP 3 (5'-TGCCTCTCAAGTGAACCCTC-3') in a reaction containing 1X SeeAmp ACP Master Mix II, 10 μM Universal Primer, and 10 μM TSP 3, in a total volume of 50 μl. A hot-start PCR program was used and the PCR machine was preheated to 94 °C. The PCR program was 94 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 100 sec for 35 cycles. A final extension was performed for 7 minutes at 72 °C. PCR products were visualized on a 1.5 % agarose gel containing ethidium bromide. A PCR product was observed using DW-ACP 2 and TSP 2 and TSP 3 primers. The PCR product obtained from TSP 2 was cloned into the TOPO 2.1 vector using the TOPO TA cloning kit (Invitrogen, San Diego, CA) as described previously. Minipreps were performed using a Qiagen miniprep kit, digested with EcoRI to confirm the presence of an insert, and sequenced at the VCU Nucleic Acids Research

Facility. The sequencing results were aligned with the DNA sequence from plasmid 5 using Vector NTI software.

RNA Isolation for Northern Blot and RT-PCR Analysis of Patatin Expression

Amebae were pelleted by centrifugation and lysed in 1 ml TRIZOL Reagent by repetitive pipetting. The samples were incubated for 5 minutes at 15 to 30 °C, then 0.2 ml of chloroform per 1 ml of TRIZOL Reagent was added. The tubes were shaken by hand for 15 seconds and incubated at 30 °C for 3 minutes. The samples were centrifuged at 12,000 × *g* for 15 minutes at 4 °C. The aqueous phase was transferred to a new RNase free-tube. The RNA was precipitated by adding 0.5 ml of isopropyl alcohol. The samples were incubated at room temperature for 10 minutes and centrifuged at 12,000 × *g* for 10 minutes at 4 °C. The supernatant was removed and the pellet was washed with 1 ml 75 % ethanol. The samples were mixed gently by inverting the tube and centrifuged at 7,500 × *g* for 5 minutes at 4 °C. The ethanol was removed using a pipette and the pellet was air-dried at room temperature for 10 minutes. The pellet was dissolved in 100 µl RNase-free water. The concentration of RNA was obtained by 260/280 ratio using a Biophotometer (Eppendorf, Westbury, NY).

DNase Treatment of RNA Isolated From Amebae for Northern Blot and RT-PCR Analysis of Patatin Expression

RNA from various amebae was DNase treated in a reaction containing 1 µg total RNA, 1 ml 10X DNase I reaction buffer (200 mM Tris-HCl, pH 8.4, 20 mM MgCl2, 500

mM KCl), 1 µl DNase I, and water to a final volume of 10 µl. Samples were incubated at room temperature for 15 minutes. The DNase was inactivated by the addition of 1 µl of 25 mM EDTA solution to the reaction mixture and heating for 10 min at 65 °C.

cDNA Synthesis of DNase Treated RNA for Northern Blot and RT-PCR Analysis of Patatin Expression

cDNA synthesis was performed using the SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, San Diego, CA) by combining 1 µg (10 µl) DNase-treated RNA with 1 µl random hexamers (50 ng/µl), and 1 µl dNTP mix (10 mM). Samples were incubated at 65 °C for 5 minute and placed on ice for 1 minute. To each sample, 2 µl 10X RT buffer, 4 µl 25 mM MgCl2, 2 µl 0.1 M DTT, and 1 µl RNaseOUT (40 U/µl) was added. For + RT samples, 1 µl SuperScript III RT (200 U/µl) was added and for –RT samples, 1 µl of DEPC-treated water was added. Samples were mixed gently, collected by brief centrifugation and incubated 10 minutes at 25°C, followed by 50 minutes at 50°C. The reactions were terminated by incubation at 85°C for 5 minutes and chilled on ice. The cDNA was stored at -20 °C.

PCR Analysis to Obtain Intron/Exon Splice Sites of Patatin

PCR was performed on genomic DNA isolated from *N. fowleri* using the CTAB method as described previously and cDNA was prepared from *N. fowleri* using the SuperScript III First-Strand Synthesis System for RT-PCR as described previously. Two different primer sets for patatin were used. The first primer set was used to determine the

junctions of intron 1. Primers used included patatin1.for (5'ATGGCAACTGTCATATCAC-3') and patatin1.rev (5'TGAGTGGACAATCTGCAGGA-3'). A second PCR assay was used to determine the junctions of intron 2 using primers patatin2.for (5'-TGGCTGCAGTCTTGTGTACC-3') and patatin2.rev (5'-TCATCACCACAATTTGCCAT-3'). PCR was performed in a 50 µl volume consisting of 1X *Taq* DNA polymerase buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 µM of deoxynucleoside triphosphates (dNTPs), 0.2-µM of primer, and 1 U of Platinum *Taq* DNA polymerase (Invitrogen, San Diego, CA). The positive control consisted of 100 ng plasmid DNA and the negative control consisted of PCR-grade water lacking DNA template. The standard temperature program was 5 minutes at 95 °C for one cycle and 30 seconds at 95 °C, 30 seconds at 60 °C, and 45 seconds at 72 °C for 35 cycles. A final extension was performed at 72 °C for 7 minutes. PCR products were visualized on a 1.5 % agarose gel stained with ethidium bromide.

Alignment of *N. fowleri* Patatin-like Protein With Other Known Patatin Proteins

The Swiss EMBnet node server (http://www.ch.embnet.org) was used to align the *N. fowleri* patatin-like protein with other known patatin-like proteins. Alignment was performed using the Tree-based Consistency Objective Function for alignment Evaluation (T- coffee) server (Notredame et al. 2000). This is a multiple sequence alignment program that combines global and local pairwise alignment by pooling the ClustalW and Lalign programs and will compare several protein sequences to each other. Sequences from known patatin proteins were obtained from the NCBI protein database

(http://www.ncbi.nlm.nih.gov/) and included those from *Solanum tuberosum* (gi 758342), *Trypanosoma cruzi* (gi 71404931), *Rickettsia felis* (gi 67004285), *Pseudomonas aeruginosa* ExoU protein (gi 2429143), and *Escherichia coli* 0157:H7 (gi 15832052).

Northern Blot Analysis for Patatin Expression

To determine the size of the patatin-like protein transcript, Northern analysis was performed using a patatin probe. Ten micrograms of DNase treated RNA was brought to a volume of 10 µl in DEPC-water. Ten microliters of formaldehyde sample buffer (Cambrex Corporation East Rutherford, New Jersey) was added and samples were heated at 65 °C for 15 minutes, cooled on ice and then loaded onto a 1.5 % denaturing agarose gel (Sambrook et al. 1989). The gel was transferred to a positively charged nylon membrane (Roche Applied Science, Indianapolis, IN) and cross-linked to the nylon membrane using a UV Stratalinker (Stratagene, La Jolla, CA). The patatin cDNA was labeled using the RadPrime DNA labeling system (Invitrogen, San Diego, CA). The membrane was incubated with the labeled patatin probe at 68 °C in ExpressHyb hybridization solution (BD Biosciences Clontech, Palo Alto, CA) according to the manufacturer's instructions. The membrane was washed two times with 2X SSC containing 0.1 % SDS (25 °C for 15 minutes) and two times with 0.1X SSC containing 0.1 % SDS (15 minutes at 50 °C). The membrane was exposed to Kodak Biomax film overnight.

PCR Analysis of Genomic DNA and cDNA Using Patatin Primers

PCR was performed on genomic DNA isolated from *N. fowleri, N. lovaniensis, N. gruberi, A. culbertsoni, A. astronyxis,* and *B. mandrillaris* using the CTAB method as described previously. cDNA was prepared from *N. fowleri, N. lovaniensis*, and *N. gruberi* using the SuperScript III First-Strand Synthesis System for RT-PCR as described previously. Two different primer sets for patatin were used. The first primer set was used to amplify cDNA using primers patatin2.for and patatin2.rev as described previously and a 928 bp product was expected. The second primer set was used on both cDNA and gDNA using primers patatin3.for (5'-TGGATGTGAACAAGTTAGCACA-3') and patatin3.rev (5'-CTGCAGCACTCCAAATCAAG-3') and a 186 bp fragment was expected. PCR was performed in a 50-µl volume consisting of 1X *Taq* DNA polymerase buffer (20 mM Tris-HCl, pH 8.4, 50 mM KCl, 1.5 mM $MgCl_2$), 0.2 µM of deoxynucleoside triphosphates (dNTPs), 0.2-µM of primer, and 1 U of Platinum *Taq* DNA polymerase (Invitrogen, San Diego, CA). The positive control consisted of 100 ng plasmid DNA and the negative control consisted of PCR-grade water lacking DNA template. The standard temperature program was 5 minute at 95 °C for one cycle and 30 seconds at 95 °C, 30 seconds at 60 °C, and 45 seconds at 72 °C for 35 cycles. A final extension was performed at 72 °C for 7 minutes. PCR products were visualized on a 1.5 % agarose gel stained with ethidium bromide.

Immunoblot Analysis of *N. fowleri* and *N. lovaniensis* Using an Anti-Human Phospholipase A_2 Antibody

Whole cell lysates, membrane preparations, and cytosolic preparations were prepared as previously described. Samples (20 μg) were subjected to polyacrylamide gel electrophoresis (4 % stacking gel and 10 % separating gel) using a Protean Slab II unit (Bio-Rad Laboratories, Hercules, CA). Samples were transferred to a nitrocellulose membrane overnight using a Trans-Blot cell unit. The nitrocellulose membranes were blocked in TBS/T containing 5 % milk for 1 hour. Membranes were incubated with a monoclonal anti-human phospholipase A_2 antibody overnight at 4 ^{o}C. Membranes were incubated in a rabbit anti-mouse immunoglobulin G secondary antibody (Sigma-Aldrich Corporation, St. Louis, MO) for 1 hour. Blots were washed with TBS/T and then developed by enhanced chemiluminescence (Western blotting detection kit; Amersham Co., Piscataway, NJ).

RESULTS

PCR Analysis on Water Samples from Richmond, Virginia

PCR analysis was performed using a nested PCR assay developed by Reveiller, et al. 2002. Primer sequences used for the PCR analysis are shown in Table 1. Four water samples collected from the James River, Richmond, Virginia, processed immediately without isolating amebae on NNA-*E. coli* plates, were positive for *N. fowleri* by PCR (Fig 3A). These four water samples, also, were enriched on NNA-*E. coli* plates and were positive by PCR analysis (Fig 3B). To confirm the results of the PCR assay, two mice were exposed to the James River isolate by intranasal instillation of water containing amebae. A lethal infection was observed in one of the two mice inoculated and amebae were isolated from the mouse brain at the time of death using standard methods as described by Martinez, 1985.

Analysis by Western immunoblotting with anti-*N. fowleri* antibody or an antibody to an *N. fowleri* recombinant protein (anti-Mp2C15) demonstrated immunoreactivity with samples from the James River (Fig 4A & 4B).

Light microscopic analysis of samples from the James River, Richmond, VA revealed the presence of limax-type (movement in a slug-like motion) amebae and cysts (Fig 5A and B). A nonpathogenic ameba, *Vannella sp.* was identified by morphological characterization using the *Illustrated Guide to the Protozoa* (2002) and found to be very prevalent in samples taken from the James River (Fig 5C).

Table 1. PCR Primers Used to Identify *Naegleria fowleri* in Environmental and Domestic Sources[a].

PCR primer	Sequence
Mp2C15.for	5'-TCTAGAGATCCAACCAATGG-3'
Mp2C15.rev	5'-ATTCTATTCACTCCACAATCC-3'
Mp2C15.for-in	5'-GTACATTGTTTTTATTAATTTCC-3'
Mp2C15.rev-in	5'-GTCTTTGTGAAAACATCACC-3'

[a]PCR primers were originally published by Reveiller et al, 2002 to the Mp2C15 coding sequence unique to *N. fowleri*.

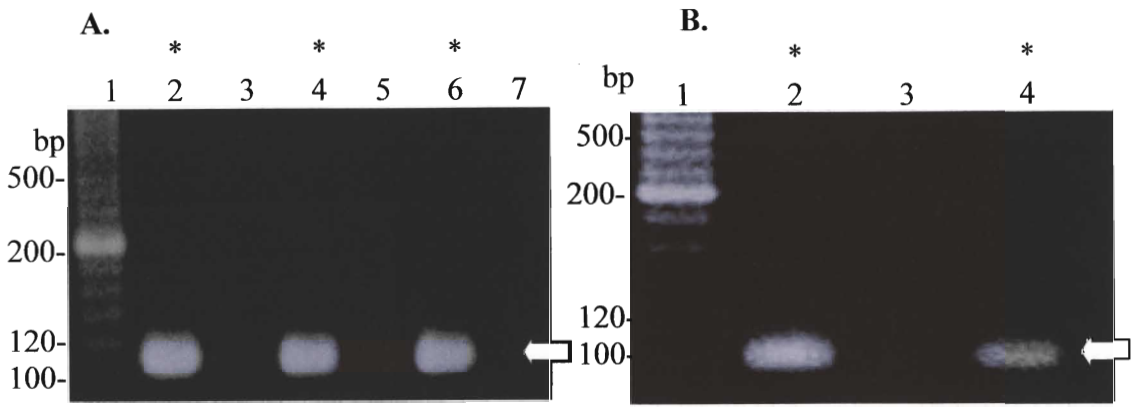

Figure 3. PCR analysis of water samples from the James River, Richmond, VA. Water samples collected from Richmond, VA were processed directly without enrichment or water was maintained for 2 d at 37 °C in 75 cm^2 tissue culture flasks. For direct analysis, amebae were harvested by centrifugation and the pellet was subjected to nested PCR analysis. Nested PCR products were detected on a Nusieve 3:1 agarose gel stained with EtBr. (A) Two of four samples processed directly from the James River were positive by PCR for *N. fowleri*. (A) Lane (1) 20 base pair ladder; (2) positive control, Mp2Cl5 plasmid; (3) negative control; (4 and 6) James River, Richmond, VA positive samples; (5 and 7) James River, Richmond, VA negative samples; (B) One sample maintained for 2 d at 37 °C was positive by PCR for *N. fowleri*. Lane (1) 100 base pair ladder; (2) positive control, Mp2Cl5 plasmid; (3) negative control; (4) James River, Richmond, VA sample maintained for 2 days at 37 °C. *Positive for *N. fowleri* by PCR analysis (MacLean et al. 2004).

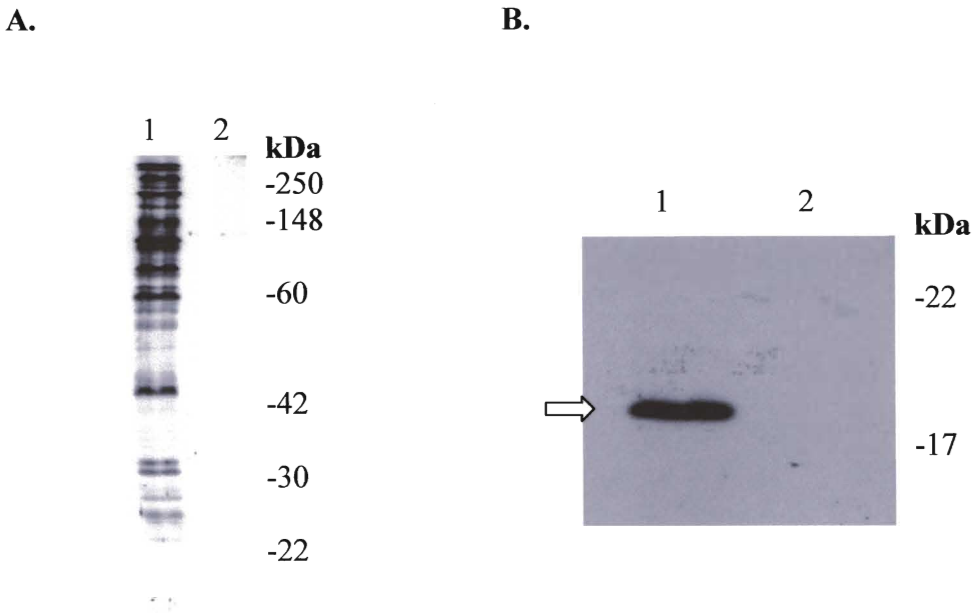

Figure 4. Western blot analysis of water samples from the James River, Richmond Virginia Samples collected from the James River, Richmond, VA, were maintained at 37 °C for 5 days, harvested, and subjected to 12 % SDS-PAGE. Separated proteins were transferred to a nitrocellulose membrane. (A) Rabbit polyclonal antiserum against a whole cell lysate of *N. fowleri* (1:500) followed by peroxidase-conjugated goat anti-rabbit IgG (1:10,000). (B) Rabbit polyclonal antiserum against the Mp2C15 recombinant protein (1:5000) followed by peroxidase-conjugated goat anti-rabbit IgG (1:10,000). Blots were developed using enhanced chemiluminescence (ECL). Lane (1) James River sample; (2) Negative sample. Arrow indicates Mp2C15 protein (MacLean et al. 2004).

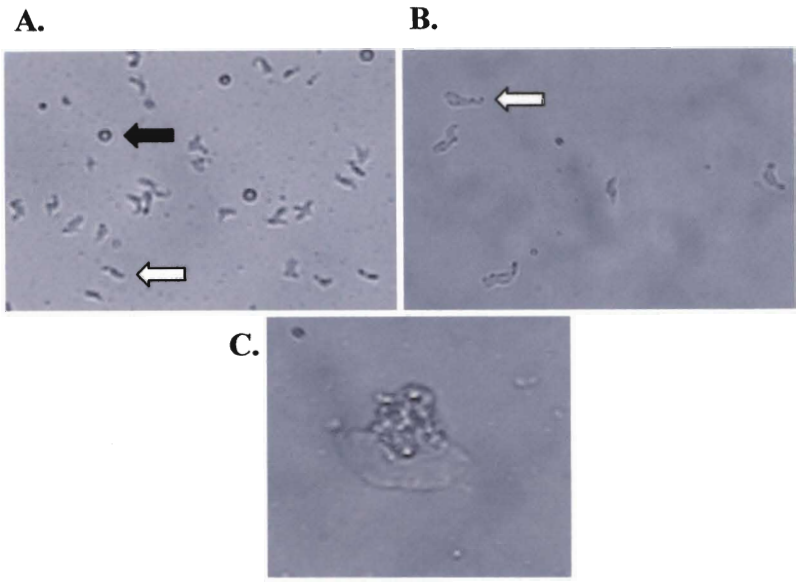

Figure 5. PCR positive cultures from Richmond, VA were observed for the presence of trophozoites, cysts, or flagellates. Limax-type amebae and cysts (A and B) were observed. (C) A prevalent nonpathogenic ameba, *Vannella* sp. also was present. Open arrows indicate limax-type amebae and closed arrows indicate round cysts. Magnification, x400.

PCR on cultures from ATCC to confirm the specificity of the PCR assay

PCR was performed on other free-living ameba found in the environment to confirm that the nested PCR assay was specific for *N. fowleri*. Amebae cultures purchased from ATCC which included *Naegleria galeacystis*, *Vahlkampfia avara*, *Hartmannella vermiformis*, *Willaertia magna*, *Vannella aberdonica*, and *Balamuthia mandrillaris* were negative by nested PCR analysis (Fig 6).

PCR Analysis on Environmental Samples from Connecticut

In order to determine whether *N. fowleri* was present in an area not previously reported, samples were collected from a variety of areas in Connecticut. Clark's Pond and Wharton Brook in Connecticut are small lakes resulting from impoundment of small rivers. The maximum depth of each is approximately 3 meters. The small pond on Quinnipiac University Campus is a man-made pond that collects runoff and is not a part of a stream system. Its maximum normal depth is about 1-1.3 meters. Total coliform and *E. coli* levels were determined for each study site at each collection period. All ameba isolates that were obtained at 42 °C were kept for further study. No significant correlations were noted in the number of thermotolerant isolates collected when compared with mean air temperature, water temperature, coliform levels or *E. coli* levels after regression analysis was performed (Table 2). Samples collected in Connecticut were stored in sealed plates containing NNA-*E. coli* for 12 months as cysts. PCR was performed on the samples after excystation to identify *N. fowleri*. Eighty-six isolates were tested by nested PCR. Samples testing positive by PCR as *N. fowleri* are shown in bold in the last column of

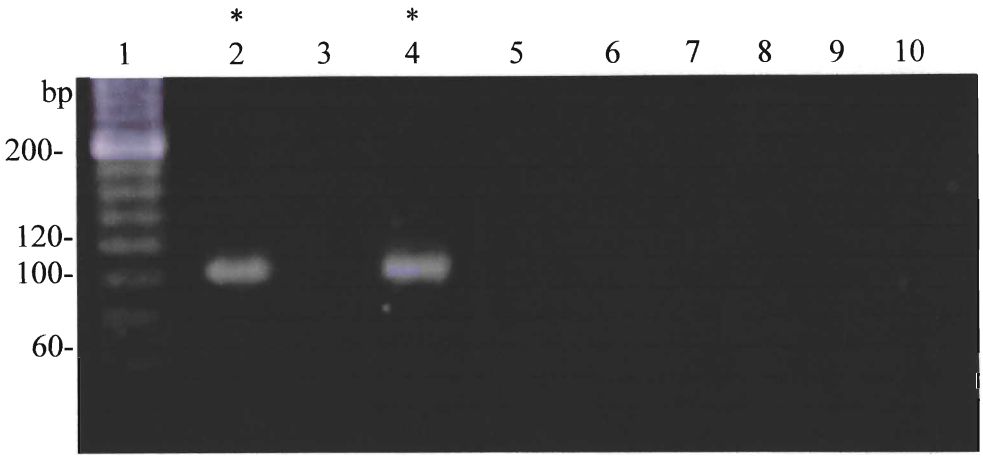

Figure 6. Specificity of the PCR assay. PCR was performed directly on free-living ameba found in the environment to confirm that the nested PCR assay was specific for *N. fowleri*. Samples were subjected to electrophoresis on a Nusieve 3:1 agarose gel. The positive controls, Mp2Cl5 plasmid DNA and *N. fowleri* DNA were positive. All others were negative by nested PCR analysis. Lane (1) 20 base pair ladder; (2) positive control, Mp2Cl5 plasmid; (3) negative control (4) *Naegleria fowleri*; (5) *Naegleria galeacystis*; (6) *Balamuthia mandrillaris*; (7) *Hartmannella vermiformis*; *(8) Vahlkampfia avara*; (9) *Vannella aberdonica*; (10) *Willaertia magna*. *Positive for *N. fowleri* by PCR analysis.

Table 2. Data Collected on Water and Soil Samples from Connecticut.

Date	Source	Rainfall[a] (cm)	Water Temp (°C)	Air Temp High	Air Temp Low	Coliform[b]	*E. coli*[c]	# Isolates	Results of PCR
7/7/2000	QUP[c]	0.97	25	80	64	100	0	8	negative
7/14/2000	QUP	0.08	22	77	66	600	0	0	negative
7/21/2000	QUP	7.80	23	80	63	300	0	4	**1 water & 1 soil +**
7/28/2000	QUP	6.81	20	73	61	220	40	5	negative
8/4/2000	QUP	5.46	25	77	66	140	0	5	**1 water +**
8/11/2000	QUP	3.68	26	87	67	110	50	2	negative
8/18/2000	QUP	4.65	22	75	61	0	0	6	**2 water & 1 soil +**
8/25/2000	QUP	0.58	23	79	57	80	10	4	**2 water +**
7/7/2000	WBSA[d]	0.97	28	80	64	150	0	2	negative
7/14/2000	WBSA	0.08	---	77	66	700	50	3	negative
7/21/2000	WBSA	7.80	24	80	63	130	30	3	negative
7/28/2000	WBSA	6.81	20	73	61	890	80	5	**1 water +**
8/4/2000	WBSA	5.46	23	77	66	1,000	40	5	negative
8/11/2000	WBSA	3.68	27	87	67	150	100	1	negative
8/18/2000	WBSA	4.65	21	75	61	10	100	4	negative
8/25/2000	WBSA	0.58	23	79	57	0	0	4	**1 water & 1 soil +**
7/7/2000	CP[e]	0.97	26	80	64	10	10	3	negative
7/14/2000	CP	0.08	26	77	66	130	0	2	negative
7/21/2000	CP	7.80	23	80	63	350	40	4	**1 water +**
7/28/2000	CP	6.81	20	73	61	620	60	4	**1 water +**
8/4/2000	CP	5.46	23	77	66	100	30	2	negative
8/11/2000	CP	3.68	24	87	67	480	100	0	negative
8/18/2000	CP	4.65	20	75	61	100	10	4	**1 water +**
8/25/2000	CP	0.58	24	79	57	80	0	6	**1 water +**

[a]Rainfall-recordings indicate rainfall for 1 wk preceding isolation , [b]CFU per 1ml water, [c]Quinnipiac University Pond, [d]Wharton Brook Swimming Area, [e]Clark's Pond (MacLean et al. 2003).

