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UNIVERSITY OF THE
WITWATERSRAND,
JOHANNESBURG

Investigating diagnostic methods for histoplasmosis and the ecological niche and comprehensive population structure genomic analysis of African *Histoplasma capsulatum*.

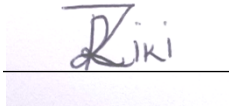
RUTENDO EUGENIA MAPENGO (563169)

Thesis submitted in fulfilment of the requirements for the degree Doctor of Philosophy in Clinical Microbiology and Infectious Diseases in the Faculty of Health Sciences, University of the Witwatersrand, Johannesburg, South Africa

Johannesburg, 2025

Declaration

The work reported in this thesis was carried out under the supervision of Professor Nelesh Govender and Dr Tsidiso Maphanga at the National Institute for Communicable Diseases and the University of the Witwatersrand, Johannesburg. All results presented unless otherwise stated are the sole work of this author, as is the composition of this thesis. The thesis has not been submitted before for any degree or examination at this or any other University. The work is being submitted for the degree of Doctor of Philosophy at the University of the Witwatersrand, Johannesburg.



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Rutendo Eugenia Mapengo **Date**

Faculty of Health Sciences, School of Pathology

Declaration of Plagiarism

I, Rutendo Eugenia Mapengo declare that:

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2. This thesis has not been submitted for any degree or examination at any other university.

3. This thesis does not contain other persons' data, pictures, graphs or other information unless specifically acknowledged as being sourced from other persons.

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Signed:  Date: 08-05-2025

Dedication

This thesis is dedicated to my husband, whose unwavering support, love, and encouragement have been my guiding light through this academic journey. To my children, whose smiles and laughter have provided me with boundless joy and motivation. To my mom, whose wisdom, strength, and sacrifices have inspired me to reach for the stars. And to my entire family, whose collective belief in me has been the cornerstone of my perseverance. This work is a testament to your love and faith in me.

Presentations arising from this PhD degree

1. **Rutendo E. Mapengo**, Tsidiso G. Maphanga, Nelesh P. Govender. Comparative genomics and population structure of South African *Histoplasma* isolates. Poster, Fungal Genetics Conference, March 15th – 20th, 2022, Pacific grove, California, United States of America.

2. **Rutendo E. Mapengo**, Tsidiso G. Maphanga, Nelesh P. Govender. Comparative genomics and population structure of South African *Histoplasma* isolates. Poster, 13th Post Graduate Symposium, July 11th, 2022, University of the Witwatersrand, Johannesburg, South Africa.

3. **Rutendo E. Mapengo**, Tsidiso G. Maphanga, Wayne Grayson, Nelesh P. Govender. Endemic mycoses in South Africa, 2010-2020: a decade-long description of laboratory-diagnosed cases and prospects for the future, British Society Medical Mycology Meeting, Poster, September 4th – 6th 2022. Exeter, United Kingdom.

4. **Rutendo E. Mapengo**, Tsidiso G. Maphanga, Nelesh P. Govender. Comparative genomics and population structure of South African *Histoplasma* isolates. Australian Microbiology Society Annual Conference, Poster, July 3rd – July 7th, 2023. Convention Centre, Perth, Australia.

5. Tsidiso G. Maphanga, **Rutendo E Mapengo**, Oresti Ventouras and Nelesh P. Govender. Molecular Detection of *Histoplasma capsulatum* in Environmental Samples Collected from South Africa. Fourth AIDS Mycoses Conference. Oral presentation, July 10th – 12th 2024, Cape Town, South Africa.

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1. **Mapengo RE,** Maphanga TG, Grayson W, Govender NP**. Endemic mycoses in South Africa, 2010–2020: A decade-long description of laboratory-diagnosed cases and prospects for the future. PLoS Negl Trop Dis. 2022 Sep 28;16 (9): e0010737. CHAPTER 3.**

Articles to be submitted for publication:

1. **Mapengo RE**, Maphanga TG, Jofre IRG, Matute DR, Teixeira M, Alanio A, Barker BM, Garcia-Hermoso D, Govender NP. **Comparative genomics, population structure and phenotypic description of African *Histoplasma spp*. CHAPTER 4.**

2. **Mapengo RE,** Maphanga TG, Alanio A, Sturny-Leclère A, Wake R, Govender NP. **Laboratory validation of *Histoplasma* antigen and molecular assays and evaluation in stored samples from adults with advanced HIV disease. CHAPTER 5.**

3. Maphanga TG, **Mapengo RE**, Ventouras O and Govender NP. **Molecular detection of *Histoplasma capsulatum* in environmental samples collected from South African caves, 2020-2021. CHAPTER 6.**

Abstract

Background: Histoplasmosis, emergomycosis, sporotrichosis and blastomycosis are endemic to southern Africa; the first two are also AIDS-related mycoses. Histoplasmosis is caused by pathogenic *Histoplasma capsulatum* fungi. Since rapid accurate diagnostic tests for disseminated histoplasmosis are not widely available in African countries, the prevalence among adults living with advanced HIV disease is mostly unknown. Knowledge about the genetic relationships of *H. capsulatum* isolates from the African continent to other global isolates and probable sources of exposure is scarce. *H. capsulatum* naturally occurs in caves occupied by bats and South African caves have been documented as probable sources of exposure for speleologists, casual visitors, or guano miners with several outbreaks of acute pulmonary histoplasmosis being reported.

Materials and methods: We analysed electronic pathology laboratory data from all public and some private facilities in South Africa from 2010-2020 to identify the number of endemic mycoses cases reported. A case was defined as a patient of any age with any specimen type submitted to a laboratory from which a thermally dimorphic fungus was cultured or detected by histopathology, *Histoplasma* antigen enzyme immunoassay (EIA), pan-dimorphic reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) and pan-dimorphic fungal PCR/sequencing. We used stored *H. capsulatum* isolates from South Africa for phenotypic description, antifungal susceptibility testing and whole genome studies of African isolates compared to other *H. capsulatum* isolates from the Americas and India. We also validated three different methods for histoplasmosis diagnosis and used them to study the prevalence in South African patients living with HIV (PLHIV), with positive results for cryptococcal antigenaemia and CD4 cell count ≤100 cells/µl. Finally, we used three molecular assays to detect *H. capsulatum* directly from soil samples collected from South African caves.

Results: We identified 682 cases of endemic mycoses reported between 2010 and 2020. Sporotrichosis, emergomycosis and histoplasmosis were the most common mycoses identified. Sequences from African isolates formed a single clade that was different from previously described *H. mississippiense*, *H. ohiense*, *H. suramericanum*, *H. capsulatum sensu stricto* and Indian clades. When screening for *Histoplasma* antigen, the EIA and lateral flow assay (LFA) yielded positive results in 6/194 (3%) and 12/194 (6%) of urine samples from PLHIV respectively and there was no reference method. The positivity rate of the RT-qPCR assay when

testing stored blood samples from PLHIV was 10/533 (2%). From the 56 soil samples collected from South African caves, *H. capsulatum* was detected in five of the seven caves included.

Conclusions: There is a need to increase awareness of histoplasmosis among healthcare workers and to develop rapid point-of-care diagnostic tools and make these widely accessible. Globally, systematic analysis of isolates from different regions is necessary to increase knowledge on the evolution and speciation of *H. capsulatum*. A novel distinct African clade was described and named *Histoplasma africanus sp. nov*, which warrants further studies to determine whether it is necessary to have specific diagnosis and treatment methods specific to this clade. Our study of the evaluation of easy-to-perform diagnostic assays for histoplasmosis on retrospective cohort samples yielded preliminary data on the disease prevalence, which is important, but larger prospective studies are now needed. The screening for *H. capsulatum* nucleic acids in soil from various caves in South Africa showed that the fungus is present in several regularly explored caves with bat populations and the methods used could now be used for further studies in understanding the broader distribution of *H. capsulatum* in the environment.

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My Sister Sharon Ziki, I do not have enough words to thank you, thank you for being a great sister and for the encouragement to follow my dreams. To James Nyamunda, May your soul continue to rest in peace, and I am glad we had time to share our academic dreams though this was too short.

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List of abbreviations and symbols

Ag	Antigen
AHD	Advanced HIV Disease
AIDS	Acquired immunodeficiency syndrome
ART	Antiretroviral Therapy
BAL	Bronchoalveolar lavage
BSL3	Biosafety Level 3
CBP	Calcium binding protein
CDC	Centers for Disease Control and Prevention
CF	Complement fixation
CLSI	Clinical and Laboratory Standards Institute
CSF	Cerebrospinal fluid
DNA	Deoxyribonucleic acid
DRK1	Dimorphism regulating kinase
EIA	Enzyme immunoassay / Enzyme immuno assays
ELISA	Enzyme-linked immunosorbent assay
FDA	Food and Drug Administration
FFPE	Formalin-fixed paraffin-embedded
GMS	Grocott's methenamine silver
Hcc	*Histoplasma capsulatum* variety *capsulatum*
Hcd	*Histoplasma capsulatum* variety *duboisii*
Hcf	*Histoplasma capsulatum* variety *farciminosum*
HIV	Human immunodeficiency virus

HMIN	Histoplasmin
HPA	*H. capsulatum* polysaccharide antigen
HSP82	Heat shock protein
ID	Immunodiffusion
IFN-γ	Interferon gamma
IL	Interleukin
ITS	Internal transcribed spacer
LFA	Lateral flow assay
MALDI-TOF	Matrix-assisted laser desorption ionization-time of flight
MAT	Mating types
MFI	Median fluorescence intensity
MIC	Minimum inhibitory concentration
MLST	Multilocus sequence typing
mL	Millilitre
MRL	Mycology reference laboratory
MS	Mass spectrometry
mtSSU	Ribosomal small subunit
NCBI	National Center for Biotechnology Information
NICD	National Institute for Communicable Diseases
NHLS	National Health Laboratory Service
NRC	Nuclear Regulatory Commission
OIDx	Optimum Imaging Diagnostics
PAS	Periodic Acid Schiff

PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PFGE	Pulse field gel electrophoresis
QC	Quality control
RAPD	Random amplified polymorphic DNA
RFLP	Restriction fragment length polymorphism
RIA	Radioimmunoassay
ROS	Reactive oxygen species
RT-qPCR	Reverse transcriptase-quantitative polymerase chain reaction
SANAS	South African National Accreditation System
SDA	Sabouraud dextrose agar
TB	Tuberculosis
USA	United States of America
WGS	Whole genome sequencing
WHO	World Health Organization
YPS	Yeast phase-specific
µg	Microgram
µL	Microliter

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CHAPTER 1: MOTIVATION AND BACKGROUND

1.1 Statement of the problem

Globally invasive fungal diseases are estimated to have an annual incidence of 6·5 million invasive fungal infections and 3·8 million deaths, primarily in patients who are already immunosuppressed (1). Over the past few decades, the number of immunocompromised individuals has been on the rise, especially those with human immunodeficiency virus (HIV) (2). Patients with Advanced HIV Disease (AHD) are mostly at risk because this subgroup is still large mostly due to people who do not know their HIV diagnosis and those who interrupt their antiretroviral therapy (ART) for various reasons. Worsening the situation is also the fact that there is not much investment in resources or for public health control efforts of such fungal infections (3). Histoplasmosis causes an estimated 100,000 disseminated cases annually, making it less common than invasive candidiasis and aspergillosis, which each exceed 1.5 million cases per year, but the former is still a major concern due to a high mortality rate (30%–50% treated; up to 100% untreated). Histoplasmosis is particularly significant in immunocompromised populations, such as those with HIV/AIDS, and is often underdiagnosed in endemic regions(4–6). Histoplasmosis is caused by a group of cryptic species in the genus *Histoplasma*. Notwithstanding the difficulties posed by histoplasmosis such as the difficulties with diagnosis and lack of awareness, it is also a neglected infection. The funding for research on histoplasmosis remains significantly lower compared to other similar fungal infections especially on the African continent as highlighted by Ocansey, *et al* in 2022 during the search for literature on histoplasmosis in Africa between 2000 and 2020 as they only found 5 original articles together with 4 reviews, 9 case series and 61 case reports (3,7). In Africa, the situation is direr due to the lack of public health resources across the continent and also the increased number of people with underlying conditions that predispose them to histoplasmosis such as HIV/ Acquired immunodeficiency syndrome (AIDS), tuberculosis (TB), is much higher (8,9). The non-specificity of the symptoms for histoplasmosis together with the above-mentioned issues lead to the under-diagnosis of the disease. Histoplasmosis is typically ranked 5th in frequency among serious fungal infections in immuno-compromised patients coming after candidiasis, aspergillosis, cryptococcosis and Pneumocystis jirovecii pneumonia (PJP) according to the

Global Action for Fungal Infections (GAFFI) website (https://www.gaffi.org). Histoplasma is a high-priority fungal pathogen on the WHO fungal pathogens priority list (FPPL).

