CHAPTER ONE

Introduction

1.1 Background of Study

Cryptococcosis is a dynamic fungal infection that continues to evolve in the second century after its initial recognition as a human pathogen in the 1890s. A little over 100 years ago, *Cryptococcus neoformans* was identified from a peach juice and very shorty the first infection was described after the yeast was later isolated from clinical case in a young German. The yeast over the next century has been extensively characterized as both a primary and secondary human and animal pathogen.

Cryptococcosis is an infection of the central nervous system (CNS) which affects both immunocompromised and immunocompetent individuals. It is the most life threatening, deep-seated fungal infection in AIDS patients, constituting a growing problem in the management of patients with AIDS complex-related diseases. Cryptococcosis is an AIDS-defining illness and, in sub-Saharan Africa, an estimated one million new cases of cryptococcal meningitis occur annually with mortality rates that may exceed 50% (Litvintseva *et al.,* 2011). The effects of cryptococcosis are devastating for both immunocompetent and immunocompromised patients, resulting in death or permanent brain damage unless the condition is diagnosed and treated at the time of onset.

The vast majority of disease in humans is caused by two *Cryptococcus* species *C. neoformans* and *C. gattii*. Genotyping methods have identified major haploid molecular types of *C. neoformans* (VNI,VNII,VNB,VNIV) and of *C. gattii* (VGI-VGIV). These molecular types may represent cryptic species (Boekhout *et al.,* 2001; Bovers *et al.,* 2008), they also differ in their geographic ranges, epidemiology, virulence and population genetics (Bovers *et al.,* 2008; Campbell *et al.,* 2005) . *C. neoformans* is ubiquitous, has a worldwide distribution and can cause meningitis in immunocompromised hosts (Litvintseva *et al.,* 2011), *C. gattii*, in contrast, has a more limited distribution, but has received increasing attention due its link

with the outbreak of cryptococcosis on Vancouver Island, British Columbia, Canada (Byrnes *et al*., 2010).). The *C. gattii* sub-species (molecular type) VGIV is of interest as it is rare with its distribution limited to India as well as having been reported to cause infections in sub-saharan Africa (Litvintseva *et al*., 2006).

Significant diversity exists within and between molecular types which can lead to varying virulence profiles. For example, VGII (further subdivided into VG11a, VGIIb and VGIIc) and VGIII(further subdivided into VGIIIa and VGIIIb) were responsible for the Vancouver outbreaks. Significant difference in the incidence of disease and antifungal susceptibility were observed between VGII sub-genotypes VGIIa, VGIIb and VGIIIc from this outbreak (Carriconde *et al*., 2011) when compared to each other and other genotypes. Generally, VGII strains have significantly higher susceptibility to flucytocine and all azole drugs. *Cryptococcus neoformans* genotypes VNII have lower susceptibility for fluconazole than VNI strains. These findings indicate that genotype and origin influence a cryptococcal strains sensitivity to antifungal agents. There is need for proper identification of isolates which includes molecular typing. Understanding the ecology of this emerging pathogen has benefits for the clinical management of cryptococcal infections in Nigeria.

1.2 Statement of Problem

The study was carried out in Jos, Plateau State, Nigeria. The setting of Jos allows for domestic raring of Pigeons and also has so many *Eucalyptus calmadulensis* trees. In some homes, human beings share the same house with Pigeons hence they are at a risk of contracting infections from Pigeons. The close proximity to human habitation is a significant risk factor for acquisition of potential pathogens. *Cryptococcus* have been isolated from pigeon guanos and captive birds in Jos (Irokanulo *et al*., 1997). The ecology of *C.gattii* is different from that of *C. neoformans, C.gattii* has been isolated from Eucalyptus trees(Saul *et al*., 2008a) The spores of *Cryptococcus neoformans* is the main sources of cryptococcosis

since spores are produced on the Pigeon guanos. Hence there is need to study Pigeon droppings and Eucalyptus trees in Jos if they also harbor *C. neoformans*/*C.gattii*.

1.3 Justification for the study

There is paucity of information as regards the phylogenetic structure of Cryptococcus in Nigeria, considering the pathogenicity of the organism. This study will provide such a database to assist in the management of Cryptococosis in Nigeria.

1.4 Aim

The aim of this study is to characterize environmental isolates of *Cryptococcus neoformans* / *Cryptococcus gattii* isolated from Jos, in order to determine its phylogenetic relationship,population structure and virulence factors.

1.5 Objectives:

1. To isolate *C. neoformans* from pigeon guanos and *Eucalyptus* trees located in Jos
2. To identify the isolates using *URA5*-RFLP PCR
3. To determine *C. neoformans* mating type (MAT **a** verses MAT **α**) allelic pattern by multiplex PCR
4. To determine the sensitivity profile of isolates against some antifungals namely fluconazole, itraconazole, voriconazole and flucytosine.
5. To compare the population structure of *Cryptococcus* isolates from Jos, Nigeria with global isolates by MultiLocus Sequence Typing (MLST) analysis using a standard set of genetic loci which have been developed to discriminate between global isolates.

6. To determine the antifungal activities of some selected plants (*Swetnia mahogany, Cassia alata and Rauvolfia vormitoria)* against *Cryptococcus*

7. To evaluate the embryonated egg model for *Cryptococcus gattii*

8. To compare virulence of *Cryptococcus neoformans* VNII and *C. neoformans* VNI using the Galleria model

CHAPTER 2

LITERATURE REVIEW

2.1 Systematics of the Genus *Cryptococcus*

2.1.1 Phylogenetic Relationship position of the Genera *Cryptococcus* and *Filobasidiella*

The biodiversity and taxonomy of the genus *Cryptococcus* has changed in recent years, with new *Cryptococcus* species continuously discovered. Following the application of advanced molecular techniques like analysis of parts of the ribosomal RNA and use of multigene analysis to generate phylogenetic relationship among the genus, the concept of the extended species concepts among the genus *Cryptococcus* have been introduced. The genus *Cryptococcus* is polyphyletic. The species belong to five major lineages within the Tremellomycetes (subphylum Agaricomycotina, Basidiomycota), namely the orders Tremellales, Filobasidales, Trichosporonales, Cystofilobasidiales, and the *Holtermannia* (Fonseca *et al*., 2011) . The orders Tremellales and Filobasidiales contains the majority of the species, whereas 10 species belong to Trichosporonales, four species belongs to the *Holtermannia*. The order Tremellales and Filobasidiales contains the majority of the species, whereas 10 species belongs to Trichosporonales, four species belong to *Holtermannia* lineage, an only one to the Cystofilobasidiales (Kwon-Chung *et al*., 2011) The genus Filobasidiella, the teleomorphic stage of *C.neoformans* and *C. gattii* is a monophyletic genus that contains *F.neoformans, F.lutea, F. baccispora, F. depauperata, F.amylolenta*

2.1.2 Nomenclature of *Cryptococcus*

The genus Cryptococcus contains 70 species (Fonseca *et al*., 2011). The nomenclature of Cryptococcus neoformans was in disarray until Benhams proposal to conserve the name *C.*

neoformans. Prior to the proposal for the conservation of the name, *C. neoformans* have had several names such as *Sachharomyces .neoformans* (Sanfelice 1985), *Torula* neoformans (Weis 1902), *Debaryomyces hominis* (Todd, 1936). Benham showed that all the pathogenic isolates assigned under *Torula, Cryptococcus, Debaryomyces* were the same species and proposed to conserve the name *C. neoformans*.

Cryptococcus organisms belong to the Filobasidiella clade of the Tremellales (Order - Tremellomycetes) (Findley *et al.,* 2009). The genus Cryptococcus comprises of at least 70 species that have been isolated from various habitats and animals on every continent. Though the species *Cryptococcus laurentii*, *C. curvatus,* and *C. albidus* have caused occasional infections, termed cryptococcosis, only two species, *C. neoformans* and *C. gattii*, have been routinely documented as pathogenic for humans. The biology and clinical significance of these two species have been reviewed (Campbell and Carter 2006; Chayakulkeeree and Perfect 2006; Lin and Heitman 2006)

Cryptococcosis is caused by two basidiomycetous yeast within the "*C. neoformans* species complex" (Lin and Heitman 2006). Based on capsular agglutination reactions of *Cryptococcus* there are five serotypes: A, B, C, D, and AD hybrid (hybrids between serotypes A and D) (Lin and Heitman 2006). On the basis of biochemical tests, such as the ability to use glycine as the sole carbon and nitrogen source, resistance to canavanine, EDTA resistant urease, and the morphology of the sexual state (such as the shape and texture of basidiospores), *C. neoformans* was originally accepted to include two varieties: *var. neoformans* (serotypes A, D, and the AD hybrid) and var. gattii (serotypes B and C) (Levitz 1991) More recently, *C. neoformans* var. gattii has been recognized to be a separate species, *Cryptococcus gattii* (Kwon-Chung *et al.,* 2002). In addition to the previously observed phenotypic differences, molecular studies and genome sequences have detected significant genetic variations between serotypes A and D, this led to the designation of serotype A as a

new variety, var. *grubii* (Litvintseva *et al*., 2011) . Currently, this organism is classified into two varieties and a sibling species: *C. neoformans var. neoformans* (D), *C. neoformans var. grubii* (A), and *C. gattii* (B, C), *C. gattii* has been proposed to have varieties (Ngamskulrungroj *et al*., 2009). Both are encapsulated yeasts, but *C. gattii* colonies on agar media tend to be more mucoid. Subtle morphological differences exist between the yeast cells of *C. neoformans* and *C. gattii*. For example, *C. neoformans* produces colonies of ovoid to spherical cells. In contrast, *C. gattii* yeasts tend to be more ellipsoidal in appearance. These two species can be differentiated via their biochemical differences. Most notable is that *C. gattii* reacts positively on CGB agar, whereas *C. neoformans* does not.

2.1.3 Population structure of *Cryptococcus neoformans* and *Cryptococcus gattii*

Population structure refers to the genetic diversity among individuals comprising the population; the operative mode(s) of reproduction and genetic exchanges; and the formation of subdivisions, which may be driving by geographical, temporal, ecological or genetic factors that distribute the individuals into separate groups.

The application of molecular genotyping methods in the study of *C. neoformans* /*C. gattii* complex has further helped us in understanding the diversities within the species. The most apt techniques used in genotyping of *Cryptococcus* based on molecular techniques include PCR finger printing, restriction fragment length polymorphism(RFLP), randomly amplified polymorphic DNA(RAPD) and amplified length polymorphism(AFLP) analysis (Boekhout *et al*., 2001; Ellis *et al*., 2000; Latouche *et al*., 2003), Many studies used different gene locus, some used single gene locus (Biswas *et al*., 2003) and Multilocus sequence types(MLST)(Litvintseva *et al*., 2006). *Cryptococcus* has been further subdivided into nine distinct molecular types (Figure 1) on the basis of DNA sequence polymorphisms detected by PCR fingerprinting, RAPD, amplified fragment length polymorphism (AFLP), restriction fragment length polymorphism (RFLP), and multilocus sequence typing (MLST) analyses(Sorrell *et al*., 1996; Litvintseva *et al*., 2006; Meyer *et al*., 1999)

Table 2.1: Nomenclature of the subpopulation of *C. .neoformans* and *C. gattii*

Species	**Serotype(Variety)**	**PCR Fingerprinting**	**AFLP genotype**
C.neoformans	A(Var. *grubii*)	VNI	1
		VNII	1B
		VNB	1A
	AD	VNIII	3
	D(Var. *neoformans)*	VNIV	2
C.gattii	B	VGI	4
		VGII	6
	C or B	VGIII	5
	C	VGIV	7

Serotype A isolates produces VNI, VNII, or VNB (so far unique to Botswana) patterns (Litvintseva *et al*., 2005); AD hybrids produce a VNIII pattern; and serotype D isolates produce a VNIV pattern. Serotype B defines VGI,VGII, while serotype C defines VGIII and VGIV. AD strains are hybrids of the two varieties. Whereas most isolates of serotypes A and D are haploid, AD strains are diploid or aneuploid, contain two sets of chromosomes, and possess two mating type alleles, one from each of the two serotype A and D haploid genome (Xu *et al*., 2002; Cogliati *et al*., 2001)

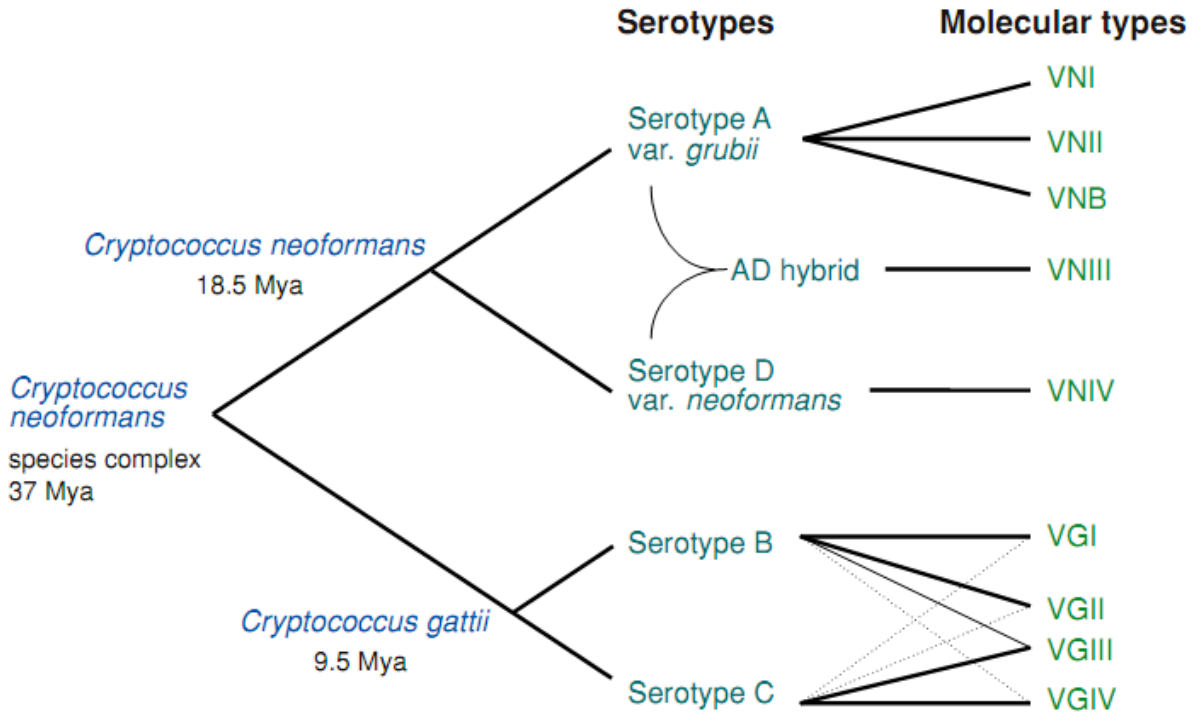


Fig 2.1: Evolution of the *C. neoformans* species complex.

The Cryptococcus species complex contains at least two subspecies, *C. neoformans* and C. gattii, which diverged from a common ancestor ~37 and ~18.5 mya, respectively. They are further divided into four serotypes consisting of at least nine molecular types. Solid, thin, and dashed lines indicate the prevalence of the respective serotype in each molecular type (Lin and Heitman 2006) MLST markers is the preferred method of genotyping *C.neoformans* and *C. gattii* (Bovers, *et al.*,2008; Litvintseva, *et al.*,2006). The most recent, universal and unambiguous set of MLST markers was developed by the *Cryptococcus* species genotyping working group (http://www.isham.org.org/groups.html.), organized by the international society for human and Animal mycology(ISHAM), the MLST markers are sufficient to assign new isolates to the major subpopulations (Meyer *et al.*, 2009).

2.1.4 Emerging Pathogen species of *Cryptococcus*

In addition to *C. neoformans* and *C. gattii*, other species of the genus *Cryptococcus* referred to as non-neoformans have been implicated in clinical situations with varying frequencies.

These includes, *C. adeliensis, C.albidus, C.laurentii,C. curvatus, C.diffuluens,C.magnus, C. humicola, C.vishniacii, C.saitoi, C.macerans*. These infections are often acquired from the environment from a variety of potential sources, these sources include bird excreta, food (cheese, fruits), soil, plants, water. They all produce capsule and melanin, however, laccase enzyme is said to be lower in the non-neoformans species(Ikeda *et al*., 2002)

Over 80% of the non-neoformans species of *Cryptococcus* isolated are *C.laurentii* and *C.albidus* (Bernal-Martinez *et al*., 2010). The use of more aggressive treatment regimen that leads to prolonged immunosuppression and the application of more sensitive identification method may lead to the increased recovery of these non-neoformans species. However, the interpretation of infections caused by *C. laurentii* and *C. albidus* should be with caution because recent studies had revealed that both species are complexes. For example, *C. albidus* senso lato was found to contain 12 species namely *C. aerius, C. albidus* senso stricto, *C. diffluens,C.saitoi, C. liquifaciens, C. uzbekistanesis* and have been found in clinical cases . However it is unknown whether or not any of these new species constitute the majority of the reported *C. albidus* isolates from clinical specimen. Species complex is not considered a zoonotic pathogen, and people and susceptible animals acquire the organism from similar environmental sources. (Blaschke-Hellmessen 2009)

2.2. **Ecology of *Cryptococcus species complex***

Current information suggests that in nature, the two species of *C. neoformans* specie complex reside in separate environmental niches.

2.2.1 Pigeons and other birds

Cryptococcus neoformans serotypes A and D have been isolated from various sources in nature. Their association with pigeon guano is well established, and the fungus has also been less commonly isolated from droppings of other avian species such as chicken, goose, duck, eagle, owl, peacock, and parrot (Irokanulo *et al*., 1997; Caicedo *et al*., 1999). The pigeons however do not develop cryptococcosis. This is because the avian body temperature does not

allow for its growth. However, the excrement of columbine birds is a natural enrichment medium for *C. neoformans var. grubii*, and the birds serve as vectors to disseminate the yeasts.The worldwide distribution of *C. neoformans* has been linked to the association between this yeast and pigeons which migrate from one place to another (Littman and Borok 1968), Pigeons are carriers of the yeast. The birds are rarely affected by the yeast, this may be due to the high body temperature of 41.5-43°C which is unfavorable for the growth of the yeast (Emmons 1960). However, about 26 cases of avian cryptococcosis have been reported, although their temperatures were not reported(Malik *et al.,* 2003).

Other than pigeon guanos, *C. neoformans* has been isolated from the body of pigeons, *C. neoformans* have been isolated from the beak, feather and legs of pigeon. These body parts are thought to be contaminated from contact with guano of the pigeon (Pal 2009). *Cryptococcus* have also been isolated from within the body of pigeons (Rosario *et al.,* 2008) Aged pigeon guano and the dirt and dust surrounding the droppings are more likely to be positive for *C. neoformans* than are fresh droppings (Granados and Castañeda 2005), suggesting either that the fungus could originate in the soil and flourish in this particular environment after the soil is contaminated with bird guano, or that the few cells originally in the guano could amplify better in the exposed environment. Airborne *C. neoformans* cells have been collected from the air above bird guano from soil, but not from air above guano deposited on a large adjacent asphalt area (Filion *et al.,* 2006), it is less likely that the fungus was originally present in the guano(Lin and Heitman 2006)

Avian habitat is a significant reservoir for *C. neoformans*. The yeast is found in aged pigeon excreta (Rosario *et al.,* 2008). Pigeon guanos offers a suitable condition and possibly less competition for the multiplication of *Cryptococcus* than other sources like plant sources (Granados and Castañeda 2005), It has been documented experimentally that the fungus multiplies well in sterilized pigeon or chicken guano(Abou-Gabal and Atia 1978). Another

reason that could suggest that pigeons are not systemic carriers of this yeast is because of the high internal temperature of pigeons which is 42°C. At such temperature, *Cryptococcus* cannot survive. Pigeons have been shown to effectively clear this yeast due to the high temperature of the gastrointestinal tract(Abou-Gabal and Atia 1978) . However, isolation of *C. neoformans* from avian environments may reflect colonization by enrichment due to the favourable conditions of guano-contaminated soil. However, this does not necessarily mean that birds do not play an active role in dissemination of *C. neoformans* in nature, since they could either pass the fungus though their body or carry the fungus on their surface and could readily transport the cells for a long distance. Birds, most notably pigeons, still remain the most probable vector for worldwide dissemination of this fungus.

Cryptococcus can be isolated from many tropical and semitropical region from avian roosting areas that are shielded from direct sunlight and UV light. In this natural environment, *Cryptococcus* secretes urease and thrives on urea and other nitrogenous substances (Mitchell *et al*., 2011). They also produce spores that are airbone that reach the aveoli if inhaled. In the laboratory, both species of *Cryptococcus* grows well in pigeon guanos and produces spores (Nielsen *et al*., 2007).

The prevalence of *Cryptococcus* in avian samples tends to corresponds with endemicity. The association of avian *Cryptococcus* presence and as a major source of human cryptococcosis has been the subject of several studies (Casali *et al*., 2003). More convincingly, the same genotype of *Cryptococcus neoformans* have been isolated from patients and pigeon faeces (Litvintseva 2005).

2.2.2 *Cryptococcus* and Tree

Cryptococcus species are found in fruits and vegetables, soil, decaying wood, and honey bee colonies during the flowering season of eucalyptus trees (Cornelissen *et al*.,2003; Ergin *et al*.,2004). However, the two species of *Cryptococcus* occupy different ecological niches.

2.2.3 Cryptococcus gattii and Eucalyptus Trees

Cryptococcus gattii have been isolated frequently from the plant detritus of eucalyptus species (Ellis and Pfeiffer 1990; Krockenberger *et al.,* 2002), establishing a preferential association between this fungus and eucalyptus trees. Increased sampling worldwide in recent years, however, suggests that there must also exist environmental sources other than the known eucalypt host (Chen *et al.,* 1997a). In Australia, C. gattii has been isolated from other gum trees, such as the turpentine gum tree (Syncarpia glomulifera), tallow wood (Eucalyptus microcorys), and flooded gum (Eucalyptus grandis). In Papua New Guinea and in regions of Australia where the prevalence of *C. gattii* cryptococcosis is highest, the known host eucalypts are not endemic (Campbell *et al.,* 2005). In Colombia, *C. gattii* isolates have been found in several new species of trees including *Terminalia catappa* (Huérfano *et al.,* 2003), *Eucalyptus globulus, Ficus soatensis, Croton bogotanus, Croton funckianus, Coussapoa*. In Brazil, *C. gattii* was isolated repeatedly from the hollow of a pottery tree (*Marlierea tomentosa*) (Lazéra *et al.,* 1998) and from the decaying wood in a hollow of a native jungle tree, Guettarda acreana, in an isolated environment with no human activity or introduced vegetation (Fortes *et al.,* 2001). Systematic surveys are needed to justify the association of *C. gattii* and specific trees

2.2.4 C. neoformans var. grubii and var. neoformans and trees.

Although *C. neoformans* var.grubii and var. neoformans are isolated more commonly from pigeon-guano-contaminated soil, they have also been isolated repeatedly from air, soil, and swabs from trees, in; (Lazéra *et al.,* 1998; Randhawa *et al.,* 2005). Thus, *C. neoformans* var. grubii and var. neoformans could share the same natural niche with C. gattii, and these sibling species could interact in their life cycle in nature. There has been no evidence for an association between *C. neoformans* var. grubii and var. neoformans and specific trees. These two varieties have been consistently isolated from decaying wood in the hollows of many species of trees from different geographic regions where the flora differ considerably

The colonization of decaying wood and tree hollows by *C. neoformans* var. grubii, var. neoformans, and *C. gattii* could be enhanced by their ability to produce the enzyme laccase, which is associated with lignin degradation by wood-rotting fungi. However, there is no evidence thus far that they can degrade wood by themselves. It is possible that they survive in the wood together with other fungi, bacteria, or in a succession of species

2.3 **Life Cycle of *Cryptococcus* / Development of *Cryptococcus***

Cryptococcus is isolated from patients and the environment as a budding yeast. During the sexual cycle, *Cryptococcus* switches from yeast growth to hyphal growth. Despite this dramatic morphological transition, Cryptococcus is not considered by some to be a dimorphic fungus because yeast cells are the predominant form in the environment and in the human host, and it is likely that the morphological transition is not involved in infection (Kozubowski and Heitman 2012) However, it can also undergo a dimorphic transition to a filamentous growth form by two distinct differentiation pathways: Mating and Monokaryotic fruiting.

There are at least three important reasons why the development of *Cryptococcus* is relevant to its pathogenicity. First, spores that result from hyphal development during mating are infectious propagules. Upon inhalation, spores (in addition to desiccated yeast) can colonize the lungs of a host. *Cryptococcus* propagates to the bloodstream and crosses the blood-brain barrier, ultimately colonizing brain tissue and leading to fatal consequences if not treated. Second, sexual reproduction contributes to the genotypic variability of *Cryptococcus* species, which may lead to increased fitness and virulence. Third, some genes located within the Mating type(MAT) locus are important during mating and during infection. Therefore, the development of Cryptococcus is not only an interesting paradigm for biologists, but is also important in the study of cryptococcal pathogenicity.

2.4 Mating Types and Virulence

2.4.1 Mating

Cryptococcus has three growth forms which manifest differently depending on the developmental phase of the yeast. The most prevalent form is its natural habitat and in clinical samples is unicellular budding yeast, which reproduce by mitotic division. Alternative vegetative forms are pseudohyphae. Pseudohyphae are linked yeast cells that do not completely separate after mitotic divisions and serve as an intermediate form between yeast and true hyphae.

Cryptococcus neoformans pseudohyphae have only occasionally been reported in clinical samples(Lin 2009) in the environment, pseudohyphal form are thought to protect the yeast from predators(Neilson *et al*., 1978). The third growth form the hyphal growth is formed as a result of mating.

During mating (Fig 2.2), two compatible yeast cells fuse, **a** and **α** cells but the two parental nuclei remain separate, leading to the formation of diakaryotic hyphae, a hallmark feature of basidiomycetes. When strains of the opposite mating type confront each other under these conditions, **α** cells make conjugation tubes, and **a** cells expand to form round, enlarged cells. The **a** and **α** cells fuse with one another to form elongated cells that contain two distinct nuclei (Dikaryon). Another basidiomycete-specific characteristic is the specialized clamp cells that form between each cellular compartment in the hyphae to maintain the dikaryotic state. The ultimate developmental stage in the sexual cycle of *C. neoformans* occurs in a terminal, specialized cell called the basidium, in which the fusion of the parental nuclei and meiosis take place. Nuclear meiotic products undergo rounds of mitotic division, and the mitotic nuclei are packaged into spores that bud from the apical surface of the basidium to form four spore chains, a feature that distinguishes the genus Filobasidiella from other members of the Basidiomycota. Upon germination, spores develop into yeast, which concludes the sexual cycle.

Mating is a possible source of hybrid *C. neoformans* species. Hybrids are most frequently formed between the A and the D serotypes of either the same or opposite mating types (aADa, aADa, aADa) and are of significant clinical importance (Litvintseva *et al.,* 2005; Lin *et al.,* 2007). Diploids derived from genetically distinct strains of the same serotype (aAAa) have also been reported(Lin *et al.,* 2009). Interestingly, even hybrids between *C. neoformans* and *C. gattii* have been described from the environment and in clinical samples(Bovers *et al.,* 2008). Kwong- Chung et al.(2002) identified mating between serotypes B and D (*C. gattii*), however, she noted that the spores produced are different from those produced by A and D.

