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A COMPARATIVE STUDY OF A PATHOGENIC VERSUS A NONPATHOGENIC NAEGLERIA SPECIES

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A COMPARATIVE STUDY OF A PATHOGENIC VERSUS A NONPATHOGENIC *NAEGLERIA* SPECIES

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

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NAEGLERIA SPECIES

By Melissa June Jamerson

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

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Major Director: Dr. Francine Marciano-Cabral

Professor, Department of Microbiology and Immunology

Naegleria fowleri (*N. fowleri*) and *Naegleria lovaniensis (N. lovaniensis)* are closely related amebae found in the environment. *N. fowleri* causes Primary Amebic Meningoencephalitis (PAM), a fatal disease of the central nervous system, while *N. lovaniensis* is nonpathogenic. *N. fowleri* infection occurs when amebae enter the nasal

passages, and migrate to the brain. The molecular mechanisms involved in the pathogenesis of PAM are not well-defined. Therefore, the purpose of this study was to define phenotypic characteristics that may be functionally linked to the pathogenicity associated with *N. fowleri.* Studies revealed that *N. fowleri* has a faster growth rate and is more resistant to complement-mediated lysis when compared to *N. lovaniensis*. Additionally, contact-independent cytotoxicity was observed only for *N. fowleri*. The ability to invade tissues can be a characteristic that distinguishes pathogens from nonpathogens. Therefore, adhesion to extracellular matrix components (ECM), laminin-1, fibronectin, and collagen I, was assessed. *N. fowleri* exhibited a higher level of adhesion to ECM components and was shown to invade tri-dimensional ECM scaffolds (matrigel and collagen I) to a greater extent than *N. lovaniensis.* Scanning electron microscopy revealed that *N. fowleri* attached on ECM substrata exhibited a spread-out appearance that included the presence of focal adhesion-like structures. Attachment of *N. fowleri* to ECM components was decreased significantly when amebae were pretreated with trypsin, suggesting a role for a surface protein in this process. Pretreatment of *N. fowleri* amebae with periodate, a sugar oxidant, led to a decrease in attachment to laminin-1 and fibronectin suggesting that the surface component contained a sugar moiety. Western immunoblotting revealed two integrin-like proteins for both species. However, one with a molecular mass of approximately 70 kDa, was detected at a higher level for *N. fowleri*. Confocal microscopy indicated that the integrin-like proteins co-localized to the focal adhesion-like structures. An anti-integrin antibody decreased adhesion of *N. fowleri* to ECM components. Zymographic analysis demonstrated differential expression of proteases occurs when *N. fowleri* and *N.*

lovaniensis invade ECM components using an in vitro invasion assay. These results indicate a distinction in adhesion to, and invasion of, extracellular matrix proteins between *N. fowleri* and *N. lovaniensis.*

Chapter 1: Introduction

Naegleria species.

The genus *Naegleria* consists of species of free-living amebae that are found worldwide in soil and freshwater lakes and ponds (Marciano-Cabral & Cabral, 2007; Martinez & Visvesvara, 1997). Although numerous species from this genus have been identified in the environment and in domestic water supplies (Anderson & Jamieson, 1972; Craun *et al.,* 2005; Gyori, 2003, Jamerson *et al.,* 2009; Marciano-Cabral *et al*., 2003; Yoder *et al.,* 2004), only one species, *Naegleria fowleri* (*N. fowleri*), has been linked to disease in humans (Carter, 1968; Cerva & Novak, 1968; Martinez, 1985). Other species of *Naegleria* have been shown to lyse tissue culture cells in vitro and cause disease in experimental animals but have never been linked to human infection (Marciano-Cabral & Fulford, 1986; De Jonckheere, 2004).

Naegleria lovaniensis (*N. lovaniensis*), a thermophilic nonpathogenic species of *Naegleria,* was first described as a nonpathogenic variant of *N. fowleri* due to a positive reaction with antiserum to *N. fowleri* (De Jonckheere & van de Voorde, 1977; Steven *et al.,* 1980). However, pathogenicity tests in mice demonstrated that these isolates were nonpathogenic (Steven *et al.,* 1980) and, thus, a different species. Later studies consisting of phylogenetic analysis based on SSU rDNA sequences confirmed a

different species, yet showed that *N. lovaniensis* is the closest relative to *N. fowleri* (De Jonckheere, 1994).

Morphology.

Naegleria is an amoeboflagellate, which consists of three different morphological forms in nature, a flagellate, trophozoite, and cyst form (Fig. 1) (Carter, 1970; Martinez, 1985). The trophozoite is the active feeding, dividing, infective form. Movement is achieved through the use of lobopodia at the anterior end (Fig. 2). This form measures from 10 to 25 μm and is able to reproduce by binary fission. It is characterized by the presence of a single vesicular nucleus with a prominent nucleolus. Numerous mitochondria, ribosomes, and food vacuoles are present, and at the posterior end (uroid), the contractile vacuole and trailing protoplasmic filaments are located (Marciano-Cabral, 1988; Schuster & Visvesvara, 2004; Visvesvara *et al.,* 2007). The trophozoite is able to transform into a swimming flagellate form when their environment becomes nutrient deprived (Fig. 2) (Dingle & Fulton, 1966). This can be induced in the laboratory by placing the amebae in distilled water. The flagellate is pear-shaped with two flagella and measures in length from 10 to 16 μm. It possesses a single nucleus with a prominent nucleolus and is unable to feed or divide. The flagellate is a temporary form that usually reverts back to the trophozoite form within an hour or less. When subjected to an unfavorable environment, such as adverse growth conditions, the trophozoite can transform into a resistant cyst form. The cyst is double-walled with a thick endocyst and a thin ectocyst. Pores are present in the cell wall, out of which the

Image adapted from: Marciano-Cabral, F., and G. Cabral. 2007. The immune response to *Naegleria fowleri* amebae and pathogenesis of infection. FEMS Immunol. Med. Microbiol. 51:243-259.

Figure 1. Scanning electron micrographs of the three morphological stages of *Naegleria*. Stages include a dormant cyst, a feeding trophozoite, and a transient swimming flagellate.

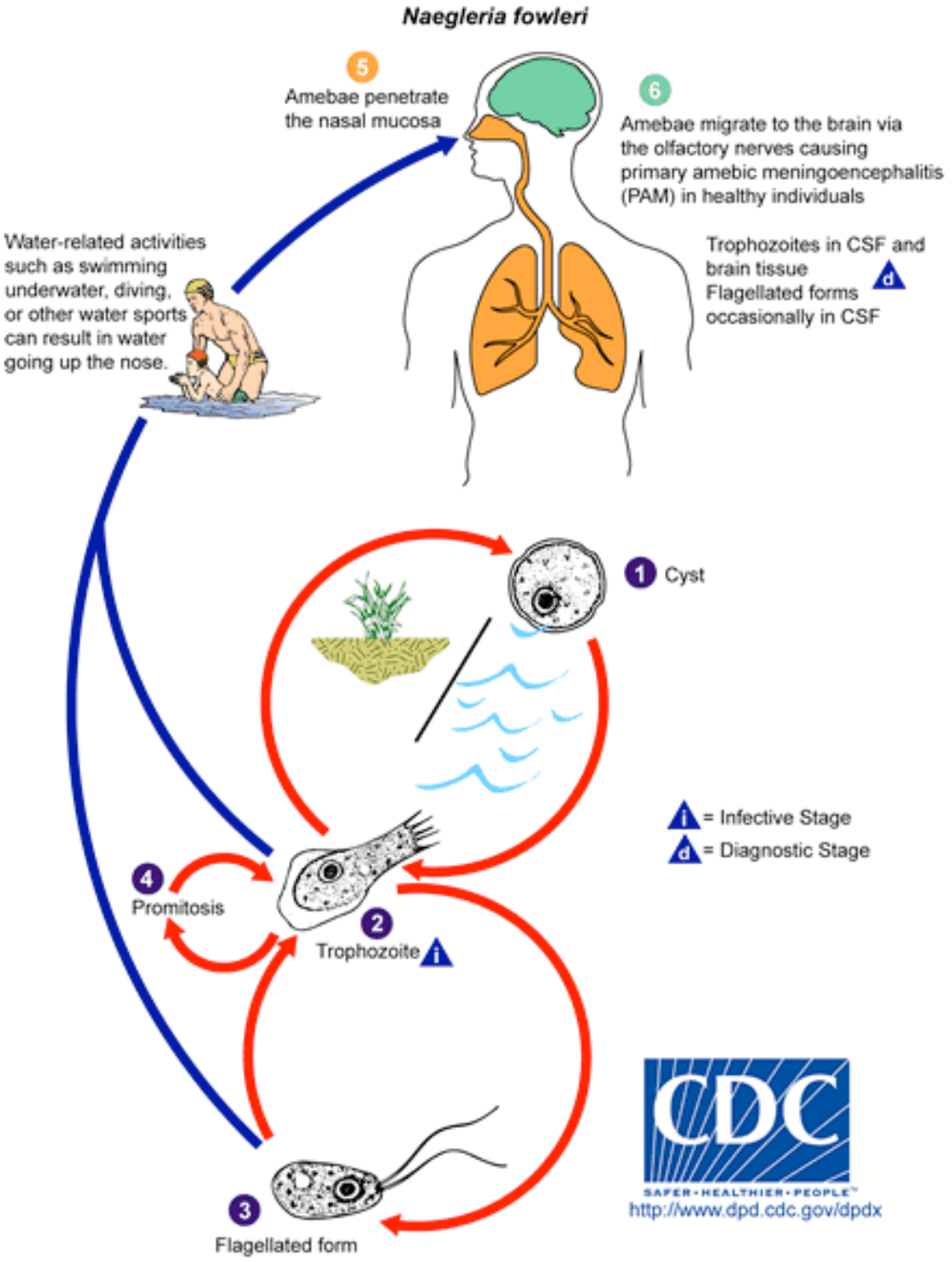

Figure 2. Image from CDC Public Health Image Library. Image # 3412, Content Provider – Alexander J. da Silva & Melanie Moser

Figure 2. *Naegleria fowleri* life cycle. *N. fowleri* has three forms including the trophozoite or ameboid form which is thought to be the infective stage. Under conditions of nutrient deprivation, the trophozoite undergoes a transitory transformation to a flagellate stage, and 'swims' to the water surface to seek a bacterial food source. The cyst of *N. fowleri* affords protection from adverse environmental conditions. Factors that induce cyst formation include food deprivation, crowding, desiccation, accumulation of waste products, exposure to toxic products of bacteria, pH changes, and salts (Marciano-Cabral,1988).

trophozoite can emerge (Fig. 2) (Marciano-Cabral, 1988; Schuster & Visvesvara, 2004; Visvesvara *et al.,* 2007).

Primary Amebic Meningoencephalitis (PAM).

N. fowleri is the causative agent of primary amebic meningoencephalitis (PAM), a rapidly fatal disease of the central nervous system (CNS) that occurs in healthy individuals engaged recently in activities such as swimming or diving in warm freshwater bodies (Carter, 1968; Martinez, 1985; Martinez & Visvesvara, 1997). Infection occurs when amebae enter the nasal passages and attach to the nasal mucosa (Carter, 1972; Martinez, 1985). Following contact with the nasal epithelium, amebae penetrate the underlying basement membrane and migrate to the brain wherein they multiply and cause extensive tissue damage (Jarolin *et al.*, 2000; Martinez *et al.,* 1973; Martinez, 1985). The destruction of tissue and hemorrhagic necrosis that occurs in the brain is accompanied by an inflammatory response consisting of neutrophils, eosinophils, and macrophages. Symptoms of PAM include severe frontal headache, fever, nausea and vomiting, stiff neck, and occasional seizures. Death usually occurs 7 to 10 days after exposure to amebae (Carter, 1970; Martinez, 1985; Barnett *et al.,* 1996; Martinez & Visvesvara, 1997; Marciano-Cabral & Cabral, 2007). There have been a few survivors of PAM, and in these cases the key to successful treatment was rapid identification of the disease followed by aggressive treatment (Schuster & Visvesvara, 2004).

Currently, the recommended treatment for PAM is Amphotericin B in combination with rifampin and other antifungal drugs (Seidel *et al.,* 1982). Survivors include a 10 year old California girl, who was aggressively treated with intravenous and intrathecal amphotericin B, intravenous and intrathecal miconazole, and oral rifampin (Seidel *et al.,* 1982). Four years after the infection, she remained completely healthy and free of any neurological deficits. Additionally, a 32 year old male with a history of waterskiing, survived after aggressive treatment with amphotericin B coupled with rifampin (Brown, 1991).

PAM epidemiology.

The first known fatal *N. fowleri* infection was reported in 1965 by Fowler & Carter in South Australia (Fowler & Carter, 1965). The disease was termed “primary amebic meningoencephalitis” to distinguish an infection caused by *N. fowleri* from the rare invasion of the brain by the intestinal ameba *Entamoeba histolytica* (*E. histolytica*) (Butt, 1966; Carter, 1968). After the initial report, accounts of patients with PAM from the United States were reported in Florida and Texas (Butt, 1966; Butt *et al.,* 1968). Additionally, a retrospective study demonstrated that an outbreak of PAM had occurred in Richmond, Virginia from 1951 to 1952 (dos Santos, 1970). Since the initial identification of the disease, infections have been reported worldwide (Martinez, 1985; Willaert, 1974) with most cases reported from developed rather than developing countries, possibly due to greater awareness (John, 1982). Areas in the United States that have reported cases include New York, Virginia, North Carolina, South Carolina,

Georgia, Florida, Mississippi, Arkansas, Texas, Arizona, Nevada, Oklahoma, Missouri, Louisiana, New Mexico, and California (Fig. 3) (John, 1982; Yoder *et al.,* 2010). Swimming in warm freshwater is the most common exposure route for humans. In Richmond, Virginia, two man-made lakes located a few miles from each other were responsible for 14 out of 16 infections (Callicott, 1968; Duma *et al.,* 1971; dos Santos, 1970). Additionally, in Czechoslovakia, 16 deaths were linked to the same heated indoor swimming pool (Cerva *et al*., 1968). Exposure to *N. fowleri* amebae in environmental bodies of water is not the only source of infection. In South Australia, water delivered to homes from above ground pipelines during a period of hot weather was determined to be the source of PAM to children in backyard wading pools or in bathtubs (Carter, 1972; Miller *et al.,* 1982). Additionally, domestic water supplies in the United States have also been linked to fatal cases of PAM (Marciano-Cabral *et al.,* 2003). In the above mentioned cases, samples were collected and *N. fowleri* was recovered from samples of tap water taken from the homes where fatal cases of PAM occurred (Carter, 1972; Miller *et al.,* 1982; Marciano-Cabral *et al.,* 2003). In two Australian cases, houses had remained unoccupied for considerable periods of time during warm weather leaving the water supplied to the homes stagnant (Carter, 1972; Miller *et al.,* 1982). It is speculated that under prolonged warm climatic conditions, *N. fowleri* can multiply resulting in a significant number of amebae in warm stagnant sections of domestic water supplies (Carter, 1972; Miller *et al.,* 1982). The presence of free-living amebae in water can be detected by removing aliquots of water and sediment and subjecting the samples to growth on nonnutrient agar plates (NNA) seeded with *Escherichia coli* (*E. coli*) (Fig. 4) followed by specific Polymerase Chain Reaction

State	Cases (*n*)	State	Cases (*n*)	State	Cases (*n*)
Arizona (AZ)	7	Louisiana (LA)	1	North Carolina (NC)	4
Arkansas (AR)	4	Mississippi (MS)	1	Oklahoma (OK)	5
California (CA)	7	Missouri (MO)	1	South Carolina (SC)	5
Florida (FL)	29	Nevada (NV)	1	Texas (TX)	30
Georgia (GA)	5	New Mexico (NM)	1	Virginia (VA)	6

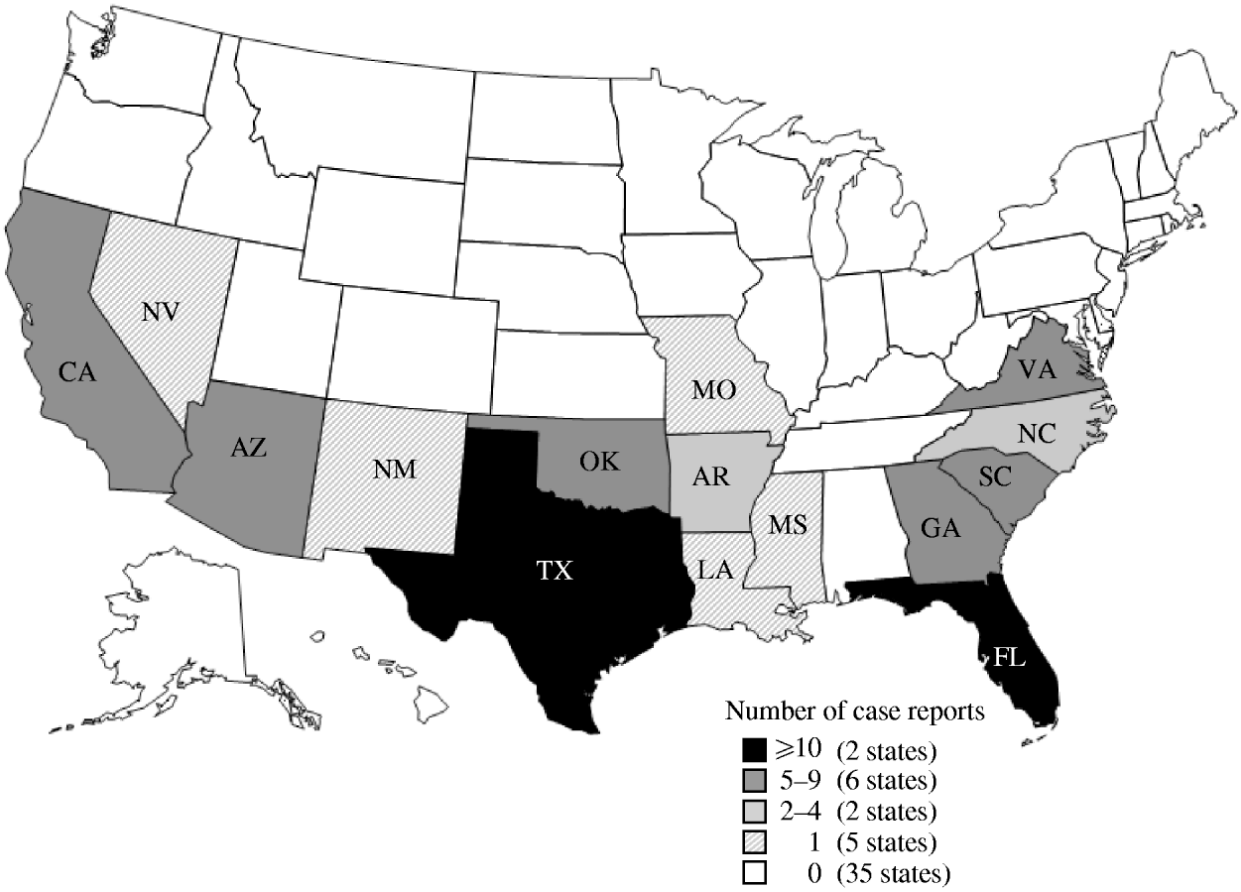

Image from. Yoder, J. S. , B. A. Eddy, G. S. Visvesvara, L. Capewell, and M.J. Beach. 2010. The epidemiology of primary amoebic meningoencephalitis in the USA, 1962 - 2008. Epidemiol. Infect. 138:968-975. Copyright © 2010 Cambridge University Press

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Figure 3. Number and location of case reports of primary amoebic caused by *Naegleria fowleri* in the United States 1962-2008. Four additional cases were reported but the sites of exposure remain unknown. (n=107)

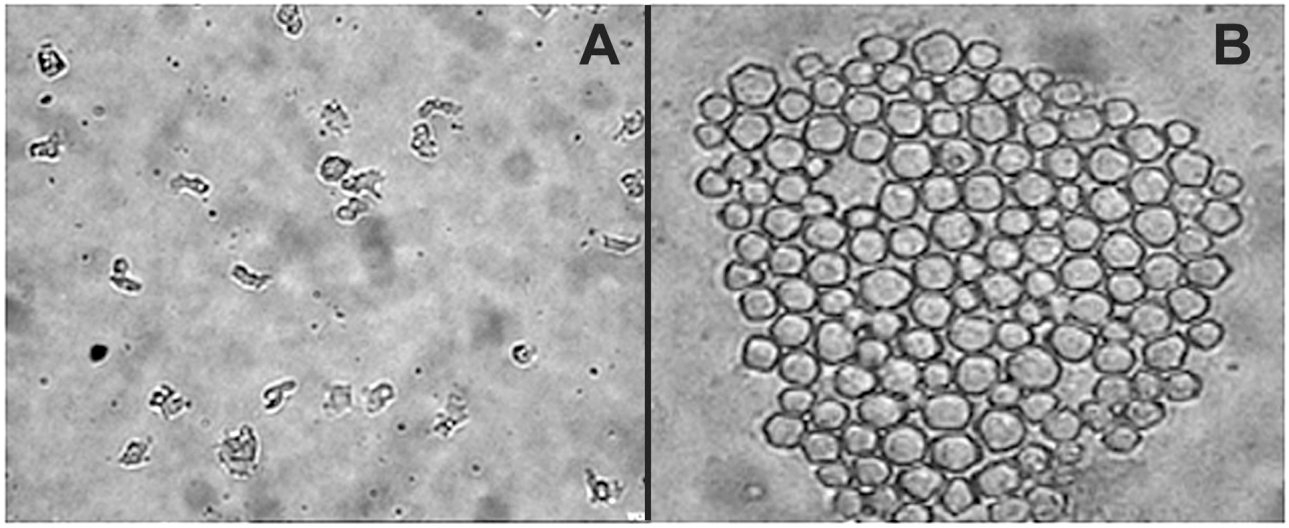

Image from: Marciano-Cabral, F., Jamerson, M., and Kaneshiro, E.S. 2010.Free-living amoebae, *Legionella* and *Mycobacterium* in tap water supplied by a municipal drinking water utility in the USA. J Water Health. 8:71-82. © IWA Publishing 2010.

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Figure 4: Light microscopic analysis of environmental samples. Amebae growth on non-nutrient agar (NNA) containing heat-killed *Escherichia coli* as a food source. (A) Trophozoites were observed after 48h growth. (B) Cysts were observed at day 7. Amebae can then be harvested and identified using PCR analysis.

assays (PCR) (Fig. 5) (Reveiller *et al.,* 2002; Jamerson *et al.,* 2009; Qvanstrom *et al.,* 2006).

Infections are more common in young people, possibly due to prolonged periods of activity in water, and engaging in diving and underwater swimming more often (Schuster & Visvesvara, 2004). The majority of infected individuals are male which may be explained by males being more likely than females to engage in water activities linked to infections. Humans are not the only known hosts to acquire infections naturally. In 1997 the first animal infection was identified in a South American tapir (Lozano-Alarcon *et al.,* 1997). Since then, infections also have been noted in cattle (Daft *et al.,* 1999; Visvesvara *et al.,* 2005).

Virulence factors.

The molecular mechanisms of pathogenesis of primary amebic meningoencephalitis are not well-defined. However, virulence factors that have been described are believed to play important roles in the pathogenicity associated with *N. fowleri* infections. Pathogenicity is the ability of a microorganism to cause disease and virulence refers to the degree of pathogenicity (Lipsitch & Moxon**,** 1997). It is important to note that the prolonged growth of *N. fowleri* in axenic culture *in vitro* leads to a decrease in virulence while serial passage through mice restores and maintains virulence (Wong *et al.,* 1977; Whiteman & Marciano-Cabral, 1987). Therefore, it is important to maintain virulence to get a true picture of what is occurring during the disease process.

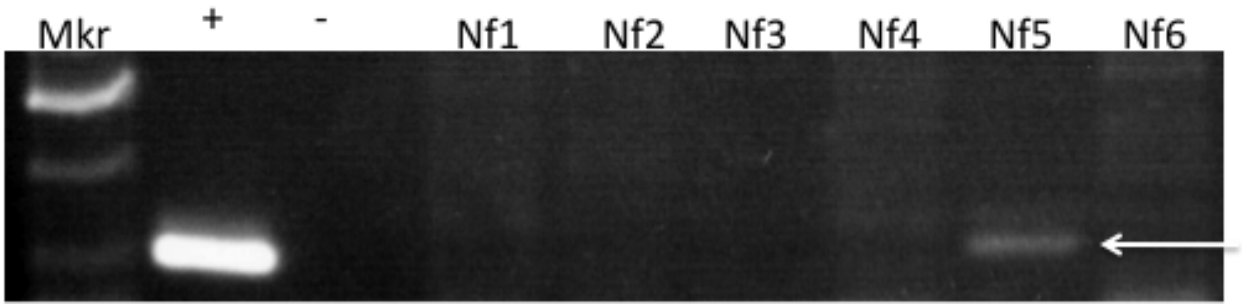


Figure 5. Nested PCR analysis for *Naegleria fowleri*. Representative agarose gel demonstrating the 110-bp nested PCR amplification product (*arrow*) for *N. fowleri*. (+) positive control, (−) negative control. Nf1–6 designates different environmental sampling sites for which material was assessed by nested PCR. The lane designated "*Mkr*" is the bp ladder markers.

