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Expression of Matrix Metalloproteinases in Naegleria fowleri and Their Role in Degradation of the Extracellular Matrix

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EXPRESSION OF MATRIX METALLOPROTEINASES IN *NAEGLERIA FOWLERI* AND THEIR ROLE IN DEGRADATION OF THE EXTRACELLULAR MATRIX

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

by

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Abstract

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Naegleria fowleri is a free-living amoeba found in freshwater lakes and ponds that is the causative agent of Primary Amoebic Meningoencephalitis (PAM). Matrix metalloproteinases (MMPs) have been described in protozoa, such as *Plasmodium falciparum*, *Trypanosoma brucei*, and *Balamuthia mandrillaris*, and have been linked to their increased motility and invasive capability by degrading components of the extracellular matrix (ECM). In addition, MMPs are often upregulated in tumorigenic cells and have been attributed as responsible for the metastasis of certain cancers. In the present study, *in vitro* experiments indicated that MMPs are linked functionally to the ECM degradation process. Gelatin zymography demonstrated protease activity in *N. fowleri* whole cell lysates, conditioned media, and media collected from *in vitro* invasion

assays. Western immunoblotting confirmed the presence of the metalloproteinases MMP-2, -9, and -14. The highly virulent mouse-passaged amoebae expressed higher levels of MMPs than the weakly virulent axenically grown amoebae. The functional relevance of MMPs found in media in degradation of ECM components was confirmed through the use of MMP inhibitors. The collective *in vitro* results suggest that MMPs may play a critical role in the invasion of the CNS. Furthermore, the expression of select metalloproteinases may serve as amenable targets for therapeutic manipulation of expansive PAM.

Chapter 1: Introduction

I. *Naegleria* species

The genus *Naegleria* is comprised of over 40 known free-living amoebae (FLA) species. However, only one species has been found to be associated with human infection, *Naegleria fowleri*, which causes the rapidly fatal disease of the central nervous system, Primary Amoebic Meningoencephalitis (PAM) (John, 1982; Marciano-Cabral, 1988). PAM was first reported as a human disease by Fowler in Australia, who mistakenly attributed the disease to an infection with *Acanthamoeba* (Fowler and Carter, 1965). *N. fowleri* is a ubiquitous thermophilic amoeba, tolerating temperatures up to 45°C and can be found worldwide in soil, dust, freshwater, and the nasal passages of some healthy, uninfected individuals (Marciano-Cabral and Cabral, 2007; Martinez and Visvesvara, 1997). *N. fowleri* has not been isolated in saltwater. Other species of *Naegleria*, such as *N. australiensis* and *N. italica*, have been shown to be weakly pathogenic in murine models, but intranasal inoculation, the usual route of infection for *Naegleria* spp., is associated with a much lower mortality rate (Marciano-Cabral and Fulford, 1986; Visvesvara *et al*., 2007). Nearly all nonpathogenic species of *Naegleria* are incapable of growing at elevated temperatures, with the exception of *N. lovaniensis*, the most phylogenetically similar species to *N. fowleri* (Jamerson *et al*., 2012). Similar to *N. fowleri*, *N. lovaniensis* thrives at 37°C. Although the two species are

very similar phylogenetically, the virulence factors of *N. fowleri* that contributes to its pathogenicity but not possessed by *N. lovaniensis* have not yet been fully elucidated.

II. Morphology

N. fowleri consists of three morphological stages, all of which display a single nucleus, 2 µm in diameter, with a prominent nucleolus (Figure 1). Amoebae may display two nuclei, and very rarely three or four (Carter, 1970). The trophozoite is the feeding, dividing, and infectious stage. Trophozoites, 10 to 25 µm in size, exhibit a varying number of amoebostomes, or “food cups,” cytoplasmic extensions on the cell surface that allow the amoebae to engulf their environmental food source, such as bacteria, yeast, algae, and organic debris (John *et al*., 1984; John *et al*., 1985; Marciano-Cabral and Cabral, 2007). Limacine locomotion is accomplished by anterior lobopodia movement, and the trophozoite replicates by binary fission. Under nutrient-poor conditions, the trophozoite can transform into a pear-shaped flagellate, from 10 to 16 µm in length (Visvesvara *et al*., 2006). The swimming flagellate stage can be induced by sudden ionic changes, such as a large influx of distilled water, but is temporary, usually lasting between 30 to 60 minutes before it reverts to a trophozoite. It harbors two flagella, both on its posterior end, for rapid motility. Under other environmentally adverse conditions, such as desiccation, overcrowding, and low temperatures, the amoeba can become a spherical cyst, about 8 to 12 µm in diameter (Marciano-Cabral, 1988; Martinez and Visvesvara, 1997). The cyst stage is resistant to many environmental conditions, including chemicals and antimicrobial drugs. It contains a

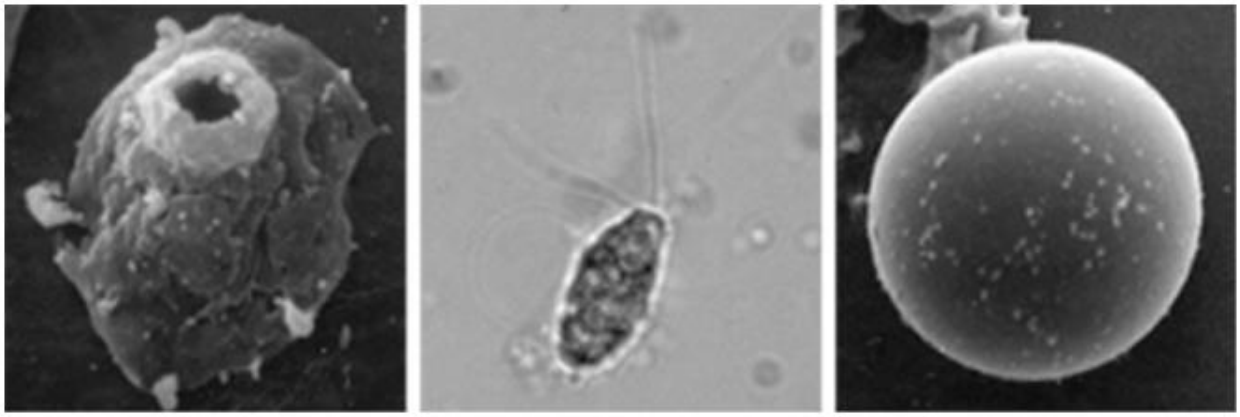


Image adapted from Marciano-Cabral, F.; Cabral, G. A. (2007). The immune response to *Naegleria folweri* amebae and pathogenesis of infection. FEMS Immunol Med Microbiol 51:243:59.

Figure 1. Microscopy images of the three morphological stages of *Naegleria*. From left to right: scanning electron micrograph (SEM) of a trophozoite, light micrograph of a flagellate, and SEM of a cyst of *N. fowleri*.

double wall, composed of a thick endocyst and a thin ectocyst, with pores through which the amoeba can excyst (Schuster and Visvesvara, 2004). When the amoebae are in the flagellate or cyst stage, they are unable to feed or divide.

III. Primary Amoebic Meningoencephalitis (PAM)

Primary Amoebic Meningoencephalitis (PAM) is an acute, fulminant, hemorrhagic meningoencephalitis caused by *N. fowleri*. Early signs and symptoms of infection include a severe headache, stiff neck, fever, nausea, and vomiting, followed by rapid progression to seizures, coma, and nearly always death (Martinez and Visvesvara, 1997; Tuppeny, 2011). Infection occurs when amoebae enter the nasal passages where they attach to the nasal mucosa (Martinez *et al*., 1971). Amoebae are able to invade the olfactory epithelium, without apparent disruption or damage to the mucosa layer or epithelial cells (Rojas-Hernández and Jarillo-Luna, 2004). The amoebae then migrate along the olfactory nerve endings through the cribriform plate. Due to the chemoattractant nature of the neurotransmitter acetylcholine on some human cells (e.g. neurons, neutrophils, and smooth muscle cells), it is believed that *N. fowleri* depends, at least in part, on the chemical gradient of acetylcholine for chemotaxis, especially due to their expression of a G-protein coupled receptor with structural homology to the human M1 muscarinic receptor that binds acetylcholine (mAChR1) (Baig, 2016). The amoebae begin dividing rapidly once they reach the olfactory and frontal lobes of the brain, although amoebae are also occasionally found in the base of the brain, the brainstem, and the cerebellum (Martinez, 1985). Trophozoites and flagellates may also be found in the cerebrospinal fluid (Butt, 1966; Vargas-Zepeda, 2005). In mice, amoebae can be

seen in the olfactory bulb as early as 30 hours after intranasal inoculation (Rojas-Hernández and Jarillo-Luna, 2004). Severe inflammation from the recruitment of neutrophils, eosinophils, and macrophage usually coincides with the hemorrhagic necrosis caused by the amoebae. Upon the arrival of amoebae to the CNS, death usually follows in 7 to 10 days (de Rocha-Azevedo *et al*., 2009). Despite 50 years of research and attempted treatments for PAM, the mortality remains above 95%, with only five well-documented survivors worldwide (Anderson and Jamieson, 1972; Cope *et al*., 2016; Linam *et al*., 2015; Seidel *et al*., 1982; Vargas-Zepeda *et al*., 2005). Treatment for all five cases involved the antifungal medication, amphotericin B, administered in varying doses both intravenously and intrathecally. Treatment typically includes a combinational therapy with rifampin, fluconazole, azithromycin, and, more recently, miltefosine, a novel antileishmanial drug (Walochnik *et al*., 2002). The successful treatment of these five patients were likely due to a combination of early detection and diagnosis, an extensive regimen of antimicrobial drugs, and, in some cases, an infection of a somewhat less virulent strain of *N. fowleri* (John and John, 1989; Cope *et al*. 2016).

IV. PAM Epidemiology

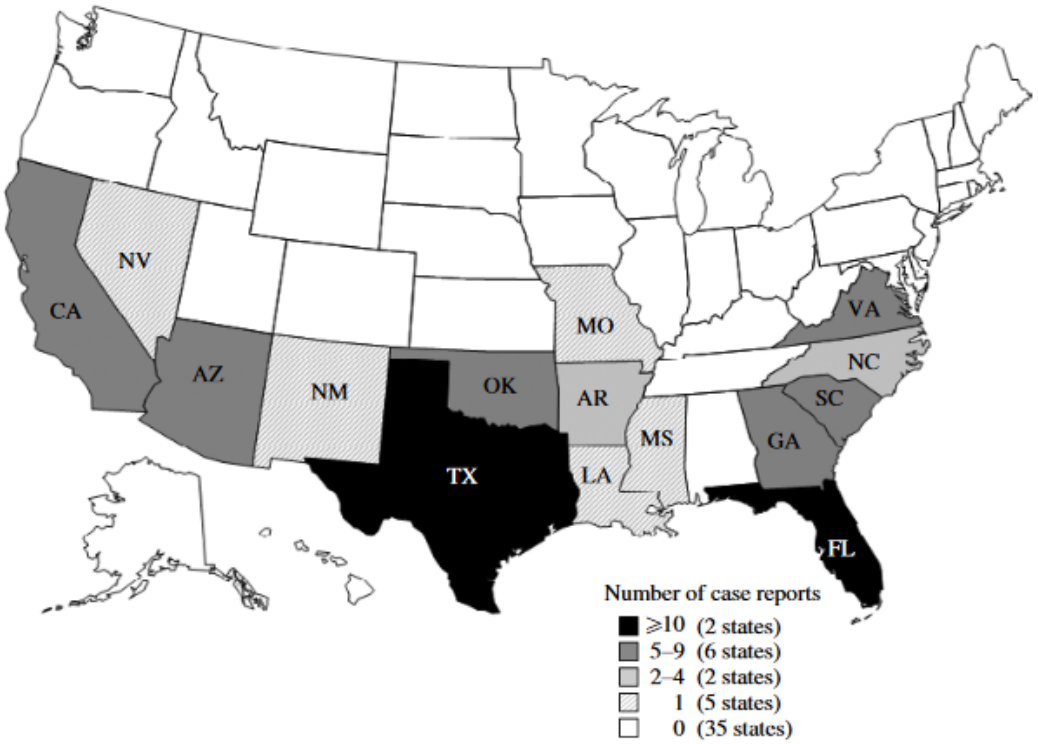
Although most reported cases of PAM occur in the United States, Australia, and Europe (France), many cases worldwide, especially in developing countries, are largely unreported (Siddiqui and Khan, 2014). Other countries with reported cases include Mexico, Great Britain, Belgium, Czech Republic, New Zealand, Nigeria, and India (Cabanes *et al*., 2011; Parija, 2015). Taiwan also recently reported its first case of PAM,

acquired from a thermal hot spring (Tung *et al*., 2011). While most cases in these countries are caused by water recreational activities, other countries, such as Pakistan, where Islam is the predominant religion, have recently reported cases of PAM due to religious practices (Hunte *et al*., 2013; Shakoor *et al*., 2011; Siddiqui and Khan, 2014). Before each of their five prayers daily, Muslims perform ablution for cleansing, which usually includes forcing water up the nostrils, potentially introducing *N. fowleri* amoebae into the nasal passages.

The United States has more reported cases of PAM than any other country. Due to the thermophilic nature of *N. fowleri*, PAM most commonly occurs in the summer months of July and August, and typically in the southern states, most notably Florida and Texas, which makes up over half of reported cases in the US between 1962 and 2008, although a fatal case was reported recently as north as Minnesota (Figure 2) (Kember *et al*., 2012; Yoder *et al*., 2010). The trends for the case reports in these 46 years include that most patients were male (79.3%), children or young adults (aged ≤19, 86.5%), and, in cases where the patient's race was documented, most were Caucasian (68.3%). However, there has been little evidence that differences in genetics, immune response, or anatomical structure could cause one sex, age group, or race to be more or less susceptible to infection of *N. fowleri*; the correlation between these populations and infection is likely due to the tendency of this demographic to partake in vigorous recreational water activities. An exception may be that the cribriform plate in children is more porous than in adults, which may allow for easier access of amoebae to the CNS for this cohort (Schuster and Visvesvara, 2004).

(a)

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



(b)

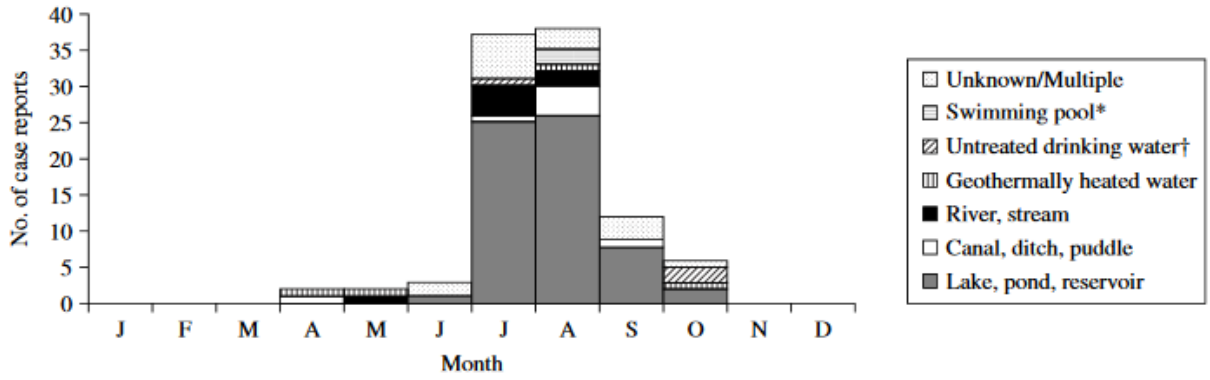


Image adapted from Yoder, J. S.; Eddy, B. A.; Visvesvara, G. S.; Capewell, L.; Beach, M. J. The epidemiology of primary amoebic meningoencephalitis in the USA, 1962-2008. 2010. Epidemiol Infect 138:968-75.

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Figure 2. Epidemiology of PAM in the United States between 1962 and 2008. (a) Map of the United States where states are shaded based on the number of reported cases of PAM. (b) Graphical representation of the months during which each case of PAM was acquired. Each bar additionally includes information about the source of infection.

In addition to environmental bodies of water, deaths have also been attributed worldwide to using domestic water supplies. Australia had multiple deaths caused by *N. fowleri*-contaminated water that was moved through overland pipes over great distances, which caused the pipes to be heated, allowing the amoebae to proliferate (Centers for Disease Control and Prevention, 2015; Dorsch *et al*., 1983). Likewise, cases have been reported of infections caused by municipal water in Arizona, Louisiana, and Pakistan (Cope *et al*., 2015; Marciano-Cabral *et al*., 2003; Shakoor *et al*., 2011; Yoder *et al*., 2012). Aside from recreational activities, most cases of infection from domestic water supply in the United States are often acquired during personal hygiene, including bathing or the use of neti pots for sinus irrigation, as were the cases in Louisiana and Arizona.

Exposure to *N. fowleri* is likely to be much higher than what is suggested by the occurrence of PAM. *N. fowleri* amoebae may be isolated from the nares of healthy, uninfected individuals, and antibodies that are reactive to *N. fowleri* are often found in adult human sera, especially in regions where PAM is more likely to occur (i.e. southern United States) (Cursons *et al*., 1980; Dubray *et al*., 1987; Marciano-Cabral *et al*., 1987; Yoder *et al*., 2010). It is unclear whether these antibodies play a protective role against *Naegleria* infection, but it has been suggested that they are able to slow the migration of the amoebae and that they may aid in complement lysis of amoebae (Cabanes *et al*., 2001).

V. Virulence Factors

The pathogenesis, host immune response, and other virulence factors of *N. fowleri* are poorly understood. Surface proteins have been found to mediate adhesion of the amoebae to components of the host extracellular matrix (ECM), including a 60 kDa fibronectin-binding protein and an integrin-like protein of approximately 70 kDa that is absent in the nonpathogenic *N. lovaniensis* (Han *et al*., 2004; Jamerson *et al.*, 2012). The directional movement of *N. fowleri* following attachment to the nasal mucosa epithelium may be attributed to the chemical gradient of acetylcholine expressed in the nervous system (Baig, 2016). Additionally, in the presence of rat B-103 neuroblastoma cells, *N. fowleri* trophozoites have been shown to exhibit an increased rate of locomotion, while three nonpathogenic *Naegleria* species displayed no response, suggesting that *N. fowleri* is unique in its neuropathogenic potential (Cline *et al*., 1986).

