Molecular epidemiology of *Cryptococcus neoformans* and *Cryptococcus gattii* in South Africa

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A Thesis submitted to the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Doctor of Philosophy.

Johannesburg, November 2021.

Declaration

I, Serisha Devi Naicker, declare that this Thesis is my own, unaided work. It is being submitted for the Degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

Serisha Devi Naicker

8th day of November 2021 in Johannesburg.

Dedication

To my family and friends that never stopped believing in me.

“Happiness is only real when shared” – Christopher McCandless

Presentations arising from this thesis

1. Serisha D. Naicker, Rindidzani E. Magobo, Tsidiso G. Maphanga, Marelize van Wyk, Nelesh P. Govender. Molecular epidemiology of *Cryptococcus neoformans* in South Africa. 19th Congress of the International Society for Human and Animal Mycology (ISHAM). 4-8 May 2015. Melbourne, Australia.
2. Serisha D. Naicker, Nelesh P. Govender. Multi-locus sequence typing of clinical *Cryptococcus gattii* isolates from South Africa. 10th International Conference on *Cryptococcus* and Cryptococcosis (ICCC-10). 26-30 March 2017. Foz do Iguacu, Brazil.
3. Serisha D. Naicker. Molecular epidemiology of *Cryptococcus* in South Africa. 20th Congress of the International Society for Human and Animal Mycology (ISHAM). 30 June-4 July 2018. Amsterdam, The Netherlands.
4. Serisha D. Naicker, Ruth S. Mpembe, Tsidiso G. Maphanga, Thokozile G. Zulu, Daniel Desanto, Jeannette Wadula, Nomonde Mvelase, Caroline Maluleka, Kessendri Reddy, Halima Dawood, Motlatji Maloba and Nelesh P. Govender for GERMS-SA. Decreasing fluconazole susceptibility of clinical South African *Cryptococcus neoformans* isolates over a decade. 8th Federation of Infection Disease Societies of South Africa (FIDSSA) Conference. 7-9 November 2019. Johannesburg, South Africa.

Publications arising from this thesis

1. Serisha D. Naicker, Ruth S. Mpembe, Tsidiso G. Maphanga, Thokozile G. Zulu, Daniel Desanto, Jeanette Wadula, Nomonde Mvelase, Caroline Maluleka, Kessendri Reddy, Halima Dawood, Motlatji Maloba, Nelesh P. Govender. Decreasing fluconazole susceptibility of clinical South African *Cryptococcus neoformans* isolates over a decade. PLOS Neglected Tropical Diseases, 2020, 14 (3), 1-11. (**Chapter 4**)
2. Serisha D. Naicker, Rindidzani E. Magobo, Tsidiso G. Maphanga, Carolina Firacative, Erika van Schalkwyk, Juan Monroy-Nieto, Jolene Bowers, David M. Engelthaler, Liliwe Shuping, Wieland Meyer, Nelesh P. Govender for GERMS-SA. Genotype, antifungal susceptibility, and virulence of clinical South African *Cryptococcus neoformans* strains from national surveillance, 2005–2009. Journal of Fungi, 2021, 7 (338), 1-17. (**Chapter 2**)

Additional manuscript in preparation

1. Serisha D. Naicker, Carolina Firacative, Erika van Schalkwyk, Tsidiso G. Maphanga Juan Monroy-Nieto, Jolene Bowers, David M. Engelthaler, Wieland Meyer, Nelesh P. Govender for GERMS-SA. Molecular type distribution and fluconazole susceptibility of clinical *Cryptococcus gattii* isolates from South African laboratory-based surveillance, 2005-2013. (To be submitted to PLOS Neglected Tropical Diseases) (**Chapter 3**)

Abstract

Introduction

Cryptococcus neoformans and *Cryptococcus gattii* are fungal pathogens found in the environment, which cause potentially life-threatening meningoencephalitis among human immunodeficiency virus (HIV) -infected and HIV-uninfected persons in South Africa. *Cryptococcus* is the most common cause of meningitis among South African adults. In South Africa, fluconazole is still used in combination with amphotericin B for induction treatment of HIV-associated cryptococcal meningitis and as monotherapy for the consolidation and maintenance treatment phases. Molecular characterisation using multilocus sequence typing (MLST) is a good discriminatory tool to genotype *Cryptococcus*. There is a high genetic diversity observed in strains isolated in southern Africa. Additionally, it was hypothesised that the molecular type VNB, that is endemic to southern Africa, may be more virulent among the *C. neoformans* molecular types. The overall aim of this study was to explore the genetic diversity, antifungal susceptibility and virulence of the two pathogenic *Cryptococcus* species complexes causing human disease in South Africa.

Materials and Methods

Multilocus sequence typing and fluconazole susceptibility testing was performed on a randomly selected sample of 251 *C. neoformans* and 146 *C. gattii* isolates collected from South African patients with cryptococcosis through laboratory-based surveillance from 2005 to 2009 and 2005 to 2013, respectively. We examined the association between clinical characteristics of patients and cryptococcal molecular type, and the effect of molecular type on in-hospital mortality. We performed whole genome phylogenetic analysis of 15 *C. neoformans* isolates with the molecular type VNB and tested their virulence in a *Galleria*

mellonella model. We also sequenced the genomes of 101 *C. gattii* isolates with the molecular type VGIV. We prospectively collected *C. neoformans* isolates from 1 January through 31 March 2017 from persons with a first episode of culture-confirmed cryptococcal disease. We determined fluconazole minimum inhibitory concentration (MIC) values (range: 0.125 μg/ml to 64 μg/ml) of 229 *C. neoformans* isolates using custom-made broth microdilution panels prepared, inoculated and read according to Clinical and Laboratory Standards Institute M27-A3 and M60 recommendations. We interpreted fluconazole MIC values using published epidemiological cutoff values (ECVs). The MIC values obtained in the 2017 survey were compared to MICs of 249 isolates from earlier surveillance (2007–2008).

Results

For *C. neoformans*, most isolates collected between 2005-2009 had the molecular type VNI (206/251, 82%), followed by VNII (25/251, 10%), VNB (15/251, 6%), and VNIV (5/251, 2%); with a total of 67 sequence types identified. There were no differences in fluconazole MIC values among molecular types and the majority of strains had low MIC values within the wild-type range (MIC_{50} of 1 μg/ml and MIC_{90} of 4 μg/ml). We did not find any associations between patients' clinical characteristics and molecular type and between molecular type and in-hospital mortality. Fifteen VNB strains were not highly virulent in a *G. mellonella* larval model. The majority of these VNB strains belonged to the VNBII clade and were very closely related by phylogenetic analysis.

In the 2017 survey, the fluconazole MIC_{50}, MIC_{90} and geometric mean MIC of 229 *C. neoformans* isolates was 4, 8 and 4.11 μg/ml compared to 1, 2 and 2.08 μg/ml in 2007–2008 (n = 249) respectively.

For *C. gattii*, most isolates had the molecular type VGIV (101/146, 70%), followed by VGI (27%, 40/146), VGII (2%, 3/146) and VGIII (1%, 2/146); with a total of 99 sequence types identified. The majority of *C. gattii* strains in our study had low fluconazole MIC values within the wild-type range (MIC_{50} of 4 μg/ml and MIC_{90} of 16 μg/ml). VGIV isolates had a lower fluconazole MIC_{50} value compared to non-VGIV isolates but still within one double-dilution. Compared to HIV-seronegative patients, HIV-seropositive patients were ten times more likely to be infected with a VGIV isolate on multivariable analysis, though with small numbers, the confidence interval (CI) was very wide (95% CI: 1.93-55.31, p=0.006). We did not find any association between molecular type and in-hospital mortality. Whole genome phylogenetic analysis of the VGIV strains in our study showed that the VGIV molecular type was highly diverse, with only some strains being closely related in two small clusters of ten and six strains, respectively.

Conclusion

Our studies showed that South African clinical *C. neoformans* and *C. gattii* isolates had a high genetic diversity with VNI and VGIV being the dominant molecular types among the two sibling species, respectively. Clinical *C. neoformans* and *C. gattii* strains collected between 2005 and 2013 mostly had low fluconazole MIC values, although the MIC_{50} and MIC_{90} values for *C. gattii* were two double-dilutions higher compared to those for *C. neoformans* and still within the wild-type range. We also observed that fluconazole MIC_{50}

and MIC_{90} values were two-fold higher in clinical South African *C. neoformans* isolates from first episodes of meningitis collected in 2017 compared to 2007–2008. Our findings led to higher fluconazole dose recommendations for induction and consolidation phases in the 2019 Southern African guideline for HIV-associated cryptococcosis. The few South African VNB strains in our study were very closely related by whole genome phylogenetic analysis, suggesting a common environmental source. Whole genome phylogenetic analysis of 98 South African VGIV strains confirmed their high genetic diversity with most strains not being closely related. However, we observed two small clusters of closely related strains, one cluster from patients living in the Gauteng and Limpopo Provinces and the other cluster consisting of patients from the Mpumalanga Province of South Africa suggesting a similar environmental source.

Acknowledgements

I would like to express my sincerest gratitude to:

- My supervisors: Nelesh Govender and Wieland Meyer for their support, guidance, patience and for always motivating me to do better.
- CHARM staff members for their technical support.
- Translational Genomics Research Institute (Pathogen and Microbiome Division) for technical assistance.
- Members of GERMS-SA for conducting the surveillance study and all participants in the study.
- Funding sources: National Health Laboratory Service Research Trust and National Institute for Communicable Diseases.

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Nomenclature

ACTA	Advancing Cryptococcal Treatment in Africa
AIDS	acquired immunodeficiency syndrome
AREC	Animal Research Ethics Committee
ART	antiretroviral treatment
AST	antifungal susceptibility testing
AT	allele type
BMD	broth microdilution
BWA	Burrows-Wheeler Aligner
CAST-NET	Cryptococcal Antigen Screening National Evaluation Team
CDW	Corporate Data Warehouse
CFU	colony forming unit
CGB	canavanine-glycine-bromothymol blue
CHARM	Centre for Healthcare Associated Infections, Antimicrobial Resistance and Mycoses
CI	confidence interval
CLAT	cryptococcal latex agglutination test
CLSI	Clinical and Laboratory Standards Institute
CNS	central nervous system
CRAG	cryptococcal antigenaemia

CSF	cerebrospinal fluid
DMP	Diagnostic Media Products
DNA	deoxyribonucleic acid
ECV	epidemiological cutoff value
EIA	enzyme immunoassay
ESS	enhanced surveillance site
GCS	Glasgow Coma Scale
GPI	glycosylphosphatidyl inositol
GXM	glucuronoxylomannan
GXMGal	glucuronoxylomannogalactan
HIV	human immunodeficiency virus
IGS	intergenic spacer
IQR	inter-quartile range
IRIS	immune reconstitution inflammatory syndrome
ISHAM	International Society for Human and Animal Mycology
LFA	lateral flow immunochromatographic assay
LSU	large subunit
MIC	minimum inhibitory concentration
MIC_{50}	minimum inhibitory concentration required to inhibit 50% of organisms
MIC_{90}	minimum inhibitory concentration required to inhibit 90% of organisms

MLST	multilocus sequence typing
NASP	Northern Arizona SNP pipeline
NCBI	National Center for Biotechnology Information
NHLS	National Health Laboratory Service
NICD	National Institute for Communicable Diseases
OR	odds ratio
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PNW	Pacific Northwest
RNA	ribonucleic acid
RPMI	Roswell Park Memorial Institute
SAHCS	Southern African HIV Clinicians' Society
SNP	single nucleotide polymorphism
ST	sequence type
STI	sexually transmitted infection
TB	tuberculosis
USA	United States of America
WFCC	World Federation for Culture Collections
WGS	whole genome sequencing
WHO	World Health Organization

WITS	University of the Witwatersrand
ml	millilitre
µl	microlitre
µg/ml	microgram per microlitre

Chapter 1: Introduction/Literature Review

1.1 Burden of cryptococcal meningitis

In 2014, the global burden of cryptococcal meningitis among people living with human immunodeficiency virus/acquired immunodeficiency syndrome (HIV/AIDS) was estimated to be 223,100 cases per year with 181,100 deaths (Rajasingham et al., 2017). Globally, cryptococcal meningitis causes 15% of AIDS-related deaths and is the second most common cause of AIDS-related mortality after tuberculosis (TB) (Rajasingham et al., 2017). The global cryptococcal antigenaemia prevalence was estimated to be 6% among people living with HIV/AIDS with a CD4 cell count of less than 100 cells per µl with 278,000 global cryptococcal antigen positive cases (Rajasingham et al., 2017). Seventy three per cent of the global cryptococcal antigen-positive cases and cryptococcal meningitis cases occur in sub-Saharan Africa (Rajasingham et al., 2017). Seventy five per cent of deaths also occur in sub-Saharan Africa (Rajasingham et al., 2017).

As a consequence of South Africa's large HIV epidemic, *Cryptococcus* is the most common cause of meningitis among adults, accounting for 62% of microbiologically-confirmed cases and is more common than bacterial meningitis (Britz et al., 2016). Based on National Institute for Communicable Diseases (NICD) data, 6,425 patients with laboratory-confirmed incident cryptococcal episodes were reported during 2019 with an incidence risk of one reported case per 1,000 HIV-infected persons in South Africa (GERMS-SA Annual Report, 2019). A third of the patients admitted to South African hospitals with cryptococcal meningitis die in hospital (GERMS-SA Annual Report, 2012, Quan et al., 2019). Cryptococcal meningitis is fatal in more than half of the treated cases in South Africa in routine hospital care (Lindani et al., 2013, Quan et al., 2019). However, a large number of patients who are successfully

treated initially also experience recurrent cryptococcal disease (Jarvis et al., 2010). Symptomatic relapse accounted for 23% of all laboratory-confirmed cases of meningitis in one South African hospital (Jarvis et al., 2010). This can be caused by a new infection, persistence of the initial infecting strain, or relapse of the infection due to the original strain or strains following apparent initial clearance (Van Wyk et al., 2014).

1.2 Clinical presentation of disease

Cryptococcal disease was first identified in 1894 (Busse, 1894) but was only recognised as a major health threat in the 1980s with the onset of the AIDS pandemic (Perfect and Casadevall, 2002). Extra-pulmonary cryptococcosis is an AIDS-defining illness but other immunocompromised patients can be infected, such as those on chronic corticosteroid treatment or those who undergo solid organ transplantation (Brizendine et al., 2011, May et al., 2016). The disease can also occur in apparently immunocompetent individuals (Kwon-Chung et al., 2014). These individuals could have immunological defects that are unrecognised or undiagnosed (Kwon-Chung et al., 2014). Infections are mainly caused after individuals inhale fungal spores or desiccated yeast cells from the environment (Kwon-Chung et al., 2014). The major body sites affected are the lungs and the central nervous system (CNS) (Maziarz and Perfect, 2016). The clinical presentation of pulmonary infection can range from subclinical disease, an incidental pulmonary nodule on a chest radiograph to severe pneumonia with respiratory distress (Brizendine et al., 2011, Maziarz and Perfect, 2016). Upon reactivation of latent infection or at the time of a new infection, cryptococcal disease can spread to the CNS through the bloodstream causing cryptococcal meningoencephalitis or meningitis (Maziarz and Perfect, 2016). There are three proposed mechanisms on how the fungus crosses the blood brain barrier; these are paracytosis, transcytosis and the “Trojan horse” hypothesis (May et al., 2016). (1) Paracytosis is where

Cryptococcus produces enzymes that breakdown the blood brain barrier thereby causing damage and allowing cells to cross between host endothelial cells that line the blood vessels in the brain (Vu et al., 2014). (2) Transcytosis involves *Cryptococcus* cells moving directly through endothelial cells (Chang et al., 2004). (3) The "Trojan horse" hypothesis proposes that *Cryptococcus* cells are transported within host phagocytes across the barrier (Zaragoza, 2019). Symptoms of meningitis include headache, fever, lethargy, coma, or memory loss, which develop over several weeks (Perfect and Casadevall, 2002, Maziarz and Perfect, 2016). Patients starting antiretroviral treatment (ART) can also experience a paradoxical worsening of cryptococcal disease symptoms called immune reconstitution inflammatory syndrome (IRIS) (Brizendine et al., 2011). An IRIS-like syndrome can also occur in non-HIV-infected individuals with cryptococcal disease on antifungal treatment (Wu et al., 2020). Other body sites less frequently affected include skin, prostate, eyes and bones/joints (Maziarz and Perfect, 2016).

1.3 Treatment of cryptococcal meningitis

There are three main antifungals used for treatment of cryptococcal disease: liposomal or deoxycholate amphotericin B, flucytosine and fluconazole (Govender et al., 2019). Antifungal treatment of HIV-associated cryptococcal meningitis consists of the induction, consolidation and maintenance phases (Govender et al., 2019). The World Health Organization (WHO) recommends that amphotericin B with flucytosine is the ideal combination for the induction phase (Guidelines, 2018). These recommendations were guided by results from the Advancing Cryptococcal Treatment in Africa (ACTA) trial (Molloy et al., 2018). This trial showed that participants on amphotericin B-flucytosine combination therapy had a 31% mortality compared to a 45% mortality of patients on amphotericin B-fluconazole combination therapy at ten weeks (Molloy et al., 2018). Although flucytosine is not registered

in South Africa, a flucytosine access programme is currently being expanded across South Africa for the treatment of cryptococcal meningitis (Shroufi et al., 2020). In the absence of flucytosine, fluconazole is recommended as an alternative in combination with amphotericin B for the induction phase (Meiring et al., 2016). Fluconazole is also recommended for the consolidation and maintenance phases (Meiring et al., 2016).

Amphotericin B is fungicidal by binding to ergosterol in the fungal cell membrane thereby generating pores in the cell wall and membrane (Gray et al., 2012, May et al., 2016). It also induces cell death by oxidative damage (Gray et al., 2012, May et al., 2016). The deoxycholate formulation of this antifungal is more toxic than the lipid formulations (Hamill et al., 2010). A new treatment strategy including a single high dose of liposomal amphotericin B is currently being explored in the Ambition-CM trial and the trial results, expected in 2021, may alter international treatment recommendations (Lawrence et al., 2018). Flucytosine can either block deoxyribonucleic acid (DNA) synthesis or disrupt protein synthesis, which leads to growth arrest (May et al., 2016). It does this by being taken up by fungal cells by the enzyme cytosine permease. Once within the cell, it is deaminated into 5-fluorouracil by the enzyme cytosine deaminase. 5-Fluorouracil can be converted by the enzyme uracil phosphoribosyl transferase into two metabolites inside the fungal cell. These metabolites are 5-fluorouridine triphosphate (FURTP) and 5-fluorodeoxyuridine monophosphate (FdUMP) that cause DNA and ribonucleic acid (RNA) damage. 5-Fluorouridine triphosphate is incorporated into fungal RNA in place of uridylic acid causing premature chain termination thereby inhibiting RNA and subsequent protein synthesis. 5-Fluorodeoxyuridine monophosphate inhibits the enzyme thymidylate synthetase that is involved in DNA synthesis therefore inhibiting DNA synthesis (Waldorf and Polak, 1983, Vermes et al., 2000). Fluconazole is fungistatic by binding to and inhibiting the lanosterol

14α-demethylase enzyme (cytochrome P450-Erg11p or Cyp51p) encoded by the gene *ERG11* in the ergosterol biosynthesis pathway. This prevents production of ergosterol for the fungal cell membrane. Depletion of ergosterol causes alteration in the permeability and fluidity of the fungal cell membrane affecting membrane-bound enzymes involved in cell wall synthesis (Odds et al., 2003, Smith et al., 2015, May et al., 2016, Chang et al., 2018).

Antifungal resistance has been encountered to all of the aforementioned drugs (May et al., 2016). There are new drugs in development or in clinical trials for the treatment of cryptococcal disease (May et al., 2016). The orally active antifungal manogepix (APX001A or E1210) inhibits the synthesis of the cell wall component, glycosylphosphatidyl inositol (GPI) – anchored mannoprotein (Shaw et al., 2018). This molecule and its close analogues have been shown to be efficacious in *in vitro* and *in vivo* studies but it is not yet in clinical trials for treatment of cryptococcal meningitis (Shaw et al., 2018). VT-1129 inhibits ergosterol synthesis and was shown to be efficacious in a mouse model (Wiederhold et al., 2018). A phase one clinical trial is currently being conducted (https://ncats.nih.gov/trnd/projects/complete/antifungal-vt1129-cryptococcal-meningitis). Arylamidine T-2307 is fungicidal and targets the fungal mitochondrial membrane (Nishikawa et al., 2017). Clinical phase one studies were completed and there were no severe adverse effects, making it a promising new candidate for cryptococcosis (Nishikawa et al., 2017). Current drugs repurposed for cryptococcosis treatment are also being investigated such as tamoxifen, a drug used to treat breast cancer that targets the fungal protein calmodulin (Ngan et al., 2019). A phase two clinical trial has been undertaken whereby the combination of tamoxifen with amphotericin B and fluconazole will be compared to the combination of amphotericin B and fluconazole (Ngan et al., 2019). Data analysis for this trial is ongoing (Ngan et al., 2019). The antidepressant sertraline is fungicidal and interferes with

Cryptococcus protein synthesis (Boulware et al., 2020). Although sertraline was shown to have *in vitro* and *in vivo* fungicidal activity against *Cryptococcus*; in clinical phase two and three trials, there was no effect on mortality (Rhein et al., 2019, Katende et al., 2019), and it also caused adverse side-effects leading to early stoppage of the trial (Boulware et al., 2020).

Mortality due to cryptococcal meningitis can be reduced by screening for cryptococcal antigenaemia (CRAG) at ART initiation or among ART-experienced patients and pre-emptively treating those patients who screen CRAG-positive (Jarvis et al., 2013, Hurt et al., 2021). Following inclusion of a cryptococcal disease screen-and-treat intervention in the National Strategic Plan for HIV/AIDS, sexually transmitted infections (STIs) and TB; South Africa conducted a phased implementation of a national screen-and-treat program from 2012-2015 (Govender, 2012). The National Health Laboratory Service (NHLS)/National Department of Health implemented reflex laboratory CRAG screening across the country in October 2016 (Govender and Glencross, 2018). A study is currently being conducted at the NICD, evaluating the effectiveness of this national program on CRAG-positive patient outcomes; this study's short title is "CAST-NET".

1.4 *Cryptococcus*, the causative agent of cryptococcal meningitis

Two species complexes within the basidiomycetous yeast genus, *Cryptococcus*, cause most human disease: *Cryptococcus neoformans* and *Cryptococcus gattii* (Pappas, 2013). Other *Cryptococcus* species are less likely to cause clinically significant infection. Only one case of invasive disease caused by a *Cryptococcus* species other than *C. neoformans* and *C. gattii* was detected from 54 American patients over an eight-year period (Cano et al., 2020). *Cryptococcus neoformans* and *C. gattii* can further be divided into three varieties, five serotypes and 11 molecular subtypes (Table 1.1) (Maziarz and Perfect, 2016). Several

differences exist between *C. neoformans* and *C. gattii* based on geographical distribution, ecological niches, epidemiology, pathobiology, clinical presentation and molecular characters (Cogliati, 2013). There is a proposal for each molecular type of *Cryptococcus* to be elevated to species level and the proposed species names are listed in Table 1.1 (Hagen et al., 2015). However, this proposal was controversial and researchers have still not reached a consensus on these taxonomic changes (Kwon-Chung et al., 2017).

About 95% of cryptococcal disease is caused by *C. neoformans* var. *grubii* (serotype A) and 4 to 5% caused by *C. neoformans* var. *neoformans* (serotype D) or *C. gattii* (serotypes B or C) (Maziarz and Perfect, 2016). *Cryptococcus neoformans* var. *grubii* (serotype A) is globally distributed whereas *C. neoformans* var. *neoformans* (serotype D) is endemic to Europe. *Cryptococcus gattii* (serotypes B or C) was previously thought to be confined to tropical and subtropical regions and was found less frequently in temperate regions (Maziarz and Perfect, 2016). However, this species-complex seems to have expanded to temperate zones, for example, the occurrence of *C. gattii* outbreaks in North America (Kidd et al., 2005). *Cryptococcus neoformans* and *C. gattii* are both known to cause disease in both HIV-infected persons and immunocompetent individuals (Pappas, 2013). Among HIV-infected persons, *C. gattii* disease is indistinguishable from *C. neoformans* disease; patients present with meningitis with or without fungaemia (Morgan et al., 2006). Among HIV-uninfected patients, disease caused by *C. gattii* frequently presents as a lesion localized in the lungs or CNS (Morgan et al., 2006).

Table 1.1: Pathogenic *Cryptococcus* species (adapted from Meyer et al., 2009, Hagen et al., 2015, Farrer et al., 2019)

Current species or variety or hybrid name	**Serotype**	**Molecular type**	**Proposed species names**
Cryptococcus neoformans var. *grubii*	A	VNI	*C. neoformans*
	A	VNB	*C. neoformans*
	A	VNII	*C. neoformans*
AD hybrid	AD	VNIII	*C. neoformans* X *C. deneoformans* hybrid
Cryptococcus neoformans var. *neoformans*	D	VNIV	*C. deneoformans*
Cryptococcus gattii	B/C	VGI	*C. gattii*
	B/C	VGII	*C. deuterogattii*
	B/C	VGIII	*C. bacillisporus*
	B/C	VGIV	*C. tetragattii*
	B/C	VGV	Not proposed yet
	B/C	VGVI	*C. decagattii*

1.5 Ecological niche of *C. neoformans* and *C. gattii*

Cryptococcal disease is not contagious; as such, human-to-human transmission is not observed (Van Wyk et al., 2014). It is likely that individuals inhale *Cryptococcus* spores or desiccated yeasts from the environment (Vanhove et al., 2017). To better understand the epidemiology of cryptococcosis, several studies have investigated the ecological niche of this fungus that is ubiquitous in the environment (Ellis and Pfeiffer, 1992, Granados and Castañeda, 2005, Litvintseva et al., 2005a, Chowdhary et al., 2012, Chen et al., 2015b, Cogliati et al., 2016, Vélez and Escandón, 2017, Vanhove et al., 2017). *Cryptococcus neoformans* is found in bird droppings, especially pigeon excreta, soil, vegetative debris, and tree hollows specifically the Mopane tree in southern Africa (Litvintseva et al., 2005a, Litvintseva et al., 2011, Vanhove et al., 2017). *Cryptococcus gattii* has been mainly associated with certain tree species, such as eucalyptus (Ellis and Pfeiffer, 1992, Vanhove et al., 2017), firs and oaks (Kidd et al., 2005). A recent environmental Zambian study showed isolation of *C. gattii* from hyrax faeces that was not reported as a niche previously (Vanhove et al., 2017).

It has been hypothesized that the global distribution of *C. neoformans* occurred due to its ability to metabolize pigeon excreta; this allowed the organism to spread worldwide along migratory and trade routes once the pigeon became domesticated (Litvintseva et al., 2011). On the other hand, the ecological niche of *C. gattii* is restricted to sedentary trees thereby restricting its global movement (Nielsen et al., 2007). However, its ability to adapt to a new temperate niche caused a geographically-restricted outbreak in North America (Kidd et al., 2005, Engelthaler et al., 2014). There are many theories on the unexpected and unexplained emergence of *C. gattii* molecular type VGII in North America. These include the shipment of eucalyptus trees or agricultural products, global warming, ocean and wind currents, animal

movement, or human/mechanical transmission due to increased international travel (Kwon-Chung et al., 2014, Engelthaler and Casadevall, 2019). One hypothesis suggests that *C. gattii* was transported by ships in contaminated ballast tanks from South America to North America in 1914 (Roe et al., 2018). This organism then established itself in coastal waters. A tsunami in 1964 then carried *C. gattii* into the coastal forests. This organism adapted to a new environmental niche (i.e. land/forest) and then caused an outbreak of human infection about three decades later (Engelthaler and Casadevall, 2019).

1.6 Pathogenesis of cryptococcal disease

Individuals inhale airborne yeast cells or basidiospores from the environment into the lungs (Kwon-Chung et al., 2014). Infection can occur in the lungs and disseminate to other organs depending on the host's immune system, the pathogen load, etc. (Maziarz and Perfect, 2016). In immunocompetent hosts, fungal cells are effectively cleared by the immune system (Kwon-Chung et al., 2014). In immunosuppressed hosts, the yeast cells proliferate and spread through the bloodstream to other tissues, especially the CNS (Kwon-Chung et al., 2014, May et al., 2016). Once in the CNS, meningitis results (Figure 1.1) (Kwon-Chung et al., 2014, May et al., 2016). Clinically asymptomatic latent infection can also occur, whereby a small lung-lymph complex is formed and yeast cells remain viable, but dormant (Kwon-Chung et al., 2014). Once the host's immunity is compromised, the dormant yeast cells are activated, leading to active disease wherein cells proliferate in the pulmonary-lymph node complex and disseminate into extra-pulmonary sites (Kwon-Chung et al., 2014).

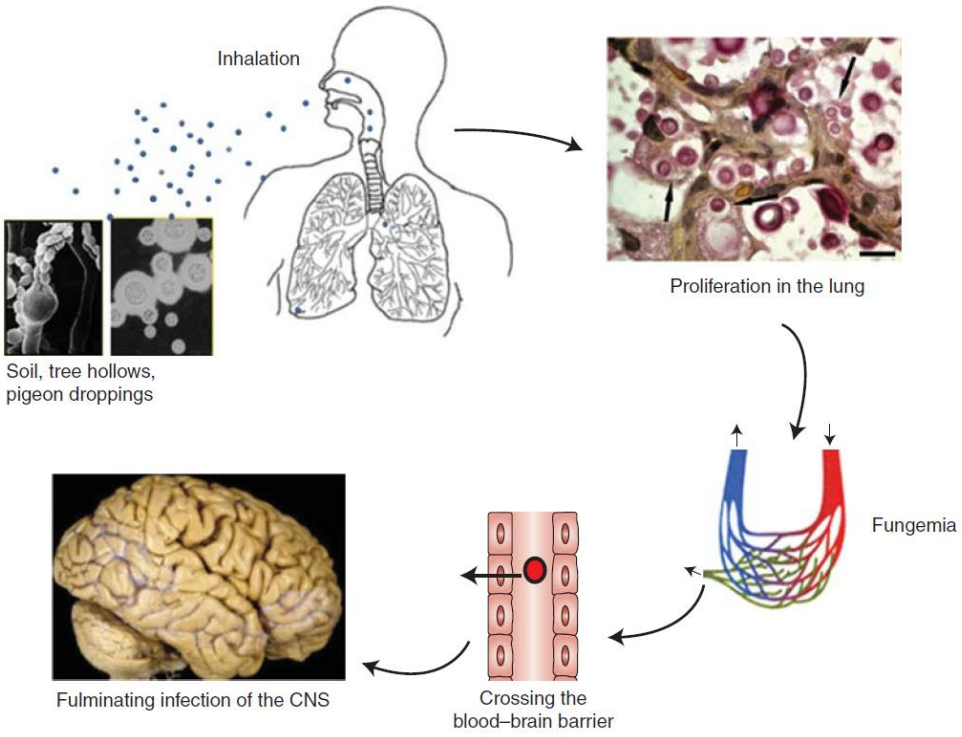

Figure 1.1: Pathogenesis of cryptococcal meningitis (Kwon-Chung et al., 2014)

The main virulence factors of *Cryptococcus* are polysaccharide capsule formation, melanin pigment production, enzyme production and thermotolerance (Maziarz and Perfect, 2016). The capsule surrounds the yeast cell and is mainly composed of two polysaccharides: glucuronoxylomannan (GXM) and glucuronoxylomannogalactan (GXMGal) (Zaragoza, 2019). Other minor components include mannoproteins and chitin-like structures (Zaragoza, 2019). In the environment, it is likely that the capsule protects the fungus from dehydration and phagocytic predators, such as amoebae (Chrisman et al., 2011). During active disease and latent infection, the capsule protects yeast cells against host phagocytic cells by "hiding" and interferes with host immune mechanisms (Casdevall et al., 2019). The capsule inhibits phagocytosis by masking the epitopes for macrophage receptors since these epitopes are found on the cell wall surrounded by the capsule (Zaragoza, 2019). Glucuronoxylomannan and GXMGal have immune modulatory abilities that degrade immune function (Casdevall et

al., 2019). Even though the capsule inhibits phagocytosis, cryptococcal cells can be phagocytosed, but are able to survive and avoid killing inside macrophages. This makes *Cryptococcus* a facultative intracellular fungal pathogen within the host as it replicates inside phagocytic cells, where it can survive for a long time (Zaragoza, 2019).

Melanin is stored in the cell wall and it protects the fungus from free radicals, ionizing radiation, heat and antifungal drug action (Zaragoza, 2019). Melanin also enables survival of the organism inside alveolar macrophages and facilitates escape from the lung thereby allowing the fungus to spread from the lungs to the brain (Noverr et al., 2004). It also changes host cytokine production and protects against macrophages (Sabiiti et al., 2014, Zaragoza, 2019).

Cryptococcus neoformans and *C. gattii* are the only species complexes within the genus *Cryptococcus* that have the ability to grow at 37°C, the most essential virulence factor, which enables these species complexes to cause disease since this is within the mammalian body temperature range (Kwon-Chung et al., 2014). Other virulence factors include production of enzymes, such as phospholipase, protease and urease (Casadevall et al., 2019, Zaragoza, 2019). These degradation enzymes allow survival of yeast cells within host cells; they can be transported to the cell surface enabling adhesion to host cells and also break down host cell membranes to penetrate host tissue (Zaragoza, 2019, Kwon-Chung et al., 2014). They could also promote sequestration of yeast cells at microcapillary vessels allowing crossing of the blood brain barrier thereby enabling brain invasion (Zaragoza, 2019, Kwon-Chung et al., 2014).

Production of capsule, modified cell wall composition and melanin synthesis are cell surface changes that have various roles in *Cryptococcus* virulence as described above (Altamirano et al., 2020). Additionally, cell size changes is another virulence factor whereby *C. neoformans* produces large titan cells and small micro and titanide cells during infection (Altamirano et al., 2020). Typical yeast cells are 5-7 µm in diameter, however, titan cells are greater than 10 µm in diameter with a cell wall thickened with a mannan layer and a highly cross-linked capsule, micro cells are less than 1 µm in diameter also with a thickened cell wall and titanides are 2-4 µm in diameter, oval-shaped and have a thinner cell wall when compared to typical yeast cells (Altamirano et al., 2020). Micro cells and titanides may promote dissemination and survival within macrophages (Altamirano et al., 2020). Titan cells may aid in pathogen survival during pulmonary infection (Altamirano et al., 2020).

1.7 Identification and antifungal susceptibility testing of *Cryptococcus*

Culture on selective/differential media, such as Niger seed agar, canavanine glycine bromothymol blue agar, and urea-containing agar, allow reliable identification of *Cryptococcus* in the microbiology laboratory (Figure 1.2) (McTaggart et al., 2011). Microscopy by staining cerebrospinal fluid (CSF) specimens with India ink and observing the cryptococcal capsule has been valuable in the past (Figure 1.3) (Nalintya et al., 2016) but this is generally no longer recommended as a diagnostic tool since it is less sensitive than culture and cryptococcal antigen detection (Dominic et al., 2009). Histopathology stains such as Alcian blue or mucicarmine (which stains the capsule) and Fontana-Masson (which stains melanin in the cell wall) have been used to differentiate *Cryptococcus* from other fungi in tissue (Lazcano et al., 1993).

Due to advances in technology, molecular methods such as Sanger sequencing of the large subunit (LSU) of the ribosomal DNA or the intergenic spacer (IGS) genes as well as a multiplex polymerase chain reaction (PCR) system were developed for the identification of *Cryptococcus* isolates (McTaggart et al., 2011, Rhein et al., 2016). Serological tests for the detection of the cryptococcal capsular antigen, GXM, include cryptococcal latex agglutination tests (CLAT), antigen capture sandwich enzyme immunoassays (EIA) and lateral flow immunochromatographic assays (LFA) (Hansen et al., 2013). The Immuno-Mycologics Ltd (IMMY) LFA is a rapid point-of-care dipstick test with a 99% specificity and sensitivity to detect cryptococcal antigen in CSF compared to traditional diagnostic tests (i.e. culture, CLAT and India ink microscopy) (Boulware et al., 2014).

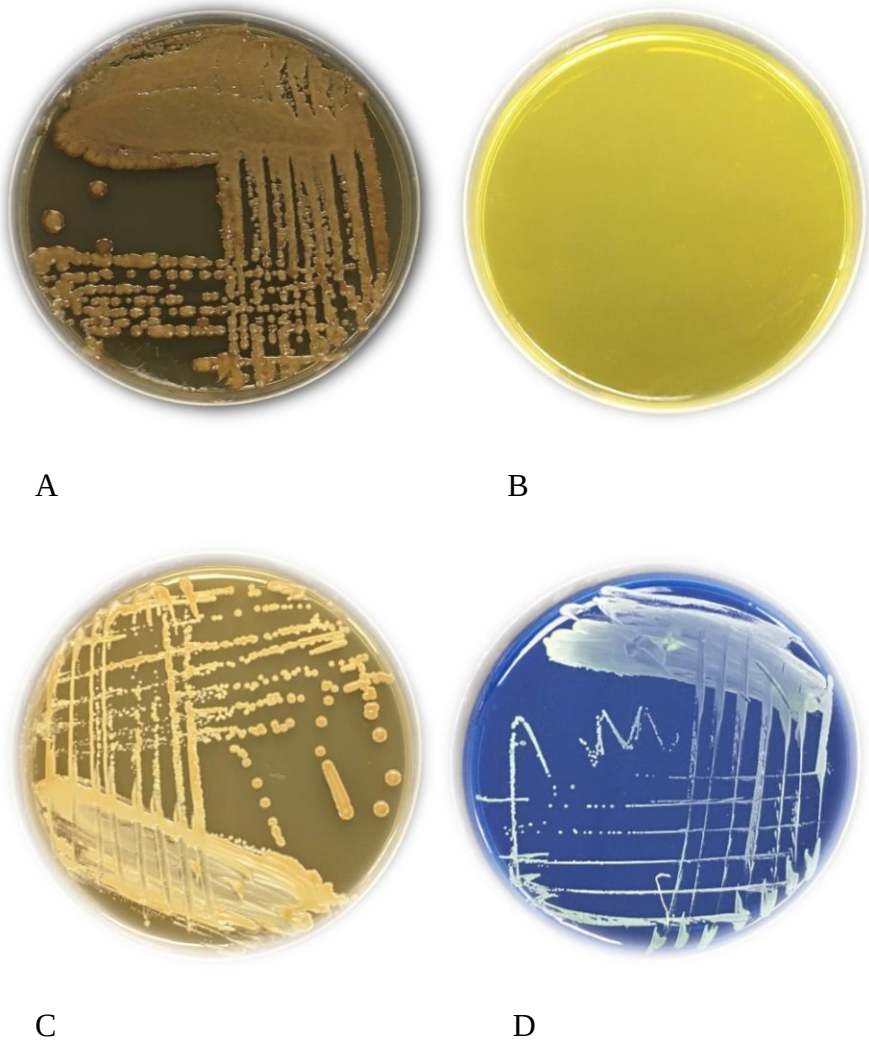

Figure 1.2: A – *Cryptococcus neoformans* sub-cultured on Niger seed agar (McTaggart et al., 2011), B – *Cryptococcus neoformans* sub-cultured on canavanine glycine bromothymol blue agar (McTaggart et al., 2011), C - *Cryptococcus gattii* sub-cultured on Niger seed agar (McTaggart et al., 2011), D - *Cryptococcus gattii* sub-cultured on canavanine glycine bromothymol blue agar (McTaggart et al., 2011) (Source: National Institute for Communicable Diseases, Centre for Healthcare Associated Infections, Antimicrobial Resistance and Mycoses)

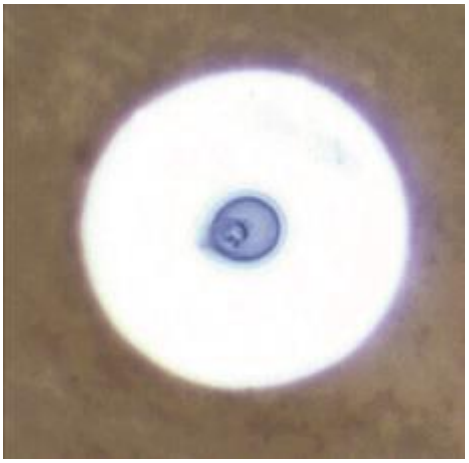

Figure 1.3: Micrograph showing the cryptococcal capsule stained with India ink (Source: https://mycology.adelaide.edu.au/descriptions/yeasts/cryptococcus/)

In vitro susceptibility testing of *Cryptococcus* is performed by reference broth microdilution (BMD) methods, for example using the Clinical and Laboratory Standards Institute (CLSI) guideline (Clinical and Laboratory Standards Institute, 2008) or commercial BMD methods, for example the Sentititre YeastOne Y10 AST plate (Thermo Fisher Scientific, UK) or Etest (bioMe´rieux, Marcy-l'E´toile, France). Antifungal susceptibility testing (AST) of *Cryptococcus* is generally not useful for incident-episode isolates, since treatment of HIV-associated cryptococcosis is standardised and AST results have shown not to correlate with fungal clearance or mortality (O'Connor et al., 2020). Antifungal susceptibility testing is however, useful for recurrent episodes of HIV-associated cryptococcosis (Govender et al., 2019).

