The epidemiology and antifungal sensitivity of clinical *Cryptococcus neoformans* and *Cryptococcus gattii* isolates from Bloemfontein, South Africa

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Submitted in accordance with the requirements for the degree

Magister **S**cientiae

in the

Department of Microbial, Biochemical and Food Biotechnology

Faculty of Natural and Agricultural Sciences

University of the Free State

Bloemfontein

South Africa

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June 2013

ACKNOWLEDGEMENTS

I wish to thank the following people, who; in some way contributed to the successful completion of this dissertation. In addition, everyone else in-between, whom because of space limitation, I may have omitted. To all of you, I'm eternally grateful.

Professional acknowledgements:

- **Dr. O.M. Sebolai,** for accepting me into his group, for his excellent supervision and mentorship and interest in my overall development. Thank you for teaching me how to be a critical researcher. It is much appreciated.
- **Prof. C.H. Pohl,** for her friendship and support. Special thanks for all the thought-provoking discussions. I have learned a lot from you.
- **Prof. J.L.F. Kock,** for his invaluable comments and immense passion for research, which is truly inspiring.
- **Prof. J. Albertyn,** for his encouragement and support and for sharing his immense molecular studies knowledge.
- **Prof. M.S. Smit,** thank you for allowing me to use your facilities.
- **Mr. S. Collett,** for preparation of figures.
- **Mrs. A. van Wyk,** for her friendship and motherly love. Thank you for also organising all the wonderful retreats.

- **My fellow students in laboratories 28 and 49,** for making the laboratory a convivial place to work. Thank you for your support and for sharing in this part of my journey.
- **My friends,** thanks a lot for your support and friendship.
- **National Research Foundation,** thank you for granting me with the Innovation Master's Scholarship towards my studies.

Personal acknowledgements:

- **My sisters, brothers and their families,** for their unflagging love and support always. You are simply the best.
- **My parents,** your unfailing love and encouragement made me who I am. I love you so much.
- **My husband: Abiodun Ogundeji, and children: Demilade and Tomisin,** I cannot ask for more from God as you are simply perfect, thank you for all your love and support. I am so blessed and honoured to have you in my life.
- **The Almighty God,** thank You for the gift of life and for Your spirit, which strengthens me always, more especially when I doubted myself during the course of my studies. You are awesome in my life!

DECLARATION

I hereby declare the work presented in the dissertation is as a result of my own independent investigations. In addition, I declare this dissertation has not been submitted, in full or part, to another institution for the granting of a masters degree. The successful completion of the dissertation has been made possible by a joint research grant from the National Research Foundation and the University of the Free State. There are no competing financial interests.

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Friday, September 13, 2013

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ETHICAL CONSIDERATION

All the studies presented in the dissertation have been ethically approved by the University of the Free State's Ethics Committee, Application No. ETOVS 173. Further approval was sought and obtained from Dr. S. Kabane, who is the Head of the Department of Health in the Free State Province in the Republic of South Africa.

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Note: The dissertation consists of different chapters, some in manuscript format. As a result, repetition of some information could not be avoided. The dissertation is written according to the reference style prescribed by the African Journal of Microbiology Research.

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DISSERTATION OVERVIEW

In nature, where over time the environment has constantly been changing, microbes have had to evolve (driven either by mutations, genetic exchanges, even epigenetics) in order to survive and flourish. Therefore, given the right conditions (i.e. opportunism; exploiting the host organism's weakened immune system), microbes can adapt and otherwise non-pathogenic microbes can become pathogenic while others can cross the species barrier to find new host species. The appearance of the human immunodeficiency virus (HIV) in the 1980s has given "old" infectious diseases such as cryptococcosis a "new" lease on life. Today, cryptococcosis is a significant cause of worldwide morbidity and mortality, more so, in Sub Saharan Africa. As a result, the causative agents of cryptococcosis, *Cryptococcus* (*Cr.*) *neoformans* and *Cryptococcus* (*Cr*)*. gattii*, have received much attention and continue to be intensely researched. This dissertation is therefore, also an attempt at advancing the current knowledge of *Cr. neoformans* and *Cr. gattii*.

The dissertation is not structured in a classical way; and as such, it is composed of a literature review section (Chapter 1) and two Research Chapters (Chapter 2 and 3). A dissertation summary section is also included at the end, which summarises all the work presented. A brief description of each Research Chapter is given below:

Chapter 2 focuses on the epidemiology of *Cr. neoformans* and *Cr. gattii*. This chapter is further divided into two sub sections, each individually dedicated to an aspect of cryptococcal epidemiology:

Sub section 2.1. The sub section examines the burden of cryptococcosis in

Bloemfontein, South Africa. It's hoped this work will also assist local health authorities and specifically, Dr. S. Kabane, Head of the Department of Health in the Free State Province and his team, in their planning and management of cryptococcosis,

Sub section 2.2. The sub section concentrates on molecular surveillance and identification of *Cryptococcus* in Bloemfontein, and proposes a simple PCR based method for the routine diagnosis of *Cr. neoformans* and *Cr. gattii*,

Chapter 3 evaluates the response of *Cr. neoformans* and *Cr. gattii* towards antimitochondrial drugs. It is hoped this study will build on the current literature on new antifungals and further facilitate the development of better antifungal strategies to better control human infectious diseases.

CHAPTER 1

LITERATURE REVIEW

1.1 MOTIVATION

Cryptococcus (*Cr.*) *neoformans* and *Cryptococcus* (*Cr*). *gattii* constitute a species complex of non-fermenting basidiomycetous yeasts (Kwon-Chung, 2011). More importantly, these yeasts are the causative or etiological agents of cryptococcosis, an often deadly inflammatory condition affecting the central nervous system (Bose et al., 2003; Bicanic and Harrison, 2004; Lin and Heitman, 2006). *Cryptococcus neoformans* is an opportunistic pathogen mainly infecting persons with an impaired cell-mediated immunity, while *Cr. gattii* is a true pathogen that can cause infectious diseases in apparently immunocompetent persons. At present, mechanisms underlying this host specificity are not yet clearly understood (Douwel, 2010).

These agents have a number of virulence factors, of these; the capsule is the most important (Buchanan and Murphy, 1998). The capsule is documented to alter the immune response in favour of the pathogen leading to disease development (Yauch et al., 2006). The discovery of oxylipins, documented elsewhere to play a role in microbial pathogenesis (Nigam et al., 1999; Ciccoli et al., 2005), on capsules of *Cr. neoformans*, has further expanded our knowledge of the known spectrum of biologically active compounds associated with this main virulence factor (Sebolai et al., 2007, 2008a).

Cryptococcosis was regarded as a rare disease during the course of 19th century. However, the appearance of human immunodeficiency virus (HV) in the 1980s brought an increase in the number of cryptococcosis cases (Levitz and Boekhout, 2006). An appreciation of the scope and extent of cryptococcosis is particularly important for

health authorities to effectively plan prevention and management strategies as well inform appropriate resource allocation. According to the Centers of Disease Control and Prevention (CDC), almost one million new cases are estimated each year (Park et al., 2009), resulting in over 600 000 deaths. Further to this, Sub Saharan Africa is reported to carry the highest burden of infections (Park et al., 2009). Despite the seriousness of this infectious disease, its actual counts continue to be under appreciated. This under appreciation may in part be due to insensitive methods that are used in the routine diagnosis of cryptococcosis.

At present, serological-based techniques and Indian ink staining are the two widely employed methods in clinical settings to diagnose suspected cases (Saha et al., 2009; Sidrim et al., 2010; Wan et al., 2011). However, these techniques are often inconsistent i.e. low sensitivity (Indian ink with 80%) and imprecise interpretation of tests due to subjectivity in reading borderline results (Casadevall and Perfect, 1998; Sidrim et al., 2010). Sidrim et al. also point out that immunological tests do often yield false-positive results due to cross-reactions with rheumatoid factors, *Trichosporon* spp., and Gram-negative rod contamination (Sidrim et al. 2010). More importantly, they cannot disclose the identity of the responsible serotype causing the infection (Ito-Kuwa et al., 2007). Such information is important in understanding, among others, the geography of isolates and, for instance, their response to drug treatment. Nonetheless, advances in molecular systematics have greatly assisted in resolving the specificity and sensitivity issues around identification (Kurtzman and Robnett, 1998; Scorzetti et al., 2002; Paschoal et al., 2004; Speers, 2006; Sidrim et al., 2010). To this end, a simple

molecular-based method that can easily be optimised for the routine identification of *Cr*. *neoformans* and *Cr. gattii*, particularly in resource-limited settings, would hold an obvious advantage.

Without the prospect of cryptococcosis treatment, life expectancy is usually less than three months in immunocompromised persons (Park et al., 2009). For many years, amphotericin B has been the drug of choice. However, its severe side effects limit its clinical application (Ghannoum and Rice, 1999). In addition, the widespread usage of drugs such as fluconazole as curative agents as well as drug non-compliance by patients, has led to a marked increase in drug resistance (Ghannoum and Rice, 1999). The challenge, therefore, remains to find effective and low cost drugs to control fungal growth. In the dissertation, consideration is given to non-traditional antimicrobial agents as possible alternatives. It is suggested in literature that antimitochondrial drugs may also possess an antimicrobial activity (Kock et al., 2007), particularly when applied against non-fermenting microbes (Leeuw et al., 2007; Sebolai et al., 2008b; Davies et al., 2009). The antimitochondrials that are of interest here are those, which specifically target mitochondrial-energy generation processes in non-fermenting microbes.

1.2 THE CLASSIFICATION HISTORY OF *CRYPTOCOCCUS NEOFORMANS*

The term *Cryptococcus* was first used by Kutzing in 1833 to describe an organism, now classified as algae, isolated from dried scrapings from a window sill (Fonseca et al., 2011). However, it was not until 1894, that *Cr*. *neoformans*, the currently recognised

basidiomycetous yeast, was first identified. From a fruit juice, the Italian, Sanfelice, made the first environmental isolation of *Cr. neoformans* and accordingly named the isolate *Saccharomyces hominis* (Casavedall and Perfect, 1998). In the same year, two German physicians, Busse and Buske, gave the first description of *Cr. neoformans* as a human pathogen, after isolating the organism from a tibia lesion (Casadevall and Perfect, 1998). Vuilleman proposed in 1901, that the genus *Cryptococcus* should be reserved to accommodate pathogenic yeasts (Fonseca et al., 2011), and as such, classified *Saccharomyces hominis* under *Cryptococcus as Cr. neoformans*. Over time, *Cr. neoformans* has been assigned to different genera (Chayakulkeeree and Perfect, 2008), since early taxonomists inconsistently classified this organism based on how it appeared, and which molecules it did or did not ferment and assimilate.

Prior to the 1950s, it was assumed that cryptococcosis was caused by an individual, homogenous species, *Cr. neoformans*. However, it was later revealed that the species could further be classified into four serotypes, A, B, C, D and a hybrid strain, AD, based on molecular and immunological properties of the capsule (Chen et al., 2010), thus; forming a species complex. In the 1970s, Kwon-Chung was the first person to observe heterothallism in *Cr. neoformans*, when she noted compatible basidiospores giving rise to the yeast phases of opposite mating types (Figure 1) (Kwon-Chung, 1975, 1976). As a result, Kwon-Chung named the teleomorphic state *Filobasidiella*, and further separated the species into two varietal forms viz. *Filobasidiella neoformans* var. *neoformans* and *Filobasidiella neoformans* var. *bacillispora,* based on basidiospore

morphological differences. This delineation was subsequently supported by molecular evidence (Litvintseva et al., 2006).

Over time, advanced molecular methods assessing the intra-specific genetic diversity have been developed and applied to further characterise the species complex (Sidrim et al., 2010). These methods include, among others, amplified fragment length polymorphisms and multi-locus sequence typing (Bovers et al., 2008; Litvintseva et al., 2006; Boekhout et al., 2001). While these methods are important in understanding the epidemiology, drug response and pathogenesis of strains, their application in clinical diagnosis may be limited by: (1) costs involved and (2) being laborious; and as such, cannot be optimised for routine use particularly, in resource limited-settings (Sidrim et al., 2010).

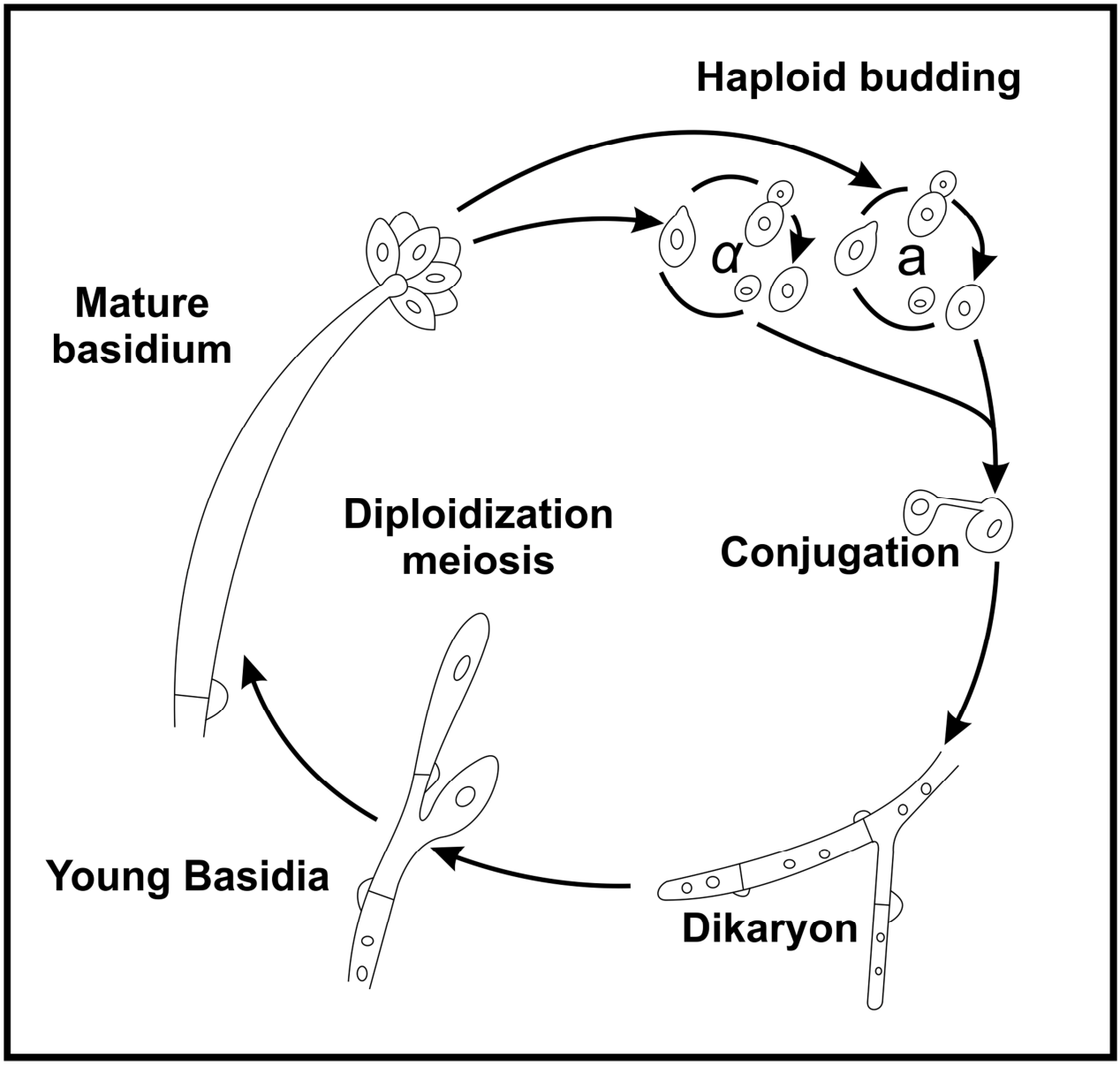

Figure 1. A pictogram illustrating two compatible mating types i.e. α and a, coming together to form a typical basidiomycetous dikaryotic hyphae. This is then followed by the rejuvenation of genes and later, the restoration of the yeast phases. The pictogram is reproduced from Kurtzman et al. (2011).