Proteases have previously been found to be expressed by *N. fowleri* as potential virulence factors. Cysteine proteases of approximate molecular weights 128 kDa and 170 kDa have been detected in amoeba whole cell lysates, and secreted cysteine proteases of 30 kDa and 58 kDa have been found with the ability to degrade host tissue (Aldape *et al*., 1994; Mat Amin, 2004; Vyas *et al*., 2014). Other degradative enzymes produced by the amoebae include phospholipase A and sphingomyelinase, which, although not unique to *N. fowleri*, have been found to be produced in much greater quantities in *N. fowleri* than in the nonpathogenic species of *Naegleria*, including *N. gruberi* (Cursons *et al*., 1978; Hysmith and Franson, 1982). *N. fowleri* has also been shown to exhibit trogocytic activity, involving two pseudopodia that pinch and detach fragments of cytoplasm from intact mammalian cells (Brown, 1979). The combination of

proteases, lipases, and other cytopathic factors contributes to the ability of *N. fowleri* to destroy relatively larger numbers of mammalian tissue cells *in vitro* (Brown, 1978).

Additionally, membrane-associated pore-forming proteins are able to mediate cytolysis of target cells (Young and Lowrey, 1988, Herbst *et al*., 2002). These naegleriapores are similar structurally and functionally to pore-forming proteins of other pathogenic amoebae. Of the two naegleriapores identified, naegleriapore A and B, both are involved in destroying human cells, while naegleriapore A also displays cytolytic activity against Gram-positive bacteria and naegleriapore B displays no bactericidal activity (Herbst *et al*., 2002). In addition to being found on the cell surface, naegleriapores are also contained within granules, presumably with other degradative enzymes, such as proteases, lysozymes, and phospholipases. It is thought that these enzymes work in conjunction to degrade bacterial cells, mammalian cytoplasmic fragments, and other cellular debris for nutrients.

Being facultative pathogens, *N. fowleri* must adapt to many different environments, including large fluctuations in temperature. *N. fowleri* has been isolated from environmental soil and water as low as 8°C, and cysts can survive for several days near freezing temperatures (Chang, 1978; Marciano-Cabral, 1988). The thermophilic nature of *N. fowleri* allows the amoebae to grow at temperatures up to 45°C, and trophozoites and cysts can withstand temperatures up to 65°C for a short period of time (1 to 3 minutes) (Marciano-Cabral, 1988). The presence of heat shock proteins (HSPs) in other pathogenic protozoa (e.g. *Plasmodium*, *Trypanosoma*, *Leishmania*, *Giardia*) suggests that HSPs play a critical role in survival within a mammalian host by assisting in protein folding and modulation of the host immune response (Ahmed *et al*., 2004;

Dobbin *et al*., 2002; Maresca and Carratú, 1992; Polla, 1991). A cytosolic heat shock protein 70 (HSP70) has been identified and characterized in *N. fowleri*, approximately 72 kDa in size with amino acid sequence homology between 41.6% to 82.9% when compared to HSP70s in other protozoa and mammals (Song *et al*., 2007). Inhibition of this HSP70 by a specific chemical compound or inhibition of the gene expression by antisense oligomers severely and adversely affects the proliferation of *N. fowleri* at 37°C (Song *et al*., 2008). Heat shock protein 70 may also be responsible for immunomodulation of the host response, which has been shown in *Toxoplasma gondii* causing the production of interleukin-10 (IL-10) and a decrease in nuclear factor-κB (NF-κB) and nitric oxide production (Ahmed, 2004; Dobbin, 2002). Although thermophily is not unique to *N. fowleri* in the *Naegleria* genus, as the nonpathogenic species *N. lovaniensis* is able to grow at 37°C, thermophily contributes to the virulence of *N. fowleri* by allowing its survival inside a mammalian host, but does not accurately confer the pathogenicity of a species (Marciano-Cabral and Fulford, 1986; Stevens, 1980).

Mechanisms of immune evasion also play a key role in the pathogenesis of *N. fowleri*. Through its evolution into becoming a successful pathogen, *N. fowleri* has developed mechanisms to resist lysis by host cytolytic factors, including tumor necrosis factor-α (TNF-α), IL-1, and the membrane attack complex (MAC) of the complement system (Fischer-Stenger and Marciano-Cabral, 1992; Marciano-Cabral and Cabral, 2007; Toney and Marciano-Cabral, 1994). Mice that are impaired in the complement system, either by deficiency in complement factor C5 or depletion of complement factors via injection of cobra venom factor, have been shown to have increased susceptibility to *N. fowleri* infection (Haggerty and John, 1978; Reilly *et al*., 1983).

However, *N. fowleri* amoebae do exhibit some resistance to complement-mediated lysis, especially those that have been passaged through mice and are thus highly virulent, while nonpathogenic species of *Naegleria* are relatively more susceptible to complement-mediated lysis (Whiteman and Marciano-Cabral, 1987). Although both pathogenic and nonpathogenic species of *Naegleria* activate the complement cascade, only *N. fowleri* is able to resist complement-mediated lysis through the presence of surface complement-regulatory receptors unique to pathogenic amoebae and shedding of the MAC by membrane vesiculation (Toney and Marciano Cabral, 1992, 1994). Additionally, a protein with structural homology to human CD59 has been found on the surface of trophozoites, suggesting that, like human CD59, which is present on human erythrocytes and leukocytes, this surface protein is a complement-regulatory protein that prevents deposition of complement factors and formation of the MAC (Fritzinger *et al*., 2006). Finally, cell-mediated immunity does not seem to play a critical role in protecting the host from *N. fowleri* infection, since there was no difference in susceptibility between euthymic and athymic mice (Newsome and Arnold, 1985).

VI. Extracellular matrix (ECM)

The extracellular matrix (ECM) is a highly dynamic three dimensional network of proteins and other macromolecules secreted by cells that form a complex structure in which cells reside in all tissues and organs. Interactions with components of the ECM is crucial for the invasion process of *N. fowleri*. Integrin-like proteins have been found in *N. fowleri* that play a role in adhesion to the ECM (Jamerson *et al*., 2012). Subsequent degradation of ECM components allows for invasion to the CNS. Components of the

ECM include collagens, proteoglycans, glycosaminoglycans, elastin, fibronectin, laminins, and many other glycoproteins (Theocharis *et al*., 2016). In addition to acting as a scaffold to provide cellular support and mechanical strength within tissues, the ECM regulates many cellular homeostatic functions through signal transduction, such as growth, migration, and differentiation. The ECM can be classified into different types of matrices, one of which includes the basement membrane, found between connective tissue and cells of the epithelium and endothelium (LeBleu *et al*., 2007). Acting as a sheet that anchors the parenchymal cells, the basement membrane is composed largely of collagen type IV, laminins, nidogens, and many proteoglycans (LeBleu *et al*., 2007; Theocharis *et al*., 2016). In the present study, *in vitro* assays that require emulation of the basement membrane utilize Matrigel, a commercially available composite of proteins produced by Engelbreth-Holm-Swarm (EHS) tumor cells that contain many components of the basement membrane (Kleinman and Martin, 2005).

VII. Matrix Metalloproteinases (MMPs)

A. Structure and Functions

Since the first discovery of matrix metalloproteinases (MMPs) in 1962 in a tadpole undergoing metamorphosis, at least 25 MMPs have been identified in mammalian and amphibian studies, with homologs varying in numbers found in other organisms (Gross and Lapiere, 1962). In humans, MMPs have some overlapping functions and play an active homeostatic role in remodeling the ECM during development and tissue repair (Mohan *et al*., 2016). They are also actively involved in the regulation of intercellular signaling by cleaving cytokines, chemokines, growth

factors, and their cognate receptors. However, certain MMPs are often found to be produced in abundance by tumorigenic cells and various pathogens to mediate metastasis and invasion by degrading components of the ECM (Bruschi and Pinto, 2013; Deryugina and Quigley, 2006; Geurts *et al.*, 2012; Gialeli *et al*., 2010; Matin *et al*., 2005).

MMPs can be classified into six groups based on the substrate of their catalytic activity (Table 1). Regardless, MMPs share a number of similar characteristics. All MMPs are Ca^{2+}-dependent endopeptidases that rely on Zn^{2+} in its catalytic site for their proteolytic activity. Additionally, MMPs are produced as zymogens (proMMP) that require cleavage of their propeptide domain for activation. This cleavage is usually performed by other proteases, including other MMPs or furin, a serine protease. The propeptide domain is approximately 80 amino acids long and contains the cysteine-switch motif PRCGXPD that maintains the proMMP form, with the exception of MMP-23, which does not contain such a motif in its propeptide domain (Rama *et al*., 2013; Visse and Nagase, 2003). Until the propeptide domain is cleaved, the cysteine residue coordinates with the zinc ion, blocking the access of water to the catalytic site, which is required for functional proteolysis (Van Wart and Birkedal-Hansen, 1990). The active site of MMPs is comprised of the two relatively conserved domains: the catalytic domain, approximately 170 amino acids long, and the Zn^{2+}-binding domain, which contains a sequence with three histidine residues that coordinate with the catalytic zinc ion: **H**EXX**H**XXGXX**H** (Mohan *et al*., 2016; Piccard *et al*., 2007; Sterchi, 2008; Zhang and Kim, 2009). Due to the structural similarity of the active site among MMPs,

Table 1. Matrix metalloproteinases

Classification	MMP	Common Name
Collagenases	MMP-1 MMP-8 MMP-13 MMP-18	Collagenase-1 / Interstitial collagenase Collagenase-2 / Neutrophil collagenase Collagenase-3 Collagenase-4 / Xenopus collagenase
Gelatinases	MMP-2 MMP-9	Gelatinase A Gelatinase B
Stromelysins	MMP-3 MMP-10 MMP-11	Stromelysin-1 Stromelysin-2 Stromelysin-3
Matrilysins	MMP-7 MMP-26 MMP-12	Matrilysin-1 Matrilysin-2 Macrophage metalloelastase
Membrane-type MMPs (MT-MMPs)	MMP-14 MMP-15 MMP-16 MMP-17 MMP-24 MMP-25	MT1-MMP MT2-MMP MT3-MMP MT4-MMP MT5-MMP MT6-MMP
Others	MMP-19 MMP-20 MMP-21 MMP-22 MMP-23 MMP-27 MMP-28	RASI 1 Enamelysin Cysteine array (CA-)MMP Epilysin

Data adapted from Bruschi, F.; Pinto, B. (2013). The significance of matrix metalloproteinases in parasitic infections involving the central nervous system. Pathogens 2:105-29. and Verma, R. P.; Hansch, C. (2007). Matrix metalloproteinases (MMPs): chemical-biological functions and (Q)SARs. Bioorg Med Chem 15:2223-68.

Table 1. Matrix metalloproteinases. The 25 known matrix metalloproteinases and the six classifications of them. Common names are also provided, with the exception of MMP-21, -22, and -27, which are unnamed. MMP-18 has no known human ortholog. All

of the MT-MMPs are membrane-associated via at least one transmembrane domain, with the exception of MMP-17 and -25, which are GPI-anchored.

substrate specificity thus depends on the hemopexin-like domain, which is more differentiated among MMPs. Nearly all MMPs, with the exception of matrilysins (MMP-7 and MMP-26) and MMP-23, contain a proline-rich hinge region and a C-terminal hemopexin-like domain, approximately 200 amino acids long, which forms variable blade-like structures that confer the divergent functions of each MMP. Some MMPs harbor additional differences in their subunit organization that provides further substrate specificity. For example, the catalytic domain of the gelatinases (MMP-2 and MMP-9) contain three repeats of a type II fibronectin domain that bind gelatin (Visse and Nagase, 2003). Although most MMPs are secreted, the membrane-type MMPs (MT-MMPs) are membrane-associated, either by containing a C-terminal transmembrane domain (75-100 amino acids long) or, in cases of MMP-17 and MMP-25, a glycophosphatidylinositol (GPI)-anchor (Sohail *et al*., 2008). MT-MMPs often additionally contain a cytosolic domain.

B. Regulation of MMPs

The regulation of MMPs exists at different levels (Figure 3). MT-MMPs are activated intracellularly in the *trans*-Golgi by the serine protease, furin (Boon *et al*., 2016; Cao *et al*., 2005). Most MT-MMPs, in addition to degrading ECM proteins, are able to activate MMP-2 by proteolytic cleavage. This proteolysis serves as the primary regulation of MMP-2; however, other MMPs are regulated primarily at the level of transcription (Ye, 2000). Activated MMPs can be further regulated by inhibitors, including matrix metalloproteinase inhibitors (MMPIs), tissue inhibitors of metalloproteinases (TIMPs), and other proteins. MMPIs include any compound, either

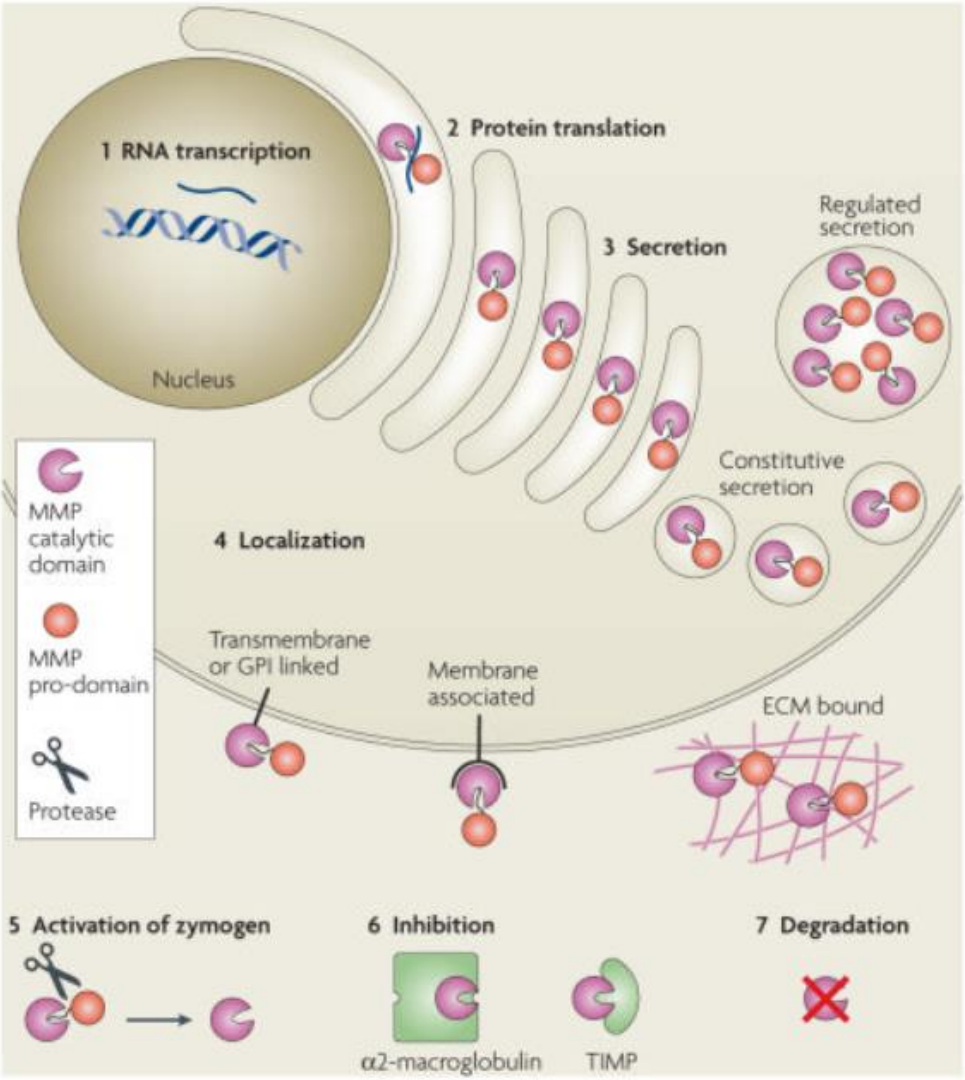

Image from Page-McCaw, A.; Ewald, A. J.; Werb, Z. (2007). Matrix metalloproteinases and the regulation of tissue remodeling. Nat Rev Mol Cell Biol 8(3):221-33.

