CHARACTERIZATION OF THE AMEBAE ISOLATED FROM THE FIRST CONFIRMED CASE OF PRIMARY AMEBIC MENINGOENCEPHALITIS IN OKLAHOMA

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LIST OF ABBREVIATIONS

CDC	**Centers for Disease Control and Prevention**
CNS	**central nervous system**
CSF	**cerebrospinal fluid**
MIC	**minimal inhibitory concentration**
MTD	**mean time to death**
NBF	**neutral buffered formalin**
PAM	**primary amebic meningoencephalitis**

CHAPTER 1

INTRODUCTION

1.1 Background

Free-living amebae are normally found in soil and freshwater sources worldwide, including sources in Oklahoma (John and Howard, 1995). Most species of amebae are part of the normal fauna, which feed on bacteria and other aquatic microorganisms. Several genera, including *Naegleria, Acanthamoeba, Balamuthia,* and *Sappina,* include species that are able to cause infection in humans and other animals. They are termed opportunistic pathogens since they are able to live as either free-living organisms or as parasites in a host. *Naegleria fowleri* is the causative agent of a rapid infection of the central nervous system (CNS) that is almost always fatal. It is called primary amebic meningoencephalitis (PAM). *Acanthamoeba* species cause a chronic form of PAM, known as granulomatous amebic encephalitis (GAE) as well as a very serious eye infection, called *Acanthamoeba* keratitis, which can cause blindness and is usually attributed to contaminated contact lens solutions (John, 1993). *Balamuthia mandrillaris* causes a form of GAE similar to that produced by *Acanthamoeba* (John, 2005). *Sappina diplodea* has recently been implicated in causing amebic encephalitis similar to PAM (Gelman et al., 2001).

In 1958, Culbertson and his colleagues first noticed the disease-causing potential of *Acanthamoeba* when they identified a contaminant destroying cell cultures. Animal models proved to be susceptible to a fatal infection when the amebae were introduced

intracerebrally, intranasally, and intravenously (Culbertson et al., 1958). However, it wasn't until 1965 that the first reported human cases appeared. These cases occurred in Australia (3 children and 1 adult) with all succumbing within 4-5 days of the appearance of symptoms (Fowler and Carter, 1965). Although no amebae were cultured from tissue, histological findings indicated amebae, tentatively identified as an *Acanthamoeba* species, as the causative agent. It was later positively identified as *Naegleria fowleri*. In 1966, the first reported cases occurred in the United States. Butt (1966) reported 3 cases in Florida, which were very similar to the Australian cases. All of the individuals had a history of swimming in fresh water before becoming symptomatic. He recognized that he was dealing with a new disease and called it primary amebic meningoencephalitis.

1.2 Taxonomy and Biology of *N. fowleri*

Naegleria spp. exist in three different forms; the trophozoite stage, flagellate stage, and cyst stage. Other ameba species have only two forms, the trophozoite and cyst stages. The trophozoite stage is the reproductive stage and has what is considered the "normal" amoeboid shape, which is the limax form, from the Latin word meaning slug. Trophozoites of different *Naegleria* species are very similar, being elongate and moving in a directional manner with eruptive pseudopodia (John, 1993). Trophozoites of *Naegleria* feed on bacteria and other organic matter by phagocytosis and exhibit aerobic respiration by their mitochondria.

The flagellate stage occurs when the organisms are exposed to ionic concentration changes in their environment, such as distilled water (Cable and John, 1986). Typical *Naegleria* flagellates are cigar or pear-shaped with one or two flagella extending from the anterior rostrum (Figures 12 and 13). Most *N. fowleri* flagellates will have two flagella,

while other species, such as *N. australiensis* may have as many as 8 flagella (John et al., 1991). Flagellates will revert back to the trophozoite stage since the flagellate is a non-feeding, non-reproductive stage.

During unfavorable environmental conditions (lack of food, cold temperatures, drought) the amebae will encyst (Figure 11). Cysts of *N. fowleri* are often spherical and clumped together. Diameter range is typically 7-15 μm (Page, 1988). They will excyst when conditions are again favorable to feed and reproduce.

N. fowleri cycles through all three of these stages to increase its chances for survival. However, it is only the trophozoite stage which is infective, producing the fatal CNS infection called PAM (John, 1993; John, 2005).

1.3 Primary Amebic Meningoencephalitis (PAM)

PAM occurs most often in healthy children or young adults who have a recent history of swimming in freshwater contaminated with *N. fowleri* (John, 1993). The amebae gain entry to the nasal cavity during swimming when droplets of contaminated water are inhaled or aspirated (John, 2005). The organisms penetrate the mucosa and migrate through the cribriform plate via the olfactory nerves and into the olfactory bulb of the brain (Jarolim et al., 2000). Once inside the CNS, they spread to more posterior regions of the brain. Within the brain, the amebae cause extensive inflammation, necrosis and hemorrhage. (John, 2005)

1.3.1 Clinical Features

The clinical course of PAM is rapidly dramatic and ultimately fatal. Initial symptoms include severe frontal headache with fever (39-40°C), anorexia with nausea and vomiting, and frequently positive Kernig's sign (an indication of meningeal

irritation) (John, 2005). Since the olfactory lobe is the first area of the brain affected, there are frequently changes in smell or taste noted early in the infection, even before the onset of headache and fever (Martinez, 1985). Visual disturbances may occur, along with irritability, restlessness, confusion, and generalized seizures, prior to lapsing into a coma. Death ensues due to pulmonary edema and cardiorespiratory arrest, usually within 3-7 days from the onset of symptoms (Parija et. al., 1999).

1.3.2 Epidemiology

Cases of PAM have been reported worldwide, including the United States (Parasitic Disease, 2002). In the late 1960s, 14 of 16 cases of PAM were identified in individuals who swam in 2 man-made lakes in Richmond, Virginia (Callicott, 1968). Also in the late 1960s, 16 young people died after swimming in the same chlorinated, heated indoor swimming pool in the Czech Republic (Cerva et al., 1968). Similar cases have been reported following swimming in pools, lakes, and streams from around the world in Australia, Belgium, England, and New Zealand and across the United States in Arkansas, Missouri, Texas, and others (Carter, 1970; Duma and Rosenblum, 1971). Prior to August of 1998, there had been no confirmed case of PAM in the State of Oklahoma (John and Howard, 1999).

1.3.3 Treatment

The work of Goswick and Brenner (2003b) is probably the most current with very interesting information regarding a new method of treatment of PAM. In this case, the use of azithromycin seemed to be much more effective in combating the pathogen, *N. fowleri*, than does the accepted method of using amphotericin B (Martinez, 1985). This could indicate a major breakthrough in the ability to successfully treat the disease. Much

more research will need to be done in this area before it becomes the accepted standard of care.

Martinez (1985) gives a thorough account of the use of amphotericin B as a chemotherapeutic agent. Seidel et al. (1982) have relevant information dealing with one of two cases of PAM which were treated successfully. With reference to the Seidel case, it is possible that success wasn't so much the drug used to treat the disease, but the aggressive and rapid form the treatment took. The little girl was treated immediately and thoroughly for PAM. Perhaps, if other cases were treated as such, there would be a higher success rate for this treatment.

1.4 Sequence of Events

On August 9, 1998, a 3-year-old girl died of PAM in Tulsa, the first confirmed case of the disease in Oklahoma (Primary Amebic Meningoencephalitis, 2001). The course of the disease was similar to that described above, in which the little girl played in water at the edge of a local lake, where she presumably inhaled some water containing the amebae, 4 days prior to the onset of symptoms. After her death, an autopsy was performed by the State Medical Examiner's Office in Tulsa, and a diagnosis of PAM was made (John and Howard, 1999). On August 13, 1998 the girl's brain tissue was received from the Medical Examiner's to test for the presence of amebae. The tissue was processed, placed in appropriate culture media and incubated at 37°C (John and Howard, 1993). The following Monday, after having incubated for less than 72 hours, the cultures were positive for the presence of amebae. This ameba isolate has been given the identification code HBT1-1998, which stands for human brain isolate from Tulsa, the

first one (1), which occurred in 1998, a designation recommended by the Centers for Disease Control and Prevention (CDC), Atlanta, Georgia.

1.5 Significance of Study

This is the first confirmed case of PAM in Oklahoma, which is one reason it is important to learn as much as we can about HBT1-1998. It is also one of few cases where amebae were positively cultured from the brain tissue of the victim. Usually, diagnosis is based on disease course and microscopic examination of brain tissue (post-mortem) stained with hematoxylin and eosin (H & E) and by the indirect immunofluorescent assay. Amebic infection can be diagnosed by trained personnel simply by observing a wet-mount of cerebrospinal fluid (CSF) if one knows to look for it. However, clinically, PAM closely resembles fulminating bacterial meningitis and the laboratory findings are similar. Therefore, PAM usually is not considered until discovered during autopsy (John, 2005).

Another reason it is important to classify and study HBT1-1998 carefully is the more we learn about this particular organism, known to be responsible for the death of a young girl, the better chances will be of saving a life in the future. This research project, entitled "Characterization of the amebae isolated from the first confirmed case of primary amebic meningoencephalitis in Oklahoma", will clearly show that the amebae cultured from the brain tissue of the 3-year-old victim were *Naegleria fowleri*. The purpose of classifying the organism thusly can have a great impact on outdoor water activities (recreation and tourism) and public health issues in Oklahoma. The Oklahoma State Department of Health has posted information on their website advising people when and how to take precautions against contracting this infection (Primary Amebic

Meningoencephalitis, 2001). Though they do make statements about the rarity of this disease, it is at least an attempt to make people aware. As of yet, sites that were determined to be positive for *N. fowleri* have not been posted by agencies in charge, though they have been made aware of the presence of these organisms at the locations. At least, as a result of this first Oklahoma case, the medical community has become more aware of the disease. This can be important in that they can be more aggressive in testing for the presence of amebae in CSF of individuals with unknown meningeal symptoms and the subsequent treatment of PAM.

Based on the typical disease course of this particular case of PAM and the initial testing of HBT1-1998, all indications are that this particular organism will prove to be *N. fowleri*. This was verified with further confirmatory testing. In the event that it is not confirmed, then the hypothesis that HBT1-1998 is *N. fowleri* would be false. This would not mean that all the work is for naught. Rather, it would support the primary aspect of the research proposal, "Characterization of the amebae isolated from the first confirmed case of primary amebic meningoencephalitis in Oklahoma". For instance, if further testing indicated the amebae were *N. lovaniensis* instead of *N. fowleri*, then that would be the result of the characterization. These two species of *Naegleria* were considered to be identical (John, 1993). The fact that they are very similar puts certain limitations on the project. These limitations deal with showing conclusively that there is a difference between HBT1-1998 (presumably *N. fowleri*) and *N. lovaniensis*. By utilizing specific experimental tests (IIF, antimicrobial sensitivity, and others), HBT1-1998 will be shown to be *N. fowleri*.

In this introduction, information has been provided about the basic life processes of free-living amebae and related information with regard to their potential pathogenicity, giving specific examples of documented cases of the disease PAM. Next, the specifics about the particular research project dealing with the first confirmed case of PAM in Oklahoma were given. The following section will detail the experimental methods used to complete the project and confirm the hypothesis. Included in this are the statistical techniques used to support the findings.

CHAPTER 2

RESEARCH DESIGN AND METHODOLOGY

2.1 Methods and Techniques

The aim of this project was to accurately characterize the ameba species, hereafter referred to as HBT1-1998, isolated from the brain tissue of a 3-year-old girl who died from the first confirmed case of primary amebic meningoencephalitis (PAM) in Oklahoma. Standard laboratory methods were used as well as experiments aimed at specifically identifying the species of ameba. The general tests pertinent to ameba identification include the following criteria: growth in axenic media, Mix, Nelson's, and Balamuth's, at 23°C, 30°C, 37°C and 42°C, for 7 days with comparison growth for *N. fowleri*, Lee strain at 23°C, 30°C, 37°C and 42°C and *N. gruberi*, EGB strain at 23°C and 30°C, the highest temperature at which the non-pathogenic *Naegleria* will grow; morphology and ameba characteristics by staining for measuring, logging data and taking photomicrographs of trophozoites, cysts and flagellates; concanavalin A agglutination; virulence of HBT1-1998, including percent mortality per inoculum, mean time to death (MTD), and the inoculum for 50% death (LD_{50}); indirect immunofluorescence antibody titers (IIF) of HBT1-1998 amebae vs. species specific *Naegleria* antisera; histology of infected mice showing the presence of amebae in the nasal mucosa early in the infection and subsequently in brain tissue later during the course of the infection; and the effectiveness of the antimicrobials amphotericin B and azithromycin on the growth of HBT1-1998 *in vitro*.

2.2 Amebae Cultivation

HBT1-1998 was cultivated axenically, without agitation, in three different media - Balamuth's medium (Balamuth, 1964), Nelson's medium (Weik and John, 1977), and Mix medium (John, 1993), which is an equal mixture of Balamuth's and Nelson's media consisting of 0.55% Panmede Liver Digest (Oxoid Ltd., Hampshire, England), 0.5% Bacto Proteose Peptone (Becton, Dickinson and Co., Sparks, Maryland), 0.25% Difco Yeast Extract (Becton, Dickinson and Co., Sparks, Maryland), and 0.3% glucose in Page's ameba saline (0.12 g NaCl, 0.004 g $MgSO_4 \cdot 7H_2O$, 0.004 g $CaCl_2 \cdot 2H_2O$, 0.142 g Na_2HPO_4, and 0.136 g KH_2PO_4 per liter of distilled water)(Page, 1988), supplemented with 4% bovine calf serum (Sigma Chemical Co., St. Louis, Missouri) and 1μg/ml hemin (Sigma Chemical Co., St. Louis, MO). All components were steam sterilized by autoclave except the bovine serum and hemin, which were sterilized by filtration. The amebae were cultivated in 25-cm^2 polystyrene Falcon tissue-culture flasks (B and D Labware, Franklin Lakes, New Jersey), and each contained 10 ml of medium with an initial inoculation of 10^4 amebae/ml.

2.3 Cell Counting

Amebae were counted with a Coulter Counter (Model Z_F, Coulter Electronics, Inc., Hialeah, FL) using the following settings: 1/amplification, 4; 1/aperture, 1; threshold, 10; sample volume, 500μl. The electrolyte used to suspend amebae for counting consisted of 0.5% (v/v) formalin, and 0.4% (w/v) sodium chloride in deionized water. Each trial was performed in triplicate, with each of the 3 flasks of the triplicate trial read 4 times. The most atypical reading was excluded and the other 9 readings were

averaged (3 readings each of 3 flasks for each medium per growth temperature) (John and Howard, 1993).

2.4 Growth Studies

HBT1-1998 was cultivated axenically, without agitation, in three flasks each of the three different media at 23°C, 30°C, 37°C, and 42°C. The counts were performed every 24 hours for a period of 96 hours, and then at 168 hours. The average counts for each trial were plotted on a semi-log growth curve.