Histoplasmosis is an AIDS-defining illness and as the number of interventions and control of HIV increased, diseases such as cryptococcosis associated with HIV decreased but little is known on the trends for histoplasmosis because there has been no routine surveillance for histoplasmosis in the African continent (1,8). Few reports have indicated that the number of histoplasmosis-infected individuals without HIV has also steadily increased (4,10). These patients include TB-infected individuals, patients taking immune-suppressing medications, cancer patients and patients who have recently received organ transplants (4,10–12). Co-infections with TB and other fungal infections have been of particular concern because most patients treated for TB alone do not improve and such cases will be classified as drug-resistant cases sometimes leading to death (11). The genetic diversity of *Histoplasma* isolates from the African continent has not been well studied due to lack of funding and difficulties in availability of the isolates caused by lack of resources required for culturing of *Histoplasma* isolates such as Biosafety Level 3 (BSL3) facilities and generally, there has been no long term storage of isolates (13). The environmental niche of *Histoplasma* in Africa has also not been well established but reports have indicated the presence of the fungus in soil associated with bats and bird droppings. This lack of clarity brings problems in the implementation of prevention measures or giving warnings to patients with risk factors to limit exposure by not visiting areas with confirmed *Histoplasma*. Such knowledge gaps have prompted the need for us to carry out studies to address the unknown epidemiology of histoplasmosis in South Africa, to investigate faster and more accurate diagnostic methods for histoplasmosis, to better understand the prevalence of histoplasmosis in people living with HIV, the genetic diversity of African *Histoplasma* isolates and to investigate the environmental sources of this infection.

1.2 Justification

Cases of disseminated histoplasmosis were expected to increase significantly with the progression of the HIV/AIDS epidemic in Africa despite the interventions with ART because many people are not tested for HIV and some do not adhere to the treatment (2,3,8). In countries that have limited access to ART and fungal diagnostic testing, HIV-associated disseminated

histoplasmosis can be fatal. For many decades the delayed diagnosis or misdiagnosis of histoplasmosis has been a persistent issue (14,15). Diagnosing histoplasmosis is difficult because its signs and symptoms often resemble those of cancer and other diseases like TB, which sometimes leads to fatal consequences (11,12). These factors have resulted in an incomplete understanding of the epidemiology of histoplasmosis in South Africa and across the African continent. We aimed in this study to close this knowledge gap by analysing cases reported over a decade (2010-2020) in South Africa for all diseases caused by thermally dimorphic fungi, with histoplasmosis included.

Histoplasma is a thermally-dimorphic fungus that grows as a mould in the environment and converts into yeast inside host tissues (16). Historically, three varieties of this fungi were known to cause disease in humans and animals (17). Two distinct organisms are responsible for histoplasmosis in humans that is *Histoplasma capsulatum* variety *capsulatum* (Hcc) and *Histoplasma capsulatum* variety *duboisii* (Hcd) (also known as African histoplasmosis). Histoplasmosis also affects animals especially the equines and it is known as Equine lymphangitis the organism responsible for that form of the disease is called *Histoplasma capsulatum* variety *farciminosum* (Hcf) (18,19). This classification was based on differences in phenotypic characteristics of the fungi such as the size of the yeast cells but the classification of *H. capsulatum* has been constantly rearranged since the introduction of the use of molecular methods in pathogens typing (20). Research into the genetic differences of *Histoplasma* isolates has been previously done using comparison of single genes or a few loci and this does not give consistent results and has led to improper classification and genetic characterisation of isolates in this genus. There had also been a few African isolates included in such studies, which warrants further investigation. We aimed in this study to investigate the classification of African isolates by comparing them with global isolates and describing the characteristics of these isolates.

Early and accurate diagnosis of histoplasmosis is critical for the rapid initiation of life-saving antifungal treatment (21). In most African countries, histoplasmosis is diagnosed by microscopic examination of tissue and other specimens such as bronchoalveolar lavage and/or culture; the latter may take several weeks to yield results. On the African continent, molecular methods and

antigen testing are mainly used for research purposes and are not available as diagnostic tools in routine pathology laboratories. Serological assays that detect *H. capsulatum* antigens such as enzyme immunoassays (EIA) and lateral flow assays (LFA) offer alternative formats that can be utilised to diagnose histoplasmosis among patients with the disseminated form of the disease (22,23). These assays provide a simple and fast diagnosis and do not require special facilities such as biosafety level 3 laboratories or extensive training, unlike culture or histology (24–26). To assess the usefulness of such diagnostic tests and understand the prevalence of histoplasmosis in South African patients, we conducted a study that involved the screening of *H. capsulatum* antigen and nucleic acids from the urine and blood of people living with HIV. In this study, we evaluated a rapid 15-minute LFA test developed by Optimum Imaging Diagnostics (Scarborough, United States of America (USA) (24) and compared the results to a commercially available EIA manufactured by IMMY (Clarus, Norman, OK, USA) (22,27,28). We also evaluated a novel reverse transcriptase-quantitative polymerase chain reaction (RT-qPCR) assay developed by the Institut Pasteur, France (29). We used these evaluated assays to screen for histoplasmosis among people living with HIV in South Africa.

The environmental reservoir of *Histoplasma* is not established in Africa but it is known that the mycelial phase of this pathogen is harboured in soil (30–32). Outbreaks have been reported in South Africa and the rest of Africa, especially among people who would have visited caves (33,34). To confirm if such caves, especially those suspected of harbouring bats as reported by cave explorers had *H. capsulatum*, we collected soil samples and used three PCR assays to screen for the presence of *H. capsulatum* nucleic acids to establish the pathogen's ecological niche.

1.3 Aims and Objectives

1. 3.1. Aim

To understand the epidemiology of histoplasmosis in South Africa, this study investigated the genetic relatedness of African *Histoplasma* isolates to those from other parts of the world and evaluated rapid diagnostic methods for detecting the pathogen in clinical specimens. It also

aimed to explore the ecological niche of the fungus by testing soil samples collected from South African caves suspected of serving as natural reservoirs.

1.3.2. Main objectives

- To describe the epidemiology of laboratory-confirmed endemic mycoses, including histoplasmosis, in South Africa.
- To perform a comprehensive phenotypic, phylogenetic and comparative genomic analysis of human and animal *Histoplasma* isolates from Africa.
- To investigate the burden of progressive disseminated histoplasmosis among people living with HIV in South Africa using *Histoplasma* antigen enzyme immunoassay (EIA), pan-dimorphic reverse- transcriptase quantitative polymerase chain reaction (RT-qPCR) assay and lateral flow assay (LFA).

1.3.3. Exploratory objective

- To investigate the natural reservoir of *Histoplasma* species in South African caves.

CHAPTER 2: LITERATURE REVIEW

2.1 Background on endemic mycoses

The impact of fungal infections on human health is not widely appreciated though they cause significant morbidity and mortality. Fungal diseases pose a substantial problem mainly driven by the HIV/AIDS pandemic, high TB burden and poverty (35). Due to a lack of resources, estimation of fungal disease burden has been challenging in South Africa and the rest of the African continent. Based on populations of people living with HIV and TB, Schwartz and colleagues estimated that around 3.2 million South Africans (about 7% of the population) develop a serious fungal infection each year although these values may not be accurate (36). The global case fatality ratios (CFRs) for endemic mycoses vary significantly depending on the specific fungal infection, geographic region, and patient factors such as immune status. An overview of key endemic fungal diseases and their associated CFRs was published by Thompson et al, in 2021(37).

Endemic mycoses namely talaromycosis, emergomycosis, coccidioidomycosis, sporotrichosis, histoplasmosis, paracoccidiodomycosis, and blastomycosis are illnesses caused by thermally dimorphic fungi that are found in certain specific ecological niches (38). Endemic mycoses are so named because they are restricted to certain regions in the world and are not cosmopolitan like other fungal diseases. Thermally dimorphic fungi grow as moulds in the environment, at temperatures around 25 degrees, and convert to the yeast form (or spherule for *Coccidioides*) at temperatures above 37 degrees inside the host. Over the past few decades, these illnesses have been reported in many countries than previously believed to be endemic (37). This has been linked to immigration, tourism, and business travel. According to Ashraf *et al*. (2020), the occurrence of endemic mycoses that were formerly thought to be uncommon, such talaromycosis and histoplasmosis, has substantially increased in recent decades due to the rising prevalence of immunocompromised people, mostly people living with HIV (37). In 2008, a study on cases of imported histoplasmosis cases in Europe documented 118 cases and a more recent review of cases reported between 2005 and 2020 documented 728 cases (39). Thermally dimorphic fungi endemic to South Africa include *Histoplasma capsulatum*, *Emergomyces africanus*,

Emergomyces pasteurianus, *Blastomyces emzantsi*, *Blastomyces dermatitidis*, *Blastomyces percursus* and *Sporothrix schenkii* (40–42).

2.2 Endemic mycoses epidemiology

Most endemic mycoses such as emergomycosis, histoplasmosis, sporotrichosis and blastomycosis have a worldwide distribution but patients with histoplasmosis are primarily diagnosed in Latin America and the Mississippi and Ohio River Valleys of the United States (43). In South Africa, there are reportedly an estimated 100, 60, 40, and 10 diagnosed cases of emergomycosis, histoplasmosis, sporotrichosis, and blastomycosis every year based on the numbers of the population at risk, though no population studies have been conducted to confirm the actual burden (41). Over the past few decades, investigations have identified new dimorphic fungi infecting South African patients such as *Emergomyces africanus*, *Blastomyces emzantsi* and *Blastomyces percursus*. An occasional examination of the epidemiology is necessary to assess changes in disease patterns and distribution caused by newly discovered fungal species. There have been a few outbreaks caused by histoplasmosis documented in Southern Africa (34,44,45). Acute pulmonary histoplasmosis cases that were recorded sporadically mostly affected new speleological society members who were exploring caves around the country (34,45). Infections linked to travel have also been documented (34,45). For instance, a South African HIV-seropositive patient who had been to mainland China was reported to have died from talaromycosis in South Africa (46).

Fungi that cause endemic mycoses are mainly present in certain environmental niches, such as soils (47,48). This makes outdoor activities the most common source of exposure and a risk for subsequent illness. Histoplasmosis, blastomycosis, talaromycosis and emergomycosis mainly develop following the inhalation of microscopic airborne microconidia that transform into the yeast form in the lungs and spread to the skin and other organs or systems (49). Typically, traumatic inoculation of the skin with vegetative material harbouring the fungus results in sporotrichosis. Sporotrichosis has also been described as an emergent zoonosis described mainly in Brazil with major reservoirs being cats.

2.3. *Histoplasma:* A global fungal pathogen

Discovered in 1905 by the pathologist Samuel Taylor Darling, *Histoplasma* is the causative agent of histoplasmosis which is sometimes termed Darling's disease to honour the discoverer of the pathogen and is a systemic disease (50). The classification of *Histoplasma* is still under review at the species level, but the fungus belongs to the phylum Ascomycota, subphylum Pezizomycotina, class Eurotiomycetes in the order Onygenales and belongs to the family Onygenaceae and genus *Histoplasma*. Classification of *Histoplasma* species has mainly been done on North American strains which were observed to be more prevalent than Central and South American strains and genetic varieties from other parts of the world. There have been no significant clinical distinctions observed among these genetic groups and further research needs to be done on the speciation of this genus (20,51–53). Initially thought to consist of three varieties Hcc, Hcd and Hcf, recent speciation studies using whole genome sequencing data have identified 6 different clades namely a group from Southern Brazil, India, *Histoplasma capsulatum sensu stricto* Darling 1906 (Panama), *Histoplasma mississippiense* (Nam 1), *Histoplasma ohiense* (Nam 2) and *Histoplasma suramericanum* (Lam A) (13,54,55).

2.4. Global distribution of *Histoplasma*

In 1906, the first case of *Histoplasma* was reported in Panama with several cases being published afterwards from all over the world (50). According to Mazi et al, 2023, there were 79749 unique histoplasmosis cases reported between 2007 and in the United States of America (USA) (56). Reports on the distribution of histoplasmosis have mainly utilised the Histoplasmin skin test which indicated previous exposure to the pathogen. Histoplasmin skin tests were positive in as much as 90% of people living in the United States in areas of high endemicity (57). The prevalence in Canada along the St. Lawrence River Valley was as much as 20% to 30%, which was an indication of frequent infections in this region (58). An estimated 60% to 90% of people living in the United States of America in areas surrounding the Ohio and Mississippi River valleys (where *H. capsulatum* is common in the environment) have been exposed to the fungus at some point during their lifetime (59). *Histoplasma capsulatum* is recognized as endemic in India, though the actual prevalence of this fungal infection remains uncertain (60,61). Most

histoplasmosis cases in India have been reported from the eastern and north-eastern regions, particularly in Calcutta (West Bengal) (60). The Gangetic West Bengal is the region with the most frequent infections with 9.4% of the population testing positive for the histoplasmin skin test (62). The presence of *H. capsulatum* in soil from a bat habitat in West Bengal was confirmed by inoculating mice with water from the soil sample and the mice then developed histoplasmosis proving the presence of histoplasmosis in this region (5,31). *H. capsulatum* has also been detected in caves in southern and East Africa using various molecular methods (8,63–66). Venezuela and Brazil have been considered hotspots for histoplasmosis due to high numbers of case reports (67,68). In other parts of the world that have been traditionally considered to be non-endemic such as in Europe, reported cases were from immigrants coming from endemic areas (6,69). The maps of histoplasmosis endemicity are being redrawn due to the recent availability of better methods for histoplasmosis diagnosis (37). In 2020, Ashraf and others published a map (Figure 1) showing the estimated areas of endemicity for histoplasmosis based on cases reported in the literature (37). Novel cryptic species in the genus are shown in coloured circles based on previous publications using whole genome sequencing data.

