Diagnostic methods for detection and characterisation of dimorphic fungi causing invasive disease in Africa: Development and evaluation

TSIDISO GUGU MAPHANGA

Diagnostic methods for detection and characterisation of dimorphic fungi causing invasive disease in Africa: Development and evaluation

by

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A thesis submitted in partial fulfilment of the requirements for the degree of

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in

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Promoter: A/Prof NP Govender

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DECLARATION

I, Tsidiso Gugu Maphanga, declare that the research thesis hereby submitted to the University of the Free State for the Doctoral Degree in Medical Microbiology and the work contained therein is my own original work and has not previously, in its entirety or in part, been submitted to any institution of higher education for degree purposes. I further declare that all sources cited are acknowledged by means of a list of references.

Signed _____ this _____ day of _____ 2020

Research is to see what everybody else has seen and to think what nobody else has thought

Albert Szent-Györgi (US Biochemist)

DEDICATION

***To my late brother, my mechanical engineer* (*Thabiso Benson Masilela*):**
I will never forget all the plans we had, the motivations, love and laughter we shared. Life is never the same without you; I miss you daily.

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LIST OF UNITS AND ABBREVIATIONS

UNITS

°C	Degrees Celsius
α	Alpha
β	Beta
g	Gram
h	Hour
kDA	Kilodalton
L	Litre
mg	Milligram
mℓ	Millilitre
mm	Millimeter
min	Minute
π	nucleotide diversity
ng	Nanogram
µg	Microgram
µm	Micrometer
U	Unit
µℓ	Microlitre
%	Percent

ABBREVIATIONS

ADP	Adenosine diphosphate
AIDS	Acquired immunodeficiency syndrome
Arf	Adenosine diphosphate-ribosylation factor 6
ATCC	American type culture collection
BAD	*Blastomyces* adhesion
B. dermatitidis	*Blastomyces dermatitidis*
B. gilchristii	*Blastomyces gilchristii*
B. percursus	*Blastomyces percursus*
B. silverae	*Blastomyces silverae*
B. helices	*Blastomyces helicus*
B. parvus	*Blastomyces parvus*
BHI	Brain Heart Infusion
BLAST	Basic Local Alignment Search Tool

BMD	Broth microdilution
Bp	Base pair
CAL	Calmodulin
CBS	Centraalbureau, Schimmelcultures
CD4	Cluster of differentiation 4
CFU	Colony Forming Unit
Chs	Chitin synthase
CLSI	Clinical Laboratory Standards Institute
CNS	Central Nervous System
CSF	Cerebrospinal fluid
Drk	Histidine kinase
dNTPs	Deoxyribonucleotide triphosphate
DMP	Diagnostic Media Product
DNA	Deoxyribonucleic acid
E. africanus	*Emergomyces africanus*
E. pasteurianus	*Emergomyces pasteurianus*
E. orientalis	*Emergomyces orientalis*
E. Canadensis	*Emergomyces canadensis*
E. europaeus	*Emergomyces europaeus*
GC	Guanine-Cytosine
GM	Geometric mean
ECV	Epidemiologic cut-off value
EIA	Enzyme immunoassay
H. capsulatum var. *capsulatum*	*Histoplasma capsulatum* var. *capsulatum*
H. capsulatum var. *duboisii*	*Histoplasma capsulatum* var. *duboisii*
H. capsulatum var. *farciminosum*	*Histoplasma capsulatum* var. *farciminosum*
H. capsulatum var. *mississippiense*	*Histoplasma capsulatum* var. *mississippiense*
H. capsulatum var. *ohiense*	*Histoplasma capsulatum* var. *ohiense*
H. capsulatum var. *suramericanum*	*Histoplasma capsulatum* var. *suramericanum*
HIV	Human immunodeficiency virus
IFI	Invasive fungal infection
IQR	Interquartile range
ITS	Internal transcribed spacer region
LSU	Large subunit
MAT	Mating
Mb	Mega base

$MgCl_2$	Magnesium chloride
MLST	Multilocus sequence typing
MIC	Minimum inhibitory concentration
MEA	Malt extract agar
MEC	Minimum effective concentration
NCPF	National Collection of Pathogenic Fungi
NHLS	National Health Laboratory Service
NICD	National Institute for Communicable Diseases
Ole	Delta-9 fatty acid desaturases
PAS	Periodic acid schiff
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
QC	Quality control
rDNA	Ribosomal DNA
SDA	Sabouraud dextrose agar
SNP	Single nucleotide polymorphism
ST	Sequence type
S. schenckii complex	*Sporothrix schenckii* complex
S. brasiliensis	*Sporothrix brasiliensis*
S. globosa	*Sporothrix globosa*
S. albicans	*Sporothrix albicans*
S. luriei	*Sporothrix luriei*
S. mexicana	*Sporothrix mexicana*
TB	Tuberculosis
TEF	Translation elongation factor
TUB	Alpha tubulin
USA	United State America
WGS	Whole genome sequencing
YPS	Yeast-phase specific-3

LIST OF PUBLICATIONS

PRIMARY PAPERS:

Published article

1. **Maphanga, T.G., Britz, E., Zulu, T.G., Mpembe, R.S., Naicker, S.D., Schwartz, I.S., et al.** 2017. *In vitro* Antifungal susceptibility of yeast and mould phases of isolates of dimorphic fungal pathogen *Emergomyces africanus* (Formerly *Emmonsia* sp.) from HIV-infected South African patients. *Journal of Clinical Microbiology* 55: 1812-1820 http://doi.org/10.1128/JCM.02524-16.
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1. **Maphanga, T.G., Naicker, S.D., Mhlanga, M., Schwartz, I.S., Bamford, C., Nel, J. & Govender, N.P.** 2020. A diagnostic accuracy case-control study to evaluate a *Histoplasma capsulatum* urine antigen enzyme immunoassay among patients with suspected histoplasmosis. PLOS Neglected Tropical Diseases.

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Diagnostic methods for detection and characterisation of dimorphic fungi causing invasive disease in Africa: Development and evaluation

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DEPARTMENT: **Medical Microbiology, Faculty of Health Sciences, University of the Free State**

DEGREE: **PhD (Medical Microbiology)**

ABSTRACT

Emergomyces, *Histoplasma*, *Blastomyces* and *Sporothrix* species complex are thermally dimorphic fungi that have emerged as the causative agents of invasive fungal infections among immunocompetent and immunocompromised patients for the past 12 years. Accurate diagnoses of the mycoses caused by these fungi are challenging, not only because as there is a lack of clinical awareness globally, but also because these thermally dimorphic fungi are difficult to identify in the laboratory by conventional methods (i.e. culture, histology). In addition, cultured isolates require specialised biosafety facilities, which pose a tremendous challenge to resource-limited, diagnostic laboratories. As a result, estimates of the prevalence and burden of disease are poor. In addition, the lack of standardised antifungal susceptibility testing methods for these pathogens makes it difficult to determine the minimum inhibitory concentration (MIC), which may jeopardise patient treatment. These challenges indicate the need for alternative laboratory methods for identification. Furthermore, molecular methods are required for the correct identification of these pathogens in clinical settings, so that efficient antifungal agents may be used appropriately.

We conducted several studies to address these challenges. In the first study, 50 *Emergomyces africanus* isolates from AIDS patients presenting with disseminated emergomycosis, from both public and private laboratories in South Africa during a nine-year period (2008 to 2016), were described and analysed. The Clinical Laboratory Standards Institute guideline (M27-A4 for yeast phase and M38-A2 for mould phase) was used to determine the MICs for the yeast and mould phase. Amphotericin B, itraconazole, voriconazole and posaconazole were more potent *in vitro*, while fluconazole, echinocandins and flucytosine were less potent agents.

In the second study, we tested and analysed 212 urine samples from patients with suspected invasive fungal disease submitted to the mycology reference laboratory from August 2014 to December 2018 by public and private sector laboratories in South Africa. Corresponding fungal culture and histopathology results from several electronic laboratory information systems were obtained. Thirty-seven cultured fungal isolates were sent in parallel with the urine specimen for species-level identification (confirmed by phenotypic and molecular identification methods). The IMMY monoclonal *Histoplasma capsulatum* galactomannan enzyme immunoassay (EIA) had a sensitivity and specificity of respectively 88% and 72% when compared to culture-confirmed histoplasmosis. In contrast, the EIA showed a sensitivity and specificity of 83% and 93% when compared to histologically confirmed histoplasmosis/ emergomycosis.

In the third study, we re-evaluated 20 cases of blastomycosis diagnosed among South African patients between 1967 and 2014. All isolates, initially identified as *Blastomyces dermatitidis* by histopathological and/or culture-based methods, were characterised phenotypically, genotypically and by whole genome sequencing. Morphological characteristics and phylogenetic analyses of multilocus sequence typing and whole genome sequences revealed two groups, both of which were closely related to but distinct from *B. dermatitidis*, and other related species. One group corresponded to the recently described *Blastomyces percursus* and the other group was described as *Blastomyces emzantsi* sp. nov. Whole genome sequencing confirmed distinct species identities and the absence of a full ortholog of *the BAD-1 gene*. Clinically, extrapulmonary disease was more common than lung involvement.

In the fourth study, 156 dimorphic isolates from immunocompromised patients presenting with systemic endemic mycoses, from both public and private laboratories in South Africa during an 11-year period (2008 to 2019), were sent to the mycology reference laboratory for species-

level identification. All isolates were initially identified by deoxyribonucleic acid (DNA) sequencing of the internal transcribed spacer (*ITS*) gene and those which failed sequencing were identified by sequencing of the large subunit (*LSU*) gene. The identity of the *Sporothrix* isolates was confirmed by sequencing the calmodulin gene. Only 63% of the dimorphic pathogens were correctly identified to genus level by culture-based methods at the diagnostic laboratories when compared to DNA sequencing methods. At the reference laboratory, culture-based methods correctly identified 95% of the dimorphic isolates. *ITS* sequencing correctly identified *Emergomyces africanus*, *Histoplasma capsulatum*, *Blastomyces percursus*, *Emergomyces pasteurianus* and *Talaromyces marneffei*. Calmodulin sequencing confirmed *Sporothrix* species and these clinical isolates belonged to *Sporothrix schenckii* sensu stricto. There was an increase in the number of cases of emergomycosis, sporotrichosis, histoplasmosis, blastomycosis and talaromycosis above what was previously reported. Skin lesions and pulmonary involvement were common.

In conclusion, the *in vitro* susceptibility data for *E. africanus* supported the management of disseminated emergomycosis with amphotericin B, followed by itraconazole, voriconazole, or posaconazole. Fluconazole was a relatively less potent agent. The *Histoplasma* EIA provided a rapid alternative for diagnosis of histoplasmosis in an endemic setting. However, it should be noted that it may cross-react with patients infected with *Emergomyces africanus*. We observed that South African blastomycosis cases were caused by species that are distinct from *B. dermatitidis*, *B. gilchristii* and *B. parvus*, which might account for clinical differences between sub-Saharan and North American blastomycosis. Lastly, DNA sequencing was a valuable tool for accurate identification of dimorphic fungi to species level when compared to culture in the current setting, which could facilitate early diagnosis and initiation of proper treatment. However, in settings where DNA sequencing is not available, training of laboratory staff is necessary for the improvement of diagnosis of these pathogens in South Africa.

CHAPTER 1

1.1 Introduction

Endemic thermally dimorphic fungi include phylogenetically related ascomycetes (van Dyke et al., 2019). These include those reported worldwide, such as *Histoplasma, Blastomyces, Sporothrix*, *Emmonsia*, and *Emergomyces*, and those acquired when travelling to endemic areas, such as *Talaromyces marneffei*, *Paracoccidioides* and *Coccidioides* (Lee and Lau, 2017, van Dyke et al., 2019). Only *Histoplasma*, *Blastomyces*, *Sporothrix* and *Emergomyces* are known to be endemic to South Africa and cause human diseases (Schwartz et al., 2017).

Surveillance of the invasive fungal infections (IFIs) caused by these fungi is limited in resource-poor facilities because of lack of experienced personnel and accurate diagnostic tests (Kozel and Wickes, 2014). Furthermore, IFIs due to these fungi are usually misdiagnosed or underreported owing to lack of clinical awareness (Schwartz et al., 2017). Infections caused by these fungi may result in similar clinical presentation, and sometimes mimic those of TB, which results in misdiagnosis (Schwartz et al., 2017). In addition, if a patient is diagnosed with an IFI, the toxicities associated with antifungal agents such as amphotericin B can make it difficult for the infection to be properly managed (Ramana et al., 2013). Despite the availability of several antifungal agents, resource-limited countries do not have easy access to certain drugs, which delays proper management of patients with fungal infection.

Traditionally, the identification of these fungal pathogens is achieved by culture-based methods, while alternative methods include histopathology, antigen detection and molecular methods (Arvanitis et al., 2014). Culture often takes two to six weeks before a final diagnosis can be made and requires experienced personnel for accurate diagnosis (Cáceres et al., 2019). This is a challenge for microbiologists, particularly in resource- limited countries owing to the limited number of experienced personnel (Cáceres et al., 2019). On the other hand, histopathology involves invasive procedures and does not enable differentiation between diseases (Schwartz et al., 2017). *Histoplasma* urine galactomannan antigen detection is the quickest assay for diagnosis of histoplasmosis (Nacher et al., 2018). Detection of the antigen is recommended in patients with progressive and/or disseminated infection (Azar and Hage, 2017). Many studies have evaluated the performance of different enzyme immunoassays

(EIAs) used to detect antigen and reported contrasting results (Nacher et al., 2018). The main problem with these EIAs is inter-laboratory variability and cross-reactivity (Theel et al., 2015, Zhang et al., 2015, Cáceres et al., 2018).

Classification of fungi to species level using phenotypic markers has become increasingly difficult because of the existence of cryptic species, which cannot be differentiated solely based on microscopy (Borman et al., 2008). For this reason, identification of fungal species has shifted towards amplification of the highly conserved regions using pan-fungal and quantitative real-time polymerase chain reaction (PCR) methods (Borman et al., 2008).

Researchers have confirmed that DNA sequence-based assays can be convenient in the identification of novel pathogenic fungi, tracking of the origin and distribution of strains and the correct identification of emergence or uncommon species (Brown et al., 2013, Kenyon et al., 2013; Dukik et al., 2017; Jiang et al., 2018). The use of new techniques and improved diagnostic tests covering a large number of fungal species may assist in understanding the true burden of these fungal pathogens in South Africa. This may also include sequencing of the entire fungal genome to allow correct taxonomic location and to explore differences in conserved regions (including virulence genes).

We therefore aimed to develop and evaluate laboratory assays for detection and antifungal susceptibility testing of dimorphic fungi in order to improve diagnosis and management of clinical disease, and to facilitate surveillance of invasive disease caused by these fungi.

The overall objective of this study was:

- To establish standardised methods to identify dimorphic fungi that cause disease in South Africa, and to determine minimum inhibitory concentrations for relevant antifungal agents.

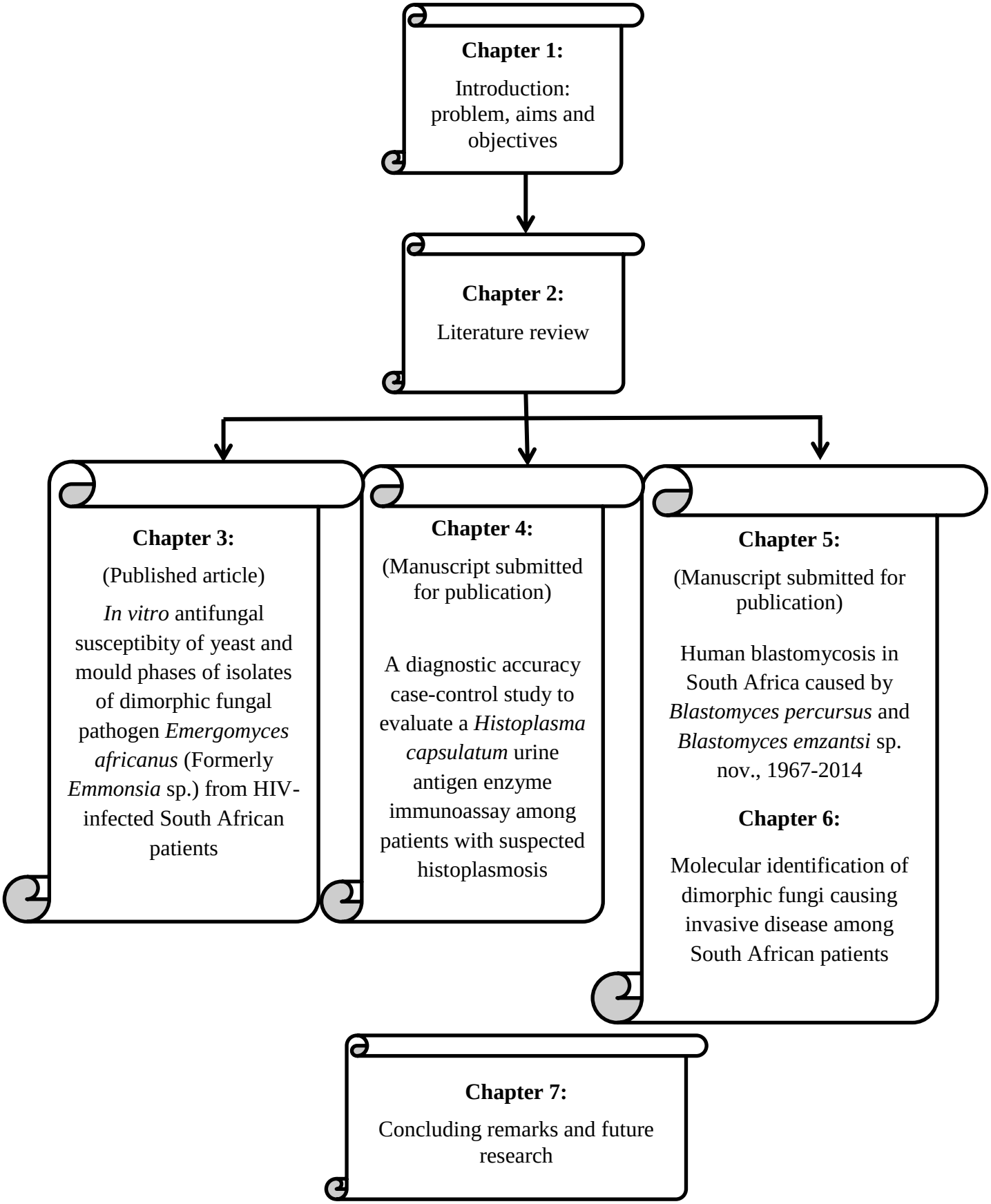
The Objectives of this study were:

- To generate yeast-phase and mould-phase minimum inhibitory concentrations (MICs) for a selected panel of antifungal agents tested against *E. africanus*
- To evaluate the use of a second-generation *Histoplasma* galactomannan antigen EIA (that uses monoclonal antibodies) and a panfungal PCR assay in diagnosing

histoplasmosis directly from urine/serum specimens compared to the reference standard (culture or histology)

- To re-examine South African *Blastomyces* human clinical isolates archived over five decades.
- To use a panfungal PCR assay and DNA sequencing to identify dimorphic fungal isolates to species level and determine the relative prevalence of these fungi submitted through laboratory-based sentinel surveillance.

1.2 Thesis Structure



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CHAPTER 2

LITERATURE REVIEW

2.1 Introduction

The field of medical mycology has become challenging and intriguing because of the increasingly extensive and taxonomically diverse array of fungi causing IFI (Pfaller and Diekema, 2004, Friedman and Schwartz, 2019). Most dimorphic fungal infections are obtained through the respiratory route, with the exception of the *S. schenckii* species complex, which can also be acquired through traumatic inoculation (Gauthier, 2017). Transmission does not occur from person to person (Gauthier, 2017).

Each year, *Histoplasma* and *Blastomyces* species are estimated to infect over 650 000 individuals in the United States (US) (Muñoz et al., 2018). This makes these pathogens the common causative agents of fungal pulmonary infections (Van Dyke et al., 2019). In South Africa, *E. africanus* is regarded as the most common pathogen causing cutaneous and pulmonary infections, predominantly in patients with the human immunodeficiency virus (HIV) (Kenyon et al., 2013, Schwartz et al., 2017a). *S. schenckii* is associated with outbreaks in South African mines (Pijper and Pullinger, 1927, Govender et al., 2015).

The major factors involved in disseminated disease caused by these fungal pathogens include acquired immunodeficiency syndrome (AIDS), cancer and pharmacologically induced immunosuppression (Kenyon et al., 2013). *Histoplasma*, *Blastomyces* and *Sporothrix schenckii* are not restricted to immunocompromised hosts, but are also reported to cause infection in immunocompetent individuals (Goughenour and Rappleye, 2017). Delay in the identification of these pathogens may result in high morbidity and mortality (Goughenour and Rappleye, 2017).

Phenotypic identification, genotypic and susceptibility data contribute to the understanding of dimorphic fungi in both diagnostic and research laboratories worldwide. Molecular methods such as conventional and real-time PCR are applied in both research and diagnostic laboratories with great advantage in the identification of fungal species. Furthermore, genotyping methods such as multilocus sequence typing (MLST) and single nucleotide polymorphism (SNP)

analysis are more useful in the determination of genetic diversity or relatedness of fungal species (Brown et al., 2013, Kenyon et al., 2013, Frost et al., 2016, McTaggart et al., 2016). Whole genome sequencing (WGS) has become the most effective tool for comparative study analysis, including correct taxa allocation, better understanding of disease outbreak, determination of virulence and resistance genes (Dukik et al., 2017, Sepúlveda et al., 2017, Muñoz et al., 2018).

2.2 Classification of thermally dimorphic fungal pathogens

Improvements in DNA sequencing technologies and diagnostic methods have facilitated rapid progress in research into fungal phylogenetics in the past decade (Van Dyke et al., 2019). These methods have provided meaningful sequence data that may be obtained through community-wide databases such as the National Centre for Biotechnology Information and Centraalbureau, Schimmelcultures (CBS) (Blackwell et al., 2006). These databases are utilised to study phylogenetic classification of many fungal taxa (Blackwell et al., 2006).

2.2.1 Phylogenetic relationships of thermally dimorphic fungal pathogens

Dimorphic fungi are represented by a major taxonomic phylum, Ascomycota (Muñoz et al., 2018). Ascomycota contains over 64 000 species and its sister phylum, Basidiomycota, contains 30 000 well-described species (Lee et al., 2010, Muñoz et al., 2018). In total, Ascomycota and Basidiomycota contain about 79% well-described true fungi, of which 0.5% (about 400 species) are known to be clinically relevant human pathogens (Bellemain et al., 2010). These fungi consist of both monophyletic and polyphyletic groups under the Ascomycetes class (Lee et al., 2010).

Based on comparative genetic sequence analysis, *Emergomyces*, *Blastomyces* and *Histoplasma* are phylogenetically related and belong to the order Onygenales (Muñoz et al., 2018). These fungi are also closely related to *Emmonsia*, *Coccidioides* and *Paracoccidioides* (Van Dyke et al., 2019). *S. schenckii* belongs to the order Ophiostomatales (Teixeira et al., 2014). Recently, MLST and WGS demonstrated that *Emergomyces*, *Blastomyces* and *Histoplasma* consist of several monophyletic and distinguishable cryptic species, while *Emmonsia* species are polyphyletic (Warnock, 2018).

MLST and WGS analysis demonstrated that *Emergomyces*, *Blastomyces*, *Histoplasma*, and *S. schenckii* genera consist of cryptic species (Marimon et al., 2007, Brown et al., 2013, Dukik et al., 2017, Sepúlveda et al., 2017, Jiang et al., 2018). These fungi have been intensely studied in terms of their morphological and phylogenetical differences.

The recently described genus *Emergomyces* consists of five species, which include *E. pasteurianus* as the type species (Gori et al., 1998), *E. africanus* (Kenyon et al., 2013), *E. orientalis* (Wang et al., 2017), *E. canadensis* (Sanche et al., 2005) and *E. europaeus* (Wellinghausen et al., 2003). The last three species are represented by very few strains: a single isolate has been studied for *E. orientalis* and *E. europaeus*, respectively; while *E. canadensis* is represented by four isolates (Wang et al., 2017, Schwartz et al., 2018d, Jiang et al., 2018). Although the three species are represented by fewer strains, concatenated sequences of *rPB2* (RNA polymerase), *TUB2* (alpha tubulin), *TEF3* (translation elongation factor), *ITS*) and *LSU* clearly separated these isolates into different clusters (Jiang et al., 2018). More isolates are required to support the stability of these species within the *Emergomyces* genus further.

The *Blastomyces* genus consisted of a single species, *B. dermatitidis* (Gilchrist, 1894). Three new species have been described, namely *B. gilchristii* (Brown et al., 2013), *B. percursus* (Dukik et al., 2017), and *B. silverae* (Jiang et al., 2018). *B. silverae* does not cause human infection, while *B. percursus* and *B. gilchristii* cause human infections (Jiang et al., 2018). In addition, formerly known as *Emmonsia helicus* (Sigler, 1996) and *Emmonsia parva* (Emmons and Ashburn, 1942), *B. helicus* and *B. parvus* have been moved to the *Blastomyces* genus, since they are phylogenetically closely related to *Blastomyces* species rather than their close relative *Emmonsia crescens* Table 2.1 (Jiang et al., 2018).

The *Histoplasma* and *Sporothrix* genera remain unchanged. Before 1999, *Histoplasma* consisted of three varieties, namely *H. capsulatum* var. *capsulatum* (Darling, 1909), *H. capsulatum* var. *duboisii* (Vanbreuseghem et al., 1953) and *H. capsulatum* var. *farciminosum* (Weeks et al., 1985). However, using concatenated and single-gene sequence analysis of *ITS*, *ARF* (ADP Ribosylation factor), *TUB*1 and *ole4* (delta-9 fatty acid desaturases), *H. capsulatum* var. *capsulatum* was found to consist of six species (Kasuga et al., 1999). This result was supported by another study that proposed six new phylogenetic species in the *Histoplasma* genus after analysing sequences of 234 isolates retrieved from GenBank (Teixeira et al., 2016).

Table 2.1: Scientific classification of dimorphic fungi (De Beer et al., 2016, Van Dyke et al., 2019)

RANK	SCIENTIFIC NAMES			
Domain	Eukaryotes			
Kingdom	Fungi			
Phylum	Ascomycota			
Class	Ascomycetes			Eurotiomycetes
Order	Onygenales			Ophiostomatales
Family	*Ajellomycetaceae*			*Ophiostomataceae*
Genus (telemorph)	*Ajellomyces*			*Ophiostoma*
Genus (anamorph)	*Emergomyces*	*Blastomyces*	*Histoplasma*	*Sporothrix*
Species	*E. pasteurianus* (type species) *E. africanus* *E. orientalis* *E. canadensis* *E. europaeus*	*B. dermatitidis* (type species) *B. gilchristii* *B. percursus* *B. parvus* *B. silverae* *B. helicus*	*H. capsulatum* var. *capsulatum* (type species) *H. capsulatum* var. *duboisii* *H. capsulatum* var. *farciminosum* *H. capsulatum* var. *mississippiense* *H. capsulatum* var. *ohiense* *H. capsulatum* var. *suramericanum*	*S. schenckii* (type species) *S. mexicana* *S. globosa* *S. luriei* *S. brasiliensis* *S. pallida* *S. chilensis*
Disease	Emergomycosis	Blastomycosis	Histoplasmosis	Sporotrichosis

Sepúlveda et al. (2017) studied the whole genome of different *H. capsulatum* var. *capsulatum* (n=28) and *H. capsulatum* var. *duboisii* (n=2) isolates and confirmed the findings of Kasuga et al. (1999) and Teixeira et al. (2016). Sepúlveda et al. (2017) reported that at least four different cryptic species that caused human infections existed. These species differed in their geographic area and their virulence (Sepúlveda et al., 2017). The differences noted led the authors to propose new species names, which were based on the area in which they were most prevalent, e.g. *H. mississippiense, H. ohiense* and *H. suramericanum* (Sepúlveda et al., 2017). This included *H. capsulatum* var. *capsulatum* as a type species. *H. capsulatum* var. *duboisii*, which represents African strains, was not changed because there were limited data to reach a conclude on the differences within this clade, while *H. capsulatum* var. *farciminosum* was not studied, since it has only been reported to cause animal infection (Sepúlveda et al., 2017).

Through the analysis of three gene sequences (chitin synthase, beta-tubulin and the calmodulin), *Sporothrix* has been considered a species complex (de Beer et al., 2003, Marimon et al., 2008, Rodrigues et al., 2014, Lopes-Bezerra et al., 2018). The complex consists of pathogenic species, which include clinical clade: *S. schenckii* (Hektoen and Perkins, 1900), *S. globosa* (Marimon et al., 2007), *S. luriei* (Marimon et al., 2008), *S. brasiliensis* (Marimon et

al., 2007) and isolates of an environmental clade: *S. mexicana* (Marimon et al., 2007), *S. pallida* (formerly known as *S. albicans*) (De Meyer et al., 2008) and *S. chilensis* (Rodrigues et al., 2016).

2.3 General characteristics of dimorphic fungi

The dimorphic fungi are eukaryotic organisms that possess chitin in their cell walls and also have a plasma membrane that contains ergosterol, but their main characteristic is the ability to exist as mould at room temperature (25°C-30°C) and convert to yeast when the temperature is raised to mammalian body temperature (35°C-37°C) (McGinnis and Tyring, 1996, Van Dyke et al., 2019). At body temperature, *Emergomyces*, *Blastomyces*, *Histoplasma* and *Sporothrix* multiply by budding; their yeasts can easily be confused under the light microscope Table 2.2 (Schwartz et al., 2017a).

Dimorphic fungi are heterotrophic, meaning that they cannot produce their own food (McGinnis and Tyring, 1996). The sexual form of *Blastomyces* and *Histoplasma* has been well studied under favourable conditions, e.g. the presence of a high nitrogen source (peptone or tryptone) and Alphacel diets (McDonough and Lewis, 1968, Kwon-Chung, 1971, Kwon-Chung, 1972). *Ajellomyces dermatitidis*, the sexual form of *B. dermatitidis*, produces cleistothecia containing asci unenclosed in a fruiting body (McDonough and Lewis, 1967, McDonough and Lewis, 1968, Kwon-Chung, 1971). The production of similar cleistothecia has also been reported in the sexual form of *H. capsulatum* var. *capsulatum* (*Ajellomyces capsulatus*) at 37°C (Kwon-Chung, 1972). The sexual form of *Emergomyces* and *S. schenckii* species complex remains unknown; however, a comparative genomic approach has suggested the presence of mating type alpha (*MAT1-1*) in *E. africanus* and *S. schenckii* and *MAT1-2* in *S. brasiliensis* (Teixeira et al., 2015, Dukik et al., 2017).

Table 2.2: Phenotypic characteristics of the endemic dimorphic fungi in Africa

Species	Histology (*In vivo*)	Culture (*In vitro*)			
		Macroscopic features		Microscopic features	
	Yeast-phase	Mould-phase	Yeast-phase	Mould-phase	Yeast-phase
E. africanus	Small round yeast with narrow-based budding on Grocott silver stain (Kenyon et al., 2013)	SDA at 25°C, colonies are white to pale brown, wrinkled. Reverse is tan at first and becomes darker with age. Moderately slow grower (7 to 14 days) (Kenyon et al., 2013)	BHI agar at 35°C, small cream to smooth grey-brown wrinkled colonies (Kenyon et al., 2013)	Branched septate hyaline hyphae (1.4 µm to 2.5 µm). Two to four round conidia (1 µm to 2 µm x 1.5 to 2.5 µm) arising at right angles to short secondary conidiophore with vegetative hyphae (0.6 µm to 1.5 µm).	Thin-walled (2 µm to 4 µm) oval cells with base buds.
E. pasteurianus	Intracellular and extracellular oval or narrow budding yeast within histiocytes on Grocott methenamine silver stain (Tang et al., 2015)	SDA at 25°C: white colonies and tan reverse. Moderately slow grower (7 to 14 days) (Malik et al., 2016).	BHI+5% sheep blood agar at 37°C, small cream yeast-like colonies (Malik et al., 2016)	Hyaline septate narrow hyphae (1 µm to 2 µm). Conidiophores are short, slender, unbranched; arise at right angles from hyphae. Conidiophores are slightly swollen at the tip, bearing one to three conidia (2 µm to 3 µm x 3 µm to 4 µm) clustered in a floret (Sigler, 2005).	Small (2 µm to 4 µm), ellipsoidal with narrow based budding (Dukik et al., 2017)
B. dermatitidis/gilchristii	Multinucleated, round or oval, (8 µm to 15 µm), may be confused with *H. capsulatum* var. *duboisii* (7 µm to 15 µm) (Azar and Hage, 2017)	SDA at 25°C to 30°C, white to off-white and cottony colonies. With age, aerial mycelia become gray to brown. Reverse is beige. Moderately slow grower (5 to 10 days) (Jiang et al., 2018)	BHI agar at 37°C, smooth, cerebriform, white to brown colonies (Jiang et al., 2018)	Delicate septate short hyaline hyphae (1 µm to 2 µm). Narrow conidiophores are short or long with pear-shaped one-celled conidia (2 µm to 4 µm) attached individually at the tip or on the sides (Saccente and Woods, 2010)	Large, round, multinucleate. unipolar broad base buds with double refractile cell walls (8 µm to 15 µm) (Guarner and Brandt, 2011)
B. percursus	Multinucleated, round or oval (8 µm to 15 µm) (Dukik et al., 2017)	SDA at 25°C to 30°C, moist and glabrous with a furrowed mycelium. Reverse is pale buff. Moderately slow grower (5 to 10 days) (Dukik et al., 2017)	BHI agar at 37°C, shiny, cream to pinkish-brown and smooth (Dukik et al., 2017)	Septate delicate short hyaline hyphae (1 µm to 2 µm). Conidiophores are short or long with oval individual-celled conidia (2 µm to 4 µm) found at the tip (Dukik et al., 2017)	Large unipolar/bipolar broad base budding (9 µm to 18 µm), presence of short and swollen hyphal elements (Dukik et al., 2017)

H. capsulatum var. *capsulatum*	Intracellular and extracellular oval thin walled narrow-based budding; may be confused with *Candida glabrata* (2 µm to 3 µm) and *C. neoformans* (4 µm to 6 µm) (Guimarães et al., 2006)	SAD at 25°C, white, cottony at first, becoming brownish with age. Reverse is pale yellow. Slow grower (4 to 6 weeks) (Guimarães et al., 2006)	BHI agars at 37°C, colonies are smooth, moist and white. Roughness is due to the presence of α-(1,3)-glucan (Sepúlveda et al., 2017)	Septate, hyaline hyphae with tuberculate macroconidia (7 µm to 15 µm) and microconidia (2 µm to 5 µm) that are smooth-walled spherical, pyriform (Azar and Hage, 2017)	Small, ovoid or round with narrow-budding (2 µm to 4 µm) (Guimarães et al., 2006)
H. capsulatum var. *duboisii*	Multinucleated giant cells, ovoid, thick-walled (7 µm to 15 µm) and lemon-shaped with narrow budding in cytoplasma with silver methenamine stain (Loulergue et al., 2007)	SAD at 25°C to 30°C, white, cottony at first, becoming brownish with age. Reverse is pale yellow. Slow grower (4 to 6 weeks) (Gugnani and Muotoe-Okafor, 1997)	BHI+5% blood agar supplemented with cysteine at 37°C, smooth, moist, white and round colonies (Gugnani and Muotoe-Okafor, 1997)	Microconidia and tuberculate macroconidia that are smooth-walled spherical, pyriform (Gugnani and Muotoe-Okafor, 1997)	Small yeast are seen at first and later convert to large, ovoid, thick walls budding (7 µm to 15 µm) (Oladele et al., 2018)
S. schenckii sensu stricto	Pleomorphic cigar or ovoid yeast (2 µm to 4 µm in diameter and 4 µm -10 µm in length), in PAS (Schwartz et al., 2017a)	SAD at 25°C to 30°C, smooth, white to creamy at first and turning brown or nearly black. Reverse is pale yellow or light. Rapid grower (3 to 4 days) (Motswaledi et al., 2011)	BHI+5% sheep blood agar at 35°C to 37°C, smooth, creamy, or white to tan colonies (Morris-Jones, 2002)	Thin septate hyphae (1 µm to 2 µm), with oval conidia around the tips of conidiophores forming a rosette-like cluster Conidiophores arise at right angle of the hyphae (Motswaledi et al., 2011)	Small round to oval, (2 µm to 8 µm), elongated, cigar-shaped (2 µm by 3 µm to 3 µm by 10 µm (de Lima Barros et al., 2011)
S. luriei	Large, thick-walled yeast cell with "eye glasses-like cells" (Ajello and Kaplan, 1969)	PDA at 30°C, colonies are moist, creamy and light tan at the centre with no growth at 40°C (Marimon et al., 2008)	BHI+5% sheep blood agar at 35°C to 37°C, smooth, creamy, or white to tan colonies (Marimon et al., 2008)	Sympodial and sessile conidia (4 µm to 10 µm) forming clusters at the end of the conidiophores, the conidium is attached on hypha (Marimon et al., 2008)	Small oval thin-walled cells, budding (Ajello and Kaplan et al., 1969)
S. mexicana	Not documented	PDA at 30°C, colonies are pale orange to grey-orange, later turns brown black (melanised) with no growth at 40°C (Marimon et al., 2007)	BHI agar at 37°C, creamy, white to tan (de Lima Barros et al., 2011)	Hyaline sympodial and sessile conidia slightly pigmented which are subglobose, ellipsoidal (3 µm to 5.5 µm), developing clusters at the end of the conidiophores (Dias et al., 2011)	Irregular budding, narrow ellipsoidal yeast (Rodrigues et al., 2015)

2.3.1 Dimorphism

As the name indicates, morphological change in dimorphic fungi is driven by temperature and this change is imperative for the development of the pathogens, pathogenesis, and virulence (Gauthier, 2015). In the environment, e.g. soil, the fungi grow as mycelium that produces infectious conidia, which become aerosolised following disturbance of the mycelia (Saccente and Woods, 2010). The conidia change to the yeast phase in the lung or enriched medium (in the laboratory) when the temperature is increased to body temperature, this conversion being due to the physiological changes that occur in the fungi when the temperature is elevated (Saccente and Woods, 2010). High temperature (40°C) has been reported to stop mitochondrial respiration in *Histoplasma* and *Blastomyces* (Gauthier, 2017).

Apart from temperature, other factors, such as carbon dioxide concentration, exogenous cysteine levels, pH and other sulfhydryl-containing compounds, influence conversion and growth at body temperature (Gauthier, 2015). In *Sporothrix*, conversion to the yeast phase is accelerated by an increase in carbon dioxide, temperature and the presence of nutrition, e.g. a medium rich in amino acids and vitamins (McGinnis and Tyring, 1996, Fisher and Cook, 1998). In *H. capsulatum* var. *capsulatum*, conversion to the yeast phase requires three stages, which include elevation of temperature, cessation of respiration and a decrease in the level of cysteine. In the first stage, an increase in temperature is categorised by incomplete or complete uncoupling of oxidative phosphorylation, which causes a decrease in ATP levels, respiration rates and transportation of electron components over 24 hours in the cell (Gómez et al., 2004). In the second stage, there is a low rate of or no respiration after 24 to 40 hours and four to six days, which allows conversion from mycelium to yeast and during this period exogenous cysteine or additional sulfhydryl-containing compounds, are needed for the activation of mitochondrial respiration (Gómez et al., 2004). In the third stage, normal respiration and cytochrome levels are maintained and completion of the transition to yeast morphology occurs (Gómez et al., 2004).

2.3.2 Virulence factors of dimorphic fungi

The conidia are converted into the yeast form in the lung (Rappleye and Goldman, 2006). The process of phase transition permits these organisms to upregulate yeast-phase specific genes

such as *Blastomyces* adhesion-1 (*BAD*-1), yeast-phase specific-3 (*YPS*3) and histidine kinase (*DRK*-1) in *Blastomyces* species and *Histoplasma* species (Van Dyke et al., 2019). There is no information on the virulence factors of *Emergomyces* species and more work is still required to understand this pathogen (Van Dyke et al., 2019). Replication of the yeast in the lungs occurs in and out of innate immune cells in *Histoplasma, Blastomyces* and *Sporothrix schenckii* (Gauthier, 2017).