N. fowleri, Lee strain, and *N. lovaniensis*, Aq/9/1/45 D strain, at 23°C, 30°C, 37°C, and 42°C and *N. gruberi*, EGB strain at 23°C and 30°C, were cultivated in like manner to compare the growth of HBT1-1998 to both known pathogenic and non-pathogenic species of *Naegleria*.

2.5 Ameba Morphology and Characteristics

Morphology of trophozoites, cysts, and flagellates is an important first step in characterization of an ameba species. While determining the most appropriate growth conditions will enhance our ability to cultivate the ameba *in vivo*, morphology is the first indicator of what genus of ameba one may be observing. For example, *Acanthamoeba* spp. have characteristic spiky pseudopodia whereas *Naegleria* spp. have smooth pseudopodia and the trophozoites are limax in appearance. *Naegleria* spp. also produce flagellates (Page, 1988); Cyst size and shape can be diagnostic for a specific genus of ameba when comparing sizes and shapes.

2.5.1 Trophozoites and Cysts

Trophozoites were grown on sterile coverslips in Bellco Leighton tubes to the exponential growth phase and then fixed in 100% methanol before being stained with

iron hematoxylin stain. These were observed and measured microscopically at 1000X magnification using an AO light microscope and ocular micrometer. Trophozoites were measured in length and width, with 100 amebae being measured. The average of the measurements and standard deviations were calculated for the trophozoites as well as for cysts.

Average cyst size was calculated based on the measurements of the diameters of 100 cysts at 1000X magnification under oil using the AO Microscope and Leitz Wetzlar ocular micrometer. Cysts were applied to poly-l-lysine coated slides, fixed and stained in the same manner as the flagellates. Cysts were observed microscopically and photographed in order to note their particular shape and structure.

2.5.2 Flagellates and Enflagellation

The enflagellation procedure of Cable and John (1986) was followed with some minor changes. Amebae were grown axenically in a tissue culture flask in Mix medium at 37°C, as in the growth studies, to the log phase of growth, approximately 60 hours. Culture medium was aspirated and the adherent amebae were gently washed three times with Page ameba saline which had been warmed to 37°C. After the third wash, amebae were suspended in 5 ml of cold (4°C) ameba saline. A cell count was made and the concentration of the suspension was adjusted to 1×10^6 amebae per ml with cold ameba saline. The final cell suspension was transferred to a 125-ml Erlenmeyer flask and agitated at 100 rpm at 37°C in a G24 Environmental Incubator Shaker (New Brunswick Scientific, Edison, NJ).

Flagellate counts were taken hourly to determine the point of maximum transformation. Time zero was defined as the point when the amebae were suspended in

the cold ameba saline. Each hour, for 8 hours, 3 drops of ameba suspension were fixed and stained with 1 drop of D'Antoni's iodine. One to 2 drops of the fixed suspension was placed on a hemacytometer slide with a coverslip and counts were taken using a phase contrast, compound light microscope (Carl Zeiss, West Germany). At least 100 cells per sample were counted to determine the percent flagellates. Cells were considered to be flagellates if flagella, in any length or number regardless of body shape, were present (Cable and John, 1986; John et al., 1991).

This procedure was repeated. The second time, at the predetermined point of maximum transformation, 10 ml of amebae was fixed, with agitation, at room temperature by the addition of 10 ml of Schaudinn's fixative working solution. The fixation process was allowed to proceed overnight. The final fixed suspension of flagellates was harvested by centrifugation (2000 x g for 10 minutes at 23°C). The pellet was suspended in 15 ml of iodine/alcohol solution (3%/70%). This suspension was allowed to stay at room temperature for 5-10 minutes. The stained flagellates were harvested by centrifugation as previously described. The pellet was then suspended in 70% ethanol for 5-10 minutes. The flagellates were once again harvested by centrifugation and suspended in a distilled water wash. A final centrifugation was preformed and the pellet was suspended in 5 ml of distilled water.

The fixed, stained, and washed flagellate preparation was applied to poly-l-lysine coated 10 x 35 mm coverslips and allowed to remain at room temperature for 10 minutes. Excess solution was shaken from the coverslips and they were placed in vials containing a working solution of iron hematoxylin stain for 4-5 minutes. The coverslips were transferred to a vial of tap water, with a gentle flow of water, for 2 minutes. Coverslips

were dehydrated through a series of ethyl alcohol solutions in increasing concentrations (30%, 70%, 95%, and 100%). Coverslips equilibrated in each solution for at least 2 minutes before being transferred to the next. Dehydrated coverslips were then transferred to a vial of xylene. They remained in xylene until they were applied to 1 x 3 inch slides with Permount®.

Slides were examined at 1000X magnification under oil using an AO Microscope equipped with a Leitz Wetzlar (Germany) ocular micrometer, calibrated with an American Optical slide (stage) micrometer. One hundred flagellates were observed for number of flagella present and their lengths measured.

2.5.3 Concanavalin A Agglutination

Concanavalin A (Con A) is a plant lectin which can cause certain species of amebae to agglutinate. Pathogenic *Naegleria* spp., such as *N. fowleri*, will not agglutinate in the presence of Con A, whereas non-pathogenic *Naegleria* spp., like *N. gruberi*, will.

HBT1-1998 was inoculated into 75 cm^2 tissue culture flasks (T75s) containing 50 ml of Mix medium as per the growth studies. After 72 hours of growth, the medium was aspirated and the sheet of cells was washed 3 times with Page's ameba saline. The washed amebae were harvested by centrifugation (2000 x g; 10 minutes; 23°C) and suspended in phosphate buffered saline (0.15 M NaCl, 0.01 M phosphate buffer) to a concentration of 1 x 10^6 amebae per ml. Con A agglutination was accomplished by the addition of concanavalin A, Grade IV (Sigma Chemical Co., St. Louis, Missouri) at a concentration of 100μg Con A/ml and incubated with agitation at 37°C for 30 minutes (John and Howard, 1996; Josephson et al, 1977). HBT1-1998 was observed for

agglutination using a Nikon, phase-contrast, inverted microscope (model #TMS-F). Cultures of *N. fowleri* Lee strain and *N. gruberi* EGB strain were processed in a similar manner for comparison. A negative control for each strain was included without Con A.

2.6 Histology

Histology of the brain tissue of mice was performed following the methods of Jarolim, et al (2000). HBT1-1998 was cultivated axenically in Mix medium to early stationary-growth-phase (John, 1993), harvested by centrifugation (1200 x g, 10 min, at 23°C), washed three times in Page's ameba saline, and suspended in Page's saline to a concentration of 1 x 10^6 amebae per 10 μl. Cell counts were made with a Coulter counter (model Z_{BI} Coulter Electronics, Hialeah, Florida) using settings previously described (John and John, 1989).

The mice used were 21-day-old, male, CD-1 outbred strain from Charles River Laboratories, Inc. (Wilmington, MA). The mice were allowed to adjust to their new environment for 2-3 days before experimentation and were given free access to water and feed (Purina Lab Chow, Ralston Purina Corp., St. Louis, MO).

Infection of the mice with HBT1-1998 was accomplished by intranasal (i.n.) instillation of a 10 μl suspension of amebae in Page's saline. Mice were anesthetized (Metofane, Pitman-Moore, Inc., Washington Crossing, NJ) and a single 10 μl drop containing the desired concentration of amebae was introduced into the left nostril using an Eppendorf pipet (Brinkman Instruments, Inc., Westbury, NY) (Bush and John, 1988; John and Howard, 1993). The right nostril was not inoculated. The mice were separated into groups of 3 and killed at time periods of 24, 48, 72, 96, and 120 hours post-inoculation.

Mice were lightly anesthetized with Metofane, and quickly decapitated. Skin and musculature was removed from the heads, the caudal aspect of the skull was opened and the heads were drop fixed for 72 hours in 10% neutral buffered formalin (NBF). Following fixation, remaining skin and muscle tissue was removed and the heads were decalcified in a solution of trisodium citrate and formic acid for 48 hours (Jarolim et al. 2002). Next, the decalcified heads were divided in a mid-sagittal plane with a razor blade. The cribriform plate was identified and a rectangular block of tissue was made by cutting away the posterior of the brain and a small portion of the anterior portion of the brain. The blocks of tissue included the olfactory bulb and a portion of the nasal cavity on each side of the cribriform plate. The tissue blocks were equilibrated in 5% trisodium citrate, washed in water, then dehydrated and embedded in paraffin. Serial sections were cut at 8 μm for light microscopy and the sections were stained with a combination of celestin blue, Harris hematoxylin, and acid fuchsin (CHF), modified from Minamisawa et al. (1990) and Auer et al. (1984). Observations were made and photographs were obtained digitally using a Boreal Digital Microscope, model number WLS48149-DXW (Science Kit and Boreal Laboratories, Tonawanda, NY) and Motic Images 2000 (Motic China Group Co., Ltd.)

2.7 Virulence

Virulence of a pathogenic ameba is a definitive indicator as to its species. *N. fowleri* will demonstrate close to 100% mortality when inoculated intranasally into mice, whereas other species of pathogenic *Naegleria* (*N. australiensis, N. lovaniensis)* may show a 50% or less mortality when inoculated into mice. The MTD can also be an important indicator as to species. For example, *N. fowleri* infection will routinely run a

disease course with a MTD of 6 days or less post exposure. *N. australiensis* infections may result in a MTD of 13 days or longer (John and Howard, 1996).

In the virulence study of HBT1-1998, amebae were cultivated as described in the histology portion of this section. Mice were anesthetized as before and a single 10 μl drop containing the desired concentration of amebae was introduced into the left nostril using an Eppendorf pipet (Brinkman Instruments, Inc., Westbury, NY) (Bush and John, 1988, and John and Howard, 1993). Inocula included a 0% and a 100% mortality concentration in order to determine the LD_{50} (Reed and Muench, 1938). The low dose inoculum was $1x10^1$ amebae per mouse (the 0% dose), then $1x10^2$, $1x10^3$, $1x10^4$, and finally $1x10^5$ amebae per mouse needed for the 100% mortality dose. For each inoculum, 3 groups of 10 mice were used (30 mice were infected with $1x10^1$ amebae, 30 mice with $1x10^2$ amebae, etc.). A control group was given a 10μl drop of Page's saline per mouse, i.n. For each inoculum, MTD in days was plotted with error bars. The percent mortality was plotted as a function of the inocula.

2.8 Indirect Immunofluorescence (IIF)

IIF is an accurate and inexpensive method used for identifying ameba species. HBT1-1998 amebae were cultivated in Mix Medium to log phase at a concentration of 1×10^6 amebae per ml at 37°C. 10 μl aliquots were applied to individual wells on Bellco 12-well multiwell slides (Cel-Line/Erie Scientific Co., Erie, Pennsylvania). The slides were incubated in moist chambers at 37°C for 30 minutes in order for the amebae to attach to the slides. After incubation, medium was absorbed and the slides were immediately immersed in a 2% formalin-anhydrous methyl alcohol fixative solution for 5-10 minutes. Each slide was rinsed in 3 consecutive solutions of fresh phosphate

buffered saline (PBS) (8.0g NaCl, 0.2 g KCl, 1.13g Na_2HPO_4 (dibasic), and 0.2g KH_2PO_4 (monobasic) per liter of distilled water) with a final rinse in distilled water. Excess moisture was blotted from the slides, which were air-dried and then either assayed immediately or stored at -20°C (John et al., 1998).

Rabbit antisera to specific ameba species were used for identification. For this project, antisera developed against *N. fowleri, N. lovaniensis, N. australiensis,* and *N. gruberi* (John et al., 1998) were used. Rabbit anti-ameba serum to each of these four *Naegleria* species was diluted serially 2-fold in PBS in a 96 well plate beginning with a 1:2 dilution. A 10µl drop of each antiserum dilution was applied to appropriately labeled wells on the prepared slides of HBT1-1998. Slides were incubated in a moist chamber at 37°C for 30 minutes. Each slide was then rinsed in three consecutive PBS rinse solutions. At this point, the fluorescent conjugate was applied to each well. Fluorescein isothiocyanate (FITC) conjugated goat anti-rabbit immunoglobulin (IgG) (Jackson ImmunoResearch Laboratories, Inc., West Grove, Pennsylvania) diluted 1:50 in PBS was used. After applying 10µl of the diluted conjugate to each well, the slides were incubated at 37C for 30 minutes. Slides were rinsed as before with PBS and counterstained with Evans blue, diluted 1:1200, rinsed in tap water, and air dried. Mounting media was applied to each well and coverslips attached. Slides were stored in the dark when not in use.

The finished slides were examined by epifluorescence using a Leitz Orthoplan fluorescent microscope equipped with an Osram HBO short arc mercury vapor lamp. The degree of fluorescence was scored from 1^+ to 4^+, with 4^+ denoting the brightest

apple-green fluorescence characteristic of FITC. The endpoint titer was the final dilution of each antiserum tested which produced a 1^+ fluorescence.

2.9 Antimicrobial Sensitivity

Based on the methods of Goswick and Brenner (2003a,b), the effectiveness of amphotericin B and azithromycin against HBT1-1998 were tested. Their work indicated that the LEE strain of *N. fowleri* was more susceptible to azithromycin, more so than to amphotericin B. The LEE strain, like HBT1-1998, is also a human isolate. This served as a good indicator that HBT1-1998 would be sensitive to treatment with azithromycin.

LEE stock and HBT-1-1998 were each tested in this drug study. 30 ml of Mix medium was inoculated with 1 x 10^4 amebae/ml of actively growing stock-cultures of either the LEE stock or HBT1-1998. A 0.2ml aliquot of diluted amphotericin B or azithromycin solution was added to experimental flasks to obtain the required drug concentrations, while control flasks received the same 0.2ml volume of sterile deionized water. Antibiotics were tested at 3 different concentrations; amphotericin B at 0.01 μg/ml, 0.1 μg/ml, and 1μg/ml, and azithromycin at concentrations of 0.01μg/ml, 0.05 μg/ml, and 0.1μg/ml with each concentration tested in triplicate.

The flasks containing amebae and experimental agent combination were vortexed, 10-ml aliquots distributed to each of three culture flasks and incubated at 37°C. Cell growth was determined daily for a period of 4 days and then again at day 7 with a Coulter Counter (model Z_F; Coulter Electronics, Inc., Hialeah, Florida). A 0.2ml aliquot of each cell suspension was added to 9.8ml of electrolyte solution containing 0.5% (v/v) formalin and 0.4% (w/v) NaCl in deionized water. Cuvettes were vortexed to separate cell aggregates and then read within 5 minutes. Four successive counts were obtained for

each cuvette. The most deviant count was excluded, and the means of the remaining nine counts (three flasks of three counts each) were determined, and ameba growth was expressed as the number of amebae per milliliter. Ameba concentrations were plotted and compared with the Student's *t* test to determine if the differences between control and treated groups were significant. The minimum inhibitory concentration (MIC) of each agent was defined as the lowest concentration of drug that significantly ($P < 0.01$) inhibited ameba growth as compared to control cultures throughout the 7-day culture period as determined by the Student's *t* test.