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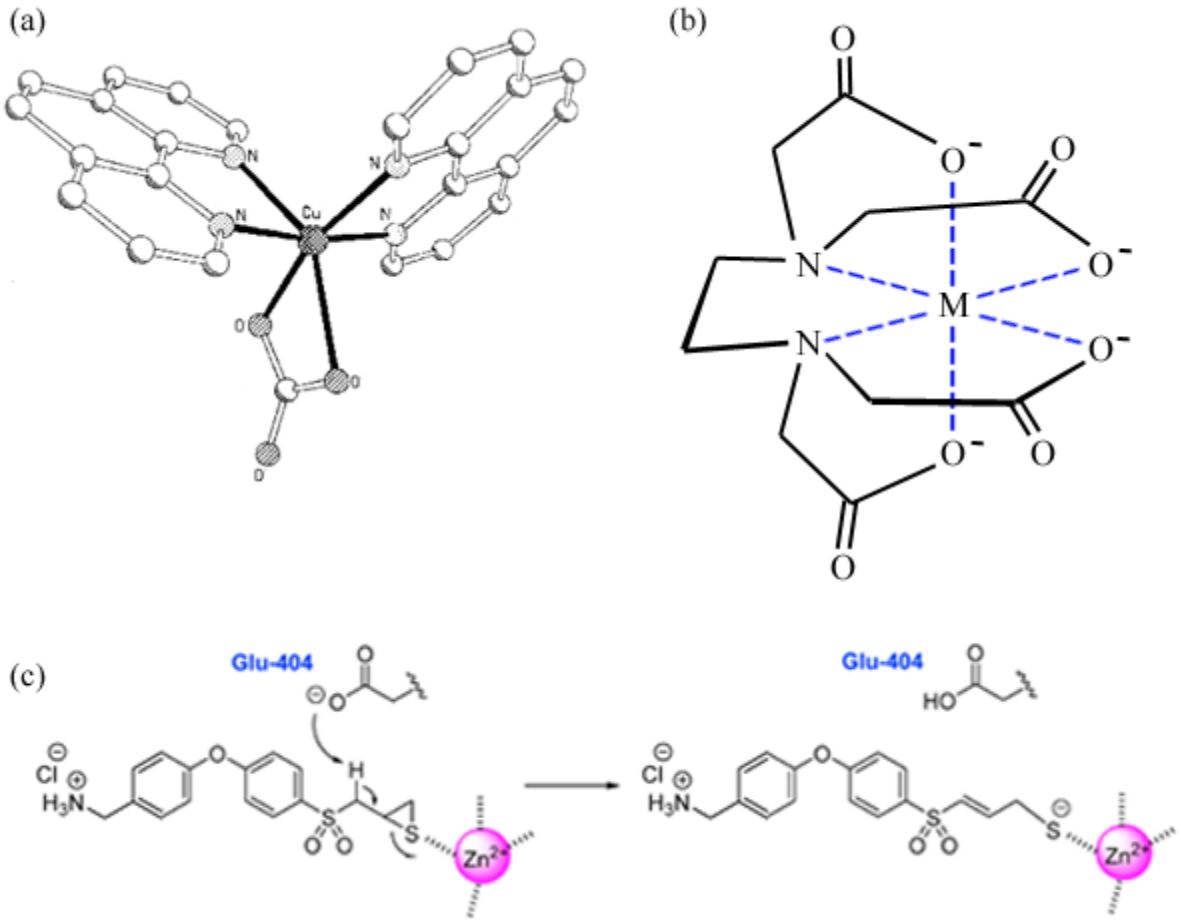
Figure 3. Regulation of matrix metalloproteinases. Seven levels of regulation exist for MMPs. In addition to (1) transcription and (2) translation, MMP expression can be regulated during (3) secretion and trafficking, (4) intracellular compartmentalization or extracellular localization in the ECM, (5) activation of the zymogen by cleavage, (6) inhibition by protein inhibitors, and (7) degradation.

synthetic or naturally occurring, that inhibits the activity of MMPs, while TIMPs are endogenous protein regulators of MMPs (Thomas *et al*., 2014; Sorsa *et al*., 2011; Zhang and Kim, 2009).

The expanding research on cancer metastasis and pathogenic invasion augments the search for naturally occurring and synthetic MMPIs. The present study utilizes various MMPIs to investigate the efficacy of MMPs on the motility and invasion of *N. fowleri*. Two such MMPIs are well documented broad spectrum inhibitors, 1,10-phenanthroline and ethylenediaminetetraacetic acid (EDTA), which function by sequestering metal ions and removing the catalytic zinc ion from the active site of MMPs, yielding inactive apoenzymes (Feder *et al.*, 1971; Hazra *et al*., 2012). 1,10-Phenanthroline interacts with zinc, iron and copper (Figure 4a), while EDTA binds various positively charged metals more nonspecifically, including zinc, calcium, copper, iron, magnesium, manganese, and lead (Figure 4b) (Brand *et al*., 1954; Felber, *et al*., 1962; Mali *et al*., 2014). Additionally, a novel specific MMPI is used in this study: ND-336 (4-[4-[(2-thiiranylmethyl)sulfonyl]phenoxy]-benzenemethanamine, monohydrochloride), which specifically inhibits MMP-2, -9, and -14 by coordinating with the catalytic zinc ion (Figure 4c) in a tight binding manner--inhibition constant (K_i) values are 85 ± 1 nM, 1504 ± 10 nM, and 120 ± nM, respectively (Gao *et al*., 2015). Moreover, the residence times of ND-336 for MMP-2, -9, and -14 are longer than those of TIMPs, rendering them to be more effective as inhibitors of these MMPs.

Four TIMPs exist in the human genome, TIMP-1, -2, -3, and -4, which are collectively able to inhibit all known MMPs, although not with the same efficacy (Bourboulia and Stetler-Stevenson, 2010). They are secreted proteins, 21 to 28 kDa in



Images adapted from (a) Mao, Z.; Heinemann, F. W.; Liehr, G.; van Eldik, R. (2001). Complex-formation reactions of Cu(II) and Zn (II) 2,2'-bipyridine and 1,10-phenanthroline complexes with bicarbonate. Identification of different carbonate coordination modes. J Chem Soc, Dalton Trans 2001(24):3652-62.; (b) Mali, S. S.; Hajare, A. A.; Karade, R. S.; Salunkhe, S. S.; Nadaf, S. J.; Honmane, S. M.; Bhatia, N. M. (2014). Expulsion by ionic complexation: benchmark therapy for atherosclerosis a review. Indian J Pharm Biol Res 2(1):103-7.; and (c) Chang, M. (2016). Restructuring of the extracellular matrix in diabetic wounds and healing: a perspective. Pharmacol Res 107:243-8.

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Figure 4. Coordination complexes of three different MMPIs with metals. (a) 1,10-Phenanthroline is an organic heterocyclic compound that sequesters metals by coordination through free electrons from its nitrogen atoms. This can be seen by the two molecules at the top coordinating with the copper ion. This example also sees coordination of the copper ion with a free carbonate molecule. (b) A single molecule of EDTA forms a complex with metal ions (M) through free electrons from its oxygen and nitrogen atoms. (c) ND-336 is initially deprotonated by a glutamate residue in the catalytic site of MMP-2, -9, or -14, opening the thiirane ring. The sulfur from the resulting thiolate then coordinates with the nearby zinc ion in the active site.

molecular weight with 0 to 2 sites for glycosylation (Baker *et al*., 2002; Stetler-Stevenson, 2008). TIMPs are crucial for modulating the balance between over- and underexpression of MMPs for proper homeostasis. TIMP-2 is unique in that it functions as both an inhibitor and activator: the N-terminal domain of extracellular TIMP-2 binds to the catalytic site of MT1-MMP (i.e. MMP-14), while the C-terminal domain recruits proMMP-2 by binding to the C-terminus of the proenzyme; a second nearby MT1-MMP that is not inhibited by TIMP-2 activates MMP-2 by proteolytic cleavage (Jones *et al.*, 2003; Overall *et al*., 2000; Strongin *et al*., 1993). Other proteins are capable of inhibiting MMPs, such as α2-macroglobulin and RECK (**re**version-inducing-**c**ysteine-rich protein with **K**azal motifs) (Baker, 2002; Takahashi *et al*., 1998). Human α2-macroglobulin is a 772 kDa plasma glycoprotein composed of four nearly identical subunits, and is expressed largely in hepatocytes and, to a lesser extent, other cell types, including macrophages (Baker, 2002; Nezu *et al*., 2013). Among its other functions, α2-macroglobulin sequesters MMPs and other endopeptidases by forming a covalent bond via transacylation (Nagase, 1997). RECK is a 110 kDa GPI-anchored glycoprotein, widely expressed in human tissues (with the exception of tumor cells), and contains protease inhibitor-like domains. In addition to inhibiting the enzymatic activity of MMP-9, RECK also downregulates expression of MMP-9 at an unknown posttranscriptional level (Takahashi *et al*., 1998).

MMPs have been shown to play a critical role in the invasion of the CNS by various pathogenic protozoa, including *Plasmodium falciparum*, *Trypanosoma brucei*, *Trypanosoma cruzi*, and *Toxoplasma gondii* (Bruschi and Pinto, 2013; Geurts *et al*., 2012; Matin *et al*., 2006). The balance between MMPs and their inhibitors, both MMPIs

and TIMPs, have been studied in experimental animal models to determine whether inhibition of MMPs ameliorated the effects of infection by these protozoa. For example, tetracyclines and their derivatives are able to inhibit MMPs by binding to zinc; thus, minocycline has been shown to reduce MMP expression by *T. brucei* and increase survival in *T. brucei*-infected mice (Masocha *et al*., 2006; Paeman, *et al.*, 1996; Stechmiller *et al*., 2010). Excessive inhibition of MMPs, however, should not be considered as therapeutic treatment for protozoan infections and other diseases. A loss of MMP-12 diminishes macrophage migration, MMP-7 plays a role in the chemoattraction of certain inflammatory cells, MMP-8 is often associated with the migration of neutrophils, and MMP-3 and -9 participate in the occurrence and resolution of T cell-dependent delayed type hypersensitivity responses (Giambernardi *et al*., 2001; Li *et al*., 2002; Shipley *et al*., 1996; Wang *et al*., 1999). Additionally, Clark *et al*. (2010) found that TIMP-1-knockout mice infected with *T. gondii* had a reduced parasite burden and a simultaneous increase of CD4+ T cells in the CNS due to the absence of the inhibitor, allowing for better tissue penetration during lymphocyte trafficking into the brain.

VIII. Research Objectives

In many cancers and infections by both pathogens and parasites, MMP-2, -9, and -14 are often found to be largely responsible for their metastatic and invasive capabilities (Bruschi and Pinto, 2007; Chiu and Lai, 2013; Deryugina and Quigley, 2006; Forsyth *et al*., 1999; Rosenberg, 2015). Thus, the objective of the present study is to determine whether MMP-2, -9, and -14 are produced by *N. fowleri*, and, if so, whether

the highly virulent mouse-passaged amoebae show higher expression of MMPs than the weakly virulent axenically grown amoebae. Next, the expression of MMPs in the amoebae should be functionally linked to the increased motility and invasion of amoebae. Higher expression of MMPs in the mouse-passaged amoebae and the role of MMPs in the invasion process would suggest that MMPs serve as a potential virulence factor for *N. fowleri*, and thus may be a target to inhibit the early invasion process.

Chapter 2: Materials and Methods

Amoebae. *N. fowleri* (ATCC 30894) was isolated from a fatal case of PAM that occurred in a 15-year old human female in Richmond, Virginia. Amoebae were grown and maintained axenically in T75 culture flasks at 37°C in Oxoid medium consisting of 0.55% liver digest, 0.30% glucose, 0.50% proteose peptone, 0.25% yeast extract, 1% calf serum, and 1 µg hemin/mL in Page amoeba saline (Band and Balamuth, 1974; Cline *et al.* 1983). Amoebae were passaged in $B_6C_3F_1$ mice by intranasal inoculation every 4 weeks to maintain virulence. Such mouse-passaged (MP) amoebae have been shown to be more virulent than axenically (Ax) cultured amoebae and exhibit greater motility and invasive capability (John and Howard, 1993). Care of animals was in compliance with the standards of the National Institutes of Health and the Institutional Animal Care and Use Committee at Virginia Commonwealth University.

Amoeba whole cell lysates. Mouse-passaged and axenically cultured amoebae grown (24 h) in Oxoid medium were detached from flasks through mechanical bumping, washed three times in 0.01 M phosphate-buffered saline (PBS) pH 7.4, resuspended in PBS, and subjected to three cycles of freezing in liquid nitrogen and thawing in a 37°C water bath.

Membrane isolation. Membrane and cytosolic proteins were isolated using the Mem-PER eukaryotic membrane protein extraction kit (Thermo Scientific, Rockford, IL), according to the manufacturer's instructions. MP and Ax amoebae grown (24 h) in Oxoid medium were detached from flasks and washed three times in PBS. The pellet (5 x 10^6 cells) was washed in a Cell Wash Solution and centrifuged (300 x g, 5 min). Following an additional wash and centrifugation, the pellet was incubated (10 min, 4°C) in Permeabilization Buffer with constant agitation. The permeabilized cells were centrifuged (16,000 x g, 15 min, 4^oC) and the supernatant containing the cytosolic protein that also includes peripheral membrane proteins was collected and stored at -80°C. The pellet was suspended in Solubilization Buffer and incubated (30 min, 4°C) with constant agitation. After centrifugation (16,000 x g, 15 min, 4°C), the supernatant containing the solubilized integral membrane proteins was collected and stored at -80°C.

Conditioned medium. Amoebae were washed in PBS and incubated in Dulbecco's minimal essential medium (DMEM) lacking serum (which acts as a protease inhibitor) (37°C, 6-24 h) (Vyas et al., 2015). To determine whether proteases were secreted, the medium was subjected to centrifugation (4X, 15,000 x g), and the supernatant was collected and subjected to zymography.

Invasion assay. Tissue culture inserts (8.0 µm, Greiner BioOne, Monroe, NC) were placed on ice before being coated by DMEM containing 5 µg/µL of Matrigel (Sigma, St. Louis, MO), a reconstituted basement membrane solution (Figure 5). The inserts were

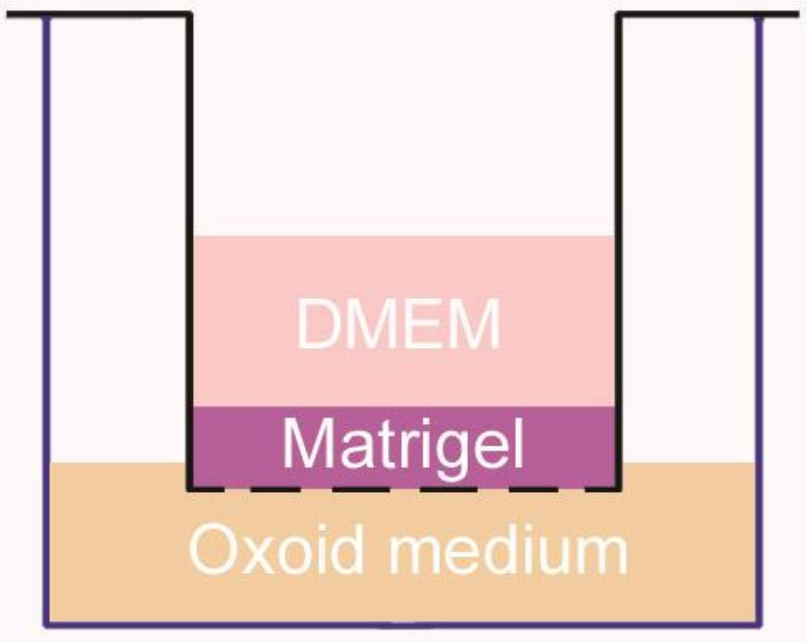


Figure 5. Design of *in vitro* invasion assay. A single well and insert is depicted, with the insert coated with Matrigel (5 µg/µL) polymerized in DMEM. Amoebae suspended in DMEM are loaded on top of the Matrigel. The bottom of the well contains a nutrient-rich Oxoid growth medium, which serves as a chemoattractant for the amoebae. This emulates the *in vivo* invasion process of amoebae in that trophozoites are in a relatively nutrient-poor condition in the nasal passages, but chemotactic factors, presumably from neurotrophic cells, draws amoebae through the ECM to the nutrient-rich environment in the CNS.

placed in either 6- or 12-well plates and incubated for 30 min at room temperature (RT). Mouse-passaged *N. fowleri* trophozoites (5 x 10^6 per 150 mm^2) were suspended in DMEM and placed onto the inserts. Oxoid medium (serum free) was placed in the well beneath the inserts to serve as a chemoattractant. The plates were incubated (37°C, 5% CO_2) for 6-24 h, at which time the media were collected separately from the upper and lower chambers. The media were centrifuged twice (15,000 x g, 15 min), and the respective supernatants were collected. For invasion assays performed under the treatment of inhibitory drug, the amoebae were prepared accordingly. Prior to placing amoebae into the inserts, the trophozoites were pretreated with 1,10-phenanthroline, EDTA, or ND-336 dissolved in DMSO for 1 hour (37°C, 5% CO_2). Trophozoites then were loaded into inserts suspended in the DMEM containing the inhibitory drug. For these inhibition assays, 1 x 10^6 cells in DMEM were used per 24-well insert.

Western immunoblotting. Trophozoite protein was subjected to electrophoresis under reducing conditions in a 10% SDS-polyacrylamide gel. The samples were prepared by mixing three parts of the sample protein with one part of 4x Laemmli loading buffer (240 mM Tris-HCl (pH 6.8), 8% SDS, 40% glycerol, 5% β-mercaptoethanol, 0.04% bromophenol blue) and boiled for 5 min. Following electrophoresis, the sample protein was transferred onto a nitrocellulose membrane, which was then placed in blocking buffer consisting of non-fat dry milk (5% (w/v) in Tris-buffered saline with 0.1% Tween 20 (TBST)) for 1 h. The nitrocellulose membrane then was incubated overnight (4°C) in 5 mL of the blocking buffer containing 10 µL of rabbit polyclonal antibody directed against MMP-2 (Cell Signaling Technology, Danvers, MA), MMP-9 (Cell Signaling

Technology, Danvers, MA), or MMP-14 (Thermo Scientific, Rockford, IL). The membrane then was washed six times (5 min each) in TBST, followed by a 1 h incubation of 10 mL of blocking buffer containing secondary HRP-conjugated goat anti-rabbit polyclonal antibody (Thermo Scientific, Rockford, IL). Following an additional six washes of five minutes each, protein bands were detected on film using the Amersham ECL Prime Western Blotting Detection Reagent kit (GE Healthcare, Little Chalfont, UK), according to the manufacturer's instruction.

Gel zymography. Enzyme activity was assessed by gel zymography. Amoebic protein was subjected to electrophoresis under nonreducing conditions in a 10% SDS-polyacrylamide gel containing 1% gelatin. The samples were prepared by mixing three parts of the sample protein with one part of 4x nonreducing Laemmli loading buffer (240 mM Tris-HCl (pH 6.8), 8% SDS, 40% glycerol, 0.04% bromophenol blue). Following electrophoresis, the gel was incubated with gentle agitation (15 min, RT) in enzyme renaturing buffer (200 mM NaCl, 5 mM $CaCl_2$, 2.5% (v/v) Triton X-100, 0.02% (w/v) NaN_3, 50 mM Tris-Cl, pH 7.5), refreshing with new buffer for a total of four times. The gel then was incubated overnight in developing buffer (200 mM NaCl, 5 mM $CaCl_2$, 0.02% (w/v) NaN_3, 50 mM Tris-Cl, pH 7.5) at 37 °C. Following incubation, the buffer was replaced with Coomassie R-250 for 1 h. Clear bands were visualized after several washes in destain solution (7% acetic acid, 5% ethanol).