The yeast forms survive by being ingested by alveolar macrophages in the lower respiratory tract (Benson et al., 2011). In an immunocompromised host, the pathogenic yeast cells persist inside the macrophages and travel via the pulmonary lymphatic system to peribronchial and mediastinal lymph nodes (Benson et al., 2011). This normally results in haematogenous dissemination (Saccente and Woods, 2010). An immunocompetent host with primary pulmonary infection does not suffer extrapulmonary infection. This is because the immune system of the host is capable of fighting or containing the infection. In contrast, an immunocompromised host with progressive pulmonary infection may develop disseminated infection because of the inability of the immune system to fight infection (Gauthier, 2015).

BAD-1 (formerly WI-1) is a multifunctional 120 kDA protein expressed by the *B. dermatitidis* strains from North America (Klein et al., 1997). The *BAD*-1 antigen has not been reported among African *Blastomyces* strains (Meece et al., 2013). In Israel and India, only a single isolate was found to possess this gene (Frean et al., 1989). The protein is made of a short N-terminal region, a core of 30 tandem repeats and C-terminal epidermal growth factor domain (Brandhorst et al., 2005). This protein is found on the outer part of the yeast of *B. dermatitidis* and is absent in the mould form (Meece et al., 2013). *BAD*-1 supports lung infection by attaching yeast cells to lung tissues (Meece et al., 2013). Brandhorst et al. (1999) showed that *BAD*-1 deletion rendered *Blastomyces* yeast non-pathogenic in a murine model of lethal lung infection.

2.4 Clinical manifestations of dimorphic fungal infections

Dimorphic fungal infections are sometimes misdiagnosed because their clinical features are usually indistinguishable from TB (Schwartz, 2015a). According to Hage et al. (2012), 10% of cases of fungal infections caused by *Histoplasma* or *Blastomyces* may be fatal or result in

hospitalisation (Hage et al., 2012). The establishment of infection, and the resultant clinical manifestations, are mainly dependent on the quantity of organism inhaled and the immune status of an individual (Saccente and Woods, 2010). Dimorphic fungal infections may disseminate via the bloodstream to cause extrapulmonary infections (Schwartz, 2015a). Widespread disseminated infections usually involve multiple anatomical sites such as skin, soft tissue, bones, joints, brain/meninges and genitourinary systems (Dukik et al., 2017). The skin lesions are indistinguishable from common features resulting from infection caused by these pathogens Figure 2.1 (Schwartz, 2017a).

2.4.1 Emergomycosis

The disease has only been diagnosed in immunocompromised patients, particularly those who are HIV-infected with a low cluster of differentiation 4 (CD4) T-cell lymphocyte count (e.g mean of 16 cells/μL) (Schwartz et al., 2017a). In the first case series of *E. africanus*, cutaneous disease and pulmonary disease were the most common features among 13 HIV-infected South African patients (Kenyon et al., 2013). In a retrospective review of 52 cases of emergomycosis, which included the original 13 cases of emergomycosis, 95% and 88% of the cases presented with cutaneous and pulmonary diseases, respectively (Schwartz et al., 2015a). Clinicians experience difficulties in distinguishing emergomycosis from TB, with three-quarters of the patients being treated for TB instead of emergomycosis (Schwartz et al., 2015a).

The disease has been associated with a 50% crude mortality rate (Schwartz et al., 2015a). The first case of emergomycosis caused by *E. pasteurianus* was reported in an Italian patient with AIDS who presented with cutaneous disease in 1998 (Gori et al., 1998). Thereafter, several cases caused by *E. pasteurianus* were reported in a renal transplant recipient patient, in a patient with cytomegalovirus enteritis and, in a patient with cutaneous disease (Feng et al., 2015, Tang et al., 2015, Malik et al., 2016). More recently, *E. pasteurianus* has been reported in two immunocompromised patients with pulmonary and subcutaneous diseases and in a Ugandan HIV patient presenting with skin lesions (Gast et al., 2019, Rooms et al., 2019).

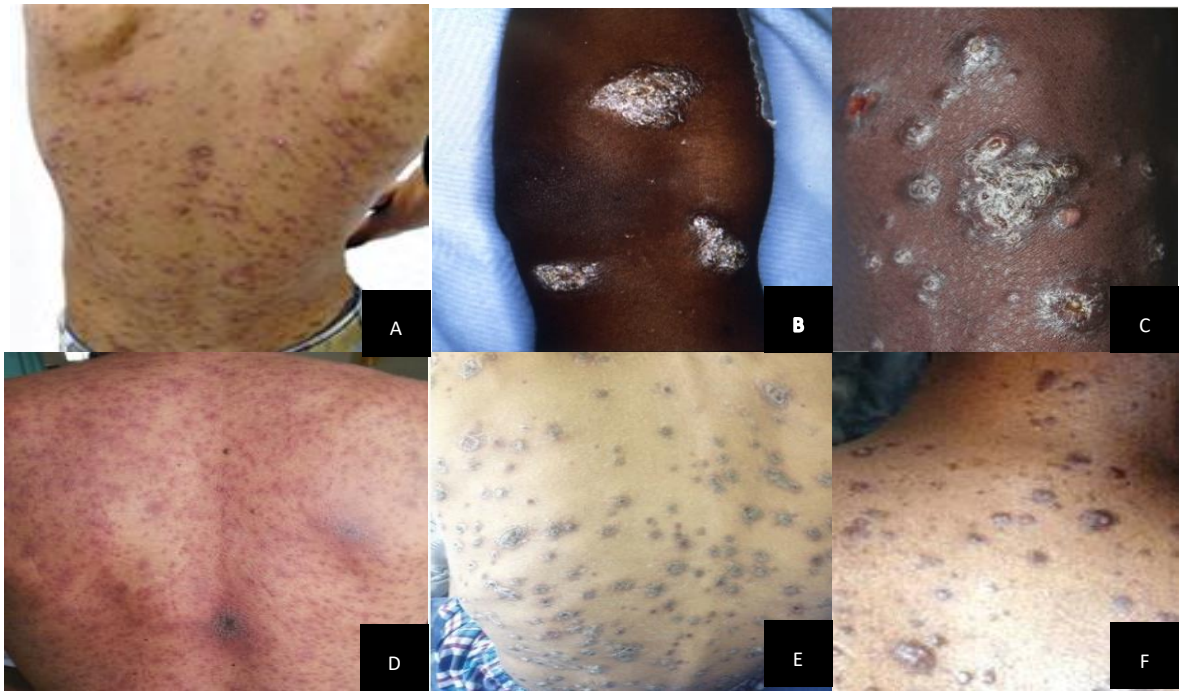

Figure 2.1: **Common skin lesions in patients with disseminated infections caused by dimorphic fungi and *Mycobacterium tuberculosis*: A- *Mycobacterium tuberculosis* (A: reproduced image from Mann et al., 2018); B- *Blastomyces dermatitidis* (B: reproduced image from Kauffman, 2017); C-D *Emergomyces africanus*; E- *Sporothrix schenckii*; F- *Histoplasma capsulatum* (C-F: reproduced image from Schwartz et al., 2017a).**

A case of emergomycosis caused by *E. orientalis* has been reported from an immunocompetent patient presenting with type-2 diabetes mellitus, who presented with cutaneous lesions, productive cough and fever (Wang et al., 2017). Recently, Schwartz et al. (2018d) reported four cases of emergomycosis caused by *E. canadensis* in North America in patients who presented with systemic disease, which involved the skin, cervix, blood, lung and lymph node (Schwartz et al., 2018d). Chest radiographs may demonstrate consolidation of the lung and lymphadenopathy in emergomycosis cases caused by *E. africanus* (Schwartz et al., 2018b).

2.4.2 Blastomycosis

African blastomycosis caused by *B. percursus* and North American blastomycosis caused by *B. dermatitidis* and *B. gilchristii*, may present with similar symptoms (Carman et al., 1989). For example, in immunocompetent hosts, primary pulmonary blastomycosis infections are

usually asymptomatic or present with flu-like symptoms, which spontaneously heal without treatment (Jehangir et al., 2015). Symptoms of pulmonary blastomycosis may be acute onset of high fever, chills, productive cough, myalgia, arthralgias and pleuritic chest pain, which are similar to those of pulmonary TB (Jehangir et al., 2015).

In 2018, Koroscil and Skabelund reported a case of chronic pulmonary blastomycosis that mimicked pulmonary TB in a Louisianan patient who was a heavy smoker and presented with hemoptysis, fatigue, weight loss and fever. This patient was initially treated with antituberculosis drugs before a diagnosis of blastomycosis was made (Koroscil and Skabelund, 2018). Another study reported two cases of disseminated blastomycosis that mimicked TB, both patients presented with lung and vertebral infection and one patient had superficial skin lesions (Frean et al., 1993). The two patients were initially treated with antituberculosis drugs for several months before a correct diagnosis could be made (Frean et al., 1993). In both African and North America blastomycosis, chest radiography often demonstrates abnormal changes, which typically manifest as a diffuse interstitial infiltrate, but focal airspace disease also occurs (Frean et al., 1989).

Clinical differences have been reported, however, between African and North American blastomycosis (Carman et al., 1989). In a review of 81 cases of blastomycosis that occurred between 1951 and 1989 in Africa, the involvement of the pulmonary and central nervous system was less common than skin lesions and bony destruction (Carman et al., 1989). African blastomycosis is more often associated with immunocompetent rather than immunocompromised patients, as noted with North American blastomycosis (Carman et al., 1989).

Cases of cutaneous blastomycosis have commonly been reported in Africa and present with verrucous “wart-like” lesions (Landay and Schwarz, 1971). These cases follow haematogenous dissemination from the lungs and are sometimes misdiagnosed as malignant neoplasms (Kuzel et al., 2018). In rarer cases, primary cutaneous blastomycosis, which results from accidental transcutaneous infection, is reported in Africa (Landay and Schwarz, 1971). According to Saccente and Woods (2010), the third common site for blastomycosis is osseous tissue, following pulmonary and cutaneous diseases.

2.4.3 Histoplasmosis

Histoplasmosis has a very heterogeneous clinical appearance, which ranges from asymptomatic infection or chronic pulmonary infection to disseminated infections (Oladele et al., 2018). About 50% to 90% of immunocompetent individuals with histoplasmosis go unrecognised (Wheat et al., 2005, Trofa and Nosanchuk, 2012). In rare cases, immunocompetent patients may also develop the disseminated form of histoplasmosis (Gonzalez et al., 2019). When immunocompetent individuals have an acute form of histoplasmosis they present with weight loss, fever, fatigue, hepatosplenomegaly and pancytopenia (Gonzalez et al., 2019). In some case, laboratory results of the immunocompetent individuals may show increased liver enzymes and pancytopenia, while clinically the patients may present with gastrointestinal, oropharyngeal lesions and adrenal involvement, which are reported in about 80% to 90% of the cases (Gonzalez et al., 2019).

In contrast, immunosuppressed individuals have a greater risk of progressing to disseminated histoplasmosis because of poorly functioning cell-mediated immunity, and these patients normally experience severe symptoms, which may include respiratory distress, a decrease in blood pressure, ulcers in the skin, gastrointestinal bleeding and enlargement of the kidney, spleen and liver (Gauthier, 2017, Mabiala et al., 2017, Gonzalez et al., 2019). A multicentre prospective study conducted in Brazil among HIV/AIDS patients with histoplasmosis showed overall mortality of 22%; disseminated histoplasmosis was more common when compared to TB in patients with a low CD4+ count (Falci et al., 2019). In countries were HIV/AIDS is prevalent, cases of histoplasmosis may easily be confused with TB, resulting in misdiagnosis and an increased mortality rate (Falci et al., 2019). Weight loss, hepatomegaly and pancytopenia, including absence of widespread lymphadenopathy, were found to be independently related with a diagnosis of disseminated histoplasmosis (Falci et al., 2019). Skin disease occurs in 25% of AIDS patients, while 5% to 10% of immunocompromised patients may develop central nervous system (CNS) histoplasmosis, with an expected mortality of 20% to 40% and relapse of 50% (Gonzalez et al., 2019).

The disseminated form of histoplasmosis caused by *H. capsulatum* var. *capsulatum* is predominantly noted in those with a low CD4 count of <200/mm^3, particularly in hyperendemic areas, and as such, has been listed as an AIDS-defining illness by the Centers for Disease

Control and Prevention (CDC) in the US after the AIDS pandemic experienced since 1987 (Adenis et al., 2014, Adenis et al., 2018). Histoplasmosis caused by *H. capsulatum* var. *capsulatum* affects the liver, spleen, bone marrow, lymph nodes, gastrointestinal tract and CNS (Lehur et al., 2017, Oladele et al., 2018). In contrast, *H. capsulatum* var. *duboisii* rarely causes infection among HIV-infected individuals, possibly because it is suspected of being less virulent than *H. capsulatum* var. *capsulatum* (Carme et al., 1990, Wheat et al., 1990). Furthermore, *H. capsulatum* var. *duboisii* may also be under-recognised in HIV prevalent countries, since the fungus is only restricted in certain parts of Africa (Oladele et al., 2018). In HIV-uninfected patients, localised cutaneous infection caused by *H. capsulatum* var. *duboisii* is mostly noted, while in HIV-infected patients, the disseminated form may involve the skin, subcutaneous tissue, bone (skull, ribs and vertebrae) and lymph nodes (Gugnani, 2000, Oladele et al., 2018). *H. capsulatum* var. *duboisii* rarely presents as pulmonary disease (Oladele et al., 2018). In contrast to *H. capsulatum* var. *capsulatum* and *H. capsulatum* var. *duboisii*, strains of *H. suramericanum* caused acute granulomatous necrotising lung inflammation in a mouse model (Durkin et al., 2004, Friedman and Schwartz, 2019). *H. mississippiense* and *H. ohiense* were found to be common causes of chronic lung disease in the same study (Durkin et al., 2004, Friedman and Schwartz, 2019).

2.4.4 Sporotrichosis

Patients with sporotrichosis have a distinctive feature that differs from other systemic endemic mycoses (Orofino-Costa et al., 2017). These patients may present with cutaneous-lymphatic sporotrichosis, which is the common form that covers the upper and lower limbs and presents with painful ulcerated nodules (Orofino-Costa et al., 2017). Frean et al. (1991) described the earliest case of lymphocutaneous sporotrichosis caused by animal bites (wild rodent) in a young boy in South Africa (Frean et al., 1991). In 2015, Govender et al. (2015) reported confirmed cases of cutaneous-lymphatic disease in 10 patients who presented with nodular skin lesions with ulcers. Most of these cases healed without treatment.

Less commonly, patients may present with cutaneous-fixed sporotrichosis, which constitutes 20% of all cases of sporotrichosis (Yagnik et al., 2017). Infection is localised and may be asymptomatic or may cause slowly growing verrucous lesions and a squamous, erythematous or violaceous halo (Orofino-Costa et al., 2017). Cutaneous-disseminated sporotrichosis is the

last form, which is rare and mostly seen in immunocompromised patients, particularly HIV-infected ones (Orofino-Costa et al., 2017). Yagnik et al. (2017) described a case of disseminated infection caused by *S. schenckii* in an immunocompetent individual who presented with infection of the skin, heart, lungs and bone marrow. Although rare, osteomyelitis infection due to *S. schenckii* has been reported in four patients from KwaZulu-Natal who presented with painful bones (Govender et al., 1989).

2.5 Prevention of dimorphic fungal infections

Education is the main method of prevention of dimorphic fungal infection in endemic areas. Since the emergence of *E. africanus* in South Africa, the community has been educated about the disease through the radio and newspapers. According to researchers, *E. africanus* may be acquired through inhalation of aerosolised conidia from soil contaminated with the fungus, so the suggestion is for people to avoid activities that involve close contact with soil or dust, if possible (NICD, 2018, Schwartz et al., 2018a, Schwartz et al., 2018c) After a sporotrichosis outbreak that occurred in a South African mine, it was recommended that contaminated mine shafts be closed and contaminated timbers be treated, and the use of personal protective equipment was promoted (Govender et al., 2015).

People who are immunocompromised should avoid activities that involve disruption of soil that is possibly contaminated with the conidia of *Histoplasma* or *Blastomyces,* for example, soils beneath bat and bird roosts, and in chicken runs, as well as related activities such as caving, construction, excavation, demolition, farming and gardening (Anderson and Meece, 2018, Muhi et al., 2019). In 2003, an outbreak of histoplasmosis was reported to the Department of Public Health, Quebec in Canada. This outbreak occurred during the renovation of an old house where bats lived or birds roosted and to prevent further outbreak, the Department of Public Health recommended suspension of any further construction work and containment of contaminated materials (CDC, 2014). Furthermore, the use of protective equipment such as respirators was advised.

No human vaccine currently exists for the prevention of infections caused by *Blastomyces, Emergomyces*, *Sporothrix* and *Histoplasma*. However, in Southern Brazil, glycoprotein *Gp*70 and *Gp*60 have been suggested as potential vaccine candidates for the control of human

sporotrichosis caused by feline sporotrichosis (Rodrigues et al., 2015). Wüthrich et al. (2011) report a live-attenuated strain of *B. dermatitidis* not possessing the *BAD*-1 gene to be harmless, well tolerated and immunogenic in dogs. In the laboratory, Dukik et al. (2017) and Jiang et al. (2018) recommend handling of cultures inside a class II biosafety cabinet. Cultures of *S. schenckii* and *Emergomyces* must be processed in a biosafety level 2 facility, and *Blastomyces* and *Histoplasma* in a biosafety level 3 facility (Dukik et al., 2017, Jiang et al., 2018). Protective clothing such as N95 masks, laboratory coats and gloves should be worn at all times when handling cultures (CDC, 2014).

2.5.1 Treatment of dimorphic fungal infections

Despite the latest developments in diagnostic methods, dimorphic fungal pathogens create a number of challenges for antifungal drug therapy (Alastruey-Izquierdo et al., 2015). Treatment for mild disease may take several months, while that for disseminated disease may take more than a year (Goughenour and Rappleye, 2017). There are three main families of antifungal drugs, namely polyenes (amphotericin B), azoles (itraconazole, voriconazole, posaconazole and fluconazole), and the echinocandins (caspofungin, micafungin and anidulafungin) (Alastruey-Izquierdo et al., 2015).

Cellular immunity is crucial for host protection against fungal infections, although many of these infections may be subclinical; and do not require treatment (Saccente and Woods, 2010). The Infectious Disease Society of America (IDSA) guideline for managing histoplasmosis and blastomycosis in immunocompromised individuals recommends amphotericin B for the treatment of disseminated infection; however, in less severe cases, oral azole, e.g. itraconazole, should be used (Goughenour and Rappleye, 2017). Treatment with amphotericin B and itraconazole may be minimised by the variable absorption or metabolism and toxicity (Kathuria et al., 2014). An open-label, nonrandomised prospective trial conducted among AIDS patients with disseminated histoplasmosis reported that 200 mg of itraconazole for 12 weeks is more effective in treating mild disseminated histoplasmosis; however, in severely disseminated histoplasmosis, patients should initially be treated with amphotericin B and this should be followed by itraconazole (Wheat et al., 1995).

Authors have recommended the management of HIV-associated emergomycosis with initial treatment with amphotericin B deoxycholate and subsequently with itraconazole for a minimum of 12 months pending immune reconstitution, based on the similar IDSA guidelines used for HIV-associated disseminated histoplasmosis (Wheat et al., 2007, Kenyon et al., 2013, van Hougenhouck-Tulleken et al., 2014, Lochan et al., 2015, Schwartz, 2015a).

As with the other endemic mycoses, itraconazole is also effective against sporotrichosis (Govender et al., 2015). In a retrospective review of patients with culture-proven sporotrichosis over a 24-year period (1981-2005) in Brazil, supersaturated potassium iodide was found to be effective against cutaneous and lymphocutaneous sporotrichosis (Yamada et al., 2011). The cure rate of potassium iodide is 94.7% among patients with lymphocutaneous disease, with 5.5% reporting side effects (Yamada et al., 2011). The adverse effects (e.g hypothyroidism, metallic or bitter taste, nausea and acneiform eruption) associated with potassium iodide have limited its use in most clinical settings (Costa et al., 2013, Orofino-Costa et al., 2017).

2.5.2 Antifungal susceptibility testing of dimorphic fungi

No consensus has been reached on testing of the yeast and the mould phases of *Emergomyces*, *Blastomyces* and *Histoplasma* (Kathuria et al., 2014, Dukik et al., 2018). However, *in vitro* activities of amphotericin B, azoles and echinocandins have been established for these fungi (Goughenour and Rappleye, 2017). *Sporothrix schenckii* is the only dimorphic fungus for which a standardised method (M38-A2) for *in vitro* antifungal susceptibility testing for the mould phase has been determined (CLSI, 2008).

Antifungal susceptibility testing of both phases can be determined according to the Clinical and Laboratory Standards Institute (CLSI) documents (M27-A4 or M60 for yeast and M38-A2 or M61 for mould) (CLSI, 2008, CLSI, 2012). Broth dilution and the Epsilometer test (E-test) are different methods used to determine the MIC of antifungal drugs (Goughenour and Rappleye, 2017). These tests require a higher inoculum and longer incubation periods (six to seven days) for proper MIC endpoint determination of the antifungal drugs (Espinel-Ingroff, 1998a, Nakai et al., 2003, Kenyon et al., 2013, Kathuria et al., 2014). The longer incubation period is required because dimorphic fungi are slow growers.

Variable susceptibility results have been reported during MIC or minimal effective concentration (MEC) determination of antifungal drugs against *Emergomyces*, *Blastomyces*, *Histoplasma* and *Sporothrix* (Espinel-Ingroff, 1998b, Nakai et al., 2003, Kenyon et al., 2013, Goughenour et al., 2015, Govender et al., 2015, Dukik et al., 2018). Kenyon et al. (2013) tested the yeast phase of 13 *Emergomyces* isolates and reported high MICs for the echinocandins and low MICs for itraconazole, posaconazole, voriconazole (except fluconazole) and amphotericin B. Kathuria and colleagues (2014) tested 23 *H. capsulatum* var. *capsulatum* strains isolated from patients with confirmed pulmonary and disseminated histoplasmosis in India (Kathuria et al., 2014). There was no MIC/MEC difference between the yeast and mycelial forms of these strains (Kathuria et al., 2014). The 23 *H. capsulatum* var. *capsulatum* strains had low MICs for amphotericin B, isavuconazole, itraconazole, voriconazole, posaconazole, and high MICs for fluconazole and flucytosine (Kathuria et al., 2014). Only 13% of the 23 *H. capsulatum* yeast form had caspofungin MIC of ≥ 0.5 µg/ml, while caspofungin MEC for the mould form ranged from 0.05 to 0.5 µg/ml (Kathuria et al., 2014).

Micafugin has been reported to be potent against the mould phase of *Histoplasma* and *Blastomyces* (MEC range, 0.0078 to 0.0625 micro g/ml) (Nakai et al., 2003). A similar study that tested the efficacy of micafungin *in vitro* and *in vivo* demonstrated that the drug was effective against the mould phase of *H. capsulatum* var. *capsulatum in vitro* (Hage et al., 2011). However, this study also showed that micafungin was ineffective in treating histoplasmosis in a mouse model (Hage et al., 2011). Brilhante et al. (2018) studied 138 *H. capsulatum* var. *capsulatum* mould isolates cultured from HIV-infected patients in Brazil. The broth microdilution (BMD) method showed an epidemiologic cut-off value (ECV) of 0.5 for amphotericin B, 0.0313 for itraconazole, 128 for fluconazole, 0.5 for voriconazole and 16 for caspofungin, respectively (Brilhante et al., 2018).

In a multicentre international study involving 17 independent laboratories (including South Africa), the ECV for *S. schenckii sensu stricto* and *S. brasiliensis* was proposed to be 4 µg/ml for amphotericin B, 2 µg/ml for itraconazole and posaconazole and 64 µg/ml and 32 µg/ml for voriconazole, respectively (Espinel-Ingroff et al., 2017). These ECVs were selected after studying 875 medically important *Sporothrix* isolates, which included 301 *S. schenckii* sensu stricto, 486 *S. brasiliensis*,75 *S. globosa* and 13 *S. mexicana* (Espinel-Ingroff et al., 2017).

2.6 Eco-epidemiology of dimorphic fungal infections

Endemic mycoses are systemic fungal diseases with high rates of mortality and morbidity (Vidal et al., 2014). The prevalence of these endemic mycoses may be underestimated in resource-limited countries owing to the lack of accurate data (Oladele et al., 2018). Fungal infections due to *Histoplasma* and *Emergomyces* contribute to increased mortality among HIV-infected individuals in South Africa (Schwartz et al., 2017a). There have been reports of infections caused by *Blastomyces percursus*, *Histoplasma capsulatum* var. *capsulatum* and *Sporothrix schenckii* sensu stricto in immunocompetent individuals (Govender et al., 2015, Dukik et al., 2017, Goughenour and Rappleye, 2017).

Any person who is exposed to contaminated soil is vulnerable to developing these fungal infections (Klein et al., 1986, Kauffman, 2006, Anstead et al., 2012, Schwartz et al., 2018c). Emergomycosis and histoplasmosis are commonly associated with defects in cell-mediated immunity caused by HIV infection or cancer and the use of immunosuppressants after solid organ transplantation (Kauffman, 2006, Schwartz et al., 2015a). People working with agricultural soil, in caves, in construction sites and near old bird/bat roosts are vulnerable to developing histoplasmosis (Anstead et al., 2012). In contrast, sporotrichosis is commonly associated with diabetes mellitus, chronic alcoholism, chronic obstructive pulmonary disease and defects in cell-mediated immunity caused by HIV infection (Coles et al., 1992, Govender et al., 2015).

In South Africa, the earliest cases of emergomycosis were diagnosed in the Western Cape Province (Kenyon et al., 2013). This was due to the introduction of a molecular technique that allowed rapid identification of the fungus to species level (Kenyon et al., 2013). Furthermore, it was speculated that the fungus occupies a microenvironment in areas around the Western Cape, hence the rise in the number of cases noted (Schwartz et al., 2018a). Sporadic cases followed across five of the nine provinces, namely the Eastern Cape, Gauteng, Free State, Mpumalanga and Northern Cape, as well as the Kingdom of Lesotho (van Hougenhouck-Tulleken et al., 2014, Schwartz et al., 2015b) (Table 2.3). To date, emergomycosis cases have been reported on four continents, namely Africa, North America, Asia and Europe (Friedman and Schwartz, 2019).

Table 2.3: Summary of the ecology and geographic distribution of dimorphic fungi

Disease and pathogens	Continent (# of cases reported)	Geographic distribution Country (regions)	Reservoir	Main host	Site of infection	References
Emergomycosis						
E. africanus	Africa	South Africa (Western Cape, Eastern Cape, Mpumalanga, Free State, Gauteng, and Northern Cape); Kingdom of Lesotho	Soil	Immunocompromised individuals (HIV-infected)	Systematic (pulmonary and cutaneous)	(Kenyon et al., 2013, Schwartz et al., 2015a, Schwartz et al., 2018a)
E. pasteurianus	Europe, Africa, Asia	Italy, Spain, France, India, China, South Africa, The Netherland, Uganda	Soil	Immunocompromised individuals (HIV-infected)	Cutaneous	(Gori et al., 1998, Dukik et al., 2017, Gast et al., 2019, Rooms et al., 2019)
E. orientalis	Asia	China (Shanxi)	Presumed soil	Immunocompromised individuals (type-2 diabetes mellitus)	Systematic (pulmonary and cutaneous)	(Wang et al., 2017)
E. canadensis	North America	Canada (Saskatchewan), USA (Colorado and New Mexico)	Presumed soil	Immunocompromised individuals	Systematic (blood, cutaneous, cervix, pulmonary, lymph node	(Schwartz et al., 2018d)
E. europaeus	Europe	Germany	Presumed soil	Immunocompetent individuals	Pulmonary	(Wellinghausen et al., 2003)
Blastomycosis						
B. dermatitidis	North America, Asia, Middle East, Europe, Africa	USA (Border the in Ohio and Mississippi river valleys of the US; Midwestern and Canada bordering the Great Lakes and St. Lawrence River), India, Israel, Democratic Republic of Congo, Morocco, Tunisia, Zambia, Mozambique, Nigeria, Libya, Algeria, Egypt, The Gambia, Liberia, Malawi, Rwanda, Tanzania, Uganda, Namibia,	Moist, warm, sandy soil of rotting timbered areas that have rich organic matters such as ammonia, copper and mercury	Immunocompetent and immunocompromised individuals	Systematic (pulmonary, cutaneous, bone, genitourinary)	(Carman et al., 1989, Frost et al., 2016)
B. gilchristii	Europe, North America	Canada (Central Canada, Alberta Saskatchewan, British Columbia and Ontario), USA (New York, Minnesota, Wisconsin, Mississippi River and St. Lawrence River), South Africa	moist, warm, sandy soil of rotting timbered areas that have rich organic matters such as ammonia, copper and mercury	Immunocompromised individuals (type-1 diabetes mellitus)	Pulmonary	(Brown et al., 2013, Frost et al., 2016)

B. percursus	Asia, Africa	Israel, South Africa, Morocco, Uganda and the Democratic Republic of Congo	soil	Immunocompetent individuals	Systematic (brain, pulmonary, bones, cutaneous)	(Dukik et al., 2017)
B. parvus	North America, South America, Asia, Australia, Africa and Europe	USA (Arizona)	Soil and Mammals	Mammalian species and humans	Pulmonary	(Jiang et al., 2018)
B. silverae	North America	Canada (Alberta)	Coyote dung	Unknown	Unknown	(Jiang et al., 2018)
B. helicus	North America	Canada	Soil	Immunocompromised individuals	Pulmonary and blood	(Schwartz et al., 2017b)
Histoplasmosis						
H. capsulatum var. *capsulatum*	North America, South America, Europe, Asia, Africa	USA (Mississippi and Ohio river valleys), Brazil, Argentina, Malaysia, China, Taiwan, South Africa, Tanzania, Zimbabwe, Ghana, Nigeria, Rwanda, the Democratic Republic of Congo, Cameroon	Soil (high in nitrogen or phosphate and associated with bird and bat guano)	Immunocompromised, immunocompetent and mammalian species (baboons, mustelids, procyonids, felines, canines)	Systematic (brain, pulmonary, bones, cutaneous, endophthalmitis)	(Teixeira et al., 2016, Oladele et al., 2018)
H. capsulatum var. *duboisii*	Africa, South America, Asia, Europe (imported cases)	Congo, Cameroon, Nigeria, Ghana, DRC, Liberia, Guinea-Bissau, Ethiopia, Mexico, Colombia, Brazil, Peru and Oceania,	Soil mixed with bat guano, bowel of bats	Immunocompetent and immunocompromised (HIV-infected)	Systematic (pulmonary, cutaneous, bones, gastrointestinal, lymphatic system)	(Loulergue et al., 2007, Oladele et al., 2018)
H. capsulatum var. *farciminosum*	Europe, Africa, Asia	Morocco, Egypt, India and Delhi	Dusty soil, contaminated objects (feeding, watering utensils, grooming tools, harnesses and wound dressing)	Horse, mule, donkeys, camel, cattle	Epizootic subcutaneous lymphangitis	(Al-Ani, 1999)
H. capsulatum var *mississippiense* (Nam1)	North America	USA (Mississippi)	Soil (high in nitrogen or phosphate and associated with birth and bat guano)	Immunocompromised and immunocompetent	Systematic (brain, pulmonary, bones, cutaneous, endophthalmitis)	(Teixeira et al., 2016, Sepúlveda et al., 2017)
H. capsulatum var. *ohiense* (Nam2)	North America	USA (Ohio river valleys)	Soil (high in nitrogen or phosphate and associated with birth and bat guano)	Immunocompromised and immunocompetent	Systematic (brain, pulmonary, bones, cutaneous, endophthalmitis)	(Teixeira et al., 2016, Sepúlveda et al., 2017)
H. capsulatum var. *suramericanum* (LAm A)	South America	USA (Alabama, Arkansas, Delaware, Illinois, Indiana, Kentucky, Michigan,	Soil (high in nitrogen or phosphate and associated with birth and bat guano)	Immunocompromised and immunocompetent	Systematic (brain, pulmonary, bones,	(Teixeira et al., 2016, Sepúlveda et al., 2017)

		Minnesota, Nebraska, Pennsylvania and Wisconsin)			cutaneous, endophthalmitis)	
Sporotrichosis						
S. schenckii sensu stricto	Africa, Asia, Australia, Europe, North America, South America	South Africa (GP, Free State, KZN, WC, Mpumalanga); Botswana, Zimbabwe, Nigeria, China (Guangdong); India (sub-Himalayan and Kangra regions), Japan, New South Wales and Southeast coast, France, Italy, and Spain, Argentina, Brazil, Columbia, Guatemala, Mexico, Peru, USA, Venezuela	soil, animal excreta and decaying vegetation (mining woods, rose thorns, *Sphagnum* moss) armadillo *Dasypus septemcinctus* and *Dasypus novemcinctus*	Immunocompetent, immunocompromised, parrots, rodents, mice, bats, cats, dogs, squirrels, horses, birds, fish dolphins	Systematic (fixed cutaneous, lymphocutaneous, pulmonary, mucous membrane)	(Vismer and Hull, 1997, Govender et al., 2015, Moussa et al., 2017)
S. mexicana	Africa, North America, Australia	South Africa (Mpumalanga); Mexico (Jalisco and Puebla), Portugal	soil, rose tree, mining woods	Immunocompetent	Systematic (subcutaneous, lymphocutaneous)	(Dias et al., 2011, Govender et al., 2015)
S. globosa	North America, South America, Europe, Asia	Spain, Italy, China, Japan, India, Mexico, Colombia, Venezuela, USA, Uruguay and Guatemala	soil, animal excreta and decaying vegetation e.g. plants	Immunocompetent, immunocompromised individuals	Systematic (fixed cutaneous, lymphocutaneous)	(Marimon et al., 2007)
S. luriei	Africa, Asia, South America	South Africa, Italy, India, Brazil	soil, animal excreta and decaying vegetation e.g. plants	Immunocompetent, individuals, canine	Systematic (fixed cutaneous, lymphocutaneous)	(De Meyer et al., 2008)
S. brasiliensis	South America (Latin America)	Southern Brazil (Rio de Janeiro-São Paulo)	soil, animal excreta and decaying vegetation parrots, rodents, mice, bats, cats, dogs, squirrels, horses, birds, fish dolphins as well as insects (ants, bees)	Immunocompetent, immunocompromised individuals and cats	Systematic (fixed cutaneous, lymphocutaneous)	(Marimon et al., 2007)
S. pallida	North America	USA (Atlanta)	soil, animal excreta and decaying vegetation	Immunocompetent individual (on corticosteroids)	Corneal ulcer	(Morrison et al., 2013)
S. chilensis	South America	Chile	Soil	Immunocompromised individuals and mammals		(Rodrigues et al., 2016)

Emergomycosis caused by other species apart from *E. africanus* have been reported from Italy (n=1), India (n=1), China (n=1), Germany (n=1), Canada (n=4), the Netherlands (n=2) and Uganda (n=1) (Gori et al., 1998, Feng et al., 2015, Tang et al., 2015, Malik et al., 2016, Wang et al., 2017, Schwartz et al., 2018d, Gast et al., 2019, Rooms et al., 2019).

Blastomycosis was initially assumed to be a North American disease because of the highest reported incidence of infections that were due to *Blastomyces* on that continent (Jerray et al., 1992). In-patient sample data from the US showed the incidence of blastomycosis in both adults and children hospitalised in the Midwest and South to be 6.07 cases/1 million people and 3.10 cases/1 million people, respectively (Saccente and Woods, 2010). Additional cases were noted from Asia (India), the Middle East (Israel), Europe and Africa (Frean et al., 1989). About 81 cases of blastomycosis have been described by more than 18 countries in Africa over a 35-year period (1952-1987) (Carman et al., 1989, Frean et al., 1989). The lower number of cases reported could be due to cases being missed or underreported in these countries (Carman et al., 1989, Frean et al., 1989). These cases were diagnosed in South Africa, Zimbabwe, the Republic of Congo, Morocco, Tunisia, Zambia, Mozambique, Nigeria, Libya, Algeria, Egypt, Gambia, Malawi, Liberia, Rwanda, Tanzania and Uganda (Carman et al., 1989). In South Africa, cases have been reported provincially in Gauteng, KwaZulu-Natal, the Free State and Western Cape, while sporadic cases have been noted in North West Province and the Northern Cape (Frean et al., 1989, Koen and Blumberg, 1999).

Histoplasmosis is endemic in the North American region (Teixeira et al., 2016). Similar to blastomycosis, the disease is endemic in the USA, particularly in the Mississippi, Ohio and Missouri river valleys (Bahr et al., 2015, Teixeira et al., 2016). In these areas 80% of people test positive for the histoplasmin skin test (Bahr et al., 2015, Teixeira et al., 2016). Sporadic reports of infection by *H. capsulatum* var. *capsulatum* have been described outside America, e.g. Brazil, Argentina, India, southern Africa and western/central Africa (Teixeira et al., 2016). In 2019, Falci and colleagues reported a prevalence of 21.60% in the biggest prospective cohort study conducted among AIDS patients with disseminated histoplasmosis caused by *H. capsulatum* var. *capsulatum* from 2016 to 2018 in Brazil (Falci et al., 2019). In Africa, most cases of *H. capsulatum* var. *duboisii* infection are reported in West Africa, while sporadic cases are reported in Central and

South America, Oceania and Europe among travellers who have visited an endemic area (Oladele et al., 2018).

Based on DNA sequencing data, *H. capsulatum* var. *duboisii* infections have not been described in South Africa; all reported cases of histoplasmosis were caused by *H. capsulatum* (Craven and Benatar, 1979, Kenyon et al., 2013, Schwartz et al., 2017a). Craven and Benatar (1979) reported an outbreak of acute disseminated histoplasmosis among 10 people who undertook a caving trip in the Cape Province. Sporadic cases have been reported in KwaZulu-Natal (14 cases) and Cape Town (three cases) among HIV-infected patients (Ramdial et al., 2002; Schwartz et al., 2017a).

Sporotrichosis cases are reported worldwide. The gender ratio is 1:1 and some reports show that two thirds of the cases are reported among people of 16 to 35 years old and only a third among people between 5 and 15 years old (Schechtman, 2010, Tirado-Sánchez and Bonifaz, 2016). The biggest epidemic occurred in a South African gold mine between 1941 and 1944, when 3 300 cases were reported (Simson et al., 1947). These cases were linked to wood that was contaminated with the fungus and most patients presented with multiple pulmonary and cutaneous infections (Simson et al., 1947).

In 1931, the first case of sporotrichosis acquired outside a gold mine was reported in Pretoria (Goldberg and Pijper, 1931). Thereafter, more cases were regularly seen in Pretoria and the Free State; however, the disease was mostly diagnosed in KwaZulu-Natal and sporadically in the Western Cape (Vismer and Hull, 1997), with about 3 154 cases of sporotrichosis described in South Africa in 2015 (Zhang et al., 2015b). Apart from South Africa, other endemic areas include China (Guangdong), India (sub-Himalayan and Kangra regions), Australia (New South Wales and southeast coast) and the US (Song et al., 2013).

A multistate outbreak occurred in the US during 1988, when 84 cases of cutaneous sporotrichosis were reported across 15 states, which included 25 cases in New York, 24 in Illinois, 15 in Pennsylvania and 20 from 12 unidentified regions (Coles et al., 1992). The source of this outbreak was reported to be sphagnum moss (Coles et al., 1992). Some parts of Europe, especially France, Italy, and Spain, have reported fewer cases of the diseases, which were mostly acquired after

travelling to endemic areas (Tirado-Sánchez and Bonifaz, 2016). In Southern Brazil, epidemics are mainly associated with domestic cats, with more than 4 703 cats and 4 188 humans being affected in an epidemic that occurred from 1998 to 2015 (Sanchotene et al., 2015, Gremião et al., 2017).

2.7 Diagnostic methods for the identification of dimorphic fungi

Early identification of fungal strains remains important for optimal treatment of fungal infection. Phenotypic methods remain the traditional methods for identification of fungal strains. Although direct examination of fungal pathogens from clinical specimens (blood, skin tissue, bone marrow, liver tissue, lung tissue and lymph node) has low sensitivity compared to culture, this may facilitate early diagnosis (Guarner and Brandt, 2011; Schwartz et al., 2017a).