Table 2. Twelve of 59 thermotolerant isolates from water and 3 of 27 thermotolerant isolates from swab samples were positive for *N. fowleri* by nested PCR (Table 3, Fig 7). The PCR assay was repeated on 9 positive samples after 3 weeks of *in vitro* culture in liquid media to confirm the results of the previous PCR assay (Fig 8). All 9 samples remained positive for *N. fowleri* by PCR on repeat assay.

Samples were cultured continuously alternating with ATCC 802 medium and Page's ameba saline. After 9 months of continuous *in vitro* culture, the original PCR positive samples were analyzed by PCR for the presence of *N. fowleri*. One of 3 samples tested remained PCR positive for *N. fowleri* after 9 months of continuous culture (Fig 9A). Light microscopy (Fig 9B) indicated that *Acanthamoeba* trophozoites and cysts were more prevalent than limax-type amebae. Additionally, bacterial and fungal growth in the samples increased.

Western immunoblot analysis of samples from Connecticut confirmed the presence of *Acanthamoeba* spp. observed by light microscopy (Fig 10 and Fig 11A) as well as *Naegleria* amebae (Fig 11B).

PCR Analysis on Domestic Water from Arizona

Based on the results from Virginia and Connecticut, we determined that the nested PCR assay could be used to test environmental samples. Two cases of PAM were reported in Arizona in 2002. In order to determine the source of the infection, nineteen samples were collected from domestic water sources in Arizona and were labeled and transported to

Table 3. Summary of PCR Analysis on Swab and Water Samples from Connecticut.

Sample [a]	# Tested	# Positive by PCR
Water	59	12
Soil and Rock	27	3
Total	86	15

[a] Water and soil samples were collected and plated on non-nutrient agar-*E. coli* plates. Samples with amebae were placed in PAGE ameba saline for 24 h and harvested by centrifugation. The pellet was subjected to nested PCR analysis (MacLean et al. 2003).

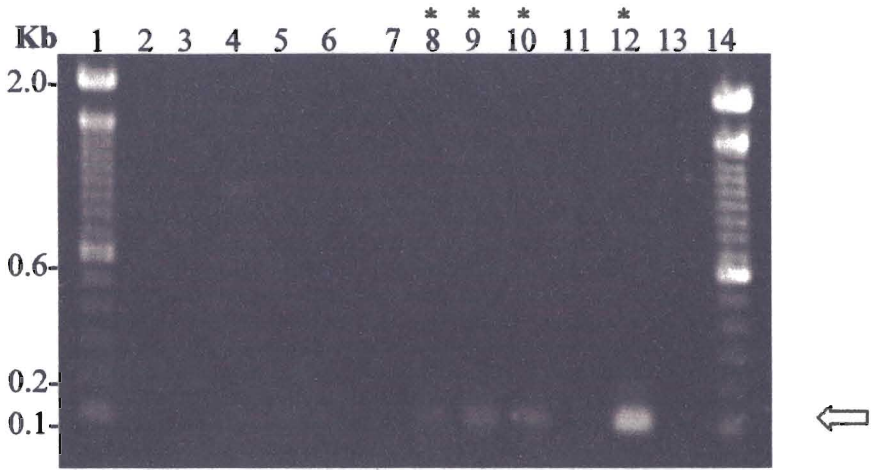

Figure 7. Nested PCR analysis on water and soil samples from Connecticut. Samples maintained on NNA-*Escherichia coli* plates were placed in liquid medium 12 months after isolation. Nested PCR products were detected on a 1.5 % agarose gel stained with EtBr. Three of ten samples tested were positive by PCR for *N. fowleri*. Lanes (1 and 14) 100 bp ladder; (2) S CP-8a; (3) W QU-14b; (4) W WB-13a; (5) S QU-16c; (6) W QU-6b; (7) W CP-15c; (8) W QU-5a; (9) W QU-14c; (10) W QU-16a; (11) W WB-7a; (12) positive control, Mp2Cl5 plasmid; (13) negative control. *Positive for *N. fowleri*. S-soil, W-water, CP-Clark's Pond, QU-Quinnipiac University Pond, WB-Wharton Brook Swimming Area. Arrow indicates 110bp PCR product (MacLean et al. 2004).

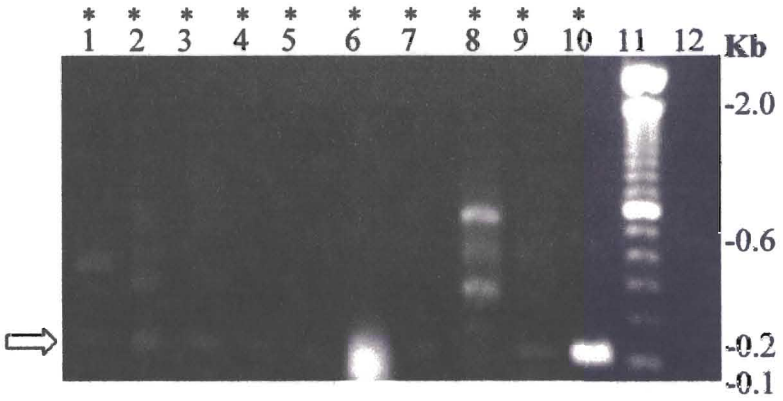

Figure 8. Repeat nested PCR on water and soil samples from Connecticut. Samples that were positive on initial PCR analysis were subjected to a repeat PCR assay after 3 wks of culture in liquid medium for confirmation of previous PCR results. Nested PCR products were detected on a 1.5 % agarose gel stained with EtBr. All samples previously positive by PCR were positive for *N. fowleri* on repeat analysis. Lane (1) W QU-5a; (2) W QU-14c; (3) W WB-8b; (4) W CP-8a; (5) S QU-6c; (6) W QU-9a; (7) W QU-13a; (8) W QU-16a; (9) W CP-5c; (10) positive control, Mp2Cl5 plasmid; (11) 100 bp DNA ladder; (12) negative control. *Positive for *N. fowleri* by PCR analysis. Arrow indicates 110bp PCR product. S-soil, W-water, CP-Clark's Pond, QU-Quinnipiac University Pond, WB-Wharton Brook Swimming Area (MacLean et al. 2004).

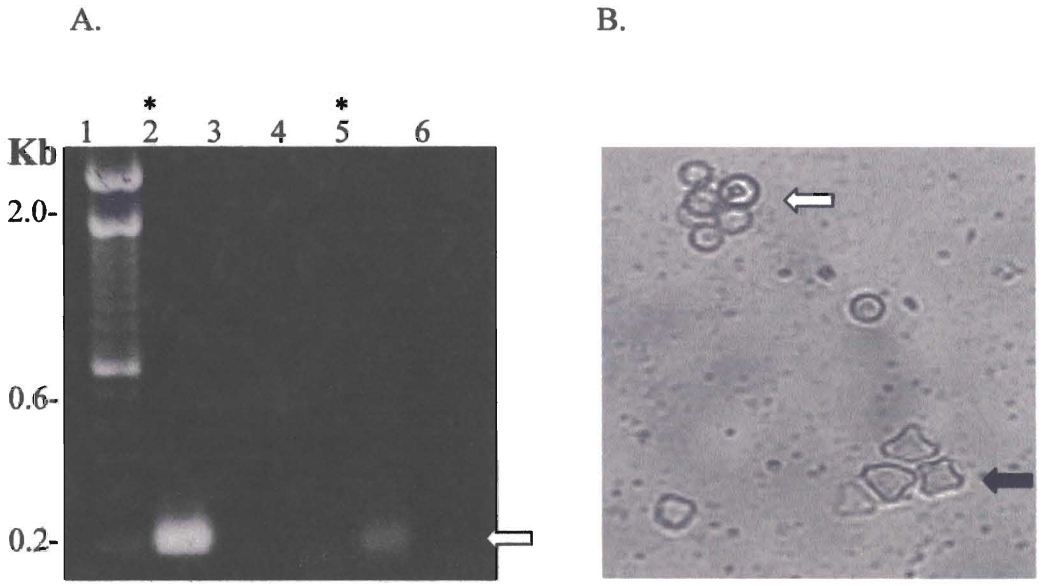

Figure 9. PCR and light microscopy of samples after 9 months of in vitro culture. Environmental samples from Connecticut were cultured in ATCC 802 medium and Page's ameba saline for 9 months. (A) Nested PCR was performed on 3 samples that were positive on the initial analysis. Nested PCR products were detected on a 1.5 % agarose gel stained with EtBr. One of three samples remained PCR positive. Lane (1) 100 bp ladder; (2) positive control, Mp2Cl5 plasmid; (3) negative control; (4) S QU-6c; (5) W CP-5c; (6) W QU-13b. * Samples positive by PCR analysis for *N. fowleri*. (B) Light microscopy was used to visualize samples at 400X. Closed arrows indicate *Acanthamoeba* cysts and open arrows indicate round cysts of unknown identity. S-soil, W-water, CP-Clark's Pond, QU-Quinnipiac University Pond, WB-Wharton Brook Swimming Area (MacLean et al. 2004).

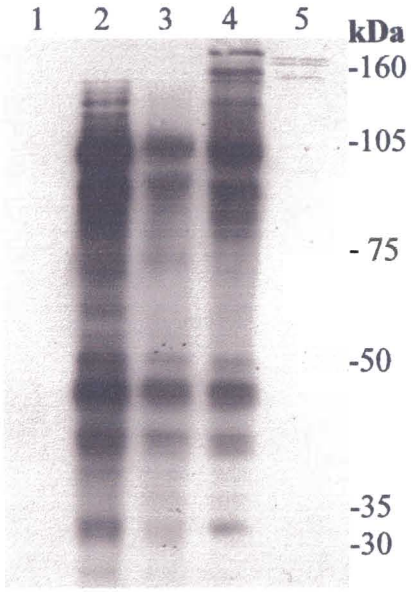

Figure 10: Western blot probed with Anti-*Acanthamoeba* antibody. Whole cell lysates of environmental samples from Connecticut were electrophoresed by 12 % SDS-PAGE. Separated proteins were transferred to nitrocellulose. Blot was incubated with a rabbit polyclonal antiserum against a whole cell lysate of *Acanthamoeba* absorbed twice on heat-inactivated *E. coli* (1:5000) followed by peroxidase-conjugated goat anti-rabbit IgG (1:10,000). Blots were developed using enhanced chemiluminescence (ECL). Lane (1) *Naegleria fowleri* (ATCC 30894) WCL; (2) S QU-6c; (3) W CP-5c; (4) W CP-15a; (5) Richmond, VA sample. Lane 1 containing a *N. fowleri* whole cell lysate was negative and lane 5 containing amebae from Richmond, VA were negative.

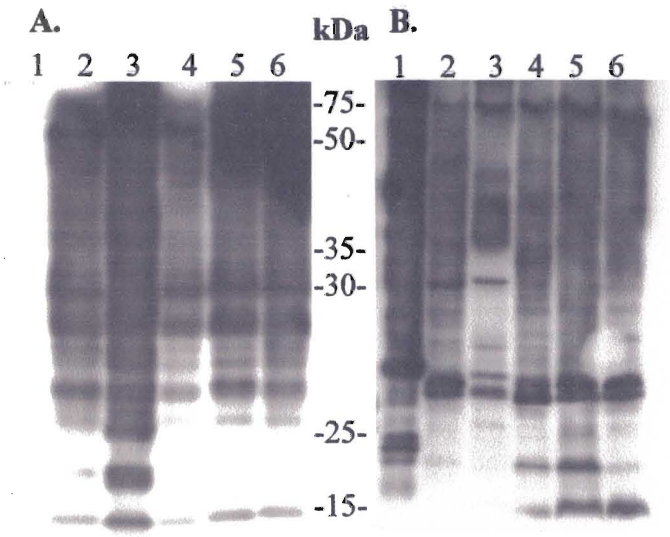

Figure. 11: Western Blot probed with polyclonal Anti-*Acanthamoeba* and polyclonal Anti-*Naegleria fowleri* antibodies. A whole cell lysate (WCL) of *N. fowleri* (ATCC 30894) amebae and environmental samples were electrophoresed by 12 % SDS-PAGE. Separated proteins were transferred to nitrocellulose. (A) Blot was incubated with a rabbit polyclonal antiserum against a whole cell lysate of *Acanthamoeba* absorbed twice on heat-inactivated *E. coli* (1:1000) followed by peroxidase-conjugated goat anti-rabbit IgG (1:10,000). (B) Blot was incubated with a rabbit polyclonal antiserum against a whole cell lysate of *N. fowleri* absorbed twice on heat-inactivated *E. coli* (1:500) followed by peroxidase-conjugated goat anti-rabbit IgG (1:10,000). Blots were developed using enhanced chemiluminescence (ECL). Lane (1) *N. fowleri* WCL; (2) S QU-6c; (3) W QU-13b; (4) W CP-14b; (5) W CP-5c; (6) W CP-14a.

to the laboratory (Table 4). Samples were dispensed into tissue culture flasks or placed on non-nutrient agar *E. coli* plates. Samples were observed daily for the presence of trophozoites or cysts. At 44°C, thermotolerant amoebae were observed, but many were encysted after 72 h. Eleven samples, numbered 2, 3, 4, 5, 11, 12, 13, 14, 15, 16, and 17, maintained for 4 days in Page's amoeba saline at 37°C, were tested by nested PCR. Of the 11 samples initially subjected to PCR, 4 were positive and 7 were negative for *N. fowleri* (Fig 12). The PCR product from sample 4, which was PCR positive for *N. fowleri*, was cloned and sequenced to confirm the identity of the product. Sequencing of the PCR product confirmed the amplification of the *N. fowleri Mp2Cl5* gene to 99 % identity.

Samples 3, 4, and 16, which were PCR positive, were subjected to a test for flagellates because of the number of amebae seen by visual observation after 4 days of culture at 37 °C. Transformation of amebae to flagellates was observed 30 minutes after the cultures were switched from ATCC medium 802 to water and were maintained in a shaker incubator. Flagellates were identified in cultures by use of an inverted light microscope. The PCR assay was repeated with samples collected after 1 week in culture in liquid medium to determine whether culturing in liquid medium at 37 °C would improve detection of *N. fowleri*, since only 10 ml of each original sample was analyzed. Figure 13A demonstrates that culturing the samples for 1 week in liquid medium at 37 °C resulted in additional positive cultures. Samples 11, 12, 13, and 14, which were initially negative by PCR, were positive after culture in ATCC medium 802. To confirm the results of the

Table 4. Identification of *Naegleria fowleri* by PCR in Water and Swipe Samples Collected from Sink Traps from the Homes of Two Children Who Died from PAM and the Home of an Adjacent Neighbor in Arizona[a].

Sample source	Assigned sample number[b]	PCR[d]	No. positive[e] / No. of Assays
Kitchen sink trap swipe	1	-	0/2
Master bathroom sink trap swipe	2	+	3/5
Master bathroom sink trap swipe	3	+	5/5
Guest bathroom sink trap swipe	4	+	3/5
Guest bathroom sink trap swipe	5	+	1/3
Guest bathroom residual sink water	6	+	4/5
Garbage disposal swipe	7	-	0/3
Bathroom sink trap swipe	11	+	2/6
Bathroom sink residual water	12	+	3/6
Double sink (bedroom) trap swipe	13	+	2/6
Double sink (bedroom) residual water	14	+	2/6
Double sink (bedroom) swipe	15	+	4/6
Double sink (bedroom) residual water	16	+	7/7
Kitchen sink swipe	17	+	3/5
Kitchen sink R.O. water	18	+	2/3
Kitchen R.O. water	19	+	1/4
Guest bathroom sink	20	+	1/5
Soil exposed to continuous water leak	23	+	2/4
WyndII filter[c]	24	+	5/5

[a]Samples were collected from domestic sites and placed in Page's ameba saline.
[b] Domestic samples-Patient one: 1-7; Patient two: 13-20, 23; Neighbor's home: 11-12.
[c]WyndII filter -water collected from both bath tubs and passed through a 1μm polypropylene filter.
[d]A nested PCR assay described in Materials and Methods was performed on each sample.
[e]Samples were tested multiple times during a 3 month period after culture at 37 °C in ATCC 802 medium alternating with Page's ameba saline (Marciano-Cabral et al. 2004).

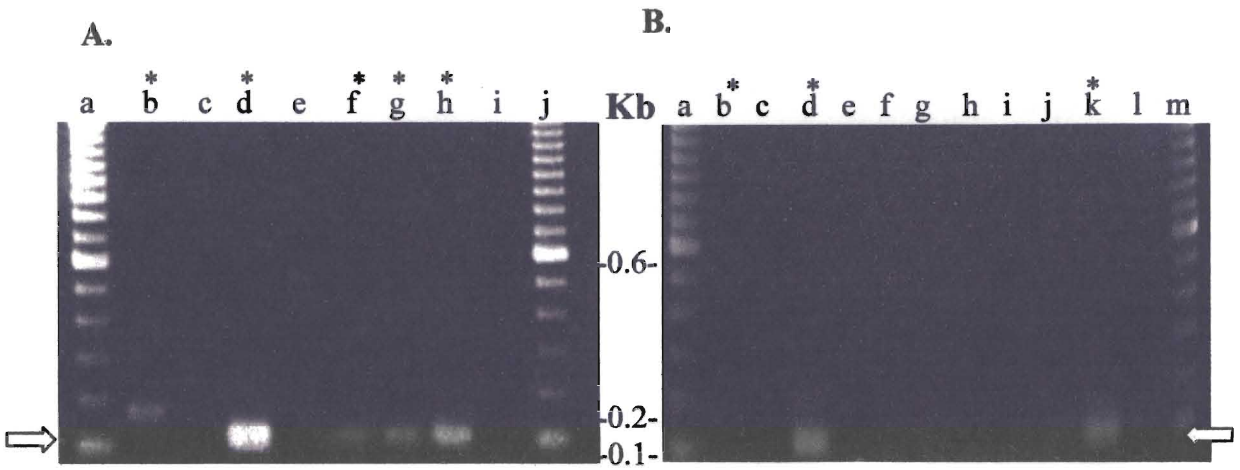

Figure 12. Nested PCR assay performed on domestic samples after culture at 37 °C for 4 days. Samples were maintained in tissue culture flasks for 4 days at 37 °C. Cultures were harvested with a cell scraper and centrifuged to obtain a pellet and supernatant. The pellet was used for nested PCR and the supernatant was discarded. Nested PCR products were demonstrated on a 3:1 Nusieve gel. (A) Lanes (a) and (j) 100 base pair ladder; (b) positive control-first PCR reaction; (c) negative control-first PCR reaction; (d) positive control-second PCR reaction; (e) negative control-second PCR reaction; (f) sample 2; (g) sample 3; (h) sample 4; (i) sample 5. (B) Nested PCR products were demonstrated on a 1.5 % agarose gel. Lanes (a) and (m) 100bp ladder; (b) positive control-first PCR reaction; (c) negative control-first PCR reaction; (d) positive control-second PCR reaction; (e) negative control-second PCR reaction; (f) sample 11; (g) sample 12; (h) sample 13; (i) sample 14; (j) sample 15; (k) sample 16; (l) sample 17. * Samples positive by PCR for *N. fowleri*. Arrows indicate 110bp PCR products (Marciano-Cabral et al. 2003).

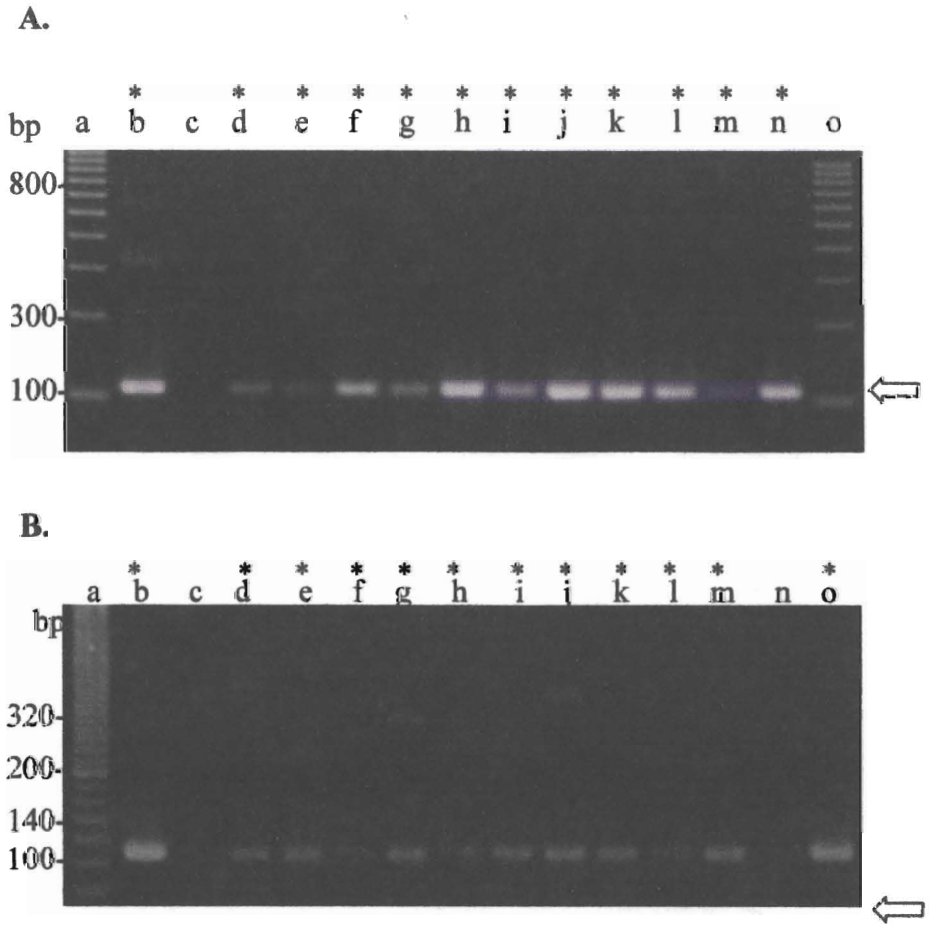

Figure 13. PCR analysis on samples from the domestic water supply cultured for one week or one month at 37 °C. The samples in tissue culture flasks were harvested after one week (A) or one month (B) after culture at 37 °C with a sterile cell scraper and centrifuged to obtain a supernatant and pellet. The pellet was used for nested PCR. PCR products were demonstrated on a 3:1 Nusieve gel. (A) Lanes (a) (o) 100 base pair ladder; (b) positive control; (c) negative control; (d) sample 11; (e) sample 12; (f) sample 13; (g) sample 14; (h) sample 15; (i) sample 16; (j) sample 17; (k) sample 18; (l) sample 19; (m) sample 20; (n) sample 23. (B) Samples for PCR were prepared after one month in continuous culture. Lanes (a) 20 base pair ladder; (b) positive control; (c) negative control; (d) sample 2; (e) sample 3; (f) sample 4; (g) sample 6; (h) sample 11; (i) sample 12; (j) sample 14; (k) sample 15; (l) sample 16; (m) sample 17; (n) sample 20; (o) sample 24. All domestic samples tested positive after one month with the exception of sample 20. * Samples positive for *N. fowleri* by PCR analysis (Marciano-Cabral et al, 2004).

previous PCR assays, samples maintained in liquid medium at 37 °C, which were originally positive, were retested within 1 month of receipt. Samples were cultured continuously, with growth medium alternating between ATCC medium 802 and Page's ameba saline. All samples remained positive by PCR on repeat assay with the exception of sample 20 (Fig 13B). Sample 20 was positive by PCR only one out of five times tested. This sample was negative by Western immunoblot analysis using polyclonal anti-*N. fowleri* antiserum (Fig 14A). The Micro Wynd filter was used for samples in which water from bathtubs where both fatal cases occurred tested positive by the PCR assay (Fig 13B). Samples testing positive for *N. fowleri* by PCR are given in Table 1. All samples tested by PCR were maintained at 37 °C in tissue culture flasks. Samples 3, 16, and 24 were positive by PCR for at least five assays. Two samples, 1 and 7, were negative for *N. fowleri* by PCR throughout the 3-month testing period. Sample 1 was a swipe sample from the kitchen sink trap, and sample number 7 was obtained from the garbage disposal. Both negative samples were swipe samples, but residual water was not obtained from these areas. Samples maintained at 37 °C for 3 months in liquid culture were harvested for Western immunoblot analysis. Select samples were reacted with anti-*N. fowleri* antibody or anti-*Acanthamoeba* antibody. Using polyclonal anti-*N. fowleri* or anti-*Acanthamoeba* antisera, both *N. fowleri* and *Acanthamoeba* were detected (Fig 14A and C). Four of five samples analyzed by Western immunoblot demonstrated reactivity with monoclonal antibody 5D12, which is specific for *N. fowleri* (Reveiller et al. 2000; Fig 14B). The organisms in sample 7, which was negative for *N. fowleri* by PCR, were also below the detection limit for *N. fowleri* by

