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DETECTION OF SECRETED PROTEASES AND A MEMBRANE PROTEASE IN PATHOGENIC ACANTHAMOEBA CULBERTSONI

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DETECTION OF SECRETED PROTEASES AND A MEMBRANE PROTEASE IN PATHOGENIC *ACANTHAMOEBA CULBERTSONI*

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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Table of Contents

Page

List of Tables

Page

List of Figures

Page

Abstract

Detection of Secreted Proteases and a Membrane Protease in Pathogenic *Acanthamoeba culbertsoni*

By Shivdeep Deo, B.S.

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Major Director: Dr. Francine Marciano-Cabral

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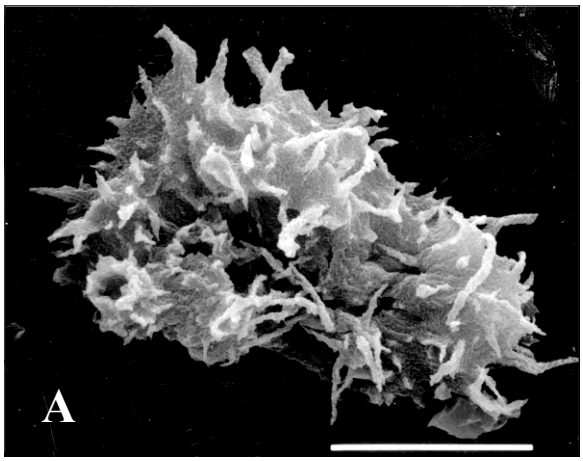
Acanthamoeba culbertsoni (*A. culbertsoni)* is an amphizoic amoeba that is the causative agent of Granulomatous Amoebic Encephalitis (GAE), an often fatal central nervous system infection that is seen most frequently in severely immunocompromised patients and is characterized by hemorrhagic and necrotic lesions of the brain as well as varying degrees of granuloma formation. *A.culbertsoni* isolates have also been identified in a few cases of Amoebic Keratitis, a painful, sight-threatening corneal infection that disproportionately affects contact lens users irrespective of immune status. Common features of both infections include amoebic interaction with host extracellular matrix (ECM) components as requisites for both attachment to, and subsequent invasion of, host tissues to facilitate disease establishment. Previous studies have demonstrated that pathogenic species of *Acanthamoeba* , such as *A.culbertsoni,* bind to the ECM proteins Laminin-1 and Collagen I to a greater extent than non-pathogenic species. It has also been documented in the literature that secreted *Acanthamoeba* proteases have the ability to degrade components of the extracellular matrix. The role of amoebic proteases in mediating the attachment and invasion processes is not entirely understood. Initial experiments conducted in the present study revealed secretion of approximately 150 and 55-kDa serine proteases during attachment as well as invasion of the ECM by *A. culbertsoni*. However, inhibition of these serine proteases using phenylmethylsulfonyl fluoride (PMSF) did not diminish the ability of amoebae to attach or invade. It was demonstrated that secretion of the observed proteases occurred in a constitutive rather than substrate-induced manner and that amoebae secrete these proteases under a number of different conditions. Additionally, a 140-kDa membrane-associated serine protease was identified which may prove to play a role in focal proteolytic degradation. Collectively, our results suggest that attachment to extracellular matrix components is mediated through non-protease-dependent mechanisms. We also suggest that ECM invasion by *A.culbertsoni* is

predominately a mechanical process that may be supplemented or enhanced by focal proteolytic degradation of extracellular matrix components by membrane-associated proteases.

Chapter 1: Introduction

Acanthamoeba is one of several genera of free-living amoebae encountered in the environment. Members of this genus enjoy a ubiquitous ecological distribution and have been isolated from a number of sites such as soil, air, natural and treated water sources, freshwater, seawater, swimming pools, sewage, sediments, hospital dialysis units, air conditioning ducts, contact lens solution and cases, as well as from the nasopharyneal mucosa of healthy individuals [Marciano-Cabral and Cabral, 2003; Khan, 2006]. Although they are free-living, several species within this genus can act as pathogens. *Acanthamoeba* has the distinction of being one of only four genera containing amphizoic amoebae that have been implicated as the causative agents of certain human diseases. Other members of this group include *Balamuthia mandrillaris*, *Naegleria fowleri* and *Sappinia pedata* [Visvesvara et al., 2007; Marciano-Cabral, 2009; Ovarnstrom et al., 2009]. A vegetative trophozoite stage and a dormant cyst stage represent the morphological stages of *Acanthamoeba* spp. The trophozoite stage also represents the infective stage in those species of *Acanthamoeba* that are capable of establishing an infection (**Figure 1**). Trophozites range in diameter from 12-35µm, are usually uninucleated with a large central



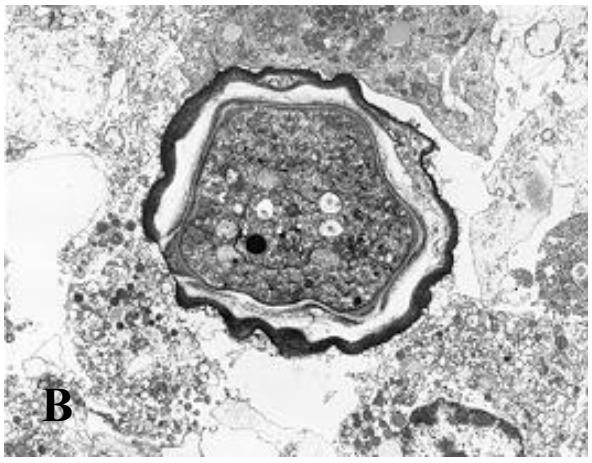


Figure 1: Morphological Stages of *Acanthamoeba* Spp. A scanning electron micrograph of *Acanthamoeba* trophozoite (A) and a transmission electron micrograph of an *Acanthamoeba* cyst (B). Courtesy of Dr. Francine Marciano-Cabral

nucleolus, and are often distinguished from amoebae of other genera by their acanthopodia, or spiny surface projections. The presence of a smooth and rough endoplasmic reticulum, mitochondria, vacuoles, ribosomes, and a Golgi complex also have been observed in these organisms. *Acanthamoeba* trophozoites feed primarily on bacteria, yeast, algae, and small organic particles in the environment through phagocytosis but also are capable of deriving nutrients from axenic growth media through pinocytosis. Reproduction in these organisms occurs asexually through binary fission [Marciano-Cabral and Cabral, 2003; Khan, 2006].

Given the plethora of ecosystems from which isolates of *Acanthamoeba* have been collected, it comes as no surprise that these amoebae are remarkably temperature and osmotolerant and exhibit the ability to grow in acidic and basic environments ranging from pH 4-12 [Khan, 2003]. However, under harsh environmental conditions or states of nutrient deprivation, *Acanthamoeba* species are able to transform into double-walled cysts which are extremely drug, desiccation, chlorination and irradiation resistant [Sriram et al., 2008; Visvesvara et al., 2007]. A wrinkled outer cyst wall, or exocyst, contains proteins and lipids while the inner cyst wall, the endocyst, is rich in cellulose and can exhibit stellate, oval or polygonal morphology [Marciano-Cabral and Cabral, 2003; Visvesvara et al., 2007]. Cyst morphology was originally used as the criterion to classify different species of *Acanthamoeba* into one of three morphological groups (I, II, III) [Page, 1967; Pussard and Pons, 1977]. Species of amoebae with cysts ranging from 16-30µm are placed in Group I. Amoebae in Group II measure approximately 18µm or less as do amoebae in Group III. However, Group III amoebae are distinct from Group I and II amoebae in that they exhibit thin, smooth exocysts and round endocysts [Marciano-Cabral and Cabral, 2003; Visvesvara et al., 2007]. This classification system has proven to be unreliable, however, due to the variability of cyst morphology induced

by different environmental conditions [Sawyer and Griffin, 1975]. Currently, the different species of *Acanthamoeba* are classified into 12 genotypes (T1-T12) established by Stothard et al., that are based on their 18S rRNA gene sequences. Of the 24 identified species of *Acanthamoeba*, only *A. castellanii, A. culbertsoni, A. divionesis, A. griffini, A. hatchetti, A. healyi, A. lenticulata, A. lugdunensis, A. polyphaga, A. quina,* and *A. rhysodes* have been implicated in human diseases [Martinez and Visvesvara, 1997;Marciano-Cabral and Cabral, 2003; Visvesvara et al., 2007]. **Table 1** lists several *Acanthamoeba* species and their corresponding genotype, group, and associated disease(s). Several species of amphizoic *Acanthamoeba* are the causative agents of Amoebic Keratitis (AK), Cutaneous Acanthamebiasis, and Granulomatous Amoebic Encephalitis (GAE).

Amoebic Keratitis is the most common infection caused by *Acanthamoeba* spp. It is a progressive, painful, sight-threatening corneal infection that occurs in both immunocompetent and immunocompromised patients. The greatest predisposing factors for AK are contact lens use and corneal trauma. In developed nations more than 80% of AK cases are seen in individuals who wear contact lenses and practice poor lens hygiene whereas in developing countries, corneal trauma and exposure to contaminated water appear to be the prominent risk factors [Clarke and Niederkorn, 2006]. During 2003-2008, a dramatic four-fold increase in AK was reported in the United States. This presumably correlated with the increasing number of contact lens wearers and demonstrates the growing importance of understanding this infection [Panjwani, 2010; Verani et al., 2009]. Early symptoms of AK generally include redness, increased tear production, and photophobia. As the infection progresses conjunctival hyperemia, inflammation, episcleritis, and scleritis also can occur. The distinguishing clinical feature of AK is the formation of a ring-like stromal infiltrate of cells of the host immune system

Table 1: *Acanthamoeba* spp. and their Respective Genotype, Group, and Associated Human Disease(s)

Species	Genotype	Group	Associated Human Disease
A. astronyxis	T7	I	NA
A. castellanii	T4	II	Encephalitis, Keratitis
A. commandoni	T9	I	NA
A. culbertsoni	T10	III	Encephalitis, Keratitis
A. divionensis	Unknown	II	
A. echinulata	Unknown	I	
A. griffin	T3	II	Keratitis
A. hatchetti	T11	II	Keratitis
A. healyi	T12	III	Encephalitis
A. jacobsi	Unknown	III	
A. lenticulata	T5	III	NA
A. lugdunensis	T4	II	Encephalitis, Keratitis
A. mauritaniensis	T4	II	Encephalitis, Keratitis
A. palestinensis	T2	III	Keratitis
A. pearcei	T3	I	Keratitis
A. polyphaga	T4	II	Encephalitis, Keratitis
A. pustulosa	T2	III	Keratitis
A. quina	Unknown	II	
A. rhysodes	T4	II	Encephalitis, Keratitis
A. royreba	T4	III	Encephalitis, Keratitis
A. stevensoni	T11	II	Keratitis
A. triangularis	T4	II	Encephalitis, Keratitis
A. tubiashi	T8	I	NA

Table 1: *Acanthamoeba* spp. and their Respective Genotype, Group, and Associated Human Disease(s). NA = no disease association has been found yet. Information in the table was obtained from Marciano-Cabral and Cabral, 2003; Khan, 2006; Visvesvara, 2007.

[Marciano-Cabral and Cabral, 2003]. An infection is thought to begin when trophozoites bind to mannose on the corneal epithelium through a 400-kDa Mannose-Binding Protein (MBP). It has been shown in animal models that contact lens use and corneal trauma upregulates mannose glycoprotein expression on the corneal epithelium. Following initial adhesion, certain species of *Acanthamoeba* have been shown to produce a 133-kDa serine protease called the Mannose-Induced Protein (MIP). MIP along with other less understood mechanisms induce cytopathic effects in the corneal epithelial cells and enable amoebae to penetrate Bowman's membrane, which is situated below the epithelial layer. Amoebae then cause degradation of the collagenous stromal layer and reach and destroy corneal nerves which may explain the radial keratoneuritis and severe pain associated with this infection [Clarke and Niederkorn, 2006; Pajwani, 2010]. Effective treatment of AK presents several challenges. Antimicrobial drugs such as miconazole, itraconazole, and ketoconazole have demonstrated limited success. The more widely accepted treatment approach involves a combination of 0.02% of a biguanide agent, such as polyhexamethylene biguanide (PHMB) or chlorhexidine, and 0.01% of a diamidine, such as propamidine isethionate or hexamidine. However, this regimen is extremely demanding. Drops must be added to the infected eye hourly for the first 72 hours of treatment and then reduced to every two hours following day 5 of the regimen. Treatment continues in this manner for 3-4 weeks and then the patient is slowly tapered off the medication in an attempt to counteract the epithelial cell toxicity that is associated with these drugs. Trophozoites also can encyst prior to being killed by these medications and as a result recurrent infections may develop [Visvesvara, 2010; Marciano-Cabral and Cabral, 2003; Khan, 2003]. Finally, if a diagnosis of AK is made once the infection has progressed significantly, treatment can be futile and corneal transplantation may be explored as a viable option [Hurt et al., 2003].

Cutaneous Acanthamebiasis is an infection of the skin caused by *Acanthamoeba* spp. It is characterized by the presence of erythematous nodules that contain purulent material and eventually develop into residual, non-healing ulcerations. This manifestation of an *Acanthamoeba* infection can occur in immunocompetent indivdiuals; however, it is primarily found in the immunocompromised; most notably in those individuals with AIDS and those undergoing immunosuppression for organ transplants or as treatment for autoimmune disorders. The skin lesions associated with Cutaneous Acanthamebiasis can either be the primary site of infection or can occur secondarily to hematogenous dissemination from primary sites in the lower respiratory tract, sinuses, or the central nervous system (CNS) [Marciano-Cabral and Cabral, 2003; Khan, 2006]. Unlike Amoebic Keratitis, Cutaneous Acanthamebiasis is associated with a poor prognosis. Cutaneous Acathamebiasis that occurs in the absence of an established CNS infection is fatal in approximately 73% of cases. In the presence of an associated CNS infection, or Granulomatous Amoebic Encephalitis, there is a 100% mortality rate. A successful therapeutic regimen does not exist for this infection, although pentamidine, isothianate, 5-flucytosine, clotrimazole, neomycin, polymyxin B, paromomycin, rifampicin, sulfadiazine, and kanamycin all have been used to treat patients with limited success [Torno et al., 2000]. Recently, the drug miltefosine has shown promising results in *in vitro* models while producing fewer undesirable side effects associated with topical applications of chlorhexidine gluconate and ketoconazole cream, which were previously deemed the most effective treatments [Walochnik et al., 2009; Visvesvara, 2010].

