IDENTIFICATION OF PEPTIDASES IN HIGHLY-PATHOGENIC VERSUS WEAKLY-PATHOGENIC NAEGLERIA FOWLERI AMEBAE

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IDENTIFICATION OF PEPTIDASES IN HIGHLY-PATHOGENIC VERSUS
WEAKLY-PATHOGENIC NAEGLERIA FOWLERI AMEBAE

A thesis submitted in partial fulfillment of the requirements for the degree of
Master of Science at Virginia Commonwealth University.

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Abstract

IDENTIFICATION OF PEPTIDASES IN HIGHLY-PATHOGENIC VERSUS WEAKLY-PATHOGENIC NAEGLERIA FOWLERI AMEBAE

By Ishan K. Vyas

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2014

Major Director: Dr. Francine Marciano-Cabral

Professor, Department of Microbiology and Immunology

Naegleria fowleri, a free-living ameba, is the causative agent of Primary Amebic Meningoencephalitis. Highly-pathogenic mouse-passaged amebae (Mp) and weakly-pathogenic axenically-grown (Ax) N. fowleri were examined for peptidase activity. Zymography and azocasein peptidase activity assays demonstrated that Mp and Ax N. fowleri exhibited a similar peptidase pattern. Prominent for whole cell lysates, membranes and conditioned medium from Mp and Ax amebae were the presence of an activity band of approximately 58kDa and 100 kDa bands susceptible to the action of cysteine and metallopeptidase inhibitors, respectively. Further roles of the peptidases during the invasion process were examined by in vitro invasion assays in the presence of inhibitors and Cysteine and metallopeptidase inhibitors were found to greatly reduce invasion through the ECM. This study establishes a functional linkage of the expressed
peptidases to the invasion process, and these peptidases may serve as a candidate target for therapeutic management of *N. fowleri* infection.
**Introduction**

The genus *Naegleria* consists of a group of free-living amoeboflagellates found in soil, freshwater lakes, and ponds. (Marciano-Cabral & Cabral, 2007; Martinez & Visvesvara, 1997; Jamerson et al., 2011). Currently, over 40 species from this genus have been identified in the environment and domestic water supplies (Craun et al., 2005; Gyori, 2003; Rothrock, 1980). Two species of *Naegleria*, *N. australiensis* and *N. italica*, are pathogenic in experimental animals, however, only *Naegleria fowleri* has been implicated in human disease (Carter, 1968; Cerva & Novak, 1968; De Jonckheere 2004, Marciano-Cabral & Fulford 1986, Marciano-Cabral & Cabral).

A study of Internal Transcribed Spacers 1 and 2 (ITS), and the 5.8s rRNA of *Naegleria* species showed a close similarity between *N.fowleri* and nonpathogenic *N. lovaniensis*, two species which are globally distributed in the same locations (De Jonckheere 2004). In contrast, ribotyping of *N. australiensis* and nonpathogenic *N. gruberi* showed significant differences with the latter species being more complex (Clark et. al 1989). While there is much variability between different *Naegleria* species, the morphology is similar across the genus (Marciano-Cabral 1988).

**Morphology**

*Naegleria* are found as free-living amoebaflagellates with three morphological forms in their life cycle: a flagellate, trophozoite, and cyst form (Fig.1) (Carter, 1970; Marciano-Cabral, 1988). The trophozoite is the feeding, replicative and infective form. The amoebae feed on bacteria, yeast, and fungi using surface structures termed “food cups” and reproduce by binary fission (Trabelsi et al., 2012; Marciano-Cabral, 1988). The “food cups” are cytoplasmic
extensions of the amebae and vary in size and number depending on the species and strain of *Naegleria* (John, 1984; John, 1985; Marciano-Cabral, 1988).

The trophozoite form, which measures 10-25 µm, moves in a sinusoid manner by producing lobopodia at its anterior end; however, the amoebae transform into a flagellated form for sustained movement (Visvesvara et al., 2007, Marciano-Cabral, 1988). The trophozoites are able to transform into a flagellated form when the ionic concentration of the milieu changes. The change can be induced in the laboratory by placing the amebae in distilled water. The flagellate is pear-shaped with two flagella at the broad end and measures 10-16 µm in length. (Visvesvara et al., 2007; Marciano-Cabral, 1988; Trabelsi et al., 2012).

Under adverse environmental conditions, the trophozoite can transform into a highly resistant cyst form. The cyst is double-walled with a thick endocyst and a closely apposed thin ectocyst. Pores present in the cell wall facilitate the exit of the trophozoite. The cyst, along with the flagellate stage, has a single nucleus with a prominent nucleolus. (Fig. 2) (Marciano-Cabral, 1988; Schuster & Visvesvara, 2004; Trabelsi et al., 2012; Visvesvara et al., 2007).

**Fig. 1.** Scanning electron micrographs of the morphological stages of *Naegleria*. The life stages include the dormant cyst, the feeding trophozoite, and a swimming flagellate.
Fig. 2. *Naegleria fowleri* life cycle. *N. fowleri* has three forms in it’s life cycle but it mostly
resides in its infective trophozoite form at ambient environmental conditions. Under conditions of nutrient deprivation, the trophozoite undergoes a transformation to a flagellate stage which enables it to swim to the water surface to seek food sources. Cyst formation can be induced by food deprivation, crowding, desiccation, accumulation of waste products, exposure to toxic bacterial products, pH changes, and salts (Marciano-Cabral, 1988).
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 recently engaged in activities such as swimming or diving in warm freshwater bodies (Carter, 1968). Infection occurs after the inhalation of water containing ameba in their flagellate or infectious forms and initiated by the trophozoites attaching to the nasal mucosa (Carter, 1972; Center for Disease Control and Prevention Nov. 9, 2012). Inhalation of cysts during dust storms has also been implicated as a means of infection (Lawande, 1979; Schuster & Visvesvara 2004). The amebae enter the submucosal plexus after penetration of the nasal mucosa and ultimately gain access to the Central Nervous System (CNS) (Jarolim et al., 2000; Martinez et al., 1973). The immune response to the hemorrhagic necrosis in the brain causes extensive inflammation due to the recruitment of neutrophils, eosinophils and macrophages. The initial symptoms of PAM are severe frontal headache, fever, nausea and vomiting which progress into seizures, altered mental status, hallucination and coma. Death usually occurs 7-10 days after exposure to the amebae (Butt, 1966; Carter, 1970; Marciano-Cabral & Cabral, 2007; Martinez, 1985). Despite the 95% mortality rate of PAM, the key to success for the few survivors of the disease were rapid identification followed by aggressive treatment (Cetin & Blackall, 2012; Schuster & Visveswara, 2004). The current treatment regimen for PAM consists of Amphotericin B in combination with rifampin and other antifungal drugs. There have been reported successes in recent cases when the experimental anti-cancer drug Miltefosine has been used (Brown, 1991; Seidel et al., 1982; Kim et al., 2012). Successful cases of treatment include a 9 year old California girl, who was aggressively treated with intravenous and intrathecal amphotericin B, miconazole and oral rifampin (Seidel et al., 1982). Overall, the prognosis for patients with PAM
is poor, but early detection and aggressive treatment have been effective in a few cases (Vargas-Zepeda, 2005).

**PAM animal model**

The mouse is used as an experimental model for the disease as the stigmata is very similar to the disease in humans. The disease was first reported in mice when ameba, which were tentatively identified as *Acanthamoeba* by Culbertson et al. in 1958, were found to be the cause of a fatal meningoencephalitis in mice and this similarity was noted after the first human case was reported by Fowler and Carter in 1965 (Culbertson et al., 1958; Fowler & Carter, 1965). Pathogenesis in the mouse model shares many similarities with the human disease such as the period and portal of entry, and the subsequent route of the invasion into the brain. In mice studies, the route of the amoebas to the brain has been shown to be through the cribriform plate of the ethmoid bone (Martinez et al., 1973). Once inside the olfactory bulb, the amebae move to the posterior region of the brain, multiplying and causing extensive tissue damage (Jarolim et al., 2000; Martinez et al., 1973). The predominantly neutrophilic response to the ameba that is noted in the human hosts is also present in the mouse model (Carter, 1972; Duma, 1972). In addition, mice develop the disease over the course of 5 to 6 days resulting in death when intranasally instilled with amoebas collected from human cases, which is similar to the incubation time for the amebae to cause disease in humans. This model thus presents the opportunity to study the mechanisms of penetration of the nasal and olfactory epithelium during the course of the disease (Martinez et al., 1973). An attenuation in virulence was noted in amebae grown axenically for a prolonged period of time. Serial mouse passage restores and maintains virulence in the amebae (Marciano-Cabral, 1988; Wong et al., 1977; Whiteman & Cabral, 1989).
PAM epidemiology

Human disease caused by the free-living amebae, *Naegleria fowleri* was first reported by Fowler and Carter, who studied four patients from South Australia with a rapidly fatal disease which presents with symptoms similar to those of viral and bacterial meningitis (Fowler, 1965; Cerva & Novak, 1968; Gyori, 2003; Marciano-Cabral, 1988). The term “Primary amebic meningoencephalitis” was first used by Butt in 1966 and later by Carter in 1968 to distinguish an infection caused by *N. fowleri* from the rare brain invasion caused by *Entamoeba histolytica* (Butt, 1966; Carter, 1968; Marciano-Cabral, 1988). After the initial report, accounts of patients with PAM in the United States were reported in Florida and Texas (Butt, 1966; Butt et al. 1968).