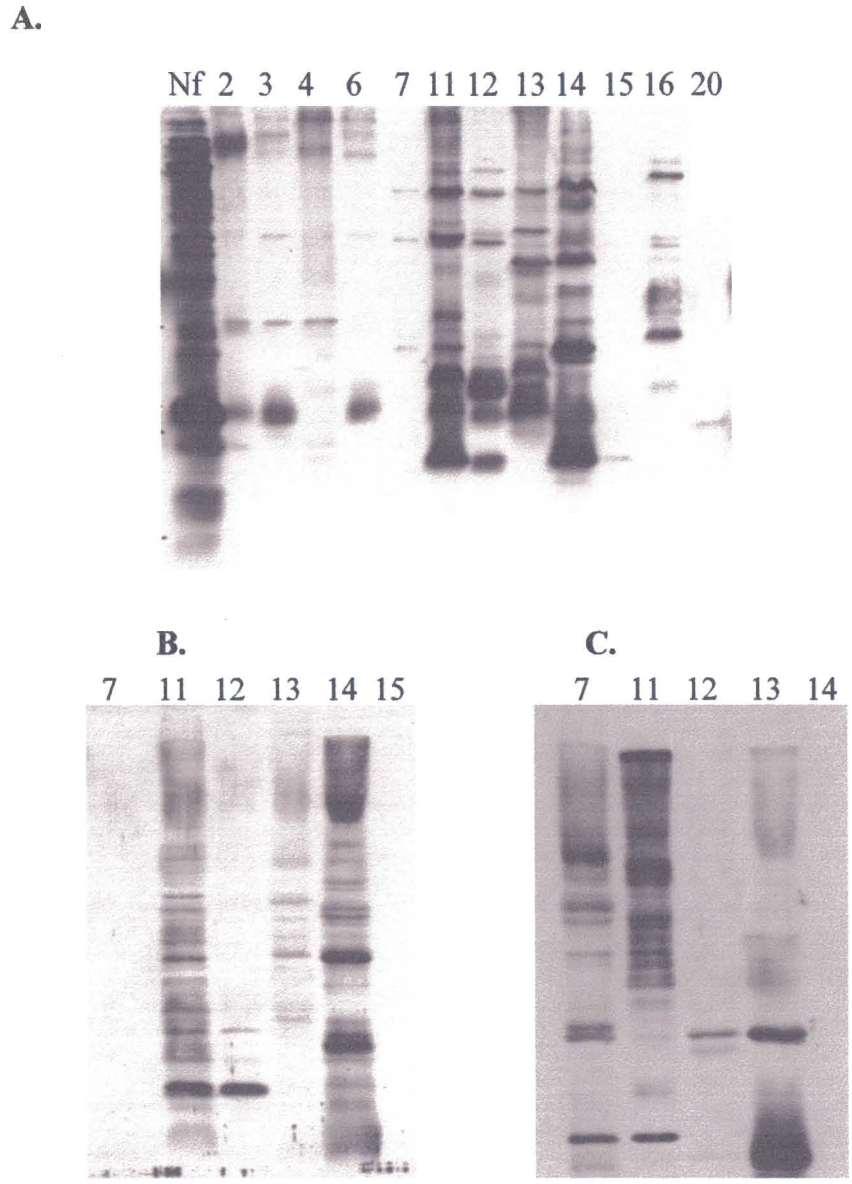

Figure 14. Western immunoblot analysis of domestic water samples from Arizona. Samples harvested at 1 month of culture at 37°C were subjected to SDS-PAGE and separated proteins were transferred to nitrocellulose membranes. The membranes were incubated in the primary antibodies rabbit polyclonal anti-*N. fowleri* (A), monoclonal 5D12 anti-*N. fowleri* (B), and rabbit polyclonal anti-*Acanthamoeba* (C). Secondary antibodies were goat anti-rabbit or rabbit anti-mouse antibodies. The blots were developed by using enhanced chemiluminescence (ECL). Lane Nf, a whole-cell lysate of *N. fowleri* used as a control. Other lanes are labeled with the sample numbers identified in Table 3 (Marciano-Cabral et al, 2004).

Western immunoblot analysis using polyclonal or monoclonal antibodies to *N. fowleri* (Fig 14A and B). However, sample 7 was positive for *Acanthamoeba* when anti-*Acanthamoeba* antiserum was used (Fig 14C). Visual observation by light microscopy confirmed that cultures contained limax-type amebae (Fig 15A) and *Acanthamoeba* cysts (Fig 15B).

PCR Analysis of Soil and Water Samples Collected from Oklahoma

Eighteen samples were collected in Tulsa, Oklahoma in 2005. The samples were transported to the laboratory within 24 hours of collection and placed into tissue culture flasks for culturing at 37 °C. Examination by light microscopy demonstrated the presence of cysts and amebae in a majority of the samples, both soil and water. The average pH of the water samples was 6.4, however, there was no correlation between pH and samples positive for *N. fowleri* (Table 5). Samples 2 and 3, which were obtained from grates that collected run-off in the "splash pad" area, were positive for *N. fowleri* by PCR analysis performed on samples after one week in culture (Fig 16A). Samples 16, obtained from one patient's home, and 18, obtained from Valley View Creek, were also positive for *N. fowleri* by PCR analysis performed on samples after one week in culture (Fig 16B). All other samples were negative. Select samples were retested by PCR analysis one week after culture at 37 °C. Sample 2, which was initially positive, was negative after one month in culture. Samples 16 and 18 remained positive. Sample 17, which was initially negative,

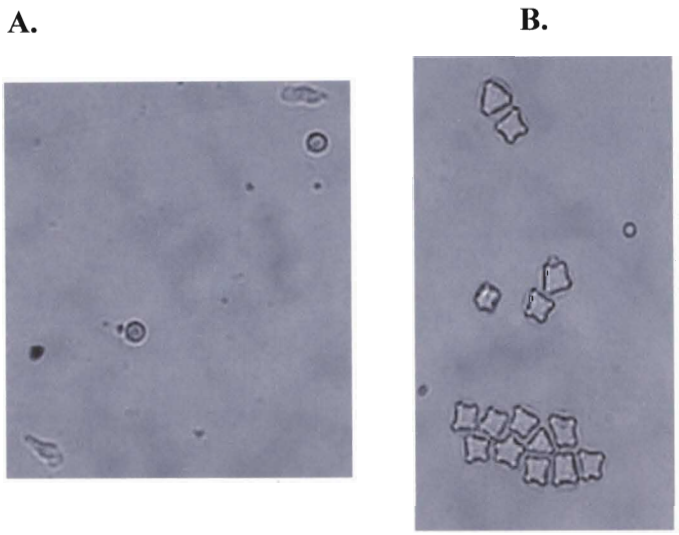

Figure 15. Cultures of domestic samples from Arizona were observed daily for the presence of trophozoites, cysts, or flagellates. Limax-type amebae were observed in Sample 16 which was positive by PCR for *N. fowleri* (A) and (B) *Acanthamoeba* cysts were observed in Sample 7 which was negative by PCR for *N. fowleri* (X400). (Marciano-Cabral et al, 2004).

Table 5. Water and Soil Samples were Collected in Oklahoma from Different Areas Including a Recreational Facility and Domestic Area Where Two Children Died of PAM.

Sample Source	Sample No.	Type[a]	pH	Cysts[b]	Amebae[c]	Results of PCR[d]
Area surrounding splash pad (L[e])	1	W	6.4	+	-	0/2
Grate 1 in Splash Pad area	2	W	6.0	+	-	1/3
Grate 2 in Splash Pad area	3	W	6.0	-	+	1/3
Area around splash pad (L)	4	S	NA	+	+	0/2
Area around splash pad[f] (L)	5	S	NA	+	+	0/2
Grassy area around splash pad (L)	6	S	NA	-	+	0/2
Area behind splash pad	7	W	6.6	+	+	0/2
Area behind splash pad	8	S	NA	+	-	0/2
Area around splash pad (R)	9	W	6.4	+	+	0/2
Creek behind splash pad	10	W	6.5	+	+	0/2
Area behind splash pad[g]	11	S	NA	+	+	0/2
Underneath the water slide	12	S	NA	+	+	1/2
Area under "whale"	13	S	NA	+	+	0/2
Area around "panda bear"	14	S	NA	+	+	0/2
Piece of mat from splash pad	15	R	NA	+	-	0/2
Patient 1 bathroom	16	W	6.8	-	-	3/3
Patient 2 bathroom	17	W	6.8	-	-	1/3
Valley View Creek	18	W	6.5	+	+	2/3

[a] "W" represents water samples, "S" represents soil samples and "R" represents rubber matting from splash pad.

[bc] Samples were observed by light microscopy for the presence of amebae or cysts after one week in culture.

[d] The top number shows the number of times the samples was tested by PCR. The bottom number shows the number of times the PCR was positive for *N. fowleri*.

[e] "L" represents a sample taken from the left side of the splash pad while "R" represents samples taken from the right side of the splash pad.

[f] Samples 4 and 5 are both from the left side of the splash pad. Sample 4 was taken from a muddy part of a pool of water and sample 5 was taken from a drier area.

[g] Samples 8 and 11 are both samples from behind the splash pad. Sample 8 was taken from behind the left side of the splash pad and sample 11 was taken from behind the right side of the splash pad.

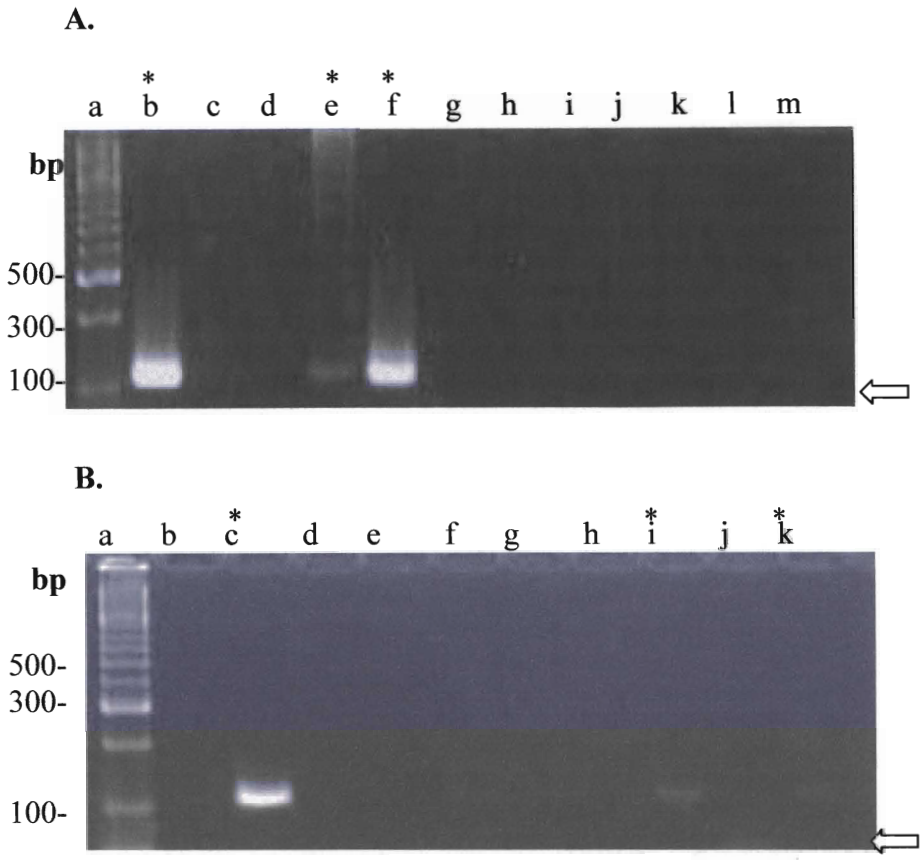

Figure 16. Samples collected from Oklahoma were cultured for one week at 37 °C and analyzed by PCR. Samples in tissue culture flasks were harvested with a sterile cell scraper and centrifuged to obtain a supernatant and pellet. The pellet was used for nested PCR. PCR products were demonstrated on a 3:1 Nusieve gel. (A) Lane (a) 100 base pair ladder; (b) positive control; (c) negative control; (d) sample 1 (e) sample 2; (f) sample 3; (g) sample 4; (h) sample 5; (i) sample 6; (j) sample 7; (k) sample 8; (l) sample 9; (m) sample 10. (B) Lane (a) 100 base pair ladder; (b) negative control; (c) positive control; (d) sample 11 (e) sample 12; (f) sample 13; (g) sample 14; (h) sample 15; (i) sample 16; (j) sample 17; (k) sample 18. * Samples positive by PCR analysis.

was obtained from the domestic water supply from the home of another patient and was positive after one week and after one month in culture, but was negative after two months in culture (Fig 18). Pictures obtained of sample 17 after one month in culture showed the presence of limax-type amebae (Fig 19), which may be a *Naegleria* species. Samples 16 and 18 remained positive after two months in culture (Fig 18B).

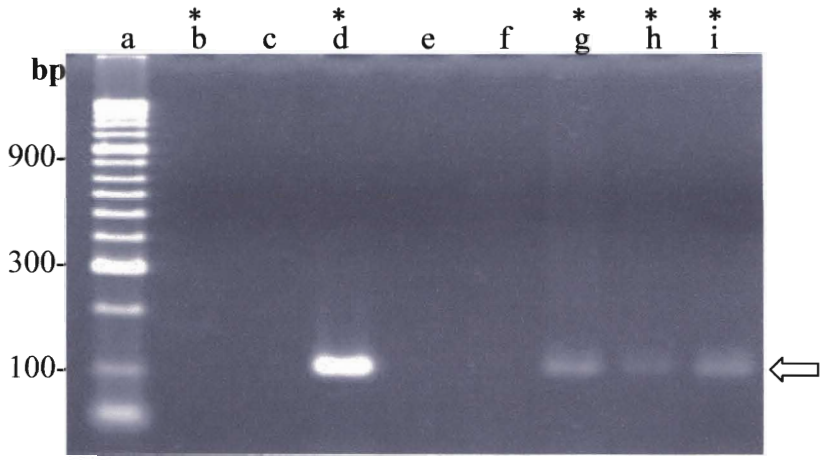

Figure 17. Water and soil samples collected from Oklahoma were cultured for one month at 37 °C and analyzed by PCR. Select samples cultured for one month at 37 °C were retested by PCR analysis. Samples in tissue culture flasks were harvested with a sterile cell scraper and centrifuged to obtain a supernatant and pellet. The pellet was used for nested PCR. PCR products were demonstrated on a 3:1 Nusieve gel. Lane (a) 100 base pair ladder; (b) positive control-first PCR reaction; (c) negative control-first PCR reaction; (d) positive control-second PCR reaction; (e) negative control-second PCR reaction; (f) sample 2 (g) sample 16; (h) sample 17; (i) sample 18. * Samples positive by PCR analysis.

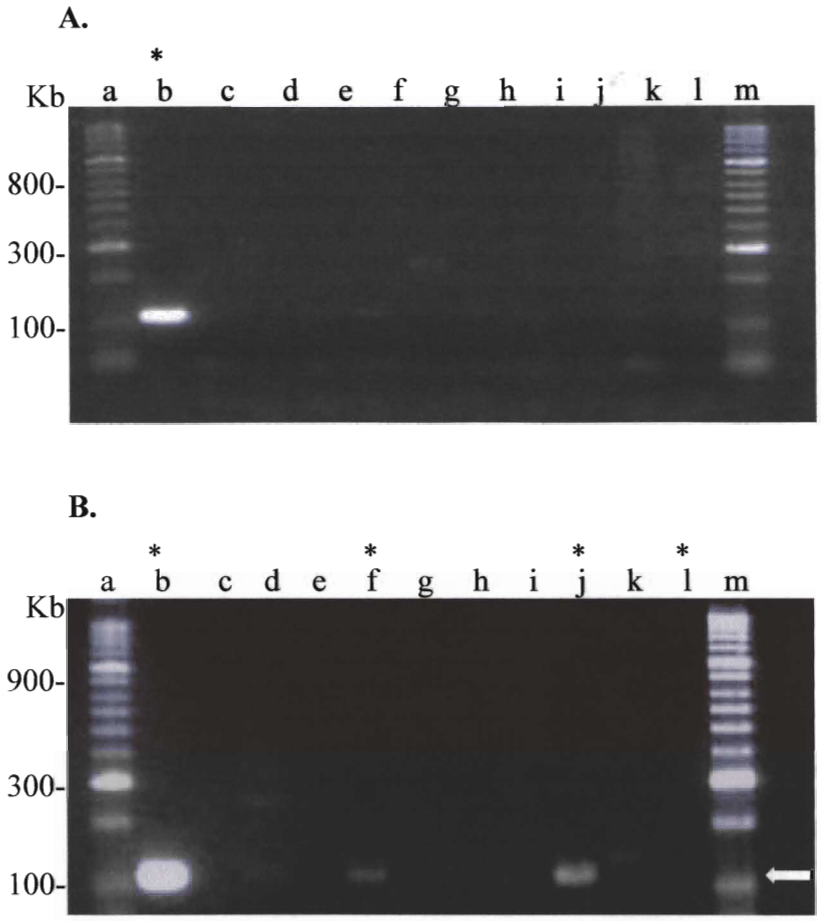

Figure 18. Samples collected from Oklahoma were cultured for two months at 37 °C and analyzed by PCR. Samples in tissue culture flasks were harvested with a sterile cell scraper and centrifuged to obtain a supernatant and pellet. The pellet was used for nested PCR. PCR products were demonstrated on a 3:1 Nusieve gel. (A) Lanes (a and m) 100 base pair ladder; (b) positive control; (c) negative control; (d) sample 1; (e) sample 2; (f) sample 3; (g) sample 4; (h) sample 5; (i) sample 6; (j) sample 7; (k) sample 8; (l) sample 9. (B) Lane (a and m) 100 base pair ladder; (b) positive control; (c) negative control; (d) sample 10; (e) sample 11; (f) sample 12; (g) sample 13; (h) sample 14; (i) sample 15; (j) sample 16; (k) sample 17; (l) sample 18. * Samples positive by PCR analysis.

A. B.

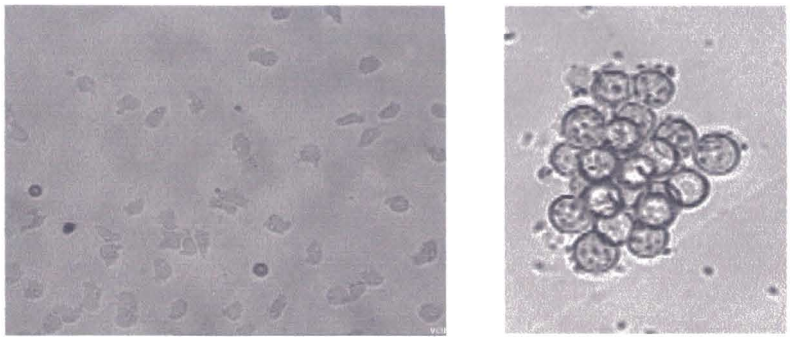

Figure 19. Cultures of samples from Oklahoma were observed daily for the presence of trophozoites, cysts, or flagellates. Limax-type amebae (A) and cysts (B) were observed in Sample 17 which was positive by PCR for *N. fowleri* (X400).

PART II: Observations of the presence of putative virulence factors in pathogenic *N. fowleri*

Southern Blot Analysis of *Naegleria sp.* for the Gene Encoding CD59

Southern blot analysis was used to determine whether a gene for the complement regulatory protein, CD59, was present in *N. fowleri*. gDNA was digested with restriction enzymes, electrophoresed, transferred to a nylon membrane, and incubated with a radiolabeled CD59 cDNA probe. Restriction enzyme digestion of *N. fowleri* LEE and LEEmp gDNA with *Eco*RI and *Hin*dIII showed a single band when hybridized with human CD59 cDNA (Fig 20). Restriction enzyme digestion with *Bam*HI showed two bands when hybridized with human CD59 cDNA (Fig 20). Hybridization of CD59 cDNA to *N. gruberi* gDNA was not observed.

Two-Dimensional Gel Electrophoresis and Immunoblot Analysis for CD59.

Two-dimensional gel analysis demonstrated that an anti-CD59 (BRIC229) antibody cross-reacted with a *N. fowleri* membrane protein at ~18kDa and an isoelectric point of ~8 (Fig 21A). In addition, an anti-Mp2C15 antibody also cross-reacted with a *N. fowleri* membrane protein at approximately the same molecular weight and pI (Fig 21B). Internal sequencing of the protein cross-reacting with the CD59 antibody was also performed. The

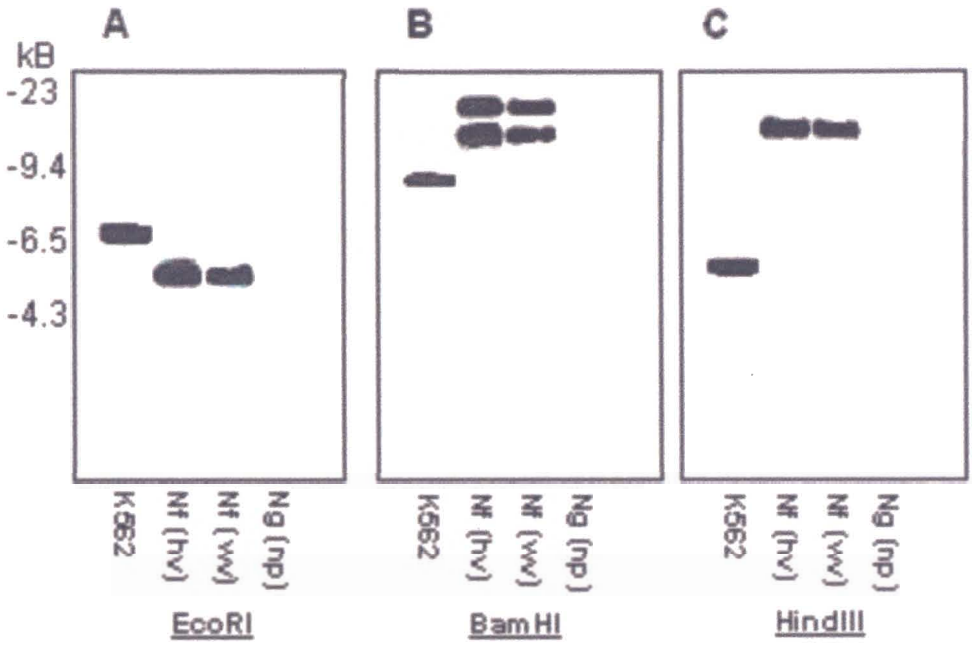

Figure 20. Southern blot analysis of pathogenic and nonpathogenic *Naegleria* genomic DNA (10 μg) hybridized with human CD59 cDNA. Genomic DNA was digested overnight with restriction enzymes EcoRI (A), BamHI (B), or HindIII (C), separated by gel electrophoresis, transferred to a nylon membrane, and hybridized with a radiolabeled human CD59 cDNA. Lane (1) K562 erythroleukemic cells, (2) highly virulent *N. fowleri* (Nf hv), (3) weakly virulent *N. fowleri* (Nf wv), (4) nonpathogenic *N. gruberi* (Ng). The human CD59 cDNA probe hybridized with the K562 control, highly virulent and weakly virulent *N. fowleri*, but not with nonpathogenic complement-sensitive *N. gruberi* (Fritzinger et al, 2006).

