This is to certify that the
thesis entitled

MORPHOLOGICAL AND ANTIGENIC STUDIES OF
PYTHIUM INSIDIOSUM HYPHAE USING TRANSMISSION
ELECTRON MICROSCOPY

presented by

RICHARD BENJAMIN GARCIA

has been accepted towards fulfillment
of the requirements for the

MASTER OF SCIENCE degree in BIO-MEDICAL LABORATORY DIAGNOSTICS

Major Professor's Signature

9/20/06

Date

MSU is an Affirmative Action/Equal Opportunity Institution

DATE DUE	DATE DUE	DATE DUE

MORPHOLOGICAL AND ANTIGENIC STUDIES OF *PYTHIUM INSIDIOSUM* HYPHAE BY TRANSMISSION ELECTRON MICROSCOPY

By

Richard Benjamin Garcia

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Bio-Medical Laboratory Diagnostics

2006

ABSTRACT

Morphological and Antigenic Studies of *Pythium insidiosum* Hyphae by Transmission Electron Microscopy

By

Richard Benjamin Garcia

For the past century *Pythium insidiosum,* the etiologic agent of pythiosis, has been reported causing disease in both humans and animals. While many diagnostic tests and immunotherapeutic treatments have been developed to diagnose this parafungal pathogen, the location and characterization of the antigens expressed during infection have yet to be described. We have mapped the antigens present in the hyphae of *P. insidiosum* using an immuno-electron microscopic approach. During this study new morphological features of the hyphal structures of *P. insidiosum* were also described using transmission electron microscopy. The immuno-electron microscopy data using sera from infected hosts (bovine, canine, equine, feline, and human) and Protein A colloidal gold (PACG), shows the specific binding of protein A colloidal gold particles to antigens within *P. insidiosum's* hyphae only with sera from cows, horse, and humans with pythiosis. Sera from infected dogs and cats did not show specific binding of the gold particles to *P. insidiosum's* hyphal structures. The finding of novel antigenic components in the cell wall and the cytosol of *P. insidiosum's* hyphae is of paramount importance for the future characterization of these proteisns to understand the immunopathology of the disease, and for the use of better immunogens for the immunotherapy of pythiosis.

Dedication

I would like to dedicate this thesis to the faculty of the Medical Technology Department, my family and friends who supported me through my education at Michigan State University, and especially Dr. Mendoza for his enthusiasm and dedication in the parafungal sciences.

ACKNOWLEDGMENTS

I would like to express gratitude to my major advisor, Dr. Leonel Mendoza, for his guidance, encouragement, and support in my degree program. I would also like to express gratitude to Dr. Alicia Pastor and Dr. Thomas Schmidt for serving on my guidance committee and all the helpful meetings and suggestions for my research. Finally, I would like to thank the Medical Technology Program faculty and staff for their assistance.

TABLE OF CONTENTS

LIST OF FIGURES

INTRODUCTION

De Haan and Hoogkamer first isolated *Pythium insidiosum*, the etiological agent of the para-fungal disease pythiosis, in 1901. For decades the treatment of pythiosis has been limited because of the obscure nature of this pathogen. Until recently, surgical removal of the infected tissues was the traditional method of treatment. However, the development of alternative ways for the management of this infection has been proposed including immunotherapy (Miller 1981). This novel approach used exo- and endo proteins antigens of *Pythium insidiosum* injected in the infected hosts. While this immunotherapeutic approach proved to be effective and was able to cure chronic and acute cases of the disease, immunotherapy showed differences in cure rate between host species. Further more, little is known about the nature of the antigens used for immunotherapy (Mendoza and Newton 2005).

Different antigens and serological assays have been used and developed to diagnose and differentiate pythiosis from similar clinical entities (Miller and Campbell 1982; Hutchens and Mendoza 2002). It is known that an exoantigen is released from the hyphae of *P. insidiosum* during infection (Mendoza et al. 1987). However, this antigen has never been characterized. The exoantigens released during infection seem to trigger a T helper 2 (Th2) response in the infected hosts, with a coordinated cascade of inflammatory cells and cell mediator proteins, mostly eosinophils, mast cells and INFγ, IL5, and IL4. The display of these inflammatory components, including eosinophil and mass cell

degranulation, are extremely destructive to the host tissue (Miller 1981; Mendoza et al. 1992b). Since nothing is known about the nature of the antigens expressed during infection and their possible location in the hyphal compartments, thus this study has the purpose of mapping those antigens in the hyphae of *P. insidiosum* using transmission electron microscopy, immunogold reagent, and sera from patients with proven pythiosis. The location of the *in vivo* antigens expressed by *Pythium insidiosum*'s hyphae, could answer questions about the immunogens expressed during infection, which in turn could help in the characterization of these important immuno-dominant proteins.

CHAPTER ONE

Literature Review

History

During the 1800's unusual cases of horses with cutaneous lesions were documented from several tropical and subtropical areas of the world (Australia, Florida, India, and Indonesia). In 1884, Smith first published a well-documented report on pythiosis. Shortly after Fish (1895-96), and Drouin (1896) also documented new cases of cutaneous granulomas in equines. The etiologic agent of pythiosis was isolated for the first time by Haan and Hoogkamer in 1901 from several horses with the disease, but it was not named. In 1925, J. Witkamp introduced several serological methods to diagnose the disease. This initial detailed report also included information on the treatment and the progression of the disease, later termed pythiosis. Because the report was published in the Dutch language, this information went unnoticed (Mendoza 2005).

In the early 20th century, *Habronema*, a pathogenic nematode with clinical features in common with pythiosis, was incriminated as the etiologic agent of the cutaneous granulomas in horses. The clinical similarities between habronemiasis and pythiosis led to some confusion on the real etiology of pythiosis. Thus the terms used to describe pythiosis were also indiscriminately used for equine cutaneous habronemiasis. In 1961 Bridges and Emmons isolated a filamentous organism from horses in Texas that they believed to be a zygomycete. They named the organism *Hyphomyces destruens*. Austwick and

Copland changed this name into the genus *Pythium* in 1974, after reporting the ability of the organism to produce biflagellate zoospores when transferred to water media. However, no scientific name was proposed. In 1987, de Cock et al., introduced the specie *P. insidiosum*, and indicated that many of the proposed epithets in the past belong to the same species. Recently, Schurko et al. (2003), using molecular tools confirmed that *P. insidiosum* is the only etiologic agent in the genus causing pythiosis in mammals.

Numerous new cases of pythiosis have been reported in humans, horses, cats, cattle, and dogs from around the world. For instance, in 2005 the first human case of pythiosis was reported in Brazil and the first case from Africa in a dog with pythiosis (de Moraes et al. 2005; Rivierre et al. 2005).

Life Cycle

P. insidiosum inhabits aquatic environments and may require symbiotic relationship with plants to complete its life cycle in nature (Mendoza et al.1992b; Shipton et al. 1982). *P. insidiosum* seems to infect plants inhabiting aquatic environments. The plant tissue serves as a reservoir for the development of sporangia and zoospores, which are then released into the surrounding environment. After the release of zoospores, they encyst developing germ tubes that reinitiate the life cycle by infecting another plant, or animal tissue (Figure 1). *P. insidiosum* has the ability to develop resistant spores that could play a major role on its capacity to survive in harsh environments. The motile zoospores are believed to be attracted by injured tissue in susceptible plants or animals. When

chemo-tactically stimulated by a suitable substrate, the zoospores secrete an amorphous sticky material, which covers their surface, which is used to attach it to the plant or animal's surface. This substance, which is believed to be a glycoprotein, has been implicated as a potential virulence factor in establishing infection (Mendoza et al. 1993). Once attached to the tissue, the zoospores encyst and developed a germ tube (hypha) that mechanically penetrates traumatic lesions causing pythiosis in the infected host (Mendoza 2005).

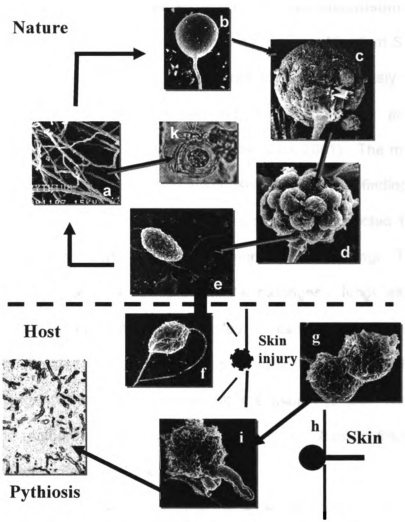

Figure 1: The life cycle of *Pythium insidiosum* in nature ,(a-e, k, in upper panel). Plant tissue is first colonized by (a) hyphae of *P. insidiosum*, and then (b-d) the differentiation of sporangium into mature stages leads to (e) zoospore release. (f) The zoospores swim to locate another plant or may be attracted by injured animal tissue. The encysted zoospores (g) were attached to tissue by a sticky substance (circle of dashes). The zoospores then germinate (h), invade the host (i) and cause pythiosis, (j) histopathology showing hyphae.

Taxonomy and Other Characteristics of *Pythium insidiosum*

P. insidiosum has been placed under the new kingdom *Straminipila*, class *Oomycetes*, order *Pythiales* . The straminipilans, previously classified in the Kingdom Fungi, are now considered to be advanced protistal microbes phylogenetically similar to plants and algae (Dick 2001). The morphological and genetic features of *P. insidiosum* are consistent with these findings.

Hyphae. *P. insidiosum* in culture and in the infected tissues develops sparsely septate hyphae similar to the members of the fungi. This is why many frequently confuse *P. insidiosum* with the pathogenic fungi, especially with the organisms from the order *Zygomycetes*. This parafungal organism produces sparsely septated hyphae, which range in diameter between 4 and 10μm with many tangent branches stretching off the main hyphae. There are usually enlarged regions of hyphae growth observed (12-28μm in diameter) in culture, termed apresorium (de Cock et al., 1987).

Zoospores. Cultures incubated in water with some ions, including Ca^{++} at 28-37°C are able to develop the production of zoosporangia containing zoospores. The hyphae produce sporangia that, under some cultural conditions, could form a vesicle (sporangium) where cytoplasm flows from the hypha. At maturity, the zoospores mechanically break the vesicle's wall and upon emergence swim for about 20 minutes and then encyst. The zoospores are biflagellate. The flagella arise from the same point in the zoospore and are unequal in length. One of the flagella has hair like structures (tinsel type) the

other is of 'whiplash' type without the hair-like structures. Following encystment the flagella are detached and the zoospore becomes spherical (cyst), producing a sticky material covering the entire surface of the cyst. *P. insidiosum* uses this strategy for the attachment of the cyst to the host surface (plant or animal tissue). This phenomenon has also been well documented in plants (Mendoza et al. 1993).

Oogonium. In contrast to other species of the genus, only a few strains of *P. insidiosum* have been reported to produce oogonia. Thus, the mechanism implicated in the process of oogonial formation in *P. insidiosum* remains obscure (Mendoza 2005). It is believe that the oogonium plays the role of the resistant stage in the members of the genus *Pythium*. Thus, this could be an important feature for survival during extremely dry conditions in the endemic areas.

Epidemiology and Distribution

While many *Pythium sp.* are primarily plant pathogens found in soils and stagnant waters, only *P. insidiosum* has been reported infecting animals. *P. insidiosum* flourishes in the summer months after large amounts of rainfall. Horses that have contracted pythiosis were usually in contact for long periods of time in stagnant water. However, some cases in humans, dogs, and horses with pythiosis never exposed to stagnant water, have suggested that the infection may be contracted from soil or grass containing the etiologic agent as well.

Rainfall increases stagnant water accumulation, which increases the number of cases of pythiosis, this fact has been used to name the disease as:

burusattee (meaning rain in India), swamp cancer, leeches, and summer sores. Pythiosis has been usually reported in most tropical and subtropical areas of the world (Figure 2). Although unusual, cases in temperate climates, such as Japan and the United states, have been also reported (Mendoza 2005).

