The use of two heterologous hosts for the study of virulence of *Cryptococcus neoformans/gattii* from Puerto Rico

By

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ABSTRACT

Cryptococcus neoformans (*C. neoformans*) and *Cryptococcus gattii* (*C. gattii*) are encapsulated, pathogenic yeasts, belonging to the Basidiomycota. They infect immune-compromised and immune-competent individuals respectively. The yeasts are usually free living, with the preferred habitats being pigeon droppings for *C. neoformans* and tree detritus for *C. gattii.* This work presents the characterization of virulence and stress response of four environmental isolates of the *Cryptococcus neoformans/gattii* species complex from Puerto Rico. Two experiments were undertaken to determine, directly and indirectly, the virulence of these isolates.

The first experiment looked to characterize the interactions between *C. neoformans/gattii* and amoebae from the genus *Acanthamoeba.* We observed an increment in capsule size after co-incubation of the organisms, with *C. gattii* showing the greatest increase in capsule size. We then undertook an experiment by infecting *Galleria mellonella* larvae with different environmental isolates and the type isolate H99, a *C. neoformans* var. *grubii* strain. The resulting data shows that at 37°C the environmental isolates are as virulent as H99 *in vivo* in a non-mammalian host. To the best of our knowledge this is the first study with environmental isolates from Puerto Rico to do studies into the virulence of said isolates, when compared to clinical isolates.

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RESUMEN

Cryptococcus neoformans (*C. neoformans*) y *Cryptococcus gattii* (*C. gattii*), levaduras patógenas encapsuladas, pertenecientes al filo Basidiomycota. Infectan a individuos inmunocomprometidos e inmunocompetentes respectivamente. Estas levaduras son generalmente de vida libre, siendo sus hábitats preferidos los excrementos secos de palomas para *C. neoformans* y el detrito de árbol de *C. gattii*. En este trabajo se presenta la caracterización de la respuesta de estrés y virulencia de cuatro aislamientos ambientales de Puerto Rico del complejo de especies *Cryptococcus neoformans/gattii*. Dos experimentos se llevaron a cabo para determinar, directa o indirectamente, la virulencia de estos aislamientos

El primer experimento se realizó para caracterizar las interacciones entre *C. neoformans/gattii* y amebas del género *Acanthamoeba*. Se observó un incremento en el tamaño de la cápsula después de la co-incubación de los organismos, con *C. gattii* mostrando el mayor aumento de tamaño de la cápsula. De igual forma se observó que la interaccion de C. Neoformans/gattii con A. polyphaga mostró el aumento mayor de capsula. El segundo método utilizado para evaluar la virulencia de los aislamientos fue la infección de larvas de *Galleria mellonella* con cada uno de los cuatro aislamientos y tipo *C. neoformans var. grubii* H99, aislado de un paciente con meningoencefalitis. Los resultados muestran que a 37°C, los aislamientos ambientales son tan virulentos como H99, evaluados *in vivo,* en un huésped no mamífero. A nuestro mejor conocimiento, este es el primer estudio que evalua la capacidad virulenta de aislamientos ambientales de Puerto Rico en comparación con los aislados clínicos usando hospederos no mamiferos.

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Dedication

Many people are part of the success of my investigation and that is why I want to dedicate my thesis work to them.

To my brother Chechi and my parents Rodney and Margarita for teir support through the years.

To Yaliz Loperena for teaching me all I know about microbiology and training me in laboratory work.

To Dr. Alejandro Ruiz Acevedo for mentoring me and permitting me to work in the world of clinical mycology.

And to Laura, for believing in me, teaching me how to experience the world with an open mind, for teaching me how to believe in myself, and for calming my frustrations with her love and understanding.

"Knowing a great deal is not the same as being smart; intelligence is not information alone but also judgment, the manner in which information is collected and used"
-Carl Sagan.

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1. Introduction and Literature Review

1.1 Introduction:

Cryptococcus neoformans and *C. gattii* are encapsulated, pathogenic yeasts, belonging to the Basidiomycota. They infect immune-compromised and immune-competent individuals, respectively. These yeasts are usually free living, with their preferred habitats being pigeon droppings for *C. neoformans* and tree detritus for *C. gattii.* The polysaccharide capsule, composed in over 90% of Galactoxylomannan (GXM), classifies the yeasts into different serotypes and is their main virulence factor.

Cryptococcus neoformans was originally isolated from fruit juices by Sanfelice (1884). Busse and Buschke (1894), both German physicians, observed the first documented case of cryptococcosis, the disease caused by these yeasts. The fungus was observed in a sarcoma-like lesion in a 31-year-old woman's tibial lesion. While it is now known that this yeast was *Cryptococcus neoformans*, it was not until 1896 that *Cryptococcus gattii* was isolated from a tumor-like infection of the hip. This is known because that isolate was similar to that of a meningitis case in Africa, studied by Gattii in 1970. By the end of the 19th century: a) the yeast was isolated from lesions in humans and other animals, establishing its capacity for disease; b) they were free living, as they were isolated from the environment, and c) they were found to cause disease in laboratory animals and could be propagated in a laboratory setting.

An increase in cryptococcosis during the 1980s was related to the appearance of the Human Immunodeficiency Virus (HIV), the cause of the Acquired Immunodeficiency Syndrome (AIDS). *Cryptococcus neoformans* has been reported as the 4th most important opportunistic fungus that affects AIDS patients in the United States, with 2-7 cases for every 1,000 persons (www.cdc.gov). In Puerto Rico, during

1980-2011 a total o f 236 cases of cryptococcosis were reported among 29,964 AIDS patients (approximately 0.7%) (OCASET, 2011).

Studies on the polysaccharide capsule have resulted in the classification of *C. neoformans* and *C. gattii* into five different serotypes, namely: A, B, C, D, and AD. A teleomorph phase was established under laboratory conditions and received the name *Filobasidiella neoformans*. During the 1970s, Dr. Kwon Chung separated the yeasts into different varieties: *neoformans and gattii*, serotypes A and D, and B and C, respectively. Today, the yeasts are divided into two species: *Cryptococcus neoformans* and *Cryptococcus gattii*.

In recent years, researchers such as Casadevall (2008) have postulated that the capacity for intracellular pathogens to survive and exploit human hosts stems from the capacity of these organisms to survive ingestion by protists such as amoebae and ingestion by metazoan hosts such as lepidopteran larva and other invertebrates.

1.2 Literature Review:

Cryptococcus neoformans and *C. gattii* are encapsulated yeasts, oval to spherical in shape, belonging to the phylum Basidiomycota in the Kingdom Fungi. In differential media such as Niger Seed Agar (NSA), the colonies appear mucoid with brown pigment. According to its teleomorph stage, the yeast belongs to the genus *Filobasidiella,* as has been known for the last thirty years, in a laboratory setting (Heitman *et al*, 2011).

The infectious route of the yeast begins with the inhalation of free-living blastospores found in the environment with a very small capsule or without one. When the yeast enters the body via the nasal cavity, it increases its capsule size or develops one to stop the attack by the host immune system. The yeast then invades

the lungs causing a subclinical chronic pneumonia known as pulmonary cryptococcosis. Later the yeasts can spread to the central nervous system (CNS) causing a life-threatening meningoencephalitis.

1.2.1 *Virulence Factors*

1.2.1.1 Capsule

In 1894, Sanfelice (1895) isolated C. neoformans from a bovine ganglion and reported the first observation of the yeast's capsule. It was 1935 when Benham first suggested that the capsule was important in virulence. Later, the capsule was found to be antiphagocytic, required for replication in macrophages, alteration of antigenic composition, affects cytokine production, inhibits leukocyte migration into infected sites and induction of complement depletion in the host (Heitman *et al.* 2011).

The capsule components were reported to be glucuronic acid, mannose, xylose, and galactose by Evans and Mehl (1950) for serotypes A, B, and C, while Bhattacharjee *et al*. (1979) reported the same for serotype D. Glucoronic acid is the main component of glucuronoxylomannan (GXM), a polymer consisting of a linear α-1,2-linked mannose backbone with ß-xylose, ß-1,2-glucuronic acid and 6-O-acetyl moieties (Heitman *et al.* 2011). Galactoxylomannan is the next most abundant polysaccharide, composed of an α-1,6-linked polymer of galactose, substituted at alternate residues with ß-1,2-linked galactose. The latter galactose is extended by an α-1,4-linked dimer of mannose, completing the trimeric side chain. Mannoproteins are the most abundant capsule components, which act as immunological modulators (Murphy, 1998).

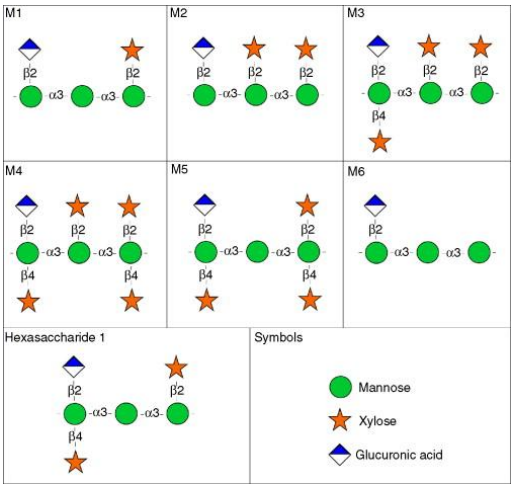

Fig. 1.1 Basic components of GXM and GalXM. (M1-M6) Structure of the six basic motifs described in GXM. Hexasaccharide 1 is GalXM structure.