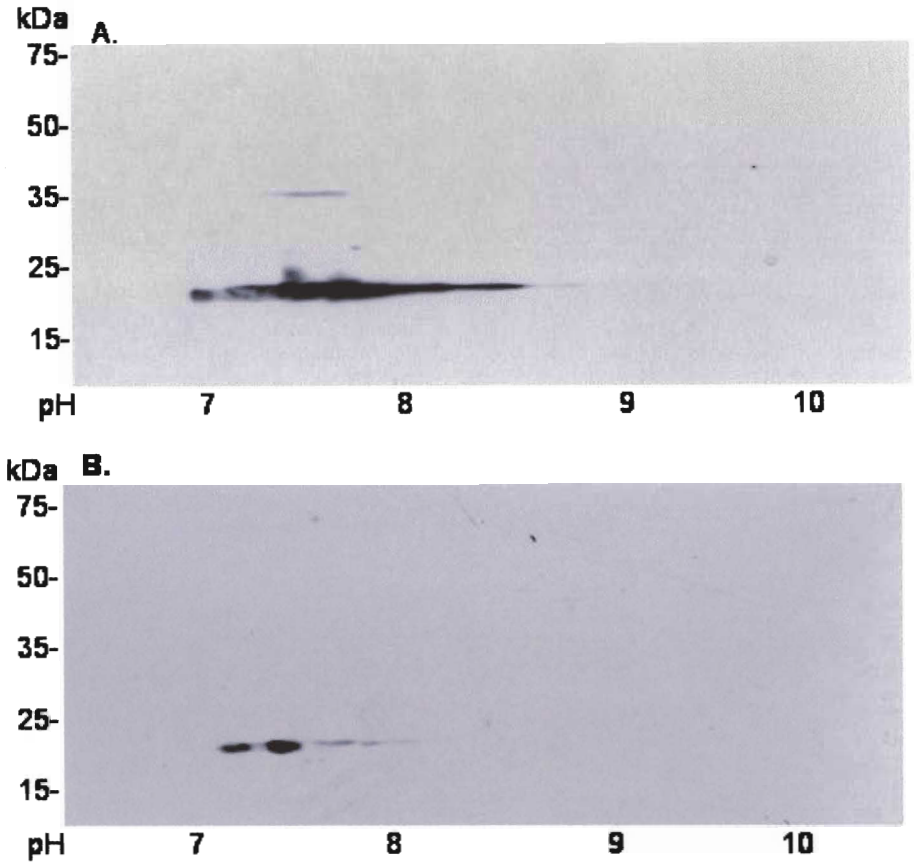

Figure 21. Two-dimensional analysis of membrane protein preparations of *N. fowleri*. *N. fowleri* membranes were prepared and separated by pH in the first dimension using IPG strips pH 7-10 and the Bio-Rad IEF separation system. Separation in the second dimension was by SDS-PAGE using the Bio-Rad Criterion system followed by transfer to nitrocellulose membrane. Membranes were incubated in (A) Mp2Cl5 polyclonal antibody (1:5000) and goat anti-rabbit HRP (1:15,000) secondary antibody or (B) CD59 monoclonal antibody (BRIC 229, 1:25) and rabbit anti-mouse (1:15,000) secondary antibody. Membranes were developed by enhanced chemiluminescence (ECL). Both antibodies reacted with proteins at approximately the same molecular weight and pH.

sequence analysis suggested that Mp2C15 was not the only protein present, but it was the only protein that matched sequences in the BLAST database.

Immunoprecipitation of the CD59 Protein

Immunoprecipitation analysis was performed using an anti-CD59 antibody (BRA-10G) cross-linked to protein G magnetic beads. Nonspecific cross-reactivity to the magnetic beads was not observed with irrelevant antibodies cross-linked to protein G magnetic beads including anti-tubulin (Fig 22A, lane 1) or anti-CD45 (Fig 22A, lane 5) by silver staining. Immunoblot analysis of immunoprecipitated proteins demonstrated cross-reactivity with both anti-CD59 (BRIC229) and anti-Mp2C15 antibodies at approximately 18kDa (Fig 22 B and C).

Immunoblot Analysis of Signal Proteins from *N. fowleri*

Signal proteins were extracted using a commercially available kit (G Biosciences), which isolates caveolin-enriched membrane proteins, glycosyl phosphitidylinositol (GPI) linked proteins, glycolipids, and GTP-binding proteins. These proteins are believed to be involved in signaling within the cell. Extracted proteins were separated by SDS-PAGE and immunoblotted. Analysis with both anti-CD59 (Fig 23A) and anti-Mp2C15 (Fig 23B) demonstrated that both proteins may be involved in signal transduction in *N. fowleri*.

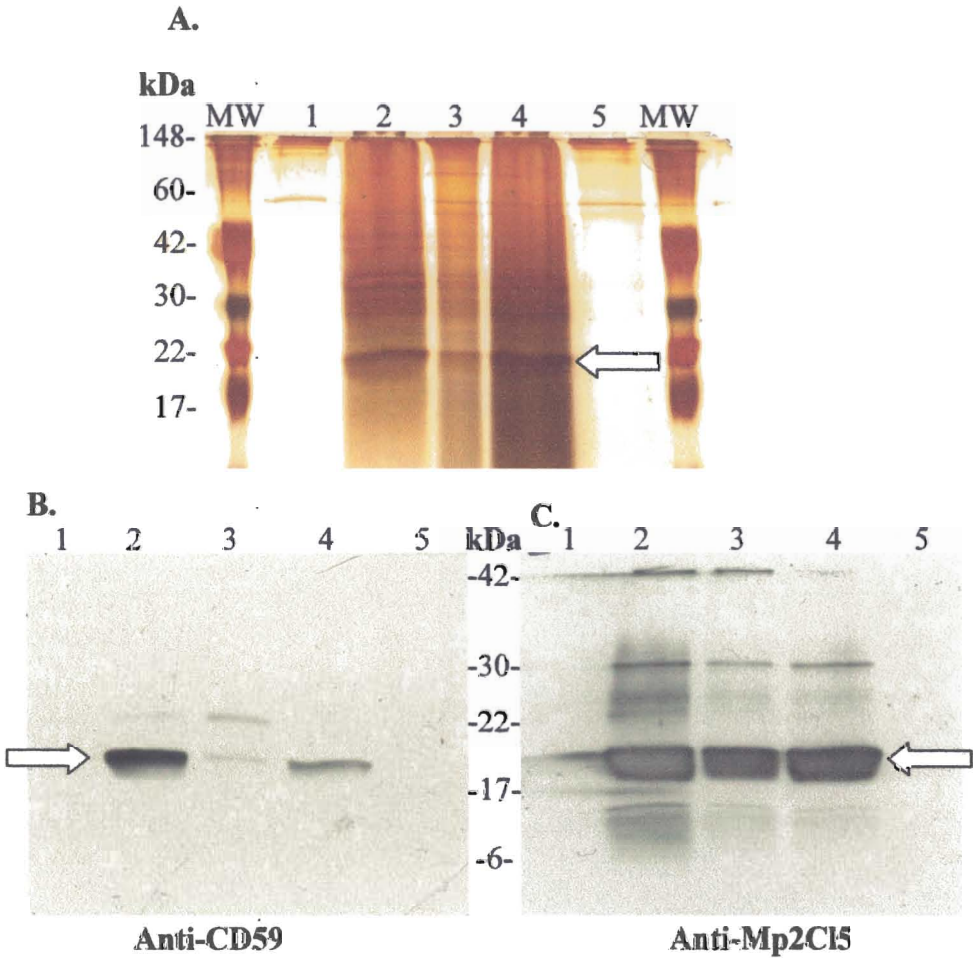

Figure 22. Immunoprecipitation of CD59 with BRA-10G (anti-CD59) antibody cross-linked to magnetic beads. Three different 100K membrane preparations of *N. fowleri* were incubated with BRA-10G monoclonal antibody cross-linked beads (2,3,4) overnight at 4 °C. As negative controls, membranes were incubated with anti-tubulin antibody (1), or anti-CD45 antibody (5) cross-linked beads overnight at 4 °C. The beads were washed 3X with immunoprecipitation buffer and eluted in 2X non-reducing lysis buffer. The eluate was separated by SDS-PAGE and (A) silver stained (B) incubated with an anti-CD59 BRIC229 (1:25) antibody. The membrane was stripped and reprobed with (C) anti-Mp2Cl5 antibody (1:5000). Both antibodies cross-reacted with ~18kDa proteins precipitated with anti-CD59 cross-linked beads. Arrows indicate the ~18kDa proteins.

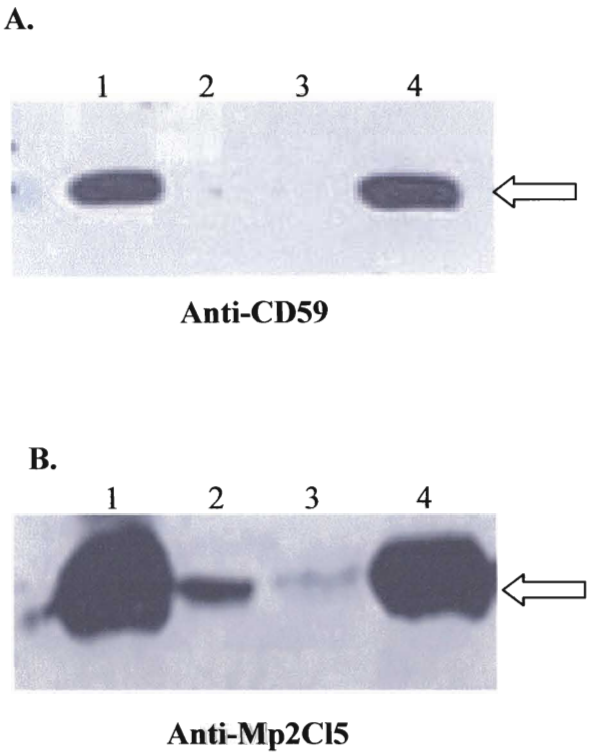

Figure 23. Isolation of signal proteins from *N. fowleri*. Signal proteins (caveolin-enriched membrane proteins, glycosyl phosphitidylinositol (GPI) linked proteins, glycolipids, and GTP-binding proteins) were isolated from *N. fowleri* amebae using the FOCUS signal proteins extraction kit (G Biosciences). Proteins were separated by SDS-PAGE and incubated with an (A) anti-CD59 BRIC229 (1:25) antibody or (B) anti-Mp2cl5 antibody. Lane (1) *N. fowleri* 100K preparation (+ control); (2) Hydrophilic portion 1; (3) Hydrophillic portion 2; (4) Signal proteins. Both antibodies cross-reacted with ~18kDa proteins, which are indicated by arrows.

Construction of a Genomic DNA Library and Mass Excision of pBLUESCRIPT Phagemids

In order to identify the gene encoding CD59, a *N. fowleri* genomic DNA library was constructed (Figure 1). Screening of this library with a cDNA encoding CD59 using Southern analysis was not successful, however sequencing of the library was performed and we identified many gene sequences in *N. fowleri* that have not been reported previously. In order to facilitate sequencing of the library, a mass excision of pBLUESCRIPT phagemids was performed (Fig 2). A total of 155 bacterial colonies were selected and minipreps were prepared for sequencing (Fig 24). Plasmids were sequenced using commercially available primers, KS and SK. Sequences were analyzed by performing translated BLAST searches. Further sequence analysis was performed on significant matches to proteins in the database by performing conserved domain searches on the BLAST website. Proteins with significant matches or conserved domains were grouped according to function. Predicted proteins included those for cell motility or chromosomal segregation (Table 6), gene regulation (Table 7), protein synthesis or degradation (Table 8), genes for protein regulation and cell signaling (Table 9A and B), genes for respiration and energy production (Table 10), genes for membrane synthesis and metabolism (Table 11), genes for the synthesis of amino acids and related molecules (Table 12), and genes with matches in the BLAST database, but have no known function in *N. fowleri* (Table 13A and B).

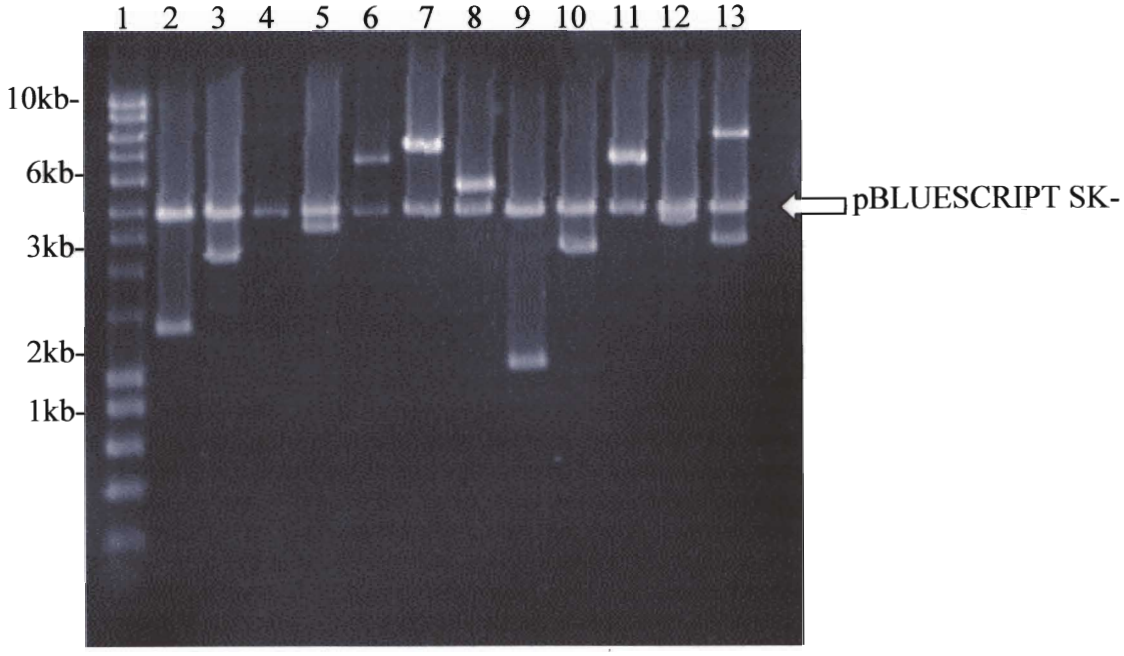

Figure 24. Representative gel of minipreps from gDNA library digested with EcoRI. Colonies were picked at random and grown overnight in LB-Amp. Minipreps were performed and 0.5μg was digested with EcoRI and run on at 1 % agarose gel and stained with Ethidium Bromide. Minipreps were sequenced using the KS and SK standard primers. Lanes (1)DNA ladder; (2) plasmid 63; (3) plasmid 64; (4) plasmid 65; (5) plasmid 66; (6) plasmid 67; (7) plasmid 68; (8) plasmid 69; (9) plasmid 70; (10) plasmid 71; (11) plasmid 72; (12) plasmid 73; (13) plasmid 74.

Table 6. Predicted Genes for Cell Motility and Chromosomal Segregation

Plasmid Number/Primer	Insert Size (kb)	Putative Protein	E value/Closest match	Putative conserved domains	Intron *
9/SK	6	Kinesin-like protein	4e-07/*Pan troglodytes* (chimpanzee)	Kinesin motor domain	N
21/KS and SK	1.5	Actinin	7e-20/*Drosophila melanogaster* (fruit fly)	SPEC, Spectrin repeats EF-hand, calcium binding motif	N
116/KS	3.5	Gelsolin (Actin-depolymerizing factor) (ADF)	7e-04/*Equus caballus* (horse)	GEL, Gelsolin homology domain Gelsolin repeat	N
132/KS	5	Truncated alpha-actinin	8e-05/*Rattus norvegicus* (Rat)	None	N
135/KS	1	Myosin heavy chain	9e-11/*Lethenteron japonicum* (arctic lamprey-fish)	Myosin motor domain	N
145/SK	4	Kinesin	2e-05/*Trypanosoma brucei* (blood flagellate)	SAM, Sterile alpha motif. Widespread domain in signaling and nuclear proteins	N
149/KS	5.5	Flagellar protofilament ribbon protein	3e-20/*Trypanosoma brucei*	RIB43A	N
149/SK		Actin	1e-07/*Oxytricha nova* (spirotrichous ciliate)	Actin	N
154/KS and SK	4	Kelch-like protein	2e-08/*Naegleria gruberi* (free-living amoeboflagellate)	None	N

*** "N" indicates there were no introns in the gene that was sequenced from plasmid DNA.**
"Y" indicates a predicted intron in the gene sequenced from plasmid DNA.

Table 7. Predicted Genes for Gene Regulation

Plasmid Number/Primer	Insert Size (kb)	Putative Protein	E value/Closest match	Putative conserved domains	Intron *
3 SK	6	Nse1 non-SMC component of SMC5-6 complex, putative	1e-04/*Oryza sativa* (rice)	None	N
8/SK	3	Sua5/YciO/YrdC/YwlC family protein	9e-37/*Tetrahymena thermophila* (ciliated protozoan)	YrdC-like domain	N
20/SK	4	ATP dependent RNA helicase	1e-08/*Homo sapiens* (humans)	None	N
34/KS and SK	1.5	Hypothetical protein DDB0183843	4e-28/*Dictyostelium discoideum* (slime mold)	DUS_like_FMN, Dihydrouridine synthase-like FMN-binding domain	N
46/SK	3	Chromatin organization domain modifier domain 8	5e-20/*Anopheles gambiae* (African malaria mosquito)	Chromatin organization domain modifier domain	N
50/KS and SK	1	Senescence-associated protein	1e-19/*Pisum sativum* (pea)	None	Y
68/SK	6	Histone deacetylase HDA110 isoform 1	2e-19/*Zea mays* (maize)	Histone deacetylase	N
69/KS	4	DEAD/DEXH helicase DDX31	1e-31/*Dictyostelium discoideum*	DEADc, DEAD-Box helicases	N
84/SK	5	RNA methyltransferase, TrmH family	5e-06/*Streptococcus agalactiae* (group B *Streptococcus*)	SpoU_methylase	N
90/KS	4	Ruv-B like DNA helicase	4e-17/*Trypanosoma brucei*	DNA helicase TIP49	N
107/KS and SK	0.6	Medea Transcription factor	1e-15/*Arabidopsis thaliana* (thale cress)	SET domain. Protein lysine methyltransferase enzyme that enhances zeste	N
109/SK	4	RNA dependent RNA polymerase family protein	8e-05/*Tetrahymena thermophila*	RNA-dependent RNA polymerases homolog 1	Y
139/KS	2.5	Modification methylase, HemK family protein	2e-08/*Synechococcus sp.* (cyanobacteria)	HemK, Methylase of polypeptide chain release factors	N

*** "N" indicates there were no introns in the gene that was sequenced from plasmid DNA. "Y" indicates a predicted intron in the gene sequenced from plasmid DNA.**

Table 8. Predicted Genes for Protein Synthesis and Degradation

Plasmid Number/Primer	Insert Size (kb)	Putative Protein	E value/Closest match	Putative conserved domains	Intron *
1/SK	5	Ribosomal protein S4	5e-59/*Naegleria gruberi*	None	Y
11/KS	2	Ribosomal protein L7/L12, putative	3e-20/*Plasmodium falciparum* (malaria parasite)	None	N
16/KS	4	Pyrrolidone carboxyl peptidase	5e-05/*Oryza sativa*	Peptidase_C15, Pytoglutamyl peptidase	N
26/SK	3	beta-1,4-mannosyltransferase	3e-07/*Dictyostelium discoideum*	None	N
38/KS	6	ribosomal protein S12	7e-33/*Naegleria gruberi*	Ribosomal_S12, Ribosomal protein	N
41/KS and SK	7	CG32379-PA	2e-15/*Drosophilia melanogaster*	Carboxy peptidase	N
57/KS and SK	1	Ubiquitin-protein ligase	3e-08/*Arabadopsis thaliana*	None	N
78/KS	1.75	Cysteine-tRNA ligase	6e-49/*Dictyostelium discoideum*	CysRS_core-responsible for the ATP-dependent formation of the enzyme bound aminoacyl-adenylate	N
111/SK	5	Putative 60S ribosomal protein L6	3e-27/*Oryza sativa*	Ribosomal protein L14E/L6E/L27E	N

*** "N" indicates there were no introns in the gene that was sequenced from plasmid DNA.**
"Y" indicates a predicted intron in the gene sequenced from plasmid DNA.

Table 9A. Predicted Genes for Protein Regulation and Cell Signaling

Plasmid Number/Primer	Insert Size (kb)	Putative Protein	E value/Closest match	Putative conserved domains	Intron *
9/KS	6	Fused-like protein kinase	2e-06/*Dictyostelium discoideum*	None	N
10/SK	2	Protein phosphatase 2A	3e-31/*Medicago sativa* (alfalfa)	Protein phosphatase 2A	Y
12/SK	3	putative protein kinase tousled	0.012/*Cabomba aquatica* (aquatic plant)	Serine/Threonine protein kinases	N
27/KS	3	dual-specificity protein phosphatase	5e-04/*Dictyostelium discoideum*	None	N
37/KS and SK	1	Heat shock protein 90	7e-46/*Toxoplasma gondii* (parasitic protozoa)	HSP90	N
43/KS	3	Serine/Threonine protein kinase	1e-11/ *Mycoplasma mobile* (gliding bacterium)	Serine/Threonine protein kinases, catalytic domain	N
43/SK		Hypothetical protein DDB0205716	1e-08/*Dictyostelium discoideum*	RhoGap, GTPase activator proteins	Y
45/KS	1.5	BCS1-like	7e-27/*Xenopus tropicalis* (western clawed frog)	ATPase family	N
76/KS	2	Dual-specificity protein phosphatase	8e-12/*Oryza sativa*	Dual-specificity phosphatase	N
76/SK		Putative protein Roco8	2e-05/*Dictyostelium discoideum*	None	N

*** "N" indicates there were no introns in the gene that was sequenced from plasmid DNA.**
"Y" indicates a predicted intron in the gene sequenced from plasmid DNA.

Table 9B. Predicted Genes for Protein Regulation and Cell Signaling

Plasmid Number/Primer	Insert Size (kb)	Putative Protein	E value/Closest match	Putative conserved domains	Intron *
86/KS	3	AGC family protein kinase	2e-07/*Dictyostelium discoideum*	Cytohesin Pleckstrin homology domain; Oxysterol binding protein; RAC-family serine/threonine-protein kinase	N
88/KS	3	MEK kinase	4e-04/ *Dictyostelium discoideum*	Serine/Threonine protein kinases, tyrosine kinase, catalytic domain	N
88/SK		Ras guanine nucleotide exchange factor	6e-31/ *Entamoeba histolytica* (anaerobic parasitic protozoan)	RasGEF	N
99/KS and SK	3	Ras related protein R-Ras	4e-05/*Rattus norvegicus*	Rab subfamily of small GTPases	N
101/SK	2.5	Serine/threonine protein kinase	0.008/*Rhodopirellula baltica* (marine bacterium)	Serine/Threonine protein kinases, catalytic domain	N
117/KS	4	Cell division control protein 48, aaa family	6e-06/*Pyrococcus furiosus* (hyperthermophilic archaebacterium)	1. ABC-type amino acid transport system, permease component 2. ATP-dependent 26S proteasome regulatory subunit 3. ATPases of the AAA+ class	N
118/KS and SK	1	Protein tyrosine phosphatase domain containing 1 protein	5e-29/*Homo sapiens*	Protein tyrosine phosphatases, dual-specificity phosphatases	N
121/KS	6	GTPase	6e-18/*Plasmodium falciparum*	Rab subfamily of small GTPases	Y
122/SK	1	Molecular chaperone heat shock protein	3e-13/*Rhizobium etli* (bacterial symbiont of plants)	GrpE, Molecular chaperone GrpE	N
135/SK	1	Hypothetical protein TTHERM_00155570	1e-05/*Tetrahymena thermophila*	Predicted GTPase	N
145/KS	4	Change to GH regulated TBC protein 1	7e-24/ *Mus musculus* (mouse)	TBC, Domain in Tre-2, BUB2p, and Cdc16p; Probable Rab-GAPs; GTPase-activating protein	N

*** "N" indicates there were no introns in the gene that was sequenced from plasmid DNA.**
"Y" indicates a predicted intron in the gene sequenced from plasmid DNA.

Table 10. Predicted Genes for Respiration and Energy Production

Plasmid Number/Primer	Insert Size (kb)	Putative Protein	E value/Closest match	Putative conserved domains	Intron *
1/KS	5	NADH dehydrogenase subunit 7	2e-125/*Naegleria gruberi*	Respiratory-chain NADH dehydrogenase	N
3/KS	6	Aconitate hydratase	6e-62/*Piromyces sp* (anaerobic fungus)	Mitochondrial aconitase	Y
25/SK	4	ATP synthase F1 subunit alpha chain	2e-76/ *Naegleria gruberi*	F1 ATP synthase alpha, central domain	N
38/KS	6	Cytochrome c oxidase (CcO)	8e-07 *Naegleria gruberi*	Cytochrome c oxidase (CcO) subunit I	N
38/SK	6	NADH dehydrogenase subunit 11 Succinate:cytochrome c oxidoreductase	5e-31/*Naegleria gruberi* 1e-47 *Naegleria gruberi*	NADH dehydrogenase Succinate dehydrogenase/fumarate reductase	N
70/KS and SK	1	Fe-hydrogenase assembly protein/small GTP binding protein domain	4e-42/*Chlamydomonas reinhardtii* (single-celled green algae)	None	N
102/SK	5	Hypothetical protein AN0346.2	4e-07/*Aspergillus nidulans* (opportunistic fungus)	Mitochondrial carrier protein	Y
125/KS	5	Cytochrome c oxidase subunit 3	2e-110/*Naegleria gruberi*	Cytochrome c oxidase subunit III	N
125/SK	5	NADH dehydrogenase subunit 11	2e-68/*Naegleria gruberi*	MopB_NADH-Q-OR-NuoG2	N
130/KS	5	ATP synthase F1 subunit alpha	5e-126/*Naegleria gruberi*	F1_ATPase_alpha, F1 ATP synthase alpha, central domain	N

*** "N" indicates there were no introns in the gene that was sequenced from plasmid DNA. "Y" indicates a predicted intron in the gene sequenced from plasmid DNA.**

Table 11. Predicted Genes for Membrane Synthesis and Metabolism

Plasmid Number/Primer	Insert Size (kb)	Putative Protein	E value/Closest match	Putative conserved domains	Intron *
5/KS and SK 5/SK	2.2	Patatin-like serine hydrolase	1e-68/*Aspergillus nidulans*	Patatin-like phospholipase	Y
7/SK	2.5	Alpha-methylacyl-CoA racemase	1e-31/*Polaromonas sp* JS666 (β-proteobacterium)	CoA_transf_3, CoA transferase family III,	N
23/KS	5	Putative cytochrome P450	8e-04/*Arabidopsis thaliana*	None	N
69/SK		similar to Lamin B receptor	3e-15/*Canis familiaris* (dog)	ERG4_ERG24, ergosterol biosynthesis	N
80KS	2	N-acyl-phosphatidyl-ethanolamine hydrolyzing phospholipase D	8e-15/*Bos Taurus* (cattle)	Zn-dependent hydrolases of the beta-lactamase fold	Y
90/SK	4	Acyl-CoA synthetase	9e-22/ *Entamoeba histolytica*	AMP-binding enzyme	N
110/KS	5	Squalene synthase	2e-05/*Botryococcus brauni* (green algae)	Squalene/ phytoene synthase	Y
129/SK	2.5	Phospholipase A2-activating protein	6e-06/*Danio rerio* (zebrafish)	None	N

*** "N" indicates there were no introns in the gene that was sequenced from plasmid DNA.**
"Y" indicates a predicted intron in the gene sequenced from plasmid DNA.

