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ACANTHAMOEBA CASTELLANII: INTRACELLULAR LOCATION OF METACASPASE

Thesis submitted to
the Graduate College of
Marshall University

In partial fulfillment of
the requirements for the degree of
Master of Science
Biological Sciences

by
Danielle Maria Desser

Approved by

Dr. Wendy Trzyna, Ph.D., Committee Chairperson
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Marshall University, Huntington, WV
December 2010

Dedication

I would like to dedicate this to my family, friends, and committee members who have always been supportive every step of the way.

ACKNOWLEDGMENT

I would like to thank Dr. Wendy Trzyna for sparking my interest in her research and in microbiology. I was given a wonderful opportunity to work in her lab and have enjoyed every minute of it. I have learned many skills and techniques under her guidance that will be very useful in years to come. I would also like to thank her for a wonderful experience in the lab, which made me realize what I really love to do and would like to make a career out of it. Also thank you for the countless supplies and patience on the number of attempts it took me to standardize the AC protein samples.

I would also like to thank Dr. Price for his time teaching me both in the classroom and techniques in the lab such as electroporation and helping me with microscopy. I would like to thank Dr. Schultz for being on my committee and helping me through the process of my thesis.

I am not sure where I would be right now on my research if it were not for the help and dedication of those in the Acanthamoeba lab. Thank you for helping me set up cultures, making reagents, and washing the countless number of flasks. Thank you to all other faculty and staff at Marshall University who aided my growth in the scientific field.

For keeping me company on the long nights working in the lab I would like to give a special thanks to Daniel Blair and Katy Wolfe.

Finally, I would like to thank my parents for their continued support.

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ABSTRACT

ACANTHAMOEBA CASTELLANII:
INTRACELLULAR LOCATION OF METACASPASE
Danielle Maria Desser

Acanthamoeba castellanii, classified as a free living protist, are found in abundance in fresh water, filtered water, and soil, feeding upon bacteria in their environment. The pathogenic strain of this organism can cause either *Acanathamoeba* keratitis that targets eyes, or more rarely, granulomatous amoebic encephalitis, with immunocompromised individuals most at risk. *Acanthamoeba castellanii* are characterized by their two different stages: the trophozoite, which is the growing and dividing form, and the cyst form, which is the dormant stage. The cyst stage is highly resistant to harsh environmental conditions due to the double layer cell wall made largely of cellulose. It is not known exactly what controls encystment at the molecular level. Prior studies in this laboratory have shown that metacaspase may be involved in the process. The intracellular location of metacaspase in the cell will provide insight as to its function. This study will be done through immunolocalization on fixed permeabilized *Acanthamoeba* cells using polyclonal antibodies specific for metacaspase. The specificity of the antibody for metacaspase will first be evaluated on western blots. Insights gained as to the function of metacaspase in *Acanthamoeba* will be widely applicable to related processes in other systems that also have metacaspases or related proteins.

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CHAPTER 1: INTRODUCTION

1.1 Acanthamoeba castellanii

Acanthamoeba castellanii are eukaryotic single cell protists. Slime mold, *Dictyostelium discoideum* has many of the same characteristics (Figure 1) (Bouzat et al., 2000). Although *D. discoideum* has a multi cellular component; its life style is not shared with *Acanthamoeba*. *Acanthamoeba* are generally free living, but some strains are characterized as parasites (Derda et al, 2009). These amoebae are abundant in water and soil, including, bottled water, and can also be found in tap water, food, and on the human body. They are phagocytic cells, feeding on other microbes such as *E. coli* and yeast in their environment. There are two different stages in which *Acanthamoeba* can exist; as trophozoite and the cyst stage. The double-layered cell wall of the *Acanthamoeba* is mainly comprised of cellulose and is highly resistant to harsh environmental conditions and is difficult to penetrate (Trzyna et al., 2006). Morphologically, there are 10-40um in size and posses acanthopodia, which are characteristic of *Acanthamoeba castellanii's* genus and are comprised largely of actin (Gordon et al, 1976).

Acanthamoeba are classified as eukaryotic cells and have various similarities in the cell structure as compared to a mammalian cell. These similarities allow *Acanthamoeba* to be a very useful model organism for studying how mammalian cells behave. *Acanthamoeba* has been a useful organism for studies based upon gene expression and on numerous components of the cytoskeleton involved in motility and other processes as well as mechanisms and

phylogeny of pathogenicity (Peng et al., 2005). In addition, *Acanthamoeba* exhibits unique stages of transitioning back and forth from trophozoite to cyst under favorable or unfavorable conditions, known as encystment. Encystment, a cellular differentiation process, is hypothesized to involve various genes and proteins regulating metacaspase that are believed to control this process. Metacaspse is closely related to caspases and paracspases, which are involved in programmed cell death. This relationship is an additional reason as to why *Acanthamoeba* is favored as a model organism for studying.

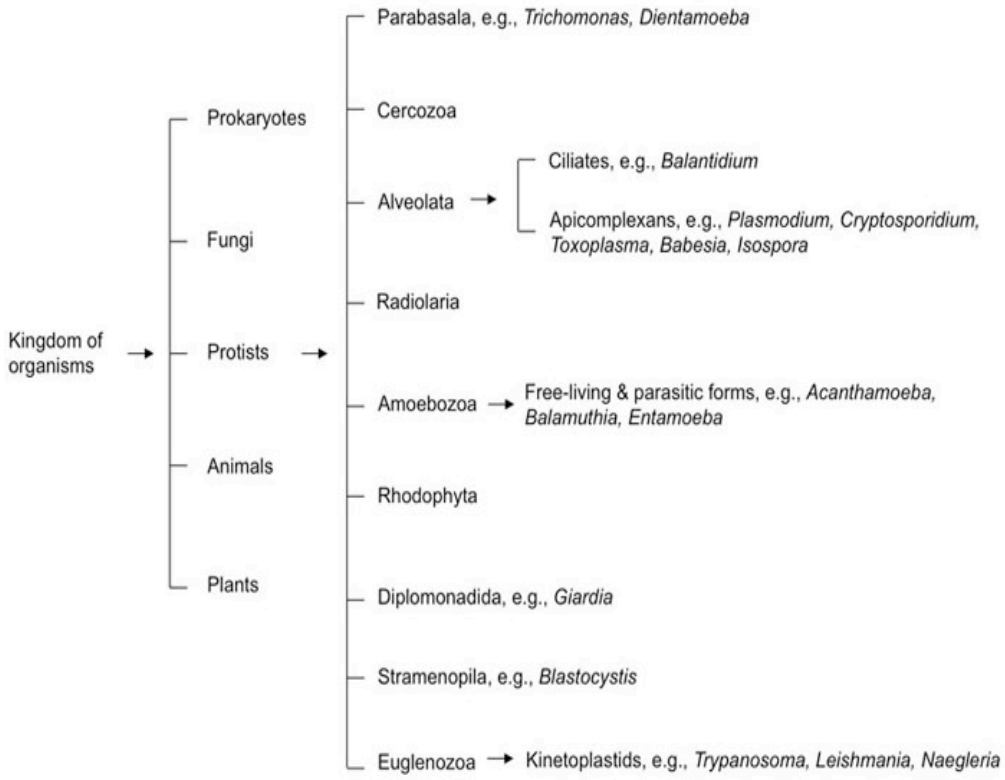

Figure 2: Phylogenetic tree of *Acanthamoeba* (Khan, 2009).

1.2 Human pathology

Some strains of *Acanthamoeba castellanii* are known to affect immunocompromised individuals and are able to cause *Acanthamoeba* keratitis or granulomatous amebic encephalitis (GAE) (CDC.gov). These infections are brought about most often in conditions in which individuals have misused contact lenses, only in *Acanthamoeba* keratitis cases, or are already in a state in which they have a decreased immune function in the case of granulomatous amebic encephalitis. Although infections are rare, if left misdiagnosed or untreated, individuals are more than likely to die with granulomatous amebic encephalitis.

Acanthamoeba keratitis affects the cornea of the eye and causes a cataract like formation over the eye inhibiting sight. This disease is a very serious condition and rarely infects healthy individuals. It is also the reason why optometrists explain to never use tap water to clean contacts (Trzyna et al., 2010). According to Trzyna et al., *Acanthamoeba* trophozoites and cysts were able to withstand harsh levels of chlorine tolerance in higher doses than normal water treatment facilities use. This study concluded that it is possible to find *Acanthamoeba* in regular tap water. Soft contact wearers are the most at risk possibly due to the depleted aeration in the make up of the soft contact. If the individual already possesses a deep wound on the eye, the risk of infection is increased (Byers, 1979). *Acanthamoeba* infected tap water is then splashed into the eye and, according to the Center of Disease Control, the trophozoite will undergo encystment under these unfavorable conditions and cause *Acanthamoeba* keratitis.

In May of 2007 *The Wall Street Journal* did a number of articles based upon an *Acanthamoeba* outbreak in contact solution by the Advanced Medical Optics with its Complete MoisturePlus contact solution. This soft contact solution that they supplied had affected 60% of their consumers with most patients complaining of eye pain or blindness. An additional study with in this article by Dr. Joslin and Tu showed that the Environmental Protection Agency happened to decrease the amount of chlorine in the water of Chicago at the time. This study led them to believe that maybe increased incidence of disease was the cause of the outbreak if soft contact wearers wore their contacts in the shower, washed their face with tap water, or went swimming.

The same article stated that researchers from Ohio State tested three different contact solutions affected with *Acathanthamoeba,* and, after 6 hours in contact solution, *Acanthamoeba* still continued to multiply. The researchers concluded that contact solution alone cannot ward of *Acathanthamoeba* in tap water. Winstein and Rundle reported The Environmental Protection Agency concluded that the Food and Drug Administration did not require chlorine levels high enough in Chicago at the time to kill amoeba in contact solution. The Environmental Protection Agency believes that water filters should have take care of amoeba and individuals should still practice proper techniques when handling soft contacts; although it is well documented by the Center for Disease Control that amoeba are small enough pass through water treatment filters. As a result of this outbreak, disposable daily soft contact sales have increased 35% in 2007 in order to reduce the risk of infection (Winstein and Rundle, 2007).

In order for a physician to detect *Acanthamoeba* keratitis is occurring, Lee et al., 2006 describe that a sheep blood agar plate is streaked from a swab of the patient's eye and is induced with *E. coli* as a food source to conclude if any colonies would grow, thus signaling *Acanthamoeba castellanii* was present and the individual is infected. The patient then would undergo a rigorous and long process of treatment because the double cell wall of the cyst is very resistant to antibiotics and could take up to a few years to treat (Lee et al., 2006).

A study done by Vural et al. in 2007 tested eighteen rats that were infected with *Acanthamoeba* keratitis and were treated with a chlorine based drug, propolis. It hypothesized that propolis was corrosive enough to penetrate the double cell wall of the cyst without harming the rat's health. The drug was administered by the use of eye drops once an hour for a total of ten days. Every day, the rat's cornea would be scraped and tested for the percent of corneal cells that were damaged due to *Acanthamoeba* keratitis. The results of this experiment proved that propolis did help degrade the cysts over the course of the ten-day trial. It also reinforced the idea that the cystic cellulose wall was too hard to penetrate and that the high doses would risk affecting the corneal epithelial cells at the price of being cured. In conclusion, many drugs are too harsh on corneas to treat *Acanthamoeba* keratitis, but if *Acanthamoeba castellanii* could somehow be coerced back into a trophozoite, the individual would be at much less a risk because trophozoites do not possess the double layered cell wall and could be lysed in a non-invasive fashion to the patient. (Vural et al., 2007).

Granulomatous amebic encephalitis is another disease that is caused by *Acanthamoeba*. It primarily affects patients who are already immunocompromised, such as patients after a surgery who are infected with MRSA. According to *Acanthamoeba* Infection webpage of the CDC, *Acanthamoeba* are first able to target the respiratory tract and then enter the central nervous system. Symptoms could include headaches and a stiff neck, which are often misdiagnosed at first as meningitis. Lesions will ultimately form on the individual's appendages and could take months or years to cure. One of the only ways to positively identify this disease, other than by visually noticing skin lesions, is to perform a test using hematoxylin and eosin, although studies have shown that identification can also be performed through streaking *E. coli* on a blood agar plate to see if *Acanthamoeba* is still present (Szenasi et al., 1998). Ultimately, polymerase chain reaction nucleic acid testing is one option that can be done to identify the disease. Although it is very rare and often misdiagnosed at first, only around four hundred cases have been reported (Jones, 1975). In order to treat this disease, multiple drugs must be taken at once to penetrate the cyst cell wall such as ketoconazole and miconazole (Gregerson, 2007).

Due to these infections being serious if left misdiagnosed or unreported, *Acanthamoeba* is an important microbe requiring further studies to better understand the infectious process and how to intervene. If *Acanthamoeba* could be controlled by inducing the cell to excyst, *Acanthamoeba* keratitis and granulomatous amebic encephalitis could be treated more urgently and less evasively, and thus save more lives.

1.3 Trophozoite

The trophozoite life stage of *Acanthamoeba* is the active metabolic state. Trophozoites are present in favorable conditions in the surrounding environment such as rich media in a laboratory setting. Trophozoites can range from 20-40um in diameter and are not uniform in shape as shown in Figure 2. Under a compound light microscope, trophozoites are easily visible under 100X exposure. A trophozoite is easily identifiable by its acanthapodia, which are 1-2um in diameter and extend for a few microns (Bowers and Korn, 1968). The function of acanthapoida is unknown at this time, but it is believed to aid the trophozoite in movement (Bowers and Korn, 1968).

Trophozoites are not morphologically similar to other eukaryotic cells. It has been noted that, under a compound microscope, *Acanthamoeba* possess golgi bodies, smooth and rough endoplasmic reticulums, a contractile vacuole, a vacuole digestive system, multiple mitochondia, and a nucleus. (Bowers and Korn, 1968). Bowers and Korn also state that if the same strain of *Acanthamoeba* is subbed over numerous generations in a laboratory setting, multiple nuclei can form. It has also been observed by the author that very few trophozoites have been able to withstand encystment. This observation could be due to some sort of mutation involving trophozoites, which are not given the chance to encyst over time. Increasing osmolarity, the addition of salt to the media, and various monoclonal antibodies that bind to the surface of the amoebae can cause the trophozoite to encyst and become a cyst (Trzyna et al., 2006).

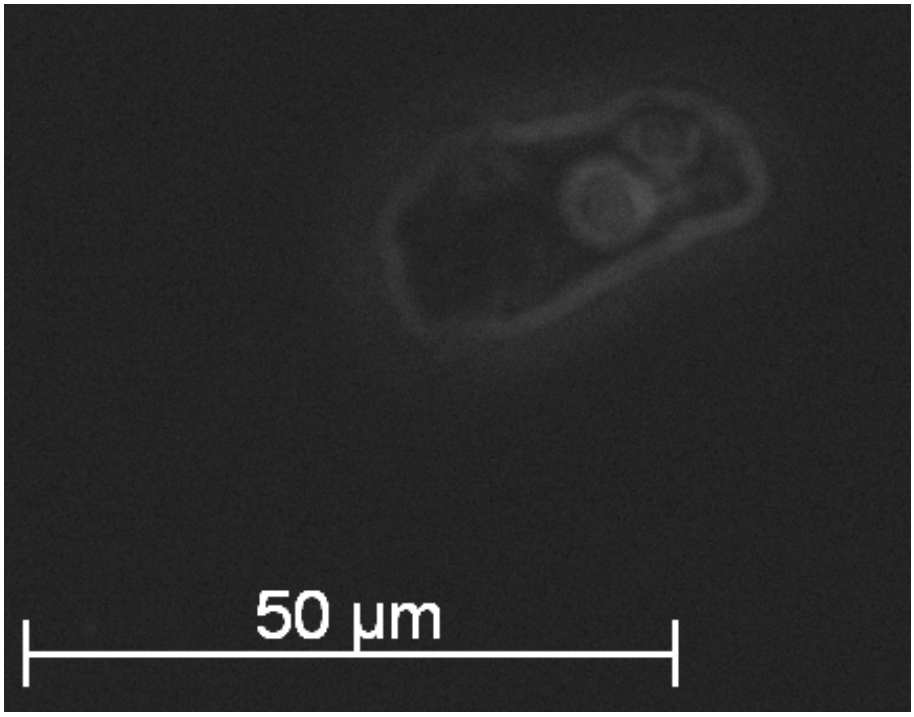

Figure 2: *Acanthamoeba* Trophozoite under 100X compound light microscope (D. Desser, 2009).

1.4 Cyst

Little is known about the cyst life stage, but it does contain a double-layered cell wall comprised of cellulose as well as an identifiable nucleus (Derda et al., 2009). The cyst life stage of *Acanthamoeba* is generally found when the trophozoite encounters low metabolic states or various environmental stresses such as increasing osmolarity (Trzyna et al., 2008) and undergoes encystment to become a cyst. The cyst is able to survive periods of “adverse” conditions that can include, but are not limited to, many environmental changes such as change

in pH, elevated temperature, and change in osmolarity (Cordingley et al., 1996). If monoclonal antibodies bind to the surface of the cyst, it has been shown that encystment can occur (Trzyna et al., 2006).

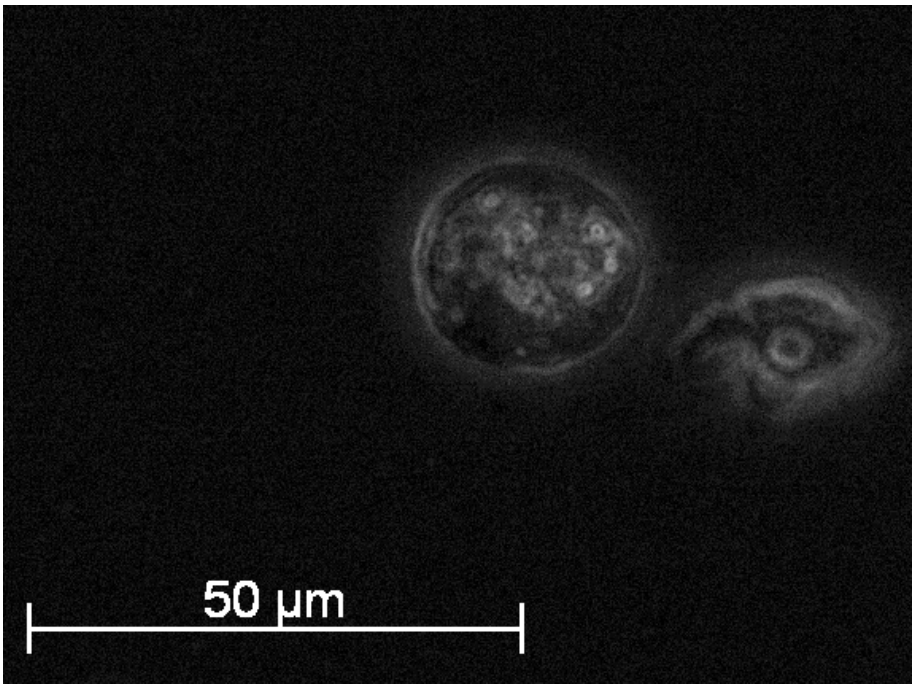

Figure 3: *Acanthamoeba* Cyst under 100X compound light microscope (D. Desser, 2009).