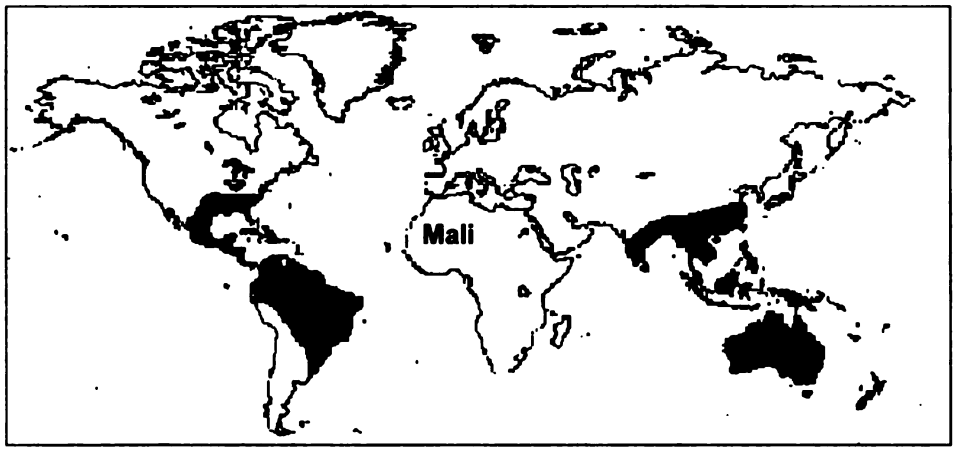

Figure 2: Distribution of *Pythium insidiosum* reported throughout the world. So far, a single case of pythiosis in Africa (Mali) has been reported.

Clinical Disease: Humans

Pythiosis of humans in Australia, Brazil, Haiti, New Zealand, Thailand, and the United States were all associated with hosts visiting aquatic environments. Open wounds, thalassemia, and occupational involvement in wet environments, have been suggested as predisposing factors of pythiosis. So far the majority of cases of human pythiosis have occurred in Thailand, with more than 90 new cases reported since the first report in 1987 (Mendoza 2005). There seems to be a correlation between the clinical forms of human pythiosis observed and the parts of the world where the disease was contracted. For

instance, in Thailand most infections are initiated as dry gangrenous lesions with pain of the infected subcutaneous tissues, especially on the extremities, with subsequent dissemination to the nearby arteries. In the United States, orbital pythiosis in young boys have been recorded (Figure 3) (Shenep et al. 1998). Similar observations were reported in Australia (Triscott et al., 1993). Other clinical forms of the disease include, periorbital swellings developing into orbital tumor, subcutaneous pythiosis, and keratitis (Triscott et al., 1993; Virgile et al., 1993). Mendoza and Newton (2005) described three main forms of pythiosis: (1) a superficial pythiosis (keratitis), (2) a granulomatous and ulcerative lesions involving the skin and subcutaneous tissues of the limbs and face, including the periorbital area, and (3) a systemic form with vascular involvement leading vasculitis, thrombosis, aneurysms, and death.

Clinical Disease: Animals

Pythiosis has been reported in different animals from the endemic areas of the world with new cases reported every year. Horses, dogs, cats, cattle, sheep, a camel, and even a jaguar have all been reported with active pythiosis. Horses and dogs are the most frequently affected animals. Horses usually develop cutaneous and subcutaneous skin lesions in the extremities. Dogs develop a gastrointestinal form of the disease as well as skin lesions (Figure 4). In addition, cases involving lungs, bones, intestines, and the nasal cavity have been reported in different animals (Mendoza and Newton 2005).

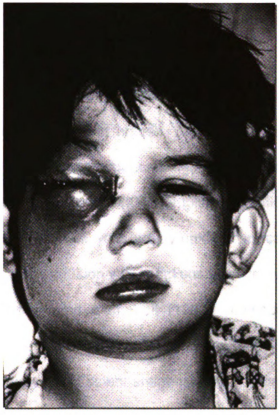

Figure 3: Orbital pythiosis in a boy from Texas, USA.

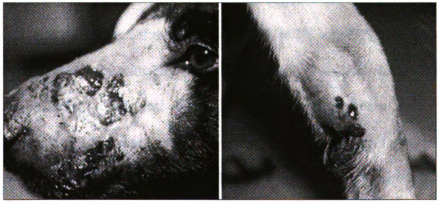

Figure 4: Cutaneous lesions in dogs with pythiosis.

Diagnosis and Serological Testing

Early in the 20th century several immuno-assays were developed to diagnose pythiosis in horses. This initial study demonstrated that there were specific antibodies produced against *P. insidiosum* hyphae. The serological tests, immunodiffusion (ID), compliment fixation (CF), and skin test, designed to diagnose pythiosis went largely unknown because the findings were published in Dutch, and confusion with reactions involving similar diseases.

Miller and Campbell (1982) developed specific antigens to diagnose pythiosis in horses based on Witkamp's findings. Miller and Campbell (1982) also reported that select antigens from *P. insidiosum* were beneficial for the cure of some infected horses. Three antigens were used for the ID, CF, and skin test. A trypsinized antigen for the ID test detected 100% of horses with pythiosis. A freeze-thawed antigen for CF test detected 82% of infected horses. Finally a soluble precipitated antigen for skin test detected 62% of horses studied. After treatment and resolve of the disease, Miller and Campbell (1982) reported that anti-*P. insidiosum* immunoglobulins could not be detected by the ID test in horses. However, CF and the skin test did detect the anti-*P insidiosum* immunoglobulins after resolve of infection. Later, evidence of cross-reaction with the antigens was reported with *Basidiobolus haptosporus*. Other ID tests using different antigens were developed over the years, but it soon became evident that the ID test was not always effective in other animals with pythiosis. A more

sensitive assay was needed for the diagnosis of pythiosis. Mendoza el al. 2003, developed a fluorescent-antibody assay that was specific for the hyphae of only *P. insidiosum*, that did not cross react with any other similar organism. Other specific tests like the indirect immuno-peroxidase assay were developed to specifically detect *P. insidiosum* in tissue (Brown et al. 1988). The immunoblot test used for diagnosis was also used to observe the antigens of *P. insidiosum* detected by host antibodies (Mendoza et al. 1992a). The ELISA test was used to diagnose pythiosis because of the sensitivity and specificity of the test, which many of the previous tests lacked. Humoral responses to pythiosis hyphae were also observed using the ELISA test (Mendoza et al. 1997; Rosa 1993). An onsite assay, called the latex agglutination test, was recently developed for rapid diagnosis of pythiosis to assist farmers' diagnoses of pythiosis (Hutchens and Mendoza 2002). These immuno-assays have contributed to the proper diagnosis of pythiosis. However, medical personnel familiar with this pathogen have to be trained to further improve the diagnosis of this disease. Because the diagnosis of pythisois is difficult, it is possible that uncommon clinical manifestations, such as ocular pythiosis, may have been under diagnosed. The disease has high morbidity, as it is evident by the many amputation or enucleation cases among the patients at Ramathibodi Hospital with active pythiosis (Krajaejun et al. 2004). Thus, early detection and an effective treatment (surgery, immunotherapy, chemotherapy) are key for successful management.

Host-Parasite Relationship

The immune response to pythiosis has been studied in a rabbit model (Miller and Campbell 1983). When rabbits were administered a suspension of motile zoospores of *Pythium sp.* isolated from a horse in Queensland by subcutaneous, intraperitoneal, and intravenous routes they developed clinical signs of pythiosis. Some of the rabbits were cortisone treated, but they developed the same infection to that in the control group. It has been suggested that one of the predisposing factors towards infection of opportunistic fungi is decrease of the hosts' immune response (Rippon, 1982). Cortisone treated and immuno-competent rabbits both were highly susceptible to *P. insidiosum* from all routes of inoculation. The histopathology of the skin lesions of the rabbits administered subcutaneously showed acute necrosis, with eosinopils and fungal hyphae in abundance, just like the changes observed during natural infection.

P. insidiosum cannot penetrate normal animal skin. However, an open wound could provide entrance for the development of pythiosis. The entrance of the pathogen initiates a humoral immune response involving a massive attack of eosinophils, giant cells, mast cells, macrophages, plasma cells, and lymphocytes. This inflammatory reaction is also similar to that triggered by different fungal pathogens including *Conidiobolus* and *Basidiobolus* (Mendoza 2005). The 'Kunkers', a mass of necrotic tissue caused by massive degranulation of eosinophils over the hyphae of *P. insidiosum,* differentiate

pythiosis from similar fungal infections. It is interesting to note that the hyphal elements of *P. insidiosum* are usually found in the center of eosinophilic micro-abscesses. This feature has been used by some investigators to postulate that a Th2 subset is probably activated by the immunogens expressed by *P. insidiosum* in the infected host (Mendoza & Newton 2005). Eosinophilic degranulation is the major factor in the pathogenesis of the disease. However, mast cells and other immunological mediators also contribute to tissue damage. The up-regulation of the inflammatory cells leads to eosinophilic degranulation that is responsible for tissue damage during pythiosis in animals and humans. Wachiwanawin et al., 1993 investigated some of the inflammatory regulators in a human with arterial pythiosis. They found that interleukin-4 (IL-4) and IL-5 were elevated, supporting the hypothesis that the immune system mounts a Th2 response against *P. insidiosum* during natural infection. This study suggested the rise of IgE levels, responsible for eosinophilic response, confirm the postulated Th2 response to pythiosis infection.

It is believed that *P. insidiosum* expresses a soluble exoantigen during infection that might be responsible for the Th2 response. In 1987 Mendoza el al. confirmed the expression of an antigen released outside the cytoplasmic compartment of *P. insidiosum* hyphae. Using fluorescent antibodies, these investigators found that the 'kunkers' present in the tissue of horses with pythiosis fluoresced, indicating that an antigen, detected by labeled anti-*P. insidiosum* immunoglobulin, had been released from the hyphae. Apparently, some of the antigen is retained in the 'kunker', from where it is released to the

surrounding tissues and then presented to local dendritic cells (Mendoza et al. 1987).

The pathogen is always presenting this antigen to the host dendritic cells resulting in high levels of IgE, which in turn triggers tropisms of eosinophils and mast cells to the site of infection, locking the immune system into a Th2 response. This immune response enables the pathogen to hide inside the inflammatory responses restricting certain antigens from being fully presented to the host immune system. The degranulation of eosinophils and mast cells are responsible for the clinical and pathological changes observed during infection. Based on the destructive effect of the inflammatory response triggered by this pathogen on the infected hosts, and the fact that it is the only mammalian oomycete pathogen, it could be speculated that *P. insidiosum* only recently developed the ability to survive in mammalian tissues, a belief also supported by current phylogenetic analysis (Mendoza and Newton 2005).

Treatment

Since most of the antifungal drugs (iodide and amphotericin B) are ineffective against *P. insidiosum*, and are very toxic, surgical removal of the infected tissue has been the main treatment for pythiosis (Mendoza 2005). Most recently a new non-invasive treatment termed immunotherapy has been introduced (Mendoza et al., 2003).

Surgical removal has been successful in numerous cases of equine pythiosis for over a century. Lesions and kunkers are removed followed by

cauterization (Habbinga 1967; McMullan et al. 1977). These surgical procedures are extremely invasive and in some cases lead to death (Fisher et al. 1994). Side effects and disease reoccurrence are commonly associated with surgical removal of infected tissue. Drastic surgical procedures like amputation of extremities have been used on humans with pythiosis. This last resort is rarely successful usually leading to death (Sathapatayavongs et al. 1989; Wanachiwanawin et al. 1993).