Table 12. Predicted Genes for Synthesis of Amino Acids and Related Molecules

Plasmid Number/Primer	Insert Size (kb)	Putative Protein	E value/Closest match	Putative conserved domains	Intron *
6/KS	2	Glutathione synthetase	2e-04/*Candida albicans* (opportunistic yeast)	None	N
45/SK	1.5	Phenylalanine hydroxylase	7e-40/*Rattus norvegicus*	Biopterin_H, PheA,	N
123/KS and SK	1	Dinucleotide-utilizing enzymes involved in molybdopterin and thiamine biosynthesis	1e-09/*Nitrosococcus oceani* (γ-proteobacterium)	None	Y

*** "N" indicates there were no introns in the gene that was sequenced from plasmid DNA. "Y" indicates a predicted intron in the gene sequenced from plasmid DNA.**

Table 13A. Predicted Genes with Unknown Functions in *N. fowleri*

Plasmid Number/Primer	Insert Size (kb)	Putative Protein	E value/Closest match	Putative conserved domains	Intron *
4/KS	2.2	Conserved Hypothetical protein	8e-12/*Solibacter usitatus* (eubacterium)	PqaA, PhoPQ-activated pathogenicity-related protein	N
7/KS	2.5	WDR23 protein	1e-20/*Homo sapiens*	WD40 domain	N
16/SK	4	Bromodomain and WD repeat domain containing 2	2e-07/ *Mus musculus*	WD 40 Domain	N
19/KS	5.5	Similar to GTPase activating Rap/RanGAP domain-like 1 isoform 1	3e-08/*Danio rerio*	None	N
24/KS	4	Hypothetical protein P9211_10507	4e-13/ *Prochlorococcus marinus* (cyanobacterium)	None	N
25/KS	4	Orf164-mitochondrial genome	2e-22/*Naegleria gruberi*	None	N
26/SK	3	Unnamed protein product	5e-07/*Mus musculus*	None	N
31/KS	6	Nodulin-like protein	1e-06/*Oryza sativa*	None	N
33/SK		Hypothetical protein LOC293059	3e-15/*Rattus norvegicus*	DUF167, YggU family	N
47/KS	4	Periplasmic phosphate-binding protein	3e-04/ *Methanocaldococcus jannaschii* (hyperthermophilic archaebacterium)	PstS, ABC-type phosphate transport system, periplasmic component	N
51/KS	2	Hypothetical protein P9211_10492	2e-29/*Prochlorococcus marinus*	None	N
52/KS	5	UV radiation resistance associated	7e-04/*Gallus gallus* (chicken)	None	N
53/SK	4	MGC68791 protein	8e-10/*Xenopus laevis* (African clawed frog)	None	N

*** "N" indicates there were no introns in the gene that was sequenced from plasmid DNA.**
"Y" indicates a predicted intron in the gene sequenced from plasmid DNA.

Table 13B. Predicted Genes with Unknown Functions in *N. fowleri*

Plasmid Number/Primer	Insert Size (kb)	Putative Protein	E value/Closest match	Putative conserved domains	Intron *
53/SK	4	MGC68791 protein	8e-10/*Xenopus laevis*	None	N
55/KS	4	Cell-wall associated hydrolase	8e-25/*Actinobacillus succinogen* (γ-proteobacteria)	None	N
63/KS	1.5	Lung seven transmembrane receptor	5e-13/*Arabadopsis thaliana*	Lung_7-TM-R	N
66/KS	2.5	Hypothetical protein DDB0218392	7e-29/*Dictyostelium discoideum*	None	N
82/KS	3	RHS Repeat family protein	3e-05/*Tetrahymena thermophila*	None	N
91/KS	6	Tuftelin interacting protein 11	6e-04/*Homo sapiens*	G-patch, RNA binding	N
98/KS	2.5	Hypothetical protein PY04656	7e-06/*Plasmodium yoelii yoelii*	None	N
120/KS	7	Putative protein	5e-26/*Magnetococcus sp.* (magnetotactic proteobacteria)	None	N
155/KS	3	Similar to ENSANGP0000 0011104	8e-05/*Apis mellifera* (honeybee)	COG3391, Uncharacterized conserved protein	N

*** "N" indicates there were no introns in the gene that was sequenced from plasmid DNA.**
"Y" indicates a predicted intron in the gene sequenced from plasmid DNA.

There were additional open reading frames that had no match in the BLAST database and may be unique to *N. fowleri*. These sequences were not included in any tables. A summary of all the groups of predicted proteins is shown in Table 14.

Primer Walking of Plasmid 5 to Obtain the Whole Sequence of the 2.2kb Insert

One of the predicted proteins from the sequencing of the genomic DNA library was a patatin-like phospholipase (Table 11) and was numbered "plasmid 5". Using KS and SK primers, we were not able to obtain the whole sequence of plasmid 5. In order to obtain the whole sequence, primer walking was performed with three primers designed to the sequence data obtained from the KS and SK primers (Fig 25). The whole sequence of the 2.2 kb insert was obtained by primer walking.

DNA Walking to Obtain the 3' End of the Patatin Gene

Analysis of the sequence of plasmid 5 revealed that the whole gene was not present and that the 3' end of the gene sequence was not known. In order to obtain the 3' end of the gene, DNA walking was performed using the DNA walking SpeedUp kit (Seegene, Rockville, Maryland). A PCR product (1.0 kb) was obtained in the second PCR reaction with the patatin specific primer 2 (TSP2) and DW-ACPN primer in the Seegene kit (Fig 26). The PCR product was purified with the Qiagen PCR purification kit and cloned into a TOPO 2.1 vector. Minipreps were performed and the cloned PCR product was sequenced. The sequence from the gene walking was aligned with plasmid 5 with

Table 14. Summary of Genomic DNA Library Sequencing. Predicted Proteins Were Grouped According to Function.

Function	Number of Predicted Genes
Cell Motility and Chromosome Segregation	9
Gene Regulation	13
Protein Synthesis and Degradation	9
Protein Regulation and Cell Signaling	21
Respiration and Energy Production	11
Membrane Synthesis and Metabolism	8
Synthesis of Amino Acids and Related Molecules	3
Unknown	21

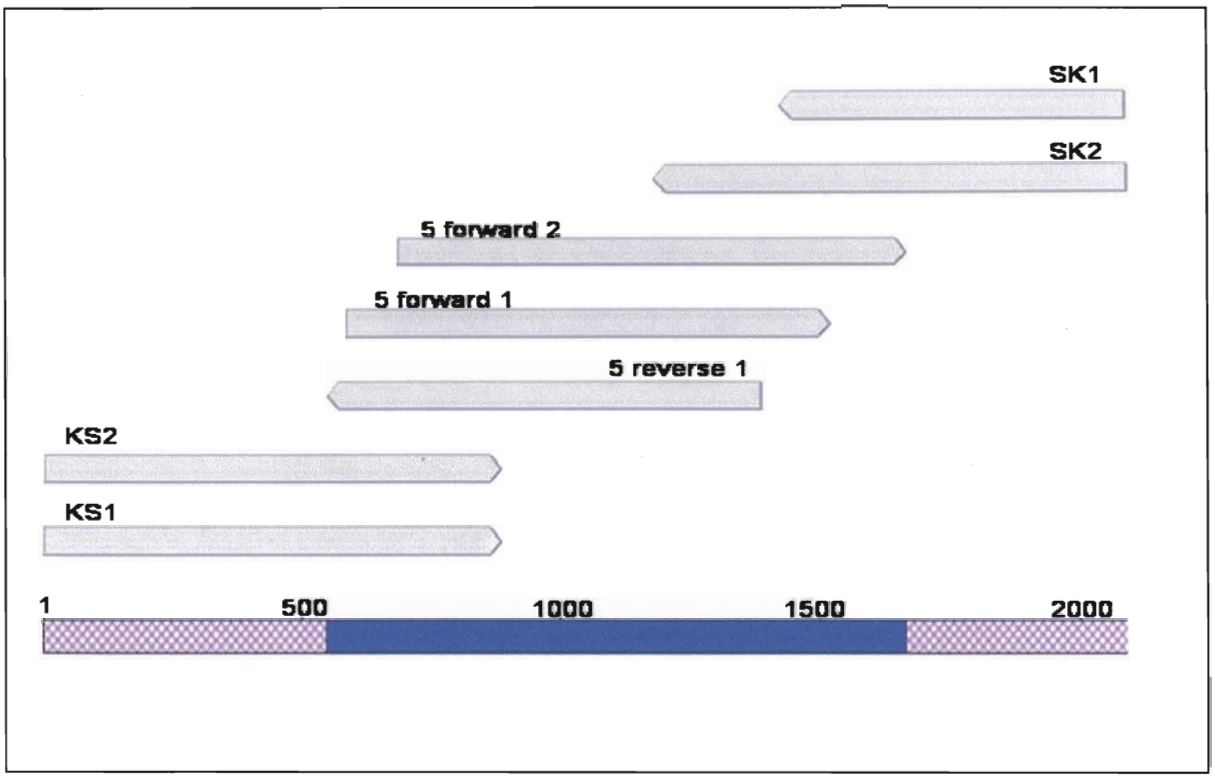

Figure 25. Primer walking strategy to obtain the whole sequence of plasmid 5. KS and SK universal primers were initially used to sequence the insert in plasmid 5, which contains a portion of the patatin gene. Sequencing of the whole insert was performed by repeating sequencing with KS and SK primers along with 3 other primers (5 forward 1, 5 forward 2, and 5 reverse 1) designed with vector NTI software. Block arrows denote sequence coverage using the primer named above the arrow. The bottom of the figure shows the probability of the predicted sequence as being correct based on the number of times the indicated area was sequenced. The blue box indicates areas where the insert was sequenced three or more times. The hatched purple box indicates areas where the insert was sequenced 2 times.

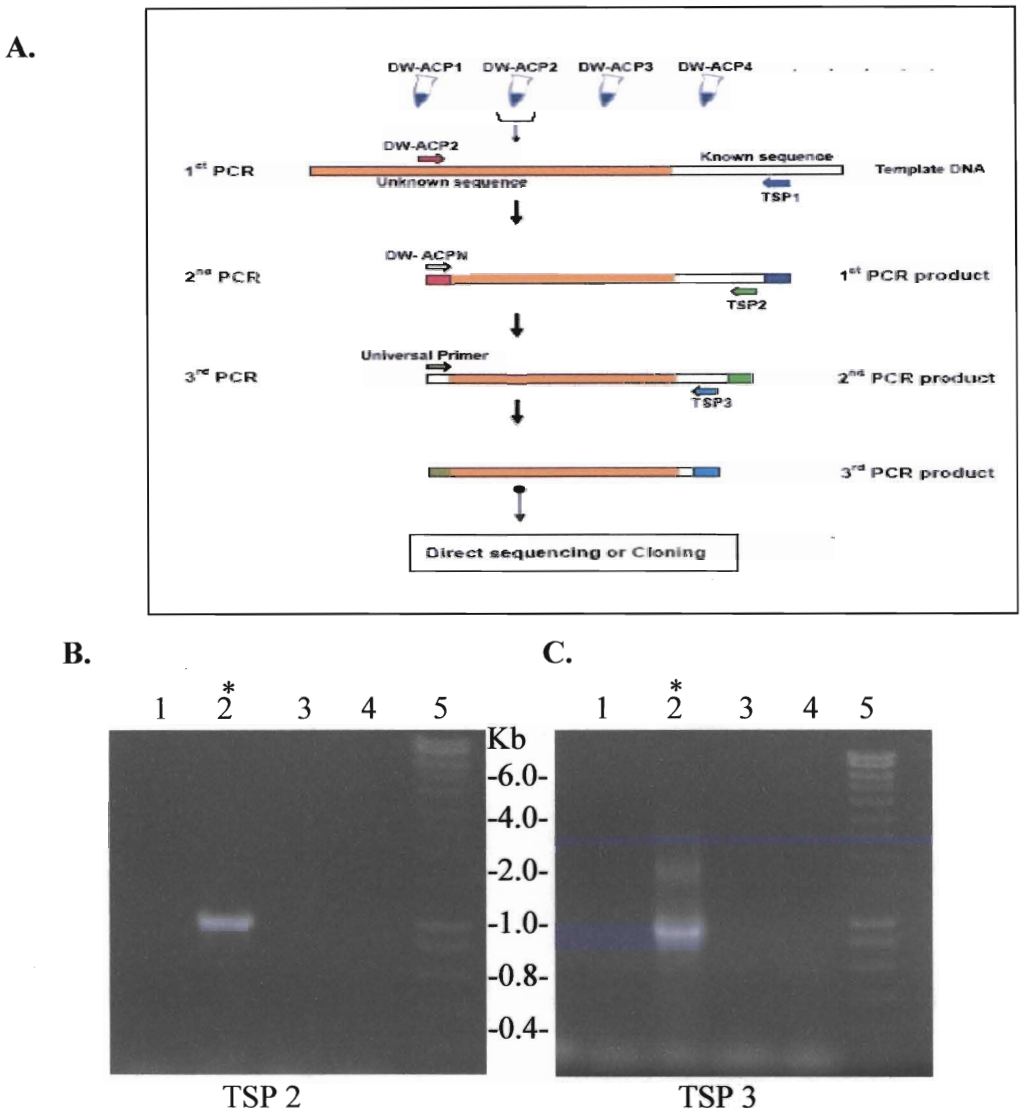

Figure 26. DNA walking of gene encoding the patatin-like protein. *N. fowleri* gDNA was isolated and PCR amplification was performed using the DNA Walking Speedup kit (Seegene). PCR was performed using DNA Walking-Annealing Control Primers (DW-ACP 1-4) and three nested target specific primers (TSP1-3) designed to the patatin sequence. PCR was performed on gDNA using DW-ACP1-4 commercial primers and TSP1 and no products were observed. (A) Diagram showing the general strategy of DNA walking (Seegene manual). PCR was performed on PCR products from the first reaction using the DW-ACPN primer and TSP2. (B) PCR was performed on the second PCR reaction using the universal primer and TSP3. Lanes (1) DNA ladder; (1) DW-ACP1; (2) DW-ACP2; (3) DW-ACP3; (4) DW-ACP4; (5) DNA ladder. Products from PCR analysis were cloned into the pCR2.1-TOPO vector and sequenced. *Products observed in PCR reactions.

Vector NTI software. A stop codon was obtained for the patatin-like phospholipase with gene walking.

PCR on cDNA, gDNA, and plasmid DNA using two different primer sets to confirm the locations of introns in the patatin-like gene sequence

PCR analysis was performed using cDNA to determine whether an intron was present and to determine the intron/exon junctions (Fig. 27). PCR products from the cDNA templates were compared to the PCR products from the gDNA templates to determine the splice sites. The first intron is 197 bp and does not have the canonical GT/AG donor acceptor sites. Instead, there is a 5' AG donor and a 3' TG acceptor. However, the intron does contain a polypyrimidine tract ($Y_6SY_2SY_6$). The second intron is 148 bp and contains the canonical GT donor and AG acceptor sites, but does not contain a polypyrimidine tract (Fig 28).

Translation of the coding sequence of the *N. fowleri* patatin-like protein

Translation of the coding sequence was performed using the ExPASy proteomics program "translate." The coding sequence is 2,391 base pairs and the protein sequence is 797 amino acids. Using the ExPASy proteomics program "compute pI/Mw," the predicted pI is 7.25 and the predicted molecular weight is 90.5 kDa (Fig 29).

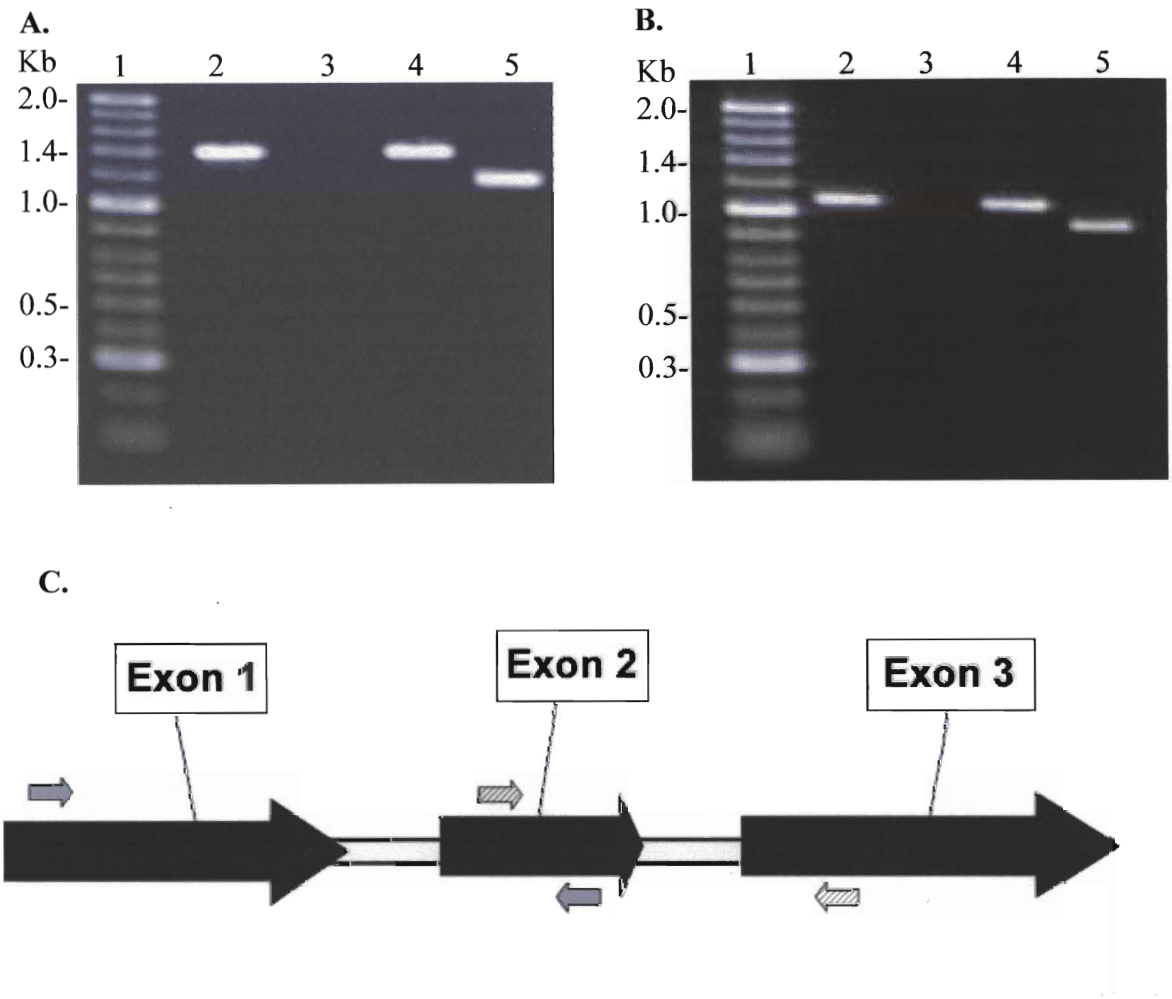

Figure 27. PCR on cDNA, gDNA, and plasmid DNA using two different primer sets to confirm the locations of introns in the patatin-like gene sequence. PCR was performed on plasmid DNA and *N. fowleri* cDNA and gDNA with primers patatin1.for and patatin1.rev spanning intron 1 (A) and primers patatin2.for and patatin2.rev spanning intron 2 (B). Lanes (1) DNA ladder; (2) plasmid DNA (+ control); (3) negative control; (4) gDNA; (5) cDNA. The PCR products were cloned into the pCR 2.1-TOPO vector and sequenced. (C) Diagram to show the predicted patatin gene exons (black arrows) and introns (bar) and to show the locations of PCR primers patatin1.for and patatin1.rev spanning intron 1 (gray arrows) and primers patatin2.for and patatin2.rev spanning intron 2 (hatched arrows) used to confirm intron/exon splice sites. Sequencing of PCR products from the cDNA templates were compared to the PCR products from the gDNA templates to determine the splice sites.

```
   1 ATGGCAACTG TCATATCACG AAGTGTCTTT GAGAGATCAC TCACTTCTCC TTCCACCACC
  61 ACCGCCACCA ATTCAAGTAC CATCAGTACT CCCAATGCAG TACTCTCTAA TTTCATACCT
 121 CATTACCATG ATGATGCGAA TGAAGGAGGA GGTGATTCAC ATGACTCACA ACAACGATCA
 181 TTACATTCTC ACCATGCTCA TCATCAACCA TGTCATGAGA GCAGCAATCA TAAGAGTACA
 241 AAACTAACGA CTCATTCTTT GGTTCCAACA ATACAACAAC AACATCAACC ACAAGAACCA
 301 CAACAAGAAC AACAACAACA ACCTCATCTA CTCGCATCAT CACCAACCAA CCACACCTCT
 361 TCTTCGATTC AACTCCCGCC CTCTACTCCC GCCAAAAAAC CCTTCTACAG GAGAGGCATT
 421 TTCAGAATCT TGGATGCCTT TTTCTTCCTC TTTCGAGTGA TACTCTTTCT ATTTAAATTC
 481 TTTTATTCTT ATATGAAATT AGTGAATCAT CAAAACAAAC AAAAACAACA TTGCAAAGTA
 541 TTATTAGAAG AGAAAGTAGA CAACTACAAT GATTACAAAC AAATTGCATT GTATTTGGAT
 601 CAATTAGAAG GTTATGATTT GTGGAAATCT GAAAATGAAA CCAAACTCTA TGACTTTGAA
 661 CAAATTGAAA ATATCAATTA CCAAATCATG GCCATGTTAA GAAAAAAAGA TATTCATGGA
 721 CTACAATGGT TGCTGAGAGC TGAATTACAT CGTAACATTG CTGGTATTAA TAATGTTCAA
 781 CTCTATGAAT GTCATACAGG TACTAAACAA CTCATTGAAG AATATATTGA TTTGGTATCC
 841 AAATCATGTC ATCTCATTCG AGAGAGTGAT GAATTGTCAT TGGAAGAGAA ATTGAAATTC
 901 TTTAGAGATA CTTGTCATGC CTTTGGAAGA AGTGCTTTAT TATTGAGTGG AGGAGGTGGA
 961 TTATCCATGT ATCATATTGG TGAGTATAAA TATATATAGA CACGATTGTA TGGATTGGAT
1021 GGACACGGTA ACATTATGAA CGATGACTAT TATTACTACT ACTATTCCTA CTATTCCTAC
1081 TATTATTACT ACTATAATTG GTATCTATTC ACTCACCCAC TCTCTCTCTT GTCACACACA
1141 CACCCCATCT ATCCCCACAC ACACACCCAC ACACAGGTGT TGTCAAATCT CTCTATGACT
1201 CTGGTATTTT ACCCAAAGTC ATTAGTGGTA GTAGTGGTGG TAGTATTGTG GCTGCAGTCT
1261 TGTGTACCAG ACGTGATGAA GATCTCTCAA AATGTTTTGA ACCTGGTTCT TTTAAATTAG
1321 AAGCATTTGG TGGTAGTGAT GATACTCCAG AGAGATCAGC CATGCGTAAA ATTAATCGAT
1381 TATTGAATAA TGGTGTGATT ATGGATGTGA ACAAGTTAGC ACAATGTATT CGTGAGAATA
1441 TTGGAGATTT AACATTTGAA GAGGCCTATA AAATTAGTGG AAGAGTATTA AATATAACAG
1501 TGAGTGGACA ATCTGCAGGA TATCAAACAC ATGAAGGTCT ATTGAATTAT TTAACTGCAC
1561 CTAATGTCTT GATTTGGAGT GCTGCAGTGG CAAGTTGTTC ATTACCAATG GTAAGTGTCC
1621 ACTGAGGACT TGTTTGACCT CAATAATAAT TATTCAATGA TGATGATGAT ATGATTTGTA
1681 TTATGGGTTG ACCTCAATGT ATTATTATAG CATGATGATG ATGAATGTAA ATGAATGTAC
1741 AATTCATTTC ATACACACAG CTCTACAAAG CAGTTCCTTT GATGGCCAAA GATCGAGATG
1801 GTAATATTGT ACCATATCAT AACTTTCCAA ATCAGAAATA TCAAGATGGT ACTCTCTTCT
1861 ATGATTTACC AATTACTCGT TTAGCAGAAT TATTTAATGT TAACTTCTAT ATTGCCTCTC
1921 AAGTGAACCC TCATGTACTT CCATTTATAT CCAATTCCAG AAAACAAAAG AATTCCGCAT
1981 TTTCCAATCT ACTTTCACTC GTATGCTCAG AAATCAAATA TCGAATTGAA CAATTATATT
2041 CTCTATCATT AATACCTGAG AGATTTCGAT GGTTTGAATT GGTCATTTCA CAATCGTATG
2101 AAGCTCATGT CACCATTGTA CCTTGTGATT TGAATTTTGA GAATATGAAA AAGATTCTCA
2161 GTAATCCAAC TGCTGAATAT ATCAATCAAG CAGTTCATCA AGGAATGAGA AGAACATTTC
2221 CTCATATGAA TCGAATTGAG AACATGTTAA AGATTGAATT GACATTGGAT TCATGTTTGA
2281 GACAAGTGAG ATTTCAATTA TTTAAATCAT CACCACAATT TGCCATTCAA ATGTCAAAAT
2341 CCTCTTCACT CTTTAATTTG GAGGAGGAAG ATGTCATTGA TCATGAAATT GTTTCATCCG
2401 ACCCGATAAT GAATTCAAAA CGTGGTGATA GTGGTAGTAC TACTACTACT ACTACTGCCA
2461 TGATGACATG CACCAATCCA CAACAACAAC ACTCTTCACA TCAACCCCAC CATTCACTTC
2521 AATATCATTC TCAACATGCT TCTCAACATC CTCCTTCTCC TTTGAGAATT TGTAGTAGTA
2581 GTACTGGTGG TGCCAATCAT CATCATCATC ACTCTCATCA TGCACACCAC CACAACAACA
2641 CCAGCCCTCG TCATTATCAC CCTTCTCATC ATGCACACCA CCACCAACAC TGCCAACACC
2701 ACCACGACCC CCCCCCCTCG CTTGGCATAC TTCTGTGA
```