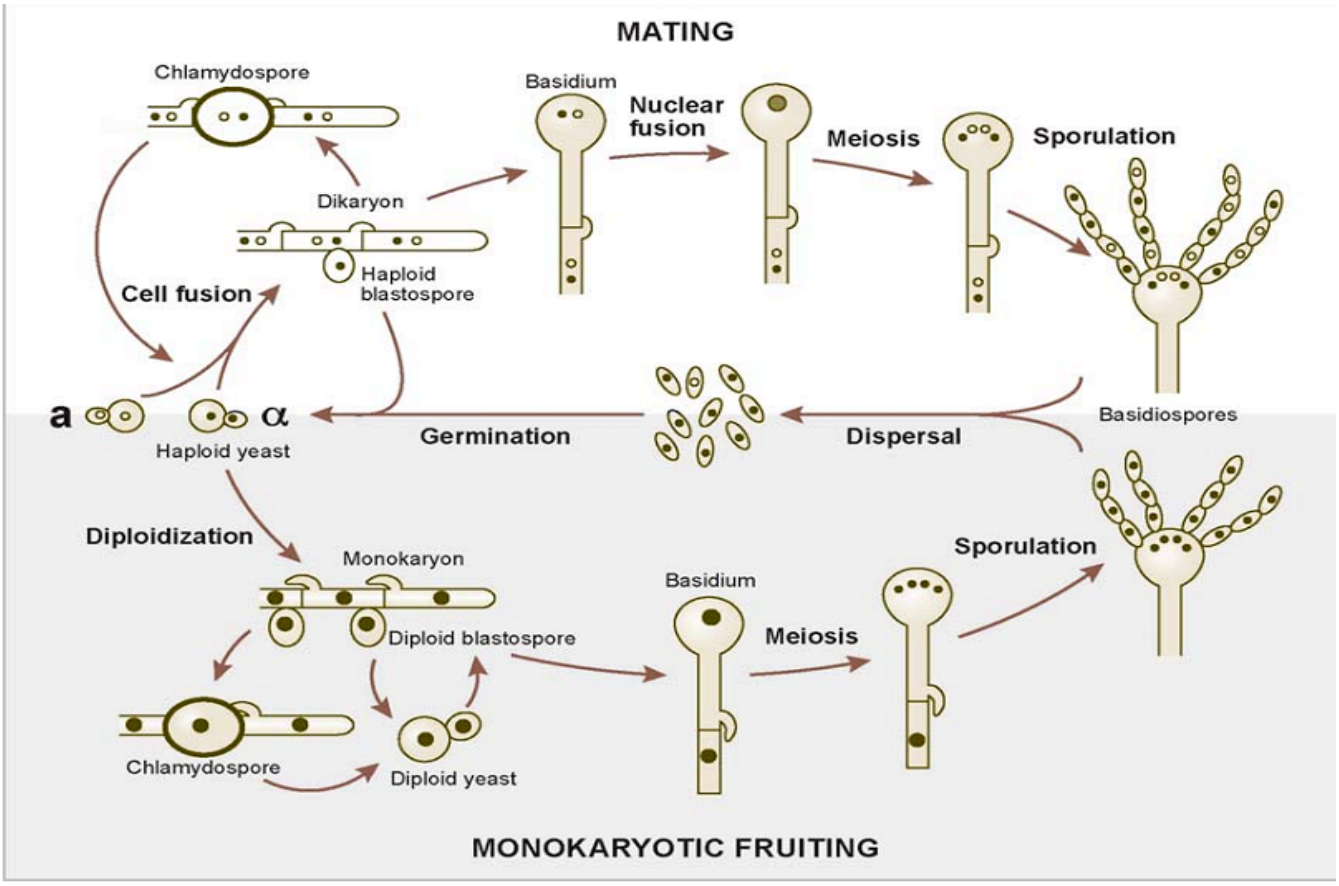


Fig 2.2 : Model of the Cryptococcus neoformans life cycle. In response to nutrient limitation, a and α yeast cells secrete peptide pheromones that trigger cell–cell fusion. Nuclear fusion is delayed, and the resulting dikaryon initiates filamentous growth. The two parental nuclei migrate coordinately in the dikaryotic hyphae, and as each septum forms to separate the cells, one nucleus is transferred to the penultimate hyphal cell through a clamp connection. At the stage of basidium development, the two nuclei fuse and undergo meiosis to produce four meiotic products that undergo mitosis and bud from the surface of the basidium to produce chains of basidiospores. During monokaryotic fruiting, cells of one mating type, for example, α cells, become diploid (α/α) cells, either by endoduplication or by nuclear fusion following fusion of two cells. The diploid monokaryotic hyphae form rudimentary clamp connections, but these are not fused to the preceding cell. At the stage of basidium development, meiosis occurs and haploid basidiospores are produced in four chains (Lin and Heitman 2006)

2.4.2 Monokaryotic fruiting or Same sex mating

Another way *Cryptococcus* undergoes filamentous growth is via monokaryotic fruiting (Fig 2.2). When exposed to environmental conditions conducive for mating under conditions of desiccation and severe nitrogen limitation (Wickes *et al*., 1996) **α** haploid *C. neoformans* strain can produce hyphae decorated with basidia and spore chains, similar to hyphae resulting from mating between opposite-sex partner(Shadomy and Utz 1966).This type of haploid-derived hyphal growth was initially considered asexual haploid fruiting that only occurred in the **α**-mating type (Wickes *et al*., 1996),but the exclusive ability of **α** cells to fruit was subsequently challenged when others demonstrated that mating type **a** cells can also undergo haploid fruiting(Tscharke *et al*., 2003; Lin and Heitman 2006) The discovery of genetic recombination and ploidy changes during haploid fruiting revealed that this form of growth is an alternative mode of sexual reproduction involving only one mating type (Lin *et al*., 2005b; Fu *et al*., 2015). Haploid fruiting is also referred to as monokaryotic fruiting, unisexual reproduction, or same-sex mating. The efficiency of same-sex mating is relatively low, and it is not a common phenomenon; in serotype D strains, fruiting of the **a**-mating type is rare, and neither of the serotype A mating types derived from H99 strain can self-filament under laboratory conditions. The biological significance of same-sex mating is evident from population genetics studies on environmental and clinical isolates (Bui *et al*., 2008; Lin *et al*., 2007; 2009; Saul *et al*., 2008b). These studies demonstrate that same-sex mating plays an important role in generating diversity in *C. neoformans* populations and contributes to the global spread of cryptococcosis. Given the predominance of the **α**-mating type in nature, same-sex mating may be one way in which *C. neoformans* can undergo genetic recombination in the environment (Lin *et al*., 2005b; Fu *et al*., 2015).

It is striking that both same-sex and opposite-sex mating are triggered and regulated by similar environmental conditions and involve the pheromone signaling pathway. However, same-sex mating is a significantly more plastic process compared with opposite-sex mating

and relies on alternative signaling pathways in addition to pheromone signaling (Wang and Lin 2011). This is exemplified by several mutants defective in bisexual mating, but capable of unisexual mating (Hsueh and Shen 2005; Hsueh *et al*., 2007; Ekena *et al*., 2007) During opposite-sex mating, a diploid nucleus is formed by nuclear fusion before sporulation, but diploidization in same-sex mating may occur via endo-replication (Lin *et al*., 2005b). Under a low magnification, hyphae produced during same-sex mating resemble those resulting from opposite-sex mating, but appear more sporadically. However, unlike opposite-sex mating hyphae, hyphal cells produced by same-sex mating contain single nuclei (monokaryotic), clamp cells do not fuse, and spores are smaller and rounder (Wickes *et al*., 1996; Lin *et al*., 2005a).

2.5 The *Cryptococcus* Mating-Type (MAT) locus

Mating in fungi is controlled by the information encoded by the MAT locus. This region of the genome is special because homologous chromosomes contain non-homologous information that specifies the genetic differences between cell types. In ascomycete fungi, this region has been studied extensively particularly in *S. cerevisiae* (O'Rourke and Herskowitz 1998) The MAT region is a specialized region of the genome that is idiomorphic or allelic between different sexes(mating types). In *S. cerevisiae* there are two different MAT genes, **a** and **α** they both regulate the transcriptional activators and repressors that govern the expression of cell-type-specific genes(Herskowitz *et al*., 1992)

Cryptococcus neoformans is a heterothallic fungus with a bipolar mating system in which a single mating locus defines each mating type (**a** and **α**) (Kwon-Chung 1976). In basidiomycetes MAT loci is made of an unlinked MAT loci; one encodes pheromones and pheromone receptors, while the other encodes homeodomain transcriptional regulators. Both MAT loci are necessary to specify cell type. The basidiomycete MAT loci are typically much

larger than the ascomycete MAT loci, encode more than one gene product, and exist in multiple alleles, giving rise to thousands of different mating types (Heitman *et al*., 2014)

Vast majority of of *C.neoformans* population obtained from the environmental and from clinical isolates are **α** mating type(Kwon-Chung and Bennett 1984). Mating type **a** are extremely rare except in a population isolated from the sub-Saharan region of Africa, in which the **a** makes up to 10% of the population (Hsueh *et al*., 2011). *MAT***α** outnumbers **a** cells by a ratio of 45:1 in environmental isolates and a ratio of 30:1 in clinical isolates(Nielsen and Kwon-Chung 2011) . The MAT**a** cells only account for 2% of the *C.neoformans* var. *neoformans* population (Nielsen and Kwon-Chung 2011). Among the var. *grubii*, only 17 of the isolates are MAT**a** (Lengeler *et al*., 2000). Also, within *C. gattii*, the same trend is observed(Nielsen and Kwon-Chung 2011).This has led to the hypothesis that the MAT**α** may be more fit than the MAT**a** under the same condition. Virulence studies conducted to compare the virulence of the two mating types showed that MAT **α** are more virulent than MAT**a** cells. Studies comparing congenic MAT**a** and MAT**α** of serotypes D JEC20/JEC21 and NIH433**a**/NIH433**α**, **α** were found to be more virulent than MAT**a** (Lengeler *et al*., 2000). In another study, the **α** were shown to cross the blood brain barrier better than the MAT**a** (Nielsen *et al*., 2003).

An early effort was made to clone the MAT region from **α** cells. Moore and Edman(1993) used a different cloning technique to identify regions of the **α** genome that were not present in the a genome . They identified an approximately 35-kb region that was present in only cells. They also defined a 2-kb fragment of this region that when transformed into MAT**a** cells conferred the ability to form conjugation tubes (mating structures) in response to conditions that support mating (V8 medium). The sequence of this fragment revealed an open reading frame with strong similarity to the mating pheromones of other fungi. This gene has subsequently been shown to encode one of three **α** pheromones used for signalling a mating

partner in *C. neoformans* (Davidson *et al*., 2002; Shen *et al*., 2002). The MAT locus also contains genes required for virulence. Some of the genes that are involved in signalling cascade include STE11, STE20 and STE12 which are all embedded in an approximately 50kb MAT region.

2.6 Sex and Virulence

The degree of genetic variations among species increases due to sex, sex within the *Cryptococcus* species also allows the population to respond to selective pressure(Butlin 2002). Sexual reproduction can disrupt harmful mutations and retain beneficial mutations. Whereas many basidiomycetous fungi posseses the tetrapolar mating system, the *Cryptococcus* species have evolved a bipolar system thereby enabling them to promote inbreeding. Thus the *Cryptococcus* species that are pathogenic to human appear to combat the possible negative attributes of recombination on virulence by limiting their sexual reproduction while still maintaining a sexual state in order to allow for adaptation to a changing environment (Nielsen *et al*., 2007). Monokaryotic fruiting/same sex mating, allows *Cryptococcus* to retain traits such as those required for virulence or environmental predation. This hypothesis suggests that sexual recombination via monokaryotic fruiting could promote virulence. Both the bipolar and the unique Monokaryotic fruiting observed in *Cryptococcus* may sustain, and possibly promote the virulence of the organism (Nielsen and Kwon-Chung 2011).

2.7 Pathogenesis of Cryptococcal Infections

2.7.1 Virulence Factors of *Cryptococcus*

2.7.1.1 Melanin

One of the defining characteristic of *C. neoformans* is its ability to synthesize a dark cell wall-associated pigment when grown in media containing phenolic compounds. This dark

substance is melanin, a negatively charged high-molecular-mass, hydrophobic, amorphous, and insoluble compound that is difficult to characterize and study (Trofa *et al.,* 2011). The structure of melanin is yet to be resolved because current biochemical and biophysical approaches are unable to provide a reliable chemical formular for this complex polymer(Henson *et al.,* 1999). At present, a substance is considered to be a melanin if it is black or brown, insoluble in aqueous or organic fluid, resistant to concentrated acid and susceptible to bleaching by oxidizing agents.(Butler and Day 1998)

Melanin in *Cryptococcus* was first described by Staib, who in 1962 documented brown cryptococcal colonies on agar containing *Guizotia abyssinica* seed extracts (Staib 1962). Melanin remains a distinctive feature used for the diagnosis of *Cryptococcus* (Pulverer and Korth 1971). The enzyme responsible for melanin synthesis was characterized as laccase (Ikeda *et al.,* 2002). The majority of melanin produced in *C. neoformans* is regulated by the gene Lac1 (Trofa *et al.,* 2011). Lac1 gene is regulated by glucose concentration, iron ions, temperature, the available nitrogen source and copper concentration, glucose starvation and lower temperature (25°C versus 37°C) increases the amount of the enzyme(Polacheck and Kwon-Chung 1988).

Melanin contributes to the environmental survival of *Cryptococcus*. In the environment, melanin is thought to protect *Cryptococcus* from various stresses including enzymatic degradation, Radiation (UV, Solar, Gamma)(Rosas and Casadevall 2001), and heavy metals (Garcia-Hermoso *et al.,* 2001; Rosas and Casadevall 2001).

Melanin is an important virulence factor that protects *Cryptococcus* from host immune response (Gómez and Nosanchuk 2003) and also functions as immunomodulator (Mednick *et al.,* 2005). It increases resistance to phygocytosis in vitro and in vivo(Martinez and Casadevall 2006). Melanin is a powerful antioxidant with broad capacity to protect fungal cells against oxygen- and nitrogen derived oxidants produced by host effector cells. The

oxidant capability of melanin is similar to those of superoxide dismutase, although melanin has greater chemical reactivity and could, at least protect against wider variety of oxidants.

Melanin absorbs microbicidal peptides which may prevent certain host-toxic compounds from reaching their final target in melanized *C.neoformans* cells (Doering *et al*., 1999). Melanized *C. neoformans* cells are less susceptible to the antifungal amphotericin B and caspofungin than nonmelanized cells (Trofa *et al*., 2011). Incubation of amphotericin B and caspofungin with melanin prior to exposure with the fungus significantly reduced their antifungal activities against *C. neoformans*, while incubation of itraconazole,fluconazole, and flycytocine had no effect (Ikeda *et al*., 2003). Melanised *Cryptococcus* cells have higher MICs.

2.7.1.2 Polysaccharide Capsule

The most distinctive feature of *C. neoformans* and *C. gattii* when visualized in India ink under the microscope is the presence of a capsule. Capsule plays important role in virulence. The mechanism by which the capsule contributes to virulence includes interference with the immune mechanisms and protection of the fungal cell from immune mechanism (Rodrigues *et al*., 2011). Other mechanisms include, apoptosis induction, inhibition of leukocyte migration, inhibition of antibody production, complement depletion, interference with phagocytosis, reduced antigen presentation, and in vitro invasive growth (Monari *et al*., 2005)

Capsule composition varies between strains. The capsule is composed mainly of the polysaccharide. Classical studies have identified two different types of glucoronoxylomannan (GXM)(Bose *et al*., 2003). During interaction with various organs of the body in vivo, the capsule changes structure thereby influencing the outcome of the interaction with the host. Capsular changes also have been correlated with the yeast crossing the blood-brain barrier (Charlier *et al*., 2005)

2.7.1.3 Thermotolerance

All human pathogenic fungi display the ability to grow at 37°C. This characteristic distinguishes them from myriads of non-pathogenic fungi in nature that prefers lower temperatures. *Cryptococcus neoformans* exhibits variable tolerance to higher temperature, and a switch from lower temperature to higher temperature causes important changes in the transcriptional regulation of many genes. In *C. gattii* unlike *C. neoformans*, the strains have been reported to be not robust in their growth at 37°C as were *C.neoformans* strains, the optimal growth temperature for *C. gattii* is 35°C (Kwon-Chung *et al.,* 1992) in tissues. *Cryptococcus gattii* exhibit mycelial phase growth instead of yeast, a feature consistent with other dimorphic fungi such as *Blastomyces dermatitidis, Histoplasma capsulatum and Sporothrix sckenkii* (Kwon-Chung *et al.,* 1992).

2.7.1.4 Phenotypic switching

Changes in colony morphology from smooth to rough cells is a known pathogenic attribute for host adaptation and microevolution. Phenotypic switching has been extensively studied for both *C.neoformans* and *C.gattii* (Jain and Fries 2008). Phenotypic switching may confer on *C. neoformans,* a mechanism for rapid change that could help the species survive adverse environmental stimuli. It allows this pathogen rapid change without the disadvantage of an increased mutation rate, thus avoiding the accumulation of disadvantageous mutations (Fries *et al.,* 2002).

2.7.1.5 Degradative enzymes

2.7.1.5.1 Phospholipases

Phospholipases are a heterogeneous group of enzymes that are able to hydrolyze one or more ester linkages in glycerophospholipids. The action of phospholipases can result in the destabilization of membranes, cell lysis, and release of lipid second messengers (Ghannoum

2000; Santangelo *et al*., 1999). *Cryptococcus neoformans* secrets a phospholipase enzyme that demonstrates phospholipase B (PLB), lysophospholipase hydrolase, and lysophospholipase transacylase activities. As with proteinases, phospholipases contribute to the degradation of host cell membrane and thus cell lysis. There is a correlation between phospholipase expression and virulence in a dose-dependent manner among the strains used to infect mice (Chen *et al*., 1997b; Ghannoum 2000). Disruption of PLB1 gene in *C.neoformans* led to reduced virulence in vivo and growth inhibition in a macrophage cell line (Cox *et al*., 2001). Phospholipase can also cleave dipalmitoyl phosphatidylcholine, one of the main components of lung surfactant, and thus assists fungal spread (Steenbergen and Casadevall 2003). Furthermore, recent studies demonstrated that phospholipase B of *C. neoformans* enhances adhesion of *C. neoformans* to a human lung epithelial cell line(Ganendren *et al*., 2006) and dissemination of cryptococcosis in a murine model(Santangelo *et al*., 2004).

2.7.1.5.2 Urease

Urease catalyzes the hydrolysis of urea to ammonia and carbamate and is an important pathogenic factor for certain bacteria (Steenbergen and Casadevall 2003). The cryptococcal urease, Ure1, is an important virulence factor and mice infected with a ure1 mutant strain live longer than mice infected with the wild type strain H99 (Cox *et al*., 2001). Although urease was not required for growth in the brain, the dissemination patterns in the brain, spleen, and other organs after intravenous inoculation differed from the wild type strain, leading to the proposal that Ure1 is important for CNS invasion by enhancing yeast sequestration within microcapillary beds (such as within the brain) during hematogenous spread, thereby facilitating blood-to-brain transmission (Olszewski *et al*., 2004).

2.8 Cryptococcosis in Humans

Although the most common clinical form of *Cryptococcus* infection in humans is meningoencephalitis, the initial infection is acquired by inhalation of fungal cells from an environmental source where mixed populations may co-exist. *Cryptococcus* is not an obligate human parasite, they can be found in both human and nonveterbrate hosts. Cryptococcosis occurs in both animals and humans, but animal-to-human or human-to-human transmission has not been documented, other than rare examples of iatrogenic transmission (Lin and Heitman 2006). Human infection is thought to be acquired by inhalation of airborne propagules from an environmental source (Currie *et al.,* 1995; Granados and Castañeda 2005).

Cryptococcus can colonize the host respiratory tract without producing any disease. Infection is typically asymptomatic, and it can be either cleared or enter a dormant, latent form. When host immunity is compromised, the dormant form can be reactivated and disseminate hematogenously to cause systemic infection. *Cryptococcus* can infect or spread to any organ to cause localized infections involving the skin, eyes, myocardium, bones, joints, lungs, prostate gland, or urinary tract (Barber *et al.,* 1995; Gurevitz *et al.,* 1994; Sobel and Vazquez 1999).

2.8.1 Latent or acute infection.

Whether cryptococcosis is caused by acute infection or reactivation of latent infection is still a debatable topic. However, accumulated evidence suggests that reactivation of latent infection may be the more common presentation. Persistent cryptococcal infection is likely due to relapse rather than reinfection (Brandt *et al.,* 1996). A survey of children 2 years and older in New York City showed that most of them had serological evidence of asymptomatic *C. neoformans* infection (Goldman *et al.,* 2001). Serial isolates of *C. neoformans* from AIDS

patients exhibit no change in genotype indicating that persistent cryptococcal infection is caused by relapse rather than reinfection (Brandt *et al*., 1996; Idnurm *et al*., 2005).

Argument supporting dormant *C. neoformans* infection was derived from a study of patients in France who acquired the fungus originally in Africa, where they lived for approximately 10 years prior to moving to Europe. All of the strains isolated from these patients had randomly amplified polymorphic DNA (RAPD) patterns different from those of isolates from European, American, or Asian patients (Garcia-Hermoso *et al*., 2001). Other clinical evidence for reactivation of latent infection, in this case with *C. gattii*, is based on the clinical history of a Cambodian who was thought to have been infected in Asia or Africa years ago before he lived in France, where he was diagnosed (Dromer *et al*., 1992). However, acute infection can also occur when immunocompromised individuals are exposed to large numbers of *C. neoformans* cells (Nosanchuk 2000).

The most common clinical manifestation of human cryptococcosis is meningoencephalitis. Although the natural route of infection by *C. neoformans* is the respiratory tract, the most common clinical form of human cryptococcosis is meningoencephalitis. Why this fungal pathogen has a predilection for the Central nervous system (CNS) is an interesting yet still unresolved question. At least three possible hypotheses could explain the neurotropism of *Cryptococcus* infection. First, specific neuronal substrates suitable for *Cryptococcus* growth could exist in the CNS. Second, the CNS might provide a refuge for *C. neoformans* from host immune responses. Third, specific receptors present on neuronal cells could attract *Cryptococcus* cells more avidly than other organs during systemic infection.

The CNS could harbor a fungal preferred substrate. Neurotransmitters such as dopamine and epinephrine can be used by *C. neoformans* for synthesis of melanin, a pigmented polymer that protects the fungal cells against oxidative stress, phagocytosis, and antifungal drugs, and also modifies host immune responses (Gómez and Nosanchuk 2003). Several neurotropic

fungi, including *C. neoformans*, produce melanin, which has been linked to virulence in both human pathogenic and phytopathogenic fungi (Langfelder *et al.,* 2003). However, the adrenal glands are also rich in neurotransmitters that can serve as substrates for melanogenesis, yet no increased predilection for colonization of this tissue site is observed during cryptococcosis. Furthermore, fungal cells can also synthesize melanin in the lungs, where the initial infection occurs (Feldmesser *et al.,* 2001). Thus, if a favorable substrate for melanin synthesis is responsible for the CNS preference, it is unlikely to represent the sole factor.

The neurotropism of *C. neoformans* may also be explained in part as an evasion of host defenses. The unique properties of the brain and the blood-brain barrier (BBB) make this privileged tissue site differ in vigorousness in immune responses compared with other organs. *C. neoformans* can cross the BBB and enter the CNS, and this site may offer a safer haven than the relatively hostile host environment in general. It is possible that in the brain fungal cells may not be under attack from host immune responses as severe as in other organs and thus can survive and amplify. Additionally, *C. neoformans* strains can survive and amplify well in hypoxic environmental conditions (1% O2 and 5% CO2 at 37◦C) similar to the brain (Bahn *et al.,* 2005).

2.9 Diagnosis

The World Health Organization (WHO) has recently released ‘‘rapid advice’’ guidelines for cryptococcal disease among persons living with HIV which are focused on resource limited settings (WHO, 2011). Early diagnosis is key to reducing mortality due to cryptococcal disease. A major WHO recommendation is to consider implementation of cryptococcal antigen screening and pre-emptive anti-fungal therapy in those with a positive diagnostic test among ART-naıve adults with a CD4 count <100 cell/ml^3 in areas with a high prevalence of cryptococcal disease.

Several tests exist for effective diagnosis of cryptococcosis in patients. Some of the methods of diagnosis include:

2.9.1 Detection of Polysaccharides

The polysaccharide capsule is a unique feature in *Cryptococcus* species that distinguishes them from other yeast of medical importance. As such, it plays an important role in the detection of the yeast through stains such as India ink, Mucin stains and serological assays.

2.9.1.1 Visual Detection of Capsule

2.9.1.1.1 India Ink

Detection of capsular polysaccharide antigen is the most valuable test for the laboratory diagnosis of cryptococcosis (Diaz and Nguyen 2011). The cryptococcal polysaccharide can be visualized in smear specimens of the cerebrospinal fluid (CSF), urine, sputum, or other infected materials by India ink or colloidal carbon preparations. The major advantage of this method is the technical simplicity and rapid turnaround time of a few samples. However, the method has poor sensitivity. For example, the sensitivity of India ink detection of *C. neoformans* in meningitis in non-HIV infected patients is approximately 30-72% when compared with culture (Champa Saha *et al*., 2008) . However, in HIV-patients the sensitivity is higher (approximately 80%) (Imwidthaya 2000)

The difference in sensitivity is attributed to the number of infectious particles in the non-HIV patients. India Ink is thought to have a lower detection limit of 10^3 to 10^4 yeast cells/ml (Cohen 1984). False positive results can also be detected using India Ink for inexperienced microscopists who may mistake lymphocytes or fat droplets for Cryptococci. Emphasis is shifting in favor of cryptococcal antigen test rather than India ink detection.

2.9.1.1.2 Cryptococcal Antigen

During infection, Cryptococcal capsular polysaccharide antigen is soluble in body fluids such as CSF and serum, and can be detected and quantified with rabbit based anti-sera. The

sensitivity however differ based on the fungal burden in the CSF and in the presence of well-encapsulated organism. Almost all AIDS patients with cryptococcal meningitis will have a positive serum cryptococcal antigen (Diaz and Nguyen 2011)) . In non-AIDS patients with cryptococcosis the sensitivity is 60%.

The use of antigen testing can be applied to urine (Chapin-Robertson *et al*., 1993; Brito-Santos *et al*., 2017) and fluid recovered from the lungs. This can serve as marker for disseminated and pulmonary cryptococcosis respectively. Quantification of Cryptococcal antigen is much lower in CSF than in urine. Cryptococcal antigen finds utility in predicting cryptococcal meningitis. This implies that cryptococcal antigen testing before initiation of antiretroviral might facilitate targeted pre-emptive antifungal strategies and improved survival.

2.10 DNA As A Molecular Tool For The Diagnosis Of Cryptococcosis

Over the past years, a myriad of molecular techniques have been employed for the detection of pathogenic fungi. Many of these techniques as opposed to the classical techniques, have the potential for rapid, sensitive and specific detection of *C. neoformans*. Some examples of some molecular method used for *Cryptococcus* species identification includes: Randomly amplified polymorphic DNA(RAPD)(Boekhout and van Belkum 1997), Restriction length polymorphism PCR(RLFP);(Chen *et al*., 2008) PCR finger printing with microsatellite (M13)(Chen *et al*., 2008) and multilocus sequence typing(Meyer *et al*., 2009).

These methods have been employed by various researchers to investigate strain relatedness and biodiversity of species complex. Due to the high discriminating ability of these methods some have been employed as a tool for epidemiological studies of *Cryptococcus* species complex

2.11 Chicken Embryonated egg model used in studying Fungal Pathogenicity

Infection models are essential tools in investigating host-pathogen interaction, pathogenicity mechanisms, virulence attributes of pathogenic fungi, and therapy studies. Thus, in vivo models, mainly using laboratory rodents, such as mice, have been developed for many microbial pathogens. These models have been critical for understanding host-pathogen interactions as well as for developing better therapeutical approaches (Clemons and Stevens 2005) and represent the current "gold standard" of in vivo virulence testing. However, ethical considerations and legal restrictions limit the use of mammals for infection studies. Alternative in vivo infection models using lower animals have therefore been developed in recent years (Perez-Nadales *et al*., 2014).

Embryonated chicken eggs provides an alternative in vivo infection model which requires little specialized equipment, it is easy to handle and is available at lower costs than murine models. embryonated bird eggs could provide an alternative model, which would fulfil the demand for refinement of animal models. Fertilized eggs are readily available from commercial breeders at low cost, are easy to handle, and require little specialized equipment and no specialized facilities or personal. This model has been successfully used in studying virulence determinants of viruses and bacteria (Adam *et al*., 2002; Townsend *et al*., 2008). This model has also been used successfully for studying the pathogens of some fungi pathogens such as *Candida* (Jacobsen *et al*., 2011; Gow *et al*., 2003) and *Aspergillus fumigatus* (Jacobsen *et al*., 2010).

Various *in vivo* models including invertebrate models, mini-hosts and mouse models have been developed for *Candida* and *Cryptococcus* research. Invertebrate models include *Drosophila melanogaster*, *Caenorhabditis elegans,* the larval stages of the moth *Galleria mellonella* and the silk worm, *Bombyx mori*. These models have often been shown to be able to recapitulate the rank virulence order of strains as assessed in mice.

The vertebrate mini-host model Zebrafish, *Danio rerio*, which has both innate and adaptive immune systems has also been used to measure *Candida* virulence, host response to infectious agents, the efficacy of antifungal drugs and the cell biology of phagocyte–pathogen interactions. However, the main vertebrate infection model is still heavily focussed on murine studies. Normally, yeast cells are intravenously injected via tail vein and mouse mortality is analysed usually over two to four weeks. Fungal organ burdens are often assessed in the kidney, which is one of the main target organs in the mouse and in other tissues and these assays can be complemented by measurements of *in vivo* cytokine levels. A chick embryo chorioallantoic membrane (CAM) assay has also been used as a pathogenicity model and invasion assay for *C. albicans*

Chapter Three

Materials and Methods

3.1 Study area

Jos is a city in Nigeria and has a population of about 900,000 based on 2006 census. The city has an elevation of about 1,238 metres above sea level. Tin mining activities left a legacy of damaged lands on the Jos, Plateau. Out of the total land areas of 316km affected, only about 2-3km has been crudely reclaimed. Both the reclaimed and un-reclaimed areas have been largely planted with *Eucalyptus camaldulensis*. The city of Jos because of its elevation is cold, the temperature can get as low as 11ºC. Jos and its environs have keep pigeons and other forms of birds; this is for both commercial purposes and personal consumption.

3.2 **Ecological survey**

The samples comprised the following;

- Pigeon (*Columba livia*), guano from their habitats close to vicinities of human dwellings; Streets, Residential buildings.
- The debris were obtained from decaying parts of Eucalyptus *calmadulensis* tree in advanced stages of decay, the inner side of the large hollows was sampled. Other parts sampled were the barks, leaves, as well as soil samples from the ground under the trees canopies.