2.7.1 Histopathology method for the identification of dimorphic fungi

Histopathological examination of the affected tissue is a defined method of diagnosis of dimorphic fungal pathogens, but has limited specificity because the morphology of some yeast-like cells of *Emergomyces* verse *H. capsulatum* var. *capsulatum* and *Blastomyces* verse *H. capsulatum* var. *duboisii* are similar (Guarner and Brandt, 2011). Emmons et al. (1964) described a case of blastomycosis in a young Ugandan boy who was mistakenly diagnosed with histoplasmosis on histology based on the similarity of the yeast, but *B. dermatitidis* was cultured from sputum and pus specimens (Emmons et al., 1964).

The yeast-like cells are more easily visualised in affected tissue, if stained with haematoxylin and eosin, Periodic acid-Schiff (PAS), Gomori's methenamine silver or Wright-Giemsa (Guarner and Brandt, 2011). On these stains, *Emergomyces* (1.5 µm to 2.5 µm) yeast-cells resemble those of *H. capsulatum* var. *capsulatum* (2 µm to 5 µm), but the size is smaller than those of *Blastomyces* species (10 µm to 15 µm) (Guarner and Brandt, 2011, Schwartz et al., 2017a, Schwartz et al., 2018b).

Humans have a low *Sporothrix* fungal load when compared to felines and this limits direct examination of the fungus (Lopes-Bezerra et al., 2018). These cells can easily be mistaken for those of *H. capsulatum* var. *capsulatum* and *C. glabrata* (de Lima Barros et al., 2011). In a PAS stain, atypical *S. schenckii* yeast cells look larger and elongated, which is useful in distinguishing these yeast cells from *H. capsulatum* var. *capsulatum* and *E. africanus* (Schwartz et al., 2017a). In 20% to 80% of sporotrichosis cases, asteroid bodies may be noted inside giant cells of granulomatous disease (Gezuele and Da, 2005).

2.7.2 Culture-based method for the identification of dimorphic fungi

Culture methods are standard methods for identification of dimorphic fungi in microbiology laboratories (Caceres et al., 2019). However, dimorphic fungi are pathogenic and require specialised laboratory infrastructure (Caceres et al., 2019). According to Scheel and Gómez (2014), areas with a low burden of histoplasmosis have advanced laboratory infrastructure while those, which does not have such infrastructure, have a high burden of the disease, which probably contributes to failure to report the disease. The most appropriate specimens for culture include blood, skin tissue, bone marrow, liver tissue, lung tissue and lymph node (Schwartz et al., 2017a). The specimen is cultured onto Sabouraud dextrose agar (SDA) or potato dextrose agar (PDA) or malt extract agar at 25°C to 30°C and transferred to brain heart infusion (BHI) agar at 35°C to 37°C once there is growth to confirm dimorphism (Dukik et al., 2017). These include longer incubation, which may take two to three weeks for *Emergomyces* and *Sporothrix* and four to six weeks for *Histoplasma* and *Blastomyces* (Saccente and Woods, 2010, Kenyon et al., 2013, Govender et al., 2015, Caceres et al., 2019). The morphology of the young mould and yeast cultures of these dimorphic fungi is indistinguishable, which may lead to misidentification. Hence highly experienced laboratory staff is required to distinguish the morphologies of these pathogens (Caceres et al., 2019).

Patients presenting with acute pulmonary histoplasmosis show a 42% rate of positive cultures rather than the 74% reported for those with disseminated histoplasmosis (Hage et al., 2015). In HIV/AIDS patients, about 90% of the histoplasmosis cases have positive respiratory cultures, followed by 50% positive blood cultures (Kauffman, 2007). In a retrospective case series of 54

patients with disseminated emergomycosis in South Africa, skin biopsy (n=20/28) was the most appropriate specimen for the isolation of *Emergomyces*, followed by blood culture (n=24/37), bone marrow (n=12/14), lung (n=1/1), lymph node (n=1/1) and brain (n=1/1) (Schwartz et al., 2015a). In another study conducted in India from 1999 to 2005, aspiration of pus from skin or soft tissue abscesses for culture was more useful in isolation of *S. schenckii* when compared to scraping or exudate (Devi et al., 2006).

2.7.3 Antigen detection method for the identification of dimorphic fungi

Galactose and mannose (galactomannan) are the two most important carbohydrates released from fungal cell walls of growing yeast and used as antigens (Reiss and Lehmann, 1979). Galactomannan antigen detection may be used for the presumptive diagnosis of blastomycosis and histoplasmosis (Kozel and Wickes, 2014). The galactomannan found in *Histoplasma* containing (1→6)-α-D-galactofuranosyl side chains is similar to that found in *Blastomyces* (Kozel and Wickes, 2014). In contrast, galactomannan found in *S. schenckii* contains (1→5)-β-D-galactofuranosyl side chains (Assi et al., 2011).

In 2011, Assi reported a case of sporotrichosis in an HIV-infected male from Texas who presented with impaired mental status and dysphagia. *Histoplasma* antigenuria was detected at 1.7 unit (U) (cut-off of 1.0 U) by the MVista *Histoplasma* antigen EIA and skin biopsy was positive for *Sporothrix* for this patient (Assi et al., 2011). This result showed that there was cross-reactivity in patients with sporotrichosis (Assi et al., 2011). In 2015, Frost and Novicki (2015) assessed the performance of the *Blastomyces* EIA in detecting antigen in urine, serum and bronchoalveolar lavage (BAL) specimens and management of blastomycosis over a 10-year period (1995-2015) in Marshfield Clinic, Wisconsin, US. The *Blastomyces* antigen EIA was more sensitive in patients with isolated pulmonary and disseminated pulmonary infection (Frost and Novicki, 2015). When compared to culture and cytology confirmed pulmonary and disseminated infection, the sensitivity of patients with antigenemia, BAL positive and antigenuria was 56%, 63% and 76%, respectively (Frost and Novicki, 2015).

In histoplasmosis, *Histoplasma* galactomannan antigen detection is the most rapid diagnostic method for the disseminated form of the disease among patients with advanced HIV (Nacher et al., 2018). In 1986, Wheat and colleagues developed the first sandwich radioimmunoassay for the detection of *H. capsulatum* var. *capsulatum* antigen in urine and serum specimens of patients with disseminated histoplasmosis (Wheat et al., 1986). This led to the development of the first EIA, which yielded semi-quantitative results in 2004 (Wheat et al., 2006). Subsequently, several *Histoplasma* assays were introduced by MiraVista Diagnostics (Indianapolis, IN) and Immuno-Mycologics Ltd. (Norman, OK, USA) (Connolly et al., 2007). Only the IMMY *H. capsulatum* var. *capsulatum* antigen EIA kits (both polyclonal and monoclonal) are approved by the Food and Drug Administration (FDA) and available for purchase (Zhang et al., 2013, Theel et al., 2015, Nacher et al., 2018, Cáceres et al., 2018).

The ALPHA IMMY EIA uses polyclonal anti-*Histoplasma* IgG antibodies and among patients with histoplasmosis has a reported sensitivity of 44% and specificity of 84% compared to MiraVista Diagnostics EIA (LeMonte et al., 2007). To improve this performance, an EIA that uses anti-*Histoplasma* monoclonal antibodies for both capture and detection steps was developed by IMMY (Theel et al., 2013, Zhang et al., 2013, Zhang et al., 2015a). Researchers have evaluated the performance of this monoclonal antibody EIA for diagnosis of histoplasmosis among clinically characterised patients and reported a sensitivity and specificity of 65% and 100% compared to MiraVista Diagnostics EIA; and a sensitivity of 91% and specificity of 96% compared to ALPHA IMMY EIA, respectively (Zhang et al., 2013, Theel et al., 2013, Zhang et al., 2015a). Most recently, a multi-centre study evaluated the performance of the IMMY monoclonal antibody EIA against culture among AIDS patients with progressive disseminated histoplasmosis, reporting sensitivity and specificity of 97% and 98% in Guatemalan and Colombian patients (Cáceres et al., 2018). Although there has been an improvement in the specificity of the monoclonal antibody EIA for *Histoplasma*, the assay may cross-react with *Emergomyces*, *Blastomyces* and *Paracoccidioides* species (Schwartz et al., 2015a, Zhang et al., 2015a, Cáceres et al., 2018).

2.8 Molecular methods for the detection and characterisation of dimorphic fungi

Molecular methods are widely used for the detection and typing of fungal isolates (Raja et al., 2017). The techniques are useful in the identification of clones (which are organisms that have the same genetic material as the original organism) of a given species (Raja et al., 2017). These methods either give a band on a agarose gel or sequence-based data about the different strains of the fungi. The information obtained from the sequences may assist in describing the genetic variation among strains or associate genotypes with clinical phenotypes (Taylor et al., 1999). There are currently no standardised species-specific molecular methods for the identification of dimorphic fungi directly from culture or clinical specimens (Caceres et al., 2019). However, different protocols have been developed and optimised, but are only utilised in certain laboratories (Maubon et al., 2007, Toranzo et al., 2009, Gago et al., 2014, Schwartz et al., 2018c).

2.8.1 Conventional and quantitative PCR for the detection of dimorphic fungi

Conventional PCR, quantitative PCR (qPCR) and DNA sequence-based (panfungal PCR) methods are the most widely used methods for fungal identification (Raja et al., 2017, Schwartz et al., 2018c). The advantage of the panfungal PCR is that the final identity of the fungus can be confirmed by sequencing, while with qPCR the presence of the probe or dye may inhibit accurate identification and are prone to the presence of inhibitory substances during amplification, which may lead to false negative results. Furthermore, pan-fungal PCR assays are less advantageous than qPCR assays because it is labour-intensive. Pan-fungal PCR assays have a longer turnaround time than qPCR ones, which take about one to two hours for results to be available.

The qPCR assays are the most rapid assay and the widely used technologies include the TaqMan probe and Sybr green dye (Borman and Johnson, 2013). The TaqMan probe has high sensitivity and specificity in comparison to Sybr green dye because it uses a species-specific probe. More recently, Schwartz et al. (2018c) have used the TaqMan qPCR protocol to isolate *E. africanus* from soil samples in the Western Cape region, South Africa. Of 12 soil samples investigated, seven were positive according to the qPCR assay (Schwartz et al., 2018c). The assay was 100% sensitive and 100% specific in detecting *E. africanus* from soil samples (Schwartz et al., 2018c). In another

study, direct detection of 797 clinical specimens (bronchial washing (n=346), BAL (n=212), pleural fluid (n=157), tracheal secretions (n=35), tissue (n=14), sputum (n=13), lung washes (n=6), blood (n=4), bone marrow (n=5), peritoneal fluid (n=3) and other body fluids (n=2) by qPCR assay using fluorescence resonance energy transfer hybridisation probes showed a sensitivity of 86% and specificity of 99% for *B. dermatitidis* and 73% and 100% for *H. capsulatum* var. *capsulatum* in comparison with culture among patients with blastomycosis and histoplasmosis (Babady et al., 2011).

Although the ITS gene is used as a barcode for fungal identification, this gene sometimes does not distinguish between cryptic species (Raja et al., 2017). Govender et al. (2015) targeted the ITS region to distinguish the *Sporothrix* species complex from cultured isolates obtained from clinical and environmental samples during an outbreak that occurred in a mine. The ITS gene could not distinguish between cryptic species; however, sequencing of the calmodulin gene revealed the presence of two *Sporothrix* species (*S. schenckii* and *S. mexicana*) (Govender et al., 2015).

2.8.2 Characterisation of dimorphic fungi using multilocus sequence typing

The limitations of using single-locus sequence data to separate closely related organisms where ITS rDNA does not provide sufficient resolution has led to the sequencing of multiple loci to delineate species (Brown et al., 2013). *Neisseria meningitidis* was the first human pathogen used to develop the MLST scheme in 1998 (Maiden et al., 1998). Since then, MLST has been applied as an instrument for the epidemiological investigation and surveillance of *Candida, Cryptococcus* and dimorphic pathogen (Matute, 2006, Odds and Jacobsen, 2008, Brown et al., 2013, Kenyon et al., 2013). MLST has laid the foundation for cryptic species recognition in *Emergomyces*, *Blastomyces*, *Histoplasma* and *Sporothrix* genera (Kasuga et al., 1999, Kasuga et al., 2003, Brown et al., 2013, Dukik et al., 2017).

With MLST, DNA sequences are used to uncover allelic variants in housekeeping genes within a species. These genes are universal (range from 4 to 10) and contain proteins of central metabolic functions (Maiden, 1998). The housekeeping genes are amplified by primer pair sets and an internal fragment sequenced is approximately 400 bp to 700 bp in length (Urwin and Maiden,

1998, Belén et al., 2009). The total number and type of housekeeping genes employed for MLST may differ from species to species or even genera to genera.

Currently, an MLST database for *Blastomyces*, *Histoplasma*, *Emergomyces* and *Sporothrix* with all reported sequence types (STs) is unavailable, which makes it difficult to assign STs to these pathogens. However, authors have used MLST data without any assignment of ST to explore the genetic diversity and population genetic structures of *Emergomyces*, *Blastomyces*, *Histoplasma* and *Sporothrix* (Kasuga et al., 1999, Kasuga et al., 2003, Brown et al., 2013, Kenyon et al., 2013, Zhang et al., 2015b, Dukik et al., 2017). After the introduction of the MLST technique, the species originally described as novel *Emmonsia* species, *H. capsulatum*, *S. schenckii* and *B. dermatitidis*, were found to include several cryptic species (Warnock, 2018).

Dukik et al. (2017) sequenced five genes and rearranged the *Emmonsia* genus to create a new *Emergomyces* genus that accommodated novel human pathogens, which were previously described as *Emmonsia* species. The newly introduced taxa included *E. africanus,* described after sequencing of five genes (*ITS*, *LSU*, beta tubulin, PRP8 and actin genes) and found to be closely related to the first *E. pasteurianus* strain isolated in Europe in 1994 (Kenyon et al., 2013). Other phylogenetically related *Emergomyces* species classified by MLST include *E. orientalis*, *E. canadensis* and *E. europaeus* (Wang et al., 2017, Jiang et al., 2018).

B. dermatitidis was considered a single species, but MLST revealed that the genus comprises five monophyletic subgroups. Brown et al. (2013) identified a novel cryptic species, *B. gilchristii* among 78 *B. dermatitidis* isolates obtained from clinical specimens and environmental samples after sequencing seven loci (*ITS-2, FADS, ARF6, DRK1, CHS2*, *PYRF* and *TUB1*). This was followed by the introduction of *B. percursus* after sequencing of five loci (*ITS*, *TUB*, *LSU*, *TEF3* and *RP 60 L1*), which showed that *B. percursus* strains were closely related to, but distinct from *B. dermatitidis* and *B. gilchristii* (Dukik et al., 2017). Thereafter, MLST was used to rename *Emmonsia helica* and *Emmonsia parva* to *B. helicus* and *B. parvus* respectively after phylogenetic data revealed closer relationships with *Blastomyces* species than *Emmonsia crescens* (Schwartz et al., 2017b, Jiang et al., 2018).

After sequencing four loci (*ARF2*, *TUB2*, *OLE* and *H-anti*), the first MLST studies on the *Histoplasma* genus showed that *H. capsulatum* var. *capsulatum* consisted of six genetically distinct phylogenetically related groups instead of three varieties (Kasuga et al., 1999). Zhang et al. (2015b) used four loci, *ITS*, *CAL*, *TEF1* and *TEF3*, to study 99 medical and 36 environmental strains from The Netherlands and showed that four species (*S. schenckii*, *S. mexicana*, *S. globosa* and *S. brasiliensis*) consisted of nine subclusters that were limited to geographic distribution.

The main advantage of MLST is that it uses sequence data, which can be employed to study changes at the DNA level, which is impossible with phenotypic methodologies (Belén et al., 2009). In addition, the assay is easy to perform and the data generated can be shared between laboratories worldwide through the internet. Overall, MLST represents a portable, reproducible and sensitive method (Urwin and Maiden, 2003, Taylor, 2003, Odds and Jacobsen, 2008).

2.8.3 Whole genome sequencing and single nucleotide polymorphism (SNP) analysis of dimorphic fungi

Genome sequencing of fungi has enabled scientists to explore questions of species identification, host interactions, genetic mutations and variations allied with virulence and drug resistance, with higher precision (Cuomo, 2017). Genomic analysis can be performed in two ways: by the generation of genome assembly *de novo* (using species that have not been sequenced and assembled previously) and by re-sequencing (using an existing reference assembly) (Cuomo, 2017). These sequences are generated using different technologies such as Illumina, which provides a short read sequence (250 bp to 300 bp), and Pacific Bioscience or Oxford Nanopore, which provides longer read sequences (obtaining fewer contigs) (Cuomo, 2017).

Draft or complete genomes of *Emergomyces*, *Blastomyces*, *Histoplasma* and *Sporothrix* have been published (Sharpton et al., 2009, Teixeira et al., 2014, Muñoz et al., 2015, D'Alessandro et al., 2016, Huang et al., 2016, Dukik et al., 2017). These genomes vary in size, GC content and protein coding genes Table 2.4 (Dukik et al., 2017). The genomic sizes of *Emergomyces* (29.7 Mb - 35.5 Mb), *Blastomyces* (30.4 Mb - 75.4 Mb), *Histoplasma* (33.3 Mb) and *Sporothrix* (32.4 Mb – 37.8 Mb) are much bigger than those reported for *Candida* species (12.3 Mb-16 Mb) and *Cryptococcus*

species (19 Mb-20 Mb) (Odds et al., 2004, Cuomo et al., 2018). Based on phylogenetical analysis, *Emergomyces, Blastomyces* and *Histoplasma* are closely related because they cluster close to each other with strongly supported (100% bootstrap replicates) clades (Dukik et al., 2017). Intrageneric variation exists though, with *dermatitidis* and *B. gilchristii* strains having higher genomic size and low GC content when compared to *B. percursus* and other closely-related genera in the *Ajellomycetaceae* family (Van Dyke et al., 2019).

Table 2.4: Sequenced dimorphic fungi with their corresponding genomic sizes

Whole genome sequenced strains	**Size (Mb)**	**GC content**	**Protein coding genes**	**References**
E. africanus (CBS136260)	29.7	43.5	8769	Dukik et al., 2017
E. pasteurianus (CBS 101426)	32.4	44.5	8950	Dukik et al., 2017
E. orientalis (CBS 124587= CGMCC2.4011)	35.5	45.6	9188	Dukik et al., 2017
B. parvus (UAMH 139)	30.4	44.7	8563	Muñoz et al., 2015
B. percursus (BP222)	32.3	47.3	10293	Dukik et al., 2017
B. dermatitidis (ER-3)	66.6	37.1	9755	Muñoz et al., 2015
B. gilchristii (SLH14081)	75.4	35.8	9692	Muñoz et al., 2015
H. capsulatum (Nam1 = AAJI01000000)	33	42.8	9390	Sharpton et al., 2009
S. schenckii (AXCR00000000)	32.4	62.0	10293	Huang et al., 2016
S. brasiliensis (AWTV00000000)	33.2	62.0	9091	Huang et al., 2016
S. pallida (JNEX00000000)	37.8	52.8	11356	D'Alessandro et al., 2016
S. globosa (CBS 120340)	33.5	54.4	7719-7760	Huang et al., 2016

The genomic size of *S. schenckii*, *S. brasiliensis* and *S. globosa* ranges from 32.4 Mb - 33.5 Mb, with *S. pallida* having a larger genome size (37.8 Mb) (Teixeira et al., 2014, D'Alessandro et al., 2016, Huang et al., 2016). Currently, the phylogenetic tree shows that all the species that cause mammalian diseases, i.e. *S. schenckii*, *S. brasiliensis*, *S. luriei* and *S. globosa*, cluster together, while the environmental fungi, i.e. *S. pallida*, *S. mexicana*, *S. chilensis*, *S. palmiculminata*, *S. stylites* and *S. humicola*, share a common ancestor (Lopes-Bezerra et al., 2018).

In 2017, Dukik *et al*. used genome sequence data to determine the phylogenetic association of the *Emmonsia* genus, which led to the creation of a new genus, *Emergomyces*. Furthermore, a different *Blastomyces* species was introduced, namely *B. percursus* (Dukik et al., 2017). Recently, Sepúlveda et al. (2017) used genome sequencing to study variation within the *Histoplasma* species based on phylogeographic patterns, which led to the description of three additional species, namely *H. mississippiense* (Nam1), *H. ohiense* (Nam2) and *H. suramericanum* (LAm A), which were restricted to different geographic areas. Most recently, Muñoz et al. (2018) characterised the level of gene family developments and contractions in order to elucidate the evolutionary mechanisms of adaptation in *Emmonsia*, *Emergomyces*, *Blastomyces*, *Histoplasma*, *Paracoccidioides*, *Coccidioides*, *Helicocarpus griseus* and *Polytolypa hystricis*. According to Muñoz et al. (2018), although the genes involved in virulence are preserved in all saprophytes investigated, including the non-dimorphic species, *Helicocarpus griseus* and *Polytolypa hystricis*, modifications in the copy numbers of proteases, kinases and transcription factors within the dimorphic fungi may have enable these pathogens to adapt rapidly to higher temperatures and to different nutritional environments (Muñoz et al., 2018).

SNPs have been studied to confirm phylogenetic relationships and recombination among dimorphic pathogens (Frost et al., 2016). This type of analysis can be used to study the association of clinical disease and geographic variation and can link it with species-specific genetic diversity (Frost et al., 2016). Sepűlveda et al. (2017) compared whole-genome assemblies of the major lineages of *H. capsulatum* var. *capsulatum* using SNPs analysis to characterise 30 isolates obtained from North America (n=21), Latin America (4), Panama (3) and Africa (2).

The authors identified five clades, with evidence of greater genetic exchange between these lineages due to the broad geographic range. Furthermore, they were able to identify SNPs within each clade that could potentially be used to diagnose histoplasmosis; e.g. *H. capsulatum* harbours four SNPs at position 1328, 1364, 1469 and 1575; *H. mississipiense* harbours seven diagnostic SNPs at position 1385, 1415, 1426, 1447, 1506, 1532 and 1556; *H. ohiense* also harbours seven diagnostic SNPs at position 631, 648, 654, 758, 782, 814 and 837; *H. suramericanum* harbours three diagnostic SNPs at position 591, 622 and 716. Phylogenetic analysis supported the existence of five clades, with four clades belonging to American species, while one was an African clade

that showed early divergence. Only one clade existed in the African strains, probably because of the small sample size (Sepűlveda et al., 2017).

2.9 Summary

Dimorphic fungi are a group of fungal species causing infections among immunocompromised and immunocompetent patients (Dukik et al., 2017). *Emergomyces, Sporothrix, Histoplasma* and *Blastomyces* are fungi that are endemic to South Africa (Schwartz et al., 2017a). These pathogens rarely cause infections; however, occurrence of infection is dependent on the fitness of the host to fight infection and the amount of conidia inhaled (Saccente and Woods, 2010). Localised infection is noted in patients infected with *H. capsulatum* var *duboisii* and *S. schenckii*, while disseminated infection is frequently noted among those infected with *H. capsulatum* var *capsulatum*, *E. africanus*, *E. pasteurianus*, *B. dermatitidis* and *B. percursus* (Angius et al., 1998, Kenyon et al., 2013, Dukik et al., 2017, Schwartz et al., 2017a).

Diseases caused by dimorphic fungi are problematic to diagnose because most patients present with clinical and radiographic features that are indistinguishable, particularly from TB (Schwartz et al., 2015a, Schwartz et al., 2017a). In immunocompromised patients, the disease may be fatal if diagnosis is delayed or incorrect treatment is provided. The first line of treatment for the disseminated form of histoplasmosis or emergomycosis is amphotericin B, followed by a triazole (Wheat et al., 2007, Schwartz et al., 2015a). The acute form of histoplasmosis or blastomycosis may sometimes resolve spontaneously without treatment (Saccente and Woods, 2010). *In vitro* susceptibility testing of the mycelial and yeast form of *Emergomyces*, *Histoplasma* and *Blastomyces* has demonstrated lower MICs for itraconazole, posaconazole and voriconazole (Espinel-Ingroff, 1998a, Nakai et al., 2003, Kenyon et al., 2013, Dukik et al., 2018).

Researchers have used different methods to provide better understanding of these pathogens (Dukik et al., 2017, Jiang et al., 2018). Phenotypic methods such as culture and histology are used as standard methods for the diagnosis of these pathogens (Schwartz et al., 2017a). Culture has low sensitivity when compared to histology, while histology does not provide a definite diagnosis, since it cannot differentiate between the yeasts of these dimorphic pathogens (Schwartz et al.,

2017a). Culture methods require longer incubations and experienced personnel for accurate identification of these pathogens (Cáceres et al., 2019). Although the use of culture and histology methods remains important in the diagnosis of endemic mycosis in clinical settings, particularly in resource- limited countries, the detection of antigen from urine specimens could be a quicker assay for histoplasmosis or blastomycosis diagnosis in these areas (Frost and Novicki, 2015, Nacher et al., 2018). However, it should be taken into account that the *Histoplasma* EIA may cross-react with *Paracoccioides*, *Blastomyces*, *Emergomyces* and *Sporothrix* species (Assi et al., 2011, Zhang et al., 2015a, Cáceres et al., 2018, Schwartz et al., 2018b).

Molecular methods provide the most valuable information about the genetic makeup of fungal pathogens (Borman and Johnson, 2013). The panfungal PCR assay targeting the *ITS* and *LSU* genes is universally used for identification of fungal pathogens (Raja et al., 2017). The ITS assay is highly discriminatory against *H. capsulatum* var *duboisii*, *H. capsulatum* var *capsulatum*, *E. africanus, B. dermatitidis* and *B. percursus;* however, it is inadequate for the discrimination of cryptic species of *S. schenckii* (Govender et al., 2015). Fewer molecular assays are available and these are currently used in different laboratories (Maubon et al., 2007, Gago et al., 2014, Schwartz et al., 2018c). qPCR assays are rapid and accurately detect fungal species directly from clinical specimens and environmental samples (Babady et al., 2011, Schwartz et al., 2018b).

Genotyping techniques such as MLST have shown discriminatory power against dimorphic fungi. However, there is currently no database for these pathogens (Kasuga et al., 1999, Kenyon et al., 2013, Zhang et al., 2015b, Dukik et al., 2017, Jiang et al., 2018). Interestingly, by targeting similar genes for MLST, authors have confirmed the close relationship among these pathogens (Dukik et al., 2017, Jiang et al., 2018). The data obtained from these MLSTs can be shared among different laboratories for further species comparison. Better than MLST, WGS is becoming an important tool for typing of dimorphic fungi worldwide (Sharpton et al., 2009, Huang et al., 2016, Dukik et al., 2017, Muñoz et al., 2018).

Development and evaluation of these methods in any clinical setting may provide timely diagnosis and proper management of these endemic mycoses in South Africa. Furthermore, understanding the antifungal susceptibility profile of these pathogens may resolve the inappropriate practice of

antifungal drug prescription and possibly prevent the emergence of resistant strains. We therefore aimed to develop and evaluate laboratory assays for detection and antifungal susceptibility testing of dimorphic fungi in order to improve diagnosis and management of clinical disease, and to facilitate surveillance of invasive disease caused by these fungi.

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CHAPTER 3

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MYCOLOGY

In Vitro Antifungal Susceptibility of Yeast and Mold Phases of Isolates of Dimorphic Fungal Pathogen *Emergomyces africanus* (Formerly *Emmonsia* sp.) from HIV-Infected South African Patients

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3.1 ABSTRACT

Disseminated emergomycosis is an important AIDS-related mycosis in South Africa that is caused by *Emergomyces africanus*, a newly described and renamed dimorphic fungal pathogen. *In vitro* antifungal susceptibility data can guide management. Identification of invasive clinical isolates was confirmed phenotypically and by sequencing of the internal transcribed spacer region. Yeast and mold phase MICs of fluconazole, voriconazole, itraconazole, posaconazole, caspofungin, anidulafungin, micafungin, and flucytosine were determined with custom-made frozen broth microdilution (BMD) panels in accordance with Clinical and Laboratory Standards Institute recommendations. MICs of amphotericin B, itraconazole, posaconazole, and voriconazole were determined by Etest. Fifty unique *E. africanus* isolates were tested. The yeast and mold phase geometric mean (GM) BMD and Etest MICs of itraconazole were 0.01 mg/liter. The voriconazole and posaconazole GM BMD MICs were 0.01 mg/liter for both phases, while the GM Etest MICs were 0.001 and 0.002 mg/liter, respectively. The fluconazole GM BMD MICs were 0.18 mg/liter for both phases. The GM Etest MICs of amphotericin B,

for the yeast and mold phases were 0.03 and 0.01 mg/liter. The echinocandins and flucytosine had very limited *in vitro* activity. Treatment and outcome data were available for 37 patients; in a multivariable model including MIC data, only isolation from blood (odds ratio [OR], 8.6; 95% confidence interval [CI], 1.3 to 54.4; *P* _ 0.02) or bone marrow (OR, 12.1; 95% CI, 1.2 to 120.2; *P* _ 0.03) (versus skin biopsy) was associated with death. *In vitro* susceptibility data support the management of disseminated emergomycosis with amphotericin B, followed by itraconazole, voriconazole, or posaconazole. Fluconazole was a relatively less potent agent.

3.2 INTRODUCTION

The family *Ajellomycetaceae* (within the order *Onygenales*) includes phylogenetically related dimorphic fungal genera such as Emmonsia, *Histoplasma*, *Blastomyces* and *Paracoccidioides* (Schwartz et al., 2016). The family was recently reorganised to include a new genus, *Emergomyces*, to accommodate several emerging *Emmonsia*-like fungi causing disseminated disease, mostly among immunocompromised patients worldwide, and to address the polyphyletic nature of fungi previously included in the *Emmonsia* genus (Schwartz et al., 2015b, Dukik et al., 2017). Unlike *Emmonsia parva* and *Emmonsia crescens*, which cause adiaspiromycosis, fungi within the genus *Emergomyces* cause disseminated emmonsiosis (or emergomycosis), a multisystem disease with a high case fatality rate (Peterson and Sigler, 1998, Kenyon et al., 2013). In addition, *Emergomyces* differs from classic *Emmonsia* species by producing budding yeasts *in vivo* rather than adiaspores (Drouhet, 1998, Kenyon et al., 2013).

Currently, the genus *Emergomyces* includes at least three species: the type species, *Emergomyces pasteurianus*, which appears to have a cosmopolitan distribution; a rarer species, *Emergomyces orientalis*, reported from China; and *Emergomyces africanus*, a species endemic to southern Africa (Dukik et al., 2017, Wang et al., 2017). The first case of *E. pasteurianus* was described in an Italian patient with AIDS (Gori, 1998). Thereafter, several reports followed from Spain, China, India, and more recently, a single case from South Africa (Pelegrin et al., 2014, Feng et al., 2015, Tang et al., 2015, Malik et al., 2016, Dukik et al., 2017). *E. orientalis* has been reported only from a single immunocompetent Chinese patient (Wang et al., 2017). At least two other unnamed species exist within *Emergomyces*,

including a strain isolated from lung tissue of a man with rheumatoid arthritis in Germany and two isolates from immunocompromised patients in Canada (Dukik et al., 2017).

E. africanus was initially described in 2013 as the causative agent of a disseminated mycosis among 13 HIV-infected South African adult patients, most of whom came from the Western Cape province (Kenyon et al., 2013). Additional cases have since been described in most South African provinces, including KwaZulu-Natal (van Hougenhouck-Tulleken et al., 2014, Lochan et al., 2015, Schwartz et al., 2015a), authors' unpublished data). To date, with 86 laboratory-confirmed cases among HIV-infected persons in South Africa, *E. africanus* is far more commonly isolated than other well-described endemic pathogens such as *Histoplasma*, *Blastomyces*, or *Sporothrix* (authors' unpublished data).

Patients with disseminated emmonsiosis often present with a syndrome of fever, widespread skin lesions of various morphologies, pneumonia, anaemia, elevated liver enzymes, and weight loss (van Hougenhouck-Tulleken et al., 2014, Schwartz et al., 2015a, Dukik et al., 2017). Misdiagnosis is common (Schwartz et al., 2015a). To date, no clinical trial has been conducted to evaluate treatment options for patients with this disease. A retrospective review suggested better outcomes for patients treated with amphotericin B, followed by triazoles, than for those treated with triazoles alone; however, many of the former patients were incidentally prescribed small doses and short courses of fluconazole to treat presumed esophageal candidiasis (Schwartz et al., 2015a). Nonetheless, authors have recommended treatment with amphotericin B deoxycholate, followed by itraconazole, for a minimum of 12 months (Kenyon et al., 2013, van Hougenhouck-Tulleken et al., 2014, Lochan et al., 2015, Schwartz et al., 2015a), pending immune reconstitution on the basis of IDSA guidelines for HIV-associated disseminated histoplasmosis (Chapman et al., 1998, Wheat et al., 2007).

We compared the *in vitro* antifungal susceptibilities of the yeast and mould phases of *E. africanus* to several antifungal agents by reference and commercially available methods and determined if there was an association between MICs and clinical outcomes in order to guide the clinical management of patients with disseminated emergomycosis.

3.3 MATERIALS AND METHODS

3.3.1 Isolates and case definition

We obtained cultured isolates of *E. africanus* during passive laboratory-based surveillance conducted by the NICD from 2008 through to 2016 at nine diagnostic medical public- and private-sector laboratories in South Africa. We defined a case of disseminated emergomycosis as a patient of any age with an isolate cultured from any normally sterile site and confirmed as *E. africanus* by phenotypic and molecular methods. We abstracted patient charts to obtain clinical details, which included demographics, history of medical conditions including HIV and coinfections, clinical presentation at the time of diagnosis, diagnostic investigations, management, and outcome.

3.3.2 Identification to the species level

The identities of 50 stored *E. africanus* isolates were initially confirmed by a detailed description of microscopic and macroscopic characteristics. While *E. africanus* has not yet been formally classified as a biosafety level 3 organisms, we prepared all slides and cultures in a class II biosafety cabinet with the use of personal protective equipment, including N95 masks. We limited our work to the mould phase as far as possible. Mould phase isolates were subcultured on SDA (Diagnostic Media Products [DMP], National Health Laboratory Service, Sandringham, South Africa) and incubated at 25°C and 30°C for up to four weeks. The typical microscopic morphology of the mould phase was observed with a lactophenol cotton blue (DMP) slide preparation: septate hyphae, slender conidiophores at right angles to hyphae, and two or three round conidia borne on each conidiophore. To convert the fungus to the yeast phase, a piece of mould obtained from SDA agar was subcultured on a BHI–5% sheep blood agar slope or a BHI agar plate (DMP) and incubated for one to two weeks at 35°C. A Gram stain was prepared to observe the typical morphology of small, oval, budding yeast cells. PCR and sequencing of the *ITS* regions of the ribosomal gene were performed with *ITS*1 and *ITS*4 primers after genomic DNA had been extracted from yeast phase isolates with the Zymo ZR fungal/bacterial DNA MiniPrep kit (Zymo Research, Irvine, CA). *Candida albicans* American Type Culture Collection (ATCC) 90028 was included as a quality control (QC) strain during PCR and sequencing of the *ITS* region. Sequences were determined by capillary electrophoresis on an ABI 3500 genetic analyzer (Applied Biosystems, USA). Identification to the species

level was done with the NCBI BLAST database (http://blast.ncbi.nlm.nih.gov/Blast.cgi) on the basis of pairwise sequence alignment.

3.3.3 Antifungal susceptibility testing

We performed susceptibility testing of both yeast and mould phase isolates by a reference BMD method and the commercial Etest method (bioMérieux, Marcy, l'Etoile, France). The BMD method was performed in accordance with CLSI-approved standards M27-A3 (for the yeast phase) and M38-A2 (for the mycelial form) by using a modified inoculum size for the latter (CLSI, 2008, CLSI, 2012). Briefly, the inoculum was prepared from fresh cultures and the turbidity was adjusted with a turbidimeter to the equivalent of a 1 McFarland standard to obtain 2.5×10^3 to 5×10^3 colony forming unit (CFU)/ml for the yeast phase and a 2 McFarland standard to obtain 2.5×10^5 CFU/ml for the mycelial phase. Customised, round-bottom, frozen, 96-well microtiter plates containing two-fold dilution ranges of itraconazole, voriconazole, posaconazole, fluconazole, flucytosine, anidulafungin, caspofungin, and micafungin were immediately inoculated (TREK Diagnostic Systems, Inc., Cleveland, OH). Yeast phase BMD MIC endpoints were read at 50% inhibition for fluconazole, voriconazole, posaconazole, itraconazole, flucytosine, caspofungin, anidulafungin, and micafungin. Mould phase BMD MIC endpoints were read at 50% inhibition for fluconazole and flucytosine and 100% for voriconazole, posaconazole, itraconazole, and amphotericin B. For the mould phase, echinocandin MEC endpoints were read macroscopically as the lowest concentration that yielded small pellets of granular growth (microcolonies) compared to the hyphal-type growth seen in the growth control well (Espinel-Ingroff, 2003). Etest MICs of amphotericin B, voriconazole, itraconazole, and posaconazole were determined with Roswell Park Memorial Institute (RPMI) 1640 medium plates containing 2% glucose (DMP) in accordance with the manufacturer's recommendations. On the basis of the very high echinocandin MICs observed during preliminary testing, the MICs of these agents were not determined by Etest. Etest MIC endpoints were read as follows: 80% inhibition for voriconazole, posaconazole, and itraconazole (i.e., microcolonies within the elliptical zone of inhibition were ignored) and 100% for amphotericin B. All plates were incubated at 35°C and read by three independent observers at seven days.

The QC strains included in each test run were *Candida parapsilosis* ATCC 22019, *Candida krusei* ATCC 6258, and *E. africanus* National Collection of Pathogenic Fungi (NCPF) 4164

for yeast phase tests and *Aspergillus fumigatus* NCPF 7097, *A. fumigatus* NCPF 7100, and *E. africanus* NCPF 4164 for mycelial phase tests. The MICs for *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were within the CLSI-recommended ranges for all runs. Quantitative colony counts were performed to assess the purity and accuracy of the final inoculum. To check the yeast phase inoculum, we spread 0.02 ml onto a SDA agar plate to determine the number of CFU per millilitre. Plates were incubated at 35°C, and after seven days, we counted 50 to 100 colonies. For the mould phase, we spread 0.002 ml of the inoculum onto the surface of a plate, and after incubation, we counted up to 500 colonies.

3.3.4 Statistical methods

We calculated a geometric mean (GM) MIC, MIC_{50}, and MIC_{90} for each MIC distribution. For each antifungal agent and test method, we used a Wilcoxon ranked-sum test to compare the MICs generated from yeast and mould phase tests. We used a multivariable logistic regression model to assess the association of age, sex, province, CD4 cell count, ART history, antifungal treatment, specimen type, and MICs of amphotericin B, itraconazole, voriconazole, posaconazole, and fluconazole with patient outcome. All analyses were performed with Stata version 14.0 (StataCorp Limited, College Station, TX). Two-sided *P* values of <0.05 were considered significant.

3.3.5 Ethics approval

Ethics clearance for this study was obtained from the Health Sciences Research Ethics Committee, University of the Free State, Bloemfontein (13/2016), and the Human Research Ethics Committee of the University of Cape Town (704/2013 and 138/2014).

3.4 RESULTS

3.4.1 Cases and isolates

Fifty-one cases of disseminated emergomycosis diagnosed from 2008 to 2016 were included. Fifty-eight per cent (28/48) of the 50 patients infected with *E. africanus* were males with a median age of 35 (interquartile range [IQR], 30 to 38) years. Sixty-nine per cent (31/45) of the cases were diagnosed in the Western Cape province, 22% (10/45) in Gauteng, 7% (3/45) in the Free State, and 2% (1/45) in the Eastern Cape. Of the 49 *E. africanus* cases with clinical data available, HIV status could be surmised for 45; all of these patients were HIV infected. Of 44

HIV-infected patients with a recorded $CD4^+$ T-lymphocyte (CD4 cell) count, the median CD4 cell count was 12 (IQR, 7 to 27) cells/µl. Of 31 cases with an available ART history, 20 (65%) were ART experienced and 9 (29%) were ART naive. Isolates were cultured from skin biopsy specimens (*n* = 23), blood samples (*n*= 18), bone marrow samples (*n* = 6), biopsy specimens from an unknown site (*n* = 1), and an unknown specimen (*n* = 1). The demographic and clinical features of patients with *E. africanus* cases with available data are summarized in Table 1.

