Detection and Identification of *Cryptococcus neoformans/gattii* complex in Novel Environmental Samples using Physiological and Genetic Tools

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Abstract

Cryptococcus neoformans/gattii complex *(C.neo/gat)* are opportunistic, encapsulated yeasts that adversely affect primarily immune-suppressed patients. The yeasts live freely in the environment, mainly associated to pigeon droppings and to eucalyptus, almond or other tree detritus. At present, the varieties belonging to *C.neo/gat* have been divided into two species according to their environmental niches and to physiological and molecular differences. Few studies on the ecology of *C.neo/gat* have been conducted in Puerto Rico. Therefore, we decided to determine the presence of the yeast on novel and diverse environmental sources. Detritus and surface samples from trees and succulent plants from western Puerto Rico were collected as well as pigeon droppings. The *C.neo/gat* isolates were preliminarily identified by melanin production on Niger Seed Agar. The identity of the isolates was confirmed using microscopical and biochemical tests and by the *in silico* analysis of the small ribosomal subunit 18S rDNA, intergenic spacer (IGS) and internal transcribed spacer (ITS) of the isolates. Genetic differences between the isolates were identified by a phylogenetic analysis using the 18S rDNA sequences, showing five homology groups. Also, strains and environmental isolates from Puerto Rico, belonging to the *C.neo/gat* complex, were analyzed using the BIOLOG Microstation System, to identify five specific common carbon sources for members of *C. neo/gat*. This research also included a preliminary phage display analysis. Through the interaction of *C. gattii* C77 strain with human brain and lung genomic libraries fused on the T7 phage, it was determined that two main peptides, which coded to nectin and telecephalin, interacted. The peptides can potentially serve as vaccine precursors to develop as a new anticryptococcal therapy and also be used to identify the interaction of the capsule with the target organs.

To the best of our knowledge, the findings present in this research correspond to the first report ever on the isolation of *C. gattii* from cacti, and from tree detritus in Puerto Rico. Also, this research corresponded to the first study of the reliability of the BIOLOG Microstation System for the rapid identification of the *C. neo/gat* complex and on the interaction of whole cells of *C. gattii* to T7 premade human genomic libraries of lung and brains.

Resumen

El complejo *Cryptococcus neoformans/gattii (Cneo/gat)* está compuesto de levaduras oportunistas, encapsuladas que afectan principalmente a pacientes inmunocomprometidos. Estas levaduras viven en el ambiente, asociadas a excreta de paloma y a detrito de árboles de eucaliptos, almendros y de otros. Actualmente, las variedades pertenecientes a *Cneo/gat* han sido divididas en dos especies de acuerdo a sus diferencias en nichos ecológicos, fisiología y biología molecular. En Puerto Rico se han realizado muy pocos estudios sobre la ecología de *C.neo/gat.* Es por esto que se ha decidido determinar la presencia de la levadura en diversas y noveles fuentes ambientales. Se colectaron y procesaron detritos y superficies de árboles y plantas suculentas del área oeste de Puerto Rico, y también se colectaron muestras de excreta de paloma. Los aislamientos de *C.neo/gat* fueron identificados preliminarmente mediante la producción de melanina en Niger Seed Agar. La identidad de los aislamientos fue confirmada mediante el uso de pruebas bioquímicas y por el análisis *in silico* de la subunidad ribosomal pequeña 18S rDNA, espacio intergénico (IGS, por sus siglas en inglés) y por el espacio interno transcrito (ITS, por sus siglas en inglés). Las diferencias genéticas entre los candidatos fueron identificadas por análisis filogenético usando las secuencias del 18S rDNA, lo que mostró 5 grupos homólogos. Además, las cepas de *C. neo/gat* A, B, C and y algunos aislamientos ambientales pertenecientes al complejo de *C.neo/gat*, fueron analizados usando el Sistema BIOLOG Microstation. En esta investigación también se incluye un análisis preliminar usando la tecnología “T7 phage display”. Por la interacción de células de *C. gattii* con bibliotecas genómicas de cerebro y pulmón fusionadas al bacteriófago T7, se lograron aislar dos péptidos recombinantes principales, nectina y telecefalina, los cuales interaccionan con la cápsula de la

levadura. Estos péptidos recombinantes pueden ser usados como posibles precursores de una vacuna contra criptococosis y para identificar la interacción de la cápsula de *C. gattii* con los órganos blancos.

Al mejor de nuestros conocimientos, los hallazgos descritos en esta investigación representan el primer reporte del aislamiento de *C. gattii* de cactus en la literatura mundial, y de detritos de árboles en Puerto Rico. Además, esta investigación corresponde el primer estudio de la que prueba la confiabilidad del Sistema BIOLOG Microstation para la identificación rápida del complejo *C. neo/gat* y de la interacción de células completas de *C. gattii* con bibliotecas genómicas prefabricadas de cerebro y pulmón codificas en el bacteriófago T7.

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 sister Beby and my parents Lissette and Yasco for their support and understanding.

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"Imagination is more important than knowledge."

Albert Einstein

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1 Introduction and Literature Review

1.1 Introduction

Cryptococcus neoformans/gattii (*C.neo/gat*) is an encapsulated, opportunistic yeast that affects immune-compromised and immune-competent patients. Generally, the yeast is a free living organism that is mainly associated with pigeon droppings (*C. neoformans*) and detritus from trees such as eucalyptus and almond (*C. gattii*). The polysaccharides capsule, mainly composed of glucuronoxylomannan, is the responsible for the serotype classification, and it is the main virulence factor of the yeast.

Cryptococcus neoformans/*gattii* was originally identified by the Italian physician Santafelice in 1884, who obtained the first isolate of the yeast from fruit juices. The description of *C. neoformans* as a human pathogen was made by the two German physicians, Busse and Buschke, who first described the infection, cryptococcosis in 1884. The research on the yeast continued until the end of 19th century, when three seminal observations were made: a) the organisms were recovered from lesions in humans and animals, establishing its potential to cause a disease; b) they were recovered from the environment, establishing that they are free-living, and c) they were propagated in the laboratory and were able to cause disease to laboratory animals. In the early half of the 20th century, all studies were made to confirm the pathogenicity of *C. neoformans*.

In 1980 the appearance of the human immunodeficiency virus (HIV), the causative agent of the acquired immunodeficiency syndrome (AIDS), brought an increase in cryptococcosis cases, awakening the "sleeping giant" (Ajello, 1971). *C. 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 1980-2005 a total of 228 cases of cryptococcosis were reported among 29,964 AIDS patients (approximately 1.1%) (OCASET,

2005). To date, in most countries, the prevalence of the infection among HIV-infected persons is about 5-10%. The infection predominates in male patients of 20-50 years old compared to in women of the same age. A possible explanation for these findings may be hormonal. This idea arises from a study by Drutz *et al.* (1981), which revealed that human sex male hormones can stimulate the growth and maturation of *Coccidiodes immitis* and that estrogen, a female hormone, can inhibit the mycelium to yeast transformation of *Paracoccidiodes brasiliensis*. In Central and South America, cryptococcosis is mainly caused by *C. neoformans* var. *grubbi* in immune-suppressed persons. There have been few reports of cryptococcosis caused by *C. gattii* (Lizarazo *et al.* 2007).

Based on studies of the antigenic characteristics of the *C. neo/gat* polysaccharides, five serotypes have been identified and named A, B, C, D and an AD hybrid. In the 1960's, the sexual phase of the yeast was discovered; basidiospores were produced under laboratory conditions. The new teleomorph was named *Filobasidiella.* In 1970, Dr. Kwon Chung found two principal varieties for *C. neoformans*: *neoformans* and g*attii*. Also, Franzot *et al*. (1999), described a third variety of *C. neoformans* named *grubbi* for serotype A. Actually, *C. neo/gat* has been classified in two species based on differences in their ecological niches, physiological and molecular traits.

Until now, the most important criterion used for the identification of the yeast is the production of melanin pigment due to the activity of the phenoloxidase enzyme (Mitchell and Perfect, 2005). The microscopic identification is made by negative staining using India ink, allowing the observation of the yeast's capsule. Also, the yeast's carbohydrates assimilation pattern and the detection of urease activity have been used as the *C.neo/gat* nutritionally identification process. The molecular and technological advances during the last three decades

have allowed the development of novel identification and differentiation techniques for the *C. neo/gat* varieties. As for example, the implementation of genetic tools has been used in understanding the epidemiology, ecological distribution, and the pathogenicity of the yeast. In the case of clinical samples, more specific and standardized methods, such as Latex Agglutination Test have been used to detect cryptococcosis. Also, the use of the luminex technology to detect IGS probes (Bloomfield *et al*., 1963 cited in Mitchell and Perfect, 1995) has been applied in the detection and identification of the serotypes of *C. neo/gat* (Mara and Fell, 2005). Recently, molecular and genetic tests have been incorporated for a fast diagnosis of cryptococcosis. The generation of molecular markers and new anticryptococcal therapy using the polysaccharide capsule as a target have been also developed using the emerging disciplines such as combinatorial chemistry (Valadon *et al*., 1996).

The actual methods for the identification of *C.neo/gat* have resulted in a late diagnosis of cryptococcosis, therefore, it is necessary to find and study more *C. neo.gat* sources that can represent infectious targets and perform the required characterization. The main aim of this investigation was to search for *Cryptococcus neoformans/gattii* in novel ecological niches and to characterize those isolates using of physiological and genetic tools. Also, we explored if *C. neo/gat* capsule specific peptides could be obtained using the combinatorial chemistry technique known as phage display.

1.2 Literature Review

The *C.neo/gat* complex is composed of encapsulated spherical to oval yeasts of the Basidiomycetes division, of Kingdom Fungi, with affinity to the Central Nervous System (Casadevall and Perfect 1998). In differential media such as Niger Seed Agar (NSA), the colonies appear mucoid with brown pigment. According to its telomorph stage, the yeast belongs to the genus *Filobasidiella*, being so far, only observed *in vitro* (Casadevall and Perfect, 1998).

The susceptibility of a host to cryptococcosis reflects a complex interplay between host and microbiological attributes. Both weak and overexuberant responses can result in host damage after infection with *C. neoformans* (Zaragoza *et al*., 2007). The infectious route of the yeast begins with the inhalation of free-living blastospores found in the environment without or with a very small capsule. When the yeast enters the body via the nasal cavity, it increases its capsule size to stop the attack by the host immune system. Then, the yeast invades the lungs causing a chronic pneumonia best known as pulmonary cryptococcosis. It is believed that once in the lungs, it can enter to the pulmonary veins until it reaches the brain. The yeast has great affinity for the brain, causing cryptococcal meningitis, lethal in most patients (Casadevall and Perfect 1998).

1.2.1 Virulence Factors

1.2.1.1 Capsule

C.neo/gat main virulence factor is its polysaccharides capsule composed mainly (90%) of glucuronoxylomannan (GXM). Galactoxylomannan (GalXM) and mannoprotein (MP), are also present (Casadevall and Perfect, 1998). The capsule is synthesized in the cytoplasm of the yeast (Garcia-Rivera *et al*., 2004), giving to the cells a negative charge. It may vary in size (between

1-50μm) depending on the strain and environment and growth conditions (Ruiz and Bulmer, 1981). Many studies of capsule modulation have been developed in order to understand its function, regulation and synthesis. Zaragoza (2004) used different factors to enhance the capsule *in vitro*. He found that high glucose concentrations inhibited the capsule, while low nutritional levels such as diluted Sabouraud dextrose broth with buffer MOPS at pH of 7.3 enhanced its production (Zaragoza and Casadevall, 2004). Capsule architecture is extremely difficult to study because the electron microscopy technique alters the structure of the yeast, which is why the order of the composition of the capsule elements [α-1, 3-mannan with monosaccharide branches of xylose and glucuronic acid] was determined, by nuclear magnetic resonance (NMR) (Cherniak *et al*., 1994).

The capsule is primarily responsible for the phagocytosis evasion, complement activation and depletion, antibody unresponsiveness, and inhibition of leukocyte migration. It is coded by the genes c*ap10, cap59, cap60* and *cap64* (Mitchell and Perfect, 1995). The first capsular gene found was *cap*59, present in all the varieties of *C. neo/gat* and associated with the capsule phenotype and virulence (Garcia-Rivera *et al*., 2004). The deletion of one capsular gene results in avirulent acapsular mutants (Perfect *et al*., 1998). Acapsular cryptococci are readily ingested by neutrophils and macrophages in the presence of serum, whereas encapsulated cryptococci are resistant to phagocytosis (Gate *et al*., 2004). The function of these capsular genes, especially *cap59*, is extracellular trafficking and/or secretion of several capsule compounds, mainly GXM (Garcia-Rivera *et al*., 2004). However the mechanism by which the genes mediate the capsule biosynthesis is not clear at this moment.

The capsule of *C. neo/gat* elicits an immune response due to cell-mediated immunity, specifically a delayed type hypersensitivity reaction mediated by the three mayor components,

GXM, GalXM and MP, being the latter the strongest one (Murphy *et al*., 1988). T cells play a predominant role in the defense against *C. neoformans* infection (Zaragoza *et al*., 2007). Neutrophils contribute to host defense against systemic infection since they can polarize the immune response toward T helper cells 2 (Th2), such that neutrophils depletion is associated with enhanced resistance to pulmonary infection (Zaragoza *et al*., 2007). The resistance to pulmonary *C. neoformans* infection is mainly associated with a Th1 response, implying that the effective inflammatory response involves granuloma formation and cellular recruitment into the lungs (Zaragoza *et al*., 2007).

Differences in the GXM structure on *C.neg/gat* strains produce antigenic differentiation providing the basis for the classical separation of *C. neo/gat* using antibodies (Casadevall and Perfect, 1998). Since many antigenic structures are shared by most strains, Ikeda *et al*. (1982) developed a serological method using eight antigenic factors (Table 1). Accordingly, *C. neoformans* has been divided into varieties which included var. *grubbi* ser. A, var. *neoformans* ser. D and a hybrid AD. On the other hand, *C. gattii* includes the serotypes B and C. Recently a hybrid between *C. gattii* and *C. neoformans* var. *neoformans* (named hybrid BD) has been reported (Bovers *et al*., 2006).

Table 1. Antigenic response of *C. neo/gat* using antibody reaction in sera (Ikeda *et al*., 1988)

	Factor Sera							
Serotypes	1	2	3	4	5	6	7	8
A	+	+	+	-	-	-	+	-
B	+	+	+	-	-	-	-	+
C	+	+	-	+	+	-	-	-
D	+	-	-	+	-	+	-	-

Note: (+) indicates positive response to the sera; (-) indicates negative response to the sera.

1.2.1.2 Mannitol as a trigger for *Cryptococcus neoformans/gattii* virulence

In vivo and *in vitro, C.neo/gat* produces and utilizes mannitol via the polyol mechanism. A *Saccharomyces cerevisiae* mannitol dehydrogenase homolog is found in *C. neo/gat* which enhanced the activity of the gene (Perfect *et al*., 1996). Mutants that produced small quantities of mannitol were susceptible to osmotic stress and heat. This finding suggests that mannitol acts as an intracellular osmolite and stress protector (Perfect *et al*., 1998). The low-producing mannitol mutants interfered with the yeast's ability to scavenge distal reactive oxygen intermediates needed to evade human polymorphonuclear phagocytosis (Chatuverdi, Wong and Newman, 1996). In conclusion, mannitol production by *C. neo/gat* and its intracellular accumulation constituted a nutritional reserve that contributed to environmental stress tolerance, and enhanced the virulence of the yeast by scavenging hydroxyl radicals and promoting brain edema (Chatuverdi, Wong and Newman, 1996; Perfect *et al*., 1998).

1.2.1.3 Phenoloxidase activity

The phenoloxidase enzyme production is the main criterion that allows the macroscopic identification of the yeast due to the fact that *C. neoformans* produces brown pigmented colonies. The pigment is produced in caffeic acid medium, dihydroxyphenylalanine (DOPA) medium (Fig.1), Niger Seed Agar (NSA) or any medium with phenolic compounds and bacterial melanin precursor such as Homogentesic Acid (HGA) (Franses *et al*., 2007). This enzyme participates in the melanin production (Williamson *et al*., 1998) and it is responsible for the wood degradation, helping in the formation of detritus. Melanins are pigments of biological origin; they are localized in the cell wall of the yeast (Nosanchuk *et al.*, 1999). The pigments have undefined chemical structure and tremendous physical stability. The phenoloxidase enzyme is a member of the laccase family in which the gene involved in its production is *cnlac1*. Transcriptional

activity of the gene was repressed in high glucose concentrations and may be also repressed in the sexual, filamentous stage of the yeast (Perfect *et al.*, 1998).

Melanin can protect *C. neoformans* against antifungal compounds, oxidants, macrophages, and extreme temperatures (Nosanchuk *et al.*, 1999). Melanin can also explain the affinity of yeast to the Central Nervous System. It is considered that high concentrations catecholamines in basal ganglia may cause the neurotropism in human hosts (Nosanchuk *et al*., 1999; Casadevall and Perfect, 1998). In *C.neo/gat*, the melanin production is extremely important when it enters in contact with the host immune system. The melanin produced by environmental *C. neo/gat* acts as a protector against UV rays and heavy cytotoxics (Rosas *et al*., 2001; Nosanchuk *et al*., 1999). In order to increase the melanin production for the macroscopic identification of the yeast, Viddotto *et al.* (2003) developed a minimum synthetic culture medium of caffeic acid. This medium was compared with the previous established culture media such as NSA (made with *Guizotia abyssinica* seed) and the original caffeic acid medium, showing that the melanin production increased in the synthetic media of caffeic acid.

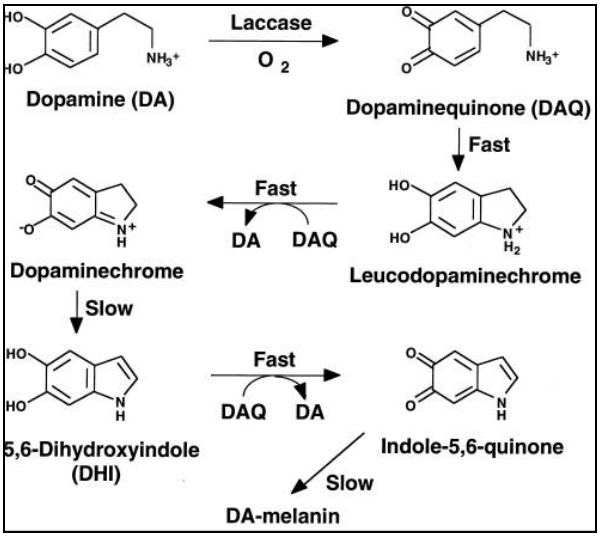


Fig1.1 Melanogenesis from dopamine in *C. neoformans* (Williamson *et al*., 1998)

1.2.2 Environmental Distribution

C. neo/gat is a saprophytic, free-living organism that can survive in a variety of environmental niches. Its presence has been reported from all regions of the world and a variety of environmental sources such as avian excreta, bat organs and guano, caves, barns, cockroaches, fruits, fermented fruit juices, horse intestinal flora, milk, bovine mastitis, rabbit pens, soils, and hollows of various trees (Casadevall and Perfect, 1998; Lazera *et al*., 2000). The complex has been also isolated from *Cassia grandis* (pink shower), *Ficus microcarpa* (fig), *Syzygium jambolana* (java plum), and *Senna multijuga* (November shower) (Lazera *et al*., 2000).

C. neo/gat can be adversely affected by abiotic factors such as high temperatures, low humidity, anaerobic conditions, direct sunlight exposure (Ishaq *et al*., 1968) and alkaline (Staib, 1962) or extreme acidic conditions (Ruiz *et al.*, 1981). Also, there are a few biotic factors that can affect the survival of the yeasts (Ruiz 1981; Ruiz and Bulmer, 1981; Ruiz *et al*., 1982). Among them, the presence of bacteria such as *Pseudomonas aeruginosa*, *Bacillus subtilis* and *Proteus vulgaris* can inhibit the growth of the yeast (Ruiz *et al*., 1982). Aditionally, it has been shown that amoebas from the species *Acanthamoeba palestinensi* and *A. polyphaga*, commonly found in pigeon excreta, can ingest and digest the yeast, making them a possible biocontrol agent (Ruiz *et al*., 1982). Fuentefria *et al*. (2006) isolated two yeasts from *Hibiscus rosa-sinensis* in Brazil that also inhibited the growth of *C. neo/gat*. The environmental distribution of *C. neo/gat* varies depending on the species studied.

1.2.2.1 Environmental distribution of Cryptococcus neoformans

C. neoformans is mainly associated with pigeon excreta; it represents the highest numbers of clinical isolates recovered from AIDS patients (Casadevall and Perfect, 1998). The first isolation of *C. neoformans* was from peach juices and after that, Emmons isolated it from a soil

contaminated with avian excreta (Casadevall and Perfect, 1998; Emmons, 1955). *C. neoformans* var. *grubii* has a global distribution and is the main common cause of cryptococcal meningitis in immune-compromised patients worldwide (Duncan, 2005). *C. neoformans* var. *neoformans* (serotypes D and AD) is less commonly recovered from the environment samples or clinical cases and it appears to be more prevalent in Europe than in other parts of the world (Dromer *et al*., 2007).

Pigeons are considered to be the main carriers, playing an important role in the dissemination of the yeast in the environment (Abou-Gabal and Atia, 1978). In this type of ecological niche, *C. neoformans* has been isolated several times from different altitudes worldwide. The predominant variety isolated from clinical and environmental samples from all over the world is *grubbi* (Casadevall and Perfect, 1998), including Puerto Rico which it is the only variety reported here (Ruiz *et al.*, 1989). The mechanism by which the pigeon excreta get contaminated with *C. neoformans* is not yet understood. The theory of cryptococcal blastospores ingestion by the pigeon, postulated by Littman and Borok (1968) was unsupported because the excreta give the yeast only the nutritional requirements and the appropriate habitat for its development. Also, the corporal temperature of the pigeon (41.5ºC-43.5ºC) inhibits the proliferation of the yeast in the pigeon digestive tract (Casadevall and Perfect, 1998). In a study by Abou-Gabal and Atia (1978), they proved that bacterial flora from the intestinal contents of apparently healthy pigeons showed a complete inhibitory effect on the growth of *C. neoformans in vitro*. They also concluded that pigeons do not favor multiplication of the fungus in their gut and consequently they do not seem to play an active biological role in dissemination of *C. neoformans* in nature. Some scientist have tried to infect pigeons with *C. neoformans* via intramuscular and orally, but the facts mentioned above made the pigeons resistant to the infection (Casadevall and Perfect,

1998). All these results support the theory that the pigeons are mechanical vectors of the yeast (Casadevall and Perfect, 1998).

In desiccated avian excreta, the yeast can be viable at least during 2 years with a very small capsule, around 1-5.5mm, a particle compatible with alveolar deposition (Littman and Borok, 1968; Powel *et al*., 1972; Ruiz *et al*., 1989). The growth of *C. neoformans* on avian excreta is possible because of the unique biochemical adaptations that the yeast has. One of this is the ability to assimilate uric acid, purines and the unique ability to assimilate creatinine as a nitrogen source, been the latter the most important ingredient in the cultured media used to select for *C. neo/gat* (Staib *et al.*, 1973).