CHAPTER 3

RESULTS

3.1 *In Vitro* Studies

The *in vitro* studies were performed in order to obtain specific growth requirements for ameba HBT1-1998 and for comparisons to other known species of *Naegleria*. All other *in vitro* experiments utilized cultures of HBT1-1998 that had been maintained at these optimum conditions.

3.1.1 Growth of HBT1-1998 and Known *Naegleria* spp

Pilot studies were conducted to determine the optimal growth conditions for ameba HBT1-1998 as well as to compare similarities in these growth conditions to known species of *Naegleria*. Growth conditions considered were the type of culture medium and different incubation temperatures. Culture media tested included Nelson's, Balamuth's, and Mix media. Incubation temperatures tested included 23°C, 30°C, 37°C, and 42°C. Cultures in each media type were tested at each temperature to optimize growth conditions. Other species of *Naegleria* that were tested for comparison were *Naegleria fowleri* Lee ATCC 30894 in all three media at all 4 temperatures, *Naegleria lovaniensis* in Mix medium only at all 4 temperatures, and *Naegleria gruberi* EGB strain in Mix only at 23C and 30C. *N. gruberi* is a non-pathogen normally found in the environment and will not grow at the higher temperatures.

As shown in Figure 1, HBT1-1998 had moderate growth in Nelson's medium at 42°C. In Figure 2, growth patterns in Balamuth's medium at 42°C were improved over

that which was seen in Nelson's medium with a slightly greater concentration by day 7 (4 x 10^5 amebae/ml in Balamuth's vs. 2 x 10^5 amebae/ml in Nelson's). There was marked improvement however in the 30°C and 37°C cultures in Balamuth's over the Nelson's with concentrations greater than 1 x 10^5 amebae/ml. Finally, in Mix medium (Figure 3), there was a dramatic increase in concentration of the amebae by day 4 in all temperatures except 23°C. Standard errors for each data point in all three studies were negligible. Amebae counts were extremely consistent for each flask on a daily basis.

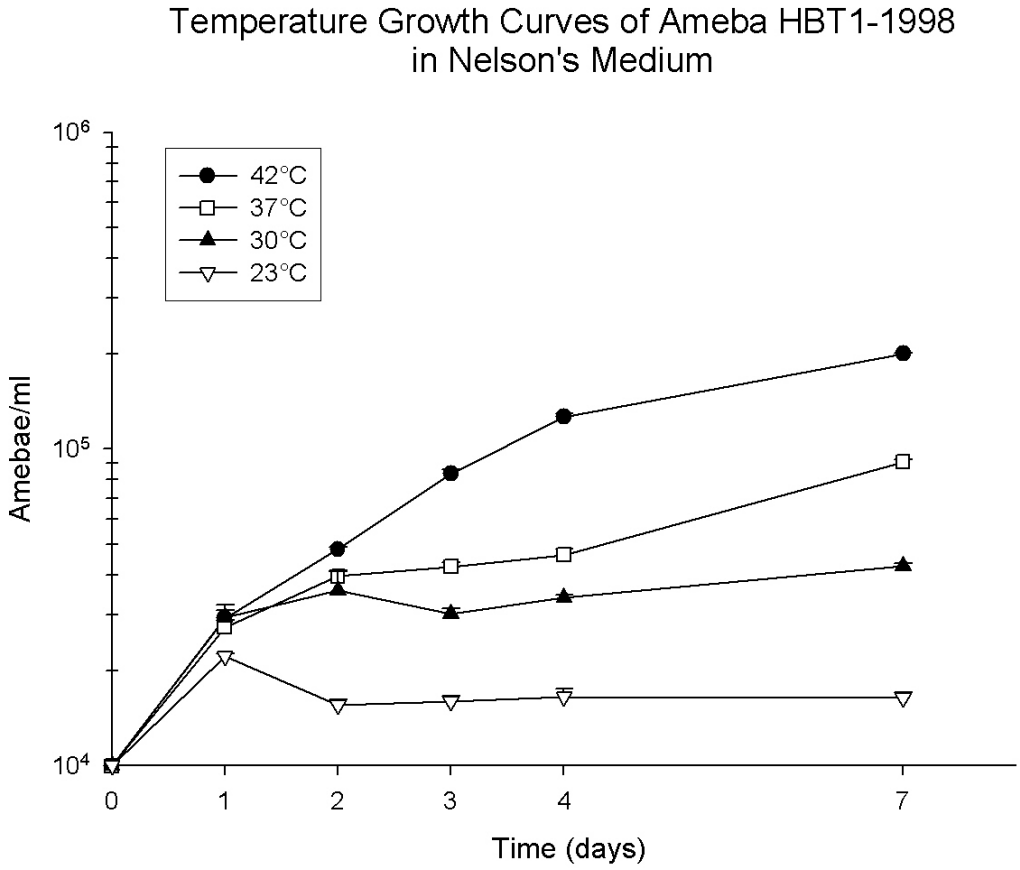

Figure 1. Growth of ameba HBT1-1998 incubated in Nelson's medium at different temperatures over a 7-day period. The data points represent the results of one experiment performed in triplicate. The error bars represent the standard error of the mean for the individual data points.

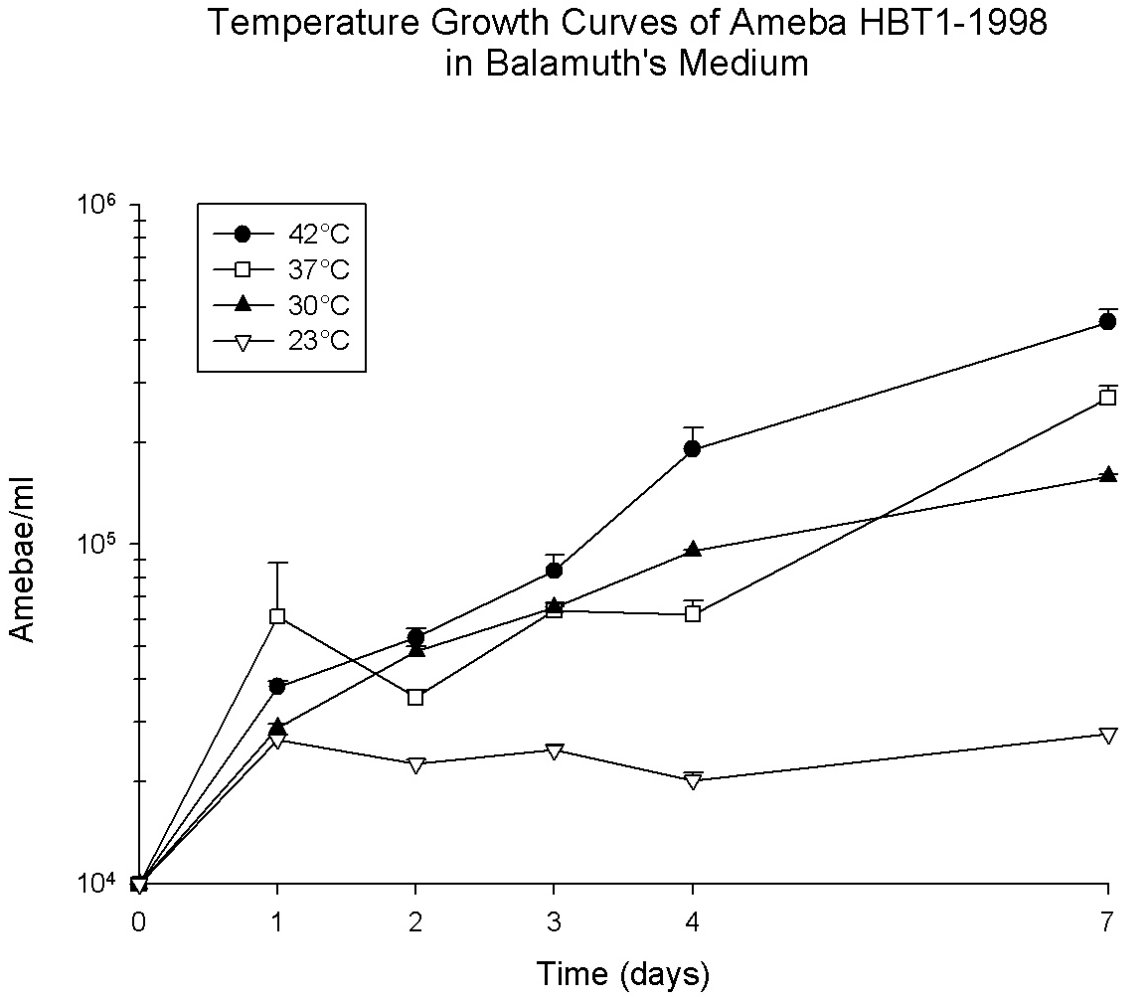

Figure 2. Growth of ameba HBT1-1998 incubated in Balamuth's medium at different temperatures over a 7-day period. The data points represent the results of one experiment performed in triplicate. The error bars represent the standard error of the mean for the individual data points.

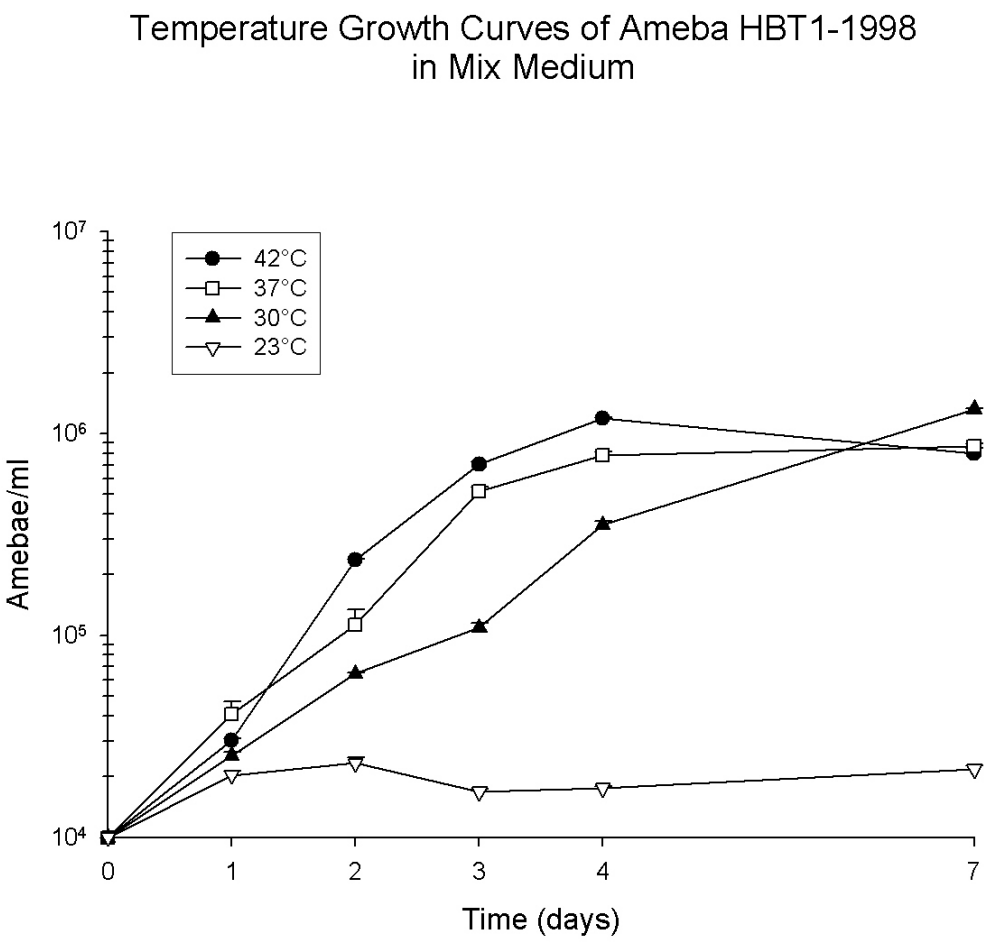

Figure 3. Growth of ameba HBT1-1998 incubated in Mix medium at different temperatures over a 7-day period. The data points represent the results of one experiment performed in triplicate. The error bars represent the standard error of the mean for the individual data points.

N. fowleri (Lee strain) demonstrated very good growth in Nelson's medium (Figure 4) at 30°C and 37°C by day 4 and moderate growth in Balamuth's medium (Figure 5) at 30°C, 37°C, and 42°C by day 4. In Mix medium, Figure 6, the *N. fowleri* control culture showed excellent growth, greater than 1 x 10^6 amebae per ml by day 4, in 30°C, 37°C , and 42°C, with the 37°C surpassing the growth of the other temperatures.

N. lovaniensis and *N. gruberi* were studied as well to compare the growth characteristics of ameba HBT1-1998 with that of another pathogen, *N. lovaniensis*, and a non-pathogen, *N. gruberi*. The results of their growth studies can be seen in Figure 7 and Figure 8. The final results of the growth studies are tabulated in Table I, indicating temperatures at which each species of *Naegleria* tested can grow.