A retrospective study indicated that an outbreak of PAM had occurred in Richmond, VA from 1951-1952 (dos Santos, 1970). Infections have been reported worldwide, with most of the reports stemming from developed countries, rather than developing ones, which may be due to increased awareness and more advanced diagnostic tools (John, 1982; Willaert, 1974). PAM has been documented in 15 southern-tier states including: Arizona, Arkansas, California, Florida, Georgia, Louisiana, Mississippi, Missouri, Nevada, New Mexico, North Carolina, Oklahoma, South Carolina, Texas, and Virginia, with over half of the infections occurring in Texas and Florida (Fig. 3) (Yoder et al., 2010). Swimming in freshwater during hot summer months is the most common route of exposure in patients. In Richmond, Virginia, infection in 14 out of 16 cases

**Fig. 3. Number and location of case reports of primary amoebic meningoencephalitis caused by *Naegleria fowleri* (n=107) in the United States from 1962–2008.** Four additional cases were reported but the sites of exposure remain unknown. Florida and Texas account for half of the cases of PAM in the United States.
were attributed to two man-made lakes located within two miles of each other. (Calicott, 1968; Duma et al., 1971; dos Santos, 1970). Additionally, 16 deaths were linked to a contaminated heated indoor swimming pool in Czechoslovakia (Cerva et al., 1968). Infections through means other than swimming have also been found, these include exposure to *N. fowleri* through face-washing, bathing, and backyard swimming pools (Carter, 1972; John, 1982; Miller et al. 1982). More recently, the increasing use of non-sterile water in nose cleansing has been thought to be causative of PAM in the 13 cases diagnosed in Karachi, Pakistan between the years 2008-2009 (Shakoor et al., 2011). Domestic water supplies in the United States have also been linked to fatal cases of PAM. Nested PCR analysis of water samples collected from homes where the cases occurred confirmed the presence *N. fowleri* (Fig 4) (Marciano-Cabral, 2003). Infections have also been noted in animals, such as cattle and a South American tapir, excluding humans as the sole hosts of PAM (Daft et al., 1999; Visvesvara et al., 2005).

**Virulence factors**

While the molecular mechanisms for the pathogenesis of *N. fowleri* are not clearly defined, virulence factors have been implicated to play a key role. Pathogenicity is the ability of a microorganism to cause disease and virulence refers to the degree of pathogenicity (Lipsitch, 1997). A variety of virulence factors such as GalNc and Kerp surface proteins, cysteine peptidases, and amoebapores have been implicated in the human pathogenesis of amebae such as *Entamoeba histolytica*, and *Acanthamoeba culbertsoni* (Faust, 2012; Ferrante, 1988; Harrison et al., 2010).

*Naegleria fowleri*, along with *Entamoeba histolytica* have been shown to damage their respective human cell targets through a process termed as trogocytosis (Brown, 1991; Ralston et

**Fig. 4. Scanning electron micrograph of a *N. fowleri* trophozoite** A Scanning Electron Microscope (SEM) image of a pathogenic *N. fowleri* (ATCC 30894) trophozoite displaying its “food cup” structures.
The amoebae express “sucker-like” structures called “food cups” that attach and endocytose parts of the target cell (Fig. 4)(Brown, 1979; Marciano-Cabral et al. 1982; Marciano-Cabral & Fulford, 1986). While these ‘food cup’ structures cause critical cellular damage, their presence does not appear to be a determinative factor in the ability of *N. fowleri* to cause disease.

Additionally, the amebae are capable of contact-induced cell destruction (Marciano-Cabral & Fulford, 1986). Initial studies found cytolytic pore-forming membrane proteins known as naegleriapores capable of lysing several tumor cell lines as well as erythrocytes (Herbst et al., 2002; Young & Lowrey, 1989). Naegleriapores, named as such for their pore-forming ability, are capable of lysing bacteria in the environment as well as brain cells in the accidental human host (Herbst et al., 2002). Chromatofocusing studies on the whole cell lysates of *N. fowleri* have also shown the presence of surface-associated hemolytic factors that may play a role in their pathogenicity (Lowry and McLaughlin, 1984; Lowry and McLaughlin, 1985).

**Immune evasion**

*Naegleria fowleri* is a successful pathogen, in part due to its wide repertoire of immune evasion strategies. The importance of the complement system in host defense against the pathogen was demonstrated when Complement 5 (C5) deficient mice and mice depleted of the complement system by cobra venom showed greater susceptibility to infection than normal mice (Haggerty & John, 1978). Mouse-passaged highly-pathogenic amebae are also more resistant to the lytic effect of the complement system and they have been shown to shed the areas of their surface where the membrane attack complex (C5-C9) has formed via vesiculation (Toney & Marciano-Cabral, 1994). This immunoevasive mechanism may be due to the presence of a “CD-
59 like” surface protein on the *Naegleria* as CD-59 has been shown to regulate complement activity and erythrocyte and leukocyte cell lysis (Fritzinger et al., 2006; Meri et al., 1990).

Microglial cells, the resident macrophages in the brain, play an important role in the primary host defense against a CNS invasion by *Naegleria* due to the secretion of a varied array of amebacidal molecules (Cleary & Marciano-Cabral, 1986). While weakly pathogenic axenic amebae were lysed by neonatal rat microglia, mouse passaged highly pathogenic amebae destroyed the microglial cells instead of serving as targets; In addition, pro-inflammatory cytokines were elicited in much greater amounts by the highly pathogenic amebae compared to the weakly pathogenic amebae, but these cytokines did not seem to augment the amebacidal activities of the microglia (Marciano-Cabral et al., 2001).

Humoral immunity does not seem to play a major role in amebacidal activities, but sera collected from almost all healthy individuals has tested positive for *N. fowleri*. A study by Cain et al. (1989) examined the serum of a patient with PAM obtained before the patient’s death and found all immunoglobulin levels were within the normal limits indicating the lack of mitigation of disease stigmata by the antibodies (Cain et al., 1979; Marciano-Cabral et al., 1987). A study by Newsome and Arnold (1985) demonstrated equal susceptibility to infection in congenitally athymic and euthymic mice indicating that cell-mediated immunity (CMI) does not play a major role in protection against *Naegleria* (Newsome & Arnold, 1985).

**Proteolytic enzymes**

Proteolytic enzymes have been investigated in several free-living amebae and are suspected to greatly facilitate invasion by destroying host tissues (Ferrante and Bates, 1988). *N. fowleri* expresses several proteolytic enzymes such as hydrolases, responsible for myelin
destruction, and elastase, responsible for breaking down elastin (Eisen & Franson, 1987; Ferrante & Bates, 1988).

Peptidases are enzymes that regulate the fate, localization, activity of proteins, modulate protein interactions, create new bioactive molecules, aid in tissue remodeling, and in the case of pathogens, act as a tool for degradation of extracellular environments to facilitate their pathogenesis (Lopez-Otin, 2008). The free-living amoeba, Acanthamoeba culbertsoni, has been shown to possess soluble peptidases that degrade cytokines and chemokines produced by microglia (Harrison et al., 2010). Additionally, cysteine proteases secreted by Entamoeba histolytica have been shown to degrade ECM, immunoglobulins, complement, and mucin, while membrane associated peptidases have also been implicated in host tissue breakdown in vivo (McKerrow et al., 1993; Serrano-Luna et al., 2012). Balamuthia mandrillaris, the causative agent of granulomatous encephalitis, has also been shown to exhibit metalloprotease activities, which were shown to break down ECM components, casein, and gelatin substrates in zymography (Matin et al., 2006). The presence of a 30 kDa secreted cysteine protease, with the ability to degrade extracellular matrix proteins in vitro and cause cellular damage to mammalian cells, was detected in N. fowleri by Aldape et al. in 1994. (Aldape et al., 1994). Additionally, two cysteine proteases with approximate molecular weights of 128 and 170 kDa, also have been observed in N. fowleri whole cell lysates (Mat Amin, 2004).

There are five classes of peptidases: Serine, cysteine, threonine, aspartic, and metallopeptidases, categorized based on their method of catalysis. Serine proteases have a nucleophilic serine residue in their active site which attacks the carbonyl moiety on a substrate to initiate the reaction (Page & Di Cera, 2008). Cysteine proteases are divided into papain-like and cathepsin-like families. The proteases found in amebae are referred to as cathepsin-like enzymes.
and possess a cysteine residue in their active site whose thiol group binds to a substrate to facilitate catalysis (Powers et al., 2002; Kissoon-Singh et al., 2011). Threonine peptidases contain an active site threonine which forms an intermediate oxyanion hole, similar to cysteine and serine proteases, which is utilized for substrate hydrolysis (Powers et al., 2002). Aspartic proteases include pepsin, renin, and Cathepsin D. These have a catalytic cleft with two aspartyl residues capable of accommodating polypeptides of up to seven amino acids (Polgar, 1987). Metallopeptidases contain zinc, and occasionally cobalt, as catalytic ions in their active site. The multifunction metal ions act as powerful electrophiles by activating the active site water molecule for a nucleophilic attack on the substrate (McCall et al., 2000).

**Extracellular Matrix**

The extracellular matrix (ECM) is a dynamic system of macromolecules including fibronectins, collagens, laminin, proteoglycans, and non-matrix proteins such as growth factors (Berrier & Yamada, 2007). During the initial stage of infection, *N. fowleri* come in contact with the host basement membrane, an extracellular structure composed of type IV collagen, laminin, enactin, and perlecan (Jarolim et al., 2000; LeBlue et al., 2007).