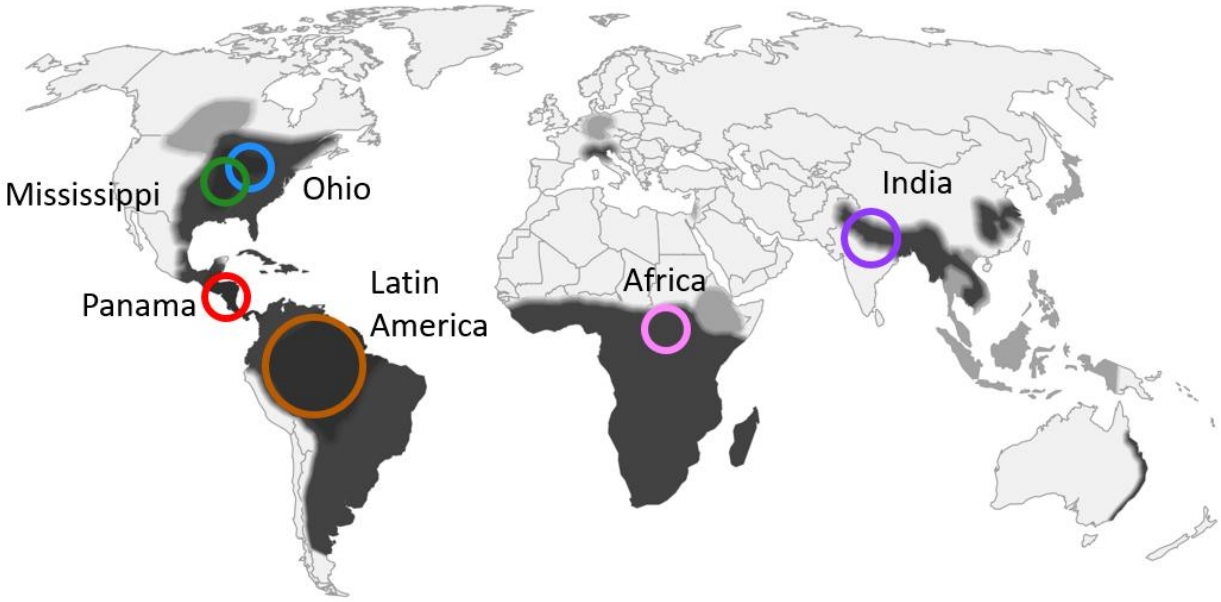

Figure 1: Map showing the estimated global distribution of histoplasmosis cases based on literature review in 2020 (Sepulveda et al., 2017; Jofre et al., 2022; Teixeira et al.; in prep; image modified from Ashraf et al., 2020). Green: *H. mississippiense*, blue: *H. ohiense*, purple: India, pink: Africa, orange: *H suramericanum*, and red: *H. capsulatum sensu stricto* circles denote the

number of *Histoplasma* whole genome sequences analysed per region respectively –circles not to map scale.

2.5. *Histoplasma* in Africa and South Africa

There have not been many studies on the epidemiology of histoplasmosis on the African continent. Cases of histoplasmosis have been reported in 32 African nations and the cases were caused by all three varieties of *Histoplasma* namely: *H. capsulatum* variety *capsulatum* (*Hcc*), *H. capsulatum* variety *duboisii* (*Hcd*) and *H. capsulatum* variety *farciminosum* (*Hcf*) (8,9). *Hcd* is primarily found in Central and Western Africa, whereas *Hcc* has been reported mostly in Southern and North Africa (66,70–73). Additionally, five confirmed cases from Madagascar have been described (74,75). Ekeng *et al*, 2022 published a map displaying the estimated cases of gastrointestinal histoplasmosis, diagnosed predominantly using histopathology of biopsied tissues/specimens, based on literature searches across world regions but they do not the show actual burden of the disease (76). Because of the unavailability of competent individuals who can correctly identify *Histoplasma* cultures and well-resourced facilities in many countries, most African countries cannot properly diagnose histoplasmosis. As a result, the number of cases that have been recorded is probably underestimated.

The majority of *Hcd* cases have been reported in West, Central, and East Africa; however, the frequency of the condition across Africa as a whole is not uniform (70,77,78). Oladele et al, 2018 asserted that the majority of *Hcc* reports came from the southern part of the continent and there were only two countries in northern Africa, Egypt and Morocco, where cases of human histoplasmosis caused by *Hcc* were documented (8). Bongomin et al, 2022 highlighted that there have been reports of apparent *Hcc* 'microfoci' which areas with unique growth requirements for *Hcc*, in the Democratic Republic of the Congo (DRC), Ghana, Kenya, Ivory Coast, Benin, and Senegal (1,79). These areas may be the result of immigration by residents from endemic areas to these specific areas. Most reviews on histoplasmosis in Africa have been in the adult population and to address the prevalence in children, Ekeng et al, 2022 published 44 cases of histoplasmosis in African children reported between 1950 and 2021 distributed across Western Africa, Eastern Africa, Southern Africa, and Central Africa (80). This study included histoplasmosis cases in children aged between 1 and 17 years including all types of

histoplasmosis. The report showed that no cases were found from Northern Africa. Cases of histoplasmosis in children were also reported by Amona et al, with 54 cases reported from Democratic Republic of Congo (71).

Outbreaks of acute pulmonary histoplasmosis in South Africa have been reported mostly in individuals who had recently explored caves (34,45). Craven, 2013 published an epidemiology report of cases of acute pulmonary histoplasmosis in Africa, with South Africa included (34). The publication summarised caves where cave explorers had reported having histoplasmosis symptoms after visiting and they included caves in Democratic Republic of Congo, Tanzania, Ethiopia, Ghana, Madagascar, Nigeria, Zambia, Zimbabwe and South Africa. The South African caves with the most cases reported were in Gauteng and Western Cape provinces with sporadic cases reported from Limpopo, North West, Mpumalanga and Northern Cape provinces. In South Africa, in-depth information from skin test prevalence surveys, which act as a proxy for environmental exposure, is seldom provided. In 1949 a histoplasmin skin test was conducted on a cohort of 140 South Africans and 8% of these had a strong positive result and 6 cases had a doubtful positive/negative result (81). Oladele and colleagues reported that 12 histoplasmin skin test surveys had been carried out in the whole of Africa up until 2017, with a positivity rate of 0% to 35% (8). Given the lengthy incubation period between exposure and clinically apparent disease and the possibility that patients could visit healthcare facilities far from their places of employment, residence, or outdoor activities, mapping of laboratory-diagnosed cases generally does not indicate the source of infection. Schwartz and colleagues investigated cases of endemic mycoses related to HIV/AIDS in South Africa and confirmed 4 cases of histoplasmosis using the urine *Histoplasma* antigen test (82). Kathali et al, 2022 published 24 cases of African histoplasmosis in the KwaZulu Natal province of South Africa; these *Hcd* cases were confirmed using histopathology and not culture. It is possible that infection have been caused by *Hcc* or other dimorphic fungi such as *Blastomyces* species and *Emergomyces* species since it is difficult to confirm identification at a genus/species level without culture or molecular methods (72). In this study, they observed both small and large fungi under the microscope and all the yeasts had various shapes with thick walls and marrow neck budding observed (72).

2.6. Clinical syndromes

Many people worldwide may get exposed to *Histoplasma* at a certain point in their lives but are not aware of it. These people develop some immunity, which reduces the severity of the disease upon re-exposure (83). The underlying health of the patient usually determines the clinical manifestations of histoplasmosis. The manifestations of the disease range from asymptomatic infections to rapidly progressive/ acute pulmonary disease and the disseminated form of the disease with complications such as respiratory failure (84). These symptoms largely depend on the level of exposure (i.e., the quantity of fungal particles inhaled), the host's immune system, and the virulence of the infectious strain, suggesting that environmental factors influence the development of the disease. If symptoms of histoplasmosis infection arise, they typically appear within 3 to 17 days after exposure, with an average onset of 12–14 days. Most individuals affected by histoplasmosis remain asymptomatic and show no clinical signs. The acute phase of histoplasmosis is marked by non-specific respiratory symptoms, often resembling a cough or flu. In 40–70% of cases, chest X-ray results appear normal. Histoplasmosis can become a chronic lung infection in some people, mainly those with compromised immune systems, or it can migrate from the lungs to other body systems, such as the central nervous system (the brain and spinal cord) (85). Those who have lung conditions like emphysema are susceptible to chronic histoplasmosis. Weight loss and a bloody cough are two indicators of chronic histoplasmosis. The signs of chronic histoplasmosis might occasionally resemble those of tuberculosis. Histoplasmosis affects HIV/AIDS patients mainly and in South Africa, the burden of histoplasmosis in this population remains unknown. In order to address this gap, we collected blood and urine samples from HIV-positive patients with CD4 cell counts less than 100 to assess the burden of histoplasmosis at a national level.

2.7. Pathogenesis

Histoplasmosis is mostly contracted by the inhalation of microconidia found in various substrates, such as bat and bird droppings, mainly in caves or other environments with high humidity. This is a systemic intracellular mycotic disease. Once the microconidia get inside the host lungs, they are deposited in the alveoli and are rapidly converted into parasitic yeast cells inside macrophages. The mycelial form stops being metabolically active when it enters the host, prompted by the mammalian body temperature. Following that, it is believed that "shunt routes"

driven by cysteine and sulfhydryl chemicals stimulate morphogenesis to the yeast form (86). Typically, this transition process takes a few hours to several days. Evaluation of genes involved in this transition showed that *DRK1* (dimorphism regulating kinase) is important for this change since silencing this gene reduces the virulence of *Histoplasma* (87,88). Other genes important for pathogenesis such as calcium binding protein (*CBP1)* and alpha-glucan synthase 1 (*AGS1)* were not expressed (42). Several genes were also revealed as factors important for the morphogenesis of *Histoplasma* namely the regulator of yeast phase 1-4 *(RYP1-4)* (89,90,90). Sulfhydryl-blocking substances, which keep the fungus in the mycelial phase, can prevent this morphogenesis. Intriguingly, employing this blocking approach, treated *Histoplasma* is unable to propagate illness in tested animal models, highlighting the requirement of the yeast form's change for pathogenicity (86). The transition from mycelial form to yeast form can happen before or after the microconidia have been ingested by macrophages in the upper airways or the alveoli (91). Once the yeasts are engulfed by macrophages, they can survive inside phagolysosomes. Macrophages are activated to kill the pathogen through the use of cytokines that include interleukin (IL) -12 and interferon (IFN) gamma in order to stop disease spread (92). In the disseminated form of the disease, spreading of the yeasts through the body is driven by phagocytes spreading firstly to lymph nodes and eventually to all other body organs (49).

2.7.1. Mucosal Barriers

When the disease first begins, aerosolised microconidia enter the host's airways; the most often reported conditions are lung and sinus infections (86). Regarding “sinus infections”, this may be part of disseminated disease (86). *Histoplasma* microconidia can elude first innate responses in the respiratory tract, including mucus, mucociliary clearance, and initial antibody defences, because of their tiny size and the negative pressure that inhalation creates. *Histoplasma* will then convert to the yeast form once it travels down the upper airways to the lungs/ alveoli. The alveoli contain the first host defences that have an impact on yeast survival.

2.7.2. Developing a niche within host macrophages

Inside the alveoli, innate immune system cells are activated. While yeast cells can interact with multiple cell types, they specifically invade alveolar macrophages which form part of the innate immune response. *Histoplasma* enters the macrophage by phagocytosis and this process of

engulfment is mediated by complement receptors and opsonisation of the pathogen is not essential. Mittal et al, 2018 reported that this is relevant since the blood vessels in the lungs have low serum opsonin levels (49). The pathogen benefits from using this phagocytosis method because it stops the activation of other fungicidal pathways, which aids in the organism's ability to stay within a cell. After entering the cell, the pathogen also alters its cell wall to avoid being detected by more macrophage receptors. After being engulfed by macrophages, *Histoplasma* is then maintained in the cytoplasm of the cell in a phagocytic vacuole. The ability of *Histoplasma* to survive in phagocytes depends on the development of virulence factors that prevent the identification of fungal cells and disarm the phagocytes' reactive oxygen species (ROS)-based defences (93–95). Another mechanism of survival for *Histoplasma* inside macrophages is by hiding immunostimulatory glucans to prevent them from activating signalling receptors such as the glucan receptor Dectin-1. The fungus generates extracellular superoxide dismutase and catalase, two oxidative stress defence enzymes, to combat reactive oxygen species produced by phagocytes. Inside leucocytes, *Histoplasma* induces an anti-apoptotic state which correlates with decreased cell death allowing the fungus to survive for longer inside the cells (96). These mechanisms allow for continued replication leading to infection of more cells.