According to Kong 2009, they were able to classify the genus *Acanthamoeba* into three groups using visual and morphological features (Figure 4).

> "Group 1 consists of Acanthamoeba spp. with relatively large cysts, distinctly stellate endocysts, and smooth spherical ectocysts. Group 2 and group 3 Acanthamoeba spp. have smaller cysts (less than 18 µm in diameter). Species in Group 2 have polygonal to satellite endocysts with irregular or wrinkled ectocysts, while the cysts of group 3 species have

> rounded or slightly angular endocysts with smaller and smooth or slightly wrinkled ectocysts. The grouping has been widely used before species identification of the amoeba" (Kong, 2009).

When conditions in the environment become favorable again, the cyst is able to excyst into a trophozoite.

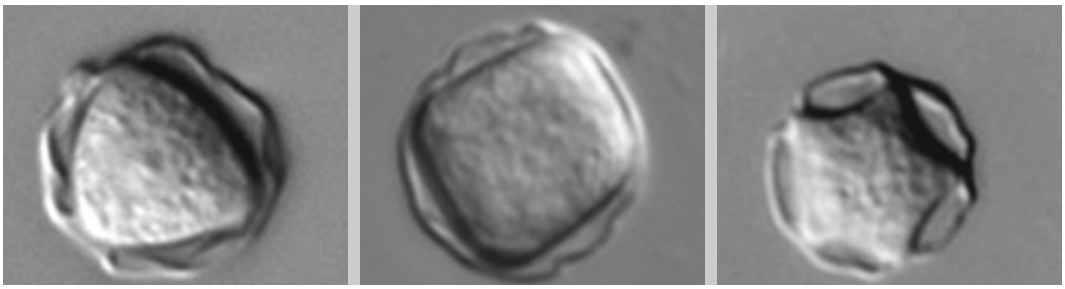

Figure 4: Cyst groups 1, 2, and 3 (Kong, 2009).

In the past, *Acanthamoeba* cysts have been observed by viewing them under transmission electron microscopy (TEM) but recently, in order to eliminate the artifacts that are generally found when fixing the cells and can lead to an unclear picture, ultrarapid freezing has been performed on cysts. This procedure involved using ultrarapid freezing, freeze-fracturing, and then deep-etching to provide a clear image. This procedure provided scientists with a 3D image of the endocyst and exocyst cell walls. The images were cleaner and clearer and proved to be a better way of studying the cellular structure. After observing the cell walls, they were able to observe many vesicles that ran through the exocyst cell wall and confirmed Pussard and Pons' observation of the three different

groups of cysts. They also observed that the endocyst cell wall was actually thinner than previously observed in other TEM studies although it was inconclusive as to where the acanthapodia lie connected to the cell. Further work must be completed, but this study showed more of an understanding of how structurally the cyst is comprised (Lemgruber et al., 2010).

As stated previously, *Acanthamoeba* cysts are highly resistant to low amount of chlorine treatments. They have also been known to be resistant to pH 2.0, freezing, irradiation of 250 rads (Chatterjee, 1968), UV irradiation of 800mJ/cm^2 (Aksozek et al., 2002), storage at room temperature for 24 months (Brown et al., 1984), and 24 years at 4°C in water (Iwanicka et al., 1995). After twenty years, *Acanthamoeba* cysts have also been found to survive after drying them out on agar plates with *E. coli* and rehydrating them with amoeba saline (Siriam et al., 2008).

In a recent study performed by researchers at Steris Research and Development labs, cysts would remain resistant even after thirty minutes of treatments by glutaraldehyde and other biocides. Other biocides that were tested on cysts and proved to have an effect on them included “peracetic acid, hydrogen peroxide, or ortho-phthalaldehyde presented greater efficacy than glutaraldehyde, as did ethanol and sodium hypochlorite” (Coulon et al., 2010). Many different incubation times were noted and proved to inactivate the cyst. Even temperature change in the *Acanthamoeba’s* environment did have an effect on the cyst when temperatures reached 65ºC for an hour. This study was very useful for hospital settings to determine better means of eliminating cysts if

hospital workers know *Acanthamoeba* is present in the environment before more patients become infected or if some already are so they can perform more rapid ways of treatment (Coulon et al., 2010).

1.5 Encystment

Encystment occurs for *Acanthamoeba* when a trophozoite's environment becomes unfavorable and it morphologically converts to a cyst in order to survive in low metabolic states. Sadoff observed that *Azotobactor* underwent encystment when glucose in the environment was replaced with B-hydroxybutyrate, an example of an unfavorable condition and loss of food source(Sadoff et al., 1970). When conditions become more favorable, as described in previous sections, cysts are able to excyst and return back to their metabolically active trophozoite form. *Acanthamoeba* are not the only protist that is able to encounter encystation. It is indicative of many protists, such as the non free living *Entamoeba* and free living parasites such as *Dictyosteluium*, as seen is Figure 1. In *Giardia*, and possibly *Acanthamoeba*, encystment is part of the life cycle. Encystment is hypothesized to be a part of the reproductive cycle of some protists by undergoing cellular division as soon as excystment occurs and new trophozoites are formed (Baron, 1996).

According to Miickimion, the earliest description of encystment is described in *Rhizomastix* when the organism first began to lose its flagellum, the body becomes rounded and the cytoplasm developed multiple vacuoles (Miickimion, 1913). This observation is very closely related to a physical

description of encystment in *Acanthamoeba*. A more recent publication by Weisman describes encystment in *Acnathamoeba* as a three-step process which includes induction, in which the trophozoite becomes rounded. The second step, of wall synthesis in which the excoyst, or the fist wall, is formed and is comprised of acid-insoluble proteins. The final step is dormancy, in which the endocyst, the second cells wall, is formed and is highly comprised of cellulose (Weisman, 1976).

A more modern approach to understanding encystment would be to look at the molecular level rather than morphology. In *Dictyostelium,* there are many cell-to-cell signaling by cell surface receptors and transmembrane signal transduction such as camp receptors (Janssens and Haastert, 1987).

> Molecularly, cyst-specific protein 21 (Csp21) has been identified as a cyst wall protein found in group II *Acanthamoebae* and was reported to be synthesized approximately 12 h after induction. The expression of the respective gene is repressed under normal growth conditions via one or more repressor elements between the TATA box and nucleotide (nt) +63. Furthermore, encystment requires serine protease activity and autophagy proteins, all of which are suggested to be involved in autolytic processes, and glycogen phosphorylase, which is necessary for the breakdown of glycogen. The glucose-1-phosphate that is thereby liberated is subsequently used for the buildup of cellulose in the cyst wall (Leitsch et al., 2010).

"Proteinases play a role in various biologic actions in *Acanthamoeba*, including host tissue destruction, pathogenesis, and digestion of phagocytosed food" (Moon et al., 2008). Leitsch's study also concluded that most of the changes in the protein profile of encysting *Acanthamoeba* occur early in the encystment process as well as the early phase of encystment can be completely inhibited by the serine protease inhibitor phenylmethylsulfonyl fluoride. Serine

proteases are indicative of caspases. He also concluded that the proteolytic processes observed during the early phase of encystment are mediated by proteases already present in the trophozoite and that the proteolytic activity localizes to the large organelle fraction and can be inhibited only by the cysteine protease inhibitor E64 (Leitsch et al., 2009). This study shows that serine protease inhibitors inhibit proteolytic activity in the cell in early encystment.

Protein expression in encystment has been growing in popularity in order to observe cell-to-cell signaling that is taking place. In France, researchers observed four specific proteins expressed in trophozoites and four specific proteins expressed in cysts, which included glycolytic pathway proteins involved in actin binding: enolase and fructose bisphosphate aldolase. Because they are associated with serine proteases and encystment, these proteins were modified during encystment and result in having links to actin morphology in encystment, glycolysis, and proteolysis (Bouyer et al., 2009).

Within the first sixteen hours of *Acanthamoeba* encystment, it has also been observed that:

> RNA polymerase II increases approximately 4-fold, whereas transcription by RNA polymerases I and III is decreased when examined by nuclear run-on and RNase protection assays. The levels of mRNAs encoding TPBF, TATA binding protein, cyclin-dependent kinase, protein disulfide isomerase, profilin, myosin II heavy chain, ubiquitin and extendin are stable during mature cyst formation, whereas mRNAs encoding actin, *S*-adenosyl methionine synthase and tubulin are substantially decreased. Control of *Acanthamoeba* differentiation is likely to be mediated by positive regulation of genes necessary for cyst maturation (Orfeo, 1998).

This observation has been useful for the use of housekeeping proteins and to observe what proteins are turned on and off in the cell at different times of the encystment process.

A number of specific conditions performed in a study found that triggering encystment in the Neff strain of *Acanthamoeba*, include starvation (Weisman 1976, Byers *et al*. 1980**),** osmolarity (Cordingley *et al*. 1996), and, in some experimental situations, several surface binding monoclonal antibodies (Yang and Villemez 1994) and MgCl2 **(**Chagla and Griffiths 1974) have also been shown to induce encystment (Cordingly and Trzyna, 2008). *Acanthamoeba* trophozoites can either grow and divide producing more vegetative cells, or, alternatively they may cease dividing and encyst, forming a cellulose-containing cyst wall around a resting stage cell, until conditions allowing excystment and replication return (Cordingly and Trzyna, 2008). The study by Cordingly and Trzyna concluded that increasing osmolarity, presence of glucose, and temperature change all play a role in inducing encystment in *Acanthamoeba*. It has also been noticed that *Acanthamoeba* have been able to lose their ability to encyst when placed in axenic cultures for long periods of time and subbed only as trophozoites (Köhsler et al., 2008). Encystment, which is a complicated process, could be brought about by multiple proteins being triggered such as possibly metacaspase (Trzyna et al., 2008), which is related to caspases in other eukaryotic cells based upon sequence data.

1.6 Caspase

Caspases received their name from being identified functionally as cystienyl aspartate-sepcific proteases (Jiang et al., 2010). Programmed cell death, also known as apoptosis, normally occurs in Eukaryotic cells when the cells have reached their expectancy and become phagocytized by their environment. Caspases are cysteine-aspartic acid proteases that trigger cellular signaling to dismantle the cell completely (Boyce et al, 2004). Recently, many scientists have become very interested in caspases because if cell death does not occur tumors will develop and some could be cancerous. Studying tumors can expand life expectancy in humans and has been an increasing field in the last few decades. It has also been shown that a faulty gene, which controls the caspases' ability to bring about apoptosis, usually affects individuals with autoimmune diseases (Cohen, 1997).

Closely related to *Acanthamoeba* is *Dictyostelium* which possesses a paracaspase, which is homologous to a caspase when it was first identified in a PSI-BLAST search (Uren et al., 2000). The paracaspase can be activated by homologous recombination (Golstein et al., 1993). Paracaspases share the Cys-His catalytic diad with caspases but the catalytic Cys resides within a context that is different from the QACXG prototypic caspase sequence, probably leading to a different specificity. An observation made by turning off the paracaspase in *Dictyostelium* showed that *Dictyostelium* did not require a paracaspase in order for cell death to occur (Golstein et al., 1993). These conclusions brought about the many unanswered questions involving the role of caspases involvement in

cell death, and are there other factors such as other regulations of other cystine proteases that are causing caspases to induce apoptosis.

This study started questions as to what exactly is the function of metacaspase and its overall purpose (Jiang et al., 2010). Because sequence information has brought about a profound similarity among, paracaspases, caspases and metacaspases, it triggers questions. Questions include what is controlling *Acanthamoeba* from triggering cell death and are the cells triggered by metacspase causing encysting or causing tumors? By intracellularlly locating the metacspase in the cell, insight to its overall function can be gained.

1.7 Metacaspase

During the process of encystment when the trophozoite morphologically changes to a cyst under unfavorable conditions, the cell possesses the ability to also return to the trophozoite stage under favorable conditions. Metacaspases are arginine and lysine specific and are found in plants, fungi, and protists (Belenghi et al, 2007). Bacterial metacaspases are cystine proteases, which are homologous to caspases. Based upon the sequence data of metacaspase, it is believed metacaspase is involved in programmed cell death. Programmed cell death has also been observed in six of the eight major groups of prokaryotes (Jiang et al., 2010). Metacaspases are not found in mammalian cells but do possess a caspase.

Plants are known to also possess a metacaspse. Woltering provided evidence in plants that type II metacaspase is associated with cell viability in the

processes. This system has shown to require proteases to induce cell death and have also shown caspase-like substrates, which would link them to the involvement (Woltering, 2010).

In some species of the human protozoan parasites *Trypanosoma spp*. and *Leishmania spp*., metacaspases have been involved in programmed cell death (Gonzalez, 2009). *Leishmania* is found to have two metacaspases known as *Leishmania donovani* metacaspase-1 (LdMC1) and LdMC2 (Lee et al., 2007). When hydrogen peroxide was added to *Leishmania* cells, LdMC levels increased to induce programmed cell death. *Leishmania* metacaspases show enzymatic characteristics of trypsin-like proteases and have been shown to be intracellularlly localized in unique acidocalcisome compartments, located in the cytoplasm, which could represent a form of sequestration of inactive enzymes in the cell (Lee et al., 2007).

Upon starvation, *Dictyostelium*, another protozoa, is able to differentiate into multicellular fruiting bodies consisting of a spore mass supported by a stalk. During this process, stalk cells die in a caspase-independent autophagic cell death (Cornillon et al., 1994). Both differentiation and cell death were demonstrated to be independent of meta-/paracaspase action (Roisin-Bouffay et al., 2004).

The opportunistic human pathogen, *Aspergillus* possesses a yeast metacaspase, Yca1p, in apoptotic-like programmed cell death (Richie, 2006). The slime mold *Plasmodium* undergoes similar cellular differentiation due to metacaspase 1 (PxMC1). The gene in *plasmodium* has been described

possessing histidine and cysteine residues that typically form the catalytic dyad in this family of proteases suggesting that this parasite species may possess a mechanism of programmed cell death (Le Chat et al., 2007).

Saccharomyces cerevisiae has only one metacaspase gene, Yor197w, and does not possess caspases or paracaspases. Although over expression of Yor197w is triggered, cell death occurs but Yor197w will prevent cell death from happening. This study suggests that metacaspase could be involved (Golstein et al., 1993). In yeast, if metacaspase is knocked out, then without metacaspase, it resulted in the activation of cell death pathways being turned on providing more insight that metacaspase does play a positive roll in cell death (Guaragnella, 2010).

Metacaspases in *Acanthamoeba* have been divided into two groups, type 1 and type 2. Type -1 metacaspases are comprised of proteins on the N-terminal region. Type-2 metacaspases are comprised of both N-terminal and C-terminal regions laying on the same length and sequence (Trzyna et al., 2006). In *Saccharomyces cerevisiae*, the metacaspase is present in this organism and has been reported that it is involved in apoptosis (Silver et al., 2005). Although the exact function of the metacaspase is unknown in *Acanthamoeba*, it is believed it plays a role in inducing the formation from trophozoite to cyst. Because type-1 metacaspase in *Acanthamoeba castellanii* was able to undergo gene expression and simulated encytment by using a monoclonal antibody, it was concluded that the metacaspase is in the direct link of events leading to encystaion (Trzyna et al., 2006). This study proved that the caspases are homologous to

metacaspases due to its His/Cyst catalytic dyad. Instead of causing apoptosis to occur, encystation occurs instead.

According to Trzyna, 2006, the metacaspase in *Acanthamoeba* castellanii is 478 AA long and 50,249 Da in size. Its molecular function is a cysteine-type, which is homologous of caspases. *Acanthamoeba* has been found to possess endopeptidase activity and is known as a protease. An upregulation of metacaspase messages when *Acanthamoeba* cells approach encystment has also been observed (Trzyna et al. 2006). There are many unanswered questions about *Acanthamoeba* metacaspase that include function, specific location, and does it turn on or off a cell metabolically.

1.8 Project overview and aims

In *Acanthamoeba castellanii*, identifying the intracellular location of the metacaspase will provide insight of its function in the cell. Previous data (Trzyna, 2006) showed an up regulation of metacaspase mRNA during encystment. Studying this protein means metacaspase may be involved in the encystment process.

The overall objective for this project is to determine the intracellular location of metacaspase in *Acanathmoeba*. Evaluating the metacaspase protein expression profile during growth and encystment and use a polyclonal antibody to metacaspase will be use for immunolocaliztion studies. Although the project still has many questions that need to be addressed, the study is interesting enough to continue further work.

CHAPTER 2: MATERIALS AND METHODS

2.1 Methods of Culturing and Growing *Acanthamoeba* in the Laboratory

In order to identify the intracellular location of metacaspase in *Acanthamoeba castellanii,* cells must first be cultured. The *Acanthamoeba* trophozoite was subcultured every few days so encystment could not take place. *Acanthamoeba castellanii*, Neff strain, cells were grown axenically using standard *Acanthamoeba* media in 30ml cultures at 30ºC in a shaking incubator at 200rpm.

Standard *Acanthamoeba* media was made by starting with 400ml of MiliQ water and adding 7.4g proteose peptone (Fisher cat. LP0085B), 7.5g yeast extract (Fisher cat. 50843369), 0.1mM ferric citrate, 1mM $MgSO_4$, and 2mM KH_2PO_4. After mixing thoroughly, the volume was brought up to approximately 700ml and pH 7 using 10X NaOH then bringing the final volume up to 900ml. The final volume was split into 450ml aliquots, weighed and autoclaved. After sterilizing, sterile water was used to replace the lost volume of water and 50ml of 15% Glucose/0.5mM $CaCl_2$ was added for a final concentration of 1.5% glucose, 0.05mM $CaCl_2$.

Acanthamoeba cells were then counted using a hemocytometer with a 1:10 dilution if the cell density was more than $1X10^6$ cells/ml. In order to determine the number of cyst to trophozoite ratio, 2% SDS (sodium dodecyl sulfate) was used in a 1:1 dilution of cells to SDS and counted. If cells were at a density of $5X10^5$ cells/ml, they were considered to be in low log growth phase,

middle log phase was around cell density of $3X10^6$ cells/ml, and late log phase approaching stationary the cells were counted at a cell density of $6X10^6$ cells/ml.