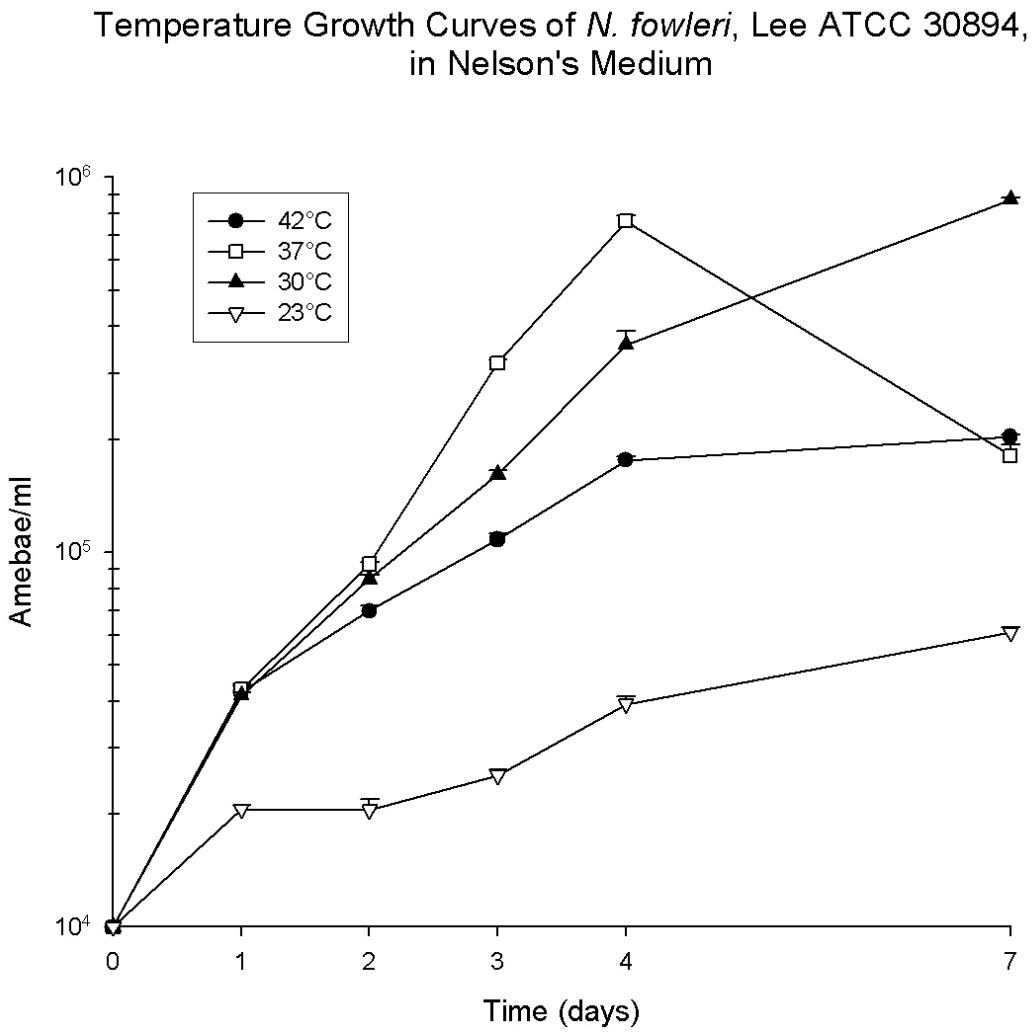

Figure 4. . Growth of *N. fowleri*, Lee strain (ATCC 30894) incubated in Nelson's medium at different temperatures over a 7-day period. The data points represent the results of one experiment performed in triplicate. The error bars represent the standard error of the mean for the individual data points.

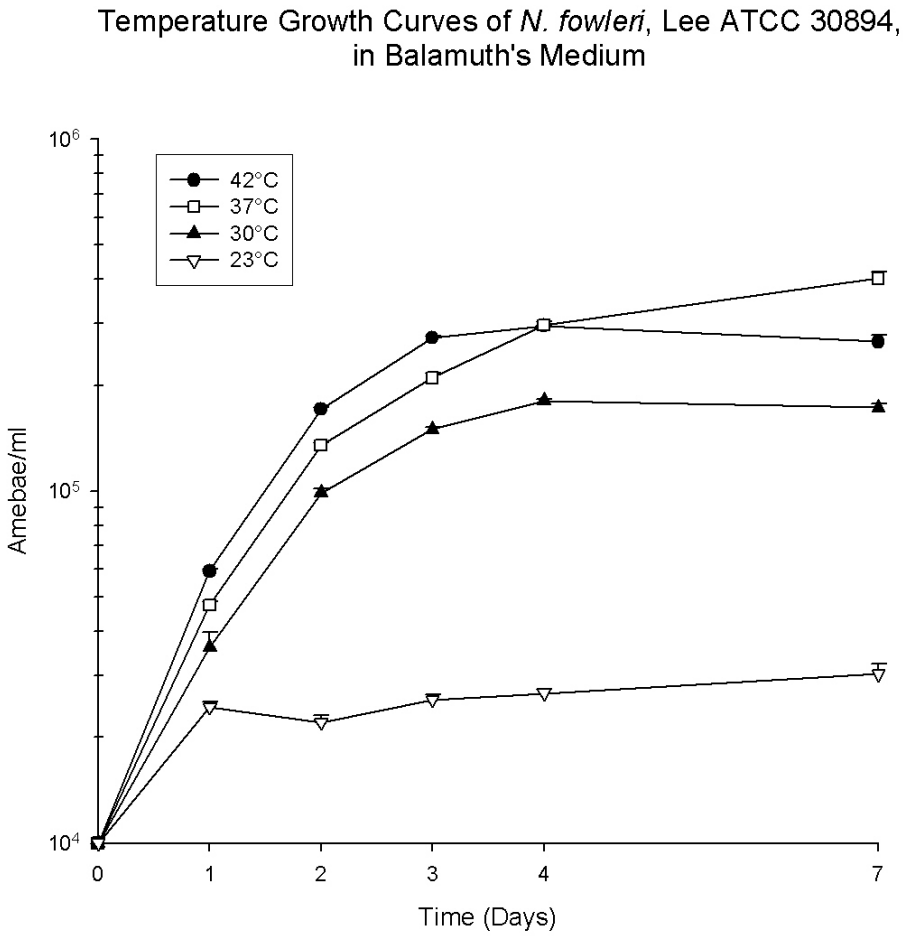

Figure 5. Growth of *N. fowleri*, Lee strain incubated in Balamuth's medium at different temperatures over a 7-day period. The data points represent the results of one experiment performed in triplicate. The error bars represent the standard error of the mean for the individual data points.

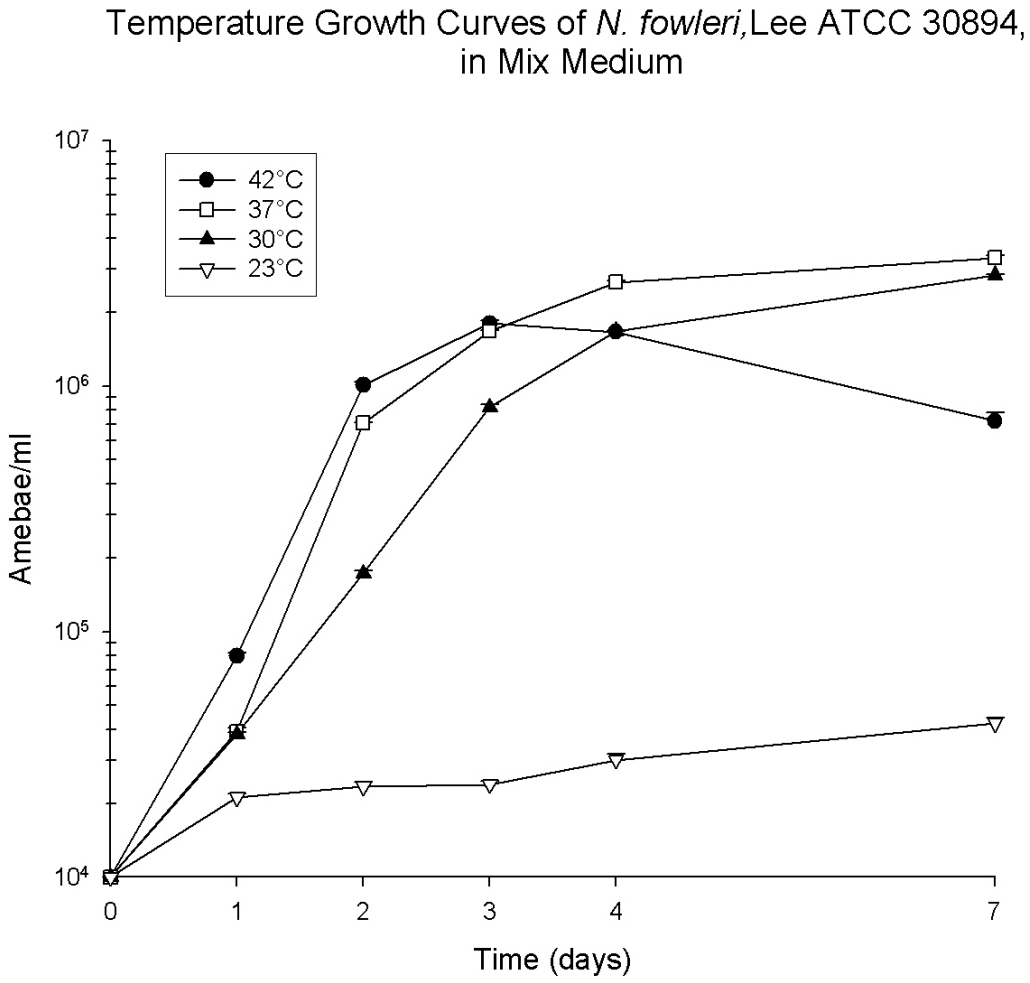

Figure 6. Growth of *N. fowleri*, Lee strain incubated in Mix medium at different temperatures over a 7-day period. The data points represent the results of one experiment performed in triplicate. The error bars represent the standard error of the mean for the individual data points.

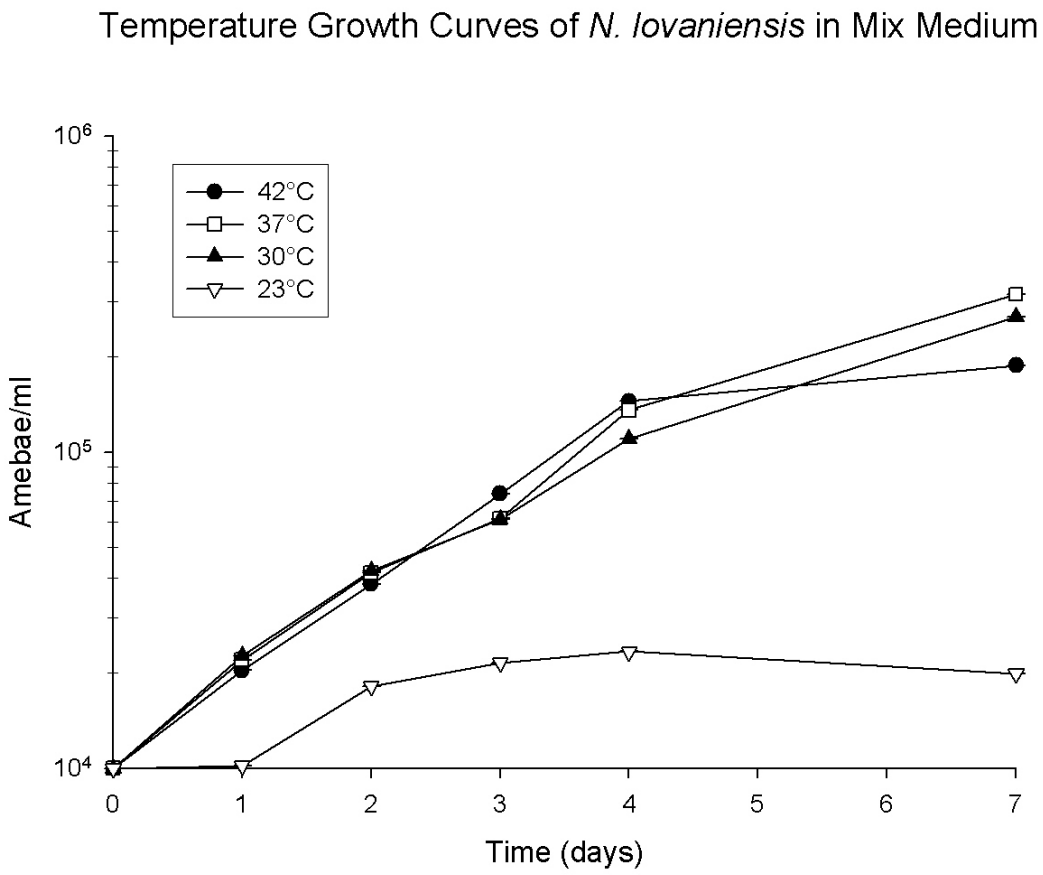

Figure 7. Growth of *N. lovaniensis* incubated in Mix medium at different temperatures over a 7-day period. The data points represent the results of one experiment performed in triplicate. The error bars represent the standard error of the mean for the individual data points.

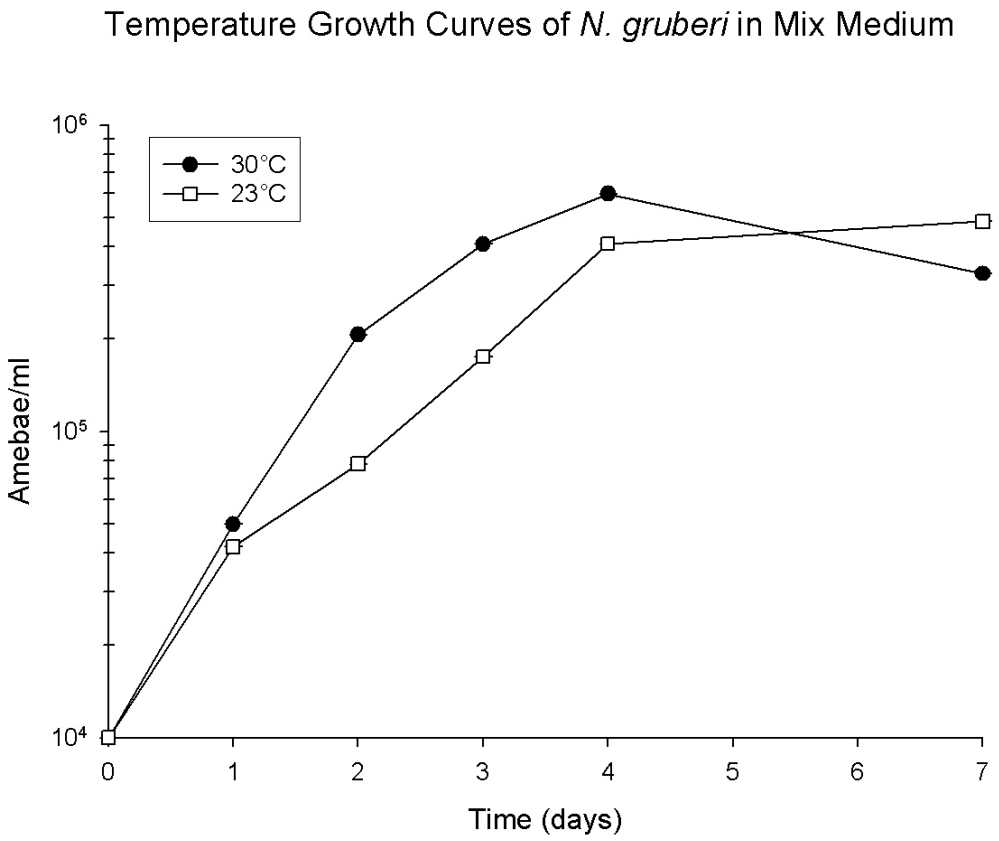

Figure 8. Growth of *N. gruberi*, EGB strain, incubated in Mix medium at 2 different temperatures over a 7-day period. The data points represent the results of one experiment performed in triplicate. The error bars represent the standard error of the mean for the individual data points.