Capsule formation is regulated by various factors, such as constituents of the growth medium such as vitamins, amino acids, and carbon sources (Littman, 1958). Granger (1985), showed that CO_2 induces capsule growth, while Zaragoza (2003) showed the same but when *C. neoformans* is incubated with mammalian serum. Capsule enlargement is associated with an apparent size increase in individual polysaccharide molecules shed from those cells, which is manifested by its reduced electromobility (Yoneda, 2008). Changes in capsule size are mediated, at least in part, at the level of individual GXM molecules (Yoneda, 2008). Other stimuli that induce a larger capsule are low iron levels and high levels of Ca^{2+}.

Bulmer *et al*., (1967) initiated simple genetic studies with *Cryptococcus neoformans*, utilizing UV mutagenesis to produce seven mutants that were acapsular and avirulent. When the mutations were reversed, capsule formation was restored as well as virulence. Jacobson, *et al*. (1982) isolated capsule mutant strains and characterized genetic complementation groups (Jacobson et. al, 1982). Yet it was in the early 1990s that the first capsule gene was cloned. Chang and Kwon-Chung (1994) cloned the first gene involved in capsule biosynthesis. This gene, *CAP59*, encodes a protein essential for GXM biosynthesis, and thus for the virulence of *C.*

neoformans. This demonstrated that the capsule was a virulence factor as defined by molecular Koch's postulates. Various *CAP* genes are now known and their function has been elucidated for most, as seen in Table 1.1

Table 1.1 Cap proteins. (Chang and Kwon-Chung, see text for reference).

Protein	Mutant	Protein Function	Comments
Cap10p	A and D	Unknown	Necessary for GXM biosynthesis
Cx1p	A and D	β-1,2-Xylotransferase	Important for GXM, GalXM, and GIPC xylosylation
Cx2p	A	β-1,2-Xylotransferase	Not known
Cap1p	A	Unknown	Not known
Cap2p	A	Unknown	Not known
Cap4p	A	Unknown	Not known
Cap59p	A and D	Unknown	Necessary for GXM biosynthesis
Cap60p	A and D	Unknown	Necessary for GXM biosynthesis
Cmt1p	A and D	α-1,2-Mannosyltransferase	Copurifies with Cas31p
Cap6p	A	Unknown	
Cap64p	A and D	Unknown	Necessary for GXM biosynthesis
Cas3p	A and D	Unknown	Regulates GXM O-acetylation and xylosilation pattern

Table 1.1 Cont.

Cas31p	A and D	Unknown	Regulates GXM O-acetylation and xylosilation pattern; copurifies with Cmt1p
Cas32p	A and D	Unknown	Regulates GXM xylosilation pattern
Cas33p	A and D	Unknown	Regulates GXM xylosilation pattern
Cas34p	A and D	Unknwon	Regulates GXM xylosilation pattern
Cas35p	A and D	Unknown	Regulates GXM xylosilation pattern and capsule size

1.2.1.2 Cell Wall

The cryptococcal cell wall contains α-1,3- glucan, β-1,3-, and β-1,6-linked glucans, and chitin (James *et al*. 1990 and Reese *et al*. 2007), as research with acapsular mutants have attested. Also, *C. neoformans* contains chitosan in its cell wall, the deacetylated derivative of chitin, in its vegetative cell wall (Banks et al. 2005).

α-1,3- glucan is localized primarily to the outer cell wall, as evidenced by immunoelectron microscopy (Reese *et al*. 2007). While termed α-1,3- glucan for its predominant linkage joining glucoses, interspaced in the polymer, about 3% of the linkages are α-1,4. A proposed mechanism for its stepwise synthesis utilizes a short primer of α-1,4-linked glucose residues. When a stretch of α-1,3- glucan has been

added, two polymers are linked with the α-1,4 primer of one polymer embedded in the middle. This synthesis is mediated by the protein Ags1p homolog of *Cryptococcus,* which was originally found in *Aspergillus fumigatus* and *Schizzosaccharomyces pombe.* When cryptococcal cells are disrupted in AGS1, the gene encoding this protein, no capsule polysaccharide is displayed on the cell wall surface. Cells lacking AGS1 and α-1,3- glucan do not contain the determinants necessary to assemble a capsule on the cell surface. The localization of α-1,3-glucan to the outer cell wall supports the possibility that this polymer participates directly in the binding of capsular polysaccharides, thus being essential for capsule formation.

β-1,6-glucan is the most abundant β-glucan in the cryptococcal cell wall. It is important because it is covalently linked to all the other cell wall polysaccharides and to the majority of the cell wall proteins. Phospholipase B 1, an enzyme responsible for the lysing of lung surfactanct in mammalian infection, is covalently attached to this glucan (Siafakas *et al*. 2007).

The cell wall contains chitin, a homopolymer of β-1,4-linked N-acetylglucosamine (GlcNAc) and is produced up to six times more in *Cryptococcus neoformans* vegetative growth, than in *Saccharomyces cerevisae* (Banks, et al., 2005). It also contains chitosan, the deacetylated form of chitin. Strains that do not contain chitosan are temperature sensitive, have an increased sensitivity to Congo Red and caffeine (cell wall stressors), with altered morphology and difficulty budding (Baker *et al*. 2007).

1.2.1.3 Phenoloxidase activity

Phenoloxidase is a laccase, an enzyme that allows for lignin degradation. Its activity allows for the macroscopic differentiation of *C. neoformans* and *C. gattii* from

other *Cryptococcus spp.,* since it produces brown colonies. This effect is seen in media containing phenolic compounds, caffeic acid or dihydroxyphenylalanine (DOPA). Phenoloxidase is the enzyme responsible for melanin production. This enzyme contains copper, is glycosylated and is composed of 634 amino acids (Williamson, 1994). This laccase is coded by the LAC1 gene, although a second enzyme is produced by LAC2. Mutation of LAC1 gene prevents melanin production, but the mutation of LAC2 does not (Walton et al. 2005). There are two locations for laccase activity. Lac1 is found in the cell wall and Lac2 is localized in the cytoplasm (Heitman *et al.* 2011).

Laccase activity can be measured through the oxidation of L-epinephrine to melanin (Rhodes and Kwon-Chung, 1985), while LAC1 is regulated by the concentration of glucose, temperature, nitrogen source, iron ion and copper concentrations. When the laccase interacts with L-dopa oxygen is consumed (Shaw and Kapica, 1972).

Melanin formation is affected by a variety of factors, such as glucose starvation and low temperatures (25°C vs. 37°C), increase the amount of laccase produced. Amino acids like glutamine, asparagine, and glycine are strong inducers of pigment synthesis (Heitman *et al*. 2011).

Laccase may serve a dual role, producing melanin and oxidizing ferrous iron during infection. For comparison, nonmelanized cells of *C. neoformans* produce ferrous iron at a rate of 0.1 nmol per 10^6 cells/hr, while melanized cells have a rate of up to 2.1 (Jung and Kronstad, 2008). Ferrous iron may also enhance *C. neoformans* pathogenicity by binding to melanin and increasing its extracellular redox buffering capacity, thus providing it better protection against oxidative killing (Jacobson and Hong, 1997).

C. neoformans produces melanin during mammalian infection, in both murine models and in human disease. The various phenolic compounds that can serve as a substrate for laccase probably facilitate melanization, while the high concentration of norepinephrine, 3,4-dihydroxyphenylacetic acid, homovanillic acid, 5-hydroxyindoleacetic acid, serotonin, and dopamine in brain tissue helps explain the neurotropism of these yeasts (Nosanchuk *et al*. 2000). The areas of the brain most frequently invaded by the yeast are rich in catecholamines (e.g basal ganglia). The laccase gene transcript (*LAC1)* has been detected in cells of the subarachnoid space during experimental cryptococcal meningitis infections in rabbits (Salas *et al*. 1996).

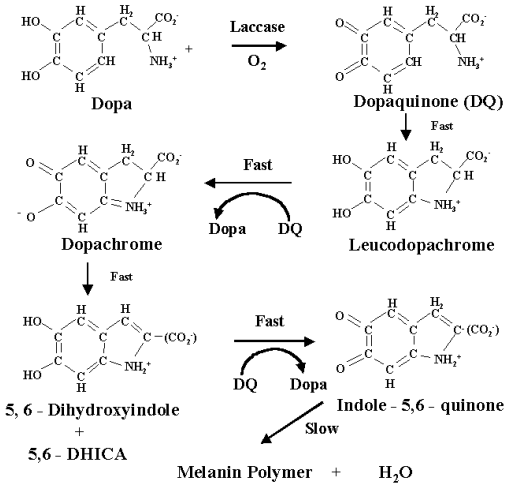

Figure 1.2 Conversion of L-Dopa to melanin polymer by phenoloxidase, a laccase. *Cryptococcus neoformans/gattii* undertake melanin synthesis in the brain with this mechanism.

Melanin is thought to protect *C. neoformans* from enzymatic degradation, radiation, and heavy metals, while providing thermotolerance and structural integrity to withstand osmotic challenges. Melanization is also associated with the ingestion of the yeast by predatory microorganisms such as *Caenorhabditis elegans* and *Acanthamoeba castellanii (*Mylonakis *et al*. 2002; Steenbergen *et al*. 2001), which produce hydrolytic enzymes to aid them in microbial digestion. It is possible that melanin protects cell wall components by directly inactivation hydrolytic enzymes and/or binding with the substrates and shielding the yeast from enzymes (Heitman *et*

al. 2011). This would allow for the survival of yeast cells within these predators and inside macrophages.