2.7.3 Virulence

Different strains of *Histoplasma* all cause respiratory and systemic disease, and the yeast form phase produces virulence factors. A number of genes mostly associated with the cell wall have been implicated in regulating the virulence of *Histoplasma* species (88). The *Histoplasma* cell wall is made up of lipids, proteins, carbohydrates and melanins and the virulence of this fungus is achieved in part through alterations of the cell wall. Figure 2 shows the schematic representation of the *H. capsulatum* cell wall (97). A protected environment is achieved through the uptake of *H. capsulatum* by macrophages through phagocytosis and to subvert this uptake yeast phase of *H. capsulatum* must avoid the activation of macrophage's antifungal defences. Table 1 below shows the components of the cell wall and their role in pathogenicity/virulence. Although it is missing in the mycelial form, α- (1, 3)-glucan is present in the cell wall of many virulent strains of *H. capsulatum* in the yeast form (49,98). This polysaccharide creates a coating that hides antigenic cell surface glucans, preventing the host phagocytic cells from recognising the fungus. The Dectin-1 receptor on macrophages recognises the β- (1, 3)-glucan present in the

cell walls of *H. capsulatum* and other fungi, which triggers the production of reactive oxygen species and proinflammatory cytokines (99). According to Gauthier (2017), the yeast phases of *Paracoccidioides spp* and *Blastomyces spp* also contain this polymer (16,100). Through genetic manipulation the loss of the α-(1, 3)-glucan in these dimorphic fungi including *Histoplasma* has resulted in the loss of virulence. It is unclear if this polysaccharide significantly influences virulence; instead, its absence may only change the structure and porosity of the cell wall. In a mouse model of infection, α- (1, 3)-glucan presence corresponds with strain-specific virulence and affects the dynamics of intracellular differentiation and proliferation. *H. capsulatum* strains control α- (1,3)-glucan synthesis so that it seems to be continuously produced when the yeasts are multiplying inside macrophages (87,90,101,102). Heat shock protein (Hsp60), which plays crucial roles in regulating proper protein folding and chaperoning intracellular proteins, has also recently been identified as a crucial surface molecule that facilitates macrophage identification and phagocytosis of yeast (97). Another crucial component of *H. capsulatum*'s normal growth and response to cellular stresses is Hsp82. This protein binds to several cellular proteins and prevents them from becoming active until they have reached the correct intracellular location or have received the right activation signal (99). The *CPB1* gene encodes a secreted metalloprotease that degrades host proteins and disrupts phagolysosome function, enabling fungal survival in macrophages. CBP is a protein that binds to calcium and is another crucial component of *Histoplasma* pathogenicity. During the yeast phase of intracellular development in the macrophage, CBP is secreted by the fungus (103). A subset of *H. capsulatum* strains have the YPS3 which has been implicated in *H. capsulatum* as a virulence factor and is important for the fungus' survival (87). The *YSP3* gene produces a yeast-phase cell wall adhesin that promotes host cell attachment and immune evasion, enhancing fungal dissemination.

Table 1: Summary of the components of *H. capsulatum* cell wall and their role in pathogenicity/virulence.

Cell wall component	**Role**
Surface carbohydrates	
Chitin	Maintenance of the cell wall rigidity and integrity and resistance of extracellular environment.

β-1, 3-glucan	Major component of the cell wall of the filamentous phase of *H. capsulatum* promotes the production of pro-inflammatory cytokines and inflammatory cell recruitment.
α-1, 3-glucan	Regulates the proliferation of the yeast phase inside host phagocytes by providing protection inside phagosomes.
Galactomannan	Inhibits macrophage migration factor release
Lectin-like components	On murine macrophages, these components bind to the 68 kDa galactosylated surface molecule, involved in macrophage activation and phagocytosis regulation.
Mannoproteins	Highly antigenic and leads to maturation of dendritic cells and activation with proinflammatory cytokine production. Helps in host tissue adherence.
Lipids	
Ceramide monohexoside	Required for fungal survival and found in both mycelial and yeast phase.
Extracellular vesicles	Contain carbohydrates, lipids and proteins and pigment-like structures required for metabolism, virulence, signaling and cell-recycling.
Proteins	
Heat shock protein 60	Mediates attachment to macrophages and monocytes CR3 integrin resulting in phagocytosis. Also an immunogenic target serving as a target for passive immunization therapy.
Heat shock protein 70	Elicits a cutaneous delayed-type hypersensitive response in mice, highly expression during transition from mycelial to yeast phase.
M-antigen	Major diagnostic antigen eliciting intense cellular and humoral responses. Important catalase for detoxification of host-derived peroxidases preventing oxidative stress.
H-antigen	Involved in nutrient acquisition and modulation of cell wall architecture.

H2B	For cell wall signaling and modulation of immune responses by the fungus.
Yeast phase synthase 3 (YPS3)	Linked to *H. capsulatum* virulence *in-vivo*. Associated with increased fungal burden in tissues and organs.
Melanin	Protects fungus from myriad insults and enhances structural strength of the cell wall. Protects against radiation, extreme temperatures, free radicals and predation in the environment.

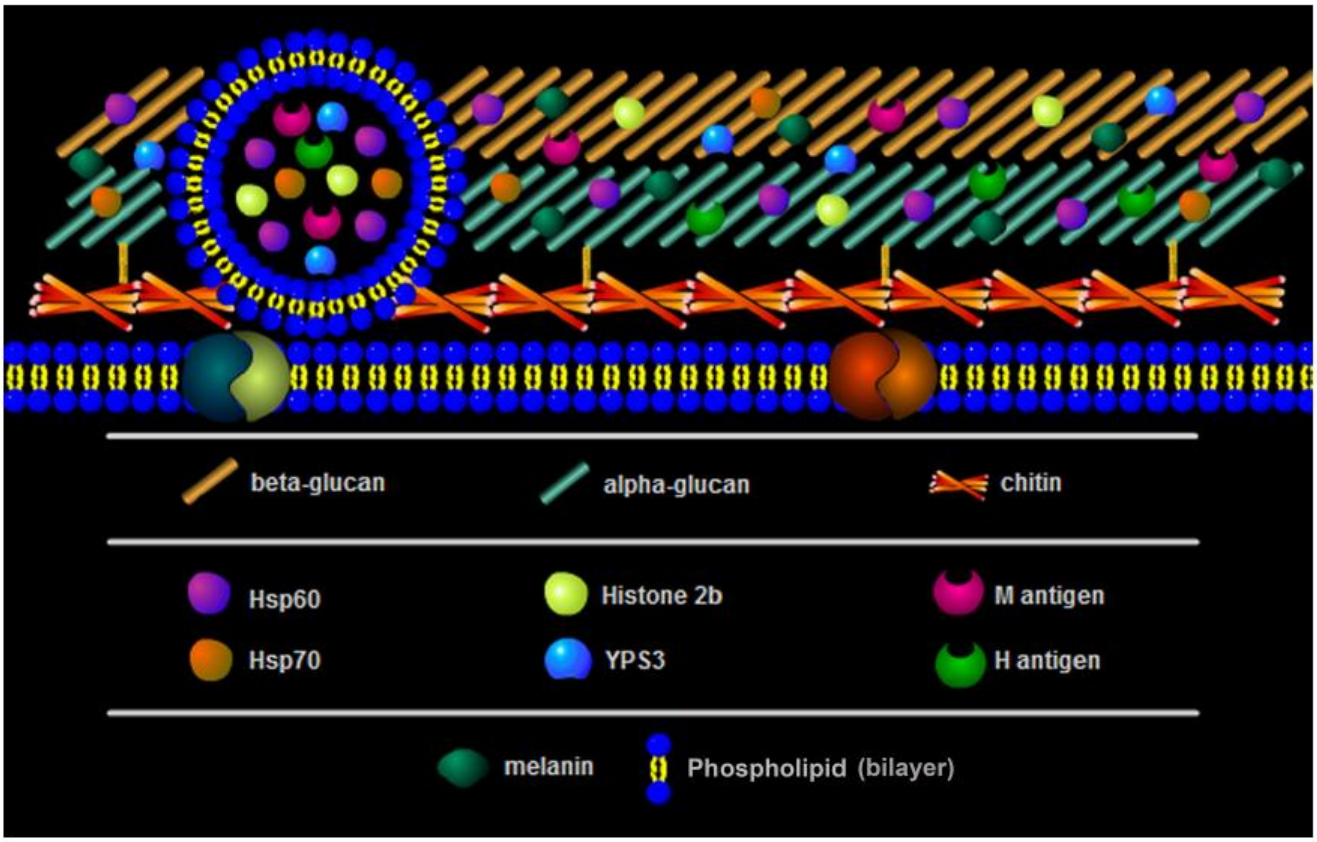

Figure 2: A hypothetical model schematic illustration of components of the *Histoplasma capsulatum* cell wall (Guimaraes *et al*, 2011)

2.8. Histoplasmosis diagnosis

The medical and travel history, signs and symptoms, physical examination, and laboratory tests are useful in diagnosing histoplasmosis (104,105). Although signs and symptoms of histoplasmosis are well known, histoplasmosis cannot be diagnosed only based on clinical evidence since it shares many of these symptoms with other illnesses. Symptoms for acute histoplasmosis and a variety of viral or bacterial respiratory tract illnesses are comparable (11). The signs of more severe pulmonary histoplasmosis, such as lung infiltrates and hilar lymphadenopathy, also manifest when *Mycobacterium tuberculosis* or other thermally dimorphic fungi are present (11,106). To make the correct diagnosis, *Histoplasma* must be isolated by culturing at 25°C in a BSL3 facility or the yeast form must be found through a direct examination of clinical samples using fungal staining procedures (100). Identification of antibodies and antigens, which are indicators of an active disease, aids in the management of infections (107). When antibody detection is questionable, antigen detection techniques are also employed and these include EIA and LFA (22,108,109). This occurs most commonly in immunocompromised patients with extensive diseases who are ordinarily unable to mount an immunological response. Most of the time, the clinical presentation and host characteristics will determine the best test to utilise for diagnosis. Although it is typically a good idea to grow the organism, culture-based methods are most effective when the fungal burden is high, as in some cases of chronic or disseminated histoplasmosis (25,110,111). In both subacute and acute histoplasmosis, culture is not always feasible. Non-culture-based methods can improve the ability to diagnose *Histoplasma* infection and assist guidance of histoplasmosis treatment. Molecular methods such as PCR can also be used for histoplasmosis detection and they allow earlier disease detection and greater diagnostic accuracy (112–115).

2.8.1. Specimen types

Clinical specimens depend on the nature of the infection, for example, sputum or bronchial alveolar lavages are the preferred specimen for pulmonary disease, or tissue biopsy if the patient has skin or mucosal lesions (116). Biopsy material from the diseased organ is useful if can be obtained and in case of swollen lymph nodes, aspirated pus may be used. Bone marrow is an excellent source of fungus in the context of disseminated disease (14). Peripheral blood is also used for culturing the fungus (in blood culture) and fungal nucleic acid extractions for

confirmation of the presence of *Histoplasma* (29). Gastric washings are also a source of *Histoplasma* mainly in children with pulmonary disease as they produce and frequently swallow their sputum. Urine and serum are used to test for antigens using EIA's and LFA's (21,28). The fungus can also be directly detected using molecular methods after extraction of nucleic acid from different specimen's types such as tissue biopsies, blood and pus aspirates (112,117,118).

2.8.2. Histopathology and direct visualisation

Techniques used to diagnose histoplasmosis include direct examination of tissues to look for yeasts after staining. It is recommended to analyse skin scrapings, exudates, and bodily fluids with 10% KOH, Parker ink, or Calcofluor white mounts, Gram stain, Grocott's methenamine silver (GMS) and Periodic Acid Schiff (PAS), these methods can also be used for staining of FFPE tissue(119). *H. capsulatum* can take the form of an oval yeast that is 2 to 4 µm (var. *capsulatum*) or 8 to 15 µm (var. *duboisii*) in size, although this can be difficult to distinguish and will need to be coupled with other diagnostic methods such as culture (120). Yeasts may be detected in clusters after being phagocytosed by macrophages, which can aid in the diagnosis (92). However, some fungi, including *Emergomyces spp*, *Blastomyces spp.*, endospores of *Coccidioides spp.*, *Nakaseomyces glabrata*, as well as the causative agents of leishmaniasis, toxoplasmosis, and Chagas' disease, can be misidentified as *Histoplasma* (121–123). In patients with disseminated disease, specimens from the site of infection such as swabs from lesions and bone marrow biopsies have increased sensitivity (105). Histopathologic tests of bone marrow, sputum, bronchoalveolar lavage fluid, biopsy material from lung or skin biopsy from lesions are frequently carried out for the diagnosis of histoplasmosis(124). In patients with disseminated histoplasmosis, peripheral blood can also be examined and intracellular organisms will be observed in white blood cells in about 50% of cases(125). A bone marrow specimen for histopathology may be the quickest way to diagnose invasive disease but it presents challenges of causing discomfort to patients and may not be possible in patients with low platelet counts (124).

2.8.3. Culture

The cornerstone for the definitive diagnosis is the culturing of *Histoplasma* from clinical samples. The fungus can be isolated on media, such as Sabouraud dextrose agar (SDA) or potato dextrose agar (PDA), MALT and *Histoplasma* Macrophage Media (HMM) after 6 to 12 weeks of incubation at 25°C(126). It also grows well on SDA and PDA-containing cycloheximide which prevents the growth of saprophytic fungi(126). Conversion is carried out using an enriched medium, such as Brain Heart Infusion (BHI) supplemented with 5% sheep blood and incubated at 35-37°C. Culturing of *Histoplasma* presents challenges such as contamination by other fungi and bacteria due to the prolonged incubation periods required for growth. Level 3 biosafety facilities are also required for safety when culturing this pathogen which is a problem in resource-limited settings (127).