Collagen is the most abundant protein in the human body and is proposed to provide mechanical strength to tissues along with its binding partners (Sweeney et al., 2008; Okuyama et al. 2006). The central feature of collagen is characterized by three parallel polypeptide chains arranged in a helical conformation. The amino acids composition consists of a repeating X-Y-Gly sequence, where X and Y can be any amino acid and Gly is found at almost every third residue (Brodsky & Persikov, 2005; Okuyama et al., 2006). The basement membranes, which are
a critical barrier to the invasion route of \textit{N. fowleri}, are composed mainly of type 4 Collagen (Labat-Robert et al., 1990; Schwarz, 1986).

Fibronectin, an adhesive glycoprotein found in connective tissues and blood, has well-defined roles in adhesion, differentiation, migration and proliferation of cells in the body (Hynes & Yamada, 1982; Hynes, 1986; Romberger, 1997). Fibronectin is generally formed as a dimer, consisting of similar but not necessarily identical chains, which fold together into an extended and flexible molecule that is folded in a series of globular domains with specific functions (Romberger, 1997). Because of its widespread presence and diverse physiological roles, several pathogens have evolved strategies to interact with the protein. Pathogenic group G streptococci exhibit tissue-specific adherence to fibronectin and this initial recognition plays a key role in their establishment and colonization. Similarly, \textit{Entamoeba histolytica} also have a fibronectin binding protein, which, upon interacting with host fibronectin, triggers a cascade of proteolytic activity required for cell migration and invasion (Kline et. al, 1996; Talamas-Rohana & Meza, 1988). The presence of an integrin-like protein with fibronectin binding ability has also been noted in \textit{N. fowleri}, and this recognition may trigger a proteolytic cascade similar to the mechanism found in \textit{Entamoeba histolytica} (Han et al., 2004).

Laminins are a family of cross-shaped molecules that contain three chains ($\alpha, \beta, \gamma$) which arrange in the shape of a flexible, four-armed glycoprotein. Laminins have high affinity for other ECM components and play a critical role in cell attachment, growth and differentiation (Kleinman & Weeks, 1989; Yurchenco & Schittny, 1990). Microorganisms have evolved several strategies to utilize this important protein to further their pathogenesis. \textit{Trichmononas vaginalis} possesses a surface receptor that interacts with laminin, increasing its adherence to epithelial cells. Similarly, \textit{Staphlococcus aureus} possesses laminin receptors which play an
important role in bloodstream invasion and their resulting dissemination through the host (Silva-Filho et al., 1988; Lopes et al., 1985). A study examining the interaction of free-living amoeba, *Balamuthia mandrillaris*, with various ECM components revealed that the amebae had the capacity to bind to laminin in the presence of divalent cations, suggesting that this interaction may play a role in their pathogenesis (Rocha-Azevedo et al., 2006).

Invasion of the extracellular matrix by a foreign organism or a tumor cell is a multi-step process which includes adhesion, degradation of the ECM by proteases such as matrix metalloproteinas (MMPs) and migration (Liotta, 1986). In tumor cell studies, proteolytic invasion by the cell consists of five stages: The initial protrusion after cell polarization, the attachment of the newly formed leading edge to ECM fibers, cell-surface proteolysis to degrade ECM components in the immediate vicinity, actomyosin contraction leading to force generation resulting in cell and ECM deformation, and the disruption of adhesion bonds in the rear of the cell, leading to a retraction of the trailing edge (Artym et al., 2006; Friedl & Wolf, 2009; Wolf et al., 2007). Proteolytic degradation of ECM components can occur via diffusive secretory enzyme, membrane-anchored contact-dependent enzymes, or by intracellular endocytosis and subsequent degradation (Fig. 5) (Wagenaar-Miller et al., 2007; Wolf et al., 2007). Alternatively, tumor cells have also been shown to invade in a protease independent manner by conformational change which allows them to pass through the gaps of the extracellular matrix meshwork (Friedl and Wolf, 2011).
**Fig. 5. An overview of the three different methods of proteolysis.** The image depicts the three different methods of proteolysis that are employed by tumor cells during their invasion.

(A) Diffuse proteolysis occurs through proteases that are secreted out as either zymogens, or as pre-cleaved active molecules that traverse through the ECM. (B) Contact-dependent proteolysis occurs when ECM bound structures that are contacted by the cell through integrins are cleaved by Matrix metalloproteinases. (C) Intracellular proteolysis occurs by internalization of substrates and their subsequent degradation in the cytosol of the cell.

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Research objectives

The objective of the present study was to identify the proteolytic profile of the weakly-pathogenic axenically grown amebae and the highly-pathogenic mouse-passaged amebae and mechanistically identify whether proteases are utilized by the amebae during the process of extracellular matrix invasion. Additionally, two strains of amoeba were compared to assess their differences.
Materials and methods

Amebae. *N. fowleri* isolates of ATCC 30894 and CDC:V212 strains were used during the course of these studies. The *N. fowleri* (ATCC 30894) isolate was obtained from cerebrospinal fluid of a 15-year old female, in Richmond, Virginia. The CDC:V212 isolate was obtained from cerebrospinal fluid of a patient from Alabama in 1990. Amebae were grown axenically in 75 cm² plastic flasks at 37°C in Oxoid medium containing 0.55% liver digest, 0.30% glucose, 0.50% proteose peptone, 0.25% yeast extract, 1% calf serum and 1µg of hemin per ml in Page ameba saline (Cline et al. 1983). In addition to growing amebae axenically in Oxoid medium, *N. fowleri* was passaged by the intranasal route in B₆C₃F₁ mice at monthly intervals to maintain their virulence. Amebae harvested from mouse brain were maintained in Oxoid medium. Amebae thus passaged in mice every 4 weeks and maintained in Oxoid medium are highly pathogenic for mice and display greater motility than axenically cultured amebae (Bradley et al. 1996; Toney and Marciano-Cabral 1992). Prolonged culture of *N. fowleri* in axenic growth medium in vitro leads to a decrease in virulence for mice, and serial passage through mice restores and maintains virulence of the amebae as described previously (Wong et al. 1977; Whiteman and Marciano-Cabral 1987; Marciano-Cabral and Toney 1994). Highly-pathogenic Mp amebae and weakly-pathogenic Ax amebae were used in comparative studies to examine peptidase activity (Barrett and McDonald 1986). Care of animals used in this study was in compliance with the standards of the National Institutes of Health and the Institutional Animal Care and Use Committee at Virginia Commonwealth University.
**Ameba whole cell lysates (WCL).** Amebae grown for 24 h in Oxoid medium were detached from tissue culture flasks, washed three times in PBS, resuspended in PBS and disrupted by three cycles of freezing in liquid nitrogen and thawing at 37 °C in PBS. Protein content was determined using the method of Lowry et al. (1951) in which Bovine Serum Albumin was used in a serially diluted manner as a protein standard to determine the protein concentration of unknown samples.

**Membrane isolation.** Amebae grown for 24 h were detached from tissue culture flasks, washed three times in PBS, and membrane proteins were isolated using the Mem-PER eukaryotic membrane protein extraction kit (Thermo Scientific, Rockford IL), according to the manufacturer’s instructions. The Mem-Per extraction kit allows for separation of soluble cytosolic proteins and membrane proteins through the use of two different detergents. The relative purity of the membrane fraction was verified by Western Immunoblot analysis using a polyclonal antibody to the phylogenetically conserved protein Glyceraldehyde-3-phosphate Dehydrogenase (GAPDH). An immunoreactive band of relative molecular weight of 37 kDa corresponding to the relative molecular weight of the fully expressed GAPDH, was identified in the WCL and the cytosolic fraction of the amebae. No comparable band was noted in the CM or membrane fraction. The absence of an immunoreactive band corresponding to the GAPDH protein in the membrane fraction and CM confirmed the absence of major contamination from the cytosolic fraction. Anti-Heat Shock Protein also was used in Western blot analysis to confirm the absence of contaminating cytosolic proteins (Fig. 16).
**Conditioned medium (CM)**. Ameba-conditioned medium that putatively contained secreted peptidases was prepared by using Dulbecco’s minimal essential medium (DMEM). Briefly, amebae were washed in PBS to remove Oxoid medium. The amebae then were incubated in DMEM at 37°C for 6-24 h. The medium was centrifuged 4 times at 13,000 rpm to remove the amebae and the supernatant was collected to obtain secreted peptidases. Ameba conditioned medium also was analyzed by Western Immunoblot to confirm that the conditioned medium was not contaminated with cytosolic proteins). DMEM was used to prepare conditioned medium since it is more physiologically relevant than Oxoid medium for examining invasion of amebae through ECM components. In addition, DMEM as opposed to Oxoid medium, contains no serum that acts as a peptidase inhibitor. Mixed glial cell cultures were maintained in non-conditioned DMEM until assessed for cytotoxic effects elicited by CM.