Cryptococcus neoformans has been also isolated from detritus of trees such as munguba, java, coconut and mango in Brazil (Lazera *et al.*, 1996). It also had been isolated from eucalyptus trees (Duarte et al., 1994). Until now, there is no specific relation between *C. neoformans* with any tree or plant, but it must be considered as a consequence due to the relationship between birds and the trees in which *C. neoformans* has been isolated (Casadevall and Perfect, 1998). The yeast has also been isolated from the digestive tract of the common cockroaches *Periplaneta americana* (Casadevall and Perfect, 1998), bat guano (Grose *et al*., 1968), dogs, cats, goats, koalas, guineas pigs, non human primates, foxes, cheetah, eastern water skink, kiwi, parrots and dolphins (cited by Duncan, 2005).

1.2.2.2 Environmental Distribution of *Cryptococcus gattii*

Almost all the isolates of *C. gattii* have been obtained from tropical and subtropical regions. There are reports of cryptococcosis from temperate and cold areas, but the source and route of infection is not yet well understood (Bottone *et al.*, 1986; Dromer *et al*., 1992; Fromtling *et al*., 1982; MacDougall and Fyfe, 2006; Quintero *et al*., 2005; St.-Germain *et al*., 1988). Dromer *et*

al. (1992), proposed that some of those cases could represent and activation of latent cryptococcosis. *C. gattii* is associated with eucalyptus and other tree detritus, and was the cause of the recent outbreak of cryptococcosis in Vancouver Island by serotype B (MacDougall and Fyfe, 2006). Ellis and Pfeiffer (1990) were the first scientists who discovered the association of *C. gattii* with tree detritus of *Eucalyptus camandulensis*. These findings originated the studies, which established the relationship between the yeast and tree products. Since then, *C. gattii* has been recovered from material associated with eucalypti species in many other parts of the world including California, India and Brazil (Chakrabarti, 1997; Montenegro and Paula, 2000; Nishiwaka, 2003; Pfeiffer and Ellis, 1991; Trilles *et al*., 2003) and from other tree species from tropical and subtropical areas worldwide (Callejas *et al.*, 1998; Fortes *et al.*, 2001; Lazera *et al*., 2000; Nishiwaka *et al.*, 2003). Most environmental isolates of *C. gattii* have been serotype B, however there are reports of serotype C isolated from almond trees (*Terminalia catappa*) in Colombia and from vegetation in southern California (Callejas *et al.*, 1998; Pfeiffer and Ellis, 1991). To date, there is no association between avian excreta and *C. gattii*, considering that *C. gattii* has the enzyme creatinine deaminase, responsible for the creatinine assimilation. One of the hypotheses is that the enzymatic pathway regulation in *C. gattii* is different from that in *C. neoformans* (Kwon-Chung, 1991; Polacheck and Kwon-Chung, 1980).

Trilles *et al*. (2003), described the isolation and characterization of *C. neo/gat* from trees such as *Casia grandis* (pink shower), *Picus microcarpa* (picus) and *Senna multijuga* (November shower) among others in Brazil. Also, in the study, the pheromones MATα and MATa were identified using the Polymerase Chain Reaction (PCR) technique using specific primers for the pheromones (Trilles *et al.*, 2003). The researchers found that both species of the complex *C. neo/gat* can live in the same tree; specifically the isolates were *C. neoformans* var. *neoformans*,

with **a** mating type and *C. gattii* serotype C, with α mating type, were both found in the same tree, but genetic material interchange was not observed. Almost all the isolates on these experiments belonged to *C. neoformans* var. *grubbi*, genotype 1 AFLP, MAT α**.** In this study it was confirmed that *C. neoformans* var. *grubbi* can live in tree detritus and in animal hosts.

As mentioned before, the serotypes of *C. neo/gat* are associated to specific ecological niches. A group of Colombian microbiologists, directed by Dr. Callejas, reported in a short communication the first isolation of *C. gattii* serotype C from *Terminalia catappa* (almond) tree from South America (Callejas *et al*., 1998). During an eleven months study, they sampled *Eucalyptus sp., Moquilia sp. (*oiti), *Delonix sp.* (canopy) and *Terminalia sp.* (almond). The only *C. gattii* isolated was from two *Terminalia* detritus. The results were confirmed by the Center for Disease Control in Atlanta (CDC). In this study, the tree was sampled for five consecutive months, in which the yeast was isolated every time, indicating its long term viability and a close and strong relationship with the environment (Callejas *et al.*, 1998). These findings tend to support the hypothesis that *C. gattii* has plant products as ecological niche. This is based on the fact that it is not likely that pigeons are the main environmental source for this species. Studies in Brazil indicated that *C. neoformans* can be isolated from *Eucalyptus*, among other trees (Callejas *et al*., 1998; Casadevall and Perfect, 1998; Lazeras, 2000). The isolation of *C. gattii* serotype B in Colombia was first made by Elizabeth Quintero as part of her Masters Thesis (Quintero, 2003). The study also demonstrated that trees are important in the yeast lifecycle but not as a primary niche, a postulate against Ellis' hypothesis.

Using the samples isolated from the almond trees by Dr. Callejas, the team of researchers infected seedlings of *T. catappa* to verify the viability of the yeast on the tree. It appears that the yeast adapted to the plant tissue. The macro to micro transition of the yeast was not observed,

suggesting that there are transitional hosts (Huérfano *et al*., 2001). Until now, there were no reports on the isolation of *Cryptococcus gattii* in Puerto Rico.

1.2.3 Physiology of Cryptococcus neoformans/gattii

Most of the physiological studies of *Cryptococcus neoformans/gattii* have been focused on the traits and pathways that cause the yeast to be virulent and to the mechanism of action of anticryptococcocal drugs (Casadevall and Perfect, 1998). The physiological knowledge related to the yeast has been skewed toward the traits of virulence. One of the traits better known is the versatility of the yeast using different carbon sources as energy. With the exception of melibiose and lactose, *C. neo/gat* can assimilate most of the sugars, making the yeast easy to adapt to different substrates (Casadevall and Perfect, 1998). In contrast, *C. gattii* can use fumaric acid and succinic acid as sole carbon sources, and malic acid and D-proline as sole nitrogen sources (Bennett *et al.*, 1978; Dufait *et al*., 1987). Also, *C. gattii* can assimilate glycine as nitrogen sources and it can resist canavanine, an arginine analog, while *C. neoformans* can not. These biochemical differences are the base for the creation and preparation of the canavanine glycine bromothymol blue medium (CGB); this medium is used to distinguish between the *C. neo/gat* species (Kwon-Chung *et al*., 1982). In order to grow on this medium, the yeast needs an aerobic environment, acidic conditions and small amounts of iron. Fluxes of iron concentration can be used to determine the capsule size of *C. neo/gat*; in low iron concentrations the capsule synthesis is stimulated (Zaragoza and Casadevall, 2004).

C. neo/gat produces various enzymes that allow the survival of the yeast not only in the environment, but in the animal or human hosts. One of those enzymes is phenoloxidase. As mentioned before, the enzyme is responsible for the melanin production in the presence of polyhydrophenolic or polyaminobencenic compounds, and catecholamines such as epinephrine,

norepinefrin, L-dopa and dopamine (Williamson *et al*., 1998). The enzyme belongs to the laccase family which plays an important role in the lignin degradation in plants and trees. Another important enzyme produced by *C. neo/gat* is the urease. This enzyme catalyzes the urea hydrolysis into ammonium and carbamate. In the host, the enzyme changes the pH, and in the environment it gives the yeast the ability to survive in low hydrogen places (Casadevall and Perfect, 1998).

Many studies for the rapid identification of yeasts have been carried out to facilitate the diagnosis of cryptococcosis. There are a series of commercially available, rapid methods, based on biochemical tests for the diagnosis of cryptococcosis. Among others, API 20C, the Flow Laboratories Uni-yeast-Tek System, the BBL Minitek System and the Vitek Yeast Biochemical Card can be mentioned. These systems use carbohydrate assimilation patterns for the identification of the yeast, and require 24 hours of incubation prior to make the proper readings (Casadevall and Perfect, 1998). Additionally, there are a few multitest systems based on detection of pre-formed enzymes that can identify the yeast within 4 hours of incubation, visualizing a chromogenic reaction (St.-Germain and Beauchesne, 1991). Another rapid identification test developed is the **BIOLOG Microstation**, a semi-automated system, connected to a computer, used for the quick identification of clinical and non clinical isolates of microorganisms, including yeasts. Until now, the database of the BIOLOG system does not include the identification patterns of *C. neo/gat*, however the telomorph stage of the yeast, *Filobasidiella neoformans/bacillisporus*, is present in the database. Mcginnis *et al*. (1996), introduced 13 genera and 38 species in the BIOLOG database after analyzing 16 genera and 159 species. They processed the samples twice to prove their results. The data obtained showed that 13.2%, 39.5% and 48.8% of the samples were correctly identified in a 24, 48 and 72 hours period

at 30ºC, respectively. The isolates incorrectly identified corresponded to a 16.7, 53.3 and 56.7% after the same amount of time mentioned before. Those isolates were not included in the BIOLOG database. The rest of the isolates were not identified at all. Praphailong *et al.* (1997) used the BIOLOG system to identify 21 species of yeasts isolated from food and wines. Using the manufacturer specifications, the group of scientists was able to identify *Saccharomyces cerevisiae, Debaryomyces hansenii, Yarrowia lipolytica, Kluyveromyces marxianus, Kloeckera apiculata, Dekkera bruxellensis* and *Schizosaccharomyces pombe*. Species such as *Zygosaccharomyces bailii* and *Zygosaccharomyces rouxii* were identified 50% of the time; and *Pichia membranaefaciens* was identified 20% of the time only. Foschino *et al*., (2004) identified and characterized 25 samples of yeasts in a wheat mass on various Italian bakeries. This work also compared the phenotypic identification techniques with the molecular techniques, showing that molecular techniques are highly reliable in comparison to phenotypic ones.

1.3 Molecular Biology of Cryptococcus neoformans/gattii

Since the 1980, molecular biology studies of *C. neo/gat* have greatly increased. The first molecular study of *C. neo/gat* involved the determination of G+C content and the DNA relatedness of various species of the genus *Cryptococcus* (Casadevall and Perfect, 1998). It was found that the percentage of G+C in *C. gattii* (56-57.3 mol%) was a bit higher than those on *C. neoformans* (53-56 mol%). Between the varieties, the deoxyribonucleic acid (DNA) association was 88-94%, reaffirming their relatedness (Aulakh *et al*., 1981). DNA isolation, PCR and ribonucleic acid (RNA) isolation of the yeast were refined in the late 1980's.

In the 1990's, most of the molecular studies and DNA typing techniques have been used to understand the epidemiology, pathogenicity and distribution of the yeast. These techniques included karyotyping, Random Amplification of Polymorphic DNA (RAPD) (Meyer *et al*., 1993;

Sorrel *et al.*, 1996), and Restriction Fragment Length Polymorphism (RFLP) of the genomic DNA using the *HaeII* and *HindIII* endonucleases (Currie *et al*., 1991). Also, especific genes such as *ura5* have been digested with *Sau96I* and *HhaI* (Meyer *et al*., 2003) and phospholipase I with *HindIII* (Latouche *et al*., 2003), in order to characterize and identify the complex. Other genetic tools used for the characterization of *C.neo/gat* are: restriction digestion of the internal transcribed spacer with *HpaII* (Mirhendi *et al.,* 2001), DNA hybridization with repetitive elements, amplified fragment length polymorphism (AFLP) using the endonucleases *MseI* and *EcoRI* (Spitzer and Spitzer, 1992; Currie *et al*., 1994, Boekhout *et al*., 2001) and PCR fingerpringting (Sorrel *et al.*, 1996). The karyotyping of *C. neo/gat* was performed using the pulse field electrophoresis technique in which differences were found around the chromosome of the two species of the complex (Polacheck and Lebens, 1989). By this technique it was found that *C. neoformans* has 8-12 chromosomes while *C. gattii* has 11-13, with a genome size of approximately 23Mb. These findings suggest a chromosome rearrangement, developed by evolutionary pressures (Perfect *et al.*, 1993). The karyotyping have been used as an epidemiological tool to distinguish among strains of *C. gattii* isolated from the environment, and from patients showing differences between karyotypes from both sources (Mitchell and Perfect, 1995).

In its genome, *C. neo/gat* has few repetitive elements. One of these elements is the rDNA. This 8kb fragment contains the 18S, 5.8S and 23S-like genes (Fan *et al*., 1994). Attempts to digest these genes with endonucleases have been made to distinguish between the two species of the *C. neo/gat* complex. Because the rDNA of *C. neo/gat* only differs in 15 nucleotides out of 5,656 bases, it can not be used to distinguish between species; therefore, specific primers have been designed to amplify the genes in *C. neo/gat* (Fan *et al*., 1994; Mitchell *et al*., 1994). In

order to better understand the yeast relationship with its ecological niche, researchers have used the 5.8S rDNA with the internal transcribed spacer (ITS) to characterize the yeast, because the 5.8S rDNA has proven to be more specific to identify the *C.neo/gat* species than the other sequence of the rDNA (Katsu *et al*., 2003). The sequencing of the ITS revealed that all the samples of *C. neoformans* var. *grubbi* are identical sequences, similar to those of *C. neoformans* var. *neoformans*. Additionally, the AD hybrid has identical sequence pattern compared to the var. *neoformans,* reflecting 2 ITS molecular types (Katsu *et al*., 2003). The sequence of *C. gattii* ITS showed five molecular ITS types, generating the ITS molecular characterization as followed: *var. grubbi* and A allele of AD hybrid as ITS1, *var. neoformans* and D allele of AD hybrid as ITS2 and *C. gattii* serotypes B and C as ITS3-ITS7 (Katsu *et al,*. 2003). The analysis of the ITS sequences of each variety of *C. neo/gat* reflects that in the nucleotides 11, 19, 108 and 221 there are a four nucleotides combination; var. *grubbi* TCTA, var. *neoformans* TTAG and *C. gattii* CCTG (Katsu *et al*., 2003). The phylogenetic tree made by this analysis showed that *C. neo/gat* is divided in two big groups: C. *neoformans* var. *grubbi* and *neoformans* with AD hybrid as one branch and *C. gattii* in the other branch, indicating differences in the ITS regions (Katsu *et al*., 2003)

Another important repetitive element is CNRE-1, present at least in seven chromosomes, with approximately 10-20 copies, with a size between 5-10 kbp (Spitzer and Spitzer, 1992). These elements have been used to generate probes in order to examine the relation between the clinical and environmental isolates. Other technique used to discriminate the *C. neo/gat* is the RFLP. One of the genes digested with endonucleases is the *ura5*, which codes for the orotidine monophosphate pyrophosphorylase. This gene is essential for the uracil synthesis and has been found in the genome in a single copy (Edman and Kwon-Chung, 1992). The *ura5* RFLP

combined with the CNRE-1 probe, can significantly be used to discriminate between *C.neo/gat* species, being more specific the CNRE-1 probe (Currie *et al*., 1994). The amplification using M13 minisatellite and the randomly amplified polymorphic DNA (RAPD) analysis demonstrated useful to classify the yeast by genotypes (Meyer *et al*., 1999). The genotype classification has been confirmed by the CNRE-1 probe, RFLP of the phospholipase gen (*plb1*) and *ura5* gen (Meyer *et al.*, 2003). The classification included clinical and environmental isolates of the yeast and is based on eight major molecular types described as follow; VNI (var. *grubbi*, serotype A), VNII (var. *grubbi*, serotype A), VNIII (serotype AD), VNIV (var. *neoformans*, serotype D) VGI, VGII, VGIII, VGIV (var. *gattii*, serotype B) (Meyer *et al*., 1999).

Several genes of *C.neo/gat* have been identified, cloned and sequenced. The Stanford Genome Technology sponsored the *Cryptococcus neoformans* Genome Project, in which the B-3501-A strain has been cloned in the bacterial artificial chromosome (BAC) and shot gun sequenced. The last update on this initiative was done on June 2004, where 70 overlapping DNA segments with a size of approximate 18.3Mb were identified. This data can be accessed at http://www-sequence.stanford.edu/group/C.neoformans/overview.html. Most of the studies of *C.neo/gat* genes have been focused on those which code for virulence factors such as *cnlac1* (laccase), *cap*59, 60, 64 and 110 (which code for the GXM capsule), sexual pheromones, urease, calcineurin, topoisomerases and cytochrome oxidase subunit 1, respectively, and among others (Casadevall and Perfect, 1998). Recently, the new emerging discipline of combinatorial chemistry, specifically phage display, has been used to identify a target protein that could be used as an anticryptococcal agent (for more details, see chapter 5, section 5.2).

2 Isolation of *Cryptococcus neoformans/gattii* on diverse environmental samples from the western region of Puerto Rico

2.1 Introduction

The western part of Puerto Rico is characterized for having diverse climate conditions such as humid and dry areas. The northern area of the Central Mountains has a moist and rainy climate; whereas in the south region predominates dry climate. Two forests are found within the western area of the Island: the Guajataca Forest, localized at 18°21'55 latitude north and 66°58'14 longitude west, and at the south, the Guánica Dry Forest localized at 17°57′56″ latitude north and 66°52′45″ longitude (http://www.srh.noaa.gov/sju/climatology/rainfall_2006.htm; Farnsworth, 1991). The Island has both introduced flora such as: *Terminalia sp*. (almond), *Persea sp*. (avocado), *Eucalyptus sp*. (eucalyptus) *Manguifera sp*. (mango) and endemic flora such as: *Crescentia portoricensis* (higuero de sierra), *Ottoschulzia rhodoxylon* (rose stick) and *Cephalocereus sp.*

The studies of *C. neo/gat* complex in Puerto Rico have been focused on the isolation of the yeast, mainly from pigeon droppings, reporting a 24.7% of recovery from the western part of the Island and 11% from the southern part (Ruiz, Vélez and Fromtling, 1989; Pérez, 1996). The species isolated from all the environmental and clinical samples in Puerto Rico were *C. neoformans* var. *grubbi* (Ruiz *et al.*, 1989). All attempts to isolate the yeast from plant material, specifically from eucalyptus trees, have been unsuccessful (Pérez, 1996). Almond trees were sampled during 2001; being *C. gattii* the one recovered (Ruiz, unpublished data). Some progress has been made in the study of the ecology of *C. neo/gat* on the Island, but still there are many aspects that remain uncertain, such as the yeasts' primary ecological niche. Therefore, a broad and in depth study about the habitat of the yeast is an important issue to be investigated. Here, it is described a study aimed to isolate the yeast from tree detritus of eucalyptus, almond, mango

and succulent plants such as cacti, both during rainy and dry seasons during the period of January 2005 thru June 2007.

2.2 Materials and Methods

2.2.1 Culture Media Preparation

2.2.1.1 Niger Seed Agar with Biphenyl (NSA+B)

The NSA+B is a differential medium that has melanin precursors and, therefore, allows to distinguish members of *C.neo/gat* macroscopically. The culture medium was made as described by Castañeda and according to a recipe developed by Staib in 1973 (Castañeda, 2002). Seventy (70)g of *Guizotia abyssinica* seeds were pulverized and added to 1L distilled water and then boiled for 15 minutes. The mixture was filtered through a sterile cheesecloth and an extract was collected. The extract was mixed with 1g of glucose, 1g of creatinine, 1g of potassium phosphate dehydrated and 20g of agar. The mixture was stirred and boiled until it was homogeneous. The medium was sterilized by the autoclave at 15psi, 15 minutes at 121ºC. The sterilized medium was cooled until reaching approximately 50ºC; at this point 20mL of antibiotic solution (penicillin at 20U/mL, streptomycin at 40U/mL and chloramphenicol at 20U/mL) and 20mL of biphenyl (5g of biphenyl salt in 20mL of absolute alcohol) were added, then mixed and poured into Petri plates.

2.2.1.2 Canavanine Glycine Bromothimol Blue Agar (CGB)

This medium, developed by Kwon-Chung *et al*. (1983), differentiates between the species of *C.neo/gat*. It consists of two solutions. Solution A contains 10g of glycine, 1g of KH_2PO_4, 1g of $MgSO_4$, 1mg thiamine-HCl and 30mg of L-canavanine sulfate diluted in 100mL of distilled water. The pH of the solution was adjusted to 5.6 and the solution was sterilized by filtration on a 0.22µm membrane filter, and finally stored at 4ºC. Solution B was made by dissolving 0.4g

bromothymol blue in 64mL 0.01N NaOH with an addition of distilled water to a final volume of 100mL.

For the preparation of 1 liter of CGB, 20mL of solution B were added to 880mL of distilled water containing 20g of Bacto agar (Difco). This solution was sterilized by autoclave as described above. After reaching 50ºC, 100ml of solution A were added and the media poured into Petri plates.

2.2.2 Sampling Areas and Sample Collection

All samples were collected from the western region of Puerto Rico which included the municipalities of Aguadilla, Aguada, Rincón, Añasco, Mayagüez, Hormigueros, Cabo Rojo, Lajas, San Germán, Maricao, Las Marías, San Sebastián, Moca, Isabela, Quebradillas, Camuy, Hatillo, Lares, Sabana Grande, Guánica, Yauco and Guayanilla (Fig. 2.1). These region has two forests which include the sub-tropical moist Guajataca Forest, with an average temperature of 24.9ºC, and annual precipitation of 192.532cm, and the sub-tropical xerophytes Guánica Forest, with an average temperature of 30ºC (on a full sunny day) and 5ºC (at nights), with an annual precipitation of 73.66 cm. The global position coordinates (GPC) of the sampled area were determined using a Garmin GPS 12 channel, software 4.58.

Samples of accumulated organic detritus were collected from holes in the plants or trees, from the ground around roots and from stems of *Eucaliptus camandulensis* (eucalyptus), *Terminalia cattapa* (almond), *Manguifera indica* (mango) and *Persea sp.* (avocado). Also, swab samples were obtained from succulent plants such as *Opuntia moniliformis* and *Cephalocereus royenii* showing visible lesions (Randhawa *et al*., 2005). Both techniques were used, depending on the availability of the detritus. Additionally, 3 samples were collected from a pigeon barn in Aguada.

2.2.3 Sample Processing

The detritus samples were processed by adding 5g of detritus to a 100mL dilution bottle with 25mL of phosphate buffer saline (PBS) at pH of 7.2. This mixture was mixed and then filtrated in a 20mL tube using sterile cheesecloth. The tube was vortexed at maximum speed (3200 rpm) for 5 minutes and with a resting time of 10 minutes, to allow the separation of the heavy particles. An antibacterial antibiotic solution (penicillin at 20U/mL, streptomycin at 40U/mL and chloramphenicol at 20U/mL) was added to the filtrate, then spreaded onto NSA+B. The NSA+B plates were incubated at 27ºC for twelve days and verified for the presence of mucoid brown colonies every three days, for twelve days. The samples collected by swabbing were also inoculated onto NSA+B plates; incubated and verified as described previously.