Lactate dehydrogenase (LDH) cytotoxicity assay. The Pierce LDH Cytotoxicity Assay Kit (Thermo Scientific, Rockford, IL) was used according to the manufacturer's

instruction to verify that concentrations of drugs used were not cytotoxic to the amoebae. Amoebae grown in Oxoid medium were detached from flasks and washed in 50/50 medium (50% Oxoid, 50% DMEM). Cells (1×10^5) in 100 µL of medium were added to a 96-well plate in triplicate, along with triplicate wells of sterile water, vehicle control, and medium (with no amoebae), which was left to incubate overnight (37°C, 5% CO_2). To one set of the triplicate wells, 10 µL of Lysis Buffer (10x) was added, and the plate was incubated for an additional 45 min (37°C, 5% CO_2). The LDH Positive Control was prepared and 50 µL was transferred in triplicate to a new 96-well plate, in addition to 50 µL of each medium sample, followed by the addition of 50 µL of previously prepared Reaction Mixture. The plate was incubated at room temperature for 30 min, protected from light. Following incubation, 50 µL of Stop Solution was added to each sample well, the absorbance was measured at 490 nm and 680 nm.

Protease inhibitors. Protease inhibitors were not used during gel zymography in order to allow for detection of enzyme activity. 1,10-Phenanthroline was obtained from Sigma (St. Louis, MO), ethylenediaminetetraacetic acid (EDTA) was obtained from Fisher Scientific (Hampton, NH), and ND-336, (4-[4-[(2-thiiranylmethyl)sulfonyl]phenoxy]-benzenemethanamine, monohydrochloride),was obtained from Cayman Chemical Company (Ann Arbor, MI).

Gel and statistical analysis. Zymograms and western immunoblots were scanned using Adobe Photoshop software (Adobe Systems, San Jose, CA). Data were

expressed as mean±SD of the mean. To determine whether results were statistically significant ($p<0.05$), a two-tailed, unpaired Student's *t*-test was used.

Chapter 3: Results

I. Detection of matrix metalloproteinases in *N. fowleri*

The first goal of this study was to determine whether MMPs are produced by *N. fowleri*, and if the highly virulent MP amoebae produced them in higher quantities than the weakly virulent Ax amoebae. Doing so would suggest that MMPs are a virulence factor that contributes to the pathogenicity of *N. fowleri*. Initial gelatin zymograms prompted further inspection of gelatinases produced by *N. fowleri*. Gelatinolytic activity between 100 and 150 kDa, and of high molecular weight extending beyond 250 kDa could be detected in conditioned media (CM) collected after 6 hours incubation in both MP and Ax amoebae (Figure 6). Following 18 hours incubation, while CM exhibited a drastic increase in gelatinolytic activity in both subpopulations, the increase from 6 to 18 hours was much more prominent in the highly virulent MP amoebae when compared to the weakly virulent Ax amoebae.

In order to determine whether MMP-2, -9, or -14 were responsible for the proteolytic activity in the gelatin zymography, Western immunoblotting was performed on whole cell lysates (WCL). Probing with polyclonal rabbit antibodies made to react against the proteins of interest detected the presence of MMP-2, -9, and -14 in WCL of *N. fowleri* trophozoites. Multiple bands appeared between 37 and 75 kDa for MMP-2 for both MP and Ax cultures, with the most prominent band at approximately 72 kDa (Figure 7a). Anti-MMP-9 antibody detected a band at approximately 48 kDa in each

lysate sample, with an additional doublet of lower molecular weight in the MP culture, but a single band of approximately 35 kDa in the Ax culture (Figure 7b). When probed with anti-MMP-14 antibody, a prominent band appeared of approximately 80 kDa in the MP lysate (Figure 7c). A faint band of the same molecular weight could be seen in the Ax lysate sample. Additionally, a faint band appeared between 100 and 150 kDa in each sample. In each immunoblot, more prominent bands appeared from the MP samples than in the Ax lysates. Densitometry analysis of the bands showed a 1.2x, 2.4x, and 3.6x greater expression respectively for MMP-2, -9, and -14 in the MP culture compared to the Ax culture.

The compartmentalization of these MMPs was also investigated by subjecting the membrane and cytosolic fractions to Western immunoblotting. MMP-2 seems to be confined in the membrane fraction of *N. fowleri* trophozoites, with higher protein expression in the mouse-passaged amoebae (Figure 8a). Densitometry analysis of the bands showed a 1.7x greater expression in the MP sample. Conversely, MMP-9 was only detected in the cytosolic fraction (Figure 8b). Anti-MMP-14 antibody detected multiple bands in the membrane fraction; the bands in the mouse-passaged sample were more prominent than in the Ax sample (Figure 8c). Immunoreactive bands similar to WCL could be seen in the cytosolic fraction: a pair of bands at approximately 80 kDa and between 100 and 150 kDa, with more prominent bands in the MP lysate.

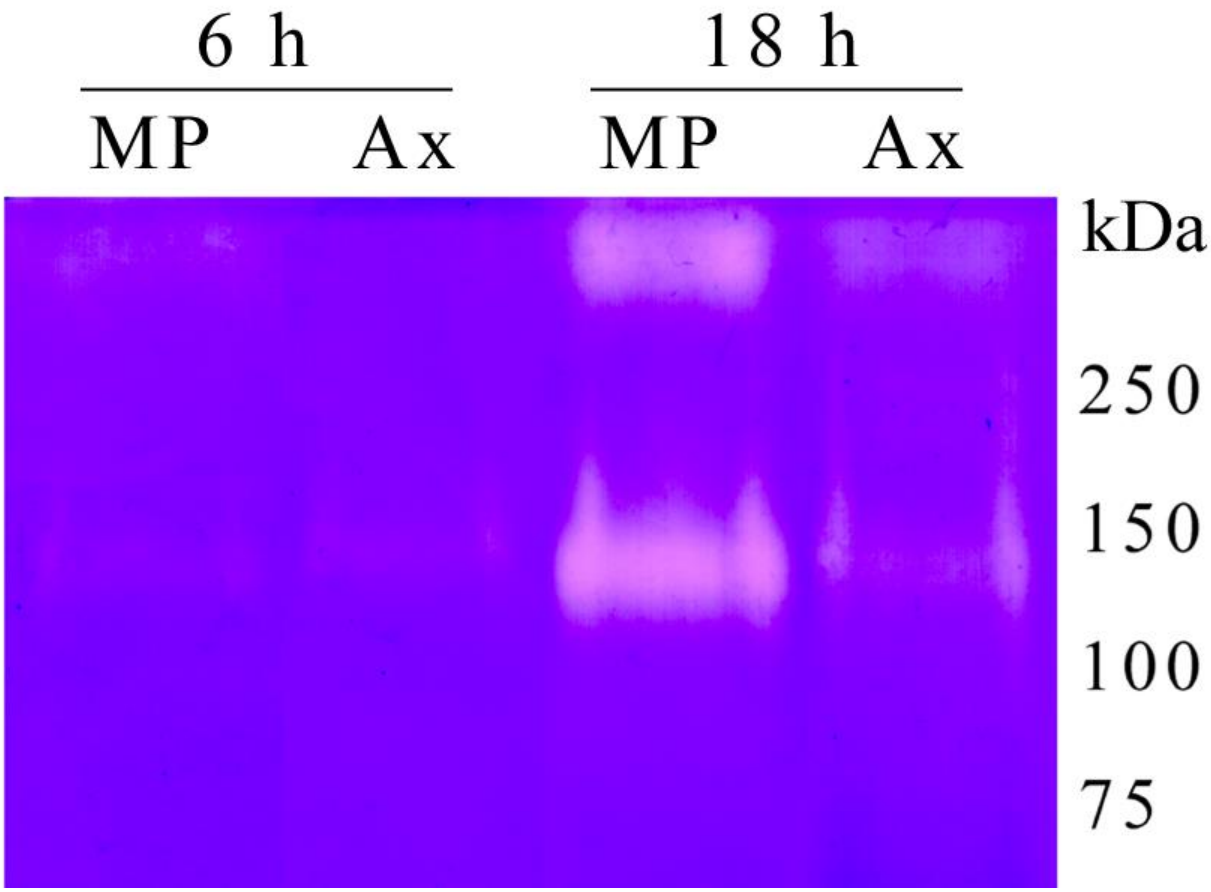


Figure 6. Gelatin zymogram of conditioned media (CM). Gelatinase activity depicted in CM for the highly virulent mouse-passaged (MP) and weakly virulent axenically grown (Ax) amoebae. Conditioned media were collected after 6 and 18 h incubation.

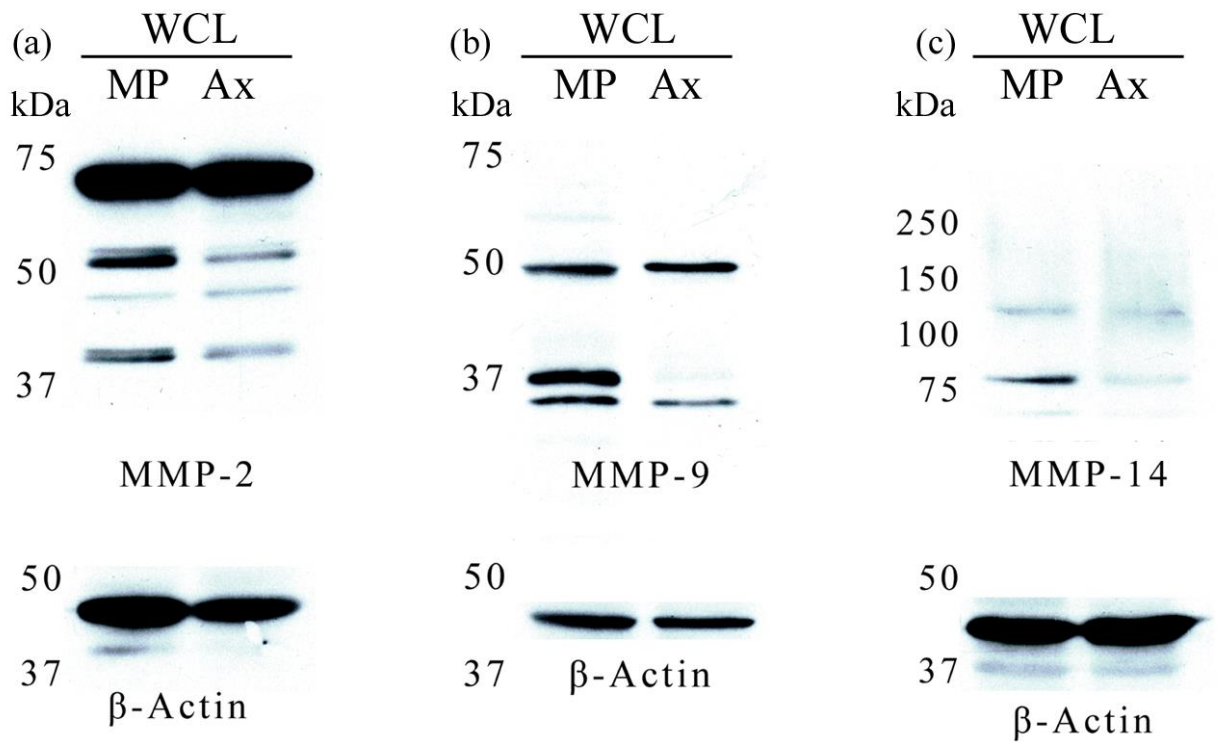


Figure 7. Western immunoblot detecting MMP-2, -9, and -14 in WCL. Whole cell lysates made from *N. fowleri* trophozoites were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated in polyclonal rabbit anti-MMP-2 (a), anti-MMP-9 (b), and anti-MMP-14 (c) antibodies. (a) Prominent bands of approximately 72 kDa were detected in WCL and additional bands at lower relative molecular weights were detected in both lysates. (b) Immunoreactive bands of approximately 48 kDa were detected in WCL, with additional bands detected at approximately 37 kDa. (c) A prominent band of approximately 80 kDa was detected in the MP sample, while a fainter band of the same molecular weight appeared in the Ax culture. Faint bands between 100 and 150 kDa were detected in both samples.

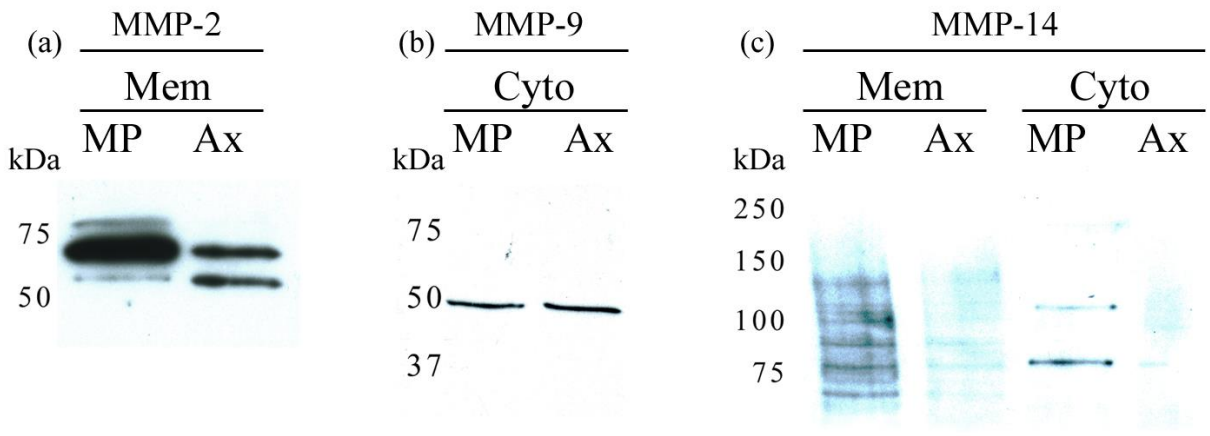


Figure 8. Western immunoblot detecting compartmentalization of MMPs. Due to using the same number of cells for the membrane protein extraction kit, equal volume was used to load each lane. (a) MMP-2 immunoreactivity was shown to be confined to the membrane fraction (Mem) and is predominant in MP amoebae. Immunoreactivity was not detected in the cytosolic fraction (Cyto). (b) Immunoreactive bands for MMP-9 appeared in the cytosolic fraction of MP and Ax trophozoites, but were not detected in the membrane fraction. (c) When probed for MMP-14, multiple bands appeared in the membrane fraction spanning from approximately 45 to 150 kDa. In the cytosolic fraction, the bands between 75 and 150 kDa are more prominent in the MP lysates than in the Ax.

II. Functional relevance of MMP expression in invasion and motility

Once *N. fowleri* was found to express MMPs, the second goal of the present study was to link this expression to the functionality of invasion and motility. This was accomplished by performing *in vitro* invasion assays with and without Matrigel, a stimulus for MMP expression, as well as in the presence or absence of metal chelators and specific inhibitors. Supernatant media collected from *in vitro* invasion assays were initially analyzed by gelatin zymography. Media were collected from the top (T) and bottom (B) chambers of invasion assays in the presence (+) or absence (-) of Matrigel after 6 and 18 hours incubation. Pairs of bands of approximately 75 kDa could be seen in samples collected from both the top and bottom chambers at each timepoint (Figure 9). A band is also apparent in the T^{+}_{18} lane between 100 and 150 kDa. Gelatin zymography on samples collected from *in vitro* invasion assays in the absence of Matrigel yielded no detectable bands (data not shown). Additionally, zymography performed on Matrigel as a sample yielded no detectable bands, indicating that the Matrigel itself does not contain gelatinases.

In an effort to investigate whether the bands seen in the gelatin zymogram of invasion assay samples were caused by the MMPs of interest, invasion assays were performed using various MMPIs. Initially, *N. fowleri* trophozoites were pretreated with a novel synthetic inhibitor, ND-336, at varying concentrations up to 1000 nM. Gelatin zymography revealed bands similar to those described above: a pair of bands could be seen in each lane at approximately 75 kDa, with additional bands between 100 and 150 kDa and beyond 250 kDa in each T^{+}_{18} lane (Figure 10). There was no apparent difference in gelatinolytic activity among the samples incubated with different

concentrations of the inhibitory drug. As a result, additional SDS-polyacrylamide gels were poured for gelatin zymography, one of which was copolymerized with the inhibitory drug throughout the gel such that during overnight incubation, the inhibitory drug in the gel would inhibit enzymatic activity. Follow zymography, the gel without ND-336 displayed similar bands as previously described (Figure 11a), while the gel containing the inhibitory drug showed only the bands between 100 and 150 kDa and an additional faint band beyond 250 kDa (Figure 11b). The pairs of bands around the 75 kDa region were no longer apparent.

An additional invasion assay was performed with the metal chelators, 1,10-phenanthroline and EDTA. The presence of 1,10-phenanthroline inhibited gelatinolytic activity such that the bands above 100 kDa were no longer apparent, while the vehicle control (DMSO) displayed the high molecular weight bands (Figure 12). EDTA also appears to have inhibited some protease activity when compared to the vehicle control (water), but its reduction in proteolytic activity was not as drastic as that of 1,10-phenanthroline. Light microscopy of the amoebae in the presence of EDTA did not show cell death (data not shown).

Western immunoblotting was performed on these media to determine whether MMP-2, -9, and -14 were responsible for the bands in the zymogram. Media were collected from invasion assays after 6, 18, and 24 hours for analysis. Probing with anti-MMP-2 antibody revealed a single band in each lane loaded with media collected from the top chambers ($T^{+}_{6\text{-}24}$) (Figure 13a). Additionally, higher protein expression could be seen for the later incubation periods. Faint bands could also be seen in the B^{+}_{18} and B^{+}_{24} lanes. Anti-MMP-9 antibody detected single prominent bands at approximately 48

kDa in each of the lanes loaded with media collected from the top chambers ($T^{+}_{6\text{-}24}$) (Figure 13b). Faint bands also appeared at higher molecular weights for T^{+}_{18} and T^{+}_{24}. Probing with anti-MMP-14 antibody revealed a single band in each lane (Figure 13c). Media collected from the top and bottom chambers at 6 and 24 hours allowed detection of an immunoreactive band at approximately 75 kDa. The most prominent band is in the T^{+}_{6} lane.