Granulomatous Amoebic Encephalitis is a rare, often fatal infection of the CNS characterized by multifocal hemorrhagic and necrotic lesions throughout the brain accompanied

by moderate to severe edema, inflammation, encephalitis, and meningeal irritation. The degree of granuloma formation around infiltrating amoebae varies with the immune fitness of the infected patient. Unlike AK and Cutaneous Acanthamoebiasis, GAE occurs almost exclusively as a secondary infection in individuals with pre-existing immune deficiencies resulting from chronic illnesses or predisposing factors. Patients with HIV/AIDS, autoimmune disorders such as systemic lupus erythematosus, poorly controlled diabetes, or those with a history of alcoholism and drug use, as well as patients undergoing organ transplants, cancer chemotherapy, and radiation are considered particularly susceptible [Marciano-Cabral and Cabral, 2003; Khan, 2006]. The exact route of infection is not entirely understood; however, several plausible modes of infection exist. GAE can occur secondarily to Cutaneous Acanthamoebiasis or through a lower respiratory infection in which amoebae subsequently are able to access the bloodstream following invasion of the intravascular space. In both cases hematogenous dissemination of amoebae through the bloodstream provides access to the blood-brain barrier, which the amoebae are thought to compromise and gain access to the brain [Visvesvara, 2007]. Access through the nasopharynx and along the olfactory tract toward the brain also has been documented in cases of GAE [Marciano-Cabral and Cabral, 2003; Visvesvara, 2007]. In vivo studies have demonstrated that intranasal inoculation of mice using *A. castellanii, A. culbertsoni,* and *A. healyi* results in the development of GAE-like disease and is associated with high fatality rates [Marciano-Cabral et al., 2001; Xuan et al., 2009]. GAE is a chronic infection that may go unnoticed for weeks or months prior to the presentation of any clinical symptoms [Martinez and Visvesvara, 1997]. The usual signs associated with GAE include headache, stiff neck, fever, nausea, vomiting, aphasia, ataxia, personality changes, seizures, hemiparesis, coma, and death [Visvesvara, 2007; Marciano-Cabral and Cabral, 2003; Khan, 2006]. Diagnosis of GAE is difficult given the

similarities in clinical presentation to bacterial and viral meningitis as well as several fungal infections of the CNS. Definitive diagnoses of GAE usually are made post-mortem. This information taken together with the fact that the vast majority of AIDS-related deaths occur in Sub-Saharan Africa makes it is very likely that the true incidence of GAE cases is grossly underestimated [Khan, 2006]. Treatment for this infection is very limited. The above mentioned drugs used to treat Cutaneous Acanthamoebiasis are often used in an attempt to treat this CNS infection. As with Cutaneous Acanthamoebiasis, drug efficacy is limited. Furthermore, the inability of several of these drugs to cross the blood-brain barrier (BBB) proves to be an additional hurdle in achieving adequate concentrations of these drugs in affected areas of the brain in order to neutralize an infection [Visvesvara, 2010; Marciano-Cabral and Cabral, 2003; Khan 2006]. However, there have been a few documented cases of successful treatment. Singhal et al. described the successful treatment of GAE in two pediatric patients using a combination of ketoconazole, rifampin, and trimethroprim-sulfamethoxazole [Singhal et al., 2001]. Surgical removal of a necrotic lesion followed by a combination of fluconzole and sulfadiazine resulted in the successful treatment of a HIV positive patient with GAE [Seijo et al., 2001]. Current treatment options being investigated include using inert Chitosan microspheres loaded with drugs such as rokitamycin. Preliminary studies have shown that this mode of drug administration results in greater localization to sites of infection and has a longer-lasting effect than administration of the free drug [Rassu et al., 2009; Visvesvara, 2010].

A common feature of all three *Acanthamoeba* infections is a compromise in the integrity of the extracellular matrix (ECM). The extracellular matrix is a meshwork of glycoaminoglycans and fibrous proteins, such as collagen, laminin, fibronectin, and elastin. The ECM comprises connective tissues, the basement membrane and is a constituent of the BBB [Alberts et al., 2002;

Persidsky et al., 2006]. In addition to being the structural component of the network surrounding cells in various tissues of the body, the ECM plays a critical role in mediating several cellular processes such as cell differentiation, adhesion, migration, wound healing, and overall tissue homeostasis [Berrier and Yamada, 2007; Lukasher and Werb, 1998]. The extracellular matrix components that are often damaged during *Acanthamoeba* infections include collagen I, collagen IV, laminin, and heparin sulfate. Collagen I is the form of collagen most frequently encountered in the human body and is found in particularly high concentrations in the corneal stroma [Michelacci, 2003]. Laminin, collagen IV, and heparin sulfate are the primary constitutes of the basement membrane which serves as an anchor for epithelial and endothelial cell layers while also separating them from other layers of tissue [Yurcheno et al., 2004; Sage, 1982]. Mutations in genes encoding ECM proteins and ECM degrading proteases have been associated with diseases of the eyes, brain, skin, and cardiovascular system [Lukasher and Werb, 1998]. Disruption of the ECM layer in the BBB leads to increased permeability of the BBB resulting in greater access by pathogens and can lead to pathological states [Persidsky, 2006]. *Acanthamoeba* infections appear to occur in ECM rich areas of the body and therefore it is critically important to understand the interactions of pathogenic species of *Acanthamoeba* with components of the ECM.

Establishment of disease by *Acanthamoeba* is a complex and multi-faceted process that most likely involves several determinants of pathogenicity. It has been reported in the literature that these amoebae possess several contact-dependent and contact-independent means of adhering to, invading, and causing overall damage of host tissues. Contact-dependent factors include the 400-kDa Mannose Binding Protein isolated and characterized from *A. castellanii*. This protein mediates adhesion of the amoeba to the host cell surface and subsequently induces

cytopathic effects which lead to host cell destruction. Induction of the MBP during adhesion has been demonstrated *in vivo* using Chinese hamsters and rabbits and *in vitro* using pig and Chinese hamster epithelium as well as rabbit and human corneal epithelial cells. This protein also has been elicited upon interaction of amoebae with rat microglial cells and human brain microvascular endothelial cells suggesting a potential role in both the AK and GAE disease processes [Panjwani, 2010; Clarke and Niederkorn, 2006; Khan 2006]. Adhesion of amoebae to components of the ECM also has been investigated. It has been demonstrated that pathogenic species of *Acanthamoeba* bind to the ECM proteins collagen, laminin, and fibronectin to a greater extent than their non-pathogenic counterparts. Additionally, a 55-kD laminin-binding protein was identified in *A. culbertsoni*, a pathogenic amoeba, but was absent from a non-pathogenic species [Rocha-Azevedo et al., 2009]. *Acanthamoeba* also possess a number of contact-independent mechanisms that may be implicated in causing disease or damage. Mattana et al. (2002) demonstrated the presence of ecto-ATPases in the plasma membrane of *Acanthamoeba*, which have the potential to induce toxic effects in host cells. Interestingly, greater ecto-ATPase activity was noted in clinical isolates versus soil isolates of *Acanthamoeba* [Khan, 2006]. Constitutive and induced secretion of hydrolytic enzymes is used by a number of pathogens to mediate infection. *Acanthamoebae* spp. have been shown to possess both phospholipases and proteases. Phospholipase A, which hydrolyzes ester bonds in the glycerophospholipids of cell membranes, was shown to be present in *Acanthamoeba* and its activity was shown to be greater in pathogens such as *A. culbertsoni and A. rhysodes* as opposed to non-pathogenic isolates [Cursons et al., 1978; Misra et al., 1983]. Finally, secretion of a number of serine, cysteine, and metalloproteases by *Acanthamoeba* has been documented in the literature; however, to date there has been no evidence of the presence of aspartic proteases.

These studies have focused primarily on T4 isolates, which are considered to be most pathogenic [Khan, 2006]. Currently serine proteases of molecular weights of 27, 36, 47, 49, 55, 60, 66, 75, 97, 100, 107, and 230-kDa have been identified in *Acanthamoeba* [Mitro, 1994; Cao, 1998; Khan 2000; Alfieri, 2000]. A 33-kDa serine protease also was isolated from *A. healyi* that demonstrated the ability to degrade collagen types I and IV, fibronectin, IgG and IgA [Kong et al., 2000]. This study was particularly important because it not only demonstrated the ability of *Acanthamoeba* to induce proteolytic degradation of ECM components, but also provided insight as to how infections can be established despite the existence of human antibodies to *Acanthamoeba*. In 1995, Mitra et al. also identified a 40-kDa serine protease that activated host plasminogen, which degrades ECM components under normal physiological conditions. Data regarding cysteine proteases are limited; however proteases of 24, 43, 65, 70, and 130-kDa have been found in certain isolates although their functions have yet to be determined [Hadas and Mazur, 1993; Alfieri, 2000]. A 150-kDa metalloprotease was recovered from a GAE patient infected with a T1 isolate. This protease also exhibited the ability to degrade collagen I and III. However, in these experiments proteolytic activity was observed when purified proteases were co-incubated with the different ECM substrates. Although this suggests that *Acanthamoeba* spp possess the ability to degrade ECM proteins it does not demonstrates that these proteases are in fact used for the purposes of attachment and invasion *in vivo.*

The degradation of ECM components has been described as a critical step in the establishment of infection by several pathogens [Han et al., 2004; Rocha-Azevado et al., 2007; Jacobs et al., 1998]. The goal of this study was to determine the involvement of proteases in attachment and invasion of ECM components by a pathogenic species of *Acanthamoeba*. While the vast majority of investigations carried out thus far have focused on members of the T4

genotype, *A. culbertsoni*, a T10 isolate, was used for the purposes of this study. *A. culbertsoni* was chosen because it is a very pathogenic non-T4 isolate that has been documented as a causative agent of both AK and GAE [Marciano-Cabral and Cabral 2003; Khan 2006]. We hypothesized that there would be indications of substrate-specific induced protease activity associated with both the attachment and invasion processes. To test this hypothesis we established three specific aims: 1) to assess which proteases were secreted during the attachment of amoebae to ECM components and during invasion of ECM scaffolds, 2) to determine the class of proteases secreted during these two processes, and 3) to elucidate whether attachment to ECM components occurs in the absence of proteases by conducting inhibitor studies.

We demonstrate that *A. culbertsoni* appears to exhibit constitutive secretion of serine proteases rather than substrate-specific protease activity when it encounters components of the ECM. Furthermore, these serine proteases do not appear to be essential to the ability of *A. culbertsoni* to adhere to and invade the ECM. While it is likely that attachment and invasion by these amoebae occurs through a non-serine protease-dependent mechanism, these studies provide a basis for further investigation into the function of constitutive protease secretion by *A. culbertsoni*.

Chapter 2: Methods and Materials

Amoebae

The pathogenic species of amoeba used for this investigation was *A. culbertsoni*. These amoebae were obtained from the American Type Culture Collection (ATCC 30171) and were originally isolated in 1957 from a primary monkey kidney tissue culture [Singh and Das, 1970]. Amoebae were grown in 75-cm² tissue culture flasks at 37°C without shaking and were maintained either in Oxoid medium consisting of (0.55% [wt/vol] Oxoid neutralized liver digest, 0.3% [wt/vol] dextrose, 0.5% [wt/vol] proteose peptone, 0.25% [wt/vol] yeast extract, 1% fetal bovine serum [FBS], and 0.1% hemin) or PYG containing (0.75% [wt/vol] protease peptone, 0.75% yeast extract [wt/vol] and 1.5% glucose [wt/vol]) [Visavera and Balamuth, 1975; Alfieri et al., 2000]. Two strains of *A. culbertsoni*, mouse-passaged or axenically-grown, were used for each experiment unless otherwise specified. Mouse passaged *A.culbertsoni* were collected from mice that had been infected intranasally (Marciano-Cabral et al., 2001). Axenic amoebae are those that have been growing in either Oxoid or PYG for an extended period of time and have

not been mouse-passaged. Mouse passaged amoebae have been shown to be more virulent than axenically-grown amoebae as previously assessed by an LD50 determination in mice (Marciano-Cabral et al.,2001). For use in experiments, amoebae were dislodged from the tissue culture flask by gentle bumping. Contents of the flasks were poured into 50mL conical tubes and centrifuged at 1250 rotations per minute (rpm) for 7 minutes at 25°C using an Eppendorf 5810 R centrifuge (Eppendorf, Hauppauge, NY). Pellets then were washed and re-suspended twice in Phosphate Buffered Saline (PBS), pH 7.4. Amoebae were counted using a hemacytometer.

Extracellular Matrix Coating

In order to simulate amoebic attachment to components of the extracellular matrix *in vitro* Collagen-I (from rat tails, Sigma, St. Louis, MO) and Laminin-1 (from Engelbreth-Holm-Swarm mouse sarcoma, Invitrogen, Grand Island, NY**)** were used. Collagen type I is the most abundant form of Collagen encountered in the human body and is present in high concentrations in the corneal stroma. Laminin-1 is a component of the basement membrane which serves as an anchor for epithelial cell layers and also serves as a barrier between tissue layers; for example, the basement membrane separates the alveoli from pulmonary capillaries [Alberts et al., 2002]. Stock solutions of each protein were re-suspended in PBS, pH 7.4, to a concentration of 10µg/mL. Six-well plates were coated with either 500µL Collagen-I or Laminin-1 and allowed to set overnight at 4°C. Plates were washed once with PBS prior to use for attachment assays in order to remove unassociated proteins.

In vivo, invasion involves three-dimensional interaction of a pathogen with components of the extracellular matrix. This was replicated *in vitro* through the creation of an invasion chamber as follows: Stock solutions of Collagen I (9.03mg/mL) and Matrigel (8.33mg/mL) were

diluted to a concentration of 2.7mg/mL using Hank's Balanced Salt Solution (HBSS). For Collagen-1 solution, a neutral pH was maintained by adding approximately 1µL of 1M NaOH. 0.8µm pore-size tissue culture inserts (Greiner BioOne, Monroe, NC) were added to a 24-well plate. 30uL of either Collagen I or Matrigel were added to the inserts and the ECM components were allowed to polymerize for 2 h at 37°C prior to use. Matrigel is a commercially available reconstituted basement membrane solution, containing Collagen IV and Laminin, which forms a sheet-like layer at 37°C.

Attachment

In order to determine the degree of amoebic attachment to ECM components and to assess whether the attachment process elicited the production of secreted proteases mouse-passaged or axenically-cultured amoebae ($5x10^6$), suspended in 500µL of Hank's Balanced Salt Solution, pH 7.4, were added to pre-coated wells for 30 minutes at 37°C. Uncoated, plastic wells served as the control for attachment of amoebae to the two ECM components, Laminin-1 and Collagen-1. Following the 30 minute incubation period, supernatant from the 6-well plates was collected and centrifuged twice at 5,000 x g for 3 minutes. The supernatant and pellets were separated. Aliquots of supernatant were frozen at -80°C until analyzed for the presence of secreted proteases using zymography. The pellets were re-suspended in 1mL of 2.5% glutaraldehyde and counted using a hemocytometer. The number of amoebae obtained during this count represented the number of amoebae that did not attach during the allotted attachment period. Attachment to the control condition, plastic, or the two ECM components, Laminin-1 and Collagen-1, was determined by subtracting the number of unattached amoebae from the original number of amoebae added to the assay.

Invasion

In an attempt to evaluate the production of secreted proteases during the invasion process, tissue culture inserts coated with either Matrigel or Collagen-I were placed into 24-well plates. Seven-hundred microliters of serum-free Oxoid media were added to the bottom chamber to serve as a chemo-attractant for the amoebae [Rocha-Azevedo et al., 2009]. Mouse-passaged amoebae ($5x10^6$) re-suspended in 100µL of Hanks Balanced Salt Solution, pH 7.4, were added to the coated insert. The 24-well plates then were incubated for 3 hours at 37°C. Following this incubation period supernatant from the inserts, the top chamber, as well as the bottom chambers was collected and centrifuged at 5,000 x g for 3 minutes; the supernatant was separated from the pellet of amoebae and centrifuged again. Zymography then was performed on the supernatant samples.