2.2.4 Detection of bacteria in detritus samples

Since it has been proven that biotic factors, such as bacteria, can affect the viability of *C. neo/gat* in the environment (section 1.2.2), a preliminary test to isolate them was performed. Detritus samples collected during the rainy season were processed by serial dilutions as follow: 1g of each detritus was diluted to 10mL PBS; the mixture was shaken by hand and then filtrated in a 20mL tube using sterile cheesecloth; the tube was vortexed at maximum speed for 5 minutes and with a resting time of 10 minutes to separate the heavy particles. From this filtrated aliquots of 100μL and 10μL were spreaded onto nutrient agar (NA) and MacConkey agar plates to have a final sample dilution of 10^{-3} and 10^{-4}, respectively. The plates were incubated at 27ºC for 24hrs. The morphology of the bacterial isolates was determined by Gram staining. The MacConkey positive isolates were tested for the ability to ferment glucose, sucrose and lactose and to produce hydrogen sulfide (H_2S) using the triple sugar iron agar medium (TSIA).

2.2.5 C. neo/gat candidate Selection

The selection of the candidates belonging to the *C. neo/gat* was made at macroscopic level by testing the activity of the phenoloxidase enzyme. In NSA, *C. neo/gat* grows brown pigmented and also mucoid. To confirm that the brown pigmented colonies belonged to the genus *Cryptococcus*, a negative staining technique was used to determine the presence of capsule (Harley, 2005). To observe the capsule, 10 μL of a cell suspension were mixed with an India Ink drop and observed in an Olympus BX41TF microscope. The sample was photographed using a RT Spot Digital Camera (Diagnostic Instrument). For better capsule observation, the Nomarsky Microscopy technique was performed in the Center of Microscopy at the Biology Building at the RUM. For this technique, the sample was processed by adding a drop of water on a glass slide and spreading the sample on it. After genus confirmation, samples were tested for the ability of the yeast to grow at 37ºC. The purified positive candidates were streaked onto NSA plates and also Sabouraud Dextrose Agar (SDA) slants, and then incubated at 27ºC for three days for further biochemical tests.

2.2.5.1 Biochemical tests

Biochemical tests were performed to confirm the identities of the positive candidates. The tests included urease enzyme activity and the carbohydrate assimilation tests, following the protocols described in the Difco Manual. In order to determine the species of the *C. neo/gat* isolates, growth in the canavanine glycine bromothymol blue (CGB) medium was determined. *Cryptococcus gattii* resists the canavanine and grows well in CGB due to the ability to use glycine as a sole carbon and nitrogen source. A positive reaction is observed when the color of the medium changes from yellow to blue. *Cryptococcus neoformans* is sensitive to canavanine and cannot use glycine as a sole carbon and nitrogen source, therefore does not grow in CGB,

keeping the medium yellow. The candidates were stored in SDA slants at 4ºC and at -80ºC in 20% glycerol as cryoprotectan.

2.3 Results

After analyzing 710 samples from *C. royenii, M. intortus, O. moniliformis* (Fig. 2.2), *T. cattapa* (almond), *M. indicant* (mango), *Persea. sp*, (avocado) and *Pisonia albida* (corcho bobo) (Fig. 2.3), a total of 23 *C. neo/gat* candidates were isolated (Table 2.1). Table 2.3 shows the isolates distribution based on the municipality and the material sampled. The identification was made according to the phenoloxidase (Fig. 2.4) and urease activities, presence of capsule (Fig. 2.5), growth at 37ºC and carbohydrate assimilation. These isolates represent a 3% of total recovery from the southwestern region of Puerto Rico (Table 2.1). Twenty of the candidates were isolated from plant material from the southwestern part of Puerto Rico, including the Guánica Dry Forest, and other three samples came from pigeon droppings collected in Aguada. Ten of twenty three isolates (44%) were recovered from the Dry Forest, specifically from visible lesions in the surface of cactus *Cephalocereus royenii;* and ten candidates from trees. To determine the identities of the candidates, the canavanine glycine bromothymol blue medium was used. The differential medium showed that nineteen (83%) of the candidates belonged to *C. gattii* species while only four (17%) were *C. neoformans*.

Table 2.1 Distribution of the samples collected in this study to detect the presence of *C. neo/gat* at the Western area of Puerto Rico

Sample	Sample Collected	Positive Samples	*Percentage of Recovery (%)
Terminalia catappa	250	3	1.0
Eucalyptus sp.	45	0	0.0
Manguifera sp.	150	5	3.0
Persea sp.	50	1	2.0
Pisonia sp.	10	1	10.0
Cephalocereus royenii	100	10	10.0
Opuntia moniliformis	50	0	0.0
Melocactus sp.	50	0	0.0
Columbia livia (feces)	5	3	60.0
TOTAL	**710**	**23**	**3.2**

***These values were calculated by dividing the total number of samples collected from each source by the number of *C.neo/gat* total of positive results for that source.**

Table 2.2 Distribution of *C. neo/gat* by municipalities and candidates identification

Candidates ID	Collection	GPC	Source	CGB
VMGc-3	Meseta, BSG	17º57’04.13”N 66º50’22.35’W	*C. royenii*	*C. gattii*
VMGc-4	Meseta, BSG	17º57’04.13”N 66º50’22.35’W	*C. royenii*	*C. gattii*
VMGar-7	Meseta, BSG	17º57’04.13”N 66º50’22.35’W	*Pisonia sp.*	*C. gattii*
PJGc-11	Jungla Beach, Guánica	17º56.33.45’N 66º57’50.24”W	*C. royenii*	*C. gattii*
VBGc11	Ballena, BSG	17º57’32.88”N 66º51’43.15”W	*C. royenii*	*C. gattii*
VBGc16	Ballena, BSG	17º57’32.88”N 66º51’43.15”W	*C. royenii*	*C. gattii*
VBGc-17	Ballena, BSG	17º57’32.88”N 66º51’43.15”W	*C. royenii*	*C. gattii*
VBGc22	Ballena, BSG	17º57’32.88”N 66º51’43.15”W	*C. royenii*	blue
VBGc-27	Ballena, BSG	17º57’32.88”N 66º51’43.15”W	*C. royenii*	*C. gattii*

VBGc28	Ballena, BSG	17º57’32.88”N 66º51’43.15”W	*C. royenii*	*C. gattii*
VBGc32	Ballena, BSG	17º57’32.88”N 66º51’43.15”W	*C. royenii*	*C. gattii*
Ym-12	Yauco	18º00’52.44”N 66º52’23.07”W	*Manguifera sp.*	*C. gattii*
Ym-42	Yauco	18º00’52.44”N 66º52’23.07”W	*Manguifera sp.*	*C. gattii*
Ym-46	Yauco	18º00’52.44”N 66º52’23.07”W	*Manguifera sp.*	*C. gattii*
Ym-47	Yauco	18º00’52.44”N 66º52’23.07”W	*Manguifera sp.*	*C. gattii*
Ym-63	Yauco	18º00’52.05”N 66º52’23.88”W	*Manguifera sp.*	*C. gattii*
Yag-44	Yauco	18º00’52.05”N 66º52’23.88”W	*Persea sp.*	*C. gattii*
La-13	Lajas	18º02’00.80”N 67º06’30.30”W	*T. catappa* detritus	*C. neo*
CRa2001 (PR)	Cabo Rojo	18º07’53.26”N 67º11’07.92”W	*T. catappa* detritus	*C. gattii*

CRa37	Cabo Rojo	18º07’53.26”N	*T. catappa*	*C. gattii*
		67º11’07.92”W	detritus	
Ap1	Aguada	18º20’59.95”N	*C. livia*	*C. neo*
		67º11’59.20”W	excreta	
Ap3	Aguada	18º20’59.95”N	*C. livia*	*C. neo*
		67º11’59.20”W	excreta	
Ap5	Aguada	18º20’59.95”N	*C. livia*	*C. neo*
		67º11’59.20”W	excreta	

GPC= global positioning coordinates; CGB= canavanine glycine bromothymol blue

Table 2.3 Sources and municipalities where positive isolation of *C. neo/gat* was obtained

Municipality	Source and Quantity	*Percentage of Recuperation by tree/plant species (%)	◈Percentage of Recovery by municipality (%)
Cabo Rojo	*T. catappa* (3)	13	13
Guánica	*C. royenii* (10)	44	48
	Pisonia sp. (1)	4	
Yauco	*Manguifera sp.* (5)	22	26
	Persea sp. (1)	4	
Aguada	*C. livia* excreta (3)	13	13
TOTAL	**23**	**100**	**100**

***These values were calculated by dividing the number of each positive sample by the total of positive samples collected.**

◈ These values were calculated by adding the percentage of recuperation by tree/plant of each municipality

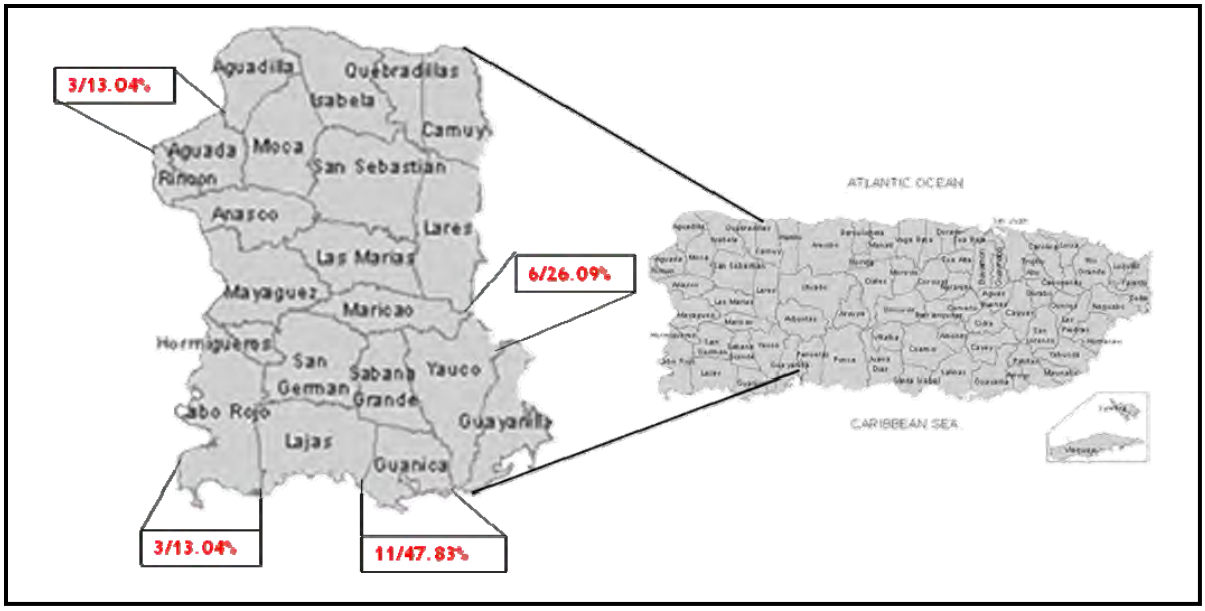


Figure 2.1 Municipalities of the western area of Puerto Rico that were sampled. The numbers on the municipalities correspond to the *C. neo/gat* positive isolates found.

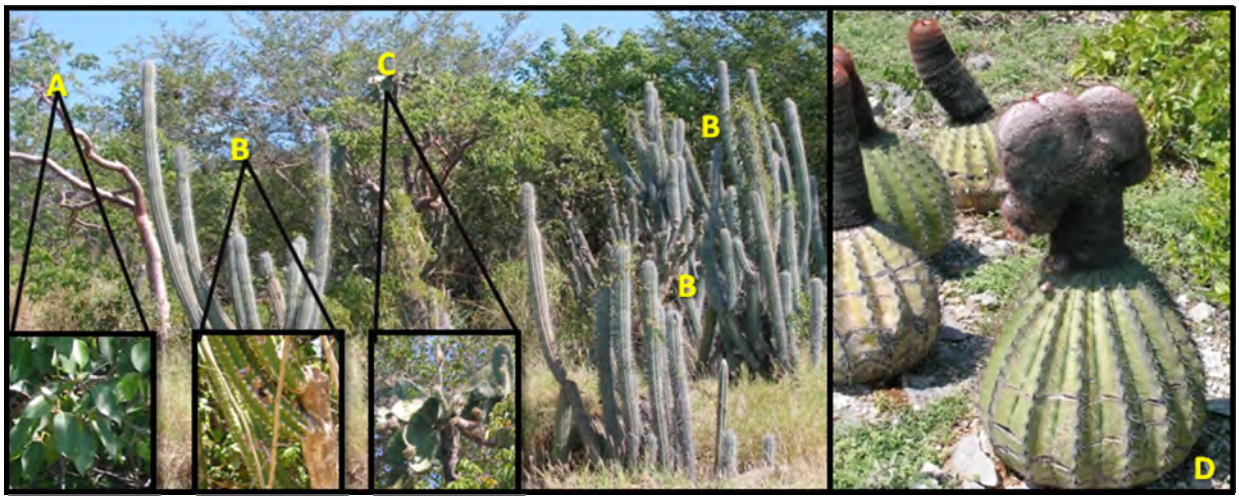


Figure 2.2 Succulent plants and trees sampled from the Dry Scrub Forest at the Guánica Dry Forest. A, represents a corcho bobo tree (*Pisonia albida*); B-D, represent cacti: (B) *Cephalocereus royenii*, (C) *Opuntia moniliformis* and (D) *Melocactus intortus*, respectively.

Figure 2.3 Trees and succulent plants sampled and sample collection techniques performed in this study. A, *C. royenii* lesion (arrow indicates sample lesion); B, *Pisonia sp.* tree; C, *T. catappa* tree and D, *Manguifera sp*. tree. E-F Collection of samples using the acquisition of tree detritus and with the swabbing technique, respectively; the arrows indicate the detritus collected and the hole sampled.

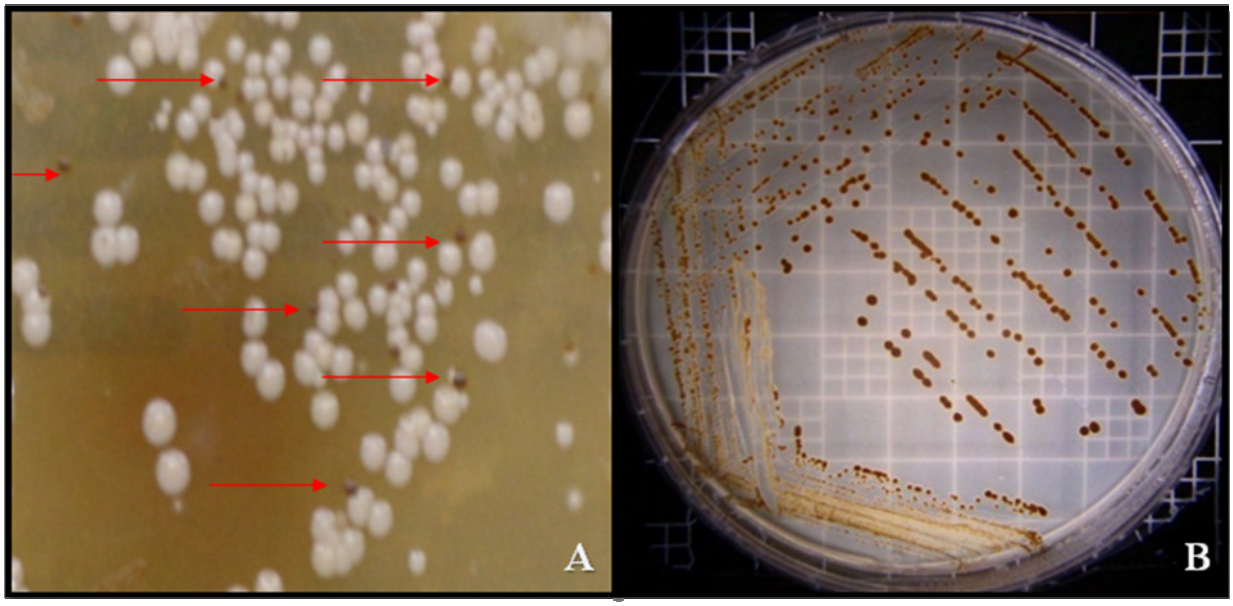


Figure 2.4 *C. neo/gat* grows as brown pigmented colonies on Niger Seed Agar (NSA). (A) The arrows show a typical pigmented colony of *C. neoformans* var. *grubbi* on NSA. B shows a *C.neo/gat* candidate streaked onto the same culture medium.

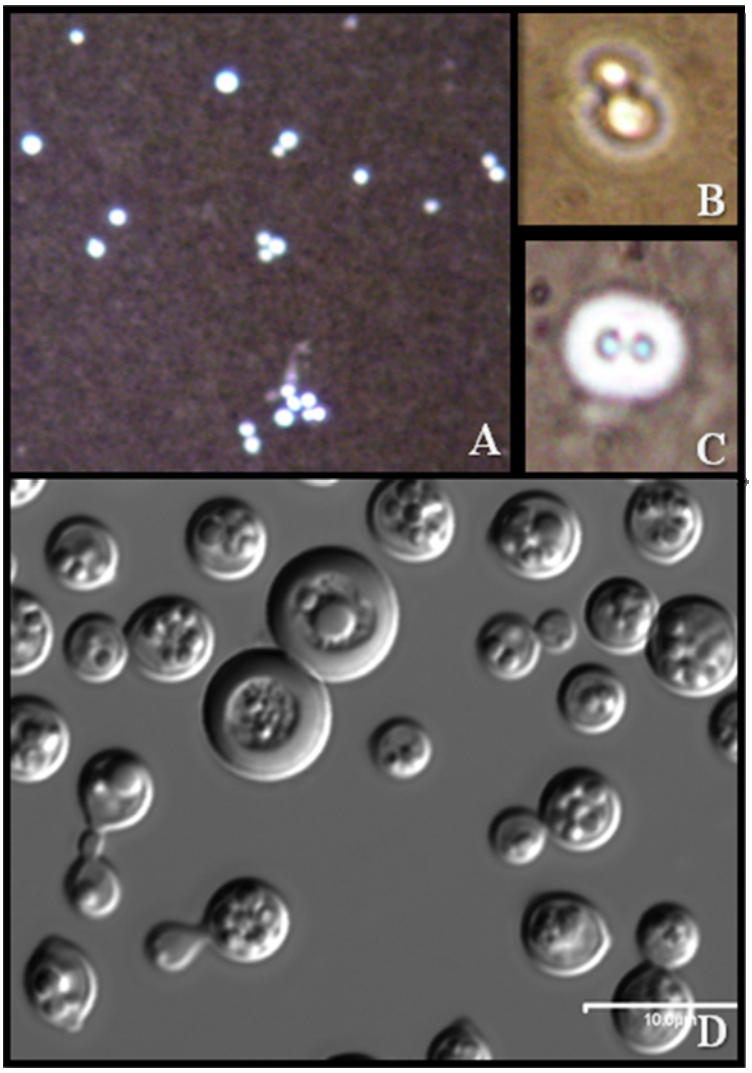


Figure 2.5 Typical microscopy of *C. neo/gat.* Capsules observed by negative staining (A-C) and with (D) Nomarsky Microcopy technique performed at the UPR-Mayagüez (Biology Department Microscopy Center)

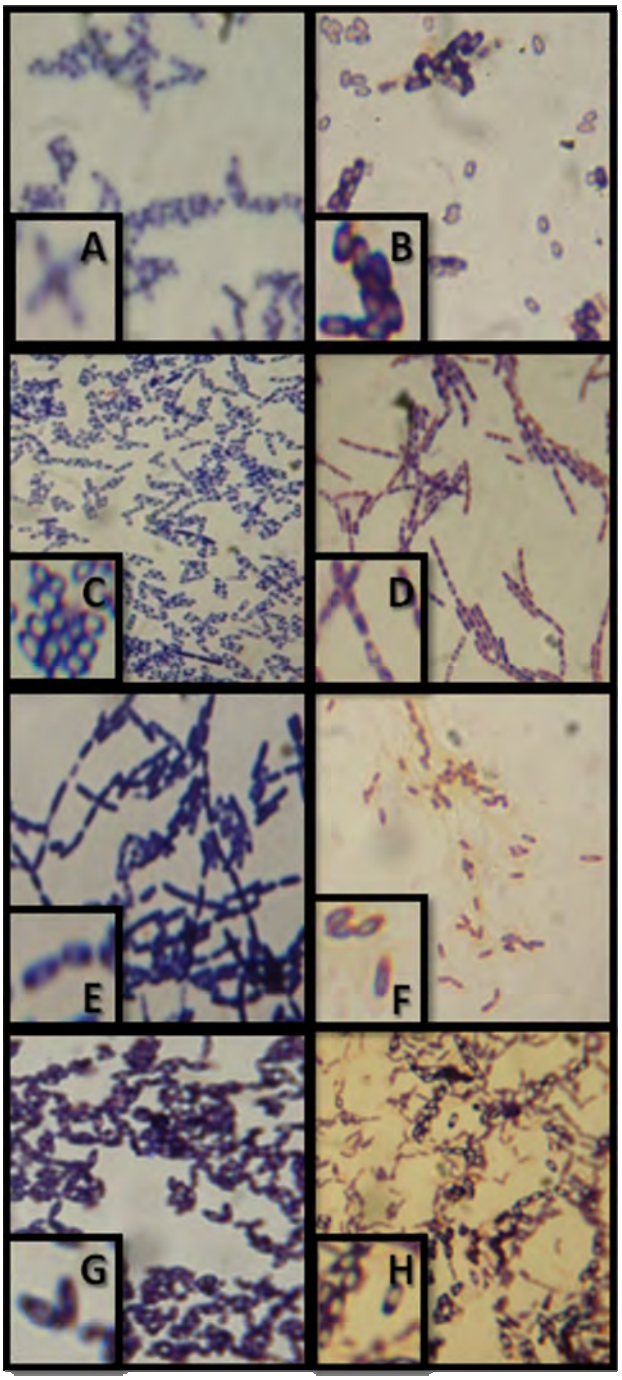


Figure 2.6 Gram staining of the bacteria isolated from cacti detritus during the rainy season at the Guánica Dry Forest. All bacterial specimens were gram-positive bacilli. The spore staining (data not shown) suggests that the isolated bacteria belonged to the genus *Bacillus*.