Currently, there are no breakpoints for *Cryptococcus* but epidemiological cutoff values (ECVs) for fluconazole, itraconazole, posaconazole, voriconazole, amphotericin B and flucytosine have been published for *C. neoformans* and *C. gattii* (Table 1.2) (Espinel-Ingroff et al., 2012a, Espinel-Ingroff et al., 2012b, Clinical and Laboratory Standards Institute, 2018).

A systematic review of 21 papers reported an increase in fluconazole minimum inhibitory concentration (MIC) distributions for clinical cryptococcal isolates from 2000–2012 to 2014–2018 (Chesdachai et al., 2019). A few African reports on susceptibility testing of *Cryptococcus* isolates have shown varying results. For example, *Cryptococcus* isolates from Zimbabwe had low CLSI BMD MIC values for fluconazole (range: 0.25-8 µg/ml), amphotericin B (range: 0.125-1 µg/ml) and flucytosine (range: <0.063-64 µg/ml) using published ECVs (Espinel-Ingroff et al., 2012a, Espinel-Ingroff et al., 2012b, Nyazika et al., 2016b). A Kenyan study also showed that *Cryptococcus* isolates had low CLSI BMD MIC values to fluconazole (range: 0.25-16 µg/ml), amphotericin B (range: 0.5-1 µg/ml) and flucytosine (range: 1-16 µg/ml) using published interpretive criteria (Nguyen and Yu, 1998, Mdodo et al., 2011). However, Smith and colleagues showed an increase in fluconazole MIC_{50} values in Ugandan *C. neoformans* isolates from 4 µg/ml in 1998-1999 to 8 µg/ml in 2010-2014 with 31% of isolates having a fluconazole MIC of ≥16 µg/ml using the CLSI BMD method (Smith et al., 2015). In a South African study, 0.6% of incident *C. neoformans* isolates from 2002–2003 had a fluconazole MIC of ≥16 µg/ml, and these isolates also had very low MICs to amphotericin B, voriconazole and posaconazole (Govender et al., 2011). Additionally, *C. neoformans* isolates from HIV-seropositive patients were less susceptible to fluconazole compared to those from HIV-seronegative patients using the CLSI BMD method (Trilles et al., 2012). It is possible that HIV-seropositive patients are more likely to be exposed to antifungal treatment for oropharyngeal candidiasis prior to a diagnosis of cryptococcal meningitis, require longer maintenance therapy and relapses are also common (Trilles et al., 2012).

Table 1.2: Epidemiological cutoff values (in μg/ml) for *Cryptococcus neoformans* and *Cryptococcus gattii* isolates (Espinel-Ingroff et al., 2012a, Espinel-Ingroff et al., 2012b, Clinical and Laboratory Standards Institute, 2018)

Antifungal agent	***Cryptococcus neoformans***					***Cryptococcus gattii***				
	Nontyped	**VNI**	**VNII**	**VNIII**	**VNIV**	**Nontyped**	**VGI**	**VGII**	**VGIII**	**VGIV**
Fluconazole	16	8	-	16	-	8	16	32	8	16
Itraconazole	0.5	0.25	-	-	-	0.5	0.5	1	0.5	1
Posaconazole	0.25	0.25	-	-	-	0.5	0.5	-	-	-
Voriconazole	0.25	0.25	-	0.25	0.12	0.25	0.5	0.5	-	-
Amphotericin B	1	0.5	-	-	-	1	0.5	1	-	-
Flucytosine	16	8	-	-	-	4	4	32	-	-

The amphotericin B and flucytosine MIC values of *C. gattii* isolates differs significantly between geographical regions, for example European *C. gattii* strains were more susceptible to amphotericin B compared to African, Asian, Australian, North American and South American *C. gattii* strains (Hagen et al., 2010). Additionally, North American *C. gattii* strains were less susceptible to flucytosine than African, European, Asian and South American *C. gattii* strains (Hagen et al., 2010). The fluconazole MIC values of Brazilian, Spanish and Indian *C. gattii* isolates were significantly higher than *C. neoformans* isolates as tested by the CLSI BMD method (Trilles et al., 2004, Torres-Rodriguez et al., 2008, Chowdhary et al., 2011). The susceptibility of *C. gattii* to azole antifungals has been described in a previous South African study (Morgan et al., 2006); the MICs were approximately one double-dilution higher than those for *C. neoformans* isolates (Govender et al., 2011).

Cryptococcal strains exhibit heteroresistance to fluconazole and with longer exposure to fluconazole, clinically relevant resistance may emerge (Trilles et al., 2012). Heteroresistance is a chromosomally mediated phenomenon in which a resistant subpopulation can emerge from a single susceptible strain and exhibit transient adaptation to fluconazole at higher concentrations (Sionov et al., 2009). This acquired resistance can then revert to susceptibility when the isolate is no longer exposed to the drug (Sionov et al., 2009). It is also possible that cryptococcal strains can develop resistance to azoles in the environment when exposed to azole fungicides that are used to treat crops and it has been speculated that some people may then develop infections with primarily resistant strains (Smith et al., 2015). Fluconazole resistance can be caused by aneuploidy whereby chromosome 1 is duplicated relating to heteroresistance (Sionov et al., 2012, Stone et al., 2019). Two genes present on chromosome 1 are involved in fluconazole resistance; these are *ERG11* that encodes the fluconazole target enzyme lanosterol 14α-demethylase (Rodero et al., 2003) and *C. neoformans* Antifungal

Resistance 1 (*CnAFR1*), an adenosine triphosphate (ATP) binding cassette (ABC) efflux pump that is a major transporter of azoles in *C. neoformans* (Posterato et al., 2003, Stone et al., 2019). Point mutations such as G484S (Rodero et al., 2003) and Y145F (Sionov et al., 2012) in *ERG11* have been associated with fluconazole resistance. Overexpression or upregulation of *CnAFR1* also results in fluconazole resistance (Posterato et al., 2003).

1.8 Molecular typing of *Cryptococcus*

Molecular characterisation using multilocus sequence typing (MLST) is a good discriminatory tool to genotype *Cryptococcus* and is established as the gold standard for identifying the major molecular types of *C. neoformans* and *C. gattii* (Van Wyk et al., 2014). A standardised MLST protocol for *C. neoformans* and *C. gattii* has been established by the working group for "Genotyping of *C. neoformans* and *C. gattii"* of the International Society for Human and Animal Mycology (ISHAM) to reduce inter-laboratory variability (Meyer et al., 2009).

A comprehensive review consisting of published *C. neoformans* and *C. gattii* genotyping studies from South America (2353 isolates), Asia (1707 isolates), North America (1706 isolates), Europe (1266 isolates), Oceania (543 isolates) and Africa (505 isolates) summarised the molecular type distribution of *C. neoformans* and *C. gattii* on a global scale (Cogliati, 2013). *Cryptococcus neoformans* is divided into four molecular types: VNI, VNII, VNIII and VNIV (Meyer et al., 2009). VNI is distributed worldwide and is the most prevalent molecular type (Cogliati, 2013). VNB is predominately found in sub-Saharan Africa (clinical and environmental isolates) but has been found in the South American environment (Ngamskulrungroj et al., 2009, Rhodes et al., 2017b). VNII and VNIII are also globally distributed, but found less frequently (Cogliati, 2013). VNIV is found to a lesser extent

globally but is more frequent in Europe (Cogliati, 2013). Interestingly, African environmental isolates of VNI and VNII are associated with urban areas, pigeon excreta and non-Mopane trees whereas VNB is mostly associated with Mopane trees (Litvintseva et al., 2011, Chen et al., 2015b, Vanhove et al., 2017). Studies done in Zambia and Botswana further suggest that VNI infections are associated with urbanized populations, while VNB is associated with Mopane trees in rural settings (Chen et al., 2015b, Vanhove et al., 2017). Litvintseva and colleagues proposed the "out of Africa" hypothesis to explain the evolution of VNI (Litvintseva et al., 2011). Since VNI is mainly associated with pigeon excreta, it was postulated that pigeons enabled the global spread of *C. neoformans* from a genetically diverse southern African population (Litvintseva et al., 2011). A lower genetic diversity has been observed in global VNI strains from Southeast Asia (Simwami et al., 2011) and South America (Ferreira-Paim et al., 2017), which further supports this theory. An alternative hypothesis is that the VNI lineage originated elsewhere and became more diverse in Africa (Litvintseva et al., 2011).

Cryptococcus gattii is divided into five molecular types: VGI, VGII, VGIII, VGIV and VGV (Meyer et al., 2009, Farrer et al., 2019). The fifth molecular type VGV was recently identified from six environmental isolates collected from a specific location in Zambia (Farrer et al., 2019). VGI is mostly found in Australia and Papua New Guinea (Campbell et al., 2005). VGII is prevalent in Canada and the United States of America (USA), where it has caused a major outbreak (Kidd et al., 2005). However, this molecular type has subsequently also been found in Australia (Carriconde et al., 2011), Brazil and Colombia (Firacative et al., 2018). VGIII has been found in the USA (Lockhart et al., 2013), Mexico, Brazil and Colombia (Firacative et al., 2018). VGIV has, until now, been mainly identified from HIV-infected individuals from India (Cogliati et al., 2012), Colombia, Mexico (Meyer et al.,

2003), Botswana, Malawi (Litvintseva et al., 2005b) and Zimbabwe (Nyazika et al., 2016a). Since VGII was the major molecular type causing the Pacific Northwest (PNW) outbreak in North America, the ancestral source of the outbreak was investigated. The VGII isolates from this outbreak were most closely related to isolates from Brazil leading to the theory that *C. gattii* VGII could have originated from South America. Researchers have termed this the "out of South America" hypothesis (Souto et al., 2016).

One can use genotyping to carefully study the molecular epidemiology over the long term on condition that the fundamental principles of epidemiology are adhered to, for example, selection of clinical cases (and corresponding isolates) from a defined study population to answer a research question. Multilocus sequence typing is a valuable tool since it is able to determine the molecular differences between isolates that show similar phenotypic characteristics (Desnos-Ollivier et al., 2010). Multilocus sequence typing analysis of *C. neoformans* isolates from paediatric patients in South Africa showed that there were 24 distinct sequence types (ST), each ST corresponding to specific molecular types of *C. neoformans* i.e. VNI, VNII and VNB (Miglia et al., 2011). It has also been observed that a large proportion of *C. neoformans* strains from Africa have unique genotypes (Litvintseva et al., 2011, Ferreira-Paim et al., 2017). Genotyping analysis of global *C. neoformans* var. *grubii* molecular type VNI isolates by MLST revealed that the highest genetic diversity was observed in African isolates and the lowest genetic diversity in South American isolates (Ferreira-Paim et al., 2017). There were 65 unique genotypes in an African study, 26 of which were found only in southern Africa (Litvintseva et al., 2011). Among *C. gattii* isolates, VGIV is most common in Africa (Lockhart et al., 2013); 24 of 176 *Cryptococcus* strains were determined to be *C. gattii* (serotype C) and all were molecular type VGIV in a previous study (Litvintseva et al., 2005b).

Multilocus sequence typing analysis was performed on *C. neoformans* and *C. gattii* isolates from HIV-infected patients in South Africa with recurrent cryptococcal infection (Van Wyk et al., 2014). Clinical isolates were obtained through an active, population-based national cryptococcosis surveillance programme (Van Wyk et al., 2014). Serial isolate pairs or triads were identified from patients who were diagnosed with cryptococcal disease from 2005 to 2009 at enhanced laboratory surveillance sites (i.e. sites where a nurse interviewed case patients or reviewed medical records to obtain clinical information) and who had a second or third strain isolated more than 110 days after the first strain (Van Wyk et al., 2014). It was found that 157 *C. neoformans* isolates from 72 of the 81 patients and all eight *C. gattii* isolates from four patients had identical genotypes per patient, thereby suggesting that the same strain had caused the initial and subsequent infection (Van Wyk et al., 2014). It was also found that there was a high genetic diversity, since there were 23 genotypes obtained from 170 clinical isolates (Van Wyk et al., 2014). Compared to a previous study, there were 12 genotypes obtained from 650 environmental isolates (Litvintseva et al., 2005a). Beale and colleagues analysed 230 *C. neoformans* isolates from HIV-seropositive South African clinical trial patients and obtained 50 different STs (Beale et al., 2015). They found that patients infected with the VNB molecular type, had a significantly worse survival outcome, suggesting that genetic lineage of the pathogen may play an integral role during infection (Beale et al., 2015). The authors also observed that there was a link between ST and various clinical variables, such as survival outcome and altered mental status (Beale et al., 2015). The molecular diversity of clinical *C. neoformans* isolates was assessed in a French study using MLST in order to observe its biology in the host (Desnos-Ollivier et al., 2010). The authors found that 20% of patients diagnosed with cryptococcosis had mixed infections, with more than one genotype (Desnos-Ollivier et al., 2010). This may suggest that multiple strains are acquired by the host from the environment (Desnos-Ollivier et al., 2010).

A new approach to genotyping *Cryptococcus* is whole genome sequencing (WGS), through which potentially more genetic information, such as virulence and antifungal resistance markers can be obtained together with strain relatedness data in a shorter space of time compared to MLST, even though the overall costs are higher. This technique presents unique opportunities to further study these species complexes. In addition, one is able to extract MLST sequences from WGS data, which has been found to be a useful approach in genotyping *Cryptococcus* (Chen et al., 2015a). It also allows analysis of a large number of genes to determine diversity within a population and its evolution (Engelthaler et al., 2014, Rhodes et al., 2017b). Whole genome sequencing was used to study *C. gattii* isolates that were confined to the temperate North American Pacific Northwest region and had increased virulence and a different clinical presentation from isolates in tropical regions (Engelthaler et al., 2014). Whole genome sequencing was performed on these isolates to determine the source and genomic adaptations to this temperate region (Engelthaler et al., 2014). The authors were able to study the relationship between global *C. gattii* isolates and the North American isolates to determine the potential role of unique genes in virulence expression, environmental adaptation and clinical presentation (Engelthaler et al., 2014). There were specific regions within sugar transport, metabolic and virulence genes that were more likely to be present in the North American isolates than in the global isolates (Engelthaler et al., 2014). It was proposed that some of these extra gene regions allowed the organism to utilise a carbohydrate source in a temperate environment enabling it to survive in this region while other genes enabled certain subtypes of the outbreak strains to become more virulent and display a different pattern of clinical disease compared to its global counterparts (Engelthaler et al., 2014). Whole genome sequencing thus provided a more comprehensive view of phylogenetic relationships and genomic evolution of an emerging pathogen, such as *C. gattii* (Engelthaler et al., 2014). Whole genome sequencing analysis of *C. neoformans* var. *grubii*

serotype A isolates with the molecular types VNI, VNII and VNB revealed its genomic diversity (Rhodes et al., 2017b). These authors intensively studied the phylogenetic relationships and traced the evolution of these molecular types at the global population level (Rhodes et al., 2017b). They found that there was a much higher diversity observed in isolates with the molecular type VNB compared to isolates that had the molecular types VNI and VNII (Rhodes et al., 2017b). Further WGS studies of cryptococcal isolates from South Africa would not only reveal genetic diversity, but also allow for a better understanding of virulence traits and clinical outcomes (Ferreira-Paim et al., 2017).

Whole genome sequencing of recurrent isolates has been performed to determine if more than one genotype may be responsible for infection and to study microevolution in *Cryptococcus* (Altamirano et al., 2020). In a study by Rhodes and colleagues, a clonal relationship was observed for the majority of the 17 cases investigated (Rhodes et al., 2017a). However, there was a high level of genetic diversity observed for cryptococcal strains from two cases; this diversity was either due to infection by mixed genotypes or genetic mutations (Rhodes et al., 2017a). Whole genome sequencing was done on two serial *C. neoformans* isolates from an HIV-seropositive patient who had suffered an initial and relapse episode of cryptococcal meningoencephalitis (Ormerod et al., 2013). These two isolates were found to be identical by MLST, but were shown to have several differences by whole genome analyses that explained the presence of microevolution of the organism in the host (Ormerod et al., 2013). Whole genome sequencing was performed to investigate the microevolution of incident and relapse *Cryptococcus* isolates from South African patients (Chen et al., 2017). These isolates were genotyped by MLST previously (Van Wyk et al., 2014). The authors had found that there were changes in the virulence genes and antifungal resistance genes between the incident and relapse isolates (Chen et al., 2017). This study enabled a further study of microevolution of

strains that underwent pressure from the host immune response and antifungal treatment (Chen et al., 2017).

Due to the differences between molecular type and traits, such as virulence, geographic range, epidemiology and population genetics, the identification of the cryptococcal genotype is important. Identifying the molecular type in a particular patient also has the potential to guide treatment because the molecular type may be related to antifungal susceptibility patterns (Firacative et al., 2016). Studies have been performed to determine the differences in virulence between the various cryptococcal genotypes, using both *in vivo* and *in vitro* models (Firacative et al., 2014). It was shown that using *Galleria mellonella* as an *in vivo* model to study the virulence of *Cryptococcus* was comparable with studies performed in mammalian models (Firacative et al., 2014). Additionally, using *G. mellonella* to study virulence is easy, less costly and requires less ethical considerations than mammalian models (Firacative et al., 2014, Smith and Casadevall, 2021). This invertebrate model effectively showed that certain molecular types of *C. gattii* are more virulent than others (Firacative et al., 2014). Previous studies mainly focused on *C. neoformans* have found that there is a link between *Cryptococcus* genotype and patients' clinical characteristics such as gender (Miglia et al., 2011), clinical outcome (Wiesner et al., 2012, Beale et al., 2015), altered mental status (Beale et al., 2015), HIV status (Khayhan et al., 2013) and host immune response (Wiesner et al., 2012). Sequence type 8 was significantly associated with infection in male patients (Miglia et al., 2011). The molecular type VNB was associated with a worse survival and altered mental status (Beale et al., 2015). Sequence types 93 and 77 were associated with a higher patient mortality compared to ST63 and ST5 (Wiesner et al., 2012). Sequence type 5 has also been associated with infection among HIV-seronegative patients (Khayhan et al., 2013). Sequence

type 93 also elicited a greater type 2 helper T-cell (Th_2) cytokine response (Wiesner et al., 2012).

The work from this thesis did not cover exploring the molecular mechanisms of resistance to the three antifungals (i.e. liposomal or deoxycholate amphotericin B, flucytosine and fluconazole) used to treat cryptococcal disease. Additionally, this thesis did not involve determining virulence and antifungal resistance markers from WGS data.

1.9 Study aim

The overall aim of this study was to explore the genetic diversity, antifungal susceptibility and virulence of the two pathogenic *Cryptococcus* species complexes causing human disease in South Africa.

1.10 Study objectives

- Using MLST, to determine the sequence types of *C. neoformans* and *C. gattii* isolates from a first episode of cryptococcal disease, detected through national laboratory-based surveillance.
- To determine if patient clinical characteristics are associated with cryptococcal molecular type, and the effect of molecular type on in-hospital outcome.
- To determine the antifungal susceptibility pattern of *C. neoformans* and *C. gattii* isolates to fluconazole from a first episode of cryptococcal disease, detected through national laboratory-based surveillance.
- To determine the virulence of *C. neoformans* molecular type VNB using a *Galleria mellonella* model.

- Using whole genome sequencing, to describe the population genetic structure of *C. neoformans* molecular type VNB and *C. gattii* molecular type VGIV.

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Chapter 2: Genotype, antifungal susceptibility and virulence of clinical South African *Cryptococcus neoformans* strains from national surveillance, 2005-2009. Journal of Fungi 2021; 7 (338), 1-17.

Journal of Fungi

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Genotype, Antifungal Susceptibility, and Virulence of Clinical South African *Cryptococcus neoformans* Strains from National Surveillance, 2005–2009

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Citation: Naicker, S.D.; Magobo, R.E.; Maphanga, T.G.; Firacative, C.; van Schalkwyk, E.; Monroy-Nieto, J.; Bowers, J.; Engelthaler, D.M.; Shuping, L.; Meyer, W.; et al. Genotype, Antifungal Susceptibility, and Virulence of Clinical South African *Cryptococcus neoformans* Strains from National Surveillance, 2005–2009. *J. Fungi* **2021**, *7*, 338. https://doi.org/10.3390/jof7050338

Academic Editor: David S. Perlin

Received: 12 March 2021
Accepted: 6 April 2021
Published: 27 April 2021

Abstract: In South Africa, *Cryptococcus neoformans* is the most common cause of adult meningitis. We performed multi locus sequence typing and fluconazole susceptibility testing of clinical *C. neoformans* isolates collected from 251 South African patients with cryptococcosis through national surveillance from 2005 to 2009. We examined the association between clinical characteristics of patients and genotype, and the effect of genotype on in-hospital mortality. We performed whole genome phylogenetic analysis of fifteen *C. neoformans* isolates with the molecular type VNB and tested their virulence in a *Galleria mellonella* model. Most isolates had the molecular type VNI (206/251, 82%), followed by VNII (25/251, 10%), VNB (15/251, 6%), and VNIV (5/251, 2%); 67 sequence types were identified. There were no differences in fluconazole minimum inhibitory concentration (MIC) values among molecular types and the majority of strains had low MIC values (MIC_{50} of 1 µg/mL and MIC_{90} of 4 µg/mL). Males were almost twice as likely of being infected with a non-VNI genotype (adjusted odds ratio [OR]: 1.65, 95% confidence interval [CI]: 0.25–10.99; $p = 0.61$). Compared to patients infected with a VNI genotype, those with a non-VNI genotype had a 50% reduced adjusted odds of dying in hospital (95% CI: 0.03–7.57; $p = 0.62$). However, for both these analyses, our estimates had wide confidence intervals spanning 1 with large *p*-values. Fifteen VNB strains were not as virulent in a *G. mellonella* larval model as the H99 reference strain. A majority of these VNB strains belonged to the VNBII clade and were very closely related by phylogenetic analysis.

Keywords: *Cryptococcus neoformans*; molecular type; South Africa; MLST; whole genome sequencing; fluconazole; antifungal resistance

1. Introduction

Cryptococcus is a more common cause of meningitis among adults in South Africa than bacteria or *Mycobacterium tuberculosis*, accounting for 62% of microbiologically-confirmed cases [1]. Two species-complexes within the genus *Cryptococcus* cause most human disease: *Cryptococcus neoformans* and *Cryptococcus gattii* [2]. Both are known to cause disease in HIV-infected persons and immunocompetent individuals [2]. The ecological niche of these sibling species overlaps in southern Africa including soil, bird droppings, and certain tree species [3]. For induction treatment of HIV-associated cryptococcal meningitis, flucytosine is now recommended in combination with amphotericin B, followed by fluconazole monotherapy for consolidation and maintenance treatment [4].

Multi locus sequence typing (MLST) is a good discriminatory tool to genotype strains of the two closely-related sibling *Cryptococcus* species and is the gold standard for identifying the major molecular types of *C. neoformans* and *C. gattii* [5]. Molecular typing subdivides *C. neoformans* into five molecular types: VNI, VNB, VNII, VNIII, and VNIV [6]. New species names that correspond to each molecular type were proposed due to differences in virulence, geographic range, epidemiology, and population genetics [7]. While this proposal may not be formalized because some members of the scientific community foresaw issues of potential taxonomic instability, others have adopted this nomenclature in the published literature [8]. A comprehensive review of published *C. neoformans* genotyping studies from South America (1766 isolates), Asia (1444 isolates), Europe (1217 isolates), North America (938 isolates), Africa (475 isolates), and Oceania (189 isolates) summarized the molecular type distribution of *C. neoformans* on a global scale [9]. VNI is distributed worldwide and is the most prevalent molecular type [9]. VNB is predominately found in sub-Saharan Africa (clinical and environmental isolates), but recently-sequenced environmental and clinical isolates from Brazil had this genotype [10,11]. VNII and VNIII are less frequently found globally [9]. VNIV also has global distribution but is more frequently found in Europe [9].

African environmental isolates of VNI and VNII are associated with urban areas, pigeon excreta, and non-Mopane trees whereas VNB is mostly associated with *Colophospermum mopane* (Mopane) trees [12–14]. Studies done in Zambia and Botswana further suggest that VNI infections are associated with urbanized populations while VNB is associated with Mopane trees in rural settings [13,14]. Whole genome sequencing (WGS) has revealed that VNB isolates have a higher diversity compared to VNI and VNII isolates [15]. Two groups have proposed that the VNB lineage be split into two clades, namely VNB-A and VNB-B [14] or VNBI and VNBII [16]. Population genomic analyses of VNB isolates showed that the VNB lineage migrated bi-directionally between Africa and South America before its diversification into these two clades [15]. Additionally, patients infected with VNB in a South African study had a significantly worse survival outcome, suggesting that the genetic lineage plays an integral role during infection and that there is possibly a link between clinical characteristics and genotype [17].

We aimed to describe the molecular type distribution and fluconazole susceptibility of clinical South African *C. neoformans* isolates collected through national laboratory-based surveillance during 2005–2009. We examined the association between clinical characteristics of patients and molecular type, and the effect of molecular type on in-hospital mortality. We also sequenced the genomes of fifteen VNB isolates from our study and compared the virulence of these strains to the H99 reference strain in a *Galleria mellonella* model. We focused our Whole Genome Sequencing (WGS) and virulence studies on this molecular type since this genotype is endemic to southern Africa and based on previous work, hypothesized that this genotype may be more virulent than the other molecular types [17].

2. Materials and Methods

2.1. Study Design and Sample Selection

We conducted a cross-sectional study nested within national laboratory-based surveillance for cryptococcosis in South Africa from 2005 through to 2009 [18]. A case was defined as a person diagnosed with cryptococcal disease by any one of the following

positive laboratory tests during a 30-day period: India ink microscopy on cerebrospinal fluid (CSF), cryptococcal antigen test on blood or CSF, and/or culture of *Cryptococcus* from any specimen. At least 50 *C. neoformans* isolates from each of the surveillance years, 2005 to 2009, were randomly selected for genotyping using a random-integer generator (https://www.random.org/integers/, accessed on 4 January 2010), totaling 251 cases. In some years, more than 50 isolates were selected to account for non-viability and/or contamination. Recurrent isolates from the same patients were excluded and in such instances, the incident isolate was the one selected. We also excluded isolates that were identified as *C. gattii* or other *Cryptococcus* species. We genotyped 251 strains from a total of 18,040 cases between 2005 and 2009. We then estimated the prevalence and 95% CI for each genotype in this random sample of the study population. We applied the calculated 95% CIs to the study population of 18,040 cases to determine the range of cases per genotype in the study population.

2.2. Subculture and Identification of Surveillance Isolates

Following primary isolation in culture at diagnostic laboratories, a sweep of the culture plate was inoculated onto Dorset medium (Diagnostic Media Products (DMP), NHLS) and transported to a reference laboratory at the National Institute for Communicable Diseases (NICD) in Johannesburg, where the isolates were identified and stored at −70 °C. For this study, the *C. neoformans* isolates were retrieved from −70 °C storage and sub-cultured onto Sabouraud dextrose agar (DMP) to check for purity and viability. We characterized isolates phenotypically as previously described [5].

2.3. Multi Locus Sequence typing (MLST) Experiments and Data Analysis

DNA was extracted from single yeast colonies using the Zymo ZR Fungal/Bacterial DNA MiniPrep Kit (Zymo Research Corp, Irvine, CA, USA) following the manufacturer's instructions. Thereafter, DNA amplification and sequencing was performed for six housekeeping genes *CAP59*, *GPD1*, *LAC1*, *PLB1*, *SOD1*, *URA5*, and the intergenic spacer region *IGS1*, as described by Meyer et al. [6]. For each locus, DNA sequences in both forward and reverse orientations were obtained and edited using Sequencher 5.4.6. Allele numbers and sequence types (STs) were assigned using the MLST database (http://mlst.mycologylab.org). Concatenated DNA sequences for the seven loci were aligned using the program ClustalW (BioEdit Sequence Alignment Editor). A phylogenetic tree was generated in the program MEGA using the neighbor-joining method with a bootstrap analysis of 100 replicates and the Jukes–Cantor model. With the number and frequency of STs, the Simpsons diversity index (*D*) was calculated to estimate the genetic diversity of *C. neoformans* strains from this study. High scores (close to 1) indicate high diversity and low scores (close to 0) indicate low diversity [19]. Mating type identification was performed by polymerase chain reaction (PCR) amplification using MFα primers (upper and lower sets) and the $STE20_{SF}$a primer sets, which are specific to the mating type regions of α and a mating-type cells, respectively, according to a previous study [20].

2.4. Fluconazole Susceptibility Testing

Of the 251 *C. neoformans* isolates, antifungal susceptibility testing was performed for 105 isolates: 60 randomly-selected VNI (from a total of 206), 25 VNII, 15 VNB, and five VNIV. We determined fluconazole minimum inhibitory concentration (MIC) values (range: 0.125 μg/mL to 64 μg/mL) using custom-made broth microdilution panels (NICD, Johannesburg) prepared, inoculated, and read according to susceptibility testing methods published previously and Clinical and Laboratory Standards Institute M27-A3 and M60 recommendations [21–23]. We interpreted fluconazole MIC values using published epidemiological cutoff values (ECVs) [24].

2.5. Virulence Studies

To assess the virulence of the 15 VNB strains in our study, we used the moth *G. mellonella* model of fungal disease [25]. Larvae in the final instar stage of larval development were used in experiments according to methods published previously [26,27]. Briefly, ten larvae per VNB strain were injected with 10 µL of a 10^8 yeast cells/ml inoculum prepared using normal saline. Ten larvae were also inoculated with a highly-virulent reference strain (*C. neoformans* H99). We also included two sets of control groups, whereby we injected 10 larvae each with phosphate-buffered saline (PBS) and another 10 larvae each that were not injected. Following injection, larvae were incubated in Petri dishes at 37 °C in the dark and the number of dead larvae were recorded daily for ten days. Larvae were considered dead when they did not move in response to a physical stimulus such as a sterile forceps. We performed three experimental replicates and an average from the three replicates was calculated. We generated a Kaplan–Meier survival curve for larvae infected with each VNB strain, the reference strain H99, and the controls. As a proxy for virulence, we compared the median survival times of larvae infected with each VNB strain to larvae infected with the well-studied reference strain H99 (genotype VNI). Details of the statistical analysis are below.

2.6. Whole Genome Sequencing and Phylogenetic Analysis

Whole genome sequencing was performed on 15 isolates with the molecular type VNB using Illumina MiSeq sequencing technology (Illumina, San Diego, CA, USA). DNA samples were prepared for paired-end sequencing using the NEBNext Ultra II DNA Library Prep Kit for Illumina followed by 2 × 300 bp sequencing on a MiSeq instrument. FASTQC and PrinSeq were used to check the quality of read data and perform read filtering. Sequenced genomes were then aligned to a known reference VNB genome (Bt 85, NCBI BioSample number: SAMN01162765) to compare and call single nucleotide polymorphism (SNP) variants using Burrows-Wheeler Aligner (BWA) and SAMtools. BWA and SAMtools were performed using the publicly-available Northern Arizona SNP Pipeline (NASP) [28]; filtering parameters involved removing positions that had <10x coverage, <90% variant allele calls, and those within duplicated regions in the reference. Downstream filtering by NASP produced the final BestSNP alignment that was then used for maximum parsimony inference. Only positions present in all genomes with at least 10x depth of coverage and 90% agreement were included. Maximum parsimony trees with 500 bootstrap replicates were constructed and visualized using MEGA software. In our first analysis, we included whole genome sequences of publicly-available VNB strains from the study conducted by Rhodes et al. in 2017; this included 10 strains from Botswana, seven from South Africa, and four from Brazil [15]. From this initial analysis, we were able to calculate the distances of samples most closely related to our 15 VNB strains using MinHash. This allowed us to determine a cut-off distance *p*-value of 0.0157. There were 95 publicly-available VNBII isolates that were equal to or less than this value, which we included in our next analysis. These strains were from Botswana (n = 56), South Africa (n = 20), Zambia (n = 16), and Brazil (n = 3) [15,16]. In this analysis, we used a draft assembly of one of the 15 VNB strains from our study (isolate number 1239) as a reference using BWA and SAMtools in NASP in order to find publicly-available VNB genomes closely related to our strains.

2.7. Statistical Analyses

We used logistic regression models to determine associations between patient characteristics and genotype. Patient characteristics were obtained by nurse surveillance officers at the surveillance sites who either interviewed patients or reviewed patients' medical charts to obtain detailed case information. Mental status was categorized as "Alert" (Glasgow Coma Scale [GCS] score of 15) or "Not alert" (GCS score < 15 or recorded as disorientated, stuporose, or comatose). We defined genotype as VNI vs. non-VNI (including VNII and VNB), since there were too many sequence types with low frequencies. We therefore compared cases of *C. neoformans* serotype A infection (which corresponds to molecular types

VNI, VNII, and VNB) in this analysis and excluded *C. neoformans* serotype D (corresponds to molecular type VNIV). We included the following variables in the final regression model with genotype as the dependent variable: sex, age, year of diagnosis, province, specimen type, mental status at diagnosis, CD4+ T-cell (CD4) count at diagnosis, antiretroviral treatment, CSF India ink result, CSF white cell count, current antifungal treatment, and tuberculosis treatment. This was an exploratory analysis whereby we adjusted for age (added a priori), mental status, CD4 count at diagnosis, and CSF white cell count since these variables have been associated with molecular type in previous studies [17,29]. We also modeled the effect of molecular type on in-hospital mortality, adjusting for sex, age, mental status at diagnosis, CD4 count at diagnosis, antiretroviral treatment, and current antifungal treatment. We also compared the fluconazole MIC_{50} values of the VNI isolates and non-VNI isolates using a Wilcoxon rank-sum test. We performed a log rank test of equality to compare survival curves of larvae infected with H99 and the 15 VNB isolates, where the null hypothesis was that there was no difference between the population survival curves. Statistical analyses were performed using Stata statistical software (Version 14.1; StataCorp LP, College Station, TX, USA).

2.8. Ethics Approval

Ethics clearance for this study was obtained from the Human Research Ethics Committee (Medical), University of the Witwatersrand. We obtained an ethics waiver from the Animal Ethics Research Committee, University of the Witwatersrand (Registration number: AREC-101210-002) for the *G. mellonella* virulence studies.

3. Results

3.1. Descriptive Analysis of National Laboratory-Based Surveillance

From 2005 to 2009, 18,756 viable *Cryptococcus* isolates were collected from 18,534 cases of cryptococcal disease. Ninety-seven per cent of viable isolates (18,260/18,756) were identified as *C. neoformans* originating from 18,040 patients. Due to changes in surveillance methodology, the proportion of culture-confirmed *C. neoformans* cases by year was: 2005: 22% (3933/18,040), 2006: 26% (4686/18,040), 2007: 26% (4726/18,040), 2008: 15% (2768/18,040), and 2009: 11% (1927/18,040). The difference in the proportion of culture-confirmed cases was due to the number of surveillance sites decreasing in 2008–2009. The median age of patients was 34 years (inter-quartile range [IQR]: 29–41 years) and 53% (9616/18,040) were female. Of the 18,040 cases, most patients were diagnosed in Gauteng Province (33%, 5971), followed by KwaZulu-Natal Province (23%, 4194), Mpumalanga Province (9%, 1547), Eastern Cape Province (9%, 1639), North West Province (9%, 1554), and Western Cape Province (7%, 1282). The rest were diagnosed in the Free State (6%, 1051), Limpopo (3%, 599), and Northern Cape (1%, 203) provinces. CSF was the most common specimen type (17,288/18,040, 96%), followed by blood culture (715/18,040, 4%).

3.2. Genotyping of 251 Clinical C. neoformans Isolates

We genotyped 251 strains from a total of 18,040 cases between 2005 and 2009, which equaled 1.4% of all cases. The molecular type distribution of 251 *C. neoformans* isolates (see Supplementary Materials for metadata) were as follows: VNI (206/251, 82%; 95% CI: 0.77–0.87), VNII (25/251, 10%; 95% CI: 0.07–0.14), VNB (15/251, 6%; 95% CI: 0.03–0.10), and VNIV (5/251, 2%; 95% CI: 0.01–0.05). The characteristics of the five cases infected with the VNIV strains are described in Table S1. Most isolates had the mat-α mating type (n = 249, 99%) whereas 1% (n = 2 VNI isolates) were mat-a. There were 67 different STs observed from 251 *C. neoformans* isolates and these are shown in Figure 1. ST5 (53/251, 21%), ST93 (30/251, 12%), and ST23 (26/251, 10%) were the most commonly-observed types. The genetic diversity of South African *C. neoformans* isolates from this study was high, as determined by calculating the Simpsons diversity index (D = 0.9205). We calculated that among the 18,040 cases, VNI would have accounted for 13,891–15,695 cases, VNII for 1263–2526 cases, VNB for 541–1804 cases, and VNIV for 180–902 cases.

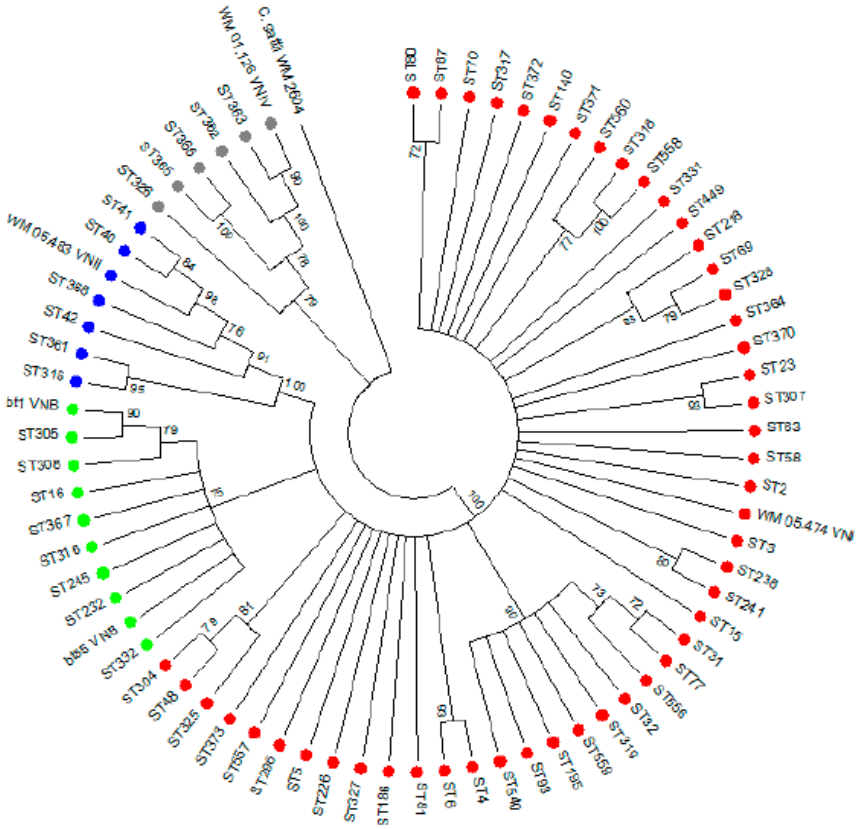


Figure 1. Neighbor joining phylogenetic tree showing clustering of molecular types for 67 different sequence types (STs) from 251 South African clinical *Cryptococcus neoformans* isolates. *Cryptococcus gattii* WM 2604 serves as the outgroup. Red circles indicate the STs with the VNI molecular type, blue circles indicate the STs with the VNII molecular type, green circles indicate the STs with the VNB molecular type, and grey circles indicate the STs with the VNIV molecular type. Bootstrap values are shown next to the branches.