The presence of secreted MMPs stimulated by Matrigel can be linked to the motility and invasion of *N. fowleri* by inhibiting the MMPs and seeing a decrease in invasive capability. Enzyme inhibition studies were performed using 1,10-phenanthroline, an inhibitor of MMPs, and light microscopy was used to compare its effect with untreated and vehicle controls. Invasion assays were performed with *N. fowleri* trophozoites pretreated with 10 mM 1,10-phenanthroline, which was confirmed by lactate dehydrogenase cytotoxicity assay not to be cytotoxic to the trophozoites (data not shown). For the untreated and vehicle controls, microscopy revealed a much greater number of trophozoites in the bottom chambers of invasion assays after 18 hours incubation compared to 6 hours (Figure 14). There was no significant difference in invasive capability between the untreated and vehicle controls. In the drug-treated samples, following 6 hours incubation, relatively few amoebae could be seen in the bottom chambers of the invasion assay. After 18 hours incubation, invasion through the Matrigel-coated insert was still significantly inhibited by 1,10-phenanthroline.

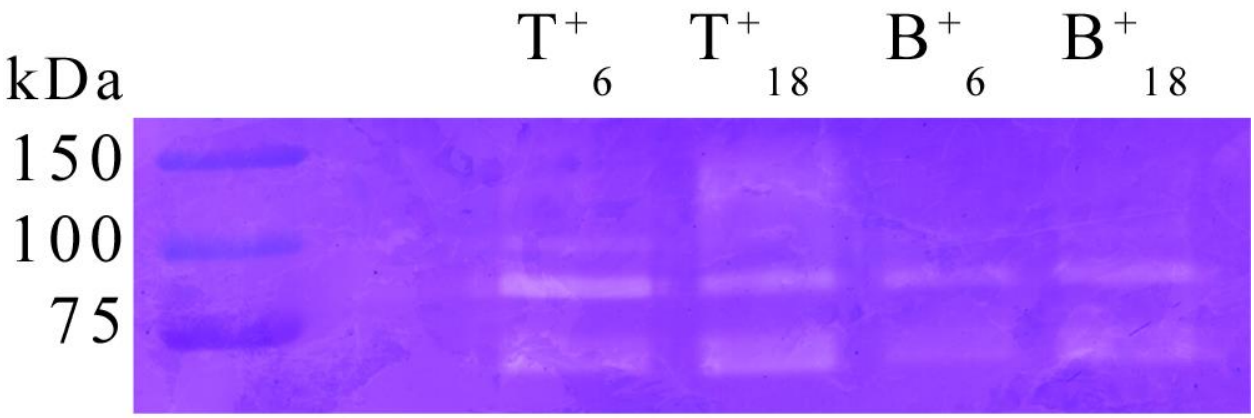


Figure 9. Gelatin zymogram of samples collected from invasion assay. Media was collected from top/upper chambers (“T”) and bottom chambers (“B”), where the inserts were coated with Matrigel (“+”) at 6 h and 18 h. Media collected from invasion assays in the absence of Matrigel yielded no detectable protease activity.

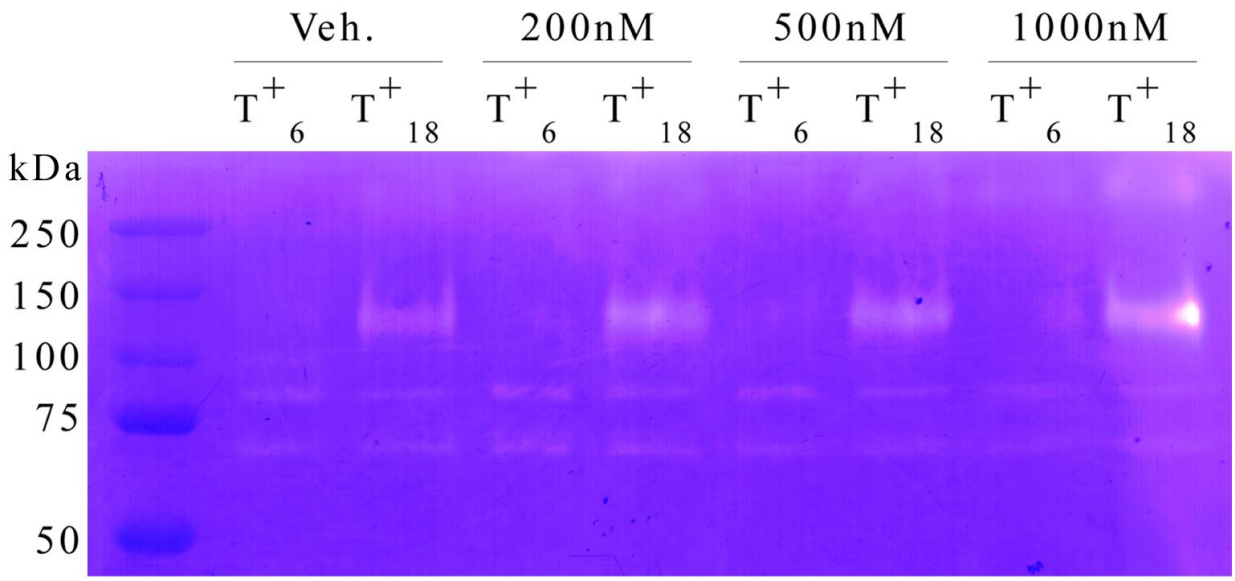
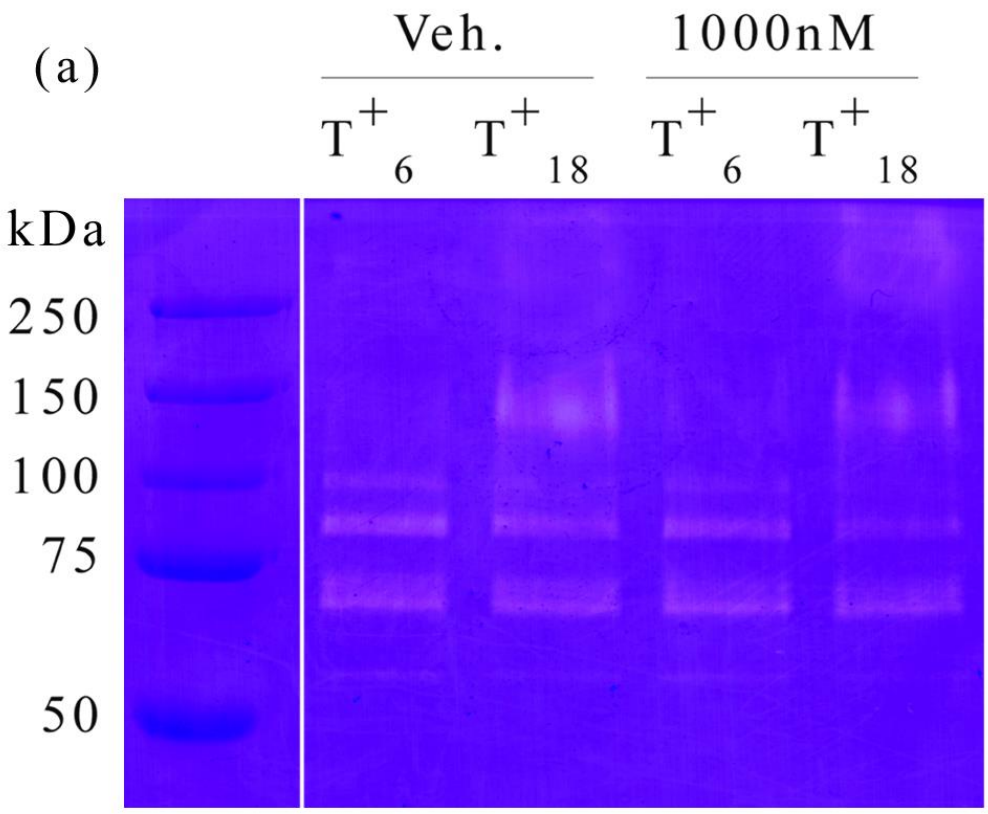


Figure 10. Gelatin zymogram of invasion assay samples with varying concentrations of ND-336. MP amoebae were treated with varying concentrations of ND-336 up to 1000 nM and vehicle control (DMSO) for invasion assays. All samples were collected from the top chamber (T) coated with Matrigel (+), collected after 6 and 18 h.


(a)
Veh.
1000nM
T+6
T+18
T+6
T+18
kDa
250
150
100
75
50

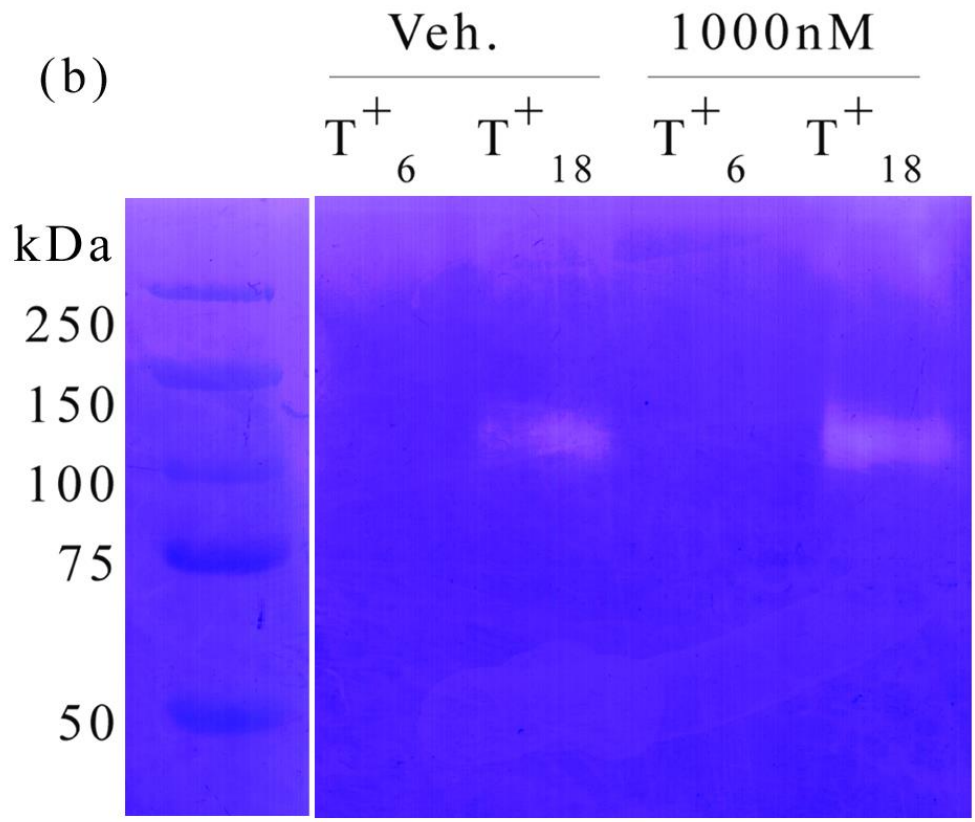

(b)
Veh.
1000nM
T+6
T+18
T+6
T+18
kDa
250
150
100
75
50

Figure 11. Gelatin zymograms on the effect of ND-336. MP amoebae were treated with varying concentrations of ND-336 up to 1000 nM and vehicle control (DMSO) for invasion assays. All samples were collected from the top chamber (T) coated with Matrigel (+), collected after 6 and 18 h. (a) SDS-polyacrylamide gel containing 1% gelatin, with samples collected from an invasion assay. (b) Identical samples used in a second SDS-polyacrylamide gel containing 1% gelatin with the addition of ND-336 copolymerized in the gel.

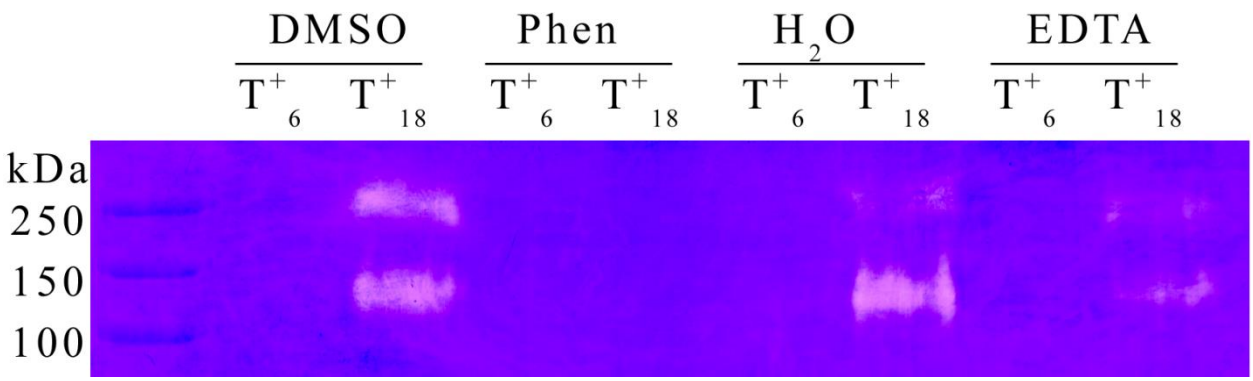


Figure 12. Effect of metal chelators on media collected from invasion assays. *N. fowleri* trophozoites were treated with 1,10-phenanthroline (Phen) and ethylenediaminetetraacetic acid (EDTA) and their respective vehicles, DMSO and water, prior to performing an invasion assay in the presence of matrigel (+). Media were collected from the top chambers (T) after 6 and 18 hours incubation.

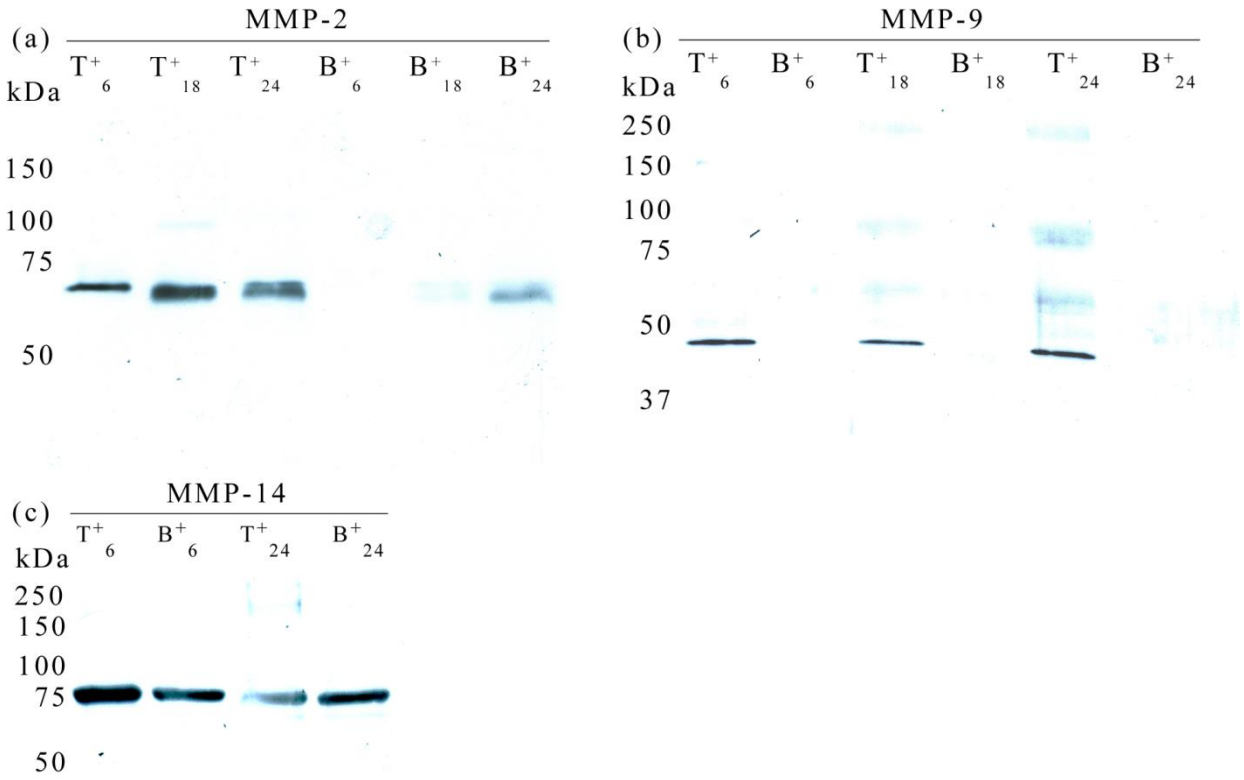
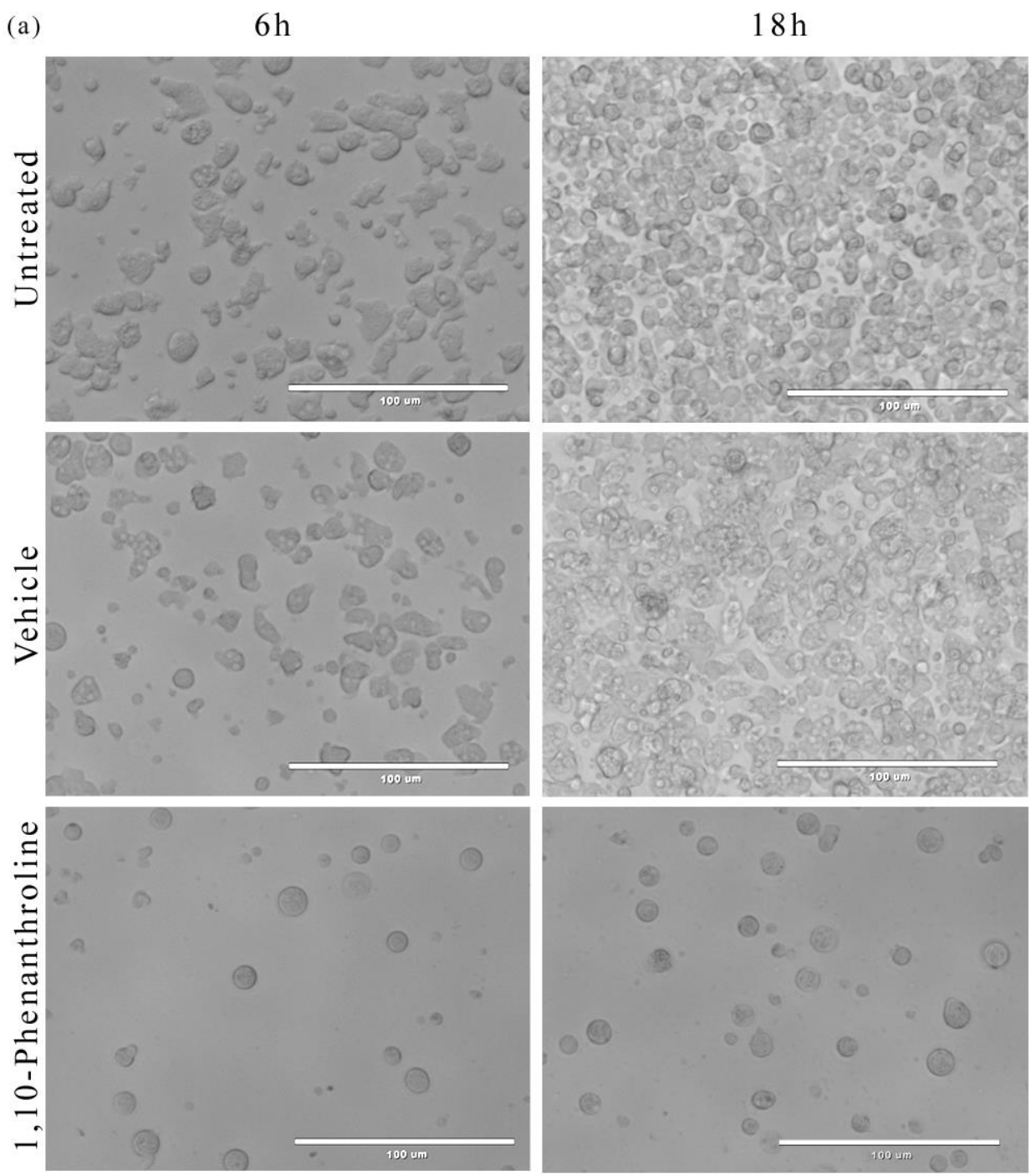