2.2 *Acanthamoeba* Purification

2.2.1 DNA Miniprep of *Acanthamoeba*

To obtain purified DNA from *E. Coli* strains on a 1% agarose DNA gel, a Qiagen QIprep Spin Miniprep Kit (50) (cat. 27104) was used. Cultures were first single colony picked from an LB ampicillin plate. Cultures were then grown in 5ml of LB plus ampicillin and were placed over night in 37ºC at 200 rpm. Following the kit's instructions, a single colony sample of *Acanthamoeba* was also grown on LB ampicillin plates to retain for use later if the colony was successful. This procedure was performed for *E. coli* plasmid samples full-Length metacaspase colony 1 in PGEX, c-term metacaspase colony 3 A, and full-length metacaspase GFP. These samples were selected and sent off to be sequenced at 200-400 ug/ul. These strains were selected because they possessed the c-terminus full-length sequences of the metacaspase done in previous work.

2.2.2 Endo-Free Plasmid Purification of *Acanthamoeba*

Quiagen Endo-Free Plasmid Giga Kit (5) (cat. 12391) was used on *E. coli* plasmids FLM-GFP and FLM colony 11 in LB ampicillin medium in a 10ml starter culture. The protocol supplied was followed and test samples were taken at the appropriate steps. The final pellet was let to air dry for 20minutes in an air hood

and re-dissolved in 1.2ml of endotoxin-free Buffer TE supplied. A 1% agarose DNA gel was run to test each sample recovered from the appropriate steps.

2.3 Restriction Digest and Gel Electrophoresis

To prepare a 1% agarose gel, 490ml miliQ water and 10ml of 50X TAE, a 1X TAE solution was constructed. Then 50ml of the 500ml 1X TAE solution was added to 0.5g of agarose. The original weight was recorded, the flask was covered with plastic wrap and microwaved for a 45 seconds, stirred, and placed back into the microwave for an additional 15 seconds. The final weight was recorded and MiliQ water was added to replace the volume of water lost. The melted agarose was then placed into a gel box and the combs were placed. Once the gel set, the combs were removed and the samples were added to wells.

For a digested sample of DNA, 3ul of DNA were placed in a separate centrifuge tube, 1ul 10X restriction enzyme buffer, 1ul 10X BSA, 4.5 ul of miliQ water, and 0.5ul enzyme were then added. For a double digest, 0.5ul of each enzyme were added and 4ul of miliQ water were adjusted. The samples were then placed on a heat block at 37ºC for 1 hour to 1.5 hours, spun briefly for 30 seconds at 2,000rpm, and 3ul of 6X loading dye was added to the sample bringing the total volume to 13ul, and then loaded.

The undigested samples of DNA consisted of 3ul undigested DNA sample, 7ul miliQ water, and 3ul 6x loading dye making 13ul total. The loading dye consisted of 3ul marker, 7ul miliQ water, and 3ul 6x loading dye. Gels were then

run at 100v for 60 minuets. The gel was then processed with a 1:100 dilution of 10ug/ml ethidium bromide (Bio-Rad cat. 161-0433) added to 1X TAE buffer and placed on a rocker overnight and then viewed.

FLM in PEGEX and C-term colony 3A showed the correct insert and were then single colony streaked onto LB ampicillin plates in order to save. C-term colony 3A and was digested using ECOR I (New England Bio Labs cat. B0101S). FLM colony 11 was ran using NDELI (Fermentas cat. ER0585) restriction enzyme and FLM-GFP was ran using Sca I (New England Biolabs cat. R0122S).

2.3.1 Nanodrop

A Nanodrop was performed on all DNA samples after purification. A 0.2ul of DNA sample from a 1:200 dilution was tested each time.

2.4 Transfection of *Acanthamoeba*

2.4.1 Transfection of *Acanthamoeba* by Electroporation

Acanthamoeba cells were cultivated at early log phase of approximately cell density of $1X10^6$ cells/ml in a 30ml shaking culture. After cells were harvested at 5,927 rfc for 10 minutes, the supernatant was then carefully poured off and the pelleted cells were resuspended in 30ml of PBSU (phosphate buffered sucrose, 272mM sucrose, 7mM $NaP0_4$ PH=7, and 1mM $MgCl_2$) and spun again for 10 minuets at 5,927 rfc and the supernatant was then carefully poured off again. The cell pellet was then resuspended to have a final cell

density of 2.5X10^6 cells/ml (The cell density was divided by 2.5X10^6 cells) by adding the appropriate amount of PBSU. The concentration of plasmid DNA from an earlier nanodrop was performed; the appropriate ug/ml of DNA was added to each samples and a 2-minute timer was set as soon as the DNA was added. Cells were then subject to electroporation after 2 minutes at 4,000v, 2,000v, 1,500v, 1,000v, 500v and 0 volts and the time constant was recorded each time. 3ml of AC media was placed into each well of a 6 well dish and 1ml of electroporated cells were placed into each appropriately labeled dish following incubation on ice for 10 minutes for a total of 4ml in each well. The 6-well dish was then incubated at 26ºC without shaking. Cells were then viewed 2 days later by fluorescent microscopy.

2.4.2 Transfection of *Acanthamoeba* using Superfect

Acanthamoeba cells were cultivated at approximately 1X10^6 cell/ml and the cell density was calculated. The 30ml of cells were then spun at 5,927 rfc for 10 minutes and then resuspended in AC media. After, 5ml from the resuspended cells in AC media were placed into 25ml of fresh AC media in a 50ml tube. Then 0.5ml of 1X10^6 cell/ml cells and 2.5ml of AC media were placed into a 6-well dish with a total volume of 3ml per well and placed into a stationary incubator over night at 25ºC. The next day, three 1.7ml centrifuge tubes were used. The first was for the control of no DNA, which consisted of 100ul AC media and 20ul Superfect transfection reagent 3mg/ml (Quiagen cat 301305), the second was for 20ug/ml FLM-GFP with 100ul AC media and 200ul superfect, the last was for 20ug/ml FLM with 100ul AC media and 20ul superfect. The tubes then were let to

incubate in the air hood for 10 minutes at room temperature. Meanwhile, the AC media was washed away from the 6-well dish, rinsed with 2ml 1X PBS, and then washed away again. 600ul of AC media was then added to the centrifuges tubes after the 10-minute wait and the cells were then placed into a 25ºC stationary incubator for 24-48 hours. Before microscopy was performed, the AC media as washed away and replaced with 3ml of fresh media in each well.

2.4.3 Transfected cells with antibiotics (G418)

After viewing cells under the fluorescent microscope, the AC media was removed from each of the 6- wells. Fresh 3ml of AC media was replaced back into each well and, using a cells scrapper, the cells were then placed into a 25ml flask. The volume was brought up to 10ml with fresh AC media and 10ul (1:1000 dilution) of G418 was added to each of the culture flasks. They were then placed into a shaking incubator at 37ºC and able to divide over a week and then looked at again under fluorescent microscopy.

2.5 Microscopy

Acanthamoeba cells were viewed under fluorescent microcopy and confocal microscopy after cells were transfected. Under fluorescent microscopy, cells were observed directly using the 6-well dish, and, while under confocal microscopy, cells were observed by placing a drop onto a lens cover. Using software Image J, pictures of the cells were obtained and observed.

2.6 Recombinant *E. Coli* protein isolation

In order to obtain recombinant protein to use as controls on protein gels, BL21 (*E.*coli control), PGEX colony 1 (vector control), and C-term colony 3A (metacaspase control) were used. From -80ºC frozen stocks, single colony streaking was done on LB ampicillin plates and placed in a 37ºC incubator over night. A single colony was then picked, placed on an LB ampicillin plate, and then inoculated in 5ml of LB ampicillan broth at 37ºC over night in a shaking incubator at 200rpm. The 5ml of inoculated culture was then placed into a 45ml LB ampicillin culture, for a 1:20 dilution, and let to shake for 2 hours. After 2 hours, an OD reading was taken at 600nm and, if the sample was between 0.08 and 0.1nm, then 50ul of IPTG (Invitrogen cat. 15529-019) was added. Every hour a 1ml sample was taken out and placed into an 8ºC refrigerator and an OD reading was taken and recorded. After six hours, the culture was spun at 7,000rpm for 10 minutes, the supernatant was discarded, and the cell pellet was placed into the freezer.

As the cell pellet was thawing on ice for 30 minuets as directed from the kit: Qproteome Bacterial Protein Prep Kit (Quiagen cat. 37900), 40ul of 5X protease inhibitor cocktail for use with bacterial cell extract (Invitrogen cat. P8465-5ML) was added. The instructions provided by the Isolation of Protein kit was followed but 1/5 of the volume was used for the 50ml culture.

FLM in PGEX, another vector insert, was used to extract protein. The same procedure was performed as stated above with addition to a few steps. After six hours of IPTG induction, the 50ml culture was spun at 10,000rpm for 8

minutes, the supernatant was discarded, and the cell pellet was then resuspended in 1.250ml of 1X PBS, and the mixture was transferred to a 50ml tube. Then 25ul of a 10mg/ml lysozyme (Boehringer Manneim cat. 84093121) mixture was added and let to sit at room temperature for 15-20 minutes and then vortexed. The samples were then divided in two; one-half received 1.3ml 1X PBS, 17ul 20%SDS, and 125ul beta-mercaptoethanol (Bio-Rad cat. 161-0710) for a total of 2.5ml total. The other half received 1.3ml of SDS-PAGE buffer, 175ul 20%SDS, 125ul beta-mercaptoethanol for a total of 2.5ml. The samples were then vortexed and placed in 2ml tubes then boiled at 100ºC for 10 minutes. After, the samples were spun for 5 minutes at 13,000pm and then froze at -20ºC.

2.7 Bradford Assay

To confirm equal loading in each lane on the protein gel that was being loaded, a Bradford Assay was performed. Four different standards, 5ug/ml, 10ug/ml, 15ug/ml, and 20ug/ml, were used to conduct the Bradford Assay. In a 2ml centrifuge tube, the appropriate volumes of 0.5ug/ml BSA (Ameresco cat. E531-1.5ML), 0.15N NaCl and then 1.5ml of Bradford (Ameresco cat. E530-1.0ML) were added. The tubes were vortexed and then sat for 2 minutes before being run at 595nm, and the readings were recorded. A blank was constructed using 150ul of 0.15N NaCl and 1.4ml Bradford. These readings were then plotted on an Excel graph of absorbency/concentration.

2.8 *Acanthamoeba* protein standardization

2.8.1 *Acanthamoeba* protein standardization using SDS or Salt to Lyse cells

Cells were taken at both early and late log phases around $8X10^5$ cell/ml and $5X10^6$ cell/ml grown in 60ml cultures in AC media in a shaking incubator at 30ºC at 200rpm. The 60ml cell culture was then split into two-30ml cell culture in which one was inoculated with 125mM NaCl and harvested identically to that of culture that did not obtain salt16-18 hours later. The culture that was not inoculated with 125mM NaCl was then harvested. A cell count was performed and recorded along with a 2% SDS cyst count. Then 1ml of cells were placed in a 1.7ml centrifuge tube and spun for 3 minutes at 13,000rpm and the supernatant was drained. The cells were then resuspended in 1ml of 50mM EDTA and spun for 3 minutes at 13,000 rpm. The supernatant was then drained and the cells received an additional 1.5-minute spin at 13,000rpm to remove any additional 50mM EDTA. The cell pellet was then resuspended to have a final cell concentration of $2.5X\ 10^5$ or $1.0X\ 10^6$ cell/lane. In a fresh centrifuge tube, 9ul of cells were placed along with 9ul SDS PAGE buffer, 1ul 20%SDS, and 1ul of Beta mercaptoethanol. The tubes were then heated at 100ºC in water for 10 minutes and stored at -20ºC.

2.8.2 *Acanthamoeba* protein standardization using Urea to Lyse cells

A more effective way to lyse trophozoite *Acanthamoeba* cells is by using an urea-based detergent. Comprised of 2% SDS, 6M Urea, 62.5mM Tris (1M Tris solution pH 6.8) and 160mM DTE. *Acanthamoeba* were then counted at cell

densities of $5X10^5$ cells/ml, $1X10^6$ cells/ml, $2.4X10^6$ cells/ml, and then $4.5X10^6$ cells/ml up to 12 hours of stationary phase. The last sample that was taken was 100% cysts. Every count was checked with a 2% SDS count to make sure the samples remained trophozoite. After the cell count of cell density, *Acanthamoeba* were then harvested to be at $5X10^6$ cells/lane. Cells were spun down for 3-minutes at 13,000rpm, decanted, washed with 1ml of 50mM ETDA, spun again for 3-minuites at 13,000rpm, and decanted. A final spin at 1 additional minute at 13,000rpm was performed and final volume of cells was resuspended using 100ul of the urea mixture and stored at -20ºC.

2.9 Western Blot Analysis of Metacaspase Antibodies

After the cells were harvested, a standard 12% separating protein mini-gel was assembled by adding 4.5ml of 40% acrylamide (Bio-rad cat. 161-0148), 6.45ml miliQ water, 3.75ml 1.5M tris-HCl pH=8.8, 150ul 10% SDS, 150ul APS, and 6ul TEMED. The 4% stacking gel was assembled by adding 0.625ml of 40% acrylamide, 0.625ml 1M tris-HCl pH=6.8, 50ul 10% SDS, 50ul, APS, and 5ul TEMED. The 40% acrylamide was always added last to prevent premature solidification of the gel. Approximately 150ul of 75% ethanol was placed over the separating gel while it set and then blotted off with a wipe before the stacking gel was placed. Each part of the gel was let to sit for 20-30 minutes. The samples were then loaded and run in 1X running buffer, which was comprised of 90ml of miliQ water and 10ml of 10X running buffer. The 10X running buffer was made from 29.0g tris base, 144.0g glycene, 10.0g SDS, and the volume was brought

up to 1 liter. The gel box was assembled with the wells facing in, and the running buffer completely filled the inside chamber and about 200ml of the outside chamber. The samples were then run for 1 hour at 200v. The gel was then placed in a container and 50ml of coomassie blue was used to stain the gel over night. The next day, the coomassie blue was drained off and the gel was then destained for 1-3 hours with protein destain made up of 800ml miliQ water, 100ml acetic acid, and 100ml methanol.

While the gel was being prepared, the *Acanthamoeba* samples were taken from the -20ºC and thawed. Then 3ul of 20% SDS was added to each sample, if it was an SDS protein standardized sample, and placed in a hot water bath at 100ºC for 10 minutes. The samples were then taken out and spun at 2,000rpm for 2 min. Before loading the wells the samples were pipetted up and down to make sure there was consistency and there was no pellet.

When transferring the gel to a membrane, in a container, 4 filters, 5 pads, and 2 membranes were soaked for approximately 30 minutes in 700ml of tris-glycine SDS transfer buffer. The tris-glycine SDS Transfer Buffer was made from 3.0g tris base, 14.40g glycine, 200ml methanol, and the volume was brought up to 1 liter with miliQ water. The nitrocellulose membrane, filter paper sandwhich 0.45ul pore size, 20/pk, (Invitrogen cat. 625411) was dated with a pen before being soaked. The assembly of transfer was done by starting with the negative side, adding 2 blotting pads, 1 filter paper, first gel, 1 transfer membrane, 1 filter aper, 1 blotting paper, 1 filter paper, second gel, 1 transfer membrane, 1 filter paper, and 2 blotting pads. This gel was then run for 1 hour at 30v.

Many different western blot kits were used such as Ameresco Western kit (cat. K205-KIT) horseradish peroxide based and The Sigma Western Breeze kit (cat. WB7105) to identify if the metacaspace was present in the *Acanthamoeba* samples that were standardized as described previously. The chromogenic substrate in the Western Breeze kit was left on for 1-2 hours in order to see the developing bands rather than 0-60 minutes as instructed. The SDI western protocol provided by SDI who processed and developed the antibody Rabbit 1 and Rabbit 2 was followed and was also horseradish peroxide based. Dilutions were made from 1:50 up to 1:500. The secondary antibody used in all the kits for metacaspase was an antirabbit from Sigma (cat. no A6154-1ML). The developmental stage of the western was changed by using one DAB tablet (Ameresco cat. E733) provided from the Ameresco western kit along with one drop of H_2O_2 provided from the Ameresco western kit (cat. E882) and 10ml of miliQ water.

The above protocol was revised using PVDF membrane and chemiluminecence in order to confirm if the metacaspase was present in the cells. When using PVDF membrane (Milipore cat. IPVH00010), the membrane was soaked in methanol on a rocker for 5 minutes then followed by 30 minutes of miliQ water wash also on a rocker prior to being transferred. After the transfer step had occurred, a 7% milk/TBS/T mixture was used to block the membrane on a rocker for 2 hours and then the appropriate antibody was diluted and placed on the rocker overnight. Around 14-16 hours later, the membrane was then washed with 1X TBS/T with 6-10, 10-minute washes. The secondary antibody was then

applied to the membrane for 2 hours. Another wash with 1X TBS/T with 6-10, 10-minute washes was performed. Finally, the membrane was taken to a dark room. An equal 1:1 mixture of 1.5ml of ECL1 and ECL 2 (Fisher cat. RPN2106V1) was added for1 min and rocked to obtain uniformity over the membrane. The membrane was then blotted in between 2 layers of blotting paper and then placed onto plastic wrap in a film cassette. Film was then layered directly on top of the membrane and exposure time was between 12-15 minutes.

2.10 Cell fixing and Immunocytochemistry

2.10.1 Cell Fixing-pre permiablizing *Acanthamoeba*

In order to view where the metacaspase protein is located in *Acanthamoeba*, cells first have to be fixed so they do not digest the antibody. *Acanthamoeba* cells were harvested at $2X10^5$ cells/ml and 1ml was placed in 10 centrifuge tubes. The cells were then spun down at 13,000rpm for 5 minutes and then washed. The first wash consisted of 1ml of 1XPBS, and the second wash consisted of 500ul of 1XPBS. The cells were then resuspended with the slurry left over after draining off the supernatant. Next, using 20ml PBS added to 0.8g of paraformaldehyde, it was then placed on a hot plate and stirred in a fume hood until the mixture was clear made 4% paraformaldehyde and stored at 4ºC. Using the 4% paraformaldehyde was then added to the tubes in increasing increments beginning with 10ul to 500ul. Then 7ul of the mixture was placed on a slide and 7ul of 50% glycerol was added on top of the samples. After allowing the samples to dry for 30 minutes, the samples were then viewed under a microscope.