Table 3.1: Demographic and clinical features of 50 patients with emergomycosis (disseminated emergomycosis)

Demographic or clinical feature (total no. of patients)	Value
Median age, yr (IQR) (45)	35 (30–38)
No. (%) of males (48)	28 (58)
No. (%) in province (45)	
–Western Cape	31 (69)
–Gauteng	10 (22)
–Free State	3 (7)
–Eastern Cape	1 (2)
No. (%) HIV infected (45)	45 (100)
Median no. of CD4 cells/µl (IQR) (44)	12 (7–27)
ART history (31)	
–No. (%) naïve	9 (29)
–No. (%) experienced	20 (65)

*The number varies owing to missing data.

3.4.2 Antifungal susceptibility testing of *Emergomyces* isolates

All isolates grew sufficiently for MIC determination after seven days of incubation. Tables 3.2 and 3.3 summarise the MIC/MEC distribution, range, GM MIC/MEC, MIC_{50}, and MIC_{90} of nine antifungal agents for the yeast and mould phases of 50 *E. africanus* isolates. The BMD and Etest MICs of itraconazole were higher than those of voriconazole and posaconazole for both the yeast and mold phases (Tables 3.2 and 3.3). The yeast and mould phase GM MICs of fluconazole (BMD MICs, 0.19 versus 0.18 mg/litre; $P = 0.06$), amphotericin B (Etest MICs, 0.03 versus 0.01 mg/litre; $P = 0.06$), or any other antifungal agent/method tested (data not shown] were not significantly different.

Table 3.2: Yeast phase MIC distribution of 50 *E. africanus* clinical isolates

Antifungal agent	Test method	No. of isolates with MIC (mg/litre) of:														GM MIC*a*	MIC_{50}*a*	MIC_{90}*a*	MIC range*a*
		≤0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	≥64				
Itraconazole	BMD	36	5	4		5										0.01	0.008	0.008	0.008–0.12
Voriconazole	BMD	45	5													0.01	0.008	0.015	0.008–0.015
Posaconazole	BMD	37	6	3	1	2	1									0.01	0.008	0.03	0.008–0.25
Fluconazole	BMD					28	11	10	1							0.18	0.12	0.5	0.12–1
Caspofungin	BMD				1	1	2	2	25	10	7	2				1.18	1	4	0.06–8
Micafungin	BMD					1	4	1	11	15	9	9				1.85	2	8	0.12–8
Anidulafungin	BMD					1	1		20	24	3	1				1.34	2	2	0.12–8
Flucytosine	BMD										1	1	1	1	46	171.74	256	256	4–256
Amphotericin B	Etest	13	1	7	11	7	9	1	1							0.03	0.06	0.25	0.002–1
Itraconazole	Etest	35	5	6	1		3									0.01	0.008	0.03	0.002–0.25
Voriconazole	Etest	49	1													0.001	0.002	0.002	0.002–0.012
Posaconazole	Etest	49	1													0.002	0.002	0.006	0.002–0.012

a= All MICs are in milligrams per millilitre.

Table 3.3: Mould phase MIC distribution of 50 *E. africanus* clinical isolates

Antifungal agent	Test method	No. of isolates with MIC/MEC (mg/litre) of:														GM MIC*a*	MIC_{50}/MEC_{50}*a*	MIC_{90}/MEC_{90}*a*	MIC range*a*
		≤0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	≥64				
Itraconazole	BMD	35	4	5	2	1	1	2								0.01	0.008	0.06	0.008–0.5
Voriconazole	BMD	37	5	5	3											0.01	0.008	0.03	0.008–0.06
Posaconazole	BMD	32	8	4	4				2							0.01	0.008	0.06	0.008–1
Fluconazole	BMD					34	8	5	1	1		1				0.18	0.12	0.5	0.12–8
Caspofungin	BMD						3		20	23	3	1				1.67	2	2	0.25–8
Micafungin	BMD			1			1		20	11	13	4				1.72	2	4	0.03–8
Anidulafungin	BMD								13	32	5					1.72	2	2	1–4
Flucytosine	BMD														50	208.42	256	256	64–256
Amphotericin B	Etest	24		7	3	6	7	2	1							0.01	0.03	0.25	0.002–1
Itraconazole	Etest	36	1	10	1		1	1								0.01	0.006	0.03	0.002–0.5
Voriconazole	Etest	50														0.001	0.002	0.002	0.002–0.008
Posaconazole	Etest	48	1	1												0.002	0.002	0.004	0.002–0.03

a=All MICs are in milligrams per millilitre.

In contrast, the BMD method yielded significantly higher GM MICs than the Etest method for voriconazole (BMD MIC of 0.01 mg/litre versus Etest MIC of 0.001 mg/litre; $P < 0.001$) and posaconazole (BMD MIC of 0.01 mg/litre versus Etest MIC of 0.002 mg/litre; $P < 0.001$) for both the yeast and mold phases. There was no difference between the BMD and Etest GM MICs of itraconazole (data not shown).

3.4.3 MIC distribution and patient outcome

Of 51 cases of emergomycosis, the clinical outcome of 37 (73%) could be ascertained and the management of 34 (67%) was known. The overall case fatality ratio was 38% (14/37). Both clinical outcome and management could be ascertained for 33 patients (65%). Of the 23 patients who survived, management was known for 22; 18 (82%) were treated with amphotericin B, followed by itraconazole (Chapman et al., 1998), fluconazole (Schwartz et al., 2016), or a combination thereof (Schwartz et al., 2016); three were treated with triazole monotherapy (two received itraconazole, and one received a small dose of fluconazole for 14 days); and one received fluconazole for >12 months (possibly with amphotericin B). In contrast, management details were available for 11 of 14 patients who died (79%); three received amphotericin B, two received triazole monotherapy (one each received itraconazole and low-dose fluconazole), and six received no antifungal treatment. In the multivariable model, only isolation from blood (odds ratio [OR], 8.6; 95% confidence interval [CI], 1.3 to 54.4; $P = 0.02$) or bone marrow (OR, 12.1; 95% CI, 1.2 to 120.2; $P = 0.03$) (versus a skin biopsy specimen) was associated with death (Table 3.4).

Table 3.4: Outcome-associated characteristics of 37 patients with emergomycosis

	Outcome		**Univariate analysis**		**Multivariable analysis**	
Variable	**Survival**	**Death**	**OR (95% CI)**	***P* value**	**aOR*a* (95% CI)**	***P* value**
Median age (yr)	33 (28–36)	35 (31–38)	1.0 (0.9–1.2)	0.59		
Sex						
No. (%) of males	15/23 (65.2)	5/14 (35.7)	Reference		Reference	
No. (%) of females	8/23 (34.8)	9/14 (64.3)	3.4 (0.8–13.6)	0.08	2.14 (0.44–10.36)	0.34

	Outcome		Univariate analysis		Multivariable analysis	
Variable	**Survival**	**Death**	**OR (95% CI)**	***P* value**	**aOR*a* (95% CI)**	***P* value**
Province						
Gauteng	1/22 (4.6)	4/12 (33.3)	Reference			
Western Cape	19/22 (86.4)	8/12 (66.7)	0.11 (0.01–1.1)	0.06		
Eastern Cape*						
Free State	2/22 (9.1)	0/12 (0)				
Median no. of CD4 cells/µl (IQR)	13 (9–32)	14 (6–27)	1.0 (0.98–1.04)	0.50		
ART history						
No. naive/total (%)	6/21 (28.6)	3/10 (30)	Reference			
No. experienced/total (%)	15/21 (71.4)	7/10 (70)	0.93 (0.17–4.87)	0.94		
Any antifungal treatment						
No. without/total (%)	0/22 (0)	6/11 (54.6)	Reference			
No. with/total (%)	22/22 (100)	5/11 (45.5)	1			
Specimen type						
No. of skin biopsy samples/total (%)	15/23 (65.2)	2/14 (14.3)	Reference		Reference	
No. of blood cultures/total (%)	6/23 (26.1)	8/14 (57.1)	10 (1.62–61.47)	0.01	8.57 (1.35–54.33)	0.02
No. of bone marrow aspirates/total (%)	2/23 (8.7)	4/14 (28.6)	15 (1.58–142.18)	0.02	12.1 (1.21–120.13)	0.03
Antifungal MIC_{50}						
Amphotericin B***b***	0.06	0.06	0.16 (0.001–20.82)	0.47		
Posaconazole***c***	0.008	0.008	1.91 (0.10–34.82)	0.66		
Itraconazole***c***	0.008	0.008	0.26 (0.0004–140.69)	0.67		
Voriconazole***c***	0.008	0.008	72.3 (9.89–5.30)	0.87		

Variable	Outcome		Univariate analysis		Multivariable analysis	
	Survival	Death	OR (95% CI)	*P* value	aOR*a* (95% CI)	*P* value
Fluconazole*c*	0.12	0.20	0.75 (0.29–1.92)	0.54		

a aOR, adjusted OR.

b Determined by Etest, yeast phase.

c Determined by BMD, mould phase.

3.5 DISCUSSION

Thermally dimorphic fungi of the genus *Emergomyces* have emerged as a cause of disseminated, sometimes-fatal disease among HIV-infected South Africans with very low CD4 cell counts. We have reported the antifungal susceptibility profile of a large series of *E. africanus* isolates. Voriconazole, posaconazole, itraconazole, and amphotericin B had the most potent *in vitro* activity against both the mould and yeast phases of *Emergomyces*. While fluconazole is far more easily accessible to clinicians in the South African public health sector, this agent was less potent than other azoles (Motsoaledi et al., 2014). This confirms the findings of an earlier antifungal susceptibility study of six *E. africanus* yeast phase isolates (Kenyon et al., 2013).

There are no well-standardised methods for MIC determination for thermally dimorphic fungi (Goughenour et al., 2015); nevertheless, *in vitro* activities of polyenes (i.e., amphotericin B), azoles, and echinocandins, have been established for some of these organisms (Goughenour and Rappleye, 2017). We used CLSI-approved standards as a guide to yeast and mould phase testing, but used a larger inoculum for the mould phase and a prolonged incubation period to facilitate growth and endpoint determinations, in line with previous studies (Espinel-Ingroff, 1998, Nakai et al., 2003, Kenyon et al., 2013, Kathuria et al., 2014, Goughenour et al., 2015). Antifungal susceptibility testing for thermally dimorphic fungi is often limited to the mould phase, results of which may be misleading because the yeast phase is responsible for human disease (Goughenour and Rappleye, 2017).

We tested both phases by reference and commercial methods and found no statistically significant differences. Although conversion of the mould phase to the yeast phase increases

the turnaround time, there are fewer laboratory safety concerns with the yeast phase versus the potentially infectious mold phase and so we recommend that this phase be used for susceptibility testing. In contrast, significantly higher BMD MICs of voriconazole and posaconazole (but not itraconazole) were generated for both phases. Since, for most of these isolates, the BMD MICs of these agents were at or below the lower limit of the testing range, we speculate that this difference may merely have been an artifact caused by testing different ranges of antifungal concentrations with the two methods. Therefore, we recommend that either the Etest or BMD method be used for susceptibility testing. In this study, we also report relatively high MIC_{50} and MIC_{90} of the various echinocandins and flucytosine. These agents are probably of no value in the management of patients with dimorphic fungal infections and need not be included in a susceptibility testing panel.

The case fatality rate was high in our series and consistent with previous reports (Kenyon et al., 2013, Schwartz et al., 2015). Only culture of the fungus from blood or bone marrow (versus a skin biopsy specimen) was significantly associated with death on multivariable analysis. There are two possible explanations for this finding. First, we speculate that a positive blood or bone marrow culture is a proxy for a greater *in vivo* fungal burden. Second, isolation from blood or bone marrow alone (and not skin tissue) implies that a skin biopsy may not have been performed and the diagnosis of a deep fungal infection may not have been considered early enough. Antifungal MICs were not associated with outcomes in our current series, although a larger study may be needed to detect such an association. Currently, there are no published treatment guidelines for patients with disseminated emergomycosis (Lochan et al., 2015).

On the basis of retrospective data and international guidelines for the management of immunocompromised hosts with disseminated diseases caused by other dimorphic fungal infections (Kauffman et al., 2007, Wheat et al., 2007, Chapman et al., 2008), some authors have recommended that patients with suspected disseminated emergomycosis be treated with amphotericin B, followed by an azole (either itraconazole or fluconazole), after reporting good clinical outcomes among patients treated with these agents (Schwartz et al., 2015). Among the triazoles, fluconazole is much more accessible in South Africa because it is cheaper and included in hospital-level essential medicine lists (versus posaconazole, voriconazole, and itraconazole, which are far more expensive and require a formal application for procurement by the treating physician). Moreover, itraconazole is sometimes avoided because of interactions with rifampin among patients with comorbid TB and the unavailability of therapeutic drug

monitoring in South Africa. On the basis of the limited *in vitro* susceptibility data presented here, we believe that itraconazole, voriconazole, or posaconazole may be superior to fluconazole for the oral step-down phase following amphotericin B therapy for disseminated emergomycosis.

3.5.1 Study limitations

This study had some limitations. Clinical data could not be obtained for some patients. We could not exclude the possibility that prescription of antifungals by outside clinicians would evade our data capture and could influence our findings on the clinical effects of antifungals. A larger-than-recommended inoculum was used for MIC determination to allow us to read endpoints by seven days. Despite this, we found very low MICs of most antifungal agents. There are no currently published interpretative clinical breakpoints for any dimorphic fungus, including *Emergomyces*. Nevertheless, given the paucity of published clinical experience with these newly recognized pathogens, knowledge of *in vitro* MIC data should inform the management decisions of clinicians caring for patients with emergomycosis.

3.6 CONCLUSION

In conclusion, *in vitro* susceptibility data support the management of disseminated emergomycosis with amphotericin B, followed by itraconazole, voriconazole, or posaconazole. Fluconazole was a less potent agent. When indicated for epidemiological purposes in a reference laboratory, we recommend that the yeast phase and either the commercial Etest or a reference BMD method be used to generate MICs for *E. africanus*.

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3.9 CONFLICTS OF INTEREST

For unrelated work, Nelesh P. Govender received speaker honoraria/travel grants from Pfizer, Astellas, and MSD (Pty.) Ltd.; has provided educational materials for TerraNova; and has acted

as a temporary consultant for Fujifilm Pharmaceuticals. The other authors have no conflicts of interest to declare.

3.10 AUTHORS' CONTRIBUTIONS

T.G.M., I.S.S., and N.P.G. collected the data. T.G.M., T.G.Z, and R.S.M. performed antifungal susceptibility testing. T.G.M., E.B., and N.P.G. analysed the data and wrote the manuscript. T.G.M., I.S.S., E.B., S.D.N., and N.P.G. critically reviewed the manuscript.

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CHAPTER 4

A diagnostic accuracy case-control study to evaluate a *Histoplasma capsulatum* urine antigen enzyme immunoassay among patients with suspected histoplasmosis in South Africa

This chapter was submitted to PLOS Neglected Tropical Diseases for consideration for publication

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Keywords: histoplasmosis, emergomycosis, enzyme immunoassay (EIA), antigen, cross-reaction

4.1 ABSTRACT

Introduction: *Histoplasma* urine antigen detection is a rapid diagnostic method for disseminated histoplasmosis, particularly useful for patients with advanced HIV disease. We evaluated a monoclonal antibody enzyme immunoassay (EIA) for detection of *Histoplasma capsulatum* galactomannan in urine in South Africa.

Materials and Methods: We analyzed 212 urine samples from patients with a suspected invasive fungal disease, submitted to a South African national mycology reference laboratory from August 2014 to December 2018. Corresponding fungal culture and histopathology results were obtained from an electronic laboratory information system, where possible. Thirty-seven cultured fungal isolates were sent in parallel with the urine specimen for species-level identification (confirmed by phenotypic and molecular identification methods).

Results: Of 212 cases, 41 (19%) were excluded since they either had no clinical history (n=1), had alternative diagnoses (n=2) or had no fungal culture or histopathology results (n=38). Of the remaining 171, only 87 (51%) had laboratory evidence of an invasive fungal disease. Of these 87 cases, 37 (43%) were culture-confirmed: emergomycosis (n=18), histoplasmosis (n=8), sporotrichosis (n=6), cryptococcosis (n=2), talaromycosis (n=1), other fungi isolated (n=2). The sensitivity and specificity of the EIA compared to culture-confirmed histoplasmosis was 88% (7/8, 95%CI: 47%-100%) and 72% (21/29, 95%CI: 53%-87%), respectively. The sensitivity and specificity of the *Histoplasma* EIA, compared to histologically-confirmed (and indistinguishable) histoplasmosis/emergomycosis, was 83% (29/35, 95%CI: 66%-93%) and 93% (14/15, 95%CI 68%-100%), respectively.

Conclusions: This *Histoplasma* EIA is a rapid assay for the diagnosis of histoplasmosis, particularly in a high HIV prevalence area. However, in settings where histoplasmosis and emergomycosis are both endemic, such as in South Africa, the specificity of the *Histoplasma* EIA may decrease.

4.2 INTRODUCTION

Histoplasma capsulatum var. *capsulatum* is a causative agent of histoplasmosis worldwide, including South Africa, while *Histoplasma capsulatum* var. *duboisii* is reportedly restricted to Madagascar, central and west Africa (Oladele et al., 2018, Schwartz et al., 2017). Based on phylogenetic analyses, *Histoplasma capsulatum* var. *capsulatum* is closely related to *Emergomyces africanus*, a causative agent of disseminated emergomycosis in Southern Africa (Oladele et al., 2018, Schwartz et al., 2017). *H. capsulatum* var. *capsulatum* and *E. africanus* both cause disseminated mycosis among HIV-seropositive patients with a low CD4 count (Schwartz et al., 2015, Dukik et al., 2017, Maphanga et al., 2017, Govender and Grayson, 2018). Though these fungi can be distinguished after isolation in culture, the histopathological features of infection are indistinguishable (Schwartz et al., 2017).

Traditionally, disseminated histoplasmosis is diagnosed by histology and culture methods (Nacher et al., 2018). Histology involves invasive procedures for specimen collection, while culture requires longer incubation (four to six weeks) and skilled personnel for the identification of the fungus (Schwartz et al., 2017, Nacher et al., 2018). These delay the diagnosis and initiation of antifungal treatment, which may result in increased mortality (Nacher et al., 2018). *Histoplasma* galactomannan antigen detection is a rapid and non-invasive diagnostic method for disseminated histoplasmosis in patients with advanced HIV disease (Schwartz et al., 2017, Nacher et al., 2018). An EIA that yielded semi-quantitative results was first developed in 2004 (Wheat et al., 2006). Thereafter, several *Histoplasma* assays were introduced by MiraVista Diagnostics (Indianapolis, IN) and Immuno-Mycologics Ltd. (IMMY, Norman, OK, USA) (Connolly et al., 2007, LeMonte et al., 2007, Zhang et al., 2013). The ALPHA IMMY *H. capsulatum* antigen EIA was previously the only commercially-available and FDA-cleared assay (Zhang et al., 2015, Cáceres et al., 2018).

The ALPHA IMMY EIA uses polyclonal anti-*Histoplasma* IgG antibodies and among patients with histoplasmosis has a reported sensitivity of 44% and specificity of 84% compared to MiraVista Diagnostic EIA (LeMonte et al., 2007). To improve this performance, an EIA that uses anti-*Histoplasma* monoclonal antibodies for both capture and detection steps was developed by IMMY (Theel et al., 2013, Zhang et al., 2015, Theel et al., 2015). Most recently, the CLARUS monoclonal antibody EIA has become commercially available and FDA-cleared. Previously, researchers have compared the performance of this IMMY monoclonal

Histoplasma galactomannan EIA with other *Histoplasma* galactomannan EIA's for diagnosis of histoplasmosis and reported different results. Theel et al. (2013) evaluated the performance of the monoclonal *Histoplasma* galactomannan EIA among patients with suspected histoplasmosis and reported a sensitivity of 65% and a specificity of 100% when compared to the MiraVista *Histoplasma* EIA. Another study compared the performance of this monoclonal *Histoplasma* galactomannan EIA and ALPHA *Histoplasma* galactomannan EIA against MiraVista *Histoplasma* EIA using 50 patients with confirmed histoplasmosis and 50 controls, reporting a sensitivity and specificity of 72% and 98% and 22% and 84%, respectively (Zhang et al., 2015). Although the monoclonal antibody EIA had high specificity, cross-reactivity was reported with the fungal species causing emergomycosis, blastomycosis and paracoccidioidomycosis in other studies (Zhang et al., 2013, Cáceres et al., 2018, Govender et al., 2018, Schwartz et al., 2018). We aimed to evaluate the routine use of this monoclonal antibody-based *Histoplasma* EIA in detecting *H. capsulatum* galactomannan in urine samples of patients with suspected histoplasmosis compared to histopathology and culture in South Africa, a country with limited resources and a high HIV prevalence.

4.3 MATERIALS AND METHODS

4.3.1 Participants, specimens and case definitions: Physicians submitted urine specimens from their patients with suspected histoplasmosis to a national mycology reference laboratory for *Histoplasma* antigen testing from August 2014 to December 2018. During the study period, this assay was available only at the NICD. Each specimen request was screened by a pathologist (N.P.G) and testing was performed at no cost. Urine specimens were transported on ice, stored at 4°C to 8°C at the reference laboratory and batched for weekly processing. Physicians were requested to submit appropriate specimens in parallel for fungal culture and histopathological examination; the latter tests were performed at the diagnostic pathology laboratory to which the clinician routinely submitted specimens. These specimens were collected when histoplasmosis was suspected and when considered appropriate by the attending clinician. We defined a confirmed case of histoplasmosis as a patient of any age who had a diagnosis of histoplasmosis confirmed by either fungal culture or histopathology of any specimen. Since histoplasmosis or emergomycosis are indistinguishable by histology, participants were defined as having either disseminated histoplasmosis or emergomycosis if small oval budding yeasts were seen, ranging in size from 2 µm to 5 µm, with an intracellular or extracellular location and staining positive with Periodic-acid Schiff or methenamine silver stains but negative for

capsular stains (e.g. mucicarmine or Alcian blue) and with no reported evidence of hyphae or pseudohyphae. Participant demographics, a history of medical conditions including HIV and co-infections, clinical presentation at the time of diagnosis and treatment were obtained from specimen request forms submitted to the NICD. Fungal culture and histopathology results were extracted from several electronic laboratory information systems. We excluded cases with no concomitant fungal culture or histopathology results or a clinical history that was not consistent with a systemic fungal infection.

4.3.2 Detection of *Histoplasma* antigen: We purchased components of an EIA for *Histoplasma* galactomannan detection from IMMY including a positive control, negative control, substrate and stop solution. Undiluted urine specimens were processed in weekly batches, according to the manufacturer's instructions. The urine specimens were run in duplicate. Briefly, 100 µl of a vortexed urine specimen was added into a microtiter well coated with a rabbit anti-*H. capsulatum* monoclonal antibody. Plates were incubated at 37°C for 60 min and washed. Horseradish peroxidase-conjugated anti-galactomannan monoclonal antibody was then added and plates were incubated for 30 or 45 min at 25°C. The plates were washed again and a substrate (3, 3, 5, 5 – tetramethylbenzidine) was added and incubated for 30 min at 25°C. A stop solution was then added and the optical density of the reaction was measured at dual excitation wavelengths of 450 nm, with a reference wavelength of 620 nm in a BioTek microplate reader (Analytical & Diagnostic Products, Randburg, South Africa). All tests were performed between August 2014 and December 2018 and the concentrations of the seven calibrator solutions used were 0.4, 0.8, 1.6, 3.1, 6.25, 12.5 and 25 ng/ml of galactomannan, with a cut-off value of >0.5 ng/ml considered positive. We further reanalysed all results using the recently FDA-approved cut-off value of >0.2 ng/ml with the same seven calibrator solutions for the monoclonal antibody-based *Histoplasma* EIA. The standards were performed to determine the quantitative values and to obtain the associated qualitative interpretation. Results were reported as indeterminate if the EIA yielded discordant results on the same specimen after two runs had been performed.

4.3.3 Species confirmation of cultured isolates: Thirty-seven cultured clinical isolates were submitted in parallel with 212 urine specimens for species-level identification. We performed a detailed description of microscopic and macroscopic characteristics of these isolates. Mould-phase isolates were sub-cultured onto SDA (DMP, NHLS, Sandringham, South Africa) and incubated at 25^{o}C and 30^{o}C for up to four weeks. To convert the fungus to the yeast phase, a

piece of mould obtained from SDA was sub-cultured on BHI + 5% sheep blood agar slope or a BHI agar plate (DMP) and incubated for one to four weeks at 35ºC. Subsequently, PCR and sequencing of the *ITS* regions of the ribosomal gene and calmodulin gene were performed using *ITS*1 and *ITS*4 primers and the *CAL*1 and *CAL*2A primers, respectively, after genomic DNA had been extracted from yeast-phase isolates using the Zymo ZR fungal/bacterial DNA MiniPrep Kit (Zymo Research, Irvine, USA) (Rodrigues et al., 2013, Govender et al., 2015). The sequences were determined using capillary electrophoresis on an ABI 3500 genetic analyser (Applied Biosystems, USA). The identity of the fungal species was obtained from the NCBI database based on pairwise sequence alignment using BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

4.3.4 Statistical methods: We calculated sensitivity and specificity with 95% confidence intervals for the EIA compared to either culture or histology using Stata version 14.0 (StataCorp, USA). Sensitivity was calculated as the index test positive divided by reference test positive multiplied by 100. Specificity was calculated as the index test negative divided by reference test negative multiplied by 100.

4.3.5 Ethics approval

Ethics clearance for the study was obtained from the Health Sciences Research Ethics Committee, University of the Free State, Bloemfontein (UFS-HSD2018/0992).

4.4 RESULTS

4.4.1 Cases with suspected histoplasmosis

Two hundred and twelve urine specimens from patients with suspected histoplasmosis were referred to the NICD from 2014 to 2018. Of 212 referred cases, 41 (19%) were excluded (no clinical history, n=1; alternative diagnoses: hydatid cyst, bacillary angiomatosis, n=2; no fungal culture or histopathology results, n=38) (Figure 4.1). Of the remaining 171 participants, 66% (113/171) were male, and their median age was 36 years IQR, 30-42 years). HIV status was available for 163 participants and of these, 96% (156/163) were HIV-seropositive. Of 152 HIV-seropositive patients with a recorded CD4 count, the median CD4 count was 20 cells/µl (IQR, 6-61 cells/µl). Fungal culture results were obtained from skin biopsy (n=92), blood (n=48), aspirate of a superficial abscess (n=4), respiratory material (n=5), bone marrow (n=3), cerebrospinal fluid (n=3), tissue biopsy of unknown origin (n=2), liver biopsy (n=2), oral ulcer/palate biopsy (n=1), superficial swab (n=1), colonic biopsy (n=1) and unknown (n=9).

Histology results were available for skin (n=115), bone marrow (n=15), lymph nodes (n=6), lung biopsy (n=3), liver (n=3), colonic biopsy (n=1), oral ulcer/palate biopsy (n=1), renal allograft biopsy (n=1), nasal mass (n=1) and unknown (n=25). The demographic and clinical characteristics of participants with available data are summarised in Table 4.1.

4.4.2 Clinical details of 171 participants with or without invasive fungal disease

Of 171 participants, 87 (51%) had laboratory evidence of invasive fungal disease, while 84 (49%) did not, i.e. culture- and/or histology was negative for invasive fungal disease. Fifty-two of 84 (62%) cases classified as being invasive fungal disease-negative were negative by both culture and histology, 26 (31%) were negative by fungal culture alone and six (7%) negative by histology alone. Of the 87 cases with confirmed invasive fungal disease, 37 (43%) had a cultured fungal isolate with identification (34 confirmed by both histology and culture and three by culture alone) (Figure 4.1). Among the 37 with a culture-confirmed diagnosis, *E. africanus* was cultured in 18 (49%), followed by *H. capsulatum* var. *capsulatum* in eight (21%), *Sporothrix schenckii* sensu stricto in six (16%), *Cryptococcus neoformans* in two (5%), *Talaromyces marneffei* in one (3%), *Acremonium* species in 1 (3%) and *Epicoccum* species in 1 (3%). Among these 37 cases, fungi were cultured from skin biopsy (*E. africanus*, n=14; *H. capsulatum* var. *capsulatu*m, n=7; *S. schenckii*, n=4; *C. neoformans*, n=1; *Acremonium*, n=1; *Epicoccum*, n=1), blood (*E. africanus*, n=4; *H. capsulatum*, n=1; *S. schenckii*, n=1; *T. marneffei*, n=1), superficial swab (*S. schenckii* sensu, n=1) and cerebrospinal fluid (CSF) (*C. neoformans*, n=1). The remaining 50 cases were confirmed positive by histology alone. Thirty-five (70%) of these 50 cases were confirmed to have either histoplasmosis or emergomycosis (by histopathological description), while 15 (30%) had another fungal infection.

4.4.3 Detection of *H. capsulatum* antigen from urine

All 171 participants were tested for *Histoplasma* antigenuria (and results re-calculated using updated cut-off values). Diagnostic accuracy calculations were performed only for confirmed cases of fungal infection with culture identification (n=37) or histology results with or without fungal identification (n=50) (Figure 4.1 and Table 4.2). Fifteen of the 37 cases with culture-confirmed disease had a positive *Histoplasma* EIA result; seven of the 15 had histoplasmosis, seven had emergomycosis and one had *Acremonium* cultured from skin (a probable contaminant). The seven patients with emergomycosis were all HIV-seropositive, the majority having widespread skin lesions indicative of disseminated disease, while the participant from whose skin biopsy *Acremonium* was cultured was also HIV-seropositive with widespread skin

lesions. Histology demonstrated evidence of intracellular yeasts for the seven cases of emergomycosis and the *Acremonium* case (Table 4.3). The EIA was negative for one participant (3%) with confirmed histoplasmosis by culture. This participant was HIV-seropositive, had widespread skin lesions and evidence of intracellular yeasts on histology. Among the 22 culture-confirmed cases with a negative *Histoplasma* EIA, one had histoplasmosis (mentioned above), 18 had emergomycosis, six had sporotrichosis, two had cryptococcosis and one patient each had talaromycosis and *Epicoccum* cultured from skin biopsy (a possible contaminant) (Figure 4.1). The sensitivity and specificity of the EIA was 88% (95% Cl, 47%-100%) and 72% (95% Cl, 53%-87%) compared to culture-confirmed histoplasmosis (Table 4.2). When we combined culture-confirmed histoplasmosis or emergomycosis cases, the EIA had a lower sensitivity (54% [95% Cl, 33%-73%]) but much higher specificity (91% [95% Cl, 59%-100%]; data not shown).

Thirty of the 50 cases with histology results had a positive *Histoplasma* EIA result, 29 of whom had either histologically confirmed histoplasmosis or emergomycosis. Among the 20 with a negative *Histoplasma* EIA, six had histologically confirmed evidence of either histoplasmosis or emergomycosis. All six participants were HIV-seropositive with a low CD4 count; three were currently receiving antiretroviral therapy and five had widespread skin lesions (Table 4.4). The sensitivity and specificity of the EIA compared to histologically-confirmed histoplasmosis or emergomycosis was 83% (95% Cl, 66%-93%) and 93% (95% Cl, 68%-100%), respectively.

4.5 DISCUSSION

We evaluated the performance of a commercially available *Histoplasma* galactomannan EIA in routine practice for detection of antigenuria among patients with suspected histoplasmosis in a high HIV prevalence setting. Compared to culture-confirmed histoplasmosis, the EIA had a sensitivity and specificity of 88% and 72% and compared with histologically confirmed histoplasmosis or emergomycosis, a sensitivity and specificity of 83% and 93%.

Galactomannan antigen detection has become a widely used method for confirming disseminated histoplasmosis among patients with advanced HIV disease because it is simpler and faster than conventional methods (Nacher et al., 2018). A high fungal antigen concentration in body fluids also facilitates diagnosis in patients with advanced immunosuppression (Wheat, 2003, Adenis et al., 2014). In 2019, a *Histoplasma* antigen detection assay was included in the

2nd World Health Organization Essential Diagnostics List and recommended by the global mycology community as an important diagnostic test for HIV-associated histoplasmosis in low- and middle-income countries (Nacher et al., 2018).

The monoclonal *Histoplasma* antigen assay is an expensive test compared to culture and histology. The estimated cost for *Histoplasma* antigen assay is ZAR2000 per test when compared to ZAR53.46 for culture and ZAR70.00 to ZAR270.00 for histology. We evaluated this monoclonal *Histoplasma* antigen assay because it offered a non-invasive and rapid means for diagnosis of histoplasmosis in our setting. Two different multi-centre studies evaluated the performance of this monoclonal *Histoplasma* galactomannan EIA using a cut-off value of 0.5 ng/ml (Cáceres et al., 2018, Falci et al., 2019). In the first study, Cáceres et al. (2018) evaluated the performance of the monoclonal *Histoplasma* galactomannan EIA against culture in AIDS patients with disseminated histoplasmosis, reporting an increase in sensitivity and specificity of 97% and 98% in Guatemalan and Colombian patients. A similar study evaluated the monoclonal antibody EIA against standard methods (microscopy, culture, antibody detection and histopathology) in people living with HIV/AIDS in Brazil, reporting a lower sensitivity of 72% (Falci et al., 2019). Similarly, we observed a decrease in the sensitivity of the monoclonal *Histoplasma* galactomannan EIA when compared to culture and histology in our study. According to Falci et al. (2019), the decrease in sensitivity was possibly due to the previous use of antifungal agents.

Among the 37 participants with culture-confirmed invasive fungal disease, eight had a false-positive histoplasmosis EIA. All cases had a positive histology report and clinical presentation, which was suggestive of histoplasmosis. However, seven cases were confirmed to have emergomycosis and we cultured *Acremonium* species from skin biopsy of one case. *Acremonium* is a mould found in the environment and we hypothesise that this was a probable contaminant because intracellular yeasts (but no hyphae) were observed on histological examination. To date, cross-reactivity of the IMMY monoclonal *Histoplasma* galactomannan EIA has only been reported in fungal species causing emergomycosis, blastomycosis, or paracoccidioidomycosis, but not in patients infected with *Acremonium* spp. (Zhang et al., 2015, Cáceres et al., 2018, Schwartz et al., 2018). In 1993, *Acremonium* spp. was found to cause false-positive results with the Pastorex *Aspergillus* galactomannan antigen latex agglutination test among patients with invasive aspergillosis (Kappe and Schulze-Berge, 1993). We observed

no cross-reaction in six *S. schenckii* sensu stricto-infected patients; however, a previous study reported cross-reactivity in an HIV-seropositive patient with the MiraVista *Histoplasma* EIA (Assi et al., 2011). Furthermore, urine specimens from patients with cryptococcosis did not cross-react with the IMMY monoclonal *Histoplasma* EIA, which is similar to what has previously been reported among eight patients with cryptococcosis who had a negative IMMY monoclonal *Histoplasma* EIA result (Cáceres et al., 2018).

To improve the sensitivity of the IMMY monoclonal *Histoplasma* EIA, Theel et al. (2015) modified the cut-off value, introduced an indeterminate range and reported an increase in the sensitivity of the assay from 65% to 82% (Theel et al., 2015). In our study, two cut-off values were used (0.5 ng/ml and 0.2 ng/ml) and similar results were obtained with these cut-off values. The urine specimen from a single case of histoplasmosis remained EIA-negative even after we used a cut-off value of 0.2 ng/ml. The positivity and the negativity of the EIA could depend on the severity of the disease and amount of antigen present in the urine specimens of patients with either disseminated histoplasmosis or emergomycosis.

Histology alone was not sufficient to allow a definitive diagnosis in patients with invasive fungal disease in our setting. This has previously been reported among AIDS patients presenting with skin infection in the Western Cape, South Africa (Schwartz et al., 2017). Of the 20 cases with a positive histology and a negative IMMY monoclonal *Histoplasma* galactomannan EIA in our study, six were HIV-seropositive with a low CD4 count and their clinical presentation was consistent with histoplasmosis or emergomycosis. Only two had confirmed TB, while others had widespread skin lesions. Although all six cases had either presumptive disseminated histoplasmosis or emergomycosis histologically, it is possible that the negative EIA result could have been due to lower antigen levels in these patients.

Based on culture alone, emergomycosis is a more common disease in South Africa among AIDS patients than histoplasmosis (Kenyon et al., 2013, Schwartz et al., 2017). We reported a ratio of 18:8 for emergomycosis and histoplasmosis in this study, while other South African studies had previously reported a ratio that ranged from 14:3 to 13:4 for emergomycosis and histoplasmosis, respectively (Kenyon et al., 2013, Schwartz et al., 2017). Phylogenetic analyses have shown that *Emergomyces* species are closely related to *Histoplasma* species and other genera within the *Ajellomycetaceae* family, such as *Paracoccidioides*, and *Blastomyces* (Dukik

et al., 2017). This could possibly explain why the IMMY monoclonal *Histoplasma* galactomannan EIA would cross-react with *Emergomyces*. Of 14 cases with a positive IMMY monoclonal *Histoplasma* galactomannan EIA, seven had confirmed emergomycosis and seven had histoplasmosis. In clinical settings where *Histoplasma* and *Emergomyces* are both common among HIV patients, it should be noted that the specificity of the assay may decrease owing to the inability of the assay to distinguish between these two pathogens. In contrast, the IMMY monoclonal *Histoplasma* galactomannan EIA may also result in over-estimation of the number of cases of histoplasmosis, especially in areas where emergomycosis is endemic. However, in clinical settings where histoplasmosis is the only disease endemic among HIV-seropositive patients, the specificity of the assay may remain high (Cácere et al., 2018).

In cases where the fungal culture is negative, but histology and the clinical presentation are both suggestive of an invasive fungal disease, the EIA may be useful to confirm an early diagnosis. Since the recommended treatment of histoplasmosis and emergomycosis is the same, (i.e. amphotericin B followed by itraconazole) (Kenyon et al., 2013, Schwartz et al., 2015, Maphanga et al., 2017, Govender et al., 2018), the inability of this assay to distinguish between the two diseases may have limited clinical relevance.

In our setting, skin and blood were the most common specimens used for the diagnosis of histoplasmosis and emergomycosis by culture, while skin and bone marrow were commonly submitted for histology.

4.5.1 Limitation of the study

Only 37 cases of invasive fungal disease had a positive culture, which limited the power of our diagnostic accuracy calculations for this IMMY monoclonal *Histoplasma* galactomannan EIA. We assumed that patients with negative fungal culture or histology results from routinely collected specimens did not have invasive fungal infection. Furthermore, we may have misclassified some patients who had invasive fungal disease as not having invasive fungal disease because appropriate specimens were not collected for culture/histology. When compared to other thermally dimorphic fungi, *H. capsulatum* is a slow-growing fungus on standard medium (requires a four to six-week incubation period) and requires experienced personnel for identification. We hypothesise that lack of mycology experience and culture plates incubated for only seven days for routine culture could explain some negative fungal culture results. In contrast, *Emergomyces* grows within seven to 14 days of incubation.

Furthermore, we could not differentiate cases of histoplasmosis and emergomycosis based on histology alone.

4.6 CONCLUSION

This is the first report to evaluate the IMMY monoclonal *Histoplasma* galactomannan EIA for diagnosis of culture-confirmed histoplasmosis, emergomycosis and other diseases in South Africa. The IMMY monoclonal *Histoplasma* galactomannan EIA proved useful for rapid diagnosis of disseminated HIV-associated histoplasmosis, even though it cross-reacted with *Emergomyces*-infected patients in South Africa. The EIA is relatively easy to use and reduces turn-around time, which decreases the time to diagnosis and potentially, the mortality associated with *Histoplasma* and *Emergomyces* infections in South Africa.

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4.9 CONFLICTS OF INTEREST

For unrelated work, Nelesh P. Govender received speaker honoraria/travel grants from Pfizer, Astellas, and MSD (Pty) Ltd; has provided educational materials for TerraNova, and has acted as a temporary consultant for Fujifilm Pharmaceuticals. The other authors have no conflicts of interest to declare.