Table I. Summary of Growth at Various Temperatures for Species of *Naegleria*

Incubation Temperature	*N. gruberi* (EGB)[1]	*N. fowleri* (Lee)	*N. lovaniensis*	*N. australiensis*	HBT1-1998
23°C (20°C)	+	+	+	(+)[2]	+
30°C	+	+	+	+	+
37°C	-	+	+	+	+
42°C	-	+	+	-	+

[1.] *Naegleria gruberi* (EGB) is a non-pathogenic strain of *Naegleria* found in the environment.

[2.] (Page, 1988) *N. australiensis* was cultivated at 20°C.

3.1.2 Measurements of Trophozoites, Cysts, and Flagellates

Part of the standard protocol for identification of ameba species is to characterize their morphology. Table II shows the results of these methods, providing information of cyst size, trophozoite size and flagellate sizes as well as the number of flagella.

Table II. Morphological Characteristics of Ameba HBT1-1998 and *N. fowleri*, Lee Strain

	HBT1-1998		*N. fowleri*, Lee strain	
	μm	Range	μm	Range
Trophozoite				
Ave. length ±SD	12 ± 2.2	8-18	12	9-15*
Ave. width ±SD	8 ± 1.4	10-12		
Cyst				
Ave. diameter ± SD	8 ± 1.1	6-11	11	7-15*
Flagellate (flagella)				
Ave. length ± SD	17 ± 2.7	11-24		
Ave. number ± SD	2 ea ± 0.42	1-2 each	2 each**	

*Carter, 1970

**Page, 1988

Figure 9 shows a photomicrograph at 1000x of stained trophozoites of ameba HBT1-1998. Figure 10 shows a stained trophozoite emerging from a cyst, while Figure 11 shows a cluster of cysts. Flagellates are shown in figures 12 and 13. They were stained using a modified iron-hematoxylin procedure.

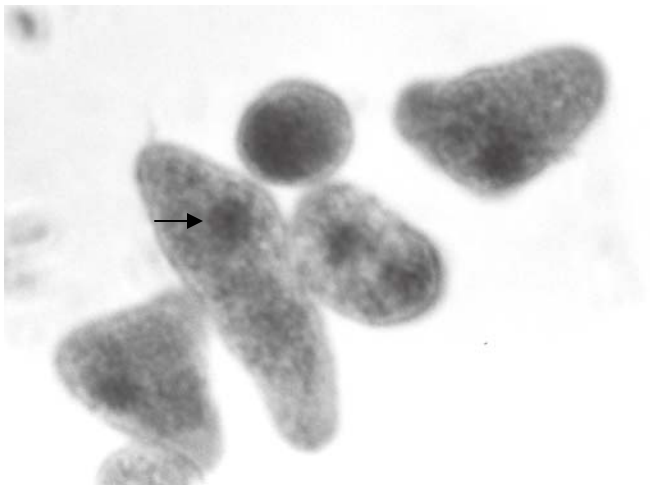

Figure 9. Iron hematoxylin-stained trophozoites of ameba HBT1-1998. Arrow indicates nuclei with karyosome.

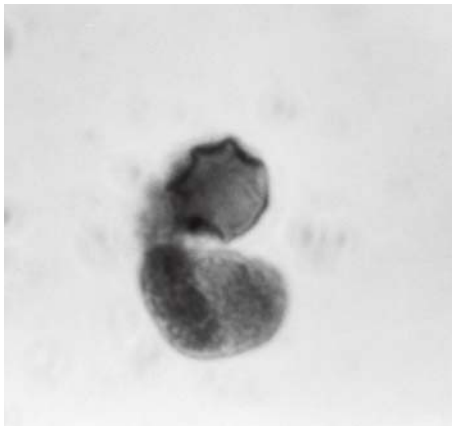

Figure 10. A iron hematoxylin-stained trophozoite of ameba HBT1-1998 emerging from an empty cyst.

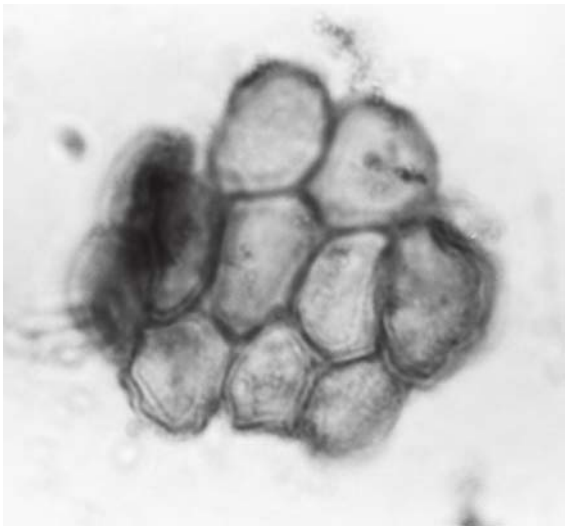

Figure 11. A cluster of iron hematoxylin-stained cysts of ameba HBT1-1998.

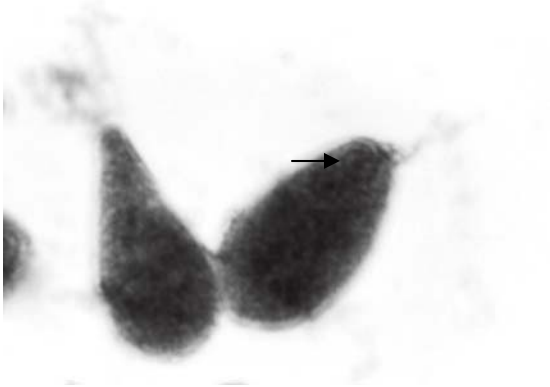

Figure 12. Two flagellates of ameba HBT1-1998, iron hematoxylin-stained. Arrow indicates visible nuclei.

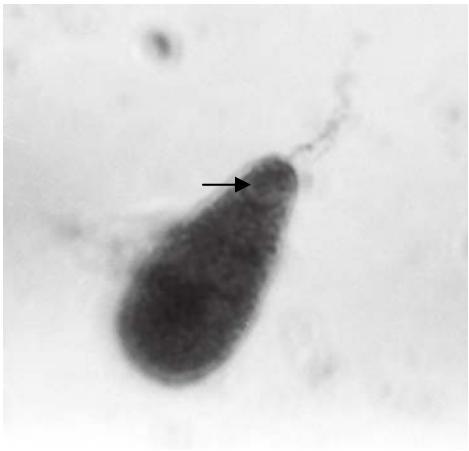

Figure 13. A flagellate of ameba HBT1-1998, iron hematoxylin-stained. The nucleus is visible (arrow) at the narrow anterior portion of the flagellate.

Flagellate transformation was done using the method of John et al. (1991). Maximum transformation to flagellates was 26% after 5 hours. A comparison of transformation of ameba HBT1-1998 and *N. fowleri* is shown in Figure 14.

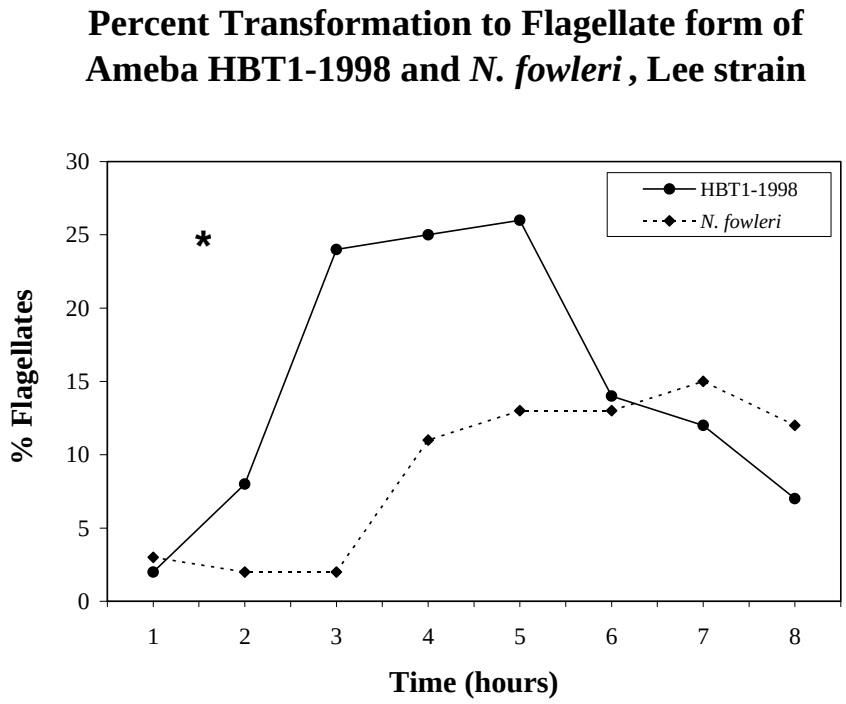

Figure 14. Comparison of percent transformation of ameba HBT1-1998 and *N. fowleri*, Lee strain. * indicates maximum enflagellation for a non-pathogen, *N. gruberi* (EGB), of 25% flagellates in 90 minutes (Cable and John, 1986).

3.1.3 Concanavalin A agglutination

Ameba HBT1-1998 did not agglutinate in the presence of Con A. A control of *N. fowleri* also failed to agglutinate. A positive agglutination control of *N. gruberi* agglutinated to a very high degree, indicating that the Con A was effective in causing agglutination (Figure 15). Ameba HBT1-1998 was a non-agglutinator as was the pathogenic control. Table III shows a compilation of this information.

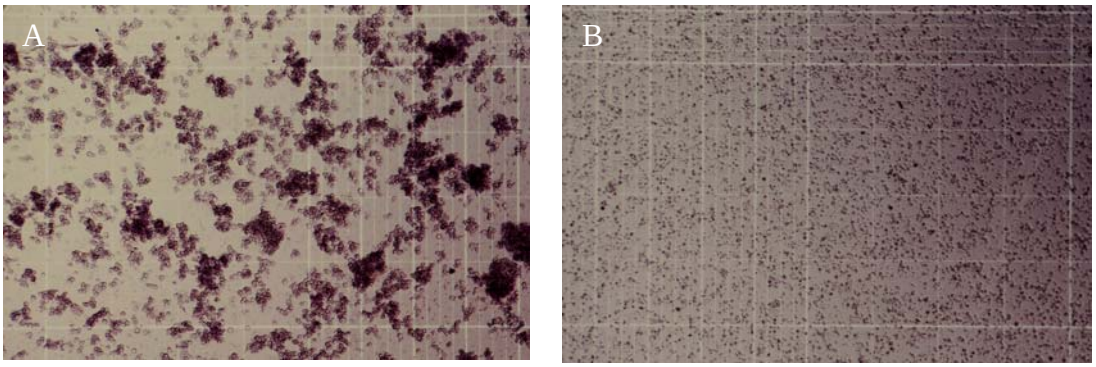

Figure 15. Concanavalin A agglutination:

A. *N. gruberi*, positive for Con A agglutination

B. *N. fowleri*, negative for Con A agglutination

Table III. Concanavalin A Agglutination of *Naegleria* Species and HBT1-1998.

	Con A agglutination[1]	Pathogenic for mice
N. fowleri	-	+
N. gruberi	+	-
N. australiensis	+	+
N. lovaniensis	+	±[2]
HBT1-1998	-	+

1. Con A at 100 μg/ml of ameba saline.
2. *N. lovaniensis* is considered nonpathogenic for mice. However, one weakly virulent strain for mice has been isolated from the environment (John et al., 1998).

3.1.4 Indirect Immunofluorescence Titers

In the IIF portion of the study, there were replicate slides of each antiserum dilution series, at least triplicates. Titers from each individual slide, for each antiserum, were tabulated and averaged. For this variable the titer closest to the average is reported. Serially, 1:2 dilutions gave the following dilution series: 1:2, 1:4, 1:8, 1:16, 1:32, 1:64, 1:128, 1:256, 1:512, 1:1024, 1:2048.

Most antisera will titer out before one reaches the 1:2048 dilution (John et al., 1998). In this study the titer with the greatest frequency was reported. This eliminated any outlier results getting averaged into the final result, possibly giving a more accurate picture of the actual titer of a particular antiserum. However, if only 3 slides were assayed, and the resulting titers were 1:16, 1:32, or 1:64, which would be the correct titer? There isn't one with the greatest frequency. For this particular example, the median would be the best choice as to the actual titer.

Table IV shows a positive comparison of titers of HBT1-1998 with only *N. fowleri*. There was some similarity to the *N. lovaniensis* antisera, but the *N. fowleri* control shows the highest affinity for this particular antiserum.

Table IV. Indirect Immunofluorescence Antibody Titers of HBT1-1998 and Ameba Controls vs. Known Ameba Antisera.

	Antibody Titers*			
Antiserum	**HBT1-1998**	***N. fowleri*, Lee strain**	***N. australiensis*, strain pp397**	***N. gruberi*, EGB strain**
N. fowleri	**1024**	**1024**	4	8
N. australiensis	16	8	**512**	64
N. gruberi	32	2	4	**2048**
N. lovaniensis	256	64	2	8

*Reciprocal endpoint titers.

3.1.5 Antimicrobial Sensitivity

Figures 16 and 17 illustrate that the control and the lowest dose of amphotericin B (0.01 μg/ml) did not inhibit the growth of either ameba HBT1-1998 or *N. fowleri*, Lee strain. These results indicate that there is only inhibition of growth of the ameba at the higher doses of amphotericin B, 0.1 μg/ml and 1.0 μg/ml.

Figures 18 and 19 presented with similar results in that the control and the lowest concentration of azithromycin (0.01 μg/ml) did not inhibit the growth of either ameba HBT1-1998 or *N. fowleri*, Lee strain. However, in the azithromycin study, in both amebae tested, there was an increase of growth after 4 days of incubation. These results indicate that there was inhibition of growth of the amebae only at the higher doses of azithromycin, 0.05 μg/ml and 0.1 μg/ml, and that azithromycin would have to be supplemented after 4 days of treatment.