Melanin protects from the host immune response and functions as an immunomodulator (Casadevall *et al*. 2000). Melanin inhibits phagocytosis, which in part may be a result of the negative charge imparted to the cryptococcal cell wall by melanin (Nosanchuk and Casadevall, 1997). Melanization protects *C. neoformans* from macrophage killing and permits survival in alveolar macrophages by providing resistance to oxidative stress and facilitating extrapulmonary dissemination to the brain. These characteristics can be seen as an adaptation for survival of microbial depredation (Casadevall, 2008).

1.2.1.4 Phospholipase B1

Phospholipase 1 is an acidic, highly glycosylated protein w/ PI values of 5.5 and 3.5. It degrades all phospholipids except phosphatidic acid. Its preferred substrates are dipalmitoyl phosphatidylcholine (DPPC) and dioeloyl phosphocholine (intermediate in phosphatidylcholine synthesis in tissue). These substrates were followed by phosphatidylethanolamine (also known as cephalin: in human physiology it is found particularly in nervous tissue), phosphatidylserine (found in the cytosolic side of cell membrane), and phosphatidylinositol (found in the cytosolic side of cell membrane). DPPC is a major constituent of the pulmonary surfactant (Surfactant keeps the airways dry, amongst other things), a lipoprotein complex formed by type II alveolar cells (these compose 60% of the alveolar lining cells.)

Plb enzymes are of particular interest in disease pathogenesis, as they are the only proven cryptococcal “invasins” (proteins that facilitate entry into mammalian cells). They are essential for virulence of *C. neoformans* and *Candida albicans,* and are produced by several pathogenic fungi. It was shown that PLB1 is essential for the

initiation of interstitial pulmonary infection and dissemination from the lung, by way of the blood and lymphatics, to the brain. It does not facilitate penetration of blood-brain barrier. There is evidence that Plb1 is produced in human infection, as specific antibodies are present in sera of patients with cryptococcosis.

DPPC and phosphatidlglycerol, which are abundant in lung surfactant (fluid lining in alveoli) and phospholipids suach as dioleoyl PC, which is present in the outer leaflet of mammalian cell membranes, are preferred substrates of Plb1.

1.2.1.5 Mannoproteins

Mannoproteins are proteins bearing branched N-linked glucan structures containing as many as 200 mannose residues and short O-linked chains of up to five mannoses. In *C. neoformans* they are mainly localized in the inner cell wall (Vartivarian *et al*., 1989). These proteins have been implicated in infection and immune response modulation of the host. They are immunogenic (Reis *et al*. 1984), stimulate the production of cytokines and proliferation of lymphocytes, induce human and murine macrophages to produce tumor-necrosis factor alpha and interleukin-12.

1.2.2 *Cryptococcus neoformans* interactions with heterologous hosts

As with any pathogen, Cryptococcus neoformans virulence and pathogenesis has been studied utilizing different animal models. Nonvertebrate hosts have been used to investigate virulence traits and therapies for several bacterial and fungal pathogens. Amongst these heterlogous hosts we have amoebae, specifically those belonging to the genus *Acanthamoeba* and the Greater Wax Moth, *Galleria mellonella* (Lepidoptera:*Galleridae*).

Amoebae are some of the most common soil protists known. Most are known predators of fungi, bacteria, and other microorganisms. They share habitats with *C.*

neoformans and *C. gattii*, and have been reported as a means of biological control of *C. neoformans* (Ruiz *et al.* 1981,1982).

The genus *Acanthamoeba* is the most widely utilized genus of amoebae utilized for interaction studies with *C. neoformans* and *C. gattii.* The first isolate of *Acanthamoeba castellanii* was found as a contaminant of a cryptococcal culture (Castellani, 1931). During the 1950s, Castellani showed that *A. castellanii* ingested and destroyed a strain of *C. neoformans* (Castellani, 1955). In the 1970s, Bulmer and colleagues demonstrated that *Acanthamoeba polyphaga* is a predator of *C. neoformans*, yet those colonies of the yeast that survived showed a morphological variant, a pseudohyphal form not ingested by *A. polyphaga* (Neilson *et al*, 1978).

The Greater Wax Moth, *Galleria mellonella,* overcomes some limitations of other heterologous hosts, such as amoebae, in that the fungal inoculum can be administered and precisely measured. This is in contrast with the inocula needed for infection of amoebae, which tends to be a 1:1 cell to effector ratio, with limited precision in the measurement.

2. Environmental Interactions between *Cryptococcus neoformans/gattii* with amoeba from the genus *Acanthamoeba*

2.1 Introduction:

Amoebae are some of the most common soil protists known. Most are known predators of fungi, bacteria, and other microorganisms. They share habitats with *C. neoformans* and *C. gattii*, and have been reported as a means of biological control of *C. neoformans* (Ruiz *et al.* 1981 and 1982). The genus *Acanthamoeba* is the most widely used genus of amoebae for interaction studies with *C. neoformans* and *C. gattii.* The first isolate of *Acanthamoeba castellanii* was found as a contaminant of a cryptococcal culture (Castellani, 1931). During the 1950s, Castellani showed that *A. castellanii* ingested and destroyed a strain of *C. neoformans* (Castellani, 1955). In the 1970s, Bulmer and colleagues demonstrated that *Acanthamoeba polyphaga* is a predator of *C. neoformans*, yet those colonies of the yeast that survived showed a morphological variant, a pseudohyphal form not ingested by *A. polyphaga* (Neilson *et al.* 1978).

We began a series of experiments with three species of *Acanthamoeba*, as to study the interactions between the amoebae and *C. neoformans* and *C. gattii*. The three species were *A. castellanii, A. polyphaga,* and *A. palestinensis. Acanthamoeba castellanii* was chosen, as it is the species whose interactions with *C. neoformans* and *C. gattii* have been most observed and studied. *A. polyphaga* was chosen as to try and replicate the findings of Bulmer and his collegues, and see if pseudohyphal variants depend on the species of amoeba used. *A. palestinensis* was chosen as pseudohyphal variants were observed by Ruiz (1980) during his Doctoral Research. The cryptococcoal strains utilized, AP3 and VBGc22 are environmental isolates from Puerto Rico, were chosen as to be able to observe the reaction of isolates that had not come from human infection and had already shown virulence in human hosts.

2.2 Materials and Methods:

2.2.1 *Cryptococcus neoformans* and *Cryptococcus gattii* culture acquisition

The samples of *C. neoformans* (named AP3) and *C. gattii* (named VBGc22), are both strains isolated from Puerto Rico and are genotypically characterized as M13 genotypes VN and VGII, respectively (Loperena *et al.* 2010). Both strains were taken out of -80°C storage and grown on Staib medium, until pigmented colonies were visible.

2.2.2 Acquisition of *Acanthamoeba spp*.

All three species of *Acanthamoeba* were acquired from the American Type Culture Collection (ATCC) with the numbers 30011 for *Acanthamoeba castellanii* (Ac), 30870 for *Acanthamoeba palestinensis* (Ap), and 30486 for *Acanthamoeba polyphaga* (Apo). They were grown as per ATCC instructions on ATCC Medium 711 for Apo and Ac, and ATCC Medium 712 for Ap. After trophozoites were observed, they were let to encyst by leaving the culture for fourteen extra days. When cysts were observed, they were collected by flushing the surface with 5 mL ATCC medium 1323, as per ATCC instructions. They were then centrifuged at 13,000 rpm for 5 minutes and supernatant was discarded. The resulting pellet was washed with HCl (1% w/v) as described by Singh.

2.2.3 Culture medium preparation.

2.2.3.1 *Staib Medium with Biphenyl (SM+B)*

Staib medium, also known as Niger Seed Agar, is a differential medium containing caffeic acid, a melanin precursor, which allows the macroscopic differentiation of members of the *Cryptococcus neoformans/gattii* from other *Cryptococcus* species. The medium was prepared following Castañeda's description, a modified version of the original version prepared by Staib (Castañeda, 2002).

Seventy (70)g of *Guizotia abbysinica* seeds were pulverized and added to 1L of distilled water, which was then autoclaved for 15 minutes at 121°C and 15 psi. The resulting mixture was then filtered through sterile cheesecloth and the extract was collected. This extract was then mixed with 1g glucose, 1g creatinine, 1g potassium phosphate, and 20g agar. This mixture was boiled and stirred until homogenous. Afterwards it was autoclaved at 121°C and 15 psi, for 15 minutes. After the mixture was autoclaved and cooled to 50°C, 20mL of an antibiotic solution (penicillin at 20U/mL, streptomycin at 40U/mL, and chloramphenicol at 20U/mL) was added, along with 20 mL of biphenyl (5g biphenyl in 20mL absolute alcohol). Afterwards the solution was poured into sterile Petri Plates.

2.2.4 Interactions of *Cryptococcus neoformans* and *Cryptococcus gattii* with members of the genus *Acanthamoeba.*

Acanthamoeba castellanii (Ac), *Acanthamoeba palestinensis* (Ap), and *Acanthamoeba polyphaga* (Apo) were grown according to ATCC established protocol, and were added to liquid 5 mL of Trypan Blue medium (Tbl) at 1.0 x 10^4 cells per milliliter. Cn cells and Cg cells were grown in liquid Staib medium, and 1.0 x 10^4 cells per milliliter were added to Tbl, giving an effector to target ratio of 1:1. They were then incubated at 30°C for 14 days at 150 r.p.m,, with 300 μL taken daily and spread unto Staib medium plates. These were incubated at 30°C and colonies were observed for changes in melanin production and samples were observed after staining with India Ink under a light microscope. Capsules were measured at the Microscopy Center of the Biology Building at the University of Puerto Rico-Mayagüez Campus.