2.8.3.1. *Histoplasma* morphology

In vitro at lower temperatures around 25°C, the fungus grows in the mould form. The macroscopic features of this form include cream to tan colonies that are characterised by slow growth, and they also have a granular to cottony appearance. The reverse side of the mould phase when viewed under the plate has an appearance showing a cream-to-yellowish colour. These features can be observed in SDA/ PDA. Morphological differences between the three varieties of *Histoplasma* are shown in Table 1.

Table 2: Comparison of morphological and clinical characteristics between the different varieties of *Histoplasma*.

Groups	*Histoplasma capsulatum* Sensus stricto	*Histoplasma capsulatum* var *duboisii*	*Histoplasma capsulatum* var *farciminosum*
Hosts	Humans	Humans	Equines
Yeast size in tissue	2 to 4 µm	8 to 15 µm	3 to 5 µm
Yeast morphology in tissue	Narrow-necked budding yeasts with a pseudocapsule, found in the cytoplasm of the large histiocytes*	Pear-shaped spores, clustered within giant cells and histiocytes	Small ovoid structures, surrounded by a clear halo, inside the macrophages and monocytes
Incidence	Occurs in 2-25 % of AIDS patients(128)	Reported in 20.8% of HIV/AIDS patients in 94 documented *H. duboisii* cases (1993-2019)	Common in equines
Epidemic area	Mostly reported in the Americas but has been reported in Africa, Asia and imported cases in Europe	Localised in Africa	Reported mainly in North Africa
Inflammatory cell infiltrate in tissue	Dense dermal infiltrate as well as suppurative granulomatous infiltrate in ulcerative lesions and neutrophils and eosinophils seen in oral lesions.	Suppurative granulomatous dermal infiltrate	Loose granuloma, lympho-histiocytic aggregates, or mononuclear diffuse infiltrates

* Histiocytes are tissue-resident macrophages and giant cells are formed from by union of several distinct cells usually histiocytes

The microscopic features of *Histoplasma* at 25°C show hyphae that are septate and hyaline. This form is a multicellular mycelium formed of vegetative hyphal networks that are connected to one another. The fungus produces hyphae-like conidiophores which arise at right angles to the parent hyphae. The fungus forms both macroconidia and microconidia by the formation of terminal phialides developing from conidiophores or they can arise directly from hyphae. Conidia are formed from asexual reproduction producing non-motile aleurioconidia developing externally from the conidiophore. Macroconidia are tuberculate or smooth, thick-walled, round, unicellular and hyaline. Microconidia are smaller, unicellular, hyaline and round with a smooth or rough wall. Depending on the strain and development circumstances such as the amount of nutrients available in the environment, macroconidia have been seen to range in size from 8 μm to 14 μm whereas microconidia are typically 2 μm to 6 μm in size.

Stimulation by mammalian body temperature is sufficient to initiate the developmental switch from hyphal growth to yeast-phase growth; this is one of the most crucial features of *Histoplasma* biology. At 37°C, *Histoplasma* forms creamy, slow-growing, moist and yeast-like colonies. Narrow-based, ovoid, budding yeasts can be observed under the microscope. This phase is mainly clearly observed in infected tissues or *in vitro* on enriched media such as BHI agar with 5% sheep blood and HMM (129). Morphological differences between the yeast phase of cryptic species in the genus *Histoplasma* were also described for 5 groups including *H. mississippiense*, *H. ohiense*, *H suramericanum*, *H. capsulatum sensu stricto* and Africa. The species-specific phenotypic variation was observed on the surfaces of the colonies of these species with *H. ohiense* having a rough surface which was different from the smooth surfaces observed on *H. mississippiense*, *H suramericanum*, *H. capsulatum sensu stricto* and Africa. This difference has been reported to be due to the lack of α-1, 3-glucan in *H. ohiense* and can be used as a diagnostic trait differentiating the different species (130). The *H. mississippiense* group was the only species reported to possess extracellular protease ability (130). Cell size differences were also noted with *H. ohiense* having the smallest yeast cell size compared to the rest of the species in this same study. These differences in phenotypic traits between the various species can be further explored for manufacture of species-specific diagnostic methods without employing molecular methods.

2.8.4. Non-culture-based diagnosis of histoplasmosis

The gold standard for histoplasmosis comes from positive cultures; however, culture diagnosis has considerable drawbacks. Sputum culture yields positive results in 15% of patients with acute pulmonary histoplasmosis and blood cultures give positive results in only 50-70 % of patients with disseminated histoplasmosis (104). To increase the rate and speed of diagnosis, non-culture approaches have been developed. These tests include the detection of antibodies and antigens as well as more recent molecular diagnostic methods for the better identification of *H. capsulatum* in clinical specimens (107,111). Any of the test results mentioned can lead to the presumptive diagnosis of histoplasmosis, and consideration of the patient's clinical history is necessary for an accurate assessment and identification of the true diagnosis (126).

2.8.4.1. Antibody detection

Detection of antibodies provides an alternative for histoplasmosis diagnosis with quick turnaround times (131). At present, antibody detection techniques are important for non-culture diagnosis of histoplasmosis, and they are mainly used in the USA. Due to their accessibility and availability, complement fixation (CF) and immunodiffusion (ID) were previously the two common techniques used (122,132). Nevertheless, due to false-negative findings, these techniques are not useful for patients with most types of histoplasmosis (133). Moreover, individuals with infections from other fungal diseases such as emergomycosis and blastomycosis experience serologic cross-reactions to *Histoplasma*-like antigens (107,123). The major focus of the serologic diagnosis of histoplasmosis is on anti-H and anti-M antibodies (107). These antibodies can be recognised using histoplasmin (HMIN) which is an antigenic extract from the mycelial culture of *H. capsulatum*. HMIN is mainly composed of C, M and H antigens (134). The carbohydrate C antigen is galactomannan which is responsible for cross-reactivity observed with other fungal species, M is catalase and H is β-glucosidase (135,136). Due to their higher specificity to *H. capsulatum*, antibodies to the M and H antigens are especially useful in the diagnosis (137).

A qualitative assessment of precipitating antibodies is made using the immunodiffusion test (H and M precipitin lines or bands). A commercially available kit is available from Miravista Diagnostics (Indianapolis , USA) (132). In the USA, ID testing has become more and more commonplace in laboratories, mostly due to its dependability, simplicity, and potential cost-effectiveness. While ID has a low sensitivity, especially when a patient presents with an acute illness, it is quite specific for detecting anti-M and anti-H antibodies. According to Buitrago & Martín-Gómez et al. (2020), generally 4-6 weeks following infection, ID can be used to detect antibodies; therefore the test is not useful in real-time diagnosis. The H band used in antibody detection for ID is found in 7% of patients with acute infection (126). In sera from individuals with acute and/or progressing illness, the H band can be seen followed by the M band. When the sickness has resolved, antibodies to the H antigen may still be present, although they normally decrease more rapidly than anti-M antibodies. The presence of both the H and M precipitins is regarded to be conclusive for the diagnosis of histoplasmosis, even if a clinical assessment of the patient is required to evaluate the severity of the illness (134).

The complement-fixation (CF) test is an additional commercially available technique for detecting antibodies in histoplasmosis, mostly available in the USA (137). This technique looks for antibodies to either the mycelial (HMIN) antigen or the entire yeast form. Comparison of the CF to immunodiffusion test using sera in Kentucky, USA showed CF using histoplasmin antigen detected *H. capsulatum* in 68% of 70 confirmed histoplasmosis cases while CF using yeast antigen detected 94% of the confirmed cases versus 90% for ID (137). A 10-year retrospective review (2011-2020) comparing ID and CF to culture-confirmed cases among 67 patients showed 65.7% positive for CF and 62.7% for ID(133). The *H. capsulatum* CF test has the disadvantage of not being able to identify cross-reactive antibodies in blood from patients who have a variety of mycoses, such as emergomycosis, blastomycosis, candidiasis, coccidioidomycosis, and paracoccidioidomycosis (104). Another problem with CF is that occasionally false-negative results are observed due to the presence of inhibitory effects of rheumatoid factor and cold agglutinins that may accumulate during histoplasmosis. After acquiring the infection with *H. capsulatum,* 95% of patients with histoplasmosis may generate complement-fixing antibodies 3 to 6 weeks (and possibly as early as 2 weeks), and subsequent tests may continue to provide

positive results for months (137). Antibody titres between 8 and 16 IU/ml are considered to be mildly positive. Although such titres can also be discovered in the serum of healthy people from locations where histoplasmosis is widespread, they are indicative of a previous infection. Other tests such as latex agglutination are more sensitive than CF and have lower cross-reactivity (138). A commercial latex agglutination test manufactured by Thermo Fisher Scientific (Waltham, Massachusetts, USA) is currently available for screening for histoplasmosis although people with TB may have false-positive findings (139,140).

2.8.4.2. Antigen detection

Antigen detection methods such as Radioimmunoassay (RIA), EIA and LFA may be more reliable than antibody screening for the diagnosis of disseminated histoplasmosis(141). Antigens can be found in bodily fluids such as serum, blood, pleural fluid, bronchoalveolar lavage fluid, cerebrospinal fluid, and urine when a person has disseminated histoplasmosis (135). The World Health Organisation together with the Pan American Health Organisation (PAHO) released guidelines aimed at providing recommendations for the diagnosis, treatment and management of histoplasmosis in HIV patients in 2020 (142). The recommendation was that for patients with disseminated histoplasmosis especially HIV patients, antigen testing should be done because the assay provides high diagnostic accuracy and the testing is non-invasive as well as easy to implement in resource-limited settings (WHO guidelines, 2020). Commercial tests for the detection of *Histoplasma* antigen have been available during the last ten years, offering the possibility of transferring this technology to resource-constrained nations where histoplasmosis is endemic and where the capacity to diagnose disease is constrained (22,28).

Radioimmunoassay

An early method for antigen detection was an RIA. The assay relied on the detection of a polysaccharide antigen from *H. capsulatum* using polyclonal antibodies from rabbit serum. The RIA was useful in the diagnosis of histoplasmosis as well as in monitoring response to treatment. *H. capsulatum* polysaccharide antigen (HPA) levels can be determined using an RIA in samples of a patient's urine, serum, and other bodily fluids. However, because it has not been feasible to

produce significant amounts of the high-quality rabbit polyclonal antibody required for the test, the performance of this test is constrained to in-house laboratory use (131). The RIA was previously useful in detecting outbreaks as it provided results in 1 working day. In an outbreak in Indianapolis, USA in 1988, the RIA was used as a diagnostic assay and detection in urine was 92% and 82% in serum in patients with disseminated histoplasmosis (141). The major disadvantage of this assay is that it exposed laboratory workers to radioactivity and it required licencing by the Nuclear Regulatory Commission (NRC) (129?). The assay was also used for screening of disseminated histoplasmosis using urine samples and 18 out of 19 (94.7%) patients with disseminated histoplasmosis tested positive(141). In the same study, the RIA was used to test patients with acute pulmonary histoplasmosis and 12 out of 25 (48%) patients tested positive. In another study comparing RIA to complement fixation assay, 19 out of 20 (95%) serum samples from HIV/AIDS patients tested positive (143). The RIA was compared to the EIA in screening for histoplasmosis using 86 urine specimens from patients with histoplasmosis and 96 control specimens. From patients with previously confirmed histoplasmosis, 71% tested positive and 1 patient from the control group also tested positive. The test filled a critical need for a test with short turn-around times as results are available within 24 hours (131). Other thermally dimorphic fungi such as Blastomyces and Emergomyces have cross-reactivity with *Histoplasma* when using this test. In a study by Durkin et al, 1997, the RIA was compared to the EIA and the two assays had the same specificity and sensitivity and they detected histoplasmosis in 89% of patients with disseminated disease and 37% with non-disseminated disease (144). The RIA has now become unpopular as the EIA became commercialised with wider availability.

Enzyme immunoassays (EIA)

In 1986, the first sandwich radioimmunoassay was developed testing for *Histoplasma* antigen by Wheat and colleagues and in 1989, it was reformulated into an EIA developed by Immuno-Mycologics Ltd (IMMY, Norman, Oklahoma). This first IMMY Alpha EIA used a polyclonal capture antibody and had poor diagnostic performance in humans giving false-positive results, especially in patients who had recently received solid organ transplants (27). A second-generation EIA was then developed by MiraVista in 2004 and it provided semi-quantitative results although it required expressing results in comparison to the negative control which was

sometimes confusing to the ordering physicians (104). In 2007, a third-generation *H. capsulatum* EIA was also developed by MiraVista providing semi-quantitative results that allowed monitoring of histoplasmosis treatment (145). These assays relied on testing for samples in a centralised laboratory in Indianapolis, USA and that led to Immuno-Mycologics Ltd developing a modification of IMMY Alpha EIA using a monoclonal capture antibody, the Clarus *Histoplasma* galactomannan EIA (IMMY Clarus EIA); this was approved by the US Food and Drug Administration (FDA) for use on urine specimens and used for testing in the USA (28). The improved IMMY Clarus EIA was an analyte-specific reagent *H. capsulatum* antigen EIA with improved performance characteristics as well as high agreement with the MiraVista EIA (146). The CDC also developed an in-house double-polyclonal-antibody sandwich ELISA using a polyclonal antibody to detect the *H. capsulatum* polysaccharide antigen but the reagents were not available for distribution to other endemic areas (147,148) . The CDC polyclonal ELISA and the IMMY Alpha EIA were compared in a study including 78 patients with suspected histoplasmosis against culture/histopathology results and the results were 10.3%, 16.7% and 17.9% positivity rates for culture/histopathology, IMMY ALPHA EIA and CDC polyclonal ELISA respectively (149).