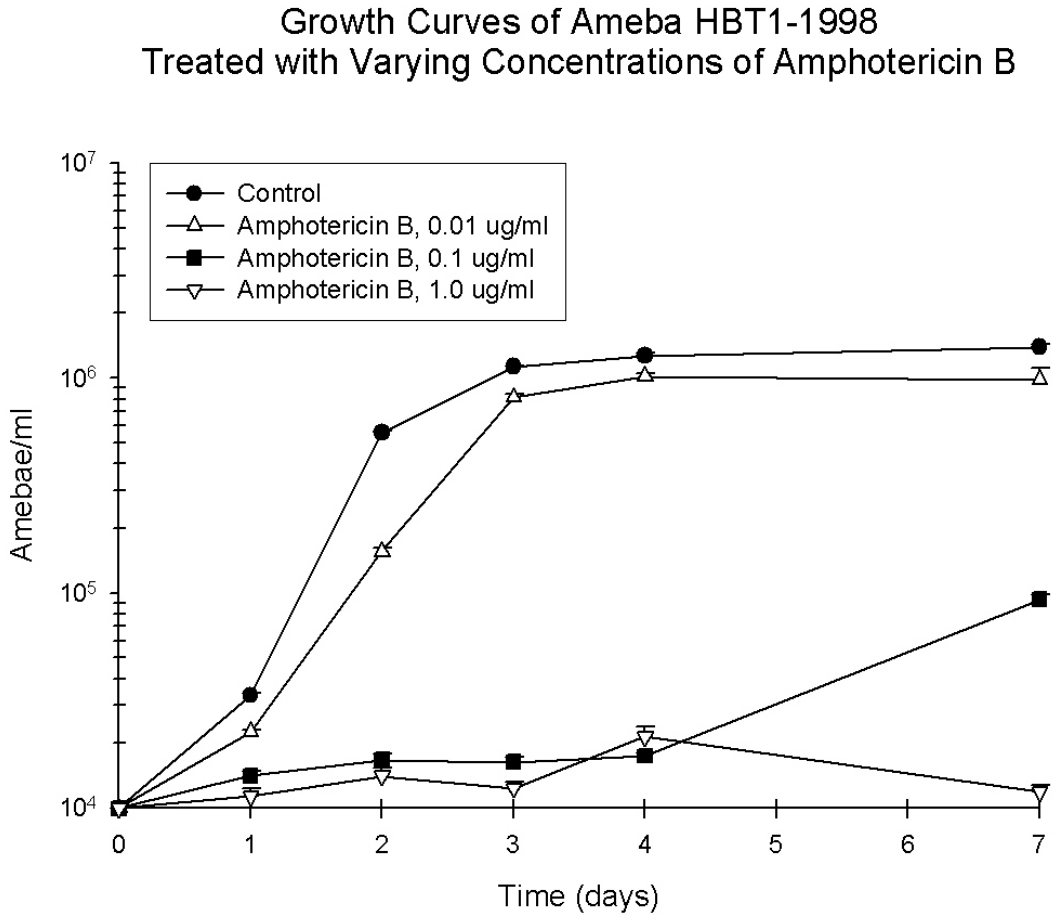

Figure 16. Ameba HBT1-1998 in Mix medium treated with amphotericin B.

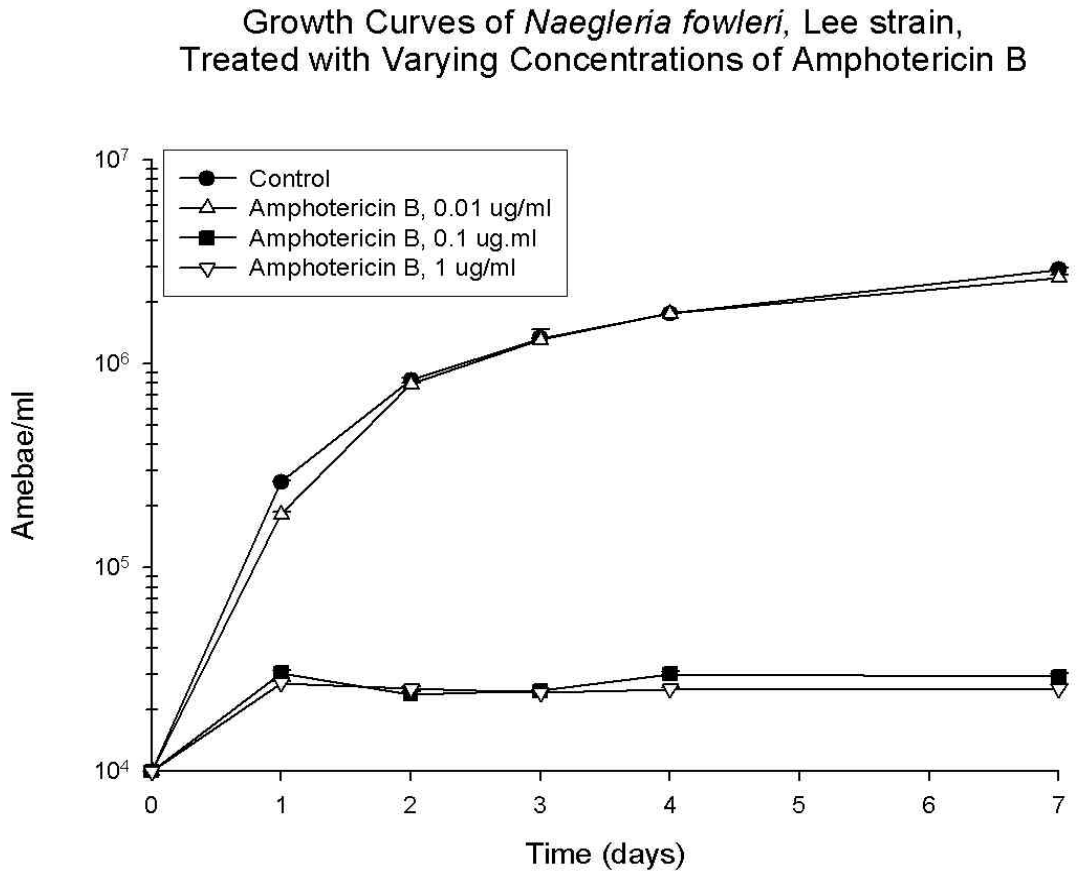

Figure 17. *N. fowleri*, Lee strain, in Mix medium treated with amphotericin B.

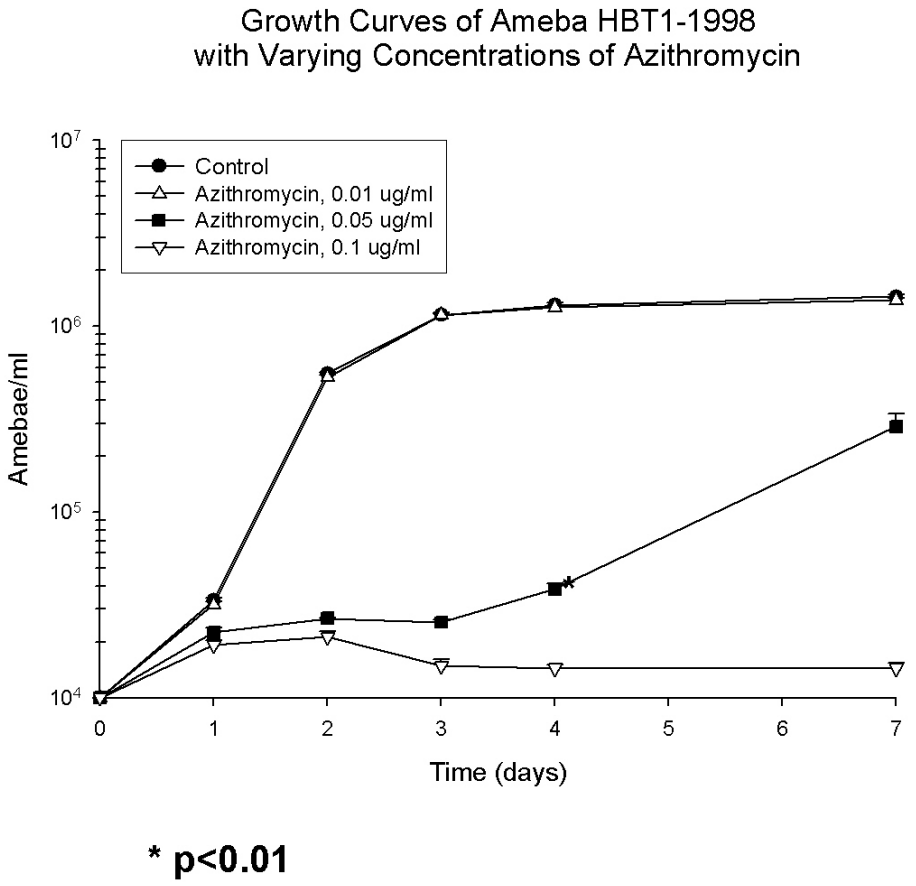

Figure 18. Growth of ameba HBT1-1998 in Mix medium treated with varying concentrations of Azithromycin.

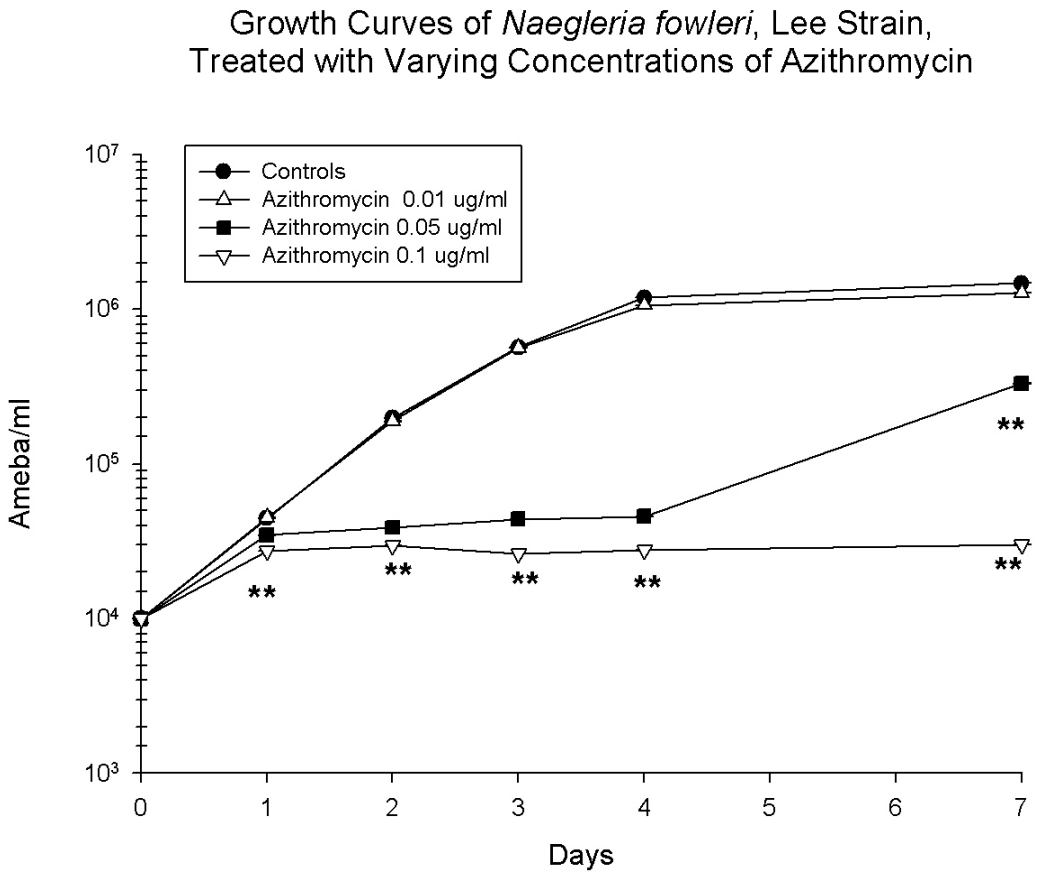

Figure 19. *N. fowleri*, Lee strain, grown in Mix medium with varying concentrations of Azithromycin.

3.2 *In Vivo* Studies in a Murine Model

A murine model was utilized to study the disease course and to monitor the path the amebae took to enter the brain. In the histological analysis, there is evidence of the amebae first in the nasal mucosa, and secondly in the brain tissue itself, thus confirming the intranasal route of infection. The virulence study portion of this research calculates the LD_{50} and the length of time required to cause death in its host.

3.2.1 Histological Analysis

In Figure 20, at 24 hours post infection, a trophozoite of HBT1-1998 can be seen migrating from the nasal mucosa to the olfactory bulb of the brain via the olfactory nerve fila. Figure 21 at 48 hours post infection shows areas of inflammation, however amebae are difficult to see at this magnification. Figure 22 illustrates abundant ameba in the brain tissue at 48 hours. Therefore, it is concluded that the disease process is well underway at this early point in the infection.

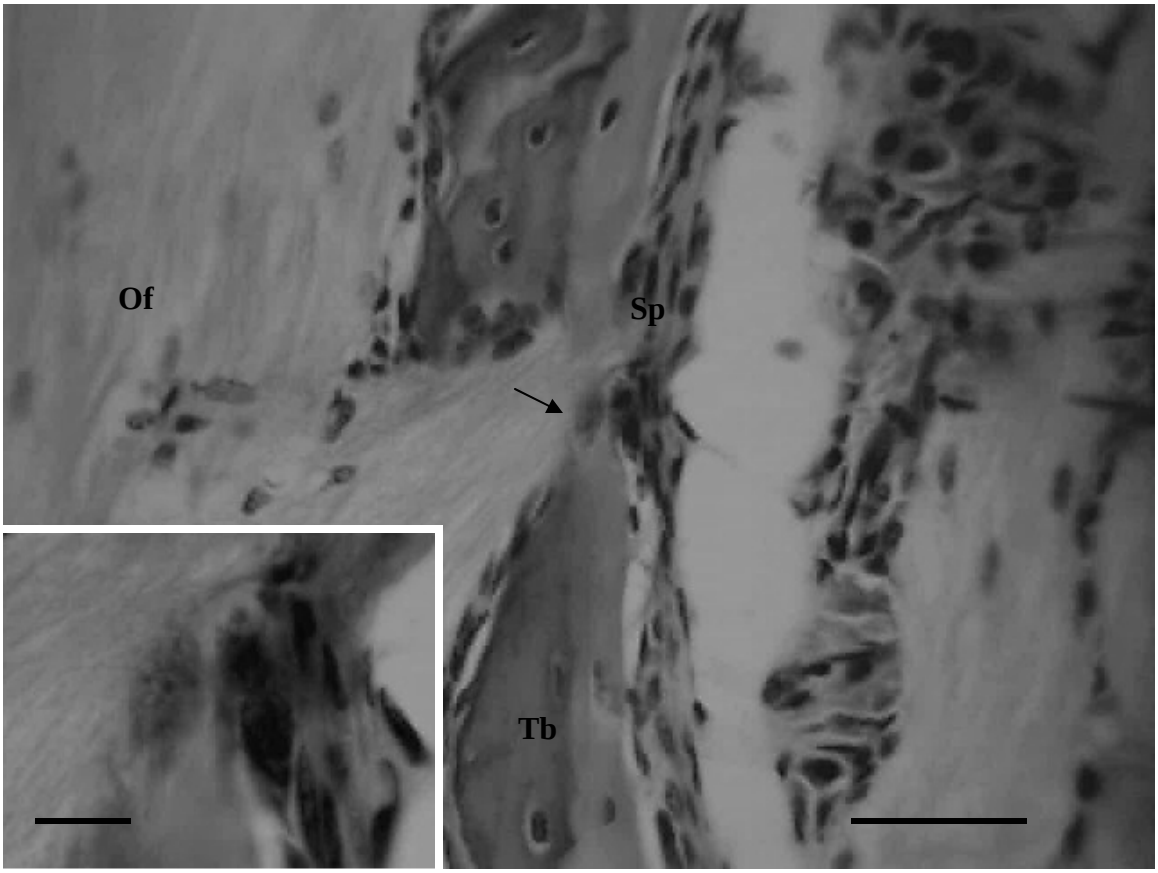

Figure 20. Section of nasal mucosa and submucosa in mouse brain tissue 24 hours post infection with HBT1-1998 (400x magnification). Arrow indicates trophozoite of HBT1-1998 passing the turbinate bone (Tb) entering the olfactory fila (Of) from the submucosal nerve plexus (Sp); bar = 50 μm. The inset is a 1000x magnification of the same ameba; bar = 15 μm. The prominent karyosome and the elongate morphology are characteristic of *N. fowleri*;