2.10.2 Cell Fixing- post-permiablizing *Acanthamoeba*

Acanthamoeba were harvested at a cell density of $1.1X10^6$ cell/ml at early and late log phases. One ml of cells were placed into a single well of a 24 well dish due to the cell's ability to adhere to the well. The cells were placed in a stationary incubator at 30ºC for 24 hours and allowed to grow and divide to reach mid log phase density of $3X10^6$ cells/ml. Cells were then carefully asperated from the wells. The cells were then fixed with a mixture of 500ul of 3% formaldehyde and 0.25% glutaraldehyde in PBS for 45 minutes at room temperature. Cells were then washed in 1ml of PBS, pH 7.4 for 5 min. The cells were then permiablized with 500ul of 0.2% saponin in PBS for 15 min. Cells were again washed with 1ml of PBS, pH 7.4 for 5 min. After, the cells were treated with 1mg/ml of sodium borohydride in PBS for 10 min to reduce the free aldehydes. To block nonspecific binding of antibodies, cells were incubated in 1% BSA and 50mM L-lysine in PBS, pH 7.4 for 2 hours in room temperature. Cells were then incubated with a 1:200 primary metacaspase antibody in BSA/lysine/PBS buffer overnight at room temperature. The next day, five washes with PBS were performed 5 min apiece. The secondary florescent antibody was then incubated with a 1:20 dilution for 2 hours in BSA/lysine/PBS buffer in the dark room. An additional five washes with PBS for 5 min apiece were performed and then the cells were viewed under fluorescent microscopy either immediately or the following day (Bowers and Korn, 1990).

CHAPTER 3: RESULTS

3.1 DNA Restriction Digests

Several plasmids were used throughout these studies for transfecting *Acanthamoeba* cells and for expressing recombinant proteins in *E. coli*. Initially, it was necessary to be certain each of the constructs contained the metacaspase insert of the correct size. For this purpose, a series of plasmid preps and DNA restriction digests were carried out. Prior to analysis, DNA was first purified utilizing a Qiagen Mini Prep Kit. Purified DNA was then restriction digested and analyzed on 1% agarose gels. Plasmids used in these studies are listed in Table 1 below and include full-length metacaspse-p-110EGFP (FLM-p-110EGFP), the vector with full-length metacaspse insert, p-110EGFP without the metacaspase insert to be used as a transfection control,and c-terminus-p-110EGFP, vector with c-terminus metacaspase insert. Other plasmids that were used for preparing recombinant proteins were pGEX (no insert control), c-terminus (of metacaspase) in pGEX, and FLM (full-length metacaspase) in pGEX (figure 5 and 6). The full-length metacaspase insert is shown at 1700bp (Fig. 5, lane 3).

Restriction digests with EcoR1 and Xho1 to release the insert were analyzed on a 1% agarose gel to confirm the presence and size of insert. The Qiagen Plasmid Giga Kit (for endotoxin free DNA) was used to purify with p-110EGFP, FLM-0-110EGFP, and c-terminus-p-110EGFP. These purified DNA samples were restriction digested with NdeI (to release the insert) followed by electrophoresis on a 1% agarose gel to confirm the size and presence of the inserts (figure 6). Figure 6 shows a 1% Agarose gel stained of restriction

digested plasmids with NdeI with plasmids p-110EGFP and FLM-p-110EGFP, which confirmed the metacaspase insert at ~1700bp. Purified DNA was later used for transfection of *Acanthamoeba* via electroporation.

Plasmid	**Description**	**Purpose**
p-110EGFP	Vector only, no insert	Transfection control
FLM-p-110EGFP	Vector with full-length metacaspase insert	Transfection of *Acanthamoeba*
C-terminus-p-110EGFP	Vector with c-terminus metacaspase insert	Transfection of *Acanthamoeba*
FLM-pGEX	pGEX Vector with full-length metacaspase insert	Expression of recombinant protein

Table 1: Represents the different plasmids used in this study.

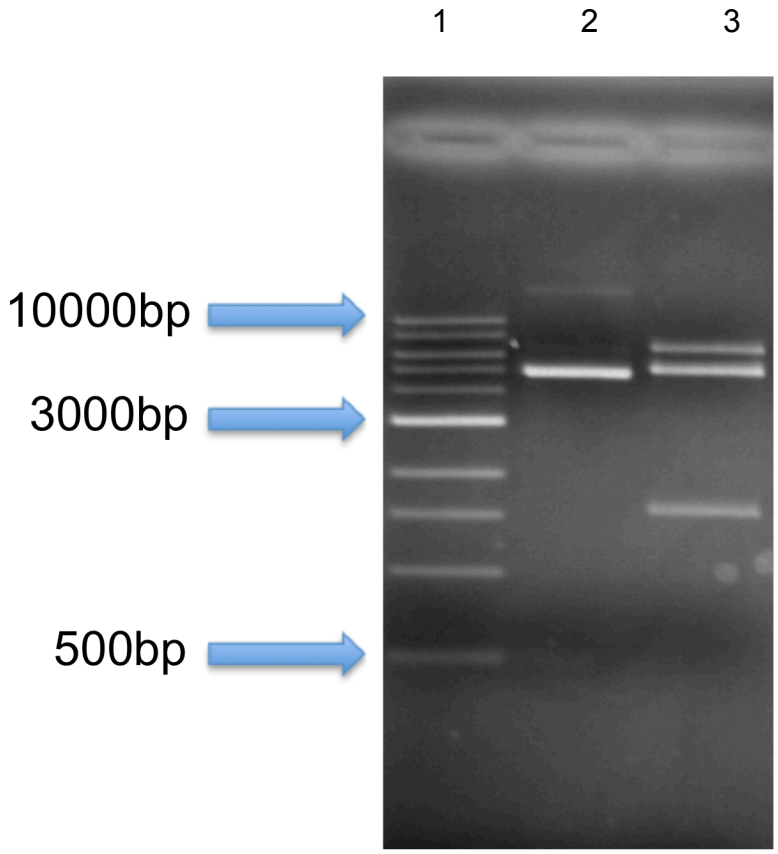

Figure 5: 1% Agarose gel of restriction digested plasmids. Lanes are as follows: lane 1) Molecular Weight Marker, lane 2) undigested FLM in PGEX, lane 3) FLM in PGEX digested with double digest ECoR1 and Xho1. FLM in PGEX is at full-length metacaspase at 5,000bp and FLM in PGEX is digested at 1,700bp.

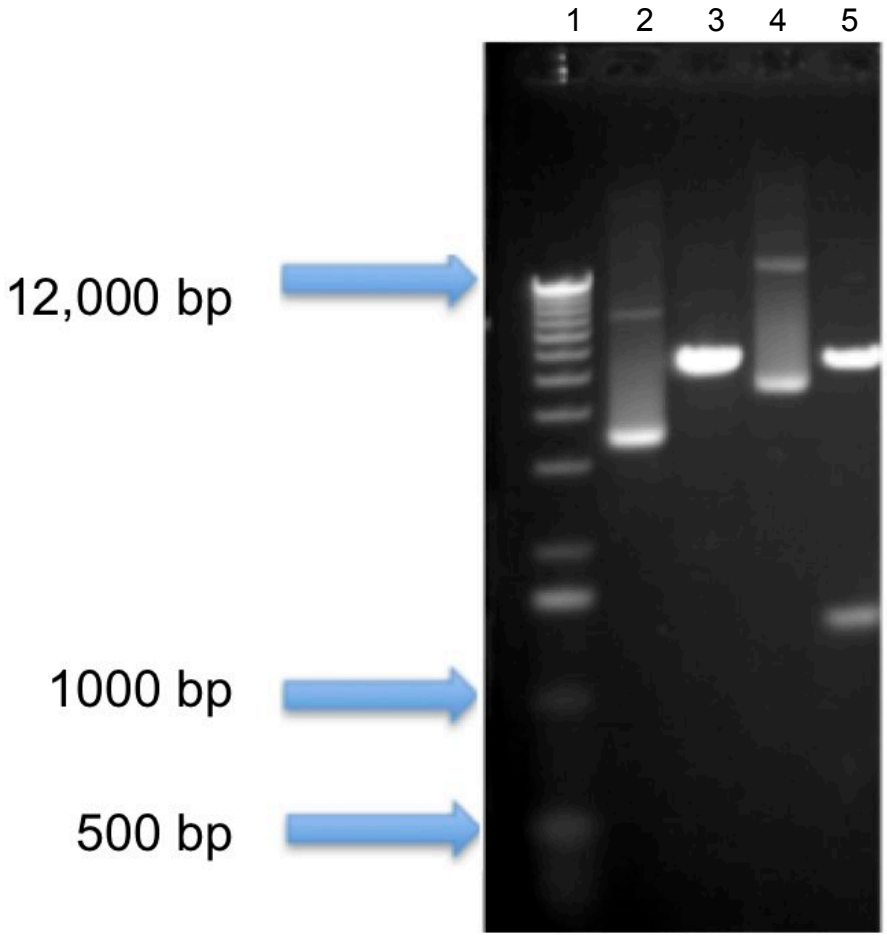

Figure 6: 1% Agarose gel stained of restriction digested plasmids with NdeI. Lanes are as follows: lane 1) Molecular Weight Marker, lane 2) p-110EGFP undigested, lane 3) p-110EGFP digested (~5-6kbp) lane 4) FLM-p-110EGFP undigested, and lane 5) FLM-p-110EGFP digested (~1700bp).

3.2 Transfection of *Ancanthamoeba*

After the plasmids were purified and the correct metacapase inserts were confirmed by using restriction digests, the plasmids were ready to be tranfected into *Acanthamoeba*. The previously purified DNA from the Qiagen Plasmid Giga Kit, p-110EGFP and FLM-p-110EGFP samples were then transfected using various voltages by utilizing both electroporation and chemical transfection methods in order to determine the optimal conditions. The results from the electroporation of cells were too damaging, and many cells were either lysed or killed. In order to enhance cell viability, transfection via superfect was then performed.

The cells transfected via superfect were then harvested into shaking *Acanthamoeba* media and treated with antibiotic G418. After incubation of *Acanthamoeba* over several days only those cells carrying the plasmid and those developing resistance to G418 were selected and were observed again under GFP flourescence and found to still have a very weak fluorescent signal.

Acanthamoeba trophozoite cells under bright field 40X transfected with p-110EGFP (figure 7) show a lot of cellular debris in the background and the cells do not look very intact. The same cells under GFP flourecence (figure 8) were imaged. As predicted the cells did not show fluorescence due to the fact that there was not a metacaspase insert present. The cells also confirmed that the cells themselves did not self fluoresce. *Acanthamoeba* cells under bright field 40X transfected with the FLM–p-110EGFP (figure 9) showed the same amount of cellular debris in the background, meaning the total number of cells had been

diminished. The *Acanthamoeba* cells still did not look like “healthy” trophozoites when transfected with FLM–p-110EGFP. The same cells transfected with FLM–p-110EGFP and under GFP frequency (figure 10) were also imaged and showed a very inconclusive result by having a relatively faint fluorescent glow. Because the cells were not fixed, many times the GFP was easy to locate but would fade very quickly. Figure 11 is a breakdown of *Acanthamoeba* cells transfected with p-110EGFP and FLM-p-110EGFP. Panels “b” and “e” show the faint merged epifluorescence and white light images of *Acanthamoeba* trophozoite cells. Panel “b” shows p-110EGFP where there is no fluorescence except in vacuoles. Panel “e” shows FLM-p-110EGFP and how there is minimal fluorescence in what appear to be vacuoles. Panel h shows an *Acanthamoeba* cyst with the same minimal fluorescence when transfected with FLM-p-110EGFP. Exposure time was between 200 and 400ms with GFP Ex 470nm/Em 525nm filter set at 20X or 40X LD plan-Neofluor objective for all pictures. Overall, these images showed only a very weak fluorescent in any case.

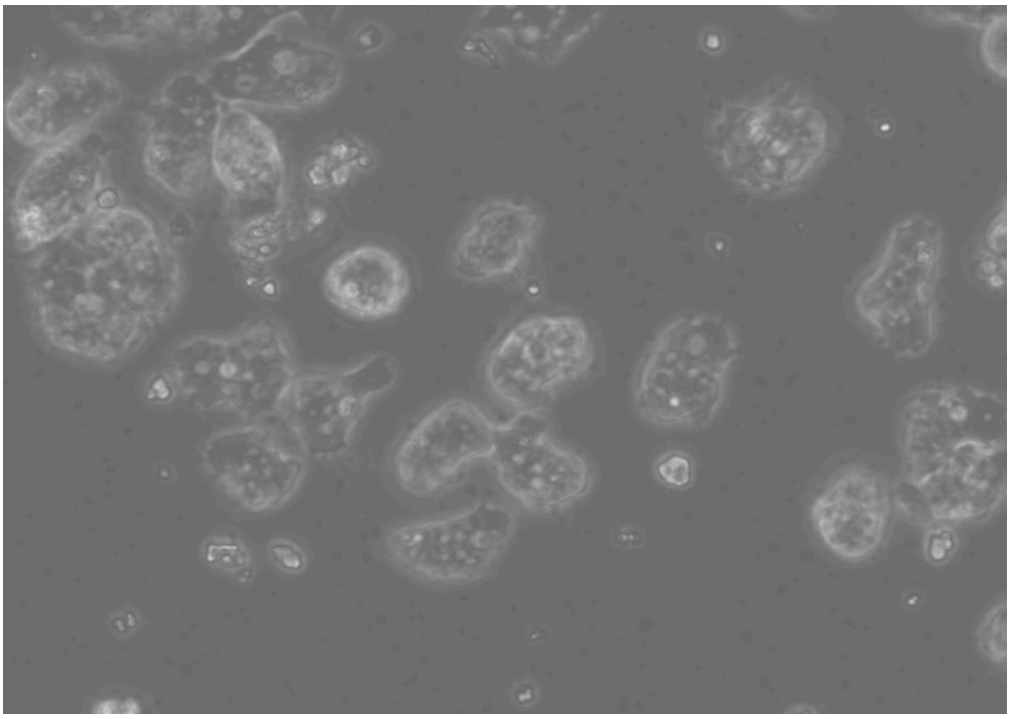

Figure 7: *Acanthamoeba* cells under brightfield 40X transfected with p-110EGFP.

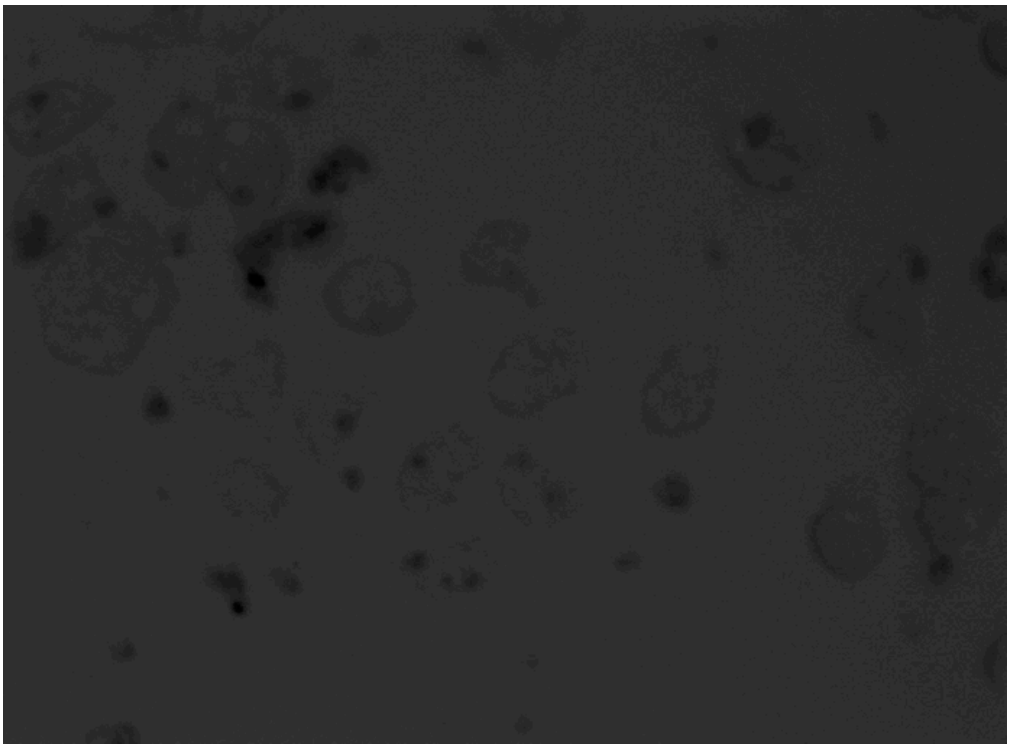

Figure 8: *Acanthamoeba* cells under GFP filter 40X transfected with p-110EGFP

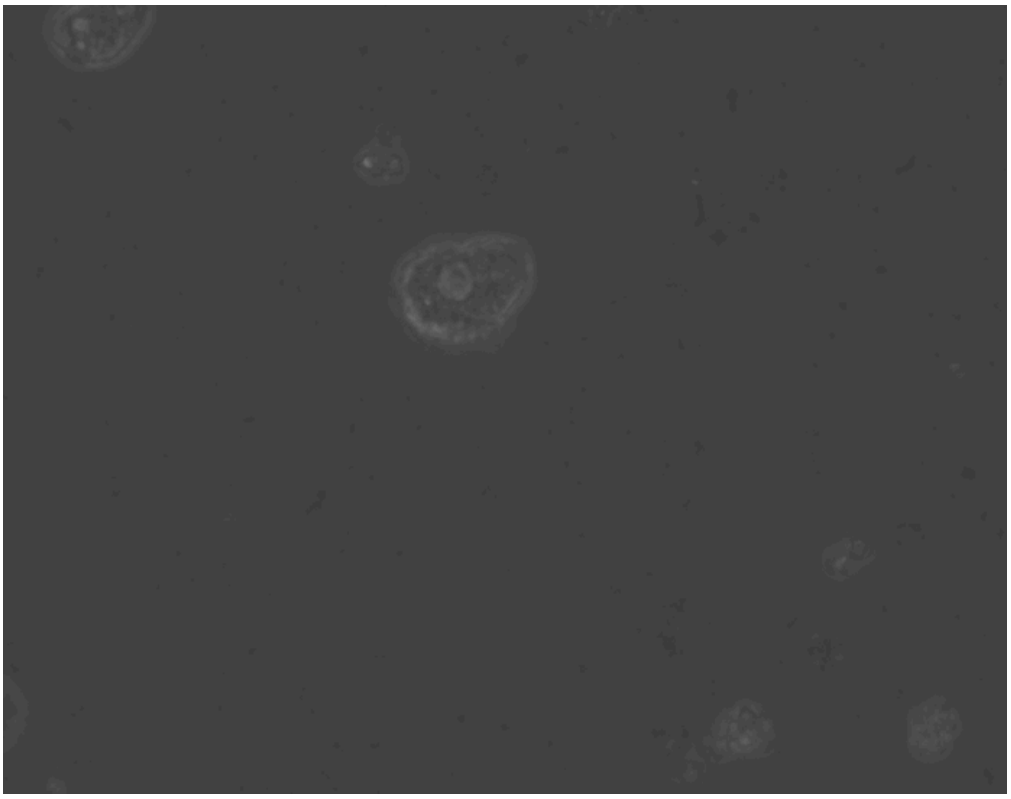

Figure 9: *Acanthamoeba* cells transfected with Full Length Metacaspase-p-110EGFP plasmid inserted cells under brightfield 40X