Figure 13. Western immunoblot of media collected from invasion assays. Secreted protein was separated by 10% SDS-PAGE and transferred to nitrocellulose membranes, which were incubated with polyclonal rabbit anti-MMP-2 antibody (a), anti-MMP-9 (b), and anti-MMP-14 (c), followed by HRP-conjugated goat anti-rabbit antibody. Media were collected from the top (T) and bottom (B) chambers; the numerical subscripts designate the time at which medium was recovered; + indicates that Matrigel was used as protein substrate.


(a)
6h
18h
Untreated
Vehicle
1,10-Phenanthroline
100 um
100 um
100 um
100 um
100 um
100 um

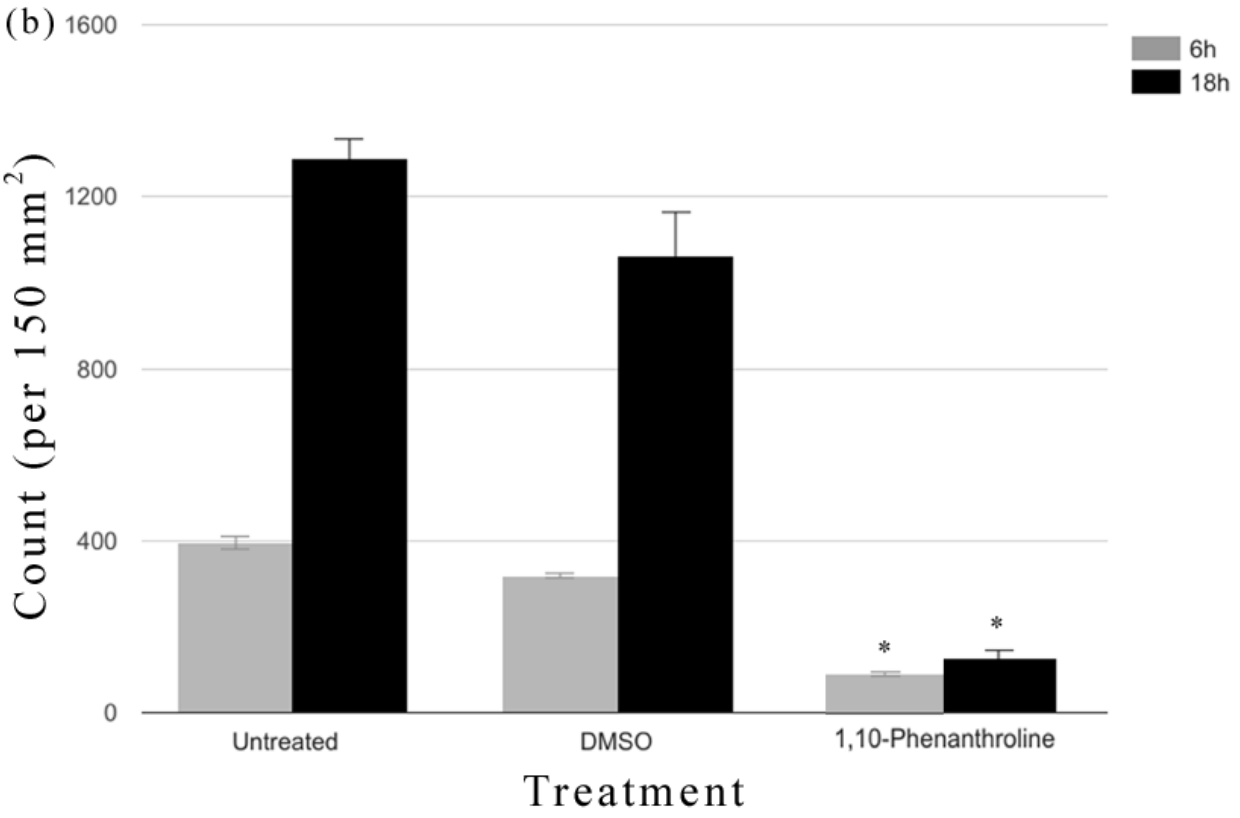


Figure 14. Effect of 1,10-phenanthroline on invasive capability of *Naegleria fowleri*. (a) Representative light microscopy images of the bottom chambers of invasion assays. Untreated control, vehicle control (DMSO), and 1,10-phenanthroline samples were incubated for 6 and 18 h. Scale bars are 100 µm. (b) Triplicate wells were counted to determine the invasive capability of *N. fowleri* trophozoites. Continuous variables were compared using a two-tailed, unpaired Student *t*-test with significance set at $p < 0.05$.

Chapter 4: Discussion

Matrix metalloproteinases have been found in pathogenic protozoa and implicated in degrading a wide range of connective tissue proteins primarily for the purpose of invasion through the extracellular matrix. Metalloproteinases are often found in abundance in infections of *Plasmodium falciparum* and *Trypanosoma brucei*, which rely on the expression of these enzymes to gain access to the CNS (Bruschi and Pinto, 2013; Masocha *et al*., 2006). Infection and dissemination of the obligate intracellular pathogen *Toxoplasma gondii* induces the production of human MMP-2, -9, and -14 in infected cells in order to pass through the ECM and other biological barriers, such as the intestinal epithelium and the placenta (Buache *et al*., 2007; Geurts *et al*., 2012; Seipel *et al*., 2010). Similarly, *Entamoeba histolytica* relies on exploiting human MMPs to degrade the ECM and invade the mucosa (Thibeaux *et al*., 2014). The apparently critical role of MMPs in the invasion and motility of other pathogenic protozoa provokes the thought that *N. fowleri* might also express these enzymes for invasion. Also, knowing that amoebae that have been passaged through mice are more virulent than amoebae that are grown axenically in media, it is expected that the mouse-passaged *N. fowleri* would express MMPs at higher levels. This phenomenon suggests that host components serve to induce differential expression and activation of virulence factors

and proteolytic enzymes by the amoebae, allowing them to penetrate the ECM and invade tissue more readily than Ax *N. fowleri*.

Cysteine proteases of approximate molecular weights 30, 58, 128, and 170 kDa have previously been found in *N. fowleri* through gelatin zymography (Aldape, 1994, Mat Amin, 2004; Vyas *et al*., 2014). Thus, in the present study, in order to limit gelatin zymography to exhibit bands linked to MMPs, SDS-polyacrylamide gels were incubated under constricted conditions at which MMPs have the highest activity: pH 7.5, 37°C, 5 µM $ZnCl_2$, 5 mM $CaCl_2$, 200 mM NaCl (Matin *et al*., 2005; Troeberg and Nagase, 2003). These conditions represent the most favorable conditions for the activity of MMPs and are in contrast to those that favor optimal activity for cysteine proteases that exhibit maximum activity at pH 5 (Vyas *et al*., 2014). Initial gelatin zymography of conditioned media yielded bands of high molecular weights, between 100 and 150 kDa, and above 250 kDa. The pairs of bands in the 75 kDa region that could be seen in the zymograms of invasion assay samples were not visible in the zymogram of conditioned media, indicating that the enzymes responsible for that proteolytic activity require a protein substrate, such as Matrigel, to induce production. Furthermore, supernatant media collected from invasion assays performed without Matrigel yielded no detectable bands in gelatin zymograms. Indeed, expression of MMP-2 and -14 have been shown to be upregulated in mammalian cells when in contact with collagen (Ellerbroek *et al*., 2001; Gilles *et al*., 1997). An increase in protease activity could be seen in the conditioned media following a longer incubation time. This increase in activity is especially drastic in the mouse-passaged amoeba culture, suggesting that the proteases that are responsible for this activity may be considered to be virulence factors. These high

molecular weight bands could also be seen in the zymograms of invasion assay samples. Initially, one of these bands, between 100 and 150 kDa, may be caused by the 128 kDa cysteine protease described by Mat Amin (2004) even though the conditions for incubation in this study were optimized for MMPs. Indeed, these high molecular bands were not inhibited by the specific inhibitor, ND-336, suggesting that they were not caused by MMP-2, -9, or -14. They were, however, no longer apparent under treatment of 1,10-phenanthroline and a reduction in protease activity could be seen with EDTA treatment, indicating that this proteolytic activity is most likely not caused by the 128 kDa cysteine protease but could be a result of either other MMPs or other proteases that require metals for their catalytic function.

Gelatin zymograms of samples collected from invasion assays displayed a pair of bands in the 75 kDa region that were no longer apparent in the presence of ND-336. Considering this specific inhibition of protease activity in addition to the consistency of finding immunoreactive bands of approximately 72 and 80 kDa by Western immunoblotting for MMP-2 and -14, respectively, these bands in the gelatin zymograms were most likely caused by the activity of MMP-2 and -14. It is less likely that bands of both molecular weights are caused by the activity of MMP-2. Multiple bands for a single enzyme may be detected as zymography is able to detect activity from both the inactive proMMP and the active cleaved MMP, but it is more likely that activated MMPs are secreted for the invasive process rather than proMMPs (Staun-Ram *et al*., 2004; Toth *et al*., 2012). Notably, the expected band of approximately 48 kDa corresponding to MMP-9 is not apparent by zymography. Consistent findings of bands at approximately 48 kDa as reported by anti-MMP-9 antibody suggests that although the structure of *N. fowleri*

MMP-9 is markedly different from the 92 kDa human MMP-9, it contains enough sequence homology to be recognized by the polyclonal antibody made to react against human epitopes. Thus, although a 48 kDa band is not apparent by zymography, one at approximately 100 kDa can be seen in zymograms of invasion assay samples. Again, this band is inhibited by ND-336, suggesting that this band may be caused by a homodimer of MMP-9, which is reportedly a unique characteristic of MMP-9 in humans (Dufour *et al*., 2010). Two monomers are tethered together intracellularly by a disulfide bond at unknown cysteine residues that is broken under reducing conditions (Olson *et al*., 2000). Thus, Western immunoblotting detected only the monomeric form of MMP-9, while under nonreducing conditions, zymography detected the dimeric form.

Western immunoblotting of membrane and cytosolic fractions detected compartmentalization of MMPs in *N. fowleri*. The reagents of the Mem-PER eukaryotic membrane protein extraction kit contain a solution of Triton X-114, which partitions proteins based on their hydrophobicity such that proteins with one or more spans of hydrophobic amino acid residues (i.e. integral membrane proteins) are solubilized by this detergent, separating them from hydrophilic proteins (i.e. cytosolic and peripheral membrane proteins) through phase partitioning with less than 10% contamination between fractions (Qoronfleh *et al*., 2003). Membrane protein extraction of lysates of *N. fowleri* trophozoites revealed that MMP-2 is localized in the cell membrane, while MMP-9 is found intracellularly. This localization is to be expected since gelatinases, being hydrophilic in nature, are more likely to be found in the cytosolic fraction, with the exception of MMP-2, a known integral membrane protein (Toth *et al*., 2012). Additionally, the locale of these gelatinases may correspond with their regulation. MMP-

2 is primarily regulated by proteolytic cleavage of its propeptide domain, performed largely by MMP-14, while MMP-9 is regulated at the level of transcription, as are most other MMPs (Ye, 2000). Thus, proMMP-2 may be exported to the cell surface prior to activation and secretion, while proMMP-9 is produced and sequestered intracellularly, only to be activated under stimulus, leading to either direct secretion or noncovalent tethering to the cell surface by complexing with other proteins. In mammalian cells, MMP-9 is not an integral membrane enzyme but may be expressed on the cell surface by associating with CD44 (Yu and Stamenkovic, 1999, 2000). If *N. fowleri* expresses MMP-9 on the cell surface in a similar fashion, membrane protein extraction as performed by the Mem-PER eukaryotic membrane protein extraction kit would not be able to detect it to be present on the cell surface.

Anti-MMP-14 antibody unexpectedly detected multiple bands in both the membrane and cytosolic fractions, as MMP-14 is characterized as a membrane-type metalloproteinase. Contamination of the fractions is unlikely as immunoreactive bands were not apparent in the cytosolic fraction for MMP-2 or in the membrane fraction for MMP-9. Due to the basis of hydrophobicity for phase partitioning in the membrane protein extraction, an abundance of hydrophilic residues or hydrophilic posttranslational modifications (e.g. glycosylation) may cause integral membrane proteins to be found in the fraction thought to contain only cytosolic and peripheral membrane proteins. Known integral membrane proteins, such as acetylcholine receptors and flotillin, which respectively contain two and four membrane-spanning domains, have been shown to partition solely in this hydrophilic phase or with 50% efficiency (Maher and Singer, 1985; Qoronfleh *et al*., 2003). As such, glycosylation may be responsible for the appearance

of MMP-14 in the cytosolic fraction, as mammalian MMP-14 contains four O-glycosylation sites (Boon *et al*., 2016). Alternatively, MMP-14 may indeed be found intracellularly in amoebae. Mammalian cells have shown internalization of MMP-14, a critical process for promoting cell migration (Itoh, 2006). In the membrane fraction, in addition to glycosylation, the multiple immunoreactive bands may be a result of other modifications, such as lipid interaction and posttranslational cleavage.

In order to investigate the secretion of proteases during the infection process of *N. fowleri* trophozoites, *in vitro* invasion assays were performed. The weakly virulent axenically grown amoebae were not used in invasion assays as they are less representative of the virulent environmental amoebae. Bands were not apparent following gelatin zymography for samples collected from invasion assays in the absence of Matrigel, suggesting that the basement membrane-like protein composite served as a substrate to induce production of gelatinases. Inhibition of protease activity by ND-336 in gelatin zymography of invasion assay samples (in the presence of Matrigel) suggests that MMP-2, -9, and -14 were secreted by the trophozoites. Western immunoblots confirmed the presence of these enzymes in the supernatant media collected from invasion assays. Following longer incubation periods, there appeared to be higher expression of MMP-2 in the top chambers, as the trophozoites were in physical contact with the Matrigel for a longer period of time, further stimulating its production. By 24 hours, MMP-2 was present in the bottom chamber, likely due to diffusion of the protease from the top chamber. Conversely, MMP-14 expression was highest at 6 hours and wanes by 24 hours, indicating that Matrigel induced production of MMP-14 prior to MMP-2. This earlier expression corresponds with its function of activating other

proteases, including MMP-2. On the other hand, expression of MMP-9 does not seem to change over time, suggesting that *N. fowleri* relies on the proteolytic activity of MMP-2 more than MMP-9 for invasion through Matrigel.

In order to link the presence of MMPs in the media of invasion assays to the reliance of *N. fowleri* on MMPs for *in vitro* invasion, the MMPI, 1,10-phenanthroline, was used in invasion assays. The significant decrease of amoebae that were able to traverse the Matrigel layer indicates that *N. fowleri* largely depends on the activity of MMPs for the degradation of components of the basement membrane. Although 1,10-phenanthroline is well documented to be an inhibitor of metalloproteinases and to have minimal interaction with other proteases, its function does not allow specificity to individual MMPs or even to groups of MMPs (Feder *et al*., 1971). As a result, the possibility exists that MMPs not examined in this study could be responsible for the degradation of Matrigel. In order to investigate this, ND-336 was used in an attempt to replicate the effects of 1,10-phenanthroline in *in vitro* invasion assays. However, the concentrations of ND-336 used in the assays were suspected to be inadequate in inhibiting a sufficient amount of MMP-2, -9, and -14 such that invasion through the Matrigel would be hindered.