4.10 AUTHORS' CONTRIBUTIONS

Specimen collection: ISS, JN, CB

Processing of urine specimens: TGM, SDN, MM

Data analysis and manuscript writing: TGM, NPG

Critical review of manuscript: TGM, BG, ISS, SDN, JN, RSM, CB, NPG

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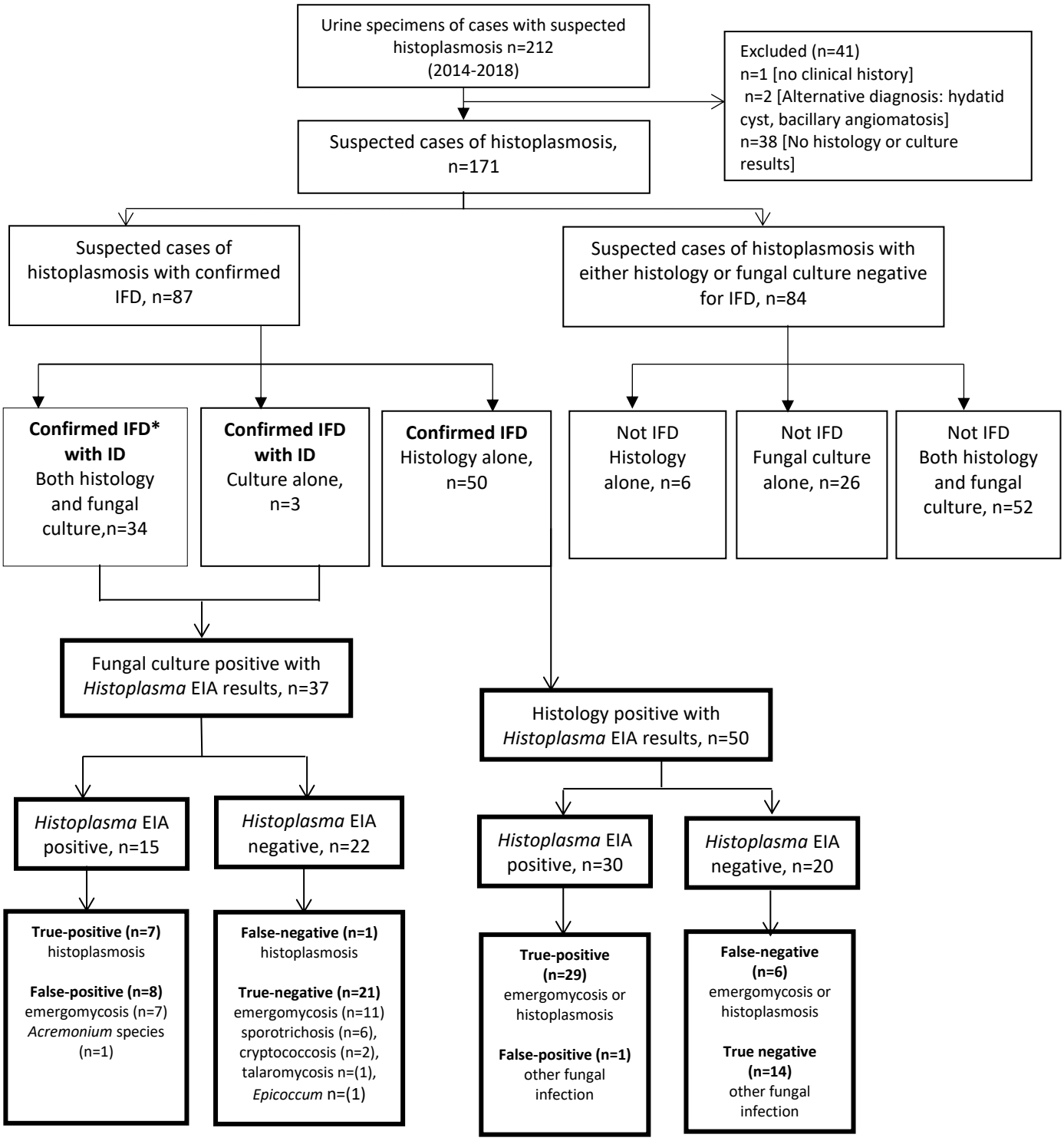

Figure 4.1: Flowchart of patients with suspected histoplasmosis who had urine specimens submitted for *Histoplasma* antigen testing, 2014-2018, n=212

***IFD=Invasive Fungal Disease; ID=Identification; EIA=Enzyme immunoassay**

Table 4.1: Demographic and clinical characteristics of suspected cases of histoplasmosis, 2014-2018, n=171*

Demographic and clinical features			
	Culture- or histology-confirmed IFD, n=87 (%)	No IFD, n=84 (%)	Overall n=171 (%)
Median age (IQR), years	38 (31-42)	35 (29-42)	36 (30-42)
Sex			
Male	65/87 (75)	48/84 (57)	113/171 (66)
Province			
Gauteng	33/87 (38)	48/84 (57)	81/171 (47)
Western Cape	24/87 (27)	18/84 (22)	42/171 (25)
Eastern Cape	25/87 (29)	17/84 (20)	42/171 (25)
KwaZulu-Natal	5/87 (6)	1/84 (1)	6/171 (3)
Sector			
Public	86/87 (99)	77/84 (92)	163/171 (95)
Private	1/87 (1)	7/84 (8)	8/171 (5)
HIV status and CD4 count			
HIV-infected	85/86 (99)	71/77 (92)	156/163 (96)
Patients with known CD4 count	82/87 (94)	70/84 (83)	152/171 (89)
Median CD4 count (IQR), cells/ µl	18 (5-52)	24 (7-79)	20 (6-61)
Specimen type (culture)			
Blood	19/84 (22)	29/78 (37)	48/162 (29)
Skin	58/84 (69)	34/78 (44)	92/162 (57)
Respiratory	1/84 (1)	4/78 (5)	5/162 (3)

Abscess (superficial)	1/84 (1)	3/78 (4)	4/162 (2)
Bone marrow	1/84 (1)	2/78 (2.5)	3/162 (2)
Cerebrospinal fluid	1/84 (1)	2/78 (2.5)	3/162 (2)
Liver	0/84 (0)	2/78 (2.5)	2/162 (1)
Tissue biopsy of unknown origin	0/84 (0)	2/78 (2.5)	2/162 (1)
Oral ulcer/palate	1/84 (1)	0/78 (0)	1/162 (1)
Colonic biopsy	1/84 (1)	0/78 (0)	1/162 (1)
Superficial swab	1/84 (1)	0/78 (0)	1/162 (1)
Specimen type (histology)			
Skin	73/86 (85)	41/58 (71)	115/146 (78)
Bone marrow	8/86 (9)	7/58 (12)	15/146 (10)
Lymph node	2/86 (2)	4/58 (7)	6/146 (4)
Liver	0/86 (0)	3/58 (5)	3/146 (2)
Lung	0/86 (0)	2/58(3)	3/146 (2)
Oral ulcer/palate	1/86 (1)	0/58 (0)	1/146 (1)
Renal	0/86 (0)	1/58 (2)	1/146 (1)
Colonic	1/86 (1)	0/58 (0)	1/146 (1)
Nasal mass	1/86 (1)	0/58 (0)	1/146 (1)

*41 cases were excluded from the 212 specimens submitted because they had either no histology or culture results or, no clinical history suggestive of invasive fungal diseases

Table 4.2: Diagnostic accuracy of a second-generation *H. capsulatum* enzyme immunoassay compared to culture-confirmed fungal disease (n=37) and histology-confirmed fungal disease (n=50)

Enzyme immunoassay	**No. of cases with culture-confirmed fungal disease (n=37)**			**No. of cases with histology-confirmed fungal disease (n=50)**		
	Histoplasmosis	Other fungal infections	Total	Histoplasmosis or emergomycosis*	Other fungal infections	Total
Positive	7	8	**15**	29	1	**30**
Negative	1	21	**22**	6	14	**20**
Total	**8**	**29**	**37**	**35**	**15**	**50**
Sensitivity % (95% Cl)	88 (47-100)			83 (66-93)		
Specificity % (95% Cl)	72 (53-87)			93 (68-100)		

*Histology-confirmed histoplasmosis or emergomycosis: description of deep tissue pathology consistent with either disseminated histoplasmosis or emergomycosis, i.e. small oval budding yeasts (ranging in size from 2 to 5 μm), positive staining with Periodic acid Schiff or methenamine silver, intracellular or extracellular location of yeasts, no reported evidence of hyphae or pseudohyphae, negative for capsular staining (mucicarmine) when reported

Calculation of sensitivity and specificity:

EIA vs. culture-confirmed cases: **Sensitivity** = 7/7+1 = 7/8*100 = 88%

Specificity = 21/8+21 = 21/29*100 = 72%

EIA vs. histology confirmed cases: **Sensitivity** = 29/29+6 = 29/35*100 = 83%

Specificity = 14/1+14 = 14/15*100 = 93%

Table 4.3: Patient characteristics of culture-confirmed cases of invasive fungal disease with a false-positive EIA result using histoplasmosis cases as reference (n=8)

Age (years)/ Sex	Clinical presentation	Culture site	Culture result	Histology site	Histology result	HIV-status	CD4+ T-cell count (cells/ul)	ART	Specimen collection date	EIA result (ng/ml)*
41, Female	Widespread skin rash	Skin	*E. africanus*	Skin	Small yeasts	Positive	97	ART-experienced	2014	4.62
26, Male	TB with widespread skin rash	Skin	*Acremonium*	Skin	Numerous narrow-based budding yeasts	Positive	50	ART-experienced	2015	2.9
30, Male	Widespread skin rash	Skin	*E. africanus*	Skin	Numerous budding yeasts	Positive	38	Unknown	2015	12.00
34, Male	Widespread skin rash, weight loss and cough	Skin	*E. africanus*	Skin	Oval and round yeasts	Positive	12	ART-experienced	2015	5.39
34, Male	Disseminated TB	Skin	*E. africanus*	Skin	Numerous narrow-based budding yeasts	Positive	69	ART-experienced	2016	3.31
30, Female	Widespread skin rash	Skin	*E. africanus*	Skin	Numerous yeasts	Positive	7	ART-experienced	2018	0.62

41, Male	Widespread skin rash	Blood	*E. africanus*	Skin	Intracellular yeasts	Positive	4	Unknown	2018	0.98
40, Male	Pancytopenia, diarrhea, severe ascites and hepatomegaly	Blood	*E. africanus*	N/A	N/A	Positive	321	Unknown	2018	2.67

*cut-off value = 0.5 and 0.2

Table 4.4: Patient characteristics of histology-confirmed IFD cases with false-negative IMMY EIA results using histoplasmosis and emergomycosis cases as reference (n=6)

Age (years), sex	Clinical presentation	Histology site	Histology result (P/N)	HIV-status (P/N)	CD4+ T-cell count/ul	ART	Specimen collection date	EIA result (ng/ml)
27, Male	Sepsis, disseminated intravascular coagulation and renal failure. Erythematous papules on the forehead and cheeks	Skin	Ovoid narrow-based budding yeasts with a clear halo	Positive	37	ART-experienced	2015	0.00
29, Male	Widespread skin rash and bilateral lung infiltrate on chest X-ray	Skin	Scattered tiny yeasts with narrow-based budding	Positive	3	Unknown	2015	0.00
41, Female	Severe loss weight, TB and pancytopenia	Bone marrow	Scattered small yeasts	Positive	584	ART-experienced	2016	0.00
40, Male	Disseminated TB with widespread skin rash	Skin	Small round yeasts	Positive	39	U	2017	0.09
42, Male	Widespread skin rash	Skin	Small intracellular narrow-based budding yeasts	Positive	1	ART-experienced	2017	0.00
56, Male	Widespread skin rash	Skin	Small clusters of ovoid/ round small yeasts	Positive	13	Unknown	2018	0.09

*cut-off value = 0.5 and 0.2

CHAPTER 5

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MYCOLOGY

Human Blastomycosis in South Africa Caused by *Blastomyces percursus* and *Blastomyces emzantsi* sp. nov., 1967 to 2014

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5.1 ABSTRACT

We re-evaluated 20 cases of blastomycosis diagnosed among South African patients between 1967 and 2014, in light of recently described species associated with deviating clinical features. All isolates, initially identified as *Blastomyces dermatitidis* by histopathological and/or culture-based methods, were characterised phenotypically, genotypically and by whole genome sequencing. Morphological characteristics and phylogenetic analyses of MLST and whole genome sequences revealed two groups, both of which were closely related to but distinct from *B. dermatitidis*, *Blastomyces gilchristii* and *Blastomyces parvus*. Group 1 corresponded to the recently described *Blastomyces percursus* and group 2 is described here as *Blastomyces emzantsi* sp. nov. Whole genome sequencing confirmed distinct species identities and the absence of a full ortholog of *BAD-1* gene. Extrapulmonary disease was more common than lung involvement. South African blastomycosis cases were caused by species that are distinct from *B. dermatitidis*, *B. gilchristii* and *B. parvus*, which might account for clinical differences from North American blastomycosis.

5.2 INTRODUCTION

The first human case of blastomycosis was described by Gilchrist in 1894 from skin tissue (Gilchrist, 1894). Blastomycosis was initially believed to be restricted to North America (Jerray et al., 1992). However, since the first case was reported from Tunisia in 1952, cases of blastomycosis have also been reported throughout Africa, and less commonly from India, the Middle East, and occasionally Europe (Carman et al., 1989, Frean et al., 1989). Differences have been noted between North American and African cases of blastomycosis, both in the isolates and the clinical presentation of disease. For instance, blastomycosis in North America primarily involves the lungs, while more African cases have involved skin and bone (Carman et al., 1989).

The genus *Blastomyces* belongs within the family *Ajellomycetaceae* and order Onygenales which includes other dimorphic fungi such as *Histoplasma*, *Emmonsia*, *Emmonsiellopsis*, *Paracoccidioides*, and a newly described genus, *Emergomyces* (Dukik et al., 2017). Initially, the *Blastomyces* genus was thought to comprise a single species, *Blastomyces dermatitidis* (Gilchrist and Strokes, 1898). Three other species have since been described within *Blastomyces*, including

B. gilchristii (a cryptic species phenotypically indistinguishable from *B. dermatitidis*), *B. percursus* and *B. silverae* (Brown et al., 2016, Dukik et al., 2017, Jiang et al., 2018). In addition, *Emmonsia parva* (*Blastomyces parvus*) and *Emmonsia helica* (*Blastomyces helicus*) have been assigned to the genus (Jiang et al., 2018). *B. dermatitidis* produces abundant large broad-based budding yeasts at 37°C, *B. percursus* produces large yeast-like cells from fragmented, swollen hyphal cells, *B. helicus* produces variably-shaped yeast cells in short chains, while thin-walled giant cells and occasional broad-based budding yeast-like cells are seen in *B. parvus* and *B. silverae* strains (Dukik et al., 2017, Schwartz et al., 2017, Jiang et al., 2018).

In prior studies, mycological differences were noted between North American and African *Blastomyces* strains (Kwo-Chung, 1971, Kaufman et al., 1983, Lombardi et al., 1988, Mercantini et al., 1995, Klein et al., 1997, Guého et al., 1997, Meece et al., 2013). African strains were more difficult to convert from the mould to the yeast phase (Kwo-Chung, 1971, Kaufman et al., 1983, Lombardi et al., 1988). While North American and African isolates shared the K antigen, North American strains additionally possessed an A antigen, which is absent from most African strains (Kaufman et al., 1983). When tested for sexual compatibility, African strains failed to mate with North American strains of *B. dermatitidis* (Kwo-Chung, 1971). Furthermore, DNA melting curve analyses separated the North American and African strains into groups that differed sexually, histologically, epidemiologically and clinically (Guého et al., 1997).

In view of the recent description of new pathogenic species within the genus *Blastomyces* and the potential molecular epidemiological, clinical and public health implications, we re-examined South African *Blastomyces* human clinical isolates archived over five decades.

5.3 MATERIALS AND METHODS

5.3.1 Case detection and isolate archive

We conducted passive laboratory-based surveillance for dimorphic fungi associated with human disease from 2008 to 2014 at nine South African diagnostic pathology laboratories in the public- and private-sectors. We defined a case of blastomycosis as a patient with an isolate cultured from any specimen, initially identified as *B. dermatitidis* by standard phenotypic culture-based or histological methods. In addition, we included 17 clinical isolates from South Africa, which had

been cultured from 1967 to 1999, previously identified as *B. dermatitidis* by phenotypic methods and archived at the NICD, South Africa or in the NCPF, United Kingdom. For ten cases, we obtained detailed information on patient demographics, medical conditions including HIV and co-infections, details of the patient's clinical presentation, diagnostic investigations, management and outcomes from previously-published case reports (Martin and Berson, 1973, Simon et al., 1977, Carman et al., 1989, Frean et al., 1989, Frean et al., 1993, Heys et al., 2014, Schwartz et al., 201, Dukik et al., 2017). Limited information was available on the other ten archived isolates. In total, we studied 20 *Blastomyces* isolates. Examination of isolates included morphological characterisation by light and electron microscopy, determination of MIC for nine antifungal agents and phylogenetic analyses using sequences of 11 genes (including six genes based on a published multi-locus sequence typing [MLST] scheme) (Supplementary appendix) (Kenyon et al., 2013, Brown et al., 2016). We also performed phylogenetic analysis using whole-genome SNPs for 18 isolates (Supplementary appendix).

5.3.2 Morphological description and electron microscopy

All isolates were sub-cultured onto SDA or PDA (DMP, Sandringham, South Africa) and incubated at 25ºC and 30ºC for up to four weeks for the mycelial phase. Isolates were transferred onto BHI agar with or without 5% sheep blood (DMP) and incubated for one-three weeks at 37ºC and 40ºC for conversion to the yeast phase. Mycelial growth was also inoculated onto a urea agar slope (DMP) and incubated at 37ºC for six days. Yeast and mycelial isolates were examined using light microscopy and electron microscopy (Supplementary appendix). The descriptive terminology used follows that of Jiang *et al*. (2018) (Jiang et al., 2018).

5.3.3 Antifungal susceptibility testing

Susceptibility testing was performed for both yeast- and mould-phase isolates using a reference BMD method and the commercial Etest method (bioMérieux, Marcy, l'Etoile, France). The BMD method was performed according to CLSI M27-A3 and M38-A2 with a modified inoculum size for the latter as described by Maphanga *et al*. 2017 (CLSI, 2002, CLSI, 2008, Maphanga et al., 2017). QC strains were included in each test run (Supplementary appendix).

5.3.4 DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from 20 isolates using the Zymo ZR fungal/bacterial DNA MiniPrep Kit (Zymo Research Corp., USA) according to the manufacturer's instructions. For five genes *ITS*, *LSU*, actin, β-tubulin and intein *PRP8*), purified amplicons were subjected to sequencing PCR as described previously (Kenyon et al., 2013). The samples were sequenced in a 3130 Sequencer (Applied Biosystems, Life Technologies Corporation, USA). Sequences were subjected to BLAST analyses in GenBank (www.ncbi.nlm.nih.gov) for identification. MLST targeting the *CHS2*, *DRK1*, *FADS*, *PYRF*, *TUB1* and *ARF6* genes was performed as described by Brown *et al*. (2013), although two genes were non-typeable using this scheme (*CHS2* gene for *B. percursus* and *PYRF* for *B. emzantsi*) (Brown et al., 2016). All six gene sequences were then extracted from the WGS data to complete the MLST dataset.

5.3.5 MLST phylogenetic analysis

DNA sequences were aligned with MAFFT version 7 and datasets for the five genes and the six MLST loci were trimmed with BioEdit version 7 (Hall et al., 1999, Katoh et al., 2017). The phylogenetic tree was generated by a maximum likelihood statistical method using 1,000 bootstrap replications on MEGA version 6 from the concatenated dataset and individual genes (Supplementary) (Tamura et al., 2013). A clade was defined as isolates from different patients which shared a common ancestor with >80% support values in the maximum likelihood analyses.

5.3.6 Whole genome sequencing and *de novo* assembly

Paired-end libraries were prepared using the Nextera XT DNA library kit, followed by 2 × 300-bp sequencing on an Illumina MiSeq sequencer (Illumina, San Diego, CA, USA). The sequenced paired-end reads were quality trimmed and *de novo* assembled using Qiagen CLC Genomics Workbench version 10 (Qiagen, The Netherlands) (Supplementary appendix).

5.3.7 Phylogenetic analysis using whole-genome single nucleotide polymorphisms

Reads were aligned to the *B. percursus* assembly strain BP222 (GenBank accession GCA_003206225.1_ASM320622v1) using BWA-MEM version 0·7·12 (Dukik et al., 2017, Li et al., 2013). Variants were then identified using GATK version 3·7 (Supplementary appendix) (McKenna et al., 2010). For phylogenetic analysis, the 1,712,033 sites with an unambiguous SNP

in at least one isolate and with ambiguity in at most 10% of isolates were concatenated; insertions or deletions at these sites were treated as ambiguous to maintain the alignment. Maximum likelihood phylogenies were constructed using RAxML version 8.2.4, using the GTRCAT nucleotide substitution model and bootstrap analysis based on 1000 replicates (Stamatakis, 2014).

5.3.8 Statistical methods

We calculated a GM for each MIC distribution using Stata version 14.0 (StataCorp Limited, College Station, Texas, USA).

5.3.9 Ethics

Ethics clearance for the study was obtained from the ethics committees of the University of the Free State (13/2016), the University of Cape Town (704/2013 and 138/2014) and the University of the Witwatersrand (M140112).

5.4 RESULTS

5.4.1 Isolates and cases

Isolates were available from 20 cases of blastomycosis diagnosed from 1967 to 2014. Only the year of isolation was available for nine archived *Blastomyces* isolates: 1975 (n=6), 1992 (n=2), 1999 (n=1). For one isolate, no information was available. Clinical data were available for the ten remaining cases. Nine patients were adult males, with a median age of 37 years (IQR, 32.5 – 52 years). Isolates were cultured from skin biopsy (n=4), pus from a thoracic vertebral abscess (n=2) (Figure 5.1), pus from a subcutaneous abscess (n=2), brain tissue (n=1) and biopsy of a tongue ulcer (n=1). For the nine patients for whom this information was available, *B. dermatitidis* was initially identified as the causative pathogen by histopathological examination (Table 5.1). All 20 isolates were phenotypically identified in culture as *B. dermatitidis*.

5.4.2 Mycological examination of the isolates

Based on examination of cultures grown on SDA (at 25°C) and BHI agar (at 37°C), we distinguished two morphological groups of isolates. Twelve isolates (group 1) resembled *B. percursus* morphologically (Supplementary appendix Figure 1). The remaining eight isolates (group 2), here named *B. emzantsi*, resembled *B. parvus* in some aspects but were morphologically

distinguishable (Figure 5.2, 5.3 and supplementary appendix). No growth at 40°C was observed for any isolate from either group. Group 1 isolates were urease-negative within 24 hours of incubation, while group 2 isolates were urease-positive within 24 hours at 37°C. The broad-based budding cells characteristic of the type species of *Blastomyces*, were evident in all available isolates at 37°C, albeit interspersed with hyphal elements.

5.4.3 Antifungal susceptibility testing of isolates

All isolates grew sufficiently for MIC determination after five to six days of incubation. Supplementary Tables 1 and 2 summarise the distribution, range and geometric mean MIC/MEC for nine antifungal agents for the yeast-and mould-phases of 17 isolates. There were no differences between *B. percursus* and *B. emzantsi*. Voriconazole, posaconazole, itraconazole, micafungin and amphotericin B had the most potent in vitro activity.

5.4.4 Multilocus and whole genome phylogenetic analyses

Phylogenetic analysis of both multilocus and whole genome sequences supported that group 1 isolates belonged to *B. percursus* and group 2 isolates formed a monophyletic clade between the *B. percursus*/ *B. dermatitidis* clade and *B. parvus*. To determine the phylogenetic relationships, we used multilocus analysis of *ITS*, *LSU*, *PRP8*, β-tubulin, actin, *CHS2*, *DRK1*, *FADS*, *PYRF*, *TUB1* and *ARF6*, and variants (SNPs) from whole genome sequences aligned to the *B. percursus* assembly strain BP222. Multilocus phylogenetic analysis of the concatenated sequences of *ITS-LSU-PRP8*-β-tubulin-actin and *CHS2-DRK1-FADS-PYRF-TUB1-ARF6* genes clearly separated group 1 (*B. percursus*), and group 2 (*B. emzantsi*) isolates into separate species (Figure 5.4A and 5.4B). In addition, whole genome phylogenetic analysis of 1,712,033 variant sites identified from aligning whole genome reads to *B. percursus* strain BP222 strongly supported these phylogenetic relationships, confirming that isolates from group 1 clustered with *B. percursus* (100% bootstrap support) and that isolates from group 2 conformed to an intermediate monophyletic clade between *B. parvus* and *B. percursus/dermatitidis* that represents a novel *Blastomyces* species (100% bootstrap support; Figure 5.4C). Large genetic diversity was found in sympatric *B. emzantsi* and *B. percursus* species with an average of 900,000 SNPs between species.

5.4.5 Clinical presentation and treatment of ten cases

5.4.5.1 *B. percursus*

Four patients (cases 2-5) presented with a two- to seven-month history of symptoms including weight loss, productive cough with hemoptysis, cutaneous lesions including chronic ulcers and headache. Most of these patients had multi-system involvement. All seven patients (cases 1-7) had extrapulmonary disease, and three (case 2, 3, 6) additional patients had pulmonary disease. Four patients (case 1, 3, 4, 6) had cutaneous involvement. One patient presented with vertebral disease (case 7). *B. percursus* was isolated in Gauteng (case 1, 4, 5, 7), Western Cape (case 1, 2) and Free State provinces (case 6) of South Africa (Supplementary Table 3).

5.4.5.1 *B. emzantsi*

Patient 10 had an underlying severe combined immune deficiency, with a subcutaneous abscess, but other clinical data were lacking. Patients 8 and 9 presented with cough, weight loss, night sweats and dyspnea. Both were found to have pulmonary disease with collapsed thoracic vertebrae and patient 8 had a purulent discharge from a sinus tract at T5 vertebral level (Figure 1). Both patients were treated empirically for TB. Patient 8 died soon after starting amphotericin B, while patient 9 recovered after receiving a cumulative total of 1.8 g of amphotericin B (Table 1). *B. emzantsi* was isolated in Limpopo (case 8), North West (case 9) and Northern Cape provinces (case 10) of South Africa (Figure 4D).

5.4.6 Genome assembly sizes

The assembly genomes of *B. percursus* isolates ranged from 32·1 Mb to 34·2 Mb, whilst those of *B. emzantsi* isolates ranged from 27·4 Mb to 32·5 Mb.

5.4.7 Nucleotide diversity

The estimated genome-wide nucleotide diversity (π) for *B. emzantsi* is 0.00029, and is almost twice as large in the case of *B. percursus* 0.00052. Relative to the *Blastomyces* genus (π = 0.00905), this represents a ~31-fold and ~17-fold lower diversity for *B. emzantsi* and *B. percursus* populations than observed at the genus level, respectively.

5.4.8 Mating identification from WGS

Of the *B. percursus* strains, seven had the α mating type (*MAT1-1*) and three isolates (cases 1, 3 and 4) had a high motility group (*MAT1-2*). All *B. emzantsi* isolates belonged to the *MAT1-1*.

5.4.9 *BAD-1* gene identification from WGS

The full orthologue of the *BAD-1* gene was absent in all 18 *Blastomyces* strains; only a partial paralogue region was found.

5.4.10 Taxonomy

Blastomyces emzantsi sp. nov.: Maphanga, Govender, Birkhead, Frean; MycoBank MB828102.

Etymology: Referring to South Africa, the country of origin of the type species. Emzantsi means south in the isiXhosa language.

Morphologically, *B. emzantsi* can be differentiated on the combination of saprobic phase helical hyphae, abundant conidiogenesis, and clavate, complanate cells terminally or extending laterally from hyphal cells, together with incomplete transition to the yeast phase at 37°C (hyphae, adiaspore-like/giant cells and broad-based budding yeasts are all present in the thermotolerant phase).

Holotype: South Africa, National Institute for Communicable Diseases, dried herbarium specimen culture from subcutaneous abscess of an HIV-seronegative black African male patient with blastomycosis, described by Frean et al. in 1993; living strain NICD (SA-NICD-15; case 9)

Physiology: Minimum growth temperature 8°C, optimum 25°C reaching 55 mm on SAD, maximum 37°C; urease positive.

Differential molecular diagnosis: *B. emzantsi* can be diagnosed by the following nucleotide characters, which are fixed in *B. percursus, B. dermatitidis, B. gilchristii, B. parvus, B. helicus* and *B. silverae*: *ITS* 2 of rDNA at position 140 (T:C); 169 (A:T)

5.5 DISCUSSION

We re-examined clinical isolates from 20 human cases of blastomycosis from South Africa from 1967 to 2014, and found that these were not *B. dermatitidis*, as initially believed (Martin and Berson, 1973, Simon et al., 1977, Carman et al., 1989, Frean et al., 1989, Frean et al., 1993, Heys et al., 2014, Schwartz et al., 2015, Dukik et al., 2017), but rather different species: the recently described *B. percursus* (Dukik et al., 2017) and a novel species, described here as *B. emzantsi*.

Only four of the ten cases of blastomycosis with detailed clinical information had diffuse pulmonary infiltrates on chest X-ray, while all ten cases had extra-pulmonary disease. This is consistent with previous reports on cutaneous and bone disease being more common than pulmonary disease in African blastomycosis in contradistinction to the pattern in North America (Carman et al., 1989, Saccente and Woods, 2010). Six of ten cases had cutaneous lesions. Both *B. percursus* and *B. emzantsi* caused vertebral and bone collapse. The clinical picture of vertebral blastomycosis mimics that of vertebral TB, thus making clinical diagnosis challenging, particularly in a high-prevalence setting for TB (Saccente and Woods, 2010).

The treatment of choice for North American blastomycosis is itraconazole for mild infections and amphotericin B followed by itraconazole for severe infections (Chapman et al., 2008, Saccente and Woods, 2010). Four *B. percursus* cases, for whom treatment and outcome data were available, recovered after receiving azole or amphotericin B treatment. Although, there are no clinical trials to compare the efficacy of these regimens for treatment of blastomycosis, *in vitro* MIC data for *B. dermatitidis*, *B. percursus*, and *B. helicus* suggest that the azoles (excluding fluconazole), and amphotericin B are active (Espinel-Ingroff, 1998a, Espinel-Ingroff, 1998b, Espinel-Ingroff, 2003, Nakai et al., 2003, Dukik et al., 2017). According to previous reports, micafungin is only active against the mould- phase and weakly- active against the yeast- phase (Espinel-Ingroff, 2003, Nakai et al., 2003). Nakai *et al*. (2003) reported an MIC range of 0·0078 µg/ml to 0·0625 µg/ml for the mould-phase and 32 µg/ml to >64 µg/ml for the yeast- phase for *B. dermatitidis* with this antifungal agent. In contrast, there was no difference in micafungin activity against the two phases of *B. percursus* and *B. emzantsi* in our study.

Macroscopically, there was incomplete transition from the mould to the yeast phase, with most of the strains producing hyphal elements at 37°C. Similar results were reported by Kaufman et al. (1983) and Lombardi et al. (1988). The observation of hyphal elements in tissue warrants special mention, as histopathologists should be aware of these findings. Microscopically, the maximum size of the yeast cells of *B. percursus* (9 µm x 14 µm) were larger than those previously reported (6.5 µm x 12.2 µm) (Dukik et al., 2017, Jiang et al., 2018). *B. emzantsi* produced larger broad-based budding yeast cells that were more similar to those of *B. parvus*, which until recently were thought to produce adiaspores (Jiang et al., 2018). Helical hyphal gyres bearing conidia were present in the mycelial phase, similar to those previously described for *B. parvus* and *Emmonsia sola* (at 21°C) (Jiang et al., 2018, Sigler, 1996).

The *ITS* region is a formal fungal barcode, which cannot always distinguish closely-related cryptic species (Nguyen et al., 2015). In this study, all eight *B. emzantsi* isolates were identified as *B. helicus* or *B. parvus* based on *ITS* sequencing and a BLAST search; however, phylogenetic analysis of these *ITS* sequences separated the species into different clades. Of the 20 presumptive *B. dermatitidis* South African strains analysed, none belonged to *B. dermatitidis* or *B. gilchristii*. Concatenated sequences of the *ITS-LSU-PRP8*-β-tubulin-actin genes could not separate *B. dermatitidis* from *B. gilchristii*, but clearly differentiated *B. percursus, B. parvus,* and *B. emzantsi* from the former two species, forming four clades. In contrast, concatenated sequences of the *CHS2-DRK1-FADS-PYRF-TUB1-ARF6* genes differentiated all five *Blastomyces* species well, resulting in five clades. The concatenated sequences of the six genes also demonstrated that *B. percursus* and *B. emzantsi* differed from three African strains (two *B. gilchristii* and one *B. dermatitidis*) previously reported (Brown et al., 2016). One of those isolates identified by Brown et al. (2013) as *B. gilchristii* - CDC B1566 (UAMH10245) - was from South Africa (Brown et al., 2016). The exact provenance of the strain is not clear, but it does not appear to be among the 20 isolates currently in the NICD's collection that were included in this study. Furthermore, based on whole genome analysis, the degree of genetic variation supports that *B. emzantsi* is a single species well separated within the *Blastomyces* genus.

The genomic sizes of *B. percursus* (32·1-34·2 Mb) and *B. emzantsi* (27·4 Mb -32·5 Mb) were similar to other closely-related dimorphic fungi; however, they differed from those reported for *B.*

dermatitidis (66·6 Mb) and *B. gilchristii* (75·4 Mb) (Muñoz et al., 2015). Of the 18 *Blastomyces* strains analysed for mating type, only the three recently- diagnosed cases (case 2, 3 and 4) of *B. percursus* contained *MAT1-2* gene, while the older- diagnosed cases of both *B. percursus* and *B. emzantsi* contained *MAT1-1*. The fact that two alleles are not evenly distributed in *B. emzantsi* and *B. percursus* suggests clonal expansion of *MAT1-1* and selection favouring *MAT1-1*; however, wider sampling is needed to confirm these trends (Li et al., 2013).

BAD-1 is an important adhesion-promoting protein and virulence factor in *B. dermatitidis* strains (Finkel-Jimenez et al., 2002). We could not identify the entire *BAD-1* gene in our strains. Previous studies have also reported the absence of the *BAD-1* gene from African *Blastomyces* strains (Klein et al., 1997, Meece et al., 2010).

5.5.1 Study limitations

Our study has several limitations. Only cases reported to a reference laboratory were included, which precludes estimation of the prevalence of blastomycosis in South Africa. If *B. percursus* and/or *B. emzantsi* are more likely to cause cutaneous disease than other species, then given that skin lesions (or draining pus) may be more accessible for culture, there may be a bias towards these species being represented in NICD's culture collection. This is plausible because we suspect that cases of pulmonary blastomycosis are misdiagnosed as smear- or Xpert MTB/RIF-negative pulmonary TB in South Africa. Our analysis included only clinical isolates from humans. In fact, neither veterinary blastomycosis nor environmental isolation of *Blastomyces* species have been reported from South Africa. Small numbers and incomplete clinical details limited our comparison of the diseases caused by these species. With this in mind, the two patients infected with *B. emzantsi* for whom clinical data were available had systemic, pyogranulomatous pulmonary and vertebral blastomycosis consistent with disease observed for *B. percursus* and not unusual for North America blastomycosis (Klein et al., 1997). Whether *B. emzantsi* is more likely to be associated with this syndrome will require evaluation of additional cases.

5.6 CONCLUSION

South African cases of blastomycosis reported to a national mycology reference laboratory over five decades were caused by two species, *B. percursus* and *B. emzantsi*, and not *B. dermatitidis* as

had been presumed. Both taxa caused disease with overlapping clinical presentations, with high proportions of extra-pulmonary disease. Although histology and culture are useful methods in the diagnosis of blastomycosis, our analysis highlights the importance of molecular methods to identify emerging species correctly as causes of old diseases.