3.3 Collection and Processing of Samples

Soil sample was collected from under each sampled tree-two hundred grams (200g) of soil sample and plant debris were collected into sterile plastic bag. Two hundred grams (200g) of pigeon guanos, were collected into a sterile bag and taken to the laboratory for processing.

3.3.1 Processing Of Samples

One gram (1g) of each soil sample, Pigeon guanos or tree sample was suspended in 10ml of sterile physiological saline fortified with gentamycin and vortexed for 2min, and allowed to stand for 30 min. Aliquot of 100μl of the1:10 dilution of the supernatant was inoculated in duplicates on plates of Sunflower seed agar (SSA) and Niger Seed agar(NSA) supplemented with chloramphenicol (250 mg/l). This was incubated at 25°C and 37°C for 3-5days respectively. Brown colonies were harvested from positive plates and subsequently streaked to obtain single colonies on a Sabouraud dextrose agar (SDA) plate. Each isolate recovered from the environmental specimens were further sub cultured on SDA and a single pure colony selected, propagated and identified as *C. neoformans* by characteristic brown colony colour on birdseed agar, positive urease test, negative nitrate test, carbohydrate assimilation (Barnett *et al*.,1990).

3.3 Storage of cryptococcal cultures

Sterile distilled water was made in screw capped Bijou bottles and autoclaved. After cooling, a loop full of *C.neoformans* grown in Sabouraud dextrose agar for 24-48hrs at 37°C suspended in 10ml of sterile distilled water. Procedure was aseptically carried out. The cap was properly secured and stored at room temperature.

3.4 Phenotypic characterization of *Cryptococcus* isolates

The colony morphology of the isolates on different culture media were documented and their phenotypic properties studied:

3.4.1 Growth at 37°C

Isolates were grown on Sabourauds dextrose agar in duplicates and incubated at 37°C and 25°C. for 48 hrs. Growth at both temperature was indicative of pathogenicity.

3.4.2 Melanin production

Ability to produce melanin was detected by inoculating isolates on Niger seed agar(NSA) and Mucuna seed agar(MSA) and incubated for 37°C for 48 hours. The production of dark brown colonies indicated melaninogenesis and the presence of phenol oxidase activity

3.4.3 Production of urease enzyme

The isolates were grown on Christensen's Urease test medium by stroke culture and incubated overnight at 37°C. Colour change is noted for Urease positivity.

3.4.4 Presence of Capsule

A drop of India ink was put on a clean glass slide. A loop full of 24 hours culture of *C. neoformans* was emulsified on the India ink. A clean cover slip was placed on the mixture and viewed under x10 and x40 objective.

3.5 In vitro preparation of melanised *C. neoformans*

Melanin is a very important virulence factor. This virulence factor is both diagnostic and and indicator of virulence. It was detected using agar media sysnthesized from locally sourced *Mucuna pruriens* (Cowitch). Briefly, dried cowtish seeds were obtained locally from Rukuba farmers, located in Bassa Local Government Area, Plateau State Nigeria. The seed was identified by a botanist(Mr. Simon) at Botany Department, University of Jos, Jos, Plateau State. The seed was finely blended and sieved using 180µm pore size. The 30g of filtrate was weighed into 200ml distilled water and boiled at 100°C for 30 minutes to denature the proteins. The solution was filtered using 180µm pore size filter. The filtrate was made up to 1000ml. To the final volume 15g of agar powder was added as a solidifying agent. The mixture was autoclaved and dispensed in Petri dishes. Strains of *Cryptoccus neoformans* and *C. gattii* were streaked on the Cowitch agar and incubated for 72 hours at 37°C

3.5.1. Detection of melanin in fungal cells (Suryanarayanan *et al.,* 2004)

Following growth of isolates on the Cowitch agar, the ability of the medium to induce melanin was evaluated using chemical tests and UV Spectroscopy. The tests are stated below:

3.5.1.1 Chemical tests to determine presence of melanin

Fungal melanins have unique properties such as solubility in hot alkali, insolubility in organic solvents, insoluble in water. A few of these tests were performed to check whether the pigment produced was melanin and to evaluate their purity. The following tests were performed:

1. To check solubility in 1M KOH
   - Approximately 5 colonies of 1 mm diameter was added to test tube containing 1M KOH.
   - The tube was swirled to mix.
   - The mixture was then boiled for 2 hours at 80ºC.
2. To check solubility in DMSO (dimethylsulfoxide)
   - Melanized cells were dissolved in 10 ml of DMSO.
   - The mixture was swirled.
3. To check for solubility in water
   1. Melanized colonies of the yeast were dissolved in 10 ml water and the mixture swirled.

3.5.1.2 UV spectrophotometry to obtain melanin curve

Five (5) Melanized fungal colonies were vortexed and dissolved in 1N NaOH by boiling at 80°C for 2 hours. UV visible spectrum was measured in UV-VIS spectrophotometer (UV-

1700 PharmaSpec). The absorbance for the wavelength from 250nm to 600 nm was recorded. A graph was plotted of absorbance against the wavelength.

3.6 Minimum Inhibitory Concentration (MIC) Determination of *Cryptococcus neoformans* Using Antifungals

Broth microdilution assay was used for antifungal studies. This method has advantages such as ease of performance, economy and more rapid results to determine the susceptibility of yeasts The antifungals tested were fluconazole, itraconazole, voriconazole and flucytosine at a concentration ranging from 0.03 to 128 μg/ml.

3.6.1 Microtiter plates preparation

A modified version of the Clinical Laboratory Standards Institute(CLSI) protocol (M27-A2) was used to test the susceptibility of *C. neoformans* strains in 96 wells microtitre plate. Stock concentrations was prepared by dissolving 10mg of specific antifungal powder (fluconazole, itraconazole, flucytosine and voricinazole) in 10 ml of Yeast Nitrogen Base plus glucose (YNBG) to obtain a 1000 μg/ml working concentration. The working concentration is further diluted using two fold dilution techniques in Yeast Nitrogen Base plus glucose (YNBG) to obtain a final concentration of 128 μg/ml which is used for the assay.

Initially, 100 µl of YNBG medium was added to all the wells except the media control, where 200 µl was added.

Next, 100 µl of the antifungal drugs was added respectively to their wells in column 1. Column 1 contains 100µL of the highest concentration of the drugs (128ug/ml) and 100µL of the medium. By performing a serial dilution from column 1 to column 10, to obtain the following set of dilutions: 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.06,0.03. Column 10 had 100µL of the lowest concentration of the drugs (0.03µg/ml) and 100µL of the medium. Column 11 contained 100 μl of YNBG. Two hundred (200) μl of YNBG without antifungal

was pipetted into column 12 as blank control. Finally, 100 µl of the diluted inoculum suspension was added to wells from column 1 to column 11.

3.6.2 Inoculum preparation

Cell suspensions were made from a two days old cultures grown on SDA plates at 35 °C, the cell suspension was adjusted to 0.5 McFarlands. To obtain a working cell concentration of 1 – 5 x 10^3 cells/ml. A 1:50 dilution was made from the original suspension, this was followed by a 1:20 dilution which was made from the 1:50 cell suspension to give a final dilution of 1:1000. One hundred microliter (100 μL) of the final cell suspension was then inoculated into all but the 12^{th} column of the 96 welled plate so that the final concentration of cells tested comes to 1 – 5 x 10^3 cells/ml.

The concentration and purity of the cell inoculum were tested by plating 100 μL of the 1:20 dilution onto SDA. A correct inoculum size will yield 100 to 500 colonies. Duplicates were done for each strain and biological replicates were performed on different days.

Quality control

Strains ATCC 6258 (*Candida krusei*) and ATCC 22019 (*Candida parapsilosis*) were used as positive control strains. Column 11 was the organism control containing 100 µL of sterile YNBG medium and 100µl of organism. Column 12 was the media control, containing 200µL of the medium to determine the sterility of the medium.

Incubation and reading

Microtiter plates were incubated at 30°C for 48 hours. The absorbance was read by a spectrophotometer and the absorbance of the well with more than 50% inhibited with respect to the control growth well (well 11), was noted and the corresponding antifungal dilution is the MIC.

3.7 **Molecular characterization of fungal isolates**

3.7.1 **DNA Extraction**

Extraction of fungal DNA was done as described by Romeo *et al*., (2012). Briefly, *Cryptococcus neoformans* were cultured in Sabouraud glucose liquid medium at 37°C for 48 h. Fungal cells were collected by centrifugation at 3,000g for 10 min and then washed three times with sterile water. Each sample was lysed using glass beads (0.5 mm diameter) in a lysis buffer containing 1 mM EDTA (pH 8.0), 0.1 M NaCl, 2% Triton X-100, 1% sodium dodecyl sulphate (SDS), 0.1 M Tris hydrochloride (pH 8.0). High molecular weight DNAs were obtained from these samples by phenol and chloroform extraction. Deoxyribonucleic acid(DNA) samples were dissolved in TE buffer (10 mM Tris hydrochloride, pH 8.0 and 1 mM EDTA) and used for molecular analysis.

3.7.2 URA5 Gene RFLP

PCR of the URA5 gene was conducted in a final volume of 50 μL. Each reaction contained 50 ng of DNA, 1X PCR buffer (10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM MgCl2; Applied Biosystems, Foster City, CA), 0.2 mM each of dATP, dCTP, dGTP, and dTTP (Roche Diagnostics GmbH), 3 mM magnesium acetate, 1.5 U AmpliTaq DNA polymerase (Applied Biosystems), and 50 ng of each primer URA5(5'ATGTCCTCCCAAGCCCTCGACTCCG-3') and SJ01 (5'TTAAGACCTCTGAACACCGTACTC 3'). PCR was performed for 35 cycles in a Perkin-Elmer thermal cycler (model 480) at 94°C for 2-min initial denaturation, 45 s of denaturation at 94°C, 1 min annealing at 61°C, and 2-min extension at 72°C, followed by a final extension cycle for 10 min at 72°C. Amplification products were mixed with one fifth volume of loading buffer (15% Ficoll 400, 0.25% orange G, MilliQ water), 15 μL of PCR products were double digested with Sau96I (10 U/μL) and HhaI (20 U/μL) incubated for 3 h or overnight.

This was then separated by 3% agarose gel electrophoresis at 100 V for 5 h. RFLP patterns were assigned visually by comparing them with the patterns obtained from the standard strains (VNI-VNIV and VGI-VGIV)

3.7.3 **Determination of Molecular Types**

Molecular type was determined by multiplex PCR as previously described (Esposto *et al*., 2004)

3.7.4 Determination of Mating Type

Mating type of *C. neoformans* was identified performing a multiplex PCR of four sero-mating type specific loci embedded in the mating type locus as reported by (Esposto *et al*., 2004). Strains H99 (VNI-alphaA), JEC21 (VNIV-alphaD), IUM 96-2828 (VNI-aA), JEC20 (VNIV-aD) and WM626 (VNII-alphaA) were used as reference strains.

Table 3.1 : Primers used for Mating type determination

REGIONS	**PRIMER**	**TM (°C)**	**AMPLICON SIZE (BP)**
STE20aAF	TCCACTGGCAACCCTGCGAG	64	860
STE20aAR	ATCAGAGACAGAGGAGCAAGAC	64	860
STE20αDF	TGGGCGTATCCCAACCTACGA	64	413
STE20αDR	TAACGACTCCGGTGCCGTGAA	64	413
NAD4αAF	CATTTATCCGTATACGCATTCCC	64	320, 400
NAD4αR	GCAAATTCAGCACCAGCAATAGT	64	320, 400
NCP1αDF	GGCAGTGATGGTTTCATCCGTA	64	645
NCP1αDR	ATTGTCATCAGGGTCGACAAACA	64	645

Multiplex and conventional PCRs were performed in a 50µl mix containing 5 µl 10X PCR buffer (500 mM KCl, 100Mm Tris-HCL,pH 8.3) 3mM $MgCl_2$, 400 µM (each) dNTPs(Roche,

Monza, Italy) 20 pmol of each primer, 2.5 U AmpliTaq polymerase (Applied Biosystems, Monza, Italy) and 400 ng of DNA extract, with denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 s, 64°C for 10 s and 72°C for 1 min, and a final extension at 72°C for 5 min. PCR products were analysed on an agarose 1.4% w/v gel run at 60 V for 2 h.

3.8. Mating Assay

Twenty (20) ml of Murashige Skoog (MS) agar medium (See Appendix B) were prepared in plates. The strain to be tested was streaked on the plates and then near streak a parallel loopful of the tester strain (IUM 96-2828 (VNI-aA), H99 (VNI-alphaA)). Then with circular movements the two streaks of both tester strains and tested strain were mixed using a sterile spatula. The plate was then incubated at 25 °C in the dark for 3- 4 weeks. To determine self filamentation among strains, each strain was streaked alone in a single plate and incubated under the same condition.

After one week, the plates were then read periodically to observe filaments formation near the edge of the mixed colony. When filaments are abundant, portion of medium containing the filaments was cut and put on a slide and a cover slip gently placed. The setup is then observed with the microscope in order to confirm the presence of basidia, basidiospores, and clamp connections.

To determine the phenotypic variation generated from crossing VNI and VNII, two strains EN28 (Environmental strain VNII *MATα*) was made to mate with KN99a (clinical strain *MATa*). Basidiospores were dissected using a micromanipulator.

3.9 Multi-locus sequence type(MLST) analysis and Population structure of Nigeria Cryptococcus

Multi-locus sequence type (MLST) analysis was performed according to ISHAM consensus scheme of seven unlinked genetic loci (*CAP59*, *GPD1*, *LAC1*, *PLB1*, *SOD1*, *URA5*, and

IGS1).DNA from each isolate was amplified by PCR in a 20 µL reaction volume for each of the seven MLST loci by using the primers and protocols as described previously (Meyer *et al*.,2009) Each locus was subsequently sequenced using the Applied Biosystems 3730 sequencer with the BigDye Terminator cycle sequencing kit v3.1 (Applied Biosystems, Foster City, CA, USA). Alleles at each locus were assigned numbers (allele types [ATs]) following comparison with those previously identified in the global collection (Simwami, *et al*.,2011), resulting in a 7-digit profile for each isolate. Each unique allelic profile was concatenated and assigned a sequence type (ST) according to the MLST scheme

In order to determine the population structure of Nigerian isolates among global isolates of *C. neoformans*, 38 global strains from Italy, Germany, Thailand, and North America strains. MLST sequences were obtained from the global *Cryptococcus* MLST database hosted by university of Sydney, Australia (mlst.mycologylab.org). The generated sequences were manually edited using the software Sequencer 4.9 (Gene Codes Corporation, MI, USA) and aligned using Clustal W (Thompson *et al*.,1994), part of the program Bioedit 7.0.9.0 (Hall,1999). The concatenated alignments were then imported to the program MEGA 6(Tamura *et al*., 2007) and analyzed using the maximum likelihood method with p-distance (Saitou and Nei 1987). Bootstrap analysis (Felsenstein 1985) using 1000 replicates was used to estimate support for clades of the concatenate dataset. All allele types and subsequently the combined sequence types were assigned using the ISHAM consensus database at mlst.mycologylab.org, as described previously (Carriconde *et al*., 2011). All MLST sequences are deposited at mlst.mycologylab. org.

3.10. Distribution of *Cryptococcus* reported in Nigeria

A search was done on pubmed using keywords like " *Cryptococcus* +Nigeria", "*Cryptococcus neoformans* +Nigeria", "*Cryptococcus gattii* +Nigeria" irrespective of year of publication. The search excluded sero-prevalence study and only took into account isolation and identification of *Cryptococcus* species complex.

Table 3. 2. Primer sets and PCR conditions for MLST analysis for *Cryptococcus species*

Gene Locus	**Gene Product**	**Primer name and sequence**	**Amplification conditions**	**No. of Bases analysed (bp)**
CAP59	Capsular associated protein	CAP59F 5' CTCTACGTCGA GCAAGTCAAG 3' CAP59R 5' TCCGCTGCA CAAGTGATACCC 3'	94°C 3 min; 35 cycles: 94°C 30 s, 56°C 30 s, 72°C 1 min	559
GPD1	Glyceraldehyde-3-phosphate-dehydrogenase	GPD1F 5'CCACCGAACCC TTCTAGGATA 3' GPD1R 5' CTTCTTGGCA CCTCCCTTGAG 3'	94°C 3 min; 35 cycles: 94°C 45 s, 63°C 1 min, 72°C 2 min	543
LAC1	Laccase	LAC1F 5'AACATGTTCCCT GGGCCTGTG 3' LAC1R 5'ATGAGAATTG AATCGCCTTGT 3'	94°C 3 min; 30 cycles: 94°C 30 s, 58°C 30 s, 728C 1min	469
PLB1	Phospholipase	PLB1F 5'CTTCAGGCGGA GAGAGGTTT 3' PLB1R 5'GATTTGGCGT TGGTTTCAGT 3'	94°C 3 min; 30 cycles: 94°C 45 s, 61°C 45 s, 72°C 1 min	532
SOD1	Cu, Zn superoxide dismutase	SOD1CNF 5'AAGCCTCT CATCCATATCTT 3' SOD1CNR 5'TTCAACCAC GAATATGTA 3'	94°C 3 min; 35 cycles: 94°C 30 s, 52°C 30 s, 72°C 1.5 min	700
URA5	Orotidine monophosphate pyrophosphorylase	URA5F 5'ATGTCCTCCCA AGCCCTCGAC 3' URA5R 5'TTAAGACCTCT GAACACCGTACTC 3'	94°C 3 min; 35 cycles: 94°C 45 s, 63°C 1 min, 72°C 2 min	601
IGS1	Ribosomal RNA intergenic spacer 1	IGSF 5'ATCCTTTGCAGA CGACTTGA 3' IGSR 5'GTGATCAGTGC ATTGCATGA 3'	94°C 3 min; 35 cycles: 94°C 30 s, 60°C 30 s, 72°C 1 min	723

3.11 Antifungal Activities of Plant extract

3.11.1 Collection and preparation of plant material

Fresh leaves of *Swietenia mahogani, Senna siamea* and *Cassia alata* were collected from Vom and Barkin ladi and the roots and stem of *Rauvolfia vormitoria* were collected from Nnewi, Anambra State, Nigeria. The plants were washed with clean tap water and allowed to shade-dry at room temperature (25^0C).The dried leaves were crushed into powder. The roots were shredded into small pieces and allowed to shade-dry then pounded using mortar. The bark of the stem were scrapped and allowed to shade-dry before pounding in a mortar.

3.111.2 Extraction procedure

Dried plant leaves from each species were individually extracted by weighing samples of 1 g of finely ground plant material and extracting with 10 ml of acetone (ACE), hexane (HEX), dichloromethane (DCM) and methanol (MeOH) (technical grade-Merck) in polyester centrifuge tubes. Tubes were vigorously shaken for 3 to 5 min . After centrifuging at 3500 rpm for 10 min the supernatant was decanted into pre-weighed labelled containers. The process was repeated 3 times to exhaustively extract the plant material and the extracts were pulled together. The solvent was removed under a stream of air in a fume cupboard at room temperature, to quantify the extraction efficiency.

3.11.2 **Determination of Minimal Inhibitory Concentration of plant extract against *C. neoformans* using modified CLSI protocol**

A modified version of the CSLI protocol (M27-A2) was used to test the susceptibility *C. neoformans* strains in 96 well plates. Stock concentrations (10 mg of plant powder was dissolved in 10 ml of acetone to obtain a 1000 μg/ml final concentration). The stock solution was further diluted using 10 fold dilution with Yeast Nitrogen Base plus glucose (YNBG) (

6.7 g Yeast Nitrogen Base(YNB) 20 g Glucose ,1 L distilled water) in order to obtain a 128 μg/ml concentration.

Initially, 100 µl of YNBG medium was added to all the wells of the plate except the media control, where 200 µl was added.

Next, 100 µl of the antifungal drugs was added respectively to their wells in column 1. Column 1 contains 100µL of the highest concentration of the drugs(128ug/ml) and 100µL of the medium. By performing a serial dilution from column 1 to column 10, to obtain the following set of dilutions: 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, 0.125, 0.06. Column 10 had 100µL of the lowest concentration of the plant extract (0.06ug/ml) and 100µL of the medium. Column 11 only contained 100 μl of YNBG without antifungal. A 200 μl volume of YNBG without antifungal was pipetted into column 12 as blank control. Finally, 100 µl of the diluted inoculum suspension($3x10^6$cfu/ml) was added to wells from column 1 to column 10. A 40 µl volume of Tetrazolium chloride(TTC) was added to each well as an indicator. The MIC was determined as the lowest sample concentration at which no red color (signifying live growth) appeared.

3.11.3 Selection of plant with higher activity

To select the plant extract, total activity was calculated for each plant extract using the formula below

Total activity (ml/g) = quantity extracted (mg/g)/MIC value (mg/ml) (Eloff 2000)

3.11.4 Bioautography

TLC plates (10 × 10 cm) were loaded with 100 µg of each of the extracts with a micropipette in a line c. 1 cm wide. The prepared plates were developed using Hex:MeoH 3:1 as the mobile systems The chromatograms were dried at room temperature to remove the remaining solvent. Fungal cultures were grown on SDA for 3 to 5 days. Cultures were transferred into SD broth from agar with sterile swabs. The Thin-Layer Chromatography (TLC) plates were sprayed with a concentrated suspension containing 1.0×10^6 cells/ml of actively growing

fungi. The plates were sprayed until wet, incubated overnight, sprayed with 2 mg/ml solution p-iodonitrotetrazolium chloride(TTC) and further incubated overnight or longer at 35°C in a clean chamber at 100% relative humidity in the dark. White areas indicated where reduction of TTC to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of the test fungi. The plates were sealed in plastic to prevent the spreading of the fungi in the environment and to retain the humidity and then scanned to produce a record of the results.

3.11.5 Phytochemical analysis.

Phytochemical screening test was carried out on all the plant samples according to the procedure of the Association of Official Analytical Chemist (AOAC),2005(Chemists and Horwitz 1980) and (Harbone 1998) for phytochemical analysis.

I. Test for alkaloids:

To the extract dilute hydrochloric acid was be added and filtered. The filtrate will be treated with various alkaloidal reagents .

a. **Mayer's test**: The filtrate was treated with Mayer's reagent: appearance of cream colour indicates the presence of alkaloids.
b. **Dragendroff's test:** The filtrate was treated with Dragendroffs reagent: appearance of reddish brown precipitate indicates the presence of alkaloids.
c. **Wagner test**: A quantity of concentrated extract(3ml) was pipetted into a test tube and 1 ml HCl was added to the mixture. This was then heated gently for 20 min and allowed to cooled and was subsequently filtered. The filtrate was treated with Wagner's reagent; formation of brown reddish precipitate indicates presence of alkaloids**.**

II. Test for Saponins

Five (5) ml of aqueous extract was shaken vigorously with 5 ml of distilled water in a test tube and warmed. The formation of stable foam was taken as an indication of the presence of saponins.

III. Test for Phenol

Ferric Chloride test: Test extract were treated with 4 drops of Alcoholic $FeCl_3$ solution. Formation of bluish black colour indicate the presence of Phenol.

IV. Test for Flavonoids

Lead acetate test: To 1 ml of aqueous extract, 1 ml of 10% lead acetate solution was added. The formation of a yellow precipitate was taken as a positive test for flavonoids.

V. Test for terpenoid

Salkowski Test: Extract was treated in chloroform with few drops of concentration sulfuric acid, shaken well and allowed to stand for some time. Formation of yellow colored lower layer indicated the presence of triterpenoids.

VI. Cardial Glycosides

Keller-Killani Test: Plant extract was treated with 2 ml glacial acetic acid containing a drop of $FeCl_3$ .A brown colour ring indicated the presence of positive test.

VII. Test for Phlobatannins:

About 2 ml of aqueous extract was added to 2 ml of 1% HCl and the mixture was boiled. Deposition of a red precipitate was taken as an evidence for the presence of phlobatannins.

3.12 Phospholipase Activity Test

Phospholipase is an extracellular enzyme. In this method, egg yolk digested by phospholipase produces precipitation around colonies (Vidotto *et al*., 2005). The phospholipase activity was expressed as Pz value (a/b) as described by (Price *et al*., 1982)

The Environmental isolates of *Cryptococcus neoformans* phospholipase activity was detected by measuring the size of precipitation zone after the growth on egg yolk agar (Price *et al*., 1982) Standard inocula of the test *Cryptococcus neoformans* isolates [5 µl, with 10^8 yeast cells/ml were deposited onto the egg yolk agar medium and left to dry at room temperature. Each culture was then incubated at 37°C for 48 h. The assay was conducted in duplicate on three separate occasions for each *Cryptococcus* isolate tested.

Phospholipase activity of the isolate was considered positive when a precipitation zone was visible around the colony on the plate (Ying and Chunyang 2011)) The value of phospholipase activity (Pz) was measured by the ratio of the diameter of the colony to the total diameter of the colony plus the precipitation zone(Ying and Chunyang 2011) A Pz value of 1 denotes no activity, and less than one (Pz < 1) indicated the phospholipase activity. The lower the Pz value, the higher the enzymatic activity (Ying and Chunyang 2011).

According to Prize et al.(1982) low Pz values mean high phospholipase production and, inversely, high Pz values indicate low production.

- High Pz group between 1 and 0.700 (+)
- Moderate Pz group between 0.699 and 0.400 (++)
- Low Pz group between 0.399 and 0.100 (+++) \

3.13 Pathogenesis of Environmental isolates of *Cryptococcus neoformans* in the Alternative Chorio-allantoic Membrane(CAM) of Embryonated Egg

3.13.1 Pre-incubation of eggs and candling

In the morning of day 0, eggs were placed into the incubator, the blunt pole of all eggs facing in the same direction. From day 3 onwards, the eggs were turned lengthwise every 6 hours. In the evening prior to infection, the incubated eggs were checked for embryonic development and survival by candling. Candling was done in a darkened room, a strong light source onto one pole of the egg. The light shines through the shell and thus illuminates internal structures. In developing viable eggs, the blood vessel pattern of the CAM is clearly visible. The embryo appears as dark shape (size increasing with age) within the interior of the egg and moves frequently. Embryo movement is triggered by warmth from the light source. Non-developed or non-viable eggs were discarded.

The natural air space was marked with a pencil. The eggs were individually numbered or to allow identification of different infection groups.

3.13.2 Preparation of *Cryptococcus* strains and preparation for inoculation (Jacobsen *et al.,* 2012)

The *Cryptococcus gattii* strains used were, R272, R265 and EJB18 strains. Also, *Malassezia sympodialis* ATCC42132 were used. For infection experiments, strains were subcultured once on YPD agar plates for 24 hours at 37°C. A single colony was then inoculated into 20 ml liquid YPD and cultured for 12–14 hours at 30°C with 200 rpm. Cells were harvested by centrifugation at a speed 3,000 g for 5 min at 4°C. The cells were washed twice with cold sterile phosphate buffered saline (PBS), pH 7.4. The cell concentration was determined using the haemocytometer. the suspensions were adjusted to the desired concentration with cold, sterile PBS. Inocula were maintained on ice and used within 1–2 hours. The cell numbers in

inocula were confirmed by plating serial dilutions on YPD agar plates. Colony forming units (cfu) were determined after 36 hours incubation at 30°C. For Virulence studies the inoculum was adjusted to 10^7cfu/ml

3.13.3 Inoculation of the Chorio- allantoic Membrane

A hole was Carefully drilled in the middle of the blunt pole using a dentist drill, the hole penetrated the shell and shell membrane into the natural air space but not to disrupt the CAM. This process was repeated for all eggs of the group. Removal of the natural air space is seen as darkening of the air space. In parallel, air should flow into the second hole at the longitudinal site, causing lowering of the CAM and creation of an artificial air space. The artificial air space appears as a sharp, oval, lighter area under the shell. After the artificial air space has been constructed in all eggs the room was again lit.

Inoculation was done by introduction of 100μl of the infection inoculum through the hole at the longitudinal site directly onto the CAM using a sterile needle and twenty (20) eggs were infected per isolate(Sequence type). Both holes were sealed by warming the paraffin wax in an open flame and gently rubbing it over the perforation and placed back eggs into the incubator.

Survival was monitored for up to 7 days by candling once to twice daily, at the end of the experiment, surviving embryos were killed by chilling on ice or freezing prior to autoclaving and disposal. Survival curves were prepared using Prism 6 (GraphPad) and statistically analysed using the Log-rank (Mantel–Cox) test.

3.13.4 Determination of fungal burden

Embryonated eggs were inoculated with 10^5 cfu/egg as described above. At given time points, viable embryos (defined by embryonic movement at candling) were humanely sacrificed by chilling the eggs on ice for 30–60 min. The eggs' surface was then disinfected

with 70% ethanol. The shell was cut in half with sterile scissors. Approximately 0.5–1 g CAM (comprising 1/3 to 1/2 of total CAM), were removed. Samples were homogenized in 2 ml sterile cold PBS . This was later used for ten fold serial dilutions, after which they were plated onto YPD agar plates and incubated for 36 hours prior to colony counting.

3.13.5 Histological examination of CAM

For histological analysis, the CAM underneath and beside the membrane was removed and placed into a container half filled with and fixed in 10 times neutral formalin. Paraffin-embedded specimens were cut into 5 mm sections, stained with periodic acid-Schiff (PAS) stain according to standard protocols and examined using bright field microscopy at x20 indicated magnification.