Immunotherapy includes a treatment of products produced by *P. insidiosum*. Immunization using these products was reported to have curative properties in the early eighties (Miller 1981). Immuotheropy using Miller's vaccine was only 53% successful in horses. Using both exo and endoproteins precipitated from *P. insidiosum* hyphae Mendoza et al. 2003, developed a new more effective immunotherapeutic vaccine to treat both humans and animals.

Introduction to Transmission Electron Microscopy (TEM)

The locations of the antigens produced by *Pythium insidiosum* hyphae have yet to be mapped. The use of immuno electron microscopic approaches for identification of the antigen positioning of hyphal organisms has been successfully used in the past by some investigators (Herr et al., 1999; Sandoval el al., 1996). The identification of the antigens within *P. insidiosum* hyphae is essential for a better understanding of the immunopathology and for development of a more specific immunotherapeutic approach (Mendoza and Newton 2005). Thus, this study has the following objectives: To evaluate the

morphological structures of *Pythium insidiosum* hyphae cultured at 37°C using the Transmission Electron Microscope (TEM). To investigate the antigen-antibody reaction of host sera with sections of *Pythium insidiosum* hyphae, using Protein A Colloidal Gold (PACG) particles as a detection system.

CHAPTER TWO

Materials and Methods

Strain, Culture, and Sample Strategies

P. insidiosum strain H9 was selected based on the principle that it is the type strain of this species, and also that it is the most immunologically studied strain (Schurko et al. 2003). In addition, it is the strain used to prepare the immunotherapeutic antigens for the treatment of this disease (Mendoza and Newton 2005) (ATCC 58643; CBS 574.85; PIMT 19; originally known as H9 strain). The strain was kept at 30°C in the Medical Technology Program culture collection and then subcultured onto Sabouraud agar slants (2% agarose, 2% sucrose, and 1% peptone, in distilled water) just before transferring to broth cultures. The subculture strain was incubated at 37 °C for 1 to 2 weeks until white/clear growths were observed. A small section, around 1 cm of growth, was transferred to separate flaks 500 mL flasks containing 300 mL of Sabouraud broth (2% sucrose and 1% peptone in distilled water) using a sterile loop. The flasks were incubated 37°C while agitated using a shaker at 150 rpm for 2 to 4 weeks until the inoculated flasks showed a substantial growth of hyphae. After incubation, a spherical large mass of hyphal growth was removed from the Sabouraud broth and placed in a sterile Petri dish. The hyphal mass was then transferred into a 50 ml glass flask and approximately 10 mL of 2% melted (~45°C) water agarose was added to each flask containing the hyphal mass. After the agar solidified, the samples were placed into two separate sterile 15 mL

plastic sample tubes. The samples were fixed by adding approximately 5 ml of 2.5% formaldehyde (Electron Microscopy Sciences, EMS, Fort Washington, PA.) to each tube. The samples were then stored at 4 °C for 2 to 7 days until they were prepared for morphology or immunolabeling.

Transmission electron microscopy (TEM)

Thin sections using traditional fixative and stains in TEM were prepared to evaluate details of the cell wall and intracellular structures of *P. insidiosum*'s hyphae. A different approach was also used to evaluate the sample preparation using immuno reaction of the thin sections containing hyphae with the infected and non-infected sera from patients with proven pythiosis, and sera from apparently healthy hosts. The following are the details of the procedures used to achieve the objectives of this study.

Sample Preparation

Sample preparation for morphological analysis

The samples in solidified agar were placed in a Petri dish and aseptically cut into approximately 1 cm cubes using a sterile scalpel. Fixative (2.5% glutaraldehyde and 2.0% paraformaldehyde in 0.1 M cacodylate buffer, pH 7.4) (EMS, Fort Washington, PA.) was added in vials containing small section samples to stabilize cellular components, especially proteins, and then stored at 4°C for 5 days. Samples were then rinsed in cacodylate buffer (0.1 M, pH 7.4) (EMS, Fort Washington, PA.) three times for 15 minutes per rinse. A 2% solution

of osmium tetroxide in 0.1M cacodylate buffer (EMS, Fort Washington, PA.) was added as a postfixative agent in order to fix and preserve lipids and maintain three-dimensional structure prior to dehydration. After postfixation the samples were rinsed with cacodylate buffer for three 15 minute cycles. The samples were dehydrated in a series of acetone dilutions; 10%, 15%, 30%, 50%, 70%, 100% for 10 minutes and finally 100% acetone for 1 hour. After the acetone dehydration, sample sections were infiltrated with a graded series of Poly/Bed 812 and acetone solutions; 3:1, 2:2, 1:3, and pure resin (EMS, Fort Washington, PA.). To provide support during ultramicrotomy and retain spatial organization of the specimen section on the TEM grid, samples were placed in a silicone mold, embedded in Poly/Bed 812 resin, and polymerized in an oven (Blue M Electric Co., Blue Island, IL.) at 60 °C for 24 hours.

Sample preparation for Immuno-labeling analysis

The samples in solidified agar were placed in a Petri dish and were aseptically cut into approximately 1 cm cubes using a sterile scalpel. The samples were then washed three times with TTBS (0.05% Tween 10 in Tris-buffered-saline pH7.4) using a dropper. Three drops of TTBS were administered to the surface of the cubes in the Petri dish and then rotated with forceps and washed again. After the wash, the samples were placed in small vials and, using a dropper, the samples were dehydrated in a series of ethanol dilutions; 10%, 15%, 30%, 50%, 70%, 100% for 10 minutes and finally 100% ethanol for 1 hour. The samples were suspended in the ethanol solutions while slowly rotated using

an inclined rotation device. The samples were dehydrated for optimal resin penetration. The ethanol was removed from the vials before infiltration process. The dehydrated samples were infiltrated with LR White resin (EMS, Fort Washington, PA.) in a stepwise gradient; 25% for 2 hours, 50% for 2 hours, 75% for 10 hours, and over night in 100%. The samples were infiltrated with resin to maintain the integrity/structural-organization and provide support during sectioning. The samples were embedded and positioned near both ends of the pill-shaped mold gelatin capsules to facilitate sectioning. The samples were cast into fresh LR resin and polymerized in the oven at 60°C, for 24 hours.

Sectioning procedures

Before the samples were sectioned, the hard outer shells on the blocks were removed using a razor blade. Each pill shaped block was secured onto an MTX ultra-microtome chuck and chuck holder (RMC, Boeckeler Instruments. Tucson, AZ), for trimming. A razor blade was used to trim the sample embedded in the block. The oculars attached to the MTX ultra-microtome were used to view the location of the sample in the block during the trimming process. Manipulating the chuck holder, the top and surrounding area of the block was shaped into a pyramid-like structure in order to expose the sample. To preserve the hyphal antigens the samples were not post-fixed with osmium tetroxide during sample preparation and appeared opaque/white. This made the positioning of the sample difficult to see, so the block was held under a bright light to determine

sample location. Once the block was shaped into a pyramid it was ready for thick sectioning.

Thick Sectioning

The trimmed exposed sample was placed in the MTX ultra-microtome geared arch segment mount and adjusted for sectioning. The sample was cut into thick sections (500-1000 nm) using a glass knife, by adjusting the knife holder and geared arch segment mount. Once the knife and block were aligned, distilled water was added to the 'boat' of the glass knife. The water almost touched the inside edge of the glass knife. A cutting window and the thickness were set for the motion of the cutting arm. As the sample was automatically cut, the newly formed sections would fall and float in the water of the 'boat'. The samples were retrieved from the glass knife using a long stick with a concave indentation carved near the end like a skinny spoon. The retrieved sections were placed on a glass slide in a drop of distilled water and dried on a hot plate for approximately 30 seconds. The thick sections on the slide were stained with a drop of 1% toluidine blue and 1% boric acid mixed (1:1) and placed briefly on the hotplate for 15-30 seconds. The slide was washed with distilled water to remove the excess stain, and viewed with a microscope (Olympus CX41RF, Optical Co. Philippines) at 10x, 20x, and 40x to evaluate the presence, absence, and position of the hyphae located in the resin. Thick sections containing stained hyphae were also viewed for sample quality. Once positioning and quality of the sample was acceptable, it was ready for thin sectioning.

Thin Sectioning

The blocks were secured to the MTX ultra-microtome chuck and chuck holder and re-trimmed with a razor using the oculars to eliminate excess resin. Once all excess resin was removed and only sample of interest remained, the block was secured to the geared arch segment mount and adjusted. Thin sections were cut using a diamond knife (Delaware Diamond Knives Inc., Delaware). The diamond knife was secured to the knife holder and adjusted. After the diamond knife and the sample were aligned, the cutting window and thickness were set. Distilled water was added to the 'boat' of the diamond knife to collect the sample. As the block was cut, the sections floated into the "boat" of the knife and viewed through the oculars of the MTX ultra-microtome. The thicknesses of the sections obtained were 70nm in thickness. During thin sectioning a plastic platform covered the 'boat', knife, and sample to shield the sectioning from any environmental disruption. Thin sections were transferred to formvar and carbon coated nickel grids (EMS, Fort Washington, PA.), by touching the coated side of the grid to the sections in the boat using forceps. The Ni grids were air-dried section side up on filter paper for 5 minutes until ready for immuno-labeling.

Immuno-labeling

The Ni grids containing the sectioned hyphae of *P. insidiosum* were placed section side down on a drop (15 µL) of the blocking solution TTBS-BSA (0.05% TWEEN, TRIS-buffered-saline, 1.0% w/v bovine serum albumin, and

0.02% sodium azide pH7.4) and incubated for 15 minutes on a piece of para-film. The grids were dried using the corner pieces of filter paper by touching the outside edges of the grid to absorb the buffer. The Ni grids, section side down, were washed by placing three 15 µL drops of TTBS on para-film for 1 minute and they were then transferred to the next drop using forceps. Host sera from animals (bovine, feline, canine, equine and human) with pythiosis confirmed by culture, histopathology, and serological assays such as immuno-diffusion, agglutination, ELISA, and Western Blot were selected to be used. Sera from each of the selected species used in this study (apparently healthy animals) were used as negative controls. Following the wash the Ni grids were transferred to a 15 µL drop of each host sera diluted 1:1 with TTBS-BSA facing section side down at 25°C for 1 hour. To prevent the samples from drying out, a Petri dish was placed over the para-film and samples with a small piece of moist paper towel. Following the binding, the Ni grids were washed with TTBS as mentioned above and were placed on a 15 µL drop solution of 10% protein A colloidal gold (Ted Pella, Redding CA) (20 nm in diameter) in TTBS-BSA and incubated at 25 °C for 1 hour. Again, to prevent the samples from drying out, a Petri dish was placed over the para-film and samples with a small piece of moist paper towel. The sections were washed with TTBS as above. A final wash included a series of three 15 µL drops of water placed on para-film, the sections were transferred between drops after one minute. The samples were dried with the filter paper as above, and then stained with one drop of 2% uranyl acetate in 50% ethanol (EMS, Fort Washingyon, PA.) for 5 minutes. Samples were then washed with

distilled water as above. Using Sodium Hydroxide pellets to create an carbon dioxide free environment, the samples were stained with 15 µL of Reynolds formulation (EMS, Fort Washingyon, PA.) for 15 minutes. The samples were washed with distilled water as mentioned above and left to dry on filter paper for 5 minutes or until ready for Transmission Electron Microscopy.

Transmission Electron Microscopy Evaluation

The stained sections were then evaluated using a JEOL 100-CX Transmission Electron Microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 100 kV. The TEM was used to locate and evaluate the samples at different magnifications (5,000X-450,000X). Samples were photographed using a Mega View 2 digital camera (Soft Imaging Systems, Lakewood, Colorado).

CHAPTER THREE

Results

Electron Microscopic Features of *Pythium insidiosum* Hyphae

The presence of intact *Pythium insidiosum* hyphae and its structural components in the stained samples were documented on several digital images. These digital images revealed numerous healthy hyphae with a distinct cell wall and multiple internal organelles.