1.3 CURRENT CLASSIFICATION

At present, members belonging to the *Cr. neoformans* species complex are classified as follows, based on the latest yeast monograph by Kurtzman et al. (2011):

Kingdom:	Fungi
Phylum:	Basidiomycota
Class:	Tremellomycetes
Order:	Tremellales
Family:	Tremellaceae
Genus:	*Cryptococcus*
Type species:	*Cryptococcus neoformans*

Traditionally, members belonging to the *Cr. neoformans* species complex have been classified into three varieties and four non-hybrid serotypes, based on genetic and capsular epitopes (Fraser et al., 2003; Chen et al., 2010). These are *Cr. neoformans* var. *grubii* (serotype A) (teleomorphic state: *Filobasidiella neoformans* var*. grubii*), *Cr. neoformans* var*. gattii* (serotype B and C) (teleomorphic state: *Filobasidiella neoformans* var*. bacillispora*) and *Cr. neoformans* var*. neoformans* (serotype D) (teleomorphic state: *Filobasidiella neoformans* var*. neoformans*) (Table 1). In addition, a hybrid strain, AD (probably a diploid or aneuploid organism), has also been identified (Enache-Angoulvant et al., 2007). Today, *Cr. gattii* is recognised as a separate individual species, distinct from *Cr*. *neoformans*, based on biochemistry, ecology, genetics and phylogenetic diversity (Kwon-Chung and Varma, 2006; Kurtzman et al., 2011).

Perhaps, in future, as more research data become available, there may be a proposal to further separate *Cr. neoformans* var. *grubii* (serotype A) from *Cr. neoformans* var. *neoformans* (serotype D). To this end, other scholars (Coenjaerts, 2006; Lin and Heitman, 2006) are championing a discourse for the establishment of up to eight distinct species within the *Cr*. *neoformans* species complex. However, Cooper (2011) in the latest yeast monograph by Kurtzman et al. (2011) cautions that, …"taken alone, the genotypic variation supports the contention that the complex is in the process of evolving new species. However, what is not clear is at which point does this genotypic variation define the emergence of separate species, particularly if phenetic and biologic information is not considered"… Therefore, it will be interesting to follow developments and attempts to further define and characterise the *Cr. neoformans* species complex.

Table 1

Classification of fungal species composing the *Cryptococcus neoformans* species complex (Day, 2004; Chayakulkeeree and Perfect, 2008; Meyer and Trilles, 2010).

Species	**Variety**	**Serotype**		**Ecology**	**Source**
		Non hybrid	**Hybrid**		
Cr. neoformans	var. *grubii*	A	-	Worldwide	Pigeon excreta; Soil
	var. *gattii*	B	-	Mainly tropics	Eucalyptus trees
	var. *gattii*	C	-	Mainly tropics	Eucalyptus trees
	var. *neoformans*	D	-	Worldwide	Pigeon excreta; Soil
	var. *grubii* / var. *neoformans*	-	AD	Unknown	Unknown

1.4 *CRYPTOCOCCUS NEOFORMANS*

Cryptococcus neoformans isolates are mainly either serotype A or serotype D, and they have always been described as varieties and not as separate species (Chayakulkeeree and Perfect, 2008; Kwon-Chung, 2011). Recently, a hybrid strain, serotype AD, has also been described. In general, when cultivated on 2% malt agar, *Cr. neoformans* colonies appear to be white to cream in colour, and under the microscope, yeast cells are globose to ovoid in shape, and are between 2.5 µm to 10 µm in diameter (Kwon-Chung, 2011). Heterothallism is usually observed when compatible mating types are cultivated together in a nitrogen-poor mycological agar media. To this end, V8 juice can successfully be employed to induce formation of basidial structures (Kwon-Chung et al., 1982). *Cryptococcus neoformans* is not known to ferment any sugars, thus this species is greatly dependent on actively respiring mitochondria to produce cellular energy (Kwon-Chung, 2011).

Even though the first environmental isolation of *Cr. neoformans* was made from a fruit juice, this yeast has consistently been isolated from bird droppings (suggested natural habitat) and soil contaminated with droppings, where they also, possibly, interact with soil amoeba (Steenbergen et al., 2001; Hull and Heitman, 2002). *Cryptococcus neoformans* is documented to have a worldwide ecological distribution - possibly transported over long distances by birds (Mitchell and Perfect, 1995; Douwel, 2010). Serotype A (varietal form *grubii*) in particular, is universal in distribution; while serotype D (varietal form *neoformans*), is commonly found in central Europe (Viviani et al., 2006). In addition, serotype A is the main serotype of *Cr. neoformans* that is frequently isolated

from infected patients, particularly from those with an impaired cell-mediated immunity; possibly as a result of AIDS (Day, 2004), accounting for about 95 % of all *Cr. neoformans* infections (Hull and Heitman, 2002).

1.5 *CRYPTOCOCCUS GATTII*

Growth of *Cr. gattii* on malt extract agar (with 2% glucose) is similar to that of *Cr. neoformans* with respect to colour and shape. However, cells also appear to be highly mucoid, at times periform, marginally smaller (2.5 µm to 8 µm; diameter) and are surrounded by a thick polysaccharide capsule (Figure 2) (Kwon-Chung, 2011). When cultivated on canavanine-glycine-bromothymol blue (CGB) agar (3 to 5 days), colonies turn the media blue due to cells hydrolysing glycine (a trait not present in *Cr. neoformans*). Therefore, CGB media can be employed to distinguish *Cr. gattii* strains from *Cr. neoformans* although the test is not ideal as a point-of-care test (due to a long cultivation period before reading the test results). Moreover, even though the life cycle of *Cr. gattii* is similar to that of *Cr. neoformans*, the respective basidiospores are different when comparing size and morphology (Kwon-Chung, 2011). Furthermore, as expected and consistent with the genus *Cryptococcus*, *Cr*. *gattii* strains also lack fermentation ability.

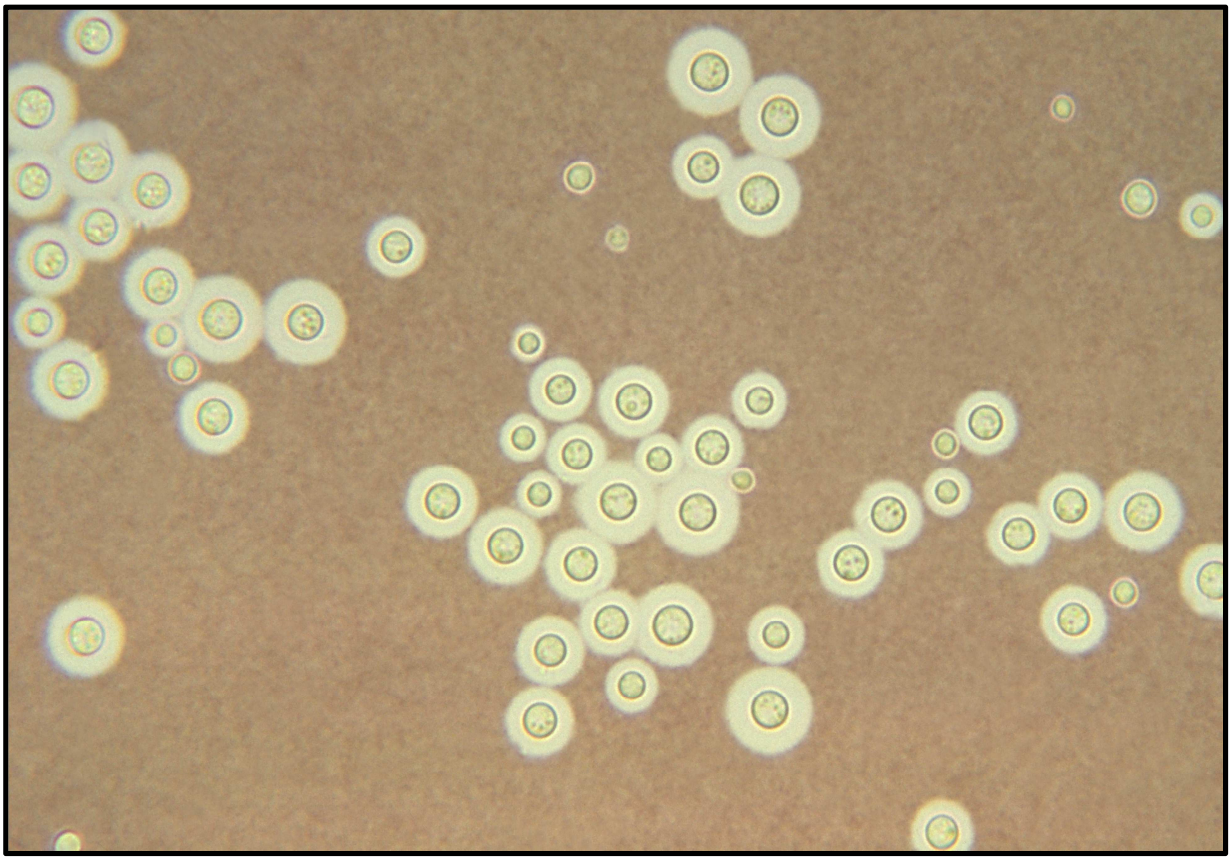

Figure 2. An Indian ink preparation. The pictogram depicts a characteristic polysaccharide layer of an encapsulated *Cr. neoformans* strain. The pictogram was obtained from Wikipedia. The image is in the public domain. Pictogram: Credit to CDC/ Dr. Leanor Haley.

Cryptococcus gattii isolates are either serotype B or serotype C based on capsular immunological epitopes (Kwon-Chung, 2011), and are regarded to constitute a singular varietal form. *Cryptococcus gattii* strains are frequently isolated from leaves and flowers of river red gumtree (eucalyptus; which is the presumed natural habitat) (Ellis and Pfeiffer, 1990), and thought to be mainly limited to tropical and subtropical regions of the world (Day, 2004). However, more recently, an unusual outbreak of *Cr. gattii* in a

temperate region of the world was recorded (Kidd et al., 2004). Interestingly, the concerned patients had no travel record to the tropics. The origin of the outbreak strains could either be traced to the US or Australia following molecular typing (Fraser et al., 2005; Kidd et al., 2004, 2005). Systematic sampling of environmental sources further indicated that the distribution of strains could be a result of human activity (Kidd et al., 2007). *Cryptococcus gattii* is documented to be a true or primary pathogen, capable to establish infectious diseases in seemingly immunocompetent persons (Chayakulkeeree and Perfect, 2008). Interestingly, *Cr. gattii* has rarely been reported to cause infections in AIDS patients even in the tropics, where it is presumed to be more prevalent (Kwon-Chung, 2011). Because of its close relationship to *Cr. neoformans*, *Cr. gattii* infections are often wrongly diagnosed as *Cr. neoformans* infections, which can then affect the course of treatment - as levels of drug susceptibility varies between *Cr. neoformans* and *Cr. gattii*.

1.6 OTHER PATHOGENIC *CRYPTOCOCCUS* SPECIES

Traditionally, the genus *Cryptococcus* has been regarded to host non-pathogenic yeast species (Chen et al., 2001). However, cases of non-*neoformans*/*gattii* cryptococcosis have increasingly been reported over the years (Pedroso et al., 2009), with species such as *Cr. albidus* and *Cr. laurentii* causing the majority (80 %) of such infectious diseases (Khawcharoenporn et al., 2007). *Cryptococcus albidus* is a saprophyte found the world over (Fonseca et al., 2011). In 2005, Labrecque and co-workers reported that *Cr. albidus* causes ocular and systemic disease in immunocompromised patients,

including AIDS patients. *Cryptococcus laurentii* is also a saprophyte (Fonseca et al., 2011), and like *Cr. albidus*, it is rarely a human pathogen. However, it has been documented to cause pulmonary and cutaneous infections in humans (Ajesh and Sreejith, 2012). *Cryptococcus laurentii* also shares some phenotypic characteristics with *Cr. neoformans* and *Cr. gattii*, for instance, the ability to produce melanin (Fonseca et al., 2011). Interestingly, *Cr. adeliens*, a species found mainly in Antarctica, has also been found in clinical samples (Rimek et al., 2004), and its proposed to also cause pathology.

1.7 INFECTION AND THE CAPSULE

Depending on the status of the host's immune system, cryptococcosis can be a potentially fatal infectious disease caused by fungal species constituting the *Cr. neoformans* species complex (Table 1) (Day, 2004; Chayakulkeeree and Perfect, 2008). Associated symptoms may be neurological or respiratory, even both in severe cases (Douwel, 2010; Voelz and May, 2010), and may appear many weeks, and potentially months, after exposure. Infection usually starts with the inhalation of airborne infectious propagules (thought to be basidiospores instead of yeast cells) from the environment, which can then be lodged in the lungs (Figure 3) (Casadevall and Perfect, 1998). The relatively small sizes of the basidiospores, usually acapsular or poorly encapsulated, allows them to easily lodge within the alveoli. There, they can remain latent (Garcia-Hermoso et al., 1999) or evoke an immune response (Hull and Heitman, 2002). Usually in immunocompetent persons, a localised lung infection can be cleared (Hull and

Heitman, 2002; Lin and Heitman, 2006). However, if not cleared (usually in immunocompromised individuals), the infection can spread to other organs, including the central nervous system, by crossing the blood-brain barrier. The resulting infection can be deadly if left untreated (Casadevall and Perfect, 1998). Fortunately, to date, there is no record of infections being transmitted from one person to the next (Litvintseva et al., 2011). Therefore, avoiding habitats where there could be a high concentration of infectious propagules may assist in minimizing infections, including possible outbreaks.

Members constituting the *Cr. neoformans* species complex have a number of virulence factors i.e. ability to grow at 37 ^{o}C and melanin production, which can assist in establishing a diseased-state (Buchanan and Murphy, 1998). However, the capsule is suggested to be the main virulence factor (Bose et al., 2003; Zaragoza et al., 2009) and thus, has been extensively studied. Chemically, the capsule is composed of glucuronoxylomannan (major polysaccharide), galactoxylomannan and mannoproteins (Bose et al., 2003; Zaragoza et al., 2009). During immunity, microbial cell wall structures, such as the cryptococcal capsule, are crucial in providing protection from the extracellular environment and for interacting with the environment (Zaragoza et al., 2009; Pommerville, 2010; Sebolai et al., 2012). Upon entering the alveolar space, the capsule acts as a physical barrier, and it (i.e. capsule structure) has been shown to swell in size leading to the formation of giant or “titan” cells, which may be as big as 80 µm in cell diameter (Casadevall and Perfect, 1998; Zaragoza, 2011).