The resulting capsule sizes were put into InfoStat Statistical Software and underwent a T-test analysis.

2.3 Results

2.3.1 Capsular enlargement of cryptococcal capsule after interaction with *Acanthamoeba spp.*

Capsular enlargement was observed after co-incubation of *Cryptococcus neoformans* (Cn) with all three species of *Acanthamoeba:* Ap, Apo, and Ac. The averages for capsule growth for each *Cryptococcus spp.* were 14.32 μm for Cn and Apo, 16.14 μm for Cn and Ap, and 8.22 μm for Cn and Ac.; 8.24 μm for Cg and Ac, 18.36 for Cg and Ap, and 15.25 μm for *Cryptococcus gattii* (Cg) and Apo. While these were the calculated averages, some cells were observed up to a diameter of 33 μm (Figures 2.1 and 2.2).

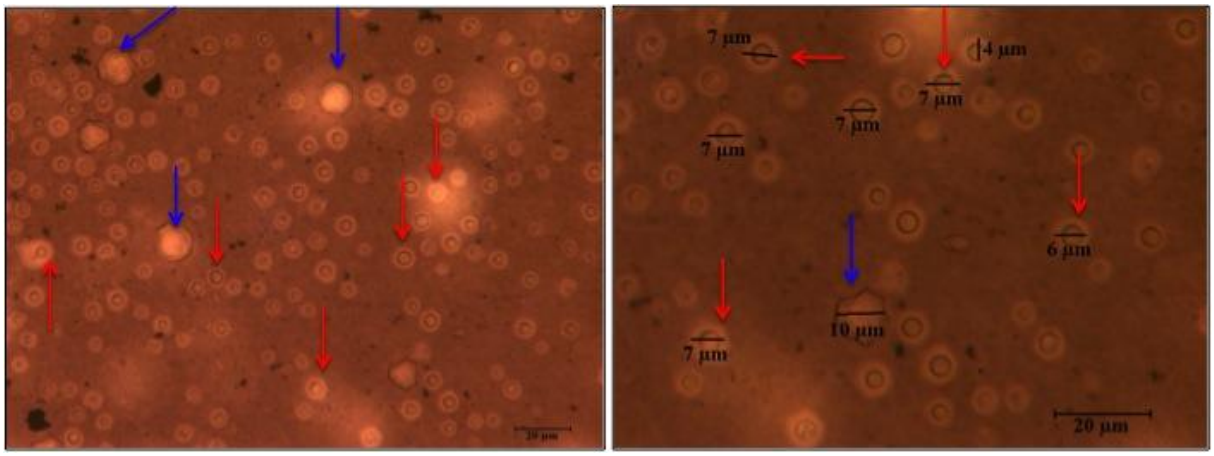

Fig 2.1 Incubation of *C. neoformans* with *A. polyphaga*. a) Day 0 showing cysts (blue arrows) and yeast cells (red arrows). b) Day 14 showing larger capsules (red arrows) and cysts (blue arrows).

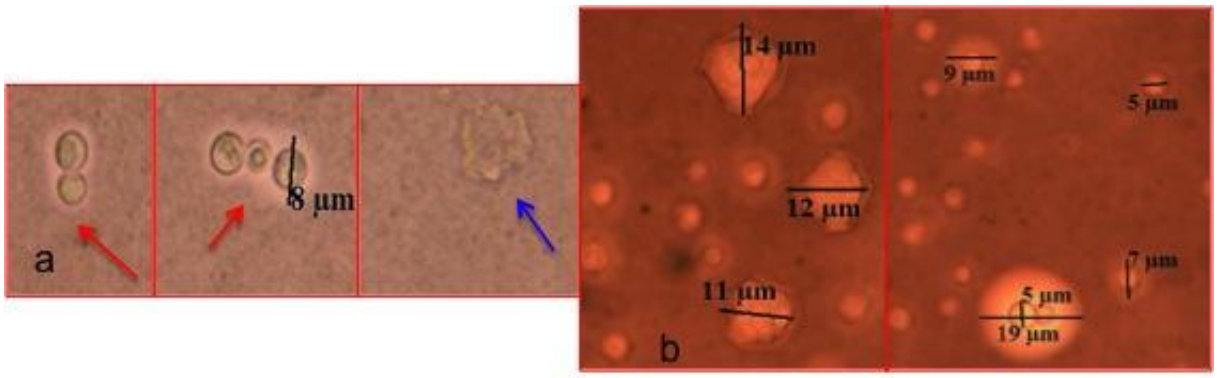

Fig. 2.2 Incubation of *C. gattii* with *A. polyphaga*. a) Day 0 of incubation. Yeast cells (red arrows) and cysts (blue arrows) are visible. b) Day 14 of incubation. Cysts are visible, some yeast cells demonstrate large capsules after incubation.

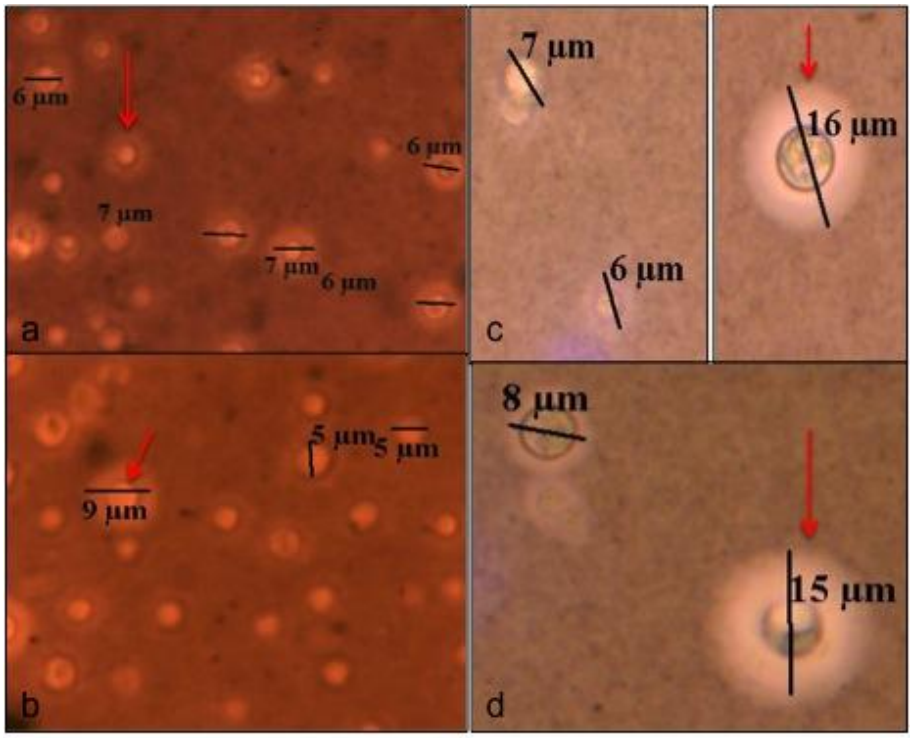

Figure 2.3 Incubation of *C. neoformans* and *C. gattii* with *A. palestinensis* a) Day 0 of incubation of *C. neoformans* and b) Day 14 of incubation with *A. castellanii*. Similar capsule sizes were observed at the start and ending of the experiment. c) Day 0 of incubation of C. gattii and b) day 14 of incubation with *A. castellanii*

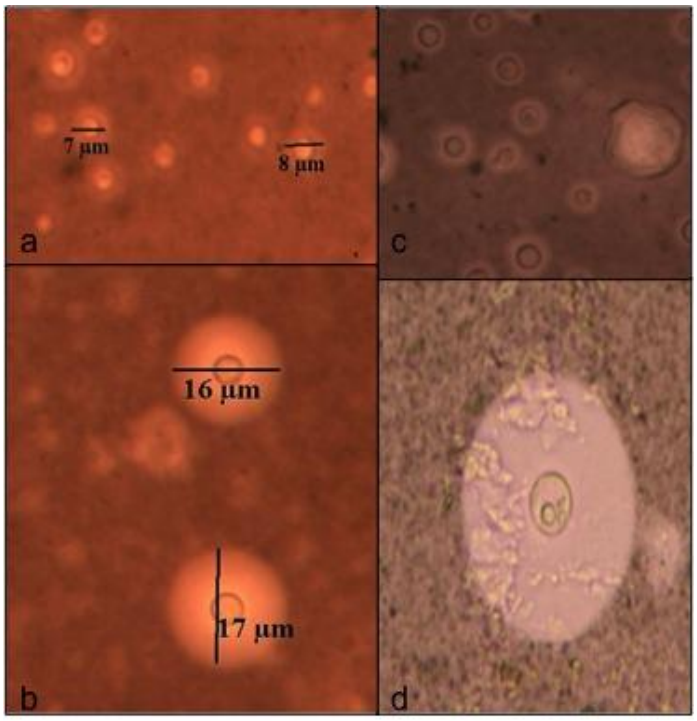

Fig. 2.4 Incubation of *C. neoformans/gattii* with *A. palestinensis* a) Day 0 and b) day 14 of incubation of *C. neoformans* with *A. palestinensis* demonstrating increased capsular phenotype. c) Day 0 and d) day 14 of *C. gattii* and *A. palestinensis.* d) shows that the largest capsule resulted from incubation with *A. palestinensis.*