Heat shock proteins of parasitic organisms are involved in multiple essential processes including protein folding, refolding of denatured proteins, and regulation of host's immune mechanisms (Polla, 1991; Joshi *et al.,* 1992; Mun *et al.,* 2000). Heat shock 70 protein has been identified in *N. fowleri* and has been demonstrated to play a role in proliferation and cytotoxicity (Song *et al.,* 2007; Song *et al.,* 2008). However, since there are thermophilic nonpathogenic species of *Naegleria* (Stevens *et al.,* 1980), temperature tolerance and, therefore, the presence of heat shock proteins do not appear to define pathogenicity.

Numerous studies have investigated the mode by which *N. fowleri* is able to destroy target cells. *N. fowleri* trophozoites have been shown to destroy a variety of target cells by trogocytosis or piecemeal ingestion, using a 'food-cup' structure located on their surface (Brown, 1979; Marciano-Cabral *et al.,* 1982; Marciano-Cabral & Fulford, 1986). Studies have demonstrated that multiple species, both pathogenic and nonpathogenic, possess 'food-cups' (Marciano-Cabral & Fulford, 1986) and that more pathogenic mouse-passaged *N. fowleri* exhibit fewer 'food-cups' than nonpassaged axenic *N. fowleri* (John *et al.,* 1984). Therefore, while multiple studies have shown that 'food-cup' structures are involved in cellular damage in vitro, there is no definitive correlation between pathogenicity and these structures.

Another method of cell destruction is amebae-mediated lysis, which is observed to a greater extent in recently mouse-passaged, highly-pathogenic *N. fowleri* (Marciano-Cabral & Fulford, 1986). Proteins that have been demonstrated to be involved in

this type of cell lysis include naegleriapores (Young & Lowrey, 1989; Herbst *et al.,* 2002). Initial studies isolated one membrane-bound pore-forming protein, which was able to lyse several tumor cell lines as well as erythrocytes (Young & Lowrey, 1989). Herbst *et al.* (2002), further investigated the role of pore-forming proteins and named these proteins naegleriapores, based on their pore forming ability. Their studies also demonstrated that naegleriapores are capable of lysing bacteria, providing a functional role for these proteins in *N. fowleri's* natural environment (Herbst *et al.,* 2002). Other proteins such as phospholipases and hydrolases are believed to be involved in the disease process through the degradation of myelin (John, 1982) but do not appear to be unique to *N. fowleri* (Eisen & Franson, 1987). Additionally, proteases have been investigated and are suspected to contribute to tissue destruction facilitating invasion. Elastase, a serine protease has been reported in several free-living amebae, including *Acanthamoeba culbertsoni, N. fowleri,* and *Naegleria australiensis* (Ferrante and Bates, 1988). Studies have revealed a 30 kDa cysteine protease that is secreted by *N. fowleri* and nonpathogenic *N. gruberi* that is able to degrade extracellular matrix proteins in vitro and causes cellular damage to mammalian cells (Aldape *et al.,* 1994). Furthermore, two high molecular weight cysteine proteases (128 kDa and 170 kDa) have been observed in *N. fowleri* whole cell lysates (Mat Amin, 2004). However, although numerous studies have demonstrated the presence of proteases in *N. fowleri* it is important to note that nonpathogenic species also possess proteases (Aldape *et al.,* 1994; Serrano-Luna *et al.,* 2007).

Immune evasion mechanisms also may play a role in the virulence of *Naegleria* amebae. To be successful in establishing an infection amebae must survive the activity

of cells and soluble mediators of the host's immune system. Studies have demonstrated that neither antibodies nor cell-mediated defense mechanisms are sufficient to provide protective immunity against *N. fowleri* (Cursons *et al.,* 1980; Newsome & Arnold, 1985; Reilly *et al.,* 1983; Thong *et al.,* 1979; Marciano-Cabral, 1988). Complement system components appear to be important mediators since complement deficient mice are more susceptible to *N. fowleri* infections (Reilly *et al.,* 1983). Whiteman and Marciano-Cabral (1987) demonstrated that the nonpathogenic species of *Naegleria* (*N. gruberi*) and weakly pathogenic species (*N. australiensis*) are more susceptible to lysis by normal human serum complement than the pathogenic species (*N. fowleri*). Furthermore, removal of surface proteins with trypsin and papain increase highly pathogenic mouse passaged *N. fowleri* sensitivity to complemented-mediated lysis (Whiteman & Marciano-Cabral, 1989). These findings suggest that surface components sensitive to proteolytic activity function to limit complement-mediated lysis. Additional studies by Fritzinger *et al.,* (2006) identified an 18 kDa immunoreactive protein that was detected on the membrane of *N. fowleri* by western immunoblot and immunofluorescence analyses with monoclonal antibodies for human CD59. CD59 is a glycosyl-phosphatidylinositol (GPI) anchored glycoprotein that is present in eukaryotic cells, including erythrocytes and leukocytes, which inhibits complement-mediated lysis through interference with the formation of the membrane attack complex of complement (Zalman *et al.,* 1986; Morgan, 1989). Interestingly, a CD59-like molecule was not detected in nonpathogenic *N. gruberi*, which is susceptible to complement (Fritzinger *et al.,* 2006). Additionally, *N. fowleri* amebae have also been shown to resist complemented-mediated lysis by removing surface associated membrane attack

complexes through membrane vesiculation (Toney & Marciano-Cabral, 1994).

Extracellular matrix.

The extracellular matrix (ECM) consists of a group of macromolecules, which include fibronectin, collagens, laminin, proteoglycans, and non-matrix proteins, (growth factors) which function in cell migration, differentiation, provide support and anchorage of cells, and segregate tissues (Berrrier & Yamada, 2007). A pivotal step during infection by *N. fowleri* is its interaction with the host extracellular matrix, including the basement membrane, a complex layer of specialized extracellular matrix (ECM) glycoproteins and proteoglycans that serves to separate the epithelium from stromal tissues (Jarolim *et al.,* 2000; LeBleu *et al.,* 2007). During migration to the brain, *N. fowleri* must transverse the epithelium and contact specified ECM components, such as laminin-1, collagen I, and fibronectin. Laminins are a family of cross-shaped molecules that contain three polypeptides (α, ß, γ), which have high affinity for other ECM components (Yurchenco & Schittny, 1990). Laminin-1 is the main laminin in the basement membrane (Yurchenco & Schittny, 1990) and was therefore utilized in the present study. Collagen is characterized by having a primary structure containing a three residue repeat, Gly-Pro-X or Gly-X-Hyp (hydroxproline), where X may be any of various other amino acid residues (Mayo, 1996; Brodsky & Persikov, 2005; Okuyama *et al.,* 2006). Proline and 4-hydroxyproline stabilize the three individual polyproline II-like helices and following post-translational modification, secreted collagen helices self-assemble to cross-linked microfibrils (Vakonakis & Campbell, 2007). Collagen I was utilized in the present study

since the majority of collagen found in the human body is collagen I (Jensen & Host, 1997). Fibronectin is an adhesive glycoprotein found in connective tissues and the blood (Hynes & Yamada). It is a heterodimeric molecule containing three types of repeating domains, termed I, II, and III, with the majority of the molecule composed of domain type III repeats. It has binding domains capable of binding fibrin, heparin, collagen, integrins, and other cell surface moieties (Tanzer, 2006).

Extracellular matrix components are usually inaccessible to an incoming pathogen. However, disruption such as tissue damage can lead to the exposure of ECM components to pathogenic organisms. Therefore, microorganisms have developed strategies to interact with ECM components, facilitating the pathogenesis of these organisms. For example, *Trichomonas vaginalis* expresses a surface receptor that binds laminin, resulting in increased parasitic adherence to epithelial cells (Silva-Filho *et al.,* 1988). Additionally, ECM interactions also have been noted for bacterial pathogens. *Staphylococcus aureus* (*S. aureus*) possesses laminin receptors, which are believed to play a role in bloodstream invasion and widespread metastatic abscess formation, characteristic of *S. aureus* infection (Lopes *et al.,* 1985). Protozoa, including *E. histolytica*, the causative agent of amebic dysentery, interacts with ECM components to spread from the gut to other organs, including the liver (Espinosa-Cantellano and Martinez-Palomo, 2000).

Integrins and other ECM binding proteins.

Many microbial pathogens express cell surface receptors that mediate the critical step of adhesion to ECM components of host tissue. Receptors possibly involved in this

interaction are integrins, a family of glycosylated heterodimeric transmembrane adhesion receptors involved in a variety of cellular processes (Johnson *et al.,* 2009). These molecules are expressed in various cells and are composed of two non-covalently associated subunits (α and β), which bind to specific peptide epitopes on ECM proteins and function as signaling receptors (Harburger & Calderwood, 2009). Currently, 18 α subunits and 8 β subunits that can assemble into 24 different heterodimers have been identified in vertebrates (Takada *et al.,* 2007). Integrins act as bi-directional signaling receptors through outside-in and inside-out signaling. The inside-out signaling acts to bring the integrin into its active conformation. Once an integrin binds its ligand, the integrin undergoes a conformational change that leads to outside-in signaling. This signaling in turn activates further signaling events that are dependent on other signaling receptors present and available in that specific cell (Barczyk *et al.,* 2010). Integrins have been studied extensively in cancer cells where they serve a dual function, assisting in adhesion to ECM components and assisting in migration (Lauwaet *et al.,* 2000). Integrin-like molecules have been identified on parasites and bacteria for interactions with the ECM (Santoni *et al.,* 2001; Hostetter, 1999; Han *et al.,* 2004; Sengupta *et al.,* 2009).

Another possible type of receptor that may be involved in attachment to ECM components are lectins. Lectins recognize and bind sugar components present on many different classes of molecules including ECM components. Microbes have been shown to bind highly glycosylated ECM components by lectin interactions. For example, Kukkonen *et al.* (1993), have demonstrated that *Salmonella enterica* and *Escherichia coli* bind to laminin and reconstituted basement membranes through the use of a

fimbrial lectin. *Entamoeba histolytica* possess a 140 kDa integrin-like fibronectin binding protein that has high sequence homology to the *E. histolytica* Gal/GalNAc-specific lectin (Sengupta *et al.,* 2009). Furthermore, Rocha-Azevedo *et al.* (2010), demonstrated that the presence of mannose decreases attachment of *Acanthamoeba culbertsoni* to both laminin-1 and collagen I, implying a possible role for the *Acanthamoeba* mannose-binding lectin in attachment to ECM components.

ECM invasion.

Invasion is a multistep process that brings an invading cell or organism from the tissue of origin into another. It has been studied in depth in terms of cancer metastasis and is described in terms of cell-cell adhesion, cell-matrix interactions, proteolysis, and motility (Marcel *et al.,* 1991). Two different strategies for invasion have been described for cancer cells: protease-dependent and protease-independent invasion. Protease-dependent invasion involves the cleavage of matrix components and amoeboid, or nonproteolytic invasion occurs through gaps in the extracellular matrix meshwork. Invasion involving proteolytic cleavage of the matrix is achieved through the use of matrix metalloproteinases, cathepsins, and serine proteases located at the cell surface, along with integrins, which provide adhesion needed for invasion. The breakdown of matrix components creates localized matrix defects, remodeling the ECM and facilitating invasion (Friedl & Wolf, 2003). In contrast, nonproteolytic invasion does not remodel the ECM, but instead requires a cell to change its shape allowing for migration by the cell gliding and squeezing through gaps present in connective tissues (Friedl & Wolf, 2003).

The process of invasion is not unique to cancer cells and has been observed for a variety of microorganisms. For example, invasive pathogens such as *E. histolytica,* invade through the enteric wall, spread to the blood, and disseminate to the liver (Espinosa-Cantellano and Martinez-Palomo, 2000). During this process the amebae interact with the enteric basement membrane, the stromal extracellular matrix, and the subendothelial basement membrane. Cysteine proteases have been suggested to be involved in this invasive process by degrading components of the extracellular matrix, including fibronectin, laminin, and collagen (Que & Reed, 2000). Studies investigating invasion of other microorganisms including *T. cruzi*, *Plasmodium falciparum*, and *Cryptosporidium parvum* have shown that invasion can be blocked by specific protease inhibitors further implying a role for proteases in invasion processes (Mayer *et al.,* 1991; de Cazzulo *et al.,* 1994; Forney *et al.,* 1996).

Research objectives.

Naegleria fowleri is a highly pathogenic free-living ameba that causes a rapidly fatal disease of the central nervous system known as primary amebic meningoencephalitis. Over 40 different species of *Naegleria* have thus far been isolated and identified, yet only *N. fowleri* has been associated with human infections. We speculate that there are distinct phenotypic differences functionally linked to the pathogenicity and high mortality that is only associated with *N. fowleri*. Therefore, the objective of the present study was to define characteristics that distinguish pathogenic *N. fowleri* from nonpathogenic *N. lovaniensis*. The assumption is that processes such as attachment and invasion are essential for *N. fowleri* pathogenicity and that these

processes require recognition and destruction of host extracellular matrix components. To undertake this objective, we compared a variety of phenotypic characteristics, including attachment and invasion of the ECM. The differences observed in these areas led to further investigations of the possible receptors involved in attachment and the possible mechanism involved in invasion of host extracellular matrix components.

CHAPTER 2: Materials and Methods

Amebae. The species of amebae utilized in this study are *N. fowleri* (ATCC 30894) and *N. lovaniensis* (ATCC 30569) obtained from the American Type Culture Collection (ATCC). The amebae were grown at 37^{o}C for 24 h in Oxoid medium (13) in 75 cm^{2} plastic flasks. *N. fowleri* (ATCC 30894) was originally isolated from a patient who died from Primary Amebic Meningoencephalitis (PAM). *N. fowleri* was passaged by the intranasal route in $B_6C_3F_1$ mice at monthly intervals to maintain their virulence and were harvested from mouse brain tissue (Toney & Marciano-Cabral, 1992). For experiments, amebae were detached from flasks by mechanical bumping and washed two times in 0.01M phosphate-buffered saline (PBS) pH 7.2.

Mammalian cell cultures. Human nasal epithelial cells (ATCC CCL-30) were cultured in 75 cm^{2} plastic flasks at 37^{o}C with 5% (v/v) CO_2 in minimal essential medium (MEM) supplemented with 10% fetal calf serum, L-glutamine, non-essential amino acids, and vitamins.

Growth rate studies. *N. fowleri* and *N. lovaniensis* (10^4 total amebae) were placed in 75 cm^{2} plastic flasks at 37^{o}C containing 10 mL Oxoid media. At designated time

intervals (24 h, 48 h, 72 h, and 96 h) the flasks were bumped to detach amebae, 10μL of the media containing amebae were removed, and amebae were counted using a hemacytometer. Images were acquired using an Olympus CK2 inverted light microscope (Opelco, Washington, DC) in concert with an attached XV-GP230 digital video camera (Panasonic, Yokohoma, Japan). A Dell Dimension XPS1450 computer (Dell, Inc., Round Rock, Texas) programmed with Videum 100 hardware and Window NT software (Winnov, Sunnyvale, CA) was used to capture images.

Contact-dependent cytotoxicity. Human nasal epithelial cells (10^5) were seeded into a 96 well plate. *N. fowleri* and *N. lovaniensis* (10^5) were added and the plates were incubated at 37^{o}C for 2.5 h. Cytotoxicity was determined by measuring lactate dehydrogenase (LDH) released from mammalian cells using a LDH cytotoxicity detection kit (Roche, Indianapolis, IN). One hundred percent cytotoxicity represented the full release of LDH by target cells, which had been solubilized with 2% (v/v) Triton X-100. Cytotoxicity was calculated according to the formula: (total LDH release — sample LDH release) / (total LDH release) × 100%

Contact-independent cytotoxicity. Twenty-four well tissue culture plates (Becton, Dickinson and Company, Franklin Lake, NJ) were seeded and grown overnight with human nasal epithelial cells (2 x 10^5) (ATCC-CCL-30). *N. fowleri* and *N. lovaniensis* (2 x 10^5) then were added to tissue culture inserts (Greiner BioOne, Monroe, NC) having a pore-size of 0.4μm and incubated for 4.5 h. After the incubation the inserts were removed, cells were fixed with 2.5% glutaraldehyde, and the wells were washed once to

remove residual fixative. Subsequently, 0.1% crystal violet in PBS was added to the wells for 30 min. Wells were washed three times with PBS, the stain associated with the attached cells was released by adding methanol and the absorbance was read at 530 nm in a SpectraMAX spectrophotometer (Molecular Devices, Sunnyvale, CA).

Complement sensitivity assay. *N. fowleri* and *N. lovaniensis* ($5x10^4$) were incubated in 96 well plates with normal human serum (dilutions 1:2-1:32). The plates were incubated at 37^{o}C for 24 h and then examined for cell lysis microscopically. Heat-inactivated serum was used as a negative control. Light micrographs were acquired using an Olympus CK2 inverted microscope (Opelco, Washington, DC) in concert with an attached XV-GP230 digital video camera (Panasonic, Yokohoma, Japan). A Dell Dimension XPS1450 computer (Dell, Inc., Round Rock, Texas) programmed with Videum 100 hardware and Window NT software (Winnov, Sunnyvale, CA) was used to capture images.

Extracellular matrix surface coating. Twenty-four multi-well plates or glass coverslips were coated with Sigmacote (Sigma, St. Louis, MO), washed once with deionized water, and air-dried overnight at room temperature. The surfaces of plates or coverslips then were incubated (2 h, 37^{o}C) with (50 μg mL^{-1}) of fibronectin from human plasma (Sigma, St Louis, MO), collagen I from rat tails (Sigma), or laminin-1 from Engelbreth-Holm Swarm mouse sarcoma (Invitrogen, Grand Island, NY) diluted in PBS. Before addition of amebae, wells or coverslips were washed gently once with PBS to remove unattached ECM glycoproteins.

Attachment assay. *N. fowleri* and *N. lovaniensis* were radiolabeled (24 h or 48 h) with 60μCi [^{3}H]-uridine (specific activity: 35.6 Ci/mmol) (Toney & Marciano-Cabral, 1992). Labeled amebae then were detached from flasks, washed two times with PBS, and counted using a hemacytometer. Amebae (2 x 10^5) then were added to ECM-coated wells (50 ug/ul). Following incubation (50 min, 37^{o}C), wells were washed once with PBS to remove non-adherent amebae. Wells coated with bovine serum albumin (BSA) were used as a control for non-specific attachment. Attached amebae were solubilized using 2% (v/v) Triton X-100 to release the incorporated radiolabel that was quantitated by liquid scintillation counting (Packard 2200CA TRI-CARB Liquid Scintillation Analyzer, Packard Instrument Co., Downers Grove, IL). Counts per minute (CPM) were converted to percent attachment, where 100% represented the amount of radioactivity present in 2 x 10^5 amebae: % attachment = [CPM of attached amebae]/[CPM of 2 x 10^5 amebae] x 100%.

Pretreatment of amebae. *N. fowleri* amebae (2x10^5) were preincubated for 30 min in PBS, galactose (100mM), mannose (100mM), alpha-mannopyranoside (100mM), trypsin (0.25%), or periodate (1mM). Amebae preincubated with trypsin and periodate were washed once with PBS to remove the enzymes and then were added to ECM coated wells (laminin-I, collagen 1, and fibronectin) as well as to BSA control wells. Amebae preincubated in PBS, galactose, mannose, and alpha-mannopyranoside were added directly to the ECM coated wells without a wash step to achieve co-incubation. After a 50 min coincubation (37^{o}C) wells were washed once with PBS and attached

amebae were fixed with 2.5% glutaraldehyde. Wells then were washed once (PBS) to remove residual fixative. Subsequently, 0.1% crystal violet in PBS was added to the wells for 30 min. Wells were washed three times with PBS, the stain associated with the attached cells was released by adding methanol and the absorbance was read at 530 nm in a SpectraMAX spectrophotometer (Molecular Devices, Sunnyvale, CA).

Amebae plasma membrane isolation. Amebae grown for 24 h were detached from tissue culture flasks, washed twice in PBS, and membrane proteins were isolated using Mem-PER eukaryotic membrane protein extraction kit (Pierce, Rockford IL), according to the manufacturer's instructions.

Indirect immunoblotting. A total of 30μg of *N. fowleri* or *N. lovaniensis* plasma membrane fractions, quantified using the Bradford assay (Bradford, 1976) were subjected to a 12% polyacrylamide SDS-PAGE. Following electrophoresis, proteins were transferred to nitrocellulose membranes (Towbin *et al.,* 1979) and then membranes were rinsed in Tris-buffered saline (5 min) containing tween 20 (TBST) and blocked for 1 h at room temperature in blocking buffer composed of 5% (w/v) non-fat dry milk in TBST. Following blocking, the membranes were rinsed and incubated for 2 h at room temperature in either mouse laminin-1 or human collagen I solutions (50mg/mL) in TBS containing 1% BSA, 0.01 M CaCl2, 0.01 M MnCl2, and 0.01 M MgCl2. Membranes were washed in TBST for 6 times, 5 min each wash. Membranes were incubated overnight with either a mouse monoclonal antibody against human laminin-1 (clone LAM-89, Sigma) in TBST (diluted 1:500) or a mouse monoclonal antibody against

human collagen I (MAB3391, Millipore) in TBST (diluted 1:500). Membranes were washed in TBST (6 times per 5 min) and incubated with a peroxidase-conjugated goat anti-mouse antibody (diluted 1:5000). Protein bands were visualized using a chemiluminescence detection kit (Perkin Elmer, Waltham, MA), according to the manufacturer's instructions. Negative controls consisted of nitrocellulose membranes incubated in the absence of laminin-1 and collagen I.

Ameba whole cell lysates. Amebae grown for 24 h were detached from tissue culture flasks, washed twice in PBS, and disrupted by three cycles of freezing in liquid nitrogen and thawing at 37^{o}C in lysis buffer containing protease inhibitors (50mM Tris-HCl, pH 7.4, 1mM PMSF,1.5mM pepstatin A, and 1.5mM leupeptin). Cell lysates were used for western immunoblot analysis.

Western immunoblot. Thirty micrograms of *N. fowleri* or *N. lovaniensis* whole cell lysates, as quantified by the Bradford method (Bradford, 1976), were subjected to 12% polyacrylamide SDS-PAGE under non-reducing conditions. Following electrophoresis, proteins were transferred to nitrocellulose membranes (Towbin *et al*., 1979). The membranes were rinsed (5 min) in Tris-buffered saline containing 0.1% tween 20 (TBST) and treated for 1 h at room temperature (RT) with blocking buffer consisting of 5% (w/v) non-fat dry milk in TBST. Nitrocellulose membranes then were rinsed and incubated overnight with chicken polyclonal antibody directed against human β1 integrin (GW22754, Sigma) diluted in blocking buffer (1:500). Membranes were washed 6 times (5 min each) in TBST and incubated with a peroxidase-conjugated rabbit anti-chicken

antibody (diluted 1:10,000). Protein bands were visualized using a chemiluminescence detection kit (Perkin Elmer, Waltham, MA), according to the manufacturer's instructions. A human monocyte lymphoma whole cell homogenate (U937) (10μg) served as a positive control for integrin protein.

Confocal microscopy. *N. fowleri* amebae were incubated on uncoated, collagen I-coated, laminin-1-coated, or fibronectin-coated coverslips (25 min 37^{o}C), fixed in 4% paraformaldehyde (60 min, 37^{o}C), and rinsed with Dulbecco's Phosphate Buffered Saline (DPBS, containing Ca^{++} and Mg^{++}). Cells were permeabilized (20 min) with 0.1% Triton X-100 in DPBS, blocked (60 min) with 5% BSA in DPBS, and incubated (2h, RT) with a FITC-conjugated monoclonal anti-integrin ß1 antibody (P4G11, Millipore, Billerica, MA). Coverslips were washed with DPBS (1% BSA), incubated with Alexafluor 594 phalloidin (1:500, A12381, Invitrogen) and washed with DPBS. Coverslips were incubated with DAPI (1:20,000) to identify nuclei. Images were acquired by spinning disc confocal microscopy using a BX51 microscope (Olympus, Center Valley, PA, USA) affixed with an Olympus disk spinning unit and an Orca-R2 CCD camera (Hammamatsu, Japan). Images were processed using the Slidebook software package (Intelligent Imaging Innovations, Denver, CO, USA).

Competitive attachment assays. *N. fowleri* amebae were radiolabeled (24h) with 60μCi [^{3}H]-uridine (specific activity: 35.6 Ci/mmol) (Thong & Ferrante, 1986). Following labeling, amebae were incubated (20 min) with the peptide sequence Gly-Arg-Gly-Asp-Thr-Pro (500 μg.mL^{-1}, MP Biomedicals) which contains an integrin recognition site, to

determine whether the peptide could inhibit attachment (Ruoslahti, 1996). Amebae also were incubated (20 min) with the peptide sequence Ser-Asp-Gly-Arg-Gly (Sigma), which represents a non-specific control. Amebae then were used in the attachment assay using collagen I. Counts per minute (CPM) were converted to percent attachment, where 100% represented the amount of radioactivity present in 2 x 10^5 amebae: % attachment = [CPM of attached amebae]/[CPM of 2 x 10^5 amebae] x 100%. Also, *N. fowleri* (2 x 10^5) were preincubated (10 min) with a monoclonal anti-Integrin β1 antibody (P4C10, Billerica, MA) (1:100) or with anti-Keyhole Limpet Hemocyanin (KLH12B4.G3.A8, Abcam, Cambridge, MA) (1:100) an irrelevant antibody used as a control, prior to addition to ECM components for 50 min to assess for blocking of attachment. At the end of the incubation period, unattached amebae were collected, fixed with glutaraldehyde and counted using a hemacytometer to determine whether the anti-Integrin β1 antibody inhibited attachment.