Attachment in the Presence of Inhibitors

The role of proteases in mediating attachment was assessed as follows: mouse-passaged or axenically-cultured amoebae ($5x10^6$), suspended in 500µL of Hank's Balanced Salt Solution were pre-treated for 20 minutes at 37°C with either 1mM PMSF, 1µM 1,10-Phenanthroline, 1ug/uL E-64, .5% by volume of 100% ethanol, or 1% by volume of Phosphate Buffered Saline, pH 7.4, with the latter two solutions representing vehicle controls for the three protease inhibitors. Concentrations used were found to be non-toxic to the amoebae as assessed visually by microscopy and by using the MTT Assay [Mossman, 1983]. Following the 20 minute pre-treatment period, amoebae were added to uncoated plastic wells, Laminin-1 coated wells, or Collagen -1 coated wells in a 6-well plate for 30 minutes. The supernatant was collected and separated from the pellet of amoebae in the same manner as previously described. Supernatants

from the various conditions were examined for the presence of proteases using zymography. Pellets were re-suspended and counted to determine percent attachment in the presence of the different classes of inhibitors and their vehicle controls.

Invasion in the Presence of Inhibitors

The ability of *A.culbertsoni* to invade a three-dimensional ECM scaffold in the absence or diminished production of secreted proteases was examined using mouse-passage amoebae and Collagen I-coated tissue culture inserts (Collagen I invasion chambers). Mouse-passaged amoebae ($5x10^6$), suspended in 100µL of Hank's Balanced Salt Solution were pre-treated for 20 minutes at 37°C with either 1mM PMSF, 1µM 1,10-Phenanthroline, 1ug/uL E-64, .5% by volume of 200 proof ethanol (PMSF and 1,10-Phenanthroline vehicle), or 1% by volume of Phosphate Buffered Saline, pH 7.4, (E-64 vehicle). Untreated amoebae served as the controls to the different inhibitor and vehicles conditions. Subsequently, amoebae were added to Collagen I invasion chambers, described earlier, for 3 h at 37°C. Following this invasion period, tissue culture inserts were removed and the 24-well plates were placed at 4°C overnight. This allowed the amoebae that had invaded and attached to the bottom of the well to become rounded and dislodge. 300µL of 5mM EDTA were added to the wells for 1 hour at 4°C to further dislodge amoebae. The amoebae in the wells were then counted using a hemacytometer to assess percent invasion.

Amoeba Conditioned Media

In order to obtain a profile of constitutive protease secretion $5x10^6$ recently mouse passaged amoebae were re-suspended in 5mL of HBSS, PYG, or serum-free Oxoid media and placed in 50mL conical tubes. The conical tubes were placed on their side, length-wise, at 37°C for either 45 minutes or

24 hours. Following the specified incubation period, the 50mL conical tubes were centrifuged twice at 1250 rpm for 7 minutes at 25°C to separate the pellet from the supernatant. The supernatant then was analyzed using zymography.

Whole Cell Lysates of *Acanthamoeba culbertsoni*

Whole cell lysates of mouse-passaged *A.culbertsoni* were obtained for zymographic analysis in order to determine the range of intracellular protease observed under the various experiment conditions. To obtain whole cell lysates of *A. culbertsoni*, pellets of amoebae were re-suspended in 500µL of 50mM Tris-HCl lysis buffer in a sterile microcentrifuge tube. The suspension then was subjected to three rounds of freezing in liquid nitrogen and at 37°C. The protein concentration was determined using the Bradford method [Bradford, 1976] and aliquots of the whole cell lysate were kept at -80°C until used for zymography.

Isolation of the Membrane Fraction of *Acanthamoeba culbertsoni*

To assess the presence of unique membrane-associated proteases, the membrane fraction of mouse-passaged *A. culbertsoni* was separated from the cytosolic portion using the Mem-PER Eukaryotic Membrane Protein Extraction Kit (Pierce, Rockford, IL). Protein concentration was determined using the Lowry method [Lowry, 1951].

Western Blot Analysis

Aliquots (25µl) of 24 h *A. culbertsoni* conditioned media, generated in HBSS, PYG, and serum-free Oxoid, were run out on a 10% SDS-PAGE and transferred to a nitrocellulose membrane. Membranes were probed using rabbit anti- *A. culbertsoni* and anti-*A. castellani* at a

dilution of 1:2000. Films were developed for 15 seconds using Western Lightening® Plus-ECL (Perkin Elmer, Watham, MA).

Western blot analysis was also performed using a MT1-MMP, membrane type 1 matrix metalloprotease, antibody (Rabbit Polyclonal Antibody, Fisher Scientific,Waltham, MA) against whole cell lysates and membrane fractions of mouse-passaged amoebae in order to determine whether *A. culbertsoni* possessed a membrane-associated metalloprotease.

Scanning Electron Microscopy

Mouse-passaged *A.culbertsoni* prepared for invasion of Matrigel and Collagen I, as described above, also were prepared for SEM. Following the 3 h invasion period Samples were fixed with 2.5% (v/v) glutaraldehyde diluted in PBS, washed 4 times with PBS, and post-fixed for 40 min in the dark with 2% (w/v) osmium tetroxide buffered with 0.1M cacodylate buffer, pH 7.2. Following extensive washing with PBS, samples were dehydrated in a graded series of ethanol, critical-point dried with CO_2 as the transitional fluid, mounted on stubs, and coated with a thin layer of gold(30 nm) using an ion-sputtering device. Samples were observed in a Zeiss EVO 50XVP (Zeiss, Oberkochen, Germany) SEM, operating at an accelerating voltage of 15 kV as previously described [Rocha-Azevedo, 2009]

Determination of Protease Activity

In order to visualize protease activity produced by the amoebae during the attachment and invasion processes gel zymography was performed. Evaluation of amoeba conditioned media, whole cell lysates, and membrane fractions for the presence of secreted proteases also was done using this method. Samples were collected and subjected to zymography using 10%

SDS-PAGE gels copolymerized with 1% gelatin. Samples (25µL) were loaded into each well and gels electrophoresed at 200 V for approximately 45 minutes. The gels were washed in 1X Novex® Renaturing Buffer (Invitrogen), pH 7.5, for 30 minutes at room temperature. The gels, then, were washed in 1X Novex® Developling Buffer, pH 7.5, for an additional 30 min. Renatured and developed gels were placed in fresh 1X Novex® Developling Buffer for approximately 42 h in a 37°C incubator. Following this developing process gels were washed once with deionized water and stained with Bio-Safe G-250 Coomassie Brilliant Blue overnight with gentle agitation. Subsequently, they were destained for several hours using deionized water and gentle agitation. Proteolytic activity was visualized as a white clearing on an otherwise bright blue gel. A Microtek ScanMaker 9800XL/TMA 1600 flatbed scanner (Microtek, Santa Fe Springs, CA) and Silverfast scanning software (LaserSoft Imaging Inc., Sarasota, FL) were used to scan and save the gels.

Protease Class Determination

In order to determine whether the visualized proteases belonged to the serine, metallo or cysteine classes, respectively, ,aliquots of the samples collected were incubated either in the absence or presence of 5mM PMSF, 5mM 1,10-Phenanthroline, or 5ug/ul of E-64 for 30 minutes at 37°C prior to electrophoresis. This was done in order to determine whether the visualized proteases belonged to the serine, metallo or cysteine classes, respectively. The appropriate inhibitor concentrations used were previously determined (unpublished data, Ferreira, G; Harrison et al., 2010).

Densitometry Analysis

Differences in the intensity of proteolytic activity visualized during attachment of mouse-passaged or axenically-cultured amoebae to extracellular matrix components were quantified and compared using Quanity One software (Bio-Rad, Hercules, CA).

Chapter 3: Results

Previous studies from our laboratory have shown that *A. culbertsoni* binds to the ECM components Laminin-1 and Collagen I to a greater extent than does a non-pathogenic species, *A. astronyxis* (**Figure 2**) [Rocha-Azevedo et al., 2009]. However, attachment of both the pathogen and non-pathogen to fibronectin was shown to be comparable. Thus, the potential role of proteases in mediating attachment to ECM components was explored using only Laminin-1 and Collagen I. In order to assess for the production of proteases during the attachment of *A.culbertsoni* to these proteins in comparison to a plastic substrate (ECM control), gelatin zymography was employed. Zymographic analysis of the supernatant collected following 30 minute attachment assays involving both ECM components as well as plastic revealed proteolytic profiles in which proteases of approximately 150-kDa and 55-kDa were detected (**Figure 3**). These two proteases were present in the supernatant collected from amoebic attachment to Laminin-1, Collagen I as well as plastic. Furthermore, these two proteases were also visualized when attachment assays were conducted using weakly-pathogenic axenically grown or highly virulent mouse-passaged *A.culbertsoni* (**Figures 3 A and B**). Upon visual

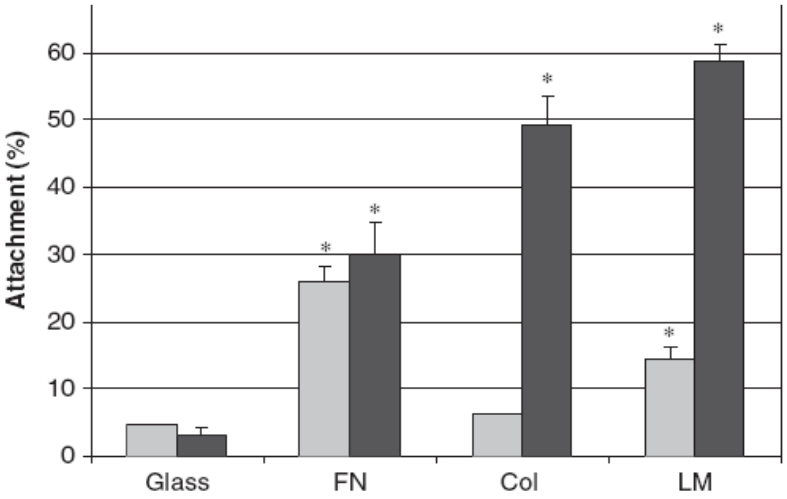


Figure 2: Previous Studies Involving the Attachment of *A. culbertsoni* and *A. astronyxis* to Extracellular Matrix Components. Previous studies conducted in our lab using *A.culbertsoni* and *A. astronyxis*, grown axenically in PYG media, demonstrated a similar degree of attachment to Fibronectin. However, the pathogen, *A. culbertsoni*, displayed significantly greater attachment to Collagen I and Laminin-1 than did the non-pathogen, *A. astronyxis.* Attachment of *A. culbertsoni* is represented by the black bars whereas attachment of *A. astronyxis* is presented by the gray bars. Asterisks represent statistical significance ($P < 0.05$, Student's t-test, compared to the control).

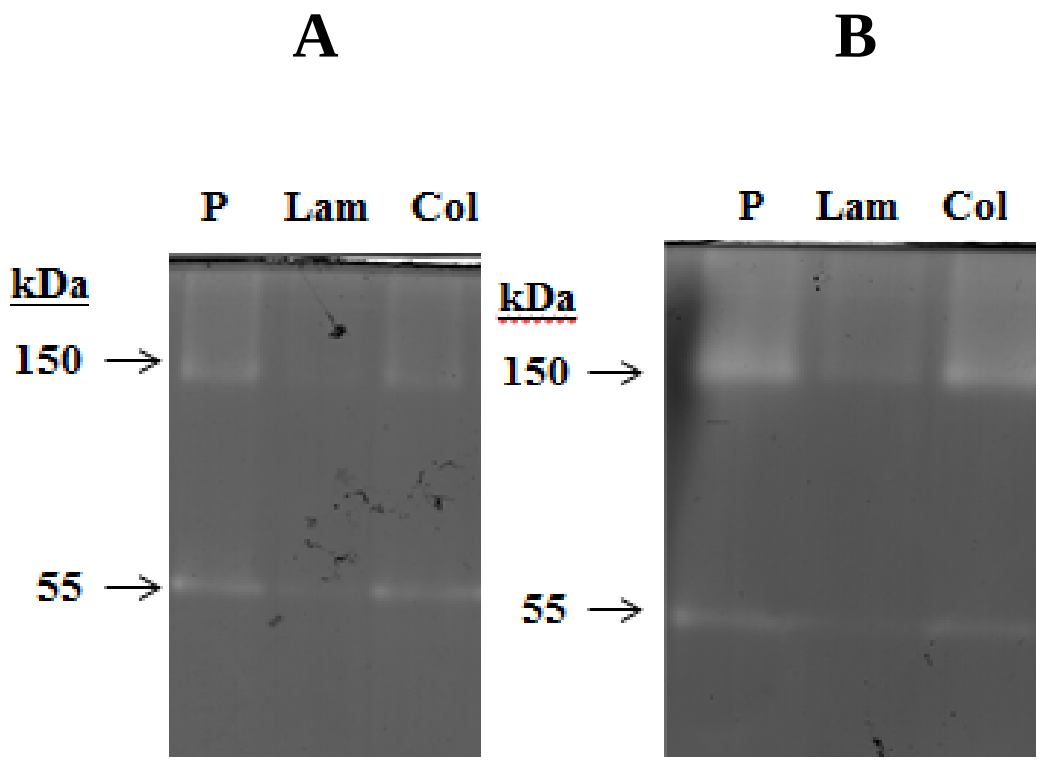


Figure 3: Profile of Protease Secretion by Mouse-Passaged or Axencially-Cultured *A. culbertsoni* during Attachment to ECM Components. Mouse-passaged (A) or axenically-cultured (B) *A. culbertsoni* ($5x10^6$) were added to 6-well tissue culture plates coated with either Laminin-1 or Collagen I. Uncoated, plastic wells served as the control. Following a 30 minute incubation period at 37°C the supernatants were collected, filtered and equal volumes of 25µL were analyzed using zymography. The arrows indicate 150-kDa and 55-kDa proteases that were visualized in supernatants collected during attachment of amoebae to uncoated, plastic wells (P), Laminin-1-coated wells (Lam) and Collagen I-coated wells (Col).

inspection, axenically-grown amoebae appeared to display greater protease activity, as seen in the zymograms, than their mouse-passaged counterparts; however, a statistically significant difference was not observed when the protease bands were analyzed using densitometry.

To further characterize these proteases, a number of inhibitor studies were performed in which aliquots of the supernatants from attachment assays involving axenic and mouse-passaged amoebae were pre-treated with cysteine, serine, or metalloprotease inhibitors for 30 minutes prior to analysis by zymography. Incubation with 5µg/µl of E-64, a cysteine protease inhibitor, as well as with saline (E-64 vehicle) failed to diminish or eliminate the protease bands seen at 150-kDa and 55-kDa indicating that the two bands did not represent cysteine proteases (**Figures 4 A-F)**. Treatment with 1,10-Phenanthroline, a metalloprotease inhibitor also failed to eliminate the 150-kDa and 55-kDa protease bands as did anhydrous ethanol (vehicle control for 1,10 Phenanthroline and PMSF). However, treatment of supernatants collected during the attachment of axenic and mouse-passaged *A. culbertsoni* to plastic, Laminin-1 and Collagen I with the serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF) resulted in complete abolishment of protease activity (**Figures 5 A-H**).