3.14 Generation Progenies from EN28 X KN99a Cross

Two parents with different temperature phenotype were selected. The parents are EN28(*C. neoformans* VNII, *MATa* From this study) and KN99a (*C. neoformans* VNI, *MAT*α Control). The two parent strains were grown overnight in Yeast Extract Peptone Dextrose (YPD) broth (see Appendix B) at 30°C. Following incubation, cells were centrifuged at 4766 x *g* for 10 minutes after which the cell pellet was re-suspended in fresh YPD broth and diluted to get 0.5 Mcfarlands. Ten(10) fold dilution was made on microtitre plates. Three (3) μL of each dilution was dropped onto YPD agar. Plates were inverted and incubated at 30°C (control) and 37°C for 72 hours, after which growth was recorded and photographed.

Mating was setup for both parents as described in 3.9 Spores were dissected from the cross using a micromanipulator and incubated on YPD for 7days.

3.15 Determination of Ploidy of F1 progenies from EN28 XKN99a Cross using Fluorescence- activated cell Sorting(FACS)

To determine the ploidy of progenies from cross, the cells were grown overnight at 30°C on YPD plates. After 24 hours of growth, the cells were fixed by scrapping them from the patch and suspended in 1ml of PBS, this was then centrifuged at 12000rpm for 1 minute and supernatant discarded. The cells were re-suspended in 1 ml of 70% ethanol and incubated at 4°with agitation overnight. The cells were then centrifuged at 12000rpm for 1 min and supernatant discarded. The residue was then re-suspended in NS buffer and centrifuged for 1 min. The supernatant discarded. The cells were re-suspended in 200μl of NS-RNase-P1 solution and covered with foil. This was then incubated at room temperature with agitation for 2-3 hours. 50μl of stained cells were pipetted into 500 μl of Tris-PI solution in a 5ml falcon tube. Flow cytometry was performed with 10,000 cells, and the results were analyzed on the FL1 channel with a BectonDickinson FACScan

3.16 Phenotypic variations among F1 progenies dissected from KN99a and EN28 Cross

3.16.1 High-Temperature Growth Assay

All *C. neoformans* F1 progeny strains were grown overnight in Yeast Extract Peptone Dextrose (YPD) broth at 30°C. Following incubation, cells were centrifuged at 4766 x *g* for 10 minutes after which the cell pellet was re-suspended in fresh YPD broth. Serial 10-fold dilution of each strain were prepared in a 96 well microtitre plate with distilled water and 5μL of each dilution was dropped onto YPD agar. Plates were inverted and incubated at 30°C (control) and 37°C for 72 hours, after which growth was recorded and photographed and scanned.

3.16.2 Temperature Phenotyping for Progenies

F1 Progenies from EN28 x KN99a cross were grown to saturation on YPD media on 96 well plates for 48 hours. They were then pinned onto Omni trays containing YPD agar and incubated in duplicates for 48 hours at 30°C and 37°C respectively. After 48 hours, plates were scanned. Plate quantification was done using gitter (Wagih and Parts 2014) in R studio. Variations in temperature was determined using Python scripts. The mean for each plate was determined and mean centred (The average X and Y coordinates of all the sizes in the plate, mean centre is useful for tracking changes in the distribution or for comparing the distributions of different features). Then the mean centred 37°C data and the mean centred data at 30°C were regressed. The approach was to determine which progenies grew worse or better than expected at 37°C once the differences in growth at 30°C have been accounted for.

3.16.3 UV Radiation

All *C. neoformans* strains were grown overnight in Yeast Extract Peptone Dextrose (YPD) broth (see Appendix B) at 30°C. Following incubation, cells were centrifuged at 4766 x *g* for 10 minutes after which the cell pellet was re-suspended in fresh YPD broth. Serial 10-fold dilutions (10^6-10^1 cells/3μL) of each strain were prepared with distilled water and 3μL of each dilution was dropped onto YPD agar. Plates were exposed to various doses of UV radiation using the UV stratalinker 2400 (Stratagene) and incubated at 30°C for 72 hours, after which growth was recorded and photographed.

3.16.4 Melanin Production Assay

All *C. neoformans* strains were grown overnight in YPD broth at 30°C. Following incubation, cells were centrifuged at 4766 x *g* for 10 minutes after which the cell pellet was re-suspended in fresh Yeast peptone dextrose (YPD) broth. A dilution of 10^6 cells/3μL was prepared for each strain and 3μL of the dilution was dropped onto an L-DOPA agar plate (pH

5.6) (see Appendix 1). The plate was inverted and then incubated at 30°C for 72 hours after which the degree of melanisation of the drop was recorded and photographed.

3.16.5 Hyphal Cell Wall Visualization and Nuclear Staining

Yeast colony was grown on MS agar for three weeks to allow hyphal growth. After which 1 cm^2 MS agar containing hyphae was cut and placed in a small Petri dish. The agar was fixed with 5mls of 3.7% Formaldehyde for one hour. The fixed agar block was washed once with PBS for 5 minutes, and permeabilized with 10 mls 1% Tritox x-100 for 1 hour. The agar was washed three times with PBS. The hyphae was stained with 3μg/ml Hochest 33342 (Invitrogen) and 1 μg/ml Calcoflour white for 10 minutes. The stain was washed off with PBS and transferred to glass slide for microscopic observation.

3.17 *Galleria mellonella* model for Study of Virulence for *Cryptococcus neoformans(Mylonakis et al., 2005)*

Twenty similar sized *G. mellonella* larvae (about 3 g each) were selected, placed in a 90 mm plastic Petri dish, weighed, and used for inoculation. Each *Cryptococcus neoformans* strain was previously grown on YPD for 48 h at 27°C and used for the study. To determine the microbial density to use, a Neubauer Chamber was used. A final inoculum density of 10^5 yeast cells/ml was prepared in Phosphate Buffered Saline (PBS) and 4 μl were inoculated into the hemocoel of each larva by injection into the last left pro-leg, using a sing a Hamilton syringe (26S gauge, 50 μl capacity). A group of 20 larvae was also inoculated with PBS to monitor potential effects on survival due to physical injury. After injection, the larvae were incubated in Petri dishes at 37°C for 10 days and checked daily for any mortality.

3.18 **Draft Genome Sequencing of *Cryptococcus neoformans* ST43**

3.18.1 DNA Extraction Protocol for Whole genome Sequencing using Cetrimonium bromide(CTAB)

Fifteen (15)ml of liquid culture incubated overnight in a shaking incubator was Centrifuge and supernatant discarded. Residue was freeze dried over night at -80ºc. Following the drying of the cells, 5 mL of 4 mm glass beads were added to the tube and vortexed until the cells turn into fine powder. Six(6) mL of Cetrimonium bromide (CTAB) buffer was added to the tube along side 60 µL of β-mercaptoethanol and vortexed. The mixture was incubated at 65º c in a water bath for 30 min. and vortexed every 10 min. The tube was cooled in a refrigerator. The liquid was transferred to a new 15 mL tube and 6ml of chloroform was added and vortexed until thoroughly mixed. The tube was Centrifuged at 5,100 rpm for 10 minutes. Six (6) ml of isopropanol was added to a new 15 mL tube and the top layer (aqueous phase) of the centrifuged tube was transferred to the new tube containing isopropanol. Tube was mixed by inversion and cooled -20ºc freezer. Tube was centrifuged at 5,100 rpm for 10 minutes and supernatant discarded. One (1)ml of 70% ethanol was added and gently agitated. Tube was centrifuged at 5,100 rpm for 2 minutes and ethanol discarded . remaining ethanol was removed and the DNA pellet was allowed to dry.

Five hundred (500) µL of water was then added to the pellet and incubated for 15mins at 65º in a water bath. DNA was transferred to a new 1.5ml microcentrifuge tube and 2 µL of RNase A (10 mg/mL) was added to it, tube was and incubate at 37º for 30 minutes . Two hundred (200) µL of 6 M sodium acetate and 500 added µL of chloroform was added respectively, the tube was vortexed to thoroughly mix. DNA mixture was then centrifuged at max speed for 15 mins. Five hundred (500) µL of isopropanol was the added to a new 1.5 mL

microcentrifuge tube and top layer transferred to the new microcentrifuge tube and mixed by inversion.

The tube was cooled in -20ºc freezer and centrifuged at max speed for 10 mins. The supernatant was discarded and DNA precipitated using 500 µL of 70% ethanol. Tube was centrifuged and ethanol removed. DNA was allowed to dry. To store, DNA pellet was dissolved in water and incubated in 70º heat block for 10 minutes. DNA at 20ºC. DNA quality was determined using nanodrop method and Agar gel diffusion method

3.18.2 DNA Sequencing

The whole genome of the Nigerian *C. neoformans* EN28 strain as paired end (2x150 bp) sequenced using the Illumina HiSeq platform. High quality genomic DNA of the EN28(ST43) strain as used to generate a paired end library with insert sizes of 300bp. DNA sequencing was done at the University of North Carolina University DNA facility

3.18.3 Genome Assembly and Annotation

Before assembling, raw reads were processed using Trimmomatic(Bolger *et al.,* 2014) to remove adapters and sequences with low Phred-scores (cutoff: ≥ 30). IMR- DENOM program was used for assembling the whole fungal genome using the following sequence GCA_000149245.3 as reference. Protein-encoding genes and tRNAs were predicted by AUGUSTUS(Stanke and Morgenstern 2005) and tRNAscan-SE (Lowe and Eddy 1997) programs respectively.

CHAPTER FOUR

RESULTS

In this study, a total of 100 pigeon droppings and 300 *Eucalyptus calmadulensis* tree samples were collected at different locations of Jos City.A total of 3 isolates yielded brown colour effect (BCE) on Niger seed agar (NSA) and Sunflower seed agar (SSA). As shown in table 4.1. The three isolates were all from pigeon guanos. None was isolated from *Eucalyptus calmadulensis* trees.

Further tests to confirm identity of the three isolates were performed, the three isolates has the presence of capsule, urease positive and assimilated inositol which differentiated it from *Rhodotorula* species (Table 4.1). The three isolates showed results consistent with *Cryptococcus* species.

The three isolates represent a prevalence of 3%. No *C. gattii* was isolated from either the pigeon guanos or from the *Eucalyptus calmadulensis* trees.

Restriction length fragment polymorphism(RLFP)- PCR of URA5 gene with the restriction enzymes Sau96I and HhaI in a double digestion gave two bands 500bp and 300bp which were consistent with the digestion of the URA5 gene double digestion(Plate 4.2).

Table 4.1 : Phenotypic identification of *Cryptococcus* isolates from Pigeon guanos, from Jos, Plateau State Nigeria

		Assimilation tests			
Strain	**Capsule presence**	**Inositol**	**Maltose**	**Glucose**	**Urease Reaction**
27	**+**	+	+	+	+
28	**+**	+	+	+	+
326	**+**	+	+	+	+

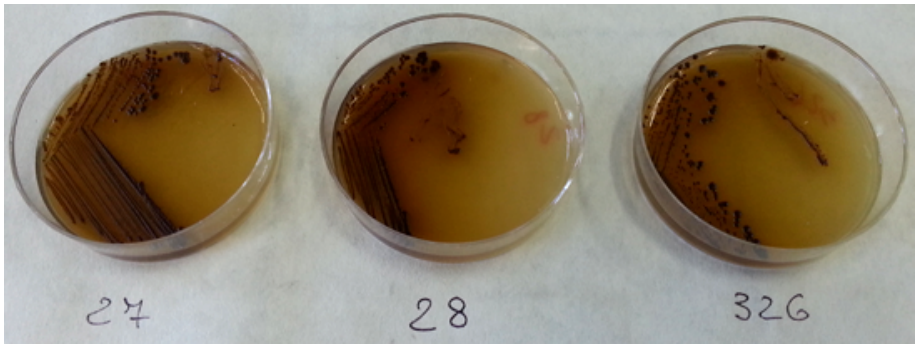


Plate 4.1. Brown color effect of the three isolates of *Cryptococcus neoformans* on Niger seed agar media.

Figure 4.3A shows the bands products obtained by the simultaneous amplification of 4 loci located in the mating type locus, NAD4 specific for αA allele. NAD4 produce two bands namely the expected 320-bp fragment and an expected 400-bp fragment and 413 bp for Dα (Plate 4.3A) The result was confirmed using a control isolate H99 an αA.

Plate 4.3B shows the determination of the Molecular types of the three isolates. The molecular type specific multiplex PCR amplifies simultaneously four DNA regions dispersed in the genomic DNA of *Cryptococcus neoformans*, the fragment yielded were 679bp, 427bp and 420bp fragments these combinations are specific for *C. neoformans* VNI . The result of the molecular study showed that the three isolates, 27, 28 and 326 were all *Cryptococcus neoformans* var. *grubii* all belonging to the mating type αA and to the rare molecular type VNII.

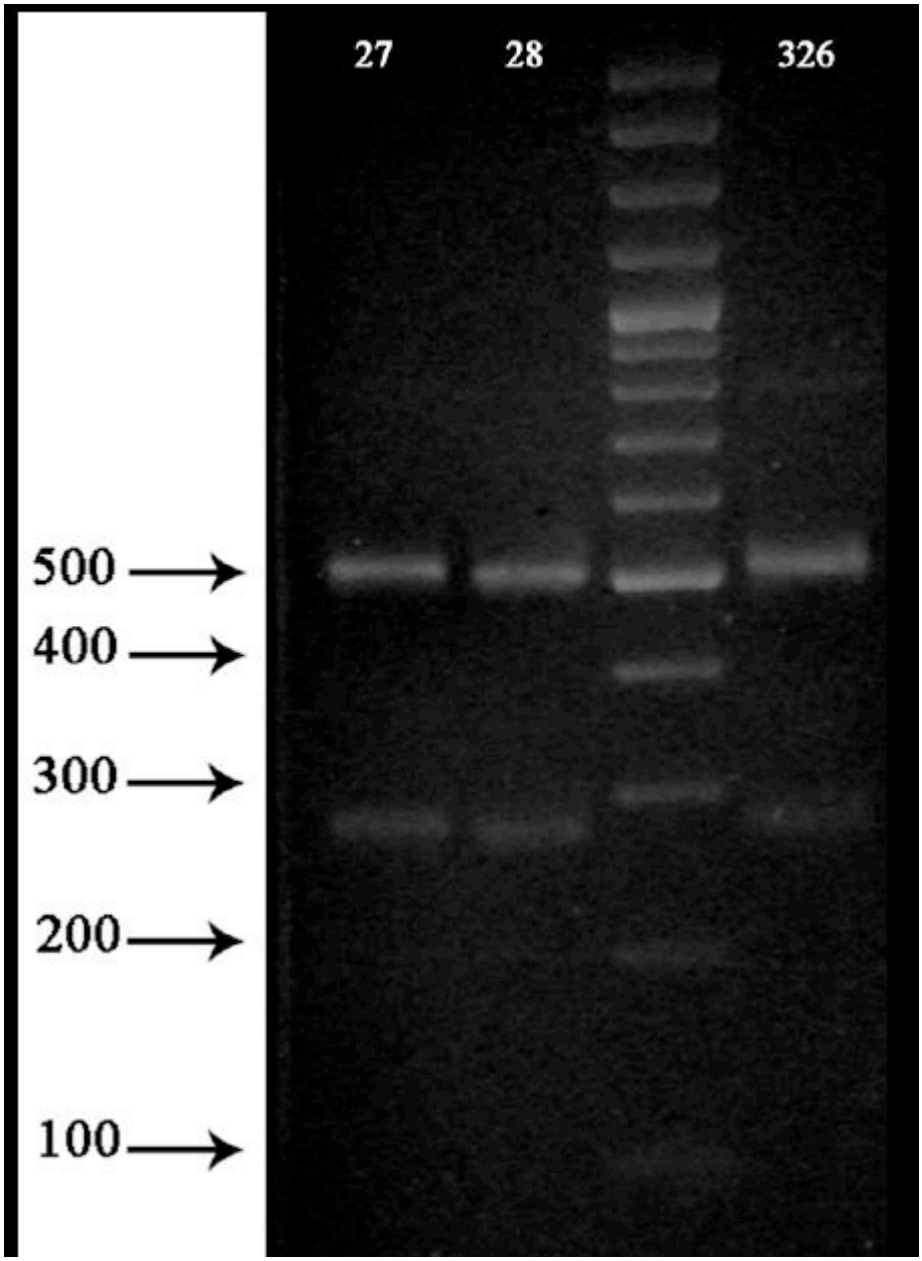


Plate 4.2 URA5 RLFP-PCR of isolate 27,28 and 326, RFLP pattern gave bands of 500bp and 300bp

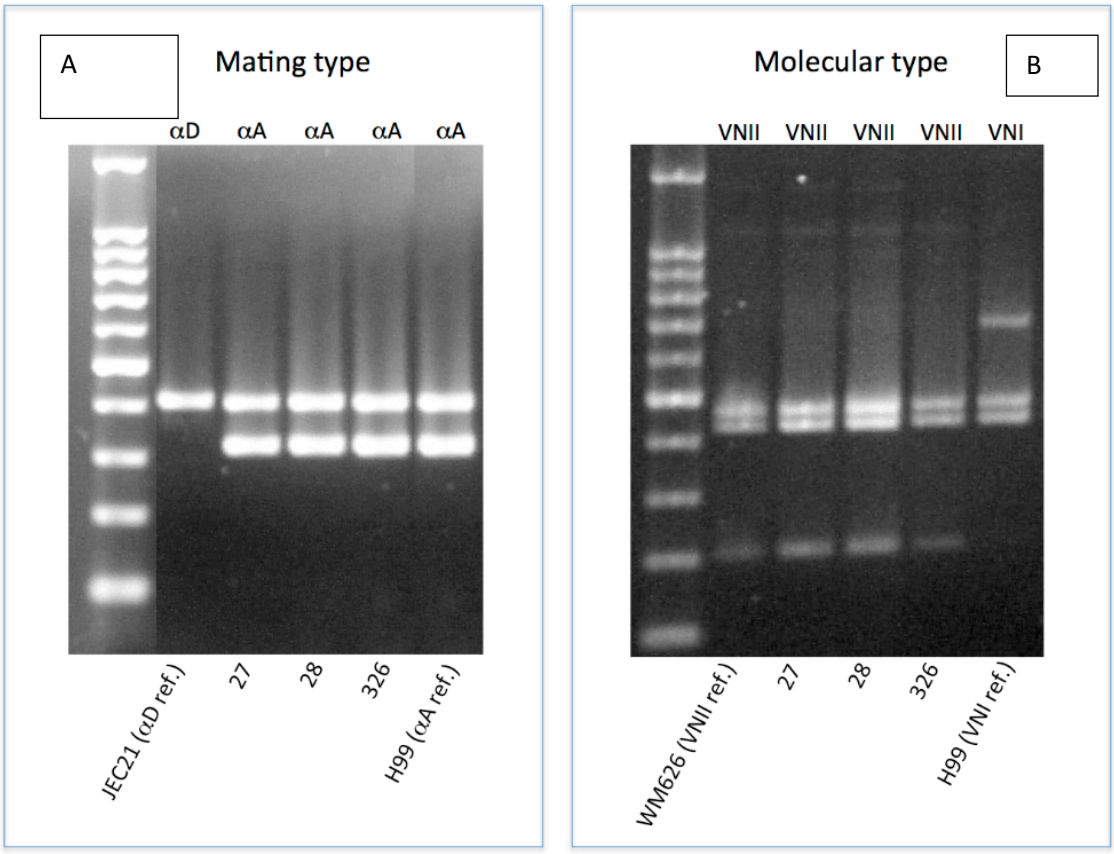
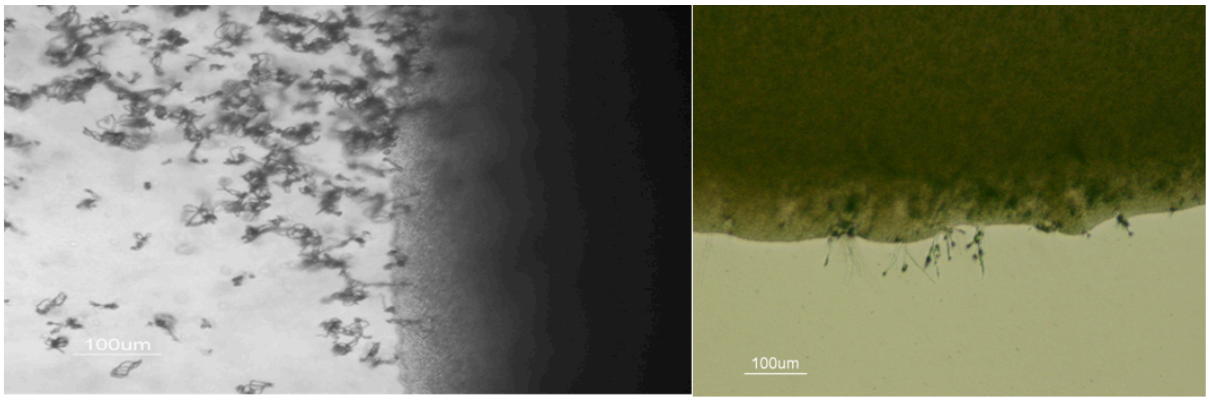


Plate 4.3 : Results obtained by multiplex PCR with *Cryptococcus neoformans* strain isolated from Jos, Plateau State. 100- bp molecular size ladder (Invitrogen, Milano, Italy); A). Mating type PCR with JEC21(Serotype D, Mating type α); H99(Serotype A Mating Type α) as Controls B).Molecular Type detection with H99(Molecular type VNI)and WM626(Serotype A Mating Type α) were used as control isolates.

Mating plates were examined by microscopy for typical mating structures, including filaments, clamp connections, basidia, and basidiospores, and the results for each isolate are presented in Plate 4.4.1-4.4.2. All of the isolates tested produced mating structures when mixed with the congenic KN99**a** strain and the IUM 96-2828 **a**A strains, confirming them to be of the α mating type (Plate 4.4.1-4.4.2). The isolates did not show any sign of self-filamentation when cultured alone (Plate 4.4.3). DAPI staining showed the presence of clamp connection and basidium (Plate 4.4.4).



Mating assay between isolates 27XKN99**a** congenic strain

Mating assay between isolates 28XKN99**a** congenic strain

Plate 4.4.1 Mating between KN99a(*MATa)* and Environmental strains EN28 and EN27

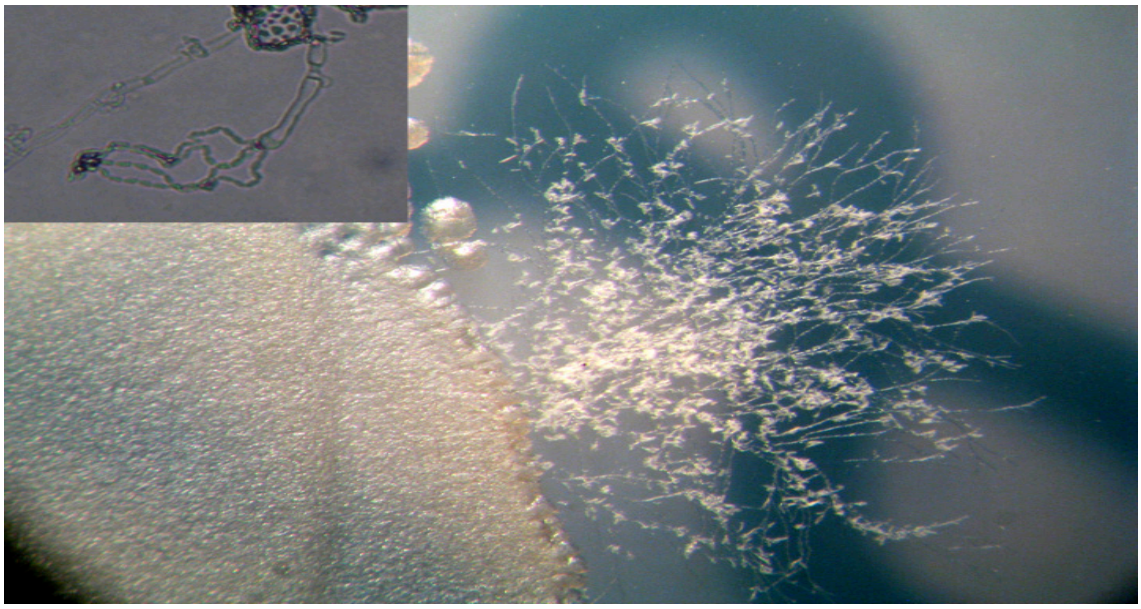


Plate 4.4.2: Filaments formation from the edge of the mixed culture of strain 27 (VNII-alphaA) and tester strain IUM 96-2828 (VNI-**a**A) . The magnification on the top right side shows a basidium bearing four chains of Basidiospore

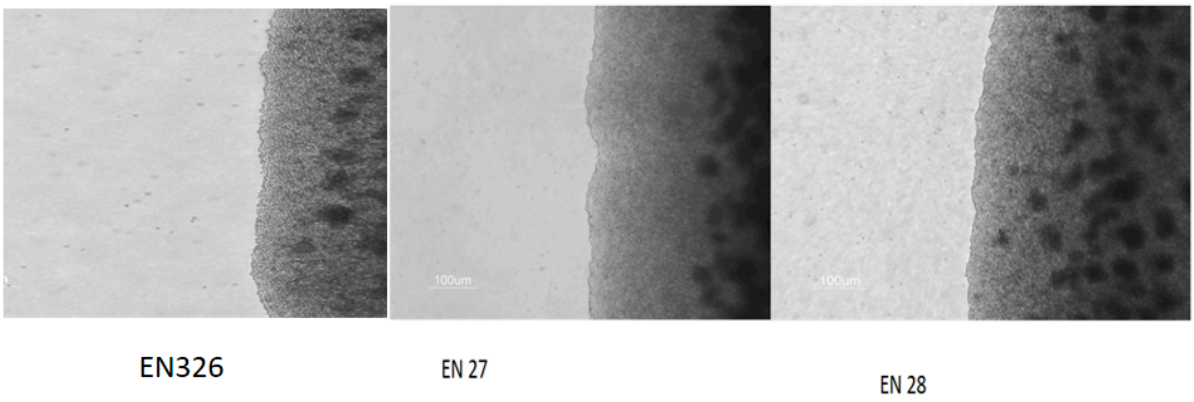


Plate 4.4.3. *Cryptococcus neoformans* strains EN326, EN27 and EN 28 cultured alone to detect self filamnetation ability among these strains

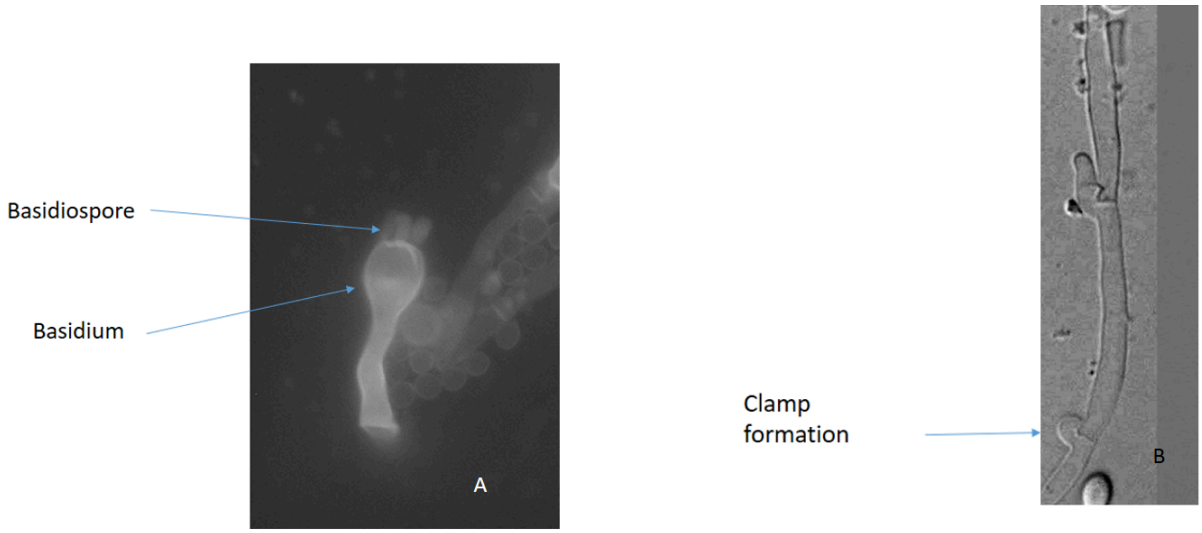


Plate 4.4.4 Clamp connection and basidiospores formation from EN28 and KN99**a** cross. A.)Shows the presence of basidium and spore chains budding from the basidium. B). Shows clamp connection typical of a-α cross.

Table 4.2 shows the alleic profile of the 3 *C. neoformans* isolates. The three isolates belonged to sequence type(ST) 43.

Figure 4.1 shows a phylogenetic tree for the *C. neofomans* strains using the MLST CAP59, GPD1, IGS1, LAC1, PLB1, SOD1, URA5 sequenced loci in comparison with 38 global isolates of *Cryptooccus neoformans* deposited in the MLST database. The phylogenetic tree shows two clusters within the VNII molecular types, the VNII ST43 clustered together irrespective of isolation source and were separate from other VNII (Figure 4.1). MLST was able to demarcate and identify the various clusters within the *C. neoformans* grubii which includes VNI, VNB and VNII.