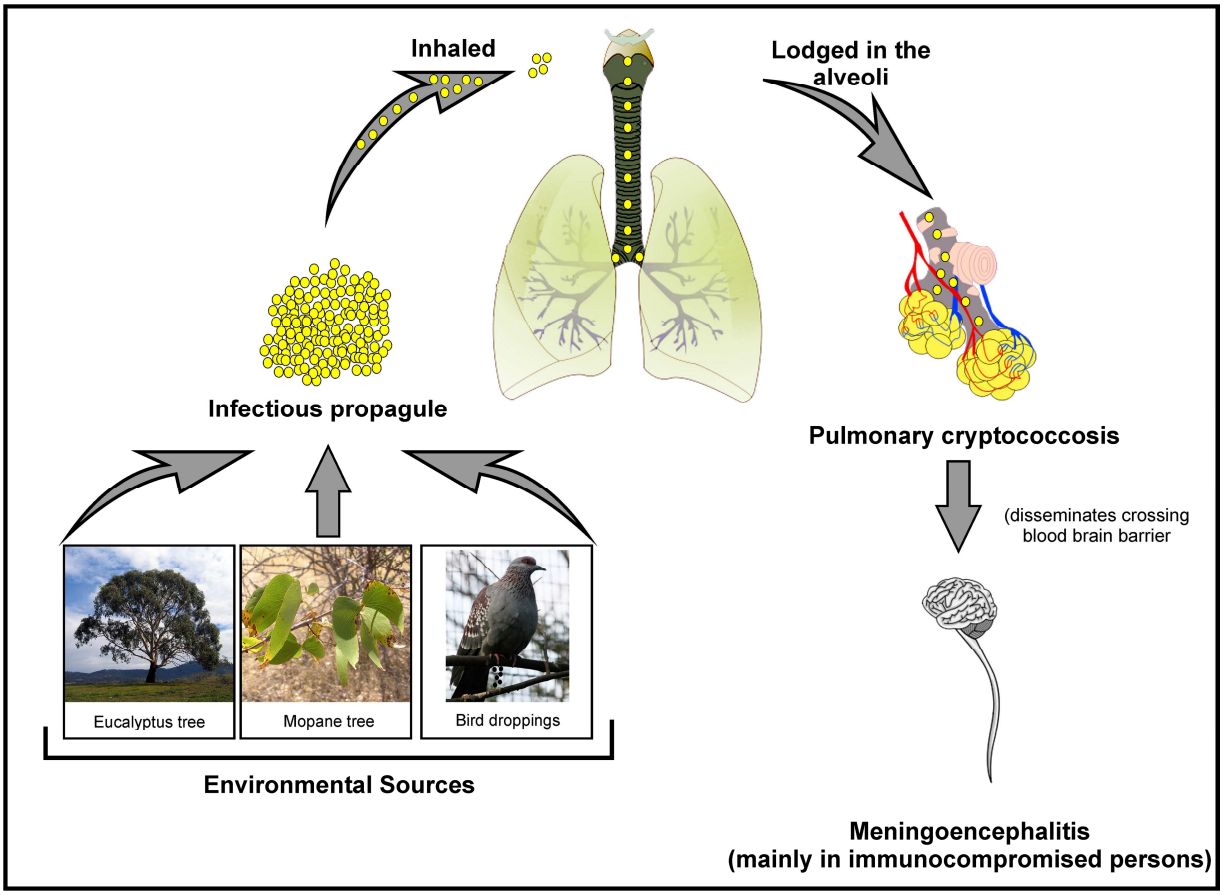

Figure 3. A schematic representation of cryptococcal infection. Infection usually starts with inhalation of an infectious propagule leading to a diseased-state either in the lungs or, in disseminated cases, in other body parts. The scheme was constructed using pictures obtained from Wikipedia. All images are in the public domain.

Bird: Credit to Rklawton;

Mopane tree: Credit to Teo Gomez;

Eucalyptus tree: Credit to Alexander110;

Lungs: Credit to Rastrojo;

Alveoli: Credit to Rastrojo;

Brain: Credit to Grm wnr.

Here, the invading cryptococcal cells come up against phagocytic cells such as macrophages, dendritic cells and neutrophils (Voelz and May, 2010). The capsule is reported to prevent phagocytosis (Buchanan and Murphy, 1998); a receptor-mediated process, in which foreign bodies such as cryptococcal cells, are taken up into phagocytic cells (Del Poeta, 2004). As a result, cryptococcal cells can escape immunoprocessing and remain dormant (Garcia-Hermoso et al., 1999), until an immune imbalance leads to further development. A unique aspect of host-pathogen interactions called nonlytic exocytosis or phagosome extrusion/expulsion, has also been observed during *Cryptococcus*-host interactions. In this case an invading cryptococcal cell, can reside and replicate inside a host macrophage, exit and enter the next macrophage without: (1) destroying the macrophages, and (2) evoking inflammation and an immune response (Ma et al., 2006; Nicola et al., 2011). However, when processed or disseminated, the capsule can be shed and circulate in the blood and cerebrospinal fluid (Vecchiarelli, 2000; Yauch et al., 2006). When processed, the characteristic cell wall epitopes, usually masked by the capsule, are revealed. This, allows for opsonization, which further leads to the activation of T cells (Vecchiarelli et al., 2003) or that of anti-pathogen serum proteins constituting the complement system (Janeway et al., 2001), which in turn, cause inflammation and attract more phagocytic effector cells to the infection site. However, what makes *Cryptococcus* unique and difficult to control is that, even when under siege like this; it can still employ the capsule to further mediate the immune response in its favour. To this end, the capsule is documented to also down regulate proinflammatory cytokine secretion from monocytes, inhibit leukocyte migration, inhibit T-cell proliferation and enhance production of anti-inflammatory

cytokines (Buchanan and Murphy, 1998; Vecchiarelli et al., 2003; Ellenbroek et al., 2004; Yauch et al., 2006).

1.8 EPIDEMIOLOGY

Formerly, the majority of cryptococcosis cases were mainly noted in persons with other predisposing risk factors i.e. organ transplantation, which results in an immunosuppressed state (Chayakulkeeree and Perfect, 2008). However, the appearance of HIV has led to a marked increase in the number of cryptococcosis cases worldwide (Levitz and Boekhout, 2006). In the 1980s, cryptococcosis occurred in 5 % to 10 % of HIV-infected persons in the developed world (Jarvis and Harrison, 2007). However, at present, the majority of cases are concentrated in central and southern Africa (Jarvis and Meintjes, 2011) with case fatality rate of between 35 % and 65 %, compared to between 10 % and 20 % in developed countries (WHO, 2011). Members belonging to the *Cr. neoformans* species complex have a worldwide distribution (Table 1), and more importantly, can establish infectious diseases in both immunocompromised and immunocompetent individuals (Day, 2004). This implies that, given the right conditions, everyone across the world is at risk of acquiring this infectious disease. Perhaps, the most comprehensive study documenting the epidemiology of cryptococcosis, was led by Park of the Centers of Disease Control and Prevention (Park et al., 2009). In this widely cited text, Park and co-workers placed the global burden of cryptococcal infections at almost one million cases, resulting in just over 620 000 deaths annually. The study further documents the highest burden of

infections to be in Sub Saharan African (720 000 cases), where it's claimed that cryptococcosis causes more deaths (504 000) compared to tuberculosis (350 000). The study also estimates South and South-east Asia to carry a burden of 120 000 cases; Oceania - 100 cases; Western and Central Europe - 500 cases North Africa and Middle East - 6500 cases; and North America with 7800 cases (Park et al., 2009). These authors, however, acknowledge that more epidemiological studies still have to be carried out in order to have a true global picture of the extent and scope of infections.

1.9 CURRENT DIAGNOSIS, TREATMENT AND ANTIMITOCHONDRIALS

The discovery of characteristic epitopes on the cell wall of *Cr. neoformans* implies commercial immunological products can be developed, which could potentially be employed to correctly classify *Cr*. *neoformans* isolates into its respective serotypes. To this effect, a commercial kit, developed by a company in Japan (Crypto Check, Iatron Laboratories), was until recently available for use (Ito-Kuwa et al., 2007). However, it has since been discontinued. This has left a gap in the market, but more importantly as an unintended consequence, infections caused by *Cr. neoformans* can potentially be misdiagnosed as *Cr. gattii* infections and vice versa. At present, many of the current immunological products on the market cannot reveal the serotype of the infection-causing agent (Jarvis et al., 2011). This can have an effect on the treatment prescribed to patients. In addition, microscopy also cannot reveal the identity of the responsible serotype causing the infection (Saha et al., 2008). Microscopy and serology (although ideal as point-of-care screening tests) often yield inconsistent test results (Casadevall

and Perfect, 1998; Saha et al., 2008, 2009). One-way of overcoming this is through cultivation of isolates; however; growth is obtained only after a few days (Kwon-Chung, 2011). Advances in molecular techniques have assisted in resolving sensitivity and specificity issues around identifying *Cr. neoformans* serotypes (Sidrim et al., 2010). However, most of these typing techniques are expensive and laborious (Sidrim et al., 2010), and as such cannot be optimised for routine clinical use particularly in resource-limited settings such as South Africa. Therefore, a simple PCR-based method or serological kit that can reveal the identity of the responsible infectious serotype would hold an obvious advantage.

According to Perfect et al. (2010), management of cryptococcal infections is strongly dependent on resources available in a specific country. Multi-national companies, through drug donation programmes, have made medicines such as fluconazole readily available in developing countries such as South Africa (Wertheimer et al., 2004; Collett and Parrish, 2007). This, in conjunction with the expanded antiretroviral programme in South Africa, has greatly assisted in managing infections. However, fluconazole is known to be fungistatic and thus can allow treated cells to persist. The reader is referred to an excellent text detailing the current treatment guidelines for the management of cryptococcosis (Perfect et al., 2010). The discussion in the following sub section is therefore restricted to the general usage of antimicrobial agents, drug resistance and antimitochondrials.

Microbes can develop either resistance or tolerance (the latter is a phenomenon usually displayed by persister cells) towards antimicrobial agents as a mechanism for survival (Ghannoum and Rice, 1999; Wakamoto et al., 2013). These survival mechanisms are activated via biological processes such as mutations or epigenetics. To compound this, drug non-compliance by patients has also made managing microbial infections even more difficult. Ghannoum and Rice (1999) highlighted that amphotericin B, which binds to ergosterol leading to membrane damage, has been the drug of choice for many years in combating fungal infections. However, its clinical application is limited by its severe side effects. Azoles such as fluconazoles, which inhibit ergosterol biosynthesis, tend to be fungistatic (Jarvis and Meintjes, 2011) thus highlighting the need to find new alternative drugs to combat fungal infections.

Over time, non-traditional antimicrobials have been employed to treat non-infectious human diseases (Cederlund and Mårdh, 1993). In 2007, Kock and co-workers proposed that antimitochondrial drugs could be used to control microbial growth particularly the growth of strict aerobes, which cannot switch between aerobic respiration and fermentation. Here, the mode of action is thought to be through the disruption of mitochondrial energy generating processes (Glasgow et al., 1999; Norman et al., 2004). This theory was later tested and proven to be true during two independent *in vitro* growth inhibition studies (Sebolai et al., 2008b; Leeuw et al., 2007) leading to a patent (Davies et al., 2009). However, the study of many strains, particularly strains representing medically important pathogenic microbes such as *Cryptococcus* and *Mycobacterium*, which are highly aerobic, should further test the veracity of this theory.

1.10 PURPOSE

With the preceding discussion providing a background and context for the studies presented in the dissertation, the aims became:

1. To establish the incidence rates of cryptococcosis in Bloemfontein, South Africa (Chapter 2; sub section 2.1);

2. To assess the usefulness of simple PCR techniques in the routine diagnosis of *Cr. neoformans* and *Cr. gattii* (Chapter 2; sub section 2.2); and

3. To evaluate the *in vitro* response of *Cr. neoformans* and *Cr. gattii* towards antimitochondrial drugs alone and in combined therapy with fluconazole (Chapter 3).

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CHAPTER 2

EPIDEMIOLOGY

Note: This chapter consists of two sub sections, each independently dedicated to one aspect of cryptococcal epidemiology in Bloemfontein, South Africa.

Sub section 2.1: Incidence rate

Note: This sub section constitutes part of a study into HIV and associated co-infections (tuberculosis and cryptococcosis) in Motheo District, Free State Province, South Africa. The now presented cryptococcal part has been included with permission in the current M.Sc. study (section 2.1). The candidate independently performed this study.

2.1.1 ABSTRACT

Background: A successful health prevention and management strategy is usually influenced by research-based evidence. Often, an inappropriate management strategy may results in unintended consequences. The human cost as a result of inappropriate management strategies is incalculable, particularly, for poor families who rely on the state for health services. Therefore, a study examining the incidences of cryptococcal infections could assist health officials in their planning.

Objective: We sought to draw an epidemiological sketch of cryptococcosis in Bloemfontein, South Africa, through determining the incidence rate of cryptococcal infections over a two-year period viz. 2011 and 2012. We also sought to further define incidences based on age; gender and race in order to better define and understand the scope and extent of cryptococcosis cases.

Methods: Data used in the study was obtained from Universitas Academic Hospital, which serves as a central facility for testing in Bloemfontein. The formula: [number of reported cases in one year] / [Bloemfontein population at risk] x 100 000 was used to

calculate the incidence rates. The population data was based on the latest 2011 South African Census.

Results: In 2011, 16 in 100 000 persons in Bloemfontein had cryptococcal infection. This number decreased to 13 per 100 000 in 2012. On average, this represents less than 0.1 % of the Bloemfontein population acquiring cryptococcal infection over the two-year period of this study. Cases were estimated to be higher among the sexually active age group i.e. 20 to 49 and fewer cases were noted among 10-19 and in persons over 50. No cases were observed among small children (0 to 9). Moreover, cases were only observed among Blacks (Africans, Coloureds and Indians) and not in other population groups.

Conclusion: In this study, cases of cryptococcal infections mirrored the biographic distribution pattern of HIV-infected persons in South Africa. Contrary to expectation, considering the huge population living with HIV that is at risk of acquiring this AIDS-defining illness, few cases of cryptococcosis were noted for the study period - possibly due to misdiagnosis of cases hence, the under reporting. Nonetheless, we hope this study will shed light on and draw much needed attention to cryptococcosis, an infectious disease, which has for many years received inadequate attention in the public space and discourse compared to infectious diseases such as tuberculosis.

2.1.2 INTRODUCTION

Acquired immunodeficiency syndrome (AIDS), a syndrome characterised by a systematic loss of immunity, is often associated with an insidious onset of life-

threatening infectious diseases such as cryptococcosis (Kwancharoenporn et al., 2007). Cryptococcosis may present in both immunocompetent and immunocompromised individuals (Day, 2004; Chayakulkeeree and Perfect, 2008).

For most of the 1800s and early 1900s, few cases of cryptococcosis were reported (Levitz and Boekhout, 2006). However, the appearance of the human immunodeficiency virus (HIV), the causative agent of AIDS, brought an increase in the number of cryptococcosis cases. Today, cryptococcosis is a significant cause of worldwide morbidity and mortality especially in Sub Saharan Africa (Jarvis and Harrison, 2007; Jarvis and Meintjes, 2011), where according to the Centers of Disease Control and Prevention, over 700 000 new cases are reported each year (Park et al., 2009).

In South Africa, where just over 5 million people are estimated to be living with HIV (Statistics South Africa, 2010), cryptococcosis (which is an AIDS-defining illness) has become a serious public health concern. In light of the above, we sought to establish incidences of cryptococcosis in Bloemfontein, South Africa and to study incidences across age, gender and race. Such information may assist health officials in their management strategies directed at controlling current cryptococcal infections, and possible future outbreaks.

2.1.3 METHODOLOGY

Population data.

This study was limited to Bloemfontein (BFN); the administrative seat of Mangaung Metropolitan Municipality as well the capital city of the Free State Province in South Africa. The population data for Bloemfontein was based on figures obtained from the 2011 National Census (Statistics South Africa, 2011).

Apart from a number of private hospitals, three public hospitals, Pelonomi Secondary Hospital, National District Hospital, and Universitas Academic Hospital are present where residents can access and receive health care services. Universitas Academic Hospital serves as a central facility for testing suspected cases of cryptococcocal infections. Laboratory technicians (at Universitas Academic Hospital) maintained positive cryptococcal cultures after performing screening tests i.e. Indian ink staining and antigen determination. The corresponding patient information (married to positive cryptococcal cultures), which is reported in this study, excludes persons who received private care. Table 1 in the appendix section shows how culture and patient data were recorded. In this study, the HIV status of concerned patients was unknown. Ethical clearance was obtained from the University of the Free State's Ethics Committee (ETOVS 173) as well as from Dr. S. Kabane, the Head of Health in the Free State Province of South Africa.

Confidentiality.

Confidentiality was maintained in respect of all information gained during the course of the study.

Statistical note.

The following formula was used in calculating estimations: incidence rate = [number of reported cases in one year] / [Bloemfontein population at risk] x 100 000 (Last, 2001; Dorland, 2011). In this study, incidence refers to new cases per unit time of cryptococcal infections, and this should not be confused with the prevalence rate. All fractions were rounded to the nearest whole number.