A large multicentre study by Caceres et al in 2018 evaluated the commercial monoclonal IMMY Clarus EIA in two cohorts of people living with HIV/AIDS from Guatemala and Colombia, in comparison to the CDC polyclonal ELISA, with a sensitivity and specificity of 98% and 97% respectively and a cut-off value of 0.5 ng/ml (111). Reports of cross-reactions of the IMMY Clarus *Histoplasma* EIA with blastomycosis, talaromycosis, emergomycosis and paracoccidiodomycosis have been published (121,150). A sandwich EIA for the detection of *Histoplasma* antigens in urine, was also developed by Optimum Imaging Diagnostics Scarborough, ME (OIDx) and an evaluation of this assay showed sensitivity of 92%, specificity of 32% and accuracy of 51% compared to culture results (108). This assay showed high sensitivity but it had false-positive results in 68% of the specimens tested in non-histoplasmosis cases (108).

Lateral flow assays (LFA)

Lateral flow assays have also been developed for the detection of *Histoplasma* antigen in both serum and urine. An LFA to detect *H. capsulatum* antigen in serum developed by MiraVista was used to screen 75 serum samples (culture-proven disseminated histoplasmosis n=24 and patients with other fungal infections n=45) and showed a sensitivity of 96% and specificity of 90% in comparison with the culture results(25). Cross-reactions were observed principally in samples from patients with proven diagnosis of paracoccidioidomycosis. The MVista *Histoplasma* antigen LFA was also compared to the MiraVista *Histoplasma* EIA in screening for *Histoplasma* antigen in 100 urine samples from Colombian patients where 26 had HIV and previously confirmed histoplasmosis and 42 were living with HIV and other infectious diseases and 32 were non-HIV-patients (26). The sensitivity and specificity of the MVista *Histoplasma* antigen LFA were 96% and 77% compared to the MiraVista *Histoplasma* EIA. Concordance analysis between MVista *Histoplasma* antigen LFA and the MiraVista *Histoplasma* EIA displayed an 84% agreement and a Kappa of 0.656. Optimum Imaging Diagnostics (IODx) also developed an LFA for rapid detection of *Histoplasma* within 15 minutes. Screening for *Histoplasma* using the OIDx *Histoplasma* LFA compared to the IMMY *Histoplasma* EIA in 150 people living with HIV from Ghana showed prevalence rates for *Histoplasma* antigen of 4.7% (5 of 107) (151). The OIDx *Histoplasma* LFA showed a high concordance (98.4%) with the IMMY *Histoplasma* EIA. The LFA format has several benefits, including the use of urine specimens, simplicity of use, short turnaround times with results in about 15 min, and lack of need for sophisticated laboratory infrastructure or highly skilled laboratory workers.

2.8.5. Skin testing

The histoplasmin (HMIN) skin test is generally used to determine a person's history of *H. capsulatum* infection although this test is not used often in recent years. This test resembles a delayed-type hypersensitivity tuberculin skin test. Histoplasmin antigen, a culture filtrate of the mycelial phase of growth, is injected intradermally into the forearm as part of a skin test. As an epidemiological tool, it is helpful. Two to four weeks after a person has been exposed to *H. capsulatum*, a histoplasmin skin test results in a positive result. After 48 hours, the test is positive with a 5 mm skin induration. A positive skin test suggests either recent or historical exposure to *H. capsulatum*. If a person has had no previous exposure to *H. capsulatum*, there will be no reaction. A past *H. capsulatum* infection can offer some protection. This method may be utilised

to identify initial *H. capsulatum* infections in immunocompetent patients, whether they are symptomatic or asymptomatic, according to multiple studies (57,59,62). Nonetheless, HMIN skin testing has previously been used more commonly for research than for diagnostic needs. Testing with HMIN is particularly useful for doing epidemiological studies in non-endemic areas on persons who occasionally go to endemic areas or during epidemics. Previous studies in Africa show that skin tests show a positivity rate of between 0 and 35% (8). In Uganda, the prevalence of HMIN-positive skin tests ranges from 0 to 10%, with the Busoga region in eastern Uganda having the highest frequency at 12% (152). In 1949, the test was used to assess the prevalence of histoplasmosis among 140 White males in South Africa with a positivity rate of 8%(81). During the annual National Speleological Society Convention in Bracketville, Texas, USA in June 1994, cave exploration as part of the event led to the development of histoplasmosis in 18 individuals, which was confirmed using the HMIN skin test (153).

2.8.6. DNA-based *Histoplasma* identification methods

2.8.6.1. Cultured isolate identification

Histoplasma colonies grow slowly and the differentiation from other dimorphic or saprophytic fungi with similar colony or microscopic morphology is challenging. PCR-based *Histoplasma* identification has been published in several papers targeting various regions of the genome (29,112,113,154). Most established techniques used are conventional, nested, or qPCR formats and focused on particular multicopy ribosomal DNA regions or the single-copy Hcp100 gene (117,155). Rapid advances in molecular techniques, affordability of sequencing costs and availability of more sequences in the public domain (such as NCBI) could help to design simple assays with high-throughput capabilities for the identification of *H. capsulatum*. Single-gene sequencing has added value in the detection of *Histoplasma* because of the high accuracy that sequencing provides. The identification of *H. capsulatum* follows the extraction of nucleic acids from pure fungal culture followed by amplification of the internal transcribed region (*ITS*)/ Hc100/ other housekeeping genes and then Sanger sequencing of the same genes to confirm the identification of *Histoplasma*. PCR assays with a variety of molecular targets have been developed but currently, there are no FDA-approved molecular assays directly applied to clinical specimens for diagnosis due to lack of standardisation (112–114,154). The sensitivity of

Histoplasma PCR assays targeting various regions of the *Histoplasma* genome ranges between 67% and 100% compared to culture methods and histology (156).

2.8.6.2. Clinical specimens

Molecular methods based on the detection of fungal DNA directly from clinical samples are currently used mainly in the mycology reference laboratory (MRL) in South Africa and a few diagnostic laboratories have access to such methods (44,72,157). Most PCR assays for the diagnosis of histoplasmosis are designed for in-house use for certain laboratories with none of them available for commercial use (112,114,154). The type of sample tested, the clinical characteristics of the patients, and the PCR format employed for DNA detection all affect the sensitivity and specificity of these assays. These assays demonstrated an outstanding analytical performance (overall sensitivity of 95% and specificity of 99%) when testing specimens such as Formalin-Fixed Paraffin-Embedded (FFPE) and Bronchoalveolar-lavage fluid (BALF) from HIV patients. However, sensitivity is decreased to 25-90 % and specificity of 85-100 % when testing blood and serum samples (29,110,112–114,117,154,155) .The added benefit that real-time PCR (qPCR) formats have is that they enable the determination of the fungal load in blood and urine samples from patients by using non-specific DNA-binding dyes or fluorescently labelled probes. The other advantages of such assays centre on their simplicity, high specificity, and quick turnaround times. The limited DNA content in minimally invasive samples such as blood and urine, the lack of standardisation, and the scarcity of generally accepted commercial systems are some of the disadvantages of this technology. The use of chemiluminescence-labeled DNA probes for the detection of specific sequences of rRNA has allowed the detection and confirmation of the presence of *H. capsulatum* in clinical specimens used for the evaluation (158). A real-time PCR assay that correctly identifies cultured *H. capsulatum* from among a variety of other fungi appears promising (29). Using such assays for the positive identification of *H. capsulatum* was shown in tissue biopsies and bronchoalveolar lavage fluid from patients who had documented histoplasmosis (155,159). Recently, the Mycology Laboratory at the Institut Pasteur (Paris, France) developed an RT-qPCR assay that targets the ribosomal small subunit gene (*mtSSU*) for *Histoplasma* (29). The report indicates that this assay does not cross-react with other dimorphic fungi except for *Blastomyces* species, *Emergomyces pasteuriana*,

Nannizziopsis and Emergomyces crescens. This RT-qPCR method may provide a rapid tool for the diagnosis of histoplasmosis and could be offered as a test in South African laboratories if it becomes commercialised.

2.8.6.3. Environmental samples

Generally, it is challenging to culture *Histoplasma* from the environment due to the high number of contaminants found in the soil (30). The lack of accurate and fast diagnostic methods has limited the ability to detect *Histoplasma* in environmental samples. *Histoplasma* is a slow grower and attempts to culture soil samples has been hindered by the overgrowth by other fungi (32). Suspending soil in water, followed by inoculation into mice and then harvesting their lungs for culturing has been employed with success before (30). The indirect detection of *Histoplasma* through mice inoculation takes 6-8 weeks to get results and is an expensive method. Polymerase chain reaction methods have been developed and evaluated to the detection of *Histoplasma* in environmental samples. A two-stage PCR was evaluated and used for *Histoplasma* detection with an estimated limit of detection of 10 spores targeting the *ITS* region of the 5.8S rRNA gene of *H. capsulatum* (160). Bialek et al, 2002 published a nested PCR capable of detecting *Histoplasma* in clinical samples with fast and reliable results (155). This nested PCR was specific to *H. capsulatum* targeting the 100 kDa protein (Hc100). In 2018, Gomez and other optimised and standardised the use of this PCR in environmental samples with 100% specificity and a limit of detection of 0.1 pg/µL using 239 organic fertilisers (161). Various studies have used molecular assays to test for the presence of *H. capsulatum* nucleic acids in soil and demonstrated the usefulness of such tools in countries such as Brazil, Mexico, and the United States of America (155,160,162–164). The 100 kDa *Histoplasma* nested PCR was compared against a real-time 100 kDa PCR and was able to identify *Histoplasma* nucleic acids in 80% of raw organic samples and 62% of organic fertilisers (165). The two PCR methods had 100% concordance and sequencing the products identified had 100% identity with the 100 kDa gene of *H. capsulatum* (165). Such assays can be used to identify high-risk areas such as bat-inhabited caves. They can also direct the development of strategies for preventing infection among people at increased risk of severe histoplasmosis. The optimisation of PCR methods initially developed for detection of nucleic acids in clinical samples to now be used in testing environmental

samples is useful in provision of fast and reliable results (30,31,163,164). A new reverse transcriptase qPCR (RTqPCR) assay real-time PCR method was developed for detection of *H. capsulatum* and evaluated in clinical samples by Alanio and others in 2021(29). The assay allows the amplification of whole nucleic acids of *Histoplasma spp.* The study evaluated the use of this method in detection of *Histoplasma* nucleic acids in soil samples collected from caves in South Africa.

2.8.7. *Histoplasma* taxonomy

There are several genotypes of *Histoplasma* with global geographic distribution. *Histoplasma* taxonomy has been under consistent and vigorous exploration resulting in several reclassifications of this genus. Traditional assessments of the genus utilised the geographical distributions, clinical symptoms, morphological features and host associations to differentiate the different strains. These studies suggested grouping these strains into three different varieties namely *Histoplasma capsulatum* variety *capsulatum, Histoplasma capsulatum* variety *duboisii* causing African histoplasmosis and *H. capsulatum* variety *farciminosum* causing epizootic lymphangitis in equines. *H. capsulatum* var *capsulatum* had worldwide distribution while var *duboisii* was only found in Africa characterised mainly by granulomatous lesions in cutaneous, subcutaneous and osseous tissues with observations of larger yeasts compared to var *capsulatum* (51). Classification of *H. capsulatum* has been revolutionalised by the use of molecular tools in the genetic analysis of global isolates, highlighting the high diversity within this genus (51,166). The genetic characterisation of *H. capsulatum* by DNA-based techniques differs according to the genome region being targeted and the methodologies applied (167). Results from a Brazilian study performed in 2001 using PCR-based random amplified polymorphic DNA (RAPD) analysis revealed that this method was useful in typing of isolates from Rio de Janeiro and those from other Brazilian states (164). The same method was also used in the typing of isolates from Thailand showing genetic discrimination with isolates from the USA (168). In a study by Kasuga et al in 1999, 46 *Histoplasma* isolates were analysed using partial DNA sequences of four protein-coding genes; *arf*, *H-anti*, *ole* and *tub1* (20). Analysis of the genes using parsimony and distance analysis were generally congruent and led to the identification of 6 clades: (i) class 1 North American (*var capsulatum*), (ii) class 2 North American (*var. capsulatum*), (iii) Central American (*var. capsulatum*), (iv) South American (*var. capsulatum*) group A, (v) South

American (*var. capsulatum*) group B, and (vi) *H. capsulatum var. duboisii*. In 2003, Kasuga and team analysed 137 *Histoplasma* isolates from 6 continents representing all 3 varieties using DNA sequence variation in four independent protein-coding genes namely *arf, H-anti, ole* and *tub1*(169). They identified 8 different clades namely: (i) North American class 1 clade; (ii) North American class 2 clade; (iii) Latin American group A clade; (iv) Latin American group B clade; (v) Australian clade; (vi) Netherlands (Indonesian?) clade; (vii) Eurasian clade and (viii) African clade. Komori et al in 2005 analysed partial sequences of the large subunit (28S) ribosomal gene (D1/D2 region) of 49 *Histoplasma* isolates and classified them into 9 groups(170). Many other reports were published with different classifications for *Histoplasma* based on a variety of DNA regions (17,51–53,73,166,170,171). This led to several inconsistencies in the phylogenetic relations within this genus, which were resolved through whole genome sequencing studies.