3.3. Association between Patients' Clinical Characteristics and Genotype

On multivariable analysis, males were almost twice as likely to be infected with a non-VNI genotype compared to females, after adjusting for age, mental status at diagnosis, CD4 count at diagnosis, and CSF white cell count. Although the point estimate for the odds ratio was 1.65, the 95% CI spanned 1 (0.25–10.99) and the *p*-value was large (0.61) (Tables S2 and S3). This also makes our findings consistent with males having a 75% reduced odds to an eleven-fold increased odds of being infected with non-VNI (95% CI: 0.25–10.99; $p = 0.61$).

3.4. Association between Genotype and In-Hospital Mortality

The overall crude in-hospital case fatality ratio was 36% (64/180). The crude case fatality ratio among patients infected with each molecular type was as follows: VNI 37% (54/145), VNII 35% (7/20), VNB 18% (2/11), and VNIV 25% (1/4). Patients infected with a non-VNI cryptococcal molecular type had a case fatality ratio of 29% (9/31) versus 37% (54/145) for patients infected with a VNI genotype (crude odds ratio [OR]: 0.69, 95%CI: 0.29–1.61, $p = 0.39$) (Table S4). Compared to patients infected with a VNI genotype, those with a non-VNI genotype had a 50% reduced odds of dying after adjusting for sex, age, mental status at diagnosis, CD4 count at diagnosis, antiretroviral treatment, and current

antifungal treatment. Although the point estimate for the odds ratio was 0.50, the 95% CI spanned 1 (0.03–7.57) and the *p*-value was large (0.62) (Table S5).

3.5. Fluconazole Susceptibility Testing

The fluconazole MIC_{50} and MIC_{90} values for 105 *C. neoformans* isolates was 1 µg/mL and 4 µg/mL, respectively, with a geometric mean of 1.32 µg/mL (Table 1). The MIC_{50} value for five VNIV isolates was 2 µg/mL compared to 1 µg/mL for 60 VNI isolates, 25 VNII isolates, and 15 VNB isolates, respectively (Table 1). There was no difference in the MIC_{50} values between the VNI isolates and non-VNI isolates ($p = 0.17$). Most isolates had a MIC value of less than or equal to 8 µg/mL, which is considered to be wild-type according to published ECVs [24]. There was only one VNI isolate that had a MIC of 32 µg/mL.

Table 1. Fluconazole minimum inhibitory concentration (MIC) distribution (in µg/mL) by molecular type of 105 South African *Cryptococcus neoformans* isolates from national laboratory-based cryptococcosis surveillance collected between 2005 and 2009.

Molecular Type	Total	MIC Value										MIC_{50}	MIC_{90}	Geometric Mean	Range
		0.125	0.25	0.5	1	2	4	8	16	32	64				
VNI	60	0	2	10	20	19	3	5	0	1	0	1	6	1.43	0.25–32
VNB	15	0	1	2	6	3	3	0	0	0	0	1	4	1.26	0.25–4
VNII	25	0	0	5	15	4	1	0	0	0	0	1	2	1.03	0.5–4
VNIV	5	0	0	0	0	5	0	0	0	0	0	2	2	2	2
Total	105	0	3	17	41	31	7	5	0	1	0	1	4	1.32	0.25–32

3.6. Virulence and Whole Genome Sequencing (WGS) of 15 C. neoformans Isolates with the VNB Molecular Type

We plotted survival curves for *G. mellonella* larvae inoculated with 15 *C. neoformans* VNB isolates and H99 (Figure 2). Median survival times and *p*-values are shown in Table 2. The 15 VNB strains showed some heterogeneity in their virulence, as some strains killed as quickly as H99, while others killed the larvae more slowly. The larvae inoculated with one VNB strain (isolate number 5873) had a survival time of two days, which was the same as shown for the highly virulent reference strain H99. Nine VNB strains resulted in a median survival time of the inoculated larvae of three days, four VNB strains resulted in a median survival time of the inoculated larvae of four days, and one strain in a median survival time of the inoculated larvae of five days (Figure 2).

The WGS results of the 15 VNB isolates are shown in Figures 3–5. Figure 3 shows the 15 VNBs including two reference strains for the VNBI and VNBII clades. All 15 VNB isolates clustered in the VNBII clade. Figure 4 shows the 15 VNBs and 21 publicly-available clinical and environmental samples obtained from the study conducted by Rhodes and colleagues [15] with reference strains for the two representative clades. The cluster clade at the top of the tree in Figure 4 consists of nine of our VNB strains with fewer than 35 SNPs among them. Two patients from whom these strains were cultured were female and seven were male. Six patients were from Gauteng Province and had been diagnosed at the same health care facility, another two were from the Free State Province, and one from the Eastern Cape Province. The cases did not cluster in time: two patients were diagnosed in January and August of 2005, four patients in February, April, and November of 2006, and one patient each in March 2007, October 2008, and January 2009, respectively (Table S6). Four other isolates were scattered throughout the tree within the VNBII clade. Two patients were male, one patient was female, and the gender was unknown for one patient. Three patients were from Gauteng Province and one patient was from the North West Province. The remaining two VNBs (sample numbers 637 and 2890) clustered closely with an environmental Brazilian isolate (WM1408 Hamden C3-1 BR); there were fewer than 160 SNPs among the three isolates, but fewer than 30 SNPs between the two South

African VNBs. Both patients were male and one was from the Free State Province and one from KwaZulu-Natal Province.

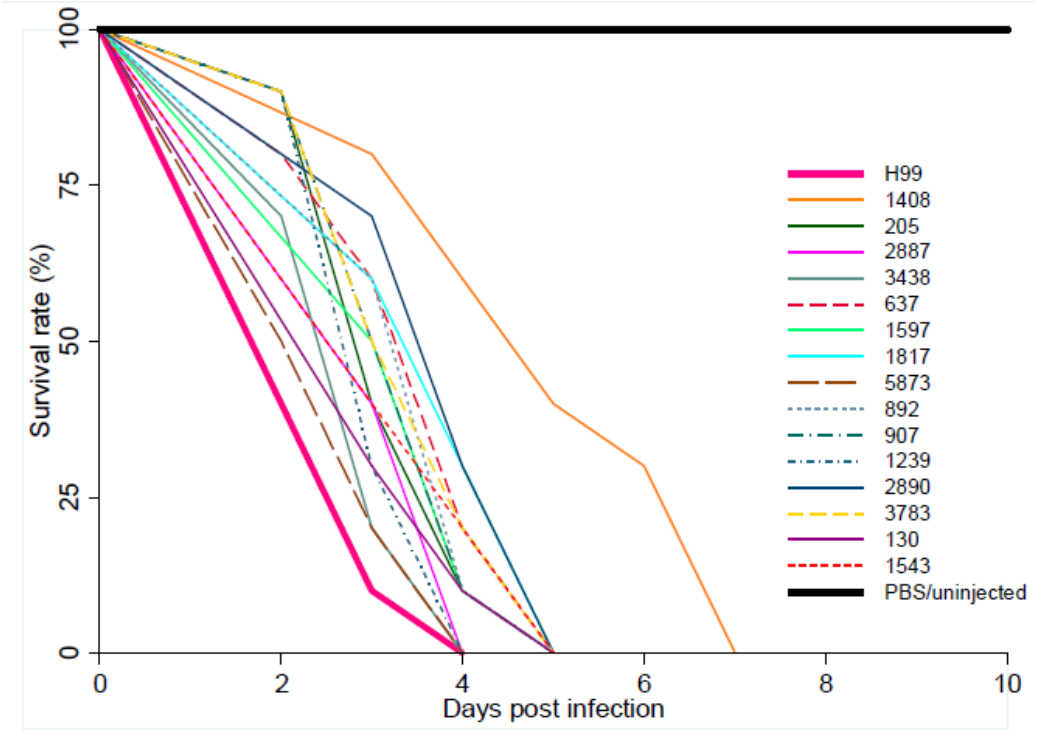


Figure 2. Survival curves of *Galleria mellonella* larvae (30 larvae per strain from three experimental replicates) infected with 15 clinical South African *Cryptococcus neoformans* molecular type VNB isolates. Larvae (n = 10 each) were also inoculated with H99 (pink solid line), which is a highly virulent reference strain, or phosphate buffered saline (PBS) or not injected (black solid line).

Table 2. Virulence of *Cryptococcus neoformans* molecular type VNB strains inoculated into *Galleria mellonella* larvae compared to larvae inoculated with the highly-virulent H99 strain.

Strain	Median Survival Time	*p* Value
H99 *	2	NA
5873	2	0.54
205	3	0.02
2887	3	0.01
3438	3	0.24
1597	3	0.01
907	3	0.02
1239	3	0.04
3783	3	0.01
130	3	0.01
1543	3	0.01
637	4	0.02
1817	4	0.002
892	4	0.003
2890	4	0.001
1408	5	0.001

* H99 was used as the comparison strain in the log rank test of equality.

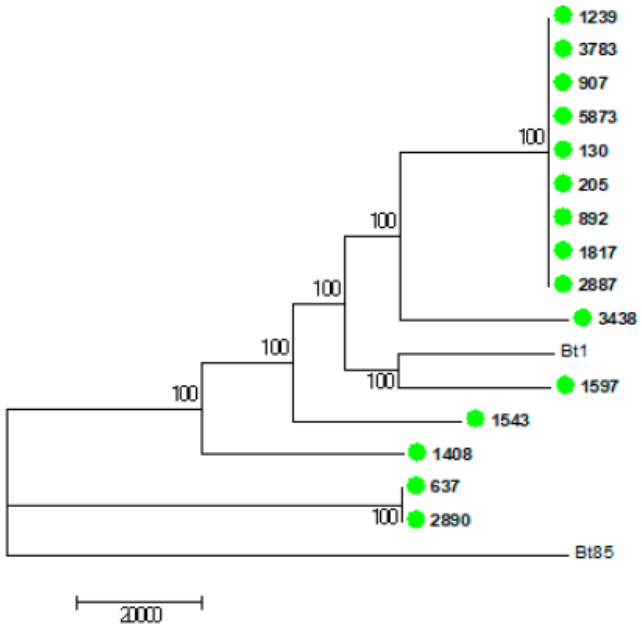


Figure 3. Whole genome sequencing single nucleotide polymorphism (WGS SNP) analysis of 15 South African clinical *Cryptococcus neoformans* isolates with the VNB molecular type (green circles and bold). Bt 85 (VNBI) was used as the reference and Bt 1 (VNBII) was added as an external genome. BWA was used to align reads to the reference and SAMtools was used to call SNPs. There were 293,484 SNPs in total with a coverage breadth of 88% and this is a midpoint-rooted maximum parsimony tree with 500 bootstrap replicates. The bootstrap values are shown next to the branches, the consistency index was 0.65, and the number of phylogenetic sites was 293,484.

We then performed a comprehensive analysis consisting of 95 publicly-available VNBII isolates from Botswana, South Africa, and Brazil. Six South African strains isolated from patients with recurrent cryptococcosis from previous studies [30,31] were found to be closely related to nine of our strains within the cluster clade with fewer than 12 SNPs between them. Figure 5 shows the WGS SNP analysis using BWA and SAMtools that zoomed in on these 15 closely-related VNB strains (nine from this study and six from previous South African studies). There were fewer than 30 SNP differences among these strains, further confirming that these strains are very closely related. In the comprehensive analysis, we also observed that two of our isolates were closely related to two Brazilian isolates (one being environmental and one being clinical) with fewer than 48 SNPs among the four isolates, while there were fewer than nine SNPs between our two VNB strains.

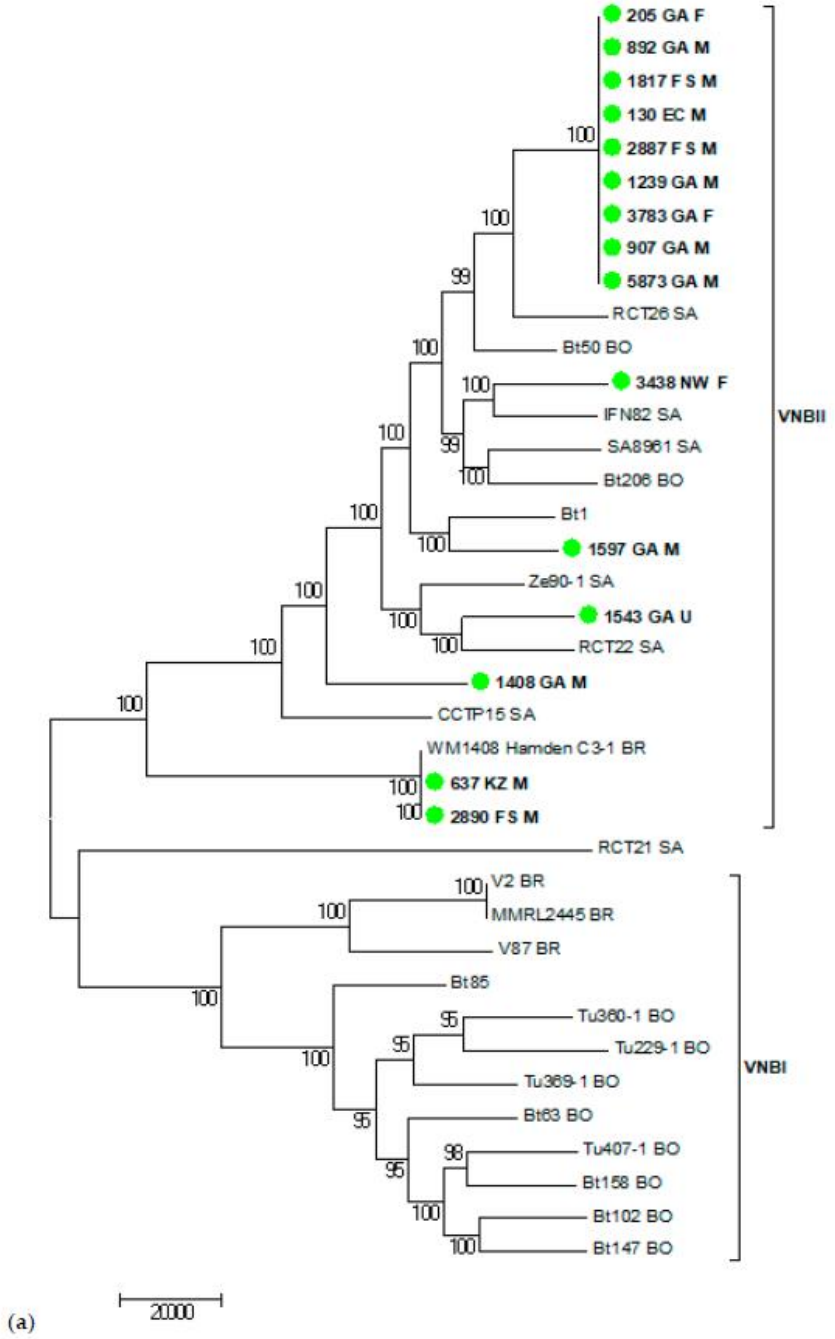
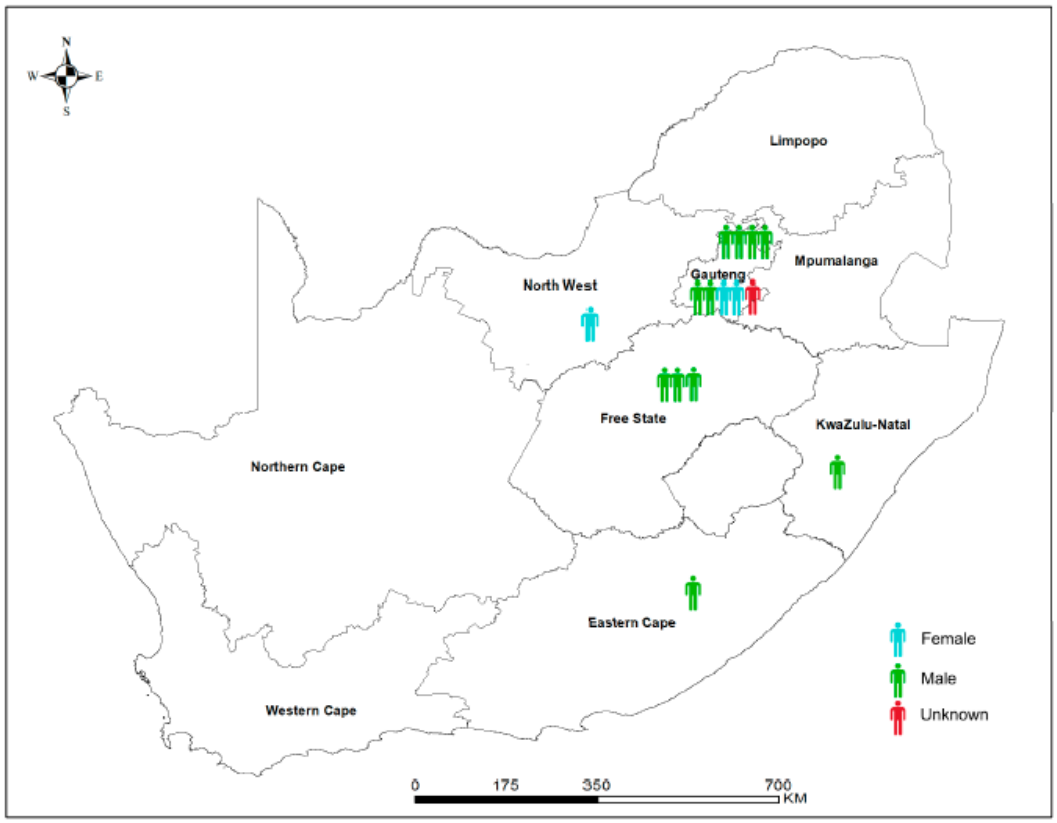


Figure 4. *Cont.*



(b)

Figure 4. (**a**) WGS SNP analysis of 15 South African clinical *Cryptococcus neoformans* isolates with the VNB molecular type (green circles and bold) and 21 publicly-available clinical and environmental VNB isolates [15]. Bt 85 (VNBI) was used as the reference and Bt 1 (VNBII) was added as an external genome. BWA was used to align reads to the reference and SAMtools was used to call SNPs. There were 488,188 SNPs in total with a coverage breadth of 79% and this is a midpoint-rooted maximum parsimony tree with 500 bootstrap replicates. The bootstrap values are shown next to the branches, the consistency index was 0.39, and the number of phylogenetic sites was 488,188. GA—Gauteng, FS—Free State, EC—Eastern Cape, NW—North West, KZ—KwaZulu-Natal, M—Male, F—Female, U—Unknown, SA—South Africa, BO—Botswana, BR—Brazil. (**b**) The map shows the geographical and gender distribution of the 15 South African patients infected with *C. neoformans* molecular type VNB in our study.

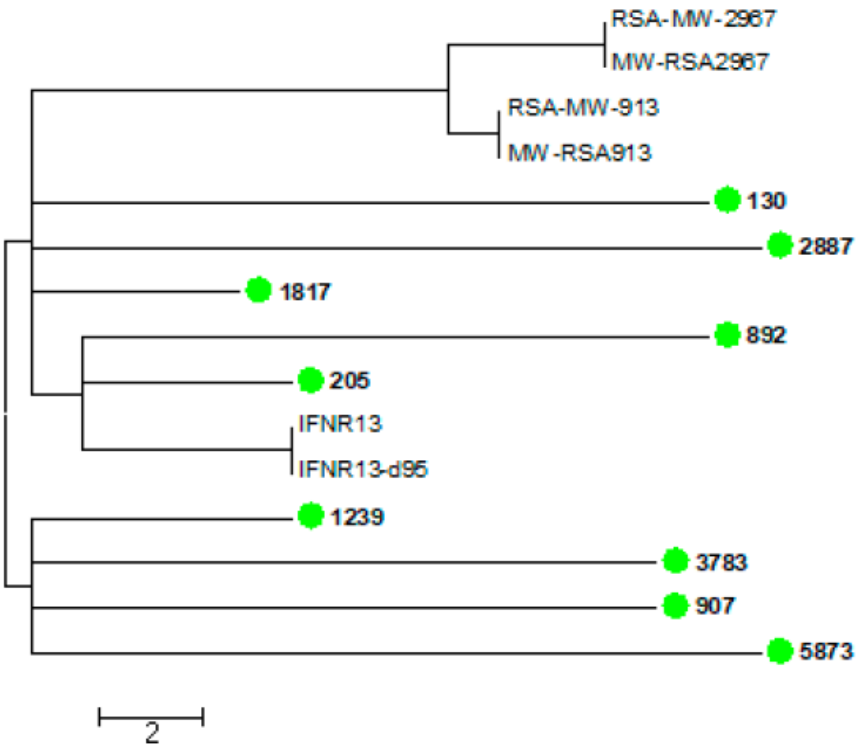


Figure 5. WGS SNP analysis of 15 South African clinical *Cryptococcus neoformans* isolates (nine from this study and six from previous South African studies) with the VNB molecular type. Isolates from this study are indicated with green circles and bold. Sample number 1239 was used as the reference for the VNBII clade. BWA was used to align reads to the reference and SAMtools was used to call SNPs. There were 111 SNPs in total with a coverage breadth of 73% and this is an unrooted maximum parsimony tree. The consistency index was 1.00 and the number of phylogenetic sites was 111.

4. Discussion

In summary, most of the 251 South African clinical *C. neoformans* strains in our study had the VNI molecular type, followed by VNII, VNB, and VNIV. There were no differences in fluconazole MIC values among molecular types and the majority of strains had low MIC values within the wild-type range. Males were more likely to be infected with a non-VNI genotype and patients infected with the non-VNI genotype were less likely to die in hospital, though for both of these analyses, our estimates had wide confidence intervals spanning 1 with large *p*-values. There was some heterogeneity in virulence between 15 VNB isolates in a *G. mellonella* larval model compared to the highly-virulent H99 strain. WGS data analysis showed that the 15 VNBs isolated in our study were very closely related and belonged to the VNBII clade.

VNI was the major molecular type identified in our study, which is consistent with the global molecular type distribution [9]. In Africa, VNI accounts for the highest percentage, followed by VNB and VNII, and a small percentage of VNIII [9]; this has been further confirmed by recent molecular epidemiology studies from Cameroon [32], Kenya [33], Ivory Coast [34], and Zimbabwe [35]. VNIV is mostly found in Europe and we found a few isolates with this molecular type in our study. VNIV has also been found in low percentages in Oceania, Asia, and in the Americas [9].

We observed 67 genotypes among these clinical South African *C. neoformans* strains, suggesting a high genetic diversity (D = 0.9205). There were 65 unique genotypes in a southern African study, of which 26 genotypes have only been found in this region

previously [12]. MLST analysis of *C. neoformans* isolates from pediatric patients from the same South African surveillance system used in our study showed 24 distinct sequence types (ST) [36]. Genotyping analysis of global *C. neoformans* var. *grubii* molecular type VNI isolates by MLST revealed that the highest genetic diversity was observed in African isolates [37]. Beale and colleagues analyzed 230 *C. neoformans* isolates from HIV-seropositive South African clinical trial patients and obtained 50 different STs [17]. Due to the high genetic diversity of African *C. neoformans* clinical and environmental strains, it has been hypothesized that this organism originated in Africa and then spread globally to the rest of the world. Litvintseva et al. termed this the "Out of Africa" hypothesis [12].

The *C. neoformans* isolates in this study, collected between 2005 and 2009, had fluconazole MIC_{50} and MIC_{90} values of 1 μg/mL and 4 μg/mL, respectively, and were therefore considered wild-type. We have recently shown that fluconazole MIC values of isolates from first episodes of meningitis increased between 2007–2008 and 2017 [23], providing evidence for higher fluconazole dose recommendations in the 2019 Southern African guideline for HIV-associated cryptococcosis [4]. In our current study, we found no association between MIC value and genotype. A Zimbabwean study found similar results [35]; whereas other studies have found that VNI isolates had a higher geometric mean fluconazole MIC compared to VNII [38] and VNIV isolates had a lower fluconazole MIC than VNI and VNIII isolates [39].

Previous studies have shown an association between HIV status [40], clinical outcome [10,17,29], altered mental status [17], host immune response [29], and genotype. More specifically, patients infected with the VNB genotype had a worse survival compared to those patients infected with VNI [17], suggesting that this molecular type is more virulent. However, in our study, we could not conclude that our 15 VNB strains were more virulent than H99 in a *G. mellonella* larval model. We only found one strain with a median larval survival time to H99. Furthermore, we observed heterogeneity in virulence between the 15 VNB strains. While we observed that patients infected with the non-VNI genotype (VNII and VNB) were less likely to die compared to patients infected with the VNI genotype, our sample size for patients infected with the non-VNI genotype was small and the 95% CI crossed 1 with a large *p*-value. In a previous study, patients infected with ST93 and ST77 isolates had a 79% mortality when compared to patients infected with ST5 and ST63 within the VNI genotype [29]. Miglia and colleagues found an unadjusted association between gender and ST in a South African pediatric population (84% of males compared to 16% of females, $p = 0.01$). This finding was subject to confounding due to lack of adjusting [36]. We found that males were more likely to be infected with a non-VNI genotype on adjusted analysis, but again, the 95%CI for this effect spanned 1 with a large *p*-value.

Phylogenetic analysis showed that nine of our VNB isolates clustered closely together, suggesting a common environmental source. However, these nine patients resided in the Gauteng, Free State, and Eastern Cape Provinces and therefore spread across different parts of South Africa, thus making it difficult to draw a firm conclusion on how these strains may be related or may have originated. Six South African strains isolated between 2005 and 2010 from patients with recurrent infection from two previous studies [30,31] were very closely related to our nine strains, suggesting a possible outbreak of *Cryptococcus*. Alternatively, it is also possible that the isolates were related by chance considering that there was an estimated total of between 541 and 1804 VNB isolates in 2005–2009.

Two of our VNB strains were more closely related to two Brazilian isolates than to the other South African isolates in our study, suggesting an association between the two South African strains and the two Brazilian strains. There may be more similarity between geographically-distinct VNB isolates and as more VNB isolates are identified elsewhere, especially from South America, we can study this further. Genomic evidence suggests that there were ancient migrations of the VNB molecular type bi-directionally between Africa and South America, and that South American VNB isolates are highly diverse [15]. In addition, VNB isolates from South America have experienced ancestral recombination and donated genetic material to all molecular types across different geographical areas [15].

In conclusion, our study shows that South African clinical *C. neoformans* isolates collected through national laboratory-based surveillance had a high genetic diversity with VNI being the dominant genotype and these clinical strains also had low fluconazole MIC values. South African isolates with the molecular type VNB were not highly virulent in a *G. mellonella* larval model, but more research needs to be done to confirm this. The few South African VNB strains in our study were very closely related by WGS SNP analysis, suggesting that this genotype may be able to cause outbreaks. Two South African VNB strains were related to Brazilian VNB strains, suggesting some level of relatedness and donation of genetic information between strains across geographical regions. As we learn more about the relationships between molecular types of *C. neoformans* from different geographical locations, we will gain a better understanding of this fungus that is globally distributed throughout the world.

Supplementary Materials: The following are available online at https://www.mdpi.com/article/10.3390/jof7050338/s1, Spreadsheet: Supplementary information for metadata, Table S1: Characteristics of five South African patients infected with the *Cryptococcus neoformans* VNIV molecular type (serotype D) collected during national laboratory-based surveillance for cryptococcosis, 2005–2009, Table S2: Univariable logistic regression analysis to determine associations between clinical characteristics and infecting strain molecular type among South African patients infected with *Cryptococcus neoformans* serotype A (n = 246), 2005–2009, Table S3: Multivariable logistic regression analysis to determine associations between clinical characteristics and infecting strain molecular type among South African patients infected with *Cryptococcus neoformans* serotype A (n = 246), 2005–2009, Table S4: Univariable logistic regression analysis to determine associations between infecting strain molecular type and in-hospital outcome among South African patients (n = 180) infected with *Cryptococcus neoformans* serotype A, 2005–2009. Table S5: Multivariable logistic regression analysis to determine associations between infecting strain molecular type and in-hospital outcome among South African patients (n = 180) infected with *Cryptococcus neoformans* serotype A, 2005–2009, Table S6: Characteristics of 15 South African patients infected with the *Cryptococcus neoformans* VNB molecular type collected during national laboratory-based surveillance for cryptococcosis, 2005–2009.

Author Contributions: Conceptualization, S.D.N., W.M., and N.P.G.; Methodology, S.D.N., R.E.M., and T.G.M.; Formal analysis, S.D.N., C.F., E.v.S., J.M.-N., J.B., D.M.E., and L.S.; Investigation, S.D.N., R.E.M., T.G.M., C.F., J.M.-N., J.B., and D.M.E.; Writing—original draft preparation, S.D.N. and N.P.G.; Writing—review and editing, S.D.N., C.F., D.M.E., L.S., W.M., and N.P.G.; Supervision, W.M. and N.P.G.; Funding acquisition, S.D.N. and N.P.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research was funded by the National Health Laboratory Service Research Trust (grant number 94712).

Institutional Review Board Statement: The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the Human Research Ethics Committee (Medical), University of the Witwatersrand (protocol code M160375 and 22 April 2016). An ethics waiver was obtained from the Animal Ethics Research Committee, University of the Witwatersrand (Registration number: AREC-101210-002) for the *G. mellonella* virulence studies.

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available in the supplementary information for metadata.

Acknowledgments: We acknowledge Samantha Bush from the Forestry and Agricultural Biotechnology Institute, University of Pretoria for providing the *Galleria mellonella* starter colony, Shune Oliver from the Center for Emerging, Zoonotic, and Parasitic Diseases at the NICD for use of the NICD insectary, Mushal Ali from the Core Sequencing Facility at the NICD for bioinformatics queries, and Rudzani Mathebula from the Center for Healthcare-Associated Infections, Antimicrobial Resistance and Mycoses at the NICD for creating the map in Figure 4. Members of GERMS-SA (2005–2009): Sandeep Vasaikar, Vivek Bhat (Eastern Cape); Gene Elliot (Free State); Anwar Hoosen, Kathy Lindeque, Olga Perovic, Trusha Nana, Charlotte Sriruttan, Charles Feldman, Alan Karstaedt, Jeannette Wadula, David Moore, Sharona Seetharam, Maphoshane Nchabeleng, Bonnie Maloba, Tlou

Chephe (Gauteng); Nomonde Dlamini, Yacoob Coovadia, Halima Dawood, Sumayya Haffejee, Meera Chhagan (KwaZulu Natal); Ken Hamese (Limpopo); Greta Hoyland, Jacob Lebudi (Mpumalanga); Pieter Jooste, Eunice Weenink (Northern Cape), Andrew Rampe (North West); Elizabeth Wasserman, Siseko Martin, Andrew Whitelaw (Western Cape); Keshree Pillay (Lancet laboratories), Adrian Brink, Maria Botha, Peter Smith, Inge Zietsman, Suzy Budavari, Xoliswa Poswa (Ampath laboratories), Marthinus Senekal (PathCare); Anne Schuchat, Stephanie Schrag (CDC); Keith Klugman, Anne von Gottberg, Linda de Gouveia, Karen Keddy, Arvinda Sooka, John Frean, Desiree du Plessis, Jaymati Patel, Vanessa Quan, Susan Meiring, Penny Crowther, Cheryl Cohen, and Nelesh Govender (NICD).

Conflicts of Interest: The authors declare no conflict of interest.

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Chapter 3: Molecular type distribution and fluconazole susceptibility of clinical *Cryptococcus gattii* isolates from South African laboratory-based surveillance, 2005-2013. (To be submitted to PLOS Neglected Tropical Diseases)

Abstract

Background: As is the case globally, *Cryptococcus gattii* is a less frequent cause of cryptococcosis than *Cryptococcus neoformans* in South Africa.

Methodology/Principal Findings: We performed multilocus sequence typing (MLST) and fluconazole susceptibility testing of isolates from 146 (of 750) South African patients with *C. gattii* disease identified through enhanced laboratory surveillance, 2005 to 2013. The dominant molecular type was VGIV (101/146, 70%), followed by VGI (27%, 40/146), VGII (2%, 3/146) and VGIII (1%, 2/146). There were 99 different sequence types (STs) among 146 *C. gattii* isolates, with ST294 (14/146, 10%) and ST155 (10/146, 7%) being most commonly observed. The fluconazole MIC_{50} and MIC_{90} values of 105 (of 146) randomly selected *C. gattii* isolates were 4 µg/ml and 16 µg/ml respectively. VGIV isolates had a lower MIC_{50} value compared to non-VGIV isolates, but these values were within one double-dilution of each other. HIV-seropositive patients had a ten-fold increased adjusted odds of a VGIV infection compared to HIV-seronegative patients, though with small numbers (99/136; 73% vs. 2/10; 20%), the confidence interval (CI) was wide (95% CI: 1.93-55.31, p=0.006). Whole genome phylogeny of 98 isolates of South Africa's most prevalent molecular type, VGIV,

identified that this molecular type is highly diverse, with only some strains being closely related within two small clusters of ten and six strains, respectively.

Conclusions/Significance: South African clinical *C. gattii* strains were highly diverse with VGIV being the dominant molecular type. Most VGIV strains had fluconazole MIC values within the wild-type range. HIV-seropositive patients were more likely to be infected with VGIV. Whole genome phylogeny of 98 VGIV strains confirmed their high genetic diversity, though two small clusters of closely related strains, with one cluster consisting only of patients from the Mpumalanga Province in South Africa, suggest a similar environmental source.

Author summary:

Cryptococcus is the most common cause of meningitis among adults in South Africa. Two species complexes within the fungal genus, *Cryptococcus neoformans* and *Cryptococcus gattii*, cause most human disease. The environmental range of these species complexes, both found in soil, overlaps in southern Africa though *C. gattii* is a less common human pathogen. *C. gattii* is divided into five molecular types: VGI, VGII, VGIII, VGIV and VGV. In earlier molecular epidemiology studies including relatively few isolates, most southern African strains were confirmed as molecular type VGIV. We aimed to determine the molecular diversity of *C. gattii* in South Africa by genotyping patient isolates obtained through laboratory surveillance, 2005-2013. We confirmed that VGIV was the dominant molecular type and that HIV-seropositive patients were more likely to be infected with VGIV compared to those HIV-seronegative. Analysis of the genomes of South African VGIV strains revealed that they spanned the whole VGIV clade and confirmed that most strains were not clustered.

However, we observed two small clusters of closely related strains, consisting of patients from three neighbouring provinces in South Africa, suggesting a similar environmental source. Further studies of clinical and environmental African *C. gattii* strains are needed to gain a better understanding of this pathogen.

3.1 Introduction

Basidiomycetous fungi within the genus *Cryptococcus* are the most common cause of meningitis among HIV-seropositive adults in southern Africa (Britz et al., 2016). Human disease is mostly caused by two species complexes within the genus, *Cryptococcus neoformans* and *Cryptococcus gattii* (Cano et al., 2020), with *C. gattii* causing fewer than 1% of cases of cryptococcal disease globally (Altamirano et al., 2020). While more often reported in the literature as causing disease in immunocompetent hosts without underlying medical conditions, *C. gattii* also causes disease in immunocompromised people (Altamirano et al., 2020, Montoya et al., 2021). People infected with *C. gattii* appear to present with pulmonary cryptococcosis more often than cryptococcal meningitis, with the opposite pattern being observed for *C. neoformans* disease (Montoya et al., 2021, Baddley et al., 2021) . Among HIV-seropositive adults with advanced immunosuppression, *C. gattii* disease (meningitis with or without fungaemia) has been reported to be indistinguishable from disease caused by *C. neoformans* (Morgan et al., 2006, Baddley et al., 2021). Regardless of the pathogen, flucytosine is recommended in combination with amphotericin B for induction treatment of HIV-associated cryptococcal meningitis, followed by fluconazole monotherapy for the consolidation and maintenance phases of treatment (Govender et al., 2019).

Cryptococcus gattii was historically thought to be confined to tropical and subtropical regions and was found less frequently in temperate regions (Maziarz and Perfect, 2016). The environmental range of the two closely related species overlaps in southern Africa, though *C. gattii* is a less common pathogen (Edwards et al., 2021). *Cryptococcus gattii* is mainly associated with decomposing plant matter and certain tree species, such as eucalyptus in Australia and California (Ellis and Pfeiffer, 1992, Vanhove et al., 2017), firs and oaks in the Pacific Northwest region of North America (Kidd et al., 2005) and numerous tropical tree species (e.g. *Ficus* spp.) (Springer and Chaturvedi, 2010), with a new niche for *C. gattii* recently described after its isolation from hyrax faeces in an environmental Zambian study (Vanhove et al., 2017).

Since *C. gattii* is mainly restricted to sedentary trees, it is not as widely distributed as *C. neoformans*, which is hypothesized to have a global distribution owing partially to its ability to metabolise pigeon droppings (Litvintseva et al., 2011). *Cryptococcus gattii* has also demonstrated the ability to adapt to new temperate niches, causing a geographically-restricted outbreak in North America (Kidd et al., 2005, Nielsen et al., 2007, Litvintseva et al., 2011, Engelthaler et al., 2014). There are many theories on the unexpected and unexplained emergence of *C. gattii* in North America. A recently-published hypothesis suggests that *C. gattii* was transported by ships in contaminated ballast water tanks from South America to North America in 1914 (Roe et al., 2018). This organism then established itself in coastal waters. A tsunami in 1964 may then have carried *C. gattii* into the coastal forests. It adapted subsequently to a new environmental niche (i.e. land/forest) causing human infections about three decades later (Engelthaler and Casadevall, 2019). While *C. neoformans* has been hypothesized to originate from Africa, *C. gattii* could have originated from South America

considering the high genetic diversity of South American *C. gattii* strains (Casadevall et al., 2017, Edwards et al., 2021).

Cryptococcus gattii is divided into five molecular types: VGI, VGII, VGIII, VGIV and VGV (Meyer et al., 2009, Farrer et al., 2019), which have also been proposed as separate species (Hagen et al., 2015), with the molecular type VGV recently identified from six environmental isolates collected from the Zambian Central Miombo woodlands (Farrer et al., 2019). VGI is mostly found in Australia and Papua New Guinea (Campbell et al., 2005). VGII is prevalent in Canada and the United States of America (USA), where it has caused a major outbreak (Kidd et al., 2005). However, this molecular type has subsequently also been found in Australia (Carriconde et al., 2011), Brazil and Colombia (Firacative et al., 2018). VGIII has been found in the USA (Lockhart et al., 2013), Mexico, Brazil and Colombia (Firacative et al., 2018). VGIV has until now been mainly identified from India (Cogliati et al., 2012), Colombia, Mexico (Meyer et al., 2003), Botswana, Malawi (Litvintseva et al., 2005) and Zimbabwe (Nyazika et al., 2016). In earlier molecular epidemiology studies including relatively few isolates, most southern African strains were confirmed as molecular type VGIV (Ellis et al., 2000, Litvintseva et al., 2005), with this molecular type mostly associated with disease in HIV-infected individuals (Montoya et al., 2021).

To expand on these earlier findings, we aimed to: (1) determine the molecular diversity of *C. gattii* in South Africa by genotyping a subset of clinical isolates from enhanced laboratory-based surveillance conducted between 2005 and 2013; (2) describe the fluconazole susceptibility of these isolates, and (3) determine if there was an association between patient clinical characteristics and the infecting molecular type, and the effect of molecular type on

in-hospital mortality. In addition, we analysed the genomes of 98 isolates of the most common South African *C. gattii* molecular type VGIV to place them into the global context.