The majority of hyphae observed in the sections possessed an intact cell wall structure followed by a combination of inner layers. The cell wall had a very smooth profile of the plasmalemma which surrounded the entire hyphae (Figure 5). The inner layers beneath the cell wall interlaced and meshed with the other layers forming a coarse thick inner layer (Figure 6). Cytoplasmic vesicles appeared to be transporting proteins to the cytoplasmic membrane, outside the inner layer, and then integrating them into the thick inner layers. Following integration, the cytoplasmic bilayer membrane maintained its integrity.

Within the cytoplasmic membrane many internal structures were observed. Large vacuoles were characterized by the presence of electron dense bodies (EDBs). The EDBs appeared as large dark black structures within the large vacuoles (Figure 7). Numerous small vacuoles were also observed dispersed through out the entire hyphae. These vacuoles ranged in size and shape, many posses a finely granular content (Figure 8). Numerous

mitochondria with tubular cristae were observed in some of the stained samples (Figure 9). All observed mitochondria possessed intact tubular cristae. Multiple nuclei were observed in some hyphae, each surrounded by a bilayered membrane (Figure 8). The chromatin appeared granular within the membrane. Several golgi bodies were also observed throughout the hyphae (Figure 10), with numerous golgi vesicles pinching off from the golgi apparatus (Figure 10 and 11). Relatively large microtubules were observed dispersed throughout the hyphae (Figure 6 and 10). Endoplasmic reticula (ER) were sometimes observed near the mitochondria, multiple ribosomes were also present around the ER (Figure 12).

The mergence of multiple golgi vesicles and the cytoplasmic membrane near the cell wall were observed (Figure 6). Golgi vesicles were also observed integrating into the intermediate cell wall layer (Figure 6).

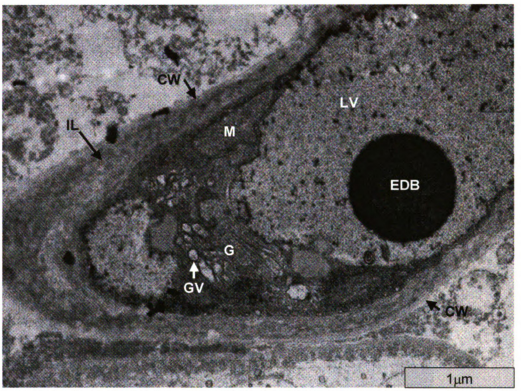

Figure 5: Electron Micrograph of Stained *Pythium insidiosum* Hyphae; Displays a defined cell wall and inner layer structures The components of the hypha are as follows: Cell Wall (CW), Inner Layer (IL), Mitochondria (M), Large Vacuole (LV), Electron Dense Body (EDB), Golgi Body (G), Golgi Vesicle(GV).

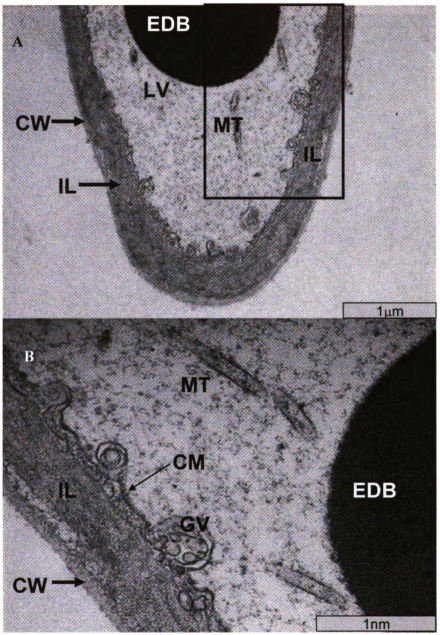

Figure 6: Electron Micrograph of Stained *Pythium insidiosum* Hyphae. Showing the integration of the golgi vesicles into the cytoplasmic membrane and Inner layers. The components of the hypha are as follows Cell Wall (CW), Inner Layer (IL), Cytoplasmic Membrane (CM), Electron Dense Body (EDB), Golgi Vesicle (GV), Microtubule (M). Panel B is an enlargement of Panel A represented by a box.

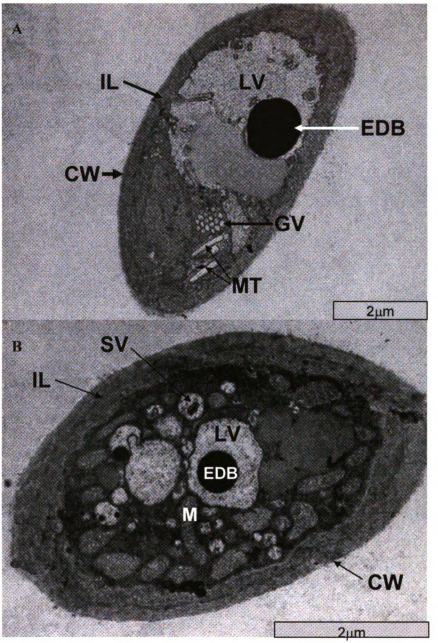

Figure 7: Electron Micrograph of Stained *Pythium insidiosum* Hyphae; Panel A, shows Cell Wall (CW), Inner Layer (IL), Large Vacuole (LV), Electron Dense Body (EDB), Golgi Vesicle (GV), Microtubules (MT). Panel B, depicts also several Mitochondria (M), and Small Vacuoles (SV).

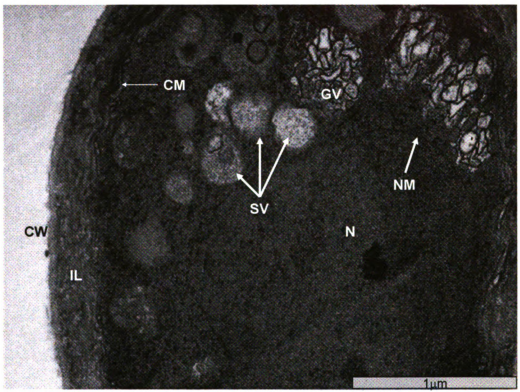

Figure 8: Electron Micrograph of Stained *Pythium insidiosum* Hyphae; This figure depicts the presence of Small Vacuoles (SV), Nucleus (N), Nuclear Membrane (NM), Cell Wall (CW), Inner Layer (IL), Cytoplasmic Membrane (CM).

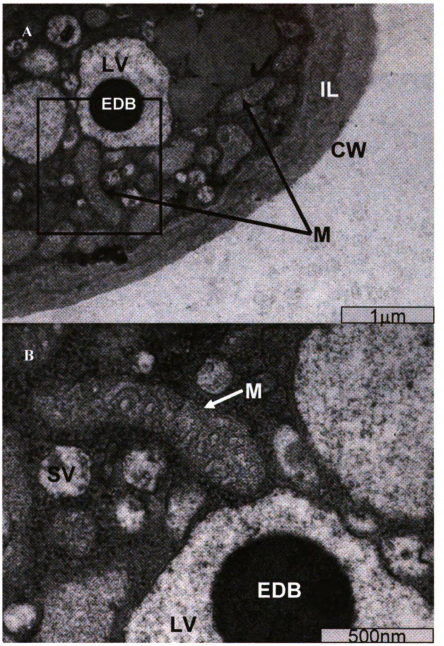

Figure 9: Electron Micrograph of Stained *Pythium insidiosum* Hyphae;
Panel A and B contain Mitochondria (M), Large Vacuole (LV), Electron Dense Body (EDB), Cell Wall (CW), Inner Layer (IL), Small Vacuole (SV), Panel B is an enlargement of Panel A represented by a box. Note the presence of mitochondria (M) with tubular cristae.

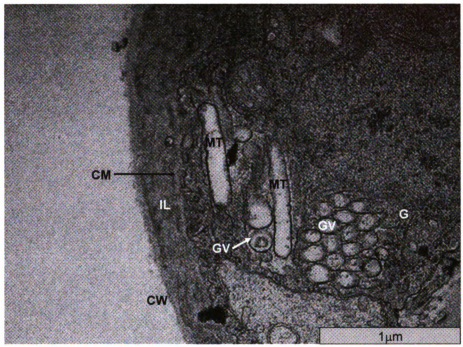

Figure 10: Electron Micrograph of Stained *Pythium insidiosum* Hyphae; The micrograph shows the presence of Cell Wall (CW), Inner Layer (IL), Cytoplasmic Membrane (CM), Microtubule (MT), Large Vacuole (LV), Electron Dense Body (EDB), Golgi Body (G), Golgi Vesicle(GV).

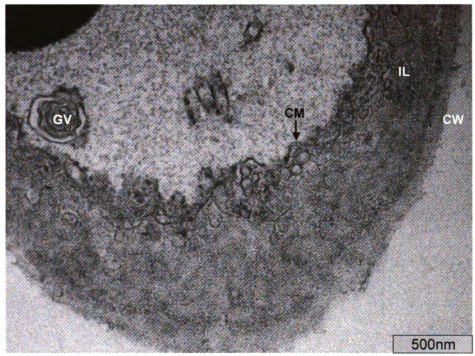

Figure 11: Electron Micrograph of Stained *Pythium insidiosum* Hyphae; This figure shows the presence of Golgi Vesicle (GV), Cytoplasmic Membrane (CM), Inner Layer (IL), Cell Wall (CW). A well developed Inner Layer is clearly observed in this figure.

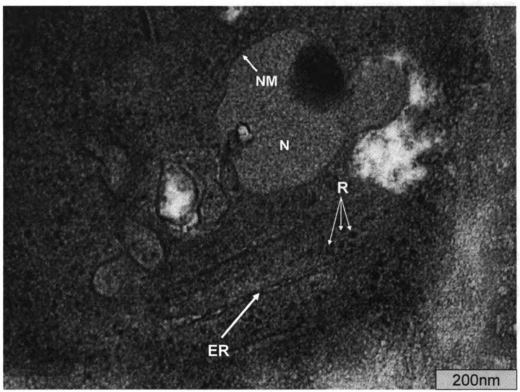

Figure 12: Electron Micrograph of Stained *Pythium insidiosum* Hyphae; This figure depicts Endoplasmic Reticulum (ER) with Ribosomes ®, Nuclear Membrane (NM), Nucleus (N).

Immuno-labeling of *Pythium insidiosum's* Hyphal Antigens

Digital images of the immuno-labeled structures in the hyphae of *Pythium insidiosum* were photographed for interpretation of the binding immuno-globulins present in the sera from the above hosts to PACG particles. *P. insidiosum* hyphae used for immuno-labeling were not osmium stained to preserve the antigenic properties of the immuno-reactive hyphal proteins. Thus, many of the structures observed in the unstained samples for immuno-labeling were not clearly defined.

A series of negative controls (sera from apparently normal bovine, canine, equine, feline, and human) were first tested to validate the specificity of the immuno-labeling. The control samples showed some gold particles in each of the tested sera randomly bound throughout the examined fields (Figures 13 and 14). Control samples with only protein A colloidal gold showed few randomly bound gold particles after rinsed with TTBS buffer (Figure 15).

The antigenic proteins in the cell wall of mature hyphae reacted with the Bovine, Human, and Equine sera containing anti-*P. insidiosum* antibodies, as shown by the binding of protein A colloidial gold to those specific areas. All of the examined mature hyphae cell wall structures were found to contain great quantities of bound PACG particles. In those samples, random PACG binding were rarely observed (Figure16). In samples reacted with sera from infected feline and canine resulted in random distribution of the PACG particles with no specific binding (Figure 17).