2.4 Discussion

The main objective on this section was to determine *C. neo/gat* complex new ecological niches in the western area of Puerto Rico. The majority of the isolates (11) were from Guánica, specifically from Ballena and Meseta trails, in the southern part of the Guánica Dry Forest. A previous study indicated that the composition of detritus positive for the presence of *C.neo/gat* was mainly sandy (Callejas *et al.*, 1998). Both trails of the Forest were located within the Dry Scrub Forest, which is composed mainly on sandy red, thinly covered by limestone bedrock soil (Farnsworth, 1991). These data contrast with the previous findings, since different soils may provide different nutrients and humidity conditions. In both trails, the vegetation that predominates is twisted trees and cacti. All the remaining candidates, but one, were isolated from lesions on the surface of *C. royenii* (Table 2.2; Fig. 2.1 A). The CGB medium suggests that all the candidates isolated in the Forest belonged to *C. gattii*. These findings were unexpected because literature states that *C. gattii* is present mainly in humid tropical and subtropical areas (MacDougall and Fyfe, 2006; Duncan C., 2005). In contrast, our findings suggest the presence of *C. gattii* related to dry and hostile environment exposed to direct sunlight, the ones that predominates in the sampled area. According to the definition of water activity (measurement of the energy status of the water in a system and defined as the vapor pressure of water divided by pure water at the same temperature), when temperature increases the Aw increase, providing microorganisms the right conditions for growing. For fungi, the inhibitory Aw is 0.70, indicating that in the cacti, the Aw may be higher for the proliferation of *C.neo/gat*. According to this fact, cacti may provide a suitable water source, which can maintain the *C. neo/gat* cells viable. Also, the melanin production by *C. neo/gat* protects the cells from UV radiation, giving also, viability to the cells, helping them to survive on the cacti. We propose that the presence of *C.neo/gat* in

the Guánica Dry Forest is due to a colonization process in lesions initiated or birds or insects. Just recently, an interview with the entomologist Dr. Alex Segarra, reveals the risk that insects such as *Hypogeococcus pungens* represent to the cacti in Puerto Rico (Del Valle, 2008). *H. pungens* is well known to produce the visible lesions on cacti between Cabo Rojo to Guayama, maybe providing better access to the yeast to colonize and extract the cacti nutrients. The arrival of this insect from South America may support the hypothesis of insects acting as mechanical vectors for the yeast. Literature has described that members of the genus *Cryptococcus* had been previously found on some species of cacti, but never the pathogenic species *C. neoformans* nor *C. gattii* (Rosa *et al*., 1994). A possible explanation for the presence of *C. gattii* in such hostile environment could be that the cacti lesion provides water, nutrients and protection from sunlight, needed for the yeast to survive on it. We also isolated members of the *C.neo/gat* from previously reported ecological niches such as *T. cattapa* (Fig. 2.1 C) in the municipalities of Cabo Rojo and Lajas, *M. indicans* (Fig 2.1 D) from Yauco, and in *C. livia*, excreta from Aguada (Table 2.1). Also, isolation of the yeast from novel ecological niches such as *Persea sp*. from Yauco, and *P. albida* from the Guánica Dry Forest was achieved. The CGB suggest that one of the isolates of *T. catappa* and all the isolates from *C. livia* belong to *C. neoformans*, while the remaining isolates from trees belong to *C. gattii*.

C. neo/gat was isolated from four municipalities located at the southwestern and one from the northern region of Puerto Rico, only during the dry season. Preliminary data suggest the idea that bacteria are present in cacti and as a consequence, affect the viability of the *C. neo/gat* during the rainy season. Another study related to the inhibitory capability of bacteria to *C. neo/gat* was performed by Abou-Gabal and Atia (1978). They proved that isolated bacterial flora from the intestinal contents of apparently healthy pigeons showed a complete inhibitory

effect on the growth of *C. neoformans in vitro.* To determine whether or not the samples contain the bacterial flora described above, detritus was processed (section 2.2.4) in order to determine the predominant bacterial flora present. The majority of the colonies isolated were identified as gram-positive, sporulating bacilli, suggesting that they belonged to the genus *Bacillus* (Fig 2.6). This is one of the genera reported to have the ability to inhibit *C. neo/gat*. The bacteria that grew on MacConkey plates were tested for their ability to ferment glucose, lactose and galactose, including the hydrogen sulfate production (data not shown). The tests showed that one of the candidates had a non fermentative pattern for the three sugars, suggesting the presence of *Pseudomonas*, another bacterium that may inhibit *C.neo/gat* present in detritus during the rainy season.

The recovery of *C.neo/gat* from tree detritus has been extensively reported but not from tropical or dry climates. These findings represent a novel adaptation of the yeast to dry environments, being the cacti a realized or transitory ecological niche. Unexpectedly, almost all the isolates of *C. neo/gat*, including those from the Guánica Dry Forest, were recovered using the "swabbing" technique. This phenomenon could indicate that the concentration of the yeast in those areas was low. Direct swabbing may be the best technique to access all the possible areas in which the yeast can not be reached, avoiding the yeast dilution during the detritus processing. If we found *C. gattii* in those hostile areas, then is it possible that plants represent mechanical vectors of the yeast. According to our results and to preliminary studies performed in Mona Island, it is very likely to find *C. gattii* all over the Caribbean.

2.5 Findings and Conclusions:

- *Cryptococcus neoformans/gattii* complex isolates were recovered from trees and cacti from the southwestern part of Puerto Rico; nineteen isolates of *C. gattii* and four of *C. neoformans*.
- Preliminary studies of bacteria present in detritus during the rainy season showed the presence of genera previously reported as inhibitory for *C.neo/gat*, such as *Bacillus spp*.
- The isolation of *C. gattii* from dry and hostile environments such as the Guánica Dry Forest, specifically from the cactus *Cephalocereus royenii*, is the first isolate reported in the literature.
- It was confirmed that *C. gattii* can be isolated from *Terminalia catappa* and *Manguifera indica* and that *C. neoformans* was isolated from *Columbia livia* excreta.
- This is the first time that *C. gattii* is isolated from trees such as *Persea sp*. in Puerto Rico.
- Swab technique proved to be the most appropriated approach to isolate the yeast from trees and plants instead of detritus collection.

3 Comparison between the common biochemical and physiological methods for the identification of *Cryptococcus neoformans/gattii* with BIOLOG Microstation System, a rapid identification method

3.1 Introduction

Before the implementation of molecular techniques in diagnostic microbiology, morphology and biochemical tests were the only techniques used to characterize and identify microorganisms. In clinically important microorganisms, waiting for the test reactions resulted in a delayed diagnosis and in many cases in the death of the patients. Cryptococcosis diagnosis is one of these examples. In the case of *C.neo/gat*, the traditional biochemical tests used to identify and diagnose the mycosis are the urease and phenoloxidase activities, assimilation of most sugars with the exception of lactose and melobiose; mannitol production and reaction on CGB medium (for more details, see sections 1.2.1.2, 1.2.1.3 and 1.2.3). In order to reduce the waiting time for the results, therefore, for the diagnosis, the development of new identification techniques has arisen. The VITEC Yeast Biochemical Card, API 20C, RapID Plus System and Minitek System are some of the rapid identification systems designed for yeasts identification (Smith *et al.*, 1999). Among these methods, the API20C has been proved to be more reliable in order to correctly identify isolates of *C. neo/gat*, with the disadvantage of about 72 hr waiting time of incubation. One of the recent technologies developed for this purpose is the BIOLOG Microstation System. The system consists of a 96 wells on plate containing different carbon sources that can be assimilated or oxidized by the tested microorganism. The plate is read by a computer which has a database containing the different reactions patterns of known microorganisms. The reading corresponds to a change in optical density on each carbon source as a result of a positive reaction with tretrazolim violet as indicator of oxidation present in each well. Then, the system searches its internal database and identifies the microorganism according to its reaction pattern on each carbon source. The system can provide a genus and species identification pattern, being above 95% a reliable value for a correct identification. A

disadvantage of the BIOLOG System is that less than 25 positive wells is not enough to generate an identification result. The system can identify medically important and environmental yeasts at a time range of 48 to 72 hrs. In the case of *C. neo/gat*, the BIOLOG database does not have the biochemical identification patterns of the anamorph stage (*Cryptococcus*) but it has the biochemical patterns for the telomorph stage (*Filobasidiella*) (BIOLOG Microstation Manual: Yeast Data Base 3.5).

The objective of this section is to determine whether or not the BIOLOG Microstation System is reliable for the identification of *C. neo/gat* in its anamorph stage. This will be achieved by comparing the results of different assays performed on *C. gattii* C77 strain, as well with other strains of *C. neo/gat* isolated as part of this investigation. Also, the Biolog Microstation will be used as a tool to identify specific carbon sources that are unique to *C. neo/gat* (serotypes A, B C) in order to further develop a rapid identification tool that may be used with environmental and clinical samples.

3.2 Materials and Method

3.2.1 Description of the strains used and traditional biochemical test

Strains A11, B92 and C77, used as control, were provided by Mrs. Elizabeth Quintero, isolated from Colombia as part of her thesis work. They correspond to *C. neoformans* var. *grubii* and *C. gattii*, serotypes B and C, respectively. The strain A11 was isolated from pigeon droppings; and strains B92 and C77 were isolated from eucalyptus trees from Colombia (Quintero *et al.*, 2003). The control strains were tested for their ability to produce the enzymes phenoloxidase and urease, their assimilation of lactose, inositol and melibiose, and reaction on CGB medium. Also, other isolates of *C.neo/gat* (CRa37, CRa2001- obtained from almond tree

from Cabo Rojo and Ap1- obtained from *C. livia* dropping from Aguada) were included in this section as experimental strains, to determine the reliability of the system.

3.2.2 BIOLOG Microstation Test

The BIOLOG test was performed according to the manufacture's instructions, with few modifications. Briefly, strain C77 was streaked onto a SDA plate and incubated at 27ºC for 72hrs. Then a colony was streaked on a BIOLOG Universal Yeast medium (BUY) and incubated as mentioned above. When the yeast showed visible growth on the medium, cells were removed with a sterile swab and suspended in 15ml of a sterile 0.85% saline solution until it reached 46% transmittance using a turbidometer provided by the BIOLOG System. A 150µL of the solution were dispensed on the BIOLOG Yeast MicroPlate wells (YT), each containing a different carbon source. The MicroPlate was incubated at 27°C; spectrophotometric readings were made at 24, 48, 72 hrs, or until a sufficient metabolic pattern was observed (change of color from transparent to violet). The plates were read using the Biolog MicroStation Reader, which has a database with 267 species of yeasts represented (Biolog Manufacturer Manual: Yeast Database 3.50). The reading step was performed eight times to obtain an average result. Another modification of this protocol consisted of the addition of cells to the saline solution until it reached 30% of transmittance, which means a higher cell concentration than recommended by the manufacturer.

3.3 Results

All the *C. neo/gat* strains analyzed using the BIOLOG Microstation System, oxidized L-aspartic acid, L-glutamic acid, L-proline, D-gluconic acid, gentibiose, maltose and N-acetyl-glucosamine (Table 3.1). Therefore, this set of carbon sources could be used to generate a test to identify members of the complex. Also, the BIOLOG analysis allows us to detect any unique carbon source metabolized by the *C. neo/gat* strains. The analysis of the three serotypes of *C. neo/gat* showed unique oxidation pattern for D-sorbitol (ser. A), Tween 80 (ser. B), and salicin (ser.C). The experimental strains also oxidized unique carbon sources, corresponding to turanose (CRa2003) and sucrose (CRa37) (Table 3.2). Experimental strain Ap1 did not oxidize any distinctive carbon source. Strain CRa2001 was the only one the Biolog System was able to identify as the telomorph stage *Filobasidiella bacillisporus*. These patterns of carbon sources oxidation correspond to a 46% of transmittance, the one that the manufacturer suggests. A 30% of transmittance showed a reduction in oxidation capabilities.

Table 3.1 Common carbon sources oxidized by *C. neo/gat* strains

Strain	Carbon sources oxidazed
A, B, C	L-aspartic acid, L-glutamic acid, L-proline, D-gluconic acid, gentibiose, maltose, N-acetyl-glucosamine
CRa2001, CRa37, Ap1	Formic acid, succinic acid, L-aspartic acid, L-glutamic acid, L-proline, D-gluconic acid, dextrin, cellobiose, gentigiose, maltose, maltotriose, D-melezitose, D-melobiose, palatinose, D-raffinose, stachiose, N-acetyl-D-glucosamine, a-D-glucose, D-galactose, D-psicose, L-sobose, D-sorbitol, D-arabitol, xylitol, glycerol

Table 3.2 Unique carbon sources oxidize by strains CRa2001, CRa37 and Ap1 *C. neo/gat*

Sample	Carbon sources oxidized
A 11	L-glutamic acid, D-sorbitol
B 92	Tween 80
C 77	Maltotriose, salicin
CRa2001	Turanose
CRa37	Sucrose

YT MicroPlate™

A1 water	A2 acetic acid	A3 formic acid	A4 propionic acid	A5 succinic acid	A6 methyl succinate	A7 L-aspartic acid	A8 L-glutamic acid	A9 L-proline	A10 D-gluconic acid	A11 dextrin	A12 inulin
B1 cellobiose	B2 gentiobiose	B3 maltose	B4 maltotriose	B5 D-melezitose	B6 D-melibiose	B7 palatinose	B8 D-raffinose	B9 stachyose	B10 sucrose	B11 D-trehalose	B12 turanose
C1 N-acetyl-D-glucosamine	C2 a-D-glucose	C3 D-galactose	C4 D-psicose	C5 L-sorbose	C6 salicin	C7 D-mannitol	C8 D-sorbitol	C9 D-arabitol	C10 xylitol	C11 glycerol	C12 tween 80
D1 water	D2 fumaric acid	D3 L-malic acid	D4 methyl succinate	D5 bromo succinic acid	D6 L-glutamic acid	D7 g-amino butyric acid	D8 a-keto-gluturic acid	D9 2-keto-D-gluconic acid	D10 D-gluconic acid	D11 dextrin	D12 inulin
E1 cellobiose	E2 gentiobiose	E3 maltose	E4 maltotriose	E5 D-melezitose	E6 D-melibiose	E7 palatinose	E8 D-raffinose	E9 stachyose	E10 sucrose	E11 D-trehalose	E12 turanose
F1 N-acetyl-D-glucosamine	F2 D-glucosamine	F3 a-D-glucose	F4 D-galactose	F5 D-psicose	F6 L-rhamnose	F7 L-sorbose	F8 a-methyl D-glucoside	F9 b-methyl D-glucoside	F10 amygdalin	F11 arbutin	F12 salicin
G1 maltitol	G2 D-mannitol	G3 D-sorbitol	G4 adonitol	G5 D-arabitol	G6 xylitol	G7 i-erythritol	G8 glycerol	G9 tween 80	G10 L-arabinose	G11 D-arabinose	G12 D-ribose
H1 D-xylose	H2 methyl succinate + D-xylose	H3 N-acetyl-L-glutamic acid + D-xylose	H4 quinic acid + D-xylose	H5 D-glucuronic acid + D-xylose	H6 dextrin + D-xylose	H7 a-D-lactose + D-xylose	H8 D-melibiose + D-xylose	H9 D-galactose + D-xylose	H10 m-inositol + D-xylose	H11 1,2-propanediol + D-xylose	H12 acetoin + D-xylose

The layout of tests in the YT MicroPlate is shown above.

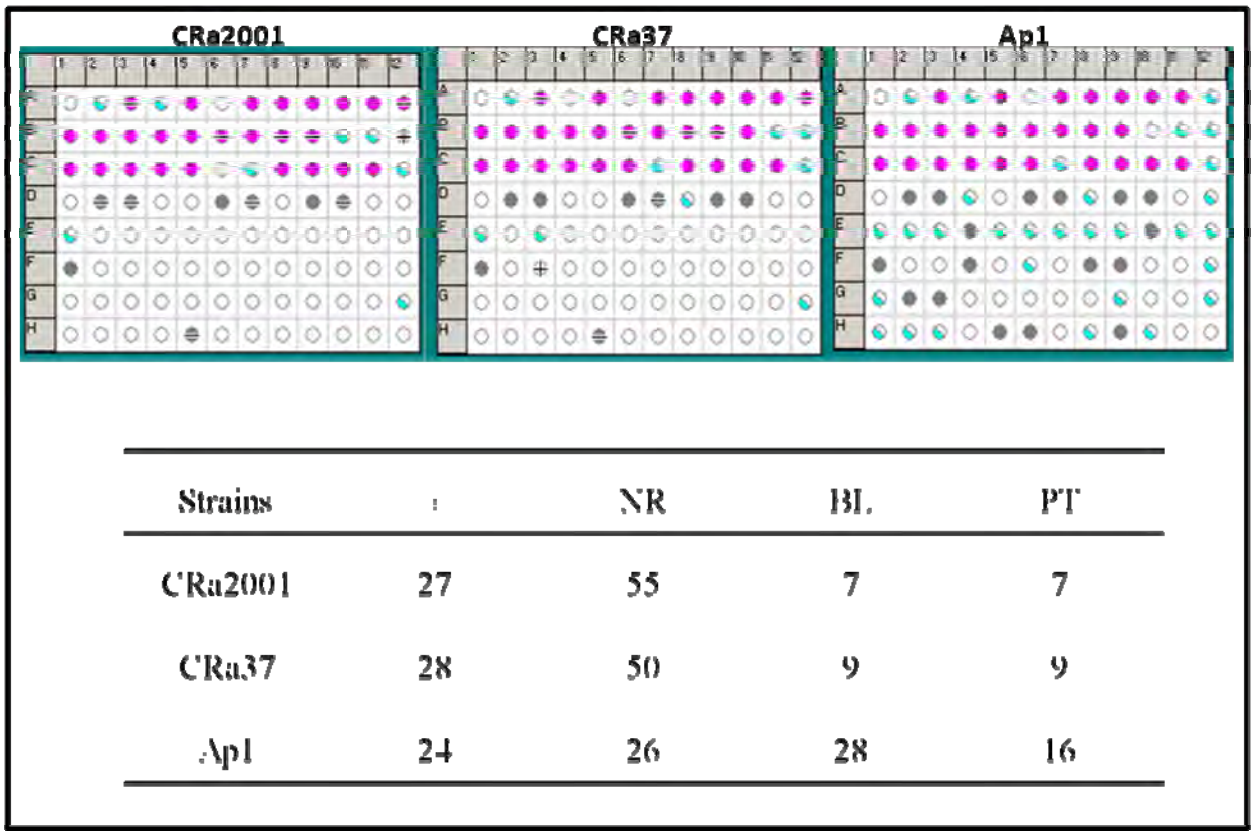
Oxidation Tests Assimilation Tests

Figure 3.1 BIOLOG Microstation Yeast Microplate showing the different carbon sources in the test.

Legend:

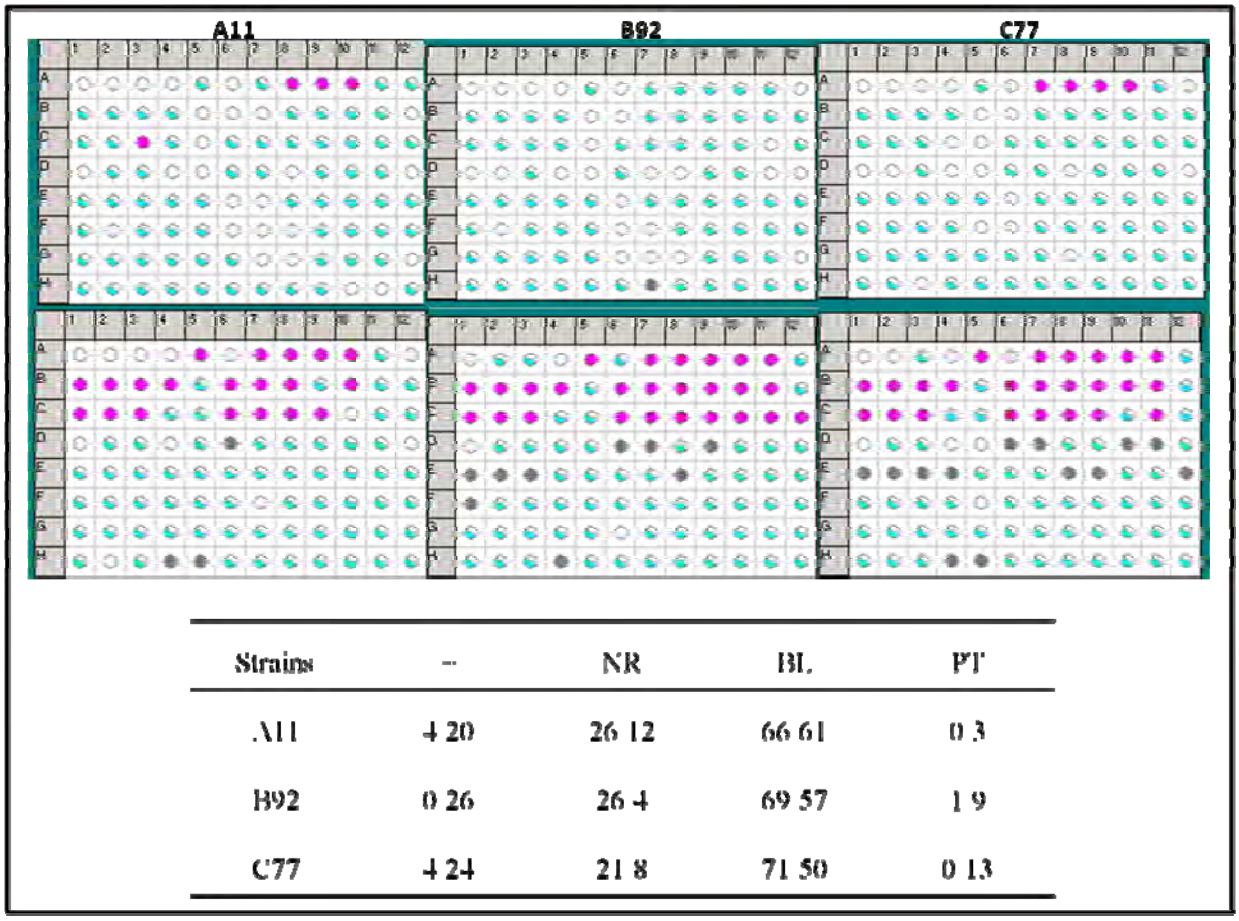
A1-water; A2- Acetic acid; A3-Formic acid; A4-Propionic acid; A5- Succinic acid; A6-Methyl succinate; A7- L-aspartic acid; A8-L-glutamic acid; A9-L-proline; A10-D-gluconic acid; A11-dextrin; A12-inulin; B1-cellobiose; B2-gentibiose; B3-maltose; B4-maltotriose; B5-D-melezitose; B6-D-melibiose; B7-palatinose; B8-D-raffinose; B9-stachyose; B10-sucrose; B11-trehalose; B12-turanose; C1-N-acetyl-D-glucosamine; C2-a-D-glucose; C3-galactose; C4-D-psicose; C5-sorbose; C6-salicin; C7-mannitol; C8-D-sorbitol; C9-arabitol; C10-xylitol; C11-glycerol; C12-tween 80; D1-water; D2-frumaric acid; D3-L-malic acid; D4-methy succinate; D5-bromo succinic acid; D6-L-glutamic acid; D7-g-amino butyric acid; D8-a-keto-glutamic acid; D9-2-keto-D-gluconic acid; D10-D-gluconic acid; D11-dextrin; D12-inulin; E1-cellobiose; E2-gentibiose; E3-maltose; E4-maltotriose; E5-D-melezitose; E6-D-melibiose; E7-palatinose; E8-D-raffinose; E9-stachyose; E10-sucrose; E11-D-trealose; E12-turanose; F1-N-acetyl-D-glucosamine; F2-D-glucosamine; F3-a-D-glucose; F4-D-galactose; F5-D-psicose; F6-L-rahmnose; F7-L-sorbose; F8-a-methyl-D-glucoside; F9-b-methyl-D-glucoside; F10-amygdalin; F11-arbutin; F12-salicin; G1-maltitol; G2-D-mannitol; G3-D-sorbitol; G4-adonitol; G5-D-arabitol; G6-xylitol; G7-i-erythritol; G8-glycerol; G9-tween80; G10-L-arabinose; G11-D-arabinose; G12-D-ribose;H1-D-xylose; H2; methyl succinate+ D-xylose; H3-N-acetyl-L-glutanic acid+ D-xylose; H4-qunic acid+ D-xylose; H5-D-glin+uconic acid+ D-xylose; H6-dextrin+ D-xylose; H7-a-D-lactose+ D-xylose; H8-D-melibiose+ D-xylose; H9-D-galactose+ D-xylose; H10-m-inositol+ D-xylose; H11-1,2-propanediol+ D-xylose; H12-acetoin+ D-xylose

Figure 3.2 YT microplate showing each of the carbon sources analyzed by the BIOLOG System. The plate assay shows the results obtained with strain C77. White circles represent a negative reaction; pink circles represent a positive reaction; grey circles correspond to exceed incubation time reaction; and aqua and white circles correspond to a border line reading.