Having determined that serine proteases were secreted during attachment, the role of these proteases in mediating this process was assessed by pretreating amoebae with either E-64, PMSF, 1,10-Phenanthroline or the vehicle controls, saline and anhydrous ethanol, for 20 minutes prior to attachment to Laminin-1 or Collagen I. Both the percent attachment of amoebae to the ECM components as well as proteolytic profiles of secretion under each treatment condition were obtained. Pretreatment of amoebae with the various inhibitors and vehicles did not produce a significant decrease in attachment to either Collagen I or Laminin-1 as compared to their

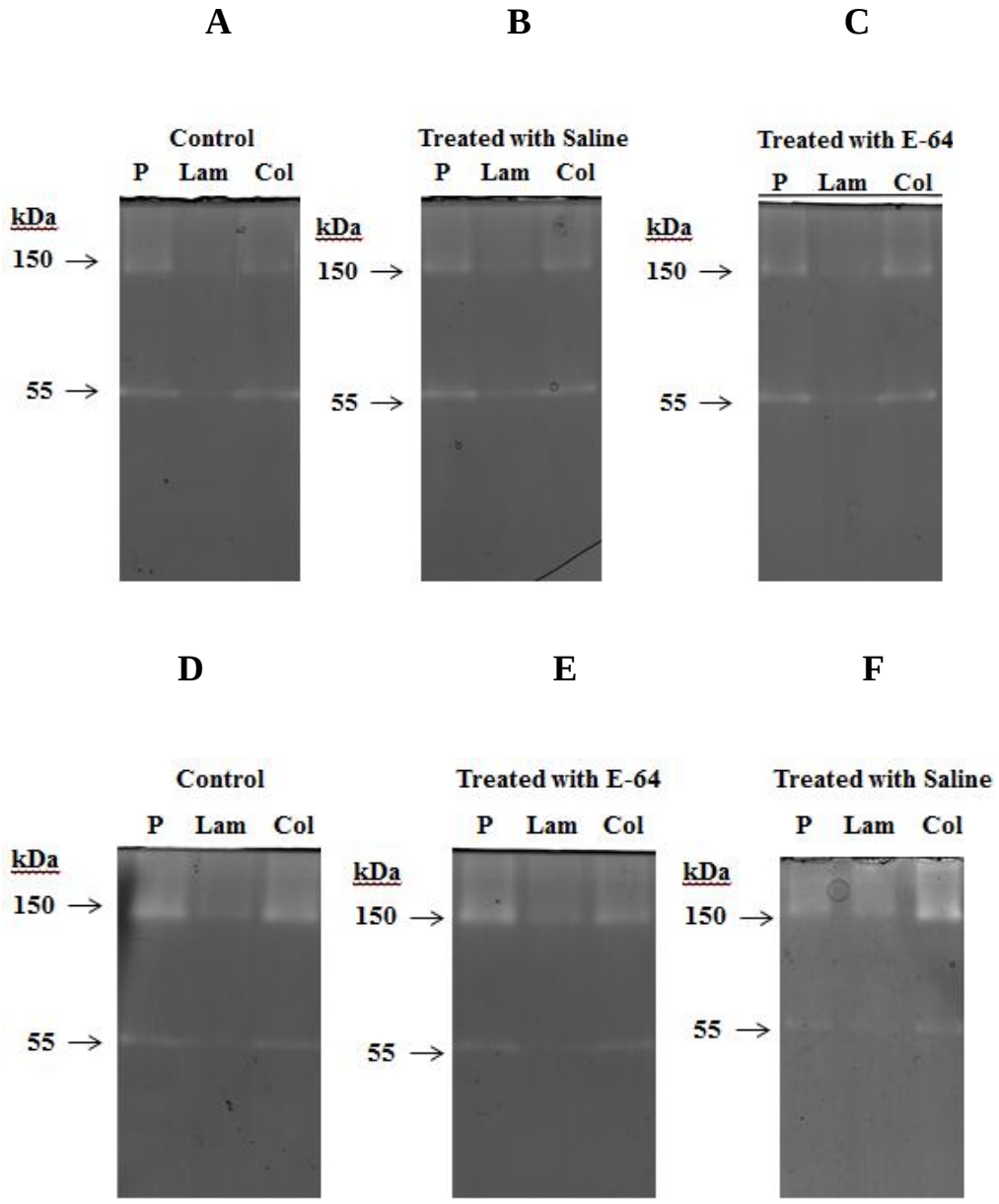


Figure 4: Proteases Secreted by Mouse-Passaged or Axenically-Cultured *A. culbertsoni* during Attachment to Plastic, Laminin-1 and Collagen I are not Inhibited by E-64. Aliquots of supernatants collected during the attachment of mouse-passaged (A-C) and axenically-cultured (D-F) *A. culbertsoni* ($5x10^6$) to ECM components Laminin-1 and Collagen I for 30 minutes at 37°C were treated with 5µg/µl of E-64, a cysteine protease inhibitor, or an equivalent amount of saline (E-64 vehicle) for 30 minutes. Subsequently, 25µl of treated supernatants were analyzed using zymography. Gels were developed at pH 7.5. Gels A & D represent untreated supernatant collected during attachment to plastic (P), Laminin-1 (Lam), and Collagen I (Col). Gels B & F depict proteolytic activity of theses samples as seen following a 30 minute incubation period with saline and gels C & E demonstrate the proteolytic activity visualized following a 30 minute treatment of supernatant samples with E-64.

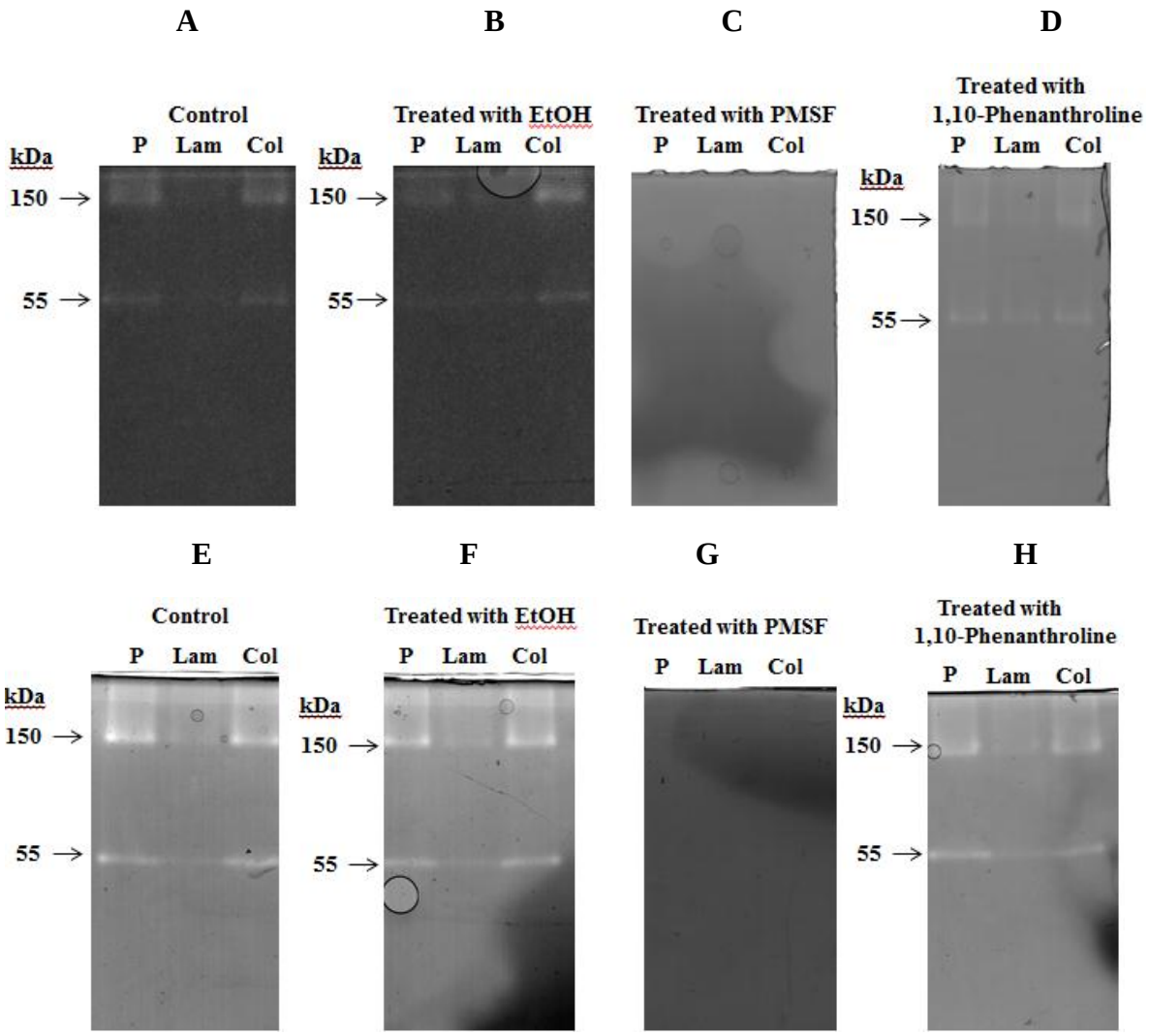


Figure 5: Proteases Secreted by Mouse-Passaged or Axenically-Cultured *A. culbertsoni* during Attachment to Plastic, Laminin-1 and Collagen I are not Inhibited by 1,10-Phenanthroline but are Inhibited by PMSF. Aliquots of supernatant collected during the attachment of mouse-passaged (A, B, C, D) and axenically-cultured (E, F, G,H) *A. culbertsoni* ($5x10^6$) to ECM components Laminin-1 and Collagen I for 30 minutes at 37°C were treated with either 5mM 1,10-Phenanthroline, a metalloprotease inhibitor, 5mM PMSF, a serine protease inhibitor, or an equivalent amount of anhydrous ethanol (1,10-Phenanthroline and PMSF vehicle) for 30 minutes. Subsequently, 25µl of treated supernatants were analyzed using zymography. Gels were developed at pH 7.5. Gels A & E represent untreated supernatant collected during attachment to the control condition -plastic (P), Laminin-1 (Lam), and collagan I (Col). B & F depict the proteolytic bands that were seen when supernatant samples were treated with the anhydrous ethanol vehicle. Gels C & G demonstrate a complete inhibition of both proteolytic activity bands when the three supernatant samples were incubated with PMSF prior to zymography. D & H demonstrate that proteolytic activity is visualized following a 30 minute treatment of supernatant samples with 1,10-Phenanthroline. Only PMSF results in inhibition of both the 150-kDa and 55-kDa bands indicating the presence of serine proteases.

respective controls. This was seen for both axenic and mouse-passaged amoebae (**Tables 2-3**). Furthermore, zymographic analysis of the supernatants collected from attachment assays performed with pretreated amoebae revealed the presence of both the approximately 150-kDa and 55-kDa protease activity bands under all experimental conditions. However, secretion of these two protease bands was greatly diminished when amoebae were pretreated with PMSF (**Figures 6-7)**.

The attachment of axenic and mouse-passaged amoebae to Laminin-1 and Collagen I was not decreased significantly despite diminished secretion of the approximately 150-kDa and 55-kDa serine proteases. In order to determine whether secretion of these proteases was specific to the attachment process or whether their production was constitutive, 45 minute and 24 hour *A. culbertsoni*-conditioned medium generated using amoebae growth medium, PYG and serum-free Oxoid, or Hank's Balanced Salt Solution (HBSS), was analyzed for proteases. While constitutive secretion of proteases appeared more robust in 24 hour-conditioned medium as compared to 45 minute-conditioned medium, approximately 150-kDa and 55-kDa serine proteases were again the only two bands present at both time periods. This was determined by treating aliquots of conditioned medium with 5mM PMSF, 5mM 1, 10-Phenanthroline, 5µg/µl E-64, or the vehicle controls for 30 minutes prior to use for zymography (**Figures 8-11**). Furthermore, 24 hour *A. culbertsoni*-conditioned PYG, serum-free Oxoid, and HBSS media were subjected to western blot analysis using anti-*A.culbertsoni* and *A.castellani* antibodies. Proteins bands of approximately 55, 58, 65, and 75-kDa were recognized by both the anti-*A.culbertsoni* and *A.castellani* antibodies (**Figure 12**).

The proteolytic profiles generated from the attachment assays involving untreated and treated amoebae, as well as the different conditioned media, were compared to those obtained by

Table 2: Attachment of Axenic and Mouse-Passage *A. culbertsoni* to Laminin-1 Following Pretreatment with Inhibitors

Attachment Substrate	Pre-treatment	% Attachment by Axenic *A. culbertsoni*	% Attachment by Mouse-passaged *A. culbertsoni*
Plastic	None	96%	92%
Laminin-1	None	98%	98%
Laminin-1	1mM PMSF	94%	92%
Laminin-1	0.5% Ethanol (by volume)	97%	97%
Laminin-1	1µM 1,10-Phenanthroline	97%	96%
Laminin-1	1µg/µl E-64	97%	96%
Laminin-1	1% Saline (by volume)	97%	97%

Table 2: Following a 20 minute pre-incubation of *A. culbertsoni* ($5x10^6$) with the above mentioned inhibitors and vehicles, treated amoebae were added to tissue culture wells in 6-well plates that were coated previously with Laminin-1. Uncoated plastic wells served as the ECM control. Pellets of unattached amoebae were separated out from the supernatant that was collected following a 30 minute attachment period. These pellets were resuspended in 1mL of 2.5% glutaraldehyde, unattached amoebae were counted using a hemocytometer, and percent attachment was calculated using the following formula:

$$\frac{5x10^6 \text{ Amoebae} - \#\text{ of Unattached Amoebae}}{5x10^6 \text{ Amoebae}} \times 100\%$$

Table 3: Attachment of Axenic and Mouse-Passage *A. culbertsoni* to Collagen I Following Pretreatment with Inhibitors

Attachment Substrate	Pre-treatment	% Attachment by Axenic *A. culbertsoni*	% Attachment by Mouse-passaged *A. culbertsoni*
Plastic	None	97%	94%
Collagen I	None	97%	96%
Collagen I	1mM PMSF	97%	95%
Collagen I	0.5% Ethanol (by volume)	97%	97%
Collagen I	1µM 1,10-Phenanthroline	97%	96%
Collagen I	1µg/µl E-64	98%	96%
Collagen I	1% Saline (by volume)	98%	97%

Table 3: Following a 20 minute pre-incubation of *A. culbertsoni* ($5x10^6$) with the above mentioned inhibitors and vehicles, treated amoebae were added to tissue culture wells in 6-well plates that were previously coated with collagen I. Uncoated plastic wells served as the ECM control. Pellets of unattached amoebae were separated out from the supernatant that was collected following a 30 minute attachment period. These pellets were resuspended in 1mL of 2.5% glutaraldehyde, unattached amoebae were counted using a hemocytometer, and percent attachment was calculated using the following formula:

$$\frac{5x10^6 \text{ Amoebae} - \#\text{ of Unattached Amoebae}}{5x10^6 \text{ Amoebae}} \times 100\%$$