Samples reacted with sera from an infected bovine showed specific binding on the cell wall and in the cytosol (Figures 18-24). Some other indistinguishable internal structures in the cytosol did not display specific binding (Figures 18-24). Within the hyphae a substantial number of PACG particles were specifically bound to the antigens of the inner layers beneath the cell wall (Figures 18-24). In addition, gold particles were heavily bound to specific areas in the cytosol (Figures 19 and 20). One section of this sample showed a group of dead hyphae without cytoplasm, but with intact cell wall structures (Figure 25). The skinny cell wall structure of these dead hyphae showed specific binding of gold particles (Figure 26).

The serum sample from an infected human reacted in the same fashion to that of bovine. The cell wall and the inner layers beneath the cell wall were heavily bound with gold particles around the entire hyphae (Figures 27-30). There was a significant binding within the cytosol to the hyphal antigens. However, it was considerably less compared the binding observed in bovine samples (Figures, 27-30). Most structures within the cytosol were almost free of gold particles in most samples reacted with the human serum (Figures 27-30).

The binding of gold particles in samples reacted with the horse sera were significant, especially in the cell wall regions of the hyphae (Figures 17, and 31-41). On the outside of the cell wall surface gold particles were specifically bound to the antigens, sometimes forming chains (Figure 32). Other sections of the cell wall surface showed specific binding in clusters as well (Figure 33). Cytoplasmic structures and sections of the cytosol where the structures were embedded were

rarely observed bound to gold particles (Figures 17, and 31-41). A long single hypha approximately 100 μm in length was studied in detail (Figures 34). This long hypha was intact, and possessed a very thick inner layer structure beneath the cell wall on only one side of the hypha (Figures 34-41). This section of the hyphae was heavily bound to the gold particles along the entire 100 μm length of the hypha (Figures 34-41). The thick-sided inner layer structures possessed two layers located immediately beneath the cell wall (Figures 34-41). Both layers were specifically bound by the gold particles (Figures 39-41). The opposite side of the hypha did not possess inner layers and appeared dark. However, it displayed the binding of PACG particles along its entire surface of the cell wall (Figures 39-41). The cytosol of the long hypha showed some sections with few gold particles binding to it (Figures 34-41).

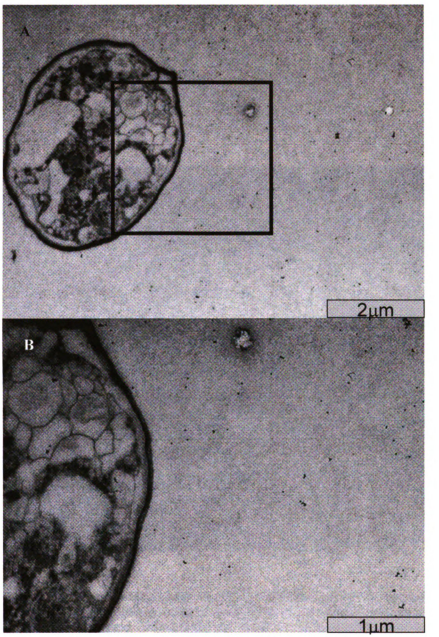

Figure 13: Electron Micrograph of *Pythium insidiosum* Hyphae reacted with sera from healthy equine; Protein A colloidal gold particles randomly bound to sections of the field. Panel B is an enlargement of Panel A represented by a box. Sera from all healthy hosts (bovine, canine, equine, feline, human) displayed the same random binding of gold particles (not shown).

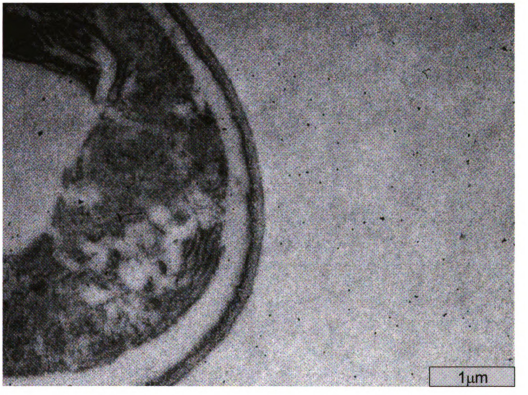

Figure 14: Electron Micrograph of *Pythium insidiosum* Hyphae reacted with sera from healthy equine; Protein A colloidal gold particles are shown randomly bound to some sections of this field. Sera from all healthy hosts (bovine, canine, feline, human) displayed the same random binding of gold particles (Not shown).

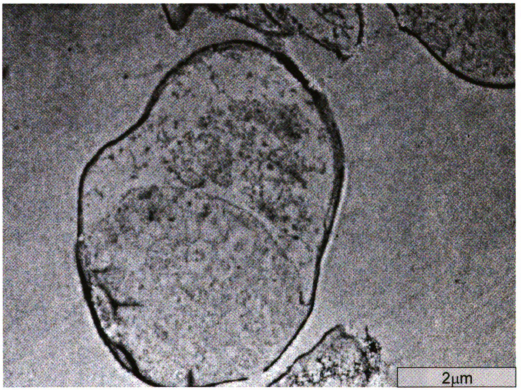

Figure 15: Electron Micrograph of *Pythium insidiosum* Hyphae reacted with only colloidal gold particles. Most gold particles were washed off by TTBS buffer.

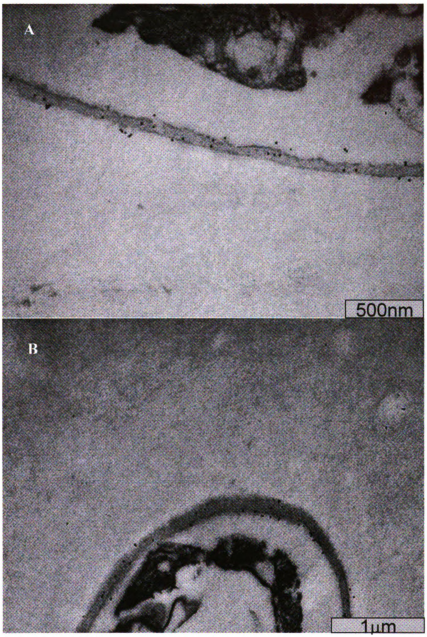

Figure 16: Electron Micrograph of *Pythium insidiosum* Hyphae reacted with sera from equine with pythiosis. Protein A colloidal gold particles Specifically bound to cell wall and inner layer structures on both Panel A and B. Note the lack of binding in the cytosol (See also Figure 31).

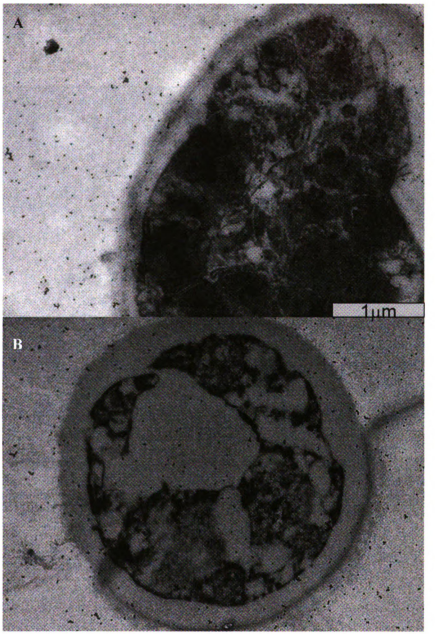

Figure 17: Electron Micrograph of *Pythium insidiosum* Hyphae reacted with sera from Feline Panel A and Canine Panel B with pythiosis. Protein A collodial gold particles displayed random binding.

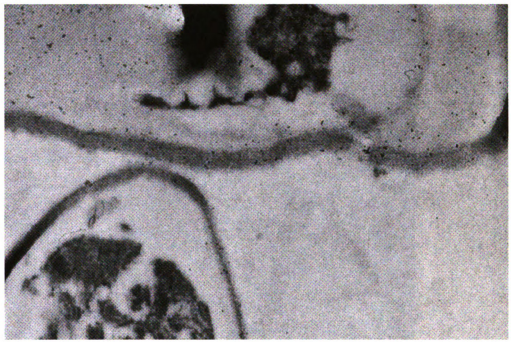

Figure 18: Electron Micrograph of *Pythium insidiosum* Hyphae reacted with sera from bovine with pythiosis. Protein A collodial gold particles specifically bound to the cell wall, inner layers, and cytosol of the hyphae. Note no gold binding outside the hyphae.

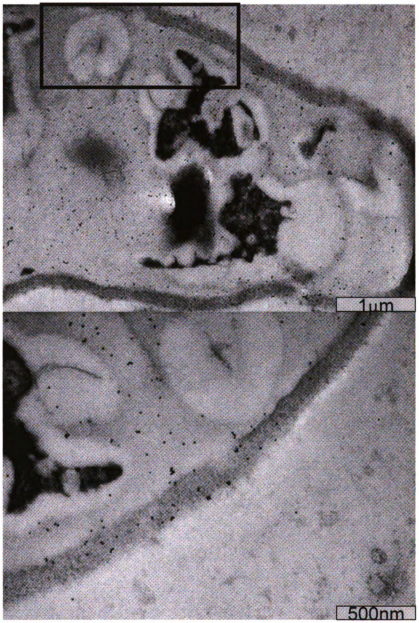

Figure 19: Electron Micrograph of *Pythium insidiosum* Hyphae reacted with sera from bovine with pythiosis. Panel A shows Protein A collodial gold particles Specifically bound to cell wall, inner layers, and cytosol on the hyphae, Panel B is an enlargement of Panel A represented by a box. Note the large congruity of gold particles bound to both the cytosol and cell wall of the hypha.

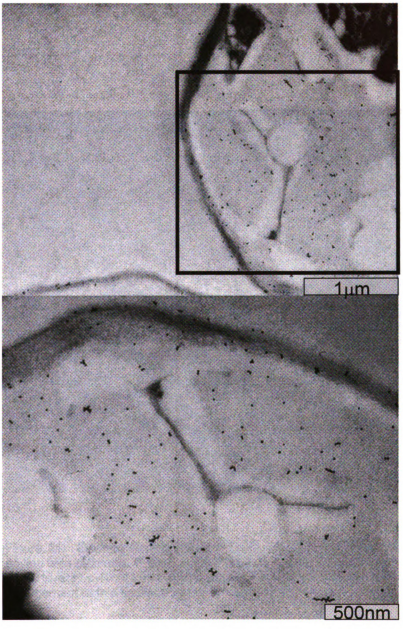

Figure 20: Electron Micrograph of *Pythium insidiosum* Hyphae reacted with sera from bovine with pythiosis. Panel A depicts Protein A collodial gold particles Specifically bound to cell wall, inner layers, and cytosol on the hyphae, Panel B is an enlargement of Panel A represented by a box. Note the prominent binding of PACG particles in the cytosol and cell wall.

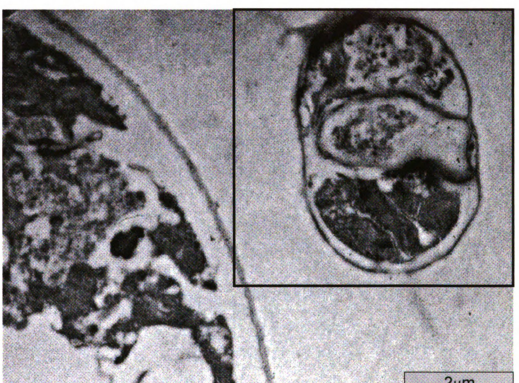

Figure 21: Electron Micrograph of *Pythium insidiosum* Hyphae reacted with sera from bovine with pythiosis. Protein A collodial gold particles specifically bound to the cell wall, inner layers, cytosol, and some internal structures of the hyphae. Figure 21 is an enlargement of this figure represented by the box.