Strains	+	NR	BL	PT
CRa2001	27	55	7	7
CRa37	28	50	9	9
Ap1	24	26	28	16

Figure 3.3.Carbon sources assimilated by experimental strains using BIOLOG System. Symbols represents: (+) positive; (NR) no reaction; (BL) border lines; (PT) past time reaction



Strains	--	NR	BL	PT
A11	4 20	26 12	66 61	0 3
B92	0 26	26 4	69 57	1 9
C77	4 24	21 8	71 50	0 13

Figure 3.4 Reaction patterns of strains A11, B92 and C77 on YT plate in different assays. The table corresponds to the different reactions of each plate. Symbols represents: (+) positive; (NR) no reaction; (BL) border lines; (PT) past time reaction

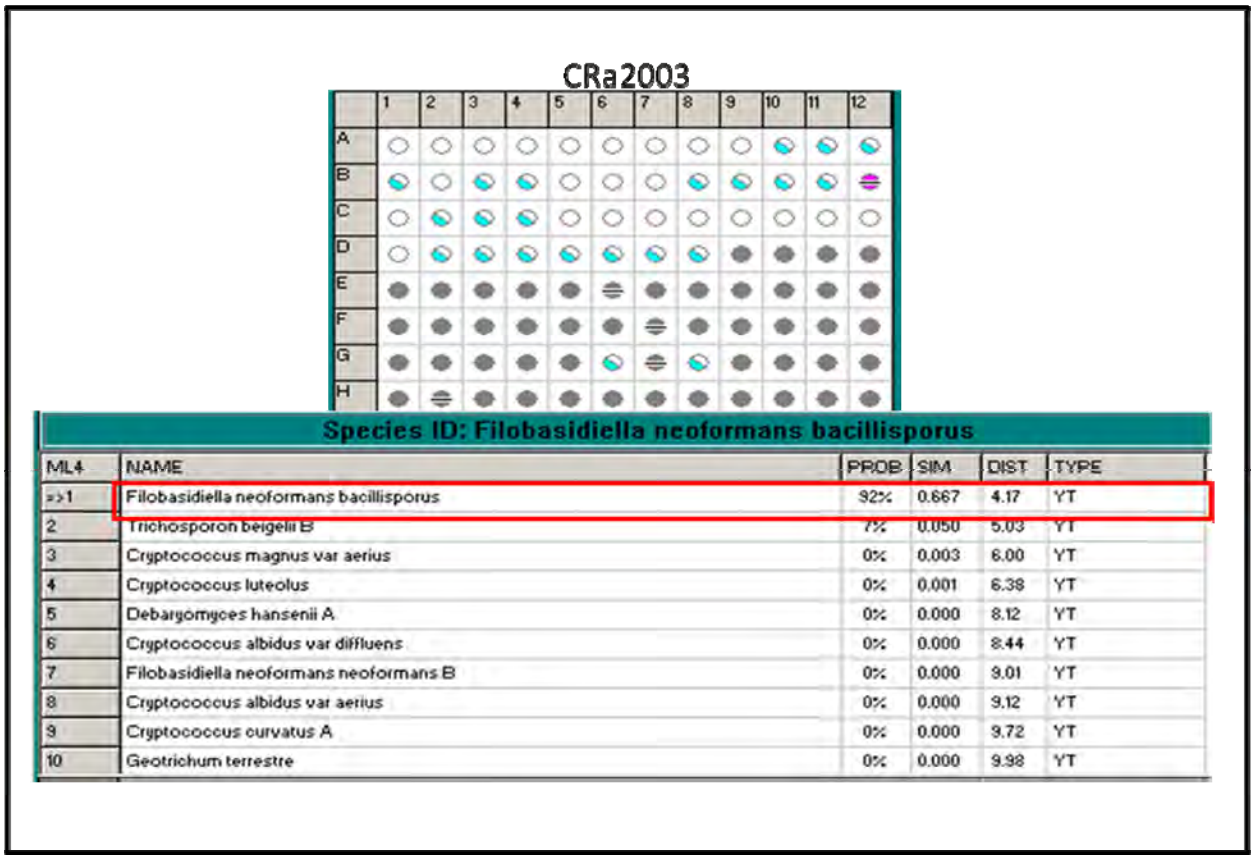


Figure 3.5 Reaction pattern of CRa2003. This figure shows the only specific identification obtained using the BIOLOG database, which specifically relates to *Cryptococcus/neoformans/gattii* telomoph stage, *Filobasidiella*.

3.4 Discussion

The purposes of this thesis objective is: to determine whether or not the BIOLOG Microstation System can identify different strains of *C. neo/gat*, and to identify carbon sources that may be used as a taxonomic tool to distinguish *C. neoformans* vs. *C. gattii*. After the analysis it has been observed that all the carbon sources that showed positive reactions in the BIOLOG MicroPlates were metabolized by *C. neo/gat* by oxidation rather than assimilation. Our data also suggest that the best optic density of the inoculum (when the 0.85% saline solutions is used) is a 46% transmittance as recommended by the manufacturer. However, it was found that better pattern readings occurred at 72 hrs incubation period, longer thatn what it has been established by the manufacturer.

During the first part of the experiment, *C. neo/gat* strains were analyzed both with standard biochemical test and with the BIOLOG Microstation System. As expected, the biochemical tests showed a correct identification of members of *C. neo/gat* within 48 hrs. As for the BIOLOG analysis the assays were performed on five occasions on each strain showing no identifications and irregular patterns. However, a positive reaction to L-aspartic acid, L-glutamic acid, L-proline, D-gluconic acid, gentibiose, maltose and N-acetyl-glucosamine was consistently observed. Therefore, this set of carbon sources can be used to generate a new panel for the identification of members of *C. neo/gat.* However, further specificity analysis is needed in order to rule out other important genera of yeast such as *Candida*, *Rhodoturula* and *Saccharomyces*. If the carbon sources mentioned above are specific to the complex, then it would be necessary to include these results in the BIOLOG System Database to improve the correct identification of the yeasts complex.

The System was also used to identify unique carbon sources between similar strains, in order to identify a possible molecular marker for the rapid identification of the yeast. Table 3.2 indicates which carbon source was unique for each of the strains analyzed. The BIOLOG analysis showed that serotype A11 oxidizes sorbitol and L-glutamic acid (Table 3.3; Fig 3.4). Since L-glutamic acid is oxidized also by strain C77, the appropriate carbon source to be tested as a molecular marker could be sorbitol. In serotype B92 the unique carbon source is Tween 80 whereas in serotype C the oxidation occurs in maltotriose and salicin (Table 3.4; Fig 3.4). Strains CRa2001 and CRa37 oxidized turanose and sucrose, respectively. Any of these unique carbon sources can be used as a starting point to determine the enzymes that catalize the corresponding oxidation. This can be performed by determining the gene which encodes for this activity, by *in silico* analysis. Hypervariable could be used in an array as a discriminatory tool to detect the *C. neo/gat* complex specifically the serotypes of the yeast, directly from environmental and clinical samples. In order to generate this tool, additional experimentation will be required. It is important to test if the putative molecular marker can identify yeasts other than *C. neo/gat*.

Also, *C. neo/gat* strains and candidates were tested to determine if the System can be used as a fast identification tool. In the analysis of the samples isolated from Puerto Rico (Table 3.4) only CRa2001 was identified, but as its telomorph stage, *Filobasidiella neoformans bacillisporus* (Fig. 3.5). When the YT plate of the sample was observed, almost all the carbon sources were assimilated and the only carbon source oxidized was the disaccharide turanose. These results are consistent with the fact that *C.neo/gat* can assimilate almost all carbon sources with the exception of lactose and melobiose (Casadevall and Perfect, 1998). All the candidates and samples belonging to *C. neo/gat* were tested at its anamorph stage. Five replicas of these assays were made with these samples and only once the identification pattern indicated that the sample

belongs to *C. neo/gat* complex. In order to verify if the BIOLOG was designed to discriminate between the anamorph and telmorph stage of the yeast, the assay should be repeated using also the telomorph stage of *C. neo/gat*. By using three to six traditional biochemical tests, the correct identification of these candidates was made in approximately 48hrs. In contrast, the BIOLOG Microstation System, which is designed to identify microorganism in a faster and broader way, only one sample was identified. The data showed, suggest that the BIOLOG Microstation System is not the best system for rapid identification of *C. neo/gat*.

3.5 Findings and Conclusions

1. It was proven that the best optic density and time to analyze *C.neo/gat* with the BIOLOG Microstation System is the one indicated by the manufacturer.
2. The metabolic reaction made by *C. neo/gat* to the carbon sources in the YT plate was oxidation.
3. Five common carbon sources were oxidized by the *C.neo/gat* strains, corresponding to L-aspartic acid, L-glutamic acid, L-proline, D-gluconic acid, gentibiose, maltose and N-acetyl-glucosamine.
4. The assay of the *C. neo/gat* strains unique oxidation pattern to sorbitol (ser. A), Tween 80 and maltotriose (ser. B), salicin (ser. C), turanose (CRa2001) and sucrose (CRa37).
5. Only one sample was identified by the BIOLOG Microstation as a member of *C. neo/gat*, indicating that the System is not the best approach to rapid identify members of the *C. neo/gat*.

4 Characterization of the environmental isolates of *Cryptococcus neoformans/gattii* by the use of genetic tools

4.1 Introduction

The molecular and genetic studies related to *C.neo/gat* have been focused on the understanding its pathogenicity and molecular epidemiology. The latter has recently been defined as the science that searches for the contribution of potential genetic and environmental risk factors, identified at the molecular level, to define the etiology, distribution and prevention of diseases within families and across populations (http://www.pitt.edu/~kkr/task.html). This new field has emerged from the integration of molecular biology into traditional epidemiologic research. In the last decade, several DNA techniques have been used to study the epidemiology of *C.neo/gat*. Among these techniques, karyotyping, Restriction Fragment Length Polymorphism (RFLP), DNA hybridization studies, Amplified Fragment Length Polymorphism (AFLP) and Polymerase Chain Reaction (PCR) fingerprinting (for more details, refer to section 1.3) can be mentioned. PCR targeted at ribosomal operons has been used to identify the species accurately, but has provided insufficient resolution for epidemiological research. A major step forward was the discovery of a plasmid isolated from a *URA5* transformant of *C. neoformans* that could be used to detect genetic polymorphisms. Each of these techniques has shown marked genetic differences among clinical and environmental isolates of *C.neo/gat*, even within small geographic areas. Additionally, knowledge of genetic variations is essential for understanding the population structure of any given microorganism, in this case of the *C.neo/gat* complex (Franzot *et al*., 1997).

This section will describe how the positive isolates of *C.neo/gat* obtained from plants and trees in Puerto Rico were characterized using the amplification of coding and non coding ribosomal regions such as the small ribosomal subunit18S rDNA and the internal transcribed spacer (ITS) in order to determine genetic differences among the isolates.

4.2 Materials and Methods

4.2.1 *DNA extraction*

For the genomic DNA extraction, the candidates were grown in potato dextrose broth (PDB) enriched with 1% yeast extract (YEPD) and incubated for 72 hours at 27ºC with constant, orbital shaking at 150rpm. After incubation, 1.5mL of the culture was transferred into a 1.5 mL microcentrifuge tube and centrifuged for two minutes at 24,907 Xg. This step was repeated twice. The resultant pellet cells were resuspended in 200µL of sterilized distilled water. The cell suspension was used to extract the genomic DNA following the protocol and specification designed by Q-Biogene Company. The DNA extracted was treated with 10µg/mL of RNAse and incubated for 30 minutes at 37ºC.

4.2.2 18S rDNA, ITS and IGS amplifications

Seven of twenty three *C.neo/gat* isolates were used to perform PCR analysis of 18s rDNA, ITS and IGS (Table 2). Approximately 50ng/µL of the clean DNA was used to amplify the small ribosomal subunit 18S rDNA with the primer NSI (forward) [5'GTAGTCATATGCTTGTCTC3'] (López, 2004) and the primer NS8 (reverse) [5'TCCGCAGGTTCACCTACGGA3'] (López, 2004). The amplification reaction was made using the Green *Taq* Master Mix by Promega using the following parameters: initial denaturalization at 94ºC for 3 minutes; 30 cycles of denaturalization at 94ºC for 30 seconds, annealing at 44.5ºC for 30 seconds, extension at 72ºC for 105 seconds and final extension at 72ºC for 10 minutes. Also, the intergenic spacer (IGS) was amplified with the primers LB25BI (forward) [5'GCTACGATCCGCTGAGGTTAA3'] (López, 2004) and 5SA (reverse) [5'CAGAGTCCTATGGCCGTGGAT 3'] (López, 2004) using the parameters described below: initial denaturalization at 94ºC for 30 seconds; 30 cycles of denaturalization at 94ºC for 30

seconds, annealing at 60ºC for 30 seconds, extension at 72ºC for 1 minute; and final extension at 72ºC for 10 minutes. The internal transcribed spacer (ITS) was amplified using the primers ITS1 (forward) [5'TCCGTAGGTGAACCTGCGG3'] (López, 2004) and ITS4 (reverse) [5'TCCTCCGCTTATTGAT3'] (López, 2004) using the following parameters: initial denaturalization at 94ºC for 3 minutes; 30 cycles of denaturalization at 94ºC for 30 seconds, annealing at 55ºC for 30 seconds, extension at 72ºC for 1 minute; and final extension at 72ºC for 10 minutes (López, 2004). The amplicons were sent to the biotechnological company Macrogene (www.macrogenusa.com) for sequencing, followed by an *in silico* analysis using available nucleic acid databases such as BLAST.

4.2.3 Phylogenetic analysis

The candidate 18S rDNA sequences were edited using the program Chromas Lite 2.0.0 and then aligned and edited with Clustal W server (http://www.ebi.ac.uk/Tools/clustalw/) and BioEdit 7.0.5.3, respectively. The phylogenetic analysis was performed using Phylip 3.67 program and Seqboot that generated a set of 100 bootstrap data sets. The distance matrix was calculated using dnadist. The creation of a consensus tree was made by Neighborhood Joining method, minimizing the bias by randomizing the analysis. The final tree was draw with the use of treeview program.

4.3 Results

The size of the PCR products lengths were determined and confirmed by agarose electrophoresis. All the amplicons generated (18S rDNA, ITS and IGS) showed the expected size (Figures 4.1, 4.2 and 4.3). The sequences of 18S rDNA, ITS and IGS fragments gave us values of 1700, 536 and 1000bp respectively, indicating that the sequences represent only a fragment of the rDNA subunit and the non coding parts of the rDNA. The results obtained by the macroscopic, microscopic and biochemical analysis (section 2.3) were confirmed by the *in silico* analysis of the amplicons of 18S rDNA, ITS and IGS, showing that the candidates belong to either *C. neoformans or C. gattii.* After the generation of a phylogenetic tree, the analysis showed five different homology groups (Fig. 4.4). One of the branches groups isolate Ym63 with CRa2001 but with approximately 95 base pairs differences. The analysis also shows that isolates Ym63 and CRa2001 differentiate from Y12 by a difference of 105 nucleotides. The differences between isolate Ym12 were also observed in the alignment of the sequences of ITS and IGS, showing different alignments among the other isolates. Samples VMGc4 and VMGar7 represents different branches but related with each other. Isolate Ym44 and VGMc3 represent a branch in which both isolates are closely related with one another (94% of similarity).

Table 4.1 Candidates of the complex *C. neo/gat* used to amplify 18S rDNA, ITS and IGS and its source of recovery

Candidates	Collection Site	Source	Sampled Technique
Ym-12	Yauco	Tree (*Manguifera sp*)	S
MBGc-3	Meseta, BSG	Cacti (*Cephalocerus sp*.)	S
MBGc-4	Meseta, BSG	Cacti (*Cephalocerus sp*.)	S
MBGar-7	Meseta, BSG	Tree (*Bucida bucera*)	S
Yag-44	Yauco	Tree (*Persea sp.)*	S
Ym-63	Yauco	Tree (*Manguifera sp.*)	S
PR	Cabo Rojo	Tree (*Terminalia sp.*)	DE

S- swab; DE- detritus

Table 4.2 18S rDNA, and ITS accession numbers of the candidates of *C. neo/gat* complex placed on Gene Bank database

Candidates	18S rDNA	ITS
MBGc-3	EU402428	EU402435
MBGc-4	EU402429	EU402436
MBGar-7	EU402430	EU402437
CRa2001	EU402431	N/P*
Ym-12	EU402432	EU402438
Yag-44	EU402433	EU402439
Ym-63	EU402434	EU402440

N/P- not performed

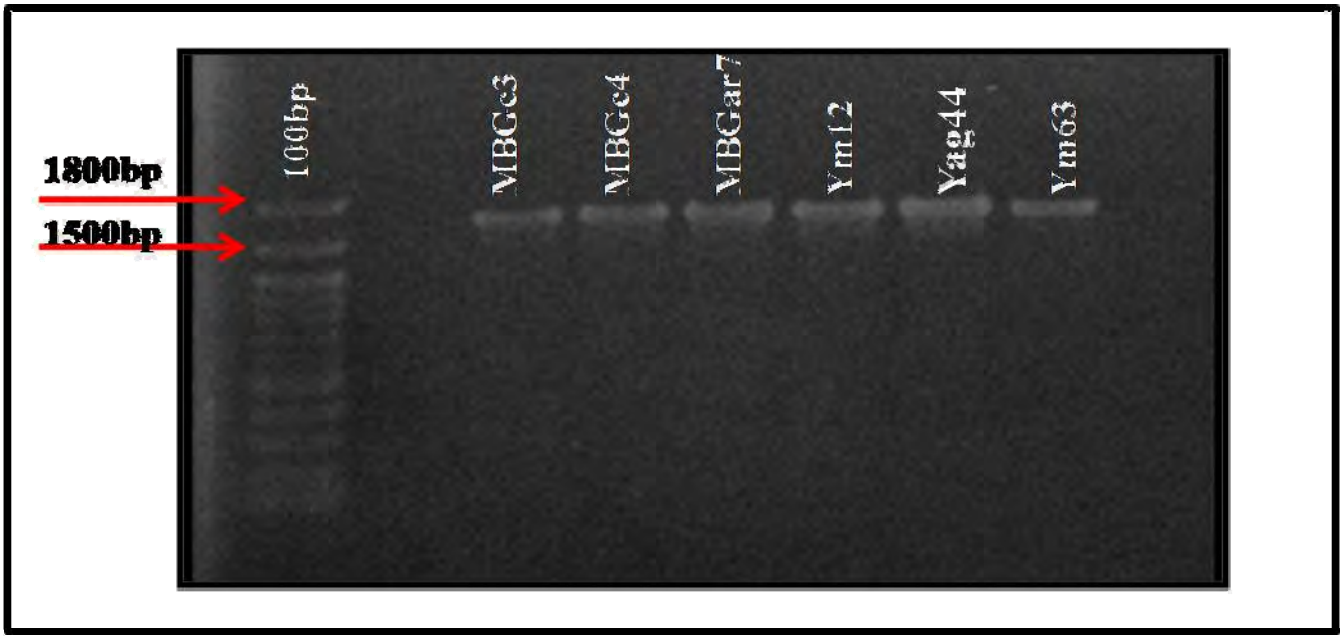


Figure 4.1. 18S rDNA amplification of *C. neo/gat* complex isolates in 1.2% of agarose. The arrows represent the two bands of 100bp molecular marker used in order to determine the approximate size of the isolates 18S rDNA fragment, approximately 1700 bp. According to the literature, *C.neo/gat* fragment size is approximately 1800 bp.

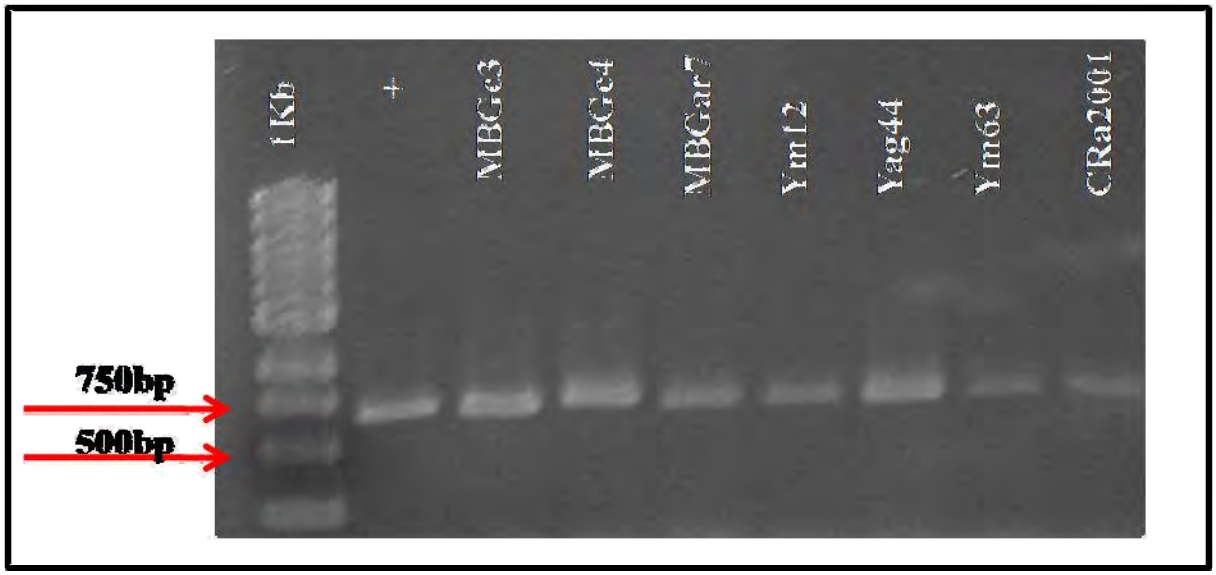


Figure 4.2. Internal transcribed spacer (ITS) amplification of *C. neo/gat* complex isolates in 1% of agarose. The arrows represent two bands of 1Kb molecular marker used in order to determine the approximate size of the isolates ITS fragment, approximately 700 and 800 bp. According to the literature, *C.neo/gat* fragment size is approximately 800 bp.