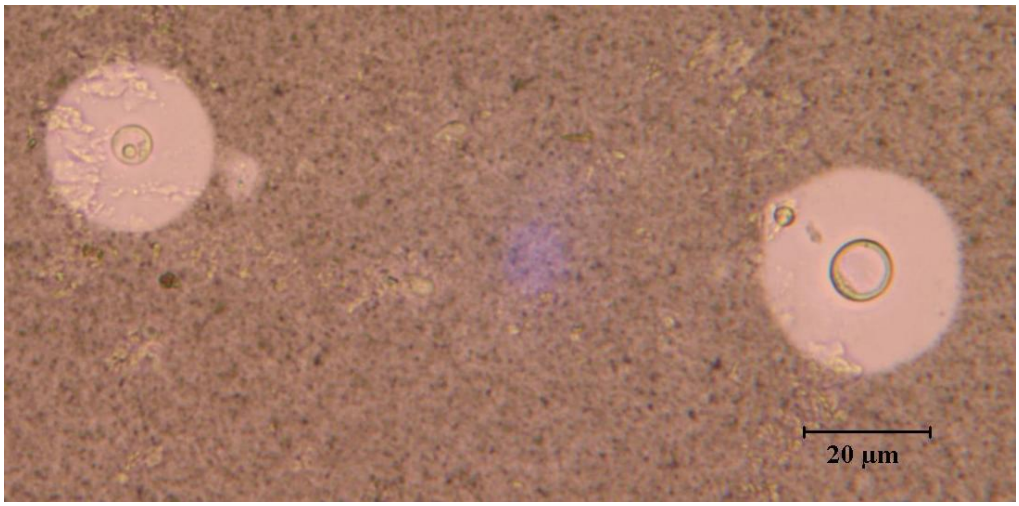

Figure 2.5 *Cryptococcus gattii* incubated with *Acanthamoeba palestinensis* demonstrating extreme capsular phenotype.

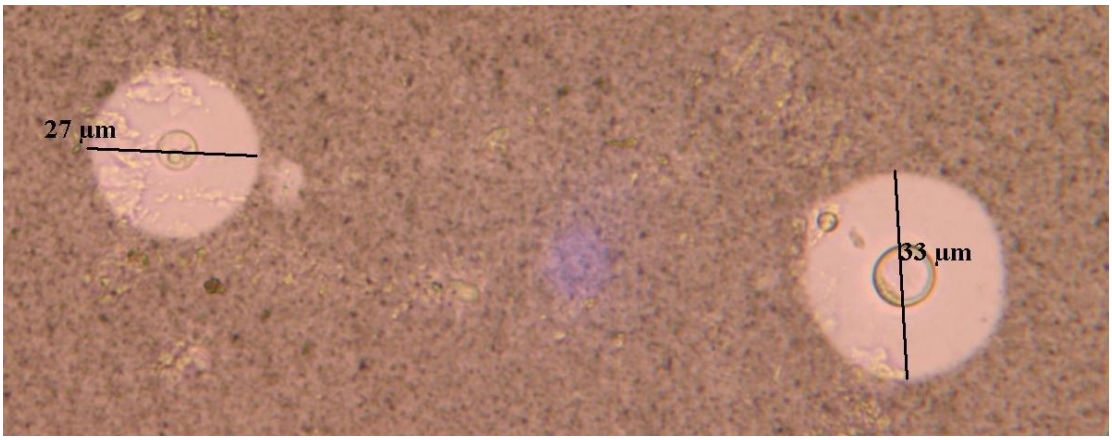

Figure 2.6 *Cryptococcus gattii* incubated with *Acanthamoeba palestinensis* demonstrating the biggest capsules observed in this study.

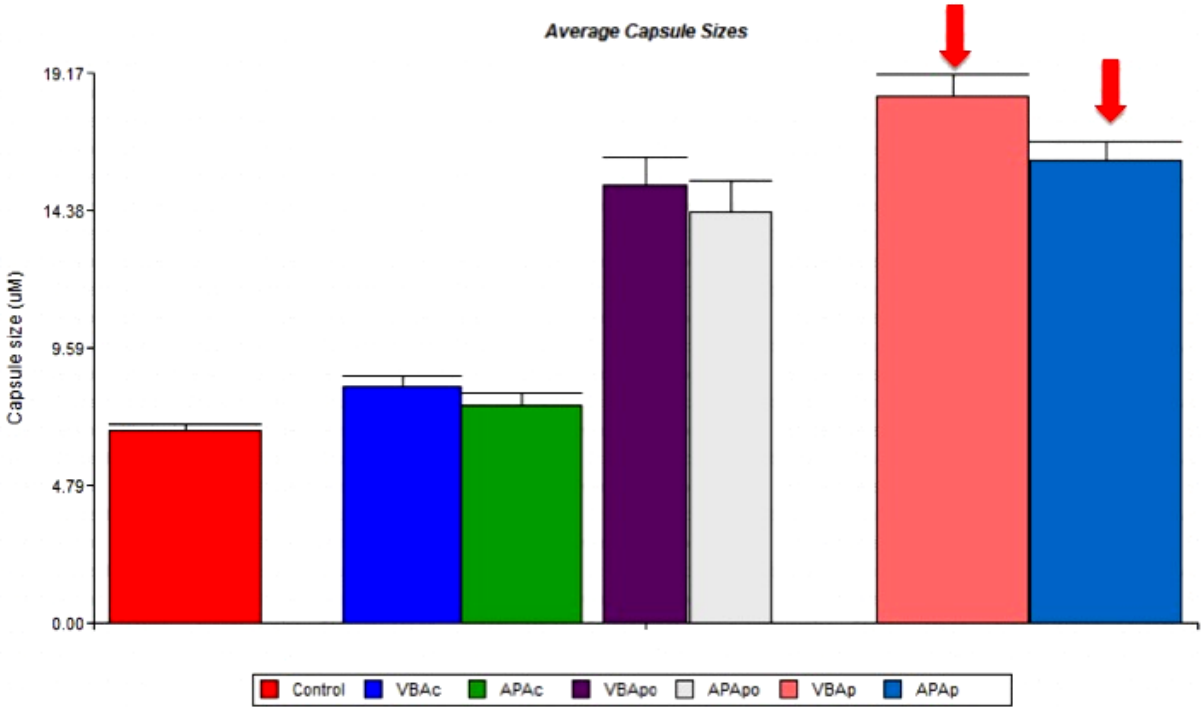

Fig. 2.7 Average capsule sizes of *C. neoformans/gattii.* Red bar is control (H99), blue and green are VBGc22 and Ap3, incubated with *A. castellanii* respectively. Purple and grey bar are VBGc22 and Ap3 incubated with *A. polyphaga.* Pink and light blue bars are VBGc22 and Ap3 incubated with *A. palestinensis.*

2.4 Discussion

While previous studies had shown that cryptococcal isolates from human infection show an increase in capsule size when grown with amoebae (Chrisman, et al., 2011; Steenbergen, et al. 2001; Malliaris, et, al., 2004), but no previous studies

have shown the interactions of purely environmental isolates of both pathogenic cryptococcal species with amoebae from the genus *Acanthamoeba.*

Cn and Cg do not need a mammalian host for replication or survival, indicating that there must be an evolutionary origin that predates mammals and other animal hosts. The cryptococcal isolates worked with on this study, show that known virulence factors in mammalian infection are shown with amoebae and environmental isolates that have never been in metazoan hosts.

Studies of the interactions of Cn and Cg with Ac have been previously done and results presented (Malliaris, et, al., 2004; Chrisman, et, al., 2011), yet limited in interactions between the fungi and other *Acanthamoeba* species.

The cryptococcal capsule is one of the determining virulence factors for Cn and Cg infections. Capsular enlargement is an early response during infection (Feldmesser, et al., 2001). While the role of capsule growth is not known in this stage, it has been shown that capsule enlargement interferes with complement-mediated phagocytosis (Zaragoza, et al., 2003), and this same inhibitory effect can be seen in heterologous host model infections, like those presented in this study (Zaragoza, et al., 2008).

3. *Cryptococcus neoformans* and *Cryptococcus gattii* interactions with the Greater Wax Moth (*Galleria mellonella*)

3.1 Introduction

Cryptococcus neoformans and *Cryptococcus gattii* (*C. neoformans* and *C. gattii*, respectively) are the causative agents of cryptococcosis, a fungal infection of the lungs and central nervous system in humans and other animals (Heitman *et al.* 2011). Various models have been utilized for the study of pathogenicity of these yeasts, such as murine models (Carroll *et al*., 2007 and Capilla *et al*. 2006). Because of the costs and ethical considerations when it comes to experimenting with vertebrates, different models have been proposed and tested for studies about *C. neoformans/C. gattii* pathogenesis and the origin of virulence (Coleman *et al.* 2011).

Nonvertebrate animals and other heterologous hosts such as amoebae have been used to study the virulence traits and therapies for fungal and bacterial pathogens. They are now used extensively as experimental systems for the screening of virulence characteristics because they are less costly, are exempt from vertebrate animal study regulations, and are amenable to high-throughput protocols (Coleman *et al.* 2011).

Amongst these nonvertebrate animals we have *Galleria mellonella* (Lepidoptera: *Galleridae*), the Greater Wax Moth. Larvae of this species have been used to examine the pathogenicity of various yeasts such as *Candida spp.* (Cotter *et al.* 1999), *Saccharomyces cerevisae* (Cotter *et al.* 1999), and *Cryptococcus neoformans/gattii* (Mylonakis *et al.* 2005). *G. mellonella* is amenable to studies at mammalian temperatures, are susceptible to local and systemic infection, literature is less extensive, and wild-type individuals are susceptible to fungal infections (Mylonakis, 2008).