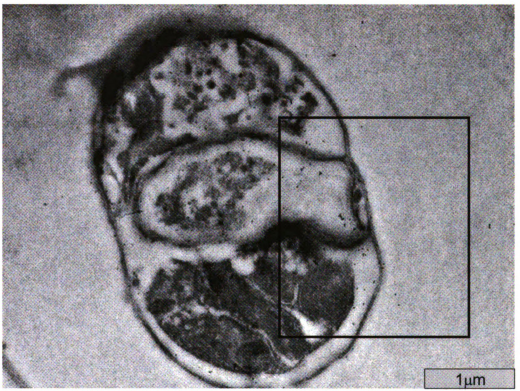

Figure 22: Electron Micrograph of *Pythium insidiosum* Hyphae reacted with sera from bovine with pythiosis. Protein A collodial gold particles specifically bound to the cell wall, inner layers, cytosol, and some internal structures of the hyphae, This Micrograph is an enlargement of figure 21. Figure 23 is an enlargement of this figure, represented by the box.

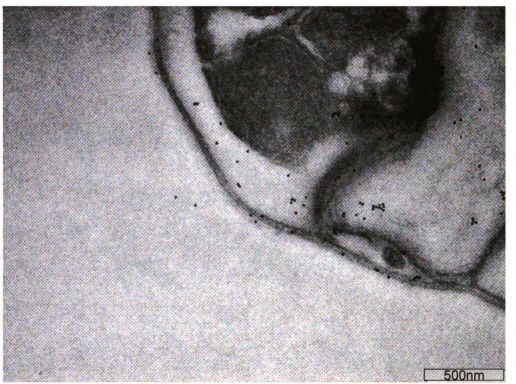

Figure 23: Electron Micrograph of *Pythium insidiosum* Hyphae reacted with sera from bovine with pythiosis. Protein A collodial gold particles specifically bound to cell wall, inner layers, and cytosol of the hyphae, This Micrograph is an enlargement of figure 22.

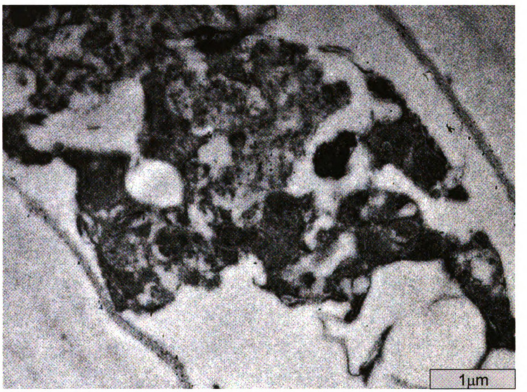

Figure 24: Electron Micrograph of *Pythium insidiosum* Hyphae reacted with sera from bovine with pythiosis. Protein A collodial gold particles specifically bound to internal structures, cell wall, inner layers, and cytosol of the hypha.

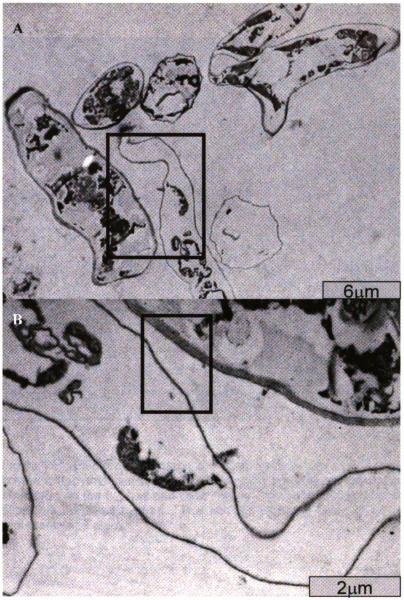

Figure 25: Electron Micrograph of *Pythium insidiosum* Hyphae reacted with sera from bovine with pythiosis. Panel A Protein A collodial gold particles specifically bound to cell wall structures on the healthy hypha and dead hypha. (Gold particles are difficult to see because of low magnification). Note the presence of cytosol in the healthy hypha versus the dead hyphae. The dead hypha is characterized by the thin cell wall and lack of inner layer Panel B is an enlargement of Panel A represented by a box.

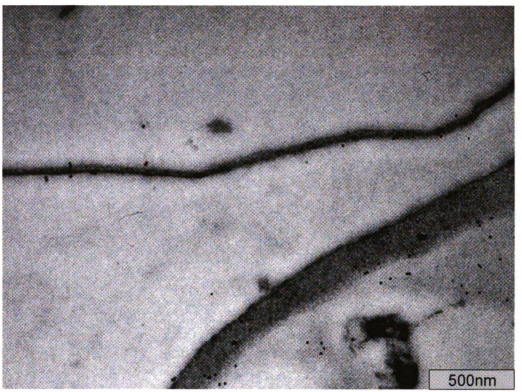

Figure 26: Electron Micrograph of *Pythium insidiosum* Hyphae reacted with sera from bovine with pythiosis. Protein A collodial gold particles specifically bound to the cell wall structures on the hyphae and dead hyphae, Note the presence of cytosol in the healthy hypha vs the dead hyphae. This Micrograph is an enlargement of figure 25 Panel B represented by a box.

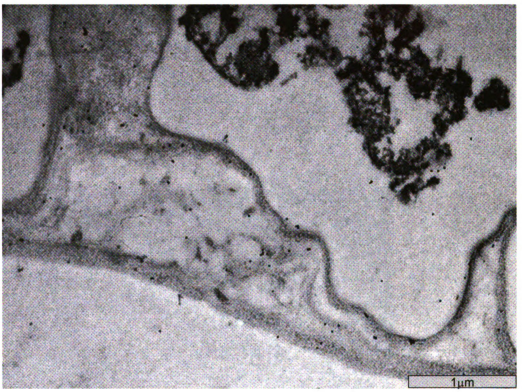

Figure 27: Electron Micrograph of *Pythium insidiosum* Hyphae reacted with sera from a human with pythiosis. Protein A collodial gold particles Specifically bound to cell wall, inner layers, and cytosol of the hyphae. Note, no gold particles are observed outside of the hypha.

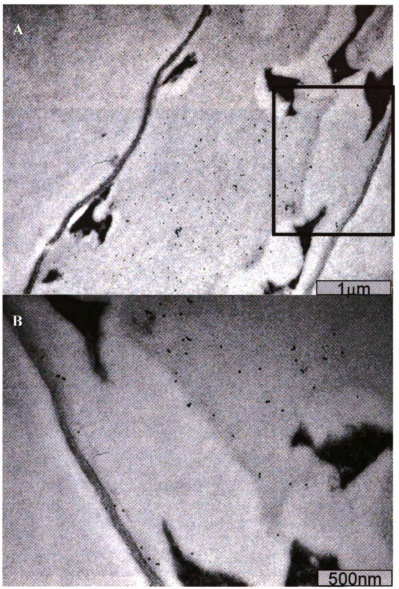

Figure 28: Electron Micrograph of *Pythium insidiosum* Hyphae reacted with sera from a human with pythiosis. Panel A depicts Protein A collodial gold particles specifically bound to cell wall, inner layers, and cytosol of the hyphae. Panel B is an enlargement of Panel A represented by a box.

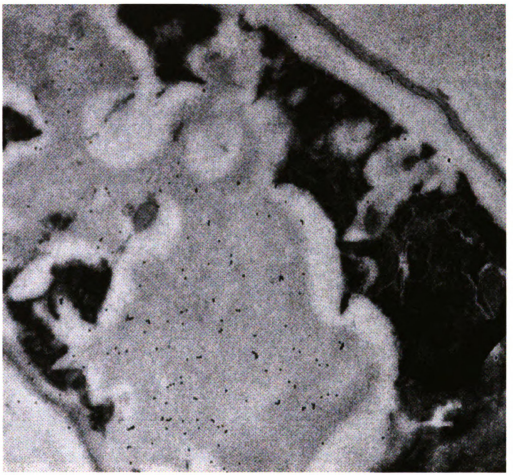

Figure 29: Electron Micrograph of *Pythium insidiosum* Hyphae reacted with sera from a human with pythiosis. Protein A collodial gold particles Specifically bound mainly to the cytosol of the hyphae. Note the heavy binding in the cytosol. Few particles are bound to the cell wall.

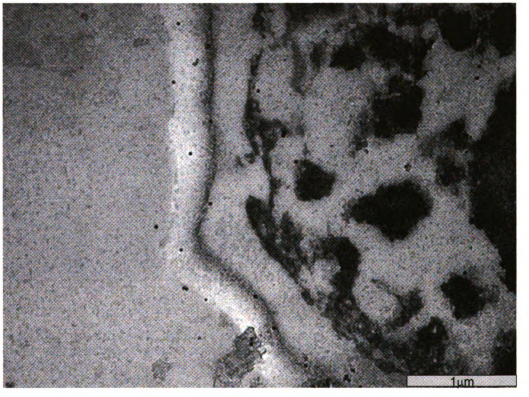

Figure 30: Electron Micrograph of *Pythium insidiosum* Hyphae reacted with sera from a human with pythiosis. Protein A collodial gold particles Specifically bound to two different inner layer structures on the hypha.

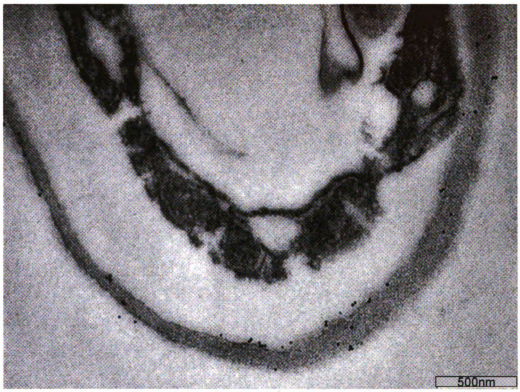

Figure 31: Electron Micrograph of *Pythium insidiosum* Hyphae reacted with sera from a horse with pythiosis. Protein A collodial gold particles specifically bound to the cell wall structures on the hypha. Note the lack of binding in the cytosol and outside the hypha (Enlargement of Figure 16).

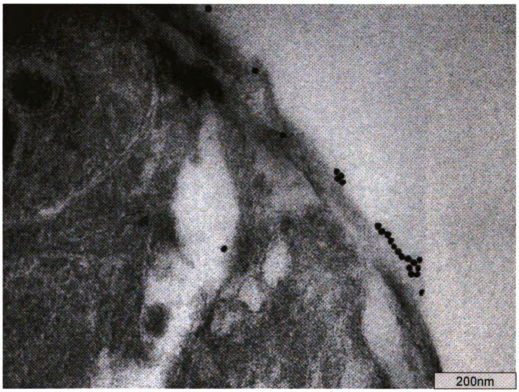

Figure 32: Electron Micrograph of *Pythium insidiosum* Hyphae reacted with sera from a horse with pythiosis. Protein A collodial gold particles Specifically bound forming a chain on the cell wall of the hypha. Note the lack of binding in the cytosol and outside the hypha.

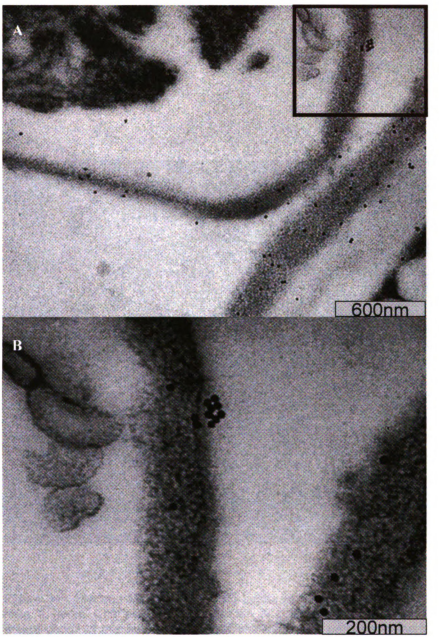

Figure 33: Electron Micrograph of *Pythium insidiosum* Hyphae reacted with sera from a horse with pythiosis. Panel A shows Protein A collodial gold particles specifically bound to the cell wall and inner layer structures on the hypha. Note the lack of binding in the cytosol., Panel B is an enlargement of Panel A represented by a box.