3.2 Materials and Methods

3.2.1 Study design and sample selection

We conducted a cross-sectional study nested within laboratory-based surveillance for cryptococcosis in South Africa from 2005 to 2013. We used this time period since routine collection and storage of cryptococcal isolates through surveillance was terminated in 2013. From 1 January 2005 to 30 June 2008, cryptococcal isolates were submitted from all laboratories to the National Institute for Communicable Diseases (NICD). The surveillance methodology changed from 1 July 2008 to 31 December 2013 when only enhanced surveillance sites (ESS) (29 hospitals in nine provinces), National Health Laboratory Service (NHLS) laboratories in KwaZulu-Natal Province, and pathology laboratories in the private, mining, and military sectors were required to submit isolates. A case was defined as a person diagnosed with cryptococcal disease by any one of the following positive tests during a 30-day period: (1) India ink microscopy on cerebrospinal fluid (CSF) or (2) a positive cryptococcal antigen test on blood or CSF or (3) culture of *Cryptococcus* from any clinical specimen. Recurrent isolates from the same patients were excluded and only cases with cultured and viable isolates were included (Naicker et al., 2021). We also excluded isolates that were identified as *C. neoformans* or other *Cryptococcus* species. Following these inclusion and exclusion criteria, there were 750 cases of *C. gattii* infection in total that were available for analysis. HIV infection status was recorded for 387 of these patients at enhanced surveillance sites; we excluded all other patients with an unknown HIV infection status. Among these 387 patients, 374 were HIV-seropositive and 13 were HIV-seronegative. One hundred and thirty-six *C. gattii* isolates were randomly selected from the 374 HIV-

seropositive cases using a random-integer generator (https://www.random.org/integers/) and all 13 isolates from HIV-seronegative persons were selected but only ten were viable. In total, 146 unique cases were selected from 387 cases of *C. gattii* disease between 2005 and 2013 and the corresponding isolates were genotyped (Supplementary Figure 1 in Appendix C).

3.2.2 Subculture and identification of surveillance isolates

Following primary isolation of *Cryptococcus* species at diagnostic pathology laboratories, a sweep of the culture plate was inoculated onto Dorset medium (Diagnostic Media Products (DMP), NHLS, Johannesburg, South Africa) and transported to a reference laboratory at the NICD in Johannesburg, where the isolates were identified by phenotypic methods (Van Wyk et al., 2014) and stored at -70°C. The patient metadata that accompanied each isolate were captured in a surveillance database. The selected *C. gattii* strains were retrieved from -70°C storage and sub-cultured onto Sabouraud dextrose agar (DMP, NHLS, Johannesburg, South Africa) to check for purity and viability (Naicker et al., 2021). We phenotypically characterised isolates as previously described (Van Wyk et al., 2014).

3.2.3 Multilocus sequence typing (MLST) experiments and data analysis

DNA was extracted from single yeast colonies using the Zymo ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research Corp, USA) following the manufacturer's instructions. The only modification from the manufacturer's instructions was the starting material for DNA extractions since single yeast colonies grown on Sabouraud agar (DMP, NHLS, Johannesburg, South Africa) at 30°C after 48h was used. The International Society for Human and Animal Mycology (ISHAM) MLST consensus scheme for *C. neoformans* and *C. gattii* containing three housekeeping genes: *GPD1*, *SOD1* and *URA5*; three virulence genes:

CAP59, *LAC1* and *PLB1*; and the intergenic spacer region *IGS1* was used (Meyer et al., 2009). Primer sequences were obtained from a previous study (Meyer et al., 2009). DNA was amplified using a conventional PCR with DreamTaq Hot Start DNA Polymerase (Thermo Fisher Scientific, Waltham, Masschusetts, USA) in an Applied Biosystems 2720 Thermal cycler (Thermo Fisher Scientific, Waltham, Masschusetts, USA) using PCR cycling conditions described previously (Meyer et al., 2009). Amplicons were visualised on a 1% agarose gel and Sanger sequencing was performed. For each locus, DNA sequences in both forward and reverse orientations were obtained and edited using the program Sequencher ver. 5.4.6 (http://www.genecodes.com). Allele types (ATs) and sequence types (STs) were assigned using the online *C. gattii* MLST database (http://mlst.mycologylab.org). The concatenated DNA sequences for the seven loci were aligned using the program ClustalW (BioEdit Sequence Alignment Editor). A phylogenetic tree was generated with the program MEGA5 using the neighbor-joining method with a bootstrap analysis of 100 replicates and the Jukes-Cantor model (Tamura et al., 2011).

Mating type identification was performed by conventional PCR amplification using primer sets (Table A.3 in Appendix A) and cycling conditions from previous studies (Yan et al., 2002, Bovers et al., 2006), which are specific to the mating type regions of a and α mating-type cells. Amplicons were visualised on a 1% agarose gel and the mating type was determined using amplification that related to each mating-type's expected amplicon size (Table A.3 in Appendix A).

3.2.4 Fluconazole susceptibility testing

Of the 146 *C. gattii* isolates, fluconazole susceptibility testing was performed for 105 isolates: 60 randomly selected VGIV (from a total of 101), 40 VGI, three VGII and two VGIII. We

determined the fluconazole minimum inhibitory concentration (MIC) values (range: 0.125 µg/ml to 64 µg/ml) using custom-made broth microdilution (BMD) panels (NICD, Johannesburg) prepared, inoculated and read according to susceptibility testing methods published previously and the Clinical and Laboratory Standards Institute (CLSI) M27-A3 and M60 recommendations (Clinical and Laboratory Standards Institute, 2008, Clinical and Laboratory Standards Institute, 2017, Naicker et al., 2020). We interpreted fluconazole MIC values using published epidemiological cutoff values (ECVs) for the VGIII and VGIV isolates (Espinel-Ingroff et al., 2012), and the CLSI ECVs for the VGI and VGII isolates (Clinical and Laboratory Standards Institute, 2018).

3.2.5 Whole genome sequencing and phylogenetic analysis

Whole genome sequencing (WGS) was performed on 101 isolates of the molecular type VGIV using Illumina MiSeq sequencing technology (Illumina, San Diego, California, USA). Previously-extracted DNA samples (extraction procedure described above) were prepared for paired-end sequencing using the NEBNext Ultra II DNA Library Prep Kit for Illumina followed by 2 × 300 bp sequencing on a MiSeq instrument at the Translational Genomics Research Institute (Pathogen and Microbiome Division). Three genome sequences were of low quality; these genomes were excluded from further analysis and therefore we only analysed 98 VGIV genomes. Single nucleotide polymorphisms (SNPs) were detected from raw read data using the publically available Northern Arizona SNP pipeline (NASP) (Sahl et al., 2016). The pipeline was set to align reads for every sample to the known assembled reference VGIV genome (IND 107, NCBI BioSample number: SAMN01932842) using Burrows-Wheeler Aligner (BWA) (Li, 2013). SNP variants were detected using SAMtools (Li et al., 2009). Posterior filtering parameters involved removing positions that had <10x coverage, <90% variant allele calls, and those mapping to duplicated regions in the reference.

Downstream filtering by NASP produced the final high-quality or BestSNP alignment that was then used for maximum parsimony inference. Only positions present in all genomes with at least 10x depth of coverage and 90% agreement were included. Maximum parsimony trees with 100 bootstrap replicates were constructed using the phangorn library (Schliep, 2010) and visualized using MEGA 6 software (Tamura et al., 2013). We included 47 additional public VGIV whole genome sequences, five of which were sequenced in previous studies (Chen et al., 2017, Farrer et al., 2019) and 20 VGIV strains were obtained from the Australian Medical Mycology Culture Collection (WFCC registration number: WM-1205) sequenced herein. The 47 additional VGIV genomes included 19 strains from Botswana, 16 strains from South Africa, four strains from Colombia, three strains from India, two strains from Australia, one strain from Zambia and the geographical origin of one strain was unknown. For assessment of specific clusters, trees were drawn separately to maximize the high-quality comparable positions between samples of each apparent clade. For this, draft assemblies of one of the samples in each clade were created *de-novo* using SPAdes (https://cab.spbu.ru/software/spades/) in careful mode and then used as references for the cluster trees.

3.2.6 Statistical data analysis

For the 2005-2013 surveillance period, we compared demographic characteristics of patients with *C. gattii* and *C. neoformans* disease to determine if there were any differences between these two groups. For *C. gattii* infection, we used logistic regression models (Sperandei, 2013) to determine associations between patient characteristics and molecular type. Patient case data were collected by nurse surveillance officers at enhanced surveillance sites who either interviewed patients or reviewed their medical charts (Naicker et al., 2021). We defined a molecular type group as VGIV vs. non-VGIV since VGIV was the dominant molecular type

in South Africa. We included the following variables in the analysis with molecular type as the dependent variable: sex, age, year of diagnosis, geographical region, specimen type, HIV infection status, mental status at diagnosis, CD4+ T-cell (CD4) count at diagnosis, antiretroviral treatment, current antifungal treatment, and tuberculosis treatment. Mental status was categorised as "alert" (a Glasgow Coma Scale [GCS] score of 15) or "not alert" (a GCS score <15 or a patient was recorded to be disorientated, stuporose or comatose at the time of diagnosis) (Naicker et al., 2021). We divided the geographical regions of South Africa, based on the Koppen-Gieger climate classification zones (Adesina et al., 2017), into mostly temperate (Gauteng, Mpumalanga, KwaZulu-Natal and Western Cape provinces) vs. mostly arid (Northern Cape, Free State, Limpopo, North West and Eastern Cape provinces). In the final multivariable analysis, we only adjusted for factors that were associated with geographical region and were risk factors for VGIV infection; the variables age, sex and HIV infection status all met these criteria. We also separately modelled the effect of molecular type on in-hospital mortality, adjusting for sex, age, mental status at diagnosis, CD4 count at diagnosis, antiretroviral treatment, and current antifungal treatment. We compared the fluconazole MIC_{50} values of the VGIV isolates and non-VGIV isolates using a Wilcoxon rank-sum test (Kim, 2014). We also compared the association between MIC value (≤8 μg/ml vs >8 μg/ml) and outcome data of patients infected with *C. gattii*. Statistical analyses were performed using Stata statistical software (Version 14.1; StataCorp LP Texas USA).

3.2.7 Ethics approval

Ethics clearance for this study was obtained from the Human Research Ethics Committee (Medical), University of the Witwatersrand with clearance certificate numbers: M160375 and M1809107.

3.3 Results

3.3.1 Descriptive analysis of national laboratory-based surveillance

From 2005 to 2013, 25,676 viable *Cryptococcus* isolates were collected from 24,286 cases of cryptococcal disease. Only 3% of the viable isolates (781/25,676) were identified as *C. gattii* originating from 750 patients compared to 97% (24,871/25,676) of viable *C. neoformans* isolates originating from 23,512 patients. Owing in part to changes in surveillance methodology, the number of culture-confirmed *C. gattii* cases varied by year: 2005: 114 (15% of total), 2006: 133 (18%), 2007: 91 (12%), 2008: 61 (8%), 2009: 75 (10%), 2010: 65 (9%), 2011: 58 (8%), 2012: 56 (7%) and 2013: 97 (13%). The median age for patients infected with *C. gattii* or *C. neoformans* was 35 years, though the inter-quartile ranges (IQR) differed slightly (*C. gattii*: IQR, 30-42 years vs. *C. neoformans*: IQR, 29-41 years). Fifty-seven per cent (426/748) of male patients were infected with *C. gattii* compared to 48% (11,057/23,206) infected with *C. neoformans* (p=0.0001). More patients diagnosed with either *C. neoformans* or *C. gattii* disease were from the mostly temperate provinces of South Africa (*C. gattii*: 565/750, 75%; *C. neoformans*: 17,682/23,512, 75%) compared to the mostly arid provinces (*C. gattii*: 185/750, 25%; *C. neoformans*: 5,830/23,512, 25%). For both *C. gattii* and *C. neoformans* infections, CSF was the most common specimen type from which the pathogen was cultured (*C. gattii*: 712/750, 95%; *C. neoformans*: 22,279/23,512, 95%) followed by blood culture (*C. gattii*: 32/750, 4%; *C. neoformans*: 1179/23,512, 5%).

3.3.2 Genotyping of 146 clinical *C. gattii* isolates

We genotyped 146 isolates from a total of 387 cases between 2005 and 2013, equaling 38% of all cases from enhanced surveillance sites. The molecular type distribution of 146 *C. gattii* isolates (Supplementary information for metadata in Appendix C) was as follows: VGIV (101/146, 70%; 95% CI: 0.61-0.77) followed by VGI (40/146, 27%; 95% CI: 0.20-0.35),

VGII (3/146, 2%; 95% CI: 0.004-0.06) and VGIII (2/146, 1%; 95% CI: 0.002-0.05). Most isolates had the MATα mating type (n=139, 95%), whereas 5% (n=7, 5 VGIV and 2 VGI) were MATa. There were 99 different STs observed amongst 146 *C. gattii* isolates (Figure 3.1). ST294 (14/146, 10%) and ST155 (10/146, 7%) were the most commonly observed STs.

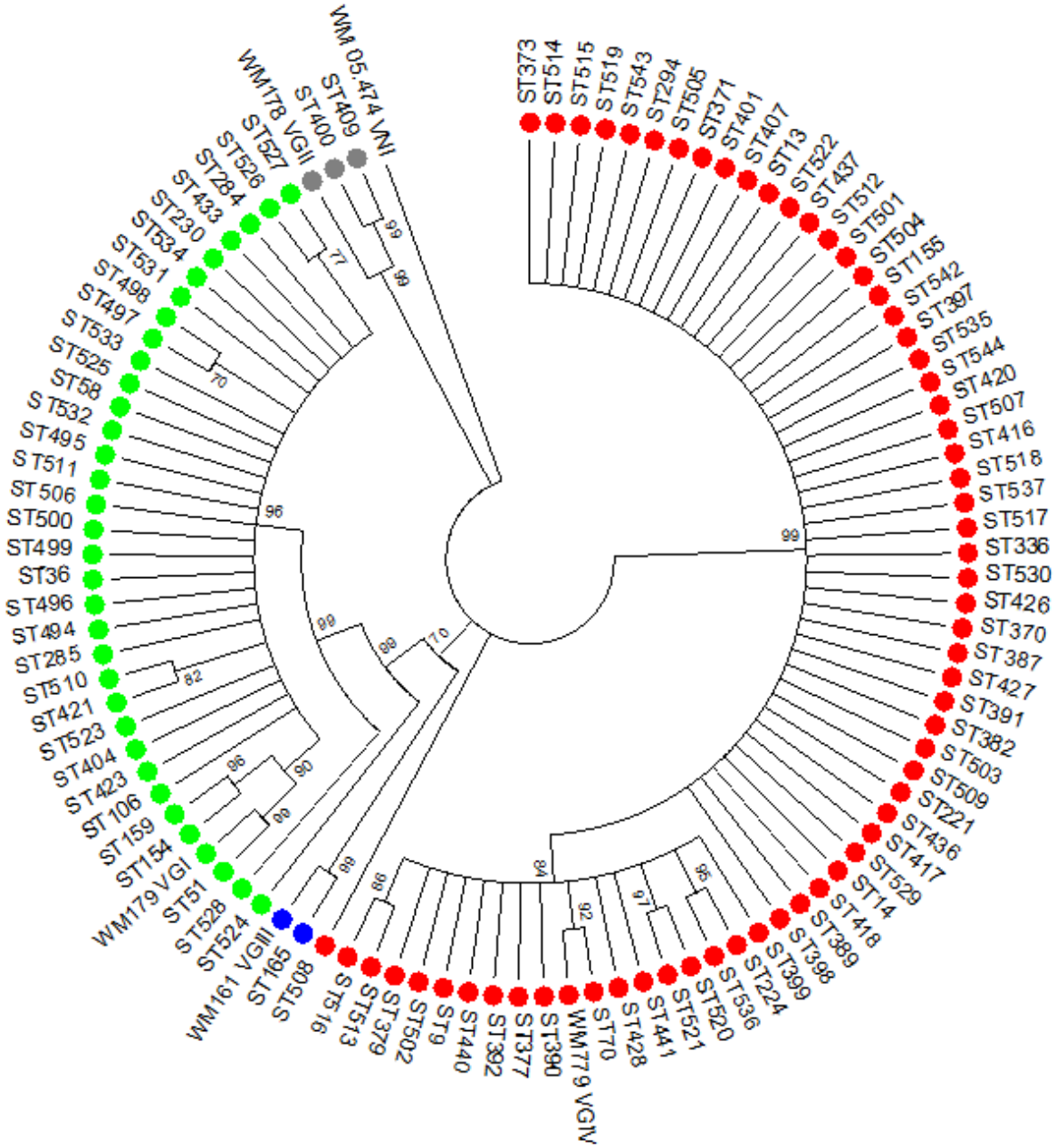

Figure 3.1: Neighbour joining phylogenetic tree based on multilocus sequence typing data showing clustering of molecular types for 99 different sequence types (STs) from 146 South African clinical *Cryptococcus gattii* isolates. *Cryptococcus neoformans* strain WM 05.474, molecular type VNI serves as the outgroup. Red circles indicate the STs with the VGIV molecular type, green circles indicate the STs with the VGI molecular type, grey circles indicate the STs with the VGII molecular type and blue circles indicate the STs with the

VGIII molecular type. Bootstrap analysis was performed using the program MEGA 5 with 100 bootstrap replicates. Bootstrap values are shown next to the branches.

3.3.3 Association between patients' clinical characteristics and molecular type

After adjusting for sex, age and HIV infection status, patients diagnosed in the mostly temperate provinces of South Africa had a 43% reduced odds of a VGIV infection than patients from mostly arid provinces, though the 95% CI spanned 1 (0.21-1.58) and the p-value was large (0.28) (Supplementary Tables 1 and 2 in Appendix C). We also found that HIV-seropositive patients had a ten times increased odds of a VGIV infection compared to those who were HIV-seronegative in the same adjusted analysis, though with small case numbers, the CI was very wide (95% CI: 1.93-55.31, p=0.006) (Supplementary Tables 1 and 2 in Appendix C).

3.3.4 Association between molecular type and in-hospital mortality

The overall crude in-hospital case-fatality ratio was 28% (40/142). The case-fatality ratio was 30% (29/98) for patients infected with *C. gattii* isolates of the VGIV molecular type versus 25% (11/44) for patients infected with a non-VGIV molecular type (crude odds ratio [OR]: 1.26, 95% CI: 0.56-2.84, p=0.57) (Supplementary Table 3 in Appendix C). On multivariable analysis, patients infected with the VGIV molecular type had a 43% reduced adjusted odds of dying than those infected with other molecular types, though the 95% CI crossed 1 (0.07-4.43) and the p-value was large (0.59) (Supplementary Table 4 in Appendix C).

3.3.5 Fluconazole susceptibility testing

The average fluconazole MIC_{50} and MIC_{90} values for all 105 *C. gattii* isolates (60 VGIV, 40 VGI, 3 VGII and 2 VGIII) were 4 µg/ml and 16 µg/ml, respectively, with a geometric mean of 4.53 µg/ml (Table 3.1). There was a statistical difference in the MIC_{50} values between the VGIV isolates and non-VGIV isolates (p=0.001), though these differed by only one double-dilution (4 µg/ml for VGIV isolates vs. 8 µg/ml for non-VGIV isolates) (Table 3.1). Most of the VGI, VGII and VGIV isolates had a MIC value of ≤8 µg/ml, which is considered to be wild-type according to published ECVs (Espinel-Ingroff et al., 2012, Clinical and Laboratory Standards Institute, 2018). However, the two VGIII isolates, six VGIV isolates and six VGI isolates were considered to be non-wild-type (Espinel-Ingroff et al., 2012, Clinical and Laboratory Standards Institute, 2018). On unadjusted analysis, patients with a cryptococcal isolate that had a fluconazole MIC value of greater than 8 µg/ml were more than one and a half times more likely to die, though this estimate also had a 95% CI spanning 1 and a large p-value (95% CI: 0.47-5.64; p=0.43).

Table 3.1: Fluconazole minimum inhibitory concentration (MIC) distribution (in µg/ml) by molecular type of 105 South African *Cryptococcus gattii* isolates from enhanced laboratory-based surveillance collected between 2005 and 2013

Molecular Type	**Total**	**MIC Value (µg/ml)**							**MIC_{50} (µg/ml)**	**MIC_{90} (µg/ml)**	**Geometric Mean**	**Range**
		0.5	1	2	4	8	16	32				
VGI	40	0	1	5	9	19	5	1	8	16	6.17	1-32
VGII	3	0	0	0	0	3	0	0	8	8	8	8
VGIII	2	0	0	0	0	2	0	0	8	8	8	8
VGIV	60	3	7	14	17	13	5	1	4	12	3.52	0.5-32
Total	**105**	**3**	**8**	**19**	**26**	**37**	**10**	**2**	**4**	**16**	**4.53**	**0.5-32**

3.3.6 Whole genome sequencing of 98 *C. gattii* VGIV isolates

Figure 3.2 shows the WGS SNP analysis of 98 VGIV isolates, the VGIV reference strain IND 107 and 47 global VGIV isolates. There were 123,891 SNPs called. The minimum, median and maximum number of SNPs were 0; 13,309 and 21,804, respectively. Scattered throughout the tree, there were nine clusters overall and the clusters are labelled in Figure 3.2. There were five clusters that only included isolates from our study. Cluster 1 consisted of three isolates (339, 1866 and 1788). Isolates 339 and 1866 had zero SNPs separating them and were isolated from patients from two different provinces. However, isolate 1788 was separated by more than 30 000 SNPs from the other isolates in cluster 1. Cluster 2 consisted of four isolates. The median number of SNPs was 198 between three isolates (924, 1428 and 274). The fourth isolate (4102) was separated by 850 SNPs from the other isolates in this cluster. Isolate numbers 924 and 1428 were from patients living in the same town in the Mpumalanga Province diagnosed at the same facility just over two months apart in 2011. Isolates 3096, 786

and 3470 were in cluster 3 and the median number of SNPs was 404. All three patients from whom these strains originated lived in the Gauteng Province and two patients were diagnosed in the same facility less than two months apart in 2013. There were two interesting clusters of closely related isolates observed (clusters 4 and 5 in Figure 3.2). Cluster 4 consisted of ten isolates from patients living in the Gauteng and Limpopo provinces named the Gauteng/Limpopo cluster (Supplementary Table 5 in Appendix C) and cluster 5 consisted of six isolates from patients living in the Mpumalanga Province named the Mpumalanga cluster (Supplementary Table 6 in Appendix C). Kmer-based typing of these genomes *in-silico* using a custom kraken database indicated that none of our isolates belonged genetically to the new molecular type VGV.

There were four clusters that included isolates from our study and some of the global strains (including other South African strains either from Chen et al., 2017 or from the Australian Medical Mycology Culture Collection). There were fewer than 30 SNPs between two previously-published sequences of South African isolates cultured from a South African patient with recurrent cryptococcosis between 2005 and 2009 (Chen et al., 2017) and South African isolate 500 from our study cultured from a patient living in the KwaZulu-Natal Province diagnosed in January 2007 (cluster 6 in Figure 3.2). There were fewer than 700 SNPs between isolate number 1005 and two South African samples sequenced from the Australian Medical Mycology Culture Collection in cluster 7. The minimum, median and maximum number of SNPs were 0, 6 and 478 respectively; between four Colombian strains (Escandón et al., 2006), a publically available sequence from a South African animal isolate and isolate number 3994 (cluster 8 in Figure 3.2). The patient infected with isolate 3994 was from the Gauteng Province and was diagnosed in September 2008. The minimum, median and maximum number of SNPs were 0, 318 and 908 respectively between isolates 547 (patient

from Gauteng Province) and 3882 (patient from Mpumalanga Province) and fifteen Botswanan samples that were isolated from CSF in 2000 and 2012 (cluster 9 in Figure 3.2).

We then analysed the Gauteng/Limpopo cluster and Mpumalanga cluster separately using the draft assembly of one of our strains as a reference in NASP. There were 1600 SNPs called within the Gauteng/Limpopo cluster of ten isolates (Figure 3.3a and c). The minimum, median and maximum number of SNPs were 52, 426 and 574 SNPs, respectively. Six patients from whom these strains originated were male and four patients were female. Seven patients were from the Limpopo Province; four of which were diagnosed at the same facility; and three were from the Gauteng Province. The cases did not cluster in time: one patient each was diagnosed in December 2006 and February 2008, three patients each in January, November and December of 2009, one patient each in May 2010 and August 2011, and one patient each in January, April and November 2013 (Supplementary Table 5 in Appendix C).

The Mpumalanga cluster of six isolates from our study and two from a previously published South African study (Chen et al., 2017) contained 1649 SNPs among all genomes (Figure 3.3b and c). The minimum, median and maximum number of SNPs were 0, 598 and 699 respectively. The six isolates from our study originated from five male patients and one female patient; five patients were diagnosed at the same facility. The cases did not cluster in time: one patient each was diagnosed in May 2006, April 2008, June 2009, January 2010, December 2010 and July 2011. The two previously-published sequences were from isolates cultured from another South African patient with recurrent cryptococcosis between 2005 and 2009 (Chen et al., 2017). The two isolates 70 and 2153 within this cluster were distanced by zero SNPs between them. These patients lived in two towns that were about 30 kilometers

apart, but both were diagnosed at the same facility two years apart (2008 and 2010) (Supplementary Table 6 in Appendix C).

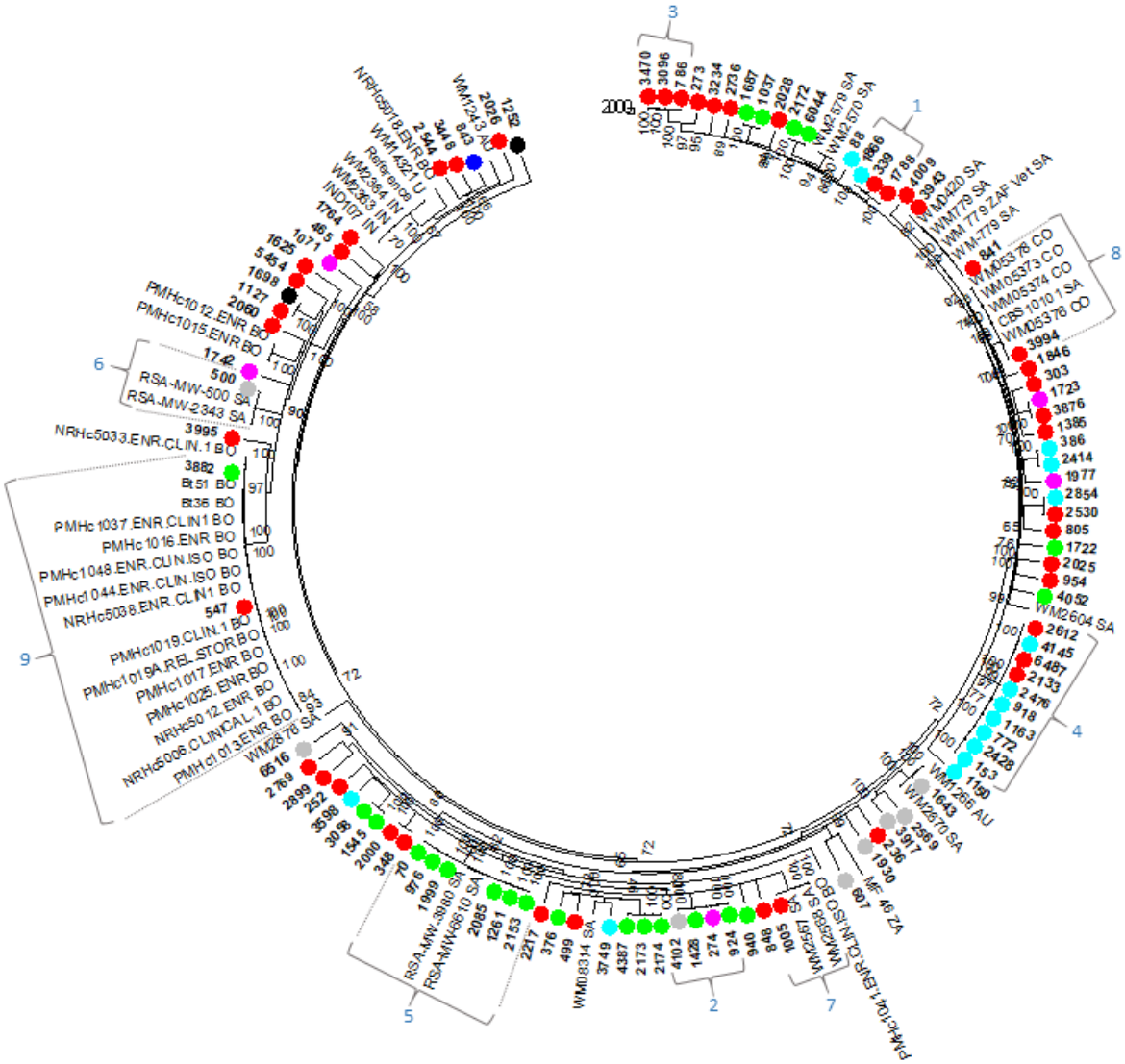

Figure 3.2: Whole genome sequencing single nucleotide polymorphism (SNP) analysis of 98 South African clinical VGIV *Cryptococcus gattii* isolates (coloured circles and bolded tip labels) and 47 previous samples. IND107 (labelled in the tree as "Reference") was used as the reference. Burrows-Wheeler Aligner was used to align reads to the reference and SAMtools (Li et al., 2009) was used to call SNPs. There were 123,891 SNPs in total with a coverage breadth of 61% and this is a rooted maximum parsimony tree with 100 bootstrap replicates. The bootstrap values are shown next to the branches, the consistency index was 0.35 and the number of phylogenetic informative positions were 123,891. BO – Botswana, SA – South Africa, CO – Colombia, AU – Australia, IN – India, ZA – Zambia, U - Unknown. Red –

Gauteng, green – Mpumalanga, light blue – Limpopo, purple – North West, grey – KwaZulu-Natal, black – Western Cape, dark blue – Northern Cape.

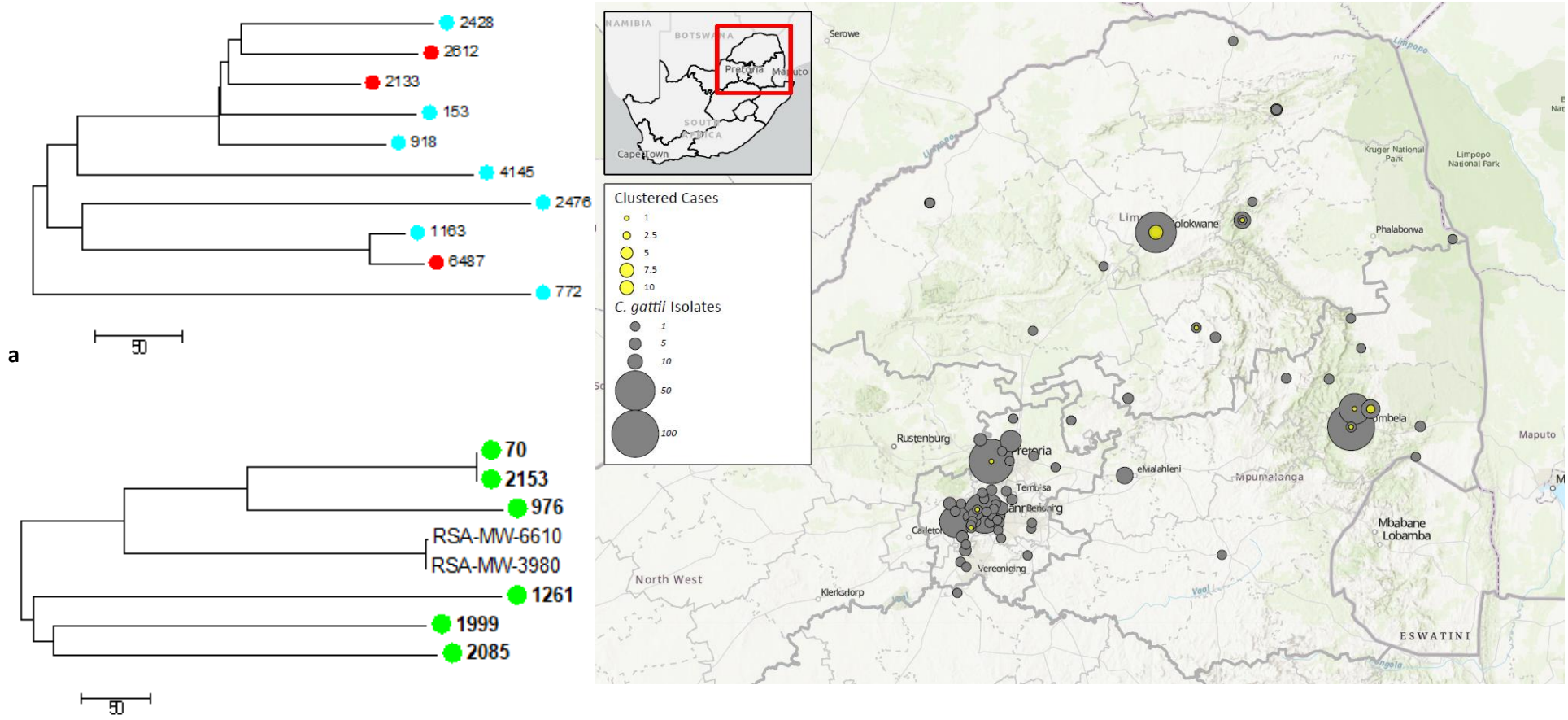

Figure 3.3: All clinical cases of *Cryptococcus gattii* infection (n=508) collected through laboratory-based surveillance during 2005-2013 from three South African provinces highlighting the cases that clustered closely by genomic data. a: Whole genome sequencing single nucleotide polymorphism (SNP) analysis of ten South African clinical VGIV *C. gattii* isolates from patients living in the Gauteng and Limpopo provinces. Strain number 918 was used as the reference. There were 1600 SNPs in total with a coverage breadth of 96% and this is an unrooted maximum parsimony tree constructed using MEGA6 software. The consistency index was 0.99 and the number of phylogenetic sites were 1600. Red – Gauteng, light blue – Limpopo. b: Whole genome sequencing SNP analysis of eight South African clinical VGIV *C. gattii* isolates [six from this study and two from a previously-published South African study (Chen et al., 2017)]. Isolates from this study are indicated in bold. Strain number 976 was used as the reference. There were 1649 SNPs in total with a coverage breadth of 96% and this is an unrooted maximum parsimony tree constructed using MEGA6 software. The consistency index was 1.00 and the number of phylogenetic sites were 1649. Green – Mpumalanga Province. c: Map of South Africa showing the clustered cases in yellow and all *C. gattii* cases (n=508) in grey from Gauteng, Limpopo and Mpumalanga provinces.

3.4 Discussion

Laboratory-based surveillance for cryptococcosis, a common HIV-associated opportunistic infection in South Africa, provided a platform to determine the molecular epidemiology of *C. gattii*. Only 3% of viable isolates were identified as *C. gattii* species-complex with the remainder identified as *C. neoformans* species-complex. Previous clinical studies from neighbouring southern African countries have reported a much higher proportion of *C. gattii* infections such as 13% (Litvintseva et al., 2005) or 17% (Nyazika et al., 2016). However, these studies included small case numbers, where cryptococcal isolates were obtained from patients admitted to a single facility or cases were not randomly selected from a defined study population.

We observed 99 STs among our clinical *C. gattii* isolates indicating a high genetic diversity, with VGIV being the dominant molecular type in South Africa. This confirms the results of studies undertaken in Zimbabwe, Botswana and Malawi (Litvintseva et al., 2005, Nyazika et al., 2016). In an earlier South African study on isolates from episodes of recurrent cryptococcosis, there were eight *C. gattii* isolates recovered from four patients and four isolates each had the molecular type VGI and VGIV, respectively (Van Wyk et al., 2014). In our larger study, we were able to describe the molecular type distribution of *C. gattii* in South African patients. Twenty seven per cent of the clinical isolates in our study had the VGI molecular type; this molecular type has been described in a previous study as being dominant among Kenyan clinical and environmental strains (Kangogo et al., 2015). In a study of 350 clinical, veterinary and environmental *C. gattii* strains from Africa, Asia, Europe, North America and South America, VGII was the dominant molecular type followed by VGI (Hagen et al., 2010). There were lower percentages of VGIII and VGIV (Hagen et al., 2010).

For the twenty-eight African isolates (countries unspecified), accounting for 8% of the total number of strains included in this global study, VGI was the dominant molecular type, followed by VGIV (Hagen et al., 2010). The authors concluded that VGI and VGII are globally distributed whereas VGIII and VGIV are minor molecular types that seem to be geographically restricted to specific locations (Hagen et al., 2010). However, a relatively larger number of strains was obtained from Europe, North America and South America, and therefore this collection was not representative of a global molecular type distribution (Hagen et al., 2010). The fluconazole MIC_{50} values, MIC_{90} values and ranges overlapped for isolates with all four molecular types in our single-centre study. Our study therefore does not provide sufficient evidence for any meaningful difference in fluconazole susceptibility by molecular type.

We found incidentally that HIV-seropositive patients had an increased odds of a VGIV infection compared to those HIV-seronegative, after adjusting for age, sex and climatic region. With a small sample size, our estimate of this effect is likely to be imprecise. However, previous studies in southern Africa have also shown that the VGIV molecular type is a major cause of *C. gattii* meningitis in HIV-seropositive patients (Litvintseva et al., 2005, Nyazika et al., 2016). VGIV and VGIII have been reported to cause infection in HIV-seropositive patients whereas VGI and VGII are reported to cause infection mainly in immunocompetent hosts (Byrnes et al., 2011, Chen et al., 2014, Montoya et al., 2021). We are uncertain of what could explain this difference but Harris and colleagues previously speculated that *C. gattii* molecular type, sequence type, fungal population genetics and environmental distribution "probably interact to mediate infection in patients with varying degrees of immune competence" (Harris et al., 2014). Based on the point estimate, patients

infected with the VGIV molecular type were less likely to die in hospital compared to patients infected with the non-VGIV molecular types, although we may have found this difference entirely by chance with a large p-value. Recent phenotypic variation results suggest that the VGIV molecular type may be a less virulent pathogen (Fernandes et al., 2018). These authors observed that the VGIV molecular type is similar to other molecular types in terms of capsule production and cell size (Fernandes et al., 2018). Both VGIV and VGIII molecular types were more sensitive to temperature compared to VGI and VGII that are considered most virulent (Fernandes et al., 2018). VGIV also produces irregular cells, a variant phenotype not shown to be related to patient death when compared to other variant phenotypes, such as giant cells, micro cells and shed extracellular capsule produced by *Cryptococcus* (Fernandes et al., 2018). There is also evidence that virulence is not specifically associated with molecular type (Firacative et al., 2014, Firacative et al., 2016). In one of these studies, *Galleria mellonella* larvae were infected with the four *C. gattii* molecular types and highly virulent strains were observed from all molecular types, suggesting that virulence may be related to specific strains regardless of the molecular type (Firacative et al., 2014).