**Gel zymography.** Whole cell lysates, membrane fractions and CM were subjected to electrophoresis under non-reducing conditions in a 10% SDS-polyacrylamide gel containing 1% gelatin, a peptidase substrate. Prior to electrophoresis, three volumes of sample were mixed with one volume of Laemmli loading buffer (Laemmli 1970; Alfieri et al. 2000). Following electrophoresis, gels were incubated (30 min) at RT in 1x sodium acetate renaturing buffer (0.1M, pH 5.0), equilibrated (30 min, RT) in 1x sodium acetate developing buffer (0.1M, pH 5.0) containing dithiothreitol (DTT) (1mM), and incubated overnight in 1x sodium acetate developing buffer (0.1M, pH 5.0) containing DTT (1mM). Peptidase activity was assessed at pH 5 and pH 7. Gels then were washed once with ultrapure deionized water and stained with Coomassie blue R-250.
**Assay for azocaseinolytic activity.** Whole cell lysates and CM were used to assay for azocaseinolytic activity by the procedure of Sarath et al. (1989), with modifications (Alfieri et al. 2000). Reaction mixtures contained in a final volume of 200 µl azocasein (Sigma) (5 mg/ml for CM or 10 mg/ml whole cell lysates), 400 mM buffer solution (sodium acetate, pH 5; or PBS, pH 7), and up to 0.15 mg of protein of WCL or 100 µl of CM. The reducing agent DTT, and/or peptidase inhibitors, were added 10 min before the addition of substrate. In addition, samples were incubated with inhibitors for 1 h prior to the addition of azocasein. In each experiment, a cell lysate was tested against a battery of peptidase inhibitors. After incubation at 37 °C (60 min for WCL, 4 h for CM), the reaction was terminated by the addition of 600 µl of 10% (v/v) Trichloroacetic acid and tubes were placed on ice for 30 min. After centrifugation (5 min, 8,000 x g), supernatants were collected and 0.5 ml of each sample was mixed with 0.7 ml of 1 M NaOH. The absorbance was read at 440 nm in a Spectramax model 250 (Molecular Devices) and proteolytic activity was expressed in mU/mg (total extracts) or mU/ml (culture supernatants). One unit (U) of peptidase activity was defined as the level that hydrolyzed 1 mg of azocasein min⁻¹.

**Western immunoblot.** Whole cell lysates at equal concentration, as quantified by the Bradford method (Lowry, 1951), or conditioned medium and membrane fractions from equal number of amebae were subjected to 10% polyacrylamide SDS-PAGE under non-reducing conditions. Following electrophoresis, proteins were transferred to nitrocellulose membranes (Towbin et al., 1979). The membranes were rinsed (5 mins) in Tris-buffered saline containing .1% tween-20 (TBST) and treated for 1h 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 with
primary antibodies of interest (chicken anti-GAPDH, mouse anti-Hsp83, rabbit anti-MMP14) diluted in blocking buffer to optimal dilutions. Membranes were washed 6 times (5 mins each) in TBST and incubated with the relevant peroxidase-conjugated secondary antibodies (rabbit anti-mouse, goat anti-rabbit, goat anti-chicken) at 1:10,000 dilution in blocking buffer. Protein bands were visualized using a chemiluminescence detection kit (GE healthcare, Pittsburgh, PA), according to the manufacturer’s instructions.

**Scanning electron microscopy (SEM).** Brain tissue from infected mice was examined by SEM to observe overt tissue damage caused by amebae. Infected brain tissue was placed on a glass coverslip and incubated for 24 h in DMEM. The tissue was fixed with 2.5% glutaraldehyde. To examine for microscopic evidence of cytopathic effects in cultured cells, primary mixed glial cells obtained from rat pup brains were co-incubated with CM from *N. fowleri* for 6 – 24 h on glass coverslips at 37 °C. Primary mixed glial cells were provided by Dr. Babette Fuss, Department of Anatomy and Neurobiology, Virginia Commonwealth University. The co-cultures were fixed with 2.5% glutaraldehyde in 0.1M cacodylate buffer, pH 7.2, washed four times with PBS, post-fixed (40 min in the dark) with 2% (w/v) osmium tetroxide buffered in 0.1M cacodylate buffer, pH 7.2, dehydrated in a graded series of ethanol, subjected to critical-point drying with CO₂ as the transitional fluid, mounted on stubs, and coated with gold (30 nm)(Pettit et al. 1996). Samples were examined in a Zeiss EVO 50XVP scanning electron microscope (Zeiss, Oberkochen, Germany) operating at an accelerating voltage of 15 kV.

**Peptidase inhibitors.** Peptidase inhibitors were added to ameba WCL, membrane fractions, or CM, 1h prior to use. The inhibitors and concentrations used were: for cysteine peptidases, 10μM
trans-epoxysuccinyl-L-leucylamido(4-guanidino) butane (E64); for serine peptidases, 5mM and 10mM phenylmethlysulfonylfluoride (PMSF); and for metalloproteases, 10mM phenanthroline. Stock solutions of PMSF and phenanthroline were prepared using dimethylsulfoxide (DMSO). A stock solution of E64 was prepared in distilled water. An equal volume of DMSO or dH2O was added to control preparations and peptidase inhibition was based relative to this control.

**Extracellular matrix surface coating.** Twelve multi-well plates were coated with Sigmacote (Sigma, St. Louis, MO), washed once with deionized water, and air-dried overnight at room temperature. The surface of the plates then were incubated (2h, 37˚C) with 50 ug mL\(^{-1}\) of Laminin-1 from Engelbreth-Holm Swarm mouse sarcoma (Invitrogen, Grand Island, NY) diluted in PBS and DMEM.

**Invasion assays.** Tissue culture inserts (Greiner BioOne, Monroe, NC) having a pore-size of 8 \(\mu\)m were coated (50\(\mu\)l, 2 h, 37 ˚C) with 1.7 and 5 mg.ml\(^{-1}\) of matrigel (Sigma), a reconstituted basement membrane solution that forms a matrix and has been used extensively for in vitro invasion studies (Kleinman and Jacob 2001; Kleinman and Martin 2005). 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 (Jamerson et al. 2012). Mouse-passaged *N. fowleri* (2 \(\times\) 10\(^5\)) suspended in DMEM alone or incubated with DMEM containing E64, PMSF, and 1,10-Phenanthroline were added to the upper chamber, and the plates were incubated for 4 h at 37 ˚C. Amebae that passed through the ECM-coated inserts and into the bottom chamber were counted and photographed as described previously (Fraga et al. 2011).
Gel and statistical analysis. Zymogram images were acquired using Adobe Photoshop software. Images were saved as tiff files at 300 dpi and analyzed using Quantity One version 4.5 software (Bio-Rad Laboratories). A positive over negative ratio of reverse-density images greater than or equal to 2.1 was considered statistically significant. Azocasein assay data were obtained and analyzed for standard deviation using Excel software (Microsoft).
Results