Invasion assays. Tissue culture inserts (Greiner BioOne, Monroe, NC) having a pore-size of 8 μm were coated (100μL, 10 min) with 1.7mg.mL^{-1} of either type I collagen or matrigel (Sigma), a reconstituted basement membrane solution that forms a matrix that has been used extensively for in vitro invasion studies (Kleinman & Jacob, 2001; Kleinman & Martin, 2005). Fibronectin and laminin-1 were not used for invasion studies since they do not allow for formation of a tri-dimensional matrix structure that can be utilized for invasion studies. Following the coating period, residual ECM solutions were removed, and the inserts were allowed to dry (2 h) at room temperature. The coated inserts were placed in 24 well plates and were used as an upper chamber. Oxoid

medium was added to the bottom chamber of the tissue culture well to serve as an ameba attractant. *N. fowleri* or *N. lovaniensis* (2 x 10^5 amebae) suspended in PBS were added to the upper chamber, and the plates were incubated for 4 h at 37^{o}C. Amebae that passed through the ECM coated inserts and into the bottom chamber were collected and counted using a hemacytometer. A second type of invasion assay was performed which incorporated the use of human nasal epithelial cells to mimic the in vivo site of *N. fowleri* infection. Tissue culture inserts were coated with matrigel as previously described, and matrigel was allowed to polymerize (2 h) under humid conditions at 37^{o}C. Human nasal cells (10^5) were then added to form a monolayer of cells on the top of the matrigel layer. Oxoid growth medium was added to the bottom of the tissue culture wells to serve as a migration attractant for amebae. *N. fowleri* or *N. lovaniensis* (10^5 amoebae) suspended in MEM were added to the insert top containing the nasal cell-matrigel scaffold and plates were incubated for 5 h at 37^{o}C with 5% (v/v) CO_2. Amebae that were able to migrate through the nasal cells and matrigel layer into the tissue culture wells were counted using a hemacytometer.

Gel zymography. Gel zymography was performed to detect protease activity during in vitro invasion assays. Invasion assays were performed as previously described with the addition of collecting the medium after 4 h of invasion. Aliquots of this medium were subjected to electrophoresis under non-reducing conditions on a 10% SDS-polyacrylamide gel containing 1% gelatin. Following electrophoresis, gels were incubated (30 min) at RT in 1x acetate renaturing buffer (0.1M, pH 5.0), equilibrated (30 min, RT) in 1x acetate developing buffer (0.1M, pH 5.0) containing DTT (1mM) , and

incubated overnight in 1x acetate developing buffer (0.1M, pH 5.0) containing DTT (1mM). Gels then were washed once with ultrapure deionized water and stained with Coomassie blue R-250.

Scanning electron microscopy. To examine for attachment to ECM components**,** *N. fowleri* and *N. lovaniensis* were incubated (50 min) on coated glass coverslips as described in the attachment assay and then fixed with 2.5% glutaraldehyde. To examine for invasion of the ECM matrix, *N. fowleri* and *N. lovaniensis* were incubated (2h) at 37 ºC on collagen I or matrigel (5 mg mL^{-1}) coated tissue culture inserts. Inserts then were immersed (1 h) in 2.5% (v/v) glutaraldehyde in 0.1M cacodylate buffer, pH 7.2, washed 4 times with PBS, and postfixed (40 min in the dark) with 2% (w/v) osmium tetroxide buffered in 0.1M cacodylate buffer, pH 7.2. The inserts were washed with PBS, dehydrated in a graded series of ethanol, subjected to critical-point drying with CO_2 as the transitional fluid, mounted on stubs, and coated with gold (30 nm) (Rocha-Azevedo *et al*., 2007). Samples were examined in a Zeiss EVO 50XVP scanning electron microscope (Zeiss, Oberkochen, Germany) operating at an accelerating speed of 15 kV.

Two dimensional gel electrophoresis protein preparation. Amebae (*N. fowleri* mouse-passaged and axenically maintained *N. fowleri* or *N. lovaniensis*) grown for 24 h were detached from tissue culture flasks, and the cell suspensions were placed in 50 mL conical tubes. The cells were washed twice in PBS. After the second wash the cells were resuspended in Cydye compatible 2D lysis buffer (7M urea, 2M thiourea, 4% CHAPS, 30mM Tris, 5mM magnesium acetate). Endonuclease (Sigma,150-300U) and a

protease inhibitor cocktail (Sigma, 10uL) were added to each sample. The protein mixture was then incubated on ice for 10 min. Samples were sonicated with three 30 second bursts and the protein preparation was placed on ice for 10 min after each burst of sonication. Samples were centrifuged for 10 min at 12,000 x g (or maximum speed on Eppendorf microcentrifuge) at 4^{o}C and then placed in a Spin-x filter microcentrifuge tube (Corning, Corning, NY) and centrifuged for 2 min at 12,000 x g (or the maximum speed on an Eppendorf microcentrifuge) at 4^{o}C. The protein concentration was determined using the RC/DC protein assay (BioRad, Hercules, CA).

Reconstitution of Cydyes. Cydyes (1nM of each, Cy2, Cy3, and Cy5) (GE Healthcare, Amersham, Little Chalfont, Buckinghamshire, UK) were removed from the -80°C freezer and left at room temperature to warm for 5 min. After 5 min, 1.5uL of DMF (Dimethylformamide) was added to each Cydye, leading to a concentration of 400pmol/µL. Tubes were vortexed vigorously for 30 sec and centrifuged for 30 sec at 12 000 × g (4^{o}C) in a microcentrifuge. Fluors then were used for labeling. Unused CyDye stock solution was stored at -20^{o}C.

Cydye labeling for Difference In Gel Electrophoresis (DIGE). Cell lysates were thawed on ice and microcentrifuged for 30 secs at 12,000 × g (4^{o}C). A volume of protein sample (*N. fowleri* or *N. lovaniensis*) equivalent to 50µg was added to a microcentrifuge tube and 1µl (400pmol) of diluted *N*-hydroxy succinimidyl ester-derivates of the cyanine dyes (Cy2, Cy3, Cy5) was added to the same microcentrifuge tube. Cy3 was added to the *N. lovaniensis* protein sample or axenic *N. fowleri* and Cy5 was added to the

mouse-passaged *N. fowleri* protein sample. A volume of protein sample equivalent to 25µg of each sample was added and mixed to a microcentrifuge tube. Cy2 was added to this mixture to serve as an internal standard. The microcentrifuge tubes then were mixed and centrifuged for 30 sec at 12,000 × g (4^{o}C). Tubes were placed on ice and incubated for 30 min in the dark. Lysine (1µl, 10mM) was added to each tube to stop the labeling reaction. Tubes were mixed, centrifuged (30 secs,12,000 × g, 4^{o}C), and incubated on ice for 10 mins in the dark.

First dimension. Fifty micrograms of protein from each of the Cydye labeled samples (Cy2, Cy3, and Cy5) were combined and the final volume was adjusted to 300µl using Ready Prep rehydration/sample buffer (Bio-Rad). Three hundred microliters were added to a rehydration/sample buffer tray. A 17cm IPG strip (Bio-Rad, pH range 5-8) was thawed (5 min) and forceps were used to remove the coverslip. The strip gel was placed facedown into the sample, making sure that air bubbles were not introduced. The strip then was overlayed with 2 mL mineral oil (Bio-Rad) and rehydrated overnight (RT). Paper wicks (Bio-Rad) were dampened with ultrapure water and placed at both ends of the focusing tray channel, covering the electrodes. The mineral oil was removed from the rehydrated IPG strip and allowed to drain off of the strip for 10 sec. The strip was transferred to the focusing tray channel containing the paper wicks so that the gel side with the "+" was in line with the "+" mark on the tray. Mineral oil (2 mL) was pipetted over the IPG strip and the tray was covered. The tray was then focused using a PROTEAN IEF cell (BioRad) for a total of 40,000 volt-hours. The IPG strips were removed from the focusing tray, mineral oil was allowed to drain for 10 secs, and

transferred gel side up to a clean rehydration/equilibration tray. Strips then were frozen at -80^{o}C until needed for the second dimension.

Second dimension. IPG strips that were frozen at -80^{o}C were allowed to thaw at RT for 10-15 min. The strip then was placed gel side up in a clean rehydration/sample buffer tray and covered with Equilibration Buffer I (Bio-Rad), containing 375 mM Tris-HCl, pH 8.8, 6 M urea, 2% SDS, and 2% DTT. The tray was placed on an orbital shaker shaking gently for 25 min. At the end the incubation period the buffer was removed carefully via a transfer pipet so as to not disturb the gel. The IPG strip was covered with Equilibration Buffer II (Bio-Rad), placed on an orbital shaker shaking gently for 25 min, and the buffer was removed as previously described. The IPG strip was then dipped in a graduated cylinder containing 1X Tris-glycine-SDS running buffer and placed gel side up onto the back plate of a 10% SDS-PAGE gel. Using forceps the IPG strip was pushed into the IPG well and overlayed with agarose. The gel then was run at 40mA/gel until the dyefront ran off the bottom of the gel (approx. 5 h). The gel was removed from the glass plate, scanned in using a Molecular Imager FX Pro Plus (Bio-Rad) and fluorescence was visualized using PDQuest ver.8.0 differential analysis software (Bio-Rad). Excitation filters used were as follows, Cy3: 532 nm, Cy5: 635 nm, and Cy2: 488 nm.

Statistical analysis. Data were expressed as the mean $\pm$ the standard deviation of the mean. To determine statistical significance ($P<0.05$), a two-tailed, unpaired Student's t-test and one-way analysis of variance (ANOVA) tests were used.

CHAPTER 3: Phenotypic differences between *Naegleria fowleri* and *Naegleria lovaniensis*

The first goal of this study was to compare a variety of phenotypic characteristics between pathogenic *N. fowleri* and nonpathogenic *N. lovaniensis* in order to gain insight as to why these two similar species are different in terms of pathogenicity. To accomplish this goal we examined multiple characteristics, which included growth rate, sensitivity to complement-mediated lysis, contact-dependent and contact-independent cytotoxicity, attachment to different extracellular matrix components, and invasion of different extracellular matrix components.

RESULTS:

Growth rate.

The growth rates of *N. fowleri* and *N. lovaniensis* were compared by counting the ameba density at different time intervals (Fig. 6). The generation time for *N. fowleri* was 1.5 h and the generation time for *N. lovaniensis* was 6.5 h. Light micrographs which were taken after 72 h of growth to visualize the difference in growth between the two species, demonstrated a difference in number of amebae (Fig. 7)

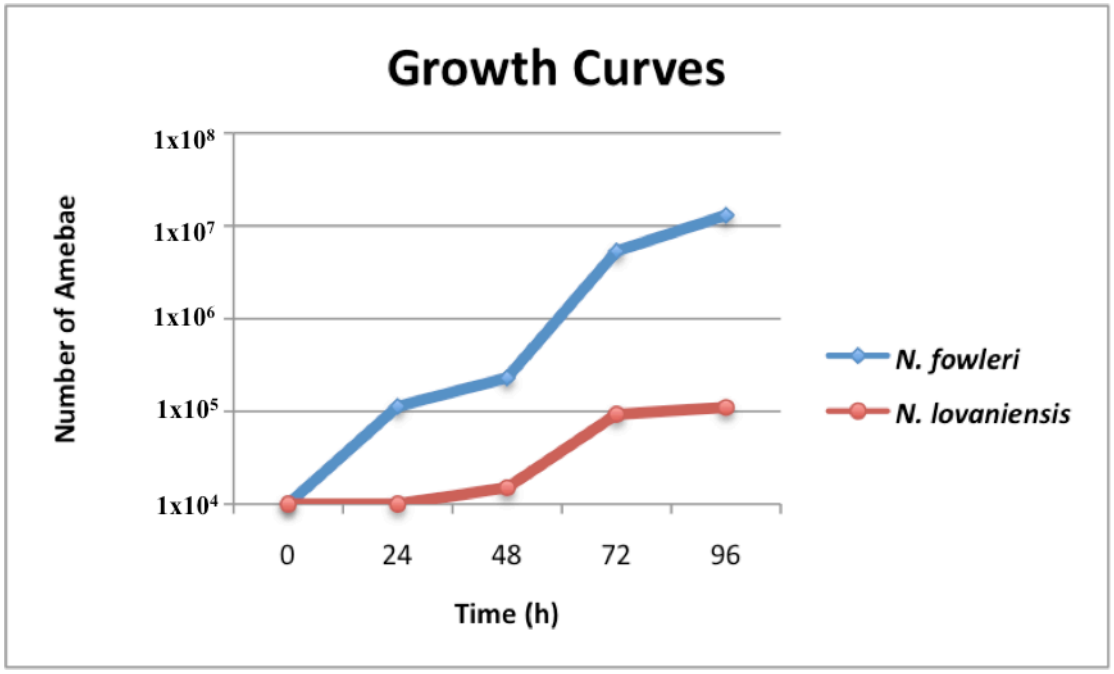


Figure 6: Representative growth curves of *Naegleria fowleri* and *Naegleria lovaniensis*. Amebae (10^4) were added to 75 cm^2 plastic flasks at 37^oC containing 10 mL of complete Oxoid media. Amebae were collected and counted using a hemacytometer. Diamonds represent *N. fowleri* and circles represent *N. lovaniensis*.

A B

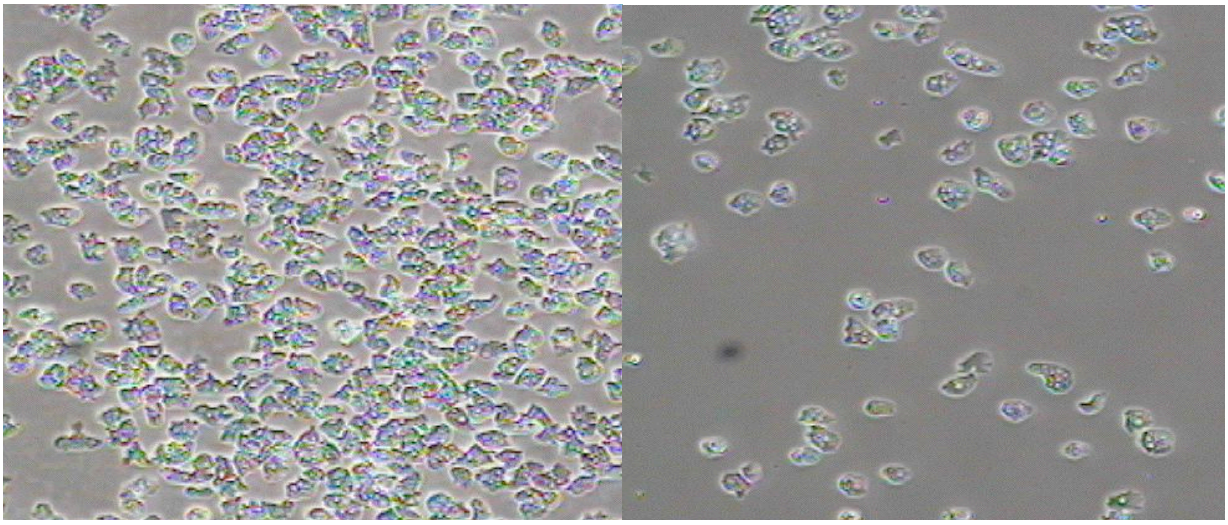


Figure 7: Representative micrographs of *Naegleria fowleri* and *Naegleria lovaniensis* during growth studies. Amebae (10^4) were added to 75 cm^2 plastic flasks at 37^{o}C containing 10 mL complete Oxoid media. Amebae (A) *N. fowleri* and (B) *N. lovaniensis* were visualized by light microscopy after 72 h of growth. Images are magnified 20x.

Contact-dependent cytotoxicity.

Contact-dependent cytotoxicity assays were utilized to determine whether there was a difference between *N. fowleri* and *N. lovaniensis* in contact-dependent lysis of human nasal epithelial cells. Release of cytoplasmic LDH was measured as an indication of plasma membrane damage. After 2.5 h both species were able to lyse human nasal epithelial target cells. However, no significant difference in contact-dependent cytotoxicity was observed (Fig. 8).

Contact-independent cytotoxicity.

Contact-independent cytotoxicity assays were used to investigate whether there was a difference between *N. fowleri* and *N. lovaniensis* in products secreted in response to the presence of human nasal epithelial cells. Assessment of target cell integrity using a crystal violet staining method revealed a 10% difference after a 4.5 h incubation period (Fig. 9). Although the difference appears minor it is statistically significant (Student's t-test). Light microscopy also demonstrated a difference in morphology of target cells suggesting a difference in secreted products, with more cytotoxic activity secreted by *N. fowleri* (Fig. 10).

Complement sensitivity.

Susceptibility to complemented-mediated lysis was examined to determine whether *N. fowleri* was more resistant than *N. lovaniensis*. Examination by light microscopy determined that pathogenic *N. fowleri* was resistant to complement-

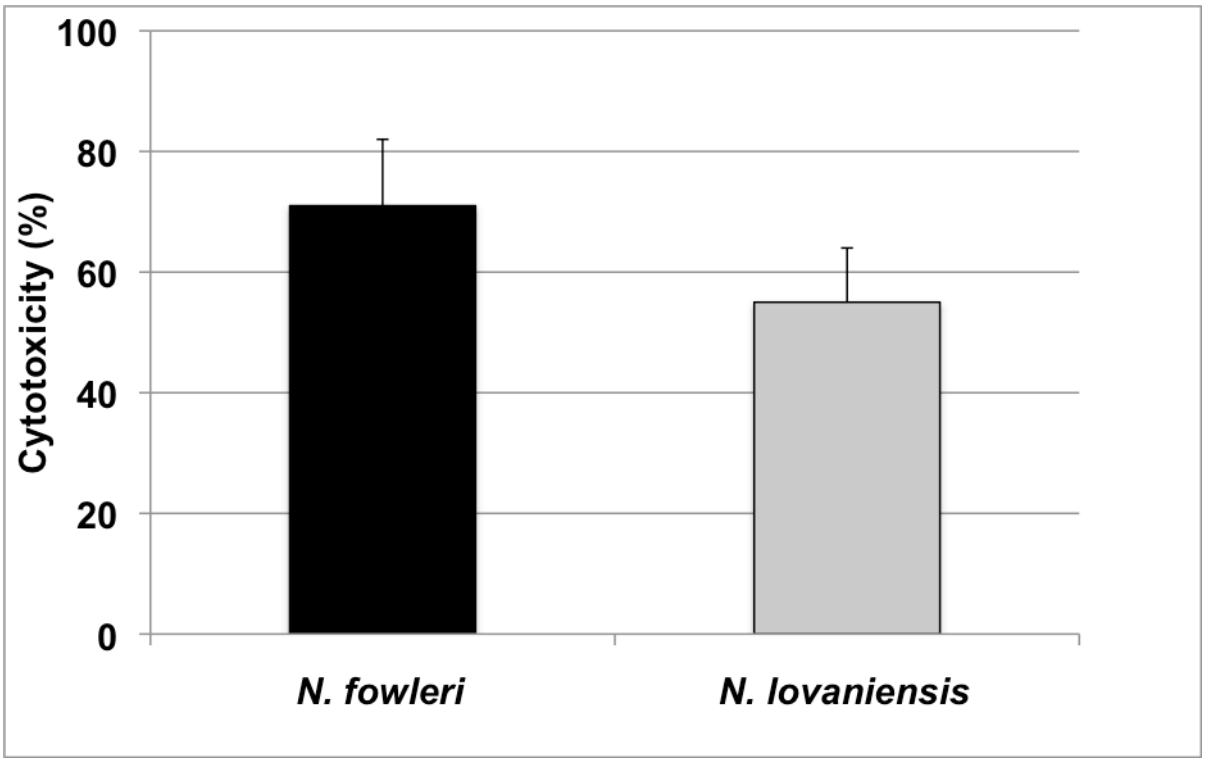


Figure 8: Effect of *Naegleria spp*. contact-dependent cytotoxicity on human nasal epithelial cells. Human nasal epithelial cells (10^5) were seeded into tissue culture plates. *N. fowleri* and *N. lovaniensis* (10^5) were added and allowed to interact for 2.5 h (37^oC). Cytotoxicity was determined by measuring lactate dehydrogenase (LDH) released from target cells. One hundred percent cytotoxicity represented the full release of LDH by target cells, which were solubilized with 2% (v/v) Triton X-100. Cytotoxicity was calculated as follows: (total LDH release - sample LDH release) / (total LDH release) × 100%. Black bar represents *N. fowleri* and gray bar represents *N. lovaniensis*.

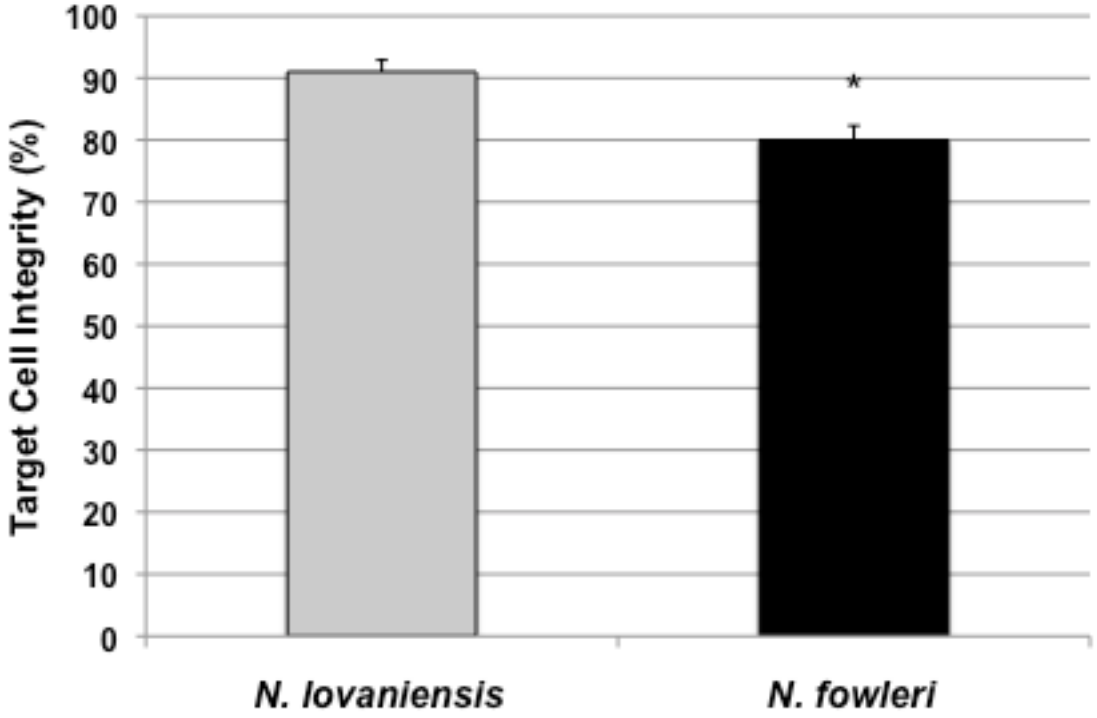


Figure 9: Effect of *Naegleria fowleri* and *Naegleria lovaniensis* contact-independent cytotoxicity on human nasal epithelial cells. Human nasal epithelial cells were seeded into tissue culture plates and *N. fowleri* and *N. lovaniensis* (2×10^5) were added to tissue culture inserts. Inserts were placed in tissue culture plate wells and incubated for 4.5 h. Inserts were removed, cells were fixed (2.5% glutaraldehyde), and wells were washed once to remove residual fixative. Crystal violet (0.1%) was added to the wells (30 min). Wells were washed, the stain associated with the attached cells was released by adding methanol and the absorbance was read (530 nm, SpectraMAX spectrophotometer). Black bar represents *N. fowleri* and gray bar represents *N. lovaniensis*. (*$P < 0.05$ Student's t-test).