In our WGS analysis, we observed two small clusters consisting of ten and six isolates respectively. The median number of SNPs for each of these two clusters were 426 and 598 respectively. However, the remaining isolates were highly diverse with a median of 13,309 SNPs. A previous study described nine VGI isolates isolated from patients living in the south-eastern states of the USA that clustered together with 41,000 SNPs and 4558 SNPs between any two isolates (Lockhart et al., 2016). Firacative and colleagues were able to define two major clusters of VGIII isolates in their study, separating serotype B from

serotype C isolates. There were 88,337 SNPs for one cluster and 79,945 SNPs for the second cluster (Firacative et al., 2016). Three clusters were observed amongst the VGII outbreak isolates in the PNW region of North America, made up of VGIIa, VGIIb and VGIIc, with 107, 132 and 137 SNPs within each of those subtypes, respectively, indicating that these VGII populations in that region were clonal with a low genetic diversity (Engelthaler et al., 2014, Lockhart et al., 2016). Our results suggest that, like VGI and VGIII, the VGIV molecular type also has a high genetic diversity. We observed that one of the isolates from our study and a previously-isolated South African sample were related to all four previously-isolated environmental Colombian VGIV strains with <480 SNPs (Escandón et al., 2006). This suggests some level of relatedness between VGIV strains from different geographical regions or a similar environmental exposure, although we do not have the travel history of the patients to confirm this. Our surveillance data also consisted of the patients' residential addresses and healthcare facility at the time of clinical diagnosis. However, this does not take into account the long latency period of cryptococcosis in some cases and relocation of residence between provinces. The Gauteng, Limpopo, North West and Mpumalanga Provinces are located in the northern parts of South Africa. These provinces are inland. However, climatic differences between these provinces do exist. The Gauteng Province is elevated and temperate, whereas parts of the Mpumalanga Province are low-lying, subtropical to tropical and humid but also dry in some areas. The North West Province and the Limpopo Province are hot and dry. There was a cluster of six strains isolated from patients living in the Lowveld region of Mpumalanga Province in South Africa, suggesting a common environmental source. Based on our findings, we can speculate that the VGIV molecular type may be widely distributed in this subtropical environment. In a Zambian study, *C. gattii* was mostly associated with the Zambian Central Miombo woodlands that is dry and tropical (Vanhove et al., 2017). The genomes of six of these isolates were recently

sequenced and found to represent a new molecular type, VGV (Farrer et al., 2019). This molecular type was found in hyrax-associated environments making this a new environmental niche for *C. gattii*. We did not find any VGV isolates in our collection of clinical *C. gattii* isolates. As Farrer and colleagues conclude, it is possible that this molecular type is strictly environmental and yet to cause clinical infection (Farrer et al., 2019). Further studies of clinical and environmental *C. gattii* strains from southern Africa should be performed to gain a better understanding of this pathogenic species-complex (Edwards et al., 2021).

In conclusion, our study showed that South African clinical *C. gattii* strains, collected through enhanced laboratory-based surveillance from 2005-2013, were highly diverse, the VGIV molecular type dominated and these strains also mostly had low fluconazole MIC values. HIV-seropositive patients were more likely to be infected with VGIV. We observed two small clusters of closely related VGIV strains, with one cluster consisting of patients from the Mpumalanga Province in South Africa, suggesting a similar environmental source. Our large study provides a broader baseline for more extensive WGS studies in southern Africa, to enable us to gain a more complete picture of *C. gattii*, the lesser known cause of HIV-associated cryptococcal meningitis in this part of the world.

Acknowledgements

We acknowledge Mushal Ali from the Core Sequencing Facility at the NICD for bioinformatics input, and Greg Greene from the Centre for Healthcare-Associated Infections, Antimicrobial Resistance and Mycoses at the NICD for creating the map in Figure 3.3. We

acknowledge the following people for supplying the additional VGIV strains in this study: Colombian strains - Elizabeth Castaneda, Patricia Luz Escandon Hernandez, Grupo de Microbiology, Institute Nacional de Salud, Bogoda, Colombia; South African strains - Valarie Davis, South African Institute for Medical Research, Johannesburg, South Africa and Melanie Anne John, University of Natal, Department of Medical Microbiology, Durban, South Africa; Australian strains - David Ellis, Mycology, Women's and Children's Hospital, Adelaide, SA, Australia and Kerry Weeks, Australian Medical Mycology Reference Laboratory, NSW Health Pathology, Sydney, NSW Australia.

Members of GERMS-SA (2005-2013)

Pieter Ekermans, Patricia Hanise, Carel Haummann, Dania Perez, Sandeep Vasaikar (Eastern Cape); Gene Elliott, Dominique Goedhals, Ute Halbauer, Anwar Hoosen, Madeleine Pieters, Justyna Wojno (Free State); Theunis Avenant, Kamaldeen Baba, Norma Bosman, Vindana Chibabhai, Jeane Cloete, Nicolette du Plessis, Adriano Duse, Charles Feldman, Alan Karstaedt, Ranmini Kularatne, Ruth Lekalakala, Kathy Lindeque, Warren Lowman, Motlatji Maloba, Caroline Maluleka, Moamekgethi Moshe, Trusha Nana, Maphoshane Nchabeleng, Gary Reubenson, Sharona Seetharam, Sheeba Varughese, Charl Verwey, Jeannette Wadula (Gauteng); Moherndran Archary, Prathna Bhola, Asmeeta Burra, Yacoob Coovadia, Halima Dawood, Khatija Dawood, Sumayya Haffejee, Prasha Mahabeer, Koleka Mlisana, Fathima Naby, Romola Naidoo, Praksha Ramjathan, Lisha Sookan, Khine Swe Swe Han (KwaZulu Natal); Andries Dreyer, Ken Hamese, Phaswe Maredi (Limpopo); Greta Hoyland, Jacob Lebudi, Barry Spies (Mpumalanga); Riezaah Abrahams, Dhamiran Naidoo, Eunice Weenink (Northern Cape); Andrew Rampe, Eduard Silberbauer, Lino Sono, Ebrahim Variava (North West); Colleen Bamford, Louise Cooke, Brian Eley, Heather Finlayson, Rena Hoffmann, Preneshni Naicker, Mark Nicol, James Nuttal, Heidi Orth, Helena Rabie, Catherine Samuel, Andrew Whitelaw (Western Cape); Maria Botha, Adrian Brink, Mark Cruz de Silva,

Chanelle Moore, Xoliswa Poswa, Peter Smith, Charlotte Sriruttan, Inge Zietsman (AMPATH); Chetna Govind, Krishnee Moodley, Keshree Pillay, Juanita Smit (LANCET); Marthinus Senekal (PathCare); Stephanie Schrag, Jennifer Verani, Cynthia Whitney (CDC); Keith Klugman (Emory); Penny Crowther-Gibson, Cheryl Cohen, Linda de Gouveia, Linda Erasmus, Melony Fortuin-de Smidt, Nelesh Govender, Nevashan Govender, Nazir Ismail, Karen Keddy, Sarona Lengana, Sonwabo Lindani, Susan Meiring, Cecilia Miller, Ruth Mpembe, Nireshni Naidoo, Ananta Nanoo, Tsakane Nkuna, Olga Perovic, Vanessa Quan, Mmakgomo Rakhudu, Languta Sibiya, Marshagne Smith, Arvinda Sooka, Anne von Gottberg, Claire von Mollendorf, Bulelwa Zigana (NICD).

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Chapter 4: Decreasing fluconazole susceptibility of clinical South African *Cryptococcus neoformans* isolates over a decade. PLOS Neglected Tropical Diseases 2020; 14 (3), 1-11.

PLOS NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Decreasing fluconazole susceptibility of clinical South African *Cryptococcus neoformans* isolates over a decade

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OPEN ACCESS

Citation: Naicker SD, Mpembe RS, Maphanga TG, Zulu TG, Desanto D, Wadula J, et al. (2020) Decreasing fluconazole susceptibility of clinical South African *Cryptococcus neoformans* isolates over a decade. PLoS Negl Trop Dis 14(3): e0008137. https://doi.org/10.1371/journal.pntd.0008137

Editor: Chaoyang Xue, Rutgers University, UNITED STATES

Received: November 6, 2019

Accepted: February 11, 2020

Published: March 31, 2020

Data Availability Statement: All relevant data are within the manuscript.

Funding: The authors received no specific funding for this work.

Competing interests: The authors have declared that no competing interests exist.

Abstract

Background

Fluconazole is used in combination with amphotericin B for induction treatment of cryptococcal meningitis and as monotherapy for consolidation and maintenance treatment. More than 90% of isolates from first episodes of cryptococcal disease had a fluconazole minimum inhibitory concentration (MIC) ≤4 μg/ml in a Gauteng population-based surveillance study of *Cryptococcus neoformans* in 2007–2008. We assessed whether fluconazole resistance had emerged in clinical cryptococcal isolates over a decade.

Methodology and principal findings

We prospectively collected *C. neoformans* isolates from 1 January through 31 March 2017 from persons with a first episode of culture-confirmed cryptococcal disease at 37 South African hospitals. Isolates were phenotypically confirmed to *C. neoformans* species-complex level. We determined fluconazole MICs (range: 0.125 μg/ml to 64 μg/ml) of 229 *C. neoformans* isolates using custom-made broth microdilution panels prepared, inoculated and read according to Clinical and Laboratory Standards Institute M27-A3 and M60 recommendations. These MIC values were compared to MICs of 249 isolates from earlier surveillance (2007–2008). Clinical data were collected from patients during both surveillance periods. There were more males (61% vs 39%) and more participants on combination induction antifungal treatment (92% vs 32%) in 2017 compared to 2007–2008. The fluconazole MIC_{50},

MIC_{90} and geometric mean MIC was 4 μg/ml, 8 μg/ml and 4.11 μg/ml in 2017 (n = 229) compared to 1 μg/ml, 2 μg/ml and 2.08 μg/ml in 2007–2008 (n = 249) respectively. Voriconazole, itraconazole and posaconazole Etests were performed on 16 of 229 (7%) *C. neoformans* isolates with a fluconazole MIC value of ≥16 μg/ml; only one had MIC values of >32 μg/ml for these three antifungal agents.

Conclusions and significance

Fluconazole MIC_{50} and MIC_{90} values were two-fold higher in 2017 compared to 2007–2008. Although there are no breakpoints, higher fluconazole doses may be required to maintain efficacy of standard treatment regimens for cryptococcal meningitis.

Author summary

Cryptococcus neoformans, a pathogenic fungal species-complex with an environmental niche, is the most common cause of meningitis among HIV-seropositive adults in sub-Saharan Africa. Fluconazole is recommended in combination with amphotericin B for induction treatment of cryptococcal meningitis and as monotherapy for consolidation and maintenance treatment. Fluconazole is also commonly prescribed to HIV-seropositive individuals for other indications; fluconazole exposure may result in secondary resistance if patients have concurrent active cryptococcal disease. Azole fungicides used in agriculture may potentially drive primary cryptococcal resistance when the fungus is exposed to these fungicides in the environment. We aimed to determine fluconazole MICs in 2017 and compare these values to those obtained in a 2007–2008 South African survey to assess whether fluconazole resistance had emerged in *C. neoformans* over a decade. We found that the proportion of isolates with an MIC of ≥16 μg/ml increased from 0% in 2007–2008 to 7% in 2017. MIC_{50} and MIC_{90} values were also two-fold higher in 2017 compared to 2007–2008. These study findings provided evidence for higher fluconazole dose recommendations (in combination with amphotericin B for the induction phase and as monotherapy for consolidation and maintenance phases) in the 2019 Southern African guideline for HIV-associated cryptococcosis.

Introduction

In South Africa, more than 6500 patients were diagnosed with a laboratory-confirmed first episode of cryptococcal meningitis during 2017, with an estimated incidence risk of 0.1% among HIV-seropositive persons [1]. A third of patients admitted to South African hospitals with cryptococcal meningitis die in hospital [2]. Cryptococcal meningitis is fatal in more than half of treated cases in routine care [3]. Fluconazole monotherapy is not appropriate as a first-line induction-phase treatment but is recommended as an alternative to flucytosine, in combination with amphotericin B. Fluconazole is also recommended for consolidation and maintenance treatment [4]. In an earlier population-based surveillance study of *Cryptococcus neoformans* in Gauteng province, South Africa, fluconazole susceptibility remained largely unchanged between 2002–2003 and 2007–2008. Only 0.6% of incident isolates from 2002–2003 had a fluconazole MIC of ≥16 μg/ml and these isolates also had very low MICs to amphotericin B, voriconazole and posaconazole [5]. In contrast, in a recent Ugandan study, Smith

and colleagues documented a substantial increase in fluconazole MICs in 2010–2014 compared to a previous study in 1998–1999. The MIC_{50} and MIC_{90} values were 8 μg/ml and 32 μg/ml respectively in 2010–2014 compared to an MIC_{50} of 4 μg/ml and an MIC_{90} of 8 μg/ml in 1998–1999 [6]. In a systematic review of 21 papers reporting fluconazole MIC distributions for clinical cryptococcal isolates, the median MIC_{50} value increased from 4 μg/ml in 2000–2012 to 8 μg/ml in 2014–2018 [7]. In a US study, 37% of isolates collected between 2001 and 2011 had an MIC ≥16 μg/ml, which the authors considered elevated based on a literature review. This study reported an association between elevated fluconazole MIC and prior azole use [8]. Fluconazole is a broad-spectrum antifungal agent with several indications, including cryptococcal antigenaemia, candidaemia, mucosal candidiasis and common fungal skin infections [9]. Fluconazole is commonly prescribed to HIV-seropositive patients; therefore, fluconazole exposure for other indications could result in development of secondary resistance if patients have concurrent active cryptococcal disease [10]. In agriculture, azole fungicides are used to treat crops. Of 229 pesticides registered in South Africa, 22 are azole-based fungicides [11]. Analogous to the emergence of azole-resistant *Aspergillus fumigatus* [12], it is also possible that cryptococcal strains develop resistance to azoles when exposed to fungicides in the environment and some people develop infections with primarily-resistant strains. The aim of this study was to assess whether there was an increase in fluconazole MIC values in South African clinical cryptococcal isolates since the last survey was performed almost ten years ago.

Materials and methods

Study design

Since 2002, the National Institute for Communicable Diseases (NICD) has conducted laboratory-based surveillance for cryptococcosis. A case has been consistently defined as a person diagnosed with cryptococcal disease at any South African laboratory, based on any one of the following positive tests: India ink test on cerebrospinal fluid (CSF), cryptococcal antigen test on blood or CSF and culture of *Cryptococcus* from any specimen. Using a standardised case report form, study nurses at enhanced surveillance sites collected detailed information from participants who met the laboratory case definition. In an earlier survey, Govender et al. had reported that 3/467 (0.6%) clinical *C. neoformans* isolates had fluconazole MICs ≥16 μg/ml [5]. In the 2017 survey, we needed to perform antifungal susceptibility testing on at least 220 *C. neoformans* isolates to show a difference in prevalence based on 80% power and an alpha of 0.05, if we hypothesized that the proportion of isolates with a fluconazole MIC ≥8 μg/ml had increased from <1% to 5%. In the earlier survey, 280 of 571 first episodes of cryptococcosis from 2007–2008 had been randomly selected from four enhanced surveillance hospitals in Gauteng province; 249 viable isolates were then tested for antifungal susceptibility using the same laboratory methods (with no modifications) as detailed in the section below. We prospectively collected *C. neoformans* isolates from 1 January through to 31 March 2017 from persons with a first episode of culture-confirmed cryptococcal disease at 37 enhanced and non-enhanced surveillance hospitals across South Africa. Only incident cases were included and these were defined as participants of any age with first isolation of *C. neoformans* from any specimen. We thus compared the antifungal susceptibility profiles of *Cryptococcus* isolates collected during 2 surveillance periods: 1 March 2007 through 28 February 2008 (n = 249, Gauteng province) and 1 January 2017 through 31 March 2017 (n = 229, national [9 provinces]).

Laboratory methods for 2017 survey

Isolates were received on Dorset transport medium (Media Mage, Johannesburg, South Africa) at NICD, were confirmed as *C. neoformans* species-complex by standard phenotypic methods,

then stored at -70˚C [13]. Canavanine-glycine-bromothymol blue (CGB) agar has a reported 100% specificity for identification of *C. neoformans* and was read after 96h of incubation at 30˚C [14]. No *C. gattii* isolates were identified. Antifungal susceptibility testing was performed on 229 randomly-selected *C. neoformans* isolates from first episodes of cryptococcal disease. All phenotypically-confirmed *C. neoformans* isolates submitted to NICD during 2017 were labelled consecutively and we randomly selected isolates (based on our required sample size) using a random integer generator (https://www.random.org/integers/). The 229 *C. neoformans* isolates were sub-cultured from freezer storage at least twice on Sabouraud agar (Diagnostic Media Products, National Health Laboratory Service [DMP], Johannesburg, South Africa) before antifungal susceptibility testing was performed. We determined fluconazole MICs (range: 0.125 μg/ml to 64 μg/ml) using custom-made broth microdilution panels prepared at NICD and inoculated and read according to Clinical and Laboratory Standards Institute M27-A3 and M60 recommendations [15, 16]. No technical and biological repeats were performed. However, two independent observers blinded to each other's readings manually read broth microdilution MICs using a reading mirror after 72h of incubation at 35˚C. MICs were read at a 50% inhibition endpoint. Each new batch of broth microdilution plates was tested using *Candida parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 as quality control strains. MIC values of *C. neoformans* isolates from 2017 were then compared to the MIC values of previously tested isolates from 2007–2008 [5]. For isolates with a fluconazole MIC ≥16 μg/ml, we also determined voriconazole, posaconazole and itraconazole MICs by Etest (bioMérieux, Marcy-l'Étoile, France) using RPMI agar (Diagnostic Media Products, NHLS, South Africa). MIC endpoints were also read at 50% inhibition for the above antifungal agents after 72h of incubation at 30˚C. A small subset of isolates (n = 20) were randomly selected (using the same random selection mentioned above) from 2007–2008, retrieved from -70˚C storage and re-tested using the custom-made fluconazole broth microdilution plates to determine whether MIC values matched values recorded previously [5].

Statistical analyses

We used chi-square or Fisher's exact tests to compare proportions and the Wilcoxon rank sum test to compare medians between the two surveillance periods using Stata version 14.1 (StataCorp LP, College Station, Texas, USA). We also determined whether prior hospitalisation (as a proxy for exposure to antifungals) was associated with a fluconazole MIC of ≥16 μg/ml. We performed a sensitivity analysis by varying the cut-offs for an elevated fluconazole MIC (≥8 μg/ml and ≥32 μg/ml) [7].

Ethics approval

Ethics clearance for this study was obtained from the Human Research Ethics Committee (Medical), University of the Witwatersrand. All data analysed was anonymized.

Results

In 2007–2008, there were 571 first episodes of cryptococcosis (Gauteng province), 280 of which were randomly selected; 249 viable isolates from these cases were tested for antifungal susceptibility [5]. From January through to March 2017, there were 260 *C. neoformans* isolates from first episodes (national survey) that met the inclusion criteria for antifungal susceptibility testing, 229 of which were randomly selected for antifungal susceptibility testing. In 2007–2008, 55% of participants were female with a median age of 35 years (IQR: 30–40 years). In 2017, 59% were male with a median age of 36 years (IQR: 30–43 years). There were more participants on combination induction antifungal treatment (fluconazole and amphotericin B) included in the

2017 survey compared to 2007–2008 (Table 1). The 20 *C. neoformans* isolates from the 2007–2008 period that were re-tested on the custom-made broth microdilution plates all had fluconazole MIC values within a double dilution of previously-reported MICs (S1 Table). Table 2 and Fig 1 show the MIC values for the two surveillance periods; in 2007–2008, the geometric mean was 2.08 μg/ml and 4.11 μg/ml in 2017. The MIC_{50} value increased from 1 μg/ml in 2007–2008 to 4 μg/ml in 2017. The MIC_{90} value increased from 2 μg/ml in 2007–2008 to 8 μg/ml in 2017. Voriconazole, itraconazole and posaconazole Etests were performed on 16 of 229 (7%) *C. neoformans* isolates with a fluconazole MIC value of ≥16 μg/ml. One isolate had MIC values of >32 μg/ml for voriconazole, itraconazole and posaconazole. The MIC ranges for the other isolates were 0.0004–1.5 μg/ml, 0.012–2 μg/ml and 0.064–8 μg/ml for voriconazole, itraconazole and posaconazole respectively (S2 Table). We found no evidence of an association between prior hospitalisation and a fluconazole MIC of ≥8 μg/ml (crude odds ratio 1.33, 95% CI: 0.59–3.01; p-value = 0.5) and ≥16 μg/ml (crude odds ratio 0.84, 95% CI: 0.17–4.08; p-value = 0.8). Clinical outcome data for participants in both surveillance periods are shown in Table 3. We found an 11% increased unadjusted odds of death among those infected with strains with an MIC ≥8 μg/ml (versus <8 μg/ml), though a 34% reduction to an 86% increase is also compatible with our data (crude odds ratio for death, 1.11; 95% CI: 0.66 to 1.86; p-value = 0.15).

Discussion

Over the last decade, the fluconazole susceptibility of *C. neoformans* isolates from a first episode of disease has decreased overall in South Africa. Fluconazole MIC_{50} and MIC_{90} values were two-fold higher in 2017 compared to 2007–2008. The proportion of isolates with a

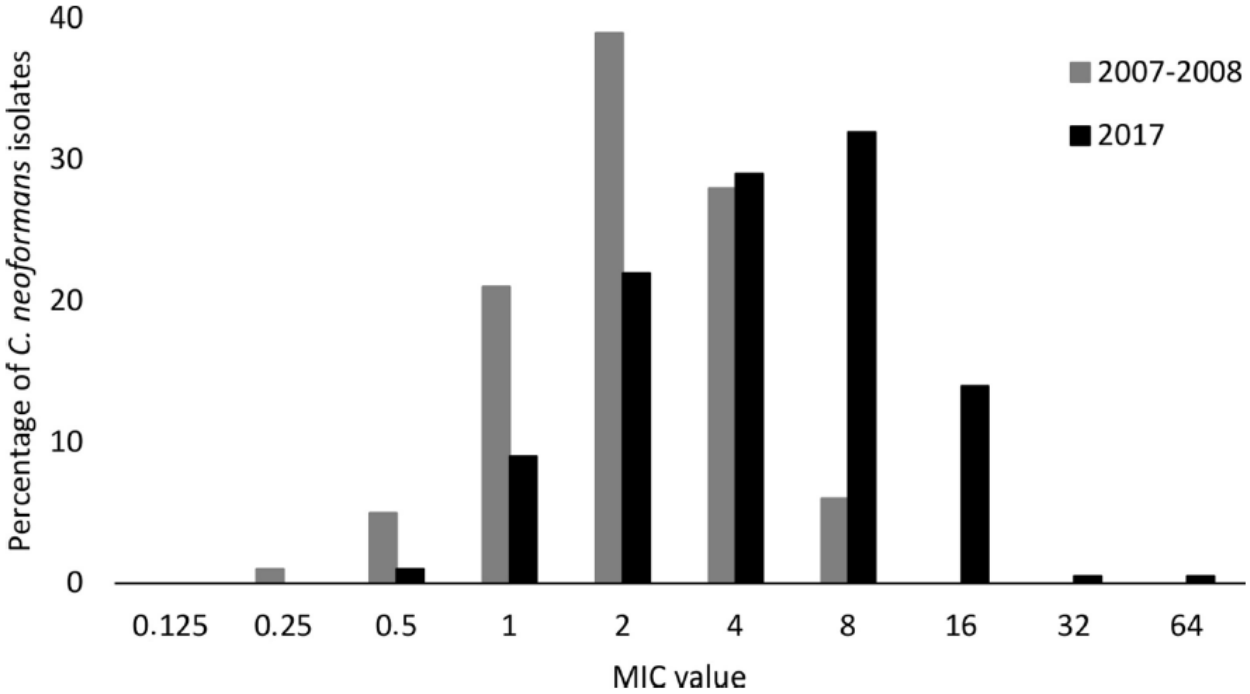

Fig 1. Fluconazole MIC values of cryptococcal isolates between 2 surveillance periods: 1 March 2007–28 February 2008 (n = 249) and January 2017 – March 2017 (n = 229).

https://doi.org/10.1371/journal.pntd.0008137.g001

Table 1. Comparison of patients with cryptococcosis and antifungal susceptibility results during two surveillance periods: 1 March 2007–28 February 2008 and January 2017–March 2017.

Characteristic	Number (%)		p value*
	2007–2008 (n = 249)	2017 (n = 204+)	
Sex (n = 453)	249	204	<0.001
Male	98 (39)	124 (61)	
Female	151 (61)	80 (39)	
Age, years (n = 450)	247	203	0.21
Median (IQR)	35 (30–40)	36 (30–44)	
HIV status (n = 375)	223	152	0.09
HIV-seropositive	223 (100)	150 (99)	
HIV-seronegative	0 (0)	2 (1)	
CD4 count, cells/μl (n = 299)	172	127	0.17
0–50	108 (63)	87 (69)	
51–100	35 (20)	22 (17)	
101–200	24 (14)	10 (8)	
>200	5 (3)	8 (6)	
Specimen type for positive cryptococcal culture (n = 453)	249	204	0.39
CSF	218 (88)	172 (84)	
Blood	31 (12)	31 (15)	
Other	0 (0)	1 (1)	
Antifungal treatment during admission (n = 365)	235	130	<0.001
Fluconazole alone	121 (51)	5 (4)	
Amphotericin B alone	20 (9)	6 (4)	
Fluconazole and amphotericin B	75 (32)	119 (92)	
No treatment	19 (8)	0 (0)	
In-hospital outcome (n = 384)	226	158	0.68
Died	84 (37)	62 (39)	
Survived	142 (63)	96 (61)	

*Wilcoxon rank sum test, chi-squared test or Fisher's exact test.

+ Number of cases after deduplication performed.

https://doi.org/10.1371/journal.pntd.0008137.t001

fluconazole MIC ≥16 μg/ml increased from 0% in 2007–2008 to 7% in 2017. This trend is consistent with studies from Uganda [6], USA [7] and Taiwan [17]. Epidemiological cut-off values currently exist for *C. neoformans*: non-typed isolates with an MIC value of ≥16 μg/ml are considered to be non-wild-type [18]. These findings have provided impetus for new fluconazole dosing recommendations from the Southern African HIV Clinicians' Society (SAHCS) for management of cryptococcal meningitis [19, 20]. Consistent with the antifungal regimens used in the ACTA trial [21], the 2019 SAHCS guideline recommends a fluconazole induction dose of 1200 mg per day (versus 800 mg per day, as previously recommended) in combination with amphotericin B, and a consolidation dose of 800 mg per day (versus previously-recommended 400 mg per day). Based on a MIC_{50} of 4 μg/ml, this will ensure that the area under the curve: MIC ratio (approximated by a daily dose: MIC ratio) is more than 100 for the first 10 weeks of treatment in most cases [7, 22]. Flucytosine should ideally be included in combination with either amphotericin B or fluconazole in the induction-phase regimen for meningitis [21]. Although flucytosine is not registered in South Africa, it is currently available through a clinical access programme [29]. The 2019 guideline also recommends that isolates from the first relapse episode be tested for fluconazole susceptibility rather than waiting for repeated

Table 2. Fluconazole MIC values of cryptococcal isolates collected during 2 surveillance periods: 1 March 2007–28 February 2008 and January 2017 –March 2017.

MIC value (μg/ml)	Number (%) of isolates	
	Feb 2007—Mar 2008 (n = 249)	Jan–Mar 2017 (n = 229)
0.125	0 (0)	0 (0)
0.25	1 (1)	0 (0)
0.5	14 (5)	3 (1)
1	53 (21)	20 (9)
2	97 (39)	50 (22)
4	70 (28)	67 (29)
8	14 (6)	73 (32)
16	0 (0)	14 (6)
32	0 (0)	1 (0.5)
64	0 (0)	1 (0.5)
Total	**249**	**229**
Range	0.25–8	0.5–64
MIC_{50}	1	4
MIC_{90}	2	8
Geometric mean	2.08	4.11

https://doi.org/10.1371/journal.pntd.0008137.t002

relapses to occur before testing [19, 23]. Fluconazole inhibits the lanosterol 14α-demethylase enzyme encoded by *ERG11* in the ergosterol biosynthesis pathway and targets the same cell processes as amphotericin B [6, 24]. The molecular basis of resistance includes multidrug efflux pump proteins, decreased affinity to target enzymes or overall decreased drug uptake [7]. Heteroresistance which is a phenomenon where strains express a transient adaptation to the drug also occurs [24, 25]. These mechanisms of resistance will need to be investigated further in the few *C. neoformans* isolates with a high fluconazole MIC from our study. Secondary resistance may occur in patients with active cryptococcal disease who are exposed to azoles or as a consequence of primary infection with resistant cryptococcal strains from the environment due to triazole fungicide exposure [6]. To support the latter hypothesis, a study has shown that cryptococcal strains exposed to the pesticide tebuconazole caused resistance to fluconazole both in vitro and in vivo [26]. We observed low MICs to voriconazole, posaconazole and itraconazole in most *C. neoformans* isolates with a fluconazole MIC ≥16 μg/ml. In a Zimbabwean study, isavuconazole, voriconazole, itraconazole and posaconazole had the most potent in vitro activity against *Cryptococcus* isolates from a cohort of patients with cryptococcal meningitis using the CLSI broth microdilution method [27]. These azoles could be used as alternatives to fluconazole though are associated with much higher costs, more adverse effects, more erratic pharmacokinetics and limited availability in most resource-limited settings [28].

Table 3. Association between fluconazole MIC values and clinical outcome of patients with cryptococcosis during two surveillance periods: 1 March 2007–28 February 2008 and January 2017 –March 2017.

MIC value (μg/ml)	Case-fatality ratio, n/N (%)	
	2007–2008	2017
≤2	50/165 (30)	17/66 (26)
2–4	27/70 (39)	22/62 (35)
≥8	7/14 (50)	23/76 (30)
All	84/249 (34)	62/204 (30)

https://doi.org/10.1371/journal.pntd.0008137.t003

Nasri et al. reported that prior azole exposure was associated with a higher fluconazole MIC in immunocompromised persons especially those with HIV infection. They also found that isolates with a fluconazole MIC ≥16 μg/ml were more likely to be cultured from people with central nervous system complications [7]. We could not determine an association between high fluconazole MIC and prior fluconazole exposure due to data sparsity. Although we did not genotype the *C. neoformans* isolates collected over the two surveillance periods, most isolates are likely to belong to the VNI genotype since this was the dominant genotype observed in a random sample of South African cryptococcal isolates over a 5-year surveillance period (Naicker SD, unpublished data). We also compared the fluconazole susceptibility profile of *C. neoformans* isolates from a national survey in 2017 to those from Gauteng (provincial) surveillance in 2007–2008 which is a limitation. However, nearly half (100/229) of the isolates tested for antifungal susceptibility in 2017 were from Gauteng province, five (31%) of which had a fluconazole MIC ≥16 μg/ml. We did not determine the molecular mechanism of resistance for the 16 *C. neoformans* isolates with a MIC of ≥16 μg/ml.

In conclusion, fluconazole MIC_{50} and MIC_{90} values were two-fold higher in clinical South African *C. neoformans* isolates collected in 2017 compared to 2007–2008. The resistance mechanisms of these isolates need to be studied further. These study findings provided evidence for higher fluconazole dose recommendations (in combination with amphotericin B for the induction phase and as monotherapy for consolidation and maintenance phases) in the 2019 Southern African guideline for HIV-associated cryptococcosis. Further efforts are needed to make flucytosine available for induction phase treatment.

Supporting information

S1 Checklist. STROBE checklist.
(PDF)

S1 Table. Fluconazole MIC values of 20 *C. neoformans* isolates from 2007–2008 that were obtained using custom-made broth microdilution plates.
(DOCX)

S2 Table. MIC values of voriconazole, itraconazole and posaconazole Etest MIC values for 16 *C. neoformans* isolates with a fluconazole MIC of ≥16 μg/ml.
(DOCX)

Acknowledgments

N.P.G. and D.D. were supported by the National Institute of Allergy and Infectious Diseases of the National Institutes of Health under Award Number R01AI118511. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health. Members of GERMS-SA: John Black, Vanessa Pearce (Eastern Cape); Masego Moncho, Motlatji Maloba (Free State); Caroline Maluleka, Charl Verwey, Charles Feldman, Colin Menezes, David Moore, Gary Reubenson, Jeannette Wadula, Merika Tsitsi, Maphoshane Nchabeleng, Nicolette du Plessis, Nontombi Mbelle, Nontuthuko Maningi, Prudence Ive, Theunis Avenant, Trusha Nana, Vindana Chibabhai (Gauteng); Adhil Maharj, Fathima Naby, Halima Dawood, Khine Swe Swe Han, Koleka Mlisana, Lisha Sookan, Nomonde Mvelase, Praksha Ramjathan, Prasha Mahabeer, Romola Naidoo, Sumayya Haffejee, Surendra Sirkar (Kwazulu Natal); Ken Hamese, Ngoaka Sibiya, Ruth Lekalakala (Limpopo); Greta Hoyland, Sindi Ntuli (Mpumalanga); Pieter Jooste (Northern Cape); Ebrahim Variava, Ignatius Khantsi (North West); Adrian Brink, Elizabeth Prentice, Kessendri Reddy, Andrew Whitelaw (Western Cape); Ebrahim Hoosien, Inge Zietsman, Terry Marshall, Xoliswa Poswa

(AMPATH); Chetna Govind, Juanita Smit, Keshree Pillay, Sharona Seetharam, Victoria Howell (LANCET); Catherine Samuel, Marthinus Senekal (PathCare); Andries Dreyer, Khatija Ahmed, Louis Marcus, Warren Lowman (Vermaak and Vennote); Anne von Gottberg, Anthony Smith, Azwifarwi Mathunjwa, Cecilia Miller, Charlotte Sriruttan, Cheryl Cohen, Desiree du Plessis, Erika van Schalkwyk, Farzana Ismail, Frans Radebe, Gillian Hunt, Husna Ismail, Jacqueline Weyer, Jackie Kleynhans, Jenny Rossouw, John Frean, Joy Ebonwu, Judith Mwansa-Kambafwile, Juno Thomas, Kerrigan McCarthy, Liliwe Shuping, Linda de Gouveia, Linda Erasmus, Lynn Morris, Lucille Blumberg, Marshagne Smith, Martha Makgoba, Mignon du Plessis, Mimmy Ngomane, Myra Moremi, Nazir Ismail, Nelesh Govender, Neo Legare, Nicola Page, Nombulelo Hoho, Ntsieni Ramalwa, Olga Perovic, Portia Mutevedzi, Ranmini Kularatne, Rudzani Mathebula, Ruth Mpembe, Sibongile Walaza, Sunnieboy Njikho, Susan Meiring, Tiisetso Lebaka, Vanessa Quan, Wendy Ngubane (NICD).

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Chapter 5: Discussion

This series of studies investigated the genotype, fluconazole susceptibility, and virulence of clinical *Cryptococcus neoformans* and *Cryptococcus gattii* strains from South Africa, collected through national laboratory-based surveillance during 2005-2013. We also compared fluconazole susceptibility patterns of *C. neoformans* isolates from 2007-2008 to isolates prospectively collected in 2017.

We studied a large number of clinical strains that made up a nationally representative sample. This enabled us to determine crude differences in the demographic and clinical characteristics of patients infected with *C. neoformans* and *C. gattii*. There was a much higher percentage of South African patients infected with *C. neoformans* than with *C. gattii* in our study. This is in keeping with global data (Altamirano et al., 2020) and consistent with the high prevalence of HIV-associated cryptococcosis in South Africa. In our study, meningitis was the most common syndrome regardless of the infecting *Cryptococcus* species. This finding is commonly observed among HIV-infected persons, among whom *C. gattii* disease is indistinguishable from *C. neoformans* disease and patients present with meningitis with or without fungaemia (Morgan et al., 2006). The median age of patients in our study population was 35 years regardless of infection with *C. neoformans* or *C. gattii*. For patients infected with *C. neoformans*, more females were infected than males (52% vs. 48%). However, for *C. gattii*-infected patients, more males were infected than females (57% vs. 43%). This is in contrast to one recent study comparing the two species complexes where *C. gattii*-infected patients were more frequently female (Baddley et al., 2021). Most patients that present with cryptococcosis are males (Montoya et al., 2021). Initially, it was thought that the reason for this could be that males are more exposed to *Cryptococcus* in the environment, however, it

was observed that males and females are exposed at equal rates (McClelland et al., 2013). There could be a possible interaction between *C. neoformans* and testosterone resulting in increased capsule shedding and *C. neoformans*-mediated macrophage death that make males more predisposed to cryptococcal disease (McClelland et al., 2013). We also observed that there were more *C. neoformans* isolates collected from males in 2017 compared to the 2007 study period. A possible reason for this could be that males changed their healthcare behaviour in 2017 and decided to seek care. More females being identified in 2007 could be due to the intervention of prevention of mother to child transmission (PMTCT) practices.

There were differences in the provincial distribution of patients infected with *C. neoformans* and *C. gattii* in our study, since more patients from the Mpumalanga and Limpopo Provinces were infected with *C. gattii* than with *C. neoformans*. There are possible ecological reasons such as the Mpumalanga Province being subtropical to tropical or that the Limpopo Province is hot and dry that could provide a more suitable environment for *C. gattii*. However, more environmental studies are required as suggested by Edwards and colleagues in a recently published review (Edwards et al., 2021) to confirm the above hypothesis. During our study period 2005-2013, we found that the case fatality ratio for South African patients infected with *C. neoformans* was 36% compared to 28% for *C. gattii* (p=0.16). An overall in-hospital case-fatality ratio of 34% was observed in 2019 (GERMS-SA Annual Report, 2019). Possible reasons for the high mortality rate could be due to South African patients entering routine hospital care with advanced HIV disease and advanced cryptococcal disease, as well as a failure of integrated care following diagnosis of cryptococcal meningitis (Quan et al., 2019). The fluconazole MIC_{50} and MIC_{90} values were two double-dilutions higher for *C. gattii* compared to those for *C. neoformans*; a previous study showed that there was a higher level of heteroresistance to fluconazole in *C. gattii* than in *C. neoformans* (Varma and Kwon-

Chung, 2010). These *C. gattii* strains were also more resistant to xenobiotics than *C. neoformans* which could be due to the ecological niche *C. gattii* inhabits since tree hollows may harbour various ubiquitous saprophytic microorganisms that produce xenobiotics (Varma and Kwon-Chung, 2010). All *C. gattii* strains showed heteroresistance in this one study therefore this is an intrinsic property of *C. gattii* that the authors speculate reflect an acquisition from its ecological niche but this needs to be studied further (Varma and Kwon-Chung, 2010).

While other South African studies have focused on genotyping a subset of *Cryptococcus* strains from patients enrolled into clinical trials or from clinical surveillance (Miglia et al., 2011, Van Wyk et al., 2014, Beale et al., 2015), we randomly selected and genotyped a nationally representative sample of clinical *Cryptococcus* isolates. This strength in our study design allowed us to explore the molecular diversity of clinical *Cryptococcus* strains in South Africa at a population level. We were able to confirm that there is a high genetic diversity among South African clinical *C. neoformans* and *C. gattii* isolates. Previous genotyping analysis of global molecular type VNI isolates by multilocus sequence typing (MLST) revealed that the highest genetic diversity was observed in African isolates (Ferreira-Paim et al., 2017). *Cryptococcus gattii* VGII isolates that caused the outbreak in the Pacific Northwest (PNW) in North America were clonal with low genetic diversity (Engelthaler et al., 2014), whereas isolates with the VGI and VGIII genotypes had a high genetic diversity (Lockhart et al., 2013, Firacative et al., 2016, Lockhart et al., 2016). We confirmed that VNI is the dominant genotype in our setting in Africa, which has been observed in previous studies (Cogliati, 2013, Kangogo et al., 2015, Nyazika et al., 2016b, Ngouana et al., 2016, Kassi et al., 2016). We also confirmed that VGIV is the dominant genotype in South Africa, which also confirms previous data (Litvintseva et al., 2005, Nyazika et al., 2016a).

Our whole genome phylogenetic analysis of VNB and VGIV isolates provided more information on these molecular types since there are a few studies on the whole genome sequencing (WGS) of African *Cryptococcus* isolates (Vanhove et al., 2017, Chen et al., 2017, Rhodes et al., 2017a, Rhodes et al., 2017b, Desjardins et al., 2017, Farrer et al., 2019). About 80% of VNB and VGIV isolates were found to be closely related by whole genome phylogenetic analysis in our study with the same sequence type (ST) demonstrating that MLST is able to determine relatedness at an imperfect level compared to WGS. Our study further confirmed that WGS is more discriminatory than MLST. However, in resource-limited settings, MLST will still be used to identify similarities between strains and to study associations with patients' clinical characteristics (Montoya et al., 2021).