Figure 28. Sequence of gene encoding the patatin-like protein. Introns are underlined and intron/exon donor and acceptor sites are boxed. There are two introns and three exons in the sequence.

```
   1 ATGGCAACTGTCATATCACGAAGTGTCTTTGAGAGATCACTCACTTCTCCTTCCACCACCACCGCCACCAATTCAAGTACCATCAGTACT
   1  M  A  T  V  I  S  R  S  V  F  E  R  S  L  T  S  P  S  T  T  T  A  T  N  S  S  T  I  S  T
  91 CCCAATGCAGTACTCTCTAATTTCATACCTCATTACCATGATGATGCGAATGAAGGAGGAGGTGATTCACATGACTCACAACAACGATCA
  31  P  N  A  V  L  S  N  F  I  P  H  Y  H  D  D  A  N  E  G  G  G  D  S  H  D  S  Q  Q  R  S
 181 TTACATTCTCACCATGCTCATCATCAACCATGTCATGAGAGCAGCAATCATAAGAGTACAAAACTAACGACTCATTCTTTGGTTCCAACA
  61  L  H  S  H  H  A  H  H  Q  P  C  H  E  S  S  N  H  K  S  T  K  L  T  T  H  S  L  V  P  T
 271 ATACAACAACAACATCAACCACAAGAACCACAACAAGAACAACAACAACAACCTCATCTACTCGCATCATCACCAACCAACCACACCTCT
  91  I  Q  Q  Q  H  Q  P  Q  E  P  Q  Q  E  Q  Q  Q  Q  Q  P  H  L  L  A  S  S  P  T  N  H  T  S
 361 TCTTCGATTCAACTCCCGCCCTCTACTCCCGCCAAAAAACCCTTCTACAGGAGAGGCATTTTCAGAATCTTGGATGCCTTTTTCTTCCTC
 121  S  S  I  Q  L  P  P  S  T  P  A  K  K  P  F  Y  R  R  G  I  F  R  I  L  D  A  F  F  F  L
 451 TTTCGAGTGATACTCTTTCTATTTAAATTCTTTTATTCTTATATGAAATTAGTGAATCATCAAAACAAACAAAAACAACATTGCAAAGTA
 151  F  R  V  I  L  F  L  F  K  F  F  Y  S  Y  M  K  L  V  N  H  Q  N  K  Q  K  Q  H  C  K  V
 541 TTATTAGAAGAGAAAGTAGACAACTACAATGATTACAAACAAATTGCATTGTATTTGGATCAATTAGAAGGTTATGATTTGTGGAAATCT
 181  L  L  E  E  K  V  D  N  Y  N  D  Y  K  Q  I  A  L  Y  L  D  Q  L  E  G  Y  D  L  W  K  S
 631 GAAAATGAAACCAAACTCTATGACTTTGAACAAATTGAAAATATCAATTACCAAATCATGGCCATGTTAAGAAAAAAAGATATTCATGGA
 211  E  N  E  T  K  L  Y  D  F  E  Q  I  E  N  I  N  Y  Q  I  M  A  M  L  R  K  K  D  I  H  G
 721 CTACAATGGTTGCTGAGAGCTGAATTACATCGTAACATTGCTGGTATTAATAATGTTCAACTCTATGAATGTCATACAGGTACTAAACAA
 241  L  Q  W  L  L  R  A  E  L  H  R  N  I  A  G  I  N  N  V  Q  L  Y  E  C  H  T  G  T  K  Q
 811 CTCATTGAAGAATATATTGATTTGGTATCCAAATCATGTCATCTCATTCGAGAGAGTGATGAATTGTCATTGGAAGAGAAATTGAAATTC
 271  L  I  E  E  Y  I  D  L  V  S  K  S  C  H  L  I  R  E  S  D  E  L  S  L  E  E  K  L  K  F
 901 TTTAGAGATACTTGTCATGCCTTTGGAAGAAGTGCTTTATTATTGAGTGGAGGAGGTGGATTATCCATGTATCATATTGGTGTTGTCAAA
 301  F  R  D  T  C  H  A  F  G  R  S  A  L  L  L  S  G  G  G  G  L  S  M  Y  H  I  G  V  V  K
 991 TCTCTCTATGACTCTGGTATTTTACCCAAAGTCATTAGTGGTAGTAGTGGTGGTAGTATTGTGGCTGCAGTCTTGTGTACCAGACGTGAT
 331  S  L  Y  D  S  G  I  L  P  K  V  I  S  G  S  S  G  G  S  I  V  A  A  V  L  C  T  R  R  D
1081 GAAGATCTCTCAAAATGTTTTGAACCTGGTTCTTTTAAATTAGAAGCATTTGGTGGTAGTGATGATACTCCAGAGAGATCAGCCATGCGT
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1171 AAAATTAATCGATTATTGAATAATGGTGTGATTATGGATGTGAACAAGTTAGCACAATGTATTCGTGAGAATATTGGAGATTTAACATTT
 391  K  I  N  R  L  L  N  N  G  V  I  M  D  V  N  K  L  A  Q  C  I  R  E  N  I  G  D  L  T  F
1261 GAAGAGGCCTATAAAATTAGTGGAAGAGTATTAAATATAACAGTGAGTGGACAATCTGCAGGATATCAAACACATGAAGGTCTATTGAAT
 421  E  E  A  Y  K  I  S  G  R  V  L  N  I  T  V  S  G  Q  S  A  G  Y  Q  T  H  E  G  L  L  N
1351 TATTTAACTGCACCTAATGTCTTGATTTGGAGTGCTGCAGTGGCAAGTTGTTCATTACCAATGCTCTACAAAGCAGTTCCTTTGATGGCC
 451  Y  L  T  A  P  N  V  L  I  W  S  A  A  V  A  S  C  S  L  P  M  L  Y  K  A  V  P  L  M  A
1441 AAAGATCGAGATGGTAATATTGTACCATATCATAACTTTCCAAATCAGAAATATCAAGATGGTACTCTCTTCTATGATTTACCAATTACT
 481  K  D  R  D  G  N  I  V  P  Y  H  N  F  P  N  Q  K  Y  Q  D  G  T  L  F  Y  D  L  P  I  T
1531 CGTTTAGCAGAATTATTTAATGTTAACTTCTATATTGCCTCTCAAGTGAACCCTCATGTACTTCCATTTATATCCAATTCCAGAAAACAA
 511  R  L  A  E  L  F  N  V  N  F  Y  I  A  S  Q  V  N  P  H  V  L  P  F  I  S  N  S  R  K  Q
1621 AAGAATTCCGCATTTTCCAATCTACTTTCACTCGTATGCTCAGAAATCAAATATCGAATTGAACAATTATATTCTCTATCATTAATACCT
 541  K  N  S  A  F  S  N  L  L  S  L  V  C  S  E  I  K  Y  R  I  E  Q  L  Y  S  L  S  L  I  P
1711 GAGAGATTTCGATGGTTTGAATTGGTCATTTCACAATCGTATGAAGCTCATGTCACCATTGTACCTTGTGATTTGAATTTTGAGAATATG
 571  E  R  F  R  W  F  E  L  V  I  S  Q  S  Y  E  A  H  V  T  I  V  P  C  D  L  N  F  E  N  M
1801 AAAAAGATTCTCAGTAATCCAACTGCTGAATATATCAATCAAGCAGTTCATCAAGGAATGAGAAGAACATTTCCTCATATGAATCGAATT
 601  K  K  I  L  S  N  P  T  A  E  Y  I  N  Q  A  V  H  Q  G  M  R  R  T  F  P  H  M  N  R  I
1891 GAGAACATGTTAAAGATTGAATTGACATTGGATTCATGTTTGAGACAAGTGAGATTTCAATTATTTAAATCATCACCACAATTTGCCATT
 631  E  N  M  L  K  I  E  L  T  L  D  S  C  L  R  Q  V  R  F  Q  L  F  K  S  S  P  Q  F  A  I
1981 CAAATGTCAAAATCCTCTTCACTCTTTAATTTGGAGGAGGAAGATGTCATTGATCATGAAATTGTTTCATCCGACCCGATAATGAATTCA
 661  Q  M  S  K  S  S  S  L  F  N  L  E  E  E  D  V  I  D  H  E  I  V  S  S  D  P  I  M  N  S
2071 AAACGTGGTGATAGTGGTAGTACTACTACTACTACTACTGCCATGATGACATGCACCAATCCACAACAACAACACTCTTCACATCAACCC
 691  K  R  G  D  S  G  S  T  T  T  T  T  T  A  M  M  T  C  T  N  P  Q  Q  Q  H  S  S  H  Q  P
2161 CACCATTCACTTCAATATCATTCTCAACATGCTTCTCAACATCCTCCTTCTCCTTTGAGAATTTGTAGTAGTAGTACTGGTGGTGCCAAT
 721  H  H  S  L  Q  Y  H  S  Q  H  A  S  Q  H  P  P  S  P  L  R  I  C  S  S  S  T  G  G  A  N
2251 CATCATCATCATCACTCTCATCATGCACACCACCACAACAACACCAGCCCTCGTCATTATCACCCTTCTCATCATGCACACCACCACCAA
 751  H  H  H  H  H  S  H  H  A  H  H  H  N  N  T  S  P  R  H  Y  H  P  S  H  H  A  H  H  H  Q
2341 CACTGCCAACACCACCACGACCCCCCCCCCTCGCTTGGCATACTTCTGTGA
 781  H  C  Q  H  H  H  D  P  P  P  S  L  G  I  L  L  *
```

Figure 29. Translation of the coding sequence of the *N. fowleri* patatin-like protein. Translation was performed using the ExPASy proteomics program "translate." The coding sequence is 2,391 base pairs and the protein sequence in 797 amino acids. Using the ExPASy proteomics program "compute pI/Mw," the predicted pI is 7.25 and then predicted molecular weight is 90.5 kDa. *Indicates the stop codon.

Alignment of *N. fowleri* Patatin-like Protein With Other Known Patatin Proteins

The *N. fowleri* patatin-like protein was aligned to other known patatin proteins in the BLAST database including *Solanum tuberosum* (gi 758342), *Trypanosoma cruzi* (gi 71404931), Rickettsia felis (gi 67004285), *Pseudomonas aeruginosa* ExoU protein (gi 2429143), and *Escherichia coli* 0157:H7 (gi 15832052) using the "T-Coffee" multiple sequence alignment program. The *N. fowleri* patatin-like protein contains conserved domains, including a glycine rich motif, G-S-X-S-G conserved motif, and conserved DG motif, as well as conserved proline residues (Fig 30). The T-coffee program also gives the alignment a score based on how similar the sequences are to each other. The *N. fowleri* patatin-like protein was most closely related to the *T. cruzi* patatin-like protein, followed by *E. coli* 0157:H7, ExoU (*P. aeruginosa*), *R. felis*, and *S. tuberosum* (potato).

Northern blot analysis of patatin expression

Northern blot analysis using a patatin cDNA probe generated by PCR demonstrated that the message encoding the patatin-like phospholipase is present in the three strains of *N. fowleri* tested at approximately 3 kb (Fig 32). There was a smaller, weak product observed in *N. lovaniensis.* There was no hybridization observed between the patatin probe and RNA isolated from *N. austrailiensis, N. gruberi, A. culbertsoni, A. astronyxis,* or *B. mandrillaris* (Fig 32).

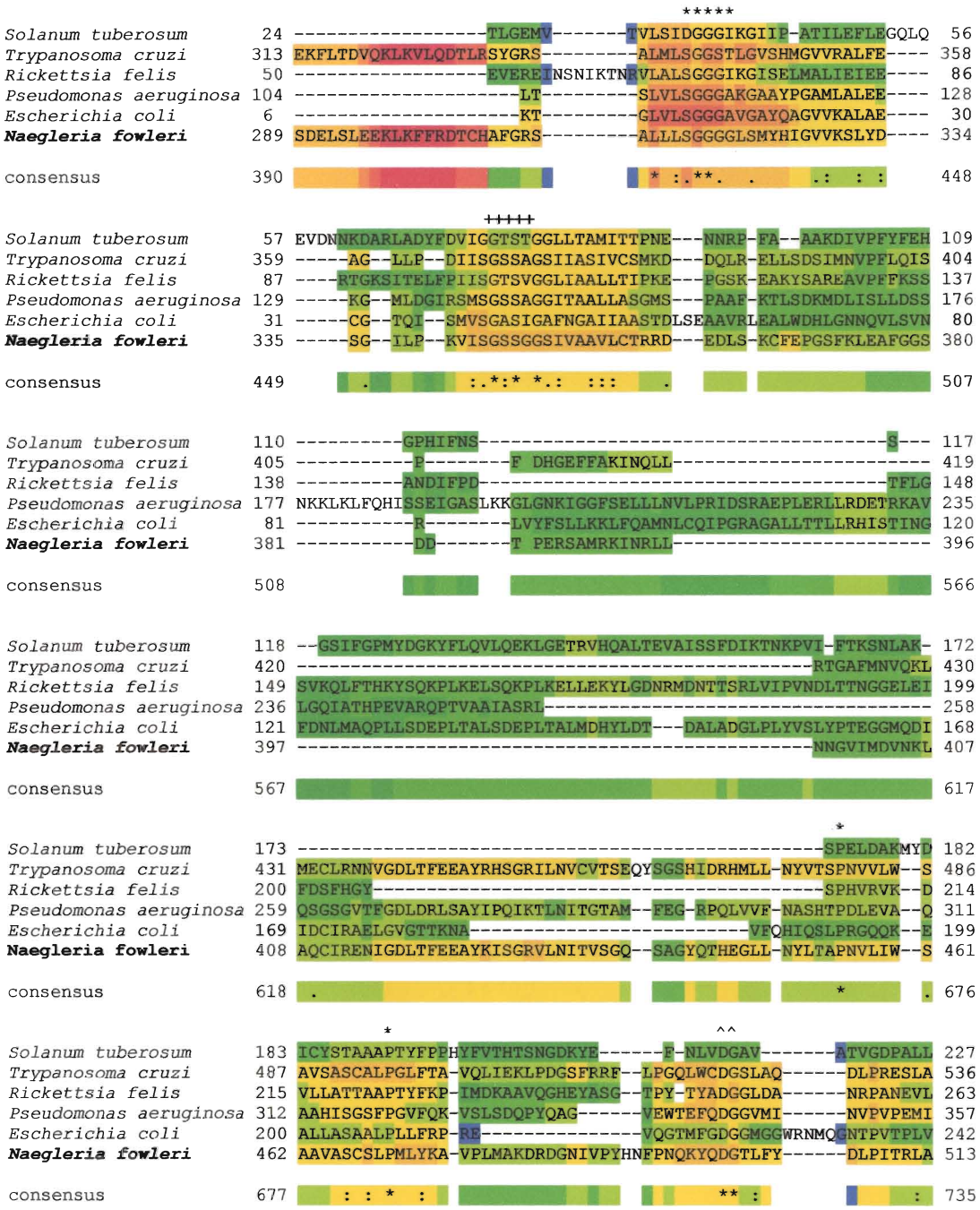

Figure 30. Alignment of patatin-like proteins. The *N. fowleri* patatin-like protein was aligned to other known patatin proteins in the BLAST database including *Solanum tuberosum*, *Trypanosoma cruzi, Rickettsia felis*, *Pseudomonas aeruginosa* ExoU protein, and *Escherichia coli* 0157:H7. Protein alignment was performed with the "T-Coffee" multiple sequence alignment program. ***** glycine rich motif, +++++ GSXSG conserved motif, ^^conserved DG motif, * conserved proline residues, BAD AVG GOOD:legend for the scoring by the T-Coffee software.

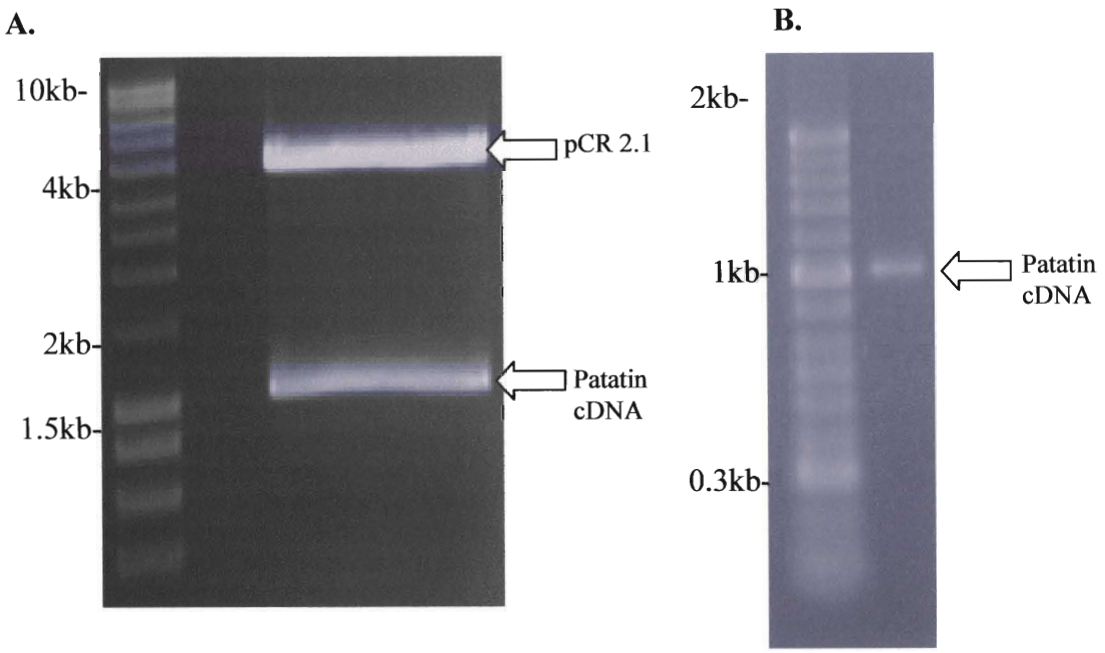

Figure 31. Generation of patatin probe for Northern analysis. PCR was performed using the PCR primers patatin2.for and patatin2.rev (shown in Figure 27) on cDNA from *N. fowleri*. PCR products were cloned into the pCR 2.1-TOPO vector and sequenced. (A) Five micrograms of miniprep DNA was digested with NotI and HindIII and the fragment containing the patatin cDNA was gel extracted. (B) The extracted fragment was run on an agarose gel to determine the concentration of the DNA.

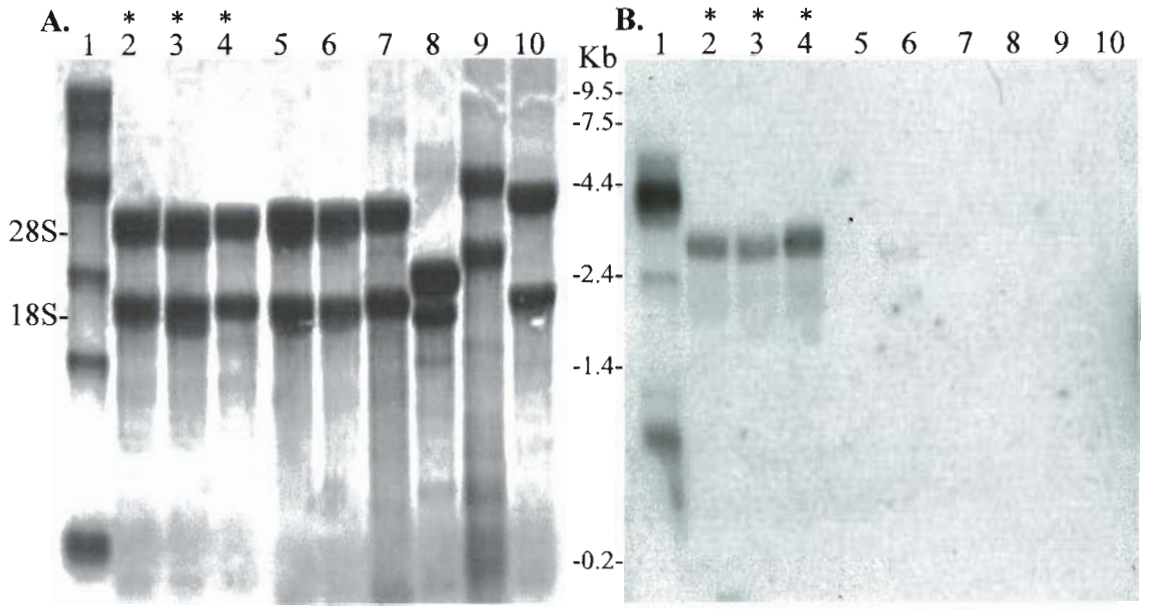

Figure 32. Northern blot analysis of patatin expression. Total RNA (10ug) from pathogenic and nonpathogenic *Naegleria* as well as *Acanthamoeba* spp. and *Balamuthia mandrillaris* was separated by denaturing formaldehyde gel electrophoresis, transferred to a nylon membrane, and stained with methylene blue to show equal loading (28S and 18S ribosomal bands are visible) (A) or hybridized with a patatin cDNA probe (B). The position of 28S and 18S ribosomal bands is indicated. (1) RNA ladder; (2) *N. fowleri* LEEmp; (3) *N. fowleri* LEE; (4) *N. fowleri* Northcott; (5) *N. australiensis*; (6) *N. lovaniensis*; (7) *N. gruberi*; (8) *A. culbertsoni*; (9) *A. astronyxis*; (10) *B. mandrillaris*. The patatin probe hybridized with *N. fowleri* strains, but not with *N. australiensis*, *N. gruberi*, *Acanthamoeba* spp., or *B. mandrillaris*. There was a slightly smaller weak band observed in *N. lovaniensis*. * Indicates *N. fowleri* RNA.