2.1.4 RESULTS AND DISCUSSION

The incidence rates over the study period.

For 2011, 16 cases of cryptococcal infections in 100 000 population were recorded (Figure 1). In the following year (2012), the figure decreased to 13 cases per 100 000. On average, for the two-year period under review, less that 0.1 % of the Bloemfontein population was infected with cryptococcosis. This is very curious since cryptococcosis is an AIDS-defining illness and there is a large population in South Africa, and by extension in Bloemfontein, living with HIV that is at risk of contracting this infectious disease. Nonetheless, our data seem to suggest that cryptococcosis is not prevalent in Bloemfontein. This could be attributed to: (1) lack of active surveillance programmes, and (2) inconsistent diagnostic methods used in the routine diagnosis of cryptococcosis

in clinical settings. The latter is further expanded on in sub section 2.2 of the dissertation.

Biographic analysis.

HIV and associated infectious diseases such as cryptococcosis, are regarded as diseases prevalent amongst the poor (WHO, 2011), and in South Africa, Blacks (Africans, Coloureds and Indians) constitute the largest population grouping, which in the main is afflicted by poverty. The above assertion holds true when studying incidences across race (Figure 2). To be specific, all cases were detected in Blacks and not in other population groups over the two-year period. As all cases were noted among the Black population group, case distribution per gender and age were determined as a function of the Black population group alone. Therefore, further analysis revealed that females (2011: 11 cases and 2012: 9 cases), in general, carried the highest burden of infections compared to males (2011: 10 cases and 2012: 7 cases) (Figure 3). This skewed disease burden (in favour of females) is similar to that noted among HIV-infected persons (Statistics South Africa, 2011).

In addition, this infectious disease seems to be prevalent among the 20-49 age groups (Figure 4). This distribution pattern is similar to the burden of HIV observed among the sexually active group 15-49 years in South Africa (UNAIDS, 2010; Statistics South Africa, 2011). In addition, no cases were recorded among the 0-9 age group. However, a notable burden of cryptococcosis cases among 10-19 and over 50 age groups was detected. The observed adolescent burden maybe as a result of children who become

immunocompromised during their early childhood, and acquire this infections disease. With respect to the over 50 age group, it could be as a result of acquiring cryptococcosis via normal modes or as a result of a weakened immune system due to old age.

2.1.5 CONCLUSIONS

In conclusion, the following points are noted, which underpin the important observations from the study:

1. For the study period, cases of cryptococcosis in Bloemfontein were rare, with less than 0.1 % of the population infected. This is contrary to what can be extrapolated, considering that 10 % of the South African population, and by extension Bloemfontein, is living with HIV. This under-reporting perhaps highlights the fundamental error of how cases are diagnosed and recorded within the national health care system, which should further signal a review on methods employed to achieve positive diagnosis.

2. The cryptococcosis demographic profile i.e. gender, race and age, mirrors the profile of HIV-infected persons in South Africa. With respect to race, the observed high burden among Blacks may be qualified, as this AIDS-defining illness is considered an infectious disease prevalent among the poor.

We trust health authorities in the Free State Province will find this study useful. More importantly, we trust that the study will guide the authorities in their planning to control this infectious disease within the Province.

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FIGURES

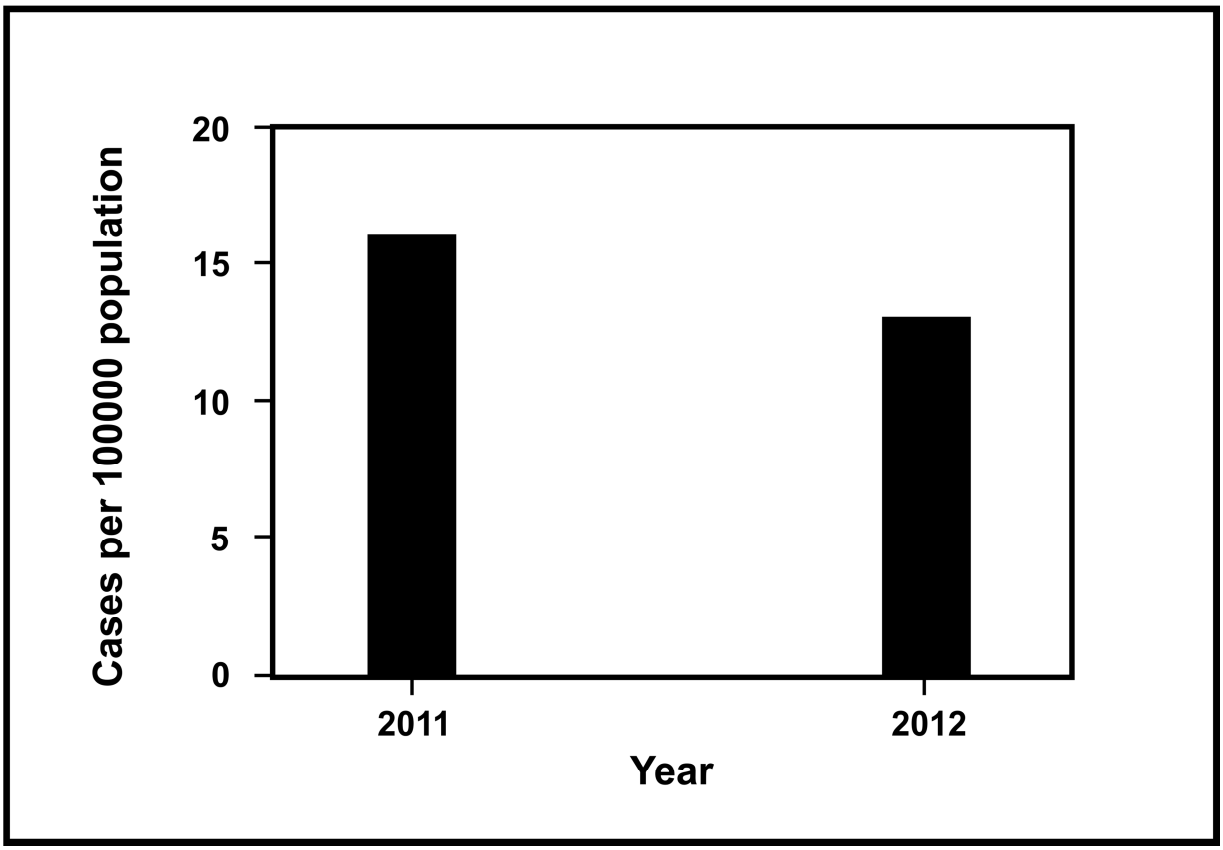

Figure 1. The incidence rate of cryptococcosis in Bloemfontein, South Africa. The study period is 2011 and 2012.

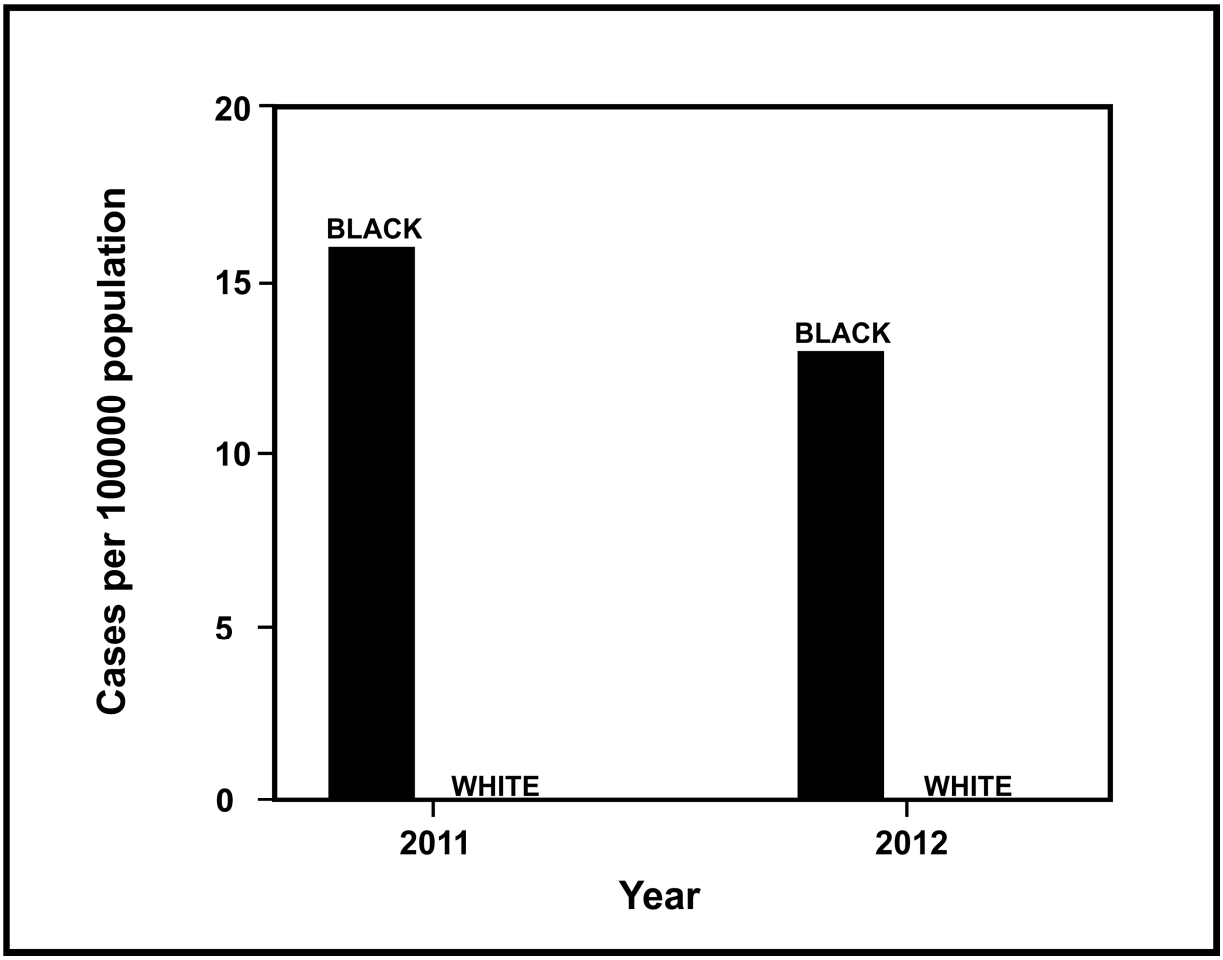

Figure 2. The incidence rate of cryptococcosis as measured per race in Bloemfontein, South Africa. The study period is 2011 and 2012.

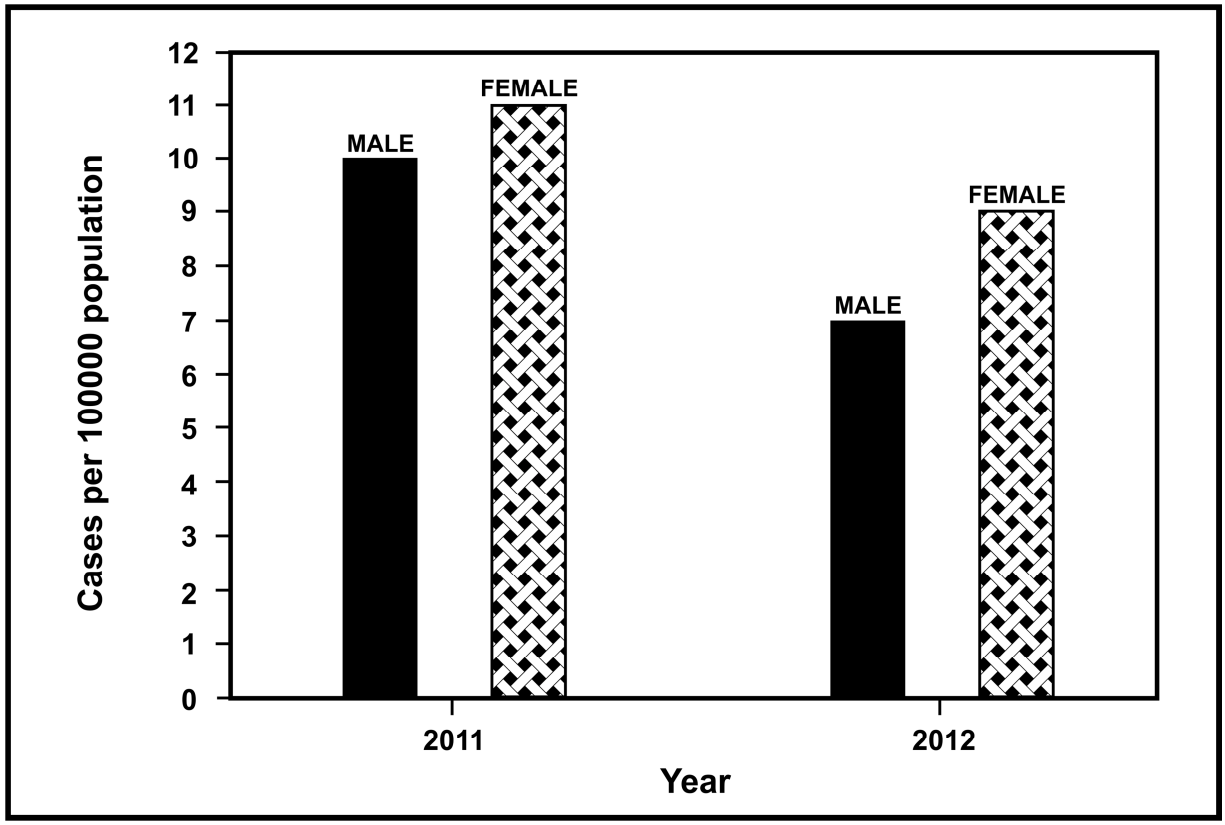

Figure 3. The incidence rate of cryptococcosis as measured per gender in Bloemfontein, South Africa. The study period is 2011 and 2012.

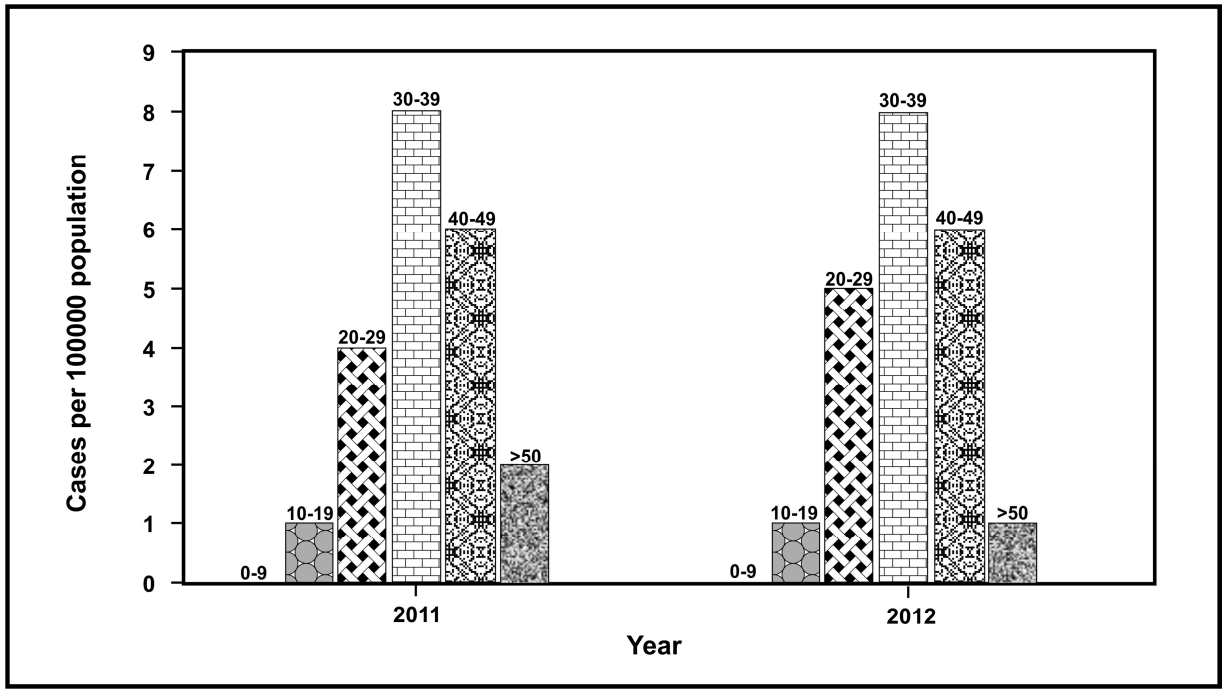

Figure 4. The incidence rate of cryptococcosis as measured per age in Bloemfontein, South Africa. The study period is 2011 and 2012.