We observed two interesting clusters of closely related *C. gattii* VGIV isolates, one cluster of ten isolates from South African patients living in the Gauteng and Limpopo Provinces and the other cluster of six isolates from patients in the Mpumalanga Province suggesting a similar environmental source, which prompted us to speculate that this genotype may be widely distributed in the environment. Sequencing *Cryptococcus* isolates from rarely studied geographical regions provided useful information (Vanhove et al., 2017, Rhodes et al., 2017b). Vanhove and colleagues studied Zambian *Cryptococcus* isolates and found that both *C. neoformans* and *C. gattii* adapted to occupy different ecological niches, observing differences at the species-complex level (Vanhove et al., 2017). *Cryptococcus gattii* was associated with the tropical Central Miombo Woodlands that has a wetter climate with a higher altitude, whereas *C. neoformans* was associated with the drier, lower altitude Zambezi Mopane Woodlands (Vanhove et al., 2017). Furthermore, Farrer and colleagues discovered a

new molecular type of *C. gattii* named VGV from these Zambian Central Miombo Woodlands region and a new hyrax-associated environmental niche for this particular genotype (Farrer et al., 2019).

There were <30 single nucleotide polymorphisms (SNPs) between most of the South African *C. neoformans* VNB isolates in our study, suggesting a common environmental source. A population genomics study on *C. neoformans* included VNB isolates from South America; this molecular type experienced ancestral recombination and donated genetic material to all molecular types across different geographical areas (Rhodes et al., 2017b). Additionally, it was hypothesised that the VNB molecular type may be more virulent than the other *C. neoformans* molecular types (Beale et al., 2015). However, we were unable to conclude that this molecular type was more virulent in a *Galleria mellonella* larval model in our study. Our whole genome phylogenetic analysis showed that South African *C. neoformans* VNB and *C. gattii* VGIV isolates were also related to Brazilian and Colombian isolates, respectively, suggesting that geographically-distinct VNB and VGIV isolates show some similarities. *Cryptococcus* has the ability to adapt to different environments (Nielsen et al., 2007, Litvintseva et al., 2011, Vanhove et al., 2017). Certain factors, such as increased urbanization (Vanhove et al., 2017), shipment of trees/agricultural products, increased international travel and global warming (Kwon-Chung et al., 2014, Engelthaler and Casadevall, 2019) may have also contributed to the global distribution of these fungal pathogens.

In our logistic regression analyses, we found associations between male sex and the non-VNI genotype. We can merely speculate that males travel more frequently away from their rural

home towns to the larger suburbs to find work. Since *Cryptococcus* is acquired from the environment and since VNI is mostly associated with urban populations (Litvintseva et al., 2011, Chen et al., 2015, Vanhove et al., 2017), it is possible that males obtain the less dominant non-VNI genotype from rural areas. We also found a direct association between HIV-infected patients and the VGIV genotype. We had a small number of HIV-seronegative patients therefore the estimate of this effect is likely to be imprecise. However, we can speculate that the *C. gattii* molecular type together with other factors such as fungal population genetics and environmental distribution all interact to cause infection in patients with varying immune competence (Harris et al., 2014). We could not find any association between molecular type and in-hospital mortality for both *C. neoformans* and *C. gattii*. It is important to note that cryptococcal molecular type is not the only factor influencing the outcome of South African patients. Other factors include the host (such as advanced HIV disease), appropriate antifungal treatment and access to healthcare in South Africa.

We also observed that fluconazole resistance has increased in incident cryptococcal isolates between 2007-2008 and 2017. Our findings led to higher fluconazole dose recommendations for induction and consolidation phases in the 2019 Southern African guideline for HIV-associated cryptococcosis (Govender et al., 2019). In South Africa, fluconazole is used in the absence of flucytosine in combination with amphotericin B for the treatment of HIV-associated cryptococcal meningitis (Guidelines, 2018). However, flucytosine remains the best combination with amphotericin B (Molloy et al., 2018). Trial participants receiving amphotericin B-flucytosine combination therapy had a lower mortality (31%) compared to the 45% mortality of patients on amphotericin B-fluconazole combination therapy at ten weeks (Molloy et al., 2018). These findings provided strong additional evidence that flucytosine together with amphotericin B should be made more accessible in resource-limited

settings. In South Africa, flucytosine is only currently available for cryptococcal meningitis through the clinical access programme. Certain patients at clinical access sites received flucytosine-based induction treatment in 2019 (Shroufi et al., 2020), however; the overall case fatality ratio still remained high among patients with cryptococcosis (GERMS-SA Annual Report, 2019). A new and better treatment is urgently needed for treatment of HIV-associated cryptococcal meningitis. Candidate drugs in development or in clinical trials, such as fosmanogepix (Shaw et al., 2018), VT-1129 (Wiederhold et al., 2018), tamoxifen (Ngan et al., 2019) and arylamidine T-2307 (Nishikawa et al., 2017) are currently being considered. However, these options are still far in the future. In the near future, the Ambition-CM trial results can lead to implementation of a new treatment strategy (Lawrence et al., 2018). The results released in July 2021 showed that the 10-week mortality was 24.82% in the AmBisome arm (single high-dose liposomal amphotericin B with two weeks of flucytosine plus fluconazole) compared to 28.75% in the control arm (amphotericin B deoxycholate plus flucytosine) (Lawrence et al., 2021). These results showed that liposomal amphotericin B in combination with flucytosine and fluconazole was non-inferior compared to the standard treatment regimen (Lawrence et al., 2021). However, liposomal amphotericin B needs to be made more accessible to countries with the highest burden of cryptococcal disease (Lawrence et al., 2021).

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Chapter 6: Conclusion, strengths, limitations and recommendations

We studied the molecular epidemiology, fluconazole susceptibility profile and virulence of clinical South African *Cryptococcus neoformans* and *Cryptococcus gattii* isolates collected through laboratory-based surveillance. Our study showed that South African clinical *C. neoformans* and *C. gattii* isolates had a high genetic diversity with VNI and VGIV being the dominant genotypes among the two sibling species, respectively. Males were more likely to be infected with a non-VNI genotype and patients infected with the non-VNI genotype were less likely to die in hospital, though for both of these analyses, our estimates could be a result of chance. HIV-seropositive patients had a ten times increased odds of a VGIV infection on multivariable analysis, though with small numbers in our analysis, the confidence interval (CI) was very wide. Patients from the temperate regions of South Africa were less likely to be infected with a VGIV genotype. Patients infected with the VGIV genotype were less likely to die in hospital and patients with a fluconazole minimum inhibitory concentration (MIC) value of greater than 8 µg/ml were more than one and a half times more likely to die. For the latter three analyses, our estimates could again be a result of chance. Clinical *C. neoformans* and *C. gattii* strains collected between 2005 and 2013 mostly had low fluconazole MIC values, although the MIC_{50} and MIC_{90} values for *C. gattii* were two double dilutions higher compared to those of *C. neoformans*. We also observed that fluconazole MIC_{50} and MIC_{90} values were two-fold higher in clinical South African *C. neoformans* isolates collected in 2017 compared to 2007–2008.

South African isolates with the molecular type VNB were not highly virulent in a *Galleria mellonella* larval model, but more research needs to be done to confirm this. The few South African VNB strains in our study were very closely related by whole genome phylogenetic

analysis, suggesting a common environmental source. Two South African VNB strains were related to Brazilian VNB strains, suggesting some level of relatedness and donation of genetic information between strains across geographical regions. Whole genome phylogenetic analysis of 98 South African VGIV strains confirmed their high genetic diversity with most strains not closely related. However, we observed two small clusters of closely related strains with one cluster consisting of patients from the Mpumalanga Province of South Africa suggesting a similar environmental source.

Strengths

1. A representative sample of clinical *Cryptococcus* isolates from laboratory-based surveillance was randomly selected and genotyped. This strength in our study design allowed us to explore the molecular diversity of clinical *Cryptococcus* strains in South Africa at a population level.
2. In order to compare whether fluconazole susceptibility patterns have changed between 2007-2008 and 2017, the same antifungal susceptibility testing (AST) methods were used in the same reference laboratory allowing for a reliable comparison.

Limitations

1. There were 16 *C. neoformans* isolates collected from persons with a first episode of culture-confirmed cryptococcal disease in 2017 with a fluconazole MIC of ≥16 μg/ml, according to epidemiological cutoff values (ECVs), these isolates were considered as non-wild-type. However, we did not determine the molecular mechanism of resistance.

2. In our 2017 fluconazole susceptibility study of *C. neoformans* isolates collected from persons with a first episode of culture-confirmed cryptococcal disease, we could also not determine an association between high fluconazole MIC and prior fluconazole exposure due to data sparsity.
3. Cryptococcal isolates were collected for national laboratory-based surveillance of cryptococcosis between 2005 and 2013. We only genotyped *C. neoformans* isolates from 2005-2009 and *C. gattii* isolates from 2005-2013; we therefore did not have recent genotyping data available to make a comparison.
4. Clinical isolates were collected from patients who may have re-located between the time of acquiring infection from the environment and the time of clinical diagnosis. We had the patients' residential addresses at the time of clinical diagnosis; this enabled us to determine hospital and residence. However, this does not take into account the long latency period and if patients re-located.
5. We only performed single nucleotide polymorphism (SNP) analysis on the whole genome sequencing (WGS) data of the VNB and VGIV isolates and we did not determine virulence and antifungal resistance markers from this data.
6. There were also missing patient data from laboratory-based surveillance thereby providing small datasets for the logistic regression analyses thereby reducing power and contributing to the lack of associations.
7. Phenotypic and genotypic variation in fungi can occur whereby performing susceptibility and typing studies on isolates stored for a long time at -70°C may not be a true reflection.

Recommendations

1. Some of the findings from this thesis provided evidence for higher fluconazole dose recommendations (in combination with amphotericin B for the induction phase and as monotherapy for consolidation and maintenance phases) in the 2019 Southern African guideline for HIV-associated cryptococcosis. However, further efforts are needed to make flucytosine widely available for induction phase treatment.
2. Since 2013, the National Institute for Communicable Diseases (NICD) has conducted electronic laboratory surveillance for cryptococcal disease. Isolate-based surveillance should be initiated again at the NICD so that we can analyse recent data in a continuing perspective and observe trends. Surveillance should be initiated at certain enhanced surveillance sites in South Africa such as the Gauteng, KwaZulu-Natal, Mpumalanga and Limpopo Provinces to focus on finding more VNB isolates and more *C. gattii* cases.
3. We should determine the molecular mechanism of resistance of 16 *C. neoformans* isolates with a fluconazole MIC of ≥16 μg/ml from the 2017 survey.
4. More virulence studies (possibly in a mouse or rabbit model) are needed to determine whether the VNB genotype is more virulent among the *C. neoformans* molecular types.
5. Future work should include exploring the virulence and resistance genes of the VNB and VGIV isolates from this study using WGS data.
6. Further studies should be performed to determine the genetic differences between the VNB and VGIV isolates from our study. As more VNB and VGIV isolates are identified globally, we can include these isolates into a larger study to determine the meaning of genomic similarities between geographically distinct isolates.

7. Further studies should include the determination of the geographic origin of the VGIV molecular type.
8. Additional environmental studies focused on areas in the Limpopo, Mpumalanga, and Gauteng Provinces of South Africa in collaboration with other researchers in the southern African regions should be performed to determine the overall environmental distribution of *C. gattii* in the region, which should also include environmental strains in a larger genome-wide association study to determine any differences in virulence or stress response genes between environmental and clinical isolates.

Findings from this thesis confirmed that there is a high genetic diversity among clinical *Cryptococcus* strains from South Africa. Whole genome phylogenetic analysis showed certain cryptococcal strains in closely related clusters suggesting that people in South Africa may acquire infection caused by these organisms from a common environmental source. South African cryptococcal isolates were also related by whole genome phylogenetic analysis to isolates from geographically distinct regions such as Brazil and Colombia suggesting a genetic link between African and South American strains. Exposure to *Cryptococcus* in the environment is almost universal and cannot be prevented. However, high-risk patients with advanced HIV disease should be screened for cryptococcal antigenaemia and started quickly on appropriate antifungal treatment to prevent mortality. The induction regimen of amphotericin B and fluconazole is sub-optimal based on clinical trial data; our data also showed that fluconazole resistance has increased for South African *C. neoformans* isolates from first episodes of cryptococcal disease when compared to ten years ago.

Appendix A: Materials and Methods

A.1 Cryptococcal disease surveillance in South Africa

Since 2005, population-based national surveillance for cryptococcosis has been conducted by the National Institute for Communicable Diseases (NICD). A case has been consistently defined as illness in a person diagnosed with cryptococcal disease at any South African laboratory, based on any one of the following positive laboratory tests during a 30-day period: India ink test on cerebrospinal fluid (CSF), antigen test on blood and CSF and culture of *Cryptococcus* from any specimen. Using a standardised case report form, study nurses at enhanced surveillance sites (ESS) collected detailed information from participants who met the laboratory case definition. The surveillance methodology changed between 1 July 2008 to 31 December 2013 whereby only ESS (29 hospitals in nine provinces), National Health Laboratory Service (NHLS) laboratories in the KwaZulu-Natal province, and laboratories in the private, mining, and military sectors were required to directly report case patients and/or send isolates to the NICD. Viable cryptococcal isolates collected from patients at ESS were characterised by phenotypic and/or genotypic tests through to 2013. From 2015 onwards, no laboratories were required to directly report case patients or send isolates to the NICD. For these cases of cryptococcosis, data were obtained directly from the NHLS Corporate Data Warehouse (CDW), which stores information from laboratory information systems (GERMS-SA Annual Report, 2019). From 1 January through to 31 March 2017, *Cryptococcus* isolates from persons with a first episode of culture-confirmed cryptococcal disease at both enhanced and non-enhanced surveillance hospitals across South Africa were specifically collected for a fluconazole susceptibility testing project.

A.2 Genotyping of clinical *Cryptococcus neoformans* and *Cryptococcus gattii* isolates by multilocus sequence typing (MLST)

A.2.1 Study design and sample selection

This was a cross-sectional study nested within national laboratory-based surveillance for cryptococcosis. Only cases diagnosed at an ESS were included in the study. In addition, recurrent isolates from the same patients were excluded. At least 50 *C. neoformans* isolates from each of the surveillance years, 2005 to 2009, were randomly selected for genotyping, using a random-integer generator (https://www.random.org/integers/) totalling 251 cases. In some years, more than 50 isolates were selected to account for non-viability and/or contamination.

Based on a preliminary analysis of the number of cases and isolates submitted to the NICD, 750 viable *C. gattii* isolates were available for further study. Of these cases, HIV infection status was recorded for 387, 374 patients with *C. gattii* disease were HIV-infected and 13 were HIV-seronegative. One hundred and thirty six *C. gattii* isolates were randomly selected from the 374 HIV-infected cases using a random-integer generator (https://www.random.org/integers/) and all 13 isolates from HIV-seronegative persons were selected but only ten were viable. While other studies have focused on genotyping a subset of *Cryptococcus* strains from patients enrolled into clinical trials or diagnosed at a single hospital in South Africa, we randomly selected a representative sample of clinical *Cryptococcus* isolates from an isolate bank created through national population-based surveillance. This study design allowed us to explore the molecular diversity of the whole population of clinical *Cryptococcus* strains in South Africa.

A.2.2 Subculture and identification of surveillance isolates

Following primary isolation in culture at diagnostic laboratories, a sweep of the culture plate was inoculated onto Dorset medium (Diagnostic Media Products (DMP), NHLS, Johannesburg, South Africa) and transported to the NICD, where the isolates were identified by phenotypic methods (described below) and stored at -70°C. The patient metadata (Table A.1) that accompanied each isolate were captured in a surveillance database. The selected *C. neoformans* and *C. gattii* strains were retrieved from -70°C storage and sub-cultured onto Sabouraud agar (DMP, NHLS, Johannesburg, South Africa) to check for purity and viability. Once these strains were grown, colonies were sub-cultured onto Niger seed agar (DMP, NHLS, Johannesburg, South Africa) to confirm melanin production. Brown colonies on Niger seed agar were considered as positive for *C. neoformans* and *C. gattii* and white colonies were negative for melanin production. Brown colonies were inoculated onto a canavanine-glycine-bromothymol blue (CGB) agar plate (DMP, NHLS, Johannesburg, South Africa) and if the agar remained yellow, the isolate was phenotypically confirmed as *C. neoformans* and if the CGB agar turned blue with growth present then it was confirmed as *C. gattii* (Kwon-Chung et al., 2014). Canavanine-glycine-bromothymol blue agar was read after 96h of incubation at 30°C. Canavanine-glycine-bromothymol blue agar has a reported 100% specificity for identification of *C. neoformans* and 93% sensitivity for identification of *C. gattii* compared to DNA sequencing (McTaggart et al., 2011).

Table A.1: Patient metadata that accompanied each *Cryptococcus neoformans*/*Cryptococcus gattii* isolate for the South African laboratory-based surveillance programme, 2005-2013

Patient metadata
Sex
Age
Year of diagnosis
Province
Specimen type
Final outcome
HIV status
CD4+ T-cell count at diagnosis
Antiretroviral treatment
Mental status at diagnosis
CSF India ink result
CSF white cell count
Current antifungal treatment
Current TB treatment

*Note: HIV – human immunodeficiency virus, CSF – Cerebrospinal fluid, TB – tuberculosis

A.2.3 Multilocus sequence typing experiments and data analysis

DNA was extracted from single yeast colonies grown on Sabouraud agar (DMP, NHLS, Johannesburg, South Africa) using the Zymo ZR Fungal/Bacterial DNA MiniPrep kit (Zymo Research Corp, USA) following the manufacturer's instructions. The only modification from the manufacturer's instructions was the starting material for DNA extractions since single yeast colonies grown on Sabouraud agar (DMP, NHLS, Johannesburg, South Africa) at 30°C

after 48h was used directly. The International Society for Human and Animal Mycology (ISHAM) MLST consensus scheme for *C. neoformans* and *C. gattii* containing three housekeeping genes: *GPD1*, *SOD1* and *URA5*; three virulence genes: *CAP59*, *LAC1* and *PLB1*; and the intergenic spacer region *IGS1* was used (Meyer et al., 2009). Table A.2 shows the primer sequences used (Meyer et al., 2009). DNA was amplified using a conventional PCR with DreamTaq Hot Start DNA Polymerase (Thermo Fisher Scientific, Waltham, Masschusetts, USA) in an Applied Biosystems 2720 Thermal cycler (Thermo Fisher Scientific, Waltham, Masschusetts, USA) using PCR cycling conditions described previously (Meyer et al., 2009). Amplicons were visualised on a 1% agarose gel and Sanger sequencing was performed. For each locus, DNA sequences in both forward and reverse orientations were obtained and edited using Sequencher 5.4.6 (http://www.genecodes.com). Allele numbers and sequence types were assigned using the online MLST database (http://mlst.mycologylab.org). Concatenated DNA sequences for the seven loci were aligned using ClustalW (BioEdit Sequence Alignment Editor). Phylogenetic trees were generated with the programs MEGA5 and MEGA6 using the neighbor-joining method with a bootstrap analysis of 100 replicates and the Jukes-Cantor model (Tamura et al., 2011, Tamura et al., 2013).

Table A.2: Primer sequences used for multilocus sequence typing of *Cryptococcus neoformans* and *Cryptococcus gattii* (Meyer et al., 2009)

Primer	**Sequence (5'-3')**
CAP59F	CTCTACGTCGAGCAAGTCAAG
CAP59R	TCCGCTGCACAAGTGATACCC
GPD1F	CCACCGAACCCTTCTAGGATA
GPD1R	CTTCTTGGCACCTCCCTTGAG
LAC1F	AACATGTTCCCTGGGCCTGTG
LAC1R	ATGAGAATTGAATCGCCTTGT
PLB1F	CTTCAGGCGGAGAGAGGTTT
PLB1R	GATTTGGCGTTGGTTTCAGT
SOD1CNF (for *C. neoformans*)	AAGCCTCTCATCCATATCTT
SOD1CNR (for *C. neoformans*)	TTCAACCACGAATATGTA
SOD1CGF (for *C. gattii*)	GATCCTCACGCCATTACG
SOD1CGR (for *C. gattii*)	GAATGATGCGCTTAGTTGGA
URA5F	ATGTCCTCCCAAGCCCTCGAC
URA5R	TTAAGACCTCTGAACACCGTACTC
IGSF	ATCCTTTGCAGACGACTTGA
IGSR	GTGATCAGTGCATTGCATGA

A.2.4 Mating type analysis

Mating type identification was performed by conventional PCR amplification using primer sets (Table A.3) and cycling conditions from previous studies (Yan et al., 2002, Bovers et al., 2006), which are specific to the mating type regions of a and α mating-type cells. Amplicons

were visualised on a 1% agarose gel and the mating type was determined using amplification that related to each mating-type's expected amplicon size (Table A.3).

Table A.3: Primer sequences used for mating type analysis of *Cryptococcus neoformans* and *Cryptococcus gattii* (Yan et al., 2002, Bovers et al., 2006)

Primer	**Sequence (5'-3')**	**Expected amplicon size (bp)**
STE20Aa Forward	TCCACTGGCAACCCTGCGAG	865
STE20Aa Reverse	ATCAGAGACAGAGGAGCAAGAC	
STE20Aα Forward	CCAAAAGCTGATGCTGTGGA	588
STE20Aα Reverse	AGGACATCTATAGCAGAT	
STE20Da Forward	GATCTGTCTCAGCAGCCAC	440
STE20Da Reverse	AATATCAGCTGCCCAGGTGA	
STE20Dα Forward	GATTTATCTCAGCAGCCACG	443
STE20Dα Reverse	AAATCGGCTACGGCACGTC	
STE12α-F809	TTGACCTTTTRTTCCGCAATG	760
STE12α-R1607	TTTCTTCTCCCCTGTTTATAGGC	
STE12a-F537	GTTCTTTGGAATGGCTTATTTCATAT	800
STE12a-R1299	GMCTTGCGTGGATCATATCTA	

A.3 Antifungal susceptibility testing of *Cryptococcus* isolates

Fluconazole minimum inhibitory concentration (MIC) values were tested at different concentrations using the Clinical and Laboratory Standards Institute (CLSI) broth microdilution method (BMD). Broth microdilution panels were prepared at the NICD

containing two-fold dilution ranges of fluconazole (Himedia Laboratories, India) according to the CLSI standard M27-A3 (Clinical and Laboratory Standards Institute, 2008). The fluconazole concentrations tested ranged from 0.12 µg/ml – 64 µg/ml. Varying concentrations of fluconazole were dispensed into wells of a 96-well U-shaped microtitre plate at 2X the desired final concentration. An inoculum was prepared from normal saline using a few yeast colonies grown on Sabouraud agar (DMP, NHLS, Johannesburg, South Africa) at 30°C after 48h. Turbidity was adjusted to a 0.5 McFarland standard of approximately 1 x 10^6 CFU/ml to 5 x 10^6 CFU/ml using a MicroScan turbidity meter (Dade Behring Inc., Deerfield, Illinois, USA). A 1:100 dilution of the inoculum was made in sterile water and thereafter a 1:20 dilution was made in Roswell Park Memorial Institute (RPMI) broth (Lonza, Switzerland) to obtain a desired inoculum size of 1-5 x 10^3 CFU/ml. A final volume of 200 µl was used per well whereby 100 µl of the above inoculum in RPMI broth was added to 100 µl of each diluted fluconazole solution in RPMI broth. Broth microdilution MIC endpoints were read at 50% inhibition for fluconazole after 72h of incubation at 35°C. Each new batch of microdilution plates were tested using either *Candida parapsilosis* ATCC 22019 or *Candida krusei* ATCC 6258 as quality control strains read after 24h of incubation.

A.4 Virulence studies

The moth *Galleria mellonella* model of fungal disease was used to assess the virulence of 15 VNB strains in this study (Mylonakis et al., 2005). Larvae in the final instar stage of larval development were used in experiments according to methods published previously (Firacative et al., 2014, dos Santos et al., 2020). Briefly, ten larvae per VNB strain were injected with 10 µl of a 10^8 yeast CFU/ml inoculum prepared using normal saline and a few yeast colonies grown on Sabouraud agar (DMP, NHLS, Johannesburg, South Africa) at 30°C after 48h. A 50U insulin syringe with a 29-gauge needle was used to inject larvae in the

haemocoel of each caterpillar via the last left pro-leg. Ten larvae were also inoculated with a highly-virulent reference strain (*C. neoformans* H99, molecular type VNI). Two sets of control groups were also included, whereby ten larvae each were injected with phosphate-buffered saline (PBS) and another ten larvae were not injected. Following injection, larvae were incubated in Petri dishes at 37°C in the dark and the number of dead larvae were recorded daily for ten days. Larvae were considered dead when they did not move in response to a physical stimulus such as sterile forceps. Three experimental replicates were performed and an average from the three replicates was calculated. Kaplan-Meier survival curves were plotted for larvae infected with each VNB strain, the reference strain H99 and the controls. As a proxy for virulence, the median survival time of larvae infected with each VNB strain was compared to survival of larvae infected with the well-studied reference strain H99. Details of the statistical analysis are below.

A.5 Whole genome sequencing of *Cryptococcus* isolates

All *C. neoformans* isolates with the molecular type VNB and all *C. gattii* isolates with the molecular type VGIV identified by MLST in this study were processed for whole genome sequencing (WGS). These molecular types are endemic to southern Africa and have been found at a higher frequency when compared to the rest of the world. This presented a unique opportunity to study these molecular types further to determine their genetic diversity. Whole genome sequencing was performed using Illumina MiSeq sequencing technology (Illumina, San Diego, California, USA). Previously-extracted DNA samples (extraction procedure described above) were prepared for paired-end sequencing using the NEBNext Ultra II DNA Library Prep Kit for Illumina followed by 2 × 300 bp sequencing on a MiSeq instrument at the Translational Genomics Research Institute (Pathogen and Microbiome Division). FASTQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and PrinSeq

(Schmieder and Edwards, 2011) were used to check quality of read data and perform read filtering. Reads for every sample were aligned to known reference assemblies: VNB genome (Bt 85, NCBI BioSample number: SAMN01162765) and VGIV genome (IND 107, NCBI BioSample number: SAMN01932842) to compare and call single nucleotide polymorphism (SNP) variants using Burrows-Wheeler Aligner (BWA) (Li, 2013) and SAMtools (Li et al., 2009). Burrows-Wheeler Aligner and SAMtools were used within the publically available Northern Arizona SNP Pipeline (NASP) (Sahl et al., 2016); posterior filtering parameters involved removing positions that had <10x coverage, <90% variant allele calls, and those mapping to duplicated regions in the reference. Downstream filtering by NASP produced the final high-quality or BestSNP alignment that was then used for maximum parsimony inference. Only positions present in all genomes with at least 10x depth of coverage and 90% agreement were included. Maximum parsimony trees using the sub-tree-pruning-regrafting option as the maximum parsimony (MP) search method with 500 bootstrap replicates were constructed and visualized using MEGA5 (Tamura et al., 2011) and MEGA6 software (Tamura et al., 2013). For assessment of specific clusters, trees were drawn separately to maximize the high-quality comparable positions between samples of each apparent clade. For this, draft assemblies of one of the samples in each clade were created *de-novo* using SPAdes (https://cab.spbu.ru/software/spades/) in careful mode and then used as references for the cluster trees.

A.6 Statistical data analysis

Chi-square or Fisher's exact tests (Kim, 2017) were used to determine associations between patient characteristics (such as mental status at diagnosis and HIV status as shown in Table A.1) and molecular type. We used logistic regression models (Sperandei, 2013) to determine the association between patient characteristics and molecular type for cases of *C. neoformans*

and *C. gattii* disease. Patient characteristics were obtained by nurse surveillance officers at the surveillance sites who either interviewed patients or reviewed patients' medical charts to obtain detailed case information. Mental status was categorised as "alert" (Glasgow Coma Scale [GCS] score of 15) or "not alert" (GCS score <15 or recorded to be disorientated, stuporose or comatose) (Durant and Sporer, 2011). We characterised molecular types into VNI vs. non-VNI (including VNII and VNB) for C. *neoformans* since there were many sequence types with low frequencies. Similarly, for *C. gattii*, we characterised molecular types into VGIV vs. non-VGIV. We also modelled the effect of molecular type on in-hospital mortality. We compared the MIC_{50} values to fluconazole of the VNI isolates and non-VNI isolates using a Wilcoxon rank-sum test (Kim, 2014). We also compared the MIC_{50} values to fluconazole of the VGIV isolates and non-VGIV isolates using a Wilcoxon rank-sum test (Kim, 2014). We used the log rank test of equality (Bewick et al., 2004) to compare survival curves between H99 and the 15 VNB isolates. STATA version 14 (StataCorp, College Station, TX) was used for all statistical tests.

A.7 Ethics

Ethics committee approval was obtained from the University of the Witwatersrand Human Research Ethics Committee (Medical) with clearance certificate numbers: M160375 and M1809107. A waiver from the Animal Research Ethics Committee of the University of the Witwatersrand was obtained (Registration number: AREC-101210-002).

A.8 Role of candidate in study

The student was directly involved in isolate processing, data entry and data cleaning during the 2013 surveillance year. The student performed a secondary data analysis for the 2005-2013 surveillance period. The student was directly involved in and led all aspects of the

project with a few exceptions. The first being that the student only assigned allele numbers and sequence types for 251 *C. neoformans* isolates and performed phylogenetic analysis. Random selection of *C. neoformans* isolates, DNA extractions, PCR and sequencing was not done by the student for this particular aspect. The second exception was that WGS was performed at the Translational Genomics Research Institute (Pathogen and Microbiome Division). The student sent the DNA to the Translational Genomics Research Institute (Pathogen and Microbiome Division) and did not do any of the library preparation and sequencing on the MiSeq instrument. The student obtained some assistance from the Translational Genomics Research Institute (Pathogen and Microbiome Division) with acquiring some of the publicly available VNB and VGIV sequence data as well as with assembling the draft genomes used as references for the high resolution trees.

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Appendix B – Supplementary information for Chapter 2

Supplementary metadata information for 251 clinical *Cryptococcus neoformans* strains from South African national surveillance, 2005-2009

Isolate ID	Record	CAP59	GPD1	IGS1	LAC1	PLB1	SOD1	URA5	Sequence type	Molecular type	Serotype	Mating type	FLZ MIC, µg/ml	Province	Specimen
1	10	1	23	10	3	4	1	1	93	VNI	A	matα	ND	Gauteng	CSF
2	36	1	1	10	3	2	1	1	31	VNI	A	matα	ND	Gauteng	CSF
3	137	7	1	1	18	1	1	1	63	VNI	A	matα	ND	Gauteng	Unknown
4	141	1	1	10	3	2	1	1	31	VNI	A	matα	2	Gauteng	Unknown
5	167	1	3	1	5	2	1	1	5	VNI	A	matα	ND	Western Cape	CSF
6	205	4	11	7	6	6	6	3	245	VNB	A	matα	2	Gauteng	CSF
7	207	1	26	1	5	2	1	1	186	VNI	A	matα	2	Mpumalanga	CSF
8	2005-310	1	23	10	3	4	1	1	93	VNI	A	matα	ND	Gauteng	CSF
9	317	1	23	10	3	4	1	1	93	VNI	A	matα	1	Gauteng	CSF
10	2005-344	1	23	10	3	4	1	1	93	VNI	A	matα	ND	Gauteng	CSF
11	444	1	23	10	3	4	1	1	93	VNI	A	matα	ND	Gauteng	CSF
12	588	1	3	1	5	2	1	1	5	VNI	A	matα	ND	KwaZulu-Natal	CSF
13	2005-637	13	16	23	6	15	29	26	232	VNB	A	matα	1	KwaZulu-Natal	CSF
14	1038	7	1	1	18	1	1	1	63	VNI	A	matα	ND	Eastern Cape	CSF
15	1043	1	1	1	18	1	1	2	58	VNI	A	matα	0.5	Gauteng	Blood culture
16	1077	7	1	1	2	1	1	2	23	VNI	A	matα	ND	Eastern Cape	CSF
17	1408	5	11	2	6	10	16	3	305	VNB	A	matα	1	Gauteng	CSF
18	1432	7	1	1	1	1	1	2	2	VNI	A	matα	ND	Mpumalanga	CSF
19	1489	7	5	1	3	3	1	1	69	VNI	A	matα	ND	Gauteng	CSF

Isolate ID	Record	CAP59	GPD1	IGS1	LAC1	PLB1	SOD1	URA5	Sequence type	Molecular type	Serotype	Mating type	FLZ MIC, µg/ml	Province	Specimen
20	1521	1	3	1	5	2	1	1	5	VNI	A	matα	ND	North West	CSF
21	2005-1561	7	5	1	3	3	1	1	69	VNI	A	matα	1	KwaZulu-Natal	CSF
22	1575	1	1	1	18	1	1	2	58	VNI	A	matα	ND	Western Cape	CSF
23	1630	1	1	1	4	2	1	5	4	VNI	A	matα	ND	Gauteng	CSF
24	1806	7	1	1	2	1	1	2	23	VNI	A	matα	ND	Gauteng	CSF
25	1946	7	5	1	3	3	1	1	69	VNI	A	matα	2	Mpumalanga	CSF
26	1947	1	23	10	3	4	1	1	93	VNI	A	matα	0.5	North West	CSF
27	2241	1	3	1	5	2	1	1	5	VNI	A	matα	2	Gauteng	CSF
28	2361	4	11	7	6	6	1	3	304	VNI	A	matα	ND	Gauteng	CSF
29	2402	1	3	1	5	2	1	1	5	VNI	A	matα	8	Eastern Cape	CSF
30	2450	1	3	1	5	2	1	1	5	VNI	A	matα	1	Northern Cape	CSF
31	2451	7	23	1	2	1	1	2	307	VNI	A	matα	8	Gauteng	CSF
32	2776	4	11	5	6	7	5	3	325	VNI	A	matα	0.25	Mpumalanga	CSF
33	2779	2	9	14	8	11	12	4	40	VNII	A	matα	1	Mpumalanga	CSF
34	2887	4	11	7	6	6	6	3	245	VNB	A	matα	0.5	Free State	CSF
35	2899	1	3	1	5	2	1	1	5	VNI	A	matα	ND	Gauteng	CSF
36	2935	1	3	1	5	2	1	1	5	VNI	A	matα	ND	Western Cape	CSF
37	2939	7	1	1	2	1	1	2	23	VNI	A	matα	ND	Western Cape	CSF
38	3231	7	1	1	2	1	1	2	23	VNI	A	matα	ND	Gauteng	CSF
39	3236	1	3	1	5	2	43	1	296	VNI	A	matα	1	Limpopo	CSF
40	3301	7	23	1	2	1	1	2	307	VNI	A	matα	ND	Limpopo	CSF
41	3355	4	10	14	8	11	6	4	315	VNII	A	matα	4	KwaZulu-Natal	CSF
43	3438	4	11	9	6	4	6	3	316	VNB	A	matα	4	North West	Unknown
44	3466	1	5	1	18	4	34	9	317	VNI	A	matα	ND	Free State	CSF

Isolate ID	Record	CAP59	GPD1	IGS1	LAC1	PLB1	SOD1	URA5	Sequence type	Molecular type	Serotype	Mating type	FLZ MIC, µg/ml	Province	Specimen
45	3505	1	3	1	5	2	1	1	5	VNI	A	matα	ND	Eastern Cape	CSF
46	3537	7	1	1	18	1	1	1	63	VNI	A	matα	4	KwaZulu-Natal	CSF
47	2005-3586	1	23	10	3	4	1	1	93	VNI	A	matα	ND	North West	CSF
48	3729	1	5	1	44	2	4	1	318	VNI	A	matα	ND	Mpumalanga	CSF
49	3861	1	23	10	18	4	1	1	319	VNI	A	matα	ND	Western Cape	CSF
50	3896	7	1	1	18	1	1	1	63	VNI	A	matα	ND	Gauteng	CSF
51	4571	7	1	1	1	1	1	2	2	VNI	A	matα	ND	Gauteng	CSF
52	22	1	6	1	18	4	1	1	80	VNI	A	matα	ND	North West	CSF
53	97	1	3	1	5	2	1	1	5	VNI	A	matα	ND	Western Cape	CSF
54	181	1	3	1	5	2	1	1	5	VNI	A	matα	ND	KwaZulu-Natal	CSF
55	2006-637	1	3	1	5	2	1	1	5	VNI	A	matα	ND	KwaZulu-Natal	CSF
56	657	2	9	14	8	11	12	4	40	VNII	A	matα	1	KwaZulu-Natal	CSF
57	707	7	1	1	2	1	1	2	23	VNI	A	matα	32	Gauteng	CSF
58	731	1	3	1	5	2	1	1	5	VNI	A	matα	ND	Limpopo	CSF
59	868	1	1	25	3	2	1	1	77	VNI	A	matα	ND	Mpumalanga	CSF
60	892	4	11	7	6	6	6	3	245	VNB	A	matα	2	Gauteng	CSF
61	900	7	1	1	1	1	1	2	2	VNI	A	matα	ND	Gauteng	CSF
62	907	4	11	7	6	6	6	3	245	VNB	A	matα	4	Gauteng	CSF
63	968	1	23	10	3	4	1	1	93	VNI	A	matα	ND	KwaZulu-Natal	CSF
64	1220	1	1	10	3	2	1	1	31	VNI	A	matα	ND	KwaZulu-Natal	CSF
65	2006-1561	1	23	10	3	4	1	1	93	VNI	A	matα	ND	Eastern Cape	CSF
66	1562	1	23	10	3	4	1	2	540	VNI	A	matα	2	Eastern Cape	CSF

Isolate ID	Record	CAP59	GPD1	IGS1	LAC1	PLB1	SOD1	URA5	Sequence type	Molecular type	Serotype	Mating type	FLZ MIC, µg/ml	Province	Specimen
67	1564	7	5	1	3	3	1	2	218	VNI	A	matα	2	Eastern Cape	CSF
68	1597	9	11	19	6	8	6	3	308	VNB	A	matα	0.25	Gauteng	CSF
69	1604	1	23	10	3	4	1	2	540	VNI	A	matα	ND	Gauteng	Unknown
70	1710	1	23	10	3	4	1	1	93	VNI	A	matα	0.5	Gauteng	CSF
71	1817	4	11	7	6	6	6	3	245	VNB	A	matα	0.5	Free State	CSF
72	1925	1	3	1	5	2	1	1	5	VNI	A	matα	ND	Mpumalanga	CSF
73	2064	1	3	1	5	2	1	1	5	VNI	A	matα	ND	Gauteng	CSF
74	2654	2	9	14	8	11	12	4	40	VNII	A	matα	1	KwaZulu-Natal	CSF
75	2713	1	1	1	3	4	1	1	3	VNI	A	matα	ND	Gauteng	Blood culture
76	2006-2718	7	5	1	3	3	1	1	69	VNI	A	matα	ND	Gauteng	CSF
77	3022	4	11	7	6	6	1	3	304	VNI	A	matα	ND	KwaZulu-Natal	CSF
78	3437	4	11	7	6	6	1	3	304	VNI	A	matα	ND	North West	CSF
79	3562	9	23	60	23	16	1	40	326	VNIV	D	matα	2	Gauteng	CSF
80	3721	7	1	1	2	1	1	2	23	VNI	A	matα	8	Gauteng	CSF
81	3810	4	3	1	5	2	1	25	327	VNI	A	matα	0.5	KwaZulu-Natal	CSF
82	3833	1	1	10	3	2	1	1	31	VNI	A	matα	ND	Gauteng	Blood culture
83	4087	7	1	1	2	1	1	2	23	VNI	A	matα	2	North West	CSF
84	4155	1	1	1	4	2	1	5	4	VNI	A	matα	ND	KwaZulu-Natal	CSF
85	4646	2	9	14	8	11	12	4	40	VNII	A	matα	1	Eastern Cape	CSF
86	4809	1	1	10	3	4	1	1	32	VNI	A	matα	ND	KwaZulu-Natal	CSF
87	4909	1	3	1	5	2	1	1	5	VNI	A	matα	ND	Mpumalanga	CSF
88	5023	7	5	1	3	3	13	1	328	VNI	A	matα	ND	Western Cape	CSF