PCR for the patatin gene on genomic DNA isolated from different genera of free-living ameba

PCR analysis was performed on genomic DNA to determine whether other free-living amebae also had the *N. fowleri* patatin gene. PCR analysis was performed using patatin primers on genomic DNA isolated from pathogenic *N. fowleri*, nonpathogenic *N. lovaniensis*, nonpathogenic *N. gruberi*, pathogenic *A. culbertsoni*, nonpathogenic *A. astronyxis*, and pathogenic *B. mandrillaris*. A PCR product was observed for *N. fowleri* at 186 bp, the expected size, but no product was observed for the other ameba tested (Fig 33).

RT-PCR on cDNA from *Naegleria sp* with two sets of patatin primers

PCR was also performed on cDNA from pathogenic *N. fowleri*, nonpathogenic *N. lovaniensis*, and nonpathogenic *N. gruberi* grown at room temperature and at 37 °C. Two different primer sets for patatin were used. A PCR product of approximately 929 bp was observed for the first primer set (Fig 34A) only in *N. fowleri* and not in *N. lovaniensis* or *N. gruberi*. PCR performed with the second primer set also showed that the gene encoding the patatin-like phospholipase is specific for *N. fowleri.* A 186 bp PCR product was observed for only *N. fowleri* and not in the other *Naegleria* species tested (Fig 34B). The –RT controls were all negative for both primer sets, as expected.

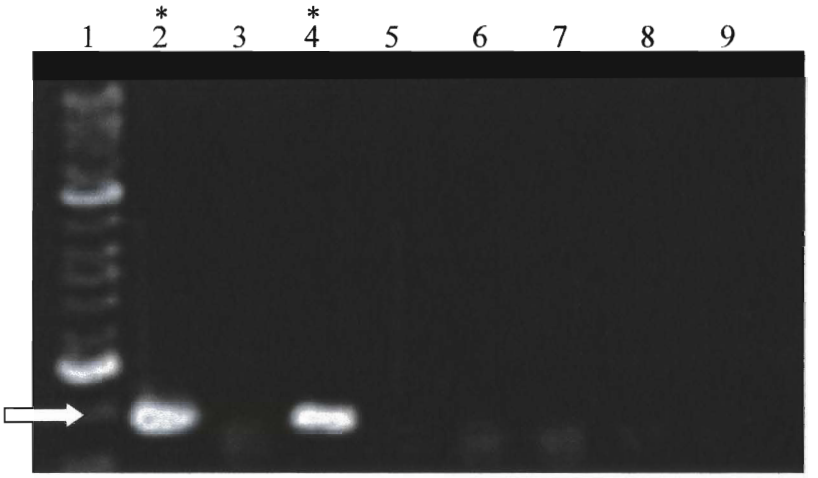

Figure 33. PCR on genomicDNA isolated from different genera of free-living ameba for the patatin gene. Genomic DNA was isolated from pathogenic and nonpathogenic ameba using the Qiagen DNeasy kit (Qiagen). PCR was performed on 100ng of gDNA with primers for patatin3.for and patatin3.rev as described in the materials and methods. (1) DNA ladder; (2) positive control (plasmid DNA); (3) negative control; (4) pathogenic *N. fowleri* (5) nonpathogenic *N. lovaniensis*; (6) nonpathogenic *N. gruberi*; (7) pathogenic *A. culbertsoni*; (8) nonpathogenic *A. astronyxis*; (9) pathogenic *B. mandrillaris*. An 186bp PCR product was observed for *N. fowleri* but no product was observed for the other ameba tested. *PCR was positive for the patatin gene.

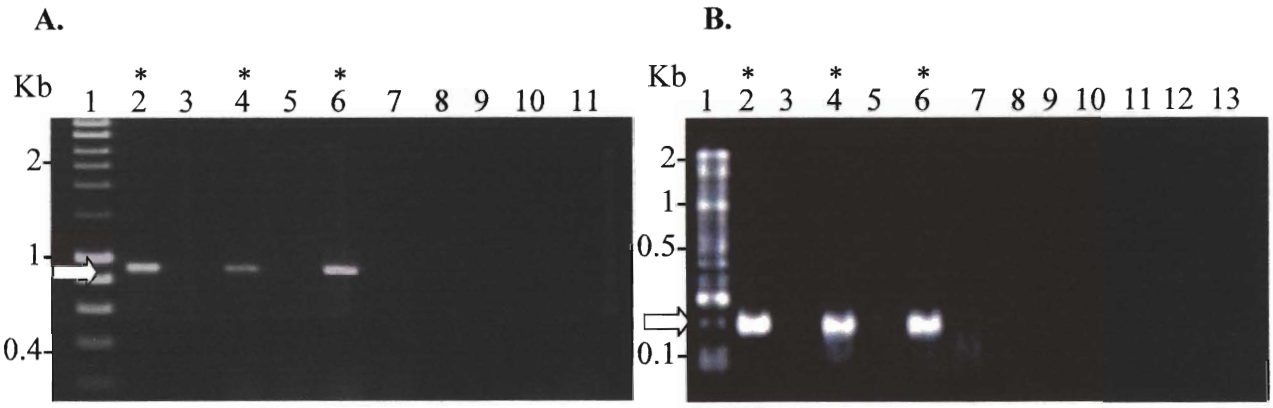

Figure 34. RT-PCR on cDNA from *Naegleria* spp. with two sets of patatin primers. RNA from nonpathogenic and pathogenic *Naegleria* amebae was extracted and reverse-transcribed into cDNA using random primers. PCR was performed on cDNA and using primers patatin2.for and patatin2.rev (A) described in figure 27 and another set of primers patatin3.for and patatin3.rev (B) described in the materials and methods. Products were visualized on a 1.5 % agarose gel. Lane (1) DNA ladder; (2) positive control (plasmid DNA); (3) – control; (4) *N. fowleri* LEEmp +RT; (5) *N. fowleri* LEEmp -RT (6) *N. fowleri* LEE + RT; (7) *N. fowleri* LEE – RT; (8) *N. lovaniensis* + RT; (9) *N. lovaniensis* – RT (10) *N. gruberi* rt + RT; (11) *N. gruberi* rt - RT; (12) *N. gruberi* 37°C + RT; (13) *N. gruberi* 37°C - RT;. PCR primers for patatin amplified cDNA from pathogenic *N. fowleri* LEEmp and LEE, but not nonpathogenic *N. gruberi* or *N. lovaniensis* with both primer sets. RT= reverse transcriptase and rt= grown at room temperature. *PCR was positive for the patatin gene.

Western blot analysis of the *N. fowleri* patatin-like protein

Western blot analysis with an anti-human phospholipase A_2 was performed on *N. fowleri* and *N. lovaniensis* whole cell lysates, as well as membrane and cytosolic preparations (Fig 35). The anti-PLA_2 antibody cross-reacted with *N. fowleri* at approximately 90-100 kDa in the whole cell lysate and in the membrane and cytosol preparations, which is the expected size of the patatin-like phospholipase. There was also cross-reactivity at ~15-20 kDa, which represents a soluble form of phospholipase A_2. There was a weak band observed in the *N. lovaniensis* whole cell lysate at ~80 kDa. The membrane and cytosolic preparations did not cross-react with the antibody.

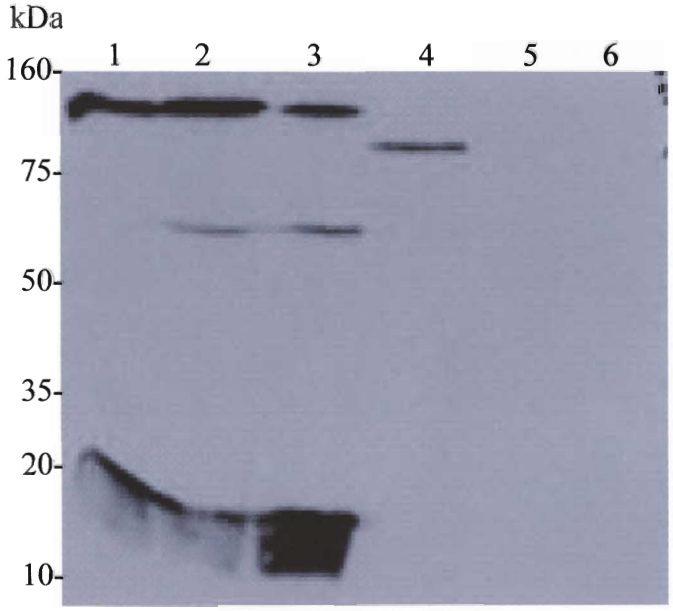

Figure 35. Immunoblot Analysis of *N. fowleri* and *N. lovaniensis* Using an Anti-Human Phospholipase A_2 Antibody. SDS-PAGE was performed on proteins isolated from *N. fowleri* and *N. gruberi*. Membranes were incubated with an anti-human PLA_2 antibody. Lanes (1) *N. fowleri* whole cell lysate (2) *N. fowleri* membrane preparation (3) *N. fowleri* cytosolic preparation (4) *N. lovaniensis* whole cell lysate (2) *N. lovaniensis* membrane preparation (3) *N. lovaniensis* cytosolic preparation. The anti-PLA_2 antibody detected three possible PLA_2s in *N. fowleri* at approximately 90kDa, 65kDa, and 15 kDa in all three fractions. *N. lovaniensis* only had one band in the whole cell lysate, which was approximately 80kDa. There were no bands observed in the membrane or cytosolic fractions of *N. lovaniensis*.

DISCUSSION

In the present study a nested PCR assay was used to identify *N. fowleri* in environmental samples from a river in Richmond, VA, three areas in Connecticut, a domestic water supply from Arizona, and three areas in Oklahoma. The sample from Virginia, a state where *N. fowleri* has been isolated previously (Martinez 1985; Ettinger et al. 2003), was positive by nested PCR (MacLean et al, 2004). Furthermore, a mouse pathogenicity test performed on two mice demonstrated that the culture positive for *N. fowleri* by PCR produced a lethal infection in one of two mice inoculated by the intranasal route. These studies indicate that the nested PCR assay developed using a laboratory strain can detect *N. fowleri* in environmental samples.

Of 86 environmental samples tested from Connecticut, 15 were positive by PCR. This is the first report of identification of *N. fowleri* in environmental samples from Connecticut. Of the 3 sampling sites examined, *N. fowleri* was isolated with the greatest frequency from a small shallow pond on the campus of Quinnipiac University. This pond is the result of runoff and contains less organic matter than other sampling sites tested. *N. fowleri* was isolated more often in this pond than in the other water samples from Connecticut (MacLean et al, 2004). Kyle and Noblet (1985; 1986; 1987) noted that soil was the ideal habitat for free-living amebae and that runoff from rains introduced amebae into water. These investigators reported that significant increases in the numbers of *Naegleria* and *Acanthamoeba* were detected in surface water from a shallow water station

after a major rainfall. Major weather events such as heavy rains and winds were more important than temperature in determining the relative abundance of free-living amebae in aquatic ecosystems (Kyle and Noblet 1985; 1986; 1987). Furthermore, "organically-rich" water does not appear to favor the growth of *N. fowleri* (De Jonckheere and Van de Voorde 1977).

Factors responsible for the increased presence of *N. fowleri* in water have been attributed for the most part to thermal pollution from industrial waste and the presence of coliforms (Brown et al. 1983; Griffin 1972; Kasprzak et al. 1982; Tyndall et al. 1989). Other studies have shown that water temperature is not the major factor affecting the distribution of *N. fowleri* since these thermotolerant amebae have been isolated from water with temperatures ranging from 16 °C to 45 °C (Griffin 1972; Stevens et al. 1980; Tyndall et al. 1989; Wellings et al. 1979). Our studies, although limited, support the proposition that water temperature and the presence of coliforms are not the only factors to influence the distribution of *N. fowleri*.

Environmental samples that were stored on NNA plates for one year prior to analysis by PCR were positive for *N. fowleri* indicating that samples can be stored in the cyst form for long periods of time. However, once samples are cultured in liquid medium other organisms in the samples can out-compete *N. fowleri*. Cultures remained positive for at least 1 month after culture in liquid medium but after 9 months of continuous culture, most samples were no longer positive by PCR for *N. fowleri*. Kilvington and White (1986) noted that accurate identification of *N. fowleri* was dependent on processing samples soon after isolation due to the close ecological relationship between pathogenic *N. fowleri* and

nonpathogenic *N. lovaniensis.* Also, it has been reported that *N. lovaniensis* proliferates more rapidly than *N. fowleri* (Griffin 1983; Kilvington and Beeching 1995; Sparagano 1993) and that constantly elevated water temperatures support nonpathogenic strains such that thermotolerant competitors are more abundant than *N. fowleri* (Sykora et al. 1983). This PCR assay can distinguish *N. fowleri* from *N. lovaniensis* (Reveiller et al. 2002). A number of free-living amebae are present in water and often interfere with detection of human pathogens. We have employed the nested PCR assay previously to distinguish *N. fowleri* from other species of *Naegleria* as well as from amebae of the genus *Acanthamoeba* (Reveiller et al. 2002). In the present study we have shown that the nested PCR assay can be used to discriminate *N. fowleri* from other species of amebae commonly found in the environment.

Chemicals present in water samples collected from environmental sites often contain inhibitors of DNA amplification (Orlandi and Lampel 2000; Sluter et al. 1997; Tebbe and Vahjen 1993). In order to avoid the possibility of inhibition of PCR products, environmental samples from Connecticut were placed on NNA-*E. coli* plates to isolate amebae prior to PCR. However, river water from Virginia, domestic water supplies from Arizona, and samples from Oklahoma were tested directly by the nested PCR assay, and inhibition of DNA amplification did not appear to be problematic. These studies indicate that environmental samples can be tested either directly or amebae can be isolated prior to testing. Additionally, intact amebae suspended in Page's ameba saline were subjected to nested PCR without isolation of DNA. A five minute incubation at 95 ^{o}C during the first PCR amplification may have caused lysis of the amebae and made the DNA accessible to

the *Taq* polymerase eliminating the necessity of extraction and purification of DNA. A previous study revealed that the nested PCR assay specifically detects as little as 5 pg of *N. fowleri* DNA or 5 trophozoites (Reveiller et al. 2002). When 5 pg of DNA or 5 cysts or trophozoites of *N. fowleri* are present in a sample, PCR can be performed directly on the sample without prior culture as was the case for the water samples from Virginia.

After determining the nested PCR assay could be used to identify *N. fowleri* in the environment, we used the assay to test samples collected from domestic sites in Arizona where two cases of PAM occurred in previously healthy children. In this study, samples were collected in approximately 250 ml volumes but only 10 ml of each sample was tested initially by PCR. Analysis of these 10 ml samples by PCR indicated the presence of *N. fowleri* in 4 of 11 samples after 4 days of culture at 37°C. Culture of the samples for 1 week followed by nested PCR increased the frequency of detection of *N. fowleri* such that 11 of 11 samples were positive. Samples were cultured prior to PCR to eliminate the possibility of the presence of inhibitors of *Taq* polymerase in the environmental samples, which would result in false negatives. Three domestic samples, labeled 3, 4, and 16, containing thermotolerant amebae, which were positive by PCR after 4 days of culture, were selected for a test for flagellates. Flagellates were detected in all three samples, indicating that a thermotolerant amoeboflagellate was present. Transformation of amebae into flagellates is a distinctive feature of the genus *Naegleria* (Martinez 1985).

Select samples were examined by Western immunoblot analysis using polyclonal antiserum elicited against *N. fowleri*. Samples analyzed by Western immunoblotting confirmed the presence of *Naegleria* amebae in the samples. However, the immunological

technique using polyclonal antiserum is genus specific and cannot distinguish the species *N. fowleri* from *N. lovaniensis*. Sufficient amounts of samples from five cultures were tested for *N. fowleri* by using a monoclonal antibody (5D12) specific to the ameba, which indicated that *N. fowleri* was present (Reveiller et al. 2000; Sparagano et al. 1993). The samples were also tested for the organisms of the genus *Acanthamoeba* by Western immunoblot analysis, since visual assays using light microscopy indicated that the cultures also contained *Acanthamoeba* cysts, which are readily recognized using morphological criteria (Martinez 1985; Page 1988; Marciano-Cabral et al. 2000; Marciano-Cabral and Cabral 2003). *Acanthamoeba* was also detected by immunoblot analysis in several domestic samples, an additional human health hazard since *Acanthamoeba* spp. have been associated with amoebic keratitis and granulomatous amebic encephalitis (Martinez 1985; Marciano-Cabral et al. 2000; Marciano-Cabral and Cabral 2003). Animal pathogenicity tests have been used to distinguish pathogenic from nonpathogenic *Naegleria* spp. Although *Naegleria australiensis* is pathogenic for mice, it has not been isolated from a human case of PAM (Martinez and Visvesvara, 1997). However, animal pathogenicity tests do not distinguish *N. fowleri* from *N. australiensis*. The specificity of the product generated by nested PCR was confirmed by cloning and sequencing the PCR product. Thus, the nested PCR assay not only eliminates the need to do animal pathogenicity testing but also constitutes a highly sensitive tool for discriminating *N. fowleri* from other *Naegleria* species as well as from amebae of the genus *Acanthamoeba* and other free-living amebae commonly found in the environment (Reveiller et al. 2002; MacLean et al. 2004). Differentiation of *N. fowleri* from pathogenic species of *Acanthamoeba* is important

because the course of the disease and the treatment regimens are different (Martinez 1985; Martinez and Visvesvara, 1997; Marciano-Cabral et al. 2000; Marciano-Cabral and Cabral 2003).

Nineteen sites in the households associated with PAM victims and a neighboring home were sampled. Seventeen of these sites were shown to be positive for *N. fowleri* by PCR. Of particular interest is that *N. fowleri* was detected in residual water from the sink pipes in both homes as well as from the Micro-Wynd filter, which was used to filter bathtub water from the homes. These results are consistent with the source of infection for these two children being the domestic water source, because neither child had a history of swimming in a natural freshwater lake or pond prior to the onset of symptoms of PAM. However, both victims routinely played in the bathtub (Okuda et al. 2004).

As a result of two children acquiring a fatal infection of PAM in 2005, more samples were tested from Tulsa, Oklahoma. It was suggested that the source of the infection was a recreational water facility or a nearby creek that was associated with both children.

Infection with *N. fowleri* has been acquired through modes other than conventional swimming or diving in ponds and lakes. Sniffing water into the nasal passages as a religious ritual prior to prayer (Lawande et al. 1980), total immersion in bathwater (Anderson, and Jamieson 1972a; 1972b; Anderson et al. 1973), playing in a warm muddy puddle after rain (Apley et al. 1970), and immersion of the head in a trough of water on a school playground (Dorsch et al. 1983) have been described as sources of infection with

these amebae. An 8-month-old infant was thought to have acquired an infection with *N. fowleri* during a full-submersion baptism ceremony in a natural body of water (Barnett et al. 1996).

The occurrence of *N. fowleri* in the domestic water supply has been reported previously (Anderson, and Jamieson, 1972a; Anderson and Jamieson, 1972b; Anderson et al. 1973). In South Australia, household water delivered via overland pipelines during a prolonged period of hot weather was attributed as the source of PAM in children in backyard wading pools or in bathtubs. *N. fowleri* was recovered from a sample of tap water taken from a home where a fatal case of PAM occurred. In two Australian cases, houses had remained unoccupied for considerable periods of time during warm weather (Anderson et al. 1973, Carter 1972, Miller et al. 1982). It has been suggested that under such climatic conditions, *N. fowleri* can multiply to significant numbers in warm stagnant sections of domestic water supplies (Anderson et al, 1973, Carter, 1972, Miller et al, 1982). In this context, a number of studies have shown that, out of several physical and chemical characteristics of water, elevated temperature has been one of the most important factors accounting for the increased incidence and higher levels of *N. fowleri* (Griffin 1972; Dorsch et al. 1983; Griffin 1983). The cases studied in the present report emphasize that PAM should be considered in the differential diagnosis of unexplained meningoencephalitis since not all cases of this disease are associated with freshwater sports. The development of a PCR assay to detect pathogenic *N. fowleri* in the environment provides a valuable surveillance tool that will aid in the potential prevention of human disease. *Naegleria* also harbor pathogenic bacteria such as *Legionella* and may act as

vectors of bacterial diseases. In addition, *Naegleria* spp. and other limax amebae should be considered in the etiology of fish and invertebrate pathology or mortalities (Franke 1982; Sawyer 1971). The nested PCR assay that we have described provides a rapid, sensitive, and specific method to determine the presence of *N. fowleri* in environmental and recreational sources.

In addition to identifying *N. fowleri* in the environment, it is also important to determine how *N. fowleri,* a free-living ameba, is able to survive in a human host and cause extensive damage to brain tissue. One characteristic of *N. fowleri* that may contribute to pathogenesis is the ability to evade complement-mediated lysis. Previous studies in our laboratory have suggested that resistance to complement-mediated lysis in *N. fowleri* is regulated at the level of MAC formation (Whiteman and Marciano-Cabral 1989). Therefore, a complement regulatory protein that acts at the level of the MAC of complement, CD59, was investigated. Northern analysis was performed using a cDNA encoding human CD59. The CD59 probe hybridized with RNA from pathogenic *N. fowleri* at ~2 kb. Western immunoblot studies demonstrated the presence of a protein reactive with monoclonal antibodies to human CD59 in the membrane fraction of *N. fowleri*. No hybridization or cross-reactivity was observed with *N. gruberi*, a nonpathogen. In addition, it was determined that the amebae "CD59-like" protein associates with pore-forming complement component C9 using immunoprecipitation analysis. In addition, immunofluorescence analysis revealed clustering of the protein on the membrane of *N. fowleri* incubated with a sublytic dose of serum complement (Fritzinger et al. 2006).

Proteins that exhibit functional and antigenic similarity to human CD59 have been demonstrated in a variety of pathogenic organisms, including *Schistosoma mansoni* (Parizade et al. 1991), *Entamoeba histolytica* (Braga et al. 1992), and *Borrelia burgdorferi* (Pausa et al. 2003). However, despite the shared characteristics of these proteins with human CD59, a large disparity exists among their molecular weights, 94 kDa, 260 kDa, and 80 kDa, respectively.

Collectively, studies in our laboratory has demonstrated the presence of a protein on the surface of pathogenic *N. fowleri* that is cross-reactive with monoclonal antibodies for human CD59. The RNA transcript and "CD59-like" protein were detected in pathogenic *N. fowleri*, therefore the current study was undertaken to determine the sequence of the gene encoding the "CD59-like" protein. Molecular analyses were employed initially to detect CD59 in *N. fowleri*. Southern blot analysis indicates there is one gene that encodes for CD59 in *N. fowleri*. Similarly, the human and rat genomes also contain one gene for CD59 (Philbrick et al. 1990; Qian et al. 2000). In contrast, the mouse genome contains two CD59 genes (Qian et al. 2000). Further studies are necessary to determine the exact structure of the *N. fowleri* CD59 gene since two bands were present in the *Bam*HI digest and the DNA sequence and restriction sites of the ameba CD59 are unknown.

Two-dimensional gel electrophoresis was performed to determine the molecular weight and pI of the "CD59-like" protein. The molecular weight of the protein is approximately 18-20 kDa, which correlates with previous reports (Fritzinger et al. 2006). There were multiple spots observed in the pH 7-8 range, which indicates that CD59 is

glycosylated in the ameba. Mammalian CD59 is highly glycosylated (Rudd et al. 1998). Protein spots cross-reacting with anti-CD59 on 2-D analysis were extracted and sent to Proteomic Research Services, Inc., (Ann Arbor, Michigan) for internal sequencing. Sequence analysis suggested that another known *N. fowleri* membrane protein, Mp2C15, was in the same area as CD59. Therefore, immunoblot analysis was performed on proteins separated by 2-D gel electrophoresis. The anti-Mp2C15 antibody cross reacted with several proteins in the pH range of 7-8 and a molecular weight of 18-20 kDa.

Immunoprecipitation was performed to separate the Mp2C15 protein from the ameba "CD59-like" protein. Silver staining of the immunoprecipitated proteins demonstrated several proteins that co-precipitated with CD59. Immunoblot analysis of the immunoprecipitated proteins with anti-CD59 and anti-Mp2C15 demonstrated that both proteins immunoprecipitated together. Further studies need to be performed to determine whether CD59 and Mp2C15 are different proteins or whether anti-human CD59 is cross-reacting with an epitope on the Mp2C15 protein.

Another approach employed to determine the sequence of the gene encoding CD59 was to construct a genomic DNA library. A CD59 cDNA hybridized with gDNA digested with EcoRI in Southern blot analysis. Therefore, EcoRI was chosen to digest *N. fowleri* genomic DNA for the construction of the genomic DNA library. Screening of the library with the same probe used in Southern analysis was employed. The sequence of the gene encoding CD59 was not found, however sequencing of 155 plasmids resulted in 95 genes that had matches in the BLAST database. Because only one round of sequencing was performed, plasmids containing more that 1 kb of insert are only partial gene sequences.