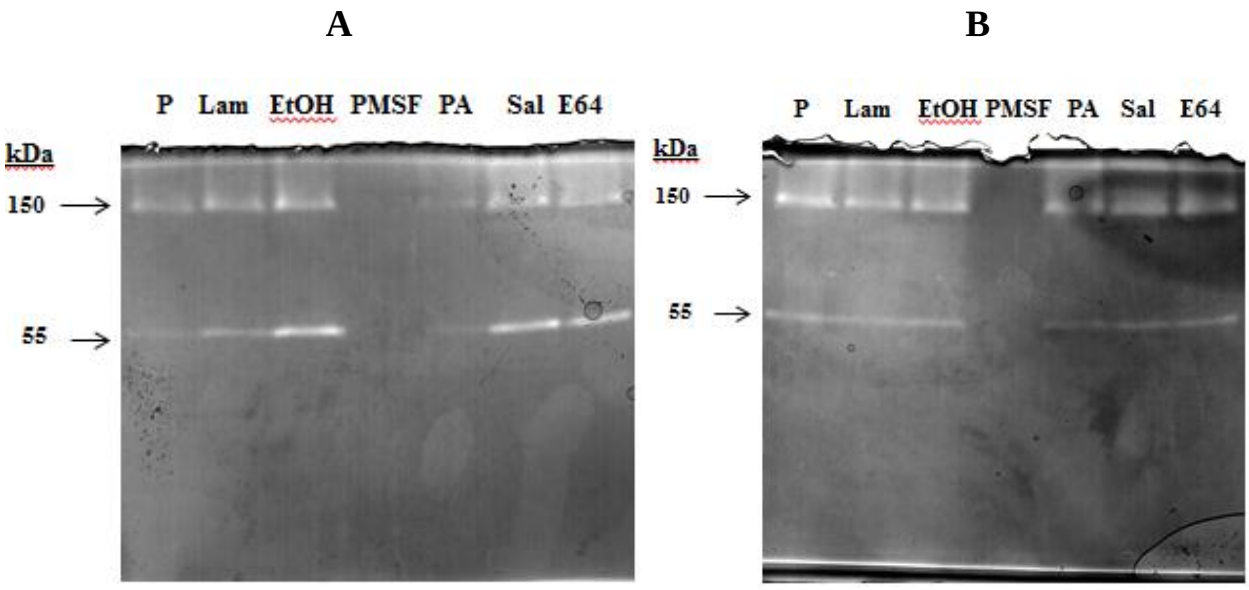


Figure 6: Proteases Secreted by Pretreated, Mouse-Passaged or Axenically-Cultured *Acanthamoeba culbertsoni* during Attachment to Laminin-1. $5x10^6$ mouse-passaged (A) or axenically-cultured (B) amoebae were pretreated for 20 minutes at 37°C with either 1µg/µl of E-64, 1% Saline by volume, 1mM PMSF, 0.5% anhydrous ethanol by volume, or 1µM 1,10-Pheanthroline. Subsequently, amoebae were added to tissue culture wells in a 6-well plate that had been previously coated with Laminin-1 for 30 min at 37°C. Uncoated plastic wells served as the ECM control. The supernatant was collected for each treatment condition, centrifuged to remove unattached amoebae and then 25µL aliquots were analyzed using zymography. Gels were developed at pH 7.5 and visualized using Coomassie Brilliant Blue. (P) represents the proteases secreted during attachment of untreated amoebae to plastic. (Lam) represents proteases visualized following attachment of untreated amoebae to Laminin-1. The remaining 5 lanes depict secreted protease present in supernatant collected following the attachment of amoebae that had been pretreated with either ethanol (EtOH), saline (Sal), PMSF (PMSF), 1,10-Phenanthroline (PA), or E-64(E64) to Laminin-1. The 150-kDa protease band and 55-kDa protease band are present under all conditions; however, band intensity was decreased significantly when amoebae were pretreated with PMSF.

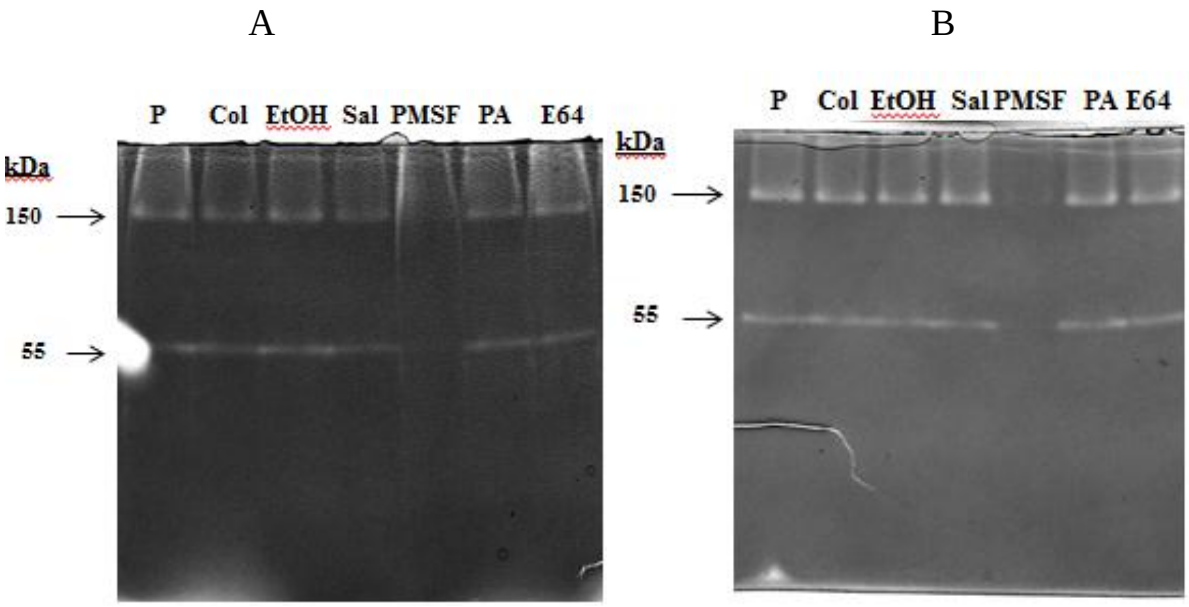


Figure 7: Proteases Secreted by Pretreated, Mouse-Passaged or Axenically-Cultured *A. culbertsoni* during Attachment to Collagen I. $5x10^6$ mouse-passaged (A) or axenically-cultured (B) amoebae were pretreated for 20 minutes at 37°C with either 1µg/µl of E-64, 1% Saline by volume, 1mM PMSF, 0.5% anhydrous ethanol by volume, or 1µM 1,10-Pheanthroline. Subsequently, amoebae were added to tissue culture wells in a 6-well plate that had been previously coated with Collagen I for 30 min at 37°C. Uncoated plastic wells served as the ECM control. The supernatant was collected for each treatment condition, centrifuged to remove unattached amoebae and then 25µL aliquots were analyzed using zymography. Gels were developed at pH 7.5 and visualized using Coomassie Brilliant Blue. (P) represents the proteases secreted during attachment of untreated amoebae to plastic. (Col) represents proteases visualized following attachment of untreated amoebae to Collagen I. The remaining 5 lanes depict secreted protease present in supernatant collected following the attachment of amoebae that had been pretreated with either ethanol (EtOH), saline (Sal), PMSF (PMSF), 1,10-Phenanthroline (PA), or E-64(E64) to Collagen I. The 150-kDa protease band and 55-kDa protease band are present under all conditions; however, band intensity was significantly decreased when amoebae were pretreated with PMSF.

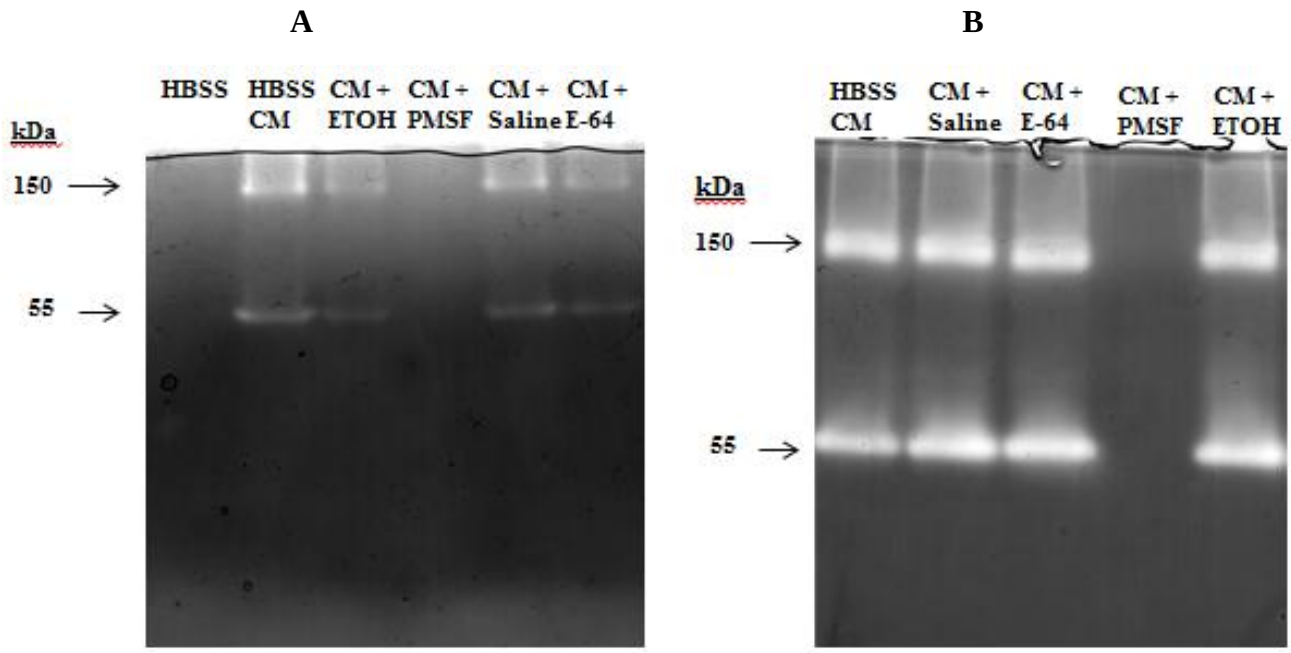


Figure 8: Proteolytic Profile of *A. culbertsoni*-Conditioned HBSS Media at 45 minutes and 24 hours Demonstrates Constitutive Secretion of Serine Proteases. $5x10^6$ of mouse-passaged amoebae were placed in a conical tube containing Hank's Balanced Salt Solution (total volume = 5mL). Amoebae were allowed to incubate in the HBBS for either 45 minutes or 24 hours at 37°C. This medium then was classified as either 45 minute amoebae-conditioned media (ACM) or 24 hour ACM. The ACM was then centrifuged twice to remove all amoebae. Aliquots of the 45 minute ACM and 24 hour ACM were then treated with either anhydrous ethanol (CM + EtOH), 5mM PMSF (CM+PMSF), saline (CM + Saline), or 5µg/µl E-64 (CM+E-64) for 30 minutes prior to analysis by zymography. Gels were developed at pH 7.5 and stained using Coomassie Brilliant Blue. Unconditioned HBSS (HBSS) was used to demonstrate the absence of proteases in the HBSS medium. HBSS CM represents *A. culbertsoni* conditioned HBSS medium that was not treated with any inhibitors or vehicles. The gel on the left, A, represents bands visualized in 45 minute HBSS-conditioned medium whereas the gel on the right, B, depicts bands visualized in 24 hour-conditioned medium. Two proteases bands were visualized at approximately 150-kDa and 55-kDa and complete inhibition of activity at both bands was only achieved following pretreatment with PMSF indicating that these bands represented serine proteases.

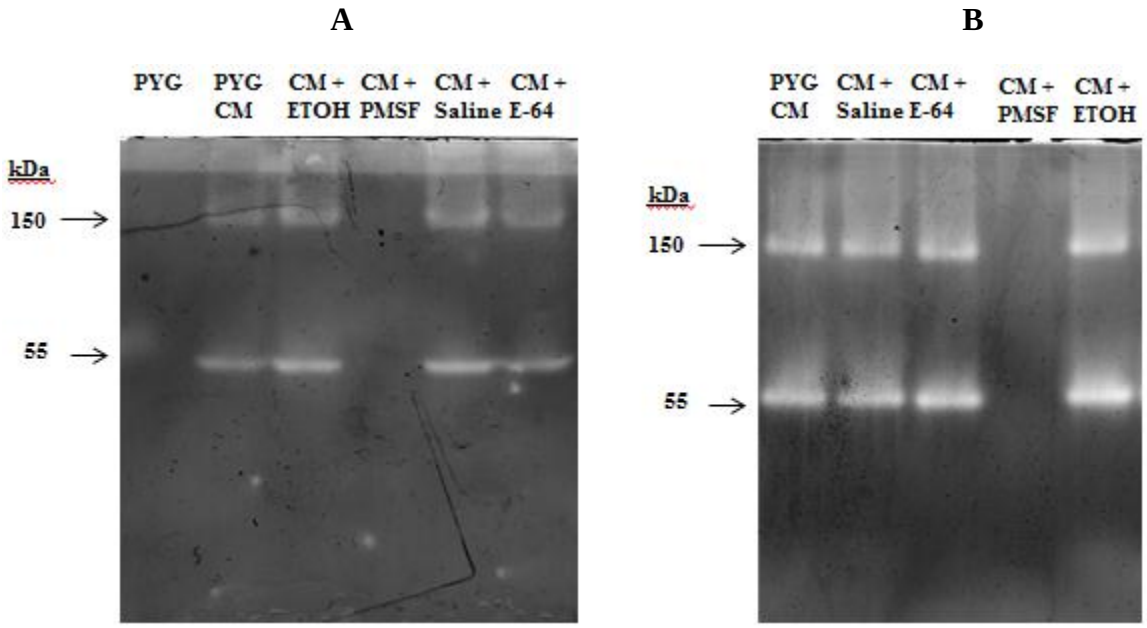


Figure 9: Proteolytic Profile of *A. culbertsoni*-Conditioned PYG Medium at 45 minutes and 24 hours Demonstrates Constitutive Secretion of Serine Proteases. $5x10^6$ of mouse-passaged amoebae were placed in a conical tube containing PYG (total volume = 5mL). Amoebae were allowed to incubate in the PYG for either 45 minutes or 24 hours at 37°C. This medium then was classified as either 45 minute amoebae-conditioned medium (ACM) or 24 hour ACM. The ACM then was centrifuged twice to remove all amoebae. Aliquots of the 45 minute ACM and 24 hour ACM were then treated with either anhydrous ethanol (CM + EtOH), 5mM PMSF (CM+PMSF), saline (CM + Saline), or 5µg/µl E-64 (CM+E-64) for 30 minutes prior to analysis by zymography. Gels were developed at pH 7.5 and stained using Coomassie Brilliant Blue. Unconditioned PYG (PYG) was used to demonstrate the absence of proteases in the PYG media. PYG CM represents *A. culbertsoni*-conditioned PYG medium that was not treated with any inhibitors or vehicles. The gel on the left, A, represents bands visualized in 45 minute PYG-conditioned medium whereas the gel on the right, B, depicts bands visualized in 24 hour PYG-conditioned medium. Two proteases bands were visualized at approximately 150-kDa and 55-kDa and complete inhibition of activity at both bands was only achieved following pretreatment with PMSF indicating that these bands represented serine proteases.