Fig. 6. ATCC 30894 WCL zymograms with inhibitors at pH 5. Gel zymography of whole cell lysates (WCL) of mouse-passaged (Mp) and axenically grown (Ax) Naegleria fowleri at pH 5. Note the higher level of activity for Mp N. fowleri of an E64 sensitive band corresponding to a relative molecular weight of 58 kDa (arrow). Ctrl=control solvent used for the respective peptidase inhibitor; A. E64=trans-Epoxysuccinyl-L-leucylamido(4-guanidino)butane, a cysteine peptidase inhibitor; B. PMSF=Phenylmethanesulfonyl fluoride, a serine peptidase inhibitor; C. Phen=Phenantroline, a metalloprotease inhibitor. Note that all proteins were equally loaded at 2 µg/µL concentration.
Fig. 7. ATCC 30894 membrane fractions zymograms with inhibitors at pH 5. Gel zymography of membrane fractions from mouse-passaged (Mp) and axenically grown (Ax) *Naegleria fowleri* at pH 5. Ctrl=control solvent used for the respective peptidase inhibitor; A. E64=trans-Epoxy succinyl-L-leucylamido(4-guanidino)butane, a cysteine peptidase inhibitor; B. PMSF=Phenylmethanesulfonyl fluoride, a serine peptidase inhibitor; C. Phen=Phenanthroline, a metalloprotease inhibitor. A prominent activity band of approximately 58 kDa relative molecular weight is sensitive to the action of the cysteine peptidase inhibitor E64.
Fig. 8. ATCC 30894 CM zymograms with inhibitors at pH 5. Gel zymography of conditioned medium (CM) from mouse-passaged (Mp) and axenically grown (Ax) *Naegleria fowleri* at pH 5. Note the prominent activity band corresponding to a relative molecular weight of 58 kDa (arrow). Ctrl=control solvent used for the respective peptidase inhibitor; A. E64=trans-Epoxy succinyl-L-leucylamido(4-guanidino)butane, a cysteine peptidase inhibitor; B. PMSF=Phenylmethanesulfonyl fluoride, a serine peptidase inhibitor; C. Phen=Phenanthroline, a metalloprotease inhibitor.
Fig. 9. ATCC 30894 WCL zymograms with inhibitors at pH 7. Gel zymography of Whole Cell Lysates (WCL) from mouse-passaged (Mp) and axenically grown (Ax) Naegleria fowleri at pH 7. Note the prominent activity bands in the high molecular weight peptidases. Ctrl=control solvent used for the respective peptidase inhibitor; A. E64=trans-Epoxysuccinyl-L-leucylamido(4-guanidino)butane, a cysteine peptidase inhibitor; B. PMSF=Phenylmethanesulfonyl fluoride, a serine peptidase inhibitor; C. Phen=Phenanthroline, a metalloprotease inhibitor.
Fig. 10. ATCC 30894 membrane fractions zymograms with inhibitors at pH 7. Gel zymography of membrane fractions from mouse-passaged (Mp) and axenically grown (Ax) *Naegleria fowleri* at pH 7. Ctrl=control solvent used for the respective peptidase inhibitor; A. E64=trans-Epoxysuccinyl-L-leucylamido(4-guanidino)butane, a cysteine peptidase inhibitor; B. PMSF=Phenylmethanesulfonyl fluoride, a serine peptidase inhibitor; C. Phen=Phenantroline, a metalloprotease inhibitor.
Fig. 11. ATCC 30894 WCL azocasein assay performed at pH 5 and 7. Azocasein activity assay of whole cell lysates of mouse-passaged (Mp) versus axenically grown (Ax) Naegleria fowleri. A. Assay performed at pH 5. B. Assay performed at pH 7. Enzymatic activity was particularly sensitive to the cysteine peptidase inhibitor E64. Control=control solvent used for the respective peptidase inhibitor; E64=trans-Epoxysuccinyl-L-leucylamido(4-guanidino)butane, a cysteine peptidase inhibitor; PMSF=Phenylmethanesulfonyl fluoride, a serine peptidase inhibitor; Phen=Phenanthroline, a metalloprotease inhibitor.
Fig. 12. ATCC 30894 CM azocasein assay performed at pH 5 and 7. Azocasein activity assay of conditioned medium from mouse-passaged (Mp) versus axenically grown (Ax) *Naegleria fowleri* (n=3). A. Assay performed at pH 5. B. Assay performed at pH 7. Enzymatic activity was particularly sensitive to the cysteine peptidase inhibitor E64. Control=control solvent used for the respective peptidase inhibitor; E64=trans-Epoxysuccinyl-L-leucylamido(4-guanidino)butane, a cysteine peptidase inhibitor; PMSF=Phenylmethanesulfonyl fluoride, a serine peptidase inhibitor; Phen=Phenanthroline, a metalloprotease inhibitor.
Fig. 13. CDC:V212 WCL zymograms with inhibitors at pH 5. Gel zymography of the whole cell lysates (WCL) of mouse passaged (MP) and axenically grown (Ax) V212 strain of amebae were analyzed in the presence of different inhibitors. Ctrl=control solvent used for the respective peptidase inhibitor; A. E64=trans-Epoxysuccinyl-L-leucylamido(4-guanidino)butane, a cysteine peptidase inhibitor; B. PMSF=Phenylmethanesulfonyl fluoride, a serine peptidase inhibitor; C. Phen=Phenanthroline, a metalloprotease inhibitor. Note the sensitivity of the V212 WCL to all peptidase inhibitors.
Fig. 14. V212 WCL CDC azocasein assay performed at pH 5 and 7. Azocasein activity assay of conditioned medium from mouse-passaged (Mp) versus axenically grown (Ax) V212 strain of *Naegleria fowleri* (n=3). **A.** Assay performed at pH 5. **B.** Assay performed at pH 7. Enzymatic activity was sensitive to all peptidase inhibitors at pH 5. Control=control solvent used for the respective peptidase inhibitor; E64=trans-Epoxysuccinyl-L-leucylamido(4-guanidino)butane, a cysteine peptidase inhibitor; PMSF=Phenylmethanesulfonyl fluoride, a serine peptidase inhibitor; Phen=Phenanthroline, a metalloprotease inhibitor.
Table 1. Inhibition percentages of ATCC 30894 and V212 WCL. Inhibition percentages of the inhibitors E64, PMSF, and 1,10-Phenanthroline were calculated based on azocasein activity assays of both 30894 and V212 strains of axenically grown (Ax) and mouse passaged (MP) amebae (n=3). E64=trans-Epoxysuccinyl-L-leucylamido(4-guanidino)butane, a cysteine peptidase inhibitor; PMSF=Phenylmethanesulfonyl fluoride, a serine peptidase inhibitor; Phen=Phenanthroline, a metalloprotease inhibitor.
Fig. 15. Comparison of the membrane fractions and Conditioned medium of ATCC 30894 and CDC:V12 strains. Gelatin zymography comparing the membrane fractions and conditioned medium of axenically grown (Ax) and mouse-passaged (MP) ATCC 30894 and CDC:V212 strains of amebae. **A.** Membrane fractions of axenically (Ax) grown 30894 and V212 amebae were compared at pH 5. Note the prominence of the 37 kDa band in the V212 membrane fraction. **B.** Conditioned medium of the two strains were compared at pH 5. Note the heightened activity of the 149 kDa activity band in the ATCC 30894 strain compared to the V212 strain. Equal numbers of amebae were used for this comparison as to establish equal loading.
Fig. 16. Western immunoblot confirming the purity of the membrane fractions. Western immunoblotting on various fractions of highly-pathogenic *N. fowleri* was performed using antibodies to cytosolic proteins to ascertain the purity of the membrane samples. The samples above are whole cell lysates (WCL), membrane fraction, membrane fraction diluted 1:2 and 1:4, conditioned medium, conditioned medium diluted 1:2, and the cytosolic fraction. Note the absence of immunoreactive bands in the membrane fraction and the conditioned medium.
Peptidase Identification and characterization

ATCC 30894 pH 5

SDS gelatin gels used in zymography assays revealed a similar peptidase pattern in whole cell lysates from Mp and Ax N. fowleri (Fig. 6). However, Mp preparations exhibited greater overall levels of peptidase activity. Phenylmethylsulfonyl fluoride and phenanthroline treatment did not have a major effect on the peptidase activity of WCL preparations derived from Mp or Ax amebae. A subset of activity bands, sensitive to the cysteine peptidase inhibitor E64, was observed in the range of 58 kDa to 75 kDa relative molecular weight. Prominent among these was a band of activity with a relative molecular weight of 58 kDa that was observed in zymograms of preparations from Mp and Ax amebae. Mp amebae WCL exhibited a greater than 2.4-fold increase level of activity at the 58 kDa band as compared with WCL from Ax amebae. In contrast, the enzymatic pattern for the membrane fraction of Ax amebae showed a greater activity that was sensitive to E64 that corresponded to the 58 kDa relative molecular weight band (Fig. 7). A similar pattern of peptidase activity was observed for CM from Ax and Mp amebae. Again, a prominent activity band that was sensitive to E64 and that migrated at a relative molecular weight of 58 kDa was observed for Ax amebae (Fig. 8). Conditioned medium from Mp amebae exhibited a 2-fold increased level of activity that was sensitive to the action of E64.

V212 strain results and comparison with ATCC 30894 strain

Analysis of the WCL of the V212 strain at pH 5 showed that multiple activity bands at the approximate molecular weights of >250, 250, 217, 171, 82, 73, 58 kDa. E64 inhibits the activity of the subset of activity bands ranging from 58 to 82 kDa. PMSF has a slightly inhibitory effect on the 58 and 75 kDa bands while heavily inhibiting the 82 and 217 kDa bands. The action
of 1,10 phenanthroline did not affect a single specific activity band but contributed to an overall reduction of protease activity.

A comparative study of axenic membrane fractions and axenic and Mp conditioned medium of the V212 and ATCC 30894 strains was performed via gelatin zymography at pH 5 (Fig. 15). Membrane fractions from axenically grown amebae were used in this study as previous studies on the axenic fractions found an increased expression of peptidases compared to Mp membrane fractions. Membrane fraction from ATCC 30894 showed activity bands at approximately 54, 58, 64, and 75, 149, 250, and >250 kDa, whereas V212 membrane fraction showed activity bands at approximately 37, 58, 75, 149, 217, and 250 kDa (Fig. 15a). Especially notable is the substantially higher level of activity noticed in the bands corresponding to the approximate molecular weights of 58, 75, and 250 kDa in the V212 strain compared to the ATCC 30894 strain. Conditioned medium (CM) from V212 and ATCC 30894 also highlighted the characteristic differences between the two strains (Fig. 15b). CM from V212 showed greater activity in the peptidase band corresponding to approximately 75 kDa, whereas the ATCC 30894 CM showed heightened activity in the activity band of approximately 149 kDa. The Mp amebae in both strains show greater activity compared to their axenically grown counterparts.

Additionally, a comparative table of differences in the percentage of inhibition by the different peptidase inhibitors on the WCL of axenic and Mp amebae of the two strains showed differences in the levels of the activity of the various classes of peptidases each strain possesses (Table 1). Axenic ATCC 30894 WCL samples have a higher percentage of their proteolytic activity derived from cysteine proteases compared to the V212 strain at pH 5, whereas the V212 samples had similar composition of activity for cysteine, serine, and metallopeptidases. Analysis of Mp samples revealed the lack of major difference in the proteolytic profiles of the two strains.
with a significant portion of activity in both strains at pH 5 stemming from cysteine proteases, though substantial inhibition was noted by PMSF and 1,10-phenanthroline (Fig. 14A). At pH 7, the profiles look distinctly different as a significant portion of the activity in the ATCC 30984 samples still stems from cysteine proteases, whereas the V212 Mp sample shows a distinct reduction in the percentage of inhibition of activity due to the action of E64, indicating an attenuation of cysteine protease activity compared to pH 5 (Table 1).

**ATCC 30894 pH 7**

Gelatin zymography done on ATCC 30894 WCL samples at pH 7 yielded protease activity corresponding to the high molecular weight bands of approximately 98, 134, 149, >250 kDa in the control (Fig. 9A). Pre-treatment of the samples with E64 resulted in the inhibition of the 98 kDa activity band (Fig. 9B). Addition of PMSF resulted in the inhibition of all the expressed activity bands (Fig. 9C). 1,10-Phenanthroline showed no significant reduction in inhibition of activity bands (Fig. 9D). Analysis of membrane fractions by zymography revealed the presence of the 134, 149, and >150 kDa activity bands, which were all susceptible to the action of PMSF. E64 had no observable effect on this fraction, while 1,10 Phenanthroline reduced the activity of all molecular weight bands (Fig 10).