Sub section 2.2: Molecular identification

Note: A patent, which is based on this part of the study, has been filed and a manuscript copy has also been prepared for publication.

2.2.1 ABSTRACT

Background: The high burden of HIV in South Africa has led to cryptococcosis becoming a public health concern. To compound this, conventional identification methods have been inconsistent in diagnosing cryptococcosis, thereby influencing the number of reported cases. By extension, this undermines efforts by local health officials to appropriately plan and manage infections.

Objective: Therefore, there is a need for more sensitive methods to obtain early and accurate diagnosis in order to improve patient outcomes as well as management strategies.

Method: In this study, we examined the usefulness of a simple PCR method followed by sequencing of the internal transcribed spacers (ITS) region including the 5.8S gene, as a reliable and rapid identification tool. The method was tested on 70 strains obtained from patients diagnosed with cryptococcosis.

Results: The intra-specific variation between the tested strains allowed for their delineation into the three traditional varieties of *Cryptococcus* (Cr.) *neoformans* viz. *Cr*. *neoformans* var. *neoformans*, *Cr*. *neoformans* var. *grubii* and *Cr*. *neoformans* var. *gattii*. Furthermore, a closer examination of the multiple alignment sequence data revealed a

restriction site that is present only in the distinct species *Cr. neoformans* (as constituted by *Cr. neoformans* var. *neoformans* and *Cr. neoformans* var. *grubii* strains), which is absent in the distinct species *Cr. gattii* (as constituted by *Cr. neoformans* var. *gattii* strains). The restriction site now allows for the identification of these two distinct species by directly digesting the polymerase chain reaction products without the need for sequencing.

Conclusion: Molecular identification proved to be reliable method. Elimination of sequencing from the identification process makes this method, similar to conventional serological techniques, much more cost effective, rapid and easy to optimise (requiring less specialised training) for routine use. This is particularly true in resource-limited settings such as South Africa.

2.2.2 INTRODUCTION

Cryptococcosis is a life-threatening fungal disease that may present in either immunocompetent or immunocompromised persons (Day, 2004). However, persons with an impaired cell-mediated immunity are more vulnerable to the disease. The advent of HIV has brought an increase in the number of cryptococcosis cases, and in Sub Saharan Africa, it is estimated that cryptococcosis annually accounts for over 500 000 deaths of HIV-infected individuals (Park et al., 2009). Although cryptococcosis can be managed, its clinical diagnosis may often be inaccurate. A diagnostic error could leave survivors with neurological problems or worse, have fatal consequences (Saha et al., 2008). Moreover, inaccurate diagnosis further undermines efforts to understand the

extent and scope of this infectious disease, and thus could have a negative impact on plans and management strategies by health authorities.

Over the years, Indian ink preparations have been used to diagnose cryptococcosis (Saha et al., 2008, 2009). However, Indian ink has poor sensitivity, and there have been reported cases of acapsular strains inducing pathogenesis (Del Poeta, 2004). Although serological tests are more specific, they have not been consistent in diagnosing cryptococcosis due to false positive or negative results (Saha et al., 2009). More importantly, these techniques cannot resolve the identity of the serotype causing the infection. This information is crucial in understanding the epidemiology, pathogenesis, clinical presentations and drug resistance of clinical isolates (Ito-Kuwa et al., 2007). In addition, it can also provide information relating to the agent's oxylipin production pattern and associated pathogenesis (Sebolai et al., 2007, 2012).

The availability of an extensive molecular database has allowed for easy identification and referencing of yeasts (Scozetti et al., 2002). Traditionally, *Cryptococcus* (*Cr*.) *neoformans* has been classified into three varieties viz. *Cr. neoformans* var. *neoformans* (serotype D), *Cr. neoformans* var. *grubii* (serotype A), *Cr*. *neoformans* var. *gattii* (serotype B and C) as well as a hybrid (AD) (Chen et al., 2010). Recently, it has been proposed that the species complex should be reclassified into two distinct species viz. *Cr. neoformans* (serotypes A, AD and D) and *Cr*. *gattii* (serotype B and C) (Kwon-Chung and Varma, 2006). Based on this proposal, this study sought to examine the usefulness of sequencing the internal transcribed spacers (ITS) including the 5.8S

gene, as a reliable method for the identification of etiological agent(s) from clinical strains obtained from patients with cryptococcosis. In resource-limited settings, a simple PCR method would have a clear competitive advantage over more elegant typing techniques that are too expensive and laborious for routine use (Sidrim et al., 2010). We also sought to confirm cases of *Cr. gattii*, found to be less prevalent in temperate regions such as South Africa, by cultivation on canavanine-glycine-bromothymol blue media.

2.2.3 MATERIALS AND METHODS

Strains.

Seventy clinical strains: 13 *Cr. neoformans* var. *neoformans* strains, 51 *Cr*. *neoformans* var. *grubii* strains and 6 *Cr. neoformans* var. *gattii* strains were examined in this study. These strains were obtained from Universitas Academic Hospital, Bloemfontein, South Africa. Strain numbers and details of origin are provided in Table 1. Strains were maintained on yeast-malt-extract (YM) agar (3 $g.L^{-1}$ yeast extract, 3 $g.L^{-1}$ malt extract, 5 $g.L^{-1}$ peptone, 10 $g.L^{-1}$ glucose, 16 $g.L^{-1}$ agar; Merck, South Africa).

PCR amplification.

DNA was extracted using a thermal shock method. In brief, 18 h cells, sub-cultured on YM agar at 30 ^{o}C, were suspended in 25 µL of triple distilled water and heated for 10 min at 96 ^{o}C (Motaung et al., 2012). To this 25 µL of a double concentrated reaction mixture was added. The reaction mixture (50 µL total volume) contained 1.5 mM

magnesium chloride, 200 µM deoxynucleoside triphosphates, 100 pmol of each primer, 0.5 U Taq DNA polymerase (New England BioLabs®). Amplification was performed in a thermal cycler (Applied Biosystems 2720) using the following steps: 94 ^{o}C for 2 min followed by 30 cycles of 30 sec denaturation at 94 ^{o}C and 30 sec annealing at 52 ^{o}C with 1 min extension followed by final extension for 5 min at 72 ^{o}C. The primer sequences used for amplification were ITS4 (59-TCCTCCGCTTATTGATATGS-39) and ITS5 (59-GGAAGTAAAAGTCGTAACAAGG-39) (White et al., 1990). The PCR products were visualised on a 0.8 % agarose gel.

Sequencing.

PCR products were purified and concentrated using the DNA Clean and Concentrator™ kit (Zymo Research) and the sequencing reaction was performed with the ABI Prism™ Big Dye terminator™ V3.1 cycle sequencing ready reaction kit (Applied Biosystems®) and data collected on an ABI 3130XL genetic analyzer (Applied Biosystems®). Data was analysed using Geneious version 5 (Drummond et al., 2011). The obtained sequences were compared to yeast sequences in the NCBI database by using the basic local alignment search tool (BLAST).

Preparation of canavanine-glycine-bromothymol blue (CGB) media and cultivation.

The media was prepared as previously described (Kwon-Chung et al., 1982). Strains were cultivated in duplicate at 30 ^{o}C for 5 days, before reading the results. The media was supplemented with carvananine (growth inhibitor) and bromothymol blue; which

turns the media blue due to a change in pH as positive strains cleave glycine to produce ammonia. As such, a negative result was interpreted as an agar plate that remained yellow after 5 days of cultivation.

2.2.4 RESULTS AND DISCUSSION

Resolving the identity of studied strains.

The gel electrophoresis patterns of ITS amplification products, obtained from 12 of the seventy clinical isolates, are shown in Figure 1. Using the primers ITS4 and ITS5 to amplify the ITS region, we obtained the expected DNA fragments of between 600 and 700 bases for all strains tested. Sequencing of these amplicons followed by editing resulted in an ITS 1-5.8S-ITS 2 sequence of 478 bases. These sequences were further multi-aligned and analysed in order to determine intra-specific variation (Figure 2). Our analysis revealed variable bases between the strains which allowed for their delineation into three varieties viz. *Cr. neoformans* var. *neoformans* (variable bases: T [position: 28], A [position: 117]), *Cr. neoformans var. grubii* (variable base: A [position: 230])as well as *Cr. neoformans* var. *gattii* (variable base: C [position: 19]) (Table 2). These variable bases were also consistent in their delineation when applied against a number of *Cr*. *neoformans* species complex sequences available from GenBank.

In this study, we resolved the tested strains into two distinct species viz. *Cr*. *neoformans* (*Cr. neoformans* var. *neoformans* and *Cr. neoformans* var. *grubii*) and *Cr*. *gattii* (*Cr. neoformans* var. *gattii*) as proposed by Kwon-Chung and Varma (2006). We further

uncovered a restriction site for *Ssp*I present only in the distinct species *Cr. neoformans* (as constituted by *Cr. neoformans* var. *neoformans* and *Cr. neoformans* var. *grubii* strains), which is absent in the distinct species *Cr. gattii* (as constituted by *Cr. neoformans* var. *gattii* strains) (Figure 3). Amplification of this region and its subsequent digestion would now allow for the identification of these two distinct species without sequencing, thus enhancing the speed at which identities can be resolved whilst eliminating sequencing costs.

Confirmation of studied strain identities.

Traditionally, *Cr. gattii* has been thought to be limited to tropical and subtropical regions of the world (Day, 2004). In this part of the study, we sought to confirm cases of *Cr. gattii* as identified from our sequencing results. All 64 *Cr. neoformans* (*Cr. neoformans* var. *neoformans* and *Cr. neoformans* var. *grubii* strains) CGB plates remained yellow; while all 6 *Cr. gattii* (*Cr. neoformans* var. *gattii* strains) plates were blue after five days (Figure 4 and Table 2). Identification of *Cr. gattii* as demonstrated in our study does not seem to be an isolated incidence, as this pathogen has also been isolated in other temperate regions viz. North America, thereby supporting the idea of a more worldwide ecological distribution (Datta et al., 2009). Speculatively, this may in part be due to the exportation of eucalyptus trees, which are suggested to be the primary source of *Cr. gattii* (Kidd et al., 2007).

In conclusion, in this study, we tested clinical isolates using a simple PCR method as a tool for the early and accurate diagnosis of cryptococcosis, thus contributing to efforts to

fully understand the extent and scope of this infectious disease. Compared to less sensitive conventional methods, PCR is an excellent tool as it can detect low fungal loads and can be used on small sample sizes. In clinical practise, it is often difficult to obtain large sample sizes particularly from paediatric patients (Jarvis et al., 2011). Using the observed variable bases and their positions within the ITS region, it was possible to delineate tested strains into three varieties viz. *Cr*. *neoformans* var. *neoformans*, *Cr*. *neoformans* var. *grubii* and *Cr*. *neoformans* var. *gattii*. The identification of a specific restriction site, *Ssp*I, implies laboratory technicians would require minimal training to carry out the protocol compared to: (1) more elegant typing methods, and (2) performing sequencing analysis. Therefore, it should now be possible to optimise this technique, especially in resource-limited settings for the routine diagnosis of cryptococcosis. It was also possible to confirm cases of *Cr*. *gattii* using CGB media. However, CGB is not ideal as a point-of-care test due to the long waiting period before reading the results. Nonetheless, it is a reliable method for confirmatory purposes.

2.2.5 REFERENCES

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TABLES

Table 1

The origin and identity of clinical isolates studied.

† Strain name	Isolation		Molecular identification
	Source	Country	†† ITS results
LMPE 001	Human	South Africa	*Cr. neoformans* var*. neoformans*
LMPE 002	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 003	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 004	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 005	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 006	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 007	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 008	Human	South Africa	*Cr. neoformans* var*. grubii*

† LMPE = Laboratory of Microbial Pathogenesis and Epidemiology. †† The above strains showed 100% similarity to homologous sequences retrieved from the GenBank.

Table 1 (continued)

The origin and identity of clinical isolates studied.

† Strain name	Isolation		Molecular identification
	Source	Country	†† ITS results
LMPE 009	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 010	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 011	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 012	Human	South Africa	*Cr. neoformans* var*. neoformans*
LMPE 013	Human	South Africa	*Cr. neoformans* var*. neoformans*
LMPE 014	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 015	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 016	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 017	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 018	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 019	Human	South Africa	*Cr. neoformans* var*. grubii*

† LMPE = Laboratory of Microbial Pathogenesis and Epidemiology. †† The above strains showed 100% similarity to homologous sequences retrieved from the GenBank.

Table 1 (continued)

The origin and identity of clinical isolates studied.

† Strain name	Isolation		Molecular identification
	Source	Country	†† ITS results
LMPE 020	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 021	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 022	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 023	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 024	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 025	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 026	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 027	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 028	Human	South Africa	*Cr. neoformans* var*. neoformans*
LMPE 029	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 030	Human	South Africa	*Cr. neoformans* var*. neoformans*

† LMPE = Laboratory of Microbial Pathogenesis and Epidemiology. †† The above strains showed 100% similarity to homologous sequences retrieved from the GenBank.

Table 1 (continued)

The origin and identity of clinical isolates studied.

† Strain name	Isolation		Molecular identification
	Source	Country	†† ITS results
LMPE 031	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 032	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 033	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 034	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 035	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 036	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 037	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 038	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 039	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 040	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 041	Human	South Africa	*Cr. neoformans* var*. grubii*

† LMPE = Laboratory of Microbial Pathogenesis and Epidemiology. †† The above strains showed 100% similarity to homologous sequences retrieved from the GenBank.

Table 1 (continued)

The origin and identity of clinical isolates studied.

[†] Strain name	Isolation		Molecular identification
	Source	Country	[††] ITS results
LMPE 042	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 043	Human	South Africa	*Cr. neoformans* var*. neoformans*
LMPE 044	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 045	Human	South Africa	*Cr. neoformans* var*. gattii*
LMPE 046	Human	South Africa	*Cr. neoformans* var*. neoformans*
LMPE 047	Human	South Africa	*Cr. neoformans* var*. neoformans*
LMPE 048	Human	South Africa	*Cr. neoformans* var*. gattii*
LMPE 049	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 050	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 051	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 052	Human	South Africa	*Cr. neoformans* var*. gattii*

[†] LMPE = Laboratory of Microbial Pathogenesis and Epidemiology. [††] The above strains showed 100% similarity to homologous sequences retrieved from the GenBank.

Table 1 (continued)

The origin and identity of clinical isolates studied.

† Strain name	Isolation		Molecular identification
	Source	Country	†† ITS results
LMPE 053	Human	South Africa	*Cr. neoformans* var*. neoformans*
LMPE 054	Human	South Africa	*Cr. neoformans* var*. gattii*
LMPE 055	Human	South Africa	*Cr. neoformans* var*. neoformans*
LMPE 056	Human	South Africa	*Cr. neoformans* var *gattii*
LMPE 057	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 058	Human	South Africa	*Cr. neoformans* var*. neoformans*
LMPE 059	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 060	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 061	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 062	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 063	Human	South Africa	*Cr. neoformans* var*. grubii*

† LMPE = Laboratory of Microbial Pathogenesis and Epidemiology. †† The above strains showed 100% similarity to homologous sequences retrieved from the GenBank.