Table 4.3 shows a distribution of reported isolates of *Cryptococcus* species complex from Nigeria. Most states of the federation do not have any report of *Cryptococcus* isolation. Most states however that reported isolated *Cyptococcus* species did not perform molecular identification but rather stopped at serological identification.

The mating experiment revealed that KN99a showed greater fitness at higher temperature than EN28(Plate 4.5) at 37°C. The F1 progenies from the mating produced progenies that grew better than expected at 37°C and those that grew badly at 37°C (Figure 4.4). A wide range of temperature sensitivity was observed among the 480 F1 progenies.

A total of 1621 Random spores were dissected from the cross. Only 480 progenies recovered, hence given a germination rate of 29.6%.

Figure 4.3 shows the cell content among the F1 progenies, a total of fluorescence activated cell sorting (FACS) was performed for 288 progenies. Out of which 2(0.007%) were diploid (P188 and P033) while others were haploid(99.3%).

Flow cytometry showed the presence of 1n haploid and 2n haploid strains (Figure 4.3A and 4.3D) and 2n and 4n diploid (Figure 4.3C and 4.3D). The M1, M2, and M3 indicate nuclear

DNA content. The *x* axis indicates fluorescence intensity reflecting the DNA content and the *y* axis indicates cell count. Haploid cells show double peaks at M1 and M2, while diploids (double DNA cell counts) showed double peaks at M2 and M3 regions

Plate 4.6 shows the PCR determination of the mating types of the diploid strains. As shown in Plate 4.6, diploid strain P033 contains the two mating alleles(STE20a and STE20α alleles) hence its an **aAAα** strain while P188 Contains only two copies of the *MAT*a(STE20a gene) allele hence its **aAAa** strain.

Table 4.2: The allelic profiles of three *C. neoformans* isolates from Jos, Plateau State, Nigeria typed by MLST

Strain	**CAP59(** 501 bp)	**GPD1** (489 bp)	**IGS 1** (709 bp)	**LAC1** (471 bp)	**PLB1** (533 bp)	**SOD1** (527 bp)	**URA5** (637 bp)	**SEQUENCE TYPE(ST)**
27	2	9	14	8	11	11	4	43
28	2	9	14	8	11	11	4	43
326	2	9	14	8	11	11	4	43

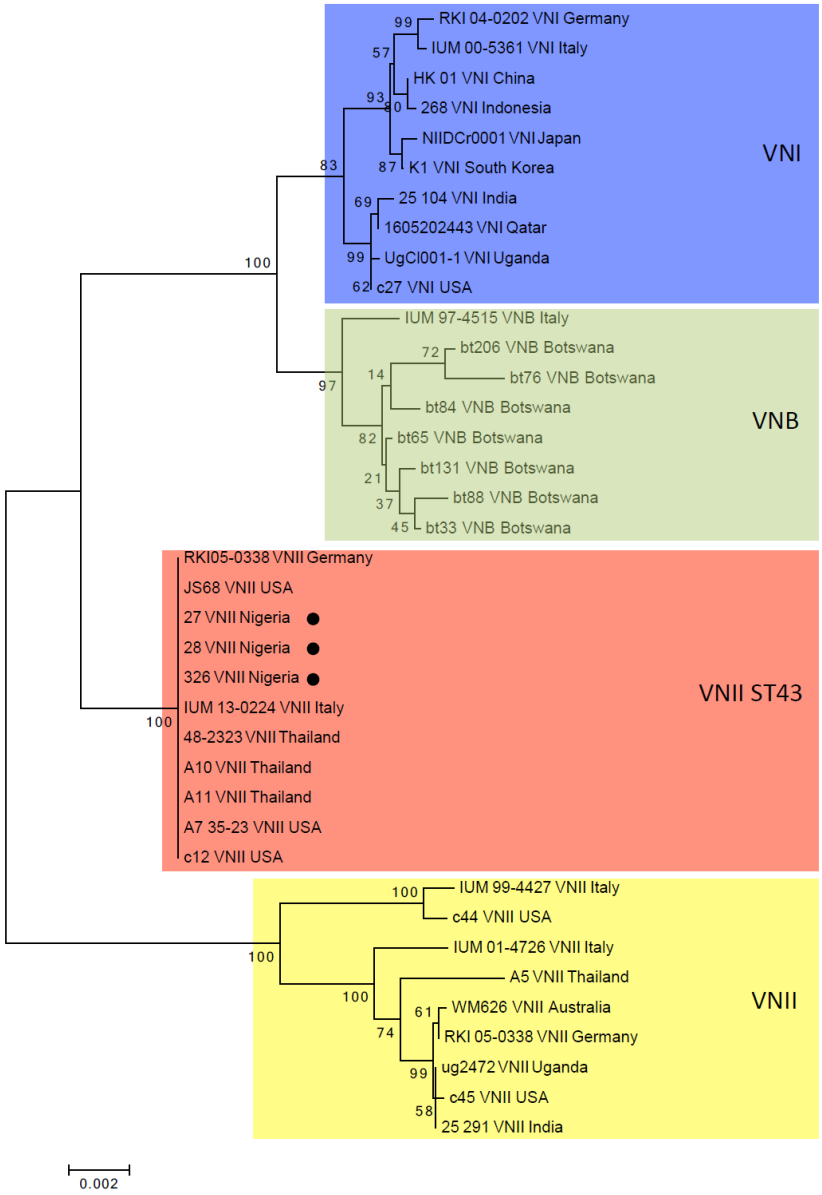


Figure 4.1: Phylogram of the Nigeria *C. neoformans* isolates within the global context. Phylogram depicting the genetic relationships between the Nigerian *C. neoformans* isolates based on Maximum likelihood phylogenetic reconstruction including 38 *C. neoformans* var. *grubii* (VNI, VNII, VNB) isolates. Black dots indicate Nigerian environmental isolates. Numbers near the nodes represent the bootstrap values obtained for 1000 replications of the concatenated seven ISHAM consensus MLST loci(*CAP59*, *GPD1*, *IGS1*, *LAC1*, *PLB1*, *SOD1*, *URA5*) using the program MEGA 6.

Table 4.3 : Distribution of *Cryptococcus neoformans* isolates reported from states in Nigeria

S/No	State	No. of *Cryptococcus* reported	References
1	Abia State	*C.neoformans 2 Isolates*	**(Nweze *et al.*, 2015)**
2	Adamawa State	No Data	2
3	Akwa Ibom State	No Data	3
4	Anambra State	*C.neoformans* 31 isolates	**(Nweze *et al.*, 2015; Mbata 2006)**
5	Bauchi State	No Data	
6	Bayelsa State	No Data	
7	Benue State	No Data	
8	Borno State	No Data	
9	Cross River State	No Data	
10	Delta State	No Data	
11	Ebonyi State	C.neoformans 4 isolates	**(Nweze *et al.*, 2015)**
12	Edo State	No Data	
13	Ekiti State	No Data	
14	Enugu State	C.neoformans 21 isolates	**(Nweze *et al.*, 2015)**
15	Federal Capital Territory	No Data	
16	Gombe State	No Data	
17	Imo State	C.neoformans 2 isolates	**(Nweze *et al.*, 2015)**
18	Jigawa State	No Data	
19	Kaduna State	No Data	
20	Kano State	No Data	
21	Katsina State	No Data	

22	Kebbi State	No Data	
23	Kogi State	No Data	
24	Kwara State	No Data	
25	Lagos State	No Data	
26	Nasarawa State	No Data	
27	Niger State	No Data	
28	Ogun State	No Data	
29	Ondo State	No Data	
30	Osun State	No Data	
31	Oyo State	No Data	
32	Plateau State	*C.neoformans 8 isolates*	**(Irokanulo *et al.,* 1997; Nnadi *et al.,* 2016)**
33	Rivers State	No Data	
34	Sokoto State	No Data	
35	Taraba State	No Data	
36	Yobe State	No Data	
37	Zamfara State	No Data	

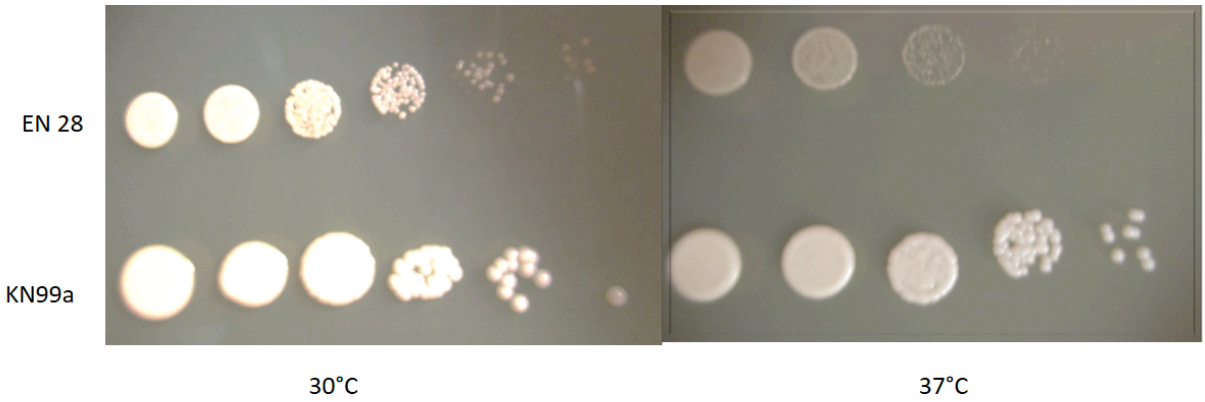


Plate 4.5 Growth at various temperature for two parents with different molecular types

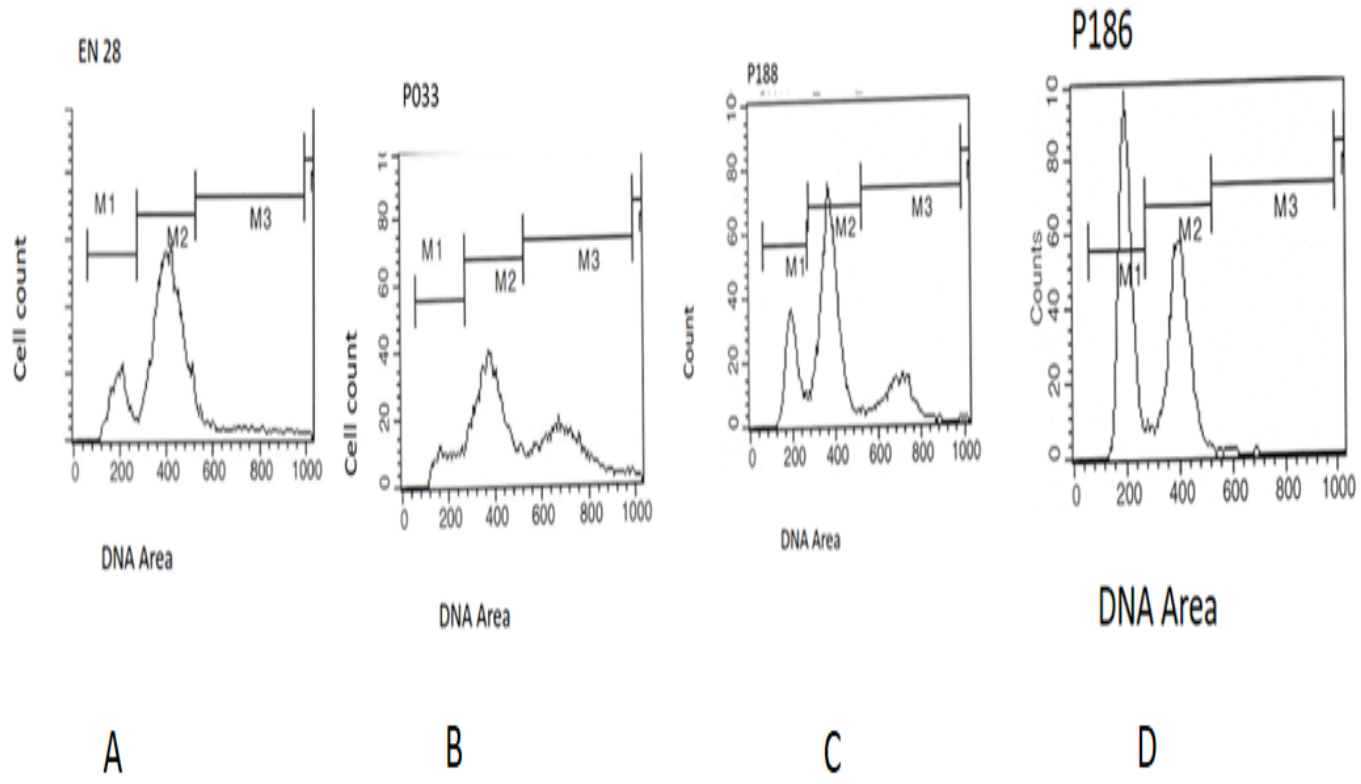


Figure 4.2 Representative ploidy variation from EN28 and KN99a Cross

Plate 4.6 shows the determination of mating type of the progenies. The diploid strains P033 gave double bands for Ste20***α*** and Ste20**a** showing heterozygosity at the MAT locus with the presence of the two alternative alleles **aAA*α***, while P188 showed homozygosity at the MAT locus with one band for Ste20a **aAAa .**One haploid progeny P165, ploidy confirmed using FACS, gave amplification for ste20***α*** gene, giving **αA.**

P033is a diploid F1 progeny strain produced filaments when cultured alone on MS agar (Plate4.7) whereas P188 did not self filament after 2weeks. This confirms the presence of heterozygosity(**aAA*α*)** of the P033 diploid. Both P188 and P033 were exposed to increasing dose of UV radiation. However, when compared with the parents, the diploid strains showed less fitness than the two parent strains at the harsh conditions. However, P188 showed greater fitness than P033 when exposed to increased radiation (Plate 4.8-4.9).Ploidy did not confer any advantage when compared to the parent strains, although among the two diploids, the homozygous **aAAa** (P188) diploid showed greater fitness than P033.

The phenotypic variation among the progeny generated through **a-α** bisexual reproduction was investigated and result shown (Plate 4.10). The phenotypes that were assessed, were temperature tolerance on YPD solid medium at different temperatures, melanin production on Niger seed medium, and resistance to UV radiation.

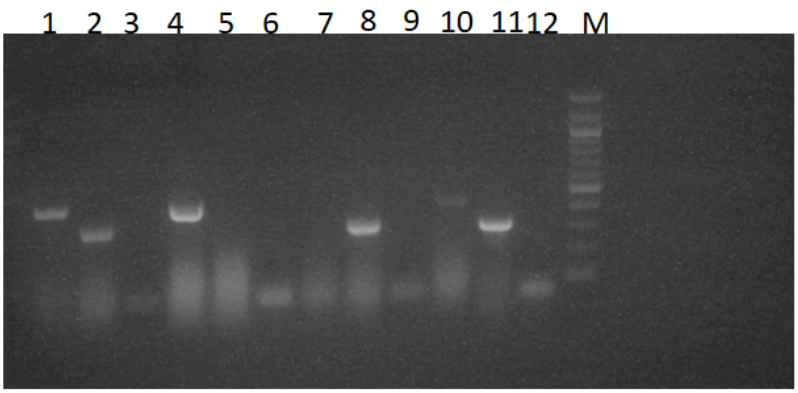
At a higher temperature, most of the progeny strains showed growth defects and hence sensitive to high temperature growth. However, strains P135(Plate 4.10C), P136(Plate 4.10B) and P175(Plate 4.10C) grew as much as KN99a parent strains.

For exposure to UV radiation, the progeny strains gave better resistance than parents to UV radiation when exposed to higher doses of UV radiation.

For melanin production, transgressive trait was observed with some progeny strains producing less melanin and others producing more melanin.

Figure 4.4shows the distribution of the 480 progenies at 30°C and 37°C. High bulk corresponds to those progenies that grew better at 37°C while those at 30°C which corresponds to low bulk were those progenies that grew worse at 30°C.

A total of 30 progeny samples were selected for mating type determination via mating assay on MS agar. 10 of the progenies belonged to *MATa* Mating type while 16 were *MAT* α to give a ratio of 1:1.6.



1=P033MAT**a**
2=P033 MAT**α**
3=Water
4=P188 MAT**a**
5=P188 MAT**α**
6=Water
7=P165 MAT**a**
8=P165 MAT**α**
9=Water
10=KN99**a** MAT**a** control
11=KN99**α** MAT **α** Control
12= Marker

Plate 4.6 Showing Mating type PCR for Diploid strains P033,P188 and a P165 a haploid strain. P033 has two alleles(**aAA*α*)**

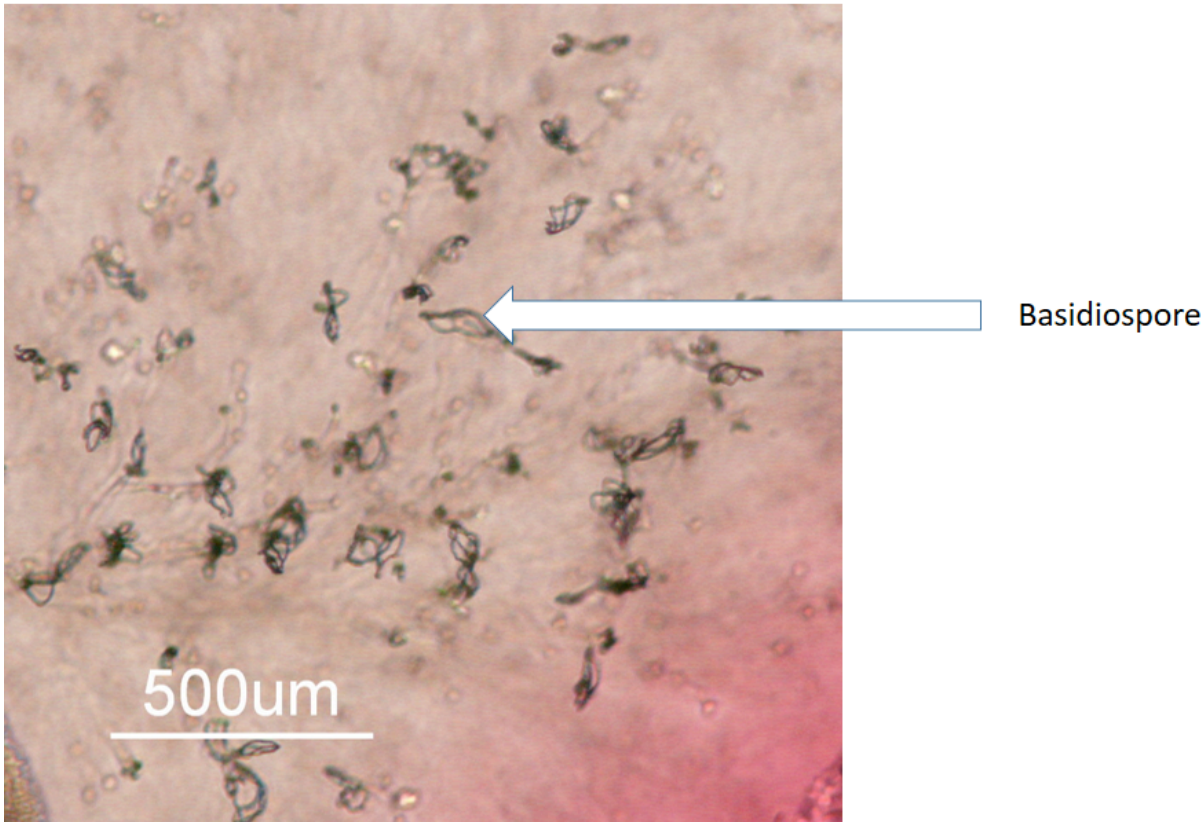


Plate 4.7: Self filamentation of Diploid strain P033

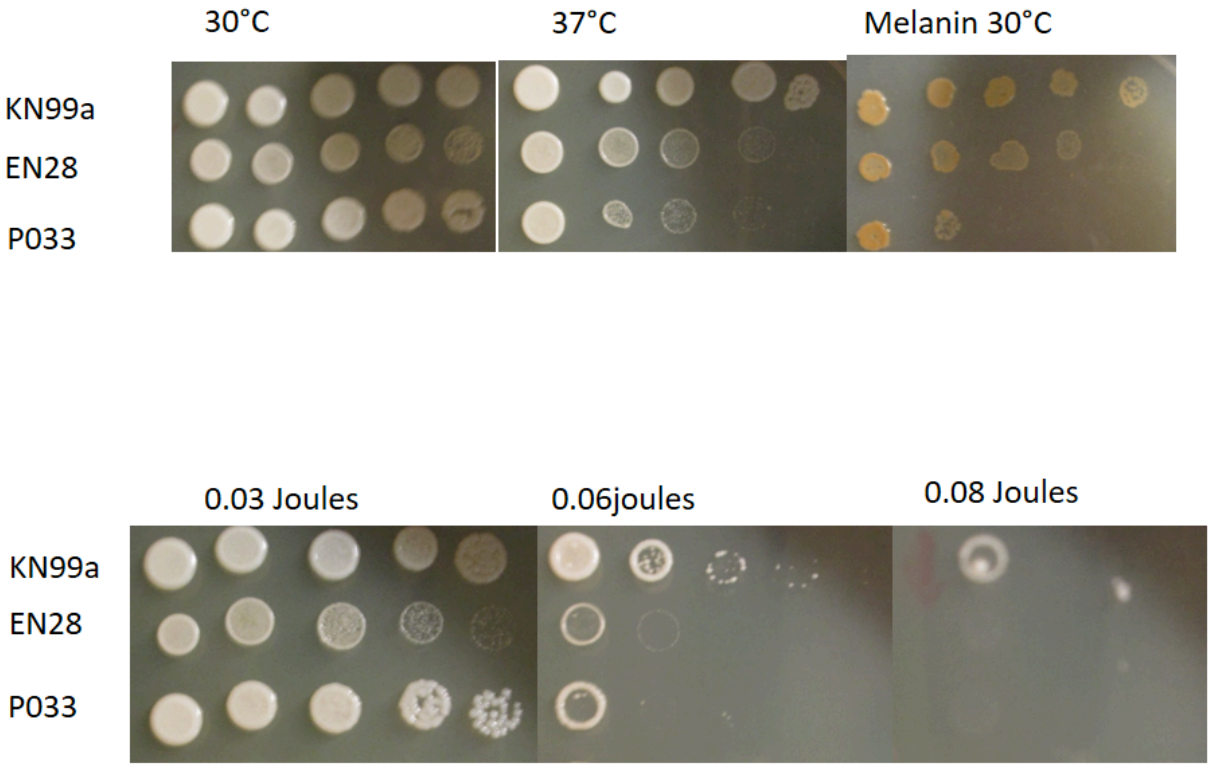


Plate 4.8 : Phenotypic variation of Diploid strain P033 to harsh environmental conditions

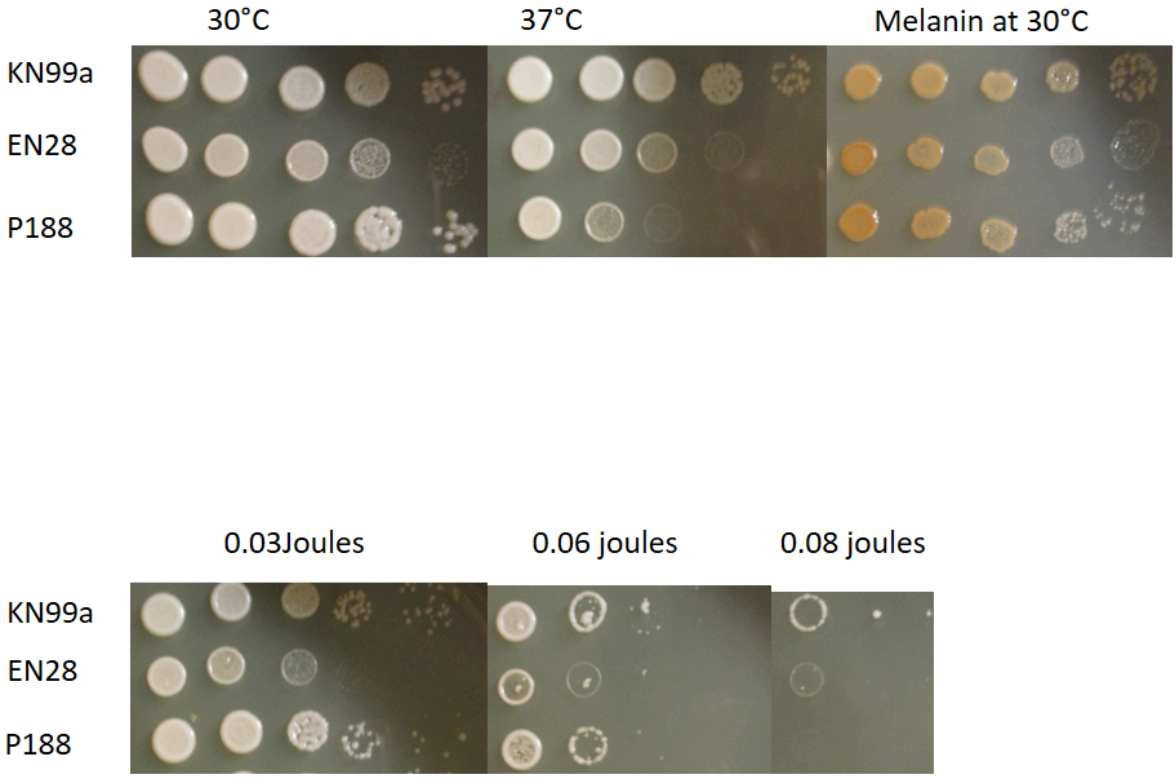


Plate 4.9 Showing isogenic **a**AA**a** diploid exposure to extreme conditions

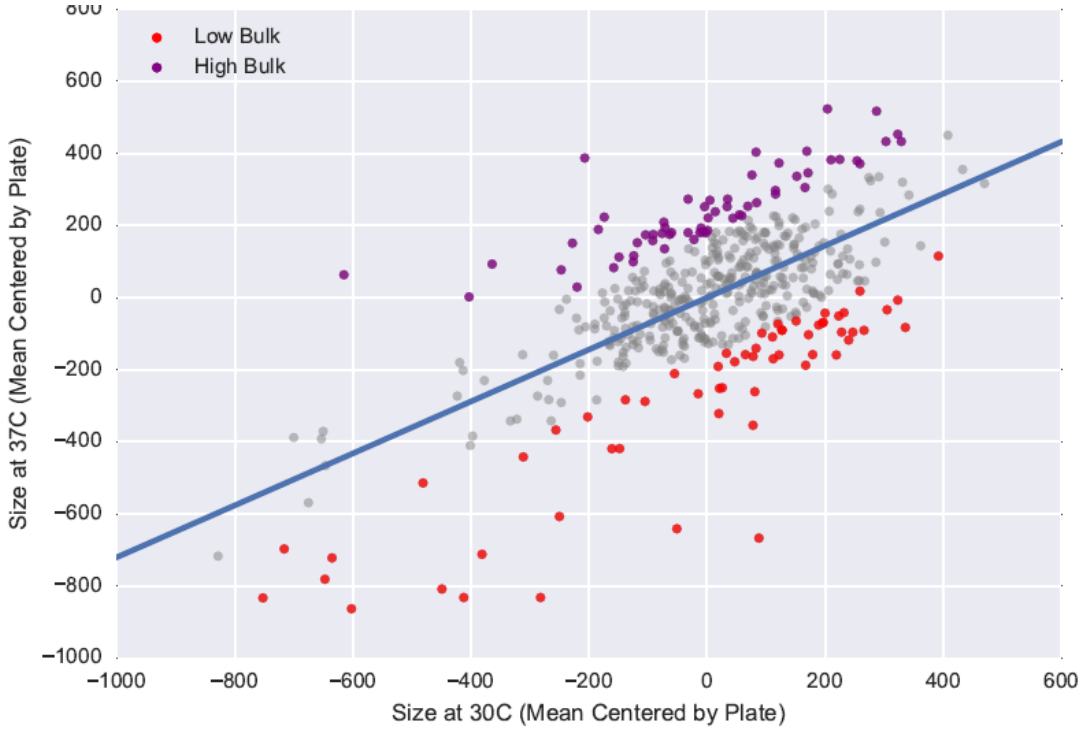


Figure 4.3 : Distribution of segregants (growth variation in progenies) generated from KN99a and EN28 cross at 30°Cand 37°C and corresponding low and high bulks

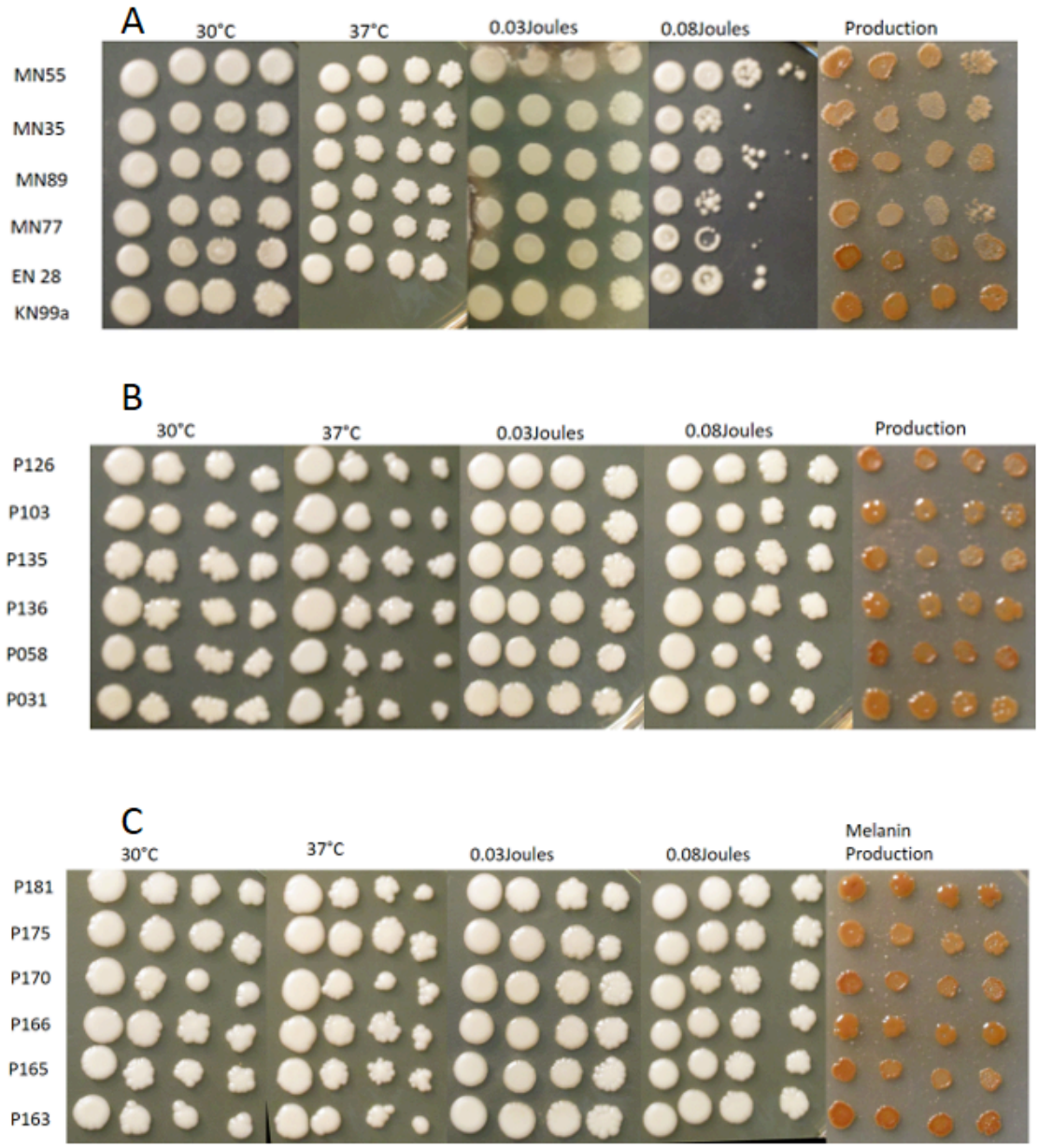


Plate 4.10 : F1 Representative strains of *C. neoformans* generated from the cross between EN28 and KN99a. A). Controls B and C. Progenies.