**Azocasein activity assay**

The results obtained by gel zymography were confirmed by the azocasein activity assay that was performed at pH 5 and pH 7. These pH conditions were selected since they mimic an environment encountered in the nasal passage (pH 5-8) (Washington et al. 2000; England et al. 1999) and CNS, respectively (Magnotta et al. 2012). Consistent with the zymography results,
greater overall peptidase activity was obtained for Mp *N. fowleri*. Furthermore, higher activity was obtained when assays were performed at pH 5. In whole cell lysates, peptidase activity for both Mp and Ax amebae was most sensitive to the action of E64, the cysteine peptidase inhibitor (Fig. 11). However, PMSF and phenanthroline showed some inhibition of peptidase activity for lysates from Mp versus Ax amebae when compared to their respective controls. Nevertheless, the preponderant inhibitory effect was obtained when E64 was used (Fig. 11). A similar result regarding E64 sensitivity was obtained when assessment was undertaken using CM under either pH condition (Fig. 12), with the exception that PMSF treatment resulted in augmentation of activity when assessed at pH 7. This enhanced activity for PMSF may have been due to a pH dependent reaction of the PMSF and DMSO vehicle with the substrate (Sheean et al. 2012; van Kampen et al. 2008). Additionally, the azocasein assay also augmented the zymography data collected for the V212 WCL samples (Fig. 14). WCL from axenically grown amebae and the mouse-passaged amebae show substantial decrease in activity in the presence of E64, PMSF and 1,10-Phenanthroline at pH 5 (Fig. 14A). At pH 7, the axenic WCL samples show a decrease in activity in the presence of the inhibitors, while the MP samples are resistant to the inhibitory action of the inhibitors in a pH dependent manner (Fig. 14B).
In Vitro studies

Fig. 17. Zymogram showing results of a co-incubation study of microglial cells with the CM of ATCC 30894 Mp amebae. Gel zymography of conditioned medium from mouse-passaged 30894 (MP) amebae and microglial cells (mg). Lane 1 contains conditioned medium collected at 6h from MP amebae. Lane 2 contains conditioned medium collected at 6h from microglial cells. Lane 3 contains conditioned medium collected after microglial cells were allowed to incubate in the presence of MP conditioned medium for 18h. Note the prominent band that is present at approximately 28 kDa.
Fig. 18. Scanning electron micrographs of a mouse brain post-infection and of microglial cells co-incubated with ATCC 30894 Mp CM. A. Brain tissue from a mouse infected intranasally (10⁴ amebae) and assessed on day 5 post infection with mouse-passaged 30894 (Mp) *Naegleria fowleri*. The arrow denotes the ameba. B. and C. Mixed glial cultures subjected to conditioned medium (CM) from Mp *N. fowleri* for 6h (B) and 24h (C). The bars represent 10 µm.
Fig. 19. Zymograms showing results of co-incubation of live amebae with Laminin-1 in different media. Highly-pathogenic 30894 mouse-passaged (MP) amebae were incubated for 18 hours with Laminin-1 in PBS or DMEM. A. Laminin-1 alone in PBS B. Amebae alone in PBS C. Amebae + Laminin-1 in PBS D. Laminin-1 alone in DMEM E. Amebae alone in DMEM F. Amebae + Laminin-1 in DMEM. Note the differences in peptidase expression in the different media.
Fig. 20. Zymograms showing invasion assay samples at various time points and comparing Axenic and Mp amebae during invasion. Gel zymography of samples collected from the top of the matrigel coated or uncoated inserts after an invasion assay after 6h, 15h, and 24h time periods. Lanes 1,2,3,4,7, and 8 are samples from 30894 mouse-passaged (MP) amebae whereas lanes 5 and 6 are samples from axenically grown amebae (Ax). Lanes 1 and 2 are samples collected at 6h from an insert without matrigel (-) and an insert with matrigel (+). Lanes 3 and 4 show a comparison of samples collected from matrigel coated inserts at 6h (6) and 15h (15). Lanes 5 and 5 show samples collected at 24h from insert without matrigel (-) and with matrigel (+). Lanes 7 and 8 show a comparison of samples collected at 15h from an insert without matrigel (-) and with matrigel (+) from an invasion assay done with axenically grown (Ax) amebae.
Fig. 21. Zymogram comparing samples collected from a comparative invasion assay of ATCC 30894 and CDC:V212 strains. Gelatin zymography of samples collected after 6 h from the top (T) and bottom (B) of an invasion assay done with V212 and ATCC 30894 strains.
Fig. 22. Zymograms of an invasion assay done with amebae pre-treated with E64. Gelatin zymography of 30894 samples collected from an invasion assay using the cysteine protease inhibitor E64 (10 µM). A. Samples collected from the top of matrigel coated inserts and the bottom of wells from the H₂O treated (Ctrl T and B) and 10 µM E64 treated amebae (E64 T and B). B. Samples collected from the top of uncoated inserts and the bottom of wells from H₂O treated (Ctrl T and B) and 10 µM E64 treated amebae (E64 T and B). Note the reduction in the activity of the lower molecular weight bands in the E64 treated amebae compared to the H₂O treated control in the matrigel coated inserts.
Fig. 23. Light micrographs depicting the amebae treated with 5 μM E64 that migrated to bottom chamber of the invasion apparatus through ECM. Migration into the bottom chamber was assessed 6h after ATCC 30894 MP amebae were introduced into the upper chamber. **A.** Amebae treated with vehicle (ie., H₂O). **B.** Amebae treated with 5 μM of cysteine peptidase inhibitor E64. Note the decreased number of amebae in the bottom chamber. The bars represent 100 μm.
Fig. 24. Light micrographs depicting the amebae treated with 10 µM E64 that migrated to bottom chamber of the invasion apparatus through ECM. Migration into the bottom chamber was assessed 6h after ATCC 30894 MP amebae were introduced into the upper chamber. A. Amebae treated with vehicle (ie., H₂O). B. Amebae treated with the cysteine peptidase inhibitor E64 (10 µM). Note the decreased number of amebae in the bottom chamber. The bars represent 200 µm.
Fig. 25. Light micrographs depicting the amebae treated with 10 µM E64 that migrated to bottom chamber of the invasion apparatus without ECM. Migration into the bottom chamber was assessed 6h after ATCC 30894 MP amebae were introduced into the upper chamber where the inserts were not coated with matrigel (Control). A. Amebae treated with vehicle (ie., H₂O). B. Amebae treated with the cysteine peptidase inhibitor E64 (10 µM). Note that there is no decrease in the number of amebae in the bottom chamber in the presence of E64. The bars represent 200 µm.
Fig. 26. Gelatin zymography of 30894 MP samples collected from an invasion assay using the serine protease inhibitor PMSF (3 and 5 mM). A. Samples collected from the top of matrigel coated inserts and the bottom of wells from the DMSO control treated (Ctrl T and B), 3 and 5 mM PMSF treated amebae (PMSF T and B). B. Samples collected from the top of uncoated inserts and the bottom of wells from the DMSO control treated (Ctrl T and B), 3 and 5 mM PMSF treated amebae (PMSF T and B). Note the reduction in the activity of high molecular weight bands in the PMSF treated amebae compared to the DMSO treated control in the matrigel coated inserts.
Fig. 27. Light micrographs depicting the amebae treated with 3 and 5 mM PMSF that migrated to bottom chamber of the invasion apparatus through ECM. Migration into the bottom chamber was assessed 6h after ATCC 30894 MP amebae were introduced into the upper chamber. A. Amebae treated with vehicle (ie., DMSO). B. Amebae treated with the serine peptidase inhibitor PMSF (3mM). C. Amebae treated with the serine peptidase inhibitor PMSF (5mM). The bars represent 200 µm.
Fig. 28. Light micrographs depicting the amebae treated with 5 mM PMSF that migrated to bottom chamber of the invasion apparatus without ECM. Migration into the bottom chamber was assessed 6h after ATCC 30894 MP amebae were introduced into the upper chamber where the inserts were not coated with matrigel. A. Amebae treated with vehicle (ie., DMSO). B. Amebae treated with the serine peptidase inhibitor PMSF (5 mM). Note that there is no decrease in the number of ameba that migrate to the bottom in the presence of PMSF. The bars represent 200 µm.
Fig. 29. Zymograms from an invasion assay done with amebae pre-treated with 1,10-Phenanthroline. Gelatin zymography of ATCC 30894 MP samples collected from an invasion assay using the metalloprotease inhibitor 1, 10-Phenanthroline (1 and 3 mM). A. Samples collected from the top of matrigel coated inserts and the bottom of wells from the DMSO control treated (Ctrl T and B) and 1 and 3 mM phenanthroline treated amebae (1mM and 3 mM T and B). B. Samples collected from the top of uncoated inserts and the bottom of wells from the DMSO control treated (Ctrl T and B) and 3 mM phenanthroline treated amebae (3mM T and B). Note the reduction in the activity of the protease bands in the phenanthroline treated amebae compared to the DMSO treated control in the matrigel coated inserts.
Fig. 30. Light micrographs depicting the amebae treated with 3 and 5 mM phenanthroline that migrated to bottom chamber of the invasion apparatus through ECM. Migration into the bottom chamber was assessed 6h after ATCC 30894 MP amebae were introduced into the upper chamber.

A. Amebae treated with vehicle (ie., DMSO). B. Amebae treated with the metallopeptidase inhibitor 1,10-Phenanthroline (3mM). C. Amebae were treated with the metallopeptidase inhibitor 1,10-Phenanthroline (5mM). Note the decreased number of amebae in the bottom chamber. The bars represent 100 µm.
Fig. 31. Light micrographs depicting the amebae treated with 5 mM phenanthroline that migrated to bottom chamber of the invasion apparatus without ECM. Migration into the bottom chamber was assessed 6h after ATCC 30894 MP amebae were introduced into the upper chamber where the inserts were not coated with matrigel. A. Amebae treated with vehicle (ie., DMSO). B. Amebae treated with the metallopeptidase inhibitor 1,10-Phenanthroline (5 mM). Note that there is no decrease in the number of ameba that migrate to the bottom in the presence of phenanthroline. The bars represent 100 µm.
Fig. 32. Light micrographs depicting the top of the invasion chamber showing amebae migrating through the ECM in the presence of 3 and 5 mM phenanthroline. Migration in the upper chamber was assessed 6h after ATCC 30894 MP amebae were introduced into the chamber.