Whole genome studies have brought in an important dynamic in understanding pathogen genomics. The first analysis of *Histoplasma* genomes using WGS data was done by Sepulveda et al, 2017 and identified relationships shown in Figure 3. They characterised 30 *Histoplasma* isolates: 21 from North America (10 NAm 1 isolates, 11 NAm 2 isolates), 7 from Latin America (4 LAm A isolates, 3 Panama isolates), and 2 isolates from the African group (53). The phylogenetic analysis classified these isolates into 5 groups. Four different species from four groups were proposed: *Histoplasma sensu stricto* Darling Panama (31Mb), *Histoplasma mississippiense* (Nam 1) (33Mb), *Histoplasma ohiense* (Nam 2) (39.41Mb) and *Histoplasma suramericanum* (Lam A) (26Mb) (13). The fifth group was not well characterised in this study, since only two isolates from Africa were included. Therefore, there is a need to study more isolates from the African continent to show how they relate genetically to other global isolates. Knowing more about the *Histoplasma* strains in Africa may assist, for example, with the design of specific antigen or antibody tests to allow a more accurate diagnosis of histoplasmosis in Africa.

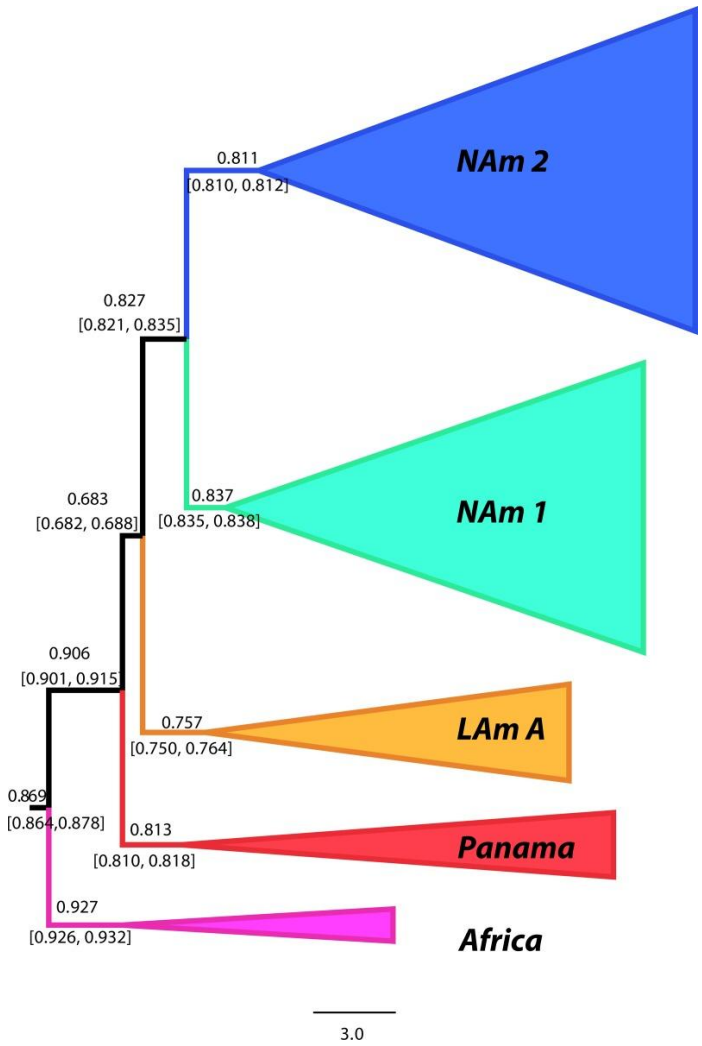

Figure 3: Species tree of the genus *Histoplasma* showing phylogenetic relations within this genus and that all species have high levels of genomic concordance (Sepulveda *et al*, 2017).

Histoplasma strains vary in their karyotype and are thought to have between five to seven chromosomes with a haploid genome, depending on the strain (172–174). Whole genome sequencing provides a platform to analyse the different karyotypes that are found in different strains. With comparative genomics and transcriptional profiling, the list of putative virulence factors for *Histoplasma* has been expanded. Factors that are of major importance for parasitism in *Histoplasma* have been studied through the analysis of both the mould and yeast phase and understanding how its existence in these two forms is important in its pathogenicity. Gene-specific studies have revealed that the *YPS-3* gene, various sulphur metabolism genes, a trichothecene C-15 hydroxylase (TRI11), α-(1,3)-glucan; and *CBP* gene contribute to *H.*

capsulatum's virulence and pathogenesis but further studies need to be done to show differences in the different species (87,101,175). *H. capsulatum* has two mating types (MAT1-1 and MAT1-2) that have been demonstrated in the laboratory to occur in the mycelial phase of the pathogen (173,176–178). The mating types are represented equally in environmental samples with a 1:1 ratio and in clinical samples they do not have an equal representation with a 7:1 (-/+) ratio being reported (178).

2.8.8. Future diagnosis of histoplasmosis

The diagnosis of histoplasmosis requires a multifaceted approach, and all the benefits and drawbacks of each diagnostic method should be considered. The patient's immunological state and the form or type of the disease have an impact on how well the diagnostic test works. The lack of availability of several of the recommended testing methods makes diagnosis more challenging. Such tests must be verified in individuals from various backgrounds before being extensively employed on a regular diagnosis. The rise in AIDS-related cases of histoplasmosis has encouraged efforts to develop innovative antigen-detection techniques with improved sensitivity and specificity. In immunocompromised patients, it is important to diagnose the disease before the disease becomes severe and for evaluation of proper course of therapy, it is important to use diagnostic methods that have quick turn-around times and are accurate. High-throughput screening approaches for the identification of fungal pathogen sequences are also anticipated using urine or blood samples from patients. Rapid identification is made possible by new methods such as matrix-assisted laser desorption ionisation (MALDI) TOF MS technology; however, research is still lacking on this method (179,180). Technology such as Luminex offers a quick and easy test with the ability to detect different fungi in clinical samples, thanks to recent developments in molecular biology such as publicly available fungal sequence databases (181). Luminex technique makes use of a microsphere-based hybridisation test that confirms target DNA from PCR-biotinylated amplicons into an assortment of microsphere beads harbouring species-relevant probes (181). The technique makes use of equipment that measures fluorescence intensity in the presence of a specific fungal pathogen. The equipment produced the raw median fluorescence intensity (MFI) signal, and the TDAS LSM software (Luminex Molecular Diagnostics) was then used to analyse the results (115). A positive result required a minimum MFI with a cut-off value of 500. Probe innovation and technique validation are still being

performed to evaluate the performance of this assay in the precise detection of clinically relevant fungi.

2.9. Treatment

For people with healthy immune systems, the symptoms of histoplasmosis will go away without treatment. However, prescription antifungal medication is needed to treat severe histoplasmosis and infections that have spread from the lungs to other parts of the body (disseminated histoplasmosis). The drug of choice in severe illness is amphotericin B (avoided in renal patients due to the risk of nephrotoxicity) for 2 weeks (182,183). Amphotericin B is available in two forms and liposomal amphotericin B is much safer than amphotericin B deoxycholate; the latter is normally avoided, or its dosage is adjusted to reduce the severity of the side effects. However, in severe cases of histoplasmosis, amphotericin B deoxycholate remains the drug of choice in low-income countries followed by maintenance with itraconazole or fluconazole for 12 months (184). Alternatives to itraconazole can also be posaconazole and voriconazole (183). Individuals taking itraconazole are monitored for hepatic function (183).

2.10. Antifungal susceptibility testing

H. capsulatum in the filamentous and yeast phases have been subjected to in vitro antifungal susceptibility testing using the microdilution method, following the Clinical and Laboratory Standards Institute (CLSI), against amphotericin B, fluconazole, itraconazole, voriconazole, and caspofungin in different studies. There are currently no published breakpoints and there has been no resistance observed for most of the drugs except for fluconazole. (185–188). Fluconazole is less effective than itraconazole for treating *H. capsulatum*, which contributed to treatment failure in certain patients (189). Twenty-three strains from pulmonary and disseminated histoplasmosis patients in India were examined for antifungal susceptibility using the broth microdilution method and there was good agreement of susceptibility profiles for both yeast and mould phase isolates for all drugs tested which included itraconazole, amphotericin B, voriconazole, posaconazole and isavuconazole (185). High minimum inhibitory concentrations (MICs) values have been reported for caspofungin (16.6 μg/ml (range, 8 to 32 μg/ml)), fluconazole (8.0 μg/ml (range 0.25 to 64.0 μg/ml)) and 5-flucytosine (16 μg/ml (range 8-64 μg/ml)) (185,185,190–193).

The mechanism of resistance to fluconazole in *Histoplasma* isolates with high MICs was found to be alterations in the cytochrome P450-dependent enzymes, 14α-demethylase (CYP51p) and 3-ketosteroid reductase, most specifically via the Y136F mutation (194). In vitro antifungal susceptibility testing for *H. capsulatum* remains largely non-standardized and there is no consensus on whether the pathogenic yeast form that occurs in tissues or the mould form present in the environment causing infection should be tested. A correlation of in vitro antifungal susceptibility testing results with clinical outcomes is also necessary to fully understand the resistance patterns of *H. capsulatum*.

2.11. Conclusion

Histoplasmosis affects over 500 000 people every year globally and the disseminated form of the disease is known to affect more than 100 000 of that population but the disease remains under-reported and neglected in many countries (195). The disease has variable levels of clinical symptoms and sometimes it is misdiagnosed as cancer or tuberculosis making its treatment challenging. Because of this lack of proper incidence of histoplasmosis in South Africa and the rest of the African continent, our study will address this gap by reviewing all cases that have been reported in all public hospitals and 2 major private diagnostic laboratories in the country between 2010 and 2020. This will give an idea of the prevalence of the disease over a decade and address the shortfalls in reporting in South Africa. Due to the sizeable population living with HIV on the African continent, it is important to address the challenges that are associated with the diagnosis of HIV infection. Histoplasmosis is an AIDS-defining infection, yet there is a dearth of fundamental knowledge on its incidence, prevalence, morbidity, and prognosis. This is a result of haphazard reporting in areas with less developed laboratory and imaging infrastructure and a lack of clarity and misdiagnosis mistaken for diseases like TB. Level 3 biosafety facilities are required for its culturing, and they are not easily accessible, and culturing takes up to 6 weeks causing delays in diagnosis and treatment of patients. Our study will address available screening assays with short turnaround times to provide faster and more accurate alternatives to culture methods (196). Histoplasmosis can also infect people, and they remain asymptomatic for many years until they are immune compromised. This means that people can travel from endemic areas and only become sick when they reside in other areas that may be non-endemic; therefore, it is crucial for doctors practising outside of endemic areas to understand this illness' symptoms and

treatment options. In regions where *Histoplasma* is endemic, individuals with weakened immune systems (such as those with HIV/AIDS, organ transplant recipients, or those taking medications like corticosteroids or TNF inhibitors) should avoid activities that increase the risk of contracting histoplasmosis. These activities include disturbing soil (e.g., digging or chopping wood) in areas with bird or bat droppings, cleaning chicken coops, and exploring caves. The presence of *H. capsulatum* in caves most visited by tourists and cave explorers in South Africa remains unknown and this poses a threat to people with compromised immune system if they go inside these cases. To get an understanding of whether these caves have *H. capsulatum* we will test for the presence of *Histoplasma* nucleic acids in soil samples collected from caves suspected of having bats carrying *Histoplasma*, that is where previous visits have resulted in people getting histoplasmosis symptoms. Another study gap for *H. capsulatum* is the lack of information on the genetic relatedness of isolates collected from patients from Africa and those diagnosed with African histoplasmosis after travelling to other parts of the world. To address this, we will collect African isolates and sequence the whole genomes of these isolates and compare their genetic relatedness to isolates from other continents.

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CHAPTER 3. ENDEMIC MYCOSES IN SOUTH AFRICA, 2010-2020: A DECADE-LONG DESCRIPTION OF LABORATORY-DIAGNOSED CASES AND PROSPECTS FOR THE FUTURE. *NEGLECTED TROPICAL INFECTIONS.* 2022 16(9), E0010737.

3.1. Introduction

Endemic mycoses are caused by thermally-dimorphic fungi that are generally restricted to certain geographical areas. Over the last few decades, reports of endemic mycoses have increased in Sub-Saharan Africa partly due to gradually increasing awareness of serious fungal diseases among healthcare workers with expanded advocacy efforts and increasing availability of improved diagnostic techniques although these are still not optimal [1-5]. There has been an expansion of areas in which these diseases are recognised to be endemic owing to climate change, migration and travel [6]. Some endemic mycoses such as histoplasmosis disproportionately affect immunocompromised hosts, for example, people living with HIV [1-6]. According to UNAIDS, the global burden of HIV in 2020 was 37.7 million, with 36.0 million adults and 1.8 million children aged <15 years [7]. UNAIDS estimated that South Africa had around 7.5 million individuals living with HIV in 2020, 92% of whom were aware of their HIV status, 70% of whom were on HIV treatment and 64% of whom were virally suppressed [8]. The medical impact and burden of endemic mycoses is unknown or underestimated in many African countries [9]. This is because endemic mycoses may mimic other diseases or occur together with other conditions resulting in few diagnoses, suboptimal patient management and poor clinical outcomes [10].