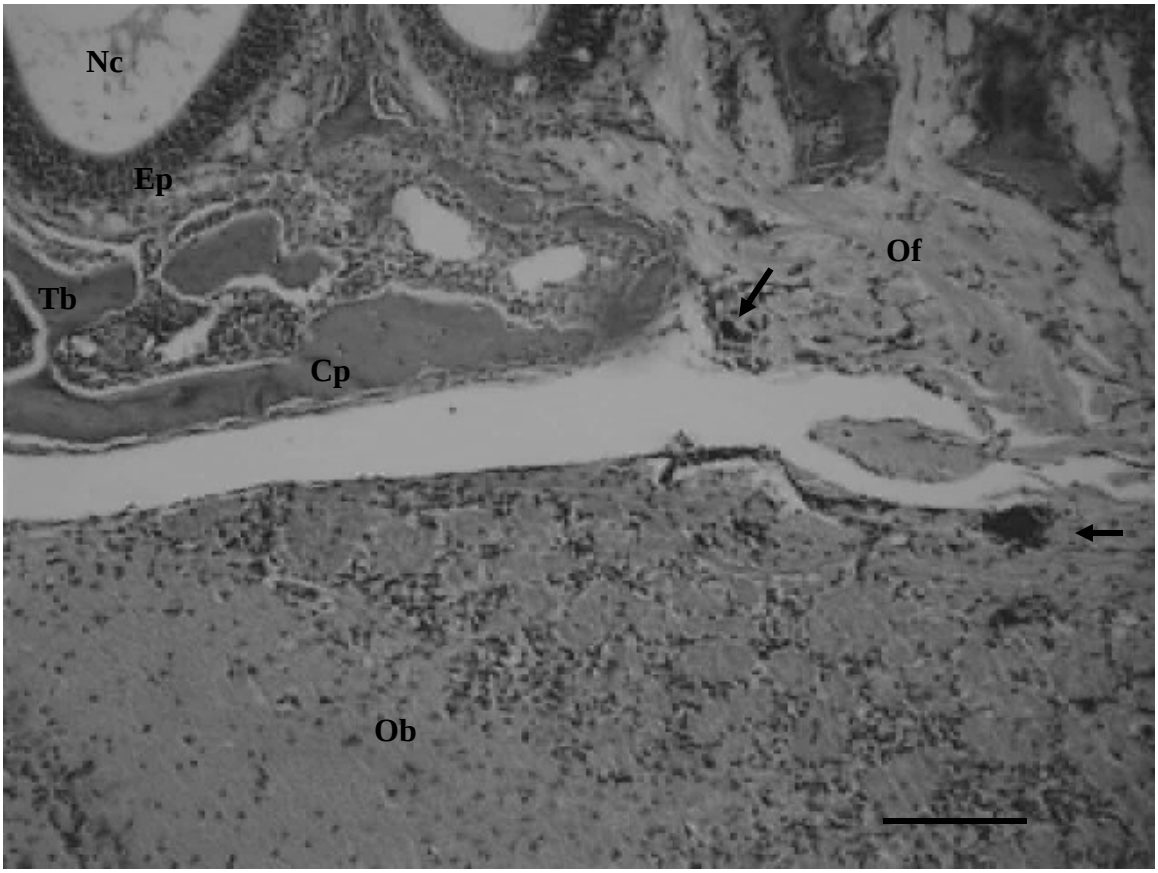

Figure 21. Midsagittal section of an area similar to Figure 19, magnified 100x, from a mouse infected for 48 hours with HBT1-1998. Focal areas of acute inflammation (arrows) are located in the olfactory fila(Of). The cribriform plate (Cp) is an incomplete barrier between the nasal cavity (Nc) and the olfactory bulb (Ob) of the central nervous system. The olfactory fila (Of) occupies areas in the openings of the cribriform plate. Ep, olfactory epithelium lining the nasal cavity; Tb, turbinate bone projecting from the cribriform plate; bar = 150 μm.

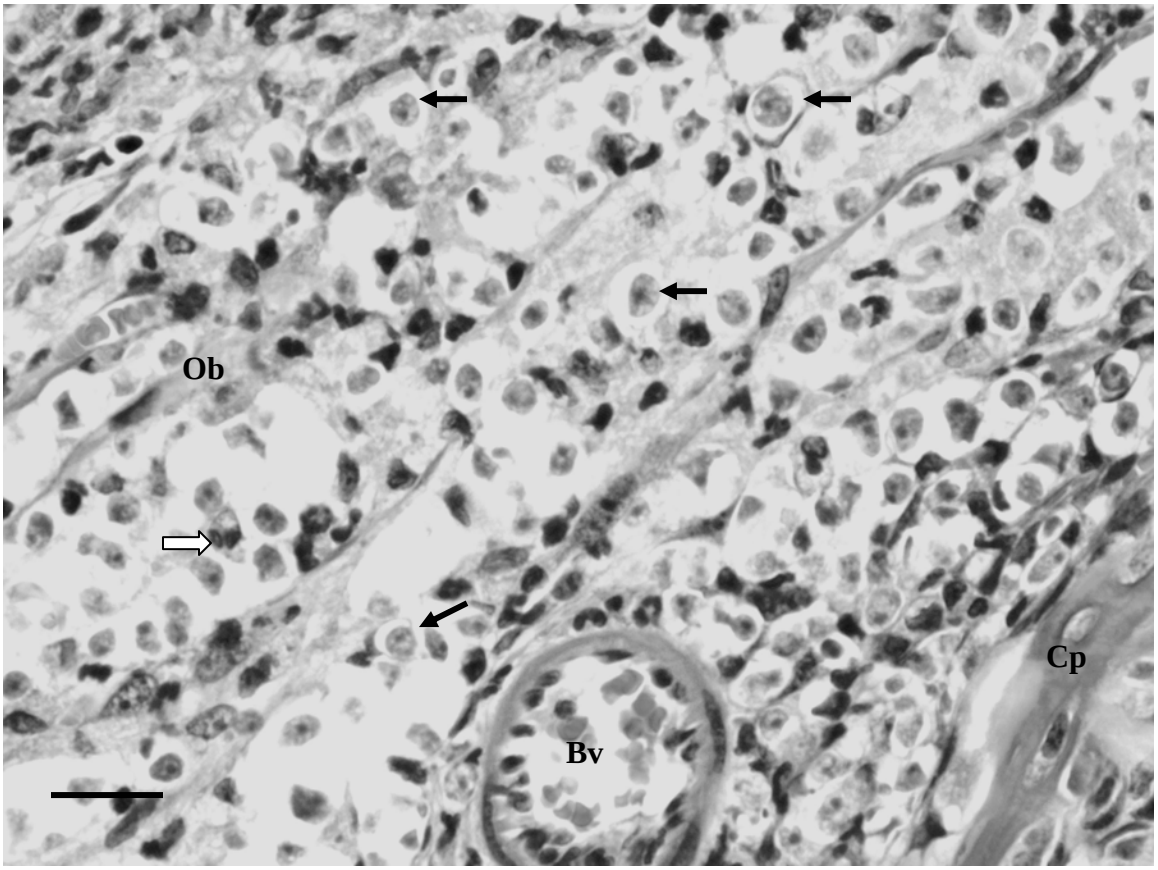

Figure 22. An area of mouse brain tissue (400x magnified) 48 hours post infection with HBT1-1998. Numerous trophozoites (arrows) are present in the olfactory bulb (Ob). A blood vessel (Bv) can be seen with multiple lymphocytes lining the wall. Note the isolated monocyte (white block arrow); Cp, Cribriform plate; bar = 50 μm.

3.2.2 Virulence

The virulence portion of this study lends itself to a greater depth of statistical analysis. Based on the numbers of amebae instilled in the mice, and the percent mortality, a lethal dose of 50% of the mice (LD_{50}) was able to be calculated (Reed and Muench, 1938) (Table V). This particular statistic emphasizes the virulence of the amebae, since, if the LD_{50} is low (meaning, the number of amebae required to kill 50% of the population is small), this would indicate a highly virulent strain. On the other hand, if the LD_{50} is high (the number of amebae required to kill 50% of the population is large), the strain is weakly virulent. This is pertinent in the characterization of HBT1-1998, since a strain of the highly virulent *N. fowleri* will have a low LD_{50} (approximately 100 amebae per mouse to cause death). Other species, such as *N. australiensis*, would have to be administered in much greater numbers (>1000 amebae per mouse) to achieve an LD_{50}.

Table V. Virulence Data of Ameba HBT1-1998
Percent Mortality and Mean Time to Death

Inoculum of amebae per mouse (3 groups of 10 mice each)	Mean Time to Death in days ± Standard Deviation	Percent Mortality* ± Standard Deviation
1×10^1	0	0.0
1×10^2	10.2 ± 3.8	43.3 ± 5.8
1×10^3	7.9 ± 2.8	93.3 ± 11.5
1×10^4	5.6 ± 0.8	96.7 ± 5.8
1×10^5	5.4 ± 0.6	100

*** Calculated $LD_{50} = 1.32 \times 10^2$ (Reed and Muench, 1938) ameba HBT1-1998 for an inoculum of 10 μl instilled intranasally in 21-day-old male CD-1 mice.**

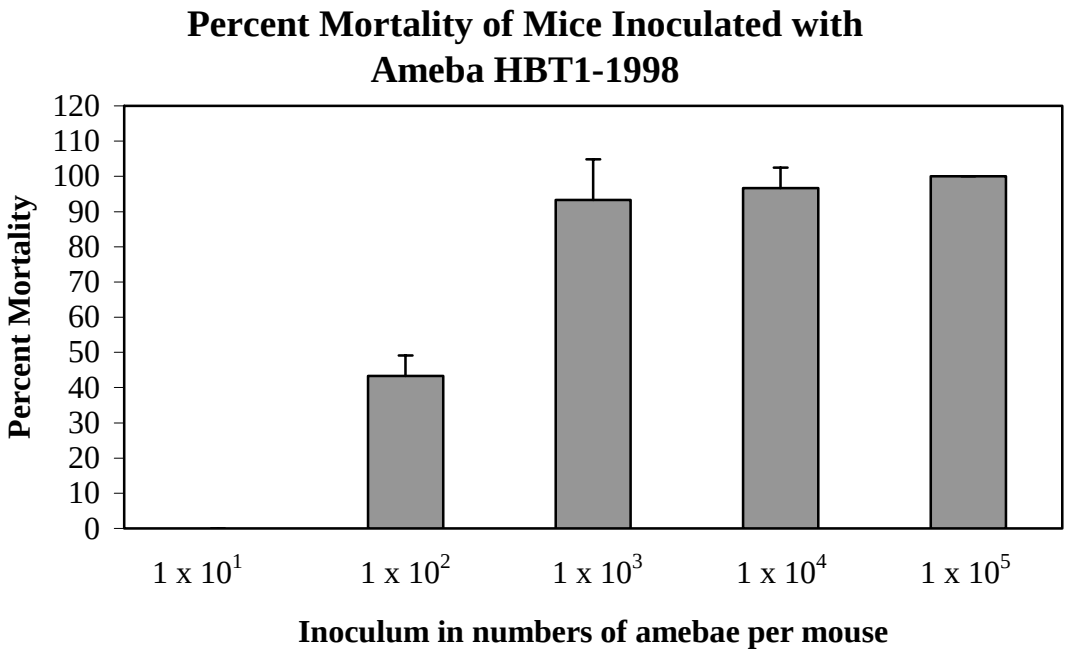

Figure 23. Percent deaths in relation to the concentration of the inoculum.

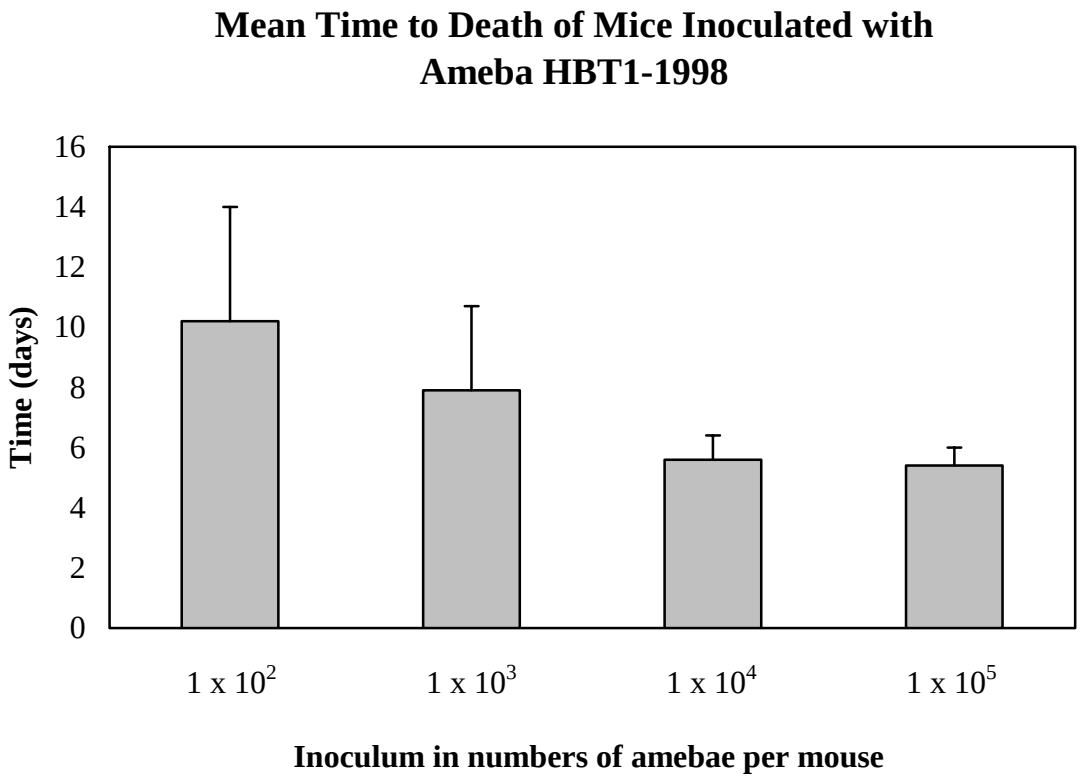

Figure 24. Decreasing mean time to death with increasing concentration of inoculum.