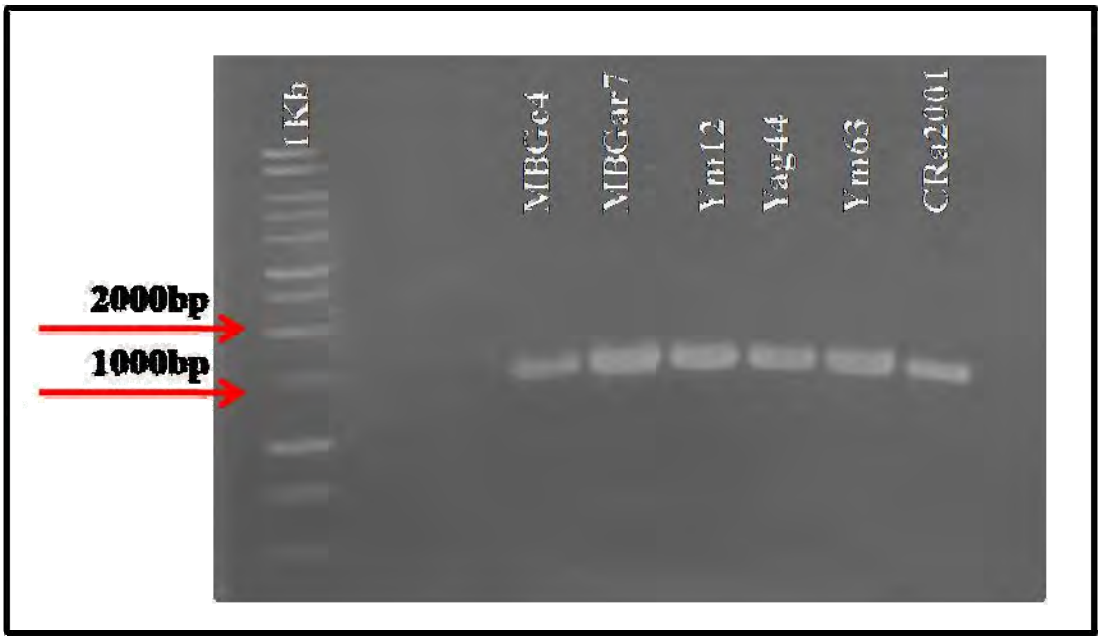


Figure 4.3. Intergenic spacer (IGS) amplification of *C. neo/gat* complex isolates in 1% of agarose. The arrows represent the two bands of 1Kb molecular marker used in order to determine the approximate size of the isolates IGS fragment, approximately 1100 bp. According to the literature, *C.neo/gat* fragment size is approximately 1200 bp.

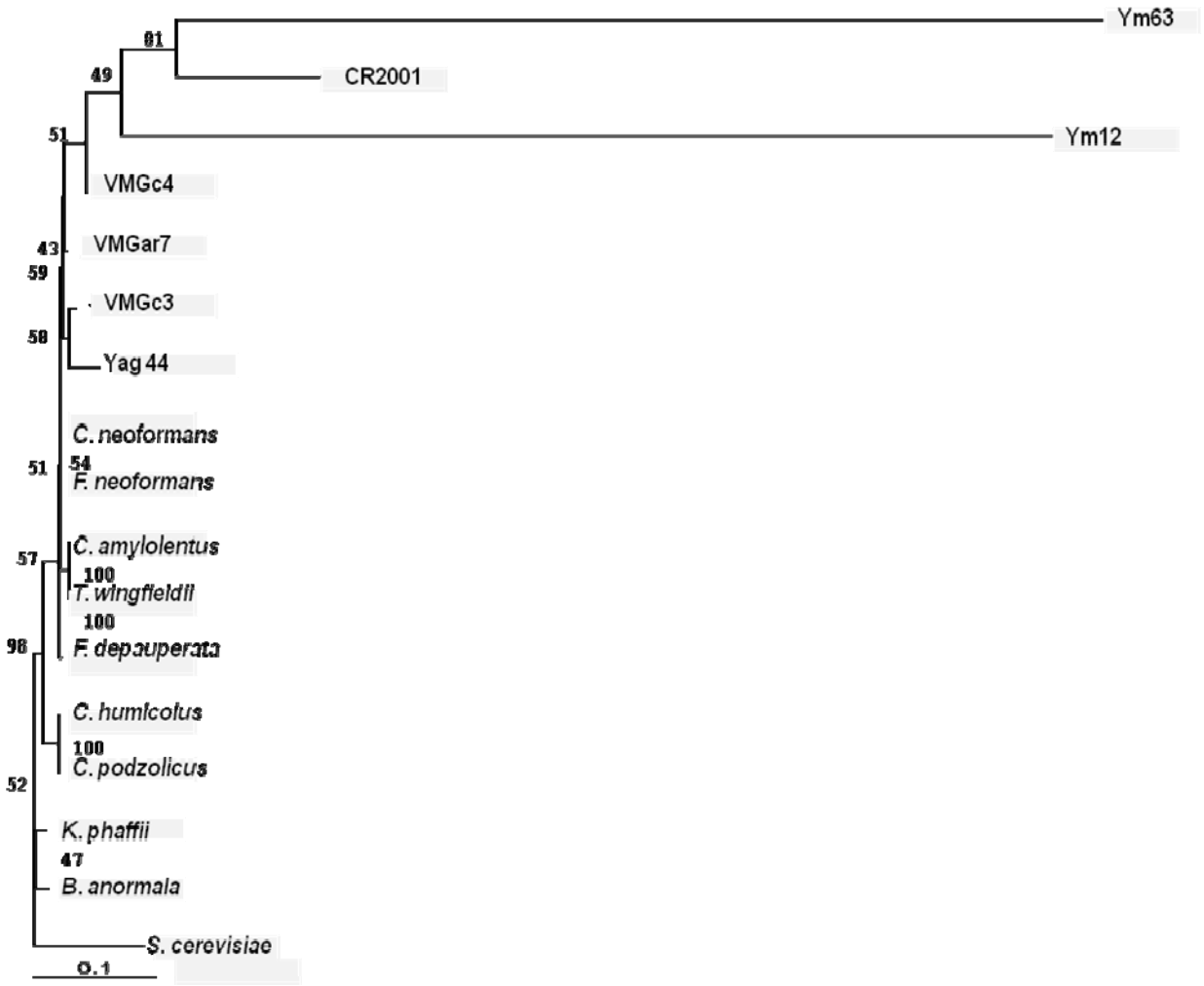


Figure 4.4 Neighbor-joining distance tree of 18S rDNA sequences for the candidates selected of the *C. neo/gat* (VMGc3, VMGc4, VMGar7, Ym12, Yag44, Ym63 and CR2001), created using Phylip 3.67 program. Bar (1.4cm) represents 10 substitutions per 100 nucleotides. Significant bootstrap values are indicated.

4.4 Discusion

The purpose of this section is to genetically characterize the environmental isolates of *C. neo/gat*, and to search for molecular differences between the isolates which could provide a tool for their differentiation. The *in silico* analysis of the 18S rDNA, IGS and ITS of the seven isolates, using the BLAST program, showed that all of them belong to *C. neo/gat* complex. The analysis of the IGS1/IGS2 region of the large-subunit ribosomal DNA (rDNA), which is widely used for phylogenetic analysis, showed that the sequences of serotype A (CBS 132; DDBJ/EMBL/GenBank accession no. AF075484) and serotype D (CBS 882; DDBJ/EMBL/GenBank accession no. AF189845) strains were identical (Biswas *et al*., 2003). In other words, these regions can not be used to distinguish between isolates at serotype level. For the study of *C.neo/gat* complex, phylogenetic analysis has been used among isolates of different countries (Meyer *et al*., 2003). In this investigation we used the partial 18S rDNA sequences for the phylogenetic analysis. The rDNA sequences alignment suggested differences among the sequences obtained of *C.neo/gat* from the BLAST database. The fact that *C.neo/gat* members have different homology groups can be explained by the micropopulation phenomenon. This phenomenon has demonstrated genetic heterogeneity among clinical and environmental isolates of *C. neoformans*, even within small geographic areas. For example, Currie *et al*. (1994) defined 18 strains among 25 environmental and clinical *C. neoformans* var. *neoformans* isolates from a single New York City borough (Currie *et al*., 1994).

The phylogenetic analysis of the isolates showed five homology groups in which sample Ym12 differs from the other isolates of *C. neo/gat*, even though the *in silico* analysis identify the sample as part of the complex. The results analyzed by the phylogenetic tree suggest that the genetic difference between the isolates could be associated with their ecological habitat. In the

sequence alignment in Clustal W server, the candidates were grouped in two branches of the phylogenetic tree: the candidates isolated from the Guánica Dry Forest and the candidates isolated from two municipalities of the southwestern area of Puerto Rico (Fig. 4.4). This observation could represent a point of further differentiation between the isolates and a possible adaptation of *C. neo/gat* according to this newly reported environmental niche. The adaptation of the *C. neo/gat* may be explained in two ways: 1) maybe the yeast is adapting to the environment by producing new enzymes and 2) maybe the yeast found a microenvironment which provided the necessary water and nutrients for its survival. To prove the first hypothesis, a genetic comparison using the fingerprinting technique (Meyer *et al*., 2003) may be performed between the environmental isolates from Puerto Rico and type sequences belonging to *C. neo/gat*. By the comparison of the fingerprints produced by the isolates and type sequences, it would be determined if there are any sequence differences between the isolates, and the sequences could be used to further characterize the isolates. To prove the second hypothesis, it is necessary to conduct a study of the conditions which the cacti may provide to the yeast such as water activity, temperature, moisture and nutrients among others.

At this investigation the amplification of 18S rDNA, IGS and ITS was used for the identification of the yeast, but these amplifications did not distinguish between isolates of *C. neoformans* nor *C. gattii*. For the best characterization of the yeast the use of at least two different genetic techniques (refer to section 1.3) is highly recommended.

4.5 Findings and Conclusions

- *In silico* analysis of the 18S rDNA, ITS and IGS amplified from the seven isolates of *C. neo/gat* were confirmed as belonging to *C.neo/gat* complex.
- The Neighbor-joining phylogenetic tree generated using 18S rDNA partial sequences showed five homology groups in which the 7 isolates of *C. neo/gat* were grouped.
- The phylogenetic analysis suggested grouping based on environmental sources among isolates.

5 Determination of the presence of specific peptides that recognize *Cryptococcus neoformans/gattii* capsule using T7 phage display technique with human genomics libraries of brain and lungs

5.1 Introduction

In order to avoid the use of genetic techniques for testing one mutation at a time, scientists have found in combinatorial chemistry, an emerging discipline, the simplicity of generating large populations of molecules, or libraries, that can be screened efficiently for desirable properties at large scale. Combinatorial chemistry has become a better way to search new drugs, catalysts, and materials (Stu Borman, 1998). The key of combinatorial chemistry is that a large range of analogues are synthesized using the same reaction conditions. In this way, hundreds or thousands of compounds are synthesized, at once instead of preparing only a few by simple methodologies. Combinatorial chemistry offers the potential to make every combination of compound A_1 to A_n with compound B_1 to B_n (http://www.combichemistry.com/index.html). Combinatorial library methods were first applied to peptides and oligonucleotides. Since then, the field has been expanded to include proteins, synthetic oligomers, small molecules, and oligosaccharides. The method of library preparation is dependent on the type of library desired. All combinatorial library methods involve three main steps: preparation of the library, screening of the library components, and determination of the chemical structures of active compounds (http://www.combichemistry.com/index.html). The main application of combinatorial chemistry is related to new drug discoveries. The increasing number of small molecules and the potential targets does not automatically lead to higher efficiency of the whole drug discovery process. The new targets will provide novel therapeutic interventions of traditional and newly emerging diseases with more specific, efficient, and safer small molecules.

Phage display is a combinatorial chemistry approach to screen for protein interactions by integrating multiple genes from a gene bank into phages. The principle of this method is

summarized as follows: (a) the function of protein X is unknown, so the protein is used to coat the surface of a small plastic surface; (b) numerous genes, often all the genes in an organism's genome, are expressed in a library as fusions with the coat protein of a bacteriophage, so that they are displayed on the surface of the viral particle; (c) expose to the target molecule and the unbound molecule is removed; (d) phage-displaying proteins that interact with protein X remain attached to the dish, while all others are washed away and the DNA extracted from interacting phage contains the sequences of interacting proteins (Sidhu *et al*., 2000). Among the phage displays technology most used are M13 bacterial filamentous phage with a non-lytic phage display and the other using lytic phages such as T4, lambda and T7. Most of the studies related to *C.neo/gat* complex have been performed using M13 phage display (Nosanchuk *et al*., 2005).

New antifungal agents are needed, and research on the molecular pathogenesis of fungal infections may identify unique targets for the development of these new agents (McDade and Cox, 2001). The company Novagen developed a novel phage display system that can be used to identify target sites for the development of such treatments. The system is based on gene fusions between different human genomic libraries with a gene that encodes for a T7 phage capsid. This system had various advantages in comparison of the traditional phage display with filamentous phages. The advantages include: easy reproduction of T7 phages; large libraries can be created by fusing large peptides on the T7 capsid; the phages are very resistant to a broad amount of agents and their amplifications are relatively fast (Rosenberg *et al*., 1996). Also, the peptides that fuse on the T7 capsid do not need to be secreted thru the cell membrane of the host bacteria (Rosenberg *et al*., 1996).

By the interaction of *C. gattii* C77 strain with human brain and lung genomic libraries fused on the T7 phage, we want to determine whether or not the whole cells, as a target, interact with

any of the human peptides displayed on the phage surface. The analysis of the peptides can be used to develop a new anticryptococcal therapy or to understand better the pathogenicity of *C. neo/gat.*

5.2 Literature Review

The needs of new therapies to prevent and treat cryptococcosis have caused a continuous search of cellular products that can be used for this purpose. The biological expression and presentation systems of phage display are powerful tools, which can identify peptides with a specific ligand affinity (Nosanchuk *et al*., 1999). This is due to the fact that the libraries can screen millions of proteins at a time. This technique has many applications if we want to create antimicrobial, antifungal and antiviral therapies.

Like the two-hybrid system, phage display is used for the high-throughput screening of protein interactions (Sidhu *et al*., 2000). The most common phage used for the study of anticryptococcal therapies is the filamentous M13 phage named fUSE5. This phage has the capsid protein III (pIII), which has a particular interest because it has missing one amino acid that is found in other M13 vectors. Additionally, it has two restriction sites recognized by endonucleases *SfiI* in the protein (Beenhouer *et al*., 2002). Some modifications have been performed to this phage with the purpose of studying the capsule of *C.neo/gat*. Among these libraries the following can be mentioned: L100 (Valadon *et al.,* 1998), L200 (Beenhouwer *et al.,* 2002), fUSE5 hexapeptide and decapeptide (Valadon *et al.,* 1996; Nussbaum *et al.*, 1997; Nosanchuk *et al.,* 1999)

The characterization of antigenic determinants that can be recognized by human GXM antibodies will help with the identification of possible anticryptococcal therapies. Until now, an

experimental vaccine against the capsule of the yeast has been developed. The vaccine glucoronoxylomannan-tetanus toxoid has shown to be functional in different biological models (Devi *et al*., 1991 cited by Zhang *et al*., 1996). Zhang *et al*., (1996) monitored arbitrarily a decapeptide phage display library with the human monoclonal antibody 2E9 against glucoronoxylomannan (anti-GXM MAb 2E9). It was found that the use of this technique allows the identification of peptides that can imitate (mimotope) many molecules, including the polysaccharide. Using ELISA, the phages which contained the 2E9 peptide and that inhibit the 2E9-GXM bond were selected. The analysis of the amino acid sequences showed an increase in the frequency a combination of the residues QTGLD. Also it was proven that the phages which contained QTG/TL/D motif inhibit the 2E9-GXM bond better than QLDRW. The peptide thirteen inhibited the GXM bond of the GXM-TT of the immune serum and antibodies in the natural serum of people that do not have HIV. In the other hand, the isolated peptide did not inhibit GXM bond of the GXM-TT.

Nussbaum *et al.*, (1997) found that there are different adhesion patterns among the serotypes A, B, C and D of *C. neo/gat* and that the localization of the adhesion site of IgM has a critical importance in the efficiency of protection. This finding suggests a relation between the ability of providing protection, and the localization of the adhesion area of the antibody and the GXM capsule. In another investigation, Valadon *et al.* (1996), using a decapeptide library of the fUSE5 phage, found four motifs TPXWM/LM/L, W/YXWM/LYE, DWXDW and (Ar)WDGQ(Ar). These motifs have a great importance because they compete with the adhesion site of the immunoglobulin 2H1, one of the most protective against cryptococcal infection. In 1998, Valadon *et al*. (1998) found that peptide P601E is mimetic but not a mimotope to the polysaccharide capsule when the phage display technique was used. Other applications of phage

display have been searching for peptides that can bind to melanine produced by *C. neo/gat*, in order to understand the melanization process (Nosanchuk *et al.,* 1999). With this study it was proven that there were proteins that bond to melanine, being compounds very similar to the pigment. This suggests the protector mechanism related to melanine *in vitro* may be applied in the same way *in vivo*.

The premade human genomic libraries consist of T7, an icosahedral phage with a capsid shell of 415 copies of T7 capsid proteins, coded by gene 10. Attached is the head-tail connector coded by gene 8, a short conical tail, coded by genes 11 and 12 and a 6 tail fiber, coded by gene 17. Specifically, the fusion of the T7 phage is in the C-terminus of 10B protein at the amino acid 341 (InNovations, 1996). Human genomic libraries of brain and lungs, expressed in T7Select10-3b vector, were used for the interaction of *C.neo/gat* cells. These libraries were created by Novagen (patented by OrientExpress). The primary recombinant clones at the end of a biopanning, of the brain and lung libraries, were $1.5x10^7$ phage/μL and $1.7x10^7$ phage/μL, respectively.

5.3 Materials and Method

5.3.1 Biopanning: Microtubes Coating

The coating of 1.5mL microtubes was necessary to block away, without the target molecule, all target unbound areas in order to inhibit the interaction of the phages with the polystyrene material. To coat the microtubes, a 3% blocking solution (non fat dry milk) was prepared with Tris Buffer Solution 1X (TBS). The microtube was filled completely and stored at 4ºC overnight.

5.3.2 Preparation of C. gattii C77 cells for the Biopannings

C. gattii C77 strain (for more details of the strain, refer to section 3.2.1) was grown in YEPD broth for 48hrs. After the incubation period, 3mL of cells were pellet by centrifugation at 1831.73xg for 1.5 min. The pellet was washed five times with TBS plus Tween80 1X (TBST), and resuspended in 100μL of TBS 1X. The resuspended cells were added to the coated microtube until the biopanning assay was performed.

5.3.3 Biopannings

The biopanning assay was performed to isolate the positive recombinants phages of brain and lungs which interacted with the capsule of C77 strain. The process was made following the protocol developed by Novagen, with a few modifications. Briefly, 100μL of the clean C77 cells were combined with 1μL of human brain and lung genomic libraries displayed on the T7 capsids. The mixture was incubated at room temperature for 45min. After the incubation period, the cells were then washed five times as previously described and resuspended in 200μL of TBS 1X.

5.3.4 Amplification of the Phages

In order to amplify the gene present, being expressed on the positive recombinant phages, an overnight culture of *E. coli* Rosseta strain (Novagene) was inoculated into 20mL of Luria Bertani broth with 50μg/mL (LB+amp) of ampicillin until an optical density of 0.025 at 600nm (OD_{600}) was reached. The sample was then incubated at 37ºC with constant shaking at 150rpm until the culture reached a 1.0 OD_{600}. Then, 200μL of isopropyl-beta-D-thiogalactopyranoside 0.1M (IPTG) was added to the culture, follow by 30 min of incubation. When the incubation time passed, 50μL of the cells of the biopanning were added to the culture, re-incubating the mixture for 3 more hours at 37ºC. Then, 1 mL of the lysate was centrifuge for

10 min at 31,168.05Xg. The resultant supernatant corresponded to the T7 phages containing the positive target specific recombinants of brain and lungs proteins. This process was repeated 2 more times to select the high affinity phages for the *C.neo/gat* cells.

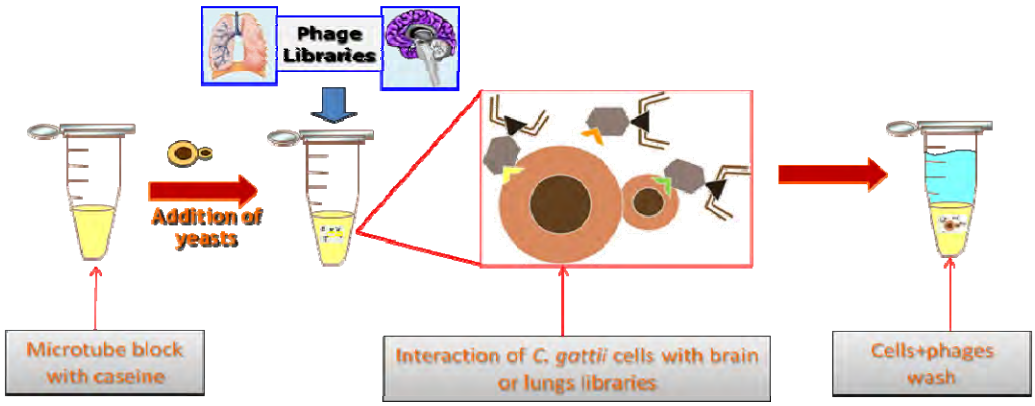


Fig. 5.1 Experimental procedure of *C. neo/gat* biopanning. This figure represents the target surface block, cell interaction with the respective human genomic libraries and amplification of the positive target recombinants. This procedure was repeated three times.

5.3.5 Plaque Assay

A plaque assay protocol was performed for the isolation of individual phages, following the specifications of Novagen. Briefly, from the biopannings, several dilutions in LB broth were made from 10^{-8} to 10^{-11}. Each dilution was combined with 250µL of a Rosetta active culture + 0.1M IPTG and 5mL of top agar and plated on LB + 50µg/mL amp agar. The plates were left at room temperature until the top agar was solidified and incubated at 37ºC for 4 hours or until plaques were formed.

5.3.6 Amplification of Selected Phages, DNA extraction and Sequencing

After plaques formation on LB + 50µg/mL amp agar, 10 plaques were chosen arbitrarily, removed by lifting and added to individual 1.5 microtubes with a Rosetta active culture at

logarithmic phase + 0.1M IPTG. After 3hr incubation at 37ºC with constant shaking at 150rpm, the microtubes were centrifuged for 10min at 31,168.05xg. The resulting lysate was placed in a clean 1.5mL microtube. For the total DNA extraction of *C. gattii* peptide specific displaying phage, 10µL of the supernatant were combined with 100mM ethylenediamine tetraacetic acid (EDTA) at pH 8 and placed in a water bath at 80ºC for 10min. Part of the sample was also stored at -20º for further analysis.