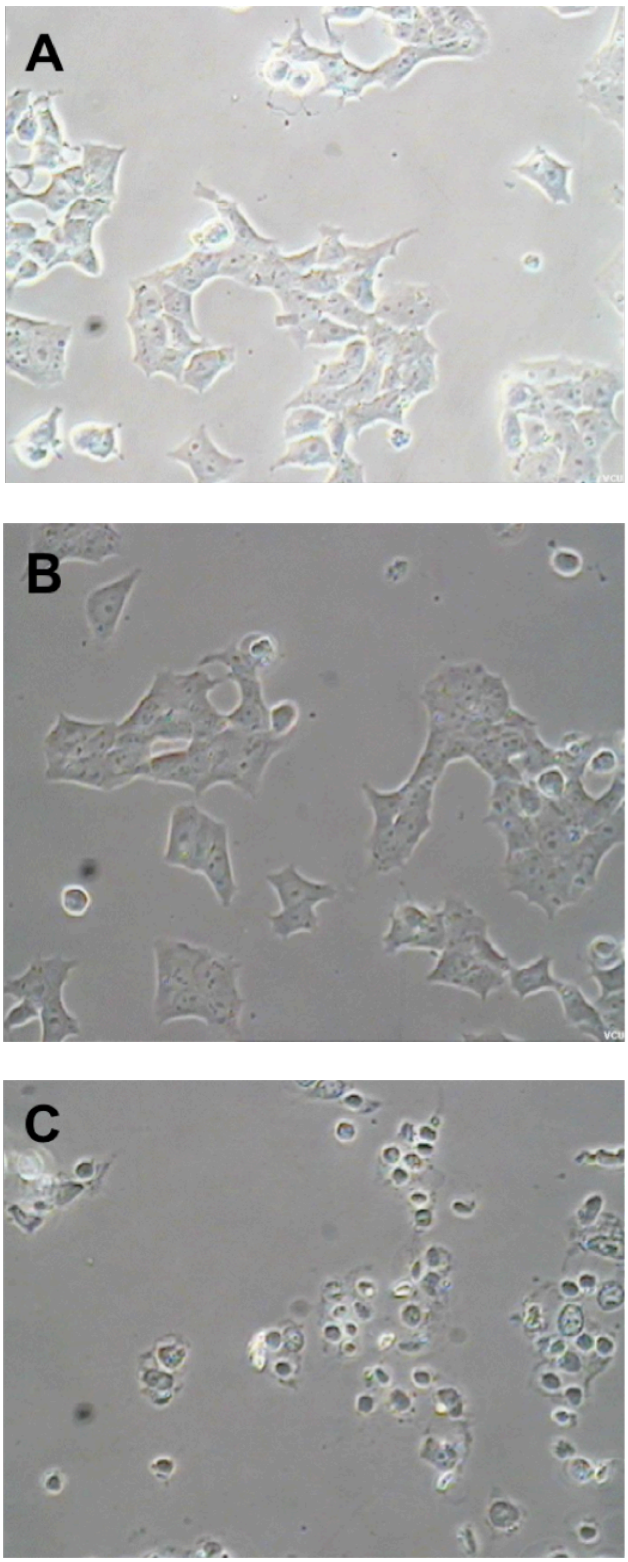


Figure 10.

Figure 10: Light micrograph of *Naegleria fowleri* and *Naegleria lovaniensis* contact-independent cytotoxicity on human nasal epithelial cells. Human nasal epithelial cells were seeded into tissue culture plates and *N. fowleri* and *N. lovaniensis* (2×10^5) were added to tissue culture inserts. Inserts were placed in tissue culture plate wells and incubated for 4.5 h. Inserts were removed, cells were fixed (2.5% glutaraldehyde), and target cells were visualized by light microscopy. Human nasal epithelial cell control (A), human nasal epithelial cells subjected to *N. lovaniensis* secreted products (B), and human nasal epithelial cells subjected to *N. fowleri* secreted products (C). Images are magnified 40x.

mediated lysis by normal human serum after 24 h in dilutions ranging from 1:32 to 1:4 (Fig. 11 and Table 1). In contrast, nonpathogenic *N. lovaniensis* were lysed by normal human serum after 24 h in dilutions ranging from 1:2 to 1:16 (Table 1 and Fig. 11).

Naegleria **attachment to ECM glycoproteins.**

The attachment of *Naegleria* to collagen I, fibronectin, and laminin-1 was assessed. *N. fowleri* and *N. lovaniensis* demonstrated differential patterns of attachment (Fig. 12). While a low level of attachment on BSA-coated wells was observed, *N. fowleri* exhibited a significantly higher level ($P<0.05$, ANOVA and Student's t-test) of attachment when exposed to laminin-1, collagen I, or fibronectin. *N. lovaniensis* exhibited minimal attachment to collagen I and laminin-1 compared to the BSA control. However, a slight increase ($P<0.05$, ANOVA and Student's t-test) in attachment of *N. lovaniensis* to fibronectin was observed. While both species exhibited attachment to fibronectin, attachment for *N. fowleri* was 30% higher than that to the BSA control while that of the nonpathogen *N. lovaniensis* was 10% higher than that to the BSA control (Figs. 12A and 12B). Similarly, attachment to collagen I and laminin-1 was increased by approximately 25% and 20%, respectively, for *N. fowleri* as compared to BSA (Fig. 12A). Attachment of *N. lovaniensis* to laminin-1 and collagen I did not differ from the BSA control (Fig. 12B). These collective results indicate a significantly higher level of attachment to ECM components by *N. fowleri* as compared to *N. lovaniensis*.

Table 1: Resistance to complement-mediated lysis

Serum Dilution (Complement Source)	*N. fowleri*	*N. lovaniensis*
1:2	+	+
1:4	-	+
1:8	-	+
1:16	-	+
1:32	-	+/-

+ = lysis observed, - = non lysis observed, +/- = partial lysis observed

Table 1: Resistance to complement-mediated lysis. *N. fowleri* and *N. lovaniensis* ($5x10^4$) were incubated in tissue culture plates with normal human serum (dilutions 1:2-1:32). The plate was incubated (37^oC, 24 h) and examined for cell lysis microscopically. Heat-inactivated serum used as negative control.

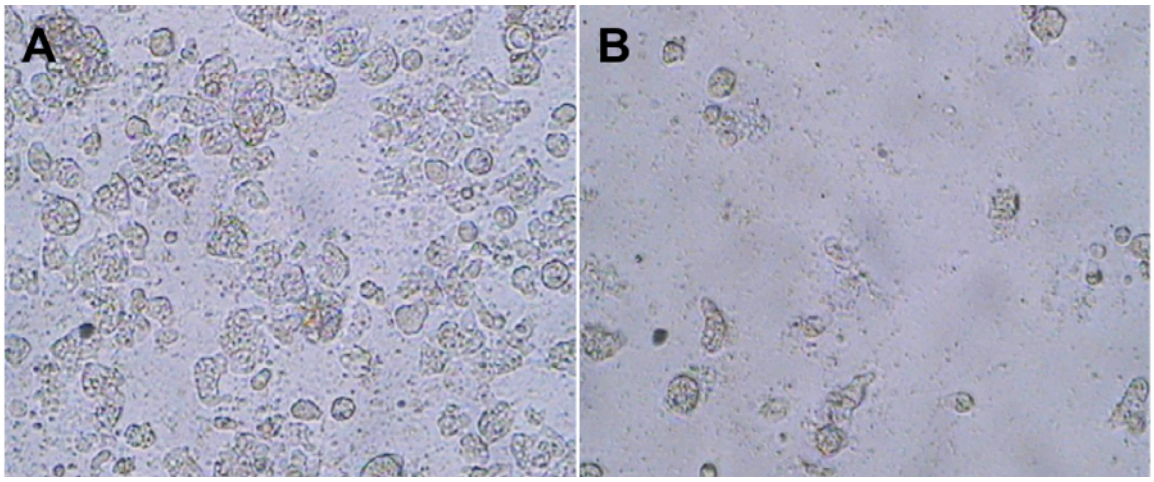


Figure 11: Light microscopy of effect of complement-mediated lysis. *N. fowleri* and *N. lovaniensis* ($5x10^4$) were incubated in tissue culture plates with normal human serum (dilutions 1:4). The plate was incubated (37^oC, 24 h). Amebae, (A) *N. fowleri* and (B) *N. lovaniensis* were visualized by light microscopy. Images are magnified 40x.

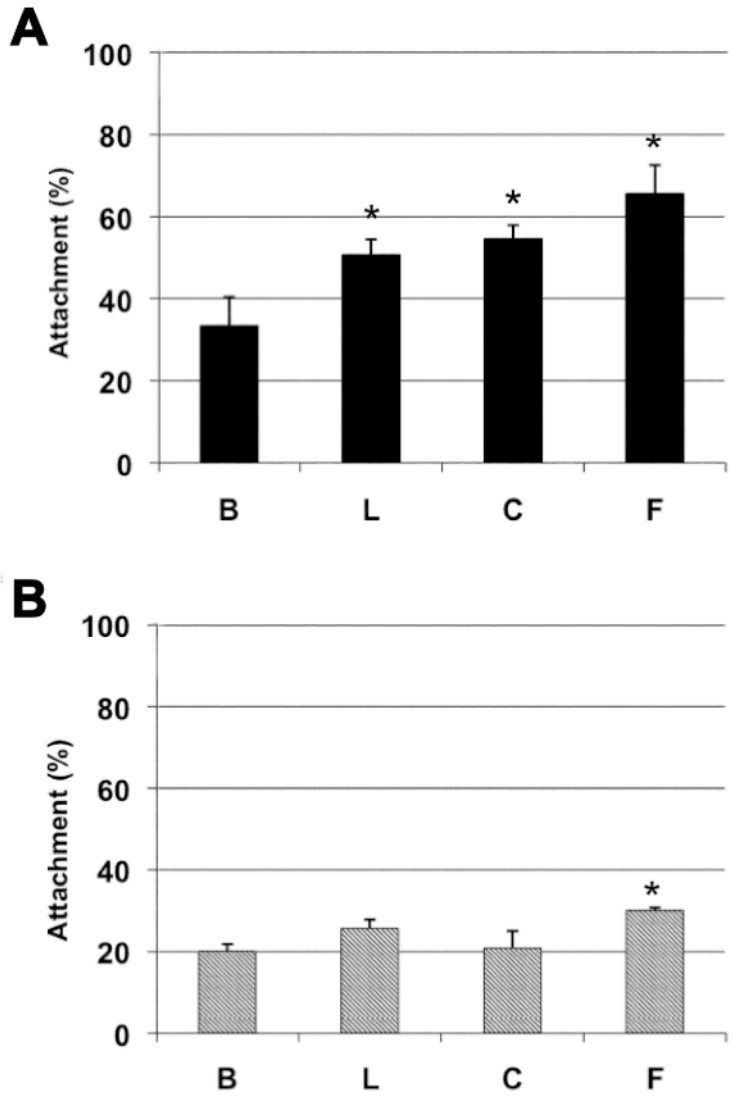


Figure 12. Attachment of (A) *Naegleria fowleri* amebae and (B) *Naegleria lovaniensis* on ECM glycoproteins. Radiolabeled amebae were added to tissue culture wells coated with 50 µg mL^{-1} of laminin-1 (L), collagen I (C), or fibronectin (F). Tissue culture wells treated with BSA (B) served as a control. The number of amebae added to each well was 2 x 10^5, which represents 100%. Experiments were performed twice in triplicate. (*P< 0.05 ANOVA with Student's t-test as compared to BSA).

Invasion of collagen I scaffolds or matrigel.

Both species of *Naegleria* were able to pass through the collagen I-and matrigel scaffolds (Fig. 13). However, evidence for only *N. fowleri* penetration of these scaffolds was demonstrated after 2 h of incubation. A very low number of *N. lovaniensis* was observed to have penetrated the ECM scaffolds and, then, only after 4 h of incubation in the upper chamber**.**

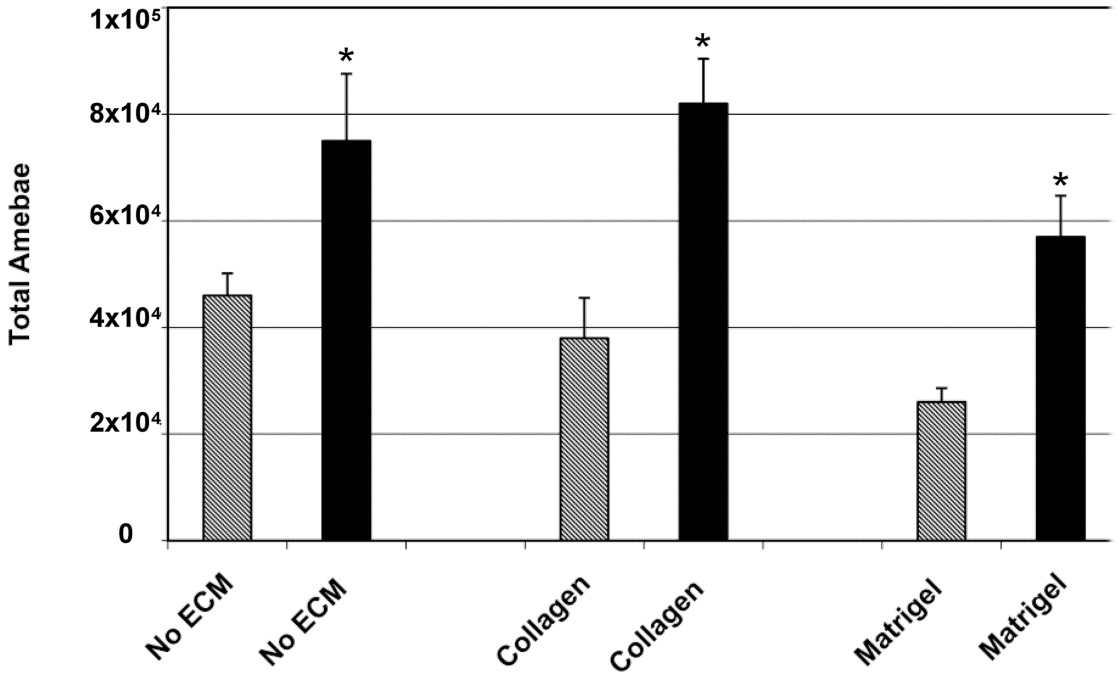


Figure 13. Invasion of *Naegleria fowleri* and *Naegleria lovaniensis* through ECM scaffolds (collagen I and matrigel). Amebae which invaded were counted using a hemacytometer. Black bars represent *N. fowleri* and gray bars represent *N. lovaniensis*. Experiments were performed twice in triplicate (*P< 0.05 Student's t-test).

DISCUSSION.

N. fowleri is the only species of the genus *Naegleria* that has been isolated from fatal CNS infections in humans (Martinez, 1985; Yoder *et al.,* 2004). Species of *Naegleria* such as nonpathogenic *N. lovaniensis and N. gruberi* are able to destroy mammalian cells in vitro (Marciano-Cabral *et al.,* 1982; Marciano-Cabral & Fulford, 1986). In addition, *N. lovaniensis* is thermotolerant and can survive at temperatures of 37^{o}C and higher (Stevens *et al*, 1980). This thermotolerance is not seen with *N. gruberi* and it is anticipated that this lack of heat tolerance leads to humans and other mammals, such as mice, being non-permissive hosts (Marciano-Cabral & Fulford, 1986). Other species, including *N. italica* and *N. australiensis*, have been shown to cause disease in experimentally-infected mice but have never been linked to human infections (De Jonckheere, 2004). Thus, the ability to elicit cytopathic effects on cultured cells in vitro and to be tolerant to temperatures of 37^{o}C or higher appears not to define pathogenicity for *Naegleria*.

Initial experiments were performed to investigate which phenotypic traits varied between a known human pathogen, *N. fowleri*, and a nonpathogen, *N. lovaniensis.* The first characteristic that was investigated was growth rate. Numerous studies involving *Acanthamoeba*, another free-living ameba, have investigated the link between growth rate and pathogenicity (Rocha-Azevedo, 2006; Walochnik, 2000). Rocha-Azevedo *et al*. (2006), compared the growth rates of an *A. polyphaga* keratitis clinical isolate and an environmental isolate of *A. polyphaga*. The study revealed that the pathogen grew at a substantially faster rate (Rocha-Azevedo *et al.,* 2006). Walochnik *et al.* (2000), showed that pathogenic strains of *Acanthamoeba* grew faster than amebae isolated from

contact lens cases. Our study revealed that the pathogen, *N. fowleri* grew at a much faster rate than the nonpathogen, *N. lovaniensis*. This difference could lead to the ability of *N. fowleri* to establish an infection more easily than *N. lovaniensis* since *N. fowleri* would reach a number of amebae sufficient to cause an infection. Additionally, once in the brain the amebae would grow more rapidly due to the faster growth rate, resulting in an increase in amebae in the brain causing cellular damage and ultimately tissue lysis and necrosis.

Cytopathogenicity of both *Naegleria* species was investigated. Studies have sought to determine the mechanisms by which *N. fowleri* is able to cause cellular damage (Marciano-Cabral *et al.,* 1982; Marciano-Cabral & John, 1983; Marciano-Cabral & Fulford 1986). Proposed mechanisms include phagocytosis of cells by pseudopod formation, trogocytosis or repeatedly nibbling on target cells by trophozoites, production of cytolytic factors (Phospholipase A and B), neuraminidase or elastase activity, production and release of pore-forming proteins that lyse target cells, and the presence of a cytopathic protein that is able to cause apoptosis in target cells (Visvesvara & Callaway 1974; Shuster & Visvesvara, 2004). To better gain insight in this area we divided cytopathogenicity into two categories, contact-dependent cytotoxicity and contact-independent cytotoxicity.

Studies investigating contact-dependent cytotoxicity have suggested that physical contact between an ameba and a target cell is a requirement for cell lyses. Brown (1979) inhibited migration and phagocytosis through the use of anti-sera and cytochalasin B to show that cytopathogenicity of *N. fowleri* on mouse embryo cell cultures was due to physical rather than biochemical mechanisms and was associated

with the phagocytic activity of trophozoites. Previous studies in our laboratory have compared the cytopathogenicity of numerous *Naegleria* species through the measurement of radiolabel release from rat neuroblastoma cells (Marciano-Cabral & Fulford, 1986). The results of this study demonstrated that both species did lyse the target cells, with more lysis seen from the cells cocultured with *N. lovaniensis*. The cellular destruction that was observed in these studies was considered contact-dependent because microscopic analysis demonstrated that the destroyed cells were those in physical contact with the amebae. To elaborate further in this area we have compared the ability of these two species to destroy human nasal epithelial cells when both species were grown on Cline's oxoid medium. The previously discussed studies utilized amebae that were cultivated in Nelson's media, a less nutrient rich medium than Oxoid. Studies have since compared the two media and have shown that the use of Cline's oxoid medium containing hemin leads to an increase in *N. fowleri* virulence resulting in a higher mortality rate and faster progression of the disease in mice (Bradley *et al.,* 1996). Our contact-dependent cytotoxicity studies demonstrated that there is no significant difference in LDH release, and therefore cell damage, when both species are placed in coculture with human nasal epithelial cells. This result correlates with the previously published notion that both species are fully capable of killing tissue culture cells in vitro. However, in comparing our data to those obtained in previous studies we observed a more rapid killing of target cells, which we attribute to the use of Cline's oxoid media.

The ability of *N. fowleri* to destroy target cells on contact is no doubt a critical aspect of pathogenicity. However, it has recently been proposed that secretory-

excretory proteins released from *N. fowleri* also play a role in this complex process (Kim *et al.,* 2008). The results from our contact-independent cytotoxicity assays as well as microscopic analysis, are in agreement with those from previous findings suggesting that contact is not a prerequisite for *N. fowleri* to cause cellular damage. Similar studies have demonstrated that other protozoa, such as *Trichomonas vaginalis*, which once were thought to only achieve cell lysis through direct cell contact, can lyse cells in a contact-independent manner (Fiori *et al.,* 1996). In the case of *T. vaginalis*, secretion of a lytic molecule was triggered by a shift in pH to a more acidic environment (from pH 7.2 to pH 5.8), This pH requirement may account for the delay in observance of contact-independent cytotoxicity in *T. vaginalis*. Fiori *et al.* (1996) also demonstrated that the lytic factor involved in *T. vaginalis* contact-independent cytotoxicity was inhibited by the presence of heat-inactivated serum in the cell media. Since most media used to grow amebae contain heat-inactivated serum, this finding, along with the pH requirement, may explain the lack of contact-independent cytotoxicity observed in prior investigations involving *N. fowleri.* Interestingly, *N. lovaniensis* was not able to cause cellular damage when inhibited from coming into direct physical contact with target cells. These findings suggest that secretion of a cytolytic molecule may be unique to *N. fowleri*, implying a possible role in pathogenicity. The ability of *N. fowleri* to cause cellular damage in the absence of physical contact would allow the amebae to impact a greater area during an infection. This could facilitate a faster migration/invasion rate from the nasal cavity to the brain and once in the brain would allow the amebae to damage a greater number of cells in a shorter time contributing to the rapid deterioration of an infected individual which is associated with *N. fowleri* infections.

Another well-studied trait associated with a variety of pathogenic microorganisms is the ability to evade complement-mediated lysis. The complement system is a proteolytic cascade involving multiple plasma proteins, which culminates in pathogen clearance from an infected host (Ricklin *et al.,* 2010). Microbial pathogens have adapted and acquired a variety of strategies to avoid complement-mediated killing. For example, *Treponema denticola*, a spirochete known to contribute to the development of periodontal disease (Simonson *et al.,* 1988), is able to bind a factor H-like protein 1 (FHL-1) facilitating the evasion of the alternative complement cascade (McDowell *et al.,* 2005). Furthermore, *Staphylococcus aureus,* a known human pathogen, secretes multiple factors that are able to block the complement cascade including staphylococcal complement inhibitor (SCIN), staphylococcal superantigen-like protein 10, and fibrinogen-binding protein (Itoh *et al.,* 2010; Rooijakkers *et al.,* 2005; Jongerius *et al.,* 2007). Protozoa also have been shown to resist complement lysis. For example, Kipnis *et al.* (1981), demonstrated that the ability of *Trypanosoma cruzi* to fail in activation of the alternative complement pathway was due to the presence of trypsin- and sialidase-sensitive regulatory surface molecules. Other species of *Trypanosoma* such as *Trypanosoma carassii,* an extracellular fish parasite, is highly resistant to complement due to the presence of calreticulin, a highly conserved multifunctional protein which is able to inhibit C1q-dependent complement activation (Oladiran & Belosevic, 2009).

Previous studies in our laboratory have demonstrated that highly pathogenic mouse-passaged *N. fowleri* are less susceptible to the lytic effects of complement in normal human serum than the weakly pathogenic, axenically grown *N. fowleri* or *N. australiensis* and the nonpathogenic amebae *N. gruberi* and *N. lovaniensis* (Whiteman

& Marciano-Cabral, 1987). Studies also have revealed that the medium in which *N. fowleri* are maintained plays an important role in complement resistance. Amebae grown in Cline's oxoid medium containing hemin exhibit a higher resistance to complement than amebae maintained in Nelson medium lacking hemin (Toney & Marciano-Cabral, 1994). Since the studies that were previously performed comparing the complement resistance of different *Naegleria* species utilized Nelson's medium, we compared amebae grown in Cline's oxoid media to ensure maximum complement resistance. Our findings demonstrated that pathogenic *N. fowleri* is much more resistant to complement-mediated lysis when compared to nonpathogenic *N. lovaniensis*, as shown in the previous study by Whiteman & Marciano-Cabral (1987). The inability of *N. lovaniensis* to escape complement-mediated lysis may be directly related to its inability to cause disease due to the clearance of the amebae before they are able to establish an infection.

The last series of experiments in our initial studies involved characterizing the interaction of *N. fowleri* and *N. lovaniensis* with host extracellular matrix components (ECM). The interaction of cells with ECM components plays an important role in mediating cell adhesion and migration (Berrier & Yamada, 2007). Binding to, and passage through, ECM components by microorganisms have been implicated in the pathogenesis of bacteria, viruses, protozoa and fungi (Alderete *et al.,* 2002; Boshuizen *et al.,* 2004; de Bentzmann *et al.,* 2004; Gozalbo *et al.,* 1998; Hostetter, 1999; Kottom *et al.,* 2008; Lama *et al.,* 2009; Silva-Filho *et al.,* 1988). For example, *Candida albicans*, a fungal pathogen, is able to bind ECM components allowing the infection to become bloodborne, which can lead to endocarditis, nephritis, or endophthalmitis (Patti *et al*,

1994). The importance of ECM binding in pathogenesis, also, has been demonstrated with viruses, such as Rotavirus. Studies suggest that the virus possesses a protein capable of binding fibronectin and laminin which leads to migration and differentiation of epithelial cells and contributes to the diarrhea associated with Rotavirus infections (Boshuizen *et al.,* 2004). In this context, the ability of *N. fowleri* to bind to and invade ECM components may be linked to its pathogenicity. For example, attachment to the nasal epithelium by *N. fowleri* appears to be an important early event in the pathogenesis of PAM (Martinez *et al.,* 1973). Studies characterizing experimentally induced PAM in mice have shown that deep invasion occurs after *N. fowleri* amebae penetrate the basement membrane (Martinez *et al.,* 1973).

Microbial attachment to host tissue is the initial critical event in the development of an infection. In the present study, *N. fowleri* and *N. lovaniensis* were assessed for differences in attachment to, and invasion of, ECM components. A differential level of binding to laminin-1, collagen I, and fibronectin was observed for *N. fowleri* versus *N. lovaniensis*. Whereas, *N. lovaniensis* exhibited a level of binding to the different ECM components that did not differ in large measure from that noted for the BSA control, a significant level of binding on all matrices was observed for *N. fowleri* when compared to the BSA control. Since attachment is a prerequisite for a successful infection it is possible that the differences in pathogenicity associated with these two species of *Naegleria* is due to their differences in attachment to ECM components. Differences in attachment to ECM correlating to differences in pathogenicity also have been noted in other amebae. For example, pathogenic *A. culbertsoni* attaches differentially to the

ECM glycoproteins laminin-1, collagen I, and fibronectin, as compared with nonpathogenic *A. astronyxis* (Rocha-Azevedo *et al.,* 2009).