Isolate ID	Record	CAP59	GPD1	IGS1	LAC1	PLB1	SOD1	URA5	Sequence type	Molecular type	Serotype	Mating type	FLZ MIC, µg/ml	Province	Specimen
89	5103	1	3	1	5	2	1	1	5	VNI	A	matα	ND	Gauteng	Blood culture
90	5116	7	1	1	2	1	1	2	23	VNI	A	matα	2	Gauteng	CSF
91	5157	7	3	1	5	2	1	1	226	VNI	A	matα	ND	KwaZulu-Natal	CSF
92	5490	7	5	1	18	3	4	9	449	VNI	A	matα	ND	Gauteng	CSF
93	5605	1	3	1	5	2	1	1	5	VNI	A	matα	ND	Gauteng	CSF
94	5782	1	5	1	3	2	1	1	331	VNI	A	matα	2	Limpopo	CSF
97	5859	1	3	10	3	2	1	1	556	VNI	A	matα	ND	Gauteng	Blood culture
98	5873	1	5	7	5	2	6	3	332	VNB	A	matα	1	Gauteng	CSF
99	5903	1	1	1	5	2	1	1	81	VNI	A	matα	ND	Mpumalanga	CSF
100	6047	7	3	1	5	2	1	1	226	VNI	A	matα	ND	Gauteng	CSF
101	6083	1	1	8	3	23	1	1	238	VNI	A	mata	ND	KwaZulu-Natal	CSF
102	6224	1	26	1	5	2	1	3	557	VNI	A	matα	2	Gauteng	CSF
103	6341	2	9	14	8	11	12	4	40	VNII	A	matα	0.5	KwaZulu-Natal	CSF
104	2006-6461	1	3	1	5	2	1	1	5	VNI	A	matα	ND	Gauteng	CSF
105	6481	7	1	1	2	1	1	2	23	VNI	A	matα	0.5	Gauteng	CSF
106	6674	10	9	14	8	11	12	4	41	VNII	A	matα	0.5	KwaZulu-Natal	CSF
107	6692	7	1	1	2	1	1	2	23	VNI	A	matα	ND	KwaZulu-Natal	CSF
108	20	1	3	1	5	2	1	1	5	VNI	A	matα	ND	Unknown	Unknown
109	52	1	3	1	5	2	1	1	5	VNI	A	matα	1	Unknown	Unknown
110	72	1	23	10	3	4	1	1	93	VNI	A	matα	8	Western Cape	Unknown
111	132	7	1	1	1	1	1	2	2	VNI	A	matα	1	KwaZulu-Natal	Unknown
112	257	7	1	1	2	1	1	2	23	VNI	A	matα	2	Gauteng	Unknown

Isolate ID	Record	CAP59	GPD1	IGS1	LAC1	PLB1	SOD1	URA5	Sequence type	Molecular type	Serotype	Mating type	FLZ MIC, µg/ml	Province	Specimen
113	265	1	23	10	3	4	1	1	93	VNI	A	matα	ND	Unknown	Unknown
114	279	1	5	1	44	39	1	1	558	VNI	A	matα	ND	Limpopo	Unknown
115	303	1	3	1	5	2	1	1	5	VNI	A	matα	2	Gauteng	Unknown
116	816	7	1	1	2	1	1	2	23	VNI	A	matα	ND	Gauteng	Unknown
117	841	1	3	1	5	2	1	1	5	VNI	A	matα	ND	Gauteng	CSF
118	872	8	10	15	8	12	3	11	42	VNII	A	matα	2	KwaZulu-Natal	CSF
119	917	4	9	14	8	11	6	3	361	VNII	A	matα	1	KwaZulu-Natal	CSF
120	1168	5	22	24	19	10	1	18	362	VNIV	D	matα	2	Gauteng	CSF
121	1239	4	11	7	6	6	6	3	245	VNB	A	matα	1	Gauteng	Unknown
122	1241	7	1	1	18	1	1	1	63	VNI	A	matα	ND	Gauteng	CSF
123	1310	1	3	1	5	2	1	1	5	VNI	A	matα	1	KwaZulu-Natal	CSF
124	1498	1	23	10	3	4	1	1	93	VNI	A	matα	ND	KwaZulu-Natal	CSF
125	1962	1	23	10	3	4	1	1	93	VNI	A	matα	ND	Unknown	Unknown
126	2328	7	5	1	3	3	1	1	69	VNI	A	matα	ND	Eastern Cape	CSF
129	2458	7	5	1	3	3	1	1	69	VNI	A	matα	2	North West	CSF
130	2671	7	1	1	2	1	1	2	23	VNI	A	matα	ND	Gauteng	CSF
131	3133	7	5	1	3	3	1	1	69	VNI	A	matα	1	Eastern Cape	CSF
132	3151	7	1	1	2	1	1	2	23	VNI	A	matα	ND	Gauteng	CSF
133	3160	1	3	1	5	2	1	1	5	VNI	A	matα	1	Gauteng	CSF
134	3192	1	3	1	5	2	1	1	5	VNI	A	matα	ND	KwaZulu-Natal	CSF
135	3276	7	5	1	18	4	1	2	70	VNI	A	matα	ND	Eastern Cape	CSF
136	3464	2	9	14	8	11	12	4	40	VNII	A	matα	1	KwaZulu-Natal	CSF
137	3476	1	23	10	3	4	1	1	93	VNI	A	matα	ND	KwaZulu-Natal	CSF

Isolate ID	Record	CAP59	GPD1	IGS1	LAC1	PLB1	SOD1	URA5	Sequence type	Molecular type	Serotype	Mating type	FLZ MIC, µg/ml	Province	Specimen
138	3483	1	23	10	3	4	1	1	93	VNI	A	matα	ND	KwaZulu-Natal	CSF
139	2007-3586	2	9	14	8	11	12	4	40	VNII	A	matα	0.5	KwaZulu-Natal	CSF
140	4189	1	4	8	3	2	37	1	241	VNI	A	matα	2	Gauteng	CSF
141	4692	1	1	1	4	2	1	5	4	VNI	A	matα	ND	KwaZulu-Natal	CSF
142	4711	1	3	1	5	2	1	1	5	VNI	A	matα	ND	KwaZulu-Natal	CSF
143	4725	7	1	1	18	1	1	1	63	VNI	A	matα	ND	Gauteng	CSF
144	4752	4	11	2	6	4	27	3	48	VNI	A	matα	ND	Gauteng	CSF
145	4768	7	7	1	18	4	1	1	140	VNI	A	matα	0.5	Gauteng	CSF
146	5011	1	23	10	3	4	1	1	93	VNI	A	matα	ND	KwaZulu-Natal	CSF
147	5130	1	3	1	5	2	1	1	5	VNI	A	matα	ND	KwaZulu-Natal	CSF
148	5297	7	1	1	1	1	1	2	2	VNI	A	matα	4	Gauteng	Blood culture
149	5400	7	1	1	1	1	1	2	2	VNI	A	matα	0.5	Gauteng	CSF
150	5532	1	3	1	5	2	1	1	5	VNI	A	matα	ND	Gauteng	CSF
151	5796	1	3	1	5	2	1	1	5	VNI	A	matα	ND	Eastern Cape	CSF
152	6058	1	23	10	5	4	1	1	195	VNI	A	matα	ND	KwaZulu-Natal	CSF
153	6236	1	3	1	5	2	1	1	5	VNI	A	matα	ND	Gauteng	CSF
154	6309	1	3	1	5	2	1	1	5	VNI	A	matα	ND	North West	CSF
155	2007-6461	1	3	1	5	2	1	1	5	VNI	A	matα	4	Gauteng	CSF
156	6529	1	1	1	18	1	1	2	58	VNI	A	matα	ND	KwaZulu-Natal	CSF
157	6534	7	1	1	2	1	1	2	23	VNI	A	matα	ND	KwaZulu-Natal	CSF
158	6613	10	9	14	8	11	12	4	41	VNII	A	matα	1	Limpopo	CSF

Isolate ID	Record	CAP59	GPD1	IGS1	LAC1	PLB1	SOD1	URA5	Sequence type	Molecular type	Serotype	Mating type	FLZ MIC, µg/ml	Province	Specimen
159	6681	7	1	1	2	1	1	2	23	VNI	A	matα	2	Gauteng	CSF
160	6836	1	3	1	5	2	1	1	5	VNI	A	matα	ND	Gauteng	CSF
161	6906	7	1	1	18	1	1	1	63	VNI	A	matα	2	Eastern Cape	CSF
162	6535	10	9	14	8	11	12	4	41	VNII	A	matα	2	Unknown	Unknown
163	469	2	9	14	8	11	12	4	40	VNII	A	matα	2	Mpumalanga	CSF
164	764	2	9	14	8	11	12	4	40	VNII	A	matα	1	KwaZulu-Natal	CSF
165	1033	2	9	14	8	11	12	4	40	VNII	A	matα	1	KwaZulu-Natal	CSF
166	1552	1	3	1	5	2	1	1	5	VNI	A	matα	ND	Unknown	Unknown
167	1344	9	22	24	19	19	1	33	363	VNIV	D	matα	2	Eastern Cape	Unknown
168	1428	1	1	8	3	23	1	1	238	VNI	A	mata	0.25	Gauteng	CSF
169	1797	1	1	1	4	2	1	5	4	VNI	A	matα	ND	Western Cape	CSF
170	1978	1	1	1	18	4	1	1	364	VNI	A	matα	ND	Gauteng	CSF
171	2038	1	5	1	18	4	1	1	87	VNI	A	matα	ND	KwaZulu-Natal	CSF
172	2155	1	1	10	3	2	1	1	31	VNI	A	matα	ND	Western Cape	Blood culture
173	2360	7	5	1	3	3	1	1	69	VNI	A	matα	ND	North West	CSF
174	2008-2394	1	23	10	3	4	1	1	93	VNI	A	matα	ND	KwaZulu-Natal	CSF
175	2487	1	3	1	5	2	1	1	5	VNI	A	matα	ND	Western Cape	CSF
176	2493	1	3	1	5	2	1	1	5	VNI	A	matα	ND	KwaZulu-Natal	CSF
177	2508	1	2	6	3	2	1	1	15	VNI	A	matα	ND	Gauteng	Blood culture
178	2530	7	1	1	1	1	1	2	2	VNI	A	matα	1	Gauteng	CSF
179	2544	7	1	1	2	1	1	2	23	VNI	A	matα	0.5	KwaZulu-Natal	Blood culture
180	2546	7	5	1	3	3	1	1	69	VNI	A	matα	1	Unknown	Unknown

Isolate ID	Record	CAP59	GPD1	IGS1	LAC1	PLB1	SOD1	URA5	Sequence type	Molecular type	Serotype	Mating type	FLZ MIC, µg/ml	Province	Specimen
181	2600	7	1	1	2	1	1	2	23	VNI	A	matα	ND	Gauteng	CSF
182	2008-2718	7	1	1	18	1	1	1	63	VNI	A	matα	0.5	North West	CSF
183	2751	7	1	1	2	1	1	2	23	VNI	A	matα	ND	KwaZulu-Natal	Unknown
184	2777	1	23	10	3	4	1	1	93	VNI	A	matα	ND	KwaZulu-Natal	CSF
185	2890	13	16	23	6	15	29	26	232	VNB	A	matα	1	Free State	Blood culture
186	2894	10	9	14	8	11	12	4	41	VNII	A	matα	0.5	KwaZulu-Natal	CSF
187	2895	7	1	1	2	1	1	2	23	VNI	A	matα	ND	KwaZulu-Natal	CSF
188	2920	1	3	1	5	2	1	1	5	VNI	A	matα	ND	KwaZulu-Natal	CSF
189	2932	1	23	10	3	4	1	1	93	VNI	A	matα	1	KwaZulu-Natal	Blood culture
190	3115	1	23	10	3	4	1	1	93	VNI	A	matα	ND	KwaZulu-Natal	CSF
191	3190	1	3	1	5	2	1	1	5	VNI	A	matα	ND	Eastern Cape	CSF
192	3269	7	1	1	1	1	1	2	2	VNI	A	matα	ND	Western Cape	CSF
195	3331	22	6	32	19	3	1	1	365	VNIV	D	matα	2	Western Cape	Blood culture
196	3381	1	23	10	3	4	1	1	93	VNI	A	matα	0.5	Gauteng	CSF
197	3482	1	3	1	5	2	1	1	5	VNI	A	matα	ND	KwaZulu-Natal	CSF
198	3500	7	1	1	2	1	1	2	23	VNI	A	matα	ND	Gauteng	CSF
199	3517	1	1	1	3	2	1	5	6	VNI	A	matα	1	Gauteng	CSF
200	3578	7	5	1	3	3	1	1	69	VNI	A	matα	ND	Western Cape	CSF
201	3670	1	3	1	5	2	1	1	5	VNI	A	matα	ND	Eastern Cape	CSF
202	3775	7	5	1	3	3	1	1	69	VNI	A	matα	2	Gauteng	Blood culture

Isolate ID	Record	CAP59	GPD1	IGS1	LAC1	PLB1	SOD1	URA5	Sequence type	Molecular type	Serotype	Mating type	FLZ MIC, µg/ml	Province	Specimen
203	3783	4	11	7	6	6	6	3	245	VNB	A	matα	2	Gauteng	CSF
204	3790	1	1	1	18	1	1	2	58	VNI	A	matα	ND	Gauteng	Unknown
205	3681	7	1	1	18	1	1	1	63	VNI	A	matα	ND	Unknown	Unknown
206	3866	10	9	14	8	11	12	4	41	VNII	A	matα	2	Unknown	Unknown
207	3986	8	10	15	8	12	3	11	42	VNII	A	matα	1	Gauteng	Unknown
208	3921	1	23	10	3	4	1	1	93	VNI	A	matα	ND	KwaZulu-Natal	CSF
209	3939	1	3	1	5	2	1	1	5	VNI	A	matα	1	Gauteng	CSF
210	3980	1	3	1	5	2	1	1	5	VNI	A	matα	ND	KwaZulu-Natal	CSF
211	3991	25	2	60	23	16	1	1	366	VNIV	D	matα	2	Gauteng	CSF
212	4114	1	23	10	3	4	1	1	93	VNI	A	matα	1	KwaZulu-Natal	CSF
213	4116	1	3	1	5	2	1	1	5	VNI	A	matα	ND	KwaZulu-Natal	CSF
214	4205	2	9	14	8	11	12	4	40	VNII	A	matα	1	Gauteng	CSF
215	4268	7	1	1	2	1	1	2	23	VNI	A	matα	ND	KwaZulu-Natal	Blood culture
217	129	1	3	1	5	2	1	1	5	VNI	A	matα	ND	Eastern Cape	CSF
218	130	4	11	7	6	6	6	1	367	VNB	A	matα	4	Eastern Cape	CSF
219	194	2	9	14	8	11	12	1	368	VNII	A	matα	1	Northern Cape	CSF
220	300	1	23	10	3	4	1	1	93	VNI	A	matα	ND	KwaZulu-Natal	CSF
221	2009-310	1	23	10	3	4	1	3	559	VNI	A	matα	ND	KwaZulu-Natal	CSF
222	329	7	5	1	3	3	1	1	69	VNI	A	matα	ND	KwaZulu-Natal	CSF
223	2009-344	1	1	1	18	3	1	2	370	VNI	A	matα	1	Gauteng	CSF
224	359	7	1	1	1	1	1	2	2	VNI	A	matα	ND	Eastern Cape	CSF
225	381	1	23	10	3	4	1	1	93	VNI	A	matα	ND	Gauteng	CSF

Isolate ID	Record	CAP59	GPD1	IGS1	LAC1	PLB1	SOD1	URA5	Sequence type	Molecular type	Serotype	Mating type	FLZ MIC, µg/ml	Province	Specimen
226	393	7	1	1	18	1	1	1	63	VNI	A	matα	ND	Gauteng	CSF
227	494	1	3	1	5	2	1	1	5	VNI	A	matα	1	Western Cape	CSF
228	511	1	1	10	3	2	1	1	31	VNI	A	matα	ND	KwaZulu-Natal	CSF
231	839	7	1	1	1	1	1	2	2	VNI	A	matα	1	Eastern Cape	CSF
232	848	1	3	1	5	2	1	1	5	VNI	A	matα	ND	KwaZulu-Natal	CSF
233	851	1	3	1	5	2	1	1	5	VNI	A	matα	8	KwaZulu-Natal	CSF
234	854	1	1	10	3	4	1	1	32	VNI	A	matα	ND	KwaZulu-Natal	CSF
235	1041	1	5	1	43	4	1	1	560	VNI	A	matα	ND	Limpopo	CSF
236	1188	1	7	1	18	4	1	30	371	VNI	A	matα	ND	Mpumalanga	Unknown
237	1200	1	3	1	5	2	1	1	5	VNI	A	matα	ND	KwaZulu-Natal	CSF
238	1252	1	1	25	3	2	1	1	77	VNI	A	matα	ND	Gauteng	Unknown
239	1255	2	9	14	8	11	12	4	40	VNII	A	matα	1	Gauteng	CSF
240	1316	2	9	14	8	11	12	4	40	VNII	A	matα	1	Gauteng	CSF
241	1322	1	3	1	5	2	1	1	5	VNI	A	matα	ND	KwaZulu-Natal	CSF
243	1383	1	3	1	5	2	1	1	5	VNI	A	matα	ND	KwaZulu-Natal	CSF
244	1407	7	1	1	18	1	1	1	63	VNI	A	matα	1	Gauteng	CSF
245	1537	1	7	1	18	4	34	1	372	VNI	A	matα	ND	Gauteng	Blood culture
246	1543	4	11	2	6	4	7	3	16	VNB	A	matα	1	Gauteng	CSF
251	2052	1	23	10	3	4	1	1	93	VNI	A	matα	ND	KwaZulu-Natal	CSF
252	2076	7	1	1	2	1	1	2	23	VNI	A	matα	ND	Western Cape	CSF
253	2110	1	3	1	5	2	1	1	5	VNI	A	matα	ND	KwaZulu-Natal	CSF

Isolate ID	Record	CAP59	GPD1	IGS1	LAC1	PLB1	SOD1	URA5	Sequence type	Molecular type	Serotype	Mating type	FLZ MIC, µg/ml	Province	Specimen
254	2133	1	23	10	3	4	1	1	93	VNI	A	matα	ND	Mpumalanga	CSF
255	2192	2	9	14	8	11	12	4	40	VNII	A	matα	0.5	Gauteng	CSF
256	2245	1	23	10	3	4	1	1	93	VNI	A	matα	ND	KwaZulu-Natal	CSF
257	2320	7	1	1	2	1	1	2	23	VNI	A	matα	ND	Gauteng	CSF
258	2322	1	8	2	6	9	1	13	373	VNI	A	matα	ND	Gauteng	Blood culture
259	2009-2394	1	5	1	18	4	1	1	87	VNI	A	matα	ND	Gauteng	CSF
260	2431	7	1	1	2	1	1	2	23	VNI	A	matα	ND	Gauteng	CSF
261	2525	1	3	1	5	2	1	1	5	VNI	A	matα	2	Gauteng	CSF
262	2529	7	5	1	3	3	1	1	69	VNI	A	matα	ND	Northern Cape	CSF
263	2558	1	23	10	3	4	1	1	93	VNI	A	matα	2	KwaZulu-Natal	Unknown
264	2611	1	1	10	3	4	1	1	32	VNI	A	matα	ND	KwaZulu-Natal	CSF
266	2619	2	9	14	8	11	12	4	40	VNII	A	matα	1	KwaZulu-Natal	CSF
267	2643	7	1	1	2	1	1	2	23	VNI	A	matα	1	KwaZulu-Natal	CSF

*Note: FLZ MIC – Fluconazole minimum inhibitory concentration, CSF – Cerebrospinal fluid, ND – Not done

Table S1: Characteristics of five South African patients infected with the *Cryptococcus neoformans* VNIV molecular type (serotype D) collected during national laboratory-based surveillance for cryptococcosis, 2005-2009

Isolate number	Home town/city	Province	Specimen collection date	Syndrome	Facility name
3562	Diepsloot	Gauteng	21/7/2006	Meningitis	Chris Hani Baragwanath
1168	Jeppestown	Gauteng	12/3/2007	Meningitis	Charlotte Maxeke Johannesburg Academic
1344	Unknown	Eastern Cape	22/2/2008	Unknown	Umtata General
3331	Cape Town	Western Cape	14/8/2008	Meningitis	Groote Schuur
3991	Jeppestown	Gauteng	26/11/2008	Unknown	Charlotte Maxeke Johannesburg Academic

Table S2: Univariable logistic regression analysis to determine associations between clinical characteristics and infecting strain molecular type among South African patients infected with *Cryptococcus neoformans* serotype A (*n* = 246), 2005-2009

Exposure variables	Non-VNI genotype N=40	VNI genotype N=206	Univariable analysis	
	n/N (%)	n/N (%)	OR (95% CI)	p-value
Sex				
Male	29/110 (26)	81/110 (74)	5.24 (2.28-12.04)	0.001
Female	8/125 (6)	117/125 (94)	reference	
Missing data (n)	3	8		
Age (years)				
<25	3/20 (15)	17/20 (85)	reference	
25-34	13/95 (14)	82/95 (86)	0.90 (0.23-3.50)	0.88
35-44	16/84 (19)	68/84 (81)	1.33 (0.35-5.11)	0.68
>45	5/34 (15)	29/34 (85)	0.98 (0.21-4.61)	0.98
Missing data (n)	3	10		
Year of diagnosis				
2005	7/50 (14)	43/50 (86)	reference	
2006	10/53 (19)	43/53 (81)	1.43 (0.50-4.10)	0.51
2007	7/52 (13)	45/52 (87)	0.96 (0.31-2.95)	0.94
2008	9/48 (19)	39/48 (81)	1.42 (0.48-4.17)	0.53
2009	7/43 (16)	36/43 (84)	1.19 (0.38-3.72)	0.76
Province				
Northern	18/123 (15)	105/123 (85)	0.81 (0.40-1.61)	0.54
Southern	20/114 (18)	94/114 (82)	reference	
Missing data (n)	2	7		
Specimen type				
Cerebrospinal fluid (CSF)	34/211 (16)	177/211 (84)	2.69 (0.34-21.14)	0.35
Blood	1/15 (7)	14/15 (93)	reference	
Missing data (n)	5	15		
CD4+ T-cell count at diagnosis (cells/µl)				
<=50	11/49 (22)	38/49 (78)	2.41 (0.61-9.52)	0.21
>50	3/28 (11)	25/28 (89)	reference	
Missing data (n)	26	143		
Antiretroviral treatment				
Yes	2/23 (9)	21/23 (91)	0.48 (0.10-2.23)	0.35
No	19/115 (17)	96/115 (83)	Reference	
Missing data (n)	19	89		
Mental status at diagnosis*				
Alert	15/104 (14)	89/104 (86)	0.63 (0.27-1.49)	0.29
Not alert	11/52 (21)	41/52 (79)	reference	
Missing data (n)	14	76		
CSF India ink result				
Positive	27/164 (16)	137/164 (84)	0.39 (0.03-4.50)	0.45
Negative	1/3 (33)	2/3 (67)	reference	
Missing data (n)	12	67		
CSF white cell count (cells/µl)				
<5	7/56 (12.5)	49/56 (87.5)	0.58 (0.22-1.50)	0.26
>=5	17/86 (20)	69/86 (80)	reference	
Missing data (n)	16	88		
Current antifungal treatment				
Fluconazole alone	6/43 (14)	37/43 (86)	reference	
Fluconazole and amphotericin B	9/51 (18)	42/51 (82)	1.32 (0.43-4.06)	0.63
Missing data (n)	25	127		
Current TB treatment				
Yes	6/36 (17)	30/36 (83)	1.22 (0.42-3.49)	0.72
No	13/92 (14)	79/92 (86)	reference	
Missing data (n)	21	97		

*Mental status was categorised as "Alert" (Glasgow Coma Scale [GCS] score of 15) or "Not alert" (GCS score of <15 or recorded to be disorientated, stuporose or comatose).

Table S3: Multivariable logistic regression analysis to determine associations between clinical characteristics and infecting strain molecular type among South African patients infected with *Cryptococcus neoformans* serotype A (*n* = 246), 2005-2009

Exposure variables	**Non-VNI genotype N=40**	**VNI genotype N=206**	**Multivariable analysis**	
	n/N (%)	**n/N (%)**	**aOR (95% CI)**	**p-value**
Sex				
Male	29/110 (26)	81/110 (74)	1.65 (0.25-10.99)	0.61
Female	8/125 (6)	117/125 (94)	reference	
Missing data (n)	3	8		
Age (years)				
<25	3/20 (15)	17/20 (85)	reference	
25-34	13/95 (14)	82/95 (86)	0.62 (0.15-2.63)	0.52
35-44	16/84 (19)	68/84 (81)	0.84 (0.20-3.50)	0.81
>45	5/34 (15)	29/34 (85)	0.47 (0.09-2.47)	0.38
Missing data (n)	3	10		
CD4+ T-cell count at diagnosis (cells/µl)				
<=50	11/49 (22)	38/49 (78)	1.01 (0.13-7.95)	0.99
>50	3/28 (11)	25/28 (89)	reference	
Missing data (n)	26	143		
Mental status at diagnosis*				
Alert	15/104 (14)	89/104 (86)	0.43 (0.06-2.96)	0.39
Not alert	11/52 (21)	41/52 (79)	reference	
Missing data (n)	14	76		
CSF white cell count (cells/µl)				
<5	7/56 (12.5)	49/56 (87.5)	0.65 (0.10-4.37)	0.66
>=5	17/86 (20)	69/86 (80)	reference	
Missing data (n)	16	88		

*Mental status was categorised as “Alert” (Glasgow Coma Scale [GCS] score of 15) or “Not alert” (GCS score of <15 or recorded to be disorientated, stuporose or comatose).

Table S4: Univariable logistic regression analysis to determine associations between infecting strain molecular type and in-hospital outcome among South African patients (*n* = 180) infected with *Cryptococcus neoformans* serotype A, 2005-2009

Exposure variables	Died N=64	Survived N=116	Univariable analysis	
	n/N (%)	n/N (%)	OR (95% CI)	p-value
Molecular type				
Non-VNI	9/31 (29)	22/31 (71)	0.69 (0.29-1.61)	0.39
VNI	54/145 (37)	91/145 (63)	reference	
Missing data (n)	1	3		
Sex				
Male	30/84 (36)	54/84 (64)	0.59 (0.24-1.47)	0.25
Female	33/95 (35)	62/95 (65)	reference	
Missing data (n)	1	0		
Age (years)				
<25	6/17 (35)	11/17 (65)	reference	
25-34	27/68 (40)	41/68 (60)	1.15 (0.38-3.49)	0.81
35-44	21/69 (30)	48/69 (70)	0.83 (0.27-2.54)	0.74
>45	10/26 (38)	16/26 (62)	1.33 (0.37-4.80)	0.67
Year of diagnosis				
2005	4/14 (29)	10/14 (71)	reference	
2006	15/42 (36)	27/42 (64)	1.47 (0.39-5.54)	0.57
2007	16/43 (37)	27/43 (63)	1.54 (0.41-5.76)	0.52
2008	12/43 (28)	31/43 (72)	0.93 (0.24-3.61)	0.92
2009	17/38 (45)	21/38 (55)	2.06 (0.55-7.77)	0.29
Hospital				
Academic	36/100 (36)	64/100 (64)	0.69 (0.30-1.60)	0.39
Regional	28/80 (35)	52/80 (65)	reference	
Specimen type				
Cerebrospinal fluid (CSF)	55/160 (34)	105/160 (66)	0.66 (0.27-1.62)	0.37
Blood	5/14 (36)	9/14 (64)	reference	
Missing data (n)	4	2		
CD4+ T-cell count at diagnosis (cells/µl)				
<=50	17/50 (34)	33/50 (66)	0.26 (0.05-1.42)	0.09
>50	8/28 (29)	20/28 (71)	reference	
Missing data (n)	39	63		
Antiretroviral treatment				
Yes	7/23 (30)	16/23 (70)	0.49 (0.15-1.58)	0.22
No	36/119 (30)	83/119 (70)	reference	
Missing data (n)	21	17		
Mental status at diagnosis*				
Alert	29/105 (28)	76/105 (72)	0.63 (0.24-1.67)	0.35
Not alert	23/54 (43)	31/54 (57)	reference	
Missing data (n)	12	9		
CSF white cell count (cells/µl)				
<5	20/41 (49)	21/41 (51)	1.22 (0.42-3.52)	0.72
>=5	16/67 (24)	51/67 (76)	reference	
Missing data (n)	28	44		
Current antifungal treatment				
Fluconazole alone	11/45 (24)	34/45 (76)	reference	
Fluconazole and amphotericin B	13/52 (25)	39/52 (75)	1.02 (0.40-2.62)	0.96
Missing data (n)	40	43		
TB treatment				
Yes	15/37 (41)	22/37 (59)	0.73 (0.24-2.23)	0.58
No	26/95 (27)	69/95 (73)	reference	
Missing data (n)	23	25		

*Mental status was categorised as "Alert" (Glasgow Coma Scale [GCS] score of 15) or "Not alert" (GCS score of <15 or recorded to be disorientated, stuporose or comatose).

Table S5: Multivariable logistic regression analysis to determine associations between infecting strain molecular type and in-hospital outcome among South African patients (*n* = 180) infected with *Cryptococcus neoformans* serotype A, 2005-2009

Exposure variables	**Died N=64**	**Survived N=116**	**Multivariable analysis**	
	n/N (%)	**n/N (%)**	**aOR (95% CI)**	**p-value**
Molecular type				
Non-VNI	9/31 (29)	22/31 (71)	0.50 (0.03-7.57)	0.62
VNI	54/145 (37)	91/145 (63)	reference	
Missing data (n)	1	3		
Sex				
Male	30/84 (36)	54/84 (64)	0.56 (0.06-5.60)	0.62
Female	33/95 (35)	62/95 (65)	reference	
Missing data (n)	1	0		
Age (years)				
<25	6/17 (35)	11/17 (65)	reference	
25-34	27/68 (40)	41/68 (60)	0.25 (0.01-9.87)	0.46
35-44	21/69 (30)	48/69 (70)	0.35 (0.01-14.84)	0.58
>45	10/26 (38)	16/26 (62)	0.55 (0.01-46.60)	0.79
CD4+ T-cell count at diagnosis (cells/µl)				
<=50	17/50 (34)	33/50 (66)	1.15 (0.13-10.29)	0.90
>50	8/28 (29)	20/28 (71)	reference	
Missing data (n)	39	63		
Antiretroviral treatment				
Yes	7/23 (30)	16/23 (70)	20.20 (0.88-464.75)	0.06
No	36/119 (30)	83/119 (70)	reference	
Missing data (n)	21	17		
Mental status at diagnosis*				
Alert	29/105 (28)	76/105 (72)	0.16 (0.01-2.10)	0.16
Not alert	23/54 (43)	31/54 (57)	reference	
Missing data (n)	12	9		
Current antifungal treatment				
Fluconazole alone	11/45 (24)	34/45 (76)	reference	
Fluconazole and amphotericin B	13/52 (25)	39/52 (75)	2.56 (0.22-30.21)	0.46
Missing data (n)	40	43		

*Mental status was categorised as “Alert” (Glasgow Coma Scale [GCS] score of 15) or “Not alert” (GCS score of <15 or recorded to be disorientated, stuporose or comatose).

Table S6: Characteristics of 15 South African patients infected with the *Cryptococcus neoformans* VNB molecular type collected during national laboratory-based surveillance for cryptococcosis, 2005-2009

Isolate number	Home town/city	Province	Specimen collection date	Syndrome	Facility name
205	Meadowlands	Gauteng	26/1/2005	Meningitis	Chris Hani Baragwanath
892	Soweto	Gauteng	25/2/2006	Meningitis	Chris Hani Baragwanath
1817	Mangaung	Free State	18/4/2006	Meningitis	Pelonomi
130	Mthatha	Eastern Cape	9/1/2009	Meningitis	Nelson Mandela Academic/Mthatha Provincial
2887	Kroonstad	Free State	29/8/2005	Unknown	Boitumelo
3783	Johannesburg	Gauteng	26/10/2008	Meningitis	Chris Hani Baragwanath
5873	Soweto	Gauteng	8/11/2006	Meningitis and pneumonia	Chris Hani Baragwanath
1239	Orange Farm	Gauteng	15/3/2007	Meningitis	Chris Hani Baragwanath
907	Soweto	Gauteng	26/2/2006	Meningitis	Chris Hani Baragwanath
3438	Unknown	North West	9/10/2005	Unknown	Bophelong/Mafikeng
1597	Lenasia	Gauteng	2/4/2006	Meningitis	Chris Hani Baragwanath
1543	Tshwane	Gauteng	16/7/2009	Meningitis	Steve Biko Pretoria Academic
1408	Boksburg	Gauteng	19/4/2005	Unknown	Tambo Memorial
637	Scottburgh	KwaZulu-Natal	9/2/2005	Unknown	GJ Crooke's/Scottburgh
2890	Rouxville	Free State	26/6/2008	Pneumonia	Pelonomi

Appendix C – Supplementary information for Chapter 3

Supplementary metadata information for 146 clinical *Cryptococcus gattii* strains from South African laboratory-based surveillance, 2005-2013

Isolate ID	Record	CAP59	GPD1	IGS1	LAC1	PLB1	SOD1	URA5	Sequence type	Molecular type	Mating type	FLZ MIC (µg/ml)	Province	Specimen
2	1252	17	4	2	1	3	62	11	13	VGIV	matα	ND	Western Cape	Blood culture
3	6185	52	11	13	13	13	74	15	230	VGI	matα	4	KwaZulu-Natal	CSF
4	500	23	4	2	1	10	35	11	14	VGIV	matα	ND	KwaZulu-Natal	CSF
5	2769	17	10	2	1	3	62	11	155	VGIV	matα	1	Gauteng	CSF
7	1031	16	20	46	13	13	63	24	23	VGI	matα	8	Mpumalanga	CSF
8	1708	36	11	13	13	13	74	15	291	VGI	matα	16	Western Cape	CSF
9	1977	63	10	8	1	37	35	11	377	VGIV	matα	8	North West	CSF
10	153	37	10	2	1	3	35	11	294	VGIV	matα	ND	Limpopo	CSF
11	954	37	19	2	44	3	35	11	378	VGIV	matα	ND	Gauteng	CSF
12	1261	23	10	2	1	3	62	11	15	VGIV	matα	ND	Mpumalanga	CSF
13	236	58	10	8	1	3	62	11	379	VGIV	matα	ND	Gauteng	CSF
14	918	37	10	2	1	3	35	11	294	VGIV	matα	1	Limpopo	CSF
15	1365	16	5	3	5	5	65	12	57	VGI	matα	16	Free State	CSF
16	1545	37	10	2	17	3	35	11	374	VGIV	matα	ND	Mpumalanga	CSF
17	273	37	10	2	17	37	30	11	387	VGIV	matα	2	Gauteng	CSF
18	303	23	10	2	1	37	62	11	16	VGIV	matα	ND	Gauteng	CSF
19	335	31	11	59	13	13	67	14	388	VGI	matα	8	Mpumalanga	CSF
20	1999	63	11	2	1	3	62	11	386	VGIV	matα	ND	Mpumalanga	CSF
21	2532	16	11	13	13	13	80	15	24	VGI	matα	4	Mpumalanga	CSF
22	2533	2	32	56	21	1	8	7	385	VGII	matα	8	Mpumalanga	CSF
23	2736	37	10	2	17	37	35	11	370	VGIV	matα	ND	Gauteng	CSF
24	2899	60	10	2	1	3	62	11	380	VGIV	matα	2	Gauteng	CSF

Isolate ID	Record	CAP59	GPD1	IGS1	LAC1	PLB1	SOD1	URA5	Sequence type	Molecular type	Mating type	FLZ MIC (µg/ml)	Province	Specimen
25	3445	16	11	46	13	13	65	24	36	VGI	matα	8	Mpumalanga	CSF
26	3876	37	10	2	1	3	35	11	294	VGIV	matα	4	Gauteng	CSF
27	4387	37	10	2	1	3	62	11	371	VGIV	matα	4	Mpumalanga	CSF
28	5454	23	19	2	1	10	30	11	389	VGIV	matα	32	Gauteng	CSF
29	5566	36	11	13	5	13	36	14	106	VGI	matα	8	Gauteng	CSF
30	5714	44	11	68	13	13	70	15	381	VGI	mata	8	Mpumalanga	CSF
31	6044	58	10	2	1	37	30	11	382	VGIV	matα	8	Mpumalanga	CSF
32	6487	37	10	2	1	3	35	11	294	VGIV	matα	ND	Gauteng	CSF
33	805	17	10	8	44	3	35	11	390	VGIV	matα	ND	Gauteng	CSF
34	1005	17	10	2	1	3	62	11	155	VGIV	matα	1	Gauteng	CSF
35	1124	37	10	2	1	3	35	11	294	VGIV	matα	8	Gauteng	CSF
36	1625	23	19	2	1	37	86	11	22	VGIV	matα	4	Gauteng	CSF
37	3917	17	10	2	1	3	30	11	391	VGIV	matα	2	KwaZulu-Natal	CSF
38	6516	61	10	2	1	10	35	11	383	VGIV	matα	1	KwaZulu-Natal	CSF
39	88	37	10	2	1	37	31	11	372	VGIV	matα	1	Limpopo	CSF
40	1163	37	10	2	1	3	62	11	371	VGIV	matα	4	Limpopo	CSF
41	1071	58	10	8	1	37	35	11	392	VGIV	matα	4	North West	CSF
42	1778	31	11	44	13	13	32	24	285	VGI	matα	8	Gauteng	CSF
43	2399	16	11	13	13	13	97	15	41	VGI	matα	8	KwaZulu-Natal	CSF
44	3448	23	10	2	17	3	35	11	393	VGIV	matα	ND	Gauteng	CSF
45	3943	45	10	8	17	37	31	11	224	VGIV	matα	4	Gauteng	CSF
46	3598	17	10	2	1	3	93	11	11	VGIV	matα	2	Limpopo	CSF
47	3994	17	10	8	18	3	37	11	70	VGIV	matα	8	Gauteng	CSF
48	3749	17	10	2	1	3	62	11	155	VGIV	matα	ND	Limpopo	CSF
49	4051	35	40	1	22	6	28	19	165	VGIII	matα	8	Mpumalanga	CSF
50	4052	37	10	2	44	3	31	11	375	VGIV	matα	ND	Mpumalanga	CSF