The DNA extracted for each plaque was used to perform a PCR reaction with the primer T7up (forward) [5′GGAGCTGTCGTATTCCAGTC3′] (Novagen, 1996) and the primer T7down (reverse) [5′AACCCCTCAAGACCCGTTTA3′] (Novagen, 1996). The amplification reaction was made using the Green Taq Master Mix by Promega using the following parameters: initial denaturalization at 94ºC for 3 minutes; 35 cycles of denaturalization at 94ºC for 50 seconds, annealing at 50ºC for 1 min, extension at 72ºC for 1 min and final extension at 72ºC for 6 min and visualized by 1% agarose gel electrophoresis. Amplicons were sent to the biotechnological company Macrogene (www.macrogenusa.com) for sequencing, followed by an *in silico* analysis using available nucleic acid databases such as BLAST. The *in silico* analysis was performed using the blastx, which searches in the protein data bank (PDB) using a translated nucleotides query.

5.3.7 Specificity Assay

In order to determine the specificity of the displayed peptides on the isolated T7 phages, that encoded for the *C. gattii* cells specific peptides, three preliminary specificity assays were performed. The different assays were done by interacting directly T7 phages display isolated with whole algae as a target and a T7 phage isolated using *C. gattii* as target by the biopanning assay previously described in section 5.3.4. The first assay consisted on amplifying both algae

and *C. gattii* specific peptides phages as previously isolated as described before (section 5.3.4). After the amplification, serial dilutions, the plating of the phages and enumeration of the phage forming units/µL (PFU/µL) were made as previously described (5.3.5).

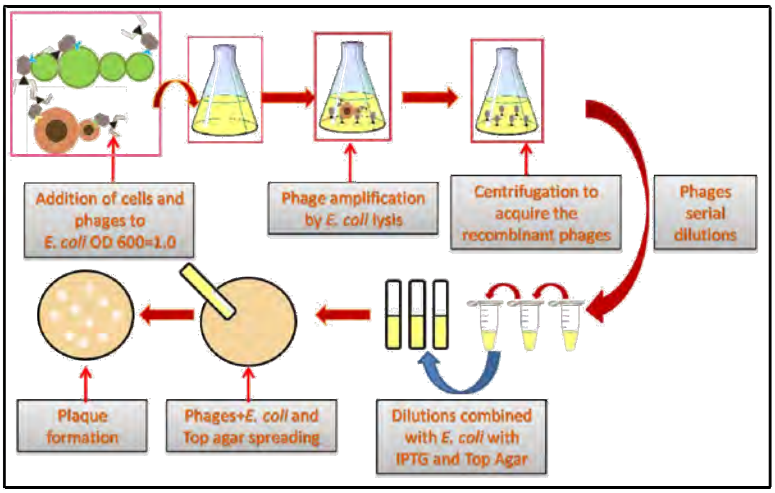


Figure 5.2 Specificity assay #1. Modified biopanning and recombinant phages amplification

The second assay consisted of using the interaction of both algae and *C. gattii* cells after the biopanning and making dilutions and plating them as described above. In this case, the dilutions contained both cells with phages (Fig. 5.3).

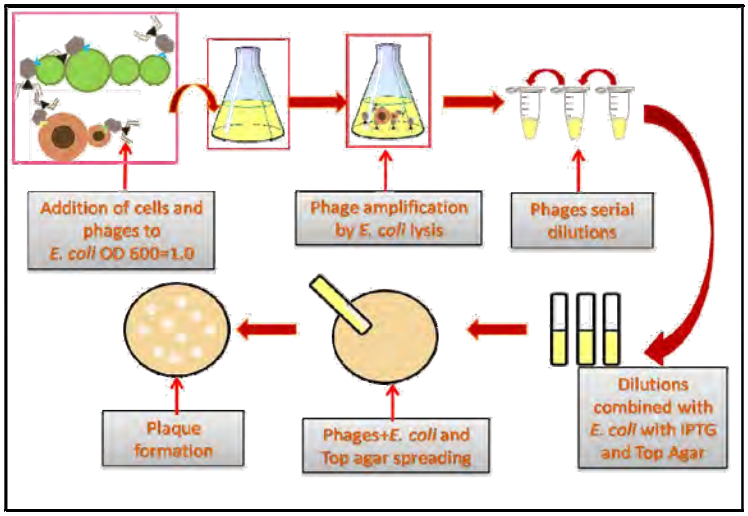


Figure. 5.3 Specificity assay. Direct amplification of recombinant phages without eluding the *C .gattii* cells

The third assay consisted of the elution, using sodium dodecyl sulfate (SDS), of both algae and *C. gattii* interactions with the recombinant bacteriophages. The elution process was performed after washing the cells with the attached phages. On the last step of the washing, the remaining pellet was resuspended in 1% SDS. The resuspeded cells were used to make dilutions and plate them as described above. The function of the SDS at this step was to remove the recombinant phages from both algae and *C. gattii* cells.

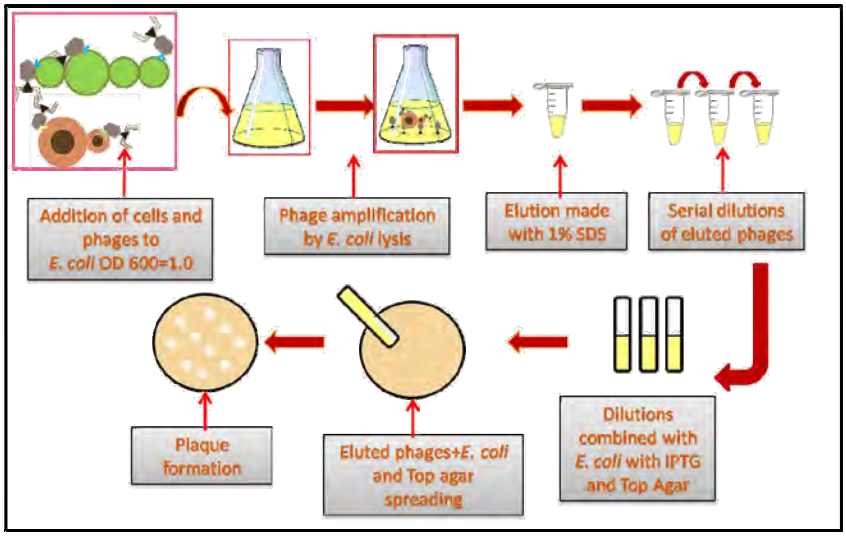


Figure 5.4 Specificity assay #3. Amplification of recombinant phages after eluding the *C. gattii* cells

5.4 Results

The modification of the biopanning process was successful; the interaction of recombinant phages with *C. gattii* cells showed cellular debris indicating infection in cultures of Rosetta (Fig. 5.5). These cultures were used to perform a plaque assay, in which infection of the T7 phages corresponding to brain and lung human libraries was observed. It is suggest that the interaction of the peptides is due to the capsule of *C. gattii* because it is the external part of the cell. Differences in size and quantity of the plaques corresponding to both lung and brain libraries, were observed. The plaques formed by the recombinant phages of lung, were more numerous and bigger when compared to the recombinant phages of brain (Fig. 5.6). Also, differences were observed in plaque size in plates of the same cDNA library indicating the interactions of peptides of lung and brain.

After the genome of the recombinant phages was amplified and *in silico* analyzed (Fig. 5.7), 21 recombinant phages, representing possible proteins that interact with *C. gattii* capsule, were identified. However, two of the peptides should be singled out: the peptides that codify for nectin (MKETAAAKFERQHMDSPKACR) and telecephalin (ARAEPWPPPQWAASLG) (Figs. 5.7, 5.8 and 5.9). Nectin is an adhesion molecule widely expressed in cell lines of different lineages, including hematopoietic cells, neuronal cells, endothelial and epithelial cells. Telecephalin is a protein only expressed in the mammalian telencephalon that controls higher-order brain functions. It promotes formation of dynamic and flexible protrusions called "dendritic firopodia", facilitating synapse remodeling and maintaining pliability of neural circuitry. Both peptides are classified as polar and positively charged according to the Kyte method (1982). The specificity assays reveled that phage $2C_2$ can infect almost the double of cells than phage A5 (Fig. 5.11).

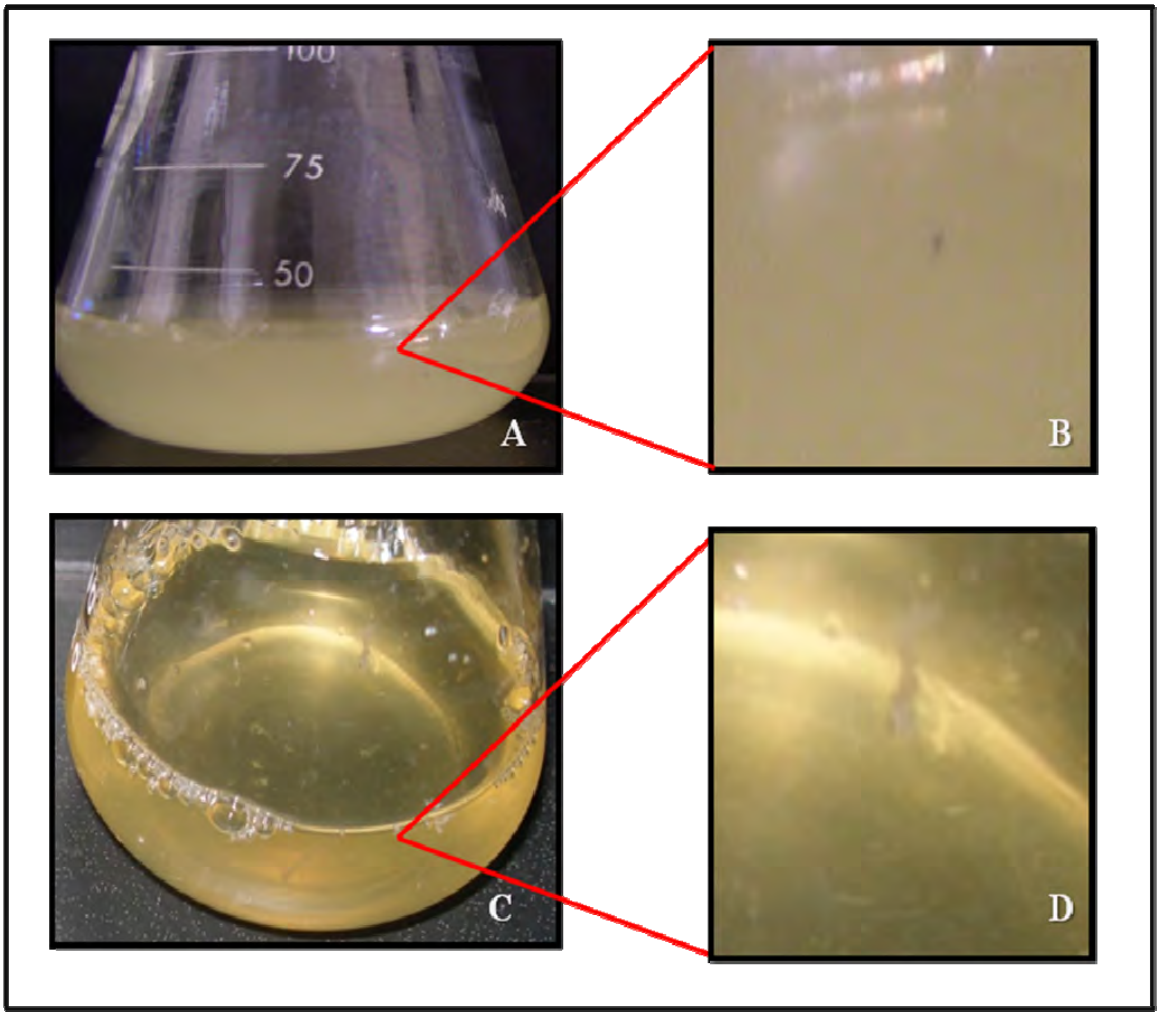


Figure 5.5 Amplification of phages in *E. coli* Rosetta strain. Figures A and B show an active culture of Rosetta strain OD_{600} of 2.5. Figures C and D correspond to a T7 phage infection culture Rosetta cultured at the same OD_{600}. When Figure B and D are compared, cellular debris is observed due to phage lysis to the Rosetta cells.

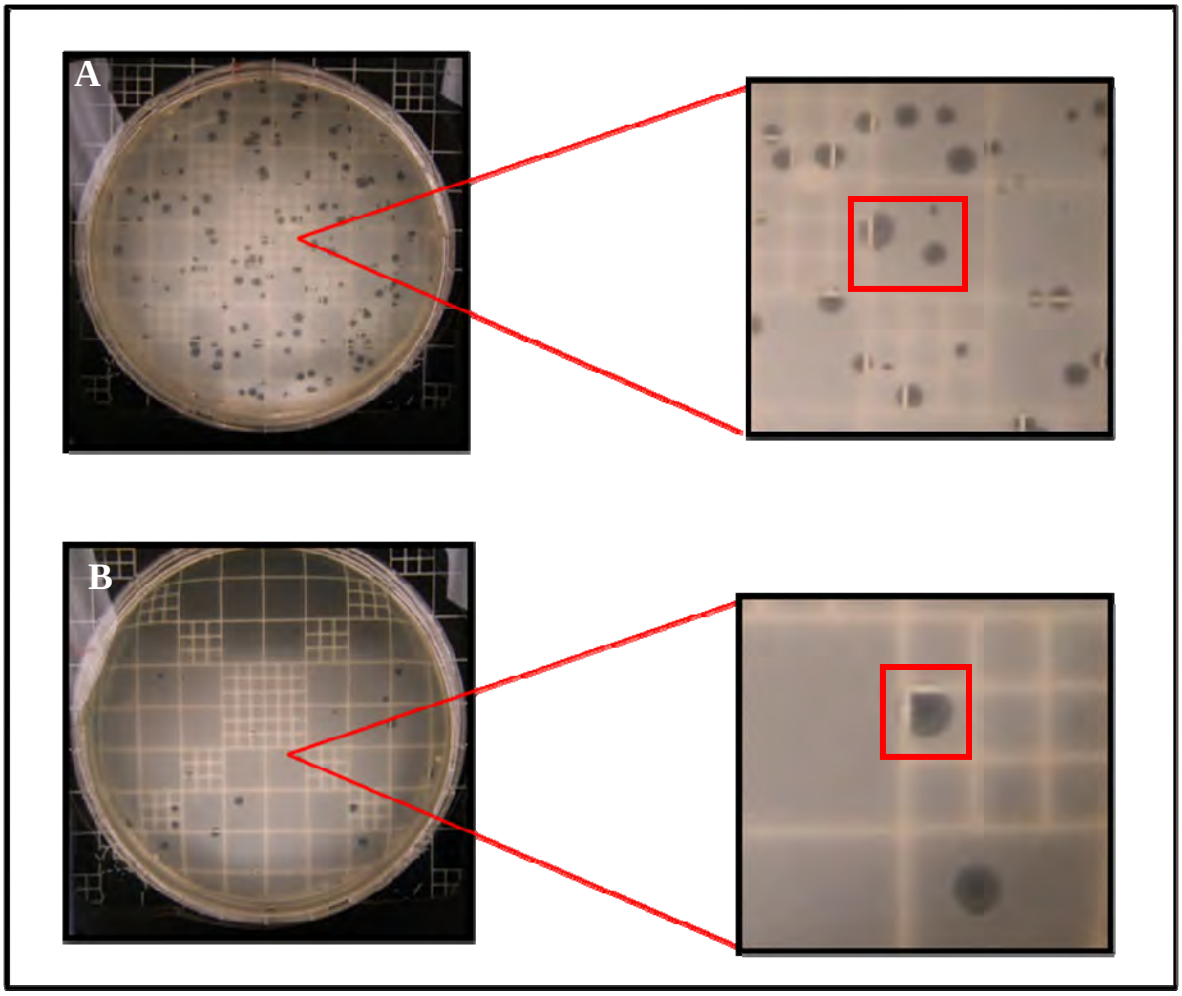
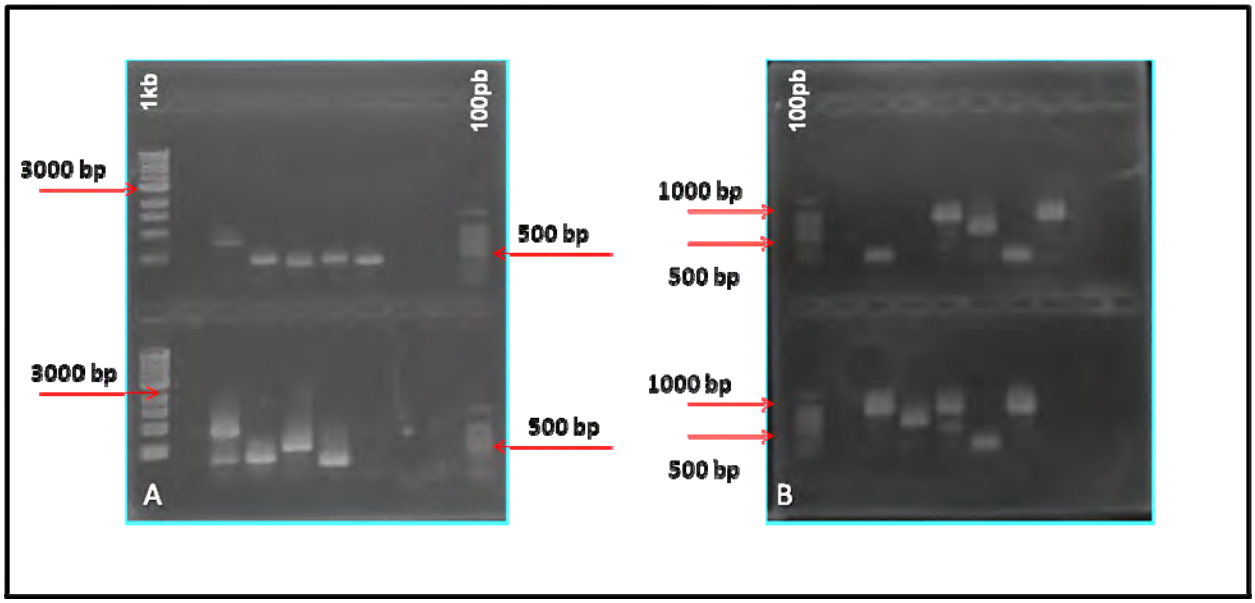


Figure 5.6 Plaque assays of premade human genomic libraries of lung and brain expressed on T7 phage after the biopanning with C77 cells. Every plaque represents a phage displaying a different peptide of lung or brain, depending on which library was used to perform the biopanning. The different sizes of the plaques indicate different recombinant phages.



Figures 5.7 Amplification of the human lung and brain peptides encoding genes that were translated and displayed on the phage capsid. Figures A and B represent the amplification of premade human T7 genomic library of lung and brain respectively.

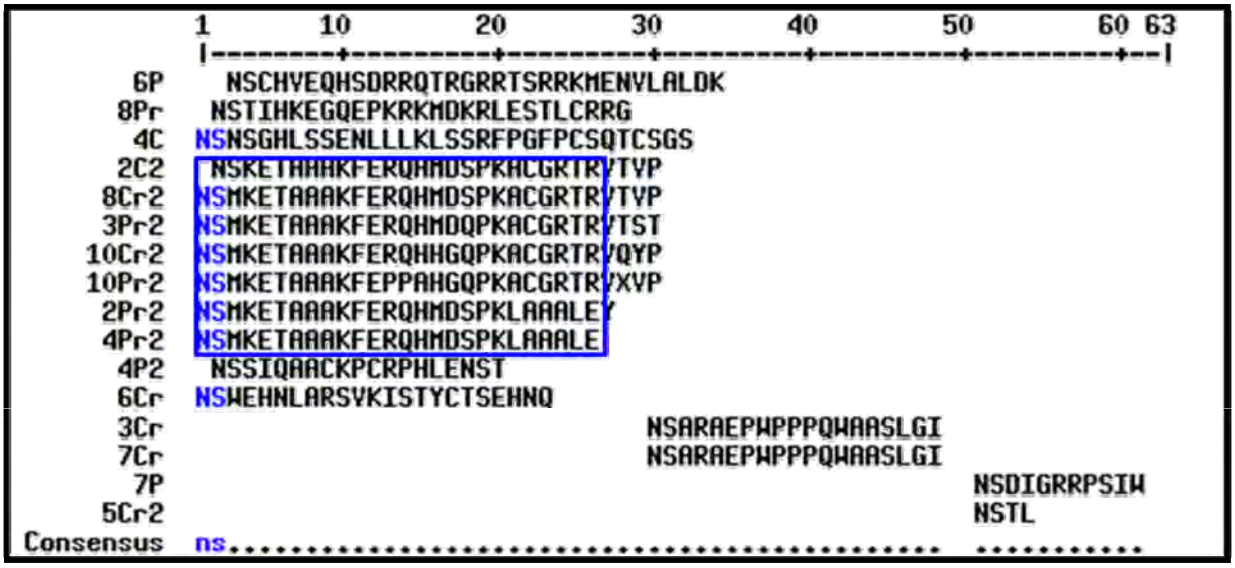


Figure 5.8. Alignment of the recombinant phages of brain and lung that interact with *C. gattii* capsule. The box represent the peptides that were displayed in the capsid of the recombinant phage, suggesting that it corresponded to nectin. Blue letters represent the only parts in which the alignment was the same for all sequences; P represents the isolated recombinant phages of lung. Pr represents the isolated recombinant phages of lungs after a repetion of the assay, C represents the isolated recombinant phages of brain while Cr represents the isolated recombinant phages of brain after a repetition of the assay.