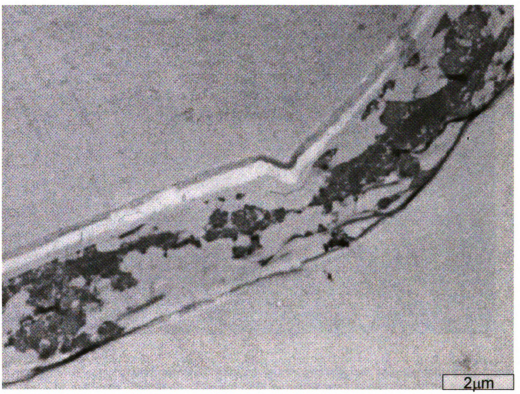

Figure 34: Electron Micrograph of long hypha of *Pythium insidiosum* reacted with sera from a horse with pythiosis and Protein A collodial gold particles. Due to the low magnification of this figure, the specific binding of gold particles to the hyphal structures could not be clearly distinguished. Figures 35-41 are of a higher magnification and depict the specific binding of gold particles to this long hypha.

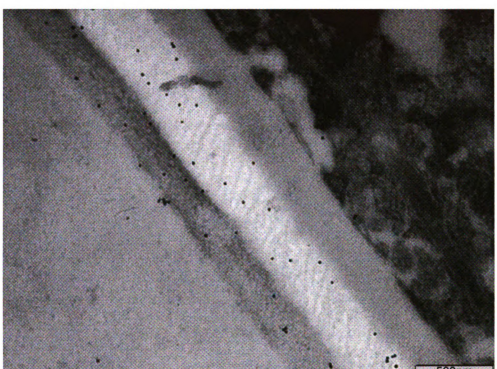

Figure 35: Electron Micrograph of *Pythium insidiosum* Hyphae reacted with sera from a horse with pythiosis. Protein A collodial gold particles specifically bound to the cell wall and at least two inner layer structures on the hypha. Note the lack of binding in the cytosol. This micrograph is an enlargement of figure 34, displaying the thick inner layer structures.

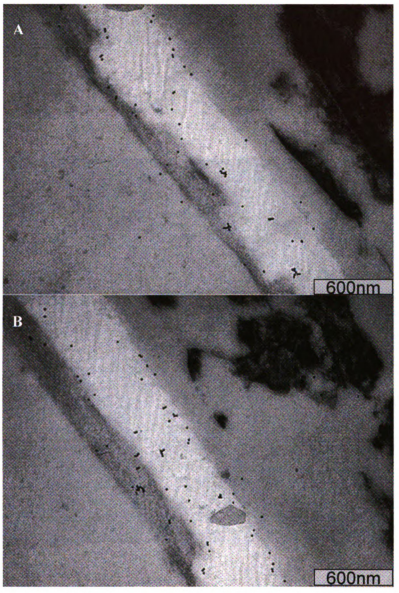

Figure 36: Electron Micrograph of *Pythium insidiosum* Hyphae reacted with sera from a horse with pythiosis. Panel A and B depicts Protein A collodial gold particles specifically bound to the cell wall and inner layer structures on the hypha. Note the lack of binding in the cytosol, This micrograph is an enlargement of figure 34, displaying the thick inner layer structures.

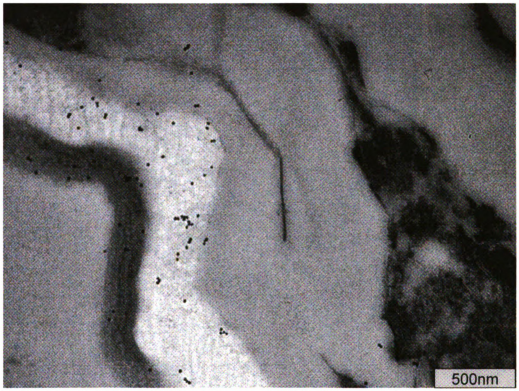

Figure 37: Electron Micrograph of *Pythium insidiosum* Hyphae reacted with sera from a horse with pythiosis. Protein A collodial gold particles specifically bound to the cell wall and inner layer structures on the hypha. Note the lack of binding in the cytosol, This micrograph is an enlargement of figure 34, displaying the thick inner layer structures.

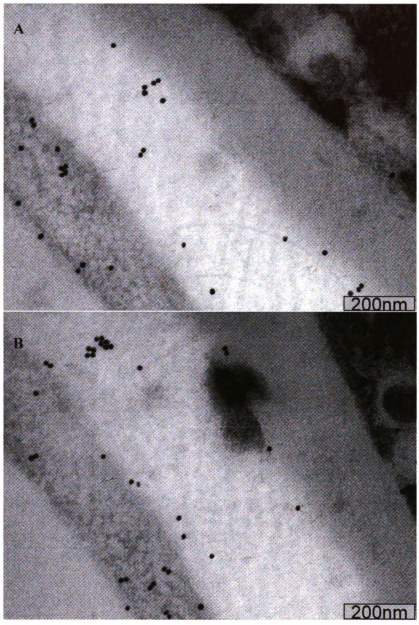

Figure 38: Electron Micrograph of *Pythium insidiosum* Hyphae reacted with sera from a horse with pythiosis. Panel A and B show Protein A collodial gold particles specifically bound to the cell wall and inner layer structures on the hypha. Note the lack of binding in the cytosol. Both Panel A and B are enlargements of figure 34, displaying the thick inner layer structures.

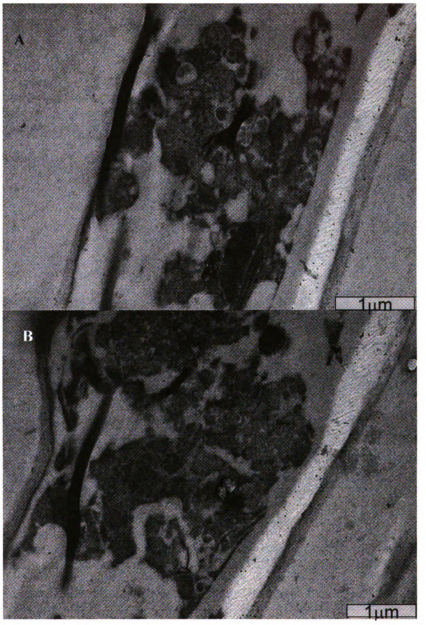

Figure 39: Electron Micrograph of *Pythium insidiosum* Hyphae reacted with sera from a horse with pythiosis. Panel A and B depict Protein A collodial gold particles specifically bound to the cell wall and inner layer structures on the hypha. Note the lack of binding in the cytosol. Both Panel A and B are enlargements of figure 34, displaying the thick inner layer structures. Note the lack of Inner layers on the left side of the hypha.

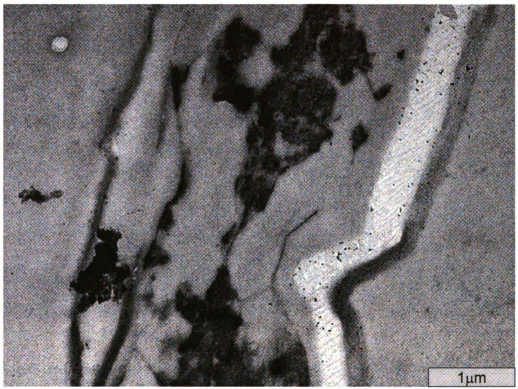

Figure 40: Electron Micrograph of *Pythium insidiosum* Hyphae reacted with sera from a horse with pythiosis. Protein A collodial gold particles specifically bound to the cell wall and inner layer structures on the hypha. Note the lack of binding in the cytosol, This micrograph is an enlargement of figure 34, displaying the thick inner layer structures. Note the binding of gold to the cell wall on the left side of the hypha. No inner layers are observed in this section of the hypha.

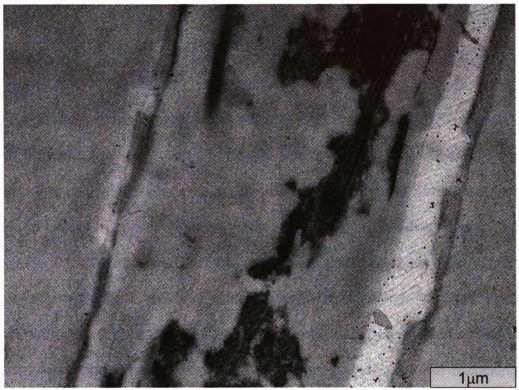

Figure 41: Electron Micrograph of *Pythium insidiosum* Hyphae reacted with sera from a horse with pythiosis. Protein A collodial gold particles specifically bound to the cell wall and inner layer structures on the hypha. Note the lack of binding in the cytosol, This micrograph is an enlargement of figure 34, displaying the thick inner layer structures. Note the binding of gold to the cell wall on the left side of the hypha. No inner layers are observed in this section of the hypha (See also Figure 36).

CHAPTER FOUR

Discussion

The electron microscopic data on the morphological features of *P. insidiosum* hyphae confirmed previous findings (Miller et al., 1985). However, the electron micrographs from this study added new information regarding the internal morphological components of *P. insidiosum's* hyphal structures, not reported in earlier studies. Internal organelles were identified based on comparisons with the internal structures of other *Pythium spp.* or other similar hyphal organisms (Hoch, 1987). The development of golgi vesicles were observed pinching off from the golgi bodies, responsible for modification of lipids and packaging of proteins. The golgi vesicles were also observed trafficking from the cytosol to the cytoplasmic membrane and then into the inner layers beneath the cell wall. Several structures involved in transportation such as microtubules, endoplasmic reticulum, and ribosomes were all observed. It is believed that key antigens of *P. insidiosum* are produced within the cytosol, packaged and modified by the golgi bodies, and then pinched off from the golgi bodies into golgi vesicles and transported by the microtubules, endoplasmic reticulum, and ribosomes through the cytoplasmic membrane, into and through the inner layers, to the cell wall and possibly to external compartments of the hyphae. These findings were confirmed by the binding of gold particles to

specific areas in the cell wall and other internal hyphal components during our immuno electron microscopic study.

Early studies have demonstrated that most immunological methods have successfully detected the antigens of *P. insidiosum*, and thus they proved to be useful for the diagnosis of pythiosis (Mendoza and Newton 2005). Other reports have documented *P. insidiosum's* lifecycle, ecology, distribution, immunological effects, and the characterization of pythiosis in both humans and animals. The recent phylogenetic study of *P. insidiosum* confirmed its placement within the members of the genus *Pythium* using molecular techniques. A novel therapeutic approach to avoid invasive surgical treatment, termed Immunotherapy, has been extremely effective (Mendoza, 2005). Although all previous studies have been of paramount importance to understand this disease, the mapping of the antigens detected during infection has yet to be achieved. Thus, the location of *P. insidosum's* putative antigens within the hyphae, involved in the pathology and immunotherapy of the disease remain a mystery. The identification and characterization of the antigens developed by *P. insidiosum* during infection is the next logical step to further understand the mechanisms of both the immunotherapy and the immunopathology of pythiosis (Mendoza and Newton 2005). This immuno electron microscopic study indicates that *P. insidiosum* expresses antigens in specific areas of the cytosol, which then migrate to the cytoplasmic membrane, and to the inner layers beneath the cell wall and then to the cell wall itself, where some of them are possibly taken outside of the hyphal compartment. Similar techniques have been used to evaluate the distribution of

antigens of some fungal and protistal pathogens. For instance, the antigens of the fungal pathogen *Paracoccidioides brasiliensis* were mapped using immunofluorescence and a protein A colloidal gold immunolabeling technique for detection of the hyphal antigens. Their immunocytochemical techniques indicated that some exoantigens were synthesized within the cytoplasm and then excreted through the cell wall (Sandoval et al., 1996). Herr et al. (1999) used a similar approach to evaluate the location of the antigenic components of *Rhinosporidium seeberi* in infected host tissue (Herr et al., 1999).