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5.8 AUTHORS' CONTRIBUTIONS

Specimen collection: ISS, JN, CB

Processing of urine specimens: TGM, SDN, MM

Data analysis and manuscript writing: TGM, NPG

Critical review of manuscript: TGM, BG, ISS, SDN, JN, RSM, CB, NPG

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5.10 CONFLICTS OF INTEREST

None declared

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Table 5.1: Clinical characteristics of 10 patients with South African blastomycosis, 1967-2014

Case #	Year of isolation/ Publication (Accession #)	Demographic information	Clinical presentation, & radiology findings	Laboratory findings	Treatment, outcome (duration of follow-up)
1	1986/ (Dukik et al., 2017). (QKWI00000000; NCPF4091)	Age: Unknown Race & sex: Unknown Province: Gauteng Comorbidities: Unknown	History: Ulcerative skin lesions Examination: Unknown Radiology: Unknown	Specimen type: Skin Histopathology: Yeasts morphologically resembling *B. dermatitidis* Culture identification: *B. dermatitidis* ITS amplification & sequencing: *B. percursus*=99%-100%)	Unknown
2	**2008/** (Heys et al., 2014, Schwartz et al., 2015) (LGTZ00000000;BP222)	Age: 52-year-old Race & sex: White, Male Province: Western Cape Comorbidities: HIV seronegative	History: 7-month history of headache with features suggestive of raised intracranial pressure, and 2-month history of ataxia Examination: Ataxic and bilateral papilloedema. Tone, power, reflexes, and sensation of extremities were normal. Respiratory examination findings were not noted Radiology: Computerised tomography brain scan: Solid contrast-enhancing cerebellar lesion with multiple small abscesses; Chest X-ray: bilateral perihilar disease and pulmonary infiltrates	Specimen type: Brain tissue Histopathology: Yeasts with broad-based budding and double refractile walls, suggestive of *B. dermatitidis* Culture identification: *B. dermatitidis* ITS amplification & sequencing: *B. percursus* (99%-100%)	Amphotericin B followed by itraconazole Recovered (3 years)
		Age: 52-year-old Race & sex:	History: 6-month history of cough with haemoptysis, weight loss, which had progressed in spite of empiric therapy	Specimen type: Skin & endobronchial tissue Histopathology:	

		Black, Male	for tuberculosis; and 3-month history of fleshy skin lesions on right lower lip and right nasolabial fold	Periodic acid Schiff (PAS) and Grocott stains; broad-based budding yeast and	
3	**2014/** (Schwartz et al., 2015) (QGQT00000000; SA-NICD-06)	Province: Western Cape Comorbidities: HIV seronegative	Examination: Digital clubbing; wart-like lesions of the naso-labial fold and right lower lip; right-ward deviation of the trachea; dullness of right upper lung zone with decreased breath sounds. Bronchoscopic examination visualized a fleshy endobronchial mass in the right main bronchus Radiology: Chest X-ray: dense right upper lobe infiltrate	double refractile walls suggestive of *B. dermatitidis* Reactive squamous epithelium and stroma with sheets of foamy histoocytes and granuloma with central microabscesses. Within these are scattered encapsulated yeasts resembling *B. dermatitidis* Culture identification: *B. dermatitidis* ITS amplification & sequencing: *B. percursus* (99%-100%)	Amphotericin B for 14 days followed by itraconazole Recovered (11 months)
		Age: 34-year-old Race & sex: White, Male	History: 5-year history of cutaneous lesions of the groin that resolved without intervention, followed by a 5-month history of skin lesions in the perineum & groin that began as pustules	Specimen type: Skin Histopathology: Dermis showed dense granulomatous inflammation with neutrophilic microabscess formation. Large yeasts with thick capsules were noted in giant cells and in the inflammatory material, resembling *B. dermatitidis*	Itraconazole
4	2014/ This study (QGQR00000000;SA-NICD-08)	Province: Gauteng Comorbidities: HIV seronegative	Examination: Partially healed cutaneous ulcer with undermined edges and cribriform scarring in the perineum Radiology: Not done	Culture identification: *B. dermatitidis* ITS amplification & sequencing: *B. percursus* (99%-100%)	Recovered (unknown)
		Age: 63-year-old Race & sex: White, Male	History: 7-month of an ulcerative growth on the tongue. Examination: Lesion measured 3 cm x 4 cm and involved the right edge and lower surface about 2 cm from the tip of the tongue. Enlarged, mobile submandibular lymph nodes were present in the right side. The rolled edge of the ulcer and its site suggested a malignant lesion.	Specimen type: Tongue biopsy Histopathology: Broad based yeasts in relation to giant cell granulomas, resembling *B. dermatitidis* Granulomas with yeasts were seen in the lung.	None
5	**1975/** (Simon et al., 1977)	Province: Gauteng Comorbidities: Unknown			Died

	(QGQS00000000; SA-NICD-05)		Radiology: Not done	Culture identification: *B. dermatitidis* ITS amplification & sequencing: *B. percursus* (99%-100%)	
6	**1983/** (Carman et al., 1989, Frean et al., 1989) (QGQQ00000000; SA-NICD-04)	Age: 34-year-old Race & sex: Black, Male Province: Free State Comorbidities: Unknown	History: Unknown Examination: Post mortem examination identified disease of lung, skin (face), kidney, liver spleen and thyroid Radiology: Not done	Specimen type: Skin biopsy Histopathology: Yeasts morphologically resembling *B. dermatitidis* Culture identification: *B. dermatitidis* ITS amplification & sequencing: *B. percursus* (99%-100%)	Unknown Died
7	**1967/** (Martin and Berson, 1973) (SA-NICD-18)	Age: 27-year-old Race & sex: Black, Male Province: Gauteng Comorbidities: Unknown	History: Swelling of scalp for 4 weeks prior to admission. Subsequently developed similar lumps over the right iliac crest, the left clavicle and the chest wall over the left axilla. Burning on micturition, pain in both thighs, a feeling of lameness and low back pain for 2 weeks. Good appetite but lost weight for the past four weeks. Examination: New lesion appeared in the fibula and there was collapse of the first lumbar vertebra Radiology: Skull X-ray showed large abscess cavity 6 cm in diameter, from which pus discharged.	Specimen type: Pus from a clavicular lesion Histopathology: Bone: enormous yeast cells associated with giant cells and acute and chronic inflammation cellular reaction. Hourglass-type yeast cells resembling *B. dermatitidis* Culture identification: *B. dermatitidis* ITS amplification & sequencing: *B. percursus* (99%-100%)	Kanamycin and ampicillin, later amphotericin B, nystatin and griseofulvin Died 3.5 months after admission
8	**1989/** (Frean et al., 1993)	Age: 40-year-old Race & sex: Black, Male Province: Limpopo	History: Cough, weight loss, night sweats and dyspnoea; Readmitted 3 months later, with chest pain and dyspnoea and paravertebral abscess at the level of T4-5	Specimen type: Paravertebral abscess Histopathology: Chromoblastomycosis; broad-based budding yeasts resembling *B. dermatitidis* on potassium hydroxide stain	Initially given TB treatment followed broad-spectrum antibiotics and amphotericin

	(QGQJ00000000; SA-NICD-13)	Comorbidities: HIV seronegative	Examination: Emaciated. Clinical signs of hepatomegaly. Paraplegic, with no sensation in the legs and in moderate respiratory distress. Discharging sinus at T5 level. Radiology: Chest x-ray showed a diffuse nodular infiltrate with paratracheal and hilar lymphadenopathy; Spine x-ray showed collapse of the T4 and T5 vertebrae	Culture identification: *B. dermatitidis* ITS amplification & sequencing: *B. helicus or B. parvus* 97%	B (30 mg daily) intravenously Died
9	Unknown/ (Frean et al., 1993) (QGQI00000000; SA-NICD-15)	Age: 31-year-old Race & sex: Black, Male Province: North West Comorbidities: HIV seronegative	History: 3-month history of productive cough, weight loss and dyspnoea. Examination: Raised erythematous patches on the skin of the face, forehead, ears and anterior chest developed 4 months admission; the skin was biopsied and blastomycosis was diagnosed Radiology: Chest x-ray showed a fluffy infiltrate in the left lung and bilateral nodular infiltrates, hilar lymphadenopathy and erosion of several ribs. Spine x-ray showed collapse of T10 and T12 vertebrae	Specimen type: Subcutaneous abscesses Histopathology: Yeasts resembling *B. dermatitidis* Culture identification: *B. dermatitidis* ITS amplification & sequencing: *B. helicus* or *B. parvus* 97%	Initially treated with anti-tuberculous agents, amphotericin B (1.8 g over 12 weeks) Recovered (3 months)
10	**1975/** (Carman et al., 1989, Frean et al., 1989) **[**3,4] (QGQK00000000; SA-NICD-14)	Age: Adult, unknown Race & sex: Black, Male Province: Northern Cape Comorbidities:	History: Unknown Examination: Unknown Radiology: Unknown	Specimen type: Subcutaneous abscesses Histopathology: Unknown Culture identification: *B. dermatitidis* ITS amplification & sequencing: *B. helicus* or *B. parvus* 97%	Unknown Died

	Severe combined immune deficiency

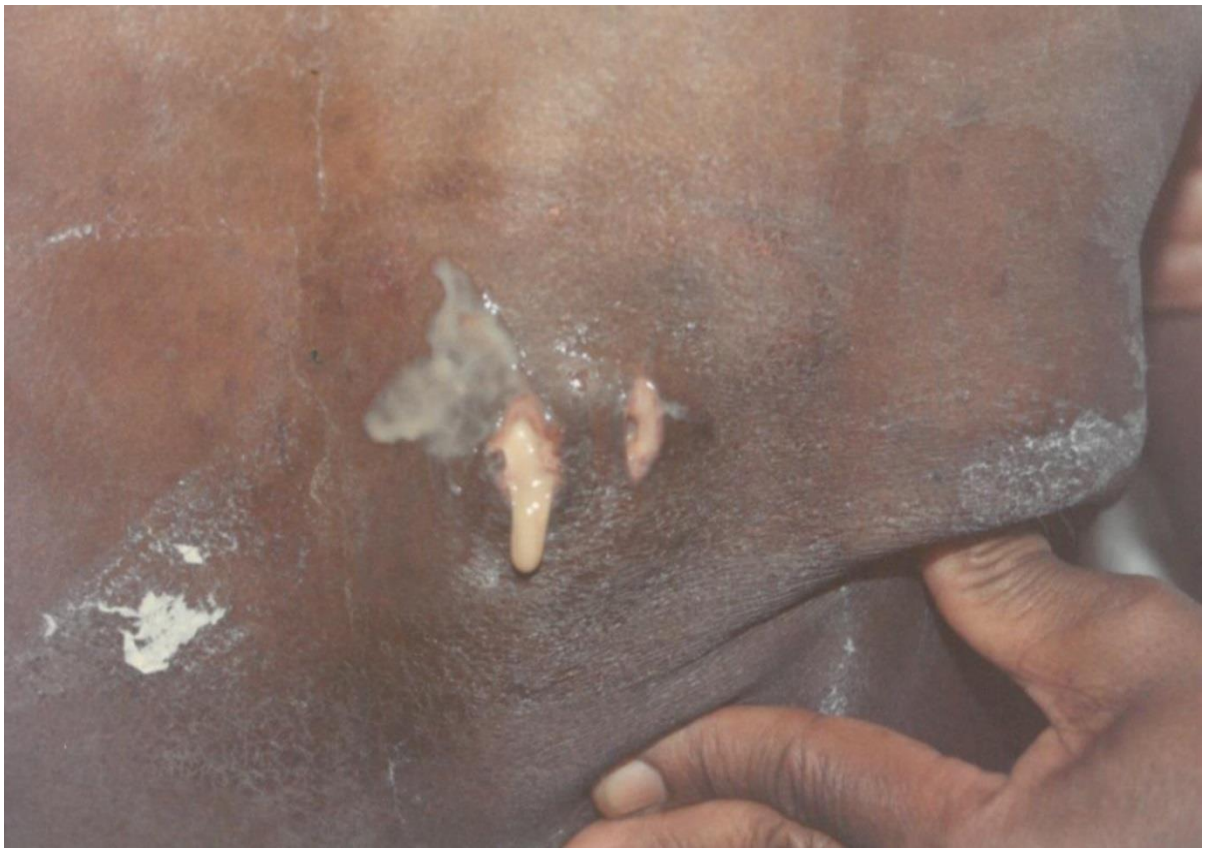

Figure 5.1. A case of disseminated blastomycosis caused by *Blastomyces emzantsi*, 1967-2014. Purulent discharge from a cutaneous sinus tract at T5 veterbral level (case 8) (reproduced with permission from Frean J, et al. J Infect 1993;26:203-206).

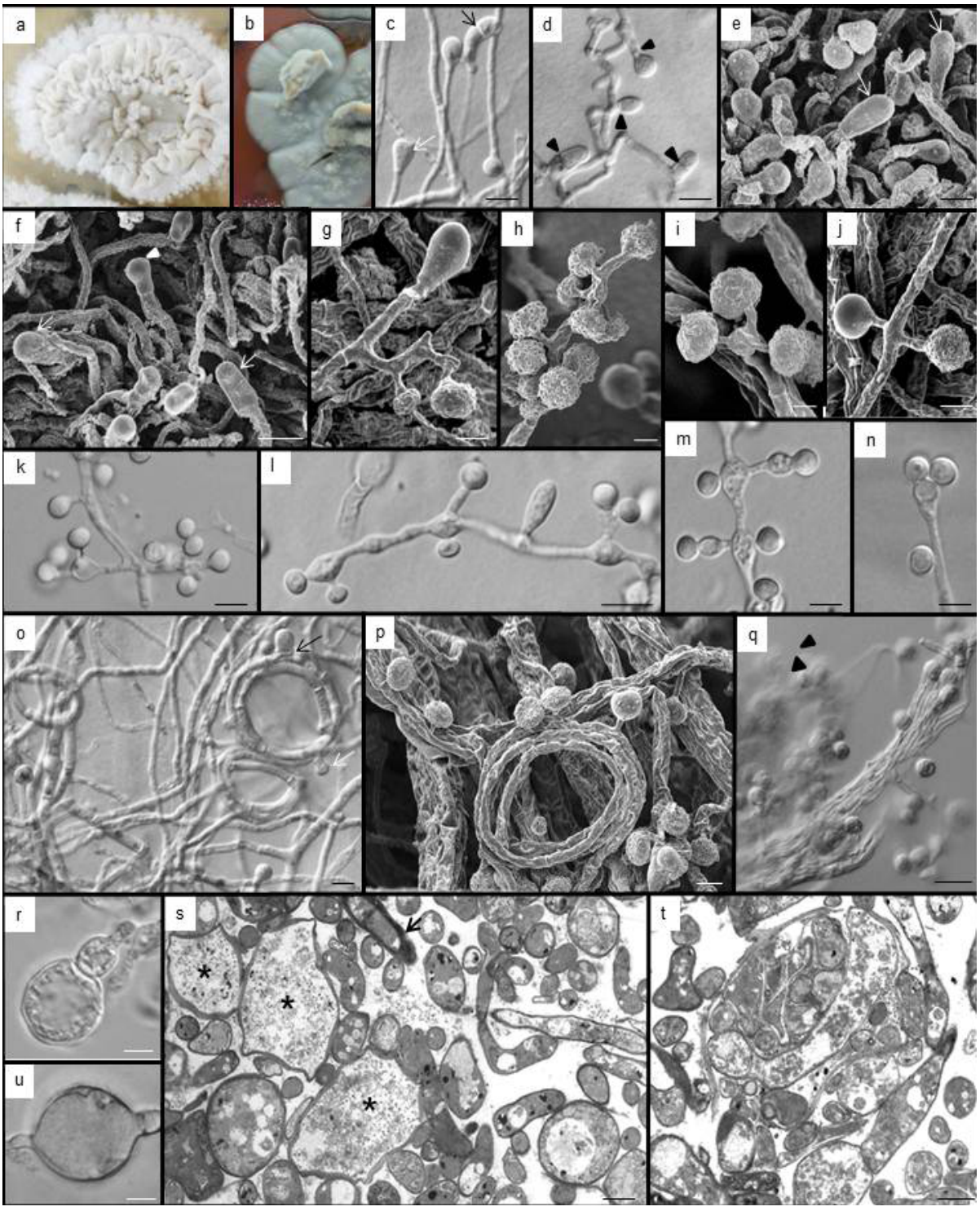

Figure 5.2. Morphology of *B. emzantsi*: saprophytic phase (25°C) on SDA after two weeks (a,c-k,o-q); intermediate phase (30°C) after three weeks on BHI agar with 5% horse blood (b) or SAD agar (l-n, r-u). (a) a floccose, white colony, with a narrow margin peripheral to the crumpled central areas; (b) at 30°C on

BHI with 5% horse blood, colonies were grey (darker grey on the reverse), with numerous aerial hyphal tufts and a flattened margin; (c) two terminal conidia, a developing clavate cell (white arrow) and a clavate cell with secondary septation (black arrow); (d) hyphal septation (arrowheads) in conidiogenesis and clavate cell formation; (e) clavate, complanate cells (arrows) clearly distinguishable from developing conidia; (f) complanate terminal cells (arrows) and a developing terminal conidia (arrowhead); (g) a hyphal cell subtending single, lateral conidia and a terminal, basally septate, clavate cell; (h) the apparent clusters of conidia are usually due to the short pedicels of many of the laterally-positioned conidia; (i) multiple conidia on short secondary conidiophores can be subtended from a single basal cell; (j) conidia ornamentation is variable, from glabrous to papillate; (k) the single conidia may be lateral or clustered on vesiculate primary conidiophores; (l) clavate cell between conidiophores, with variously arranged conidia. Some inflation of subtending cells apparent at intermediate temperature; (m) increase in temperature results in conidiophores becoming increasingly ampulliform and vesiculate, with distended hyphal cells. Light microscopy creates the illusion that one of each of the laterally paired conidia is sessile; (n) expanded primary conidiophore with two conidia on extremely short secondary conidiophores; (o) helical hyphae, one with both developing clavate cell (black arrow) and conidia (white arrow); (p) Numerous conidia developing on hyphal gyres, with bundles of older hyphae behind them; (q) abundant verrucose conidia, and hyphae aggregated into rope-like structures; (r) development of an adiaspore-like cell (inflated terminal conidia); (s) sectioned material provides accurate imaging of the diversity of cell forms, including the development of giant cells (*) from enlarged, fragmented hyphal cells, increased vacuolation of enlarging cells, numerous thin hyphal profiles, and some thick-walled cells (arrow; tangentially sectioned); (t) a section though the hyphal bundles seen with light and scanning microscopy, reveals numerous endo/intrahyphal profiles; (u) an inflated intercalary cell, suggestive of a chlamydospore. Scale bars: c, d, k, l, m, n, o, q, s, u = 5 µm; e, f, g, h, i, j, p, r , t = 2 µm.

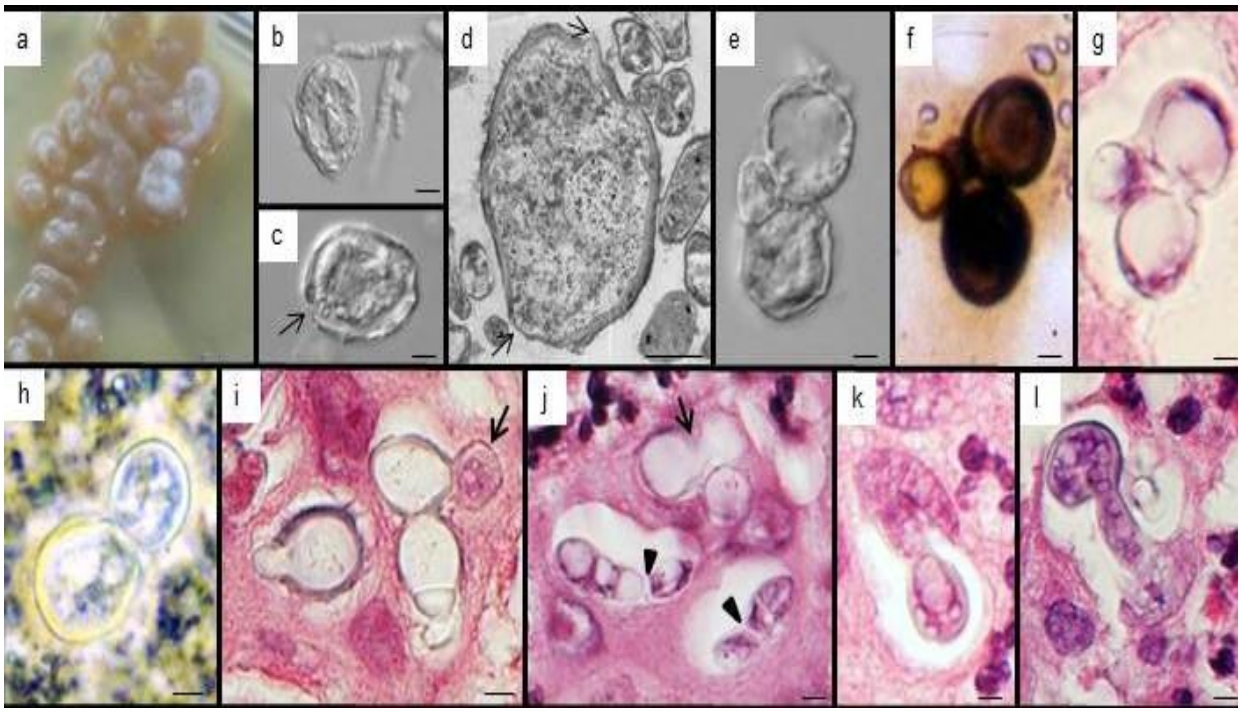

Figure 5.3. Morphology of *B. emzantsi* at 37°C: (a-e) cultured isolates on BHI agar at 4 weeks; (f) Grocott's stain of paravertebral pus smear; (g, i-l) haematoxylin+eosin stained section through sinus tissue of vertebral abscess; (h) potassium hydroxide stain of pus. (a) beige, butyrous, cerebriform colony still with some white, aerial hyphal tufts; (b) incomplete transformation in culture was typified by large, thick-walled, yeast-like cells juxtaposed to hyphal fragments; (c) thick-walled, giant cell filled with cytoplasm (indicative of active, viable cell). The slight disruption in the cell wall (arrow) may be the beginning of a budding process; (d) a section through one of the large, thick-walled cells filled with cytoplasm. A number of thin areas in the cell wall (arrows) are suggestive of the initiation of multiple budding sites. Note the many vegetative hyphal profiles surrounding the giant cell; (e) budding yeast-like cells with a smaller lateral cell, from cultured isolate; (f) similar configuration of cells as in (e) though photographed from the clinical specimen; (g) section through infected sinus tissue, again illustrating the yeast-like budding cells with a smaller laterally-positioned cell; (h) the original image mentioned in Frean et al (1993) showing a typical broad-based budding yeast cell; (i) a number of budding, thick walled cells, sectioned through various planes. It would appear that one of the daughter cells (arrow) has penetrated the tissue around the phagocytic vacuole; (j) although budding cells are apparent in the section (arrow), other budding-cell profiles appear to contain hyphal cells (arrowheads), as the cytoplasm is delimited by a cell wall on the inside of the host vacuole; (k) hyphal invasion of surrounding tissue from daughter cell within phagocytic vacuole; (l) hyphal-like extrusion of thicker-walled cell into surrounding sinus tissue. Scale bars: b, d, e, f, g, h, i, k = 2 µm; c, j, l = 3 µm

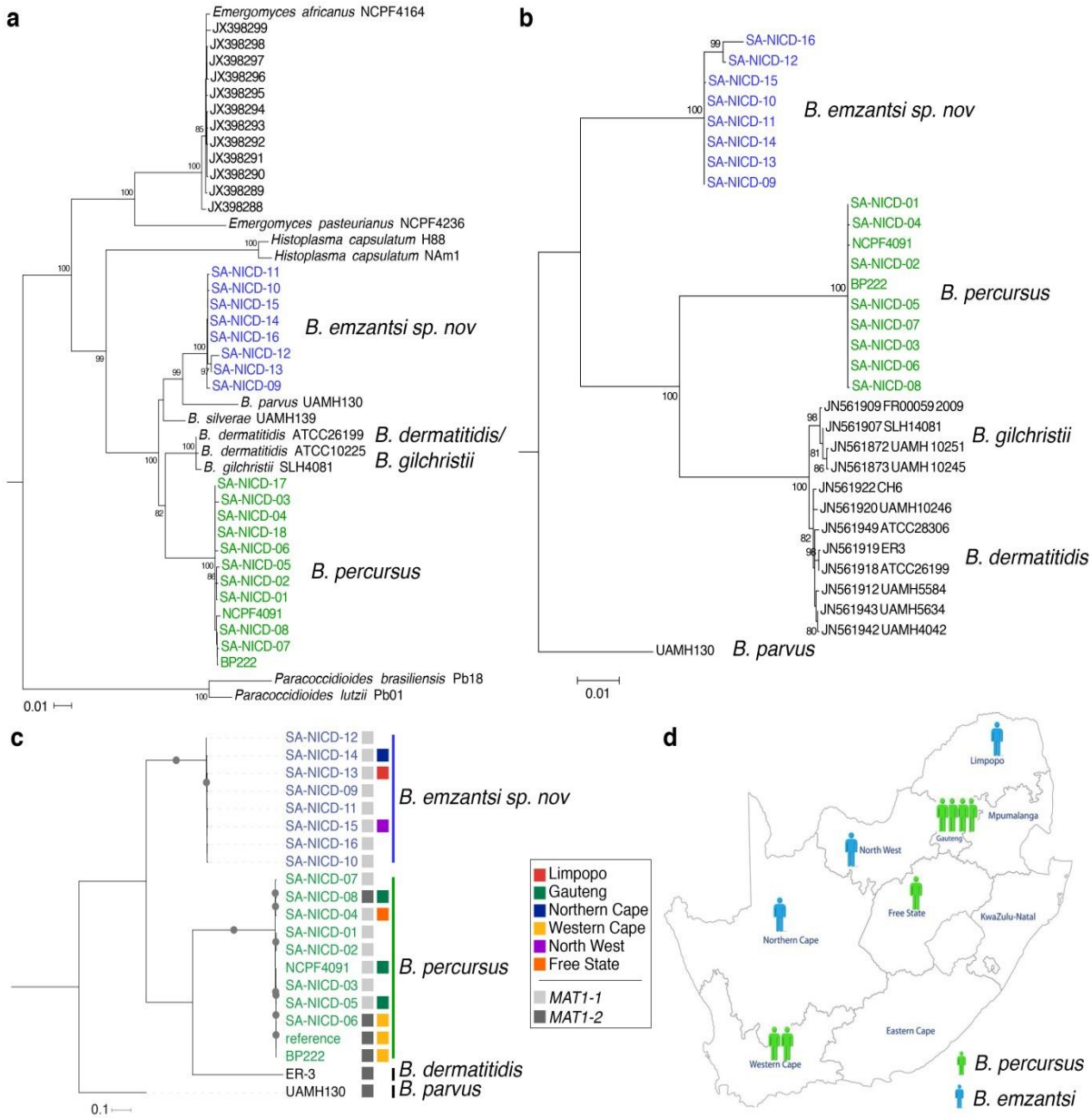

Figure 5.4. Multi-gene phylogenies of *B. percursus* (group 1; SA-NICD-01 to SA-NICD-08, SA-NICD-17, SA-NICD-18) and *B. emzantsi* (group 2; SA-NICD-09 to SA-NICD-16). 5.4A and 5.4B: Maximum likelihood tree inferred from concatenated gene alignment of *ITS2-LSU-PRP8*-β-tubulin-actin and *CHS2-DRK1-FADS-PYRF-TUB1-ARF6*, respectively based on 1000 replicates. 5.4A. Sequences of *Paracoccidioides lutzii*, *Paracoccidioides brasiliensis*, *Blastomyces dermatitidis*, *Histoplasma capsulatum* and *Emergomyces africanus* were obtained from Kenyon *et al.* (2013) and *Blastomyces silverae* and *Blastomyces parvus* were obtained from Jiang *et al.* (2018) (Kenyon et al., 2013, Jiang et al., 2018). 5.4B. Sequences of *B. dermatitidis* and *B. gilchristii* were obtained from Brown *et al.* (2013) (Brown et

al., 2013). 5.4C. Maximum likelihood tree inferred from SNP including three annotated genome assemblies of *Blastomyces* species (*B. dermatitidis* ER3, *B. parvus* UAMH 130 and *B. percursus* BP222). All nodes were supported by 100% of bootstrap replicates. The boxes indicate the mating type for each *Blastomyces* species. 5.4D. Geographic distribution of *B. percursus* (green) and *B. emzantsi* (blue) cases in South African provinces.

CHAPTER 6

Molecular identification of dimorphic fungi causing systemic mycoses among South African patients

The editorial style of Journal of Clinical Microbiology was followed in this chapter – this chapter has not yet been reviewed by co-authors or submitted for publication

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Keywords: *Emergomyces africanus*, *Histoplasma capsulatum*, *Sporothrix schenckii*, *Blastomyces percursus*, systemic endemic mycoses, dimorphic fungi

6.1 ABSTRACT

Introduction:

Although culture remains the gold standard for identification of dimorphic pathogens in resource-limited countries, sequence-based assays provide an alternative powerful tool for the accurate identification of dimorphic fungi causing systemic mycoses. We aimed to determine whether DNA- sequence based assays can be used for identification of emerging dimorphic pathogens in patients with systemic mycoses.

Materials and Methods: We conducted passive laboratory-based surveillance for systemic mycoses from July 2008 to July 2019 at 18 public and five private laboratories in South Africa. All cultured isolates from patients with systemic mycosis previously identified by macroscopic and microscopic examination at diagnostic laboratories were sent to the NICD for species-level identification. DNA-sequencing targeting the *ITS* region was perfomed for all clinical cultured isolates and *LSU* seuqncing was performed on isolates that failed amplification by *ITS* sequencing at the reference laboratory. Calmodulin sequencing was used to confirm *Sporothrix schenckii* isolates to species level. The identity of these isolates was further identified by the culture method at the reference laboratory.

Results: All 156 cases had systemic mycoses based on invasive specimens sent for fungal culture. At the diagnostic laboratories, culture-based methods correctly identified 63% (99/156) of the dimorphic isolates when compared to DNA- sequencing method performed at the reference laboratory. The isolates correctly identified included 66% (71/108) *Emergomyces*, 44% (14/32) *Sporothrix* species, 80% (8/10) *Histoplasma* species, 100% (4/4) *Blastomyces* species and (2/2) *Talaromyces marneffei*. At the reference laboratory culture-based methods correctly identified 95% (148/156) of the isolates when compared to the DNA- sequencing, the remaining seven isolates were either contaminated or had no fungal growth. Of the 156 dimorphic isolates sent for species identity, *ITS* sequencing correctly identified 86% (134/156) to species level at the reference laboratory. These included *E. africanus* (100%, 106/106), followed by *S. schenckii* (31%, 10/32), *H. capsulatum* (100%, 10/10), *B. percursus* (100%, 4/4), *E. pasteurianus* (100%, 2/2) and *T. marneffei* (100%, 2/2). Seventeen of the *S. schenckii* isolates were non-typeable by ITS PCR and LSU sequencing confirmed genus identity with a good score identity (95% to 100%). Calmodulin

sequencing confirmed species and these clinical isolates belonged to *Sporothrix schenckii* sensu stricto. Emergomycosis (n=108) was the most common mycosis followed by sporotrichosis (n=32), histoplasmosis (n=10), blastomycosis (n=4) and imported talaromycosis (n=2). Cutaneous lesion and pulmonary involvement were the most common clinical features.

Conclusions: DNA-sequencing was a valuable tool for accurate identification of dimorphic pathogens to species-level, which could facilitate early diagnosis and initiation of proper treatment. However, in settings where DNA-sequencing is not available, training of laboratory staff is necessary for the improvement of diagnosis of these pathogens in South Africa.

6.2 INTRODUCTION

Culture methods remain the mainstay for fungal identification particularly in resource-limited countries (Scheel and Gómez, 2014). However, diagnosis of endemic mycoses is often delayed by longer incubation periods for culture and the expertise needed for correct identification of dimorphic pathogens (Kozel and Wickes, 2014; Moodley et al., 2019). DNA sequencing of highly conserved regions such as *ITS* region, *LSU* and calmodulin gene from cultured isolates has increasingly been utilised for accurate identification of dimorphic fungal pathogens (Kenyon et al., 2013). DNA sequencing methods has also been used for correct taxonomy classification of dimorphic fungi (Rodrigues et al., 2013, Dukik et al., 2017a, Jiang et al., 2018). However, despite the tremendous advances in DNA sequencing, the method has not yet been widely adopted by most laboratories to identify pathogenic fungi, particularly in resource-limited countries (Schwartz et al., 2017a).

Over the past few years, the number of emerging dimorphic pathogens has increased globally (Rodrigues et al., 2013, Dukik et al., 2017, Jiang et al., 2018, Schwartz et al., 2017b, Schwartz et al., 2018). In 2013, the introduction of DNA sequencing in a reference laboratory permitted the identification of a novel pathogenic fungus (previously known as *Emmonsia*) found to be closely related to *Emergomyces pasteurianus* (previously known as *Emmonsia pasteriana*) (Kenyon et al., 2013). This novel *Emmonsia* was later named *E. africanus* and was placed within a new genus *Emergomyces* (Dukik et al., 2017, Govender et al., 2018). To date, fewer than 200 emergomycosis

cases caused by *E. africanus* have been reported in South Africa (Maphanga et al., 2017, Friedman and Schwartz et al., 2019). Several other *Emergomyces* species causing invasive fungal diseases among immunocompromised patients have been reported on four continents, i.e. Africa, North America, Asia and Europe (Friedman and Schwartz et al., 2019). In 2017, Dukik *et al*. used DNA sequencing to identify a novel *B. percursus* causing blastomycosis predominantly among immunocompetent patients in Israel and Africa. In another study, DNA sequencing was used to identify dimorphic fungi cultured from South African patients with advanced HIV who presented with skin infection (Schwartz et al., 2017a). *E. africanus* was the most common pathogen isolated followed by *S. schenckii* sensu stricto and *H. capsulatum* (Schwartz et al., 2017a).

The increasing number of these emerging dimorphic pathogens highlights the need for accurate and reliable methods for identification in laboratories, particularly in clinical settings where there is a high prevalence of HIV infection (Bongomin et al., 2017, Dukik et al., 2017a, Cacéres et al., 2019). This will allow proper diagnosis and early initiation of treatment. We therefore aimed to assess the feasibility of DNA- sequencing in the identification of emerging dimorphic fungal pathogensin a reference laboratory in South Africa when compared to phenotypic based methods and to understand the epidemiology of these pathogens in South Africa.

6.3 MATERIALS AND METHODS

6.3.1 Fungal isolates and case definition

The NICD conducted passive laboratory-based surveillance for systemic endemic mycoses from July 2008 to July 2019 at 23 laboratories in both public- (n=18) and private-sector (n=5) hospitals in South Africa. We defined a case of invasive fungal infection as a patient with an isolate cultured from any normally-sterile site (i.e. tissue, fluids, pus (not swab), blood, cerebrospinal fluid, skin biopsy, bone marrow aspirate, corneal) and confirmed to be a dimorphic fungus by culture methods in diagnostic laboratories. Diagnostic laboratories were requested to submit isolates of confirmed dimorphic infection cases accompanied by case report forms to the NICD. The completed case report form included patient details such as demographic data (age, sex etc), clinical presentation, previous medical conditions including HIV and co-infections, diagnostic investigations and treatment. In order to ensure that isolates and completed case report forms were sent to the NICD, participating laboratories were reminded to continue submitting isolates to the NICD for

identification by culture and DNA-sequencing methods. Some of these laboratories do not have microbiologist who are competent to identify dimorphic fungal pathogens.

6.3.2 Molecular identification from cultured isolates

All participating laboratories submitted cultured isolates on SDA (DMP) - NHLS, Sandringham, South Africa) or blood agar (DMP) to NICD for genus or species-level confirmation. The colony morphology was examined using the biosafety cabinet in a biosafety level 2 facility for contamination. If the cultured isolate did not have pure growth, the isolate was sub-cultured onto SDA, incubated at 25°C and monitored until pure culture was obtained. Genomic DNA was extracted from 156 dimorphic fungi using Zymo ZR fungal/Bacterial DNA MiniPrep (Zymo Research Corporation, Irvine, California, USA). Initially, PCR amplification and sequencing were performed by targeting the *ITS* domain of the multi-copy ribosomal RNA gene using *ITS1* and *ITS4* universal primers (White et al., 1990). If there was no amplification with *ITS* PCR, the *D1/D2* domain of the ribosomal RNA gene was targeted using *LROR* and *LR5* primers. Calmodulin PCR was only performed on *Sporothrix* isolates submitted for species-complex identification from July 2014, as described previously (Rodrigues et al., 2013; Govender et al., 2015). Exonuclease I and Shrimp alkaline phosphatase enzymes (Thermo Fisher Scientific) were used to purify PCR amplicons. Purified amplicons were sequenced using primers that were used during PCR amplification. The samples were sequenced using BigDye terminator version 3.1 in an ABI 3500 genetic analyser (Applied Biosystems, Foster City, California, USA). Identification of the fungi was performed by pairwise sequence comparison using the NCBI BLAST algorithm (www.ncbinlm.nih.gov/blast), If a lower score (<95%) was obtained, we used CBS-KNAW database for identity confirmation. Results greater than 95% were considered reliable for fungal species identification. Consensus sequences were generated for isolates with scores lower than 95%. A preliminary report was sent to the referring laboratory before culture results were obtained.

6.3.3 Genus confirmation by phenotypic methods

The same isolate used for molecular identification was sub-cultured onto SDA (DMP) for identification using the culture-based method by different laboratory personnel once molecular identification had been obtained and the results were sent to the referring laboratory. The staff member was blinded to the molecular results obtained for these isolates. The cultured isolates were

incubated at 25°C and 30°C until there was visible growth for identification. To confirm dimorphism, the mould phase of the cultured isolates was converted to the yeast phase by sub-culturing onto BHI agar plate (DMP). The cultured BHI plates were incubated at 35°C to 37°C for two to four weeks and checked weekly for contamination. Macroscopic examination was performed and the morphology of the mould-phase and yeast-phase was observed from the cultured isolates. Lactophenol cotton blue stain (DMP) and Gram stain (DMP) slides were prepared to observe the fungal structures of the mould and yeast phases microscopically. All testing was performed in a Biosafety level 2 laboratory and cultures were handled using a biosafety cabinet and personal protective equipment, including N95 respirators.

6.3.4 Statistical methods

Descriptive analysis was performed using STATA version 14.0 (StataCorp, Texas, USA)

6.3.5 Ethics approval

Ethics clearance was obtained from the Health Sciences Research Ethics Committee, University of the Free State, Bloemfontein (UFS-HSD2018/0992) and the Human Research Ethics Committee (Medical), University of the Witswatersrand, Johannesburg (01/2014).

6.4 RESULTS

6.4.1 Isolates and cases

One hundred and fifty-six cultured isolates from patients with systemic mycoses were submitted and tested at the NICD. The HIV status of 78% (122/156) of the patients was available, and of these, 97% (118/122) were HIV-infected. Of the 118 HIV-infected patients, 94 had recorded CD4 count with a median of 22 cells/µl (IQR, 8-48 cells/µl). The timing of CD4 count relative to fungal culture ranged from one month to 5 month. Dimorphic fungi were cultured from skin (n = 84), blood (n = 52), bone marrow (n = 8), abscess pus (n = 5), tissue from unknown origin (n = 4), tissue from brain (n = 1), CSF (n = 1) and corneal scraping (n = 1). The available demographic and clinical characteristics of the patients are summarized in Table 6.1. The geographic distribution of the dimorphic fungi causing systemic mycoses in seven of the nine South African provinces is shown in Table 6.2.

6.4.2 Identification of dimorphic fungi by phenotypic methods at the diagnostic laboratories

All 156 cases were suspected of having systemic mycoses based on the above specimen types submitted to the diagnostic laboratories. Eight of the ten *Histoplasma* species isolates were correctly identified, while all *Blastomyces* species (*n* = 4) and *Talaromyces marneffei* (*n* = 2) isolates were correctly identified (Table 6.3). Of the two *Histoplasma* isolates that were misidentified, one was reported as yeast and the other was not identified. Sixty five percent (71/108) of *Emergomyces* isolates were correctly identified to genus level, while 37 (34%) of the isolates were misidentified (Table 6.3, Figure 6.1). Most of these *Emergomyces* isolates that were misidentified were reported as *Sporothrix* species (*n* = 9), *Histoplasma capsulatum* (*n* = 6) or were reported as either *Emergomyces/Sporothrix* (*n* = 4), *Emergomyces/Histoplasma* (n = 1) or *Sporothrix/Histoplasma* (*n* = 1). The remaining *Emergomyces* isolates were reported as yeast without identity (*n* = 5), *Cryptococcus laurentii* or *Rhodotorula* (*n* = 3), *Trichosporon* (*n* = 1), mould (*n* = 3) or dimorphic (*n* = 4) (Table 6.3, Figure 6.1). Of the 32 *Sporothrix* species sent for identification, only 44% (14/32) were correctly identified to genus level, while 56% (18/32) were misidentified (Table 6.3, Figure 6.1). Misidentification of the dimorphic as *Cryptococcus laurentii* or *Rhodotorula* was obtained from the Vitek 2 (bioMérieux, Durham, NC) and Microscan (Siemens, West Sacramento, CA) systems. Overall, 63% (99/156) of the dimorphic pathogens were correctly identified by the diagnostic laboratories.

6.4.3 Identification of dimorphic fungi by phenotypic methods at the reference laboratory

The laboratory staff at the reference laboratory correctly identified 95% (148/156) of the dimorphic isolates by culture methods when compared to the diagnostic laboratories (Table 6.3). Five isolates that were previously identified at the diagnostic laboratories were contaminated with *Aspergillus* species after being subcultured on SDA (DMP) at the reference laboratories. Two isolates had no growth after three to four weeks of incubation; these isolates were previously identified as *H. capsulatum* and dimorphic fungi at the diagnostic laboratories and both were cultured from skin biopsy. One culture grew both *Yarrowia lipolytica* (previously *Candida lipolytica*) and *Emergomyces* species and the isolate was previously identified as a yeast at the diagnostic laboratories.

6.4.4 Identification of dimorphic fungi by DNA-sequencing methods at the reference laboratory

ITS sequencing at the reference laboratory correctly identified 84% (131/156) of the dimorphic fungi with a good score identity of 96% to 100% when compared to culture methods performed at both diagnostic laboratories and the reference laboratory (Table 6.3). Of the 131 dimorphic isolates, *ITS* sequencing correctly identified 106 (100%, 106/106) of *E. africanus* isolates, 10 (100%, 10/10) of *H. capsulatum* isolates, two (100%, 2/2) of *E. pasteurianus* isolates and another two (100% 2/2) of *T. marneffei* isolates (Table 6.3). Only 10 (31%, 10/32) of the *S. schenckii* sensu stricto isolates were correctly identified to species-level by *ITS* sequencing when compared to culture methods. Seventeen (53%, 17/32) of the *Sporothrix* isolates were non-typeable by *ITS* PCR and these isolates were identified by sequencing of the *LSU* region which gave a good genus score identity of 95% to 100%. Five (16%, 5/32) of the *Sporothrix* isolates did not have *ITS* or *LSU* results and species identity was confirmed by sequencing of the calmodulin gene. Overall, calmodulin sequencing was performed on 72% (23/32) of the *Sporothrix* isolates and these isolates were confirmed as *S. schenckii* sensu stricto with a good score identity (95% to 99%). *ITS* sequencing correctly identified four (100%, 4/4) *B. percursus*, of which three (75%, 3/4) were previously reported as *Emmonsia parva*. Two cultures had mixed growth, namely *Y. lipolytica* and *E. africanus,* and the other *C. neoformans* and *E. africanus.* Both *Y. lipolytica* and *E. africanus* isolates were confirmed by culture method at the reference laboratory, while *C. neoformans* and *E. africanus* cultures were contaminated before identification could be made by culture method at the reference laboratory.

6.4.5 Clinical presentation of the 156 cases with systemic endemic mycosis

Emergomycosis was the most common mycosis occurring in 68% (108/156) of all cases. Specimens from which diagnoses were obtained included blood (n=49), skin biopsy (n=49), bone marrow aspirate (n=8), nose tissue (n=1) and tissue from unknown origin (n=1). Of the 108 patients with emergomycosis, 82% (89/108) whose HIV status was available had HIV infection with a low CD4 count (median of 22 cells/µl (IQR, 8-48 cells/µl). Only 79% (31/39) received ART. Sixty seven percent 40% (43/108) of the patients presented with multiple skin lesions, 6% (6/108) with pulmonary disease and 27% (29/108) with both skin lesions and pulmonary. Five percent (5/108) of the patients presented with sepsis, while 1% (1/108) had brain lesion. The remaining 22%

(24/108) had no clinical information. One patient presented with chronic headache and a CT scan of the brain showed a non-enhancing brainstem lesion. Most of these patients (n=13) presented with similar symptoms, which included fever, weakness, weight loss, cough, nausea, diarrhoea and night sweats.