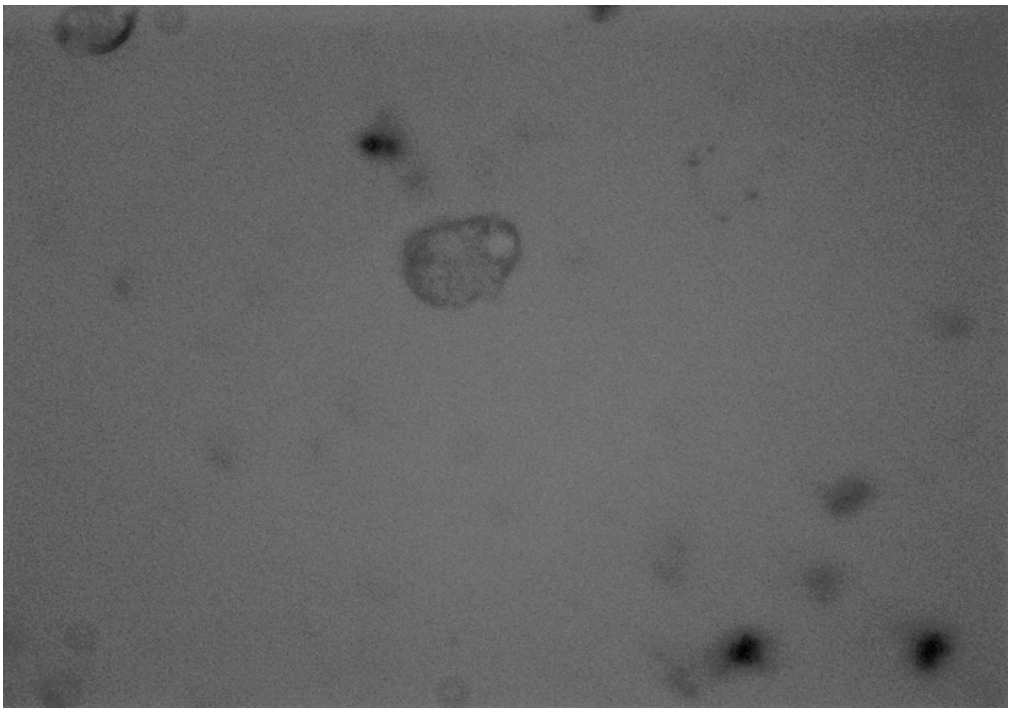

Figure 10: *Acanthamoeba* cells transfected with Full Length Metacaspase-p-110EGFP plasmid inserted cells under GFP 40X

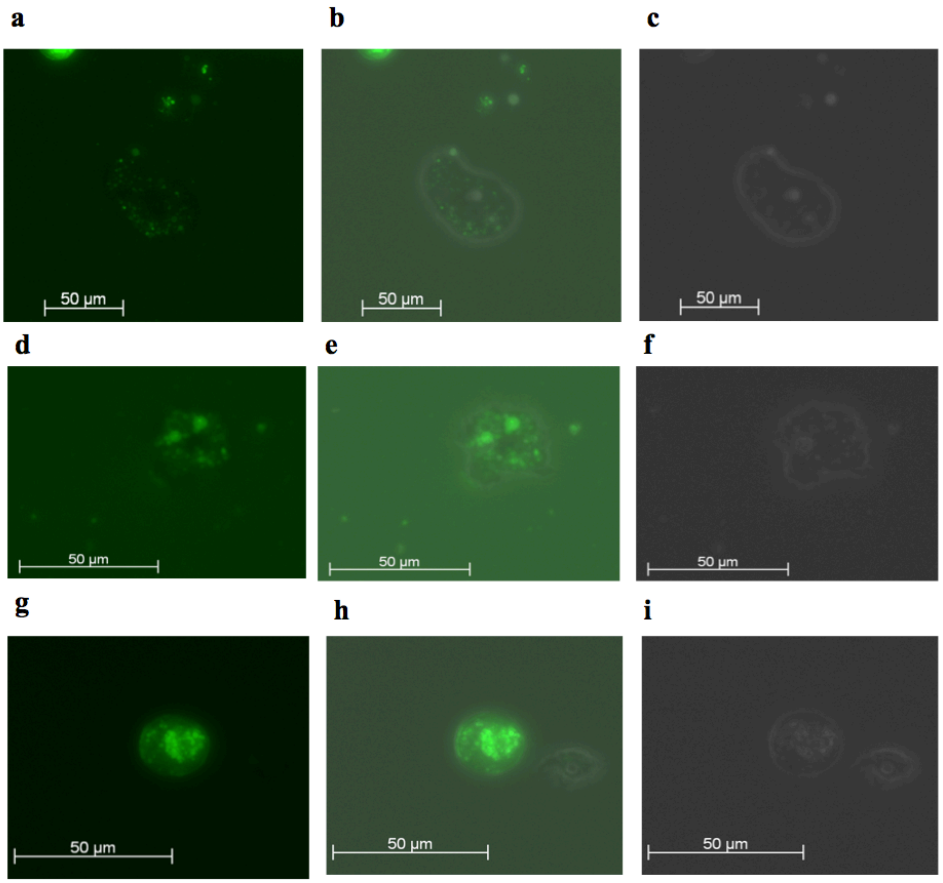

Figure 11: *Acanthamoeba* cells transfected with p-110EGFP (a-c) and FLM-p-110EGFP (d-i). *Acanthamoeba* cells were taken with Epifluorescence (a,d,g), merged (b,d,h), and brightfield (c,f,i). Exposure time was between 200 and 400ms with GFP Ex 470nm/Em 525nm filter set at 20X or 40X LD plan-Neofluar objective for all pictures.

3.3 Recombinant Protein Isolation

Following the transfection study (which gave inconclusive results), the next step was to harvest recombinant metacaspase protein and later confirm the presence of metacaspase with a protein gel. Recombinant protein plasmid was first harvested from *E. coli* BL21 expressing the c-terminal portion of metacaspase (c-terminus colony in pGEX, colony 3A), and full-length metacaspase in pGEX (colony 11). *E. coli* BL21 was harvested as a control (figure 12) and shows BL21 induced with IPTG over 6 hours. As expected, there is no effect on the cells when IPTG is added. C-terminus colony 3A in pGEX and FLM in pGEX was harvested as a positive control plasmid for metacaspase in figure 13. This gel resulted in a very strong band at ~43kDa (the expected size of the C-teminal fragment of metacaspase and the GST fusion partner). Also in figure 13, FLM in pGEX showed an expressed band after addition of IPTG. The purpose of adding IPTG was to induce the expression of proteins over a 6-hour period of time. Afterwards, a Bradford assay was completed to confirm the total amount of protein in each sample. Recombinant proteins could be used on gels as controls in western blots.

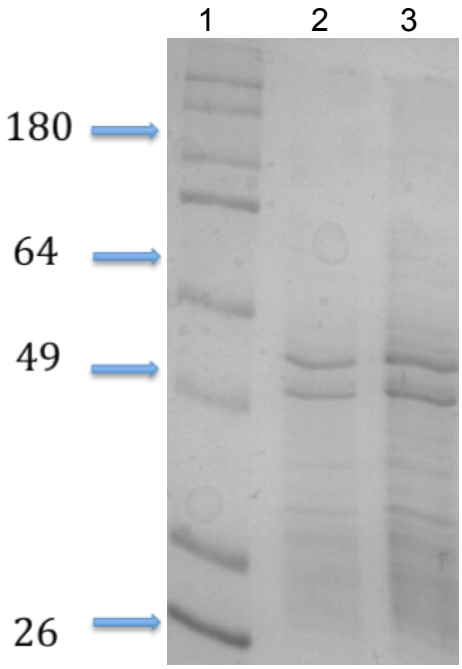

Figure 12: : Coomassie Blue Stained SDS-PAGE Gel of total *E.* coli Protein. Total protein from *Acanthamoeba* cells were extracted using SDS. Lanes are as follows: lane 2) *E.coli* BL 21 control uninduced with IPTG. Lane 3) BL21 induced with IPTG after 6 hours in lane no. 3. Lane 1) is a Molecular Weight marker 1KDa Protein Plus.

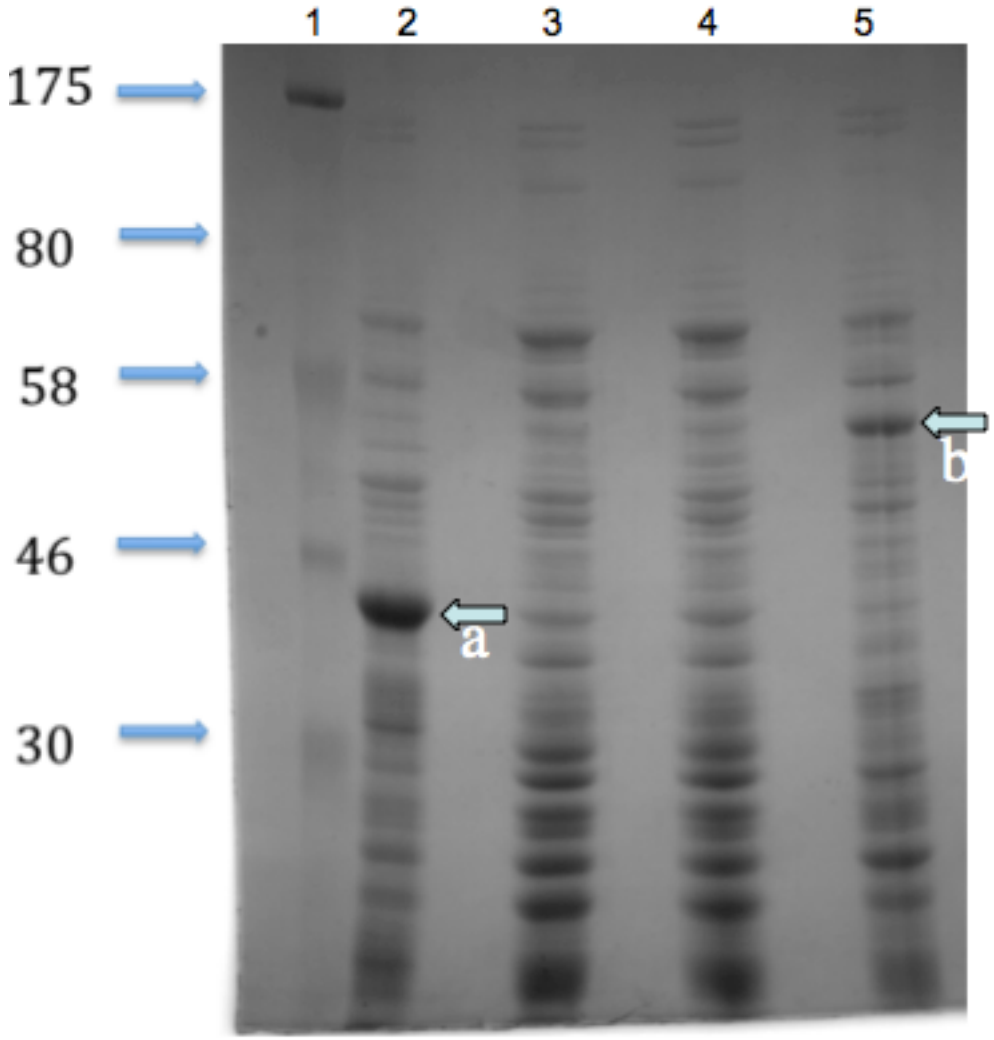

Figure 13: Coomassie Blue Stained SDS-PAGE Gel of total *E* coli Protein. Total protein from *Acanthamoeba* cells were extracted using SDS. Lanes are as follows: lane 2) C-terminus colony 3A in PGEX induded with IPTG after 6 hours (band “a”), lane 3) C-terminus colony 3A in PGEX uninduded, lane 4) FLM in PGEX unindiced with IPTG, and lane 5) FLM in PGEX induced with IPTG after 6 hours (band “b”). Lane no 1 is a Molecular Weight marker 1KDa Protein Plus Marker.

3.4 *Acanthamoeba* Protein Standardization

3.4 .1 *Acanthamoeba* Protein Standardization Using SDS to Extract Total Protein

Western blots were needed in order to determine the specificity of the metacaspase specific polyclonal antibodies. For this purpose, total Acanthamoeba proteins would be extracted from cells and run on SDS-PAGE gels. *Acanthamoeba* cells were subcultured and then harvested at early ($5x10^5$ cells/ml), middle ($3x10^6$ cells/ml), and late log phases ($6x10^6$ cells/ml), over a course of 16-18 hours. The cell densities are used to define the point at which cells are in the growth and differentiation stages of *Acanthamoeba* (Trzyna et al. 2008). Once the cells were harvested, samples were prepared to contain $1X10^6$ cells that were loaded in each lane onto a 12% protein gel (figure 14). It was determined, as in figure 14, that total protein from $1X10^6$ cells was suitable for a single lane on a gel and that *Acanathamoeba* cells were able to be lysed with SDS but a relatively high concentration was needed.

In order to see if metacaspase protein was detectable in cysts, 125mM NaCl was added to early log phase and late log phase cells. 125mM NaCl simulates cells to undergo encystment. These cells were then harvested 16-18 hours later, and samples were prepared for SDS-PAGE gel that each one contained the protein equivalent of $1X10^6$ cells. $1x10^6$ cells were loaded per lane when loaded onto a 12% protein gel (figure 15). Figure 15 resulted in a visual difference between the *Acanthamoeba* cells that were not induced with 125mM NaCl and those that were. The gels also concluded that the lanes were still able

to be equally loaded when encystment was induced. These gels were later used for western blots. The results, as shown in figures 14 and 15, were not optimal, and an alternative method for protein extraction and solubilization was used.

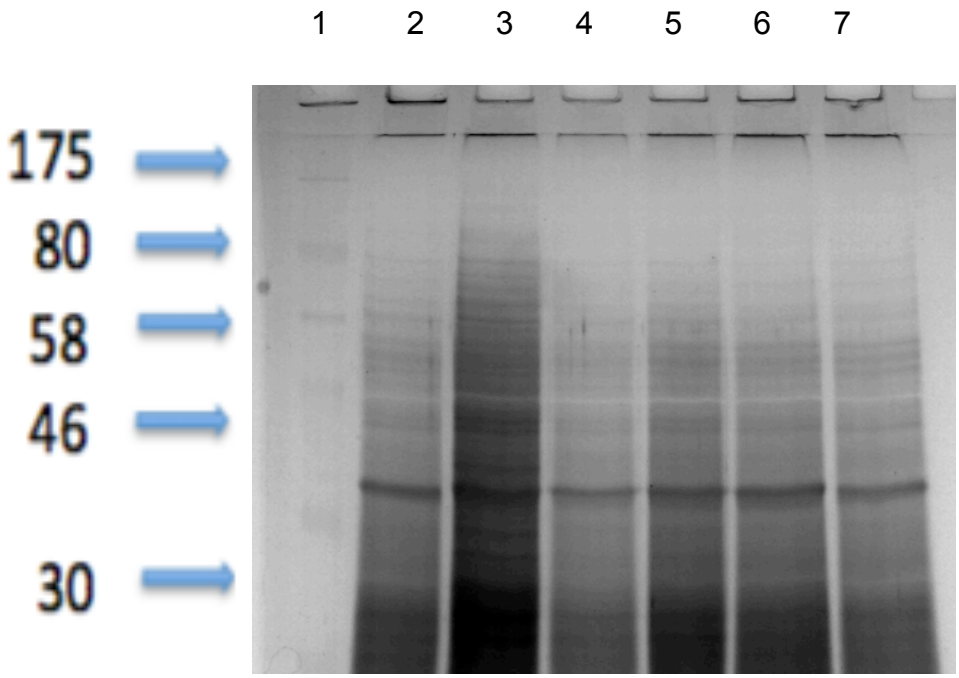

Figure 14: 12% protein gel stained with coomassie blue. Total proteins from Acanthamoeba cells were extracted using SDS. The samples in each lane contained total protein extracted from $1x10^6$ cells. Lanes are as followed: lanes 1-3 represents early log phase cells, lanes 4-5 represent mid log phase, and lanes 6-7 represent late log phase cells. Lane 1) is a Molecular Weight marker 1KDa Protein Plus.

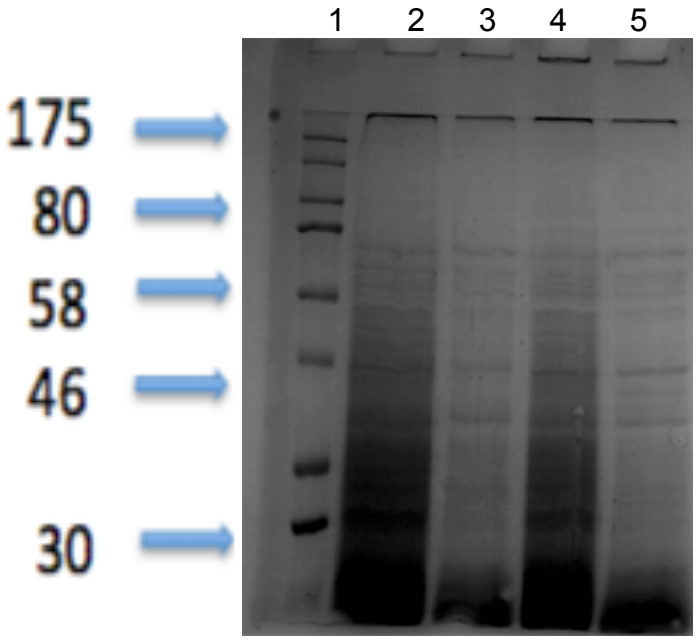

Figure 15: Coomassie Blue Stained SDS-PAGE Gel of total *Acanthamoeba* Protein using SDS extraction protocol. Certain cells were treated with 125mM NaCl at early and late log phases over 16-18 hours prior to harvesting. Lanes are as follows: Lane 1) represents total protein extracted from *Acanthamoeba* cells at $1X10^6$; cells were harvested at early log phase. Lane 2) represents total protein extracted from *Acanthamoeba* cells at $1X10^6$; cells were harvested at early log phase and induced with 125mM NaCl. Lane 3) represents total protein extracted from *Acanthamoeba* cells at $1X10^6$; cells were harvested at late log phase. Lane 4) represents total protein extracted from *Acanthamoeba* cells at $1X10^6$; cells were harvested at late log phase with 125mM NaCl. Lane 1) is a Molecular Weight marker 1KDa Protein Plus.