A. Amebae treated with vehicle (ie., DMSO). B. Amebae treated with the metallopeptidase inhibitor 1,10-phenanthroline (3mM). C. Amebae treated with phenanthroline (5mM). The bars represent 100 µm.
Fig. 33. Western immunoblot showing the expression of MT1-MMP (MMP14) by axenic and Mp ATCC 30894 *N. fowleri* amebae. Western immunoblotting analysis of the membrane fractions of 30894 axenic and Mp amebae using a rabbit anti-MT1-MMP (MMP14) antibody revealed the presence of immunoreactive bands at 63 kDa in both fractions. Densitometry analysis of the bands showed a 2.4x greater expression in Mp amebae compared to axenic amebae.
In Vitro studies

**Microglia and amebic CM**

In order to garner insight into the functional relevance of these peptidases in the CNS invasive process, SEM and invasion assays were performed. Brain tissue from infected mice demonstrated lysis of the tissue as visualized by SEM (Fig. 18A). In addition, CM containing secreted peptidases was added to mixed glial cell cultures. Scanning electron microscopy revealed that cells cultured for 6 h showed minimal damage (Fig. 18B). In contrast, cells co-cultured with CM for 24 h were lysed to an extent similar to that observed for brain tissue of mice infected with Mp *N. fowleri* (Fig. 18C). CM from the amebae, and media collected from microglia was compared to medium collected after the microglia were incubated with ameba CM cultures for 6 h (Fig. 17). A prominent increase in the activity of an approximately 28 kDa band was observed in the medium collected from the co-incubation of microglia and amoeba CM.

**Laminin-1 and live ameba**

Live ameba in PBS or DMEM were added to a layer of Laminin-1 to study whether the surrounding milieu had an effect on protease secretion and whether Laminin induced a higher secretion/activity of proteases (Fig. 19). Amebae suspended in PBS showed increased activity in the subset of the lower molecular weight bands at 43, 58, and 75 kDa. Amebae suspended in DMEM showed a high level of activity in 58, 75, and 149 kDa bands, and amebae in either media showed greater activity in the presence of Laminin compared to the amebae alone.
Invasion assay – time points and strain comparison

Analysis of samples collected from invasion assays at 6, 15 and 24 hour time points revealed that the peptidases secreted from amebae show greater peptidase activity in the presence of ECM components, and the activity increased at 15 hours compared to 6 hours (Fig. 20). Particularly prominent was the presence of the lower molecular weight bands that were found to be sensitive to the action of the cysteine protease inhibitor E64. Mp amebae showed higher proteolytic activity during invasion than their axenically grown counterparts (Fig. 20C). Additionally, samples collected from the top of the well at 24 h showed new higher molecular weight activity bands compared to the samples collected at 15 h (Fig. 20D). Additionally, the ATCC 30894 strain was compared to the V212 strain to assess differences in their proteolytic pattern during invasion. The V212 strain shows a higher activity in the ~75 and 149 kDa activity bands while the 30894 strain shows higher activity at the approximately 98 kDa activity band (Fig. 21). The activity in the bottom chamber of the assay also differed as the V212 strain expressed a higher activity in the 75 kDa band compared to the 30894 strain.

Invasion assay with E64

The absence of the subset of lower molecular weight bands of 58,64, and 75 kDa was observed when samples collected from an invasion assay done in the presence of E64 were analyzed by zymography (Fig. 22). In addition, the effect of E64 treatment on migration of Mp N. fowleri was assessed by light microscopy (Fig. 23,24). Treatment of amebae with E64 resulted in a two-fold inhibition of their migration into the bottom well (Fig. 23B) of the invasion chamber when compared to vehicle controls (Fig. 23A) at 5µM concentration. The migration was inhibited by greater than a two-fold difference compared to the vehicle controls in the
presence of 10 µM E64 (Fig. 24). Light microscopic examination demonstrated that E64 had no overt cytotoxic effect on the amebae, as seen in the control inserts without matrigel (Fig. 25).

**Invasion assay with PMSF**

Treatment of amebae with 3mM PMSF resulted in reduction in the activity of high molecular weight activity bands, and the activity observed in the amebae placed on matrigel was higher than those placed on inserts without matrigel, as shown by the controls in the absence of matrigel (Fig. 26). Light microscopy did not show a decrease in migration with 3mM PMSF treatment but a slight decrease was noticed in the presence of 5 mM PMSF (Fig. 27). Light microscopic examination demonstrated that E64 had no overt cytotoxic effect on the amebae, as seen in the control inserts without matrigel (Fig. 28).

**Invasion assay with 1,10-Phenanthroline**

Incubation of amebae with 3 mM and 5 mM 1,10-Phenanthroline resulted in a dose dependent decrease in the activity of all protease bands, with a significant decrease in the 75 kDa band compared to the vehicle DMSO control (Fig. 29). Examination of the bottom of the wells showed a greater than two-fold decrease in migration of phenanthroline treated amebae compared to the vehicle control (Fig. 30). Light microscopy examination revealed the absence of overt cytotoxic effect on the amebae by 1,3, and 5 mM phenanthroline (Fig. 31). Additionally, light micrographs from the top of the insert showed that a substantial number of amebae were inhibited from migrating compared to the vehicle DMSO control (Fig. 32). This inhibition occurred in a dose dependent manner as 5 mM concentration had a greater effect compared to 3 mM phenanthroline. Western immunoblotting was used to further explore the presence of
metallopeptidases to garner insight into the inhibition of activity due to phenanthroline, and the presence of an immunoreactive band of approximately 63 kDa was detected when a rabbit anti-MMP14 antibody was used, which corresponds to the molecular weight of a fully expressed MMP14 protein (Fig. 33).

DISCUSSION

Peptidases have been implicated as virulence factors in a number of protozoan infections and as playing a role in development, immune evasion, acquisition of nutrients, and in invasion and egress of intracellular parasites (Pina-Vazquez et al., 2012; Sio et al., 2006; Mayer et al., 1991; Que and Reed, 2000; Rascon and McKerrow, 2013; Choudhury et al., 2010). Peptidases produced by E. histolytica disrupt the epithelial barrier during the invasion process (He et al., 2010), cleave IgA and IgG, and inactivate complement components (Kelsall and Ravdin, 1993; Tran et al., 1998; Reed et al., 1995). Peptidases produced by pathogenic free-living amebae such as N. fowleri (Aldape et al., 1994), Acanthamoeba spp. (Alfieri et al., 2000), and Balamuthia mandrillaris (Matin et al., 2006), degrade ECM components in vitro. Serine proteases that degrade cytokines have been shown to be produced by Acanthamoeba culbertsoni (Harrison et al., 2010). The present studies were undertaken to determine whether highly-pathogenic mouse-passaged (Mp) N. fowleri express a distinctive proteolytic activity from weakly-pathogenic axenically-cultured (Ax) N. fowleri, and further investigate the roles of the proteases in the invasion process. Mice infected with Mp amebae exhibited characteristic stigmata of infection that culminated in high mortality. In contrast, mice infected with Ax amebae exhibited minimal
mortality and stigmata of disease (Toney and Marciano-Cabral, 1992; Whiteman and Marciano-Cabral, 1987). Mouse-passaged *N. fowleri* expressed greater overall peptidase activity as compared with Ax *N. fowleri*. This higher level of overall activity may have been in response to the cellular-rich milieu encountered during CNS passage. While both mouse-passaged and Ax *N. fowleri* expressed multiple proteolytic enzymes that included cysteine, serine, and metalloproteases, prominent among these was a cysteine peptidase of approximately 58 kDa based on its identification using E64, a selective cysteine peptidase inhibitor. This enzyme activity was observed by zymography at higher levels in whole cell lysates from Mp amebae and at higher levels in membrane preparations of Ax amebae. The presence of a higher activity of cysteine peptidases in the membrane fraction could indicate that these enzymes are compartmentalized within *N. fowleri* so as to facilitate their release upon contact with a substrate target. The 58 kDa peptidase was observed to be highly expressed in both V212 and ATCC 30894 strains of amebae, especially during the course of ECM invasion, which highlights its significance as a putative agent of pathogenicity.

A high molecular weight protease of 149 kDa susceptible to the action of serine peptidase inhibitor was especially prominent during invasion, and was inhibited by pretreatment of live amebae prior to invasion as well as pretreatment of collected samples prior to zymography. The inhibition of this activity band by PMSF was especially noticeable at pH 7 in the WCL and membrane fractions of the ATCC 30894 strain. Serine proteases exhibit optimal activity around neutral pH due to the catalytic serine residue present in their active site (Rao et al., 1998).

The variance in the expression of peptidases between the V212 and ATCC 30894 strains suggests that evolution of pathogenicity may be directly correlated to changes in the peptidase genes at a genomic level. *Naegleria fowleri* have been hypothesized to have evolved from non-
pathogenic *Naegleria* species by De Jonchkeere, 2004, and an ongoing effort to sequence the genome of *Naegleria fowleri* yielded the genome of a 60 kB nuclear DNA as well as the entirety of the mitochondrial sequence of the V212 strain (De jonckheere, 2004; Herman et al., 2012). While the mitochondrial genomes of *N. fowleri* and the non-pathogenic *N. gruberi* were found to be very similar, *N. fowleri* had a drastically different set of genes in the nuclear DNA that encoded for a variety of Cathepsin-B like peptidases hypothesized to be virulence factors during invasion. It is therefore possible that the V212 strain may also contain more peptidase oriented ORFs in its genome than the ATCC 30894 strain as V212 was isolated twenty three years after the 30894 strain.