The literature indicates that most studies with *C. neoformans* and *C. gattii* in *Galleria mellonella* have been done with clinical isolates or their mutants (Mylonakis *et al.* 2005; García-Rodas *et al.* 2011), without considering the implications of demonstrating the effects of wild-type environmental samples on a model host. As such we present our research, to the best of our knowledge, the first study of environmental isolates of *C. neoformans* and *C. gattii* from Puerto Rico with *G. mellonella.*

3.2 Methodology

3.2.1 *Strains and Media*

The *C. neoformans* and *C. gattii* strains are summarized in table 3.1 and described in the text. The fungal cultures were maintained in yeast peptone dextrose broth (YPD), Staib medium agar, and as frozen stocks. All strains were grown on YPD with aeration at 30°C.

3.2.2 *Galleria mellonella* killing assay

Galleria mellonella caterpillars in the final instar larval stage (Vanderhorst, Inc., St. Marys, Ohio) were stored in the dark and used within 7 days of shipment (Mylonakis *et al*. 2005). Caterpillars (330 $\pm$ 25 mg in body weight) were used in all six assays. Fifteen random caterpillars of the desired weight were used per assay. Larvae were a uniform color and did not have any outstanding grey markings on their body, as seen in picture 3.1.

Five μL (aliquots of a 2 x 10^7 cells/mL (1.0 x 10^5 cells/mL) dilution were injected into the larval hemocoel via the last left proleg (Choi et al., 2002), with a Hamilton 10 μL syringe.

The dilutions used were prepared from overnight cultures. One mL was taken and washed twice in sterile PBS, and a 1 in 10 dilution was prepared. 10 μL were

taken and put in a hemacytometer to count yeast cells and determine the amount of cells/mL. The amount was adjusted to 2 x 10^7 cells/mL. Five μL of the adjusted stocks were then injected into the caterpillars as described above.

Caterpillars were then incubated at 37°C for ten days and observed daily for caterpillar death. Caterpillars were considered dead when they were unresponsive to touch.

The amount of larvae that died per day was tabulated to create a table as follows: in State, 1 signifies death, 0 signifies that the larvae were alive until that moment, and 2 signifies censored data, or data eliminated because the larvae pupated before the end of study.

Figure 3.1 Dorsal view of *G. mellonella* larvae. Similar in size and coloring to those used in study.

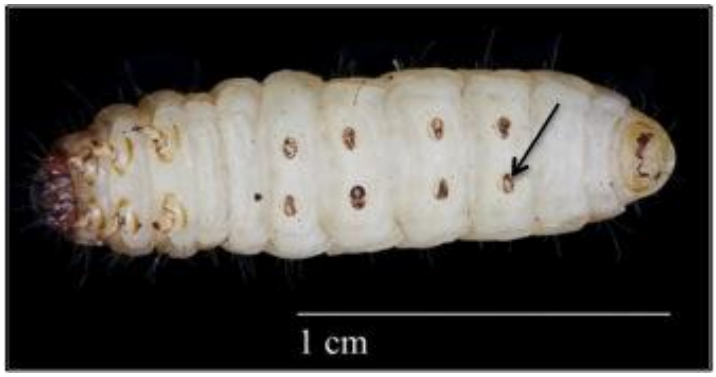

Figure 3.2. Ventral view of *G. mellonella larvae.* Black arrow demonstrates injection site, the last left proleg.

The matrix was then put into InfoStat 7.6, and a Kaplan-Meier survival analysis was undertaken, with an α value of 0.05 needed to considered the global analysis as statistically significant. Afterwards, a Multiple Comparisons Analysis was undertaken, utilizing the Kaplan-Meier Survival Analysis to compare pairs of treatments. Since multiple comparison analysis can result in errors in inference, which can include the rejection of the null hypothesis when it should be accepted. The Bonferroni Correction, as to correct for these errors. Taking the standard α, we have the formula for the correction as α= 0.05/n, where n is the total of treatments in the study. Having n as ten treatments, including the controls, we have. Having this corrected alpha, we correct for the errors previously mentioned, and gives us a stronger test.

Table 3.1 *Cryptococcus neoformans/gattii* strains used in this study. P-value calculated using Kaplan-Meier Survival Analysis:Log-Rank Test. Survival of *Galleria mellonella* injected with cells compared to those injected with DPBS.

Cryptococcus neoformans/gattii strain	Median time to mortality for *G. mellonella* killing at 37°C`	P value
H99	8 days after injection of 1.0 x 10^5 cells/mL	<0.001
AP1	7 days after injection of 1.0 x 10^5 cells/mL	<0.001
AP5	8 days after injection of 1.0 x 10^5 cells/mL	<0.001
VBGc22	8 days after injection of 1.0 x 10^5 cells/mL	<0.001
VBGc28	7 days after injection of 1.0 x 10^5 cells/mL	<0.001
AP3	8 days after injection of 1.0 x 10^5 cells/mL	<0.001
Yag44	7 days after injection of 1.0 x 10^5 cells/mL	<0.001

3.3 Results

3.3.1 Killing of *Galleria mellonella* by *Cryptococcus neoformans* and *Cryptococcus gattii* environmental strains from Puerto Rico, when compared to H99 clinical isolate.

As seen in Table 3.1, when compared with DPBS (negative control) all strains showed statistical significance. Once multiple comparison were done, their was no statistical differences between H99 (positive control) and the strains from Puerto Rico. When incubated at the same temperature, and inoculated with the same amount of cells/mL, all cryptococcal strains used in this study demonstrated the ability to cause death in *G. mellonella* larvae before ten days. The difference in median days to mortality, while demonstrating a difference of at least one day between treatments, are not statistically significant differences.

In contrast to that study, we observed a median killing time for H99 of eight days after inoculation, and all of our environmental strains show similar and statistically insignificant differences.

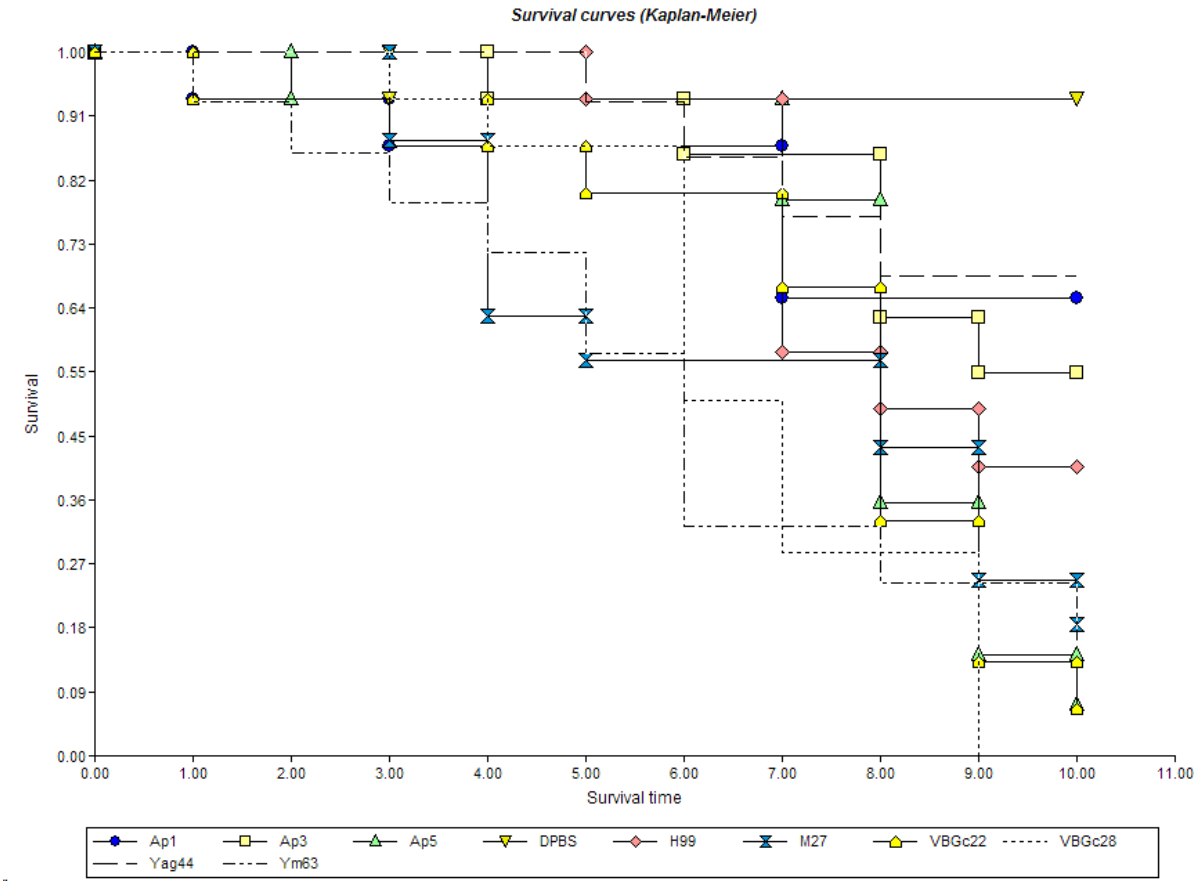

Figure 3.3 Kaplan-Meier Analysis of Survival of *G. mellonella* infected with *Cryptococcus* sp. strains.

3.4 Discussion

We report the first study on the interactions between *Galleria mellonella* and *Cryptococcus neoformans* and *Cryptococcus gattii* environmental strains from Puerto Rico. We compared four of our environmental strains, which had previously undergone molecular typing by Edmond Byrnes at Duke University (VGII for *C. gattii* isolates and VNIV for *C. neoformans* isolates), and compared them to H99. H99 has been completely sequenced and is a *Cryptococcus neoformans var. grubii* isolate, which is found in 90% of those patients suffering from cryptococcosis.