3.4 Presence of *Acanthamoeba* Metacaspase on Western Blot:

When western blots of SDS-PAGE gels of total *Acanthamoeba* protein were prepared, using SDS, they were unsatisfactory. The bands corresponding to metacaspase were not detectable even though there were sufficient amount of protein that were visible on the protein gels. *Acanthamoeba* cells were then lysed with urea as an alternative way of solubilizing and denaturing total proteins. Cells were harvested again at early, middle, and late log phases (as described earlier), and samples were prepared containing the total protein equivalent of $1X10^6$ cells for each lane. Protein was loaded onto the 12% protein gel (figure 16). A western blot was then performed. In order to see if metacaspase protein was present in cysts, 125mM NaCl was added to early log phase and late log phase cells. These cells were then harvested 16-18 hours later, and each lane represents the total protein extract from $1x10^6$ cells, loaded onto a 12% protein gel (figure 16). These gels proved to be more satisfactory than the earlier gels in which the protein was extracted using SDS.

After the SDS-PAGE gel (12% protein gel) was run to confirm all the lanes were loaded equally at $1X10^6$ cells/lane, a western blot was performed to evaluate the specificity of the metacaspase antibody. The antibody being used was previously raised to a synthetic peptide based on the 19 C-terminal amino acids of metacaspase. For the western blot, the primary antibody was diluted to 1:50 and the secondary antibody used was used at a 1:5000 dilution. After probing, the blot was visualized using chemiluminescence after 15 minutes of incubation. Placing the PVDF membrane under the microfilm and noting the

position of the pre-stained molecular weights on the film confirmed the protein ladder. The antibody bound to around 53 kDa, which is the expected size of metacaspase (figure 18). This result showed that metacaspase was positively being expressed in *Acanthamoeba* cells over a period of 16 hours. To evaluate the western blots for equal loading of *Acanthamoeba* protein, an antibody to actin was used as a control and diluted 1:5000 as seen in figure 20. The western blot confirmed the presence of actin protein, the banding patterns in each lane were different. There are considered to be as many as eight different actins present in *Acanthamoeba* over the life cycle of the cell (Bateman, 1998). This feature and the presence of multiple bands that change from sample to sample, provide evidence that actin will not be a useful “reference gene” for these studies. The pre-immune serum was also used as a control to the metacaspase antibody (figure 19). On the blot, the pre-immune sera did not detect a band at 53kDa in samples from cells at early, middle, and late log phases resulting in a positive outcome.

In order to observe if metacaspase protein was present in cysts (i.e. a culture that had completely encysted), a western blot was performed and probed with the metacaspase antibody. For this blot, cells were first induced with 125mM NaCl over 16-18 hours to stimulate encystment prior to harvesting. Total protein extracts were then prepared as described previously and loaded on the protein gel. Figure 17 shows the total amount of *Acanthamoeba* protein loaded on the gel with equal loading. Figure 21 points out the presence of metacaspase can be

detected in trophozoites and in encysting cells in early and late log phases with a band being detected at ~53kDa.

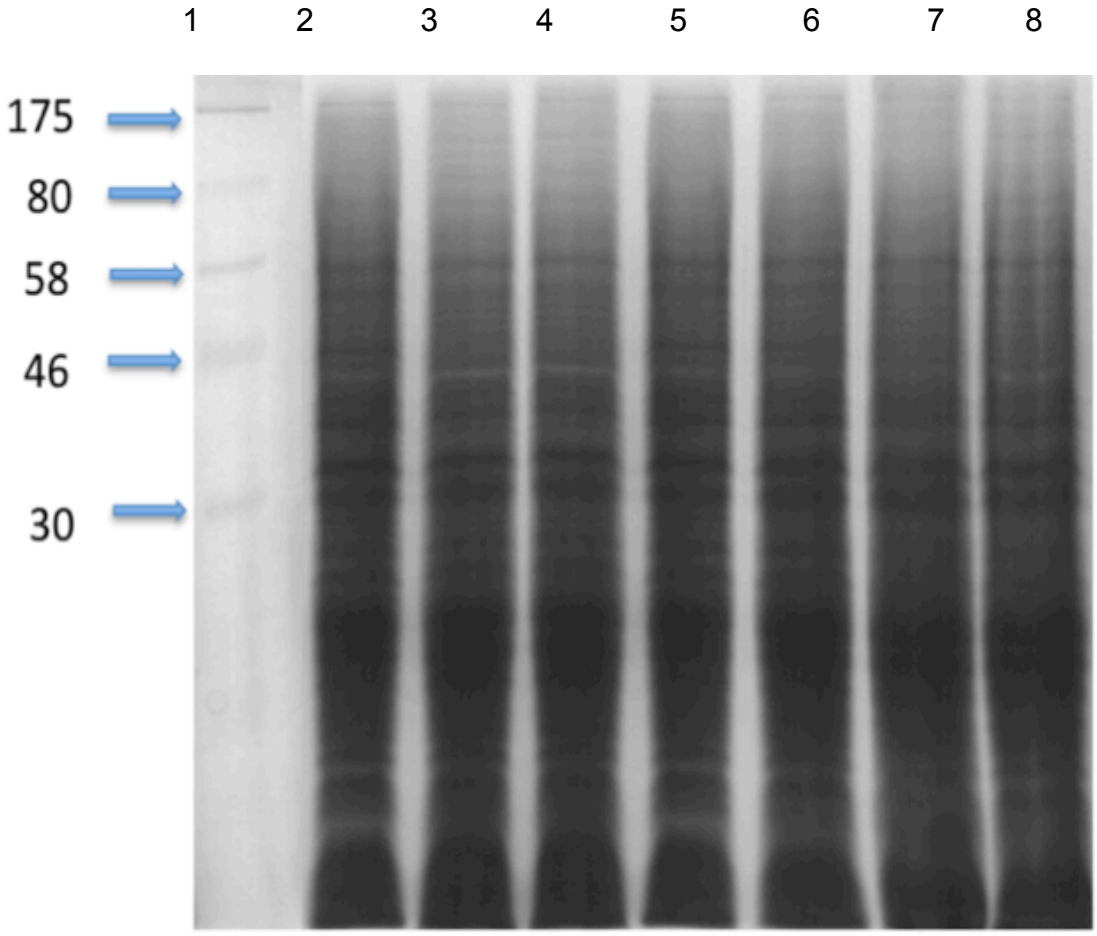

Figure 16: Coomassie Blue Stained SDS-PAGE Gel of total *Acanthamoeba* Protein. Cells were harvested with urea to lyse *Acanthamoeba* cells. Cells were harvested for total protein extracted from *Acanthamoeba* cells at $1X10^6$. Lanes are as followed: Lane no. 1-3 represents early log phase cells, lane no. 4-5 represent mid log phase, and lane no. 6-7 represent late log phase cells. Marker is: 1KDa Protein Plus Marker.

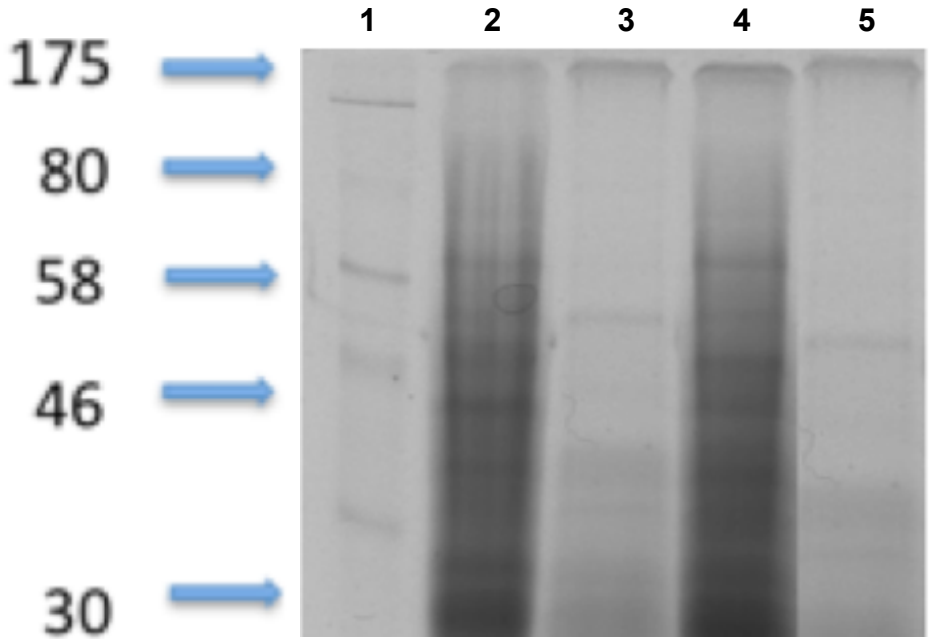

Figure 17: Coomassie Blue Stained SDS-PAGE Gel of total Acanthamoeba Protein using Urea. Cells were treated with 125mM NaCl at early and late log phases over 16-18 hours prior to harvesting. All lanes were equally at $1X10^6$ cells/lane. Lanes are as follows: Lane 2) represents *Acanthamoeba* cells at $5.5x10^5$ cells/ml with out salt induced. Lane 3) represents *Acanthamoeba* cells at $5.5x10^5$ cells/ml with 125mM salt induced. Lane 4) represents *Acanthamoeba* cells at $6x10^6$ cells/ml with out salt induced. Lane 5) represents *Acanthamoeba* cells at $6.5x10^6$ cells/ml with 125mM salt induced. Lane 1) is 1KDa Protein Plus Marker.

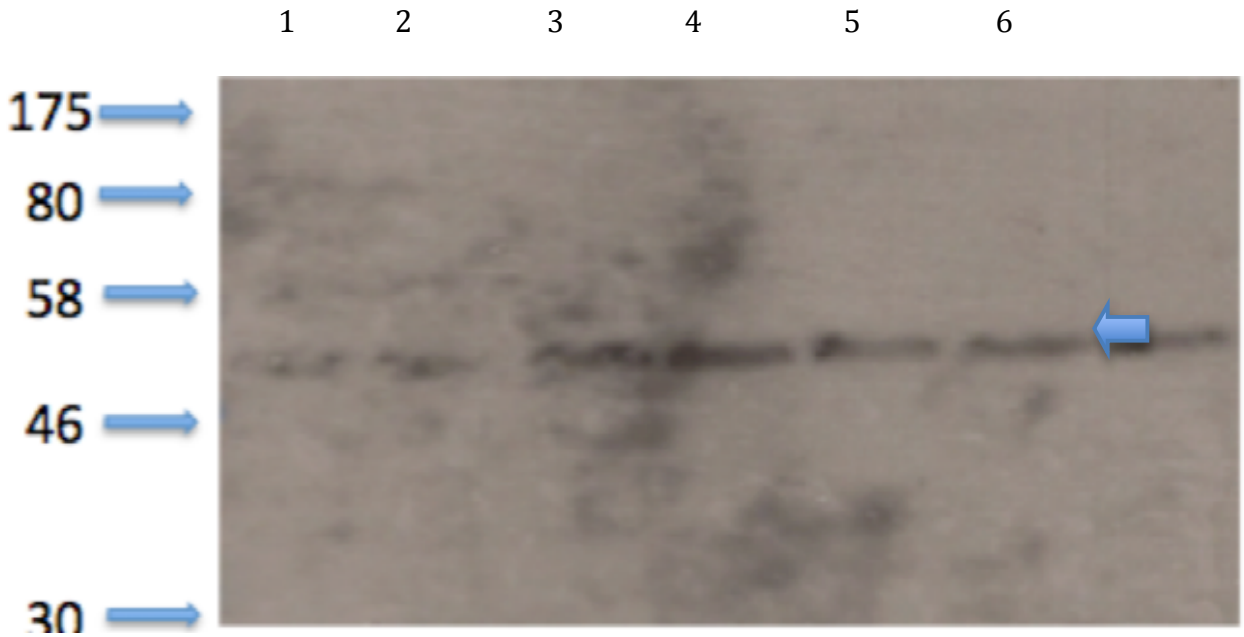

Figure 18: Western blot of total *Acanthamoeba* protein probed with the synthetic metacaspase peptide. Lanes are as follows: lanes 1-3 represents cells in early log phase, lanes 4-5 represent cells in mid log phase, and cells in lanes 6-7 represent cells in late log phase. All lanes were loaded equally ($1X10^6$ cells/lane). Metacaspase is noted at ~53kDa (arrow).

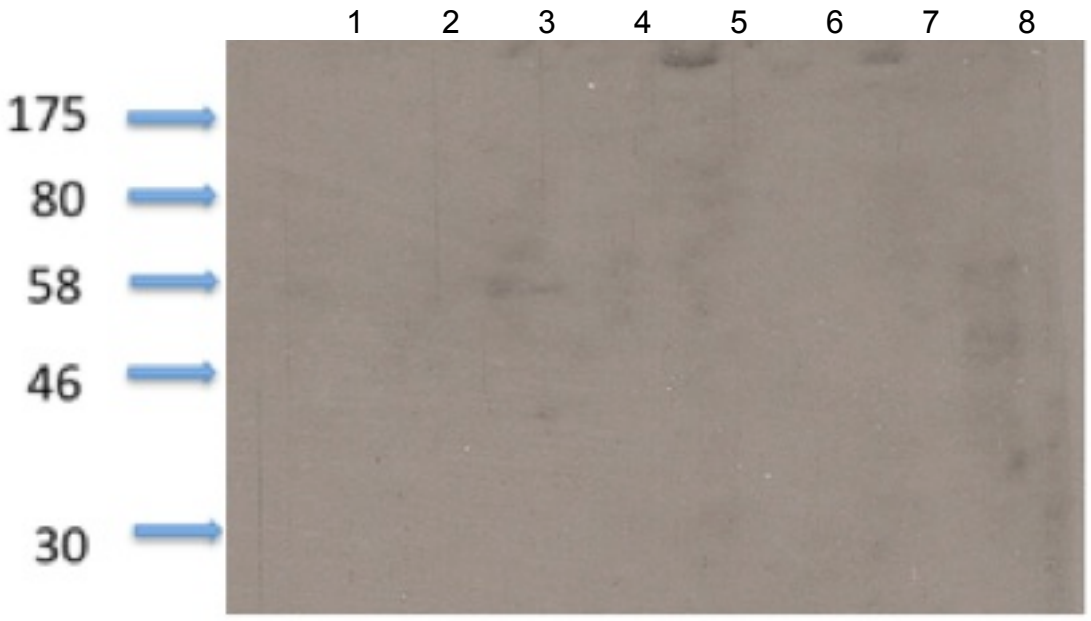

Figure 19: Western blot of total *Acanthamoeba* protein probed with pre-immune sera of the synthetic metacaspase peptide. Lanes are as follows: lanes 1-3 represents cells in early log phase, lanes 4-5 represent cells in mid log phase, and cells in lanes 6-8 represent cells in late log phase. All lanes were loaded equally ($1X10^6$ cells/lane).

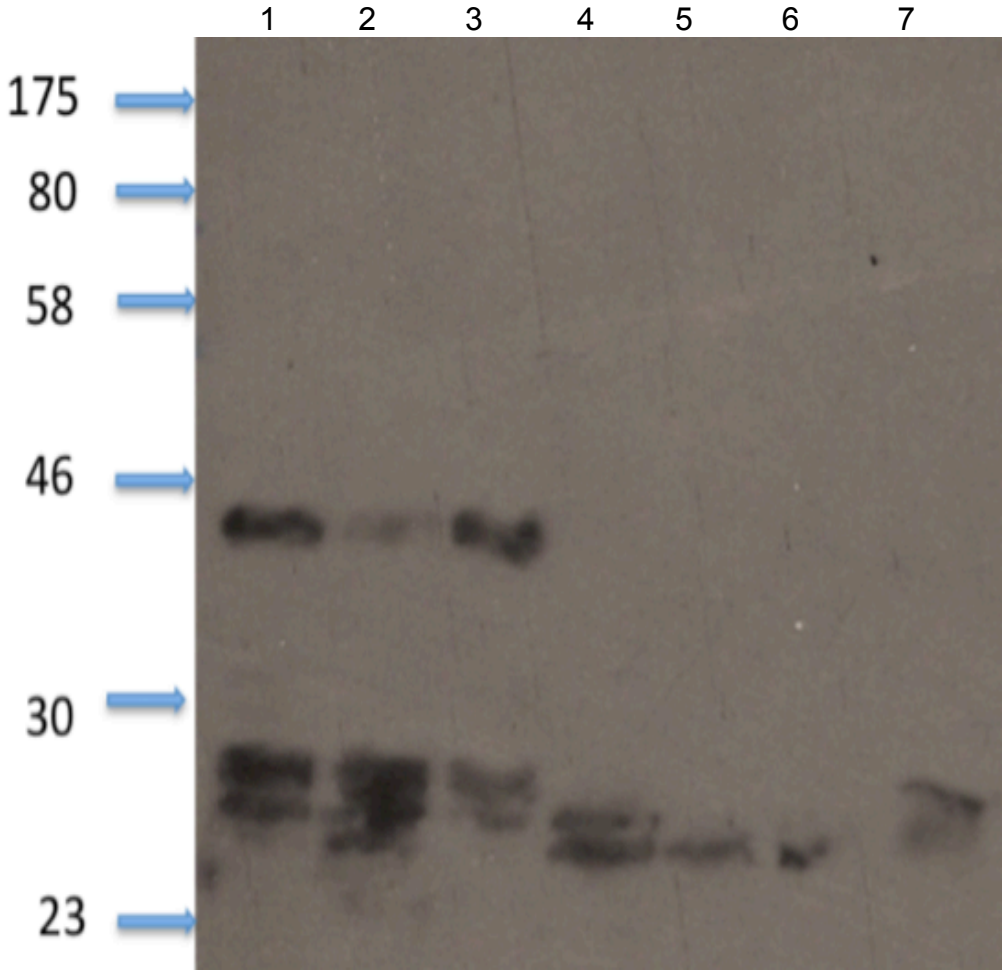

Figure 20: Western blot of total *Acanthamoeba* protein probed with C4 actin. Lanes are as follows: lane 1-3 represents cells in early log phase, lanes 4-5 represent cells in mid log phase, and cells in lanes 6-7 represent cells in late log phase. All lanes were loaded equally ($1X10^6$ cells/lane).