Finally, the ability of *N. fowleri* versus *N. lovaniensis* to invade collagen-I or matrigel scaffolds was assessed. Invasion assays have been used to measure the "invasiveness" of tumor cells (Kleinman & Jacob, 2001) and *Acanthamoeba spp*. (Rocha-Azevedo *et al.,* 2009). Microscopic examination of tissue has demonstrated that *N. fowleri* pass through the nasal epithelium and then penetrate the basement membrane as they migrate to the brain (Jarolin *et al.,* 2000). Electron microscopic analysis of infected mouse brain revealed amebae in the apparent process of engulfing collagen I fibrils during the invasion process (Martinez *et al.,* 1973). In the present study, *N. fowleri* exhibited an apparent more rapid invasion of collagen I and matrigel constructs than *N. lovaniensis*. This apparent more rapid invasion could be due to enhanced motility. For example, Thong and Ferrante (Thong & Ferrante, 1986) compared the migration patterns of *Naegleria* species under agarose and reported that their locomotive ability correlated with pathogenic potential. In addition, *N. fowleri*, but not *N. lovaniensis,* has been shown to exhibit enhanced motility when placed in proximity to rat B103 nerve cells (Cline & Marciano-Cabral, 1986), suggesting that *N. fowleri* selectively "senses" neurotropic factors as compared to *N. lovaniensis* and other free-living amebae that lack neuropathogenic potential.

Collectively, our initial experiments demonstrated that there are a number of phenotypic differences between pathogenic *N. fowleri* and nonpathogenic *N. lovaniensis.* Growth rate studies demonstrated that *N. fowleri* grows at a faster rate than nonpathogenic *N. lovaniensis* and only *N. fowleri* exhibits contact-independent

cytotoxicity. Complement studies revealed that *N. fowleri* is more resistant to complemented-mediated lysis. Lastly, ECM studies revealed that *N. fowleri* and *N. lovaniensis* attach differentially to and invade ECM components, with more attachment and invasion seen with the pathogen, *N. fowleri*. The most significant differences observed were seen in terms of attachment and invasion. Therefore, the next series of experiments sought to further define the mechanisms involved in attachment to and invasion of host ECM components.

CHAPTER 4: Characterization of Attachment to ECM Components

The attachment of a microorganism to a host or host cell is a critical step in the establishment of a successful infection. Having determined that attachment to ECM components differs greatly between *N. fowleri* and *N. lovaniensis* the next approach was to gain insight into what type, or types, of molecules might be responsible for the observed attachment. Experiments first sought to determine whether lectins, integrins, or integrin-like receptors were involved.

RESULTS:

Morphological assessment of *Naegleria* on ECM substrata.

Scanning electron microscopy demonstrated morphological differences between the two species upon exposure to ECM components. *N. fowleri* demonstrated a flattened, spread-out appearance on collagen I, laminin-1, and fibronectin (Fig. 14 A-D). Furthermore, amebae exposed to ECM components exhibited lamellipodia and morphological features that were similar to those of focal adhesions (Fig. 14E, F arrows). These features were not observed when *N. fowleri* was subjected to adherence onto non-coated glass surfaces (Fig. 14A). In contrast, *N. lovaniensis* exhibited an elongated form when placed on all substrata (Fig. 15A-D). Focal adhesion-

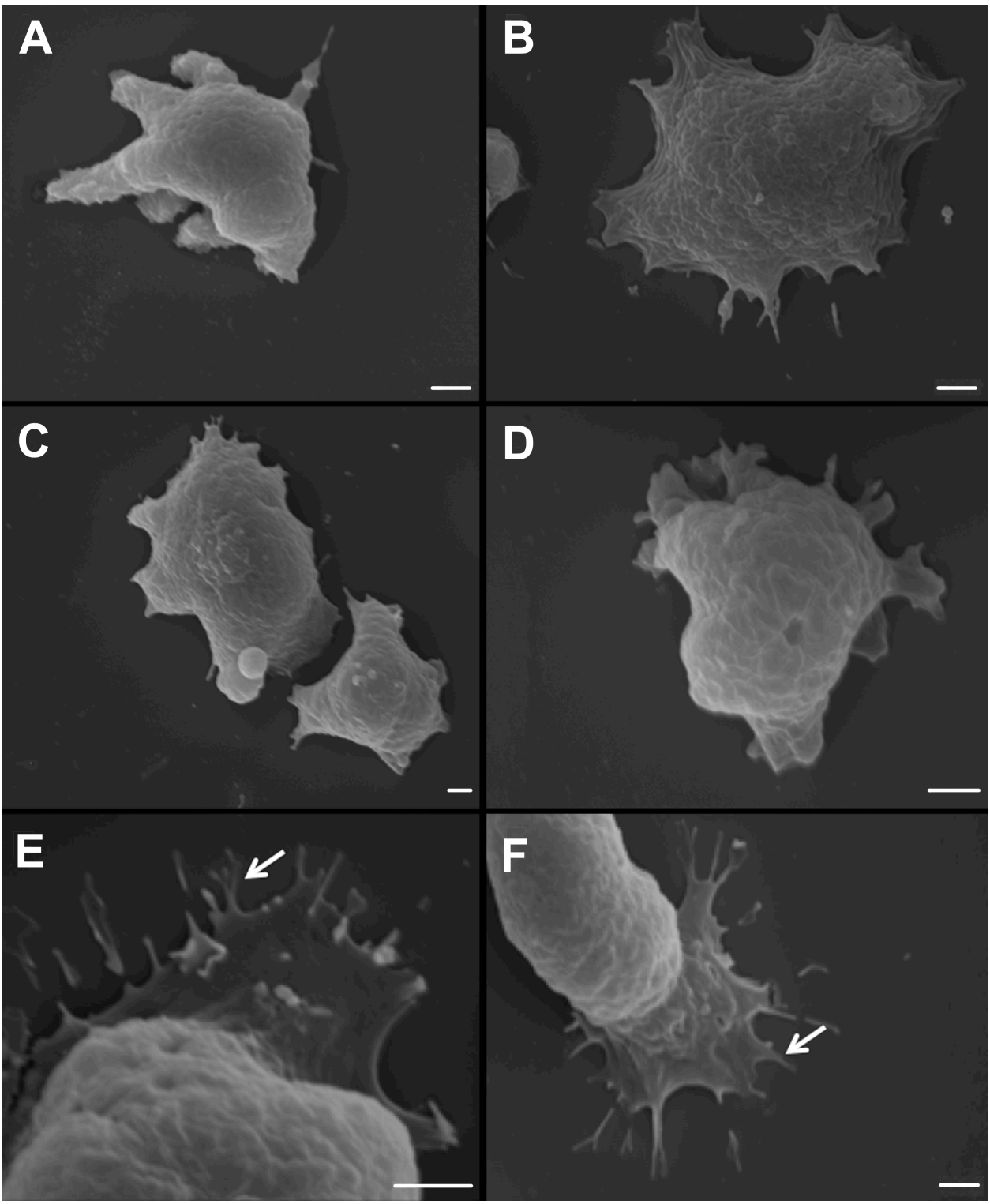


Figure 14.

Figure 14. Scanning electron micrographs of *Naegleria fowleri* attached to various substrata. Amebae interacting with (A) glass, or extracellular matrix (ECM) glycoproteins (B) collagen I, (C) fibronectin, and (D) laminin-1. Higher magnification of the focal adhesion-like structures on laminin-1 (arrows, E, F). The bar in the panels represents 2μm.

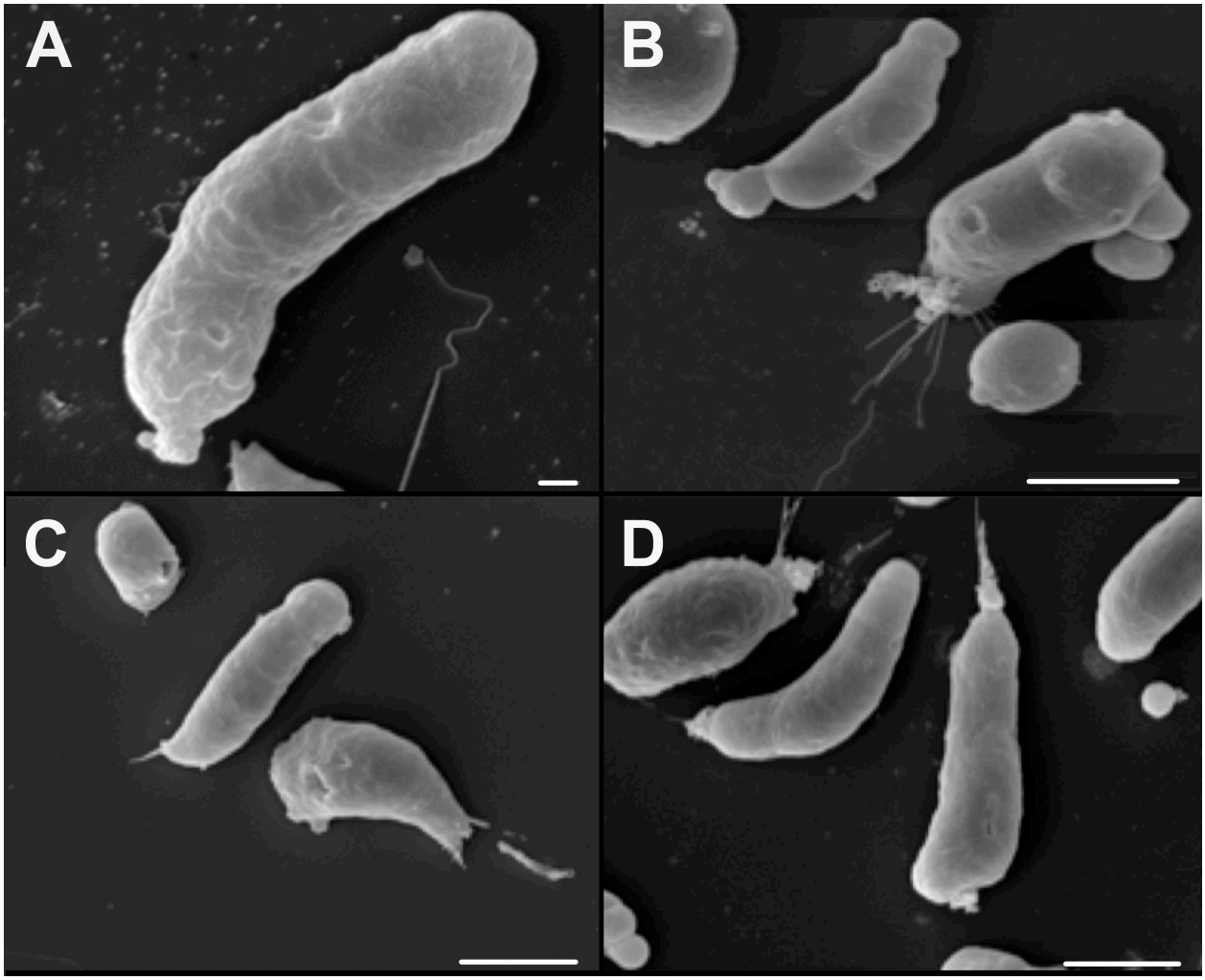


Figure 15. Scanning electron micrographs of *Naegleria lovaniensis* placed on various substrata. Amebae interacting on (A) glass, or on extracellular matrix (ECM) glycoproteins (B) collagen I, (C) fibronectin, and (D) laminin-1. The bar for panel A represents 1μm. The bar in panels B-D represents 10μm.

like structures were not observed for *N. lovaniensis* when placed on any of the substrata (Fig. 15).

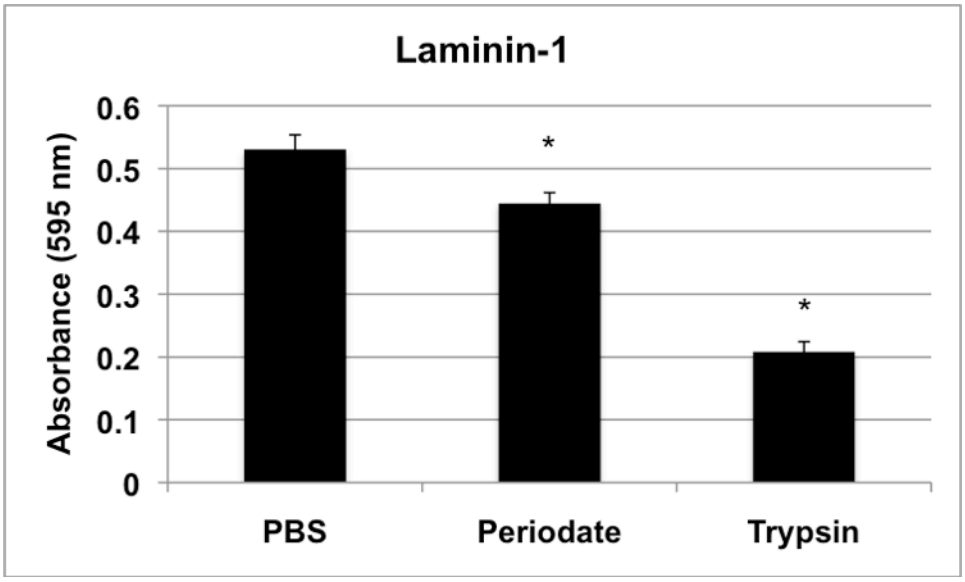
Effect of enzymatic pretreatment on attachment.

In order to obtain insight regarding which type of molecules are involved in *N. fowleri* attachment to collagen I, fibronectin, and laminin-1 attachment assays were performed using *N. fowleri* amebae that were pretreated with trypsin, a serine protease, and sodium periodate, a sugar oxidant. Attachment to all three ECM components (laminin-1, collagen I and fibronectin) was decreased significantly by trypsin pretreatment (laminin-1 61% decrease, collagen I 60% decrease, fibronectin 17% decrease, $P< 0.05$, Student's *t*-test). In contrast, periodate pretreatment resulted in a 16% decrease in attachment to laminin-1 and a 17% decrease in attachment to fibronectin while attachment to collagen was not affected (Fig. 16, $P< 0.05$, Student's *t*-test).

Influence of sugars on attachment.

The effect of exogenous sugars (methyl-α-d-mannopyranoside, d-mannose, and galactose) on *N. fowleri* adhesion on collagen I, fibronectin, and laminin-1 was assessed (Fig. 17). Adhesion of amebae on fibronectin was decreased by methyl-α-d-mannopyranoside, d-mannose, and galactose with the greatest decrease occurring when galactose was used. In contrast, treatment with exogenous sugars did not result in any significant decrease in amebae adhesion to laminin-1. For collagen I, a

A


Laminin-1
Absorbance (595 nm)
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
PBS
Periodate
Trypsin

B

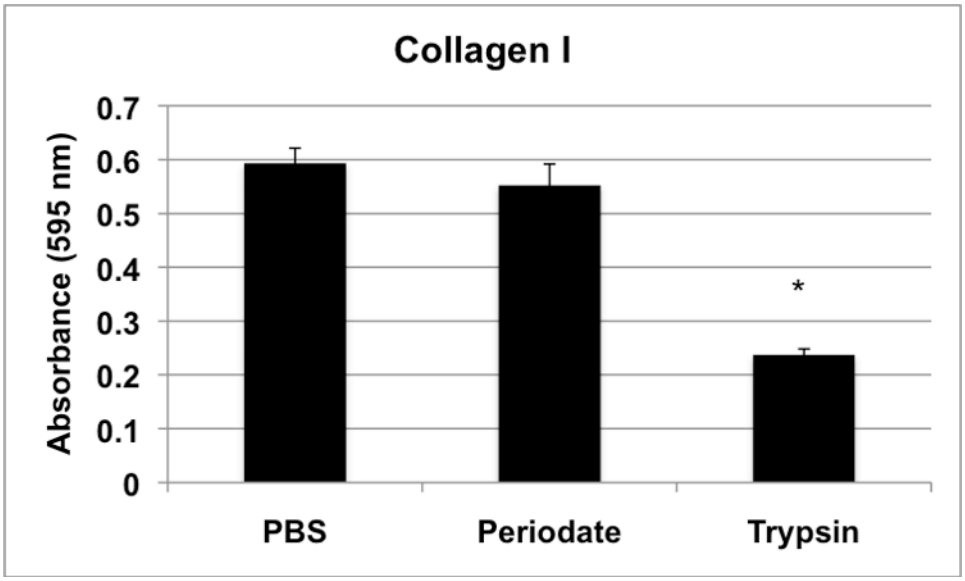

Collagen I
Absorbance (595 nm)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
PBS
Periodate
Trypsin

Figure 16.

C

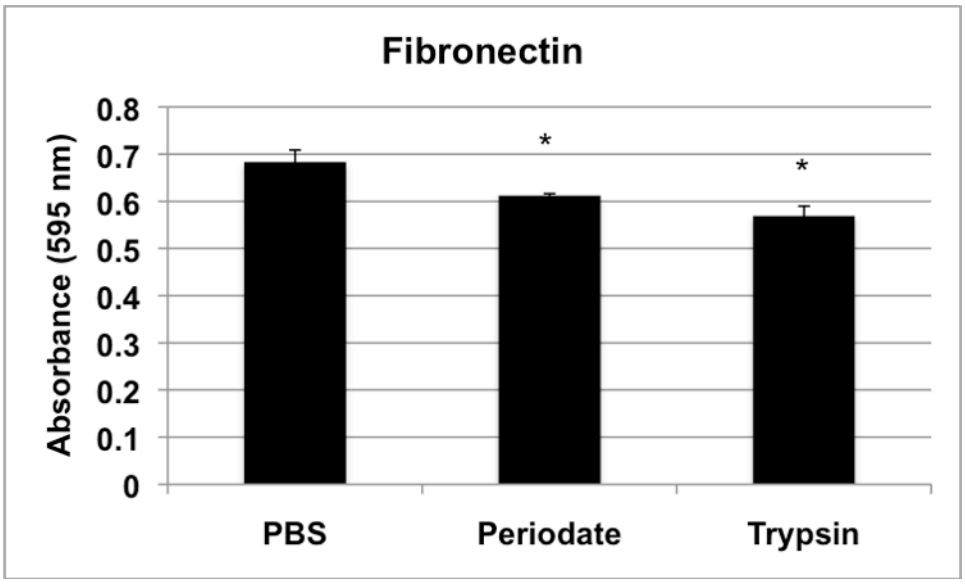
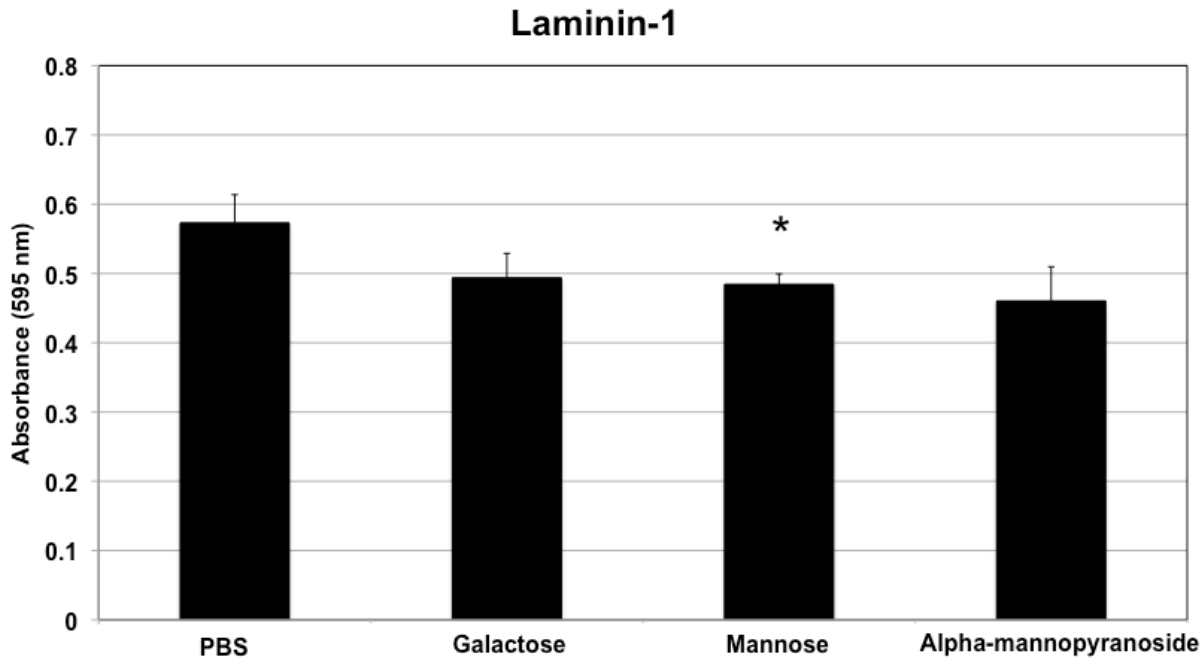


Figure 16. Effect of enzymatic pretreatment on ECM attachment. Amebae were treated with trypsin (0.25%) or periodate (1mM). Attachment assays then were performed to determine the effect on the adhesion of *N. fowleri* to laminin-1 (A), collagen I (B), and fibronectin (C). (*$P < 0.05$, Student's *t*-test, compared to the PBS control).

A


Laminin-1
Absorbance (595 nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
PBS
Galactose
Mannose
Alpha-mannopyranoside

B

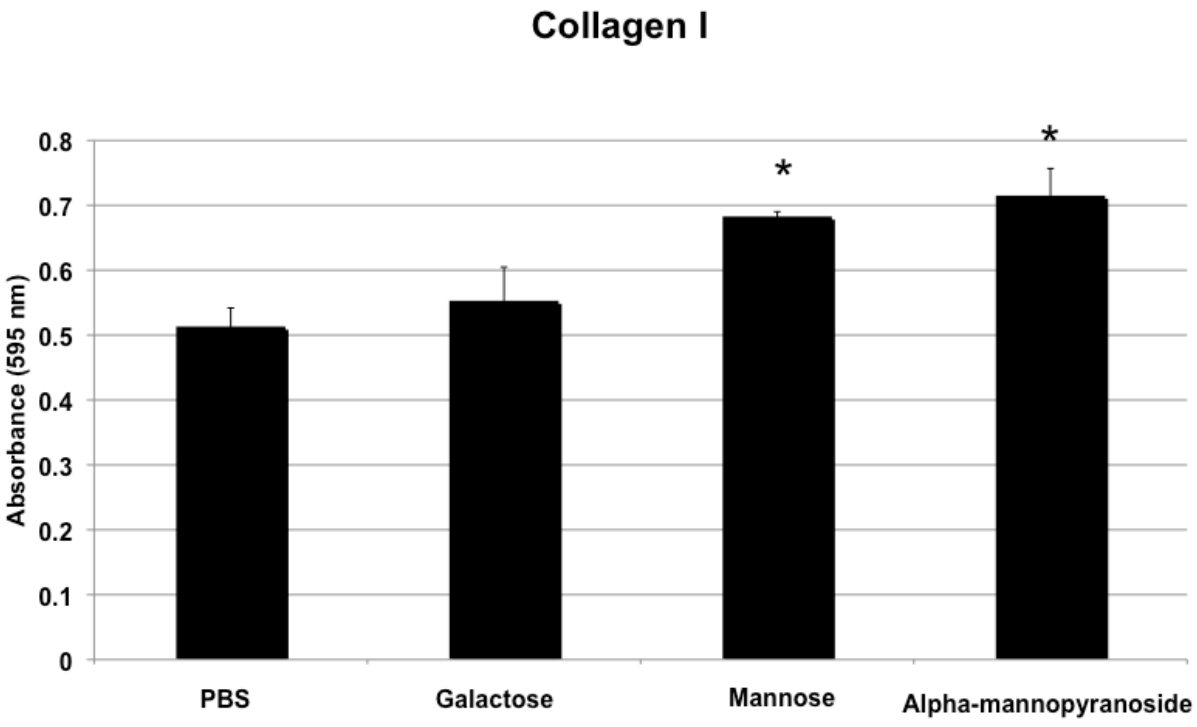

Collagen I
Absorbance (595 nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
*
*
PBS
Galactose
Mannose
Alpha-mannopyranoside

Figure 17.