Table 1 (continued)

The origin and identity of clinical isolates studied.

† Strain name	Isolation		Molecular identification
	Source	Country	†† ITS results
LMPE 064	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 065	Human	South Africa	*Cr. neoformans* var*. neoformans*
LMPE 066	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 067	Human	South Africa	*Cr. neoformans* var*. neoformans*
LMPE 068	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 069	Human	South Africa	*Cr. neoformans* var*. grubii*
LMPE 070	Human	South Africa	*Cr. neoformans* var*. gattii*

† LMPE = Laboratory of Microbial Pathogenesis and Epidemiology. †† The above strains showed 100% similarity to homologous sequences retrieved from the GenBank.

Table 2

Strain identification based on analysis of ITS sequence intra-specific variation and CGB media results.

[†††] **Identity based on ITS results**	**Intra-specific variation**		[††††]**Distinct species**	**Growth on CBG media**	
	Variable base	**Position**		**Media colour**	**CGB result**
Cr. neo var*. neo*	T; A	28; 117	*Cr. neoformans*	Yellow	*Cr. neo*
Cr. neo var*. grubii*	A	230	*Cr. neoformans*	Yellow	*Cr. neo*
Cr. neo var*. gattii*	C	19	*Cr. gattii*	Blue	*Cr*. *gattii*

[†††] *neo* = *neoformans*. [††††] Distinct species as based on the proposal by Kwon-Chung and Varma (2006).

FIGURES

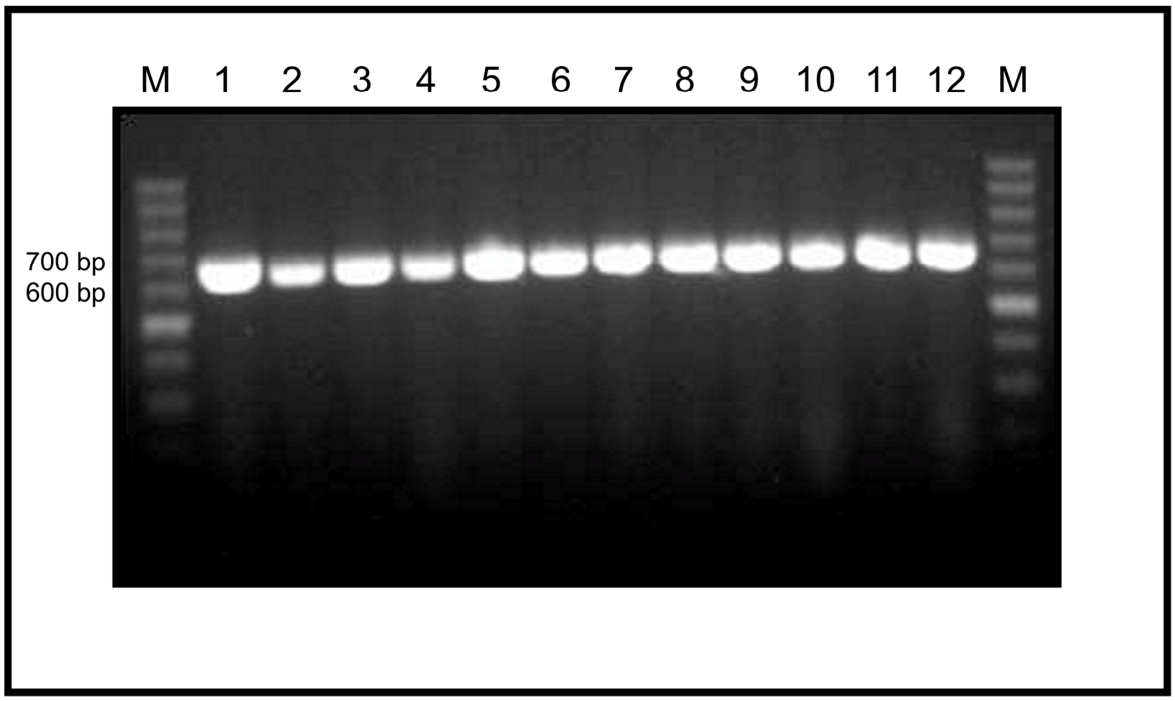

Figure 1. Electrophoregram showing PCR products. Expected DNA fragments of between 600 and 700 bases were obtained for all strains tested, depicting the approximate size of the ITS region. M = O'GeneRuler™. DNA Ladder = 1 kb.

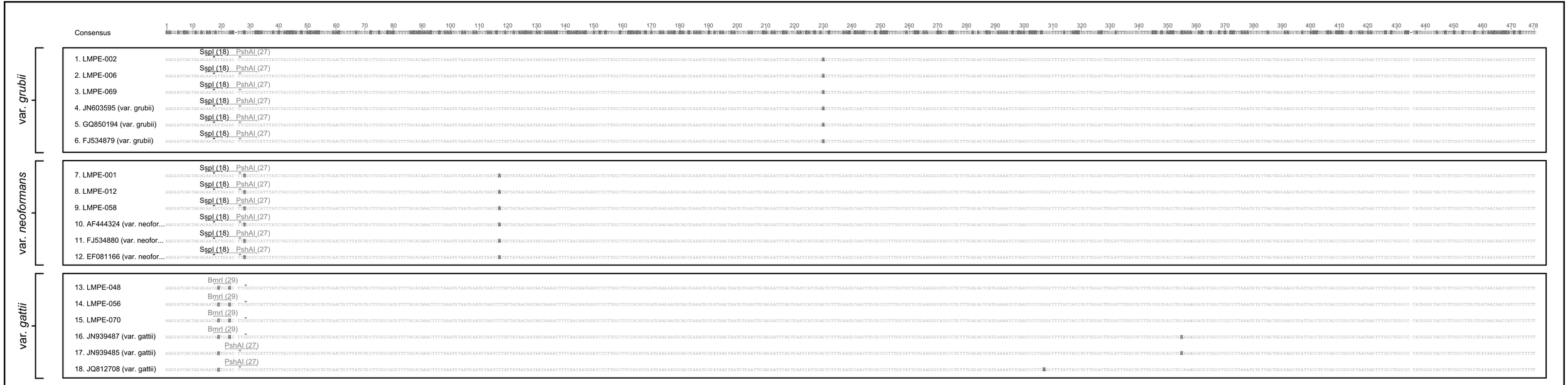
Consensus
var. grubii
1. LMPE-002
2. LMPE-006
3. LMPE-069
4. JN603595 (var. grubii)
5. GQ850194 (var. grubii)
6. FJ534879 (var. grubii)
var. neoformans
7. LMPE-001
8. LMPE-012
9. LMPE-058
10. AF444324 (var. neofor...
11. FJ534880 (var. neofor...
12. EF081166 (var. neofor...
var. gattii
13. LMPE-048
14. LMPE-056
15. LMPE-070
16. JN939487 (var. gattii)
17. JN939485 (var. gattii)
18. JQ812708 (var. gattii)
SspI (18)
PshAI (27)
BmrI (29)

Figure 2. Partial multiple sequence alignment (MSA) obtained from some of the 70 strains studied. The variable bases, highlighted in colour-coded boxes, revealed the intra-specific variation that allowed for the delineation of the studied strains into the three traditional varieties of *Cryptococcus neoformans.* These variable bases were also consistent in their delineation when applied against a number of sequences limited to *Cr*. *neoformans* species complex from the GenBank. Spaces (-) were introduced in order to improve alignment. The MSA was generated with ClustalX.

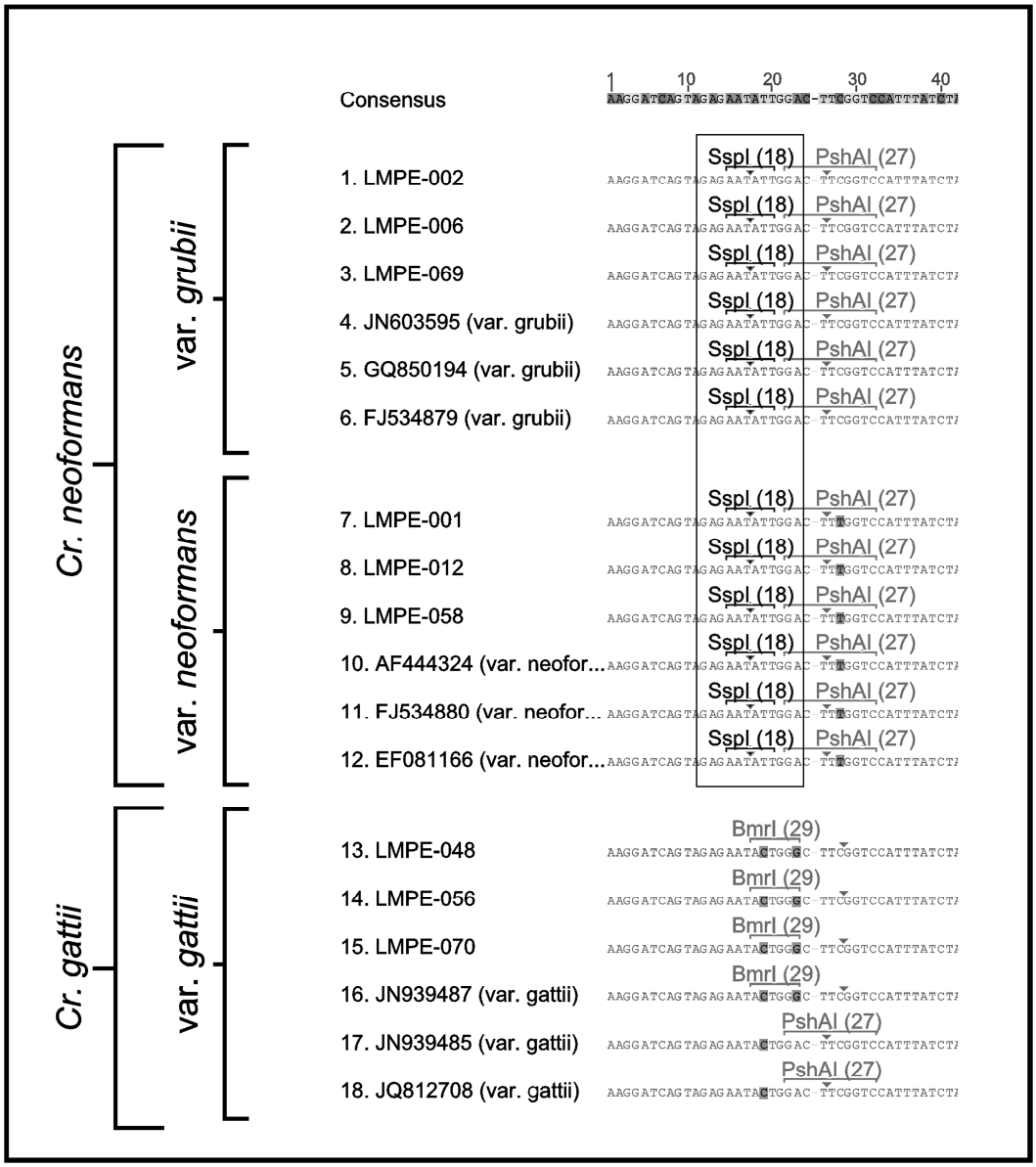

Figure 3. Detail of partial multiple sequence alignment (MSA). Closer examination of the sequence data limited to the distinct species, *Cryptococcus* (*Cr*.) *neoformans* (varietal form: *grubii* and *neoformans*) revealed a restriction site for *Ssp*I that is absent in *Cr*. *gattii* (varietal form: *gattii*). The restriction site is also observed when studying other aligned sequences limited to the distinct species, *Cr*. *neoformans* (obtained from the GenBank). Spaces (-) were introduced in order to improve alignment. The MSA was generated with ClustalX.

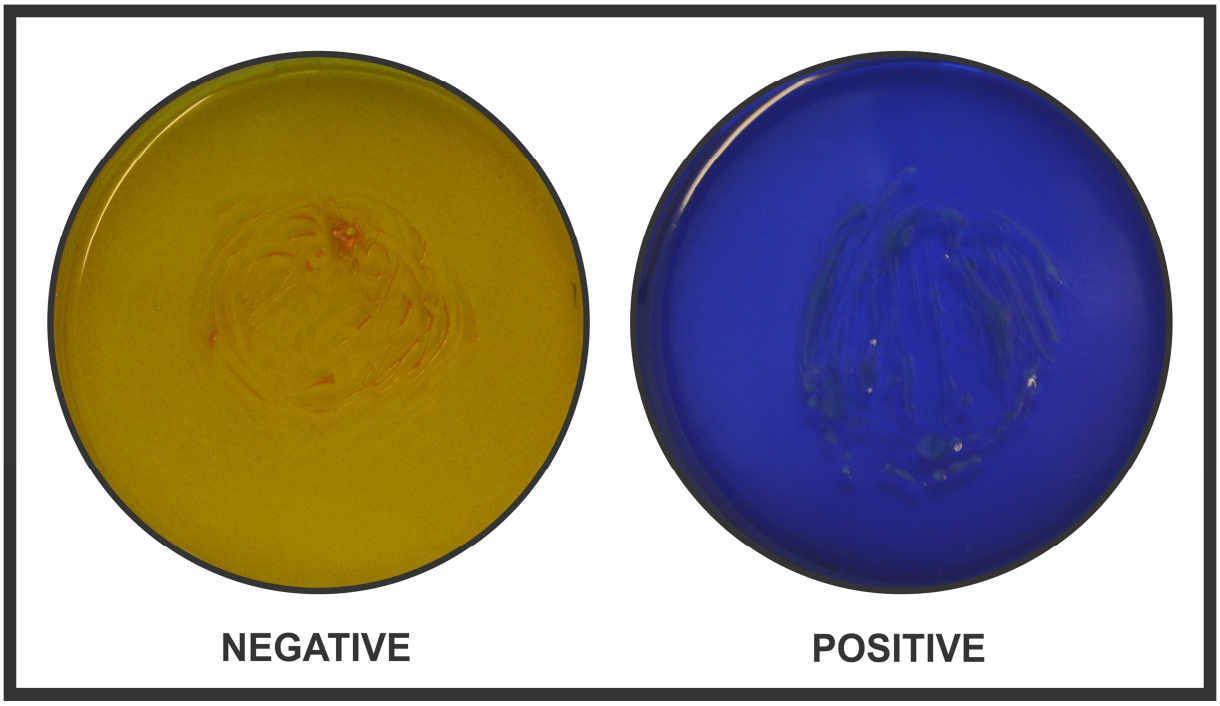

Figure 4. Growth results of *Cryptococcus* (*Cr.*) *neoformans* strains (varietal form: *grubii* and *neoformans*) and *Cr. gattii* strains (varietal form: *gattii*) following five days of cultivation on canavanine-glycine-bromothymol blue agar at 30 ^{o}C. The pictogram depicts a positive reaction (blue-coloured agar plate) - obtained after inoculation with a strain of *Cr. gattii*; and a negative reaction (a yellow-coloured agar plate) - obtained after inoculation with a strain of *Cr. neoformans*.

CHAPTER 3

ANTIFUNGAL SENSITIVITY

Note: A manuscript, which is based on this study, has been prepared for publication.

3.1 ABSTRACT

Background: Resistance, which is an inherent trait in natural selection, has necessitated the need to find new and effective antifungal drugs for the better management of fungal infections. This has led us to explore and consider non-traditional antimicrobial agents, such as antimitochondrial drugs, as possible alternatives.

Objective: We sought to examine and demonstrate the usefulness of antimitochondrial drugs (viz. aspirin and oligomycin), alone and in combined therapy with fluconazole, in controlling the growth of *Cryptococcus (Cr.) neoformans* and *Cr. gattii*.

Method: *In vitro* susceptibility tests, including a checkerboard assay, were performed to determine the response of *Cr. neoformans* and *Cr. gattii* towards test drugs. Fractional inhibitory concentration index values were also calculated to establish the nature of the interaction between aspirin and fluconazole.