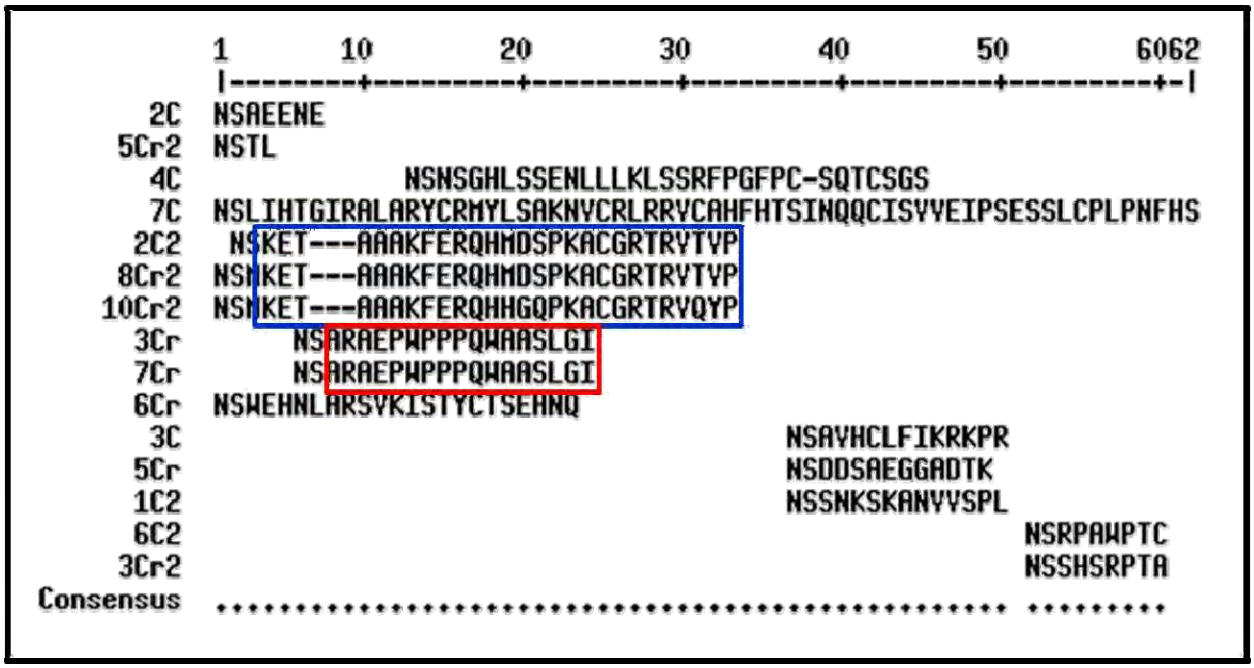


Figure 5.9 Alignment of the recombinant phages from human brain genomic library that were displayed using *C. gattii* cells as target. The blue and red boxes represents a common peptides displayed in the capsid of the recombinant phage. After the in silico analysis it was suggested that the blue box represents a peptide associated with nectin, while the red box represents a peptide associated with telecephalin. C represents the isolated recombinant phages of brain while Cr represents the isolated recombinant phages of brain after a repetition of the assay.

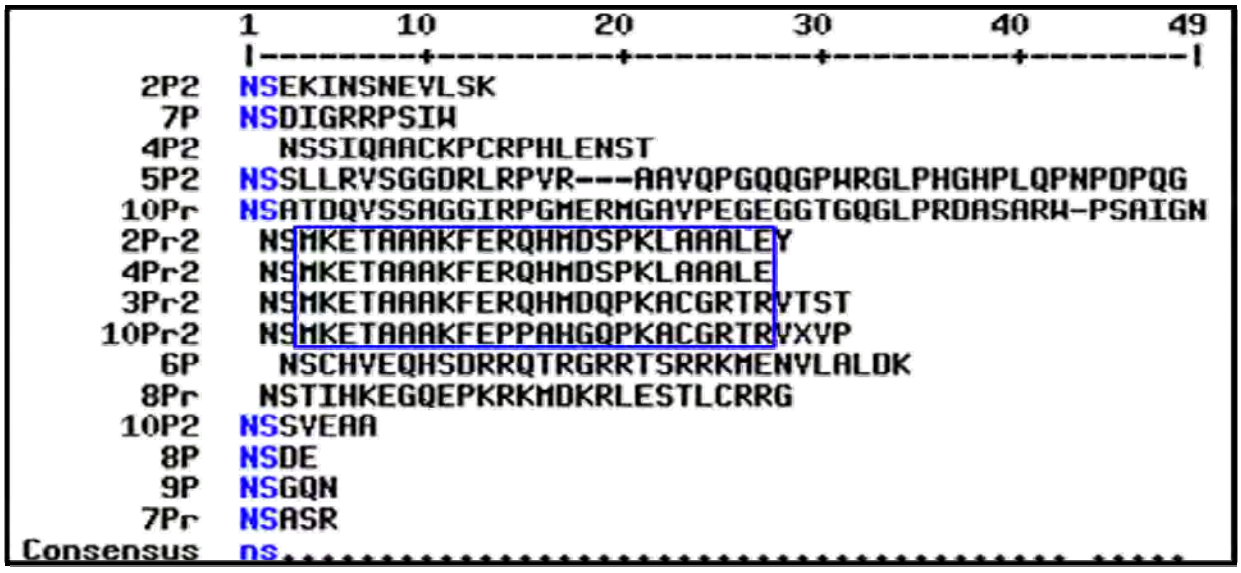
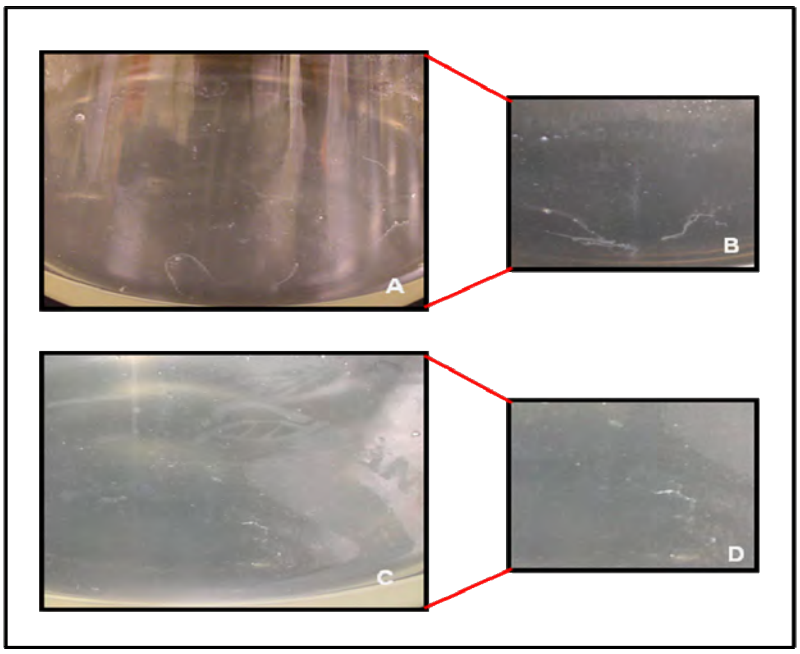


Figure 5.10 Alignment of the recombinant phages from human lung genomic library that were displayed using C77 cells as target. The blue box represents peptides that were displayed in the capsid of the recombinant phage, suggesting the presence of the nectin. Blue letters represent the only parts in which the alignment was the same in all sequences; P represents the isolated recombinant phages of lung; Pr represents the isolated recombinant phages of lungs after a repetition of the assay.



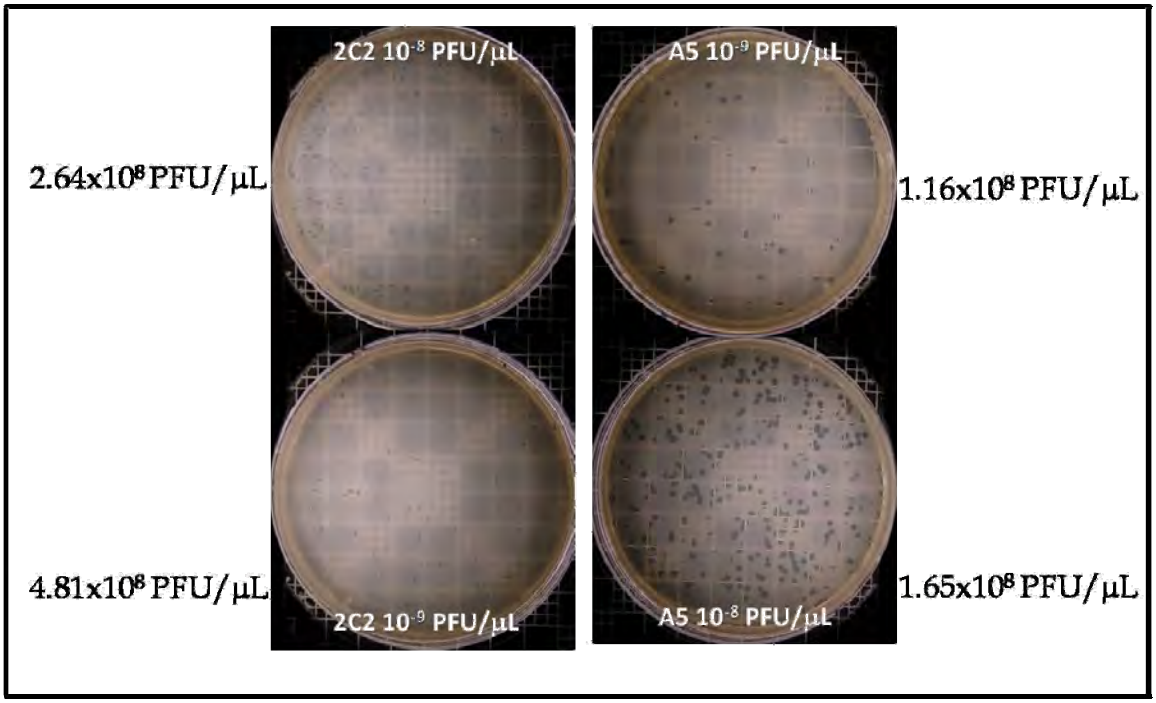


Figure 5.11 Specificity assay #1 Biopanning of C77 with phages $2C_2$ and A5. Figures A and B show cellular debris due to cell lysis by the phage. The second box corresponds to plaques from the biopanning with their respective PFU/µL.

5.5 Discussion

To isolate possible specific peptides that recognize *C. gattii* cells, the use of T7 phage display technique with premade T7 human genomic libraries of brain and lungs was performed. Because the pathogenicity of *C.neo/gat* is poorly known, particularly which areas of brain and lungs, it is necessary to make an in depth study in order to identify novel target molecules that the yeast can affect and, also, find molecules that could be used as anticryptococcal agents. Due to the fact that *C. neo/gat* whole cryptococcal cells have a capsular external layer, it is assumed that it is the area that specifically interacts with the genomic libraries displayed on the phage surfaces.

The *in silico* analysis of the DNA fragments cloned on the recombinant phages showed specificity toward to *C. gattii* target. Two main recombinant peptides were found: nectin was identified after using both cDNA libraries of lungs and brain; and telecephalin only identified when the cDNA genomic library of brain was used. Nectin is an adhesion molecule widely expressed in cell lines of different lineages, including hematopoietic cells, neuronal cells, endothelial and epithelial cells (Takai *et al.*, 2003). Nectin-based cell-cell adhesion is involved in the formation of cadherin-based adherence junctions in epithelial cells and fibroblasts. The nectin-based cell-cell adhesion induces activation of Cdc42 and Rac small G proteins, which eventually regulate the formation of adherents' junctions through reorganization of the actin cytoskeleton, gene expression through activation of a mitogen-activated protein kinase cascade, and cell polarization through cell polarity proteins (Takai *et al.*, 2003). This protein is needed in both lungs and brain, making it an excellent target for studying because is present in the target organs in which the yeast affects. The nectins are also associated with the natural killer cells (NK) in the human immune system (Bottino *et al*. 2003) and to the budding process in *S. cerevisiae* (Yamochi *et al.*, 1994). Another peptide present in the isolated T7 phage display

using premade human brain genomic library was telencephalin. Telencephalin or ICAM-5 is a protein, only expressed in the mammalian telencephalon, which controls higher-order brain functions. It also promotes formation of dynamic and flexible protrusions called "dendritic firopodia", facilitating synapse remodeling and maintaining pliability of neural circuitry (Oka *et al*., 1990). The other peptides isolated from the recombinant phages of both libraries were too small for a reliable E.value, and therefore, difficult identified.

Most of the peptides isolated in previous phage display studies can mimotope many molecules, including the polysaccharide capsule [QTGLD (Zhang *et al.*, 1997), mimic the capsule LQYTPSWMLV (Beenhouwer *et al*., 2002)] and compete with some immunoglubulins against the *C. neo/gat* capsule [SYSWMYE (Valadon, 1998)] (refer to sec. 5.2). These isolated peptides have shorter sequences when compared with the ones found in the present study. Also, the previously reported peptides are composed mainly of hydrophobic uncharged amino acid. The strategy used in the previous phage display studies included the interaction of monoclonal antibodies in order to find a peptide which serves as a vaccine against *C. neoformans* with filamentous secretory phages. To isolate recombinant phages using the filamentous phage display method, it is necessary to perform an ELISA assay with the target molecule of interest in order to select the peptide. On the contrary, the peptides isolated in present study, with emphasis on those belonging to nectin (MKETAAAKFERQHMDSPKACR) and telecephalin (ARAEPWPPPQWAASLG), were related to adhesion molecules and ICAM-5 protein, respectively, and were different no only at the length, but also at the level of amino acid sequence. The isolated recombinant peptides are composed of polar, positively charged amino acids, which could explain an interaction, possible ionic, with the *C. gattii* capsule, since this external part of the yeast also has a negative polar charge. The finding of these two peptides

could explain the affinity of the yeast for lung and brain tissues. In our study, the use of cDNA genomic libraries of lungs and brains constituted a novel approach since peptides that can serve as potential vaccine receptors could be isolated. These libraries could be used to identify the interaction of the capsule with target organs. The premade T7 genomic libraries of brain and lung used on this research had a lytic non secretory infection cycle, letting the isolation of recombinant phages by extracting one plaque after the biopannings. This advantage provided by the T7 system is faster and more efficient when compared to the filamentous phages display. Another novel approach in this study was the use of *C. gattii* in order to conduct this experiment because almost all studies focus on the pathogenicity of *C. neoformans*, due to the fact that almost all cryptococcosis cases are related with this species. Therefore, it is very important to understand how *C. gattii* can infect and cause cryptococcosis to immune-competent patients.

The interaction between the peptide and the target molecule (*C. gattii* in this case), could be explained as an immunological one. Natural killer cells have nectin receptors, which could recognize the yeast in an attempt to fight the *C. gattii* infection. To determine the effect of nectin in the capsule of *C. neo/gat*, the yeast can be pre-treated with the recombinant peptides and then inject the pretreated cells in mouse models. The absence of *C. neo/gat* symptoms in the mouse could represent that the receptor in the yeast's target organ was blocked. As for telecephalin, with the high concentration of catecholamine compounds available in the brain, this protein may reinforce the affinity that *C.neo/gat* has for the brain.

Due to the lack of the appropriated control (an acapsular *C. gattii* mutant) to rule out the interaction due to the mucilaginous capsule, the data obtained in our preliminary study are not conclusive. A quantified number of phages displaying recombinant peptides should be used to avoid binding due to sample saturation. To determine the specificity of the isolated peptide

coded on the capsid surface of the T7 phage displaying human lung and brain libraries to the capsule, one possible experiment is to treat the peptides displayed on T7 System using *Cryptococcus laurentii* and *Streptococcus pyogenes* cells. These microorganisms are good candidates because both species have polysaccharide capsules. *C. laurentii* capsule is composed of the same polysaccharides of *C.neo/gat* capsules, but in different arrangements, while the *S. pyogenes* capsule is composed of hyaluronic acid (Murray *et al*., 2002). With this interaction and the use of the T7 phage display technique, the specificity of both telecephalin and nectin as target peptides for *C.neo/gat* capsule could be determined.

5.6 Findings and Conclusion

- By using cDNA genomic libraries of brain and lungs fused on the T7 bacteriophages capsid, 21 putative capsule-specific peptides of *C. gattii* were isolated.
- Two *C. gattii* putative capsule-specific isolated peptides common to the brain and lungs: nectin with both lung and brain cDNA display libraries and telencephalin only with brain cDNA display library.
- Preliminary specificity analysis of recombinant phage was no sufficient to confirm the *C. gattii* putative capsule-specific peptides, suggesting a more definitive study.

6. General Findings, Conclusion, Recommendations and Bibliography

6.1 General Findings, Conclusions and Recommendations

- In this study, a total of 23 *C.neo/gat* members were isolated from the southwestern region of Puerto Rico where 19 belonged to *C. gattii* and 4 to *C. neoformans*. This is the first time that *C. gattii* is isolated from trees and plants in Puerto Rico. Also, *C. gattii* was recovered from cacti. This corresponds to the first report in the literature of the isolation of this species on such vegetation type. Finally, almost the entire isolation process was made using the swab technique; this factor should be considered at the moment to detect the yeast on these areas.
    - It is recommended to sample the entire Island, including Mona, Monito, and Desecheo among some of the archipelago of Puerto Rico, as well as the islands municipalities of Vieques and Culebra.
    - In order to continue searching for novel habitats for *C.neo/gat*, new tree sampling should be initiated, including common trees in Puerto Rico such as *Tabebuia rosea* (roble wood) and *Thespesia grandiflora* (maga tree).
- The characterization of the isolates using physiological analysis such as BIOLOG Microstation System, revealed seven common carbon sources oxidized by the yeast. Specific carbon sources were identified for six strains, representing the three serotypes of *C. neo/gat*. The System was not able to rapidly identify members of the *C. neo/gat* under the parameters tested.
    - For further work, it is recommended to keep testing more *C. neo/gat* isolates as well as optimizing inoculums size and incubation parameters in order to generate novel BIOLOG *C. neo/gat* identification System database.

- The unique carbon sources identified in this study could be used to develop a rapid identification tool to identify *C.neo/gat* from environmental and clinical samples.

- The amplification of 18S rDNA and the ITS, sequencing and *in silico* analysis of seven isolates revealed that all of them belonged to *C. neo/gat*.
  - To better characterize the isolates it is highly recommended the used more than one genetic technique such as sequencing of the pheromones (mating type), or the *cap 59* genes, and RFLP of *ura5* gene, among others.
- The *C. gattii* whole cell interaction with the lung, and brain PreMade genomic libraries on T7 bacteriophage, using phage display technique allowed the isolation of 21 putative *C. neo/gat*-specific peptides. The *in silico* suggested segments of nectin and telecephalin as the interacting partners. This is the first study performed using whole *C. gattii* cells as target and T7 phage display technique with human lung and brain genomic libraries.
  - It is strongly recommended that these two peptides, as well as the other peptides isolated on this study been tested analyze in dept using *in silico* analysis. Also the peptides should be tested for their specificity to *C. gatti* cells, using *Candida albicans*, *Cryptococcus laurentii*, *Streptococcus pyogenes* and other clinically important microorganisms as "competitors" target cells. If specificity is proved, the test of the peptides as possible anticryptococcal therapy in animal models is recommended.
  - Testing of other PreMade T7 Phage Display Libraries on whole *C. gattii* cells as target is also suggested.

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Appendix

Table A.1 Biolog YT Microplate analyses showing of the wells that react with the different carbon sources

C. gattii C77	Positive Wells	Carbon Source
48hrs	A3, A5, A7, A8, A10, A12, B3, B4	Formic acid, succinic acid, L-aspartic acid, L-glutamic acid, D-gluconic acid, inulin, maltose, matotriose
48hrs	All Negative	N/A
96hrs	B1, B10, B11, B12, C2	Cellobiose, sucrose, D-trealose , turanose, a-D-glucose
72hrs	A3,A5, A7, A8, A9, A10, A11, B1, B2, B3, B4, B5, B6, B7, B8, B9, B10, C1, C2, C3, C4, C5, C6, C7, C8, C9, C10, C11	Formic acid, succinic acid, L-aspartic acid, L-gluconic acid, L-proline, D-gluconic acid, dextrin, cellobiose, gentiobiose, maltose, maltoriose, D-melezitose, D-melobiose, palatinose, raffinose, stachyose, sucrose, N-acetyl-D-glucosamine, a-D-glucose, D-galactose, D-psicose, L-sorbose, salicin, mannitol, D-sorbitol, D-arabitol, xylitol, glycerol
48hrs	A8, A9, A10 (**replica has the same results**)	L-gluconic acid, L-proline, D-gluconic acid
72 hrs	A5, A7, A8, A9, A10, B2, B3, B4, B8, C1, C3, C6 A5, A7, A8, A9, A10, B2, B3, B4, B8, B9, C1, C3, C6	Succinic acid, L-aspartic acid, L-glutamic acid, L-proline, D-gluconic acid, gentiobiose, maltose, matotriose, raffinose, N-acetyl-D-glucosamine, D-galactose, salicin stachyose
120 hrs	A5, A7,A8, A9, A10, B2, B3, B4,	Succinic acid, L-aspartic acid, L-

	B8, B9, C1, C2, C3, C6, C7, C8, C9 **Replica** A5, A7,A8, A9, A10, A11, B1, B2, B3, B4, B6, B8, B9, B10, C1, C2, C3, C6, C7, C8, C9	glutamic acid, L-proline, D-gluconic acid, gentiobiose, maltose, matotriose, raffinose, stachyose, N-acetyl-D-glucosamine, a-D-glucose, D-galactose, salicin, mannitol, D-sorbitol, D-arabitol **Replica** D-gluconic acid, dextrin, maltose, matotriose, D-sorbitol, D-arabitol
144hrs	A5, A7,A8, A9, A10, A11, B2, B3, B4, B7, B8, B9, B10, C1, C2, C3, C6, C7, C8, C9, C11 **Replica** A5, A7,A8, A9, A10, A11, B1, B2, B3, B4, B6, B7, B8, B9, B10, C1, C2, C3, C6, C7, C8, C9, C11	Succinic acid, L-aspartic acid, L-glutamic acid, L-proline, D-gluconic acid, dextrin, gentiobiose, maltose, maltotriose, palatinose, raffinose, stachyose, sucrose, N-acetyl-D-glucosmine, a-D-glucose, D-galactose, salicin, mannitol, D-sorbitol, D-arabinose, glycerol **Replica** Succinic acid, L-aspartic acid, L-glutamic acid, L-proline, D-gluconic acid, dextrin, cellobiose, gentiobiose, maltose, maltotriose, D-melobiose, palatinose, raffinose, stachyose, sucrose, N-acetyl-D-glucosmine, a-D-glucose, D-galactose, salicin, mannitol, D-sorbitol, D-arabinose, glycerol
30.1, 48hrs	A10, A12, B6	D-gluconic acid, inulin, D-melobiose
30.2, 48hrs	A3, A10, A12, C1	formic acid, D-gluconic acid, inulin, N-acetyl-D-glucosamine
30.1, 72hrs	A10, A12, B6	D-gluconic acid, inulin, D-melobiose
30.2, 72hrs	A3, A10, A12, C1	formic acid, D-gluconic acid, inulin, N-acetil-D-glucosamine

46.1, 48hrs	A2, A3, A10, A12, B1, B8, B9, B10, B11, C1, C2, C6	acetic acid, formic acid, D-gluconic acid, inulin, celobiose, D-raffinose, stachyose, sucrose, trealose, N-acetyl-D-glucosamine, a-D-glucose, salicin
46.2, 48hrs	A12, B1, B2, B3, B4, B8, B9, B10, B11, C1, C2, C6	Inulin, cellobiose, gentiobiose, maltose, maltoriose, D-raffinose, stachyose, sucrose, trehalose, N-acetyl-D-glucosamine, a-D-glucose, salicin
46.1, 72hrs	A2, A3, A10, A12, B1, B8, B9, B10, B11, C1, C2, C6	acetic acid, formic acid, D-gluconic acid, inulin, cellobiose, D-raffinose, stachyose, sucrose, trehalose, N-acetyl-D-glucosamine, a-D-glucose, salicin
46.2, 72hrs	A12, B1, B2, B3, B4, B8, B9, B10, B11, C1, C2, C6	Inulin, celobiose, gentiobiose, maltose, maltoriose, D-raffinose, stachyose, sucrose, trehalose, N-acetyl-D-glucosamine, a-D-glucose, salicin