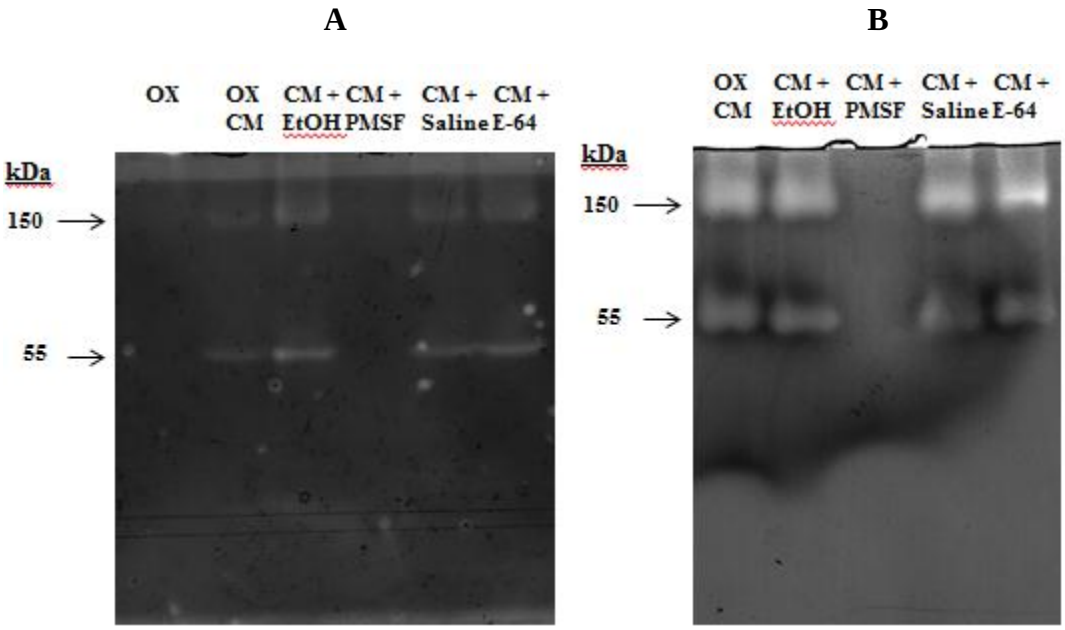


Figure 10: Proteolytic Profile of *A. culbertsoni*-Conditioned Serum-Free Oxoid Medium at 45minutes and 24 hours Demonstrates Constitutive Secretion of Serine Proteases. $5x10^6$ of mouse-passaged amoebae were placed in a conical tube containing serum-free Oxoid (total volume = 5mL). Amoebae were allowed to incubate in the serum-free Oxoid for either 45 minutes or 24 hours at 37°C. This medium was then classified as either 45 minute amoebae-conditioned media (ACM) or 24 hour-ACM. The ACM was then centrifuged twice to remove all amoebae. Aliquots of the 45 minute ACM and 24 hour ACM then were treated with either anhydrous ethanol (CM + EtOH), 5mM PMSF (CM+PMSF), saline (CM + Saline), or 5µg/µl E-64 (CM+E-64) for 30 minutes prior to analysis by zymography. Gels were developed at pH 7.5 and stained using Coomassie Brilliant Blue. Unconditioned serum-free Oxoid (Ox) was used to demonstrate the absence of proteases in the serum-free Oxoid media. Ox CM represents *A. culbertsoni* conditioned serum-free Oxoid media that was not treated with any inhibitors or vehicles. The gel on the left, A, represents bands visualized in 45 minute serum-free Oxoid-conditioned media whereas the gel on the right, B, depicts bands visualized in 24 hour serum-free Oxoid-conditioned media. Two proteases bands were visualized at approximately 150-kDa and 55-kDa and complete inhibition of activity at both bands was achieved only following pretreatment with PMSF indicating that these bands represented serine proteases.

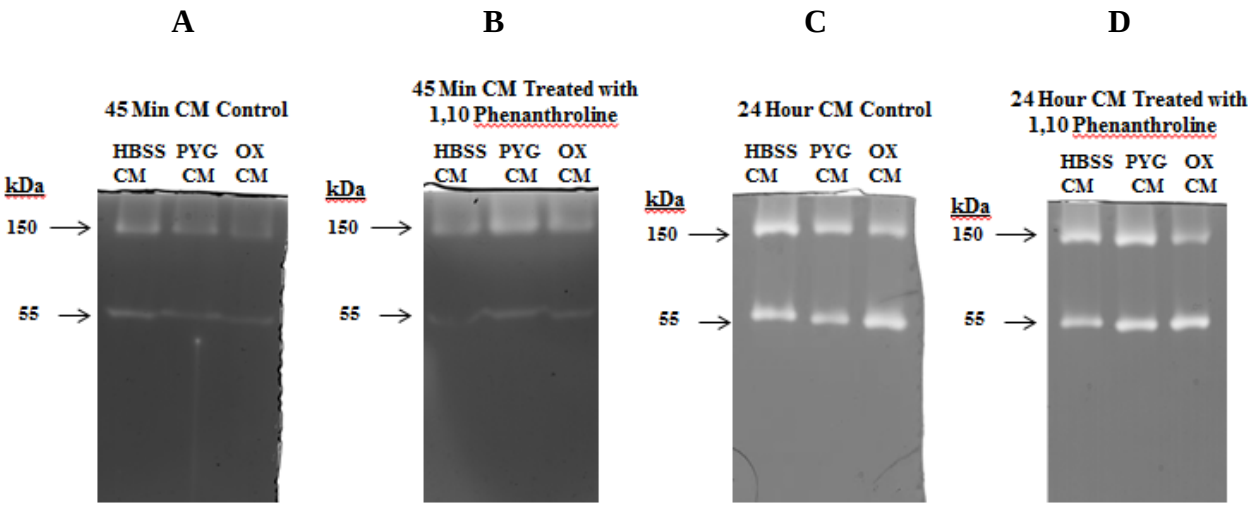


Figure 11: Proteolytic Profiles of *A. culbertsoni*-Conditioned Mediums at 45 Minutes and 24 Hours do not Demonstrate Secretion of Metalloproteases. 1, 10-Phenanthroline is a reversible inhibitor and therefore 5mM 1,10-Phenanthroline was added to aliquots of 45 minute and 24 hour HBSS, PYG, and serum-free Oxoid conditioned for a 30 minute incubation period prior to analysis by zymography and also was added to the renaturing and developing buffers during processing of zymograms. Gel A depicts proteases visualized in 45 minute HBSS-conditioned medium (HBSS CM), PYG-conditioned medium (PYG CM) and serum-free Oxoid medium (OX CM). B shows the bands seen when these three 45 minute condition medias were treated with 1, 10-Phenanthroline. Gel C represents the proteolytic profile of 24 hour HBSS-conditioned medium, PYG-conditioned medium and serum-free Oxoid-conditioned media. The last zymogram, D, shows the protease bands that were present after treatment of the three 24 hour-conditioned media with 1, 10-Phenanthroline. Under all four conditions protease bands of approximately 150-kDa and 55-kDa are present indicating. Their presence despite treatment with 1, 10-Phenanthroline is indicative of the fact that these protease bands are not due to metalloprotease activity.

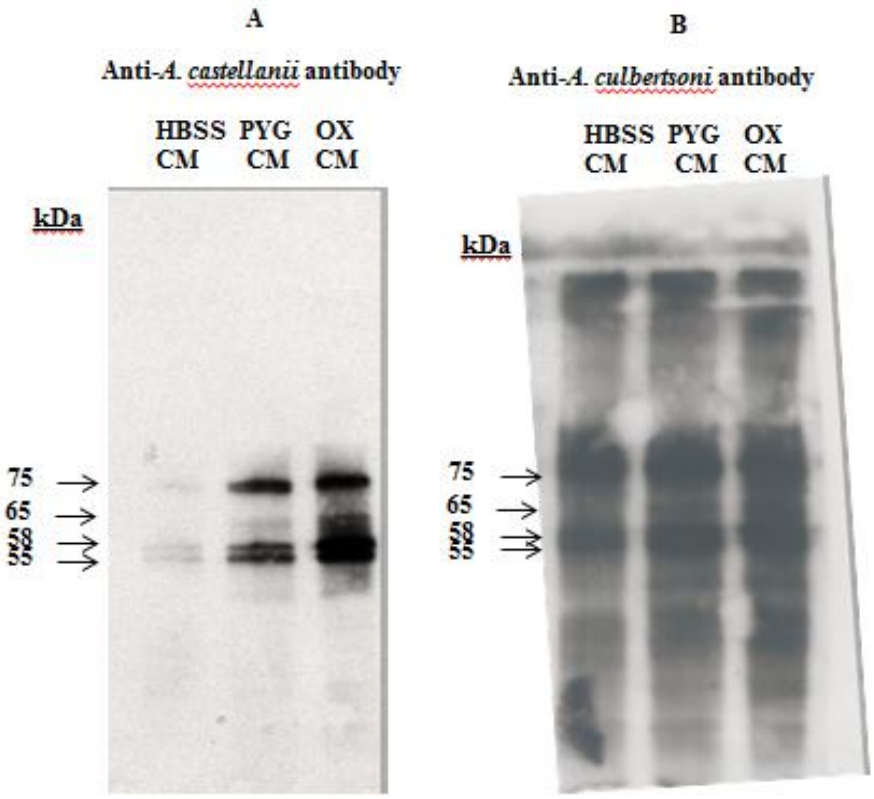


Figure 12: Western Blot of 24 hour *A, culbertsoni*-Conditioned Medium Probed Using Anti-*A.castellanii* and *A. culbertsoni* antibodies. Western blot analysis was performed on 24 hour *A.culbertsoni*-conditioned medium generated in HBSS (HBSS CM), PYG (PYG CM), and serum-free Oxoid (OX CM). Membranes were probed using either anti-*A. castellani* (A) or anti-*A.culbertsoni* antibodies (B). Both antibodies detected bands corresponding to approximately 55, 58, 65, and 75-kDa.

analysis of zymography of an *A. culbertsoni* whole cell lysate. Inhibitor studies performed on aliquots of this whole cell lysate revealed the presence of serine proteases of approximately 150, 140, 100, 55, 50, and 38-kDa (**Figure 13**). Only two of the six proteolytic bands present in *A. culbertsoni* whole cell lysate were detected in supernatants collected during attachment and in *A. culbertsoni* conditioned media.

We have determined also that *A. culbertsoni* invades Matrigel, a reconstituted basement membrane, and Collagen type I. This was visualized using Scanning Electron Microscopy (**Figure 14-15**). Amoebae also were observed by inverted light microscopy to be present in the bottom chamber following 3 hour invasion assays involving both Matrigel and Collagen I scaffolds. In order to determine whether secreted proteases participated during the invasion process of Matrigel and Collagen type I, supernatants collected following 3 hour invasion assays were subjected to inhibitor treatments in the same manner as supernatants collected from previously discussed attachment assays. Again, two serine protease bands were visualized at approximately 150 and 55-kDa. These bands were present when amoebae invaded uncoated tissue culture inserts with 8µm pores as well as following invasion of tissue culture inserts coated with 30µl layers of 2.7mg/ml of either Matrigel or Collagen I (**Figure 16**). Invasion of Collagen I by mouse-passaged amoebae that were pretreated for 20 minutes at 37°C with either 1µg/µl of E-64, 1% Saline by volume, 1mM PMSF, 0.5% anhydrous ethanol by volume, or 1µM 1,10-Pheanthroline also was explored. Treatment with the different vehicles and inhibitors did not significantly impact invasion of Collagen I and a decrease in invasion was not observed in amoebae pretreated with PMSF (**Table 4**).

The existence of membrane-associated proteases also was probed in the event that possible proteolytic degradation during invasion of either the Matrigel or Collagen I scaffolds

occurred in a localized manner rather than through constitutive secretion. Membrane fractions of mouse-passaged *A. culbertsoni* were isolated and subjected to zymography. Aliquots also were treated with E-64, PMSF, and 1,10-Phenanthroline to determine the class(s) of proteases. The 150 and 55-kDa serine protease bands were visualized in addition to an additional serine protease band of approximately 140 –kDa (**Figure 17**). Furthermore, western blot analysis of *A. culbertsoni* whole cell lysate and membrane fraction samples using a polyclonal αMT1-MMP, a membrane type matrix metalloprotease, antibody was negative; confirming that this membrane associated protease was not present in the membrane fraction at a level that was otherwise undetectable by zymography.

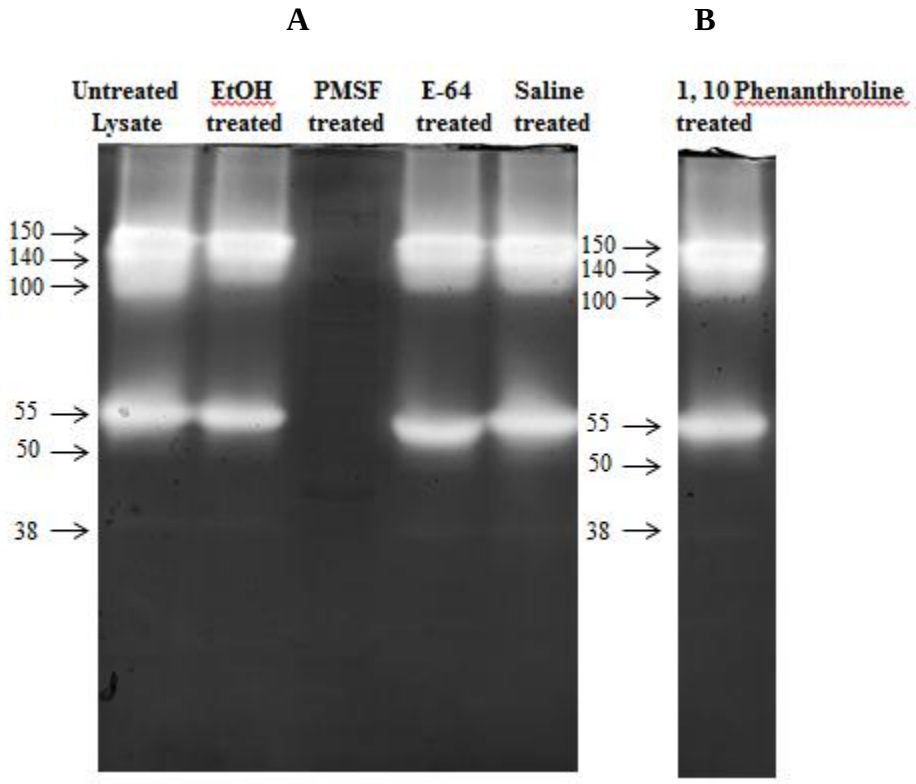


Figure 13: Proteases Present in a Whole Cell Lysate of *A. culbertsoni.* Aliquots of *A. culbertsoni* whole cell lysate were treated with either 5µg/µl of E-64, Saline, 5mM PMSF, anhydrous ethanol, or 5mM 1,10-Phenanthroline for 30 minutes prior to analysis using zymography. Following electrophoresis the lane containing 1,10-Phenanthroline-treated whole cell lysate was separated from the remainder of the gel so that 1,10-Phenanthroline could also be during the renaturing and developing process of the zymogram as 1,10-Phenanthroline is a reversible inhibitor. Gels were stained using Coomassie Brilliant Blue. Bands of approximately 150, 140, 100, 55, 50, and 38-kDa were observed. Proteolytic activity of all bands was only eliminated by PMSF indicating that these bands represented serine proteases.