C

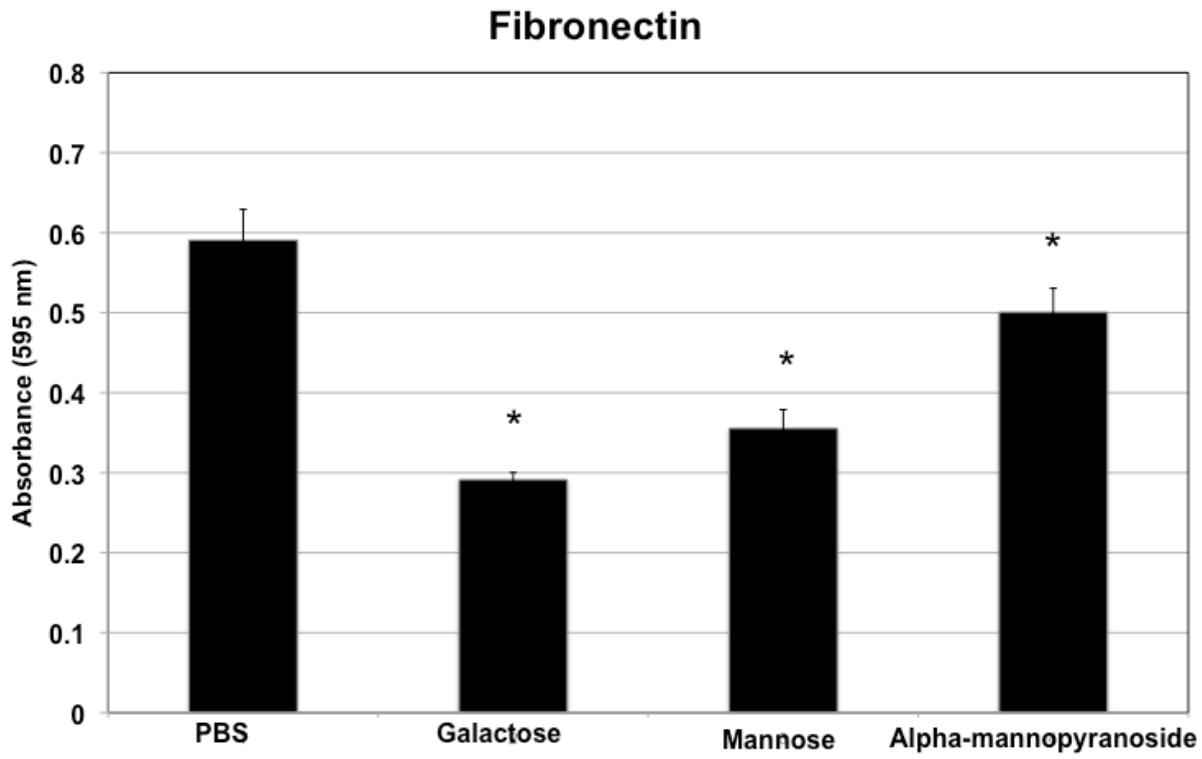


Figure 17. Effect of the exogenous sugars on attachment. Amebae were preincubated (30 min) with methyl-α-d-mannopyranoside (alpha-mannopyranoside), d-mannose (mannose) and galactose (100mM). Attachment assays then were performed to determine the effect on the adhesion of *N. fowleri* to laminin-1 (A), collagen I (B), and fibronectin (C). (*P< 0.05, Student's *t*-test, compared to the PBS control).

statistically significant increase in adhesion was observed when amebae were treated with methyl-α-d-mannopyranoside and d-mannose (Fig. 17).

Investigation of laminin-1 and collagen I-binding proteins.

Indirect immunoblotting assays were performed to identify laminin-1- and collagen I-binding proteins from plasma membrane fractions of *N. fowleri* and *N. lovaniensis.* Using mouse laminin-1 as a substrate, we were able to identify a band of approximately 32 kDa in both *N. fowleri* and *N. lovaniensis* (Fig. 18). Interestingly, a band of approximately 40 kDa was identified only in the pathogenic *N. fowleri* (Fig. 18). When human collagen I was used as a substrate a similar pattern was observed. Again, a band of approximately 40 kDa was identified in pathogenic *N. fowleri* but not in *N. lovaniensis* (Fig. 19). As an experimental control, no protein bands were observed when laminin-1 and collagen I were omitted (data not shown).

Detection of integrin-like proteins.

Confocal microscopy was used to investigate the presence of integrin-like proteins on *N. fowleri* and *N. lovaniensis.* Images shown are representative of *Naegleria* placed on all three ECM components. Both species exhibited ß1 integrin reactivity within the cell body and at the cell surface that was dispersed in a punctate pattern (Fig. 20B, E). However, a distinctive pattern of distribution was observed for *N. fowleri*. These amebae exhibited a colocalization of actin filaments and ß1 integrin-like protein at their leading edge and at focal adhesion-like structures (Fig. 20A, C). This pattern of co-localization was not observed for *N. lovaniensis* (Fig. 20D, F). Western

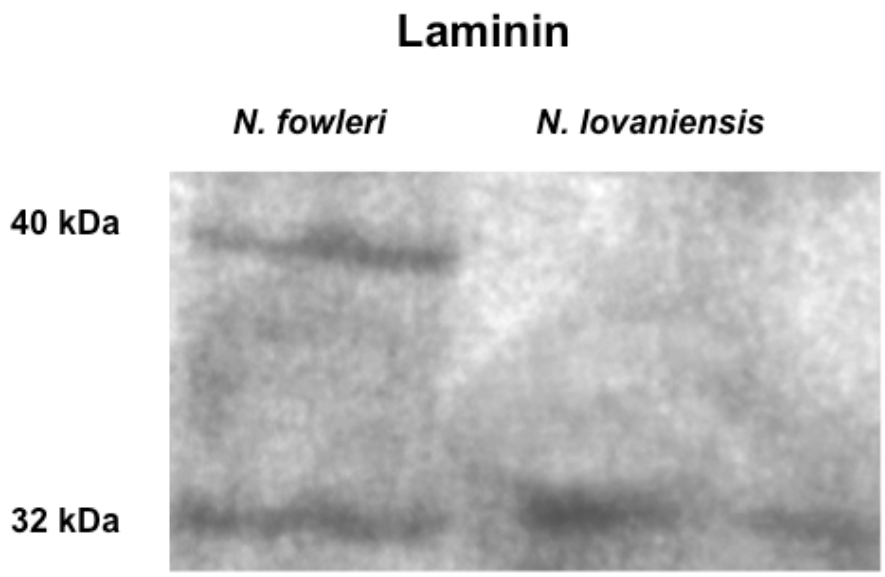


Figure 18. Detection of laminin-1-binding proteins extracted from nonpathogenic *Naegleria lovaniensis* and pathogenic *Naegleria fowleri* by indirect immunoblotting. Amebae extracts were separated in a 12% acrylamide SDS-polyacrylamide gel, and transferred to nitrocellulose membranes. Membranes then were incubated with mouse laminin-1. Laminin-1 binding was probed using a mouse monoclonal anti-human laminin primary antibody. A 32 kDa major reactive band common to *N. lovaniensis* and *N. fowleri* and a 40 kDa band only observed for *N. fowleri.*

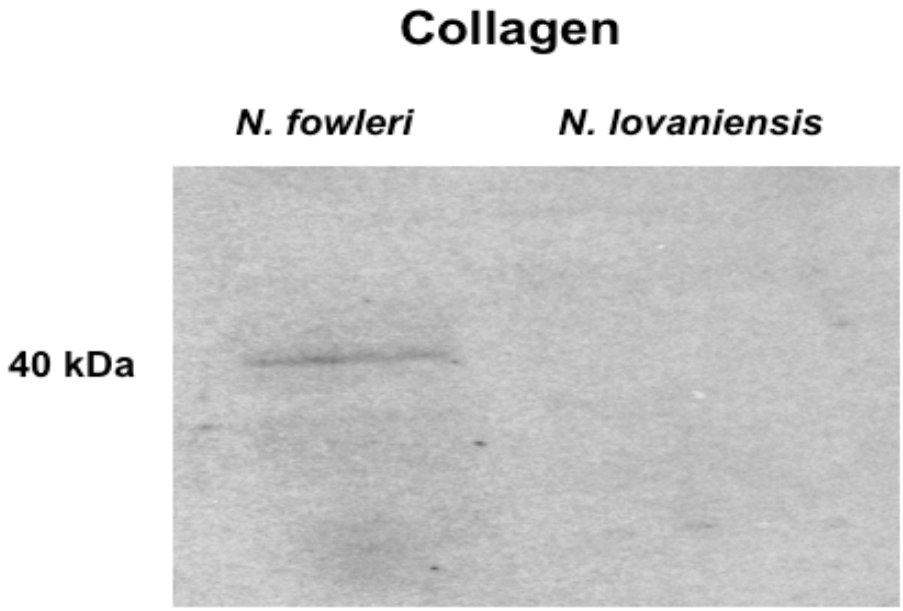


Figure 19. Detection of collagen-binding proteins extracted from nonpathogenic *Naegleria lovaniensis* and pathogenic *Naegleria fowleri* by indirect immunoblotting. Amebae extracts were separated in a 12% acrylamide SDS-polyacrylamide gel, and transferred to nitrocellulose membranes. Membranes then were incubated with human collagen. Collagen binding was probed using a mouse monoclonal anti-human collagen primary antibody. A 40 kDa major reactive band was only observed for *N. fowleri.*

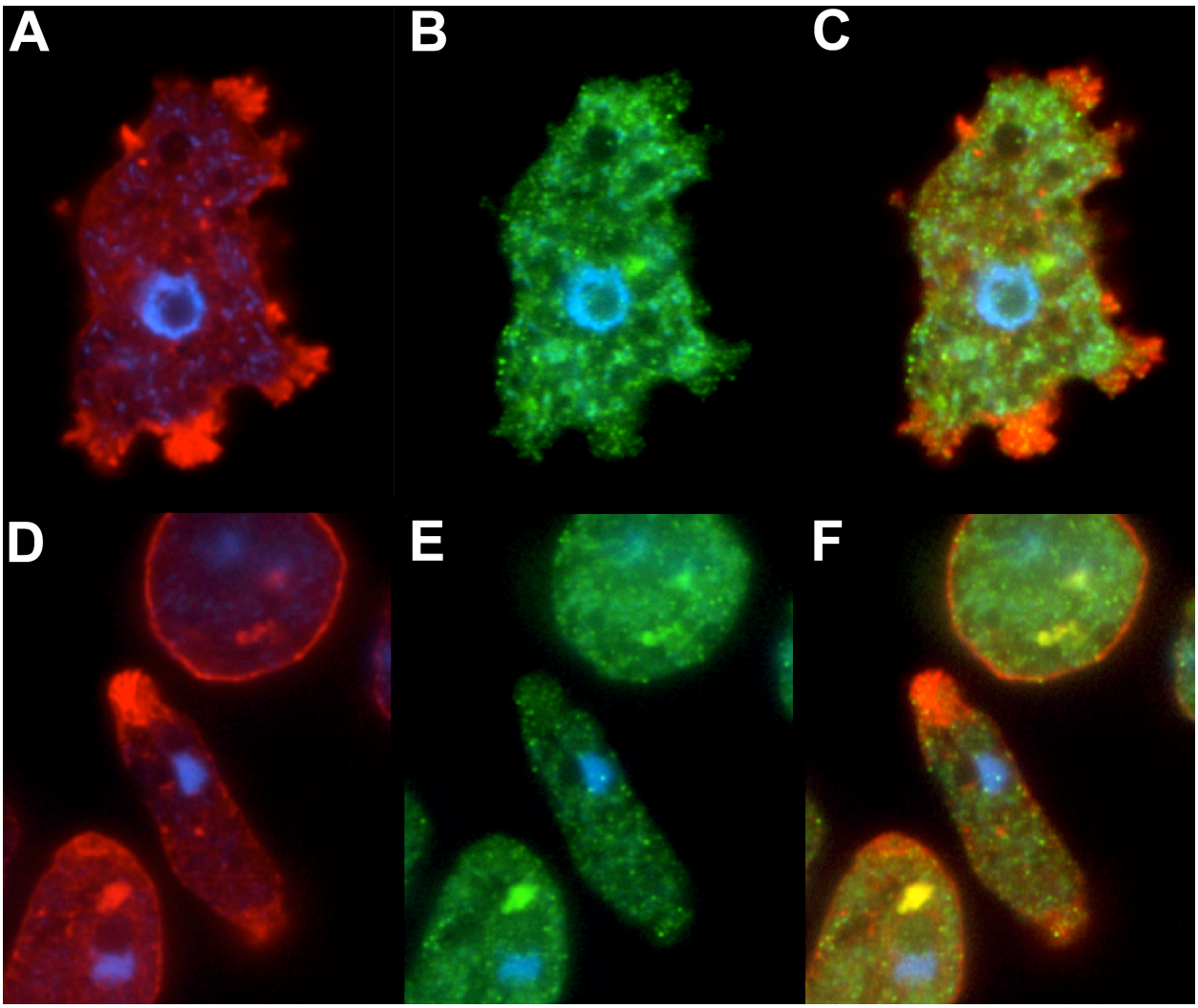


Figure 20. Confocal micrographs of *Naegleria fowleri* and *Naegleria lovaniensis* placed on collagen I. *N. fowleri* was probed with Alexafluor 594 phalloidin (A), or FITC-conjugated monoclonal anti-Integrin ß1 antibody (B). The merged fluorescent image is shown in panel C. *N. lovaniensis* was probed with Alexafluor 594 phalloidin (D), or FITC-conjugated monoclonal anti-Integrin ß1 antibody (E). The merged fluorescent image is shown in panel F. Nuclear localization is depicted in all panels by DAPI staining (A-F). All images are magnified 100x.

immunoblot analysis was performed using whole cell lysates of *N. fowleri* and *N. lovaniensis* using a polyclonal antibody directed to a human ß1 integrin subunit. A band of approximately 53 kDa was detected for both *N. fowleri* and *N. lovaniensis* (Fig. 21). However, an additional band of approximately 70 kDa was observed at a higher level for *N. fowleri* (Fig. 21). Since Western blot analysis and confocal microscopy suggested the presence of an integrin-like subunit, an RGD-containing peptide (Gly-Arg-Gly-Asp-Thr-Pro) containing an integrin binding site was used to assess its ability to inhibit attachment to collagen I. This peptide, when used in a competitive binding assay, did not inhibit attachment of *N. fowleri* to collagen I (data not shown). To further investigate whether an integrin-like protein was linked functionally to *N. fowleri* attachment, a blocking antibody was utilized in attachment inhibition studies (Table 2). Attachment of *N. fowleri* on laminin-1, collagen I and fibronectin was inhibited by the ß1 integrin antibody. In the absence of ß1 integrin antibody, 75% of input *N. fowleri* were found to bind to laminin-1 and collagen I. Also, 80% of input *N. fowleri* were found to bind to fibronectin. In contrast, in the presence of anti-ß1 antibody, 40% and 30% of input *N. fowleri*, respectively, were found to bind to laminin-1 and collagen I. Fifty percent of input *N. fowleri* were found to bind to fibronectin in the presence of anti-ß1 integrin. Thus, in the presence of the anti-ß1 integrin antibody, 1.4 - 2.2 fold less binding was obtained as compared to that obtained in the presence of the integrin-irrelevant anti-KLH antibody. An additional control consisting of preincubating the amebae with an antibody to a surface protein not involved in attachment would have been optimal. However, to date no antibody is available that recognizes a purified *N. fowleri* membrane protein.

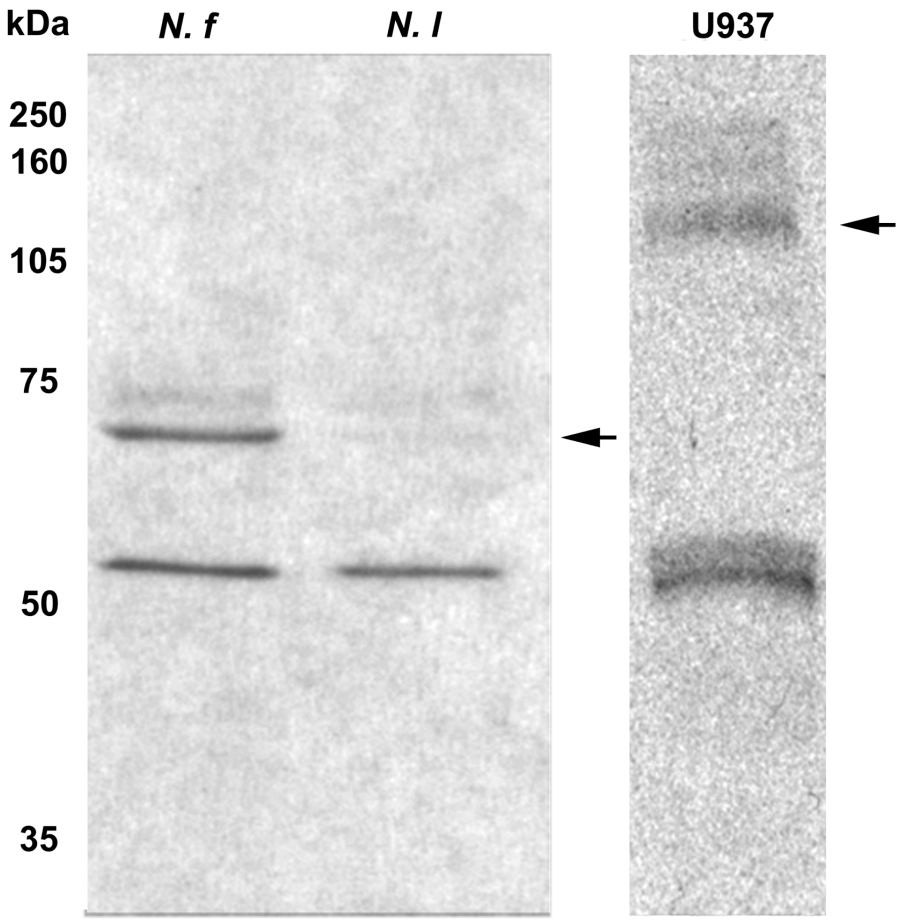


Figure 21. Western immunoblot analysis of *Naegleria fowleri* (Nf) and *Naegleria lovaniensis* (Nl) whole cell lysates for the detection of integrin-like proteins. Amebic extracts were separated by 12% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (left panel). A human leukemic monocyte lymphoma (U937) cell extract was used as a positive control (right panel). Membranes then were incubated with a polyclonal chicken anti-human integrin ß1 subunit antibody followed by horseradish peroxidase-conjugated rabbit anti-chicken. Arrowheads designate the 70-kDa (*N. fowleri* and *N. lovaniensis*) and 140 kDa (U937) major immunoreactive bands.

Table 2. Inhibition of attachment of *Naegleria fowleri* to ECM components

ECM Component	No Antibody		Anti-KLH		Anti-Beta-1 Integrin	
	Amebae Recovered	% Bound	Amebae Recovered	% Bound	Amebae Recovered	% Bound
Laminin-1	5.0×10^4	75%	7.0×10^4	65%	1.2×10^4	40%
Collagen I	5.0×10^4	75%	7.0×10^4	65%	1.4×10^4	30%
Fibronectin	4.0×10^4	80%	6.0×10^4	70%	1.0×10^4	50%

Table 2. *N. fowleri* ($2x10^5$) were preincubated (10 min) with anti-KLH, or a monoclonal anti-ß1 integrin antibody prior to addition to ECM components for 50 min. Unattached amebae then were collected, fixed with glutaraldehyde and counted using a hemacytometer. The % bound was calculated: [($2x10^5$ amebae) – (number of amebae recovered)] / [$2x10^5$ amebae] x 100%.

Discussion.

In order to gain insight as to the mode of action by which *N. fowleri* attaches to ECM components, SEM was performed. *N. fowleri* exhibited a flattened spread-out appearance and displayed numerous focal adhesion-like extrusions that appeared as principal sites of attachment to collagen I, fibronectin, and laminin-1. Additionally, structures appearing similar to lamellipodia were observed. Lamellipodia are sheet-like membrane protrusions at the leading edge of a cell, containing bundles of actin filaments (Small *et al.,* 2008). In contrast, *N. lovaniensis* maintained a rounded appearance and exhibited no focal adhesion-like structures. These morphological distinctions are consistent with *N. fowleri* versus *N. lovaniensis* differential recognition and attachment to ECM. There are multiple possible explanations to explain this adherence. Therefore, our next series of experiments sought to further describe the molecules involved in *N. fowleri* attachment to ECM components.

Initial characterization of the molecules responsible for *N. fowleri* attachment to ECM components involved treating amebae with periodate or trypsin that cleave surface sugars and proteins, respectively. Treatment with periodate demonstrated that adhesion to laminin-1 and fibronectin was dependent partially on the presence of sugars. This could be due to the presence of another molecule playing an important role in attachment, which would correlate with the results from the trypsin treatment. Furthermore, the lack of a significant effect could be explained by low levels of sugars on the surface of the amebae. This would not be surprising since *N. fowleri* has been shown to display less glucose and mannose residues on its surface when compared to nonpathogenic species (Gonzalez-Robles *et al.,* 2007).

It was observed that surface proteins appear to play a critical role in adhesion to all three ECM components. Binding to collagen I, fibronectin, and laminin-1 exhibited a differential sensitivity to trypsin treatment. Attachment to laminin-1 and collagen I was almost completely abolished, while a slight decrease in attachment was observed for fibronectin. This difference could be due to the presence of a trypsin-resistant or highly glycosylated protein(s) on the amebae that are associated with fibronectin attachment. Trypsin resistant proteins have been observed in *Mycoplasma pulmonis* which has a 46 kDa trypsin resistant membrane protein involved in adhesion to host cells (Beyers *et al.,* 1994).

Microbial pathogens express a variety of surface proteins to mediate critical interactions with ECM components. Therefore, we next sought to further define the type of protein(s) involved in *N. fowleri* adherence to ECM components. The initial molecules investigated were lectins, which have been reported to recognize and bind specific sugar residues of ECM components (Chammas *et al.,* 1994). Attachment to fibronectin was decreased in the presence of all three sugars, galactose, mannose, and methyl-α-d-mannopyranoside, suggesting that a lectin-like sugar-binding molecule is involved in this attachment. Studies on other free-living amebae also have demonstrated lectin-like molecules are involved in attachment to ECM components (Gordon *et al.,* 1993, Rocha-Azevedo *et al.,* 2009). Furthermore, a mannose-binding protein involved in this type of interaction has been isolated and characterized for *A. castellanii* (Garate *et al.,* 2004). Surprisingly, attachment to laminin-1 was only decreased minimally by the addition of methyl-α-d-mannopyranoside, and attachment to collagen I was increased with the addition of mannose and methyl-α-d-mannopyranoside. The lack of a major effect on

attachment to laminin-1 may be explained by the presence of another receptor involved in attachment that does not recognize and bind to the mannose or galactose-containing portion of laminin-1. The minimal decrease observed by the addition of methyl-α-d-mannopyranoside suggests a lectin-like molecule plays a minor role in attachment to laminin-1 but is not responsible solely for the observed attachment. Additionally, the observed increase in attachment on collagen I caused by the presence of mannose residues may be due to the existence of mannose-binding molecule on the ameba surface that serves as a co-receptor to ECM binding. Similar results have been demonstrated with *Balamuthia mandrillaris* (*B. mandrillaris*), a free-living soil amebae that causes granulomatous amebic encephalitis, a highly fatal brain infection (Schuster & Visvesvara, 2004). In the case of *B. mandrillaris*, addition of mannose resulted in an increase in attachment to laminin-1, collagen I, and fibronectin (Rocha-Azevedo *et al.,* 2007). Furthermore, proteins induced by the presence of mannose have been described for other free-living amebae. For example, a mannose-induced protein (MIP-133) that activates metalloproteinases has been described for *A. castellanii* (Alizadeh *et al.,* 2008). This same type of sugar-induced interaction could be involved in collagen I attachment.

The ability of both *N. fowleri* and *N. lovaniensis* to attach to ECM components led us to hypothesize that both species harbor ECM-binding proteins. ECM-binding proteins have been described for a variety of microorganisms. For example, a 50 kDa laminin binding protein has been identified in *Staphylococcus aureus,* which plays a role in the bacteria's ability to invade the bloodstream and cause the formation of widespread metastatic abscesses (Lopes, dos Reis, & Brentani, 1985). Additionally, a

118 kDa laminin-binding protein has been identified in *Trichomonas vaginalis,* which is thought to contribute to the initiation of an infection by aiding in the colonization of the urogenital tract (Silva-Filho *et al.,* 1988). Furthermore, studies investigating the presence of laminin binding proteins in pathogenic and nonpathogenic species of *Acanthamoeba,* have shown that pathogenic *A. culbertsoni* and nonpathogenic *A. astronyxis* possess a common laminin binding protein with a molecular weight of approximately 40 kDa (Rocha-Azevedo *et al.,* 2007). However, the pathogen, *A. culbertsoni,* was found also to express an additional binding protein of approximately 55 kDa, which may explain the difference in pathogenicity between the two species (Rocha-Azevedo *et al*, 2007). In the present study we sought to further characterize the attachment of *Naegleria spp.* to ECM components using indirect immunoblotting to identify ECM binding proteins. Since a fibronectin binding protein has been described previously (Han *et al.,* 2004) in *N. fowleri* the current study focused on whether these two species of *Naegleria* express differences in laminin-1- and collagen I-binding proteins. *N. fowleri* and *N. lovaniensis* demonstrated similar binding protein profiles with a 32 kDa laminin-1-binding protein present in both species, and a 40 kDa laminin-1-binding protein unique to the pathogenic, *N. fowleri*. This difference in binding proteins may explain the difference in attachment between the two species. Both species were able to attach, yet more attachment was seen with *N. fowleri*. The 32 kDa binding protein present in both species may allow for low affinity binding to laminin-1. The addition of a 40 kDa binding protein may lead to a higher affinity interaction and thus the increase in binding that was seen with *N. fowleri*. Investigating the presence of collagen I-binding proteins revealed similar results with a collagen I-binding protein of

approximately 40 kDa only observed in *N. fowleri*. Again, the presence of a binding protein only in *N. fowleri* may lead to the higher rate of attachment to collagen I seen with *N. fowleri*. Additionally, the presence of binding proteins unique to *N. fowleri* could result in the differences that were seen between the two species in terms of morphology when attached to laminin-1 and collagen I.