The relationship between the dose of inoculum and the percent mortality can be seen in Figure 23. As the inoculum increases, the percent mortality increases. The increase is not proportional. The first two 10-fold increases have rather dramatic effects on the percent deaths that occur, but by the time an inoculum of 1000 amebae is attained, there is almost 100% mortality. The mean-time-to-death (MTD) graph in Figure 24 shows a steady decrease in the length of time at which death occurred with 10-fold increases in the inocula, an inverse relationship.

3.3 Summary of Observations

A complete summary of observations comparing Ameba HBT1-1998 with *N. fowleri, N. gruberi, N. australiensis* and *N. lovaniensis* is in Table VI. This final table is a direct comparison of the results and illustrates the overwhelming similarities between Ameba HBT1-1998 and *N. fowleri*.

Table VI. Summary of Observations on Ameba HBT1-1998

Observation	*Naegleria fowleri*	Ameba HBT1-1998	*Naegleria gruberi*	*Naegleria australiensis*	*Naegleria lovaniensis*
Growth at 42°C	+	+	-	+	+
Flagellate stage	+	+	+	+	+
Average no. flagella	2.0	2.0	2.4	3.1	2.0
Con A agglutination[1]	-	-	+	+	+
Pathogenicity	+	+	-	+	±[2]
Highly virulent	+	+	-	-	-
Weakly virulent	-	-	-	+	+
Human isolate	+	+	-	-	-
Immunofluorescence[3]	1024	1024	32	16	256

Identity of Ameba HBT1-1998: *Naegleria fowleri*

1. Concanavalin A at 100 μg/ml of ameba saline.
2. *N. lovaniensis* is considered nonpathogenic for mice; however, one strain has been isolated from the environment that was pathogenic, but weakly virulent, for mice (John et al., 1998).
3. Indirect Immunofluorescence reciprocal endpoint antibody titers.

CHAPTER 4

DISCUSSION AND SUMMARY

The purpose of this study was to determine the species of ameba for the human isolate HBT1-1998. It was compared to both pathogenic and non-pathogenic species of *Naegleria*, this being the cause of most cases of PAM throughout the world. Upon completion of the experimental work and analysis of the data, results were presented in tabular form, plotted on graphs, and relevant photo-micrographs presented, as compared to the known *Naegleria* species, to identify the species of HBT1-1998. Based on observations and clinical manifestation, the hypothesis is that HBT1-1998 is *N. fowleri*

4.1 Growth Requirements of Ameba HBT1-1998 vs. Other *Naegleria* Species

In vitro growth studies comparing amebae HBT1-1998 with established data on various *Naegleria* species indicated strongly that HBT1-1998 was indeed *Naegleria fowleri*. Determining optimal growth conditions for HBT1-1998 was necessary to ensure successful cultivation of the amebae for various tests and studies deemed necessary to characterize this organism.

The media requirements for HBT1-1998 were the same as those that are common to the *Naegleria* species it was compared to. All three of the control organisms (*N. fowleri, N. gruberi, and N. lovaniensis)* and HBT1-1998 demonstrated their highest numbers when cultivated in Mix medium. Cultures of *N. fowleri* Lee strain and HBT1-1998 reached numbers greater than $1x10^6$ amebae/ml at the end of log phase growth in

this medium (See Figures 1-8) while *N. lovaniensis* reached a maximum growth of $3x10^5$ amebae/ml in Mix and *N. gruberi* reached a maximum of $6x10^5$ amebae/ml in Mix.

Determining the optimal temperature at which to cultivate ameba HBT1-1998 not only ensured maximum growth of organisms, it also was an indicator of the species (see Table I). By these criteria HBT1-1998 compared favorably with both *N. fowleri* and *N. lovaniensis.*

4.2 Morphological Comparisons of HBT1-1998 and Control *Naegleria* Species

The general morphology of all species of *Naegleria* is virtually identical in some ways, with noted differences in other ways. The amoeboid stage (trophozoite) tends to be similar in appearance and is referred to as being limax, from the Latin term for slug. The nuclei of *Naegleria* have a large central nucleolus, or karyosome, and a visible nuclear membrane free of chromatin granules. This feature helps to distinguish them from *Entamoeba histolytica*, a human parasitic ameba. The nucleus of HBT1-1998 seen in Figure 9 is characteristic of a typical *Naegleria*.

Naegleria tend to be elongate with directional motility. Pseudopodia are extended in an very blunt, eruptive manner and are called lobopodia (John, 1993). Comparisons of the trophozoites of HBT1-1998 and *N. fowleri* can be found in Table II. Actively moving *N. fowleri* will average 22 μm in diameter, but the rounded, less active stage ranges from 9-15 μm. HBT1-1998 averaged 12 μm in diameter for rounded trophozoites, whereas trophozoites of *N. australiensis* average about 21μm in length (De Jonckheere, 1981).

The cysts of HBT1-1998 are spherical and often clumped together (Figure 11) as are typical cysts of *N. fowleri.* The average diameter of HBT1-1998 cysts was 8 μm, with a range of 6-11 μm. *N. fowleri* Lee strain cysts are, on average, 11 μm in diameter,

with a range of 7-15 μm (Carter, 1970; Page, 1988). Cyst diameters of other species of *Naegleria* are ranges of 10-16 μm for *N. gruberi* and 9.6-13 μm for *N. lovaniensis*, and a mean of 11.6 μm of *N. australiensis*. The smaller cyst diameter of HBT1-1998 compares more closely with the cyst size for *N. fowleri* than any of the other species of *Naegleria.*

Flagellates are unique for members of the family Valkampfiidae (Page, 1988). This is a very useful tool for identifying *Naegleria*, since other infectious amebae species, such as *Acanthamoeba* and *Entamoeba*, do not transform to the flagellate stage. Also, other members of the Valkampfiidae family may form flagellates but are not known to be pathogenic. Within this family, the *Naegleria* flagellates are unique, usually having 2 flagella and being cigar or pear-shaped. But different species of *Naegleria* have very distinctive flagellate stages. Thus, this stage provides the essential distinction among genera with the ability to transform to flagellates (Page, 1988). Based on the findings of this study, HBT1-1998 is most like *N. fowleri* and *N. lovaniensis*, being a biflagellate, vs. the 2.4 flagella of *N. gruberi* and the 3.1 of *N. australiensis* (see table VI).

Concanavalin A (Con A) is a plant lectin which can cause certain species of amebae to agglutinate or clump together. The lectin binds to specific polysaccharides exposed on the surface of the amebae. Pathogenic *Naegleria* sp., such as *N. fowleri*, will not agglutinate in the presence of Con A, whereas non-pathogenic *Naegleria* sp., like *N. gruberi*, will agglutinate (Josephson et al., 1977). Again, the findings of this study confirmed the similarity of HBT1-1998 with *N. fowleri* since it does not agglutinate in the presence of Con A.

4.3 Pathogenicity and Virulence of HBT1-1998 as Compared to Other Species of *Naegleria*

Pathogenicity and virulence of the different species of *Naegleria* confirms the species of HBT1-1998. As indicated in Table VI, HBT1-1998 caused the death of Emerald Watson and was confirmed to be a pathogen by using a mouse model. The options were narrowed to it being either *N. fowleri* or *N. australiensis.* Though one isolate of *N. lovaniensis* has been reported to be a weakly virulent pathogen, it is very unlikely that HBT1-1998 is this ameba. HBT1-1998 is highly virulent, like *N. fowleri* and unlike *N. australiensis* and *N. lovaniensis*. There have been numerous cases of PAM in which *N. fowleri* was the confirmed pathogen and none attributed to other species of *Naegleria*. This information confirms that HBT1-1998 is *N. fowleri.* Non-agglutination by Con A further confirms HBT1-1998 as *N. fowleri*.

4.4 Indirect Immunofluorescence of Amebae HBT1-1998

IIF is an accurate and inexpensive method for species identification of amebae. Antisera to specific species can be acquired either by making them or by purchasing prepared antiserum from a biological supply company. Our laboratory had previously prepared antisera to 26 different species of amebae in order to test this technique for its specificity (John et al, 1998); therefore, HBT1-1998 was tested against antisera to *N. gruberi*, *N. australiensis*, *N. lovaniensis*, and *N. fowleri*. The results of these immunofluorescence assays indicated a positive match between HBT1-1998 and *N. fowleri*, with this species having the highest endpoint antibody titer (see Table VI).

4.5 Summary and Future Directions

Future work involved in characterization of these organisms should include more advanced technologies: This includes PCR for DNA analysis and species identification and isoenzyme analysis, a currently used biochemical technique in Europe. Both of these methods will be able to give accurate, reliable, and rapid results. IIF will still be useful based on its cost effectiveness, particularly for small, rural laboratories. Current trends, coupled with greater availability and understanding of PCR technology, will make this a primary method for identification of pathogenic amebic organisms. These advances, once they have been thoroughly researched, will be beneficial scientifically, but more importantly, may aid in more rapid diagnoses of infections and possibly prevent deaths due to PAM.

4.5.1 Other Confirmed Cases of PAM in Oklahoma

Since 1998 there have been 4 more cases of PAM confirmed in Oklahoma. Although there have been no more cases associated with Fort Gibson Lake, there was 1 case from Lake Texoma, 1 from Lake Konawa, and 2 from the City of Tulsa (see Table VII)(PAM, 2001). Of the 5 Oklahoma cases, Emerald Watson was the only female victim.

In comparison, there are other states that have had greater numbers of deaths over the years due to PAM. Virginia has the most with 16 confirmed cases, Florida had 15, and Texas with 12. Other states surrounding Oklahoma with confirmed cases of PAM are Arkansas (2 cases) and Missouri (1 case).

Table VII. Oklahoma Cases of PAM

(1)	• August 1998, First confirmed case Emerald Watson, 3 year old female Fort Gibson Lake, Taylor Ferry
(2)	• July 2000 Justin Baty, 13 year old male Lake Texoma, Oklahoma side
(3)	• June 2001 Hosea Delara, 11 year old male Lake Konawa, north of Ada
(4 & 5)	• August 2005 Terrell Hampton, 9 year old male Martinez Owens, 7 year old male Presumed; Mohawk Park, Tulsa; splash pad area

Wellings (1977) compiled data to determine the relative risk of swimming in Florida waters. Her statistical analysis indicated that for every 2.6 million swimming exposures in Florida lakes, there would be one case of PAM. This information leads one to think initially that the chance of contracting this particular disease is extremely small. However, given the millions of people who swim in lakes and streams annually, it is surprising that there isn't a higher incidence of PAM. Still, there may be unreported cases in rural communities in states like Oklahoma, Texas and Arkansas. Given the current capabilities for communication (internet, telemedicine) there is a greater awareness of this disease among physicians. Being aware of the disease and the correlation with water activities prior to the onset of symptoms will aid in a more rapid diagnoses and earlier treatment.

4.5.2 Treatment of PAM

To date, the current treatment for PAM, amphotericin B, has proven to be relatively ineffective. There have only been two well-documented cases where treatment

with this medication was successful (Anderson and Jamieson, 1972; Seidel et al., 1982). Seven other putative survivors have been reported; however, the reports are incomplete or have not been documented.

Unfortunately, most cases do not lend themselves to being treated for amebic infection at all, as the patients usually die before a diagnosis is made. Since HBT1-1998 was isolated from a victim of PAM who was infected by the amebae from Oklahoma waters, it would be in our best interest to explore treatments more effective in curing patients with this disease.

The work of Goswick and Brenner (2003a and 2003b) has introduced an antimicrobial alternative to amphotericin B. In their studies, the antibiotic azithromycin was tested *in vitro* and *in vivo*, using a mouse model. The results were dramatic. Amphotericin B protected 50% of mice infected with *N. fowleri* at a dose of 75 mg/kg/day for 5 days. Azithromycin at a dose of 75 mg/kg/day for 5 days protected 100% of the mice infected with *N. fowleri*. This study shows an obvious benefit to being treated with azithromycin.

The present *in vitro* studies testing the anti-amebic capacities of amphotericin B and azithromycin, the results for both HBT1-1998 and the control *N. fowleri* Lee strain were comparable. The highest treatments of 0.1 μg/ml completely inhibited amebic growth. A dose of 0.05 μg/ml inhibited growth for the first 4 days, but the cultures began actively growing after 4 days. The 0.01 μg/ml and negative controls had no effect on the growth of the amebae. Given these results, it would appear that *in vitro* there is no difference between the amphotericin B and the azithromycin in their antimicrobial capabilities towards pathogenic amebae; however, given the potential toxicity of

amphotericin B as well as the increased effectiveness in the *in vivo* study (Goswick and Brenner, 2003a) azithromycin may indeed be a useful therapy in the treatment of PAM.

4.5.3 Final Thought

After months of cultivation of organisms, treatment with antimicrobials, staining, measuring and analyzing, it can be stated that HBT1-1998 is *Naegleria fowleri*.

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VITA

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Scope and Method of Study: *Naegleria fowleri* is an opportunistic pathogen and the cause of a rapidly fatal brain disease known as primary amebic meningoencephalitis (PAM). In August, 1998 a three year old girl died after she became infected while swimming at a local lake. Prior to her death, the cause was unknown. After autopsy a diagnosis of PAM was made. Amebae were isolated and cultivated from a small amount of brain tissue provided by the Medical Examiners Office. The isolate was designated HBT1-1998 and tentatively identified as *N. fowleri*. Standard laboratory tests were performed to positively identify the species of ameba. These tests included concanavalin A (Con A) agglutination, flagellate formation, growth studies at multiple temperatures in different growth media, ameba and cyst measurement, drug studies with azithromycin and amphotericin B, and indirect immunofluorescence (IIF). *In vivo* studies included histological examination of brain tissue sections obtained from infected mice. Percent mortality as well as mean time to death was determined using a murine model.

Findings and Conclusions: The amebae of HBT1-1998were limax in appearance, elongate and slug-like, and moved with directional motility by explosive blunt pseudopodia. The amebae did not agglutinate in the presence of Con A. They were determined to be ameboflagellates. These preliminary tests were compatible with typical characteristics for *N. fowleri*. Growth conditions were also typical of *N. fowleri*, and growth curves were similar. Both HBT1-1998 and the control *N. fowleri* culture (Lee strain) had virtually identical results when cultured with azithromycin and amphotericin B. IIF positively confirmed the amebae as *N. fowleri*. The *in vivo* studies also matched positively with *N. fowleri*. The LD_{50} was 1.32×10^2 amebae per mouse, an extremely low dose, and percent mortality reached 100% with a dose of 1×10^5 amebae/mouse. Mean time to death averaged approximately 6 days post infection. After examining all of the data, HBT1-1998 was confirmed to be *Naegleria fowleri*.

ADVISOR'S APPROVAL:_____