When compared to DPBS injection, our *C. gattii* isolates were suggested to be more virulent than H99, contrasting with previous studies with H99 and other clinical isolates of *C. gattii* and *C. neoformans.* We consider that our inoculum size, of 1.0 x 10^5 cells/mL, is responsible for the difference in our results to those of previous studies that utilized anywhere from 1.5 x 10^4 to 2.5 x 10^4 CFU/larva (Mylonakis, et al., 2005). We hypothesize that when increasing the inoculum size, *C. gattii* proves to be as virulent as *C. neoformans* H99 when comparing both to DPBS (Table 4.2). When comparing H99 to VBGc28 there is no statistical difference between groups, indicating that VBGc28, a *C. gattii* strain can be as pathogenic as H99 at 37°C. While VBGc28 is an environmental isolate, H99 is an isolate from the cerebrospinal fluid (CSF) of a Caucasian male from North Carolina and is the type culture for *C. neoformans var. grubii* (Franzot, et al., 1999). When compared to a previous study (Mylonakis, et al., 2005) we observed that our *C. gattii* and *C. neoformans* environmental isolates are as virulent as H99 at 37°C.

REFERENCES:

Chapter 1: Introduction and Literature Review:

1. Baker, L.G., C.A. Specht, M.J. Donlin, and J.K. Lodge. 2007. **Chitosan, the deacetylated form of chitin, is necessary for cell wall integrity of *Cryptococcus neoformans.*** *Eukaryot. Cell* **6:**855-867

2. Banks, I.R., C.A. Specht, M.J. Donlin, K.J. Gerik, S. M. Levitz and J. K. Lodge. 2005. **A chitin synthase and its regulator protein are critical for chitosan production and growth of the fungal pathogen *Cryptococcus neoformans*.** *Eukaryot. Cell* **4:**1902-1912

3. Bhattarcharjee, A.K., K. J. Kwon-Chung, and C.P. Glaudemans. 1979. **The structure of the capsular polysaccharide from *Cryptococcus neoformans* serotype D.** *Carbohydr. Res.* **73:**183-192

4. Benham, R.W. 1935. **Cryptococci: their identification by morphology and by serology.** *J. Infect. Dis.* **57:**255-274

5. Bulmer, G.S., M.D. Sans, and C.M. Gunn. 1967. ***Cryptococcus neoformans* I. Nonencapsulated mutants.** *J. Bacteriol.* **94:** 1475-1479

6. Casadevall, A. 2008. **Evolution of Intracellular Pathogens.** Annu. Rev. Microbiol. 2008. 62:19–33

7. Casadevall, A., A.L. Rosas, and J.D. Nosanchul. 2000. **Melanin and virulence in *Cryptococcus neoformans.*** *Curr. Opin. Microbiol.* **3**:354-358

8. Castellani, A. 1931. **An amoeba growing in cultures of a yeast.** *J. Trop. Med. Hyg.* **33:**188-191

9. Castellani, A. 1955. **Phagocytic and destructive action of *Hartmanella castellanii* (*Amoeba castellanii*) on pathogenic encapsulated yeast-like fungus *Torulopsis neoformans* (*Cryptococcus neoformans*).** *Ann. Inst. Pasteur* **89:**1-7

10. Chang, Y.C. and K.J. Kwon-Chung. 1994. **Complementation of a capsule-deficient mutation of *Cryptococcus neoformans* restores its virulence.** *Mol. Cell. Biol.* **14:**4912-4919

11. Doering, T.L. 2009. **How sweet it is! Capsule formation and cell wall biogenesis in *Cryptococcus neoformans*.** *Ann. Rev. Microbiol.* **63**:223-247

12. Evans, E.E., and J. W. Mehl. 1950. **A qualitative analysis of capsular polysaccharides from *Cryptococcus neoformans* by filter paper chromatography.** *Science* **114:**10-11

13. Jacobson, E.S., D.J. Ayers, A.C. Harrel, and C. C. Nicholas. 1982. **Genetic and phenotypic characterization of capsule mutants of *Cryptococcus neoformans*.** *J. Bacteriol.* **150:**1292-1296

14. Jacobson, E.S., and J.D. Hong. 1997. **Redox buffering by melanin and Fe(II) in *Cryptococcus neoformans.*** *J. Bacteriol.* **179:** 5340-5346

15. James, PG., R. Cherniak, R. G. Jones, C.A Stortz, and E. Reiss. 1990. **Cell wall glucans of *Cryptococcus neoformans* CAP67.** *Carbohydr. Res.* **198:**23-28

16. Jung, W.H., and J.W. Kronstad. 2008. **Iron and fungal pathogenesis: a case study with *Cryptococcus neoformans.*** *PLoS Biol.* **4:**e410

17. Levitz, S.M., S. Nong, M.K. Mansour, C. Huang, and C.A. Specht. 2001. **Molecular characterization of a mannoprotein with homology to chitin deacetylases that stimulates T-cell responses to *Cryptococcus neoformans*.** *Proc. Natl. Acad. Sci. USA* **98:**10422-10427

18. Litmman, M.L. 1958. **Capsule synthesis by *Cryptococcus neoformans.*** *Trans. N.Y. Acad. Sci.* **20:** 623-648

19. Maxson, M.E., E. Cook, A. Casadevall, and O. Zaragoza. 2007. **The volume and hydration of the *Cryptococcus neoformans* polysaccharide capsule.** *Fungal Genet. Biol.* **44:**180-186

20. Murphy, J.W. 1998. **Protective cell-mediated immunity against *Cryptococcus neoformans.*** *Res. Immunol.* **149:** 373-386

21. Mylonakis, E., F. M. Ausubel, J.R. Perfect, J. Heitman, and S. B. Calderwood. 2002. **Killing of *Caenorhabditis elegans* by *Cryptococcus neoformans* as a model of yeast pathogenesis.** *Proic. Natl. Acad. Sci. USA* **99:** 15675-15680.

22. Neilson, J.B., M.H. Ivey, and G.S. Bulmer. 1978. ***Cryptococcus neoformans:* pseudohyphal forms surviving culture with *Acanthamoeba polyphaga.*** *Infect. Immun.* **20:**262-266

23. Nosanchuk, J.D. and A. Casadevall. 1997. **Cellular charge of *Cryptococcus neoformans:* contributions from the capsular polysaccharide, melanin, and monoclonal antibody binding.** *Infect. Immun.* **65:** 1836-1841

24. Nosanchuk, J.D., A.L. Rosas, S.C. Lee, and A. Casadevall. 2000. **Melanisation of *Cryptococcus neoformans* in human brain tissue.** *Lancet* **355:**2049-2050

25. Reese, A. J., A. Yoneda, J.A. Breger, A. Beauvais, H. Liu, C.L. Griffith, I. Bose, M. Kim, C. Skau, S. Yang, J.A Sefko, M. Osumi, J. Latge, E. Mylonakis, and T.L. Doering. 2007. **Loss of cell wall alpha(1-3) glucans affects *Cryptococcus neoformans* from ultrastructure to virulence.** *Mol. Microbiol.* **63:** 1385-1398

26. Rhodes, J.C., and K.J. Kwon-Chung. 1985. **Production and regeneration of protoplasts from *Cryptococcus.*** *Sabouraudia* **23:**77-80

27. Rodrigues, M.L., E. S. Nakayasu, D.L. Oliveira, L. Nimrichter, J.D. Nosanchuk, I.C. Almeida, and A. Casadevall. 2008. **Extracellular vesicles produced by *Cryptococcus neoformans* contain protein components associated with virulence.** *Eukaryot. Cell* **7**: 58-67

28. Ruiz, A. R.A. Fromtling, and G.S. Bulmer. 1981. **Distribution of Cryptococcus *neoformans* in a natural site.** *Infect. Immun.* **31:** 560-563**.**

29. Ruiz, A., J.B. Neilson, and G.S. Bullmer. 1982. **Control of *Cryptococcus neoformans* in nature by biotic factors.** *Sabouraudia* **20:**21-29

30. Salas, S.D., J.E. Bennett, K.J. Kwon-Chung, J.R. Perfect, and P.R. Williamson. 1996. **Effect of the laccasae gene CNLAC!, on virulence of *Cryptococcus neoformans.*** *J. Exp. Med.* **184:** 377-386

31. Sanfelice, F.1985. **Ueber einen neuen pathogenen Blastomyceten, welcher innerhalb der Gewebe unter Bildung kalkartig aussehender Massen degeneriert.** *Zentralbl. Bakt. Parasit.* **18:**521-526

32. Shaw, C.E., and L. Kapica. 1972. **Production of diagnostic pigment by phenoloxidase activity of *Cryptococcus neoformans.*** *Appl. Microbiol.* **24:** 824-830

33. Siafakas, A.R., T.C. Sorrell, L.C. Wright, C. Wilson, M. Larsen, R. Boadle, P.R. Williamson, and J.T. Djordjevic. 2007. **Cell wall-linked cryptococcal phospholipase B1 is a source of secreted enzyme and a determinant of cell wall integrity.** *J. Biol. Chem.* **282:**37508-37514

34. Steenbergen, J.N., H.A. Shuman, and A. Casadevall. 2001. ***Cryptococcus neoformans* interactions with amoebae suggest an explanation for its virulence and intracellular pathogenic strategy in macrophages.** *Proc. Natl. Acad. Sci. USA* **98:** 15245-15250

35. Vartivarian, S.E., G.H. Reyes, E.S. Jacobson, P.G. James, R. Cherniak, V.R. Mumaw and M.J. Tingler. 1989. **Localization of mannoprotein in *Cryptococcus neoformans.*** *J. Bacteriol.* **171:**6850-6852

36. Walton, F.J., A. Idnurm, and J. Heitman. 2005. **Novel gene functions required for melanization of the human pathogen *Cryptococcus neoformans*.** *Mol. Microbiol.* **57:** 1381-1396

37. Williamson, P.R. 1994 **Biochemical and molecular characterization of the diphenol oxidase of *Cryptococcus neoformans*: identification as a laccase.** *J. Bacteriol*. **176:** 656-664

38. Yoneda, A., and T.L. Doering. 2008. **Regulation of *Cryptococcus neoformans* capsule size is mediated at the polymer level.** *Eukaryot. Cell* **7:**456-459

39. Zaragoza, O., B.C. Fires, and A. Casadevall. 2003. **Induction of capsule growth in *Cryptococcus neoformans* by mammalian serum and CO_2,** *Infect. Immun.* **71:** 6155-6164

Chapter 2: Environmental Interactions between *Cryptococcus neoformans/gattii* with amoeba from the genus *Acanthamoeba*.