The morphology of *N. fowleri* during attachment to ECM components observed by SEM revealed focal-adhesion like structures. Attachment of focal adhesions and proteins of the ECM generally involves integrins that typically are found on mammalian cells and are classically involved in recognition of an ECM motif (Ruoslahti, 1996). Many microbial pathogens express integrin-like molecules on their surface, which mediate interactions with ECM components. For example, a 140 kDa ß1 integrin-like molecule has been identified and characterized on *E. histolytica*. This molecule has been linked to attachment of the ameba to fibronectin (Sengupta *et al.,* 2001; Talamas-Rohana & Meza, 1988; Talamas-Rohana *et al.,* 1994). Han *et al.* (2004), has reported that *N. fowleri* possesses an integrin-like molecule, which binds to immobilized fibronectin (Han *et al.,* 2004). In addition, a search of the recently published sequence of the *N. gruberi* genome has revealed the presence of a von Willebrand factor type A (VWA) domain-containing protein. It has been suggested that all integrin beta subunits contain VWA domains (Tuckwell, 1999; Whittaker & Hynes, 2002). Therefore, it is possible that the protein on *N. gruberi* containing the VWA domain is an integrin-like protein.

In the present study, western immunoblot analysis using a human ß1 integrin antibody demonstrated that *N. fowleri* and *N. lovaniensis* exhibited comparable immune

reactivity for a 53 kDa integrin-like protein. However, a 70 kDa integrin-like protein, present also in *N. lovaniensis*, was found at a much higher level for *N. fowleri*. It is also interesting to note that the protein bands observed differed from those demonstrated in the ECM-binding protein experiments discussed previously. The conditions used for the identification of laminin-1 and collagen I-binding proteins may have been too harsh to maintain integrin function, and thus integrin bands were not observed. The antibody used for detection of integrins by western immunoblot analysis was directed at the human ß1 integrin subunit, the level of cross-reactivity obtained for these ameba proteins indicated that the human ß1 integrin subunit and the putative naeglerial integrin-like proteins shared similarity. This observation is consistent with reports that both pathogenic *E. histolytica* and nonpathogenic *E. dispar* exhibit integrin-like proteins on their surface (Pillai & Kain, 2005). Similarly, less pathogenic species of *Candida* also have been reported to express integrin-like proteins that mediate binding to fibronectin (Santoni *et al.,* 1995) and an adhesion molecule with β-integrin features has been identified in free-living *Dictyostelium* ameba (Cornillon *et al.,* 2006). Thus, these observations suggest that the mere presence of cell surface integrin-like molecules may not be a distinctive feature that discriminates pathogenic *N. fowleri* from nonpathogenic *N. lovaniensis.* It is possible, that the distinctive discriminating factor for pathogenic versus nonpathogenic species may be the differential pattern of integrin-like protein expression. In particular, the context of integrin-like protein compartmentalization may be a critical element in establishment of a pathogenic phenotype. That is, while both *N. fowleri* and *N. lovaniensis* were shown to express integrin-like proteins, only *N. fowleri* exhibited a co-localization with actin filaments in focal adhesion-like structures.

Beta-1 integrin and actin reactivity was shown to co-localize at these extrusions by confocal microscopy supporting their designation as focal adhesion-like structures (Burridge & Chrzanowska-Wodnicka, 1996). In contrast, *N. lovaniensis* maintained a rounded appearance based on SEM and did not exhibit these focal adhesion-like structures. The presence of these morphological distinctions suggests that *N. fowleri* and *N. lovaniensis* differentially recognize and attach to ECM components. They also suggest that specified protein domains on the surface of *N. fowleri,* possibly integrin-like molecules, that are in close association with other elements, bind to the ECM. In this context, it has been reported that mammalian cell attachment triggers signaling that leads to formation of focal adhesions. In this structural rearrangement model, integrins link the extracellular environment with the actin cytoskeleton through the mediation of focal adhesions (Burridge & Chrzanowska-Wodnicka, 1996). Such focal adhesions, or cell matrix adhesions, as large macromolecular aggregates not only serve to anchor cells but also act as "sensors" of ECM components (Riveline *et al.,* 2001). Consistent with these observations for *N. fowleri*, it has been shown that pathogenic amebae, such as *N. fowleri* and *E. histolytica*, upon exposure to ECM components form actin plates that are involved in adhesion (Han *et al.,* 2004; Talamas-Rohana & Meza, 1988).

The attachment process mediated by the focal adhesion-like structures may set in motion a series of activation processes that result in the release of proteases and other gene products that contribute to disruption of the ECM and promote passage of amebae. It is anticipated that such attachment is selective since integrins bind to specific targets on the ECM. Thus, in order to further implicate integrin-like proteins as having a functionally relevant role in binding to the ECM, a peptide with an integrin

recognition site was used in binding inhibition studies. This peptide contained an Arginine-Glycine-Aspartic Acid (i.e., RGD) motif, an acidic amino acid domain found in the integrin interaction site of many ECM proteins. In this context, RGD motif-mediated adhesion has been described in *Neoparamoeba aestuarina* and in other protozoa (Custodio *et al.,* 1995). However, the RGD peptide did not inhibit binding of *N. fowleri* to collagen I. There are several explanations for this lack of inhibition. It has been reported that RGD-dependent attachment of cells to collagen I occurs when collagen is in the denatured state. Thus, in the native conformation of collagen I, the RGD-motif may not be accessible for binding of integrins (Barczyk *et al*, 2010). A requisite to RGD-mediated binding to collagen I would be a prior degradative step exerted on the part of the amebae. An alternate possibility is that attachment of the protein expressed on *N. fowleri* is RGD-independent. Indeed, lack of effect of the RGD peptide does not negate integrin involvement in *N. fowleri* attachment since two-thirds of integrins have been found to bind ligands in an RGD-independent manner (Barczyk *et al.,* 2010). Finally, it is also possible that the ECM component used for assessment of binding to collagen I may not be optimal for investigating RGD-dependent binding since this motif is more classically found in fibronectin (Barczyk *et al.,* 2010).

To further explore the possibility of an integrin-like protein on *N. fowleri*, linked to binding to the ECM, an anti-integrin monoclonal antibody that inhibits attachment of mammalian cells to collagen I, fibronectin, and laminin-1 was used in binding inhibition studies. This antibody decreased attachment of *N. fowleri* to ECM components supporting the presence of functional integrin-like proteins on this ameba. Two different attachment assays were used in the present study, one which assessed direct binding

of amebae to ECM proteins using radiolabeled amebae and one that assessed inhibition of binding of amebae to ECM proteins in the presence of anti-integrin antibody that involved enumeration of unattached amebae. While there was some variability due to the intrinsic design of the two assays, the two methods yielded generally comparable results with attachment to laminin-1 and collagen I recorded to be above 50% and attachment to fibronectin recorded to be above 65%. Additionally, the same pattern of attachment was noted using the two assays, with the highest level of attachment being to fibronectin.

CHAPTER 5: Characterization of Invasion of ECM Components

N. fowleri amebae gain access to the body through the nasal passages, yet the brain is the main site of infection. Therefore, the amebae must make their way from the nasal passage, invade the olfactory epithelium, penetrate the submucosal nerve plexus, cross the cribriform plate, and reach the brain (Martinez, 1985; John, 1982) to establish a successful infection. The prerequisite of invasion for an infection led us to initially investigate differences in invasion between *N. fowleri* and *N. lovaniensis*. Having determined that there is a significant difference in invasion capabilities between these two species we next sought to further investigate the mechanisms of invasion observed.

RESULTS.

Invasion on collagen I scaffolds or matrigel.

Scanning electron microscopy indicated that *N. fowleri* amebae passed through collagen I-and matrigel scaffolds (Fig. 22 and Fig. 23). Evidence for penetration of these scaffolds was obtained as early as 2 h post-incubation. A very low number of *N. lovaniensis* was observed to have penetrated the ECM scaffolds and, then, only after 4h of incubation in the upper chamber**.** On the other hand, SEM revealed a remodeling of collagen I fibers with overt extensive damage to the matrix following 2h of exposure to *N. fowleri* (Fig. 22A). Large gaps in the matrix were observed that apparently served to

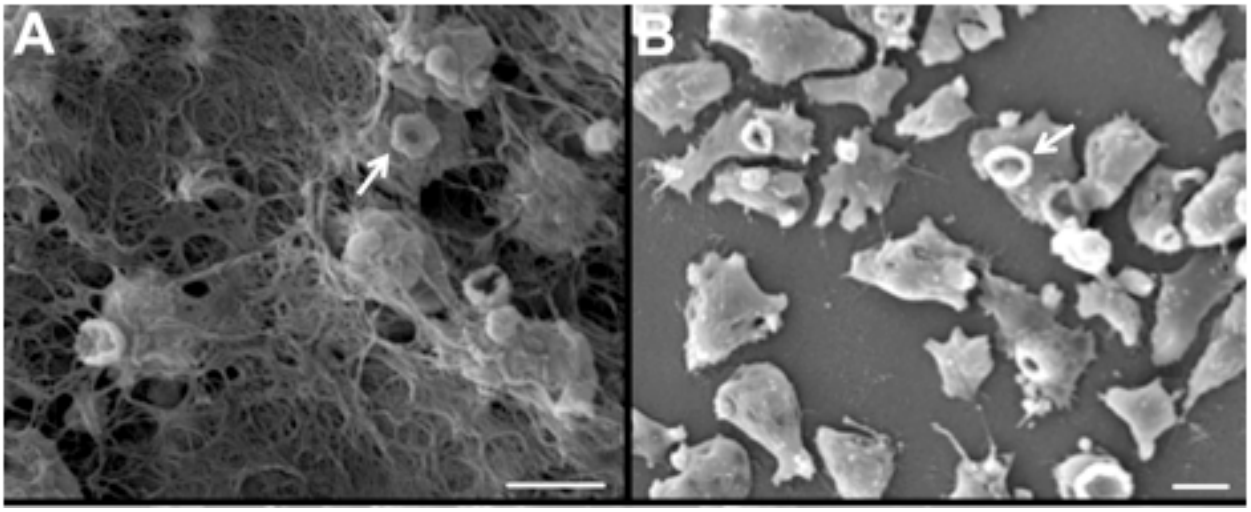


Figure 22. Interaction of *Naegleria fowleri* and *Naegleria lovaniensis* with collagen I matrices. (A) *N. fowleri* emerging through the bottom side of the collagen I scaffold and (B) *N. lovaniensis* interacting with collagen I on the surface of the scaffold. Note the presence of “food-cups” (arrows) on both *N. fowleri* and *N. lovaniensis*. The bar in the above panels represents 10μm.

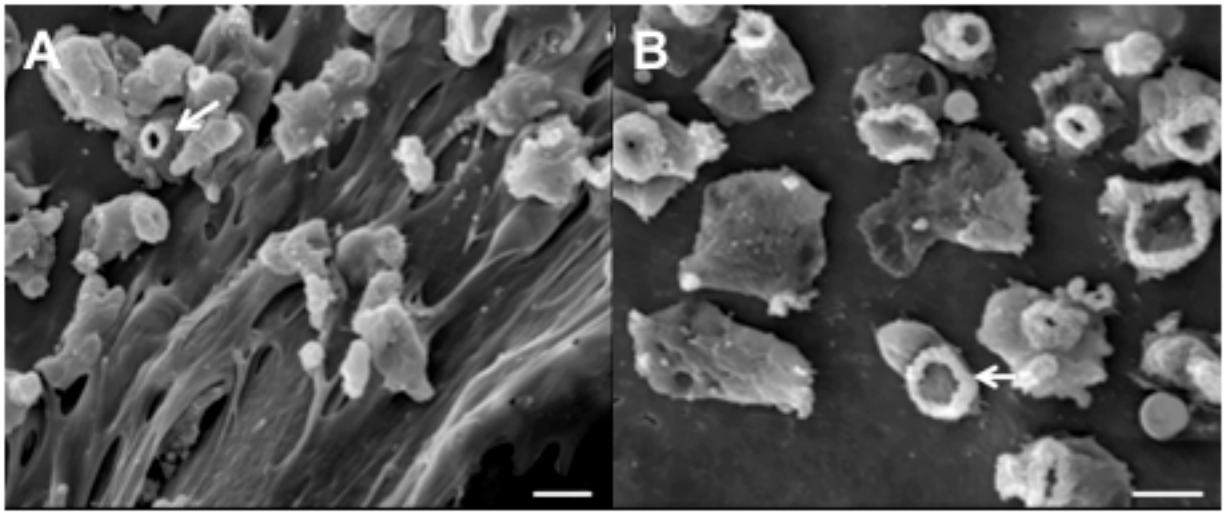


Figure 23. Interaction of *Naegleria fowleri* and *Naegleria lovaniensis* with matrigel matrices. (A) *N. fowleri* interacting with the matrigel surface. (B) *N. lovaniensis* interacting with the matrigel surface. Note the presence of “food-cups” (arrows) on both *N. fowleri* and *N. lovaniensis*. The bar in the above panels represents 10μm.

provide a passage for *N. fowleri* through the collagen I layer. No observable perturbation of the matrix was observed in the presence of *N. lovaniensis* (Fig. 22B). Both *Naegleria* species exhibited the presence of “foodcups” when in the presence of the collagen I scaffold (Fig. 22). A similar outcome was obtained for *N. fowleri* versus *N. lovaniensis* when placed on the matrigel scaffold (Fig. 23). *N. fowleri* exerted extensive damage to the matrigel matrix as evidenced by the presence of large holes or gaps (Fig. 23A). In contrast, the matrigel layer remained relatively intact when exposed (2 h) to *N. lovaniensis* (Fig. 23B).

Artificial nasal barrier.

To further investigate invasion using a model that mimics in vivo conditions, an invasion assay was performed using an artificial nasal barrier composed of a matrigel scaffold covered with a monolayer of human nasal epithelial cells (Fig. 24). After 5 h *N. fowleri* were able to pass through the nasal epithelial cell monolayer and the matrigel scaffold to a significantly greater extent than *N. lovaniensis* (Fig. 25).

Protease secretion during invasion.

To determine whether proteases were being secreted by the amebae in response to the presence of ECM components during invasion, invasion assays were performed and the media was collected for analysis. Zymographic analysis revealed that both species exhibit low levels of proteolytic activity during migration in the absence

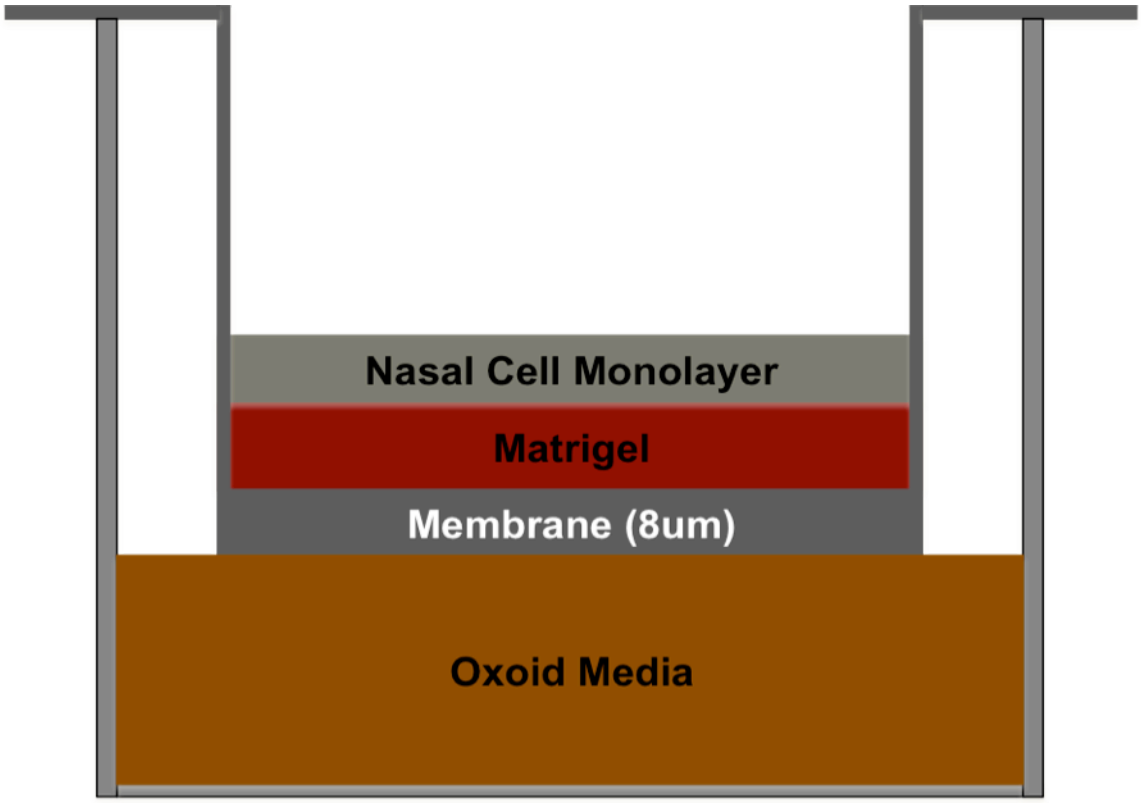


Figure 24. Schematic representation of artificial nasal barrier invasion assay.

Tissue culture inserts (pore-size of 8 μm) were coated (100μL, 10 min) with 1.7mg.mL^{-1} matrigel (Sigma). Human nasal cells (10^5) were added to form a monolayer of cells on the top of the matrigel layer and oxoid growth medium was added to the bottom of the tissue culture wells to serve as a migration attractant for amebae.

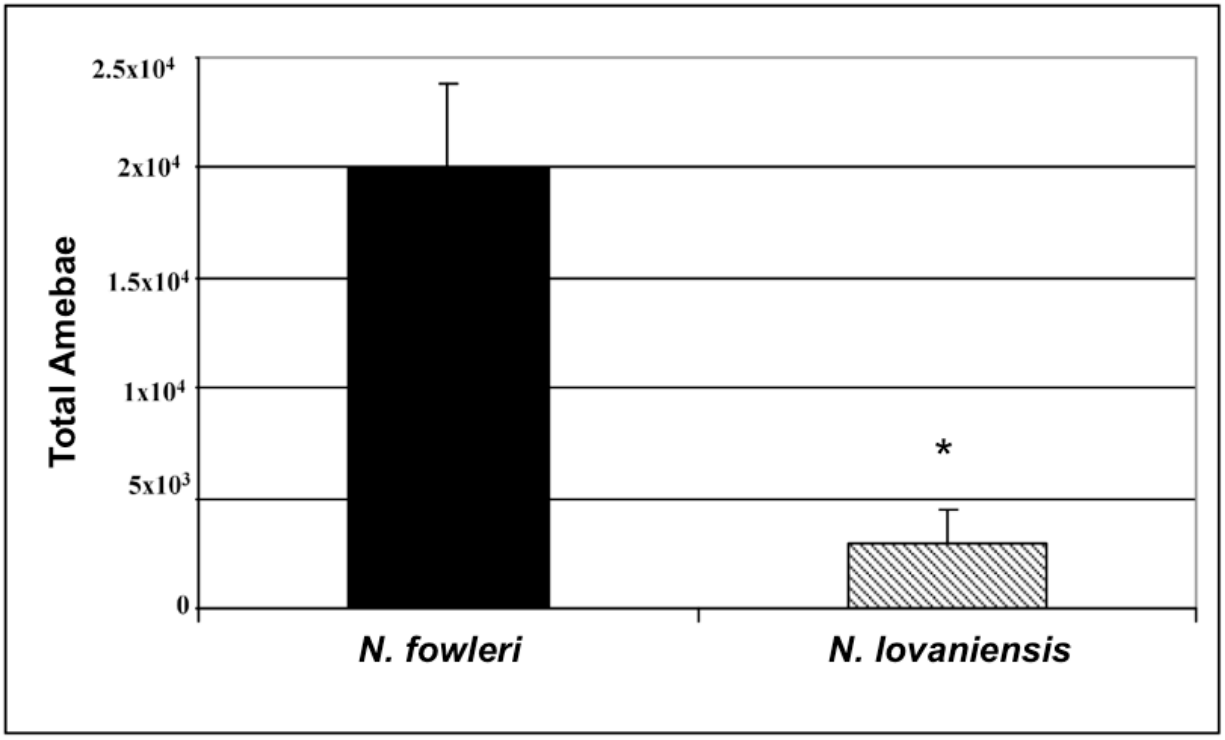


Figure 25. Invasion of *Naegleria fowleri* and *N. lovaniensis* through an artificial nasal barrier. *N. fowleri* or *N. lovaniensis* (10^5 amebae) were added to tissue culture inserts containing nasal cell-matrigel scaffolds and plates were incubated for 5 h at 37^{o}C with 5% (v/v) CO_2. Amebae that were able to migrate through the nasal cell/matrigel layer into the tissue culture wells were quantified using a hemacytometer. (*P < 0.05, Student's t-test).

of ECM components (Fig. 26 and Fig. 27). Proteolytic activity observed in media in the absence of ECM scaffolds produced by the amebae was approximately 65 kDa for *N. fowleri*, and 65 kDa and 55 kDa for *N. lovaniensis* (Fig. 26). The addition of matrigel resulted in an increase in proteolytic activity of the previously observed bands as well as an additional proteolytic band of approximately 37 kDa for *N. fowleri* (Fig. 26). Furthermore, additional bands were noted when inserts were coated with collagen I. The addition of collagen I led to the detectable presence of two new proteolytic bands for *N. fowleri* (approximately 250 kDa and 55 kDa) (Fig. 27) and one new proteolytic band for *N. lovaniensis (*approximately 50 kDa) (Fig. 27).

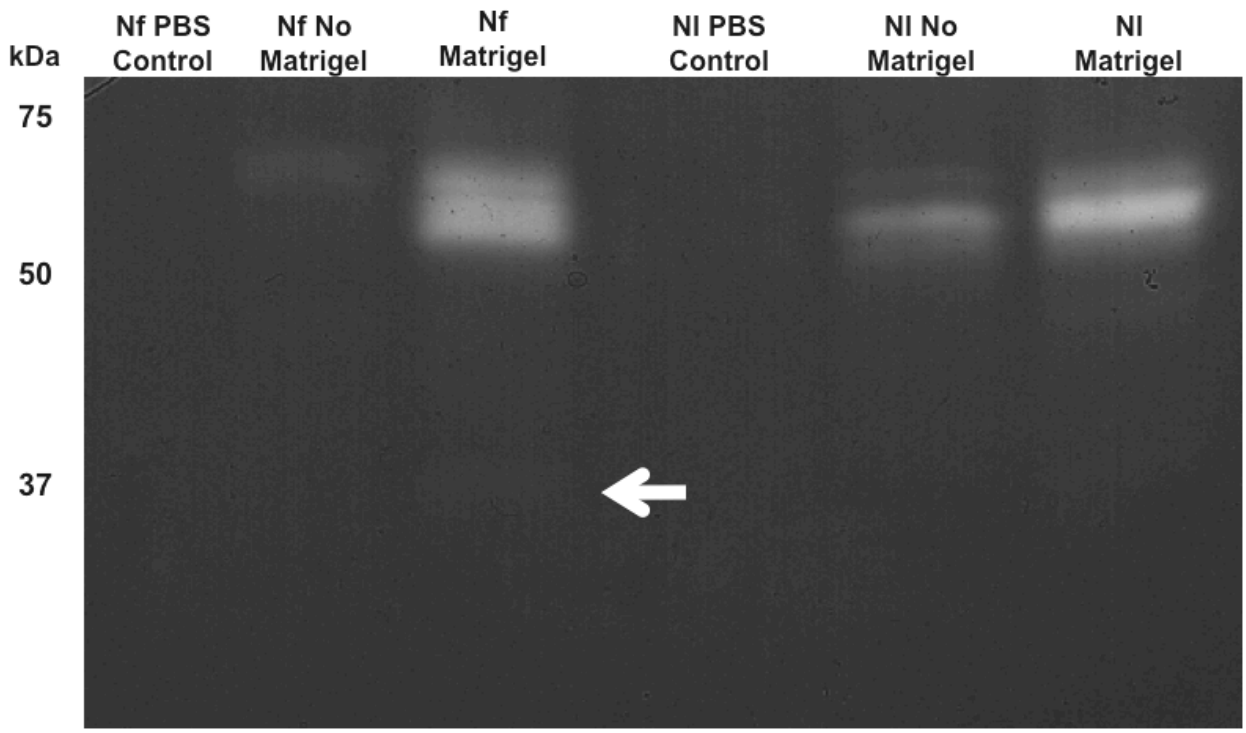


Figure 26. Detection of *N. fowleri* and *N. lovaniensis* proteases secreted during interaction on matrigel. Invasion assays were performed using matrigel coated tissue culture inserts. After 4 h the medium was collected and subjected to electrophoresis under nonreducing conditions on a 10% SDS-polyacrylamide gel containing 1% gelatin. Gels were equilibrated, developed overnight, and stained with Coomassie blue R-250 to visualize protease activity.