The V212 strain shows greater stigmata of disease in the mouse model and expresses novel peptidases such as an 82 kDa band susceptible to the action of the cysteine peptidase inhibitor E64, and a 217 kDa band susceptible to the action of the serine peptidase inhibitor PMSF compared to the 30894 strain. Peptidases in the membrane fraction of axenic V212 and 30894 strains showed a greater overall expression of activity in the V212 strain, with a 37 kDa activity band that was highly expressed which was not noted in the membrane fraction of axenic 30894 amebae. Comparison of the two strains using an in vitro invasion assay showed differences in the peptidase expression profiles in the presence of ECM which could be causative of the presentation of the higher stigmata of disease in the V212 mouse model compared to the ATCC 30894 model. Additionally, while differences in the inhibition percentages were noted in the two strains in the axenic amebae, the Mp amebae of both strains showed very similar inhibition patterns in the presence of cysteine, serine, and metallopeptidase inhibitors, indicating that the peptidases have a certain pattern of activation when the amebae are introduced into a live host.
In order to gain insight into the role of peptidases in the brain microenvironment, microglial cells were incubated in the presence of conditioned medium collected from Mp amebae. Activated microglial cells are known to secrete proteases such as calpains and MMPs, and during the course of our studies, a 27 kDa peptidase was found to be highly expressed by the microglial cells in the presence of Mp CM than in the microglial control cells (Nakanishi, 2007). Additional assessment of microglia by SEM in the presence of CM showed substantive damage to the microglia by Mp CM at 24 hours compared to mild damage done at 6 hours, which indicates that the peptidases present in the secretions of the amebae have a cytotoxic effect on microglia. This observation strengthens the argument that peptidases may play an extensive role in the survival of amebae in the human host.

A study undertaken to determine the effect of the composition of the surrounding milieu on the secreted peptidases of the amebae resulted in the expression of a varied peptidase pattern in the presence of PBS compared to a more nutrient rich DMEM. The study suggests that the secreted peptidase content depends on the conditions of the surrounding environment, as has been found to be the case in peptidases secreted by other human pathogens (Braaksma, 2009). As the genome of N. fowleri is currently being sequenced, it is not possible to observe the genetic changes that occur when the surrounding milieu of the ameba changes, but studies done with the opportunistic human pathogens *Bacteriodes fragilis* and *Porphyromonas gingivalis* show that environmental conditions effect the activation and inactivation of various peptidase genes (Lu & Mcbride, 1998; Thornton et al., 2012). Another potential factor that could account for the differences in peptidase patterns is the presence of high molecular weight zymogens that may be activated at their respective optimal pHs. Since the higher molecular weight peptidases have been shown to be susceptible to the action of the serine protease inhibitor PMSF, the
disappearance of high molecular weight bands at pH 7.4 in the PBS medium may be a resultant effect of autocatalytic activation of high molecular weight serine zymogens. The presence of serine peptidase zymogens that are activated through autocatalysis has been widely documented and these peptidases may play a valuable role in amebic survival in a hostile host environment (Khan & James, 1998).

Consistent with these observations, greater peptidase activity, based on extrapolation for E64, PMSF, and phenanthroline sensitivity, was obtained when azocasein assays were performed at pH 5. This pH condition was selected since it mimics an environment encountered in the nasal cavity, the route for *N. fowleri* infection. Although cysteine peptidase activity may be linked to a variety of *N. fowleri* functionally relevant activities such as degradation of ingested bacteria, the collective results suggest that *N. fowleri* has a reservoir of cysteine peptidases that is available upon infection that serves to facilitate passage of the amebae through the nasal cavity into the CNS. To garner support for this proposition, the studies using an enzyme activity assay were followed by an in vitro invasion assay in which matrigel served as an ECM component. Treatment of *N. fowleri* with E64 resulted in reduced migration through the ECM. These results are consistent with reports that enzymes of *Naegleria* origin target the ECM. For example, a 30 kDa cysteine protease secreted by pathogenic *N. fowleri* and nonpathogenic *N. gruberi* that is able to degrade ECM proteins in vitro and cause cellular damage to mammalian cells has been identified (Aldape et al., 1994; Serrano-Luna et al., 2007). Also, two high molecular weight cysteine proteases of 128 kDa and 170 kDa have been observed in *N. fowleri* whole cell lysates (Mat Amin 2004). In addition, a homologue of cathepsin B has been identified in a segment of the nuclear genome of *N. fowleri* (Herman et al. 2013) that was not closely related to the cathepsin in nonpathogenic *N. gruberi*. Kim et al. (2009) using N-terminal peptide sequencing
identified a cathepsin B and a cathepsin B-like cysteine peptidase from *N. fowleri*. Furthermore, purified recombinant *N. fowleri* cathepsin B and cathepsin B-like proteases have been reported to degrade human proteins including collagen, fibronectin, and immunoglobulins (Lee et al. 2014). However, it should be noted that nonpathogenic *Naegleria* species also possess a variety of peptidases (Aldape et al., 1994; Serrano-Luna et al., 2007) and that these, may serve a multiplicity of functions not linked to invasion of host tissues.

Serine peptidases play a significant role in the invasion of the free-living Acanthamoeba, and thus an in vitro invasion study was conducted using *N. fowleri* pretreated with the serine peptidase inhibitor PMSF (Moon et al., 2008; Sissons et al., 2006). Despite the substantial reduction in the activity of high molecular weight peptidases in the samples collected from the invasion assay, no inhibition of transmigration of the amebae across the inserts into the bottom chamber of the well was noticed in the presence of PMSF, suggesting that they may not be physiologically important in the host invasion process. The presence of serine peptidases had previously been ascertained by zymography, as a number of high molecular weight peptidases were found to be reduced in PMSF treated amebae compared to the vehicle control, especially at pH 7.4, due to the optimal function of serine peptidases at near neutral pH, and this finding was confirmed by an azocasein activity assay. Serine peptidases of molecular weights ranging from 100 to 310 kDa have previously been identified in *N. fowleri* and the non-pathogenic *N. gruberi*, but no functional linkage of these peptidases to the invasion or survival process has been documented prior to the present study (Serrano-Luna et al., 2007).

The presence of metallopeptidases was ascertained by gelatin zymography and activity assays, and an inhibition of a band of approximately 100 kDa was noticed in the WCL and CM of the amebae. This activity band may be attributed to the latent form of MMP-9, which is
secreted during the invasion by cancer cells and is found to be highly upregulated in cancer cell lines (Backstrom et al., 1996; Roomi et al., 2009). During the course of the present study, the presence of MT1-MMP (MMP14) in the membrane fraction was detected by western immunoblotting, confirming the expression of metallopeptidases by the amebae. MT1-MMP serves as a very important diagnostic marker for cancer prognosis due to its importance in cancer cell migration and invasion and high levels of this protease has been correlated with an extremely poor prognosis (Eisenach et al., 2012). The migration of amebae through ECM was reduced dramatically in a dose dependent manner with phenanthroline treatment, and light micrographs indicated an increasing number of amebae clustered in the matrigel layer due to their inability to transmigrate through the invasion apparatus. The inability to migrate in the absence of functional metallopeptidases has also been noticed in cancer cells, and MMP-1 inhibition led to a drastic decrease in cancer cell dissemination (Zarrabi et al., 2011). These observations suggest that metalloproteinases may play an important role in the invasion process of *N. fowleri* through the host ECM.

The results generated by these studies indicate that the peptidases generated by *Naegleria* and identified by zymography exerted bioactivity against cellular and tissue targets. In order to determine whether cysteine and metallopeptidase activity were also linked to penetration of the ECM, transmigration experiments were performed. Treatment of *Naegleria* with the selective cysteine protease inhibitor E64 and metalloprotease inhibitor phenanthroline resulted in decreased transmigration through an ECM construct. This inhibitory activity occurred in the absence of overt cytotoxicity on the part of E64 and phenanthroline treatment. Thus, while the ability to invade tissues and cause disease may be due to multiple factors, the present results suggest that cysteine and metallopeptidases play a role, at least in part, in the invasive process of
the CNS.

In summary, peptidase activity and its linkage to virulence and *N. fowleri*-linked disease progression is a complex process. The present study indicated that the pattern of peptidase activity does not serve to discriminate between highly-pathogenic and weakly-pathogenic *N. fowleri*. That is, both weakly-pathogenic axenically-cultured (Ax) and mouse-passaged highly-pathogenic (Mp) *N. fowleri* produced and secreted a similar array of bioactive peptidases, albeit at different levels. Among these were cysteine and metallopeptidases that may play a role in the invasion of the CNS. A prominent cysteine peptidase activity band was identified at a position that corresponded to a relative molecular weight of 58kDa. However, the direct involvement of the 58 kDa peptidase, and the exact role of metallopeptidases, in penetration of the ECM remains to be defined. Nevertheless, the identification of these peptidases as playing a potential critical role in invasion of the CNS indicates that this *N. fowleri*-specified gene product may serve as a candidate target for therapeutic management of *N. fowleri* infection of the CNS.

**Future studies**

The aim of the present study was to identify differences between the peptidase profiles of the highly-pathogenic mouse-passaged and weakly-pathogenic axenically grown *N. fowleri*, and to establish a functional linkage of these peptidases to the physiological invasion process. The presence of a 58 kDa activity band susceptible to the action of the cysteine peptidase inhibitor E64 was detected in all fractions of the V212 and ATCC 30894 strains of amebae. Thus, future studies will further glean insight into the molecular structure and gene expressing this 58 kDa peptidase in addition to expanding more on the role of metallopeptidases in the invasion process.
Literature cited:


VITA

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