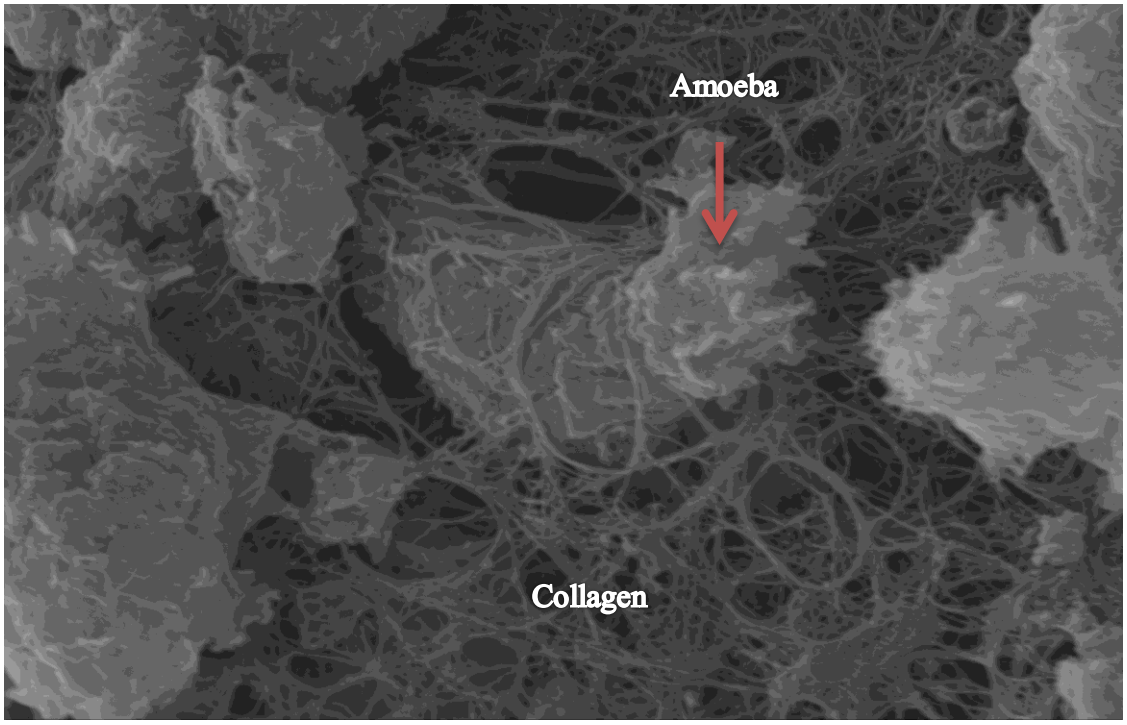


Figure 14: Invasion of a Collagen I Scaffold by *A.culbertsoni.* A 30µl layer of 2.7mg/ml of Collagen I was added to tissue culture insert of 8µm pore size, which was situated in a 24-well plate, and allowed to polymerize for 2h at 37°C. Subsequently $5x10^6$ amoebae were added to the top chamber and serum-free Oxoid was added to the bottom chamber to serve as chemoattractant. Following a 3 h invasion period, tissue culture inserts were processed for SEM. The scanning electron micrograph above shows *A. culbertsoni* invading a Collagen I scaffold during this 3 h invasion period.

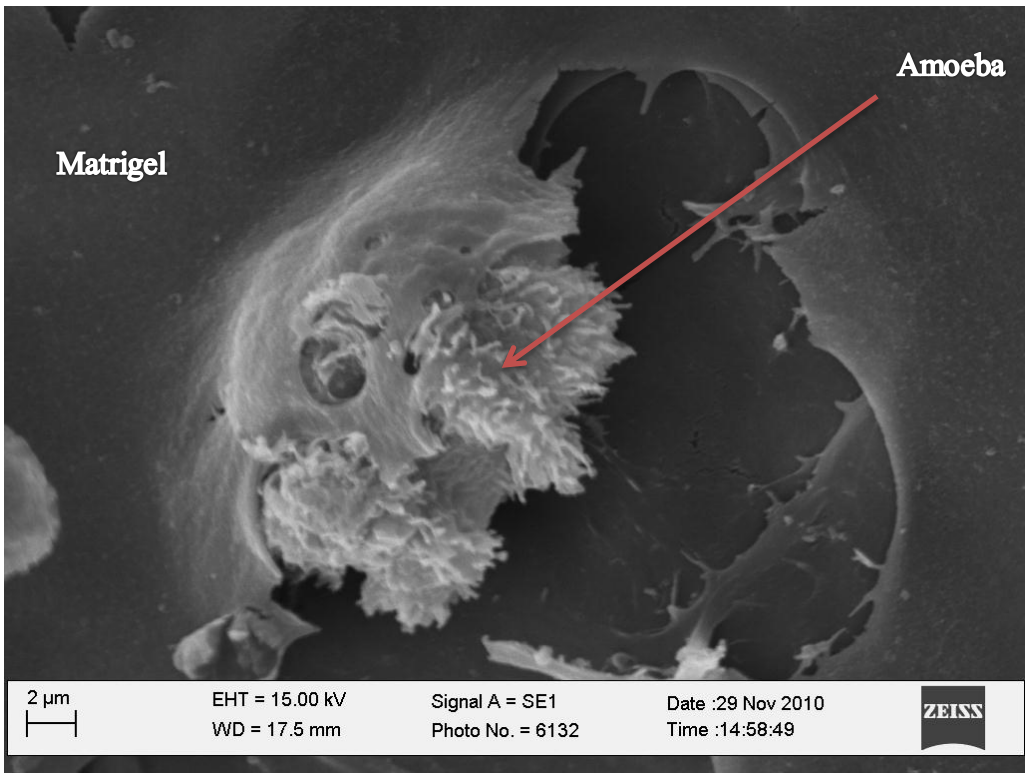


Figure 15: Invasion of a Matrigel Scaffold by *A. culbertsoni.* A 30µl layer of 2.7mg/ml of Matrigel was prepared in the same manner as Collagen I (in the previous figure). $5x10^6$ amoebae were added to the top chamber and serum-free Oxoid was added to the bottom chamber. The scanning electron micrograph here shows *A. culbertsoni* invading a Matrigel scaffold during a 3 h invasion period.

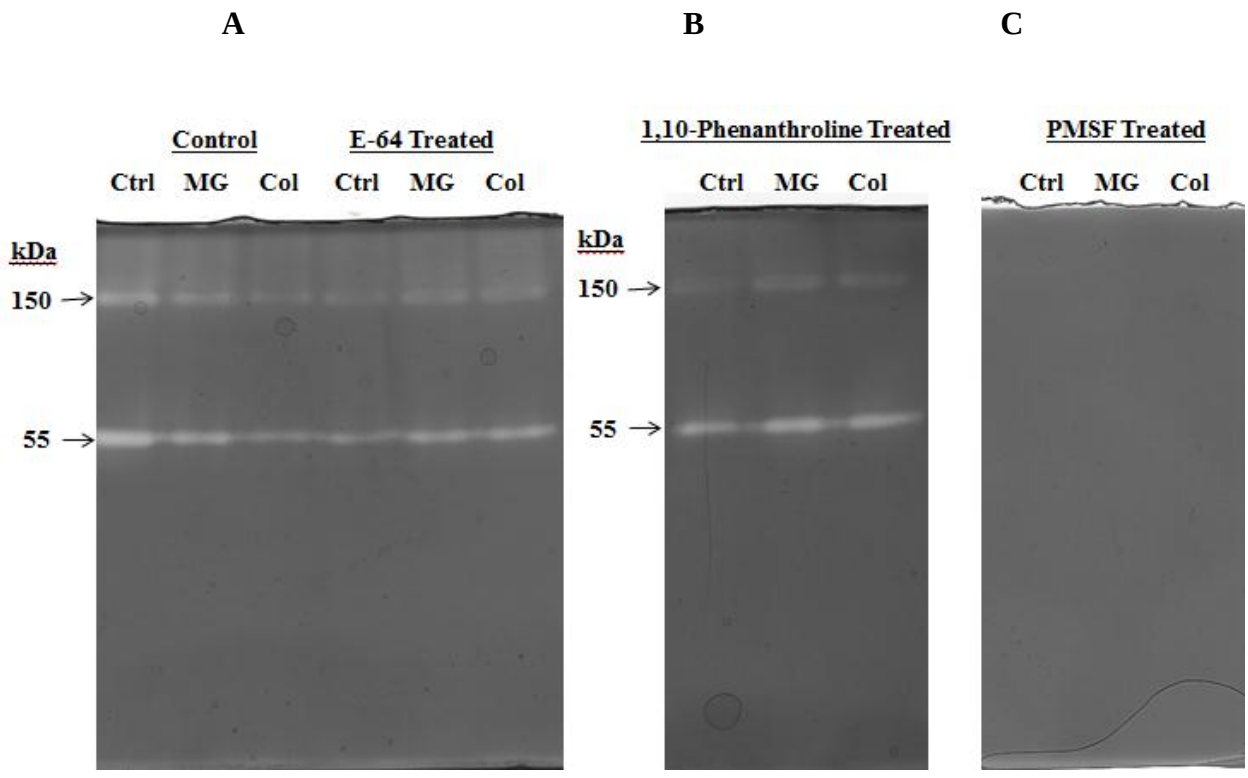


Figure 16: Proteases Secreted by Mouse-Passaged *A. culbertsoni* during Invasion of Matrigel and Collagen I. Tissue culture inserts of 8µm pore size were coated with either 30µl of 2.7mg/ml of Matrigel, Collagen I or left uncoated (control). Inserts were placed in 24-well tissue culture plates. Following a 2 hour polymerization period at 37°C, $5x10^6$ amoebae were place in the top chamber of the tissue culture inserts and 700µl of serum-free Oxoid were placed into tissue culture wells (bottom chamber) so as to surround the tissue culture inserts in liquid media that would serve as a chemoattractant for invading amoebae. Following a 3 hour incubation period at 37°C, supernatant from the top and bottom chambers was collected, centrifuged to remove amoebae, and aliquots were treated with inhibitors for 30 minutes and analyzed using zymography. Supernatant collected during the invasion of amoebae through uncoated tissue culture inserts served as the control and is designated as Ctrl. Supernatant collected during the invasion of Matrigel is designated as MG and Col represents supernatant collected from a Collagen I invasion assay. Gel A depicts the protease bands seen in untreated supernatant from three conditions and supernatant aliquots treated with 5 µg/µl of E-64. Gel B represents the proteolytic profile of control, Matrigel and Collagen I supernatant treated with 5mM 1,10-Phenanthroline. Gel C demonstrates complete inhibition of the activity of the 150 and 55-kDa protease bands seen in supernatant samples from all three invasion conditions, indicating their nature as serine proteases. No differences were noted in the proteolytic profiles of aliquots treated with inhibitor vehicles, saline and anhydrous ethanol (data not shown here).

Table 4: Invasion of Collagen I by Mouse-Passaged *A. culbertsoni.*

Invasion Substrate	Pretreatment	% Invasion
Uncoated Tissue Culture Insert	None	1.3%
Collagen I	None	0.175%
Collagen I	1mM PMSF	0.2%
Collagen I	0.5% Ethanol (by volume)	0.15%
Collagen I	1µM 1,10-Phenanthroline	0.2%
Collagen I	1µg/µl E-64	0.15%
Collagen I	1% Saline (by volume)	0.15%

Table 4: Invasion of Collagen I by Mouse-Passaged *A. culbertsoni.* Following a 20 minute pre-incubation of *A. culbertsoni* ($5x10^6$) with the above mentioned inhibitors and vehicles, treated amoebae were added to tissue culture inserts that had been previously coated with 30µl of 2.7mg/ml Collagen I. Uncoated inserts served as the control. Following a 3 hour invasion period inserts removed from wells containing serum-free Oxoid and the amoebae that had invaded during this time period. In order to dislodge amoebae from the wells, 24-well plates were placed at 4°C overnight. Subsequently 300 µL of 5mM EDTA were added to further facilitate dislodgement. The contents of the well were removed and amoebae were counted using a hemocytometer. Percent invasion was calculated as follows:

$$\frac{\text{\# of amoebe that invaded}}{5x10^6 \text{ amoebae}} \quad X\ 100\%$$

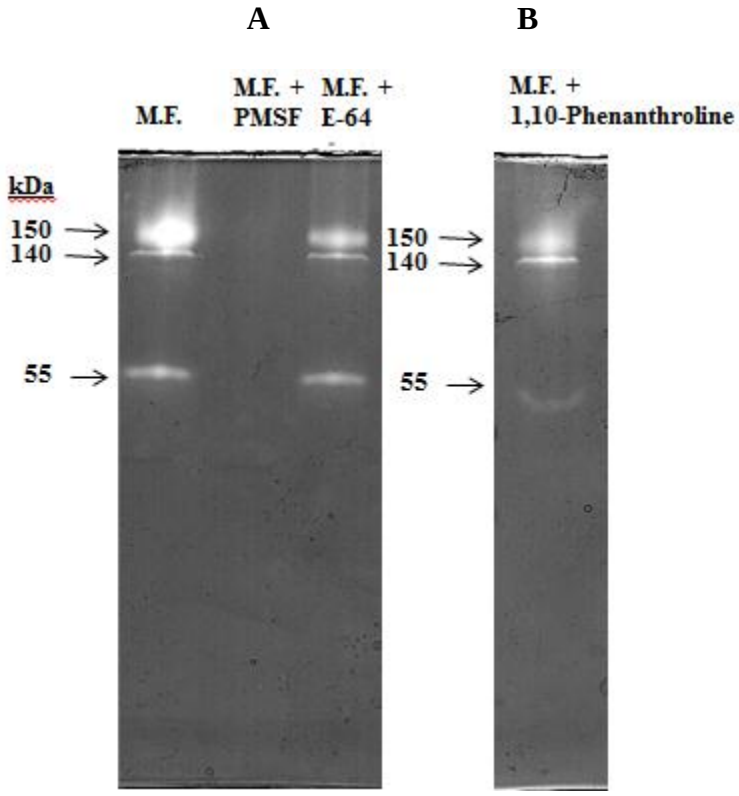


Figure 17: Protease Present in the Membrane Fraction of Mouse-Passaged *A. culbertsoni.* Membrane fractions of mouse-passaged *A. culbertsoni* were obtained using the Mem-PER Eukaryotic Membrane Protein Extraction Kit. 20µg aliquots of protein were treated with either 5µg/µl of E-64, saline (E-64 vehicle), 5mM PMSF, 5mM 1,10 Phenanthroline, and anhydrous ethanol (PMSF and 1,10-Phenanthroline vehicle) for 30 minutes and then analyzed using zymography. Protease bands of approximately 150, 140, and 55-kDa were observed in the membrane fraction (M.F.). In Gel A, zymographic analysis of untreated membrane fraction, membrane fraction treated with PMSF (M.F. + PMSF) and E-64 (M.F. + E-64) can be seen. Gel B is a zymogram representing bands seen following treatment of the membrane fraction with 1,10-Phenanthroline. No differences were noted in the proteolytic profiles of aliquots treated with inhibitor vehicles, saline and anhydrous ethanol (data not shown here). Protease activity is only eliminated when the membrane fraction is treated with PMSF suggesting that the bands represent serine proteases.

Chapter 4: Discussion

An understanding of the pathophysiological sequence of events associated with *Acanthamoeba* infections remains limited; however, common features of the three disease processes appear to be cytotoxicity and lytic protease capabilities among pathogenic species and the attachment and invasion of extracellular matrix components as requisites for amoebic dissemination and disease progression.