The results of activity of the antifungal agents Itraconazole, Voriconazole, fluconazole, and Flucytosin as range of MICs (Table 4.3). Minimium Inhibitory concentration(MIC) values were 0.03-0.12 for itraconazole, <0.03 for voriconazole, 2-4 for fluconazole, and 0.25 for flucytosine

Figure 4.4 shows table for quantity extracted from the various extracts. For *S.mahogani*, acetone extracted the least quantity 81mg(0.54%) while hexane gave the highest 185mg(1.23%).Dichloromethane and methanol gave intermediate quantity, 178mg(1.19%) 122mg(0.81%) respectively. Similarly, *S.siamea*, acetone extract also gave the least extract of 72mg(0.48%) and hexane extracted 192mg(1.28%) which is the highest. DCM and methanol gave intermediate quantity but DCM is quite higher than 169mg(1.13%) than methanol 153mg(1.02%).

On the other hand , *C.alata* showed no consistency with that of *S.mahogani* and *S.siamea* as DCM extract of *C.alata* gave the highest quantity of 264mg(1.76 %).Acetone and methanol gave almost the same quantity 250mg(1.67%) and 240mg(1.60%) but hexane gave the least quantity 51mg(0.34%).

For *R vormitoria* , the root and bark were used instead of the leaves according to the traditional medical use. For the roots of *R. vormitoria* gave the highest quantity of 170mg(1.13%)which showed consistency with that of *C.alata* but methanol gave a higher extract than acetone which is a reverse in the case of *C.alata* where acetone gave a higher yield. But hexane gave a the least extracted quantity which makes it consistent to that of *C.alata*. The bark of *R. vormitoria* also showed consistency with that of the roots and leaves of *C.alata*; DCM having the highest extracted quantity of 176mg(1.17%) followed by hexane 83mg(0.55%) then methanol 67mg(0.45%)then acetone 37mg(0.25%) which is consistent with that of *S.mahogani* and the root of *R. vormitoria* . In general, DCM extract of

C.alata gave the highest extraction of 264mg(1.76%) while acetone extract of *Ranvoltia vormitoria* (root) gave the least extraction of 30mg(0.20%).

The results is shown in table 4.4**.** Acetone extract of *C.alata* gave an MIC of 0.016mg/ml after 24hrs, however after 48hrs, the MIC changed to 0.032mg/ml. The Acetone and Dichloromethane extracts of *R. vormitoria* (root) had a very low MIC of 0.016mg /ml after 24hrs, however, the MIC value for the Acetone extract changed to 0.064mg/ml after 48 hours of incubation but Dichloromethane extract of *R. vormitoria* (root) remained unchanged from 0.032mg/ml after 48hours. The acetone, DCM and MeoH extracts of *R. vormitoria* (Bark) were promising at 24 hours with MICs of 0.032mg/ml; 0.016mg/ml and 0.032mg/ml respectively, however after 48 hours of incubation, the extracts changed to MIC of 0.128mg/ml. In order to select the best plant with the best activity against *Cryptococcus* total activity was determined

Table 4.4: MIC values of the three Nigerian isolates

	MIC (μg/ml)			
Strain	**Itraconazole**	**Voriconazole**	**Fluconazole**	**Flucytosine**
27	0.12	<0.03	2	0.25
28	0.03	<0.03	2	0.25
326	0.12	<0.03	4	0.25

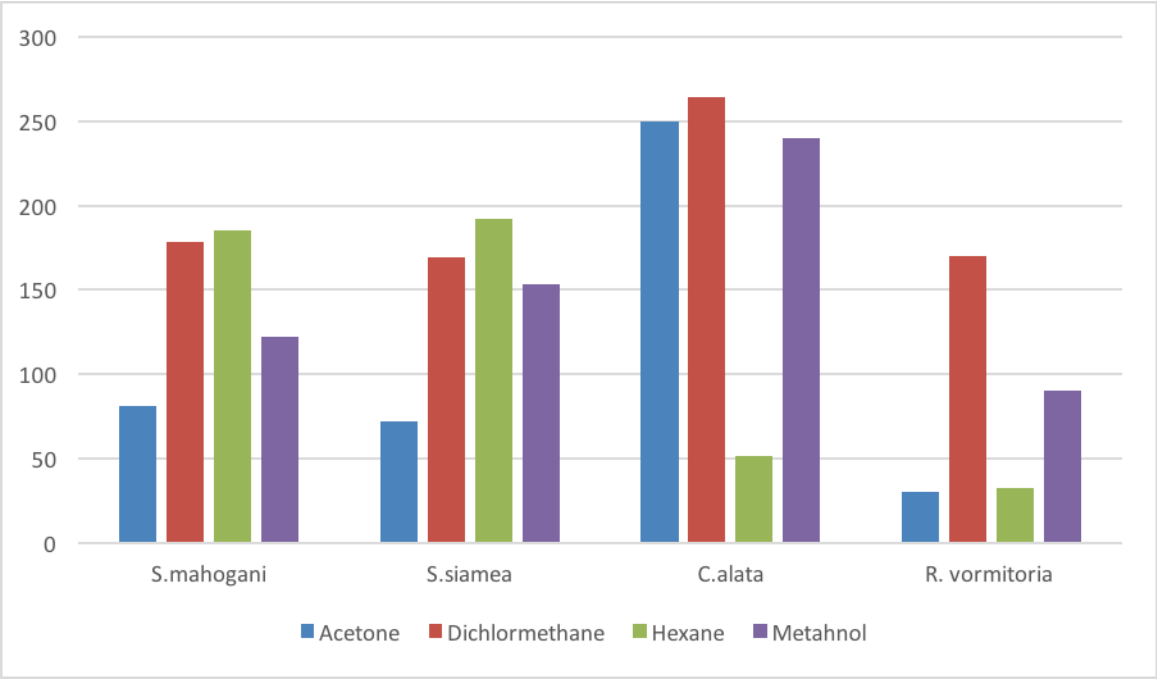


Figure 4.4 .Quantity Extracted from plant samples by different solvent

Table 4.5 Minimum inhibitory concentration (MIC) of six plant species against *Cryptococcus gattii* using different extracting solvent;acetone (A),dichloromethane(D),hexane(H),methanol(M).

Plant Species	**Time(H)**	**MIC(mg/ml)**			
		A	**D**	**H**	**M**
S.mahogany	24	0.064	0.064	0.032	0.032
	48	0.064	0.064	0.064	0.064
S.siamea	24	0.032	0.064	0.128	0.032
	48	0.064	0.128	0.128	0.064
C.alata	24	0.016	0.064	0.064	0.032
	48	0.032	0.064	0.064	0.032
R. vormitoria	24	0.032	0.032	0.016	0.032
(Root)	48	0.064	0.032	0.064	0.064
R. vormitoria	24	0.032	0.016	0.128	0.032
(Bark)	48	0.128	0.128	-	0.128

Total activity (table 4.6) is also an important parameter which made *C alata* a better plant with higher bioactivity. The acetone extract of *C.alata* gave the best activity of 1042ml/g which later dropped to 521ml/g after 48 hours. On the other hand, the methanol extract maintained a constant total activity of 500ml/g at both 24 and 48hrs. The DCM extract of *R. vormitoria* also showed high total activity of 733ml/g but because of the drastic drop in MIC after 48 hours incubation, dropping its total activity to from 733ml/g to 92ml/g. This makes *C.alata* a better option over *Ranvoltia vormitoria* (bark) which shows just slight decrease in total activity after 48 hours incubation(1042ml/g to 521ml/g) .

The acetone extract after the plate was developed by spraying with vanillin spraying agent showed more component compounds (4 compounds were spotted) on a TLC plate than methanol extract which had 3 components as shown on Plate 4.11.

Table 4.6 Total activity in ml/g of four plant species extracted with acetone(A),dichloromethane(D),hexane(H),methanol(M).

Plant Species	**Time(H)**	**Total Activity(ml/g)**			
		A	**D**	**H**	**M**
S.mahogany	**24**	84	185	385	245
	48	84	185	193	127
S.siamea	**24**	151	176	102	319
	48	75	88	100	159
C.alata	**24**	1042	275	53	500
	48	521	275	53	500
Ranvoltia	**24**	63	354	133	188
vormitoria **(Root)**	**48**	31	354	33	94
Ranvoltia	**24**	77	733	43	140
vormitoria **(Bark)**	**48**	19	92	-	35

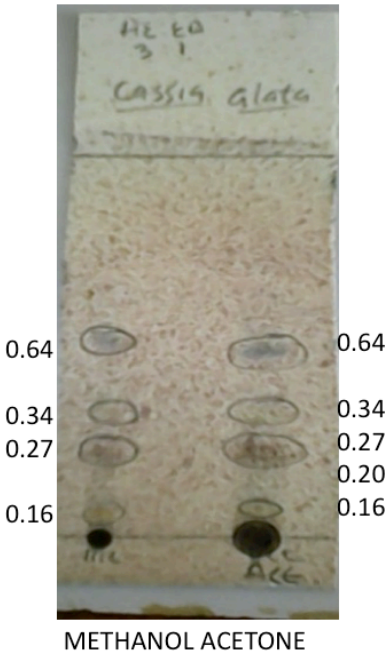


Plate 4.11. Thin-Layer finger printing of the acetone and methanol extracts of *C. alata* which showed activity against *C. gattii*.

Table 4.7 shows the phytochemical constituents of the plants. Alkaloids, saponins and flavonoids were found in the four plants used. None of the plants consisted of terpernoind and cardiac glycoside

The *Mucuna* agar did not foam while in the autoclave and also did not form clumps, and rather the media gave a fine blend consistent for other commercial media(Plate 4.13 B)..

The *Mucuna* agar plates was able to support the growth of the *Cryptococcus neoformans* and *C.gattii* producing brown color effect (Plate 4.13A) consistent with *Cryprococcus neoformans* .

Table 4.8 shows the physical tests performed on the brown color produced in the *Mucuna* agar to determine is its actually melanin. The black substance was soluble in 1M KOH, DSMO and NaOH but insoluble in water.

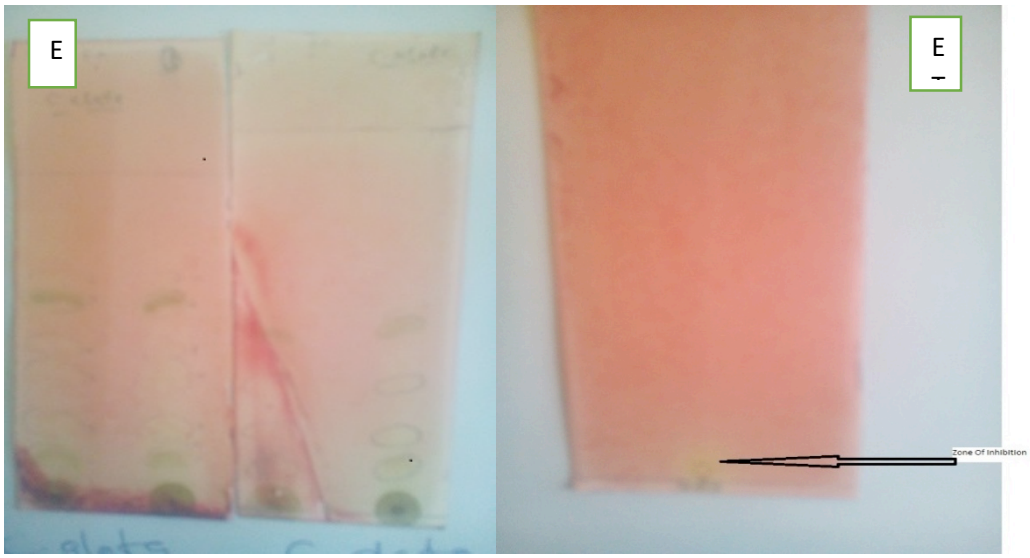


Plate 4.12 :A) Bioautography of *Cassia alata* extracted with Methanol and Acetone sprayed with *Cryptococcus*. White areas indicate where reduction of TTC to the coloured formazan did not take place due to the presence of compounds that inhibited the growth of *Cryptococcus* B.) Bioautography of DCM extract of *Ranvoltia vormitoria* .

Table 4.7. Results of phytochemical screening of the plant extracts

Plant species	***S.mahogani***	***S.siamea***	***C.alata***	***R.vormitoria (root)***	***R. vormitoria (bark)***
ALKALOIDS					
a. Dragendroff's test	**_**	**+**	**_ _**	**_ _**	**_ _**
b. Wagner's test	**+**	**+**	**+ +**	**+ +**	**+ +**
c. Mayer's test	**+**	**+**	**+ +**	**+ +**	**+ +**
SAPONINS	**_ _**	**_ _**	**+**	**_**	**_**
PHENOLS					
a. Ferric chloride test	**_ _**	**_ _**	**_ _**	**_ _**	**_ _**
FLAVONOIDS					
a. Lead acetate test	**+**	**+ +**	**+**	**_ _**	**_ _**
TERPENOIDS					
a. Salkowsk's Test	**_ _**	**_ _**	**_ _**	**_ _**	**_ _**
b. Harbone's Test	**_ _**	**_ _**	**_ _**	**_ _**	**_ _**
CARDIAC GLYCOSIDE					
Keller Kilhiamic Test	**_ _**	**_ _**	**_ _**	**_ _**	**_ _**
PHTOBATANNINS	**_ _**	**_ _**	**_ _**	**_ _**	**_ _**

The absorbance progressively decreased at the visible wavelength. In the spectral study, the absorption showed a characteristic peak in the UV region of wavelength ranging from 200 to 260 nm (Figure4.5) but none in the visible region . The absorption of light by melanin is maximum in the UV region and decreased progressively as the wavelength increases this decrease is nearly linear with increasing wavelength. The confirmation of the presence of melanin seen by the melanin curve obtained shows the importance of this virulence factor.

The three environmental isolates of *Cryptococcus neoformans var. grubii* were examined for extracellular phospholipase production after inoculation onto egg yolk agar. Each isolate was tested in duplicates. The ratio of the diameter of the colony to the total diameter of the colony plus precipitation zone (Pz) was measured as an index of phospholipase activity and result is shown in table 4.9.

Table 4.8 shows the phospholipase production of the isolates, the isolates moderately produced Phospholipase enzymes. A high Pz value means low production of phospholipase. Except for isolate 32 that had a low phospholipase production, the other two had moderate production of phospholipase enzyme.

Figure 4.6 shows the survival of Chick Embryo infected with clinical strains of *C. gattii*(R272,R265and EJB18) and *M. sympodialis*(ATCC42132). Inoculation of the CAM strains of *Cryptooccus gattii* led to mortality of the embryos. It was observed that R272 and R265 achieved <25% mortality after day 7 when the experiment was terminated. Progressive mortality was observed for these isolates, however, for R265, mortality peaked and stopped on day 4 while for R272 progressive mortality was observed till day 7 when experiment was terminated. *M. sympodialis* however, showed indisguishable mortality when compared with the control group.

Plate 4.13 : Cowitch agar media optimized for the cultivation of *Cryptococcus neoformans and C.gattii*.(a). Brown color effect that is characteristic of *Cryptococcus* species.(b). Cowitch agar dispensed on Petri dishes, the media was well formed

Table 4.8: Physical tests to detect melanin produced on Cowitch agar

Sample	**Solubity**			
	1M KOH	WATER	DMSO	NaOH
27	Soluble	Insoluble	Soluble	Soluble
28	Soluble	Insoluble	Soluble	Soluble
ENT4	Soluble	Insoluble	Soluble	Soluble

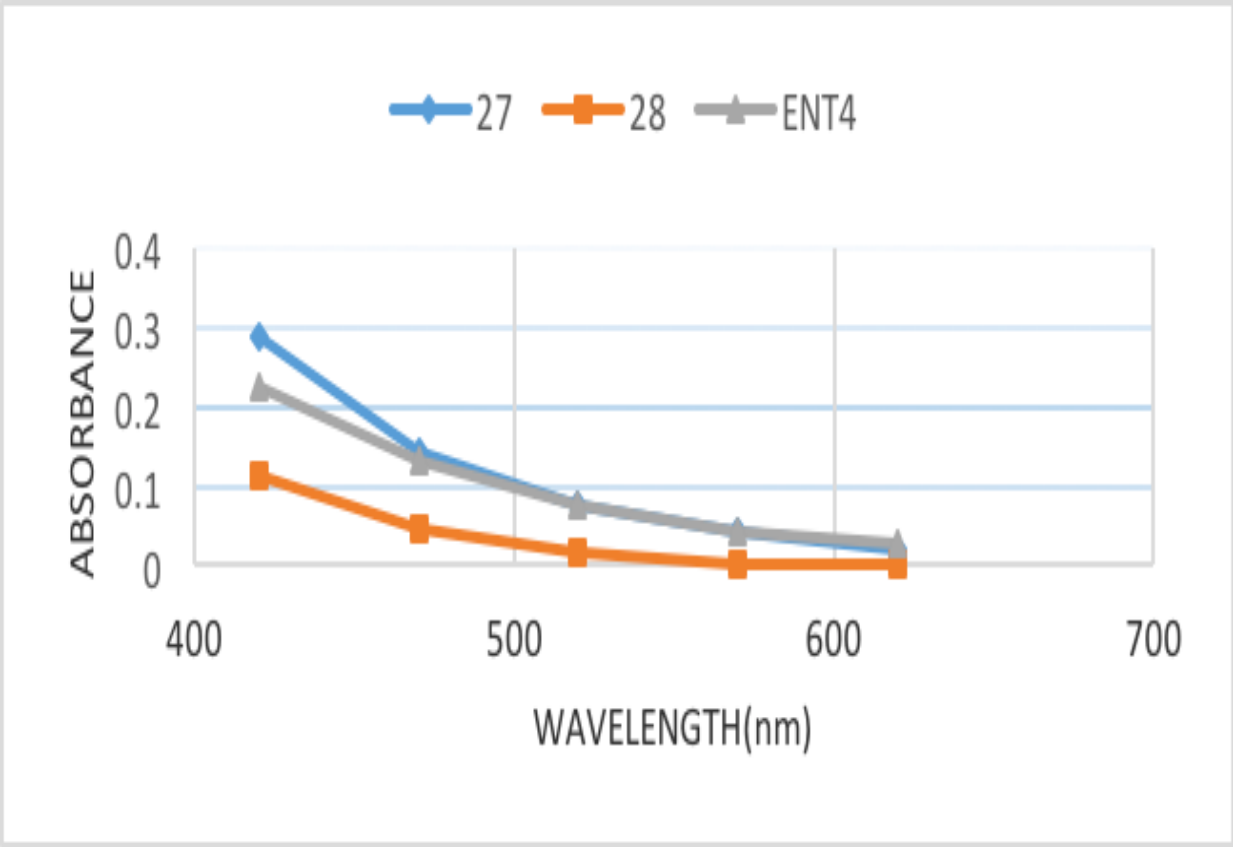


Figure 4.7 : *Absorption spectrum of melanin pigment of C. neoformans obtained in this study*

Table 4.9: Phospholipase production in environmental isolates of C. *neoformans* isolates

Isolates	**Total(cm)**	**Colony size(cm)**	**Pz**	**Class**
326	1	0.7	0.7	+
27	1	0.6	0.6	++
28	1	0.6	0.6	++

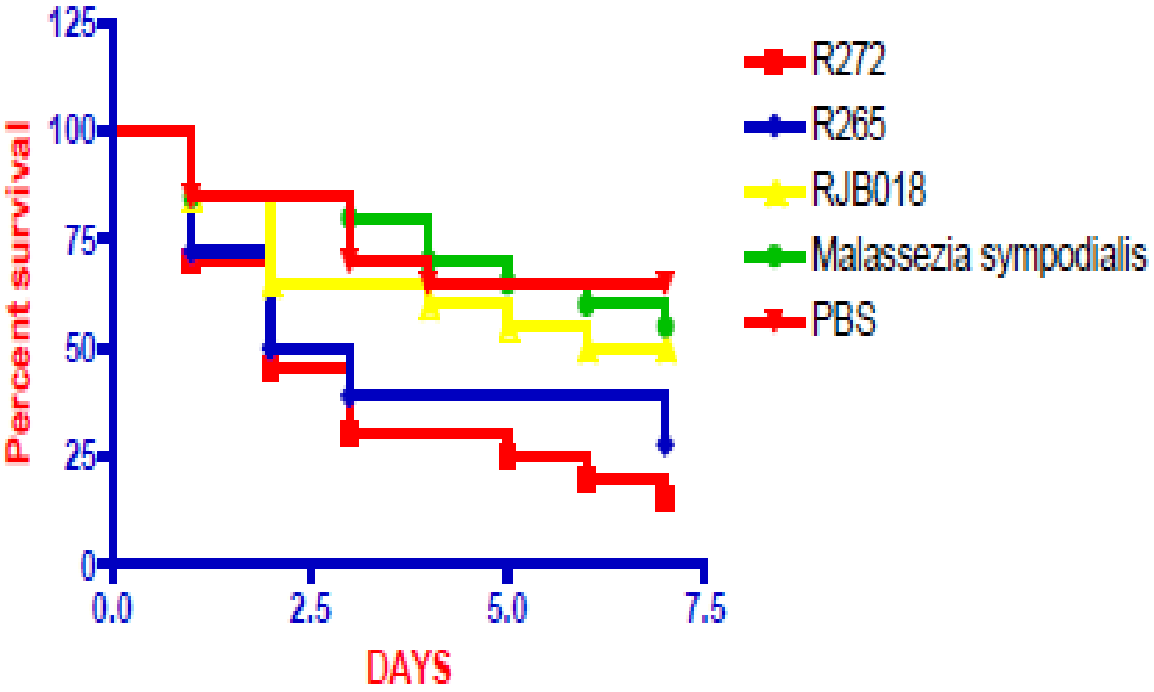


Figure 4.6 : Survival of Chick Embryo infected with clinical strains of *C. gattii*(R272,R265and EJB18) and *M. sympodialis*(ATCC42132) 20 eggs were used for each strain and phosphate buffer saline(PBS) was used as control.

From 24 hours of inoculation of CAM with 10^5cfu/ml of fungi concentration, leisons were observed on the CAM (Plate 4.14). *M. sympodialis* particularly formed a white mass on the CAM(Plate 4.15a). To evaluate what the white mass was, the white mass formed by *M. sympodialis* was carefully collected and squashed, this was stained with a drop of calcoflour white and viewed under the microscope. It was observed that the white mass was actually hyphae produced by *M. sympodialis* (Plate4.15b).The white Mass was plated PDAm to re-isolate *Malassezia sympodialis*.

To determine dissemination of fungus, attempt to re-isolate and quantify the *Cryptococcus neoformans* from CAM, liver and brain were performed. Results are shown in Figures 4.7-4.8**.** Figure 4.7 shows the number of colony forming units of *Cryptococcus* from the CAM of the embryo. All the strains could be isolated from the CAM and the fungal burden was determined as colony forming unit (cfu/ml) in the CAM after 24, 48 and 72hr after infection. A transient reduction in the burden of fungi isolated from CAM reduced on the second day, however this followed with a transient isolation of yeast for R272 and R265 from the brain (Figure4.8). EJB18 however did not disseminate to the brain. No organism was isolated from the brain after the second day as it was cleared by the embryo.

Plate 4.14 shows the macroscopic view of CAM after inoculation with *Cryptococcus gattii* cells, the formation of white plaques is indicative of fungal infection of the CAM.