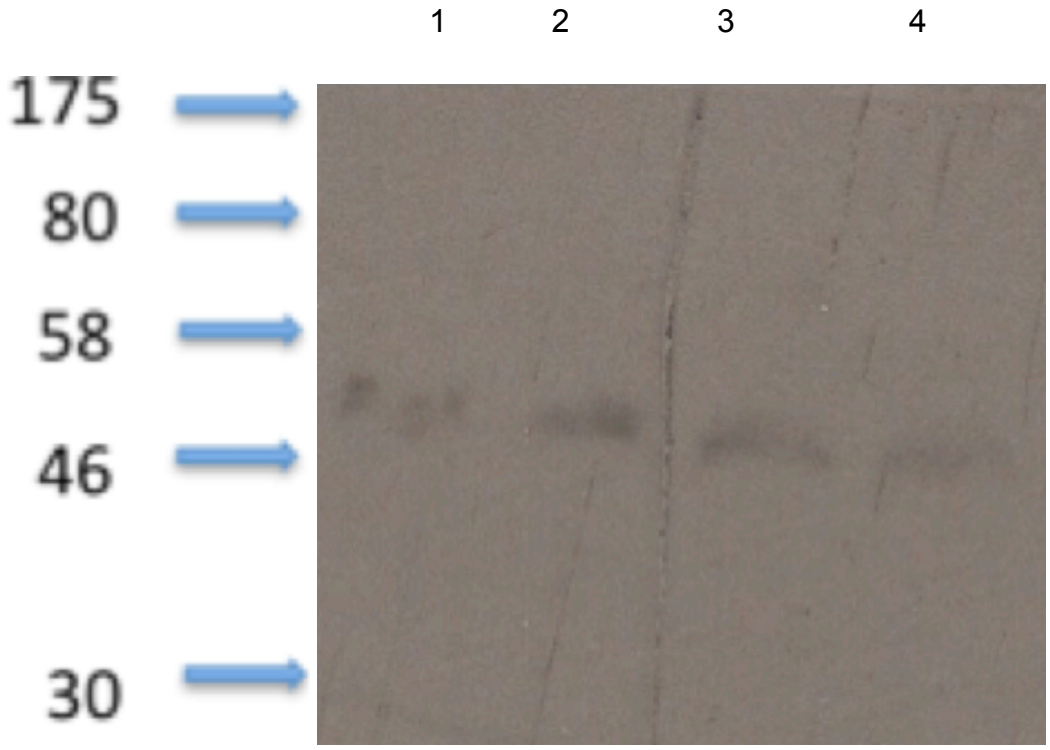

Figure 21: Western Blot of total *Acanthamoeba* protein induced with 125mM NaCl. Lanes are as follows: lane 1) represents early log phase cells with out salt. Lane 2) represents cells harvested 16-18 hours after NaCl induced. Lane 3) represents cells in late log phase with out the induction of salt. Lane 4) represents cells harvested 16-18 hours after the induction of NaCl. All lanes were loaded equally ($1X10^6$ cells/lane). Refer to figure 17 for corresponding stained 12% protein gel.

3.5 *Acanthamoeba* Immunocytochemisty:

In the previous standardized *Acanthamoeba* protein gels and western blots, the antibody to metacaspase was present at approximately 53 KDa. Because the antibody appears to be recognizing a single protein of the expected size of metacaspase, it will be used for immunolocalization studies to identify the intracellular location of metacaspase in *Acanthamoeba* cells. *Acanthamoeba* cells were harvested at mid log phase ($3x10^6$ cell density) and placed into a 12-well dish with some of the wells untreated (no NaCl) and some wells induced with 125mM NaCl to induce encystment. These cells were then fixed, blocked, and probed with primary antibody (i.e. metacaspase specific polyclonal antibodies) and secondary antibody (Alexa 568 fluorescent tagged) and observed from 0-24 hours later. Cells were also nuclear stained with DAPI prior to observation. In all conditions (panel “c”) the cells appeared intact.

In figure 22 and when the cells were in mid log phase and viewed under fluorescence, the cells did not self fluoresce and when stained with DAPI, the nuclei were visible. The same results were positive when the cells were induced with 125mM NaCl for 16-18hours and encystment was induced as seen in figure 23.

Cells were also probed with “pre-immune” sera (figure 24), with both uninduced and induced with 125mM NaCL to stimulate encystment (figure 25) and then observed. Figure 24 had faint fluorescence (panel “a”) in what looks to be a contractile vacuole as well as some “background debris”. In panel “b” the nuclei did not fluoresce meaning there was not any metacaspase protein present

in the nuclei. Figure 25 showed similar results in cells for which encystment was induced (with 125 mM NaCl). Cells appeared cyst-like and metacaspase was not detectable under the conditions used here (panel “a”). DAPI stained cells are shown in panel “b”.

When metacaspase antibody was used to probe the cells, the result was a “fluorescent perimeter” (figure 26, panel “a”). As a result, endogenous metacaspase protein was positively identified by being expressed in trophozoites in early, middle, and late log phases. When *Acanthamoeba* cells were induced with 125mM NaCl (figure 27) in early and late log phases (stimulating encystment) the results were similar and visual confirmation of metacaspase protein was apparent around the perimeter of the cells. In conclusion, the endogenous metacaspase protein appeared to be localized to the perimeter of the cells.

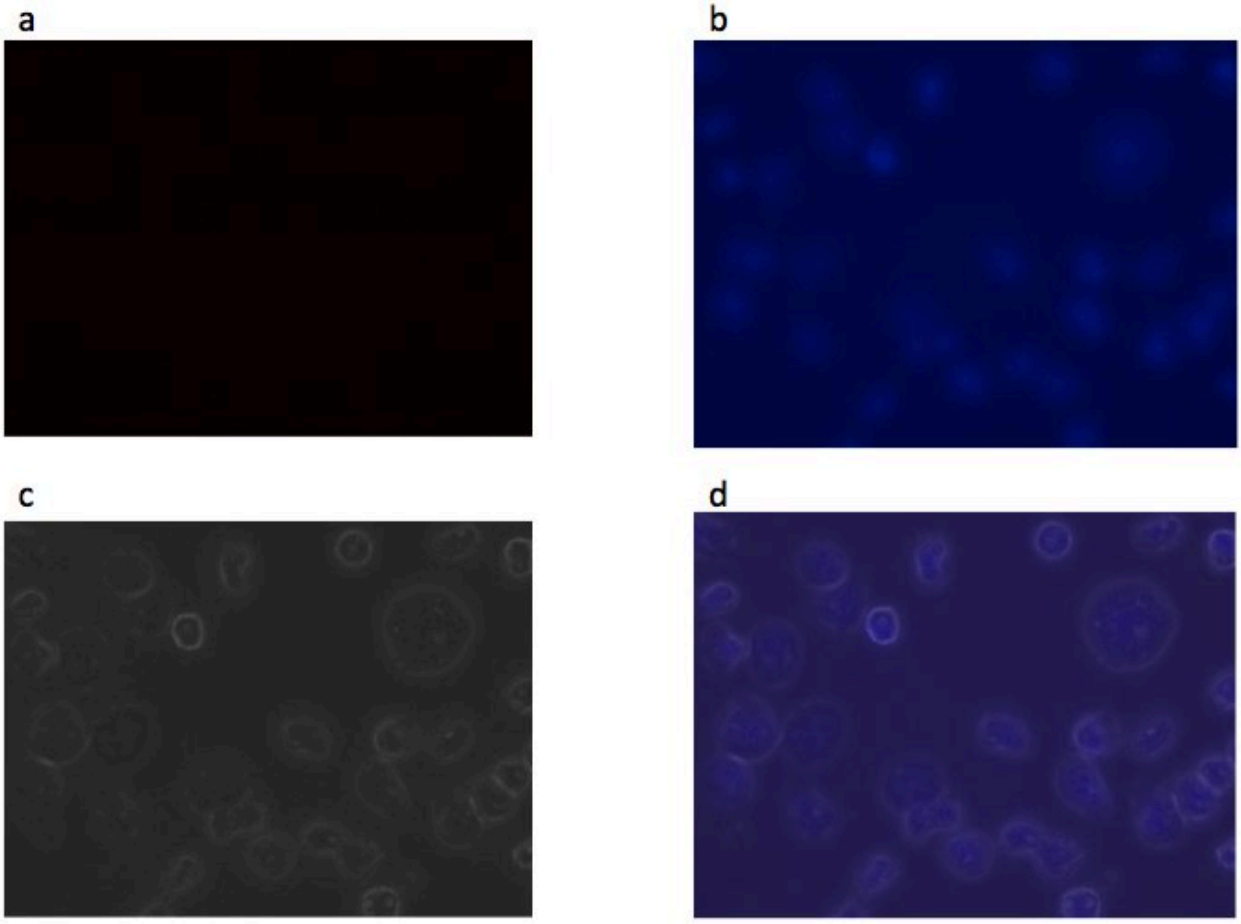

Figure 22: Negative control *Acanthamoeba* cells taken in mid log phase ($3X10^6$ cells/ml). Panel “a” represents fluorescence Alexa 558. Panel “b” represents DAPI nuclear staining. Panel “c” represents white light. Panel “d” represents a merged image of panels “a”, “b”, and “c”. Exposure time was between 200 and 400ms with Ex 470nm/Em 525nm filter set at 40X LD plan-Neofluar objective for all pictures.

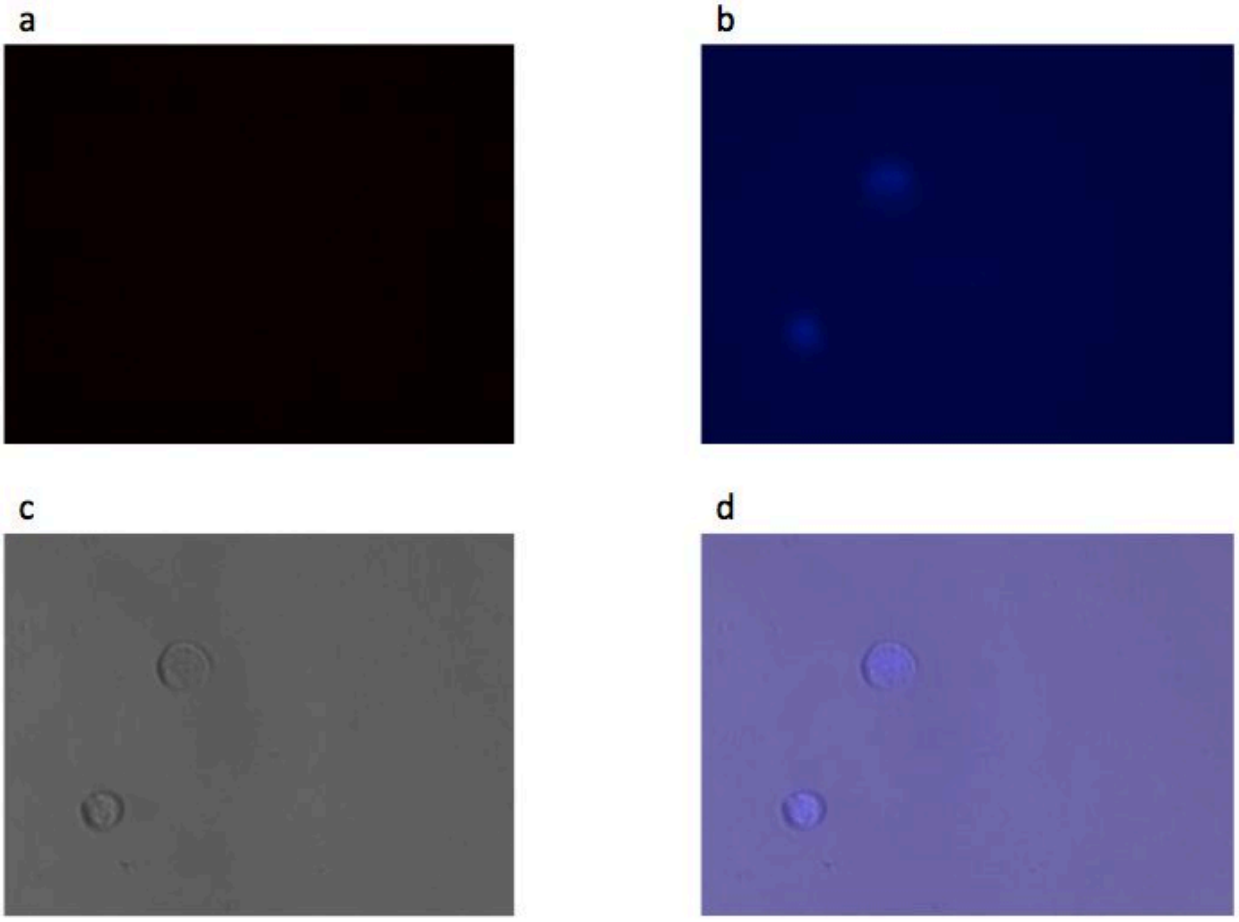

Figure 23: Negative control *Acanthamoeba* cells taken in mid log phase ($3X10^6$ cells/ml) and induced 16-18 hours with 125mM NaCl. Panel “a” represents florescence Alexa 558. Panel “b” represents DAPI nuclear staining. Panel “c” represents white light. Panel “d” represents a merged image of panels “a”, “b”, and “c”. Exposure time was between 200 and 400ms with Ex 470nm/Em 525nm filter set at 40X LD plan-Neofluar objective for all pictures.

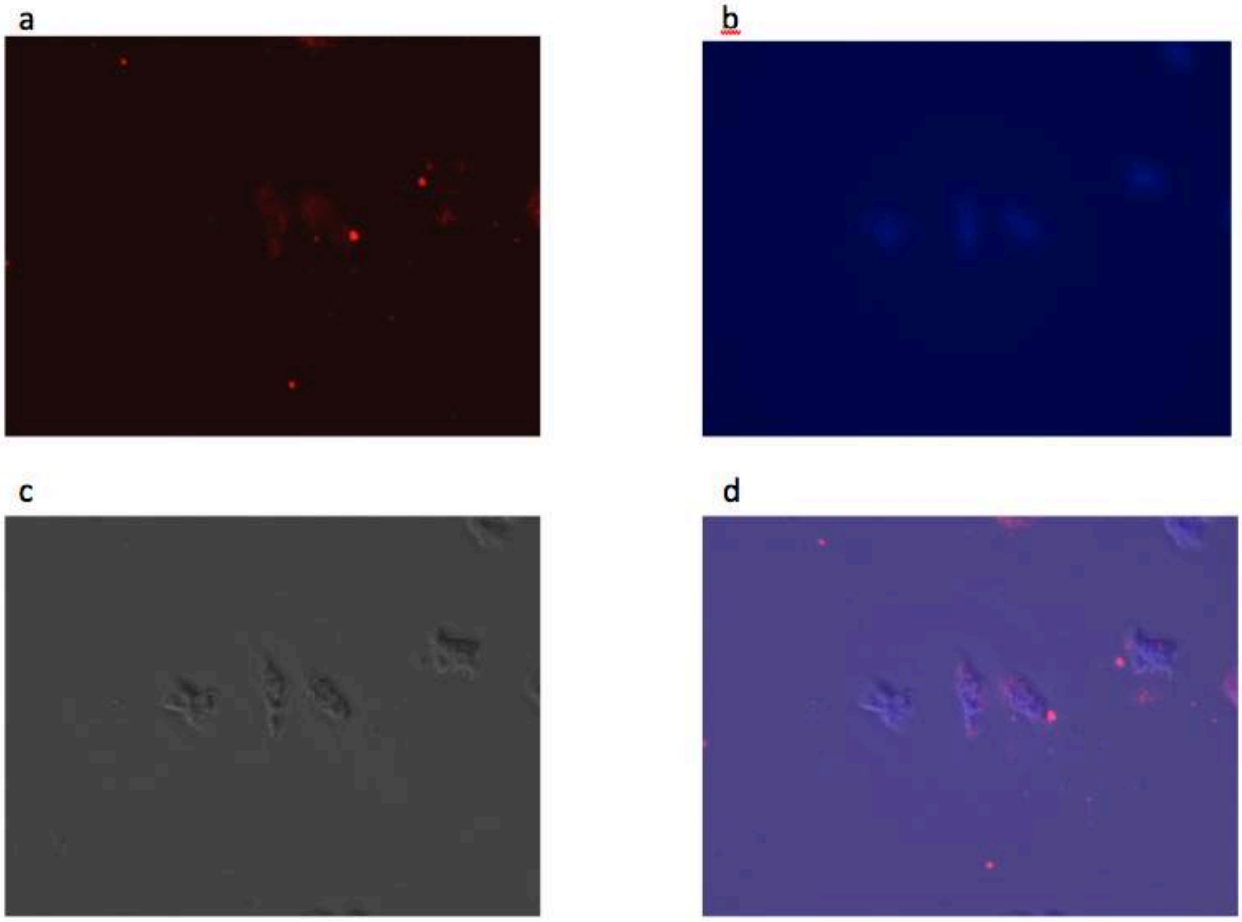

Figure 24: *Acanthamoeba* cells probed with pre-immune metacaspase antibody and secondary fluorescence. Cells were taken in mid log phase ($3X10^6$ cells/ml). Panel “a” represents florescence Alexa 558. Panel “b” represents DAPI nuclear staining. Panel “c” represents white light. Panel “d” represents a merged image of panels “a”, “b”, and “c”. Exposure time was between 200 and 400ms with Ex 470nm/Em 525nm filter set at 40X LD plan-Neofluar objective for all pictures.