Some genes that were sequenced contained putative conserved domains, others did not. The putative proteins without conserved domains may, in fact, have a conserved domain that is in the part of the gene that has not been sequenced yet. The full gene sequence needs to be known in order to determine whether they have conserved domains.

We have sequenced a small subset of the genome, however we were able to make some observations regarding the *N. fowleri* genome. Most DNA sequences obtained were AT rich and several sequences we obtained were part of the mitochondrial genome. The mitochondrial genes in *N. gruberi* are on a 50 kb plasmid and the entire plasmid has been sequenced and deposited in the BLAST database (gi 10444209). We obtained several sequences matching a number of these genes, indicating that mitochondrial plasmid DNA was also present in our library. Also, many of the sequences did not have a match in the BLAST database even though open reading frames were detected. This indicates that *N. fowleri* have novel genes that have no match to those deposited in the BLAST database (Mp2Cl5).

Of 95 genes that had matches in the database, 9 had a predicted function of cell motility or chromosome segregation. These genes included actin, myosin heavy chain, gelsolin, and a kelch-like protein that are all involved in movement or organelle transport.

Actin has been characterized in *N. gruberi* by biochemical and molecular methods. DNA sequences encoding actin in *Naegleria* have been deposited in the database. The actin that we have obtained from genomic DNA sequencing is distinct from those sequences. *Naegleria* actin is distinct from actin isolated from other eukaryotes because there are three distinct actin isoforms. In single-cell eukaryotes, only one actin isoform is

usually produced. In addition, *Naegleria* actin lacks N-methylhistidine. This residue has been found in every actin characterized, with the exception of *Naegleria*. Antibodies prepared to *N. gruberi* actin do not recognize determinants in actin from other organisms such as *Acanthamoeba* or *Dictyostelium* spp. (Sussman et al. 1984; Fulton et al. 1986).

Two kinesin proteins were sequenced, which function to pull vesicles and organelles along microtubules or in chromosomal segregation. Actinin is involved in cytoskeletal structure and the flagellar protofilament ribbon protein may be associated with forming the specialized protofilament ribbons of flagellar microtubules.

Thirteen of the 95 genes have a predicted function for gene regulation. These include RNA and DNA helicases, a methylase, a methyltransferase, a transcription factor, a RNA polymerase, among others. The presence of these genes in the *N. fowleri* genome is unremarkable, however, they may serve as potential drug targets in the treatment of PAM as these have been considered drug targets in other diseases (Seow et al. 2005 and Tuteja and Pradhan 2006).

Nine out of 95 putative genes have a predicted function involving protein synthesis and degradation. Two of the nine encode mitochondrial ribosomal proteins that have already been sequenced from the *N. gruberi* mitochondrial genome and these include ribosomal proteins S4 and S12. The majority of protist mitochondrial genomes encode large subunit and small subunit mitochondrial rRNAs (Gray et al. 1998). We also sequenced two other ribosomal proteins, L6 and L7/L12 that are unique from those in the mitochondrial genome. The rRNA genes of *Naegleria* are located on a nuclear plasmid that is approximately 14kb in size that has not been fully sequenced. Perhaps the genes

encoding the two ribosomal proteins are from this nuclear plasmid. Other predicted proteins involved in protein synthesis and degradation include a mannosyltransferase, peptidases, and ligases.

There were 21 genes that encoded putative genes for protein regulation or cell signaling. *N. fowleri* is a free-living ameba that is able to infect human and animal hosts. Therefore, it must have systems to detect environmental stresses such as cold and desiccation, bacterial pathogens (Fritzinger and Cabral 2004), or the host environment. In the genomic DNA library, we sequenced five potential serine/threonine kinases, including a predicted protein in the AGC family, which includes protein kinases A, G, and C, and consists of small, cytoplasmic kinases that mediate signaling in eukaryotic cells including phospholipid, cyclic nucleotide, and calcium signaling (Goldberg 2006). Other serine/threonine kinases found in the *N. fowleri* gDNA library could not be grouped to a particular family, but these proteins did contain serine/threonine conserved domains.

Receptor tyrosine kinases (RTKs) have an extracellular binding domain, intracellular tyrosine kinase and regulatory domains, and a transmembrane domain. RTK signaling pathways have functions including cell proliferation and differentiation, promotion of cell survival, and modulation of cell metabolism. No putative tyrosine receptor kinases were found, which is similar to reports from the *Dictyostelium* genome sequencing project (Goldberg et al, 2006), however, more sequencing needs to be performed to determine if *N. fowleri* have RTKs, however evidence suggests that *N. fowleri* may have RTKs that we have not yet identified in the library. First, *N. fowleri* has a putative gene for a tyrosine-like kinase (Roco8). The ROCO family of proteins are

considered tyrosine-like kinases because they have sequences reminiscent of both tyrosine and serine/threonine kinases, although they are known to act as serine/threonine kinases. However, it has been postulated that all members of the ROCO family of proteins can phosphorylate tyrosine (Goldberg et al. 2006). In addition, a GTP binding protein, Ras, functions downstream from RTKs in most higher eukaryotes. RTK is phosphorylated by a cytosolic protein, which activates the RTK and another cytosolic protein. This activated cytosolic protein functions as a guanine exchange factor (GEF), which helps to convert Ras-GDP to Ras-GTP, which is the active form. Genes for both Ras and Ras-GEF were found in the *N. fowleri* library.

Further evidence for a tyrosine phosphorylation signaling pathway is shown by another gene for a putative kinase, MEK, which is a dual-specificity kinase, and acts downstream from Ras and Ras-GEF. Activation of Ras to Ras-GEF by RTKs can also activate serine/threonine kinases in other signaling pathways. There were also three dual-specificity phosphatases, indicating that the ameba can phosphorylate and dephosphorylate tyrosine residues. Another gene for a putative protein, Rab, was found in the library. Rab functions as a regulator of vesicular traffic. It is thought that the hydrolysis of GTP to GDP provides the energy for vesicle movement. Previous studies have shown that tyrosine phosphorylation is important in complement-resistance of *N. fowleri* (Chu et al. 2000). Interestingly, one defense against complement-lysis is the ability of *N. fowleri* to vesiculate the MAC of complement from their surface when treated with human serum (Toney and Marciano-Cabral 1994). Perhaps the putative genes we have described aid in *N. fowleri*

resistance to serum complement by signaling the amebae in the presence of complement to remove the MAC complex from the ameba.

In addition to serine/threonine and tyrosine kinases, there are also histidine kinases, which were not found in the partial sequencing of the library. Histidine kinases, in addition to other types of kinases, are involved in environmental sensing. Histidine kinases are absent from the *Entamoeba histolytica* genome, but are found in *Acanthamoeba castellanii* and *Dictyostelium discoideum* genomes (Anderson et al. 2005). More sequencing must be performed to determine if histidine kinases are present in the *N. fowleri* genome.

Two molecular chaperones involved in signaling and stress response were also sequenced. These proteins include heat shock protein 90 (HSP90) and GrpE. HSP90 has been shown to be involved in many aspects of cell signaling including signal transduction, protein folding, protein degradation and morphological evolution (Nadeau et al. 1993; Jakob and Buchner 1994). It has been reported that lower levels of HSP90 expression in *Acanthamoeba castellanii* results in changes in cellular morphology, partial resistance to killing by bacteria, more bactericidal activity, and higher frequencies of lysosome fusion with vacuoles containing bacteria (Yan et al. 2004). The authors suggest that hsp90 plays a role in phagocytic and bactericidal pathways in the ameba that affect interactions of phagocytic cells with bacteria.

The molecular chaperone, GrpE promotes the exchange of ADP for adenosine triphosphate (ATP) and also augments peptide release from the DnaK substrate-binding

domain in an ATP-independent manner. GrpE and DnaK are proteins in the HSP 70 pathway, which assists in protein folding (Harrison 2003).

Eleven genes for predicted proteins involved in respiration and energy production were found in the library. The majority of these genes have already been sequenced by the mitochondrial DNA sequencing project. One gene of particular interest was a putative gene for an iron-hydrogenase assembly protein with a small GTP binding protein domain. Iron hydrogenases have only been found in anaerobic bacteria, some green algae, and in anaerobic protozoa (Posewitz et al. 2004). Perhaps this gene is an evolutionary link of *N. fowleri* with lower organisms.

Eight genes had a predicted function involving membrane synthesis or metabolism. Five genes have a predicted function involved in sterol or cholesterol synthesis. These include alpha-methylacyl-CoA racemase, cytochrome P450, acyl-CoA synthetase, squalene synthetase, and a protein that is similar to a lamin B receptor (ergosterol biosynthesis). The lamin B receptor is recognized as possibly playing a central role in the mitosis-related disassembly and reassembly of the nuclear envelope (Gant and Wilson 1997). This may be the function of the protein in *N. fowleri*, however this protein also contains an ergosterol biosynthesis conserved domain. Ergosterol has been shown to be a component in membranes of *N. lovaniensis* and *N. gruberi* (Raederstorff and Rohmer 1986). Amphotericin B is the most effective drug against *N. fowleri* and has been used successfully in a few cases (Schuster and Visvesvara 2004). Amphotericin B works by binding to sterols in the membrane and altering the permeability of the cell, which may explain why *N. fowleri* is susceptible to treatment with amphotericin B. Squalene

synthetase is also involved in the synthesis of ergosterol and may be another drug target to investigate as a treatment of PAM. The inhibition of this protein has been studied extensively in the treatment of infections with *Trypanosoma cruzi* and *Leishmania* (Urbina et al. 2002; Braga et al, 2004; Urbina et al, 2004; Rodrigues et al, 2005).

Other proteins involved in the metabolism of membranes include a patatin-like phospholipase, an N-acyl-phosphatidylethanolamine-hydrolyzing phospholipase D, and a phospholipase A_2 activating protein. Phospholipases are important virulence factors that may hydrolyze the host membrane and assist in invasion or destruction of tissue. The patatin-like phospholipase is a phospholipase A_2 member and will be discussed later in detail. The presence of a gene encoding N-acyl-phosphatidylethanolamine-hydrolyzing (NAPE-PLD) phospholipase D was surprising. This phospholipase cleaves N-acyl-phosphatidylethanolamines into anandamide. NAPE-PLD is structurally and catalytically distinguishable from other known enzymes, including other phospholipase Ds (Okamoto et al. 2004). Anandamide is an endocannabinoid that has been extensively studied. Cannabinoids have been shown to decrease responsiveness in infection, including granulomatous amebic encephalitis caused by *Acanthamoeba* (Cabral and Marciano-Cabral 2004). DNA encoding a predicted glutathione synthetase was also found. It has been reported that *N. fowleri* produce glutathione and its reducing enzyme glutathione reductase, which provides protection against reactive oxygen species in a majority of eukaryotes and prokaryotes (Ondarza et al, 2006). This gene may be responsible for producing glutathione to protect the ameba.

There were 21 genes that had significant matches in the BLAST database, however the function of these proteins is unknown. PqaA is a PhoPQ activated pathogenicity protein that has been reported to be responsible, in part, for melittin-resistance. Melittin is a compound produced by honey bees that has been shown to have antimicrobial activity by forming pores in cell membranes (Fennel et al. 1968). This protein has been studied for treatment of infections with *Chlamydia trachomatis, Mycoplasma hominis, Leishmania,* and *Borrelia burgdorferi* (Lazarev et al. 2005; Lubke and Garon 1997). Studies in our laboratory have suggested that *N. fowleri* is resistant to melittin-mediated lysis by observations of amebae cultured with melittin (work in progress). *N. fowleri* produce pore-forming proteins and are resistant to lysis by the membrane attack complex of *N. fowleri*, which is also a pore-forming protein. It is likely the ameba possess defenses against their own pore forming proteins as well as pore-forming proteins expressed by bacteria in the environment.

The WDR23 and bromodomain proteins have WD40 repeats, which cover a wide variety of functions including adaptor/regulatory modules in signal transduction, pre-mRNA processing and cytoskeleton assembly. There is no literature on WDR23 in any organism studied thus far, therefore the function of this protein is unknown. Proteins containing a bromodomain are implicated in both transcriptional activation and repression. Bromodomains are 110 amino acids and are found in many chromatin associated proteins. Bromodomains can interact specifically with acetylated lysine (Haynes et al. 1993 and Dhalluin et al. 2004) It is interesting to note that the most significant matches for these two *N. fowleri* putative proteins are humans and mice.

GTPase activating Rap/Ran GAP domain-like 1 isoform 1 is a protein that is found in human and mouse brain. This protein has a predicted function in neurological disorders (Schwarzbraun et al. 2002) and neurogenesis (Heng and Tan 2002).

Nodulin genes are plant genes that are specifically activated during the nodule formation process and play crucial roles in nodule genesis and symbiotic functioning. It is not known what the function of this protein is in *N. fowleri*, however, by searching several of the currently sequenced genomes, we found nodulin-like proteins found in the genomes of other protozoa, such as, *A. castellanii*, which had three nodulin-like genes (ACL00003243, ACL00000629, ACL00009427; http://amoebidia.bcm.umontreal.ca/pepdb/searches) and *D. discoideum* which has one nodulin-like gene (gi 66804677, BLAST database).

Another mechanism that *N. fowleri* may use to protect itself from the environment is UV radiation resistance. Several investigators have reported that *N. fowleri* is resistant to UV radiation. UV irradiated pools contained high levels of thermophilic *Naegleria* (De Jonckheere 1982) and encysted amebae were able to recover from UV treatment to almost 100% viability after exposure to white light (Hillebrandt 1991). Other protozoa are also resistant to UV damage including *Dictyostelium* (Yu et al. 1998), and *Acanthamoeba* (Hijnen et al. 2006). One of the genes in the library is similar to a UV radiation resistance associated gene in *Gallus gallus* (chicken). This gene may be involved in *N. fowleri*'s resistance to UV radiation.

Other proteins have less clear implications as to their function in *N. fowleri*. There is a lung seven transmembrane receptor, an RHS repeat family protein that may function in

ligand binding, and a tuftelin interacting protein 11 (TFIP11). Tuftelin is also known as enamel and TFIP11 has been shown to interact with human enamel. It has been suggested that due to the ubiquitous nature of the protein, it may have functions in other human tissues (Wen et al. 2005), however the true function of this protein is unknown. There are other proteins that are specific to bacteria including a periplasmic phosphate-binding protein and a cell-wall associated hydrolase. These proteins may have been acquired through horizontal transfer from endosymbiotic bacteria and the function of these proteins in *N. fowleri* is unclear. Other genes encoding predicted proteins that were sequenced from the library either had an unknown or uncharacterized function in any organism or had no matches in the BLAST database, indicating that these proteins may be novel to *Naegleria.*

We have obtained invaluable information regarding the general biological activities of *N. fowleri.* We have sequenced only a small fraction of the *N. fowleri* genome and found potential drug targets and virulence factors of the ameba. The putative genes and proteins that we have characterized may not produce a functional protein as a final product, therefore much more work needs to be performed to determine the expression of these genes in *N. fowleri* and to determine their function.

One of the proteins that we chose to further characterize is a patatin-like serine phospholipase. Patatin was originally described in potato tubers as a storage protein. It was first described to have enzymatic activity and catalyzed the deacylation of a large range of lipid substrates (Gaillard, 1971). Later studies demonstrated the acyl hydrolase activity was due to patatin (Racusen, 1984). Since then, this protein was characterized as having

phospholipase activity on phospholipid and lysophopholipids (Senda et al.1996; Hirschberg et al. 2001).

Patatin also exhibits another type of hydrolytic activity, acidic β-1-3 glucanase activity (Shewry and Lucas 2003). This activity has been shown in the defense of fungal pathogens (Tonon et al. 2001). β-1,3-Glucanases are thought to contribute to plant defense against fungal pathogens by digesting β-1,3-glycans in hyphal cell walls and often form part of the pathogenesis-related protein response (Shewry and Lucas, 1997; van Loon and van Strien, 1999). Other observations for patatin and defense against invading pathogens was provided by experiments where patatin was added to artificial diets of corn and inhibited the growth of the corn rootworm larvae *Diabrotica* spp. (Strickland et al. 1995). Infection of tobacco leaves with tobacco mosaic virus induced the expression of patatin-like proteins, one of which exhibited phospholipase A_2 (PLA_2) activity. The precise role of patatin in potato tubers is unknown. The data indicates that patatin may have a role in plants as a storage protein and also in defense against plant pathogens.

Comparison of patatin with other phospholipases, including human cytosolic PLA_2 ($cPLA_2$), demonstrated that patatin has a serine-aspartate catalytic dyad like human $cPLA_2$ (Hirshberg et al. 2001). The recent crystallization and mutagenesis of patatin from *Solanum cardiophyllum* confirmed the Ser-Asp conserved catalytic dyad, but also revealed that it has a α/β hydrolase fold. The α/β hydrolase family possesses a common core that consists of a well-conserved mixed β sheet, whose strands are interspersed by α helices and employ a catalytic triad similar to the one present in serine proteases (Schrag and Cygler 1997). It has been demonstrated that the folding topology of patatin is different

from the consensus α/β hydrolase fold and is more similar to the human α/β hydrolase topology (Rydel et al, 2003). Patatin is now considered to be a PLA_2 enzyme.

PLA_2 enzymes are classified on the basis of their nucleotide and amino acid sequence, calcium dependency, and subcellular localization. As of October 2000, there were 11 groups, most of which also included several subgroups, but since then, new PLA_2 enzymes have been described, leading to group XII (Gelb et al. 2000; Ho et al. 2001). Groups XIII and XIV, have been added from PLA_2s identified in parvoviruses, symbiotic fungi and *Streptomyces* (Zadori et al. 2001; Girod et al. 2002; Soragni 2001; Sugiyama et al. 2002). A broader classification utilized to divide the PLA_2 classes includes three types: secretory ($sPLA_2$), cytosolic Ca^{2+}-dependent ($cPLA_2$), and cytosolic Ca^{2+}-independent ($iPLA_2$).

PLA_2s have been shown to be activated through cell-signaling mechanisms activated by cell surface membrane receptors (Clark et al. 1991). The majority of studies suggest that these are MAP kinase pathways (Abdullah et al. 1995; Shibutani et al. 1997), however recent evidence suggests that an alternative pathway, phosphoinositide 3-kinase (PI3K) activates PLA_2 through a pathway that does not involve MAPK (Myou et al. 2003).

Phospholipase A_2 (PLA_2) is a family of enzymes that catalyze the hydrolysis of the phospholipid *sn*-2 ester bond, generating a free fatty acid and a lysophospholipid and the free fatty acid is typically arachidonic acid (AA) which produces potent inflammatory mediators called eicosinoids that include prostaglandins, thromboxanes, leukotrienes and lipoxins and are considered key enzymes in the generation of biologically active lipids during inflammation (Murakami et al. 1997; Six et al. 2000; Valentin and Lambeau 2000).

Arachidonic acid is a polyunsaturated fatty acid present in a variety of cell membranes, including those found in the brain (Farooqui and Horrocks 2006). A normal level of arachidonic acid is important for regulating many processes including signal transduction through protein kinases, neurotransmitter release, and neural cell differentiation. When AA is produced in high amounts in the brain, there are profound effects on normal brain function. AA can cause mitochondrial swelling in neurons, activates nuclear factor-κB and decreases neuronal viability. Accumulation of AA can increase levels of reactive oxygen species (ROS), which can in turn modulate cytokine expression and can cause a cascade leading to calcium influx into cells, increased cell permeability, and disruption of membranes and the cytoskeleton (Farooqui and Horrocks, 2006). In addition, AA is converted to eicosanoids (Wolfe and Horrocks, 1994), which are proimflammatory molecules. The generation of eicosanoids in the CNS results in the modulation of cerebrovascular blood flow and platelets and leukocytes, which may lead to CNS dysfunction (Wolfe and Horroks 1994). Eicosanoids in the brain contribute to cytotoxicity, vasogenic brain edema, and neuronal damage (Wolf and Horrocks 1994).

Therefore, we consider that patatin-like PLA_2 in *N. fowleri* acts as a putative virulence factor that may contribute to the invasion of *N. fowleri* into brain tissue as well as the extensive inflammation observed in PAM.

The first bacterial patatin-like protein to be characterized was ExoU in *P. aeruginosa* (Sato and Frank 2004). ExoU has been linked to lung injury, sepsis, and dissemination of the bacteria (Allewelt et al. 2000; Finck-Barbancon et al. 1997; Hauser et al. 1998, and Kurahashi et al. 1999). ExoU is a protein that has a patatin-like

phospholipase domain and has been shown to cause acute lung injury and death by cytotoxicity (Pankhaniya et al. 2004).

In addition, the recent sequencing of several bacterial genomes has led to the discovery that pathogens contain a significantly higher number of patatin-like phospholipases when compared to non-pathogens (Banerji and Flieger 2004). It was first suggested that phospholipases play a role in the pathogenesis of *Rickettsia* in 1992 (Silverman et al. 1992). Since then, the *Rickettsia felis* genome has been sequenced and a patatin-like protein was discovered (McLeod et al.; Ogata et al, 2005). It is thought that this protein is involved in invasion and the pathogenesis of Rickettsial pathogens.

All of the bacterial patatin-like proteins that have been described contain three conserved domains that are required for enzymatic activity (Banerji and Flieger 2004; Blanc et al. 2005). These domains are also found within human $cPLA_2$ and include a glycine rich region near the N-terminus of the protein, a G-X-S-X-G motif that is characteristic of esterases, and an active site aspartate residue that is part of the catalytic dyad. The serine in the ser-asp dyad is located in the G-X-S-X-G motif. Alignment of the *N. fowleri* patatin-like protein with other patatin proteins demonstrates that all of these conserved domains are found in the *N. fowleri* protein.

The patatin protein found in potato tubers is ~40kDa. The coding sequence of the *N. fowleri* patatin is 2,391 base pairs and the protein sequence is 797 amino acids and has a predicted molecular weight of 90.5 kDa. Cytosolic PLA_2s found in humans have molecular weights that vary from 26 to 114kDa (Six et al. 2000). Patatin proteins deposited in the BLAST database also display a wide range of molecular weights. In addition, patatin-like

proteins have been deposited from *T. cruzi*, *E. histolytica*, and *A. castellanii*, all protozoan pathogens. Thus far, these proteins have not been characterized.

Previous studies have demonstrated that *N. fowleri* exhibits phospholipase activity and the activity in *N. fowleri* is higher than that of nonpathogenic *Naegleria* and that phospholipase A activity degrades human myelin and may be involved in the cytolysis of nerve cells in PAM (Hysmith and Franson 1982a; 1982b). In addition, a non-specific inhibitor of phospholipase A blocks the cytolysis of nerve cells in *N.* fowleri cell extracts (Fulford and Marciano-Cabral 1986). Barbour and Marciano-Cabral (2000) have demonstrated that *N. fowleri* express a membrane associated, calcium independent form of PLA_2 and that inhibition of phospholipase A_2 activity results in the decreased release of arachidonic acid. This study also suggests that there are other forms of PLA_2 expressed by *N. fowleri.*

Northern blot and PCR analysis suggests that this protein is unique to *N. fowleri* and is expressed by different strains including LEE, LEEmp, and Northcott. Western blot analysis suggests that *N. fowleri* express multiple forms of PLA_2, including a small molecular weight protein (~20kDa) that may be a secreted form of the enzyme. A smaller product was observed in Northern and Western blot analysis for patatin in *N. lovaniensis*. This indicates that *N. lovaniensis* may have a PLA_2 similar to *N. fowleri*, however the message and protein are different from *N. fowleri* patatin. More studies need to be performed with expression of the recombinant *N. fowleri* patatin protein, production of an antibody, and functional analysis to determine its role in the pathogenesis of *N. fowleri*. It is important to note that *Acanthamoeba castellanii* has a gene encoding a patatin-like

phospholipase, however the gene sequence is not significantly similar to the *N. fowleri* patatin-like protein. The production of an antibody to *N. fowleri* patatin would be useful in determining how similar the *Acanthamoeba* patatin-like phospholipase is to the *N. fowleri* patatin protein. The *Acanthamoeba castellanii* patatin-like phospholipase does contain a glycine rich region, a G-S-X-S-G motif, as well as a conserved DG motif. This indicates that other amebic pathogens may express patatin-like phospholipases that are functionally the same, but distinct in sequence to the *N. fowleri* patatin-like phospholipase.

Collectively, we have described a method for detecting *N. fowleri* in the environment and have demonstrated its ubiquitous nature in the environment. The identification of *N. fowleri* in domestic water indicates a need for standard testing of water to determine its presence and the need for water chlorination. In addition we have identified putative drug targets and virulence factors of the ameba. Sequencing of a *N. fowleri* genomic DNA library has provided valuable information for future study of this organism.

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