Plate 4.15 shows macroscopic view of CAM after inoculation with *M. sympodialis*. Plaque formation followed the production of nodes on day 3 of infection. The nodes was made from a mass of hyphae formed by *M. sympodialis*(Plate 4.15 B). Plate 4.15C shows yeast form of *M. sympodialis* formed from plated node formed on CAM. This confirms the formation of hyphae on CAM by *M. sympodialis*

Plate 4.16 shows the histology of CAM following inoculation with *C. gattii* and PAS staining of CAM shows the presence of yeast cells and a massive disruption of CAM membrane

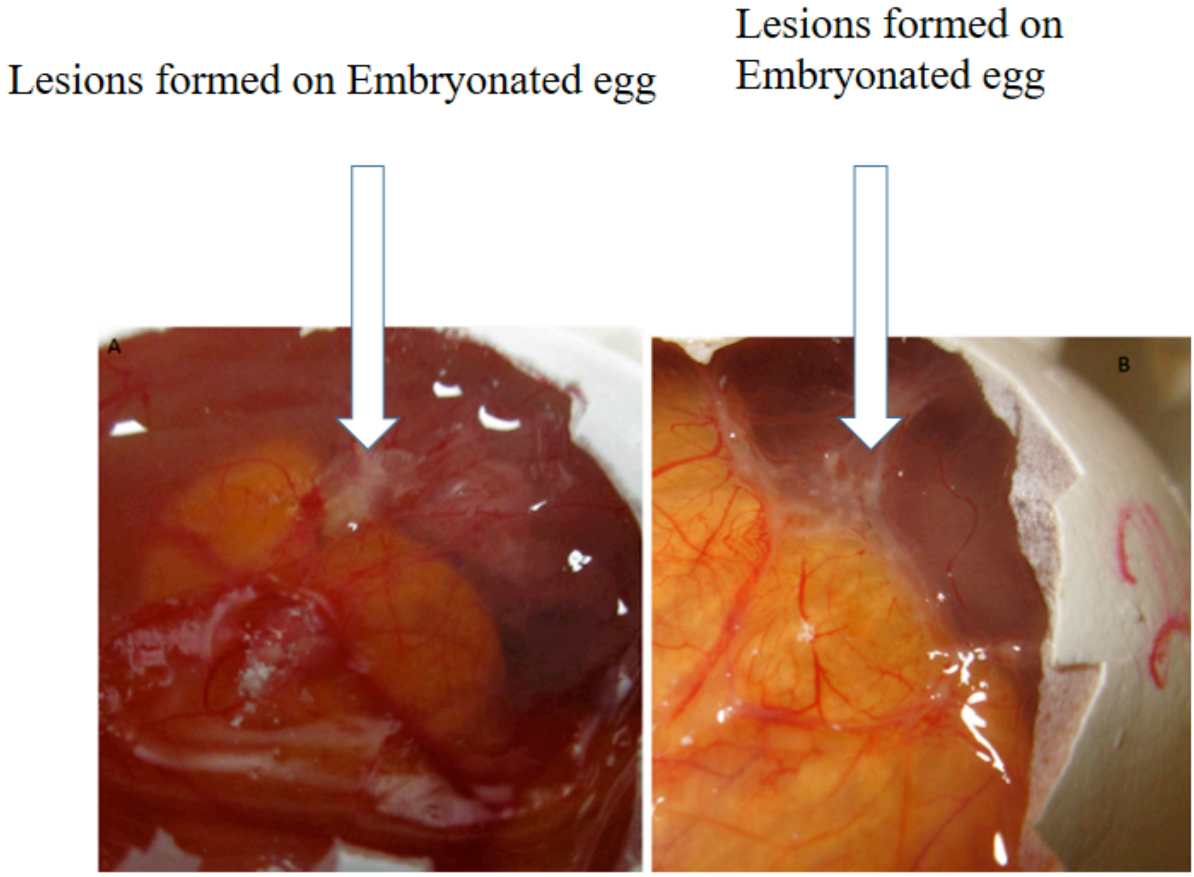


Plate 4.14: Macroscopic changes in infected embryonated eggs. Eggs were infected on developmental day 10. A and B). White lesions formed from infection with *C. gattii*

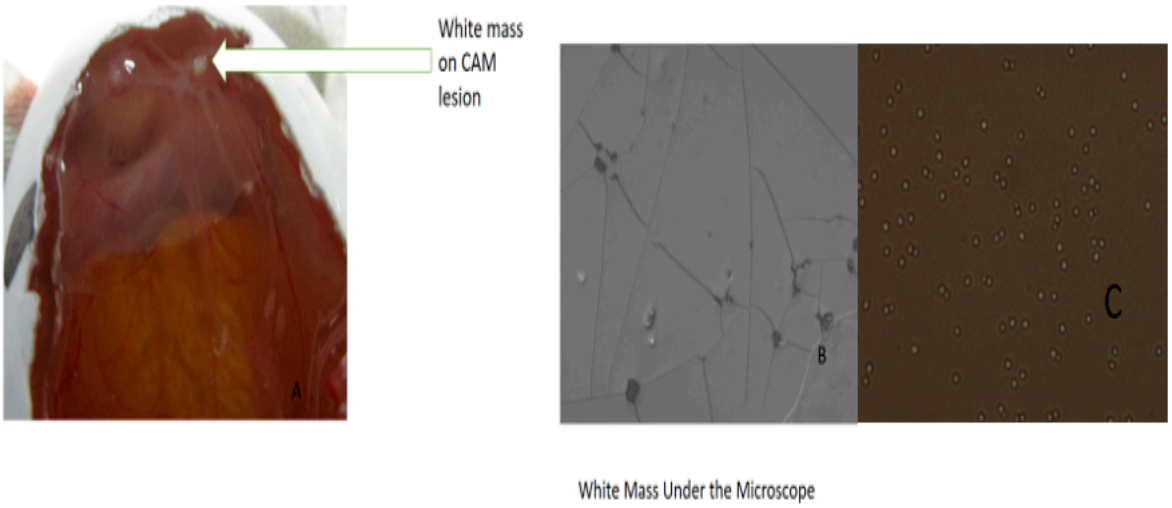


Plate 4.15: Macroscopic examination of egg CAM infected with *M. sympodialis* A.)White mass lesion observed on CAM. B.) White Mass viewed under x40 after staining with Calcoflour white.C.)White mass plated on PDA

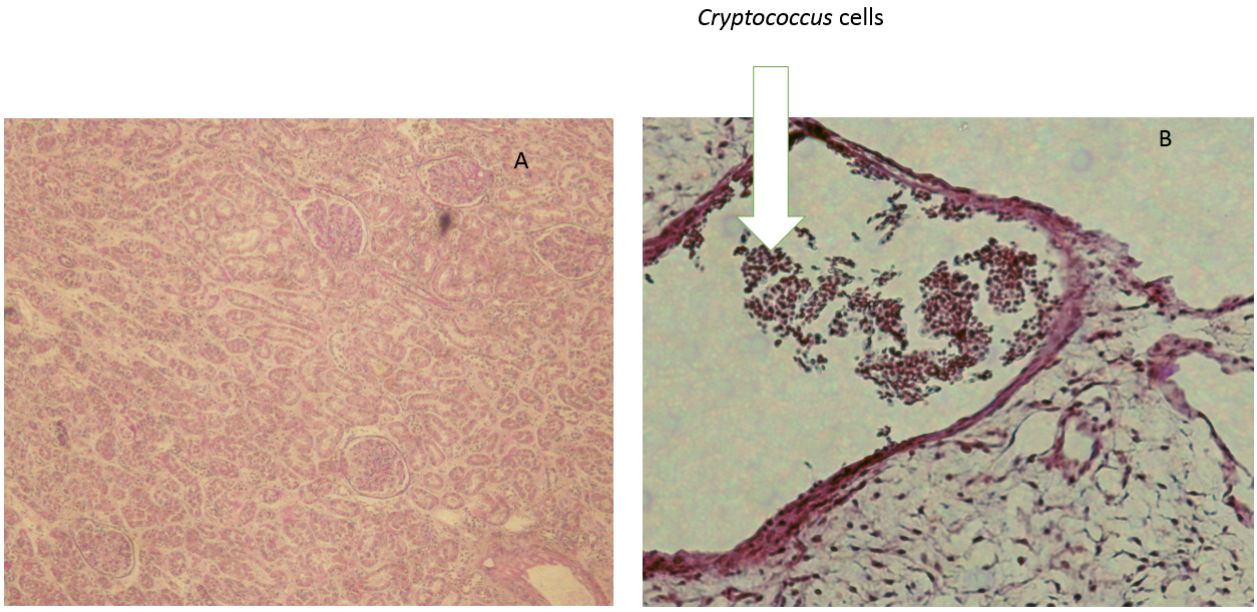


Plate 4.16 : Histology of embryos infected on developmental day 1.A. CAM control shows integrity of CAM B. Periodic acid-Schiff staining of the CAM shows the presence yeast cells and massive disruption of CAM

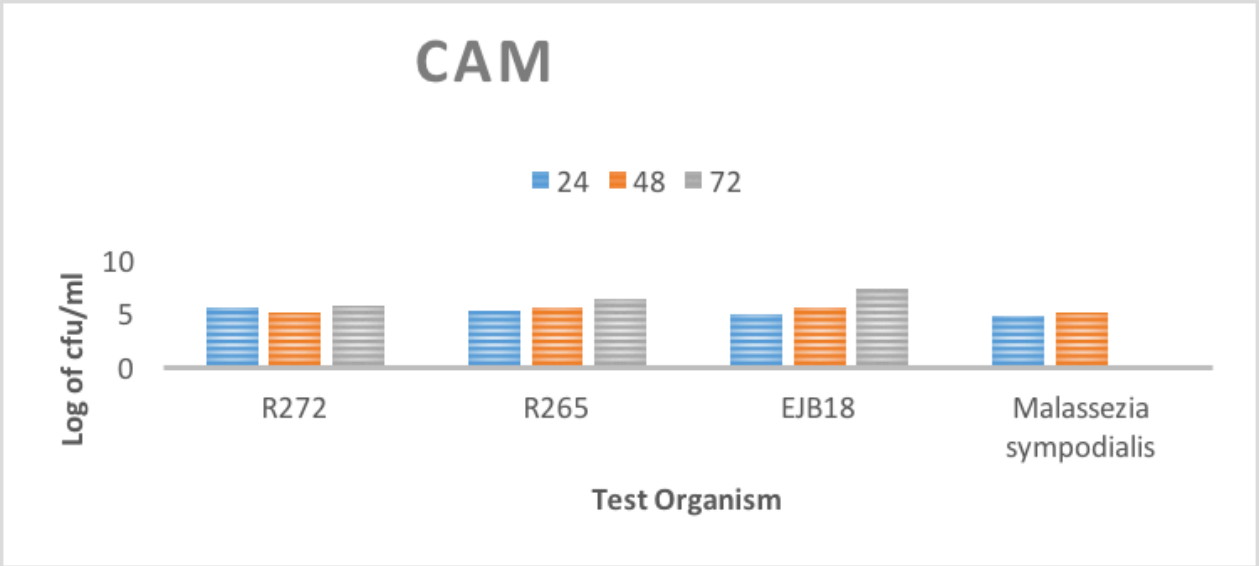


Figure 4.7 : Comparison of fungal burden of CAM at different developmental days

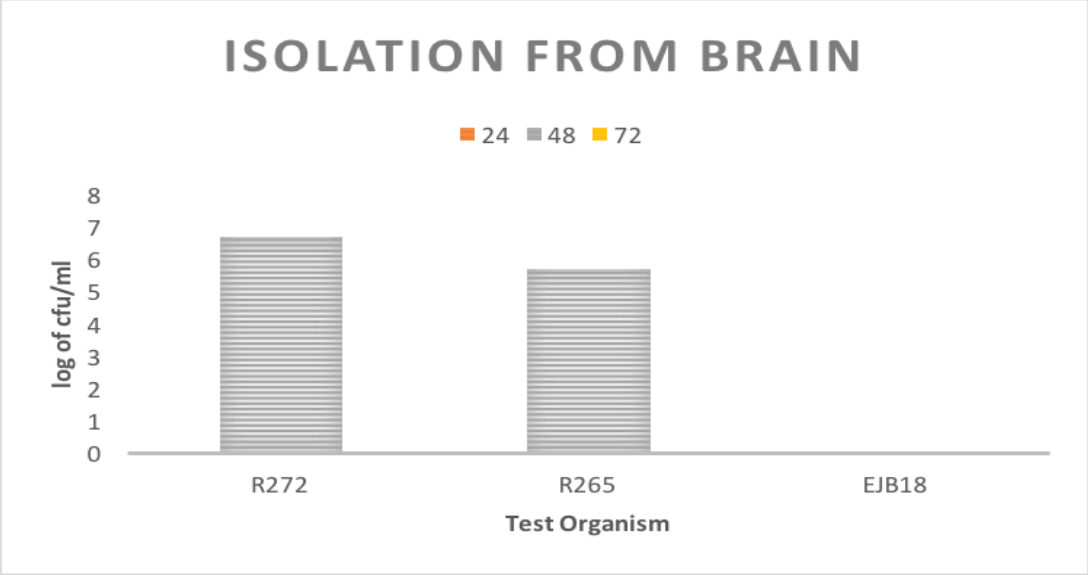


Figure 4.8 : Fungal burden of *C.gattii* from the brain of chick embryo

Figure 4. 9 shows the survival curve following the inoculation of *G. mellonella* larvae with EN326 an environmental strain of *C. neoformans* resulted in rapid death of the larvae. After the first day of inoculation and incubation at 30°C, larval death was observed. Comparing the killing rate of EN326 an Environmental strain and of the VNII molecular type with KN99a a clinical strain of VNI molecular type. KN99a was more virulent that EN326

Plate 4.17 shows a picture of *G. mellonella* larvae inoculated with *C. neoformans* in the culture plate. The plate consist of dead larvae(black) and the living larvae(cream).

Genome characteristic of *Cryptococcus neoformans* var. grubii ST 43 is found in table 4.9.
A total of 15 chromosomes(14 nuclear and 1 mitochondria) were identified with over 7,849 protein-encoding genes.

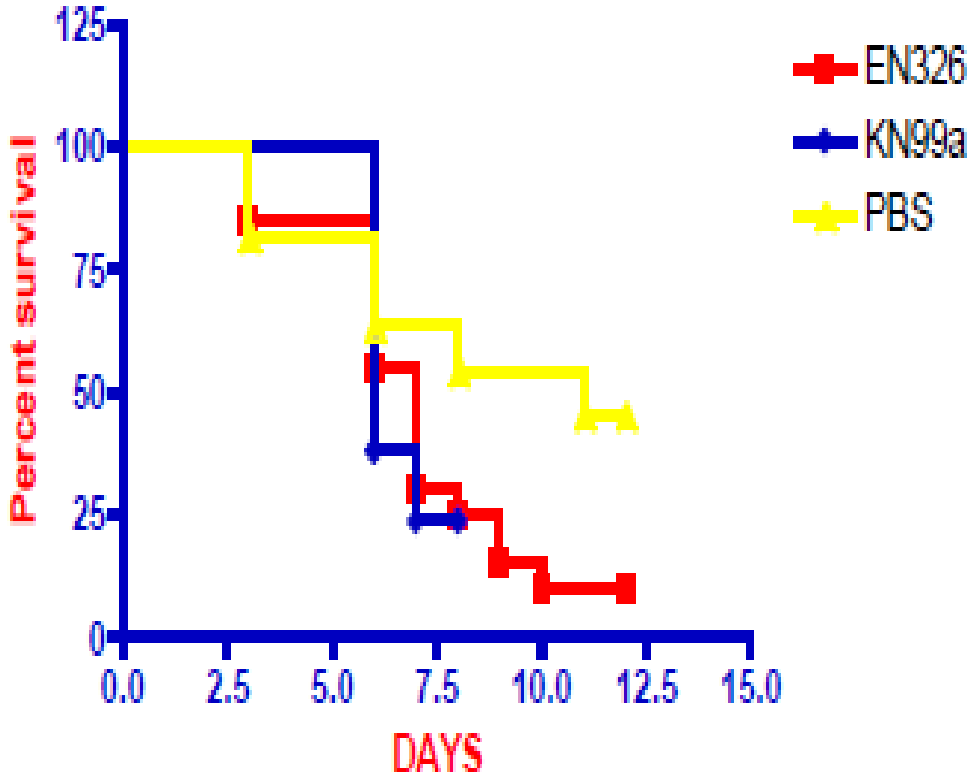


Figure 4.9. Survival curve of *Galleria mellonella* larvae inoculated with *Cryptococcus neoformans*

Plate 4.17: A plate containing *G. mellonella* the living larvae are usually cream coloured they turn black when dead

Table 4.10 : Genome summary for *C.neoformans* ST 43

Genome Characteristic	Number
putative tRNAs	**261**
protein-encoding genes	7,849
Chromosome	15 (14 nuclear and 1 mitochondrial)
Size of Genome	18.9Mbp
G+C content	48.19%

CHAPTER FIVE

DISCUSSION

This study reports the environmental occurrence of *Cryptococcus* in Jos, Plateau State, Nigeria. Three isolates of *Cryptococcus neoformans* representing a prevalence of 3% was obtained in this study. In this country, few studies have been conducted to investigate the presence and diffusion of serotypes and molecular types of *C. neoformans*. The genetic diversity of this yeast is poorly understood as no report of molecular types of *C. neoformans* or *C. gattii* has been reportedly isolated from patients or the environment in Nigeria. However, in past years, some authors reported the isolation of *C. neoformans* serotype A strains from captive birds in Jos, Nigeria (Irokanulo and Akueshi 1995; Irokanulo *et al.,* 1997) and from bat droppings in Awka, South East Nigeria (Mbata 2006), but, they did not report the molecular types of these isolates.

Isolation of *C. neoformans* from the environment is noted for its association with accumulations of avian guano, especially pigeon excreta. It has also been isolated from droppings of caged birds (Irokanulo and Akueshi 1995; Irokanulo *et al.,* 1997) and chicken guanos (Kuroki *et al.,* 2004). However, pigeons are unlikely to be the major source of *Cryptococcus* in nature, since only low concentrations of organisms are found in samples from the beak, crop, feet and rectal swabs(Emmons 1960). Pigeons and most other birds are not hosts to *C. neoformans* and they do not acquire cryptococcosis because their body temperature (average 42.5°C) is too high to allow the growth of this fungal pathogen (Kuroki *et al.,* 2004; Emmons 1960). Pigeon itself is not a reservoir of the pathogen but may serve as its mechanical carrier and disseminator in the environment (Littman and Borok 1968; Chowdhary *et al.,* 2011). The real reservoir seems likely to be trees where the fungus could find favourable microhabitats inside trunk hollows or fissures to survive. This was shown by the results obtained in a large environmental survey carried out in the Mediterranean area

(Cafarchia *et al*., 2006).This study however did not isolate any *Cryptococcus* species isolate from *Eucalyptus calmadulensis* tree sampled.

In the present study, *C. neoformans* VNII *MATα* was isolated. This molecular type is globally distributed but it has a low prevalence compared to the high occurrence of the VNI type (Cogliati 2013). *C. neoformans var. grubii* is responsible for more than 90% of all cases of cryptococcosis (Litvintseva *et al*., 2011). Recent genotype analyses of global, clinical, and environmental isolates of *C. neoformans* var. *grubii* identified three genetic subpopulations, VNI, VNII, and VB. The molecular type VNI strain is the most prevalent causative agent of cryptococcosis worldwide, while VNII is globally distributed but rare. However, all known VNII are *MATα* mating type(Litvintseva *et al*., 2011). This study corroborated the fact that all known VNII are *MATα*.

This study isolated only VNII genotype and no other genotype, this might indicate that VNII genotype has a higher prevalence in the Nigerian environment, although a more extensive survey is required to confirm this trend.

Strains of VNII have also infected people on every continent but in low frequency and there have been very few isolations from the environment. The ecology of *C. neoformans* VNII is not known. In sub-Saharan Africa, cryptococcosis caused by *C. neoformans* far exceeds those caused by *C. gattii* (Govender *et al*., 2011).The environmental reservoir of *C. neoformans* and *C. gattii* will determine who will be exposed and at risk of infection. Despite the introduction of the HAART therapy among HIV/AIDS patients, an estimated 504,000 new cases of cryptococcosis occurs per year among persons with HIV/AIDS(Park *et al*., 2009). The high number of infections due to *C. neoformans* in sub-Saharan Africa indicates that this species is widespread in this region, which must provide a rich environment for the propagation and dispersion of the yeast cells or its basidiospores.

Kaocharoen et al.(2012) in their study on Thai *Cryptococcus* strains suggested a close relationship between animal and human VNII isolates may exist, as strains from humans and

animals share the same genotype. This study however, provides a clue to the possible reservoir of the VNII serotype as being the environment. Therefore, there is need to study the ecology of *C. neoformans* VNII isolates both in the global context and in Africa where the genetic diversity of this species is lacking

Studies on the molecular epidemiology of *Cryptococcus neoformans* is poor in Nigeria and Africa at large. In the 1970s, *Cryptococcus neoformans* was isolated from pigeon guanos in Nigeria(Gugnani and Njoku-Obi 1973), twenty-four years later, Irokanulo *et al.,* (1997), isolated and reported the isolation of *C. neoformans* from captive birds in Jos, Plateau State Nigeria. However, none of these study applied molecular techniques to reveal the molecular type or mating types of the *Cryptococcus* isolates obtained in their study. In another study carried out in Enugu and other south eastern states, a prevalence of 22%(39 of 177) of *Cryptococcus neoformans* was reported from a wide scale study comprising of 5 different states in South Eastern Nigeria(Nweze *et al.,* 2015). The rate of isolation of *Cryptococcus neoformans* varies from one location to another. The reason for this is not known. No study till date has applied the use of molecular tools in the study of *Cryptococcus neoformans* in Nigeria. Hence the peculiarity of this study. Paucity of the serotypes and Molecular types exist in various states in Nigeria.

In addition, the isolates belonging to the MLST type ST43 which had been reported before, from human, animal and environmental samples, from Asia, in Thailand and Japan (Cogliati 2013; Kaocharoen *et al.,* 2012) from USA, in North Carolina(Mihara *et al.,* 2013) and Washington states(Litvintseva *et al.,* 2006), and from Germany (Singer *et al.,* 2014). This suggests that the Nigerian isolates here reported are not endemic to the region but belong to a genotype globally dispersed. In addition, our MLST analysis showed that genotype ST43 represents a separate cluster within VNII molecular type (Figure 4.4) and therefore eventual peculiar characteristics of this cluster should be investigated in future.

Mating type has an effect on the virulence of *Cryptococcus*. The vast majority of infections are caused by isolates of mating type ***α*** not mating type **a** (Nielsen *et al*., 2003). This bias is also observed in the environment, most *Cryptococcus* isolates are *MATα*. The *Cryptococcus* isolates obtained in this study is consistent with other findings that isolated more MAT ***α*** type strains . In their study, Chen et al.(2015)analyzed *Cryptococcus* strains from three different African Countries, suggesting that MAT**a** is rare. The fertility of the isolates were determined to know if they can produce basidiospores. The three strains of *Cryptococcus* were found to be fertile, producing basidiospores and clamp connection.

Several studies indicated that *Cryptococcus neoformans* basidiospores are capable of initiating infections (Giles *et al*., 2009; Velagapudi *et al*., 2009) when *MATα* isolates are found in the local environment. The present study also showed that the Nigerian environmental isolates are fertile, therefore they are potentially able to disperse in the environment by both asexual and sexual reproduction representing a menace for human health.

Since the ***α***A strains do not self-filament and mates extensively with the **a**-Mating type, this study therefore supports the hypothesis that *MATa* strain though not isolated yet may not be extinct as thought as that may be the replication pathway for the VNII in the environment. However, since cross fertilizations were observed in this study between the VNIa and VNII***α***, this may be another alternative pathway for the replication of the VNII in the Nigerian environment. This is strongly supported by the isolation of aAAα diploid from VNIa and VNIIα cross isolated from this study.

Since only VNII ***α***A strains was isolated, it may be hypothesized that the Nigerian environment may harbour the VNII **a**A strains or VNI **a**A strains through which much mating and propagation takes place. This hypothesis is however subject to further verification.

There is paucity of reports on in vitro antifungal susceptibilities of environmental isolates of C. *neoformans* and *C. gattii* and these are based on small sample size (Alves *et al*., 2001;

Souza *et al*., 2005). The isolates of *Cryptococcus neoformans* used in this study were from the environment, hence may have not been exposed to any form of antifungals, therefore the MIC value is intrinsic.

A comparison of the susceptibility profiles of environmental isolates published during 1996–2005 revealed that MIC_{90} values of antifungal agents have not changed noticeably (Currie *et al*., 1995; Tay *et al*., 2005). No Clinical and Laboratory Standards institute (CLSI) susceptibility breakpoints for *Cryptococcus* species are currently available for any of the antifungal agents and relationship of in vitro susceptibility results with clinical outcome is not yet clearly understood(Pfaller *et al*., 2011). It is clear however that patients infected with *C. neoformans* for which fluconazole MICs are ≤8μg/ml responds better to treatment with fluconazole than those infected with strains for which MICS are ≥328μg/ml(Aller *et al*., 2000). Taking into consideration the MIC distribution profiles for the various antifungals, the pharmacology of the antifungal drugs, and studies of resistance mechanisms and clinical outcomes, it is reasonable to adapt the breakpoints developed for *Candida* for use for *Cryptococcus neoformans* (Pfaller *et al*., 2011). The CLSI breakpoints for *Candida* are as follows: Itraconazole S≤0.12μg/ml, SDD 0.25 to 0.58μg/ml, R≥1μg/ml; voriconazole S≤1μg/ml, SDD 2 μg/ml, R≥4 μg/ml, fluconazole S≤8 μg/ml, SDD 16 to 32 μg/ml, R≥64 μg/ml and for flucytosine S≤4 μg/ml, Intermediate(I) 8 to 16 μg/ml. R ≥ 32 μg/ml.

It is well known that *C. neoformans* (serotypes A, D and AD) and *C. gattii* (serotypes B and C) differ from each other in several characteristics, including epidemiology, pathogenicity, and clinical manifestations. Besides, minor to significant differences in the susceptibilities of the two species have been reported. Chen *et al*., (2000) reported that *C. gattii* isolates were less susceptible than *C. neoformans* to amphotericin B ($p< 0.001$). Likewise, a difference in susceptibilities to azoles (one dilution difference) has been reported(Trilles *et al*., 2004).

In this study, only one molecular type was observed from the environment. All the strains were susceptible to all the antifungals tested. Souza et al.(2005) , obtained an MIC value of 4-

8μg/ml for *C. neoformans* against fluconazole with the exceptions of itraconazole and flycytosine, high suscpetiblity (83-100%) are seen for fluconazole, posaconazole and amphotericine B(Pfaller *et al.,* 2011). With an MIC of 2-4 μg/ml for all the environmental isolates of *C. neoformans* obtained in this study, it can be said that fluconazole can serve as a good alternative for the management of *Cryptococcus neoformans* infection in Nigeria where most other antifungals are lacking.

Isolates from resource limited settings (RLS) where fluconazole monotherapy is the only course of treatment, shows decline susceptibility to fluconazole (Hamill 2006) this makes it imperative to diversify to other azoles. A global study covering 2001-2007 involving 134 sites and 39 countries, showed that resistance to fluconazole was is less common in Europe and North-America when compared to what was seen in the Asia-Pacific, Africa, Middle East, and Latin- American regions. The possible reason may be due to the use of variety of antifungals in the management of *Cryptococcus* spp infections than is observed in Africa and Latin- American countries.

The MIC value obtained in this study for voriconazole is much lower compared to the report of Souza and colleagues (2005) in Brazil. Among the four antifungals tested, voriconazole with an MIC of 0.03μg/ml gave the best activity against the environmental isolates of *C. neoformans* used in this study. Voriconazole and itraconazole, have been shown to give excellent activity against *C. neoformans* (Souza *et al.,* 2005) *et al.*,1999. This was also corroborated by this study with both having very low MIC against *C. neoformans*. Studies have revealed that the newer triazole (posaconazole and voriconazole) retain potent activity against *C. neoformans* with more than 99% of the isolates showing MICs of $\leq$ 1μg/ml irrespective of the origin of the isolates (Pfaller *et al.,* 2011). This hypothesis is corroborated in this study with an MIC of 0.03μg/ml for voriconazole obtained for the environmental isolates of *C. neoformans*.

Acetone gave lowest extraction in 4 diffferent plant samples (*S.mahogani*, *S.siamea* ,*R. vormitoria (*root and bark), while DCM gave the highest extraction in 3 samples(*C. alata*, *R. vormitoria* (root), *R. vormitoria* (bark)). Interestingly, methanol extract and acetone extract of *C. alata* showed almost the same quantity which may be attributed to the degree of the polarity index of the two solvents which corresponds(Markom *et al.,* 2007). Akowuah et al.(2005) explained the relationship between quantity of extract and the polarity index of solvent used in extraction. Polar compounds are easier to be extracted compared to non-polar compounds. The difference in quantity extracted may be attributed to other factors including phytochemicals in plants, extraction temperature, extraction time and solvent to solid ratio.

Dichloromethane extract *R. vormitoria* (Bark) had a very low MIC of 0.016mg /ml but after 24 hour incubation changed to 0.128mg/ml this could be due to a fungistatic effect of this extract. Heterosusceptibility could be another reason for this change in susceptibility values. Acetone extract of *C. alata* on the other hand changed slightly from 0.016mg/ml to 0.032mg/ml after 48hours incubation which makes it a better option over *R. vormitoria*

Total activity is also an important parameter which made *C alata* a better plant with higher bioactivity. MIC is a function of total activity. Total activity indicates the degree to which the active compounds in 1g of plant material can be diluted and still inhibit the growth of the tested microorganism (Eloff 2000).Acetone extract of *C. alata* showed the highest total activity of 1042ml/g implying that 1g of acetone extract diluted in 1042ml will still inhibit the growth of fungi. DCM extract of *R. vormitoria* also showed high total activity of 733ml/g but because of the drastic drop in MIC after 48 hours incubation, dropping its total activity from 733ml/g to 92ml/g. This makes *C. alata* a better option over *R. vormitoria* (bark) which shows just slight decrease in total activity after 48 hours incubation(1042ml/g to 521ml/g)

Another observation is the consistency of the methanol extract of *C alata* with MIC of 0.032mg/ml and total activity of 500ml/g after 24 hours and 48 hours incubation which is

similar to that of DCM extract of *R. vormitoria* (root) with MIC of 0.032mg/ml after 24 hours and 48hours but had a lower total activity of 354ml/g compared to that of methanol extract of *C.alata*.

Also, from the phytochemical screening result, *C.alata* is the only plant species that showed positive for saponin test and according to Yang et al. (2006) steroidal saponins have therapeutic potential for treating fungal infections. This suggest that some class of saponins do have a low toxicity and may become therapeutic agents with high therapeutic index (De Lucca *et al.,* 2006).

The rationale behind the choice of a model for the study of *Cryptococcus* is that the model should be standardized, reproducible, affordable, controllable with respect to severity, and have clear end point. To better understand pathogenesis and to identify fungal virulence-associated factors, complex infection models are indispensable. The most commonly used infection models for studying fungi pathogenesis are laboratory rodents, especially mice. These models are well characterized and have been critical for understanding host-pathogen interactions, as well as for developing better therapeutic approaches. Embryonated eggs can be infected via the CAM, the albumen, the yolk sack, or the allantoic cavity or by direct injection into the embryo or major blood vessels. In this study, eggs were infected via the CAM for the following reasons. (i) The CAM is a thin, translucent membrane consisting of two epithelial layers separated by loose connective tissue (Ribatti *et al.,* 2001). (ii) Pathogens applied to the outer layer of the CAM have to invade the outer epithelial layer to access nutrients (iii) Aseptic application of solutions onto the CAM is technically straightforward, and the required manipulation is well tolerated by the embryo (Vargas *et al.,* 2007).