Sporotrichosis constituted 21% (32/156) of all systemic mycosis cases. Sporotrichosis was diagnosed from skin biopsy (n=21), pus abscess (n=5), blood (n=2), corneal scraping (n=1), CSF (n=1) and tissue from unknown origin (n=2). Seventy-eight percent (25/32) of the patients presented with multiple skin lesions, which were mostly located on the face and upper and lower limbs. *S. schenckii* sensu stricto was cultured from corneal scrapings from one of the 25 patients presenting with multiple skin lesions. Only two of the 25 patients were also diagnosed with pulmonary TB, one case being diabetic and having sepsis. Only one case was treated for TB. One of the 25 patients had had a liver transplant and was on immunosuppressive therapy. Two patients presented with no skin lesions. The first patient had chronic diarrhoea and renal failure and *S. schenckii* sensu stricto was cultured from blood. The second patient was diabetic and, on immunosuppressive therapy with after a renal transplant, *S. schenckii* sensu stricto was cultured from CSF. One patient presented with a typical case of lymphocutaneous sporotrichosis in the arm. The remaining four patients did not have any clinical information available. *S. schenckii* sensu stricto was cultured from a pus abscess in two of the patients and tissue from an unknown origin from the other patient. Eighty-eight percent (28/32) were males with a median age of 36 years (IQR, 31-42 cells/µl). Fifty nine percent (19/32) had HIV status available and all were HIV-infected. Only 95% (18/19) of those infected had a recorded CD4 count with a median of 22 (IQR, 10-55 cells/µl).

Histoplasmosis constituted 6% (10/156) of all systemic mycosis cases. All patients were HIV-infected and had a low CD4 count with a median of 22 cells/µl (IQR, 12-53 cells/µl), with only five receiving ART. All ten patients presented with skin biopsy and histoplasmosis diagnosis was made from skin biopsy. Two patients were concurrently diagnosed with pulmonary TB and both received treatment. Another patient was concurrently diagnosed with cryptococcosis. This patient had a positive *Cryptococcus* antigen on serum and her beta-D-glucan was 170 pg/ml (cut-off 60 pg/ml); the patient also and had microcytic anaemia.

Blastomycosis constituted 3% (4/156) of all systemic mycosis. *B. percursus* from the four patients was cultured from skin biopsy (n=3) and brain (n=1). All four patients were HIV-uninfected males. These patients presented with multiple skin abscesses and had pulmonary involvement, with one patient showing a brain lesion when a CT scan was conducted. Two of the four patients were smokers.

Talaromycosis constituted 1% (2/156) of all systemic mycosis. The first case was reported in 2014 from a South African woman, who travelled to mainland China. The patient was HIV-infected with a CD4 count of 20 cells/µl and on ART. The patient presented with hepatosplenomegaly and skin lesions on the face and talaromycosis was diagnosed from blood. The second case was reported in 2017 from a South African woman, who worked in Hong Kong. The patient was HIV-infected and talaromycosis was diagnosed from skin biopsy.

6.5 DISCUSSION

We evaluated the feasibility of using DNA sequencing method in a reference laboratory to identify dimorphic fungi causing systemic mycoses in South Africa. Based on the DNA-sequencing results, *E. africanus* was the most commonly identified species, followed by *S. schenckii* sensu stricto, *H. capsulatum*, *B. percursus*, *T. marneffei* and *E. pasteurianus.* These dimorphic fungi were the most common pathogenic fungi among HIV-seropositive patients. Cutaneous lesions and pulmonary involvement were the common clinical symptoms caused by these dimorphic pathogens. The pathogens were distributed in seven of the nine provinces in South Africa.

In resource-limited countries, culture remains the gold standard for diagnosis of dimorphic fungi (Kozel and Wickes, 2014, Schwartz et al., 2017a). In this study, the diagnostic laboratories were able to identify 63% of the isolates correctly using the culture method when compared to DNA sequencing. Although most of these isolates were correctly identified as dimorphic fungi, some of the isolates were misidentified as *Histoplasma* species instead of *Emergomyces* species and *Sporothrix* species instead of *Emergomyces* species. Misidentification of dimorphic fungi is common, particularly when culture or histology methods are used (Schwartz et al., 2017a). It is difficult to differentiate the yeast morphologies of these fungi, e.g. the yeast phase of *E. africanus*

may resemble that of *Histoplasma* or *Sporothrix* on culture or histology methods making accurate diagnosis difficult (Schwartz et al., 2017a, Moodley et al., 2019). Incorrect reporting of the identity of these isolates may have resulted in over-estimation of histoplasmosis cases in South Africa over the past years (Oladele et al., 2018)

The diagnosis of dimorphic fungi by the phenotypic method is a challenge because of the lack of experienced personnel (Caceres et al., 2019). Contamination of cultured isolates with overgrowth of rapid growing mould such as *Aspergillus* species may hinder accurate identification of these pathogens (Hsiao et al., 2005, Caceres et al., 2019). The prolonged incubation of these dimorphic pathogens that is required (e.g. *Histoplasma* may take four to six weeks, *Blastomyces* and *Emergomyces* two to three weeks) may result in missed cases (Dukik et al., 2017a, Caceres et al., 2019, Moodley et al., 2019). This is because some diagnostic laboratories discard cultured plates after seven days of incubation, particularly if there is no fungal growth, resulting in some cases being missed. Furthermore, confirmation of dimorphism is required before an accurate diagnosis can be made which delays patients's treatment/ management. Conversion of *Histoplasma*, *Emergomyces* and *Sporothrix* is easy on BHI agar, while *B. percursus* does not completely convert to the yeast phase (Frean et al., 1989, Carman et al., 1989, Jiang et al., 2018). The reason why *B. percursus* strains do not completely convert to the yeast phase warrants further investigation. In this study, *Emergomyces* and *Sporothrix* were misidentified as *Cryptococcus laurentii* or *Rhodutorula* species on Vitek and Microscan systems. It is important to question the morphology of the yeast isolate giving a *Cryptococcus laurentii* or *Rhodutorula* identity on Vitek or Microscan, particularly when the identity was obtained from a growing young culture isolate which grew after five to six days of incubation.

To date, few academic and private laboratories have set up molecular methods for routine identification of dimorphic fungi (Schwartz et al., 2017a, Moodley et al., 2019). *ITS* sequencing accurately identified *Histoplasma*, *Blastomyces*, *Emergomyces* and *T. marneffei* to species level but failed to amplify five of the 32 *Sporothrix* isolates. Identification of these isolates was done by *D1/D2* sequencing. The *ITS* region is not highly discriminatory for *Sporothrix* speciation (Rodrigues et al., 2013, Govender et al., 2015). We used the calmodulin gene to speciate these *Sporothrix* isolates and all isolates were found to belong to the *S. schenckii* sensu stricto. To date,

only *S. schenckii* sensu stricto has been reported to cause human disease among South African patients (Kenyon et al., 2013, Govender et al., 2015, Schwartz et al., 2017a). Species identification for *S. schenckii* isolate is important, particularly because *Sporothrix* is also a zoonotic pathogen and outbreaks of sporotrichosis caused by *S. brasiliensis* have been reported in Brazil (Rodrigues et al., 2013, Sanchotene et al., 2015). Therefore, speciation of *Sporothrix* isolates may allow identification of emerging *Sporothrix* species, which may be of public health concern. Based on *ITS* sequencing, *H. capsulatum* was the common species identified in South Africa. However, phylogenetic analysis studies are required for proper classification of this pathogen to a correct variant, particularly because *H. duboisii* has been reported as a major cause of histoplasmosis in Africa (Kenyon et al., 2013, Schwartz et al., 2017a, Oladele et al., 2018).

In addition to the 86 previously reported emergomycosis cases caused by *E. africanus* (Maphanga et al., 2017), *ITS* sequencing was useful in the identification of additional 22 patients infected with *E. africanus*. Furthermore, we reported a second case of *E. pasteurianus* from an HIV-seropositive patient who presented with multiple skin lesions and pulmonary disease. An increase in the number of *E. pasteurianus* cases has been noted in other countries, including Spain, France, the Netherlands, India and China, and more recently Uganda (Dukik et al., 2017a, Gast et al., 2019, Friedman and Schwartz et al., 2019, Rooms et al., 2019). We report a second imported case of talaromycosis caused by *T. marneffei* in South Africa. *T. marneffei* is endemic in Southeast Asia and people travelling to such areas should be informed about the disease, especially if they are immunocompromised, so that prophylaxis can be given to immunocompromised individuals before travelling (CDC et al., 2019). Although infrequently reported, we report another case of blastomycosis caused by *B. percursus*. We believe that the true burden of these dimorphic pathogens may be underestimated in South Africa owing to misdiagnosis and of these pathogens by clinicians and laboratories.

Most patients with these endemic mycoses presented with skin lesions and had pulmonary involvement, which is similar to previous reports (Kenyon et al., 2013, Schwartz et al., 2015, Schwartz et al., 2017a). The lesions were widespread and only a single patient presented with a lymphocutaneous lesion, which is a typical form of sporotrichosis (Govender et al., 2015). The clinical presentation was similar for all the dimorphic pathogens and could not be distinguished

from TB in some of the cases, which has previously been reported (Frean et al., 1993, Koen and Blumberg, 1999, Schwartz et al., 2015, Schwartz et al., 2017a, Oladele et al., 2018). We reported that some patients with these systemic mycoses were concurrently diagnosed with TB. Previous studies have reported that patients with emergomycosis, histoplasmosis and blastomycosis are concurrently diagnosed with TB, with some cases being infrequently misdiagnosed with TB before a correct diagnosis is made (Frean et al., 1993, Schwartz et al., 2015, Schwartz et al., 2017a). This is because the clinical presentation of TB sometimes mimics that of endemic mycosis. To complicate things further, patients with systemic mycoses presented with indistinguishable skin lesions. This is a challenge for clinicians in terms of accurate diagnosis (Schwartz et al., 2017a). However, amphotericin B followed by triazole has been reported to be effective in treating patients with emergomycosis or histoplasmosis (Kenyon et al., 2013, Schwartz et al., 2015, Maphanga et al., 2017, Dukik et al., 2018).

Systemic mycoses were mostly diagnosed in Gauteng and the Western Cape, possibly because these are bigger cities, more people are migrating there and there are competent staff to better identify these pathogens. Patients are being transferred from small hospitals to academic hospitals for better management of these systemic mycoses. Furthermore, it is also possible that clinical specimens was collected while the patients were on antifungal agents which may have inhibited growth of these pathogens in the laboratories. Therefore, more cases may possibly be underreported from the other provinces. In order to better detect these pathogens, identification of dimorphic pathogens directly from clinical specimens is nessassary. However, such assays are often limited to reference laboratory and are not standardised (Caceres et al., 2019).

6.5.1 Study limitations

The limitation of this study is that, this was a passive cultured-based surveillance. The laboratories were only requested to submit positive cultured isolates; therefore we may have missed cases that were positive by histology but negative by culture methods. We may have missed some of the cases owing to contamination resulting from longer incubation of cultures and incorrect identification of these pathogens as moulds or yeasts in the diagnostic laboratories. The clinicians or laboratory personnel provided clinical history that varied in detail and completeness, which may have limited detailed description of the clinical presentation of these cases. We could not compare the sensitivity

of *ITS* sequencing versus *LSU* sequencing since we only used *ITS* sequencing as a method for initial identification of these pathogens.

6.6 CONCLUSION

In conclusion, the DNA-sequencing method is a useful method for accurate diagnosis of dimorphic fungal pathogens. Although not currently implemented in most South African academic laboratories, the method may be better utilised to understand the epidemiology of systemic mycoses and facilitate appropriate diagnosis of these pathogens when compared to culture-based methods. Training of laboratory staff on the identification of these pathogens may overcome the challenges of underreporting and misidentification of these pathogens, particularly in resource-limited countries. Clinicians should be aware that these systemic mycoses may have similar clinical presentations which may lead to misdiagnosis and delay patient management.

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6.9 CONFLICTS OF INTEREST

None declared

6.10 AUTHORS CONTRIBUTIONS

A.A.H, N.M, D.N, L.B, C.C. and B.M.M. sent isolates to NICD. T.G.M. and S.D.N. performed molecular identification. T.G.Z, R.S.M., M.M. and E.M.T performed phenotypic identification. T.G.M. and N.P.G. analyzed the data and wrote the manuscript.

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Table 6.1: Demographic and clinical characteristics of individuals with systemic mycoses, 2008-2019, *n*=156

Demographic and clinical features	No of patients (%, interquartile range)
Median age, y	37 (31-41)
Paediatric (0-18y)	13 (7-16)
Adult (19-64y)	37 (32-42)
Sex	
Male	102/154 (66)
Female	52/154 (34)
Public and private-sector	
Western Cape	57/143 (40)
Gauteng	58/156 (37)
KwaZulu Natal	15/143 (10)
Eastern Cape	11/156 (7)
Free State	11/156 (7)
Limpopo	2/153 (1)
Northern Cape	2/143 (1)
Current clinical presentation based on specimen type	
Cutaneous infection	
Skin biopsy	84/152 (55)
Blood infection	
Blood	52/152 (34)
Bone infection	
Bone marrow	8/152 (5)
Subcutaneous infection	
Pus abscess	5/152 (3)
Brain infection	
Cerebrospinal fluid	1/152 (0.6)
Brain tissue	1/152 (0.6)
Eye infection	
Corneal scraping	1/152 (0.6)
Underlying condition which predisposes to the fungal disease	

Transplant	2/2 (100)
HIV/AIDS	118/122 (97)
Diabetes mellitus	2/2 (100)

Table 6.2: Geographic distribution of dimorphic fungi causing systemic mycoses in South African patients, 2008-2019, *n* =156

	Emergomycosis		sporotrichosis	histoplasmosis	Blastomycosis	talaromycosis
Provinces	*E. africanus*	*E. pasteurianus*	*S. schenckii* sensu stricto	*H. capsulatum*	*B. percursus*	*T. marneffei*
Western Cape	45	1	6	2	3	
Gauteng	38	1	12	5	1	1
Eastern Cape	10			1		
KwaZulu Natal	8		5	1		1
Free State	4*		6	1		
Northern Cape			2			
Limpopo	1		1			
Total	**106**	**2**	**32**	**10**	**4**	**2**

*one patient originally from Lesotho

Table 6.3: Isolates correctly identified by phenotypic -based methods at the diagnostic laboratories and by both phenotypic-based and molecular methods at the reference laboratory, 2008-2019, *n* = 156

Isolate ID	Diagnostic lab culture genus ID	Reference lab Culture genus ID	Reference molecular methods					
			ITS sequence genus ID	% ID	LSU sequence genus ID	% ID	Cal sequences Species ID	% ID
Emergomyces africanus (n=104)	70	100	104	(96-100)	ND		ND	
Emergomyces pasteurianus (n=2)	1	2	2	(96-100)	ND		ND	
Blastomyces percursus (n=4)	4	4	4	(96-100)	ND		ND	
H. capsulatum (n=10)	8	9	10	(96-100)	ND		ND	
Talaromyces marneffei (n=2)	2	2	2	(96-100)	ND		ND	
Emergomyces africanus/Yarrowia lipolytica (n=1)	0	1	1	(96-100)	ND		ND	
Emergomyces africanus/Cryptococcus neoformans (n=1)	0	0	1	(96-100)	ND		ND	
Sporothrix schenckii (n=32)	14	31	10	(96-100)	17	(95-100)	23*	(95-99)

ID=Identification, ITS=Internal transcribed space, LSU=Large Subunit, Cal=Calmodulin.

*Three isolates had calmodulin identity score of 95% and the LSU identity score of these isolates was above 95%

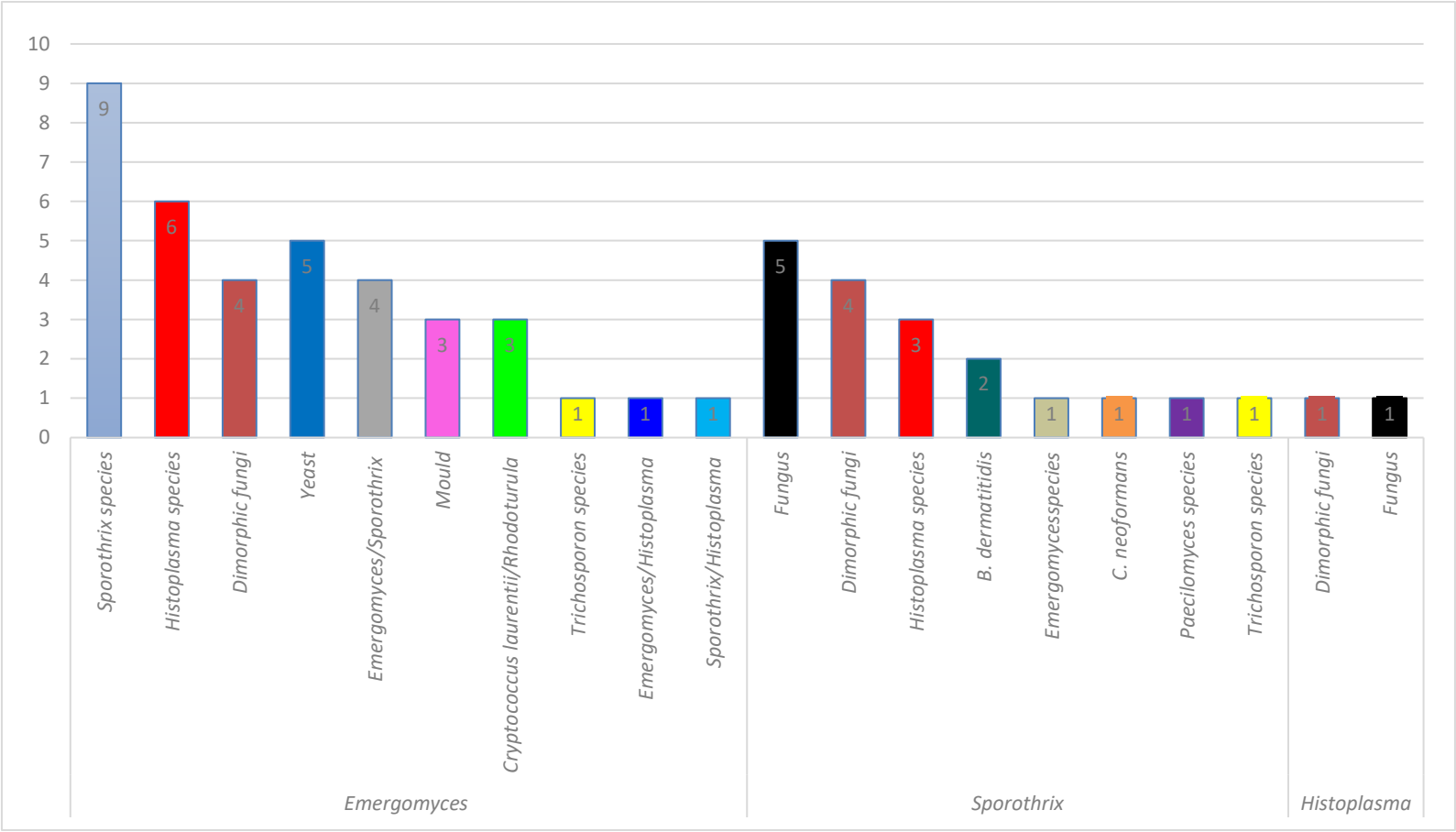

Figure 6.1: **The proportions of *Emergomyces* (n=37), *Sporothrix* (n=18) and *Histoplasma* (n=2) isolates misidentified by phenotypic methods at the diagnostic laboratories. *Emergomyces* isolates were cultured from blood (n=23), skin (n=12) and bone marrow (n=2). *Sporothrix* isolates were cultured from skin (n=14) and pus abscess (n=4), while *Histoplasma* isolates were cultured from skin.**

CHAPTER 7

7.1 Concluding remarks

Dimorphic fungi infect both immunocompetent and immunocompromised human hosts, but the reported incidence of these fungi is limited by lack of robust surveillance systems (Gauthier, 2017, Kozel and Wickes, 2014). The paucity of information can be ascribed to diagnostic challenges experienced by both clinicians and microbiologists. Clinicians are challenged when diagnosing patients because of confusing clinical presentations, and in cases where a correct diagnosis is made, effective antifungal drugs may be toxic, expensive or inaccessible in resource-limited countries (Schwartz, 2015, Bongomin et al., 2017). Microbiologists globally are challenged, in terms of accurate diagnosis of these pathogens, largely because of lack of expertise and reliable assays (Wiederhold and Gibas, 2018). The difficulties in clinical and microbiological diagnoses, compounded by complexities inherent in antifungal treatments, have a negative impact on proper management of affected patients, particularly in resource-limited settings (Bongomin et al., 2017). In the current study, we addressed a number of aspects of microbiological diagnostics, in order to facilitate the detection and characterisation of dimorphic fungi causing invasive disease among South African patients.

We compared the *in vitro* antifungal susceptibilities of the yeast and mould phases of *E. africanus* to several antifungal agents by reference (Etest method) and commercially available methods (BMD method) to determine if there was an association between MICs and clinical outcomes, in order to guide the clinical management of patients with disseminated emergomycosis. No differences between the yeast and mould phase BMD and Etest MICs were observed for any of the nine antifungal drugs tested. Voriconazole, posaconazole, itraconazole, and amphotericin B were the most potent agents against the mould and yeast phases of *Emergomyces*. Fluconazole and the echinocandins were less potent. Since fluconazole is widely used for the treatment of fungal diseases in South Africa, this azole may not be useful for proper management of dimorphic fungal infections. As recommended in the IDSA guidelines and by other authors, the *in vitro* susceptibility data in this study support the management of disseminated emergomycosis with amphotericin B,

followed by itraconazole, voriconazole, or posaconazole (Wheat et al., 2007, Chapman et al., 2008, Kenyon et al., 2013, Schwartz et al., 2015).

We evaluated the performance of an IMMY monoclonal *H. capsulatum* galactomannan EIA in routine practice in detecting antigenuria among patients with suspected histoplasmosis in a high HIV prevalence setting. The EIA is regarded as the most rapid and easy-to-use diagnostic tool when compared to culture and histology methods (Nacher et al., 2018). In July 2019 the assay was officially included in the second World Health Organisation Essential Diagnostics List as one of the assays in the diagnosis of disseminated histoplasmosis in AIDS patients, more specifically in resource-limited countries. It was also FDA-cleared in the US, meaning that it can be used as a diagnostic tool for rapid diagnosis of patients with histoplasmosis (IMMY). We reported the sensitivity and specificity of the *Histoplasma* GM EIA when compared to culture-confirmed histoplasmosis cases to be 88% and 72%, respectively. When compared to histologically confirmed histoplasmosis or emergomycosis cases, the sensitivity and specificity of the EIA were reported to be 83% and 93%, respectively. Although *Histoplasma* GM EIA is currently commercially available for diagnosis of histoplasmosis in laboratories, the results of this study showed that the assay may cross-react with *E. africanus*, as previously reported by Schwartz et al. (2017). However, in emergomycosis-endemic countries, the EIA may be useful in the diagnosis of emergomycosis and histoplasmosis cases, since these patients are treated with the same antifungal agents. Furthermore, in countries where there is a high prevalence of histoplasmosis and no access to BSL2/3 facilities, the EIA may be a useful tool for early diagnosis of histoplasmosis (Nacher et al., 2018).

The results further highlighted a low positivity rate for cultured isolates when compared to histology, which has previously been reported in other South African studies (Kenyon et al., 2013, Schwartz, 2015, Schwartz et al., 2017). These results could possibly have been due to the fact that most cases of histoplasmosis are misdiagnosed in laboratories because of the lack of skilled personnel for accurate identification of dimorphic pathogens (Nacher et al., 2018). Furthermore, histology could not distinguish between the yeasts of these dimorphic fungi in clinical specimens (Schwartz et al., 2017). However, culture and histology have remained the most useful methods for identification of histoplasmosis in resource-limited areas. The IMMY monoclonal *H.*

capsulatum EIA has proved to be a rapid and useful assay for diagnosis of histoplasmosis and emergomycosis in South Africa, which may decrease the high mortality rate associated with these diseases.

DNA sequencing is currently considered the gold standard for fungal identification, having contributed greatly to the detection and correct taxonomic assignment of dimorphic fungi and emerging fungal pathogens globally (Wiederhold and Gibas, 2018). We therefore used DNA sequencing in two studies. In the first study, we reported an atypical clinical pattern of cutaneous and osteoarticular blastomycosis seen in South Africa, caused by *Blastomyces percursus* or *Blastomyces emzantsi* sp. nov., two distinct species that can be differentiated phenotypically, molecularly and geographically (*B. percursus* being reported from Africa and Israel, while *B. emzantsi*, being a new species, has as yet only been described from South Africa). It was shown that the two species do not possess the *BAD-1* virulence gene and produce hyphal elements at 37°C. Although MLST and WGS methodologies enabled the description of a novel species, and showed the diversity among South African *Blastomyces* in this setting, the lack of expertise and an MLST database remained a challenge for sequence analysis in this setting. Nevertheless, once we were able to analyse the data using sequences available on the NCBI database as references, both MLST and WGS were found to be the most powerful tools for typing the 20 South African *Blastomyces* isolates characterised in this study.

In the second study, we assessed the feasibility of DNA sequencing for identification of emerging dimorphic fungi through laboratory-based surveillance, demonstrating that dimorphic fungi were a common cause of systemic endemic mycoses among HIV-infected patients in South Africa, with 156 confirmed cases over the past 11-year period. Although the routine diagnostic laboratories could not correctly identify dimorphic fungi to genus level using the culture method (which could possibly lead to poor management of patients), DNA sequencing was useful in the correct identification of fungal pathogens in the research setting. However, the universal ITS primers used for pan-fungal identification failed to amplify some of the *Sporothrix* isolates, so we recommend that calmodulin sequencing should always be performed in conjunction with ITS sequencing for accurate identification of *Sporothrix* isolates to species level. Furthermore, ITS sequencing was useful in the identification of more cases of emergomycosis, blastomycosis, sporotrichosis and

histoplasmosis and an imported case of talaromycosis from the previously reported cases (Kenyon et al., 2013, Govender et al., 2014, Dukik et al., 2017, Maphanga et al., 2017, Schwartz et al., 2017). These cases were widely distributed in six of the nine South African provinces. Most patients presented with skin lesions and pulmonary disease.

7.2 Future research

Susceptibility testing of dimorphic fungi remains limited to the reference laboratory because it requires trained personnel and currently there are no standardised methods for testing *Emergomyces, Histoplasma* and *Blastomyce*s. Studies should be directed at developing standardised methods for antifungal susceptibility testing of dimorphic fungi in order to monitor the antifungal susceptibility profile of these isolates. In this setting, we could not find any association between the nine antifungal agents tested against *E. africanus* and clinical outcome. *In vivo* studies (using mouse models) should be conducted to investigate if there will be any association between MICs of amphotericin B and azoles and the clinical outcome of patients with emergomycosis.

The *Histoplasma* galactomannan EIA was a rapid method for the diagnosis of histoplasmosis among immunocompromised and immunocompetent patients in the current research setting. Since the assay cross-reacted with emergomycosis patients, clinicians should be advised about this, even though the results would not have any implication for the treatment options because emergomycosis and histoplasmosis are treated in the same way, i.e. amphotericin followed by itraconazole. Future research should be directed at further evaluation of the *Histoplasma* galactomannan EIA directly from serum samples in cases where there is low antigen in urine samples of patients with disseminated histoplasmosis.

The molecular method was most useful and rapid in accurate identification of emerging and rarer dimorphic pathogens in this study. Future research should be directed at continuation of the surveillance of these pathogens in both private and public settings in order to monitor the trends in the number of cases caused by these pathogens, particularly among immunocompromised patients. In laboratories where molecular methods are not routinely used, more effort should be

directed at the implementation of training courses on how to identify dimorphic fungi correctly using phenotypic methods, for example macroscopic and microscopic examination of fungal morphology from cultured plates and longer incubation of cultures when an invasive fungal disease is suspected. This is because most dimorphic pathogens are being misdiagnosed and underreported owing to misidentification. The training courses may increase correct diagnosis of these pathogens in resource- limited countries. Furthermore, the development of qPCR species-specific assays, e.g. *E. africanus* or *H. capsulatum*, which will detect these pathogens directly from clinical specimens, could assist in early diagnosis of dimorphic pathogens in academic laboratories and limit the handling of these infectious pathogens, particularly where there is no BSL 3 infrastructure.

We used MLST and WGS to investigate close relationships among clinical isolates that were initially identified as *B. dermatitidis* based on phenotypic methods alone. The results showed that these isolates are different from *B. dermatitidis*, although closely related to *B. dermatitidis, B. gilchristii* and *B. parvus*, meaning that *B. emzantsi* is not the same as *B. dermatitidis, B. gilchristii* and *B. parvus*, but share a common ancestor that relates them to one another. Since there are currently no MLST schemes available for the assignment of sequence type for dimorphic fungi, future research should focus on developing MLST schemes that will allow classification of these fungal pathogens based on sequence types. A database should be developed that will have a list of all dimorphic fungi circulating worldwide so that it becomes easy to assign or recognise novel species. This database will be most useful for laboratories that cannot afford WGS. Furthermore, more research should be directed on investigating possibly genetic diversity of the *H. capsulatum* and *E. africanus* among South African strains using WGS in order to better understand the molecular epidemiology of these pathogens in South Africa.

In terms of emerging fungal pathogens, future research should be directed at building the capacity for WGS, creating pipelines that will be used to study these dimorphic pathogens and their virulence genes. Further investigation should also be directed at gathering all dimorphic isolates in Africa to gain detailed understanding of the molecular epidemiology that underlies the evolutionary changes in the virulence and drug resistance genes in these dimorphic pathogens. In addition, future comparative genomic studies should be done to understand if there are any

differences between the strains that infect immunocompromised patients and those infecting immunocompetent patients globally.

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SUPPLEMENTARY APPENDIX

Human blastomycosis in South Africa caused by *Blastomyces percursus* and *Blastomyces emzantsi* sp. nov., 1967 – 2014

APPENDIX A: Detailed materials and methods

Morphological description

The archived isolates had been stored in water at room temperature. All isolates were sub-cultured onto SDA or PDA (DMP, Sandringham, South Africa) and incubated at 25ºC and 30 ºC for up to four weeks for the mycelial phase. Isolates were transferred onto brain heart infusion (BHI) agar with or without 5% sheep blood (DMP) and incubated for one to three weeks at 37ºC and 40ºC for conversion to the yeast phase. The diameter of the mould-phase colonies was measured in millimetres each week using a ruler. The morphology of both phases was observed using standard light microscopic examination of lacto phenol cotton blue preparations and differential interference contrast (Nomarski) microscopic examination of unstained preparations, using an Olympus BX53 with a Nikon camera and calibrated Eclipse software. Mycelial growth was also inoculated onto a urea agar slope (DMP) and incubated at 37ºC for six days. Yeast and mycelial isolates were examined using light microscopy and electron microscopy. The descriptive terminology used follows that of Jiang et al. (2018).

Electron microscopy (EM)

Yeast and mycelial cultures were fixed by flooding with 2.5% glutaraldehyde in 0.2M phosphate buffer, pH 7.1, for several hours. Routine EM processing was as follows: pieces of agar on which the fungus was growing, were cut out and rinsed in buffer, post-fixed for 2 hours in 1% osmium tetroxide, buffer-rinsed and dehydrated in an ethanol series (Chaffey et al., 2001). For transmission EM, specimens were then infiltrated with a low viscosity epoxy resin, polymerized, sectioned at 70 nm on a Leica EM UC6 ultracut microtome, and double-stained with uranyl acetate and lead citrate. For scanning EM, dehydrated specimens were placed in hexamethyldisilazane, air dried overnight, mounted on stubs and coated with carbon. Specimens were viewed on a Zeiss Crossbeam 540 scanning electron microscope at 1kV, or at 80kV on a FEI BioSpirit Twin TEM fitted with an Olympus Quemesa CCD camera and OSIS calibrated software.

Antifungal susceptibility testing

Susceptibility testing was performed for both yeast- and mould-phase isolates using a reference BMD method and the commercial Etest method (bioMérieux, Marcy, l'Etoile, France). The BMD method was performed according to the CLSI M27-A3 (for the yeast phase) and M38-A2 (for the mycelial form) with a modified inoculum size for the latter according to Maphanga *et al*. 2017 (CLSI, 2008, CLSI, 2012, Maphanga et al., 2017). Briefly, the inoculum was prepared from fresh cultures and the turbidity adjusted using a turbidometer to the equivalent of a McFarland 1 standard (2.5×10^5) for the yeast phase and a McFarland 2 standard (5.0×10^5) for the mycelial phase. Customised, round-bottomed frozen 96-well micro-titre plates, containing two-fold dilution ranges of itraconazole, voriconazole, posaconazole, fluconazole, flucytosine, anidulafungin, caspofungin and micafungin, were immediately inoculated (TREK Diagnostic Systems, Inc., Cleveland, Ohio, USA). BMD MIC endpoints were read at 50% inhibition for fluconazole, voriconazole, posaconazole, itraconazole, flucytosine, caspofungin, anidulafungin and micafungin. Etest MICs for amphotericin B, voriconazole, itraconazole and posaconazole were determined using Roswell Park Memorial Institute (RPMI) 1640 plates containing 2% glucose (DMP) according to the manufacturer's recommendations. Etest MIC endpoints were read as follows: 80% inhibition for voriconazole, posaconazole, itraconazole (i.e. micro-colonies within the elliptical zone of inhibition were ignored) and 100% for amphotericin B. All plates were incubated at 35ºC and read by three independent observers at six days. QC strains were included in each test run: *Candida parapsilosis* ATCC 22019, *Candida krusei* ATCC 6258 and *B. dermatitidis* ATCC 10225 for yeast-phase tests and *Aspergillus fumigatus* NCPF 7097, *A. fumigatus* NCPF 7100 and *B. dermatitidis* ATCC 10225 for mycelial-phase tests. The MICs for *C. parapsilosis* ATCC 22019, *C. krusei* ATCC 6258 were within the CLSI-recommended ranges. Quantitative colony counts were performed to assess the purity and accuracy of the final inoculum: the suspension was homogenized and 20 µℓ was spread over the surface of a SDA plate and incubated at 35ºC until the number of viable colonies could be counted. Preparation of culture and AST was done under a class II biosafety cabinet in a level II biosafety laboratory. Necessary precautions were taken when handing the cultures, e.g. N95 mask, double gloves and a laboratory coat. We calculated a geometric mean for each MIC distribution using Stata version 14.0 (StataCorp Limited, College Station, Texas, USA).

DNA extraction, PCR amplification and sequencing

Genomic DNA was extracted from 20 yeast-phase isolates using the Zymo ZR fungal/bacterial DNA MiniPrep Kit (Zymo Research Corp, USA) according to the manufacturer's instructions. PCR and sequencing of five genes (*ITS*, *LSU*, actin, β-tubulin and intein *PRP8*) was performed as described by Kenyon et al. (2013). The samples were sequenced in a 3130 Sequencer (Applied Biosystems, Life Technologies Corporation, USA). Sequences were subjected to BLAST analyses in GenBank (www.ncbi.nlm.nih.gov) for identification. Isolate sequences from Kenyon et al. (2013) were added to phylogenetic trees. MLST targeting the *CHS2*, *DRK1*, *FADS*, *PYRF*, *TUB1* and *ARF6* genes was performed as described by Brown *et al*. (2013) with the following modifications: Six genes (chitin synthase (*CHS2*), histidine kinase (*DRK1*), fatty acid desaturase (*FADS*), orotidine 5'-phosphate decarboxylase (*PYRF*), alpha tubulin (*TUB1*) and ADP ribosylation factor 6 (*ARF6*) were amplified using a HotStart *Taq* polymerase (Life Technologies, Carlsbad, CA) (Brown et al., 2013). Each reaction contained 5 µl of 1X buffer (Life Technologies), 2.5 µl magnesium chloride (2.5 mM; Life Technologies), 0.5 µl deoxynucleotide triphosphate mix (0.2 mM; Life Technologies), 0.25 µl forward primer and 0.25 µl reverse primer (0.5 µM, Inqaba Biotechnical Industries, Pretoria, South Africa), 0.1 µl (1.0 U, HotStart Taq polymerase), 2 µl (genomic DNA) and 14.4 µl distilled water in a 25 µl reaction. DNA was denatured at 94°C for 2 min, followed by 35 cycles of 94°C for 30 sec, 52°C to 62°C for 1 min, 72°C for 1 min, with a final extension step of 3 min at 72°C. No amplification was observed for the *CH2* and *PYRF* genes. However, two gene were non-typeable in the Brown et al. (2013) scheme (*CHS2* gene for *B. percursus* and *PYRF* for *B. emzantsi* genes). All six gene sequences were then extracted from the WGS data to complete the MLST dataset.

MLST phylogenetic analysis

DNA sequences were aligned with MAFFT version 7 as described by Kenyon et al. (2013). The datasets for the five genes and the six MLST loci were analysed with the following settings: Q-INS-I, scoring matrix= 1PAM/K=2, Gap opening penalty=1.53, offset value=0. Threshold score=39{E=8.4e-11. Sequences were trimmed with BioEdit version seven and also checked manually (Hall et al., 1999). On MEGA we used Models to select the best DNA Models and the models for each gene region were as follows: *ITS*=TN93+I, *LSU*=JC, intein *PRP8*= K2+G, beta tubulin= K2+G, actin gene=K2, *CHS2*= K2+I, *DRK1*=K2+G, *FADS*=K2, *PYRF*=HKY+G, *TUB1*=K2+G and *ARF6*=K2. The phylogenetic tree was generated by a

maximum likelihood statistical method using 1000 bootstrap replications on MEGA version 6 from the concatenated dataset and individual genes (Tamura et al., 2013). A clade was defined as isolates from different patients which shared a common ancestor with >80% support values in the maximum likelihood analyses.

Whole genome sequencing and *de novo* assembly

Paired-end libraries were prepared using the Nextera XT DNA library kit, followed by 2 × 300-bp sequencing on an Illumina MiSeq sequencer (Illumina, San Diego, CA, USA). The sequenced paired-end reads were quality trimmed and *de novo* assembled using Qiagen CLC Genomics Workbench version 10 (Qiagen, The Netherlands). All contigs were then submitted to GenBank (QGQE00000000=SA-NICD-12, QGQF00000000=SA-NICD-09, QGQG00000000=SA-NICD-10, QGQH00000000=SA-NICD-11, QGQI00000000=SA-NICD-15, QGQJ00000000=SA-NICD-13, QGQK00000000=SA-NICD-14, QGQL00000000=SA-NICD-16, QGQM00000000=SA-NICD-01, QGQN00000000=SA-NICD-07, QGQO00000000=SA-NICD-02, QGQP00000000=SA-NICD-03, QGQQ00000000=SA-NICD-04, QGQR00000000=SA-NICD-08, QGQS00000000=SA-NICD-05, QGQT00000000=SA-NICD-06 and QKWI00000000 =NCPF4091).

Phylogenetic analysis using whole-genome single nucleotide polymorphisms

Reads were aligned to the *Blastomyces percursus* assembly strain BP222 (GenBank accession GCA_003206225.1_ASM320622v1) using BWA-MEM version 0·7·12 (Li et al., 2013, Dukik et al., 2017). Variants were then identified using GATK version 3·4 (McKenna et al., 2010). Briefly, indels were locally realigned, haplotypeCaller was invoked in GVCF mode with ploidy = 1, and genotypeGVCFs was used to predict variants in each strain. All VCFs were then combined and sites were filtered using variantFiltration with QD < 2·0, FS > 60·0, and MQ < 40·0. Individual genotypes were then filtered if the minimum genotype quality < 50, percent alternate allele < 0·8, or depth < 10. Reads from *B. dermatitidis* strain ER3 and *B. parvus* strain UAMH 130 were also included and variants were called for all samples as described above. For phylogenetic analysis, the 1,712,033 sites with an unambiguous SNP in at least one isolate and with ambiguity in at most 10% of isolates were concatenated; insertions or deletions at these sites were treated as ambiguous to maintain the alignment. Maximum likelihood phylogenies were constructed using RAxML version 8.2.4 using the GTRCAT nucleotide substitution model and bootstrap analysis based on 1,000 replicates (Stamatakis et al., 2014).

Determination of nucleotide diversity using whole genome

Genome-wide nucleotide diversity (π) was computed for *B. emzantsi*, *B. percursus* and the *Blastomyces* genus using VCFtools v0.1.12 (Danecek et al., 2011). The average nucleotide diversity (π) was computed for non-overlapping sliding windows of 10 kb.

Identification of the mating types and *Blastomyces BAD-1* from WGS data

The reverse and forward primer pairs of *Blastomyces MAT1-1* and *MAT1-2* obtained from Li et al. (2013) were BLASTed with our *Blastomyces* contigs (Li et al., 2013). For the *Blastomyces* adhesion-1 (*BAD-1*) gene determination, the genome of a reference strain of *Ajellomyces dermatitidis* (ATCC 26199: U37772) was mapped to those of our *Blastomyces* strains. Obtained sequences were visually analysed and subjected to BLAST analyses in GenBank (www.ncbi.nlm.nih.gov) for confirmation of mating types and *BAD-1* sequences.