Pathogenic species of *Acanthamoeba* have been shown to possess a vast repertoire of secreted serine, cysteine, and metalloproteases [Alfieri et al., 2000; Cao et al,, 1998; Hadas and Mazur, 1993; Han et al., 2004; Hong et al., 2002; Hurt et al., 2003; Khan 2000; Kong et al.,2000; Mitra et al., 1995; Mitro et al., 1994; Serrano-Luna et al., 2006]. Although the specific functions of secreted *Acanthamoeba* proteases continue to be investigated, several studies have suggested that they play a role in encystment, lysis of bacterial cell walls, and mediation of host cell toxicity [Leitsch et al., 2010; Moon et al., 2008; Dudley et al., 2008; Rosenthal et al., 1969; Hurt et al., 2003; He et al., 1990; Kong et al., 2000; Mitra et al, 1995]. The establishment of an initial infection by an extracellular pathogen involves two significant events. First, the pathogen must come in contact with host cells. Subsequent dissemination from an original site of infection,

as mediated by tissue invasion, requires the interaction of that pathogen with components of the host extracellular matrix. A number of studies have been conducted in which the interaction of *Acanthamoeba* spp. with corneal epithelial cells has been assessed. Using *in vivo* and *in vitro* models of Amoebic Keratitis it has been demonstrated that lectin-glycoprotein interactions contribute significantly to the attachment of amoebae to host cells. This has been reported to be mediated through a 400-kDa Mannose Binding Protein (MBP) present on the surface of *A. castellanii* [Garate et al., 2004; Panjwani, 2010]. This method of attachment is not unique to *Acathamoeba*. It has been shown that both *Pseudomonas aeruginosa* and *Entamoeba histolytica* (*E.histolytica*) employ lectin-glycoprotein binding to mediate their respective infections [Wu et al., 1995; Huston, 2004]. Additionally, *E. histolytica* demonstrates the ability to secrete cytolytic factors following this initial binding [McCoy et al., 1994]. With *A. castellani* it has been demonstrated that interaction between host mannose residues through the amoebic MBP elicits the production of a 133-kDa serine protease known as the Mannose Induced Protein (MIP) along with other cytopathic factors [Hurt et al., 2003; Clarke and Niederkorn, 2006; Panjwani, 2010]. MIP has been shown to be highly cytopathic to both human and Chinese hamster corneal epithelial cells *in vitro* [Hurt et al., 2003]. Furthermore, corneal epithelial cell cytotoxicity and disaggregation were demonstrated when human corneal epithelial cell monolayers as well as intact human corneas were placed in contact with *Acanthamoeba*-conditioned medium as well as trophozites belonging to several different pathogenic isolates [Khan et al., 2000; Omana-Molina et al., 2010].

Interactions of the extracellular pathogen, *A. culberstoni,* with components of the extracellular matrix were the major focus of the present investigation. Examining these interactions through the use of a highly-virulent, mouse-passaged strain of *A. culbertsoni*, and a

weakly-virulent, axenically-cultured strain, was of additional interest. It has been documented in the literature that virulence can be attenuated through long-term *in vitro* cultivation. Xu et al. (2009) demonstrated that axenic cultivation of *A. healyi*, a known pathogen, resulted in its inability to cause death in mice that were inoculated intranasally. However, intranasal inoculation of mice using sequentially mouse passaged *A. healyi* was fatal in 100% of the test population [Xu et al., 2009]. Unpublished data from our laboratory also has shown this to be true for *A. culbertsoni.*

It has been proposed in the literature that proteases serve as virulence markers. As a result, differential protease secretion may help to further discern pathogenic species from non-pathogenic species and may aid in differentiating strains of differing virulence within the same species [Khan et al, 2000]. In this study, serine proteases of approximately 150 and 55-kDa were visualized during attachment of mouse-passaged and axenically-cultured *A. culbertsoni* trophozoites to both Laminin-1 and Collagen I. While visual inspection of zymograms suggested greater proteolytic activity was elicited during the attachment of axenically-grown amoebae versus mouse-passaged amoebae, analysis of densitometry data revealed that this difference was not significant. Furthermore, attachment of mouse-passaged and axenically-cultured amoebae to the extracellular components was comparable while previous studies in our laboratory have shown significantly less attachment to ECM components by non-pathogenic *A. astronyxis* [Rocha-Azedvedo, 2009]. Although serine protease bands were visualized during the attachment of amoebae to Laminin-1 and Collagen I, pretreatment of amoebae with PMSF, a serine protease inhibitor, failed to inhibit attachment to either substrate despite decreased secretion of both the 150 and 55-kDa protease bands. This suggests that attachment of amoebae to ECM components likely occurs in a non-protease dependent fashion.

The attachment process may occur in a manner that is similar to the attachment of amoebae to epithelial cells. *A. culbertsoni* may possess receptors, similar to the MBP found in *A. castellanii*, that facilitate adhesion to these extracelluar matrix components. Laminin binding proteins of approximately 55-kDa and 28-kDa have been identified in *A.culbertsoni* and *A. healyi*, respectively [Rocha-Azevedo et al., 2009; Hong et al., 2004]. However, presently a Collagen I binding protein has not been identified. It is likely that the attachment process occurs through a multitude of receptor-mediated or other contact-dependent processes in which the previously identified Laminin Binding Protein may play a contributory role.

As previously discussed, induced protease secretion following receptor mediated attachment to epithelial cells has been described for *A. castellanii* [Garate et al., 2004; Hurt et al., 2003; Clarke and Niederkorn, 2006; Panjwani, 2010]. In the present study, the detection of secreted proteases in supernatants collected from the attachment assays conducted using *A. culberstoni* and extracellular matrix components presented the question of whether protease secretion was occurring secondarily to the attachment event. We have demonstrated that the two protease bands visualized in supernatants collected during the attachment of amoebae to extracellular matrix components Laminin-1 and Collagen I also were present in three different *A.culbertsoni* conditioned media, generated using Hank's Buffered Salt Solution, PYG, and serum-free Oxoid. This suggests non-specific constitutive rather than induced secretion occurred under the above-mentioned experimental conditions. *A. culbertsoni* whole cell lysates analyzed using zymography revealed the presence of proteases of approximately 150, 140, 100, 55, 50, and 38-kDa. The detection of only two of these six identified protease activity bands under our various experimental conditions suggests to us that only select *A. culbertsoni* serine proteases are constitutively secreted. The two serine proteases of approximately 150-kDa and 55-kDa

identified in this investigation are consistent with two bands of significant protease activity, corresponding to positions at 150 and 50-kDa, previously described in *A. culbertsoni* [Harrison et al, 2010]. The slight variation in molecular weight between the 55 and 50-kDa proteases, as well as the absence of other previously seen bands, is likely attributable to differences in cultivation methods, medium used to generate the amoeba conditioned media and construction of the gelatin gels used. Harrison et al. (2010), previously demonstrated that factors present in *A. culbertsoni*-conditioned Neurobasal-A medium displayed the ability to degrade chemokines and cytokines produced by a mouse microglial cell line. Additionally, the 55-kDa serine protease seen in the present study is consistent with a 55-kDa serine protease previously identified in two T4 genotype pathogens, *A. polyphaga* and *A. castellanii*, [Serrano-Luna et al., 2006]. In this investigation , western blot analysis was performed on 24 hour *A. culbertsoni*-conditioned medium, generated in HBSS, PYG, and serum-free Oxoid, using either an anti-*A. castellani* or anti-*A.culbertsoni* antibody that was generated in our laboratory. Both antibodies detected bands corresponding to approximately 55, 58, 65, and 75-kDa. The common 55-kDa protein may or may not correspond to the 55-kDa protease identified in both species. However, both the protease and protein data warrant further investigation into potential similarities that may exist among pathogenic species.

The constitutive secretion of these proteases under benign conditions, such as exposure to growth medium PYG or Oxoid, as wells as during exposure to components of the host innate immune system suggests that they may operate in a generalized manner to facilitate degradation of substrates that are foreign to the amoebae. The identification of these types of proteases in three pathogenic species may implicate a potential role in conferring overall fitness or enhanced

pathogenicity as opposed to playing a direct role in mediating attachment of amoebae to extracellular matrix components.

Invasion of host tissues and dissemination from an original site of infection are important to the survival of an extracellular pathogen in that they allow the organism to access new areas that are abundant in both space and nutrients while also providing the organism with the opportunity to evade host immune defenses by reaching immune privileged sites [Haile, 2007]. The extracellular matrix plays an important role in maintaining the integrity and separation of a multitude of host tissues. It is a component of the basement membrane, which not only serves as an anchor for epithelial cell layers but also serves as a barrier between the vascular system and the various tissues. The ECM is vital in maintaining an efficient blood-brain barrier so as to protect this important immune privileged site [Alberts et al., 2002; Persidsky et al., 2006]. It has been demonstrated previously in tumor cells as well as other pathogenic models that invasion most often occurs through both proteolytic and mechanical means as well as through interaction with cryptic binding sites [Even-Ram and Yamada, 2002; Wolf et al., 2003; Schenk and Quaranta, 2003]. The presence of cryptic binding sites located on the various fibrous proteins of the extracellular matrix has been documented in the literature. It is believed that these sites can become unveiled in two ways, through proteolytic processing or through the induction of a conformational change of a particular fiber. Serine and matrix metalloproteases as well as tension due to cell contractility have been implicated in carrying out these processes. Interaction with these sites by host cells has been noted to promote tissue and ECM reorganization, remodeling and repair [Schenk and Quarantam, 2003**]**. It may be possible that *Acanthamoeba* spp. and other pathogens use these sites to facilitate their own movement through host tissues.

Although *Acanthamoeba* spp. have been shown to possess proteases that can degrade components of the extracellular matrix such as Collagens I, III, and IV as well as Laminin, Fibronectin, and Elastin, the role of these proteases in the invasion process has yet to be demonstrated [He et al., 1990; Na et al., 2001; Hurt et al., 2003]. In this investigation the role of proteases involved in the invasion of Matrigel and Collagen I scaffolds by *A. culbertsoni* also was investigated. Secreted proteases present in supernatants collected from the invasion of ECM components were visualized at approximately 150 and 55-kDa and identified as serine proteases. These protease bands are similar to those obtained in the *A. culbertsoni*-conditioned medium as well as from attachment assays on plastic, Laminin-1, and Collagen I. Furthermore, these two proteases also were present in assays in which amoebae invaded uncoated tissue culture inserts. Therefore, it appears that these two proteases do not mediate substrate specific invasion. Their role in facilitating invasion through generalized degradation also was assessed. Highly virulent, mouse-passaged amoebae that were pretreated with PMSF prior to invasion did not demonstrate a significant inhibition in the ability to invade ECM scaffolds.

It has been previously noted in tumor cell interaction with the ECM that serine proteases, in addition to matrix metalloproteases, become localized at the invadapodia of the tumor cell [Wolf et al., 2010]. To explore the possibility of membrane-associated *A. culbertsoni* proteases, a Mem-PER Eukaryotic Membrane Extraction Kit was used to isolate the membrane fraction of *A .culbertsoni*. Subsequent zymographic anlaysis resulted in the identification of a previously unseen serine protease of 140-kDa in addition to the 150 and 55-kDa that had been previously detected throughout this study. While zymographic analysis of the membrane fraction only revealed a unique serine protease, it has been documented extensively in the literature that matrix metalloproteases are the group of proteases most closely associated with extracellular matrix

degradation, reorganization and invasion [Schenk and Quaranta; 2003, Alberts et al., 2002]. It has been reported in the literature that many of the proteases present in a particular sample, which may otherwise be detected by RT-PCR, may not be visualized by zymography [Wolf et al., 2010]. Although RT-PCR was not conducted in the present study, a whole cell lysate and membrane fraction of *A. culbertsoni* subjected to western blot analysis and probed using an MT1-MMP, membrane type 1 matrix metalloprotease, antibody, however, did not demonstrate the presence of a membrane metalloprotease**.** Although a 140-kDa serine protease was identified in the membrane fraction, any potential role that it may play in focal invasion of extracellular matrix scaffolds remains to be determined. Mechanical invasion of the ECM by *Acanthamoeba* spp. also remains a viable option. Both tumor cell invasion and T-lymphocyte mobility through tissues have been documented to occur in a protease independent fashion [Wolf et al., 2010(a); Wolf et al., 2010(b)]. In a recent study, invasion of intact human corneas by *A. castellanii* showed little to no evidence of proteolytic involvement following initial cytotoxic destruction of the epithelial layer [Omana-Molina et al., 2010]. Rather, mechanical action through the cornea was implicated in invasion.

In the present study we sought to address whether proteases were present during the attachment of *A. culbertsoni* to the extracellular matrix components, Laminin-1 and Collagen I and to determine whether the presence of these proteases appeared to be vital in mediating attachment. We also sought to determine whether proteases were involved in mediating invasion of ECM scaffolds composed of Matrigel, reconstituted basement membrane, and Collagen I. Our results indicate that *A. culbertsoni* constitutively secretes two serine proteases, of approximately 150 and 55-kDa, and while secretion of the two proteases occurs during attachment it is not limited to this process as these proteases also were identified in conditioned media generated

using two amoeba growth media and Hank's Balanced Salt Solution. The two secreted proteases also were identified during invasion of Matrigel and Collagen I scaffolds. Furthermore, inhibition of these two serine proteases using PMSF did not result in diminished attachment or invasion as compared to control conditions. This suggests that non-protease-dependent mechanisms, such as proteinaceous receptors, are likely involved in mediating attachment. Due to our results, we suggest that there is minimal, perhaps focal involvement of proteases; but that the process can occur independently of proteolytic means through the mechanical action of the amoebae themselves. Our studies, however, did unveil the presence of a novel 140-kDa membrane-associated protease whose function remains to be determined. Additionally, the 55-kDa serine protease seen in the present study appears to be consistent with 55-kDa serine proteases identified in two other pathogenic *Acanthamoeba* species and, therefore, may prove to play a role in conferring pathogenicity. Further studies are needed to determine whether these proteinsplay a role in cytoxicity of mammalian cells.

In summary, proteases are an important weapon in the arsenal of pathogenic members of the *Acanthamoeba* genus. Extensive data have implicated their cytotoxic effects against different epithelial cells layers. Secreted proteases also have been implicated in mediating the lysis of amoeba "foodstuffs" such as bacteria and in helping these organisms evade host immune system components. However, proteases are not the only tool that these amoebae use to establish infections and the present study highlights the importance of exploring multiple factors that may contribute to the pathogenicity and virulence of *Acanthamoeba* spp. We propose the following as a plausible sequence of events in the establishment of an *Acanthamoeba* infection: pathogenic *Acanthamoeba* spp. use both constitutively secreted and induced proteases to damage the various epithelial cell layers that they come in contact with during initial adhesion to host cells. This

includes the corneal epithelial layer encountered during the establishment of Amoebic Keratitis as well as skin, respiratory, and olfactory epitheliums encountered during establishment of Cutaneous Acanthamoebiasis or Granulomatous Amoebic Encephalitis. The degradation and disaggregation of epithelial cells caused as a result of the cytopathic effects of amoebic proteases, enables amoebae to gain access to the underlying basement membrane or connective tissues, which are comprised of extracellular matrix components. Amoebae then transverse through extracellular matrix layers through mechanical action and focal protease secretion. Tension due to cell contractility may induce conformational changes in the ECM and supplemental proteolytic degradation may unveil cryptic binding sites located on fibrous proteins of the ECM. Such interactions may aid in facilitating the movement of amoebae towards the underlying vascular layer. Entry of amoebae into the host blood stream would likely result in amoebic dissemination to distal tissues as well as immune privileged sites. Proteases then would be used by amoebae to evade components of the host immune system and facilitate the breakdown of nutrients to be phagocytosed.

At present no vaccines exist for infections caused by *Acathamoeba* and the efficiency of available treatments is limited at best. Currently approximately 33.3 million people worldwide are living with HIV/AIDS and this number represents on a fraction of the populations that are susceptible to *Acanthamoeba* infections. Continued investigation into the way in which these organisms are able to establish infection, disseminate, and evade host defense mechanisms may unveil successful drug targets.

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