The discovery that *N. fowleri* produces MMPs and their apparent reliance on them for invasion elicits the thought that inhibition of MMPs may hinder the early invasion process. However, use of MMPIs in the nasal passages should only be considered for very limited and temporary use, and not for long term prophylaxis, as human MMPs are constantly remodeling the ECM and performing other unique functions. For example, MMP-2 and -9 are involved in the balance between bone

formation and resorption by the recruitment of osteoblasts and osteoclasts (Engsig *et al*., 2000; Thiennu, 2001). Low levels of MMP-9 are additionally involved in wound healing by keratinocyte migration and epithelial regeneration (Chang, 2016). MMP-2 and -14 also stimulate epithelial cell migration by cleaving the γ2 chain of laminin-5 of the basement membrane, exposing a hidden epitope that induces motility of epithelial cells (Gilles *et al*., 2001; Koshikawa *et al*., 2000). MMP-14 is additionally involved in the proteolytic activation of chemokines, including IL-8 and TNF-α, and regulation of apoptosis by cleaving death receptor-6 (Rodríguez *et al*., 2010; Tam *et al*., 2004). Nevertheless, further investigation of the roles of specific MMPs in the invasion process of *N. fowleri*, including those not discussed in this study, may provide insight into introducing a specific MMPI into the current standard combinational therapy that contains amphotericin B.

In summary, the production of MMPs by *N. fowleri* facilitates their movement through physical protein barriers similar to the basement membrane. Although the presence of MMPs cannot be used to distinguish between the highly virulent MP and weakly virulent Ax *N. fowleri*, a general increase in expression of MMP-2, -9, and -14 was seen in the MP amoebae compared to the Ax amoebae, suggesting that the functionality of MMPs may aid in the virulence of *N. fowleri*. *N. fowleri* additionally seems to depend, at least in part, on the expression and secretion of MMPs for invasion and motility such that when inhibited, a significant percentage of amoebae were no longer able to traverse the Matrigel.

Future studies

The present study explored the roles of the gelatinolytic metalloproteinases, MMP-2 and -9, and the membrane-type, MMP-14, in the *in vitro* invasion process of *N. fowleri*. Future studies could be done to investigate the *in vivo* effect of inhibition of MMPs on the invasion and motility of the amoeba. Of the currently 25 known MMPs, it may also be worth examining the potential expression of other MMPs, especially those that are known to aid in the metastasis of tumor cells or invasion of pathogens. A closely related family of proteases called ADAMs (a disintegrin and metalloproteinase) may also be worth investigating as they also degrade ECM components, cytokines, chemokines, and their receptors, thereby contributing to the metastasis of cancer cells (Okazaki and Nabeshima, 2012).

Literature Cited

Ahmed, A. K.; Mun, H.; Aosai, F.; Piao, L.; Fang, H.; Norose, K.; Yano, A. (2004). Roles of *Toxoplasma gondii*-derived heat shock protein 70 in host defense against *T. gondii* infection. Microbiol Immunol 48(11):911-15.

Aldape, K., Huizinga, H., Bouvier, J., McKerrow, J. (1994). *Naegleria fowleri*: characterization of a secreted histolytic cysteine protease. Exp Parasitol 78:230-41.

Anderson, K.; Jamieson, A. (1972). Primary amoebic meningoencephalitis. Lancet 2:379.

Baig, A. M. (2016). Primary amoebic meningoencephalitis: neurochemotaxis and neurotropic preferences of *Naegleria fowleri*. ACS Chem Neurosci 7:1026-9.

Baker, A. H.; Edwards, D. R.; Murphy, G. (2002). Metalloproteinase inhibitors: biological actions and therapeutic opportunities. J Cell Sci 115:3719-27.

Boon, L.; Ugarte-Berzal, E.; Vandooren, J.; Opdenakker, G. (2016). Glycosylation of matrix metalloproteinases and tissue inhibitors: present state, challenges and opportunities. Biochem J 473(11):1471-82.

Bourboulia, D.; Stetler-Stevenson, W. G. (2011). Matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinases (TIMPs): positive and negative regulators in tumor cell adhesion. Semin Cancer Biol 20(3):161-8.

Brandt, W. W.; Dwyer, F. P.; Gyarfas, E. C. (1954). Chelate complexes of 1,10-phenanthroline and related compounds. Chem Rev 54(6):959-1017.

Brown, T. (1977). Observations by light microscopy on the cytopathogenicity of *Naegleria fowleri* in mouse embryo cell cultures. J Med Microbiol 11:249-59.

Brown, T. (1979). Observations by immunofluorescence microscopy and electron microscopy on the cytopathogenicity of *Naegleria fowleri* in mouse embryo-cell cultures. J Med Microbiol 12:363-71.

Bruschi, F.; Pinto, B. (2013). The significance of matrix metalloproteinases in parasitic infections involving the central nervous system. Pathogens 2:105-29.

Buache, E.; Garnotel, R.; Aubert, D.; Gillery, P.; Villena, I. (2007). Reduced secretion and expression of gelatinase profile in *Toxoplasma gondii*-infected human monocytic cells. Biochem Biophys Res Commun 359(2):298-303.

Butt, C. G. (1966). Primary amebic meningoencephalitis. N Engl J Med 274(26):1473-6.

Cabanes, P.; Wallet, F.; Pringuez, E.; Pernin, P. (2001). Assessing the risk of primary amoebic meningoencephalitis from swimming in the presence of environmental *Naegleria fowleri*. Appl Environ Microbiol. 67(7):2927-31.

Cao, J.; Rehemtulla, A.; Pavlaki, M.; Kozarekar, P.; Chiarelli, C. (2005). J Biol Chem 280(12):10974-80.

Carter, R. F. (1970). Description of a *Naegleria* sp. Isolated from two cases of primary amoebic meningoencephalitis, and of the experimental pathological changes induced by it. J Pathol 100(4):217-44.

Centers for Disease Control and Prevention. (2015). Public Drinking Water Systems. Retrieved from https://www.cdc.gov/parasites/naegleria/public-water-systems.html.

Chang, M. (2016). Restructuring of the extracellular matrix in diabetic wounds and healing: a perspective. Pharmacol Res 107:243-8.

Chiu, P.; Lai, S. (2013). Matrix metalloproteinase-9 leads to claudin-5 degradation via the NF-κB pathway in BALB/c mice with eosinophilic meningoencephalitis caused by *Angiostrongylus cantonensis*. PLoS One 8(3):e53370.

Clark, R. T.; Nance, J. P.; Noor, S.; Wilson, E. H. (2011). T-cell production of matrix metalloproteinases and inhibition of parasite clearance by TIMP-1 during chronic *Toxoplasma* infection in the brain. ASN Neuro 3(1):1-12.

Cline, M.; Carchman, R.; Marciano-Cabral, F. (1986). Movement of *Naegleria fowleri* stimulated by mammalian cells *in vitro*. J Protozool 33(1):10-3.

Cope, J. R.; Conrad, D. A.; Cohen, N.; Cotilla, M.; DaSilva, A. Jackson, J. Visvesvara, G. S. (2016). Use of the novel therapeutic agent miltefosine for the treatment of primary amebic meningoencephalitis: report of 1 fatal and 1 surviving case. Clin Infect Dis 62(6):774-6.

Cope, J. R.; Ratard, R. C.; Hill, V. R.; Sokol, T.; Causey, J. J.; Yoder, J. S.; Mirani, G.; Mull, B.; Mukerjee, K. A.; Narayanan, J.; Doucet, M.; Qvarnstrom, Y.; Poole, C. N.; Akingbola, O. A.; Ritter, J. M.; Xiong, Z.; da Silva, A. J.; Roellig, D.; Van Dyke, R. B.; Stern, H.; Xiao, L.; Beach, M. J. (2015). The first association of a primary amebic meningoencephalitis death with culturable *Naegleria fowleri* in tap water from a US treated public drinking water system. Clin Infect Dis 60(8):e36-42.

Cursons, R. T. M.; Brown, T. J.; Keys, E. A. Virulence of pathogenic free-living amebae. J Parasitol 64(4):744-5.

Cursons, R. T. M.; Brown, T. J.; Keys, E. A.; Moriarty, K. M.; Till, D. (1980). Immunity to pathogenic free-living amoebae: role of humoral antibody. Infect Immun 29(2):401-7.

Da Rocha-Azevedo, B.; Tanowitz, H. B.; Marciano-Cabral, F. (2009). Diagnosis of infections caused by pathogenic free-living amoebae. Interdiscip Perspect Infect Dis 2009:251406. http://doi.org/10.1155/2009/251406

Deryugina, E. I.; Quigley, J. P. (2006). Matrix metalloproteinases and tumor metastasis. Cancer Metastasis Rev 25:9-34.

Dobbin, C. A.; Smith, N. C.; Johnson, A. M. (2002). Heat shock protein 70 is a potential virulence factor in murine *Toxoplasma* infection via immunomodulation of host NF-κB and nitric oxide. J Immunol 169(2):958-65.

Dorsch, M. M.; Cameron, A. S.; Robinson, B. S. (1983). The epidemiology and control of primary amoebic meningoencephalitis with particular reference to South Australia. Trans R Soc Trop Med Hyg 77(3):372-7.

Dubray, B. L.; Wilhelm, W. E.; Jennings, B. R. (1987). Serology of *Naegleria fowleri* and *Naegleria lovaniensis* in a hospital survey. J Protozool 34(3):322-7.

Dufour, A.; Zucker, S.; Sampson, N. S.; Kuscu, C.; Cao, J. (2010). Role of matrix metalloproteinase-9 dimers in cell migration. J Biol Chem 285(46):35944-56.

Engsig, M. T.; Chen, Q.; Thiennu, H. V.; Pedersen, A.; Therkidsen, B.; Lund, L. R.; Henriksen, K.; Lenhard, T.; Foged, N. T.; Werb, Z.; Delaissé, J. (2000). Matrix metalloproteinase 9 and vascular endothelial growth factor are essential for osteoclast recruitment into developing long bones. J Cell Biol 151(4):789-89.

Ellerbroek, S. M.; Wu, Y. I.; Overall, C. M.; Stack, M. S. (2001). Functional interplay between type 1 collagen and cell surface matrix metalloproteinase activity. J Biol Chem 276(27):24833-42.

Feder, J.; Garrett, L. R.; Kochavi, D. (1971). Studies on the inhibition of neutral proteases by 1,10-phenanthroline. Biochim Biophys Acta. 235:370-7.

Felber, J.;. Coombs, T. L.; Vallee, B. L. (1962). The mechanism of inhibition of carboxypeptidase A by 1,10-phenanthroline. Biochemistry 1(2):231-8.

Ferrante, A.; Bates, E. J. (1988). Elastase in the pathogenic free-living amoebae *Naegleria* and *Acanthamoeba* spp. Infection and Immunity 56(12):3320-1.

Forsyth, P. A.; Wong, H.; Laing, T. D.; Rewcastle, N. B.; Morris, D. G.; Muzik, H.; Leco, K. J.; Johnston, R. N.; Brasher, P. M. A.; Sutherland, G.; Edwards, D. R. (1999). Gelatinase-A (MMP-2), gelatinase-B (MMP-9) and membrane type matrix metalloproteinase-1 (MT1-MMP) are involved in different aspects of the pathophysiology of malignant gliomas. Br J Cancer 79(11/12):1828-35.

Fowler, M.; Carter, R. F. (1965). Acute pyogenic meningitis probably due to *Acanthamoeba* sp.: a preliminary report. Br Med J 2:740-2.

Fritzinger, A. E.; Toney, D. M.; MacLean, R. C.; Marciano-Cabral, F. (2006). Identification of a *Naegleria fowleri* membrane protein reactive with anti-human CD59 antibody. Infect Immun 74(2):1189-95.

Gao, M.; Nguyen, T. T.; Suckow, M. A.; Wolter, W. R.; Gooyit, M.; Mobashery, S.; Chang, M. (2015). Accleration of diabetic wound healing using a novel protease-anti-protease combination therapy. Proc Natl Acad Sci USA 112(49):15226-31.

Geurts, N.; Opdenakker, G.; Van den Steen, P. E. (2012). Matrix metalloproteinases as therapeutic targets in protozoan parasitic infections. Pharmacol Ther 133:257-9.

Gialeli, C.; Theocharis, A. D.; Karamanos, N. K. (2010). Roles of matrix metalloproteinases in cancer progression and their pharmacological targeting. FEBS J 278:16-27.

Giambernardi, T. A.; Sakaguchi, A. Y.; Gluhak, J.; Pavlin, D.; Troyer, D. A.; Das, G.; Rodeck, U.; Klebe, R. J. (2001). Neutrophil collagenase (MMP-8) is expressed during early development in neural crest cells as well as in adult melanoma cells. Matrix Biol 20:577-87.

Gilles, C.; Polette, M.; Coraux, C.; Tournier, J. M.; Meneguzzi, G.; Munaut, C.; Volders, L.; Rousselle, P.; Birembaut, P.; Foidart, J. M. (2001). Contribution of MT1-MMP and of human laminin-5 gamma2 chain degradation to mammary epithelial cell migration. J Cell Sci 114:2967-76.

Gilles, C.; Polette, M.; Seiki, M.; Birembaut, P.; Thompson, E. W. (1997). Implication of collagen type 1-induced membrane-type 1-matrix metalloproteinase expression and matrix metalloproteinase-2 activation in the metastatic progression of breast carcinoma. Lab Invest 76(5):651-60.

Haggerty, R. M.; John, D. T. (1978). Innate resistance of mice to experimental infection with *Naegleria fowleri*. 20(1):73-7.

Han, K.; Lee, H.; Shin, M. H.; Shin, H.; Im, K.; Park, S. (2004). The involvement of an integrin-like protein and protein kinase C in amoebic adhesion to fibronectin and amoebic cytotoxicity. Parasitol Res 94:53-60.

Hazra, S.; Guha, R.; Jongkey, G.; Palui, H.; Mishra, A.; Vemuganti, G. K.; Basak, S. K.; Mandal, T. K.; Konar, A. (2012). Modulation of matrix metalloproteinase activity by EDTA prevents posterior capsular opacification. Mol Vis 18:1701-11.

Herbst, R.; Ott, C.; Jacobs, T.; Marti, T.; Marciano-Cabral, F.; Leippe, M. (2002). Pore-forming polypeptides of the pathogenic protozoon *Naegleria fowleri*. J Biol Chem 277(25):22353-60.

Hunte, T.; Morris, T.; da Silva, A.; Nuriddin, A.; Visvesvara, G.; Hill, V.; Mull, B.; Xiao, L.; Roellig, D.; Gargano, J.; Beach, M.; Yoder, J.; Cope, J.; Morris, J. (2013). Primary amebic meningoencephalitis associated with ritual nasal rinsing. MMWR Morb Mortal Wkly Rep 62(45):903.

Hysmith, R. M.; Franson, R. C. (1982). Elevated levels of cellular and extracellular phospholipases from pathogenic *Naegleria fowleri*. Biochim Biophys Acta 711(1):26-32.

Itoh, Y. (2006). MT1-MMP: a key regulator of cell migration in tissue. Life 58(10):589-96.

Jamerson, M.; da Rocha-Azevedo, B.; Cabral, G. A.; Marciano-Cabral, F. (2012). Pathogenic *Naegleria fowleri* and non-pathogenic *Naegleria lovaniensis* exhibit differential adhesion to, and invasion of, extracellular matrix proteins. Microbiol 158.Pt 3:731-803.

John, D. T. (1982). Primary amebic meningoencephalitis and the biology of *Naegleria fowleri*. Ann Rev Microbiol 36:101-23.

John, D. T.; Cole, T. B. Jr; Bruner, R. A. (1985). Amebostomes of *Naegleria fowleri*. J Protozool 32(1), 12-9.

John, D. T.; Cole, T. B. Jr; Marciano-Cabral, F. M. (1984). Sucker-like structures on the pathogenic amoeba *Naegleria fowleri*. Appl Environ Microbiol 47:12-4.

John, D. T.; John, R. A. (1989). Cytopathogenicity of *Naegleria fowleri* in mammalian cell cultures. Parasitol Res 76:20-5.

Jones, C.B.; Sane, D. C.; Herrington, D. M. (2003). Matrix metalloproteinases: a review of their structure and role in acute coronary syndrome. Cardiovasc Res 59(4):812-23.

Kember, S. K.; Lynfield, R.; DeVries, A. S.; Drehner, D. M.; Pomputius, W. F. III; Beach, M. J.; Visvesvara, G. S.; da Silva, A. J.; Hill, W. R.; Yoder, J. S.; Xiao, L.; Smith, K. E.; Danila, R. (2012). Fatal *Naegleria fowleri* infection acquired in Minnesota: possible expanded range of a deadly thermophilic organism. Clin Infect Dis 54(6):805-9.

Kleinman, H. K.; Martin, G. R. (2005). Matrigel: basement membrane matrix with biological activity. Sem Cancer Biol 15:378-86.

Koshikawa, N.; Giannelli, G.; Cirulli, V.; Miyazaki, K.; Quaranta, V. (2000). Role of cell surface metalloprotease MT1-MMP in epithelial cell migration over laminin-5. J Cell Biol 148:615-24.

Li, Q.; Park, P. W.; Wilson, C. L.; Parks, W. C. (2002). Matrilysin shedding of syndecan-1 regulates chemokine mobilization and transepithelial efflux of neutrophils in acute lung injury. Cell 111:635-46.

Linam, W. M.; Ahmed, M.; Cope, J. R.; Chu, C.; Visvesvara, G. V.; da Silva, A. G.; Qvarnstrom, Y.; Green, J. (2015). Successful treatment of an adolescent with *Naegleria fowleri* primary amebic meningoencephalitis. Pediatrics 135(3):e744-8.

LeBleu, V. S.; MacDonald, B.; Kalluri, R. (2007) Structure and function of basement membranes. Exp Biol Med 232(9):1121-9.

Maher, P. A.; Singer, S. J. (1985). Anomalous interaction of the acetylcholine receptor protein with the nonionic detergent Triton X-114. Proc Natl Acad Sci USA 82(4):958-62.

Mali, S. S.; Hajare, A. A.; Karade, R. S.; Salunkhe, S. S.; Nadaf, S. J.; Honmane, S. M.; Bhatia, N. M. (2014). Expulsion by ionic complexation: benchmark therapy for atherosclerosis a review. Indian J Pharm Biol Res 2(1):103-7.