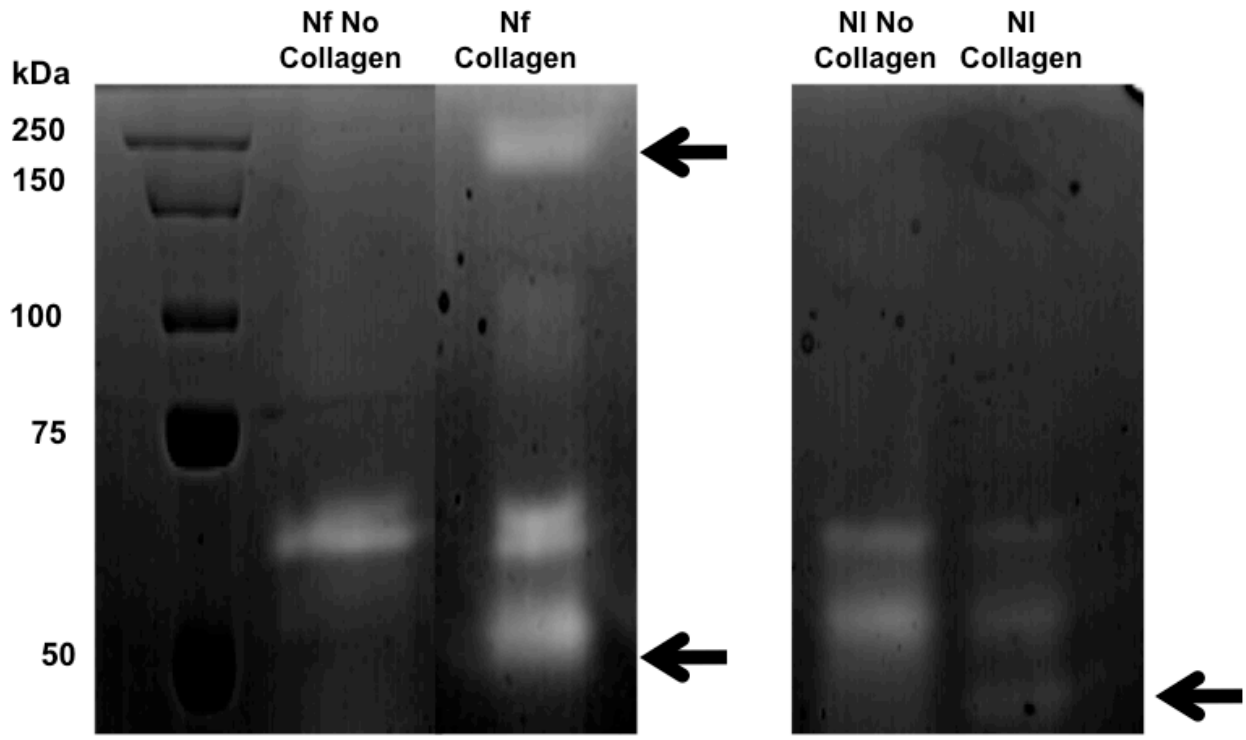


Figure 27. Detection of *N. fowleri* and *N. lovaniensis* proteases secreted during interaction on collagen I. Invasion assays were performed using collagen I coated tissue culture inserts. After 4 h the medium was collected and subjected to electrophoresis under nonreducing conditions on a 10% SDS-polyacrylamide gel containing 1% gelatin. Gels were equilibrated, developed overnight, and stained with Coomassie blue R-250 to visualize protease activity.

DISCUSSION.

Differences in invasion were evident when coated inserts were examined by SEM. Morphologically, both species exhibited a flattened spread appearance and food cups were present. This flatted, spread morphology is similar to what has been seen for *A. culbertsoni* as well as mammalian cells during invasion (Friedl, 2004; Rocha-Azevedo *et al.,* 2009). The major difference between the species was seen when the structural integrity of collagen I and matrigel were examined, revealing that only *N. fowleri* degraded and penetrated the ECM scaffolds. The presence of food-cups in both species of *Naegleria* suggests that these structures are not solely responsible for causing destruction of the ECM components. Secretory products specific to *N. fowleri* may be responsible for the observed damage. Aldape *et al.* (1994), isolated and characterized a secreted cysteine protease that catalyzed the in vitro degradation of extracellular matrix components. Therefore, this protease and/or others may be responsible for the ECM degradation observed in this study. The role of proteases was further investigated through the use of zymography. Zymographic analysis demonstrated differential protease expression in the presence of ECM components suggesting that the presence of ECM components results in the secretion of proteases, which may aid in degradation and thus invasion. Multiple studies have demonstrated that proteases play a very important role in tumor cell invasion. For example, progression of tumors results in the upregulation and activation of multiple ECM degrading enzymes, including matrix metalloproteinases (MMPs) and serine proteases (Birkedal-Hansen, 1995; Sebah *et al.,* 2004). Additionally, it has been shown using in

vitro models, that blockage of protease activity using inhibitors impairs tumor cell invasion (Mignatti *et al.,* 1986; Kurschat *et al.,* 1999; Ntayi *et al.,* 2001).

Microorganisms also express MMPs that are utilized for invasion. *Strongyloides stercoralis* larvae gain access to the body through the skin and invade the dermis. The larvae make their way to the bloodstream and eventually reach the lungs. Once in the lungs they invade through the alveolar septa, into the air spaces, and up the tracheobronchial tree, where they are swallowed, thus reaching the small intestine. The critical step of skin penetration and invasion through the dermis is facilitated by a zinc endopeptidase with elastase activity (Brindley *et al.,* 1995). Studies have shown that helminth larval extracts and secretions degrade purified elastin at a rapid rate. Additionally, specific metalloproteinase inhibitors prevented this proteolytic activity. Furthermore, inhibition of elastase activity resulted in a decrease in invasion using in vitro assays (Brindley *et al.,* 1995). Bacteria also rely on the production of proteases to support tissue invasion. *Pseudomonas aeruginosa* produces a matrix metalloproteinase called pseudomonas elastase, which has been shown to degrade laminin, type III and IV collagen, as well as elastin (Heck *et al.,* 1986). Furthermore, even microorganisms that are not capable of producing proteases on their own manage to use their activity to facilitate invasion. For example, HIV-1 is able to use a host cell MMP for its own invasion by inducing the infected carrier cell to increase secretion of collagenase IV, which in turn helps the virus invade the ECM enhancing its ability to reach other tissues (Weeks *et al.,* 1993).

Both pathogenic and nonpathogenic species of *Naegleria* possess proteases, all of which have thus far been identified as cysteine proteases (Serrano-Luna *et al.,*

2007). Studies are currently in progress using class specific inhibitors to determine to which class of proteases the observed bands belong. To further gain insight as to what role the proteases are playing during the invasion process we will perform invasion assays in the presence of inhibitors for the specific class/classes identified.

It also is important to note that we cannot rule out the importance of tensional forces created by the amebae/matrix interaction. It is possible that during this interaction the amebae can create contraction forces in order to modify the structure of the ECM scaffold, as observed in fibroblasts (Grinnell, 2008). Recent studies involving cancer cells have demonstrated that protease-independent migration is activated when proteolysis is inhibited (Friedl & Wolf, 2003; Wolf *et al.,* 2003) suggesting that proteolytic activity is not required for an invasive phenotype. This type of cancer cell migration is often referred to as “ameboid” because the cells take on an appearance similar to observed for *Dictyostelium* amebae when they migrate on 2D surfaces (Yumura *et al.,* 1984; Killich *et al.,* 1993; Wolf *et al.,* 2003). Ameboid migration is characterized further as utilizing a path finding mode of migration in which the cell adapts morphologically to overcome tissue barriers (Wolf *et al.,* 2003).

CHAPTER 6: Conclusions

The results highlight a variety of phenotypic characteristics that differ between pathogenic *N. fowleri* and nonpathogenic *N. lovaniensis.* Differences observed include growth rate, contact-independent cytotoxicity, resistance to complement-mediated lysis, as well as attachment to and invasion of ECM components. The establishment of a *N. fowleri* infection appears to be a multifactorial process with all of the above mentioned phenotypic differences playing a role in the pathogenicity associated with *N. fowleri* (Fig. 28). However, the differences in attachment and invasion, as well as the importance of these two processes in the establishment of infections led to more detailed examination of these two areas. Collectively, the results implicate integrin-like molecules as linked functionally to *N. fowleri* attachment to the ECM. Since integrins have been shown to be critical for tissue invasion by a variety of cell types, studies are in progress to characterize the integrin-like proteins and to dissect the signal transductional pathways that may be activated consequent of integrin-like protein interaction with target ECM attachment motifs. It is possible that focal adhesion-like structures may play a role in induction of signal transduction pathways consequent of ameba-ECM attachment, possibly involving release of select proteases that contribute to penetration of the ECM. Alternatively, the interaction of tensional forces exerted by the focal adhesion-like structure with the ECM may disrupt the ECM and promote passage of *N. fowleri* into the

brain. Studies are in progress to establish the extent to which these modalities are linked to attachment and invasion of the ECM.

Future Studies:

An aim of the present study was to identify differences *between N. fowleri* and *N. lovaniensis* protein profiles using 2D SDS-PAGE and mass spectroscopy analysis. However, DIGE analysis demonstrated a number of differences in these two species (Fig. 29) by 2D SDS-PAGE analysis. Proteins present only in *N. lovaniensis* appear red and proteins present only in *N. fowleri* appear as green. Proteins present in both samples at approximately the same level appear as yellow spots (Fig. 29). Upon visual examination it was determined that there were several differences in protein profiles of *N. fowleri* and *N. lovaniensis*. The abundance of protein differences led to further experiments, which analyzed differences in protein profiles of axenic *N. fowleri* versus mouse-passaged *N. fowleri* to determine gene expression during infection. Previous studies in our laboratory have determined that extended growth of *N. fowleri* in axenic conditions leads to a decrease in pathogenicity, which can be restored by passaging the amebae in mice. Studies to examine differences in protein expression linked to increase pathogenicity are currently in progress (Fig. 30).

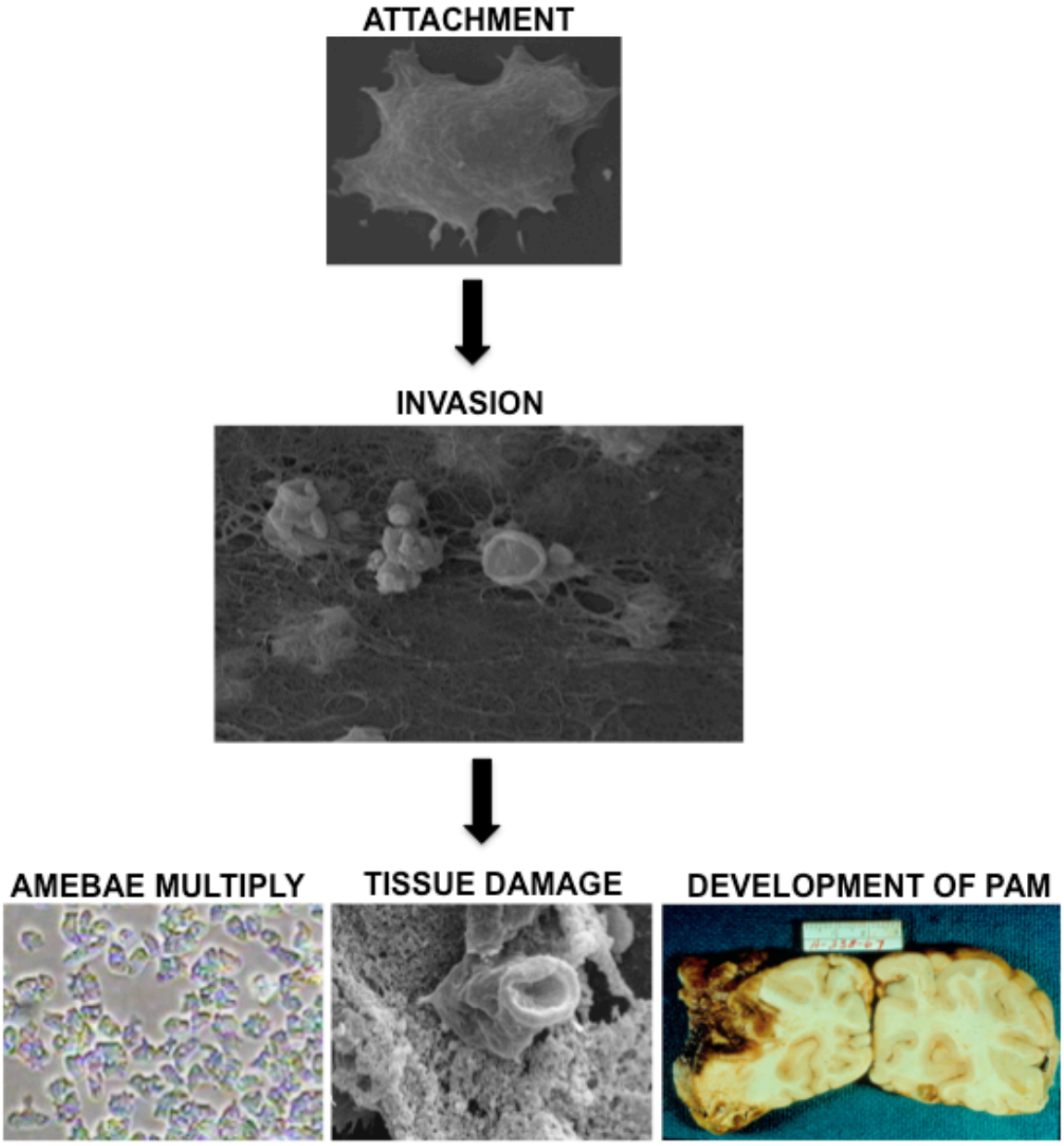

ATTACHMENT
INVASION
AMEBAE MULTIPLY
TISSUE DAMAGE
DEVELOPMENT OF PAM

Figure 28.

Figure 28. Role of investigated characteristics in PAM. Amebae enter the nasal passage, destroy the nasal epithelium, resulting in exposure of the extracellular matrix allowing the amebae to bind. Contact-dependent and contact-independent methods are involved in the destruction of the nasal epithelium, and integrin-like receptors facilitate adhesion to ECM components. Amebae invade the ECM and migrate through the cribriform plate into the brain. During invasion integrin-like molecules are utilized for attachment and movement. Amebae secrete proteases, which may aid in the breakdown of ECM components as well as cytotoxic molecules, which cause cellular damage. Amebae also interact physically with host components (including ECM) and cause an inflammatory response in the infected individual, both of which further contribute to destruction associated with PAM. Complement resistance contributes to amebae surviving the innate immune response and rapid migration/invasion allow amebae to reach the brain in a short time period, contributing to the rapid rate of death associated with PAM. Once in the brain amebae lyse cells both by contact-dependent and contact-independent methods, divide rapidly, increasing the number of amebae present, leading to extensive tissue damage and eventually death.

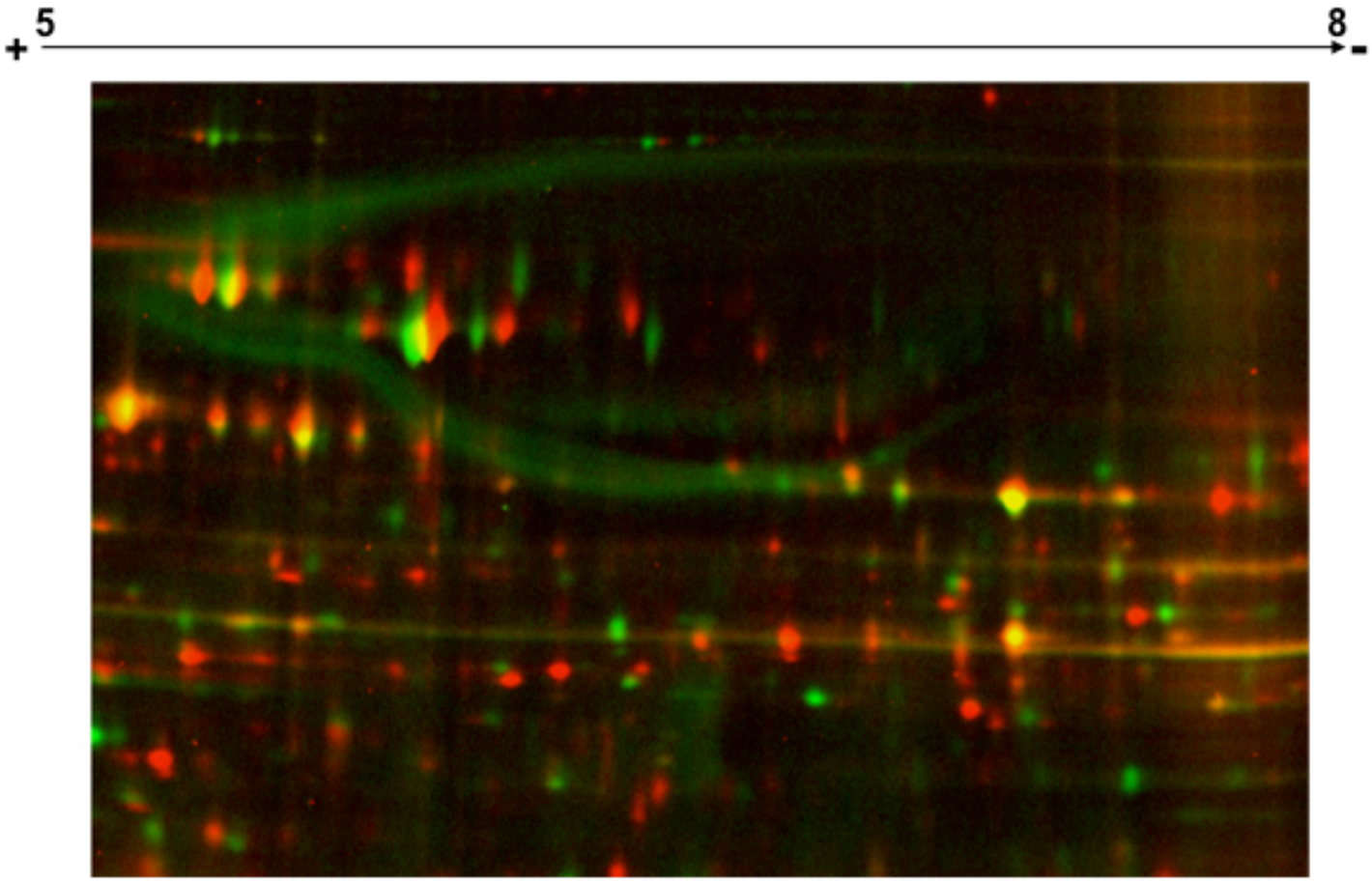


Figure 29: Difference In Gel Electrophoresis (DIGE) demonstrates varying protein profiles of *N. fowleri* and *N. lovaniensis*. Whole cell lysates (50μg) of *N. lovaniensis* and *N. fowleri* were compared by 2-Dimensional DIGE. *N. lovaniensis* was labeled with Cy3 (red) and *N. fowleri* was labeled with Cy5 (green). Protein samples were subjected to isoelectric focusing for a total of 40,000 volt hours, separated by SDS-polyacrylamide gel electrophoresis (10% gel), and visualized using Molecular Imager FX Pro Plus (Bio-Rad) and PDQuest ver.8.0 differential analysis software. Protein spots that appear red are only present in *N. lovaniensis* and protein spots that appear green are only present in *N. fowleri*. Protein spots that appear yellow are present in both *N. lovaniensis* and *N. fowleri*.

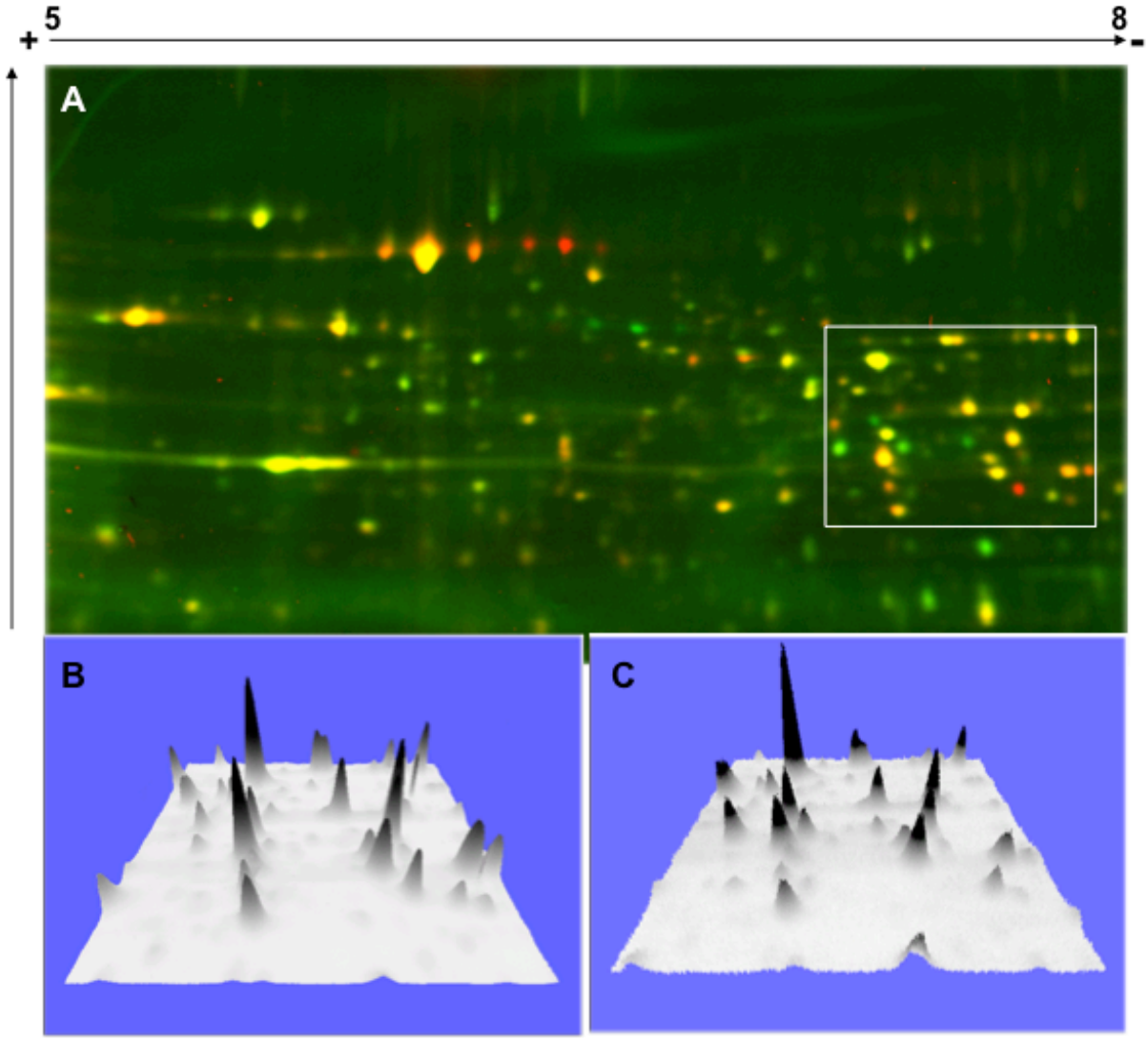


Figure 30: Difference In Gel Electrophoresis (DIGE) demonstrates protein profiles of axenic *N. fowleri* and mouse-passaged *N. fowleri.* Whole cell lysates (50μg) of axenic *N. fowleri* and mouse-passaged *N. fowleri* were compared by 2-Dimensional DIGE. Protein samples were subjected to isoelectric focusing for a total of 40,000 volt hours, separated by SDS-polyacrylamide gel electrophoresis (10% gel), and visualized

using Molecular Imager FX Pro Plus (Bio-Rad) and PDQuest ver.8.0 differential analysis software. (A) Axenic *N. fowleri* was labeled with Cy3 (red) and mouse passaged *N. fowleri* was labeled with Cy5 (green). Protein spots that appear red are only present in axenic *N. fowleri* and protein spots that appear green are only present in mouse-passaged *N. fowleri*. Protein spots that appear yellow are present in both axenic and mouse-passaged *N. fowleri*. Protein spots that appear orange are expressed in higher amounts in axenic *N. fowleri* and green/yellow proteins are expressed in higher amounts in mouse-passaged *N. fowleri*. (B) Three-dimensional view of axenic *N. fowleri* from inset. (C) Three-dimensional view of mouse-passaged *N. fowleri* from inset. Initial studies have demonstrated that there appears to be proteins are unique to both axenic and mouse-passaged *N. fowleri.* There are also proteins that appear to in increased and decreased after being passaged in a mouse. Additional studies involving mass spectrometry will be utilized to gain some insight as to the identity of differentially expressed proteins.

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