The specific reactions of the protein A colloidal gold particles were validated using a series of negative controls. Sera obtained from healthy hosts (bovine, canine, equine, feline, and human) reacted with the *P. insidiosum* hyphae and then labeled with gold particles all showed random binding. This random binding validates the specificity of the gold particles located only on the antigens developed by *P. insidosum* hyphae. Gold particles reacted with *P. insidiosum* hyphae were mostly washed away with the buffer and those few gold particles left behind displayed random binding, indicating that the gold particles themselves have no affinity to *P. insidiosum's* hyphal antigens.

The binding of the protein A colloidal gold particles, as observed by this immuno electron microscopic study using the sera from multiple hosts and *P. insidiosum* hyphae, varied between hosts. The used sera from infected hosts with pythiosis, displayed specific binding of gold particles to several areas of the hyphae. While some of the hosts displayed very similar specific binding patterns, most of the hosts were unique with different specific binding patterns observed.

For instance, reacted samples from both bovine and humans displayed large amounts of specific binding in the cytosol, the inner layers, and the cell wall. However, the equine reacted samples displayed strong specific binding on the cell wall and inner layer structures, but showed minimal binding on cytosolic structures. In contrast, the bovine and the human immunoglobulins showed similar binding on the cell wall, but it was not as significant as that on the equine samples. During this study a long laterally sectioned hypha (~100 µm in diameter) (Figures 34-41) was study in detail. This long hypha showed large amounts of PACG binding to unusually thick inner layer structures and to the cell wall. This long hypha showed three clearly inner layers, a feature did not encountered in other hyphal sections reacted with the same equine serum or with the other host's sera. Oddly, these inner layers were observed only on one side of the hyphal cell wall. The other side of the cell wall hypha was devoid of inner layers. The long hyphae appeared healthy and contained healthy cytosol. However, few PACG particles were found bound to the cytosol. In addition, the canine and the feline immunoglobulins apparently did not display specific binding in any of the hyphal structures. According to the manufacturer, the protein A colloidal gold particles used by them have never been tested in cats, dogs, or horses. Thus, it is possible that the protein A region of the PACG particles might possess low or not affinity at all for the Fc regions of these hosts immunoglobulins (cats and dogs).

It is also possible that each of the studied hosts might produce different anti-*P. insidosum* immunoglobulins against the hyphal antigens. This would

explain why the reacted sera samples from multiple hosts showed different unique binding patterns to the hyphal antigens. In our studies the bovine, equine, and human, showed good binding affinity for the Fc regions of the immunoglobulins in these species. But, the canine and feline immunoglobulins did not react. This could explain why dogs and cats immunoglabulins did not show specific biding to the PACG particles. Interestingly, cats and dogs respond poorly to immunotherapy whereas bovines, equines, and humans did respond well to immunotherapy and also displayed specific binding to *P. insidiosum* hyphae in this study. Because it is not a clear link between unresponsiveness to immunotherapy and the lack of gold binding to the hyphae of *P. insidiosum* in the sera of canine and feline, we strongly believe that the protein A low affinity for the cats and dogs immunoglobulins could well explain our negative results with the sera from canines and felines.

A recent unpublished study using western blots has identified immuno-dominate proteins using multiple host sera. Perhaps the identification of an immuno-dominant protein derived from *P. insidiosum* hyphae would encompass all the hosts for a more effective universal vaccine. There have also been reports of allergenic reactions in horses. However, *P. insidiosum* hyphae were not found in these animals (Robert Glass, Personal communication). Perhaps the antigens contained in the cell wall of the dead hyphae be still active and could mount an allergenic reaction in equines without being infected with the etiologic agent.

The results obtained during this immuno-electron microscopic mapping of *P. insidiosum* hyphae revealed that there are multiple immunodominant antigens located within the cytosolic components of the hyphae, as well as on the inner layers and cell wall. Colloquial reports indicating that some horses with positive serology, to the antigens of *P. insidiosum*, but without the typical lesions of pythiosis, had developed allergic reaction of the upper respiratory system, suggested that *P. insidiosum* could act as a potent allergen on this and other species. Thus, the finding in this study that the empty cell wall structures, found on dead hyphae, retained their antigenic/allergenic properties is rewarding. All in all, this study not only mapped for the first time the location of the immunodominant antigens of *P. insidiosum*, but also specify what of the antigens encountered in Western Blot should be first characterized using molecular tools

Bibliography

Austwick, P. K. C., and J. W. Copland. 1974. Swamp Cancer. *Nature*, 250: 84.

Bridges, C. H., and C. W. Emmons. 1961. A phycomycosis of horses caused by *Hyphomyces destruens*. *J American Vet Med Assoc*, 38: 579-89.

Brown, C. C. and J. J. McClure. 1988. Use of immunohistochemical methods for diagnosis of equine pythiosis. *American J Vet Research*, 11: 1866-1868.

De Cock, W. A. W. and L. Mendoza. 1987. *Pythium insidiosum sp.* the etiological agent of pythiosis. *J Clinical Microbiology*, 25: 344-9.

De Hann, J. and L. Hoogkamer. 1901. *Hyphomycosis destruens*. *Veeartseniij Bl v Ned Indie*, 13: 350-74.

De Moraes, S., L. Mendoza, et al. 2005. Human Pythiosis, Brazil. *Emerging Infectious Diseases*, 11: 715-718.

Dick, M.W. 2001. *Straminipilous fungi*: systematics of the *Peronosporomycetes* including accounts of the marine straminipilous protist, the plasmodiophorids and similar organisms. London: Kluwer Academic Publishers.

Fischer, J. R. and L. W. Pace. 1994. Gastrointestinal pythiosis in Missouri dogs: eleven cases. *J Vet Diagn Invest*, 6: 380-2.

Habbinga, R. 1967. Phycomycosis in an equine. *Southwest Vet*, 20: 237-8.

Herr, R. A., L. Mendoza, S. N. Arseculeratne, L. Ajello. 1999. Immunolocalization of an endogenous antigenic material of *Rhinosporidium seeberi* expressed only during mature sporangial development. *Fed of European Soc Immun and Med Micro*, 23: 205-212.

Hoch, H. C. 1987. Freeze-Substitution: A technique that yields improved ultrastructural preservation of fungi, p. 161-166. In M. Fuller, and A. Jaworski (Eds), Zoosporic Fungi In Teaching and Research.

Hutchens, M., and L. Mendoza. 2002. Development of a simplified latex agglutination test for the rapid diagnosis of the infections caused by *Pythium insidiosum.* 102nd General Meeting of the American Society for Microbiology, Salt Lake City, Utah; 124.

Krajaejun T. 2004. Ocular pythiosis: is it under diagnosed. Am J Ophthalmol, 137(2): 370-2.

McMullan, W. C., J. R. Joyce, D. V. Hanselka, J. M. Heitmann. 1977. Amphotericin B for the treatment of localized subcutaneous phycomycosis in the horse. *Journal of the American Veterinary Medical Association* 170: 1293-1298.

Miller, R. 1981. Treatment of equine phycomycosis by immunotherapy and surgery. *Aust Vet J*, 57: 377-82.

Miller, R. I., and R. S. Campbell. 1982. Immunological studies on equine phycomycosis. *Aust Vet J*, 58: 227-31.

Miller, R. I., and R. S. Campbell. 1983. Experimental pythiosis in rabbits. *Sabouraudia*, 21: 331-341.

Miller, R. I., B. M. Olcott, M. Archer. 1985. Cutaneous pythiosis in beef calves. *Journal of the American Veterinary Medical Association* 186: 984-986.

Mendoza, L. 2005. Pythiosis. p. 412-429. chap. 22. In R.J. Hay, W.G. Merz, (Eds), Topley & Wilson's Microbiology and Microbial Infections, 10th ed. Arnold, London.

Mendoza, L., F. Hernandezand, L. Ajello. 1993. Life cycle of the human and animal oomycete pathogen *Pythium insidiosum. Journal of Clinical Microbiology* 31:2967-2973.

Mendoza, L., L. Kaufman, W. Mandy, R. Glass. 1997. Serodiagnosis of hyman and animal pythiosis using an enzyme-linked immunosorbent assay. *Clinical Diagnosis Lab Immunology*, 4: 715-718.

Mendoza, L., L. Kaufman, P. Standard. 1987. Antigenic relationship between the animal and human pathogen *Pythium insidiosum. J Clinical Microbiology*, 25: 2159-2162.

Mendoza, L., W. Mandy, R. Glass. 2003. An improved *Pythium insidiosum*-vaccine formulation with enhanced immunotherapeutic properties in horses and dogs with pythiosis. *Vaccine*, 21: 2797-2804.

Mendoza, L., and C. J. Newton. 2005. Immunology and immunotherapy of the infections caused by *Pythium insidiosum. Medical Mycology J*, 00: 1-9.

Mendoza, L., V. Nicholson, J. F. Precott. 1992a. Immunoblot analysis of the humoral immune response to *Pythium insidiosum* in horses with pythiosis. *J Clinical Microbiology*, 30: 2980-2983.

Mendoza, L., J. Villalobus, C. E. Calleja. 1992b. Evaluation of two vaccines for the treatment of pythiosis insidiosi in horses. *Mycopathologia*, 118: 89-95.

Rivierre, C., C. Laprie, O. Guiard-Marigny, P. Bergeaud, M. Berthelemy, and J. Guillot. 2005. Pythiosis in Africa. *Emerging Infectious Diseases* 11: 479-481.

Rippon, J. W. 1982. Medical Mycology 2nd Edition. P 335-341, W.B. Saunders Co., Philadelphia.

Rosa, P. S. 1993. Development and evaluation of serological tests to detect pythiosis in horses. Master of Science thesis, Louisiana State University. 1-120.

Sathapatayavongs, B., P. Ledachaikul. 1989. Human pythiosis associated with thalassemia hemoglobinopathy syndrome. *J Infect Dis*, 159: 274-80.

Sandoval, M. P., G. M. B. Del Negro, M. J. S. Mendes-Giannini, T. De Brito. 1996. Distribution of exoantigens and a 43-kDa glycoprotein in the yeast and mycelial forms of *Paracoccidioides Brasiliensis. J Mycol Med*, 6: 1-6.

Schurko, A., L. Mendoza, W. A. M. de Cock, G. R. Klassen. 2003. Evidence for geographic clusters: Molecular genetic differences among strains of *Pythium insidiosum* from Asia, Australia and the Americas are explored. *Mycologia* 95: 200-208.

Shenep, J. L., B. K. English, L. Kaufman, T. A. Pearson, J. W. Thompson, R. A. Kaufman, G. Frisch, M. G. Rinaldi. 1998. Successful medical therapy for deeply invasive facial infection due to *Pythium insidiosum* in a child. *Clinical Infectious Diseases* 27:1388-1393.

Shipton, W.A., R. I. Miller, I. R. Lea. 1982. Cell wall, soospore and morphological characteristics of Australian isolates of a *Pythium* causing equine phycomycosis. *Trans Br Mycol Soc*, 79:15-23.

Triscott, J. A., D. Weedon, E. Cabana. 1993. Human subcutaneous pythiosis Journal of Cutaneous Pathology **20**:267-271.

Virgile, R., H. D. Perry. 1993. Human corneal ulcer caused by *Pythium insidiosum. Cornea*, 12: 81-3.

Wanachiwanawin, W., M. Thianprasit, S. Fucharoen, A. Chaiprasert, N. Sudasna, N. Ayudhya, N. Sirithanaratkul, A. Piankijagum. 1993. Fatal arteritis due to *Pythium insidiosum* infection in patients with thalassaemia. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 87: 296-298.