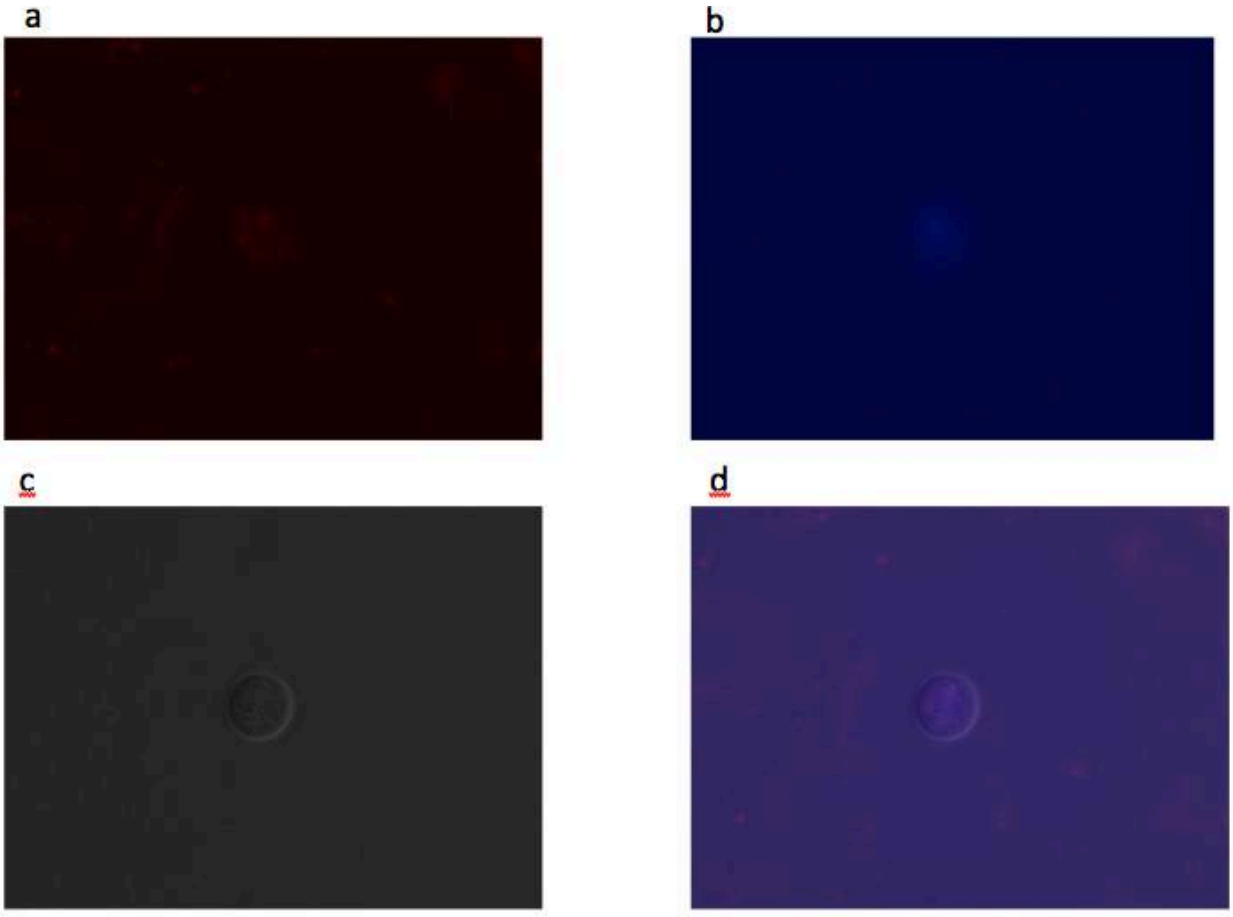

Figure 25: *Acanthamoeba* cells probed with pre-immune metacaspase antibody and secondary fluorescence. Cells were taken in mid log phase ($3X10^6$ cells/ml) and induced 16-18 hours with 125mM NaCl. Panel “a” represents florescence Alexa 558. Panel “b” represents DAPI nuclear staining. Panel “c” represents white light. Panel “d” represents a merged image of panels “a”, ”b”, and “c”. Exposure time was between 200 and 400ms with Ex 470nm/Em 525nm filter set at 40X LD plan-Neofluar objective for all pictures.

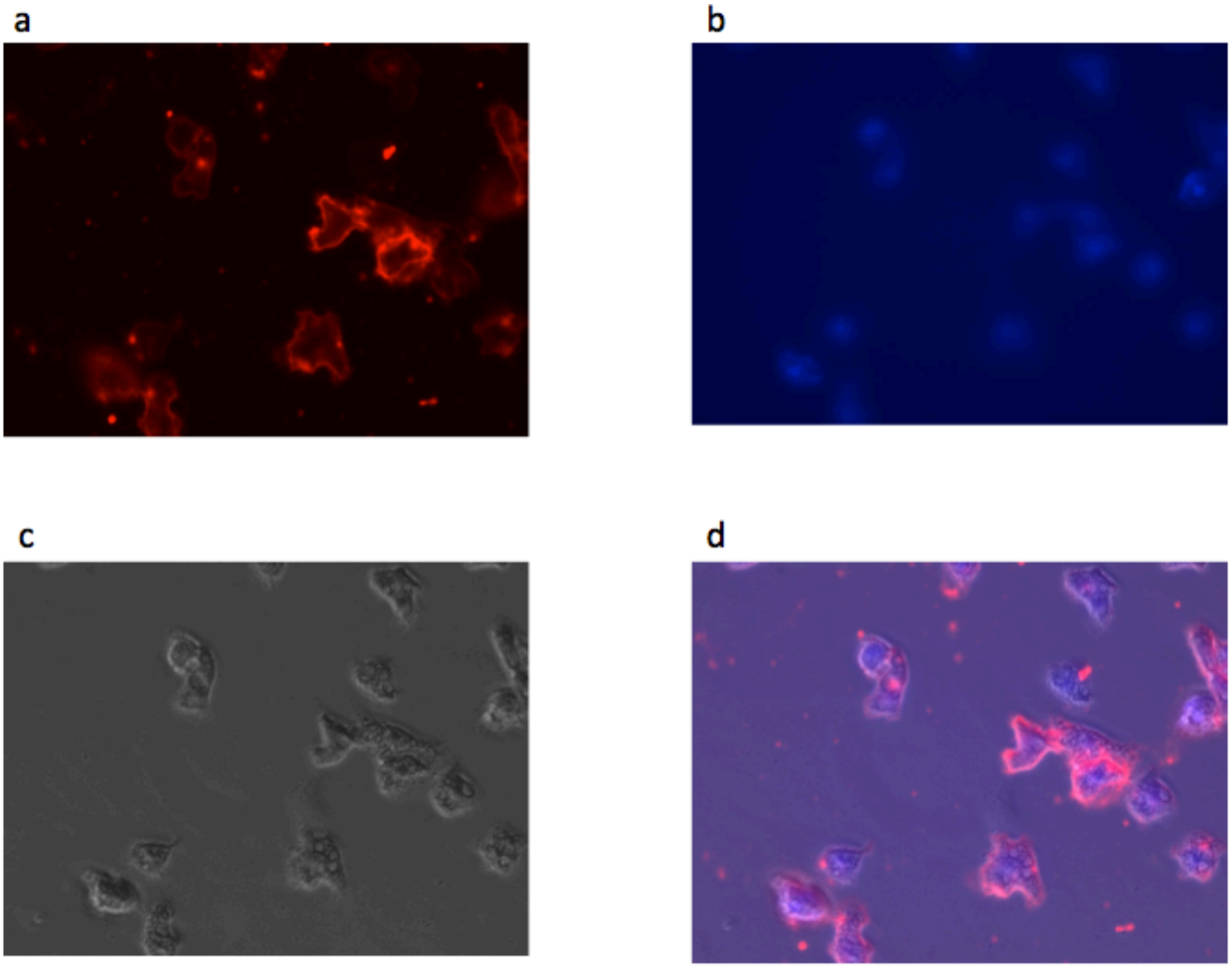

Figure 26: *Acanthamoeba* cells probed with metacaspase antibody and secondary fluorescence. Cells were taken in mid log phase ($3X10^6$ cells/ml). Panel “a” represents florescence Alexa 558. Panel “b” represents DAPI nuclear staining. Panel “c” represents white light. Panel “d” represents a merged image of panels “a”, “b”, and “c”. Exposure time was between 200 and 400ms with Ex 470nm/Em 525nm filter set at 40X LD plan-Neofluar objective for all pictures.

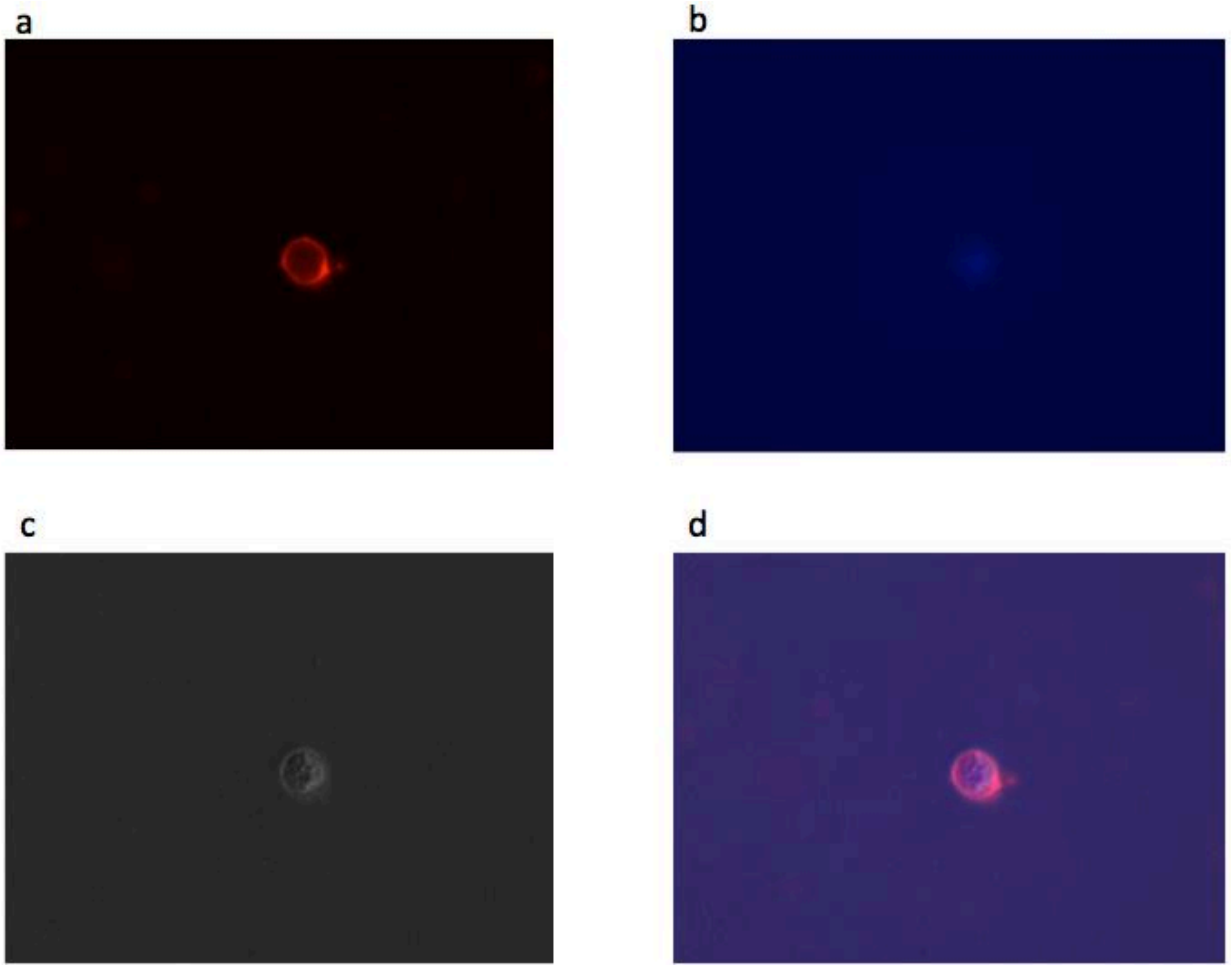

Figure 27: *Acanthamoeba* cells probed with metacaspase antibody and secondary fluorescence. Cells were taken in mid log phase ($3X10^6$ cells/ml) and induced 16-18 hours with 125mM NaCl. Panel “a” represents florescence Alexa 558. Panel “b” represents DAPI nuclear staining. Panel “c” represents white light. Panel “d” represents a merged image of panels “a”, “b”, and “c”. Exposure time was between 200 and 400ms with Ex 470nm/Em 525nm filter set at 40X LD plan-Neofluar objective for all pictures.

CHAPTER 4: DISCUSSION

As a model organism for study, *Acanthamoeba* has importance as both a single-cell model for cellular differentiation and as an important human pathogen. Some strains could potentially endanger immunocompromised individuals, such as hospital patients, as well as otherwise healthy individuals such as soft contact wearers. A single-celled eukaryotic microbe may offer insight in understanding programmed cell death. *Acanthamoeba* most likely does not undergo true apoptosis, but rather does encyst in response to stress. Metacaspases may be involved in this process. As previously discussed in the Introduction, metacaspases are closely related to caspases, which are present in the apoptotic pathway of higher eukaryotes. Studying the expression of metacaspase and where it is located in the cell can give us an understanding of its overall function and possibly even provide us with more understanding about the apoptosis pathway.

Some of the findings of this study were conclusive. Initial experiments involved standardizing quantities of *Acanthamoeba* protein for SDS-PAGE gels to be used for western blots. Two different protein extraction procedures were used: one using high concentrations of SDS and another using Urea. The extraction protocol utilizing Urea was determined to be more effective at solubilizing *Acanthamoeba* proteins. western blots of SDS-PAGE gels of total *Acanthamoeba* protein extracts prepared in this way were probed with polyclonal antibodies specific for metacaspase. A band of the expected size of metacaspase, 53 kDA, was identified. Pre-immune sera, used as a control, did

not bind to any bands on the blot. The metacaspase specific polyclonal antibodies were then to be used for immunolocalization studies to locate endogenous metacaspase in *Acanthamoeba*. The western blotting results also showed that metacaspase was being expressed in *Acanthamoeba* in the trophozoite stage in early, middle, and late log phases (i.e., while cells were actively growing and dividing and prior to encystment). Cells were also harvested in stationary phase (just prior to encystment) and also stimulated to encyst by the addition of 125mM NaCl, (a known trigger that initiates encystment (Cordingley et al. 1996)) and proteins were analyzed for the expression of metacaspase by western blotting. Metacaspase was detectable in samples from all timepoints although the levels of protein present were not rigorously quantitated.

When this study was taken one step further and cells were visualized to locate where in the cell the metacaspase was being expressed, results showed most of the fluorescence to be concentrated around the perimeter of the cells. Cells were also stained with DAPI, a nuclear stain, to confirm that metacaspase was not localized to the nucleus. The immunolocalization results also confirmed that, based upon the apparent location of the fluorescent signal in the cell, metacaspase appears to be localized to the membrane.

Certain trends were noticed in this study. When total proteins were equally loaded on gels with samples extracted from different time points collected from throughout growth and differentiation (encystment) of *Acanthamoeba* and then used for western blots, some metacaspase was detected in all samples. Initially, these experiments were done in order to determine the time during

Acanthamoeba's growth and encystment in which metacaspase was being expressed. This study was necessary so that cells that would be selected and used for immunolocalization studies would be expected to have detectable levels of metacaspase. It was confirmed visually by the immunocytochemistry photos where the metacaspase protein was positively identified. Although western blots presented in this study showed expression of metacaspase, these results were not quantitative. For quantitation, further studies could include densitometric analysis of the gels to determine expression levels. For those studies, it will be necessary to identify a constitutively expressed protein that could be used as a reference gene. As this has yet to be done, a number of questions are still unanswered from this study. Additional western blots of total proteins extracted from time points throughout growth and encystment and standardized against a constitutively expressed protein are still needed. These additional studies would allow levels of metacaspase to be measured in the cell over different life cycle stages.

Future studies that could come about from this project would include immunolocalizations viewed over time every few hours, for example. These studies would show the location of metacaspase in the cell during active growth and division, and as cells were undergoing encystment. Metacaspase, or portions thereof, may re-localize as cells undergo encystment. It may be possible to visually track these changes. Another study could include taking pictures of immunolocalized cells in a z-stack to locate more precisely where the metacaspase was expressed in the cell.

Another study could include revisiting the transfection experiments. As the initial transfections were inconclusive, due in part to only a very weak fluorescence signal being detectable, an alternative would be to use a different vector, with a more robust promoter driving GFP expression. Additional expression vectors suitable for *Acanthamoeba* are now available (Bateman 2010). New constructs containing full-length metacaspase can be prepared in these vectors and used for transfection. It might also be possible to fix the cells following transfection and prior to viewing, which might allow for the fluorescent signal to be more readily and consistently located in the cell. Also different secondary antibodies could be evaluated (e.g. horse serum or goat serum) to determine if background fluorescence for the immunocytochemistry could be reduced or eliminated, and a clearer result obtained.

The studies carried out here are very preliminary and were designed to first evaluate the specificity of metacaspase specific antibodies that were then to be used for expression studies and immunolocalization studies. The polyclonal specific antibodies were determined to be specific for a 53 kDA protein (as determined on a western blot), which corresponds to the predicted size of metacaspase. To more rigorously confirm the specificity of the antibody, it would be necessary to perform blocking experiments using the same antigen to which the antibody was initially raised. The antibody used here was raised to a 19 amino acid peptide. For the blocking experiment, this peptide would be pre-incubated with the anti-sera, which would then used to probe a western blot of total *Acanthamoeba* proteins. Absence of a signal (at 53 kDa) on the western blot

would confirm the specificity of the polyclonal antibody. This same “blocking strategy” could be used prior to immunolocalization experiments. Loss of a signal when pre-blocked sera was used to probe the cells would then confirm that endogenous metacaspase was being identified in the fixed cells.

The expression and localization of metacaspase in the cells is an initial step in evaluating the overall function of metacaspase in the *Acanthamoeba.* Further experiments might include blocking the expression of metacaspase. If metacaspase expression were turned off, the question could be asked: What would happen to the cell’s life cycle and would encystment even begin to occur? In the absence of metacaspase, would 125mM NaCl (a stimulus which triggers encystment in the Neff strain) even induce the encystment process?

Other studies that have looked at metacaspase’s involvement in programmed cell death include Bettiga et al., (2004), which studied metacaspase in yeast. They have found that metacaspase plays a vital role when other genes are turned off that are involved in the apoptotic pathway involving metacaspase. Another study by Gonzalez et al., (2007) involving the human parasite, *Leishmania major,* suggests that becasue metacaspase is arginine-specific, it expresses some peptidase activity. In that study they have replaced *Leishmania major* metacaspase with yeast metacaspase, and similar apoptotic pathways resulted when cell death was triggered. As stated previously in the introduction, it has been determined that plants also possess an identified metacaspase protein that has been shown to be involved in apoptosis. Hoeberichts et al., (2003) describes how metacaspase is linked to the programmed cell death pathway in

the tomato plant. These studies demonstrate an involvement of metacaspase in the apoptotic pathway in those systems. In this study, determining the intracellular location of the metacaspase provided more insight into its function in the cell during related processes.

The experiments described in this study are the first to show the intracellular location of metacaspase in *Acanthamoeba*. As a result of this study, metacaspase was positively identified in the perimeter of the cell and is assumed to be associated with the membrane. Further studies will be needed to confirm and expand upon this finding for a more comprehensive analysis of the functional role of metacaspase in encystment in *Acanthamoeba*.

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