Mao, Z.; Heinemann, F. W.; Liehr, G.; van Eldik, R. (2001). Complex-formation reactions of Cu(II) and Zn (II) 2,2'-bipyridine and 1,10-phenanthroline complexes with bicarbonate. Identification of different carbonate coordination modes. J Chem Soc, Dalton Trans 2001(24):3652-62.

Marciano-Cabral, F. (1988). Biology of *Naegleria* spp. Microbiol Rev 52(1):114-33.

Marciano-Cabral, F; Cabral, G. A. (2007). The immune response to *Naegleria fowleri* amebae and pathogenesis of infection. FEMS Immunol Med Microbiol 51:243-59.

Marciano-Cabral, F.; Cline, M. L.; Bradley, S. G. (1987). Specificity of antibodies from human sera for *Naegleria* species. J Clin Microbiol 25(4):692-7.

Marciano-Cabral, F.; Fulford, D. E. (1986). Cytopathology of pathogenic and nonpathogenic *Naegleria species* for cultured rat neuroblastoma cells. Appl Environ Microbiol 51(5):1133-7.

Marciano-Cabral, F.; MacLean, R.; Mensah, A.; Lapat-Polasko, L. (2003). Identification of *Naegleria fowleri* in domestic water sources by nested PCR. Appl Environ Microbiol 69(10):5864-9.

Maresca, B.; Carratú, L. (1992) The biology of the heat shock response in parasites. Parasitol Today 8(8):260:6.

Martinez, A. J. (1985). Free-living amebas: natural history, prevention, diagnosis, pathology, and treatment of disease. CRC Press. Boca Raton Fla.

Martinez, A. J.; Nelson, E. C.; Jones, M. M.; Duma, R. J.; Rosenblum, W. I. (1971). Experimental *Naegleria* meningoencephalitis in mice: an electron microscopy study. Lab Invest 24:465-75.

Martinez, A. J., Visvesvara, G. S. (1997). Free-living, amphizoic and opportunistic amebas. Brain Pathol 7:583-8.

Masocha, W.; Rottenberg, M. E.; Kristensson, K. (2006). Minocycline impedes African trypanosome invasion of the brain in a murine model. Antimicrob Agents Chemother 50(5):1798-804.

Mat Amin, N. (2004). Proteinases in *Naegleria fowleri* (strain NF3), a pathogenic amoeba: a preliminary study. Trop Biomed 21:57-60.

Matin, A., Stins, M., Kim, K. S., Khan, N. A. (2006). *Balamuthia mandrillaris* exhibits metalloprotease activities. FEMS Immunol Med Microbiol 47:83-91.

Mohan, V.; Talmi-Frank, D; Arkadash, V.; Papo, N.; Sagi, I. (2016). Matrix metalloproteinase protein inhibitors: highlighting a new beginning for metalloproteinases in medicine. Metalloproteinases in Medicine 3:31-47.

Nagase, H. (1997). Activation mechanisms of matrix metalloproteinases. Biol Chem 378(3-4)151-60.

Newsome, A. L.; Arnold, R. R. (1985). Equivalent mortality in normal and athymic mice infected with *Naegleria fowleri*. J Parasitol 71(5):678-9.

Nezu, T.; Hosomi, N.; Aoki, S.; Deguchi, K.; Masugata, H.; Ichihara, N.; Ohyama, H.; Ohtsuki, T.; Kohno, M.; Matsumoto, M. (2013). Alpha2-macroglobulin as a promising biomarker for cerebral small vessel disease in acute ischemic stroke patients. J Neurol 260(10):2642-9.

Okazaki, I.; Nabeshima, K. (2012). Introduction: MMPs, ADAMs/ADAMTSs research products to achieve big dream. Anticancer Agents Med Chem 12:688-706.

Olson, M. W.; Bernardo, M. M.; Pietila, M.; Gervasi, D. C.; Toth, M.; Kotra, L. P.; Massova, I.; Mobashery, S.; Fridman, R. (2000). Characterization of the monomeric and dimeric forms of latent and active matrix metalloproteinase-9. J Biol Chem 275(4):2661-8.

Overall, C. M.; Tam, E.; McQuibban, G. A.; Morrison, C.; Wallon, U. M.; Bigg, H. F.; King, A. E.; Roberts, C. R. (2000). Domain interactions in the gelatinase A·TIMP-2·MT1-MMP Activation Complex. J Biol Chem 275(50):39497-506.

Paeman, L. Martens, E.; Norga, K.; Masure, S.; Roets, E.; Hoogmartens, J.; Opdenakker, G. (1996). The gelatinase inhibitory activity of tetracyclines and chemically modified tetracycline analogues as measured by a novel microtiter assay for inhibitors. Biochem Pharmacol 52(1):105-11.

Parija, S. C. (2015), Nov 23. Naegleria Infection. Medscape. Retrieved 5/16/2017 from http://emedicine.medscape.com/article/223910-overview.

Piccard, H.; Van den Steen, P; Opdenakker, G. (2007). Hemopexin domains as multifunctional liganding modules in matrix metalloproteinases and other proteins. J Leukoc Biol 81(4):870-92.

Piña-Vázquez, C.; Reyes-López, M.; Ortíz-Estrada, G.; de la Garza, M.; Serrano-Luna, J. (2012). Host-parasite interaction: parasite-derived and -induced proteases that degrade human extracellular matrix. J Parasitol Res 36(6):1122-80.

Polla, B. S. (1991). Heat shock proteins in host-parasite interactions. Immunol Today 12(3):A38-41.

Qoronfleh, M. W.; Benton, B.; Ignacio, R.; Kaboord, B. (2003). Selective enrichment of membrane proteins by partition phase separation for proteomic studies. J Biomed Biotech 2003(4):249-55.

Reilly, M. F.; White, K. L. Jr.; Bradley, S. G.(1983). Host resistance of mice to *Naegleria fowleri* infections. Infect Immun 42(2):645-52.

Rodríguez, D.; Morrison, C. J.; Overall, C. M. (2010). Matrix metalloproteinases:what do they not do? New substrates and biological roles identified by murine models and proteomics. Biochem Biophys Acta 1803(1):39-54.

Rojas-Hernández, S.; Jarillo-Luna, A.; Rodríguez-Monroy, M.; Moreno-Fierros, L.; Campos-Rodríguez, R. Immunohistochemical characterization of the initial stages of *Naegleria fowleri* meningoencephalitis in mice. Parasitol Res 94:31-6.

Rosenberg, G. A. (2015). Metalloproteinases and neurodegenerative diseases: pathophysiological and therapeutic perspectives. Metalloproteinases in Medicine 2:39-50.

Schuster, F. L.; Visvesvara, G. S. (2004). Free-living amoebae as opportunistic and non-opportunistic pathogens of humans and animals. Int J for Parasitol 34(9):1001-27.

Seidel, J. S.; Harmatz, P.; Visvesvara, G. S.; Cohen, A.; Edwards, J.; Turner, J. (1982). Successful treatment of primary amebic meningoencephalitis. N Engl J Med 306:346-8.

Seipel, D.; de Lima Oliveira, B. C.; Resende, T. L.; Schuindt, S. H. S.; Pimentel, P. M. de O. Kanashiro, M. M.; Arnholdt, A. C. V. (2010). *Toxoplasma gondii* infection positively modulates the macrophages migratory molecular complex by increasing matrix metalloproteinases, CD44, and alphaveta3 integrin. Vet Parasitol 169:312-9.

Shakoor, S.; Beg, M. A.; Mahmood, S. F.; Bandea, R.; Sriram, R.; Noman, F.; Ali, F.; Visvesvara, G. S.; Zafar, A. (2011). Primary amebic meningoencephalitis caused by *Naegleria fowleri*, Karachi, Pakistan. Emerg Infect Dis 17(2):258-61.

Shipley, J. M.; Wesselschmidt, R. L.; Kobayashi, D. K.; Ley, T. J.; Shapiro, S. D. (1996). Metalloelastase is required for macrophage-mediated proteolysis and matrix invasion in mice. Proc Natl Acad Sci USA 93:3942-6.

Siddiqui, R.; Khan, N. A. (2014). Primary amoebic meningoencephalitis caused by *Naegleria fowleri*: an old enemy presenting new challenges. PLoS Negl Trop Dis 8(8):e3017.

Sohail, A.; Sun, Q.; Zhao, H.; Bernardo, M. M.; Cho, J.; Fridman, R. (2008). MT4-(MMP17) and MT6-MMP (MMP25), a unique set of membrane-anchored matrix metalloproteinases: properties and expression in cancer. Cancer Metastasis Rev 27(2):289-302.

Song, K.; Song, K.; Kim, J.; Sohn, H.; Lee, Y.; Park, C.; Shin, H. (2008). Heat shock protein 70 of *Naegleria fowleri* is important factor for proliferation and in vitro cytotoxicity. Parasitol Res 103:313-7.

Song, K.; Song, K.; Na, B.; Kim, J.; Kwon, D.; Park, S.; Pak, J.; Im, K.; Shin, H. (2007). Molecular cloning and characterization of a cytosolic heat shock protein 70 from *Naegleria fowleri*. Parasitol Res 100(5):1083-9.

Sorsa, T.; Tervahartiala, T.; Leppilahti, J.; Hernandez, M.; Gamonal, J.; Tuomainen, A. M.; Lauhio, A.; Pussinen, P. J.; Mäntylä, P. (2011). Collagenase-2 (MMP-8) as a point-of-care biomarker in periodontitis and cardiovascular diseases. Therapeutic response to non-antimicrobial properties of tetracyclines. Pharmacol Res 63:108-13.

Staun-Ram, E.; Goldman S.; Gabarin, D.; Shalev, E. (2004). Expression and importance of matrix metalloproteinase 2 and 9 (MMP-2 and -9) in human trophoblast invasion. Reprod Biol Endocrinol 2:59:71.

Stechmiller, J.; Cowan, L.; Schultz, G. (2010). The role of doxycycline as a matrix metalloproteinase inhibitor for the treatment of chronic wounds. Biol Res Nurs 11(4):336-44.

Sterchi, E. E. (2008). Special issue: metzincin metalloproteinases. Mol Asp Med 29:255-7.

Stetler-Stevenson, W. G. (2008). The tumor microenvironment: regulation by MMP-independent effects of tissue inhibitor of metalloproteinase-2. Cancer Metastasis Rev 27(1):57-66.

Stevens, A. R.; De Jonckheere, J.; Willaert, E. (1980). *Naegleria lovaniensis* new species: isolation and identification of six thermophilic strains of a new species found in association with *Naegleria fowleri*. Int J Parasitol 10(1):51-64.

Strongin, A. Y.; Marmer, B. L.; Grant, G. A.; Goldberg, G. I. (1993). Plasma membrane-dependent activation of the 72-kDa type IV collagenase is prevented by complex formation with TIMP-2. J Biol Chem 268(19):14033-9.

Takahashi, C.; Sheng, Z.; Horan, T. P.; Kitayama, H.; Maki, M.; Hitomi, K.; Kitaura, Y.; Takai, S.; Sasahara, R.; Horimoto, A.; Ikawa, Y.; Ratzkin, B. J.; Arakawa, T.; Noda, M. (1998). Regulation of matrix metalloproteinase-9 and inhibition of tumor invasion by the membrane-anchored glycoprotein RECK. Proc Natl Acad Sci USA 95(22):13221-6.

Tam, E. M.; Morrison, C. J.; Wu, Y. I.; Stack, M. S.; Overall, C. M. (2004). Membrane protease proteomics: isotope-coded affinity tag MS identification of undescribed MT1-matrix metalloproteinase substrates. Proc Natl Acad Sci USA 101(18):6917-22.

Theocharis, A. D.; Skandalis, S. S.; Gialeli, C.; Karamanos, N. K. (2016). Extracellular matrix structure. Adv Drug Deliv Rev 97:4-27.

Thibeaux, R.; Avé, P.; Bernier, M.; Morcelet, M.; Frileux, P.; Guillén, N.; Labruyère, E. (2014). The parasite *Entamoeba histolytica* exploits the activities of human matrix metalloproteinases to invade colonic tissue. Nat Commun 5:5142.

Thiennu, H. V. (2001). Don't mess with the matrix. Nature Genet 28:202-3.

Thomas, N. V.; Manivasagan, P.; Kim, S. (2014). Potential matrix metalloproteinase inhibitors from edible marine algae: a review. Environ Toxicol Pharmacol 37(3):1090-100.

Toney, D. M.; Marciano-Cabral, F. (1992). Alterations in protein expression and complement resistance of pathogenic *Naegleria* amoebae. Infect Immun 60(7):2784-90.

Toney, D. M.; Marciano-Cabral, F. (1994). Membrane vesiculation of *Naegleria fowleri* amoebae as a mechanism for resisting complement damage. J Immunol 152:2952-9.

Toth, M.; Sohail, A.; Fridman, R. (2012). Assessment of Gelatinases (MMP-2 and MMP-9) by gelatin zymography. Methods Mol Biol 878:121-35.

Troeberg, L.; Nagase, H. (2003). Zymography of metalloproteinases. Curr Protoc Protein Sci 21.15.1-21.15.12.

Tung, M.; Hsu, B.; Tao, C.; Lin, W.; Tsai, H.; Ji, D.; Shen, S.; Chen, J.; Shih, F.; Huang, Y. (2011). Identification and Significance of *Naegleria fowleri* isolated from the hot spring which related to the first primary amebic meningoencephalitis (PAM) patient in Taiwan. Int J for Parasitol 43(9):691-6.

Tuppeny, M. (2011). Primary amoebic meningoencephalitis with subsequent organ procurement: a case study. J Neurosci Nurs 43(5):274-9.

Van Wart, H. E.; Birkedal-Hansen, H. (1990). The cysteine switch: a principle of regulation of metalloproteinase activity with potential applicability to the entire matrix metalloproteinase gene family. Proc Natl Acad Sci USA 87(14):5578-82.

Vargas-Zepeda, J.; Gomez-Alcala, A. V.; Vazquez-Morales, J. A.; Licea-Amaya, L. De Jonckheere, J. F.; Lores-Villa, F. (2005). Successful treatment of *Naegleria* PAM using IV amphotericin B, fluconazole, and rifampin. Arch Med Res 36:83-6.

Verma, R. P.; Hansch, C. (2007). Matrix metalloproteinases (MMPs): chemical-biological functions and (Q)SARs. Bioorg Med Chem 15:2223-68.

Visse, R.; Nagase, H. (2003). Matrix metalloproteinases and tissue inhibitors of metalloproteinases: structure, function, and biochemistry. Circ Res 92:827-39.

Visvesvara, G. S.; Moura, H.; Schuster, F. L. (2007). Pathogenic and opportunistic free-living amoebae: *Acanthamoeba* spp., *Balamuthia mandrillaris*, *Naegleria fowleri*, and *Sappinia diploidea*. FEMS Immunol Med Microbiol 50:1-26.

Vyas, I. K.; Jamerson, M.; Cabral, G. A.; Marciano-Cabral, F. (2014). Identification of peptidases in highly pathogenic vs. weakly pathogenic *Naegleria fowleri* amebae. J Eukaryot Microbiol 62:51-9.

Walochnik, J.; Duchene, M.; Seifert, K.; Obwaller, A.; Hottkowitz, T.; Wiedermann, G.; Eibl, H.; Aspock, H. (2002). Cytotoxic activities of alkylphosphocholines against clinical isolates of *Acanthamoeba* spp. Antimicrob Agents Chemother 46(3):695-701.

Walsh, C. (1980). Appearance of heat shock proteins during the induction of multiple flagella in *Naegleria gruberi*. J Biol Chem 255(7):2629-32.

Wang, M.; Qin, X.; Mudgett, J. S.; Ferguson, T. A.; Senior, R. M.; Welgus, H. G. (1999). Matrix metalloproteinase deficiencies affect contact hypersensitivity: stromelysin-1 deficiency prevents the response and gelatinase B deficiency prolongs the response. Proc Natl Acad Sci 96:6885-9.

Whiteman, L. Y.; Marciano-Cabral, F. (1987). Susceptibility of pathogenic and nonpathogenic *Naegleria* spp. to complement-mediated lysis. Infect Immun 55(10):2442:7.

Ye, S. (2000). Polymorphism in matrix metalloproteinase gene promoters: implication in regulation of gene expression and susceptibility of various diseases. Matrix Biol 19(7):623-9.

Yoder, J. S.; Eddy, B. A.; Visvesvara, G. S.; Capewell, L.; Beach, M. J. The epidemiology of primary amoebic meningoencephalitis in the USA, 1962-2008. (2010). Epidemiol Infect 138:968-75.

Yoder, J. S.; Straif-Bourgeois, S.; Roy, S. L.; Moore, T. A.; Visvesvara, G. S.; Ratard, R. C.; Hill, V. R.; Wilson, J. D.; Linscott, A. J.; Crager, R.; Kozak, N. A.; Sriram, R.; Narayanan, J.; Mull, B.; Kahler, A. M.; Schneeberger, C.; da Silva, A. J.; Poudel, M.; Baumgarten, K. L.; Xiao, L.; Beach, M. J. (2012). Primary amebic meningoencephalitis deaths associated with sinus irrigation using contaminated tap water. Clin Infect Dis 55(9):e79-85.

Young, J. D.; Lowrey, D. M. (1988) Biochemical and functional characterization of a membrane-associated pore-forming protein from the pathogenic ameboflagellate *Naegleria fowleri*. J Biol Chem 264(2):1077-83.

Yu, Q.; Stamenkovic, I. (1999). Localization of matrix metalloproteinase 9 to the cell surface provides a mechanism for CD44-mediated tumor invasion. Genes Dev 13(1):35-48.

Yu, Q.; Stamenkovic, I. (2000). Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-β and promotes tumor invasion and angiogenesis. Genes Dev 14(2):163-76.

Zhang, C.; Kim, S. (2009). Matrix metalloproteinase inhibitors (MMPIs) from marine natural products: the current situation and future prospects. Mar Drugs. 7(2):71-84.

Vita

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