Previous studies using chicken embryos as alternative hosts vary in several technical aspects, thus hampering direct comparison of obtained results. The CAM model has been successfully used for other fungi (Jacobsen *et al.,* 2011; 2010) However, this model has not

been used for *Cryptococcus*. Therefore the study sought to characterize this model by using *Cryptococcus gattii* and *Malassezia sympodialis* (ATCC42132). The choice of CAM model is due to the huge cost of maintaining murine models, also due to the need for high competence and ethical issues; there is need to seek alternative model that may suitably substitute murine model for *Cryptococcus gattii* pathogenicity.

Varying degree of mortality rates were observed for the four test strains. Mortality has been confirmed to be dose-dependent in *Candida* model (Jacobsen *et al*., 2011; Gow *et al*., 2003). Strains R272 and R265 were more viruelent than EJB18 in the embryonated egg model. In the murine model, Cheng et al.(2009) reported that R265 was more virulent that R272. Reasons for this variation could be due to mutation in any of the strains or loss of virulence in R265 due to multiple passage. However, this reasons were not validated by this study. A transient isolation of yeast was obtained from the brain for R272 and R265 but not for EJB18 and *M. sympodialis*. This may be because *C. gattii* affects more of the lungs than CNS involvement (Ngamskulrungroj *et al*., 2012)

Buffers and chemicals used in this study were not screened for potential endotoxin contamination. Hence deaths that have been attributed to the fungal species may not be entirely attributed to it. However, care was taken to ensure sterility and all chemicals and buffers were sterilized before use.

Therefore, we cannot completely exclude that endotoxin contaminations might have influenced results obtained in this study. However, all buffers used were tested in a control group of chicken embryos (PBS control) in each individual experiment.

This study therefore hypothesizes that damages or impairment of blood flow and nutrient competition are the most likely causes of embryonic death during *C. neoformans* infection model. This hypothesis needs to be tested.

Malassezia is able to exist in both yeast and mycelial forms, with the yeast being most commonly associated with normal skin(Ashbee and Evans 2002). Less emphasis has been placed on the study of the *Malassezia* and dermatophytes because they cause only superficial infections and are more amenable to topical treatment. Nevertheless, such infections are extremely common and can cause considerable distress to chronic sufferers.

Malassezia hyphae are not well studied because they are slow growing in vitro and require specialised growth media that contains a source of lipid. In vivo, hyphae are observed only in individuals with hyperactive sebaceous-gland activity, where the presence of excess sebum appears to be the inducer of morphogenesis (Ran *et al.,* 1993). Several groups have succeeded in inducing mycelial formation in vitro using a variety of media such as Cholesterol (Porro *et al.,* 1977), and through the use of synthetic media (Dorn and Roehnert 1977).

Embryonated chicken egg is a rich source of lipids and cholesterol (Milinsk *et al.,* 2003). The ability of embryonated egg to induce morphogenesis in *Malassezia sympodialis* may be due to the high content of lipid in the egg, hence the embryonated egg model may be suitable to stimulating hyphal growth in *Malassezia* and hence used in the study of the effect of hyphae in the pathogenesis of *Malassezia* in vivo.

A variety of non-vertebrate hosts have been used to investigate the virulence of *Cryptococcus*, these includes the use of amoebas like *Acanthamoeba castellanii* (Neilson *et al.,* 1978) *Dictyostelium discoideum* (Steenbergen *et al.,* 2003). These models may not be applied to the study of various species of *Cryptococcus* as they respond differently to these models. Furthermore, Jacobsen et al.(2011) in their study on the evaluation of CAM model for *Candida albicans* and *C. glabrata*, observed a difference in virulence, *C. glabrata* did not cause mortality in the CAM model although it was re-isolated from the CAM, hence making the CAM model unsuitable for the study of *C. glabrata*

The chicken model might yield false-negative results. However, the model still provides a suitable screening tool to determine the virulence of large numbers of mutants strains to identify attenuated strains for subsequent testing in murine models. Furthermore, if specialized animal facilities are not available, the chicken embryo model can be employed as alternative in vivo system.

Studies on *C. neoformans*'s enzymatic activity is useful not only to better understand its metabolism, but in particular to establish a possible relationship between its virulence and pathogenicity. Studies on this activity, together with genetic, serological and biochemical investigations, would be very useful to characterize different *C. neoformans* strains and to elucidate the epidemiology of cryptococcosis. Extracellular phospholipase activity was first described by Chen et al.(1997b) as a zone of precipitation due to susbtrate hydrolysis, surrounding colonies growing on egg-yolk agar medium, a rich source of phospholipid.

In vitro, strains of *C. neoformans* serotype A produces more phospholipases than serotype B and *C. gattii*) (Chen *et al.,* 1997b). Chen et al.(1997b) reported that the radial zone of inhibition around colonies, and hence phospholipase secretion correlates with virulence in mice. Phospholipase activity specifically seems to be more related to *in vitro C. neoformans* virulence (Vidotto *et al.,* 1996; Chen *et al.,* 1997b; Huérfano *et al.,* 2003). According to Vidotto et al.(2005) clinical samples isolated from AIDS patients are capable of producing more phospholipase than environmental ones, suggesting that enzyme production may be associated with host-invasion mechanisms. However, da Silva (2006) did not find any correlation between the production of phospholipase and virulence in mice study, this was also corroborated by a study by Santangelo et al. (2004) . Therefore it was proposed that this enzyme acts as a helping factor in the infection process, but with no single predominant effect. The importance of phospholipase production in *C. neoformans* virulence is not yet known (Ghannoum 2000). Additional studies will be essential to understand the importance

of production of this enzyme and the correlation between enzyme production and pathogenicity and virulence.

To evaluate the production of melanin and to optimize the use of Cowitch media, *Mucuna pruriens* was used to induce melanin in the environmental isolates. Gokulshankar et al. (2009) in their study proposed the use of *Mucuna pruriens* (Cowitch) seed as a media for *Cryptococcus* isolation because of its rich L-DOPA content. This study undertook to evaluate this claim. This is because a media that has the ability to induce melanin production in *Cryptococcus* is invaluable in the management of cryptococcocosis in Nigeria, moreso that Niger seed is not indigenous to Nigeria while cowitch is indigenous to Nigeria. Preparing the media as proposed by Gokulshankar et al. (2009) produced a final media that was rough and foamed a lot making autoclaving a problem, the gradual denaturing of the protein and filtration gave a fine medium that supported the growth of *Cryptococcus neoformans and C. gattii* .

This study therefore proposes an improved protocol of preparation of Cowitch agar. The optimised media is useful for isolation of *Cryptococcus neoformans* since it supports the growth of *Cryptococcus neoformans* and *C. gattii* and also stimulated the production of melanin-like substance in the two species. Melanin production by *C. neoformans* was first documented by Staib, who in 1962 documented brown cryptococcal colonies on agar containing *Guizota abyssinica* seed extracts(Staib 1962).Melanization remains a distinctive feature used for *Cryptococcus* diagnostic microbiology (Pulverer and Korth 1971).The concept of using differential media for isolation, identification and pigment production of *C. neoformans* is not new. But considering the simplicity of the preparation of cowitch seed agar and the availability of cowitch seeds in several parts of the world, this medium could be exploited for the isolation, identification differentiation of C. *neoformans*. The characteristic brown pigment on cowitch seed agar can be attributed to the presence of L-dopa in the cowitch seeds (Gokulshankar *et al*., 2009).

Cryptococcus neoformans var. *grubii* (serotype A) is a ubiquitous saprobic yeast. Although cryptococcosis can be caused by other species of *Cryptococcus* or any of the three serotypes of *C. neoformans* (A, D, or AD), most clinical and veterinary cases worldwide are caused by isolates of serotype A, which is also the most prevalent serotype among environmental samples(Litvintseva and Mitchell 2009). Strains of *Cryptococcus neoformans* isolated from the environment have been shown to be less lethal than the clinical strains(Litvintseva and Mitchell 2009). The Use of *Galleria mellonella* has been used as an established model for study of virulence of *Cryptococcus* (Mylonakis *et al.,* 2005), the model is easy and inexpensive. The low virulence of VNII may explain why it is rare in clinical settings. However, the clinical picture of the prevalent molecular type is not fully understood, hence further study to understand the prevalent molecular type would go a long way in clarifying the understanding of the virulence of VNII. Kaocharoen et al. (2012) reported a close retaionship between VNII ST43 with animals and humans. This study however, isolated VNII(ST43) from the environment.

Mating is ubiquitous throughout biology(Fu *et al.,* 2015). Sexual reproduction provides natural selection and adaptation of the organisms to environmental conditions by allowing benefical mutations to spread and by diluting deleterious mutation (Cerikçioğlu 2009) *Cryptococcus neoformans* has a bipolar mating system and can undergo robust a-α heterothallic mating(Fu *et al.,* 2015). Environmental and clinical strains of *C. neoformans* are predominantly the α mating type (Fu *et al.,* 2015). In sub- Saharan Africa, the ratio of the **a:** **α** mating type occur in a balanced nature (Litvintseva *et al.,* 2003). Furthermore, till date no report of *MATa* mating type has been isolated for *Cryptococcus neoformans* VNII. It is therefore hypothesized that in Sub -Saharan Africa, **a- α** mating drives the phenotypic and genotypic variations of *Cryptococcus neoformans* this is supported by the fact that in Africa, **a** isolates are present at a much higher frequency and population genetics analyses have

revealed hallmarks of mating likely to involve canonical bisexual reproduction (Litvintseva *et al*., 2003; 2006)

To prove this, mating was setup between *Cryptococcus neoformans* VNI *MATa*(KN99a) and *C. neoformans* VNII *MAT* α (EN28). The two species, belongs to the same variety and have 98% genomic similarity (Blake, R and Heitman, J, Personal communication). In this study, wide range of phenotypes were generated from the cross. Sun et al.(2014) in their study comparing **a- α** bisexual mating with **α- α** unisexual mating, the frequency of occurrence of diploids was in the ratio of 6:2 the low frequency of diploid occurrence in bisexual cross was also observed in this study. The low frequency of diploids (2/480) from this cross may be due to the genetic similarities between the two mating types. Previous studies have found that diploid/aneuploid progeny can also be readily produced at high frequency during hybridization between serotypes A and D *C. neoformans,* which is likely due to the elevated sequence divergence as well as the large number of chromosomal rearrangements that are present between the parental strains (Sun *et al*., 2014).

Intra-varietal mating produced offspring's that are fit and could withstand harsh environmental conditions. Defined *C. neoformans* virulence factors include melanization, survival at UV radiation and the ability to grow at high temperature, all of which confer survival advantages in both animal hosts and the environment (Boekhout *et al*., 2001). The ability to grow at high temperature (37ºC) enables human infection (Perfect 2006), production of melanin provides protection from toxic free radicals generated by host defenses during infection and from UV irradiation in the environment (Casadevall *et al*., 2000). F1 progenies from the cross between VNI and VNII were examined for sensitivity to UV irradiation, growth at high temperature (37ºC), UV radiation and melanization. Increased fitness was observed for the haploid progenies and for the diploids.

The genome of *Cryptococcus neoformans* is slightly larger(18.9Mbp) than that of *C. gattii* VGIIa (17Mbp) (Gillece *et al*., 2011). The G+C content of *C.neoformans* ST43 is similar to *C.neoformans* ST5(Day *et al*., 2017).

5.2 CONCLUSION

In this work, environmental isolates of *Cryptococcus neoformans* were obtained, the isolates were shown to belong to molecular type VNII a rare molecular typeand Mating type αA(Plate 4.3). The isolates were shown to be fertile when crossed with a compatible mating type KN99a. Evaluation of progenies from the cross with between KN99a and EN27 showed that intra-varietal mating occur within the VNII molecular type. This may be an alternative mating pathway for *C. neoformans* VNII. The study showed that the isolates are sensitive to fluconazole, itraconazole, flucytosine and voriconazole. Further evaluation of plants with antifungal effect, Acetone and methanol extracts of *Cassia alata* and dichloromethane extract of *Ranvoltia vormitoria* are potential source of antifungal against Cryptococcus (Table 4.4-4.5). Determination of the virulence of the *C. gattii* isolates in Chorio-allantoic membrane(CAM) of embryonated chicken egg, revealed that the model can be an alternative in studying the virulence of *C. gattii* (Figure 4.7). The study further corroborated previous studies that showed the VNI is more virulent than the VNII in a *Galleria* model (figure 4.10). The study proposes the use of Cowitch seed agar for the isolation of *Cryptococcus* since the media supported the growth of *Cryptococcus neoformans* and induced the production of melanin.

The genomic dataset reported in this study has been made publicly available in the Genbank database as a resource for researchers with the accession number CP025717-CP025731.The release of this assembly will be useful for the scientific community to initiate further molecular biology work related to this important fungal pathogen.

5.3 RECOMMENDATION

More studies are needed to further answer the question, what molecular type affects patients in Nigeria? Since only *C. neoformans* VNII was isolated from the environment. To answer this question, further studies are needed on clinical isolates to get a complete picture of the predominant molecular types in Nigeria. Also, with the variations in temperature sensitivities observed in the progeny of the cross between KN99a and EN28, further studies are needed to identify the Quantitative trait loci(QTL) that regulates temperature in *Cryptococcus*

Mucuna seed agar can be used for isolation and identification of *Cryptococcus* species in Nigeria in the absence of Niger seed agar.

5.4 CONTRIBUTIONS TO KNOWLEDGE

This work contributed to the knowledge of mycology of *Cryptococcus* species as follows:

1. First molecular typing of environmental isolates of *Cryptococcus neoformans* in Nigeria
2. Intra-varietal mating occurs in between *C .neoformans* VNI and *C .neoformans* VNII as an alternative pathway for mating for VNII genotype.
3. Establishment of Chrio-allantioc membrane(CAM) as a model for studying the virulence of *C. gattii*
4. Draft genome sequence of an uncommon Sequence type(ST43) of *C.neoformans* is available for comparative genome analysis with accession number CP025717-CP025731 (https://www.ncbi.nlm.nih.gov/nuccore?term=428229%5BBioProject%5D)

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APPENDIX A

SUPPLIMENTARY MATERIALS

Strain information of isolates of *C.neoformans* used for the phylogenetic study

Code	ORIGIN	TYPE OF SAMPLE	SOURCE	DATE	MATING TYPE	MOLECULAR TYPE	SEQUENCE TYPE	REF.
48-2323	Asia, Thailand, Bangkok	Clinical	BAL	2005	-	VNII	43	Kaochaoen 2013
A10	Asia, Thailand	Environmental	Nose	2005	-	VNII	43	Kaochaoen 2013
A11	Asia, Thailand	Environmental	Nose	2005	-	VNII	43	Kaochaoen 2013
A7 35-23	North America, North Carolina	Environmental	Pigeon excreta	-	Aα	VNII	43	Litvintseva 2006
c12	North America, North Carolina	Clinical	Lung	-	Aα	VNII	43	Litvintseva 2006
RKI05-0338	Europe, Germany, Berlin	Clinical	-	2005	Aα	VNII	43	Sanchini 2014
JS68	North America, Whashington state	Veterinary	-	2010	α	VNII	43	Singer 2014
IUM 13-0224	Europe, Italy, Catania	Cllnical	-	2013	Aα	VNII	43	Cogliati (Unpublished)
27	Africa, Jos, Nigeria	Environmental	Pigeon droppings	2013	Aα	VNII	43	Nnadi (This study)
28	Africa, Jos, Nigeria	Environmental	Pigeon droppings	2013	Aα	VNII	43	Nnadi (This study)
326	Africa, Jos, Nigeria	Environmental	Pigeon droppings	2013	Aα	VNII	43	Nnadi (This study)
25_291	Asia. India, Chandigarh	Clinical	CSF	2006	α	VNII	40	Khayhan 2013
c45	North America, North Carolina	Clinical	Sputum	2001	α	VNII	41	Litvintseva 2006
c44	North America, North Carolina	Clinical	CSF	2002	α	VNII	42	Litvintseva 2006
RKI05-0338	Europe, Germany, Berlin	Clinical	-	2005	Aα	VNII	43	Sanchini 2014
IUM 01-4726	Europe, Italy, Milano	Clinical	CSF	2001	α	VNII	96	Cogliati 2013
WM626	Oceania, Australia, Sydney	Clinical	CSF	1993	α	VNII	97	Meyer 2009
ug2472	Africa, Uganda	Clinical	CSF	2001	α	VNII	100	Litvintseva 2006
IUM 99-4427	Europe, Italy, Milano	Clinical	CSF	1999	α	VNII	107	Cogliati 2013
A5	Asia, Thailand	Veterinary	Nose	2005	-	VNII	172	Kaochaoen 2013
bt131	Africa, Botswana	Clinical	CSF	2001	**a**	VNB	8	Litvintseva 2006
bt33	Africa, Botswana	Clinical	CSF	2000	α	VNB	9	Litvintseva 2006
bt65	Africa, Botswana	Clinical	CSF	2000	**a**	VNB	10	Litvintseva 2006
bt76	Africa, Botswana	Clinical	CSF	2000	α	VNB	11	Litvintseva 2006
IUM 97-4515	Europe, Italy, Bergamo	Clinical	CSF	1997	α	VNB	102	Cogliati 2013
bt206	Africa, Botswana	Clinical	CSF	2002	**a**	VNB	16	Litvintseva 2006
bt84	Africa, Botswana	Clinical	CSF	2001	α	VNB	17	Litvintseva 2006
bt88	Africa, Botswana	Clinical	CSF	2001	**a**	VNB	28	Litvintseva 2006
RKI04-0202	Europe, Germany, Göttingen	Clinical	csf	2004	Aα	VNI	2	Sanchini 2014
268	Asia, Indonesia, Jakarta	Clinical	CSF	2006	α	VNI	4	Khayhan 2013
K1	Asia, South Korea, Gyeonggi	Clinical	Urine	1994	α	VNI	5	Choi 2010
HK_01	Asia, China, Hong Kong	Clinical	-	-	α	VNI	6	Khayhan 2013
IUM 00-5361	Europe, Italy, Bergamo	Clinical	CSF	2000	α	VNI	23	Cogliati 2013
1605202443	Asia, Qatar, Doha	Clinical	CSF	2005	α	VNI	31	Khayhan 2013
c27	North America, North Carolina	Clinical	CSF	2001	α	VNI	32	Litvintseva 2006
NIIDCr0001	Asia, Japan, Hokkaido	Clinical	-	2011	α	VNI	46	Umeyama 2013
25_104	Asia. India, Chandigarh	Clinical	CSF	1999	α	VNI	77	Khayhan 2013
UgCl001-1	Africa, Uganda, Kampala	Clinical	CSF	2006-2009	-	VNI	93	Wiesner 2012

APPENDIX B

MEDIA AND SOLUTIONS

MEDIA

1. **Sunflower seed Agar**

Dried sunflower seeds were obtained from the local market. Pulverized seeds (50g) were extracted in hot water by boiling for 30 minutes. This was filtered through muslin and to this the other ingredients(Glucose 10g/l Agar 20g/l)were added. The pH was adjusted and the volume made up to 1000ml. This medium was solidified with agar (1.5%) before autoclaving. Chloramphenicol (0.05mg/mL) was amended to the medium before dispensing into plates.

2. **Egg Yolk Agar**

The egg yolk medium consisted of 13.0 g Sabouraud's dextrose agar (SDA), 11.7 g NaCl, 0.11 g CaCl2 and 10% sterile egg yolk (all in 184 ml distilled water).First, the components without the egg yolk were mixed and sterilized, then the egg yolk was centrifuged at 500 g for 10 min at room temperature and 20 ml of the supernatant was added to the sterilized medium.

3. **Christensen Urea Agar**

The urea agar base was weighed and , sterilized by autoclaving at 121oC for 15 minutes. To the cooled medium added 5 ml of filter sterilized urea solution (40%). The medium were allowed to solidify in a slanting position in such a way to get half inch butt and one inch slant in the test tube

4. V8 Media

V8 media consists of 200 ml of V8 Juice 3.0g of CaCO, Agar 5.0 g and Tap water to 1.0 L . The pH was Adjusted pH to 7.2 and autoclave at 121°C for 15 minutes.

5. Yeast Dextrose Agar

2% yeast extract, 1% peptone, 2% dextrose the composition was and autoclave at 121°C for 15 minutes.

Solutions

FACS Solution

A. NS Buffer(1L stock)

1. Water	900ml
2. Tris-HCL(1M,Ph 7.5)	10ml
3. EDTA(0.5M)	2ml
4. Sucrose	85.6g
5. $Mg.Cl_2.6(H_2O)$	$0.2033(10^{-3}M)$
6. $CaCl_2.2(H_2O)$	$0.0147g(10^{-4}M)$
7. $ZnCl_2$	$0.0136\ (10^{-4}M)$

NS-RNase-P1(200μl)

1. NS Buffer	180 μl
2. RNase A(10mg/ml)	14 μl
3. Propidium iodide(1mg/ml)	6 μl

Tris-P1(500 μl)

Tris-HCL(IM,Ph 7.5)	482 μl
Propidium iodide(1mg/ml)	18 μl

6. Periodic acid-Schiff (PAS) stain

1. 1% Periodic acid (50% vol.)

Periodic acid 2 ml , Distilled water 98 ml

2. Schiff's reagent:

Basic Fuchsin (C.I. 42500) 1 g, Potassium metabisulphite 2 g, Distilled water 200 ml, HCl concentrate 2 ml, Deactivated Charcoal 1-2 g

To a boiled distilled water, basic fuchsin was added slowly, mixed and cool to 50°C Potassium metabisulphite was then added. This was then mixed and allowed to cool to room temperature before adding HCl. This was Kept in the dark overnight for bleaching to occur. Finally, charcoal and filtered through coarse filter paper, then fine filter paper. This was then stored in the fridge.

7. Receipe for Murashige and Skoog (MS) medium preparation:

Chaoyang Xue(Xue *et al*., 2007)

1. To Prepare 10 x MS basal salt mixture

The whole bottle of Salt mixture powder was dissolved into 1000 mL dH2O and stirred to mix (will not completely dissolve) this was aliquoted into 50 mL conical tubes and frozen down in -20°C. 100 mL was used for every 1000 mL MS medium.

2. Preparation of 1000 x MS Vitamin powder

The whole bottle of Vitamin powder dissolved into 100 mL dH2O and filtered through. This was Kept in 4°C.

3. Preparation of MS medium

In a 2 L flask, 100 mL 10 x Salt mixture was added and 900 mL dH2O the pH was adjusted to 5.8, 16 g bacterial agar was added and then autoclaved. At 45 •C 1 mL 1000 x Vitamin mixture into was added to the autoclaved MS medium, stired to mix and pour plates.

Appendix C

Preservation of Isolates

Water Culture Technique

1. Two (2) ml of sterile distilled water were pipetted into small screw cap bottles and sterilized by autoclaving.
2. Two (2) or 3 colonies of yeast culture were aseptically transferred into the screw capped bottle.
3. This was stored at room temperature.

APPENDIX D

Journal de Mycologie Médicale (2016) xxx, xxx—xxx

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ORIGINAL ARTICLE/ARTICLE ORIGINAL

Molecular characterization of environmental *Cryptococcus neoformans* VNII isolates in Jos, Plateau State, Nigeria

Caractérisation moléculaire de souches de Cryptococcus neoformans *VNII isolés dans l'environnement à Jos, état du Plateau, Nigeria*

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Received 28 January 2016; received in revised form 22 March 2016; accepted 6 April 2016

KEYWORDS
Cryptococcus neoformans;
Nigeria;
Environment;
Mating types;
Molecular types

Summary *Cryptococcus neoformans* and *Cryptococcus gattii* are encapsulated yeasts able to cause fatal neurological infections in both human and other mammals. Cryptococcosis is the most common fungal *infection* of the central nervous system and has a huge burden in sub-Saharan Africa and South East Asia. Bird excreta are considered an environmental reservoir for *C. neoformans* in urban areas, therefore a study aimed at isolating and characterizing this yeast is important in disease management. In this study, one hundred samples of pigeon droppings were collected in Jos, Plateau State, Nigeria. *C. neoformans* was isolated from three samples and initially identified using standard phenotypic and biochemical tests. Molecular analysis revealed

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http://dx.doi.org/10.1016/j.mycmed.2016.04.001

Please cite this article in press as: Nnadi NE, et al. Molecular characterization of environmental *Cryptococcus neoformans* VNII isolates in Jos, Plateau State, Nigeria. Journal De Mycologie Médicale (2016), http://dx.doi.org/10.1016/j.mycmed.2016.04.001

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Abstract Book

parapsilosis isolates recovered from hands (5/7; ~71%) showed 4 unrelated genotypes whereas 2 different genotypes were obtained from remaining blood isolates. These latter genotypes were not found among those colonizing the hands of hospital personnel or environments. Sixteen environmental strains were genetically quite similar showing seven different but close related genotypes.
Conclusions: This study confirms that candidaemia in hospitalized patients is caused predominantly by *C. parapsilosis* strains colonizing various hospital surfaces including air, medical devices and hands of healthcare workers and therefore the implementation of surveillance programmes is imperative to prevent the spread of nosocomial fungal infections.

This work was funded by the Italian Ministry of Health (project code: GR-2011-02347606).

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DRAFT GENOME SEQUENCE OF A NIGERIAN *CRYPTOCOCCUS NEOFORMANS* STRAIN BELONGING TO AN UNCOMMON MULTILOCUS SEQUENCE TYPE (MLST-ST43)

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Introduction: *Cryptococcosis* is an infection of the central nervous system which affects both immunocompromised and healthy individuals. This disease is caused by members of the *Cryptococcus neoformans* and *C. gattii* species complexes which are, genetically, highly divergent and have been previously subdivided into seven haploid (termed VN and VG) and four hybrid genotypes.
C neoformans VNII genotype is globally distributed but it has a low prevalence compared to the high occurrence of the VNI type. A recent study showed that the VNII type is widely distributed in the Nigerian environment, a country where more than 20% of HIV infected patients show a positive cryptococcal antigenemia. Multilocus analysis of Nigerian *C neoformans* VNII isolates revealed that they belong to a uncommon MLST type ST43 which has only been reported from Thailand, Japan, North Carolina, Washington states and Germany. Here, we report the first draft genome assembly of a rare MLST type (ST43) of *C. neoformans* strain (VNII genotype; mating type α), isolated in Nigeria, with the aim to facilitate future comparative studies for better understand the basic biology and the evolution of this important human fungal pathogen.
Materials and Methods: The whole genome of the Nigerian *C. neoformans* EN28 strain was paired-end (2x100 bp) sequenced using the Illumina HiSeq

2500 platform. High-quality genomic DNA of the EN28 strain was used to generate a paired-end library with insert sizes of approximately 300 bp. Before assembling, raw reads were processed using Trimmomatic to remove adapters and sequences with low Phred-scores (cutoff: ≥ 30). IMR-DENOM program was used for assembling the whole fungal genome using the following sequence GCA_000149245.3 as reference. Protein-encoding genes and tRNAs were predicted by AUGUSTUS and tRNAscan-SE programs respectively.

Results: The genome of the *C. neoformans* EN28 strain was assembled in 15 chromosomes (14 nuclear and 1 mitochondrial) covering over 18,9 Mbp (G+C: 48,19%). A total of 261 putative tRNAs and over 7,849 protein-encoding genes were identified in this fungal genome.

Conclusions: The genomic dataset reported in this study will be made publicly available in the Genbank database as a resource for researchers. The release of this assembly will be useful for the scientific community to initiate further molecular biology work related to this important fungal pathogen.

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ARE WE READY FOR *CANDIDA AURIS* CLONES ATTACK? FIRST STEPS TOWARDS IDENTIFICATION AND RESISTANCE DETECTION BY MALDI-TOF MS

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Multidrug resistant clonal strains of *Candida auris* have been isolated in the last five years in many countries. The emergence of *C. auris* is alarming as this yeast, very recently implicated in cases of nosocomial fungemia and deep seated infections, exhibits resistance to azoles, amphotericin B and echinocandins, thus limiting the options of choice for the antifungal treatment and leading to clinical failure for the patients. Therefore, an accurate and rapid identification at the species-level and differentiation between susceptible and resistant clones to antifungals drugs is urgently needed.

Commercial routine systems for yeasts identification commonly misidentify these recently emerged clones and mass spectrometry systems databases lack in *C. auris* reference profiles. The aim of the present study has been the implementation of the Bruker MALDI-TOF MS database with *C. auris* mass profiles and the development of a fast and reproducible assay able to rapidly detect *C. auris* resistance to fluconazole (FLU) and anidulafungin (AFG).

All the mass measurements below mentioned were performed with a Microflex LT mass spectrometer. Briefly, protein extracts from a panel of *C. auris* clones, isolated from an Indian outbreak, have been obtained both with fast formic acid and long ethanol/formic acid extraction procedures. After the MSPs creation, a score-oriented dendrogram was generated from hierarchical cluster analysis using the integrated statistical tool of the Biotyper 3.1 software, including C. lusitaniae, C. famata, C. guillermondii and C. parapsilosis isolates.

To detect *C. auris* resistance to FLU and AFG a three hours incubation antifungal susceptibility test (AFST) was developed, after preliminary experiments aimed to find the breakpoint and