APPENDIX B: Detailed results

Mycological examination of the isolates

Based on examination of cultures grown on SDA (at 25°C) and brain heart infusion agar (at 37°C), we distinguished two morphological groups of isolates. Twelve isolates (group 1) resembled *B. percursus* morphologically (Figure 1). The remaining eight isolates more closely resembled *B. parvus*: at 25°C, colonies were white and peripherally floccose, becoming wrinkled and slightly raised in the centre. Microscopically, at 25°C, both groups had septate hyphae with conidiophores arising at right angles to the hyphae or terminally, with unicellular globose-to-subspherical conidia borne singly or in clusters on small secondary conidiophores. All isolates demonstrated cellular enlargement, swelling and giant cell formation, with an increase in temperature between 25°C and 37°C, as well as the presence of thick-walled, chlamydospore-like cells at saprobic and intermediate (30°C) temperatures. (Figure 1, *B percursus*, and figures 5.1 and 5.2 chapter five, *B. emzantsi*). As all group 1 isolates conformed to previous descriptions of *B. percursus*; they will not be described further (Dukik et al., 2017, Jiang et al., 2018). However, in *B. emzantsi*, we observed characteristic clavate, complanate cells during conidiogenesis, with occasional helical hyphae at 25°C, while at 37°C, there were adiaspore-like cells, hyphal fragments and infrequent broad-based, budding cells (Figure 5.2 chapter five). The broad-based budding cells, characteristic of the type species of *Blastomyces*, were evident in all available isolates, albeit interspersed with hyphal elements.

Description of *Blastomyces emzantsi*

On SDA at 25°C, colonies were white and granular (powdery, wrinkled), with aerial hyphal tufts on centrally- raised areas, and with distinct though narrow, floccose margins. The reverse side was white-cream. Colonies grew to between 15 – 18 mm in diameter after one week of incubation, 50 – 55 mm after three weeks. On BHI agar at 37°C, colonies were beige, butyrous and cerebriform, occasionally retaining some white, aerial hyphae. The reverse was tan in colour, and colonies reached a diameter of 10 mm after three weeks of incubation. The saprobic phase consisted of slender, hyaline, septate hyphae [(0.9)–1.3-2.8–(4.2) µm in diameter; n = 50], with limited branching, and occasional helical hyphal gyres. Abundant spherical or subspherical conidia [(1.9)-3.4-(3.8) µm; n=50] were borne singly: either terminally; on short pedicles laterally, or on longer primary or secondary conidiophores. In the latter, up to four conidia were positioned on each terminally- inflated, primary conidiophore. It was common for conidia to be arranged in opposite pairs along the hyphae. Conidia were variously ornamented, from smooth (younger spores) to tuberculate (mature spores). During conidiogenesis, there were also distinctive clavate, complanate cells terminally/extending at right angles from the hyphae, many of which developed into conidiophores by septation (may be more than once) and rounding of the terminal portion, although most of the other lateral conidiophores were slender and more delicate. Similar clavate cells were illustrated in *Emmonsia sola* (Jiang *et al*. 2018, Figure 9H, I) (Jiang et al., 2018). Laterally- borne conidia and clavate cells were less obviously septate than terminally- positioned structures. As cultures age, intra/endohyphal growth occurred, and there was a tendency for hyphal filaments to adhere, forming rope-like bundles. With an increase in temperature, there was generalised swelling and vacuolation of all cells, lipid droplets accumulated intracellularly, lateral conidiophores became ampulliform, and doliform giant cells developed from the enlarged cells of fragmenting hyphal filaments. There was incomplete transition both *in vitro* and *in vivo*, so at 37°C, hyphae, fragmented enlarged hyphal elements, adiaspore-like cells that budded multilaterally [9.0–10.2 x 13.8-20.9 µm (n=50), infrequently as large as 22.3-24.3 x 26.4-34.2 µm (n = 4)], and yeast-like budding cells were observed [5.4-8.5 x 9.6-14.3 µm (n = 50)].

Antifungal susceptibility testing of the isolates

Tables 1 and 2 summarise the MIC/MEC distribution, range and GM MIC/MEC for nine antifungal agents for the yeast- and mould- phases of 17 isolates. There were no differences

between group 1 and 2 isolates. Voriconazole, posaconazole, itraconazole, micafungin and amphotericin B had the most potent *in vitro* activity.

Multilocus and whole genome phylogenetic analysis

Phylogenetic analysis of both multilocus and whole genome sequences supported that isolates from group 1 belonged to *B. percursus* and isolates from group 2 formed a monophyletic clade between the *B. percursus*/*B. dermatitidis* clade and *B. parvus*. We included the additional *B. parvus, B. silverae*, *B. helicus* and *Emmonsia sola* on our *ITS* sequence analysis (Figure 2 [A-E]). Based on *ITS* sequences only, the 12 strains of group 1 were confirmed as *B. percursus*, while the eight strains of group 2 were very similar to *B. parvus* (97%) and *B. helicus* (97%). A phylogenetic tree of the *ITS* sequences separated the two groups from *B. dermatitidis, B. gilchristii, B. parvus, B. silverae, B. helicus* and *Emmonsia sola* (Figure 2 [A-E]). Further analysis of the five individual gene phylogenies clearly separated *Blastomyces* isolates into four monophyletic clades (*B. dermatitidis/B. gilchristii, B. percursus* [group 1; n=12] and *B. emzantsi* [group 2; n=8]). Only *LSU* gene could not separate *B. parvus, B. silverae* from *B. emzantsi* (Figure 2 [F-K]). Three (*CHS2*, *DRK*, *ARF6*) of the six individual gene phylogenies clearly separated *Blastomyces* isolates into five monophyletic clades (*B. dermatitidis, B. gilchristii, B. percursus* [group 1; n=10] and *B. emzantsi* [group 2; n=8]) that were closely related to each other, and *B. parvus* appeared as the basal species for these five clades (Figure 2 F, G, K).

Nucleotide diversity

Comparing to other dimorphic fungal pathogens from *Ajecellomycetaceae* causing endemic mycoses, the level of diversity in *B. emzantsi* is lower than that reported in *Paracoccidioides brasiliensis* S1a and PS2 (π = 0.00053 and 0.00066, respectively; but higher than the clonal diversity reported for *P. brasiliensis* PS3 that is mainly found in regions of endemicity in Colombia (π = 0.00008) (Muñoz et al., 2016). The degree of genetic variation supports that *B. emzantsi* is a single species well separated within the *Blastomyces* genus.

Mating types and *Blastomyces BAD*-1 analysis

Of the 18 *Blastomyces* strains analysed for mating type, only the three recently-diagnosed cases of *B. percursus* contained *MAT1-2* gene. Furthermore, all *B. emzantsi* were *MAT1-1* (Table 4). The population ratio for each mating type is 1:1 in other dimorphic pathogens from the

Ajellomycetaceae such as *Paracoccidioides*. Either *MAT1-1* or *MAT1-2* have been reported in other dimorphic fungal pathogens demonstrating that these strains are heterothallic (Brown et al., 2013, Dukik et al., 2017, Muñoz et al., 2016, Sigler et al., 1996, Meece et al., 2011).

Table 1: Yeast-phase MIC distribution of *Blastomyces* isolates (9 *B. percursus* isolates, 8 *B. emzantsi* isolates, 1 *B. dermatitidis* ATCC 10225)

Case # /(Accession #):	Reference	E-test MIC (mg/L)				BMD MIC (mg/L)							
		AMB	**VRC**	**POS**	**ITC**	**FLC**	**VRC**	**POS**	**ITC**	**5FC**	**CAS**	**MFG**	**ANG**
Group 1 (*Blastomyces percursus*)													
1 (QKWI00000000; NCPF4091)	(Dukik et al., 2017)	0.004	0.002	0.002	0.002	4	0.008	0.008	0.008	64	0.06	0.008	0.12
2 (LGTZ00000000; BP222)	(Heys and Orth, 2014, Schwartz et al., 2015)	0.064	0.002	0.002	0.002	8	0.03	0.25	0.25	256	0.5	0.12	0.12
3 (QGQT00000000; SA-NICD-06)	(Schwartz et al., 2015)	0.012	0.002	0.002	0.002	1	0.008	0.03	0.06	128	0.5	0.06	0.5
4 (QGQR00000000; SA-NICD-08)	This study	0.047	0.002	0.002	0.002	4	0.015	0.008	0.015	256	1	0.03	2
5 (QGQS00000000; SA-NICD-05)	(Simon et al., 1977)	0.094	0.012	0.002	0.003	8	0.015	0.008	0.008	32	4	0.12	1
6 (QGQQ00000000; SA-NICD-04)	(Frean et al., 1993, Carman et al., 1989)	0.003	0.002	0.002	0.002	0.25	0.008	0.008	0.008	32	0.5	0.03	4
7 (SA-NICD-18)	(Martin and Berson, 1973)	0.003	0.002	0.002	0.002	0.5	0.008	0.008	0.008	32	1	0.03	0.5
(QGQP00000000; SA-NICD-03)	This study	0.004	0.002	0.002	0.002	2	0.015	0.008	0.008	32	0.5	0.12	0.5
(QGQN01000000; SA-NICD-07)	This study	0.004	0.002	0.002	0.002	2	0.015	0.015	0.03	16	0.5	0.015	0.25
MIC range		0.003-0.094	0.002-0.012	0.002-0.002	0.002-0.003	0.25-8	0.008-0.03	0.008-0.25	0.008-0.25	32-256	0.06-4	0.008-0.12	0.12-4
GM*		**0.02**	**0.002**	**0.002**	**0.002**	**2**	**0.01**	**0.02**	**0.02**	**59.26**	**0.59**	**0.03**	**0.54**
ATCC*		0.002	0.002	0.002	0.002	0.5	0.015	0.12	0.06	256	2	8	2
Group 2 (*Blastomyces emzantsi*)													

8 (QGQJ00000000; SA-NICD-13)	(Frean et al., 1993)	0.25	0.002	0.002	0.006	2	0.015	0.008	0.03	128	2	0.06	1
9 (QGQI00000000; SA-NICD-15)	(Frean et al., 1993)	0.25	0.002	0.002	0.003	2	0.008	0.008	0.015	32	1	0.06	1
10 (QGQK00000000; SA-NICD-14)	(Carman et al., 1989, Frean et al., 1993)	0.023	0.003	0.002	0.002	1	0.008	0.008	0.008	16	2	0.06	1
QGQL00000000; SA-NICD-16	This study	0.002	0.002	0.002	0.002	1	0.008	0.008	0.008	64	1	0.03	0.5
(QGQE00000000; SA-NICD-12)	This study	0.032	0.002	0.002	0.002	2	0.015	0.008	0.008	128	4	0.06	2
(QGQF00000000; SA-NICD-09)	This study	0.047	0.002	0.004	0.008	4	0.03	0.008	0.12	64	0.5	0.008	1
(QGQG00000000; SA-NICD-10)	This study	0.047	0.002	0.003	0.016	2	0.008	0.008	0.008	64	4	0.03	2
(QGQH00000000; SA-NICD-11)	This study	0.64	0.002	0.002	0.002	0.25	0.008	0.008	0.008	16	1	0.03	0.5
MIC range		0.002-0.64	0.002-0.003	0.002-0.004	0.002-0.016	0.25-4	0.008-0.03	0.008-0.008	0.008-0.12	16-128	0.5-4	0.008-0.3	0.5-2
GM		**0.06**	**0.002**	**0.002**	**0.004**	**1.41**	**0.01**	**0.01**	**0.01**	**49.35**	**1.54**	**0.03**	**1**

Note.- BMD, broth microdilution; GM, geometric mean; *B. dermatitidis* ATCC 10225; AMB, amphotericin B; VRC, voriconazole; POS, posaconazole; ITC, itraconazole; FLC, fluconazole; 5FC, 5-flucytosine; CAS, caspofungin; MFG, micafungin; ANG, anidulafungin

Table 2: Mould-phase MIC distribution of *Blastomyces* isolates (9 *B. percursus* isolates, 8 *B. emzantsi* isolates, 1 *B. dermatitidis* ATCC 10225)

Case #:	Reference	E-test MIC (mg/L)				BMD MIC and MEC (mg/L)							
		AMB	**VRC**	**POS**	**ITC**	**FLC**	**VRC**	**POS**	**ITC**	**5FC**	**CAS**	**MFG**	**ANG**
(Group 1) *Blastomyces percursus*													
1 (QKWI00000000; NCPF4091)	(Dukik et al., 2017)	0.008	0.002	0.002	0.002	0.5	0.008	0.06	0.12	256	0.12	0.06	0.25
2 (LGTZ00000000; BP222)	(Heys et al., 2014, Schwartz et al., 2015)	0.004	0.002	0.002	0.012	4	0.008	0.06	0.03	64	0.06	0.008	0.008
3 (QGQT00000000; SA-NICD-06)	(Schwartz et al., 2015)	0.008	0.002	0.002	0.012	2	0.008	0.015	0.03	256	0.5	0.25	0.5
4 (QGQR00000000; SA-NICD-08)	This study	0.012	0.002	0.012	0.006	2	0.008	0.06	0.06	32	0.5	0.03	4
5 (QGQS00000000; SA-NICD-05)	(Simon et al., 1977)	0.002	0.002	0.002	0.003	2	0.008	0.008	0.015	256	1	0.03	0.25
6 (QGQQ00000000; SA-NICD-04)	(Carman et al., 1989, Frean et al., 1993)	0.002	0.002	0.002	0.003	2	0.008	0.015	0.03	64	0.5	0.03	0.25
7 (SA-NICD-18)	(Martin and Berson, 1973)	0.064	0.002	0.002	0.004	2	0.008	0.008	0.06	128	2	0.06	1
(QGQP00000000; SA-NICD-03)	This study	0.002	0.002	0.002	0.003	0.12	0.008	0.008	0.008	8	0.25	0.008	0.12
(QGQN01000000; SA-NICD-07)	This study	0.002	0.002	0.002	0.002	4	0.008	0.06	0.12	256	0.5	0.03	0.25
MIC range		0.002-0.064	0.002-0.002	0.002-0.012	0.002-0.012	0.12-4	0.008-0.008	0.008-0.06	0.015-0.12	8-256	0.06-2	0.008-0.25	0.008-4
GM*		**0.01**	**0.002**	**0.002**	**0.004**	**1.46**	**0.01**	**0.02**	**0.04**	**94.06**	**0.39**	**0.03**	**0.27**
ATCC*		0.002	0.002	0.002	0.012	0.5	0.008	0.06	0.03	256	1	8	2

Group 2 (*Blastomyces emzantsi*)

8 (QGQJ00000000; SA-NICD-13)	(Frean et al., 1993)	0.25	0.004	0.002	0.012	4	0.008	0.06	0.12	256	4	0.25	1
9 (QGQI00000000; SA-NICD-15)	(Frean et al., 1993)	0.094	0.006	0.002	0.016	4	0.008	0.015	0.06	128	1	0.25	0.5
10 (QGQK00000000; SA-NICD-14)	(Carman et al., 1989, Frean et al., 1993)	0.032	0.002	0.003	0.03	1	0.008	0.008	0.008	128	0.5	0.06	0.5
QGQL00000000; SA-NICD-16	This study	0.47	0.002	0.002	0.003	1	0.008	0.008	0.008	128	4	0.25	1
(QGQE00000000; SA-NICD-12)	This study	1	0.002	0.002	0.012	1	0.015	0.008	0.008	128	1	0.12	2
(QGQF00000000; SA-NICD-09)	This study	0.19	0.002	0.002	0.006	8	0.015	0.008	0.06	128	4	0.12	2
(QGQG00000000; SA-NICD-10)	This study	0.64	0.002	0.002	0.012	2	0.008	0.03	0.06	128	2	0.12	1
(QGQH00000000; SA-NICD-11)	This study	0.5	0.002	0.002	0.006	2	0.008	0.015	0.06	128	2	0.06	0.5
MIC range		0.032-1	0.002-0.006	0.002-0.003	0.003-0.03	1-8	0.008-0.015	0.008-0.06	0.008-0.12	128-256	0.5-4	0.06-0.25	0.5-2
GM		**0.26**	**0.002**	**0.002**	**0.01**	**2.18**	**0.01**	**0.01**	**0.03**	**139.59**	**1.83**	**0.13**	**0.92**

Note.- BMD, broth microdilution; GM, geometric mean; *B. dermatitidis* ATCC 10225; AMB, amphotericin B; VRC, voriconazole; POS, posaconazole; ITC, itraconazole; FLC, fluconazole; 5FC, 5-flucytosine; CAS, caspofungin; MFG, micafungin; ANG, anidulafungin

Table 3: Demographic and clinical features of cases of blastomycosis, 1967-2014 (n=10)

Demographic and clinical features	No. patients (%)
Median age (IQR), years: 37 (32.5-52)	
Male sex	9 (90%)
Ethnicity	
Black African	6 (67%)
White Caucasian	3 (33%)
Unknown	1 (11%)
Province	
Gauteng	4 (40%)
Western Cape	2 (20%)
Free State	1 (10%)
North West	1 (10%)
Northern Cape	1 (10%)
Limpopo	1 (10%)
HIV-seronegative*	5 (100%)

*HIV data available for 5 patients only

Table 4: Distribution of mating type, *BAD-1* gene and genomic size of *Blastomyces percursus* and *Blastomyces emzantsi* isolates, 1967-2014

Clinical strains	WGS	Nucleotide Size (Mb)	No. of contigs	Mating type	absence of a full ortholog *BAD-1* gene
Blastomyces percursus					
SA-NICD-01	QGQM00000000	32,5	5,296	*MAT1-1*	Yes
SA-NICD-02	QGQO00000000	32,5	5,053	*MAT1-1*	Yes
SA-NICD-03	QGQP00000000	32,8	6,175	*MAT1-1*	Yes
SA-NICD-04	QGQQ00000000	32,7	4,934	*MAT1-1*	Yes
SA-NICD-05	QGQS00000000	32,4	5,503	*MAT1-1*	Yes
SA-NICD-06	QGQT00000000	34,2	9,217	*MAT1-2*	Yes
SA-NICD-07	QGQN01000000	32,7	4,782	*MAT1-1*	Yes
SA-NICD-08	QGQR00000000	32,8	5,089	*MAT1-2*	Yes
NCPF4091	QKWI00000000	32,5	4,993	*MAT1-1*	Yes
BP222	LGTZ00000000	32,3	3,871	*MAT1-2*	Yes
Blastomyces emzantsi					
SA-NICD-09	QGQF00000000	27,7	3,274	*MAT1-1*	Yes
SA-NICD-10	QGQG00000000	27,4	2,739	*MAT1-1*	Yes
SA-NICD-11	QGQH00000000	27,6	2,712	*MAT1-1*	Yes
SA-NICD-12	QGQE00000000	27,5	2,597	*MAT1-1*	Yes
SA-NICD-13	QGQJ00000000	34.1	3,717	*MAT1-1*	Yes
SA-NICD-14	QGQK00000000	27,5	3,172	*MAT1-1*	Yes
SA-NICD-15	QGQI00000000	27.4	3, 249	*MAT1-1*	Yes
SA-NICD-16	QGQL00000000	27,5	2,906	*MAT1-1*	Yes

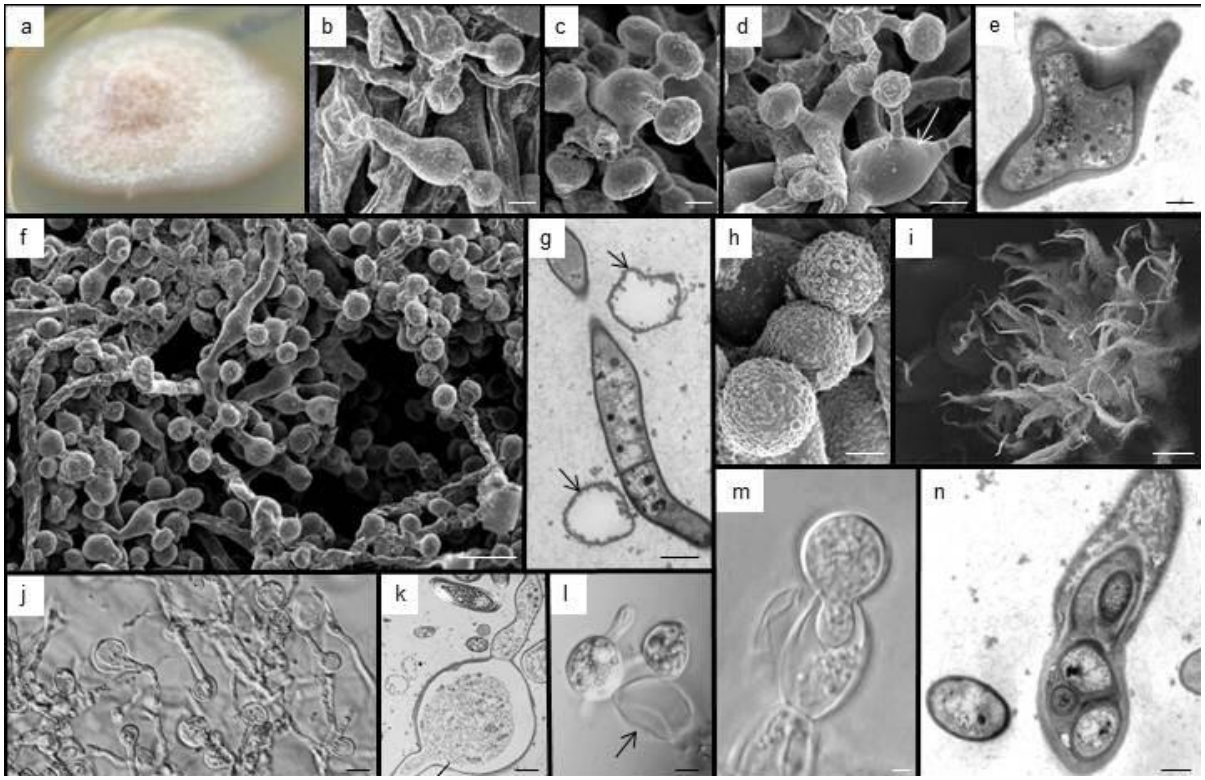
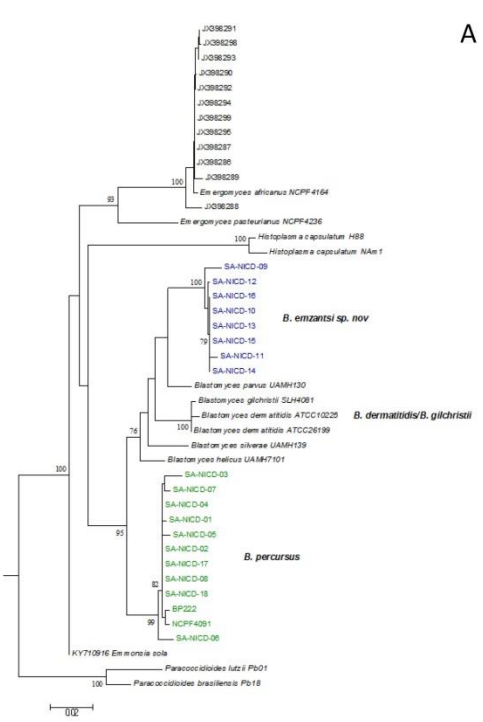
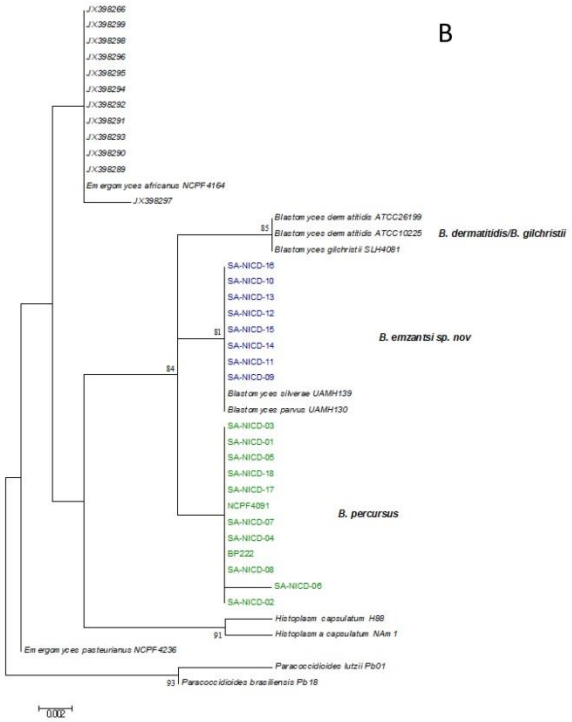


Figure 1: Microscopy of *Blastomyces percursus* (group 1 isolates), providing corroborative and supplementary information to the two published descriptions of this species [1,12]. (a – i) cultures on SDA after three weeks, 25°C; (j - n) cultures on SDA after three weeks, 30°C; (a) floccose colony with a felt of white, aerial, hyphal tufts; (b) conidia generally solitary, and may be subtended by either narrow or ampulliform primary conidiophores; (c) an inflated primary conidiophore with three delicate secondary conidiophores; (d) laterally- borne, solitary conidia may have stout or slender conidiophores, and may also extend from pyramidal, chlamydospore- like, intercalary cells (arrow); (e) tangential section through part of an intercalary, thick-walled cell; (f) abundant conidia production; (g) section through hyphal cells and two conidia (arrows); (h) variable verrucose conidia ornamentation; (i) low-power, scanning micrograph illustrating aerial hyphal tufts; (j) increase in temperature induces cellular swelling, most obviously of terminal cells; (k) cell enlargement with increased temperature is not limited to conidia and terminal cells, as this section through an enlarged, thin- walled, intercalary cell illustrates; (l) conidia swell and

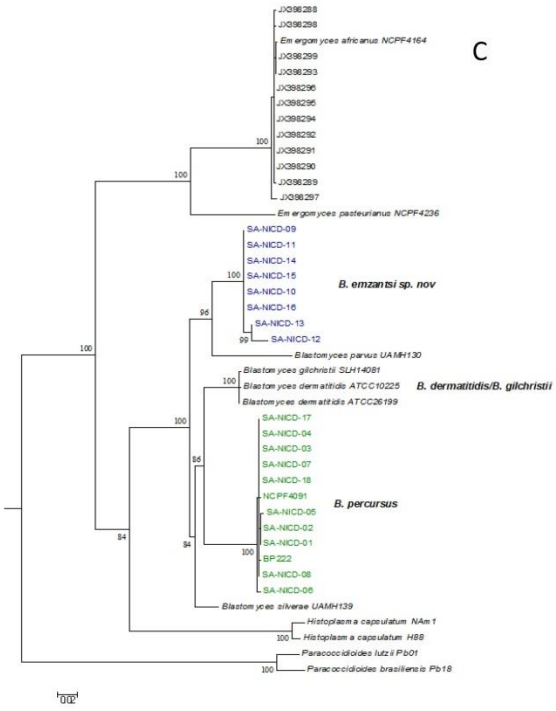
become filled with oil droplets. Note the wall of an empty enlarged cell (arrow); (m) budding of cells into hyphal lumina was common, particularly at intermediate temperatures; (n) sections through hyphal cells revealed the multiple endohyphal/intrahyphal arrangement. Scale bars: a, b, c, e, m, n = 1 µm; d, g, k = 2 µm; f, j, l = 4 µm; h = 0.5 µm; i = 200 µm.



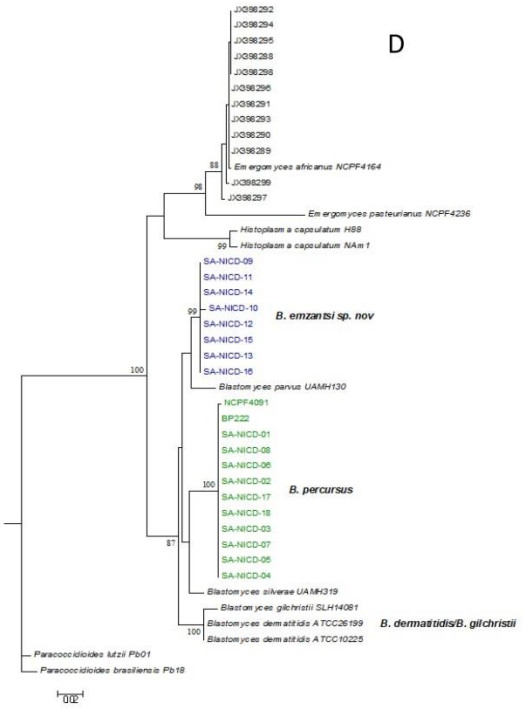
ITS: B. percursus=99%-100%; *B. emzantsi* =97% *B. parvus* and *B. helicus*



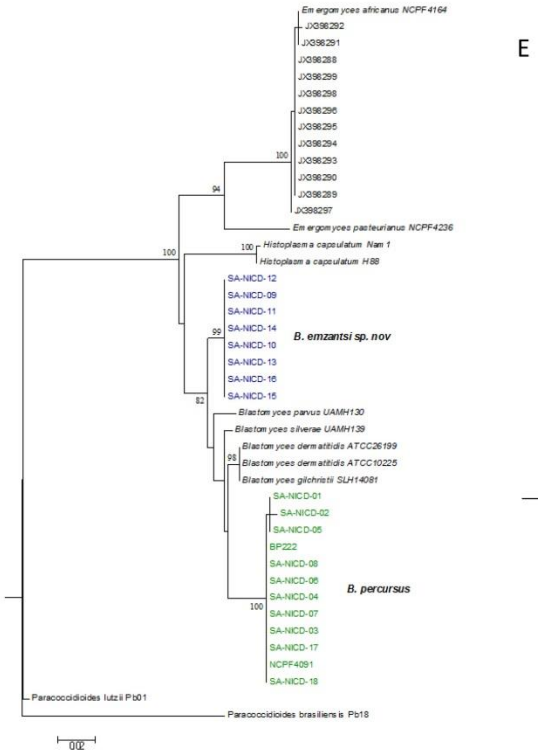
LSU: B. percursus=100%; *B. emzantsi* =100% *B. parvus* and *B. helicus*



Intein *PRP8: B. percursus*=99%-100%; *B. emzantsi* =90%-89% *B. parvus, B. dermatitidis* and *B. gilchristii*

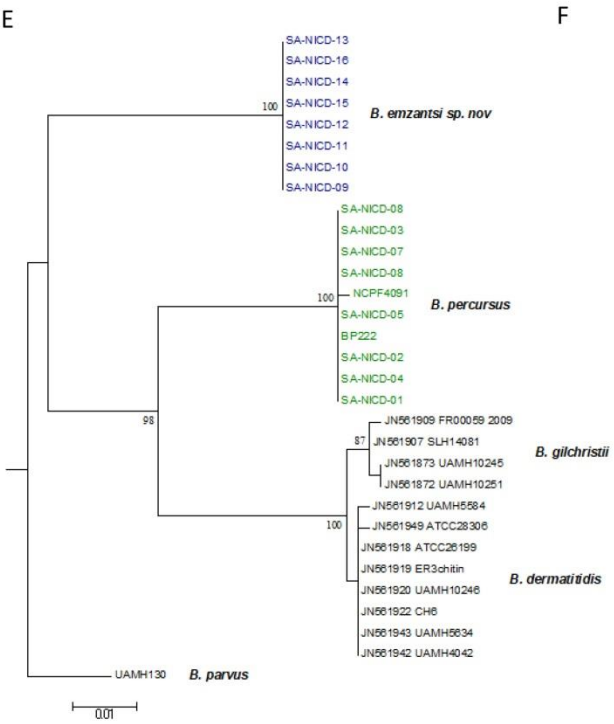


BETA TUBULIN: B. percursus=99%-100%; *B. emzantsi* =93%-96% *B. parvus*, *B. dermatitidis* and *B.gilchristii*

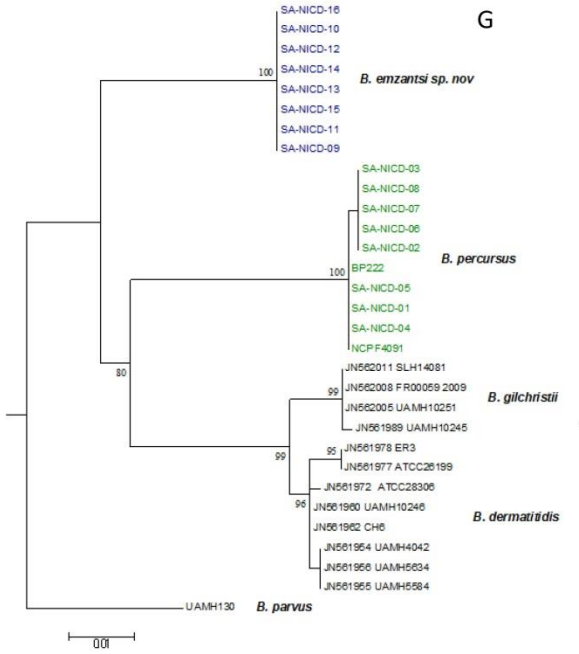


E

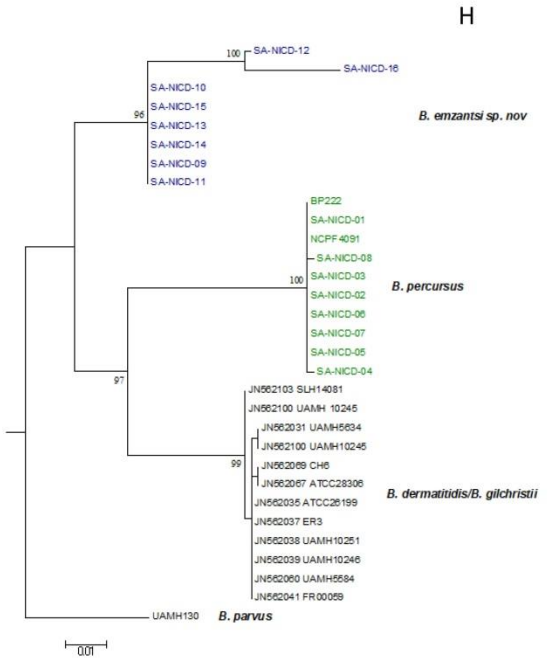
ACTIN: B. percursus=97%; *B. gilchristii*; *B. emzantsi* =97% *B. dermatitidis*



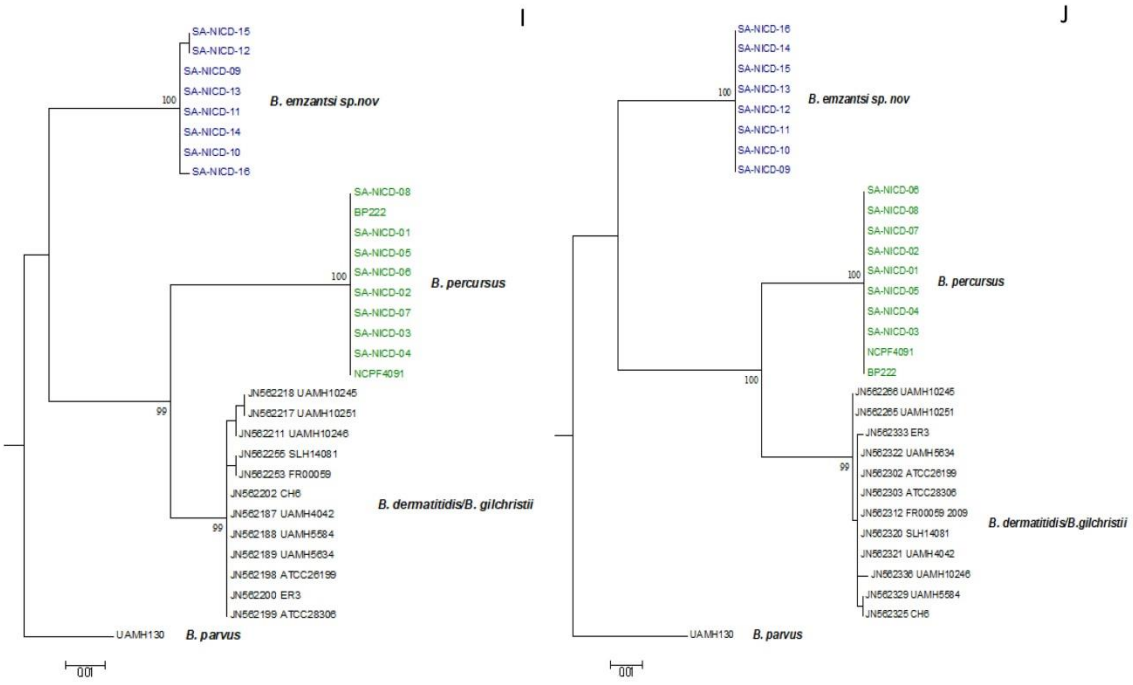
CHS2: B. percursus=95%; *B. dermatitidis/B. gilchristii*; *B. emzantsi* =95% *B. dermatitidis/B. gilchristii*



DRK1: B. percursus=95% B. dermatitidis/B. gilchristii; *B. emzantsi* =94% *B. dermatitidis/B.gilchristii*

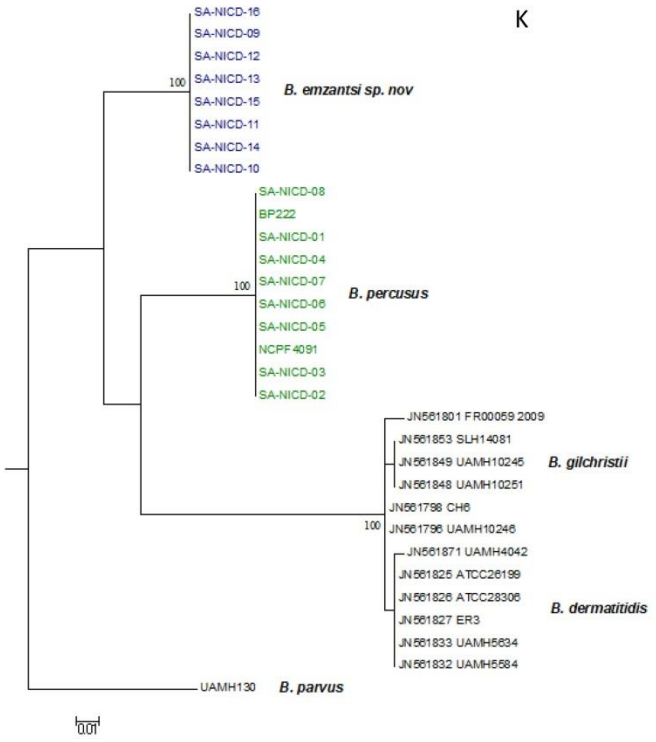


FAD: B. percursus=94% B. dermatitidis/B. gilchristii; *B. emzantsi* =90%-91% *B. dermatitidis/B.gilchristii*



PRYF: B. percursus=94% *B. dermatitidis/B. gilchristii*;
B. emzantsi =95% *B. dermatitidis/B. gilchristii*

ALPHA TUB1: B. percursus=93% B. dermatitidis/B. gilchristii;
B. emzantsi =87%-88% *B. dermatitidis/B.gilchristii*



ARF6: B. percursus=86%-87% B. dermatitidis/B. gilchristii;
B. emzantsi =89% *B. dermatitidis/B.gilchristii*

Figure 2: Phylogenetic analysis of each gene: *ITS*, *LSU*, intein *PRP8*, beta tubulin and actin gene sequences revealed 2 distinct groups **(A-E)** and each gene of *CHS2*, *DRK1*, *FADS*, *PYRF*,

TUB1, *ARF6* also revealed two distinct groups **(F-K)**. **A-E:** these groups corresponded to the recently-named *B. percursus* (group 1 strains comprising 12 isolates with only seven isolates with detailed clinical information) and a separate cluster, here as designated *B. emzantsi* (group 2 comprising eight archived isolates with only three isolates with clinical information). Both groups were closely related to, yet separate from, *B. dermatitidis, B. gilchristii, B. parvus* and *B. silverae.* Only *LSU* could not separate *B. parvus* and, *B. silverae* from *B. emzantsi*. **F-K:** these groups corresponded to the recently-named *B. percursus* (group 1 strains comprising 10 isolates with only seven isolates with detailed clinical information) and a separate cluster, here as designated *B. emzantsi* (group 2 comprising eight archived isolates with only three isolates with clinical information). Both groups were closely related to, yet separate from, *B. dermatitidis, B. gilchristii* and, *B. parvus.*

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