Results: The studied fungal strains revealed a dose dependent response profile towards both aspirin and oligomycin. However, aspirin had greater activity when directly compared to oligomycin, with 1 mM of aspirin achieving a MIC breakpoint of at least 70 % reduction in growth for both yeasts. Furthermore, an indifferent interaction was observed for both yeasts when pairing aspirin with fluconazole.

Conclusion: The levels of aspirin tested in this study were well within recommended concentrations of aspirin in the blood for the treatment of a number of physiological conditions in humans, highlighting the potential application of aspirin in the treatment of cryptococcal infections.

3.2 INTRODUCTION

Cryptococcosis is a life-threatening infectious disease, which may present in either immunocompetent or immunocompromised persons (Day, 2004). The etiological agents of cryptococcosis, *Cryptococcus* (*Cr.*) *neoformans* and *Cr*. *gattii*, are pathogenic fungal species of basidiomycetous origin (Cooper, 2011). Inhalation of these yeast cells usually leads to colonisation of the lower respiratory tract resulting in pulmonary cryptococcosis. If the local infection is not cleared, particularly in immunocompromised individuals, the cells can disseminate and cause a deadly inflammatory condition of the central nervous system (Day, 2004; Eisenman et al., 2007). A recent study by Centers for Disease Control and Prevention estimates cryptococcosis as the leading cause of death among HIV-infected persons in sub-Saharan Africa (Park et al., 2009). And the high burden of HIV in South Africa, where 10 % of the population is estimated to be living with HIV (Statistics South Africa, 2011), has now made cryptococcosis a serious public health concern.

Although cryptococcosis is relatively easy to diagnose through Indian ink staining and antigen determination, many deaths still ensue in part due to drug resistance (Saha et al., 2008; Tseng and Perfect, 2011). Resistance, which is an inherent trait in natural selection, can allow these yeasts to remain dormant and recur later, resulting in new complications such as in immune reconstitution recovery syndrome - where the host immune response can now recognise pathogenic antigens (Jarvis et al., 2007). To this end, new strategies are required to enhance management of cryptococcal infections.

Our understanding of the physiology of these yeasts indicates that they are non-fermentative (Kwon-Chung, 2011), requiring functional mitochondria to sustain growth and other energy-driven cellular processes (Litter, 2004). Mitochondria are reported to produce approximately 95 % of the cell's ATP (Nadanaciva et al., 2007). The high dependency of *Cryptococcus* on mitochondrial-derived ATP for survival highlights these organelles as target sites. Moreover, the critical role of this organelle in cellular pathology is also well documented (Ralph and Neuzil, 2009).

To test the importance of mitochondria in controlling growth of these medically important non-fermentative fungal pathogens, we assessed the antifungal activity of acetylsalicylic acid (ASA: aspirin) (anti-inflammatory drug) and oligomycin (antibiotic), both reported to induce mitochondrial damage via disruption of mitochondrial-energy production processes (Glasgow et al., 1999; Norman et al., 2004; Nadanaciva et al., 2007). When conceiving such studies, consideration should be taken in order to realise the desired therapeutic outcome without adversely affecting human mitochondria (Sebolai et al., 2012). In addition, we further examined the combined effects of aspirin and fluconazole (FCZ) against *Cr. neoformans* and *Cr. gattii*.

3.3 MATERIALS AND METHODS

Strains.

Two clinical strains: one *Cr. neoformans* and one *Cr. gattii* were examined in this study. These strains were obtained from Universitas Academic Hospital, Bloemfontein, South Africa. Strain details are shown in Table 1.

Medium.

All drug susceptibility tests, including the checkerboard assay, were performed in a chemically defined medium viz. yeast nitrogen base (YNB: 6.7 $g.L^{-1}$) (Difco Laboratories, United States) broth, which was supplemented with 40 $g.L^{-1}$ glucose (Saarchem, South Africa). The pH of the medium was adjusted to pH 5.4 ± 0.1.

Drugs.

Standard powders of aspirin (Sigma, South Africa), oligomycin (Merck, South Africa) and fluconazole (RT Corp., United States) were used in this study. Aspirin was prepared in absolute ethanol (Merck, South Africa) to obtain a stock solution of 80 $g.L^{-1}$. Oligomycin was dissolved in dimethylsulfoxide *(*Merck, South Africa) while dimethylformamide (Merck, South Africa) was used to reconstitute fluconazole in order to yield stock concentrations of 50 $mg.mL^{-1}$ and 10 $mg.mL^{-1}$, respectively. The concentrations of drug diluents, in which the stock solutions were prepared, never exceeded 1 %. In all experiments, a respective solvent control(s) were included and they did not yield a significant growth reduction when compared to a drug-free control (data not shown).

Cultivation and standardisation of cells.

The strains were maintained on yeast-malt-extract (YM) agar (3 $g.L^{-1}$ yeast extract, 3 $g.L^{-1}$ malt extract, 5 $g.L^{-1}$ peptone, 10 $g.L^{-1}$ glucose, 16 $g.L^{-1}$ agar; Merck, South Africa). A loopful of cells (1.1000 mL^{-1} loop) was taken from a 48 h old YM agar plate and cultivated in a 250 mL conical flask, containing 100 mL of YNB (6.7 $g.L^{-1}$) broth supplemented with 4 % (w/v) glucose. After a 48 h incubation period, cells were washed twice using phosphate buffered saline (PBS: Oxoid, South Africa), and then standardised spectrophotometrically (photoLab S 6: WTW, Germany) at 690 nm to reach a turbidity of 0.5 McFarland standard in fresh sterile YNB media, before further being used during drug susceptibility studies and checkerboard assay.

Drug susceptibility testing.

The effects of aspirin and oligomycin were independently assessed in duplicate for each strain, in a direct comparative study using a final drug concentration gradient in YNB media of 0.01 mM, 0.1 mM and 1 mM. Studies were performed in sterile, disposable 96-well flat-bottom microtitre plates (Greiner Bio-One, Germany). Aliquots of 100 µL of each drug, at twice the desired final concentrations as stated above, were dispensed into wells. Following this, 100 µL of the inoculum (approximately 0.5 McFarland standard) was added. The plates were incubated for 48 h at 37 ^{o}C before using a spectrophotometer (SpectraMax M2: Molecular Devices, United States) to read the optical density (OD) at 690 nm. The relationship between OD readings and colony counts of *Cr*. *neoformans* drug-free control was determined and yielded a correlation coefficient (r) of 0.92 (Figure 1). Subsequently, the OD was used to quantifying the drug response

by determination of the percentage reduction in growth. Since no interpretive breakpoints have been determined for these drugs, the minimum inhibitory concentration (MIC) was defined as the lowest drug concentration that resulted in at least 70 % reduction in fungal growth, compared to the drug-free control.

In a separate experiment, in anticipation of the checkerboard assay, OD readings were also measured for fluconazole, after treating cells for 48 h at 37 ^{o}C. Since no fluconazole interpretive breakpoints have been established for *Cr. neoformans* (Arechavala et al., 2009), for our purpose in this study; we used those documented in M27-A2 for *Candida* (CLSI, 2002). More to the point, 32 µg.mL^{-1} (considered susceptible dose-dependent [S-DD]) and 64 µg.mL^{-1} (considered resistant [R]) were chosen.

Checkerboard assay.

A checkerboard assay was designed pairing aspirin (1 mM) and fluconazole (32 µg.mL^{-1} and 64 µg.mL^{-1}) in a sterile, disposable 96-well flat-bottom microtitre plate (Table 2). A constant inoculum, concentrated to a 0.5 McFarland standard, was used. The plates were incubated for 48 h at 37 ℃ before reading the results. At the end of the incubation period, the fractional inhibitory concentration (FIC) index (FICI) was calculated. Fractional inhibitory concentration index was defined as $\Sigma FIC = FIC_A + FIC_B$ ([MIC of drug A in combination/MIC of drug A alone] + [MIC of drug B in combination/MIC of drug B alone]) (Meletiadis et al., 2005). Fractional inhibitory concentration index values were determined to establish if there was synergism (≤ 0.5), indifference (> 0.5 and ≤ 4) or antagonism (≥ 4). This was done in triplicate.

Statistical note.

Microsoft Excel 2010 was used to calculate mean values, standard deviations as well as the correlation coefficient.

3.4 RESULTS

Aspirin is more inhibitory than oligomycin.

The need for effective antifungal drugs for the improved management of cryptococcal infections necessitated this study. Over time, non-traditional antimicrobials have been used successfully to treat (often displaying synergism or antagonism when combined with traditional antimicrobials) non-infectious human diseases (Cederlund and Mårdh, 1993). Therefore, in our study, the activity of non-traditional antimicrobials i.e. aspirin and oligomycin, suggested in literature to be ideal candidate drugs for controlling non-fermentative fungal species, including fungal pathogens (Kock et al., 2007), was examined. Here, the two strains, *Cr. neoformans* and *Cr. gattii*, showed a dose dependent response profile against both test drugs when compared to their respective drug-free controls (Figure 2 and Figure 3). However, a closer examination of the comparative study results revealed that aspirin had greater activity than oligomycin, with 1 mM of aspirin achieving a MIC breakpoint of at least 70 % growth reduction against both pathogenic fungal strains. In contrast, 1 mM of oligomycin could only yield 22 % and 36 % growth reduction against *Cr. neoformans* and *Cr. gattii*, respectively. The observed greater activity of aspirin may be due to its dual action on mitochondria i.e. inhibition of beta-oxidation pathway and the uncoupling of the electron transport chain (Glasgow et

al., 1999; Norman et al., 2004) - which may lead to a complete shutdown of the mitochondria, while oligomycin only inhibit ATP synthase (Nadanaciva et al., 2007). It is important to emphasise that aspirin is a FDA approved drug, which has been proven to be important in the therapy of several physiological conditions in humans, with few reports of serious adverse effects i.e. gastro-intestinal haemorrhage (Levy, 1976; Chan, 1996; Plapp, 2008). Most encouraging about our findings is that the highest concentration of aspirin tested, 1 mM, is well within the recommended therapeutic ranges (Table 3) (Levy, 1976; Chan, 1996). This points to the potential clinical application of aspirin as an antifungal drug against *Cr. neoformans* and *Cr. gattii*.

Aspirin is not synergistic with fluconazole.

Cryptococcus neoformans, at all drug combination levels tested (1 mM and 32 µg.mL^{-1} [combined effect - 76 % growth reduction] and 1 mM and 64 µg.mL^{-1} [combined effect - 78 % growth reduction]), was shown to be indifferent meaning the combination effect was equal to the effects of the most active component, i.e. aspirin (Table 2) (Scholar and Pratt, 2000; Bharadwaj et al., 2003). According to Scholar and Pratt (2000), this is the most frequent outcome when pairing two drugs. For *Cr*. *gattii*, the combined effect was found to be indifferent at all drug combination levels tested, with the combined effect of 1 mM and 32 µg.mL^{-1} and 1 mM and 64 µg.mL^{-1} resulting in 79 % and 80 % growth reduction, respectively. Furthermore, the combined effect was also determined to be indifferent at concentrations below the chosen interpretive breakpoints for both fungal strains (data not shown).

3.5 DISCUSSION

This study was able to demonstrate *in vitro* anti-*Cryptococcus* activity of aspirin and oligomycin. The studied fungal strains responded to the test drugs in a dose dependent manner with aspirin showing greater activity when directly compared to oligomycin. The highest concentration of aspirin tested, 1 mM, was found to be well within recommended concentrations of aspirin in the blood for the treatment of indicated physiological conditions. However, further analysis revealed no synergism between aspirin and fluconazole rather, an indifferent outcome was achieved. In future, it would be ideal that a number of strains representing both *Cr*. *neoformans* and *Cr*. *gattii* are assessed to draw more concrete conclusions. In addition, a study that directly compares the inhibitory effects of aspirin and fluconazole should be conducted. Nonetheless, the findings of this study still point towards the potential application of aspirin in the treatment of cryptococcal infections. Interestingly, it was previously demonstrated that this anti-inflammatory, antimitochondrial drug, reduced encapsulation of *Cryptococcus neoformans* at 1 mM (Sebolai et al., 2008). The latter further highlights the possible application of aspirin in preventing infectious processes elicited by the cryptococcal capsule.

Over the years, previously registered presumably non-antimicrobial drugs have been given a “new lease on life” following the discovery that they also possess antimicrobial activity (Cederlund and Mårdh, 1993), and aspirin is one such drug. It will be interesting to determine if the antimicrobial activity of aspirin, as established in this study and others (Alem and Douglas, 2004), could be expanded and demonstrated in other medically

important pathogens. One such microbe is *Mycobacterium tuberculosis*, which, like *Cr. neoformans* and *Cr. gattii*, is highly aerobic. Moreover, it should be determined if aspirin, could also inhibit the production of fatty acid synthesis (FAS)-derived mycolic acid, based on the structural similarities between aspirin and acyl-portions of the FAS biosynthetic pathway (Sebolai et al., 2012).

3.6 REFERENCES

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TABLES

Table 1

The origin and identity of clinical strains used in this study.

Strain number	Strain name	Isolation	
		Source	Country
LMPE 001	*Cr. neoformans*	Human	South Africa
LMPE 070	*Cr. gattii*	Human	South Africa

Cr. = *Cryptococcus*; LMPE = Laboratory of Microbial Pathogenesis and Epidemiology.

Table 2

The *in vitro* susceptibility results of aspirin and fluconazole in combined therapy against *Cr. neoformans* and *Cr. gattii*. The FICI values are shown as an average of three independent experiments. Calculated FICI values ranging between 0.5 and 4 were interpreted (INT) as indifferent (IND).

Checkerboard assay						
Strain name	**Drug combination**		**Combined effect**			
	FCZ concentration	**ASA concentration**	**% Reduction in growth**	**INT**	**FICI value**	**INT**
Cr. neo.	32 µg.mL^{-1} (62 % +/- 0.009)	1 mM (71 % +/- 0.008)	76 % (+/- 0.005)	≥ MIC_{70}	0.98	IND
	64 µg.mL^{-1} (65 % +/- 0.005)	1 mM (71 % +/- 0.008)	78 % (+/- 0.007)	≥ MIC_{70}	1.11	IND
Cr. gat.	32 µg.mL^{-1} (50 % +/- 0.016)	1 mM (80 % +/- 0.001)	79 % (+/- 0.004)	≥ MIC_{70}	1.37	IND
	64 µg.mL^{-1} (54 % +/-0.02)	1 mM (80 % +/- 0.001)	80 % (+/- 0.007)	≥ MIC_{70}	1.41	IND

ASA = aspirin; *Cr. gat.* = *Cryptococcus gattii*; *Cr. neo.* = *Cryptococcus neoformans*; FCZ = fluconazole; FICI =Fractional inhibitory concentration index.

Table 3

Aspirin's concentration range generally accepted as optimum for safe and effective therapy in the blood (Levy, 1976).