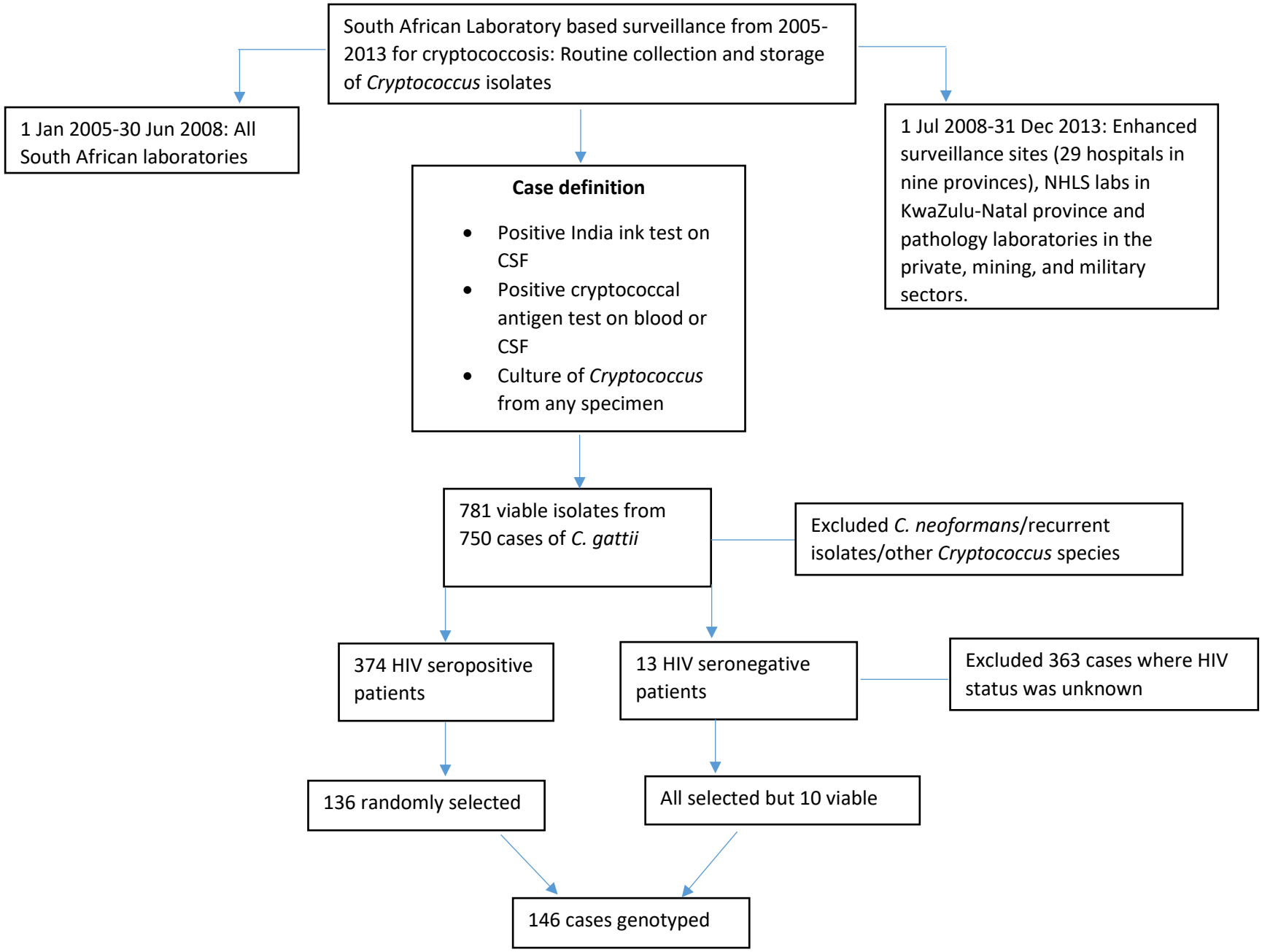
Isolate ID	Record	CAP59	GPD1	IGS1	LAC1	PLB1	SOD1	URA5	Sequence type	Molecular type	Mating type	FLZ MIC (µg/ml)	Province	Specimen
51	4069	31	11	59	13	13	71	14	394	VGI	matα	16	KwaZulu-Natal	CSF
52	4102	17	10	2	1	3	112	11	397	VGIV	matα	2	KwaZulu-Natal	CSF
53	274	17	10	2	1	3	112	11	397	VGIV	matα	1	North West	CSF
54	281	16	11	13	19	15	34	14	58	VGI	matα	8	KwaZulu-Natal	CSF
55	326	16	14	3	5	5	45	12	159	VGI	matα	4	Western Cape	Unknown
56	499	17	10	8	1	3	62	11	9	VGIV	matα	ND	Gauteng	CSF
57	547	32	4	2	1	7	85	11	221	VGIV	matα	0.5	Gauteng	CSF
58	917	16	11	24	13	13	70	15	284	VGI	matα	16	Western Cape	CSF
59	1687	37	10	8	17	3	35	11	376	VGIV	matα	ND	Mpumalanga	CSF
60	1764	37	10	2	1	3	30	11	373	VGIV	matα	ND	Gauteng	CSF
61	1846	58	10	2	17	3	30	11	384	VGIV	matα	2	Gauteng	CSF
62	2000	17	10	8	1	3	62	11	9	VGIV	matα	ND	Gauteng	CSF
63	2028	37	10	2	1	3	35	11	294	VGIV	matα	4	Gauteng	CSF
64	2174	37	10	2	1	3	62	11	371	VGIV	matα	ND	Mpumalanga	CSF
65	2428	37	10	2	1	3	35	11	294	VGIV	matα	ND	Limpopo	CSF
66	2390	37	10	2	44	3	35	11	426	VGIV	mata	2	Limpopo	CSF
67	2476	37	10	2	1	3	35	11	294	VGIV	matα	2	Limpopo	CSF
68	2544	59	10	17	17	3	35	11	336	VGIV	matα	ND	Gauteng	CSF
69	786	37	10	8	1	3	62	11	429	VGIV	matα	4	Gauteng	CSF
70	70	17	10	2	1	3	62	11	155	VGIV	matα	8	Mpumalanga	CSF
71	14	16	14	3	5	5	45	12	159	VGI	matα	2	Western Cape	CSF
72	607	37	10	17	1	3	30	11	430	VGIV	matα	4	KwaZulu-Natal	CSF
73	543	44	11	59	13	13	68	15	432	VGI	matα	8	Gauteng	CSF
74	843	23	4	2	17	7	35	34	419	VGIV	matα	8	Northern Cape	CSF

Isolate ID	Record	CAP59	GPD1	IGS1	LAC1	PLB1	SOD1	URA5	Sequence type	Molecular type	Mating type	FLZ MIC (µg/ml)	Province	Specimen
75	980	31	11	44	13	13	71	15	421	VGI	matα	4	Mpumalanga	CSF
76	1435	44	4	59	13	13	68	15	431	VGI	matα	2	Gauteng	CSF
77	376	37	10	2	1	3	62	11	371	VGIV	matα	8	Mpumalanga	CSF
78	1742	58	4	2	17	7	30	11	436	VGIV	matα	ND	North West	CSF
79	1643	37	10	8	1	3	30	11	428	VGIV	mata	1	KwaZulu-Natal	CSF
80	1787	52	11	46	13	13	34	15	434	VGI	matα	4	Gauteng	CSF
81	1788	58	10	8	17	3	30	11	441	VGIV	matα	2	Gauteng	CSF
82	1686	36	11	13	5	13	36	14	106	VGI	matα	2	Gauteng	Blood culture
83	1698	80	19	2	1	10	30	11	398	VGIV	matα	4	Western Cape	Unknown
84	1930	37	10	2	1	3	30	11	373	VGIV	matα	4	KwaZulu-Natal	CSF
85	2085	17	10	2	1	3	62	11	155	VGIV	matα	ND	Mpumalanga	CSF
86	252	17	10	2	1	3	62	11	155	VGIV	matα	8	Gauteng	CSF
87	455	16	11	59	5	13	72	15	443	VGI	matα	8	KwaZulu-Natal	CSF
88	841	45	10	8	17	37	31	11	224	VGIV	matα	4	Gauteng	Blood culture
89	848	17	10	2	1	3	62	11	155	VGIV	mata	16	Gauteng	CSF
90	940	45	10	8	17	37	31	11	224	VGIV	matα	0.5	Mpumalanga	CSF
91	924	17	10	2	1	3	112	11	397	VGIV	matα	ND	Mpumalanga	CSF
92	976	17	10	2	1	3	62	11	155	VGIV	matα	4	Mpumalanga	CSF
93	1037	37	10	8	17	3	35	11	376	VGIV	matα	ND	Mpumalanga	CSF
94	1087	31	11	70	13	13	72	15	422	VGI	matα	8	Northern Cape	CSF
95	1150	17	10	2	1	3	35	11	415	VGIV	matα	ND	Limpopo	CSF
96	1280	52	11	46	13	13	70	15	435	VGI	matα	4	KwaZulu-Natal	CSF
97	1404	35	40	1	22	6	28	19	165	VGIII	matα	8	Mpumalanga	CSF
98	1428	17	10	2	1	3	112	11	397	VGIV	matα	16	Mpumalanga	CSF

Isolate ID	Record	CAP59	GPD1	IGS1	LAC1	PLB1	SOD1	URA5	Sequence type	Molecular type	Mating type	FLZ MIC (µg/ml)	Province	Specimen
99	1723	37	10	2	1	3	35	11	294	VGIV	matα	4	North West	CSF
100	1840	36	11	13	5	13	32	14	423	VGI	matα	2	Northern Cape	CSF
101	1866	81	10	8	1	10	30	11	399	VGIV	matα	16	Limpopo	CSF
102	22	2	52	56	21	1	90	7	400	VGII	matα	8	Mpumalanga	CSF
103	339	81	10	8	1	10	30	11	399	VGIV	matα	ND	Gauteng	CSF
104	348	17	10	8	1	3	62	11	9	VGIV	matα	16	Gauteng	CSF
105	386	58	10	8	1	3	62	11	379	VGIV	matα	ND	Limpopo	CSF
106	465	23	10	17	17	3	30	11	420	VGIV	mata	2	Gauteng	Blood culture
107	561	16	11	46	13	13	34	14	411	VGI	matα	16	Gauteng	CSF
108	1127	80	19	2	1	10	30	11	398	VGIV	matα	ND	Gauteng	CSF
109	1385	58	10	2	1	3	35	11	437	VGIV	matα	ND	Gauteng	CSF
110	1783	16	11	68	13	13	34	15	412	VGI	matα	8	Gauteng	CSF
111	2025	37	10	2	44	37	30	11	427	VGIV	matα	4	Gauteng	CSF
112	2026	37	4	2	1	3	62	11	424	VGIV	matα	8	Gauteng	CSF
113	2133	37	10	2	1	3	35	11	294	VGIV	matα	0.5	Gauteng	CSF
114	2172	58	10	2	1	37	30	11	382	VGIV	matα	2	Mpumalanga	CSF
115	2173	82	10	2	1	3	62	11	401	VGIV	matα	ND	Mpumalanga	Blood culture
116	2259	25	11	46	13	13	73	11	403	VGI	matα	8	Gauteng	CSF
117	2217	17	10	2	1	3	62	11	155	VGIV	matα	16	Gauteng	CSF
119	2569	58	10	2	17	3	31	11	439	VGIV	matα	ND	KwaZulu-Natal	CSF
120	2414	58	10	8	1	3	35	11	440	VGIV	matα	4	Limpopo	CSF
121	2612	37	10	2	1	3	35	11	294	VGIV	matα	2	Gauteng	CSF
122	2843	25	11	46	13	13	73	24	403	VGI	matα	4	KwaZulu-Natal	CSF
123	2970	16	11	44	13	13	73	46	404	VGI	matα	8	Gauteng	CSF
124	3058	37	10	2	1	3	35	11	294	VGIV	matα	4	Mpumalanga	CSF

Isolate ID	Record	CAP59	GPD1	IGS1	LAC1	PLB1	SOD1	URA5	Sequence type	Molecular type	Mating type	FLZ MIC (µg/ml)	Province	Specimen
125	3096	37	10	8	1	3	62	11	429	VGIV	matα	ND	Gauteng	CSF
126	3332	16	14	3	5	5	45	12	159	VGI	matα	4	Western Cape	Unknown
127	3470	37	10	8	1	3	62	11	429	VGIV	matα	8	Gauteng	CSF
128	3772	16	5	3	5	5	32	12	51	VGI	mata	8	Western Cape	CSF
129	3796	16	5	3	5	5	45	12	154	VGI	matα	2	Free State	CSF
130	3882	32	4	2	1	7	85	11	221	VGIV	matα	8	Mpumalanga	CSF
131	4145	37	10	2	1	3	35	11	294	VGIV	matα	ND	Limpopo	CSF
133	4274	16	5	3	5	5	45	12	154	VGI	matα	4	North West	CSF
134	4009	17	4	2	1	37	30	11	414	VGIV	mata	2	Gauteng	CSF
135	75	83	11	59	13	13	34	15	405	VGI	matα	8	Northern Cape	CSF
136	2083	49	11	13	13	13	34	24	433	VGI	matα	32	Mpumalanga	CSF
137	772	37	10	2	1	3	35	11	294	VGIV	matα	8	Limpopo	CSF
138	3360	49	11	46	13	13	73	15	406	VGI	matα	8	North West	CSF
139	2060	23	4	2	1	10	30	11	418	VGIV	matα	ND	Gauteng	CSF
140	1722	17	10	2	17	3	30	11	416	VGIV	matα	ND	Mpumalanga	CSF
141	2505	16	11	68	13	13	34	24	413	VGI	matα	8	Gauteng	CSF
142	2530	82	10	2	1	3	35	11	407	VGIV	matα	8	Gauteng	CSF
143	3954	71	42	90	6	5	113	38	408	VGI	matα	1	Gauteng	CSF
144	3995	17	19	2	1	7	35	11	417	VGIV	matα	ND	Gauteng	Blood culture
145	2854	82	10	2	1	3	35	11	407	VGIV	matα	2	Limpopo	CSF
146	1751	2	52	56	21	1	8	7	409	VGII	matα	8	Mpumalanga	CSF
147	841	25	11	59	13	13	34	24	410	VGI	matα	8	Gauteng	CSF
148	2153	17	10	2	1	3	62	11	155	VGIV	matα	ND	Mpumalanga	CSF
149	3061	82	10	2	1	3	62	11	401	VGIV	matα	ND	Mpumalanga	CSF
150	3234	58	10	2	17	3	30	11	384	VGIV	matα	ND	Gauteng	CSF

*Note: FLZ MIC – Fluconazole minimum inhibitory concentration, CSF – Cerebrospinal fluid, ND – Not done



Supplementary Figure 1: *Cryptococcus gattii* isolates selected for genotyping from South African laboratory-based surveillance, 2005-2013.

Note: NHLS – National Health Laboratory Service, CSF – Cerebrospinal fluid

Supplementary Table 1: Univariable analysis to determine associations between clinical characteristics and infecting strain molecular type among South African patients infected with *Cryptococcus gattii* (n=146), 2005-2013

Exposure variables	VGIV N = 101	Non-VGIV N = 45	Univariable analysis	
	n/N (%)	n/N (%)	OR (95% CI)	p-value
Sex				
Male	56/85 (66)	29/85 (34)	0.69 (0.33-1.42)	0.31
Female	45/61 (74)	16/61 (26)	Reference	
Age (years)				
<25	11/17 (65)	6/17 (35)	Reference	
25-34	36/50 (72)	14/50 (28)	1.40 (0.44-4.52)	0.57
35-44	41/55 (75)	14/55 (25)	1.60 (0.50-5.12)	0.43
>45	13/24 (54)	11/24 (46)	0.64 (0.18-2.31)	0.50
Year of diagnosis				
2005	2/4 (50)	2/4 (50)	Reference	
2006	12/19 (63)	7/19 (37)	1.71 (0.20-15.02)	0.63
2007	9/9 (100)	0/9 (0)		
2008	12/18 (67)	6/18 (33)	2.00 (0.22-17.89)	0.54
2009	16/20 (80)	4/20 (20)	4.00 (0.42-37.78)	0.23
2010	12/21 (57)	9/21 (43)	1.33 (0.16-11.36)	0.79
2011	16/21 (76)	5/21 (24)	3.20 (0.35-28.94)	0.30
2012	7/11 (64)	4/11 (36)	1.75 (0.17-17.69)	0.64
2013	15/23 (65)	8/23 (35)	1.88 (0.22-15.93)	0.57
Geographical region*				
Temperate	79/117 (68)	38/117 (32)	0.66 (0.26-1.68)	0.39
Arid	22/29 (76)	7/29 (24)	Reference	
Specimen type				
Cerebrospinal fluid (CSF)	95/137 (69)	42/137 (31)	0.45 (0.05-3.99)	0.48
Blood	5/6 (83)	1/6 (17)	Reference	
Missing data (n)	1	2		
HIV infection status				
Positive	99/136 (73)	37/136 (27)	10.70 (2.17-52.74)	0.004
Negative	2/10 (20)	8/10 (80)	Reference	
CD4+ T-cell count at diagnosis (cells/µl)				
<=50	37/46 (80)	9/46 (20)	1.67 (0.63-4.42)	0.30
>50	32/45 (71)	13/45 (29)	Reference	
Missing data (n)	32	23		
Antiretroviral treatment				
Yes	38/55 (69)	17/55 (31)	0.80 (0.37-1.72)	0.57
No	56/76 (74)	20/76 (26)	Reference	
Missing data (n)	7	8		
Mental status at diagnosis*				
Alert	69/97 (71)	28/97 (29)	1.33 (0.61-2.91)	0.48
Not alert	26/40 (65)	14/40 (35)	Reference	

Missing data (n)	6	3		
Current antifungal treatment				
Fluconazole alone	19/30 (63)	11/30 (37)	Reference	
Fluconazole and amphotericin B	50/70 (71)	20/70 (29)	1.45 (0.59-3.58)	0.42
Missing data (n)	32	14		
Current TB treatment				
Yes	28/41 (68)	13/41 (32)	0.88 (0.39-1.95)	0.74
No	64/90 (71)	26/90 (29)	Reference	
Missing data (n)	9	6		

*Geographical region was categorised as mostly temperate (Gauteng, Mpumalanga, KwaZulu-Natal and Western Cape provinces) or mostly arid (Northern Cape, Free State, Limpopo, North West and Eastern Cape provinces). *Mental status was categorised as "Alert" (Glasgow Coma Scale [GCS] score of 15) or "Not alert" (GCS score of <15 or recorded to be disorientated, stuporose or comatose).

Supplementary Table 2: Multivariable logistic regression analysis to determine associations between clinical characteristics and infecting strain molecular type among South African patients infected with *Cryptococcus gattii* (n=146), 2005-2013

Exposure variables	VGIV N = 101	Non-VGIV N = 45	Multivariable analysis	
	n/N (%)	n/N (%)	aOR (95% CI)	p-value
Geographical region				
Temperate	79/117 (68)	38/117 (32)	0.57 (0.21-1.58)	0.28
Arid	22/29 (76)	7/29 (24)	Reference	
HIV infection status				
Positive	99/136 (73)	37/136 (27)	10.34 (1.93-55.31)	0.006
Negative	2/10 (20)	8/10 (80)	Reference	
Sex				
Male	56/85 (66)	29/85 (34)	0.57 (0.25-1.28)	0.18
Female	45/61 (74)	16/61 (26)	Reference	
Age (years)				
<25	11/17 (65)	6/17 (35)	Reference	
25-34	36/50 (72)	14/50 (28)	0.77 (0.20-3.03)	0.71
35-44	41/55 (75)	14/55 (25)	1.00 (0.25-3.93)	1.0
>45	13/24 (54)	11/24 (46)	0.42 (0.10-1.84)	0.25

Supplementary Table 3: Univariable logistic regression analysis to determine association between infecting strain molecular type and in-hospital outcome among South African patients (n=142) infected with *Cryptococcus gattii*, 2005-2013

Exposure variables	Died N = 40 n/N (%)	Survived N = 102 n/N (%)	Univariable analysis OR (95% CI)	p-value
Molecular type				
VGIV	29/98 (30)	69/98 (70)	1.26 (0.56-2.83)	0.57
Non-VGIV	11/44 (25)	33/44 (75)	Reference	
Sex				
Male	18/83 (22)	65/83 (78)	0.47 (0.22-0.99)	0.05
Female	22/59 (37)	37/59 (63)	Reference	
Age (years)				
<25	3/17 (18)	14/17 (82)	Reference	
25-34	15/48 (31)	33/48 (69)	2.10 (0.52-8.41)	0.30
35-44	15/53 (28)	38/53 (72)	1.80 (0.45-7.20)	0.41
≥45	7/24 (29)	17/24 (71)	1.97 (0.43-9.11)	0.39
Year of diagnosis				
2005	1/4 (25)	3/4 (75)	Reference	
2006	4/19 (21)	15/19 (79)	0.75 (0.06-9.48)	0.83
2007	1/9 (11)	8/9 (89)	0.30 (0.01-6.80)	0.45
2008	5/18 (28)	13/18 (72)	1.08 (0.09-13.12)	0.96
2009	3/18 (17)	15/18 (83)	0.52 (0.04-7.04)	0.62
2010	4/21 (19)	17/21 (81)	0.68 (0.05-8.51)	0.77
2011	6/21 (29)	15/21 (71)	1.07 (0.09-12.74)	0.96
2012	5/9 (56)	4/9 (44)	3.71 (0.27-51.52)	0.33
2013	11/23 (48)	12/23 (52)	2.60 (0.23-29.22)	0.44
Hospital				
Academic	18/64 (28)	46/64 (72)	1.01 (0.48-2.11)	0.98
Regional	22/78 (28)	56/78 (72)	Reference	
Specimen type				
Cerebrospinal fluid	37/134 (28)	97/134 (72)	0.80	0.80

(CSF)			(0.14-4.58)	
Blood	2/6 (33)	4/6 (67)	Reference	
Missing data (n)	1	1		
CD4+ T-cell count at diagnosis (cells/µl)				
≤50	14/45 (31)	31/45 (69)	1.36 (0.54-3.46)	0.52
>50	11/45 (24)	34/45 (76)	Reference	
Missing data (n)	15	37		
Antiretroviral treatment				
Yes	13/54 (24)	41/54 (76)	0.82 (0.36-1.84)	0.63
No	21/74 (28)	53/74 (72)	Reference	
Missing data (n)	6	8		
Mental status at diagnosis*				
Alert	15/94 (16)	79/94 (84)	0.18 (0.08-0.41)	0.001
Not alert	20/39 (51)	19/39 (49)	Reference	
Missing data (n)	5	4		
Current antifungal treatment				
Fluconazole alone	7/29 (24)	22/29 (76)	Reference	
Fluconazole and amphotericin B	15/69 (22)	54/69 (78)	0.89 (0.32-2.49)	0.82
Missing data (n)	18	26		
Current tuberculosis treatment				
Yes	18/41 (44)	23/41 (56)	3.79 (1.64-8.75)	0.002
No	15/86 (17)	71/86 (83)	Reference	
Missing data (n)	7	8		

*Mental status was categorised as "Alert" (Glasgow Coma Scale [GCS] score of 15) or "Not alert" (GCS score of <15 or recorded to be disorientated, stuporose or comatose).

Supplementary Table 4: Multivariable logistic regression analysis to determine association between infecting strain molecular type and in-hospital outcome, adjusted for potential confounders, among South African patients (n=142) infected with *Cryptococcus gattii*, 2005-2013

Exposure variables	Died N = 40	Survived N = 102	Multivariable analysis	
	n/N (%)	n/N (%)	aOR (95% CI)	p-value
Molecular type				
VGIV	29/98 (30)	69/98 (70)	0.57 (0.07-4.43)	0.59
Non-VGIV	11/44 (25)	33/44 (75)	reference	
Sex				
Male	18/83 (22)	65/83 (78)	0.72 (0.12-4.27)	0.72
Female	22/59 (37)	37/59 (63)	reference	
Age (years)				
<25	3/17 (18)	14/17 (82)	reference	
25-34	15/48 (31)	33/48 (69)	0.48 (0.02-9.71)	0.64
35-44	15/53 (28)	38/53 (72)	0.54 (0.03-9.39)	0.67
≥45	7/24 (29)	17/24 (71)	0.61 (0.02-15.40)	0.76
CD4+ T-cell count at diagnosis (cells/µl)				
≤50	14/45 (31)	31/45 (69)	2.26 (0.35-14.43)	0.39
>50	11/45 (24)	34/45 (76)	reference	
Missing data (n)	15	37		
Antiretroviral treatment				
Yes	13/54 (24)	41/54 (76)	0.64 (0.11-3.59)	0.61
No	21/74 (28)	53/74 (72)	reference	
Missing data (n)	6	8		
Mental status at diagnosis*				
Alert	15/94 (16)	79/94 (84)	0.20 (0.03-1.27)	0.09
Not alert	20/39 (51)	19/39 (49)	reference	
Missing data (n)	5	4		
Current antifungal treatment				
Fluconazole alone	7/29 (24)	22/29 (76)	reference	
Fluconazole and amphotericin B	15/69 (22)	54/69 (78)	1.65 (0.13-20.66)	0.70
Missing data (n)	18	26		

*Mental status was categorised as "Alert" (Glasgow Coma Scale [GCS] score of 15) or "Not alert" (GCS score of <15 or recorded to be disorientated, stuporose or comatose).

Supplementary Table 5: Characteristics of ten South African patients infected with the *Cryptococcus gattii* VGIV molecular type from the Limpopo and Gauteng Provinces that clustered closely together on whole genome sequencing analysis as shown in Figure 3.3a; these strains were collected during enhanced laboratory-based surveillance for cryptococcosis, 2005-2013

Isolate number	Home town/city	Province	Specimen collection date	Syndrome	Facility name
2428	Polokwane	Limpopo	18/11/2009	Meningitis	Mankweng
2612	Pretoria	Gauteng	14/4/2013	Meningitis	Dr George Mukhari
2133	Northcliff	Gauteng	23/1/2013	Meningitis	Helen Joseph
153	Polokwane	Limpopo	23/1/2009	Meningitis and pneumonia	Polokwane
918	Polokwane	Limpopo	20/5/2010	Meningitis	Polokwane
4145	Tzaneen	Limpopo	2/11/2013	Meningitis	Mankweng
2476	Polokwane	Limpopo	14/12/2009	Meningitis	Mankweng
1163	Polokwane	Limpopo	20/2/2008	Meningitis	Mankweng
6487	Eldorado Park	Gauteng	27/12/2006	Meningitis	Chris Hani Baragwanath
772	Polokwane	Limpopo	5/8/2011	Meningitis	Polokwane

Supplementary Table 6: Characteristics of six South African patients infected with the *Cryptococcus gattii* VGIV molecular type from the Mpumalanga Province that clustered closely together on whole genome sequencing analysis as shown in Figure 3.3b; these strains were collected during enhanced laboratory-based surveillance for cryptococcosis, 2005-2013

Isolate number	Home town/city	Province	Specimen collection date	Syndrome	Facility name
70	Nelspruit	Mpumalanga	14/1/2010	Meningitis	Themba
2153	Kabokweni	Mpumalanga	27/4/2008	Meningitis	Themba
976	Barberton	Mpumalanga	14/7/2011	Meningitis	Rob Ferreira
1261	Kabokweni	Mpumalanga	15/6/2009	Meningitis	Themba
1999	Kabokweni	Mpumalanga	5/5/2006	Meningitis	Themba
2085	White River	Mpumalanga	16/12/2010	Meningitis	Themba

Appendix D – Supplementary information for Chapter 4

STROBE Statement—Checklist of items that should be included in reports of ***cross-sectional studies***

	Item No	Recommendation	Manuscript section	Line numbers
Title and abstract	1	(*a*) Indicate the study's design with a commonly used term in the title or the abstract	Abstract – Methodology and Principal Findings	52
		(*b*) Provide in the abstract an informative and balanced summary of what was done and what was found	Abstract	44-70
Introduction				
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	Introduction	88-115
Objectives	3	State specific objectives, including any prespecified hypotheses	Introduction	113-115
Methods				
Study design	4	Present key elements of study design early in the paper	Materials and methods – Study design	118-139
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	Materials and methods – Study design	118-139
Participants	6	(*a*) Give the eligibility criteria, and the sources and methods of selection of participants	Materials and methods – Study design	118-139
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	Materials and methods – Statistical analyses	162-168

	Item No	Recommendation	Manuscript section	Line numbers
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	N/A	
Bias	9	Describe any efforts to address potential sources of bias	N/A	
Study size	10	Explain how the study size was arrived at	Materials and methods – Study design	126-129
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	N/A	
Statistical methods	12	(*a*) Describe all statistical methods, including those used to control for confounding	Materials and methods – Statistical analyses	162-168
		(*b*) Describe any methods used to examine subgroups and interactions	Materials and methods – Statistical analyses	162-168
		(*c*) Explain how missing data were addressed	N/A	
		(*d*) If applicable, describe analytical methods taking account of sampling strategy	N/A	
		(*e*) Describe any sensitivity analyses	N/A	
Results				
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	Results	175-181
		(b) Give reasons for non-participation at each stage	N/A	
		(c) Consider use of a flow diagram	None	

	Item No	Recommendation	Manuscript section	Line numbers
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	Results: Table 1	197
		(b) Indicate number of participants with missing data for each variable of interest	Results: Table 1	197
Outcome data	15*	Report numbers of outcome events or summary measures	Results: Table 3	205
Main results	16	(*a*) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	Results	192-195
		(*b*) Report category boundaries when continuous variables were categorized	Results: Table 1	197
		(*c*) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	N/A	
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	N/A	
Discussion				
Key results	18	Summarise key results with reference to study objectives	Discussion	210-213
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	Discussion	249-251
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	Discussion	210-256
Generalisability	21	Discuss the generalisability (external validity) of the study results	N/A	
Other information				
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	N/A	

*Give information separately for exposed and unexposed groups.

Note: An Explanation and Elaboration article discusses each checklist item and gives methodological background and published examples of transparent reporting. The STROBE checklist is best used in conjunction with this article (freely available on the Web sites of PLoS Medicine at http://www.plosmedicine.org/, Annals of Internal Medicine at http://www.annals.org/, and Epidemiology at http://www.epidem.com/). Information on the STROBE Initiative is available at www.strobe-statement.org.

Supplementary Table 1: Fluconazole minimum inhibitory concentration (MIC) values of 20 *Cryptococcus neoformans* isolates from 2007-2008 that were obtained using custom-made broth microdilution plates

Isolate number	**Previous MIC value (µg/ml) [5]**	**Repeat MIC value (µg/ml)**
1709	0.5	1
644	2	2
662	2	4
6824	4	2
6813	1	2
6625	4	4
5919	4	2
6235	2	2
6330	2	2
390	2	1
76	1	2
166	4	4
384	4	2
3164	2	2
6681	4	2
2049	1	4
3612	1	2
3635	1	1
4264	2	1
2421	4	2

Supplementary Table 2: Voriconazole, itraconazole and posaconazole Etest minimum inhibitory concentration (MIC) values for 16 *Cryptococcus neoformans* isolates with a fluconazole MIC of ≥16 µg/ml by broth microdilution

Isolate number	Voriconazole MIC (µg/ml)	Itraconazole MIC (µg/ml)	Posaconazole MIC (µg/ml)
53	32	32	32
88	0.38	0.75	0.125
93	1.5	1.5	3
138	0.38	1.5	0.125
162	0.5	1	0.75
169	1.5	2	8
170	1	1	3
177	0.75	0.75	0.75
181	0.004	0.012	0.064
208	0.38	1.5	4
210	0.38	2	1.5
211	1	1.5	4
214	0.75	2	6
227	0.38	1	0.5
241	0.25	0.75	0.25
339	0.25	1	0.5

Appendix E – Ethics approvals for thesis

R14/49 Miss Serisha Naicker

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M160375

NAME: (Principal Investigator)	Miss Serisha Naicker
DEPARTMENT:	Clinical Microbiology and Infectious Diseases National Institute for Communicable Diseases
PROJECT TITLE:	Molecular Epidemiology of Cryptococcus Neoformans and Cryptococcus Gattii in South Africa
DATE CONSIDERED:	01/04/2016
DECISION:	Approved unconditionally
CONDITIONS:	(Secondary data analysis for M140159 and M141177)
SUPERVISOR:	A/Prof Nelesh Govender

APPROVED BY: Professor P Cleaton-Jones,Chairperson, HREC (Medical)

DATE OF APPROVAL: 22/04/2016

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary in Room 10004, 10th floor, Senate House/2nd Floor, Phillip Tobias Building, Parktown, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in March and will therefore be due in the month of March each year.

Principal Investigator Signature Date 22/04/2016

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

R14/49 Dr Vanessa Quan

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M1809107

NAME: (Principal Investigator)	Dr Vanessa Quan
DEPARTMENT:	National Institute for Communicable Diseases
PROJECT TITLE:	GERMS-SA: Laboratory-based Surveillance for Pathogens of Public Health Importance in South Africa
DATE CONSIDERED:	Ad Hoc
DECISION:	Approved unconditionally
CONDITIONS:	Recertification - M140159 (M081117)
SUPERVISOR:	
APPROVED BY:	Doctor CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	08/11/2018

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office Secretary on the Third Floor, Faculty of Health Sciences, Phillip Tobias Building, 29 Princess of Wales Terrace, Parktown, 2193, University of the Witwatersrand. I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**. The date for annual re-certification will be one year after the date of convened meeting where the study was initially reviewed. In this case, the study was initially reviewed in September and will therefore be due in the month of September each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

Principal Investigator Signature

12/11/2018
Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

ANIMAL RESEARCH ETHICS COMMITTEE
Registration number: AREC-101210-002

March 23, 2018

To whom it may concern,

Re: Waiver from the Animal Ethics Research Committee of the University of the Witwatersrand

Serisha Naicker (**Student number:** 0606058D) is a PhD candidate undertaking a study titled "Molecular epidemiology of *Cryptococcus neoformans* and *Cryptococcus gattii* in South Africa".

The research is being conducted within the Department of Clinical Microbiology and Infectious Diseases, School of Pathology. The research is being supervised by

- A/Prof Nelesh Govender from the Mycology Reference Laboratory, Centre for Healthcare-Associated Infections, Antimicrobial Resistance and Mycoses, NICD and
- Prof Wieland Meyer, Mycology Research Laboratory, Center for Infectious Diseases and Microbiology, Marie Bashir Institute for Emerging Infectious Diseases and Biosecurity, Sydney Medical School, The University of Sydney, at the Westmead Institute for Medical Research, Sydney, Australia

This letter is to confirm that the research being conducted on the bacteria *Cryptococcus neoformans* and *Cryptococcus gattii* and does not require full Animal Ethics Research Committee clearance to undertake the work.

Please contact me should you require further information,

Yours sincerely

GP Candy

Geoffrey Candy
Chair : Animal Ethics Research Committee
University of the Witwatersrand

Reference : : Waiver Serisha Naicker 20180322O

Geoffrey P Candy PhD
Professor, Department of Surgery, Faculty of Health Sciences, University of the Witwatersrand, *7 York Road Parktown 2193, Johannesburg; Tel: 27-11-717-2574; Email: geoffrey.candy@wits.ac.za*

Appendix F – Declaration by student and co-authors

Declaration: Student's contribution to article(s) and agreement of co-author(s)

I, Serisha Devi Naicker, student number [0606058D], declare that this Thesis is my own work and that I contributed adequately towards research findings published in the article(s) stated below which are included in my Thesis.

Signature of Student **Date** 16/04/2021

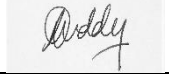
Name of Primary Supervisor Nelesh P. Govender

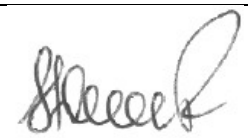
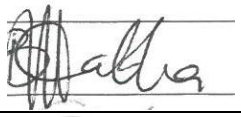
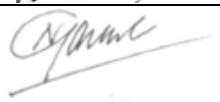
Signature of Primary Supervisor **Date** 16/04/2021

Agreement by co-authors: By signing this declaration, the co-authors listed below agree to the use of the article(s) by the student as part of her Thesis. In cases where the student is not the 1st author of a published article, the primary supervisor must explain (under comments) why the student is entitled to use the paper for her degree purposes.

Article 1: Title: Decreasing fluconazole susceptibility of clinical South African *Cryptococcus neoformans* isolates over a decade

Journal name, year, volume and page numbers: PLOS Neglected Tropical Diseases, 2020, 14 (3), 1-11

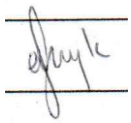
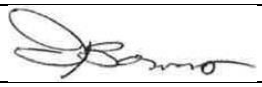
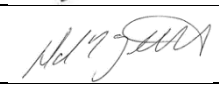
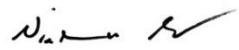
Authors	Name	Signature	Date
2nd author	Ruth S. Mpembe		
3rd author	Tsidiso G. Maphanga		16/04/2021
4th author	Thokozile G. Zulu		16/04/2021
5th author	Daniel Desanto		
6th author	Jeanette Wadula		18/04/2021
7th author	Nomonde Mvelase		19/04/2021
8th author	Caroline Maluleka		
9th author	Kessendri Reddy		16/04/2021

10th author	Halima Dawood		20/04/2021
11th author	Motlatji Maloba		16/04/2021
12th author	Nelesh P. Govender		16/04/2021

Comments by primary supervisor: The student is the first author of the paper.

Article 2: Title: Genotype, Antifungal Susceptibility, and Virulence of Clinical South African *Cryptococcus neoformans* Strains from National Surveillance, 2005–2009

Journal name, year, volume and page numbers: Journal of Fungi, 2021, 7 (338), 1-17

Authors	Name	Signature	Date
2nd author	Rindidzani E. Magobo		16/04/2021
3rd author	Tsidiso G. Maphanga		16/04/2021
4th author	Carolina Firacative		16/04/2021
5th author	Erika van Schalkwyk		19/04/2021
6th author	Juan Monroy-Nieto		
7th author	Jolene Bowers		16/04/2021
8th author	David M. Engelthaler		16/04/2021
9th author	Liliwe Shuping		
10th author	Wieland Meyer		18/04/2021
11th author	Nelesh P. Govender		16/04/2021

Comments by primary supervisor: The student is the first author of the paper.

Appendix G – Turn-it-in Report

Turnitin Originality Report

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8% match (publications) Serisha D. Naicker, Ruth S. Mpembe, Tsidiso G. Maphanga, Thokozile G. Zulu et al. "Decreasing fluconazole susceptibility of clinical South African Cryptococcus neoformans isolates over a decade", PLOS Neglected Tropical Diseases, 2020
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< 1% match (Internet from 11-Nov-2020) https://academic.oup.com/bmb/article/72/1/99/272852
< 1% match () Chowdhary, A.. "Molecular epidemiology, environmental dispersion and antifungal susceptibility of Cryptococcus grubii and C. gattii prevalent in India", [S.l. : s.n.], 2012
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< 1% match () James, Monique. "Investigating biological control agents for the management of Ceratitis capitata (Wiedemann)", Stellenbosch : Stellenbosch University
< 1% match () Marandure, Tawanda. "Sustainability of smallholder cattle production and its vertical integration into the formal beef market value chain in South Africa", Stellenbosch : Stellenbosch University, 2015
< 1% match () Bernardus Daniël, Van Biljon. "Population structure and biofilm formation of Pseudomonas aeruginosa isolated from patients with severe burn wounds at Tygerberg Hospital.", Stellenbosch : Stellenbosch University
< 1% match () Benson, Frew Gerald. "Analysing the national notifiable diseases surveillance system in South Africa", 2018
< 1% match () Green-Thompson, Lionel Patrick. "The nature of social accountability in South African medical practice and education: a qualitative reflection", 2015
< 1% match () Rhodes, J. Desjardins, CA et al. "Tracing Genetic Exchange and Biogeography of Cryptococcus neoformans var. grubii at the Global Population Level.", 'Genetics Society of America', 2017

NATIONAL INSTITUTE FOR COMMUNICABLE DISEASES
Division of the National Health Laboratory Service

Nelesh Govender
MBBCh FCPath(SA) FRCPath(UK) FECMM MMed MSc(Mycol) MSc(Epi) DTM&H DipHIV(SA)
Head: Centre for HAIs, AMR and Mycoses, NICD
Professor: School of Pathology, Faculty of Health Sciences, WITS
1 Modderfontein Road, Sandringham, 2131
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Email: neleshg@nicd.ac.za | Website: www.nicd.ac.za

29 April 2021

Postgraduate Research Administration Office

Faculty of Health Sciences

University of the Witwatersrand

To whom it may concern,

RE: Turn-it-in report for Serisha Naicker's PhD thesis (student number: 0606058D)

Miss Serisha Naicker (student no 0606058D) is a part-time student PhD student at the University of the Witwatersrand. She has published two papers in peer-reviewed journals as part of her thesis (see page v of thesis). The high similarity of 26% is partly due to a published paper included as a chapter in the thesis. The other matches have <1% similarity.

Kind regards,

Professor Nelesh Govender (supervisor)

Head: Centre for HAIs, AMR and Mycoses, NICD

Professor: School of Pathology, Faculty of Health Sciences, WITS