1. Casadevall, A. 2008. **Evolution of Intracellular Pathogens.** Annu. Rev. Microbiol. **62**:19–33

2. Castellani, A. 1931. **An amoeba growing in cultures of a yeast.** *J. Trop. Med. Hyg.* **33:**188-191

3. Castellani, A. 1955. **Phagocytic and destructive action of *Hartmanella castellanii* (*Amoeba castellanii*) on pathogenic encapsulated yeast-like fungus *Torulopsis neoformans* (*Cryptococcus neoformans*).** *Ann. Inst. Pasteur* **89:**1-7

4. Chrisman, C.J., Abuquerque, P., Guimaraes, A.J., Nieves, E., and Casadevall, A. 2011. **Phospholipids Trigger *Cryptococcus neoformans* Capsular Enlargement during Interactions with Amoebae and Macrophages.** *Plos Patog.* **5:**e1002047

5. Ellis, D.H. and T.J. Pfeiffer. 1990. **Natural habitat of Cryptococcus neoformans var. gattii.** *J. Clin. Microbiol* **28:**1642-1644

6. Feldmesser, M., Y. Kress, and A. Casadevall. 2001. **Dynamic changes in the morphology of *Cryptococcus neoformans* during murine pulmonary infection.** *Microbiology* **147:** 2355-2365
7. Malliaris, S.D., Steenbergen, J., and Casadevall, A. 2004. ***Cryptococcus neoformans var. gattii* can exploit *Acanthamoeba castellanii* for growth.** *Med. Mycol.* **42:**149-158

8. Neilson, J.B., M.H. Ivey, and G.S. Bulmer. 1978. ***Cryptococcus neoformans:* pseudohyphal forms surviving culture with *Acanthamoeba polyphaga.*** *Infect. Immun.* **20:**262-266

9. Ruiz, A., J.B. Neilson, and G.S. Bulmer. 1982. **Control of *Cryptococcus neoformans* in nature by biotic factors.** *Sabouradia* **20:**21-29

10. Ruiz, A., R.A. Fromtling, and G.S. Bulmer. 1981. **Distribution of *Cryptococcus neoformans* in a natural site.** *Infect. Immun.* **31:**560-563

11. Steenbergen JN, H.A. Shuman, and A. Casadevall. 2001. **Cryptococcus neoformans interactions with amoebae suggest an explanation for its virulence and intracellular pathogenic strategy in macrophages**. Proc Natl Acad Sci USA **98**: 15245□/15250.

12. Zaragoza, O., C.J. Chirsman, M.V. Castelli, S. Frases, M. Cuenca-Estrella, J.L. Rodriguez-Tudela, and A. Casadevall. 2008. **Capsule enlargement in *Cryptococcus neoformans* confers resistance to oxidative stress suggesting a mechanism for intracellular survival.** *Cell. Microbiol.* **10:**2043-2057

13. Zaragoza, O., C.P. Taborda, and A. Casadevall. 2003. **The efficacy of complement-mediated phagocytosis of *Cryptococcus neoformans* is dependent on the location of C3 in the polysaccharide capsule and involves both direct and indirect C3-mediated interactions.** *Euro. J. Immunol.* **33:1957-1967**

Chapter 3. *Cryptococcus neoformans* and *Cryptococcus gattii* interactions wit the Greater Wax Moth (*Galleria mellonella*)

1.

2. Capilla, J., K.V. Clemons, and D.A. Stevens. 2006. **Experimental systemic infection with *Cyptococcus neoformans var. grubii* and *Cryptococcus gattii* in normal and immunodeficient mice**. *Med. Mycol.* **44:**601-611

3. Carroll, S.F., L. Guillot, and S.T. Qureshi. 2007. **Mammalian hosts of cryptococcocal infection.** *Comp. Med.* **57:**9-17

4. Choi, J. Y., C. D. Sifri, B. C. Goumnerov, L. G. Rahme, F. M. Ausubel, and S. B. Calderwood. 2002. **Identification of virulence genes in a pathogenic strain of *Pseudomonas aeruginosa* by representational difference analysis.** *J. Bacteriol.* **184:** 952–961**.**

5. Coleman, J., C. Chrisman, A. Casadevall, and E. Mylonakis. (2011). ***Cryptococcus neoformans:* Nonvertebrate Hosts and the Emergence of Virulence. In J. Heitman, T. R. Kozel, K.J. Kwon-Chung, J. Perfect, and A. Casadevall (Eds.), *Cryptococcus* From Human Pathogen to Model Yeast** (pp.261-267*) Washington, D.C.: ASM Press*

6. Cotter, G., S. Doyle, and K. Kavanagh. 1999. **Development of an insect model for the in vivo pathogenicity testing of yeasts**. *FEMS Immun. Med. Microbiol.* **27:** 163-169

7. Franzot, S.P., I.F. Salkin, and A. Casadevall. 1999. ***Cryptococcus neoformans var. grubii*: separate variety status for *Cryptococcus neoformans* serotype A isolates.** *J. Clin. Microbial.* **37:**838-840

8. García-Rodas, R., A. Casadevall, J.L. Rodríguez-Tudela, M. Cuenca-Estrella, and O. Zaragoza. 2011. ***Cryptococcus neoformans* Capsular Enlargement and Cellular Gigantism during *Galleria mellonella* infection.** PLoS ONE 6(9): e24485 doi:10.1371/journal.pone.0024485

9. Heitman, J., T. R. Kozel, K.J. Kwon-Chung, J. Perfect, and A. Casadevall (Eds.), ***Cryptococcus* From Human Pathogen to Model Yeast** *Washington, D.C.: ASM Press* 620 p.

10. Kavanagh, K. and E.P. Reeves. 2004. **Exploiting the potential of insects for in vivo pathogenicity testing of microbial pathogens.**

11. Mylonakis, E. 2008. ***Galleria mellonella* and the Study of Fungal Pathogenesis: Making the Case for Another Genetically Tractable Model Host.** *Mycopathol.* **165:**1-3

12. Mylonakis, E., R. Moreno, J.B. El Khoury, A. Idnurm, J. Heitman, S. Calderwood, F. Ausubel, and A. Diener. 2005. ***Galleria mellonella* as a Model System to Study *Cryptococcus neoformans* Pathogenesis.** *Infect. Immun.* **73(7):3842.**

Appendix

Table A1 Multiple comparisons Procedures, utilizing the Bonferroni Correction.
Corrected $\alpha_{Corrected}=1-(1-0.05)^{1/10}= 0.0051$

Comparisons	P-value	Significant
DPBS vs H99	0.011983	Yes
DPBS vs M27	0.000251	Yes
DPBS vs VBGc22	0.000048	Yes
DPBS vs VBGc28	0.000027	Yes
DPBS vs Ym63	0.000166	Yes
DPBS vs Yag44	0.142260	No
DPBS vs Ap1	0.110723	No
DPBS vs Ap3	0.043474	No
DPBS vs Ap5	0.000115	No
H99 vs M27	0.204443	No
H99 vs VBGc22	0.186863	No
H99 vs VBGc28	0.030532	No
H99 vs Yag44	0.320808	No
H99 vs Ym63	0.037750	No
M27 vs VBGc22	0.711987	No
M27 vs VBGc28	0.403802	No
M27 vs Yag44	0.018089	No
M27 vs Ym63	0.646303	No
Ap1 vs Ap3	0.909870	No
Ap1 vs Ap5	0.086430	No
Ap1 vs H99	0.537212	No
Ap1 vs M27	0.047479	No
Ap1 vs VBGc22	0.040993	No
Ap1 vs VBGc28	0.018105	No
Ap1 vs Yag44	0.763918	No
Ap1 vs Ym63	0.020145	No

Table A1 Continued

Ap3 vs VBGc22	0.026505	No
Ap3 vs VBGc28	0.007186	No
Ap3 vs Yag44	0.645795	No
Ap3 vs Ym63	0.015185	No
Ap5 vs H99	0.346904	No
Ap5 vs M27	0.915924	No
Ap5 vs VBGc22	0.740794	No
Ap5 vs VBGc28	0.151914	No
Ap5 vs Yag44	0.029747	No
Ap5 vs Ym63	0.373574	No
VBGc22 vs VBGc28	0.317072	No
VBGc22 vs Yag44	0.012930	No
VBGc22 vs Ym63	0.604576	No