Physiological condition / Indication	**Therapeutic dosage**
Arthritis	20 $mg.dL^{-1}$ to 30 $mg.dL^{-1}$
Rheumatic fever	20 $mg.dL^{-1}$ to 30 $mg.dL^{-1}$
Antifungal activity against *Cr. neoformans* and *Cr. gattii*	18 $mg.dL^{-1}$ (approx. 1 mM)

FIGURES

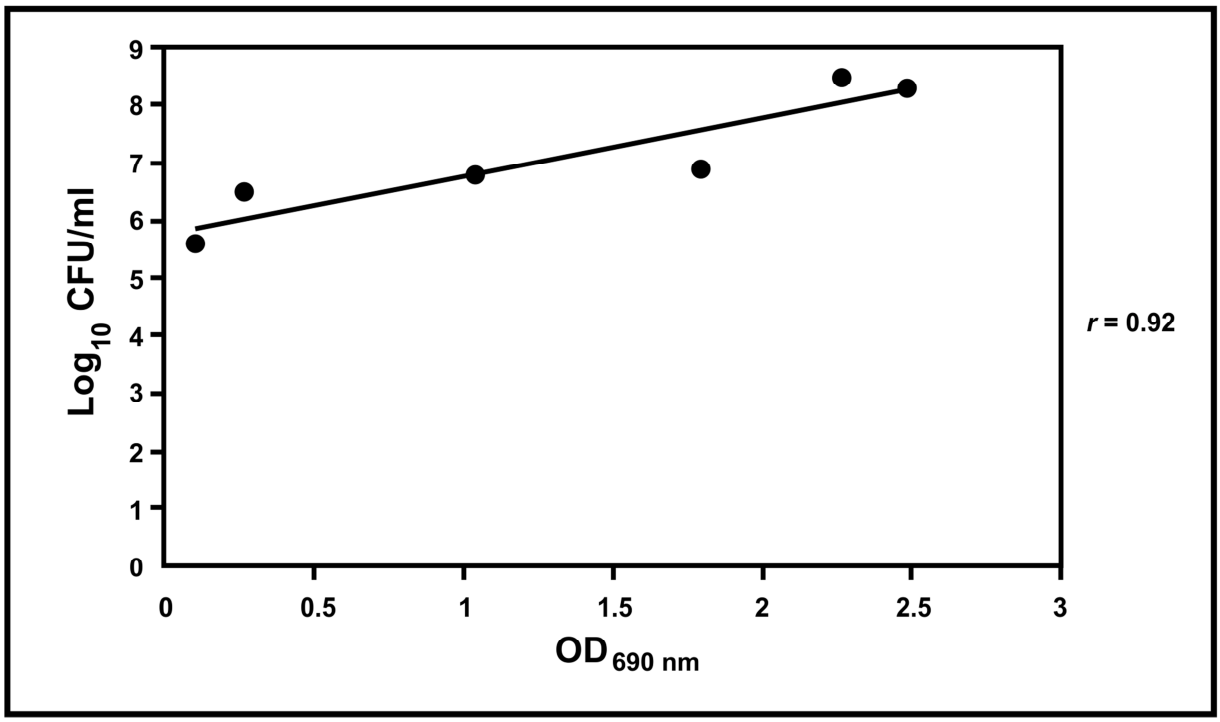

Figure 1. The relationship between optical density (OD), read at 690 nm, and colony counts when measuring growth of a drug-free control (*Cr*. *neoformans* LMPE 001), for 48 h. The correlation coefficient (r) was determined to be 0.92.

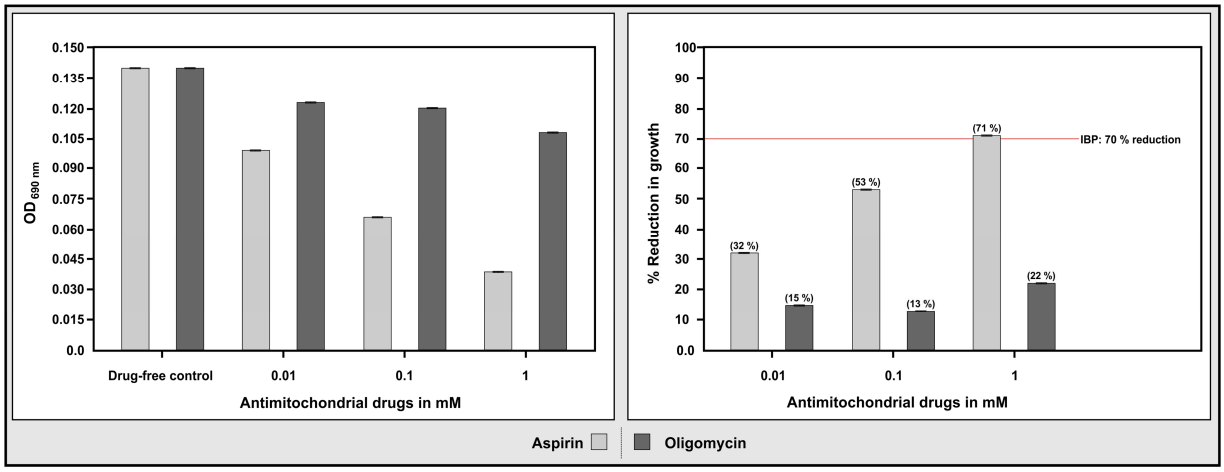

Figure 2. A comparative *in vitro* susceptibility profile of aspirin and oligomycin against *Cr*. *neoformans.* Optical density (OD) readings as well as the % reduction in growth values are shown as an average of two independent experiments with error bars. IBP = Interpretive breakpoint.

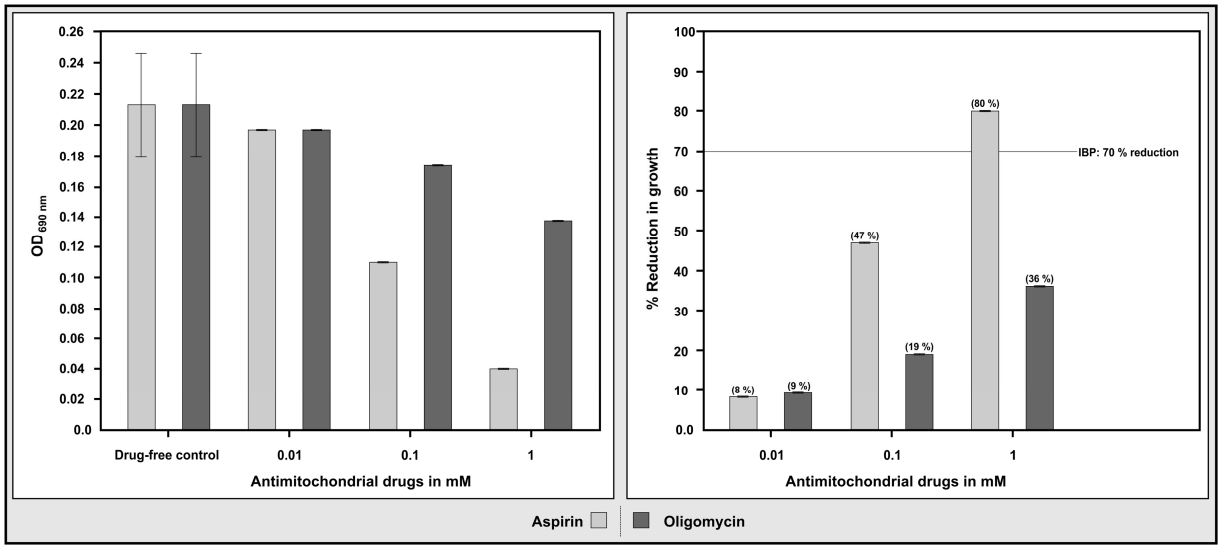

Figure 3. A comparative *in vitro* susceptibility profile of aspirin and oligomycin against *Cr*. *gattii*. Optical density (OD) readings as well as the % reduction in growth values are shown as an average of two independent experiments with error bars. IBP = Interpretive breakpoint.

DISSERTATION SUMMARY

In this dissertation, an attempt was made to study the epidemiology of cryptococcosis by first estimating the incidence rates over a two-year period, 2011 and 2012. The major findings from this part of the study included establishing that: 1) cases were more prevalent among Blacks (Africans, Coloureds and Indians), and this is in line with the assertion by WHO that diseases such as cryptococcosis are more poverty-related, 2) the distribution pattern of cryptococcosis across different age groups mirrored that of HIV-infected persons, and 3) the number of cryptococcosis cases were quite low for the study period (representing less than 0.1 % of the Bloemfontein population), this was surprising and unexpected given the huge HIV positive population in South Africa, and by extension in Bloemfontein, that is at risk of acquiring this AIDS-defining illness. It is documented in literature that the currently employed methods for the routine diagnosis of cryptococcosis often yield inconsistent test results, thereby; influencing the number of reported cases, which are important for health officials. But more importantly, these inconsistencies have far reaching consequences as they may negatively influence patient outcomes. Therefore, we sought to investigate the usefulness of molecular methods in identifying the etiological agents of cryptococcosis viz. *Cr. neoformans* and *Cr. gattii*. Here, the ITS, including the 5.8 gene, intra-specific variation between the tested strains allowed for their delineation into three traditional varieties of *Cr. neoformans*. To be specific, we identified: 1) 51 strains of *Cr*. *neoformans* var. *grubii*, 2) 13 strains of *Cr. neoformans* var. *neoformans*, and 3) 6 strains of *Cr. neoformans* var. *gattii*. Given the geographical distribution of *Cr. gattii*, thought to be limited to the tropics, we sought to confirm the six positive cases obtained from the molecular identification study by cultivating all 70 strains on CGB media. Here, only the six strains

of *Cr. gattii* (constituting Cr. *neoformans* var. *gattii*) were able to turn the media blue via hydrolyzing glycine whereas all 64 *Cr*. *neoformans* (constituted by *Cr*. *neoformans* var. *neoformans* and *Cr*. *neoformans* var. *grubii*) strains we unable to do so. Thus confirming the molecular test results. Perhaps, the important finding from the molecular study, is the uncovering of a restriction site for the enzyme *Ssp*I, which is present only in the distinct species, *Cr. neoformans* but absent in the distinct species, *Cr. gattii*. This is important as this eliminates sequencing from the identification process, thus shortening the time required to obtain test results and simultaneously cuts down the operational costs. In addition, it also makes it easier to optimise the protocol, as laboratory technicians will require no specialised training. Although a patient's outcome is dependent on the timely release of an accurate diagnosis, treatment is also crucial. Today, the widespread usage of antifungals has led to increased resistance. Therefore, there is a constant need to find alternative drugs in order to improve patient outcomes. In this part of the study, we considered antimitochondrial drugs i.e. aspirin and oligomycin, as possible candidate drugs for controlling the growth of *Cr*. *neoformans* and *Cr*. *gattii*. *In vitro* susceptibility results, based on a direct comparative experiment, revealed that aspirin was more inhibitory than oligomycin, with 1 mM aspirin yielding at least 70 % growth reduction. Meanwhile, the checkerboard assay revealed that aspirin was not synergistic with fluconazole, however; it was indifferent, which is a frequent outcome in combined therapy. In future, it will be prudent to directly compare aspirin with fluconazole. Nonetheless, in this study, aspirin was proven to be useful as an antifungal agent with the highest concentration tested, 1 mM aspirin, being well within the recommended doses in the blood.

Key words: Aspirin; Cryptococcosis; *Cryptococcus gattii*; *Cryptococcus neoformans*; Epidemiology; Fluconazole; Incidence rate; In vitro susceptibility; Sequencing; Treatment.

VERHANDELING OPSOMMING

Met hierdie verhandeling is gepoog om die epidemiologie van cryptococcose te bestudeer, deur eerstens die voorkoms daarvan oor ‘n tydperk van twee jaar, 2011 en 2012, te skat. Die hoofbevindinge van hierdie deel van die studie sluit in: 1) gevalle was meer algemeen onder swart mense (Afrikane, Kleurlinge en Indieërs), en dit stem ooreen met die stelling deur die WGO dat siektes soos cryptococcose meer armoede verwant is, 2) die verspreidingspatroon van cryptococcose oor verskillende ouderdomsgroepe is dieselfde as die van persone met MIV en 3) die hoeveelheid cryptococcosegevalle was laag vir die studietydperk (verteenwoordig minder as 0.1 % van die bevolking van Bloemfontein), dit was verrassend en onverwags gegewe die groot MIV positiewe bevolking in Suid-Afrika, en dus in Bloemfontein, wat die risiko loop om hierdie VIGS-definiërende siekte op te doen. Uit die literatuur is dit bekend dat die metodes wat tans gebruik word vir die roetine diagnose van cryptococcose, dikwels teenstrydige resultate lewer, en sodoender die hoeveelheid gerapporteerde gevalle beïnvloed, wat belangrik is vir gesondheidsbeamptes. Belangriker nog, hierdie strydighede het verreikende gevolge aangesien dit die pasiënte se uitkomste negatief mag beïnvloed. Dus het ons gepoog om die bruikbaarheid van molekulêre metodes vir die identifikasie van die veroorsakende agente van cryptococcose, nl. *Cr. neoformans* en *Cr. gattii* te bepaal.. Hier het die intra-spesifieke variasie in die ITS, insluitend die 5.8 geen, tussen die stamme hulle afbakening in die drie tradisionele variëteite van *Cr. neoformans* toegelaat. Om spesifiek te wees, het ons die volgende geïdentifiseer: 1) 51 stamme van *Cr*. *neoformans* var. *grubii*, 2) 13 stamme van *Cr. neoformans* var. *neoformans*, en 3) 6 stamme van *Cr. neoformans* var. *gattii*. Gegewe die geografiese verspreding van *Cr. gattii*, wat vermoedelik beperk is tot die trope, het ons gepoog om

die ses positiewe gevalle geïdentifiseer deur die molekulêre identifikasiestudie te bevestig deur al 70 stamme op CGB media te kweek. Slegs die ses *Cr. gattii* stamme (bestaande uit Cr. *neoformans* var. *gatti*) kon die media blou kleur via die hidrolise van glisien, terwyl al 64 *Cr*. *neoformans* stamme (bestaande uit *Cr*. *neoformans* var. *neoformans* en *Cr*. *neoformans* var. *grubii*) dit nie kon doen nie. Dus bevestig dit die molekulêre resultate. Die belangrikste bevinding van die molekulêre studie is moontlik die onthulling van ‘n beperkingsnypunt vir die ensiem *Ssp*I, wat slegs teenwoordig is in die spesie, *Cr. neoformans* maar afwesig is in die spesie, *Cr. gattii*. Dit is belangrik aangesien dit basispaaropeenvolgingbepaling uit die identifikasieproses haal, en dus die tyd wat nodig is om resultate te kry asook die operasionele koste veminder. Daar benewens vergemaklik dit die optimisering van die protokol, aangesien laboratoriumtegnici geen gespesialiseerde opleiding sal nodig hê nie. Alhoewel ‘n pasiënt se uitkoms afhang van die tydige beskikbaarstelling van ‘n akkurate diagnose, is behandeling ook noodsaaklik. Huidiglik het die algemene gebruik van antifungale middels gelei tot verhoogde weerstandbiedendheid. Dus is daar ‘n volgehoue noodsaaklikheid om alternatiewe middels te vind om pasiënte se uitkomste te verbeter. In hierdie deel van die studie is die anti-mitochondriale middels, aspirien en oligomisien, geëvalueer as moontlike kandidate vir die beheer van die groei van *Cr*. *neoformans* en *Cr*. *gattii*. *In vitro* vatbaarheidsresultate, gebaseer op ‘n direkte vergelykende eksperiment, het getoon dat aspirien meer inhiberend is as oligomisien, met 1 mM aspirien wat groei met ten minste 70 % verminder het. Intussen het die skaakbordtoets getoon dat aspirien nie sinergisties was saam met flukonazool nie, maar onverskillig, wat dikwels die uitkoms van kombinasieterapie is. In die toekoms sal dit wys wees om

aspirien direk met flukonazool te vergelyk. Nietemin is aspirien in hierdie studie bewys as ‘n nuttige antifungale middel met die hoogste getoetste konsentrasie, 1 mM aspirien, wat binne die aanbevole dosis in die bloed is.

Sleutelwoorde: Aspirien; Cryptococcose; *Cryptococcus gattii*; *Cryptococcus neoformans*; Epidemiologie; Flukonazool; Voorkoms; In vitro vatbaarheid; Basispaaropeenvolginsbepaling; Behandeling.

APPENDIX

Table 1

A record of collected sample cultures and the corresponding patient biographic information.

Sample number	Collection date	Test performed to I.D. sample		Isolation		Biographic information		
		Indian ink	Serology	Source	[§] Location	Age	Race	Gender
1	Jan 2011	Positive	Reactive	Human	BFN			
2								
3								
4								
5								
6								
7								
8								
9								

[§]BFN = Bloemfontein.