In southern Africa, emergomycosis, histoplasmosis, sporotrichosis and blastomycosis are endemic [11]. Based on case reports and series, Schwartz *et al* estimated 100, 60, 40 and 10 diagnosed cases per year for emergomycosis, histoplasmosis, sporotrichosis and blastomycosis respectively in South Africa [11]. These diseases are usually diagnosed by culture or histopathology which requires skilled personnel for accurate diagnosis and pathogen identification [12]. Several studies over the past decade have characterised novel dimorphic

fungal pathogens causing severe infections among South African patients [1,3,13-16]. The emergence of novel endemic pathogens warrants an intermittent review of the epidemiology to determine changes in disease distribution and patterns [17-20]. Relatively few outbreaks of endemic mycoses have been reported in southern Africa. Sporadic cases of acute pulmonary histoplasmosis were reported mainly among new members of speleological societies visiting caves around the country [21]. An outbreak of lymphocutaneous sporotrichosis among mine workers in South Africa was described in 2015[22]. Travel-associated infections also occur. For example, a fatal case of talaromycosis was reported in an HIV-seropositive South African patient with a history of recent travel to mainland China [23].

There are few studies on endemic mycoses on the African continent [24,25]. These infections are not included on the list of notifiable medical conditions in South Africa. The National Institute for Communicable Diseases (NICD) initiated passive surveillance for endemic mycoses in 2014, first requesting diagnostic pathology laboratories to send cultured dimorphic isolates for molecular identification and later, clinical specimens from suspected cases for reference diagnostic testing. For the latter purpose, a commercial *Histoplasma* enzyme immunoassay (EIA) and then an in-house pan-dimorphic reverse transcriptase-quantitative (RT-qPCR) assay was introduced for investigational use at NICD in 2012 and 2020 respectively. To understand the epidemiology and characteristics of endemic mycoses in South Africa, we performed a comprehensive review of cases diagnosed at public and private laboratories over a decade.

3.2. Materials and Methods

3.2.1. Ethics Statement

We obtained approval for this retrospective record review from the human research ethics committees of the University of Free State (UFS-HSD2018/0992) and the University of the Witwatersrand (M140112 and M210109).

3.2.2. Study design and setting

We performed a retrospective electronic review of laboratory-diagnosed cases of diseases caused by thermally-dimorphic fungi, which were reported by microbiology and histopathology laboratories affiliated either to the National Health Laboratory Service (NHLS) or one large national private pathology practice in South Africa, from 1 January 2010 through to 31 December 2020. Laboratory data were obtained from the NHLS corporate data warehouse, which archives pathology test results from all public-sector facilities, and from the electronic laboratory information system of the private pathology practice which serves an estimated 40% of the private healthcare market in South Africa. We also included referred cases that had been diagnosed at the NICD. Where available, we extracted data on patient demographics, clinical presentation and HIV infection status from laboratory request forms submitted to NICD or from final histology laboratory reports. In addition, for public-sector cases, we looked for NHLS haematology, histopathology, chemical pathology and microbiology test results performed within two weeks before or after confirmation of the infection. We created an online Google forms survey for NHLS laboratories to understand diagnostic practices for endemic mycoses (S1 Text).

3.2.3. Case definitions

A case was defined as a patient of any age with any specimen type submitted to a laboratory from which a thermally-dimorphic fungus was cultured or detected by histopathology, *Histoplasma* antigen EIA, pan-dimorphic reverse-transcriptase quantitative PCR (RT-qPCR) and pan-dimorphic fungal PCR/sequencing. We classified cases as: 1) proven if a thermally-dimorphic fungus was cultured (identified by phenotypic or molecular methods) from a clinical specimen or if fungal DNA was detected directly from a specimen by PCR and identified by sequencing of the fungal internal transcribed spacer (*ITS*) region, large subunit of the ribosomal RNA (LSU) or calmodulin gene [26,27], 2) probable if morphologically-consistent yeasts were observed on histopathological examination or if a urine *Histoplasma* antigen test or a pan-dimorphic (RT-qPCR) assay on several specimen types was positive, and 3) possible if a compatible clinical syndrome was reported with a consistent tissue inflammatory infiltrate but no

yeasts were observed on histology. We excluded duplicate cases and non-patient samples distributed to laboratories as part of an external quality assessment programme.

3.2.4. Culture and fungal identification methods

Fungal culture and identification were performed at diagnostic laboratories according to their standard operating procedures. Only difficult-to-identify isolates were referred to NICD for confirmation by phenotypic or molecular methods. At NICD, isolates were sub-cultured onto potato dextrose agar (Diagnostic Media Products [DMP], Sandringham, South Africa) and incubated at 25°C and 30°C for 4 weeks. To confirm thermal dimorphism, the isolate was inoculated onto brain heart infusion (BHI) agar (DMP) or BHI + 5% sheep blood (DMP) and incubated at 37°C for up to 4 weeks for conversion to the yeast phase. All cultured isolates were processed in a biosafety class II cabinet with use of personal protective equipment including N95 masks, laboratory coats and gloves. The yeast and mycelial features were examined by light microscopy after staining with lactophenol cotton blue (Difco, Becton Dickinson, Franklin Lakes, United States) or a Gram stain (DMP). For molecular identification, DNA was extracted directly from fungal isolates using the Zymo ZR fungal/bacterial DNA miniprep kit (Zymo Research Corp., Irvine, USA) following manufacturer's instructions. PCR amplification and sequencing was performed either by targeting the *ITS/LSU* genes for all dimorphic fungi and the calmodulin gene for *Sporothrix schenckii* species-complex [26,27]. The DNA amplicons were sequenced using a 3130 sequencer (Applied Biosystems, Life Technologies Corporation, USA). Sequences were subjected to BLAST analyses in GenBank (www.ncbi.nlm.nih.gov).

3.2.5. Pan-dimorphic PCR assay

We modified the published protocol for a novel *Histoplasma* reverse transcriptase-quantitative PCR assay to target the mitochondrial small subunit of *Histoplasma* and *Emergomyces* and used this pan-dimorphic assay on cultured isolates and directly on clinical specimens including fresh tissue, formalin-fixed paraffin-embedded (FFPE) tissue, bone marrow aspirates, whole blood or plasma and urine [28,29]. Briefly, DNA from tissue samples was extracted using the Qiagen DNA FFPE tissue kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. Nucleic

acids were extracted by taking 1.6 ml of liquid samples, a loopful of the cultured isolate or a small piece of FFPE tissue and adding to a 2 ml Eppendorf tube containing beads. The mixture was subjected to bead beating at 3000 rpm for 2 minutes. The samples were centrifuged at 10 0000 rpm for 5 minutes. Nucleic acids were then extracted from 200 µl of the sample using a High Pure Viral Nucleic Acid kit (Roche Molecular Systems, Branchburg, United States) according to the manufacturer's instructions. Nucleic acids were amplified using the specific *Histoplasma*/*Emergomyces* primers and probe in the LightCycler 480 Instrument II (Roche Molecular Systems). Any amplification with a Cq value of less than 40 was considered positive for *Histoplasma/Emergomyces*. Since the validation results for this in-house assay have not yet been published, patients with positive PCR results were considered as meeting the probable case definition.

3.2.6. *Histoplasma* antigen enzyme immunoassay

Urine specimens from cases with suspected histoplasmosis were submitted to the NICD for *Histoplasma* antigen testing between 2012 and 2020. Urine specimens collected from 2012 to July 2014 were tested using the Alpha *Histoplasma* antigen assay (IMMY, Norman, United States) and those collected from August 2014 to December 2020 were tested with the Clarus *Histoplasma* antigen assay (IMMY). Urine specimens were batched and processed weekly according to manufacturer's instructions and as described by Maphanga et al., 2020[30]. Since both Alpha and Clarus assays cross react in urine with dimorphic fungi such as *Emergomyces*, *Blastomyces*, *Sporothrix* and *Talaromyces marneffei*, patients with positive EIA results were considered as meeting the probable case definition [31].

3.2.7. Statistical analysis

Descriptive statistics were used. Categorical variables were reported as frequencies and proportions, while non-normally distributed quantitative variables were presented as medians and interquartile ranges (IQR). Statistical analyses were conducted using R (R studio, Boston, United States) and STATA (StataCorp, College Station, United States).

3.3. Results

3.3.1. Patient demographic and clinical characteristics

Between January 2010 and December 2020, we identified 682 cases of endemic mycoses (Fig 1). Overall, the patients had a median age of 37 years (IQR, 31-45), and 436 (64%) were male. Three hundred and seven (45%) cases were classified as proven, 279 (41%) as probable and 96 (14%) as possible (Fig 2). Eighty-six per cent (586/682) of cases were diagnosed at public hospitals. A majority were from Gauteng Province (247/682, 36%) followed by Western Cape (154/682, 23%), KwaZulu-Natal (125/682, 18%), Eastern Cape (85/682, 12%), Free State (19/682, 3%), Mpumalanga (17/682, 2%), North West (13/682, 2%), Limpopo (11/682, 2%) and Northern Cape provinces (6/682, 1%) (S1 Fig.). Specimen types were available for 569 (83%) of the 682 cases. The most commonly submitted specimens were skin tissue (276/573, 48%) followed by unspecified tissue (103/573, 18%), urine (102/573, 18%) and blood (55/573, 10%). Most cases were diagnosed by histopathology alone (273/682, 40%), followed by culture plus phenotypic and panfungal PCR/sequencing identification (167/682, 24%), culture plus phenotypic identification alone (128/682, 18%), *Histoplasma* antigen tests (100/682, 15%) and a pan-dimorphic RT-qPCR test (3/682, 0.4%) (Fig 2). The detailed clinical characteristics and demographics of all patients are summarised in Table 1.

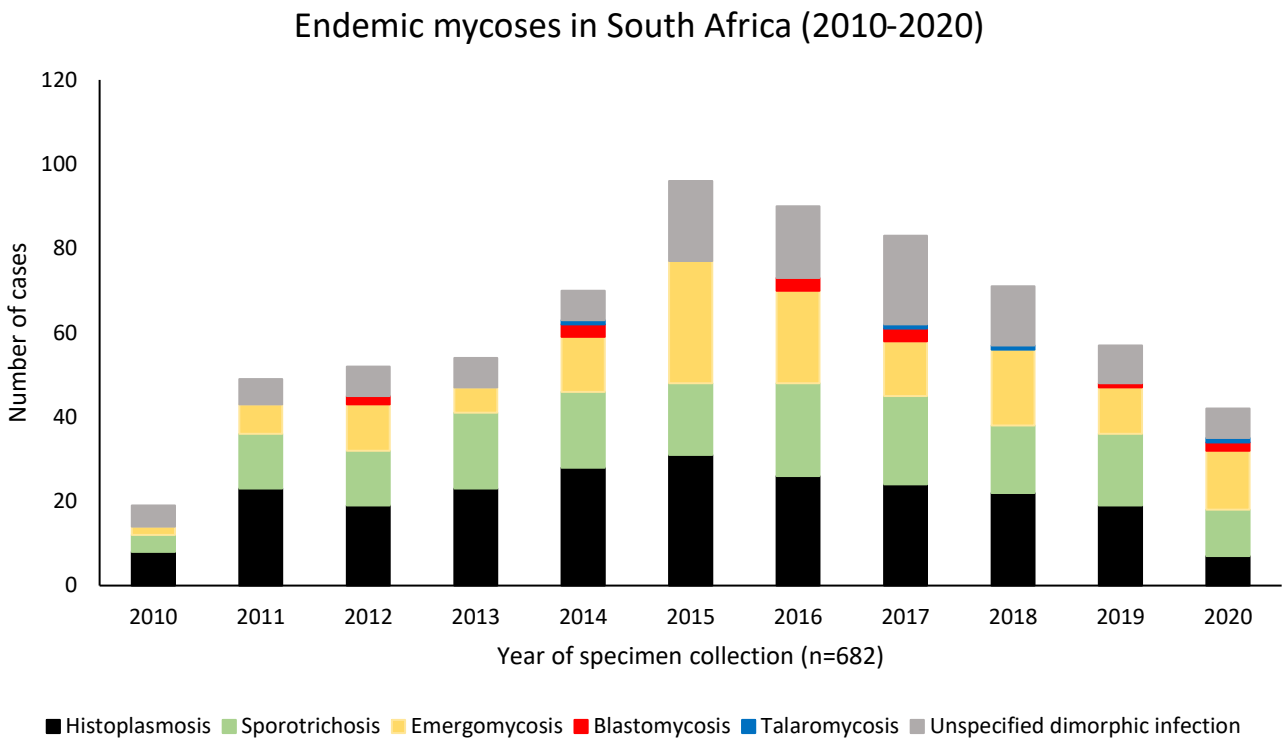

Figure 1: Frequency of laboratory-diagnosed endemic mycoses (proven, probable and possible) per year in South Africa from 2010-2020, n=682.

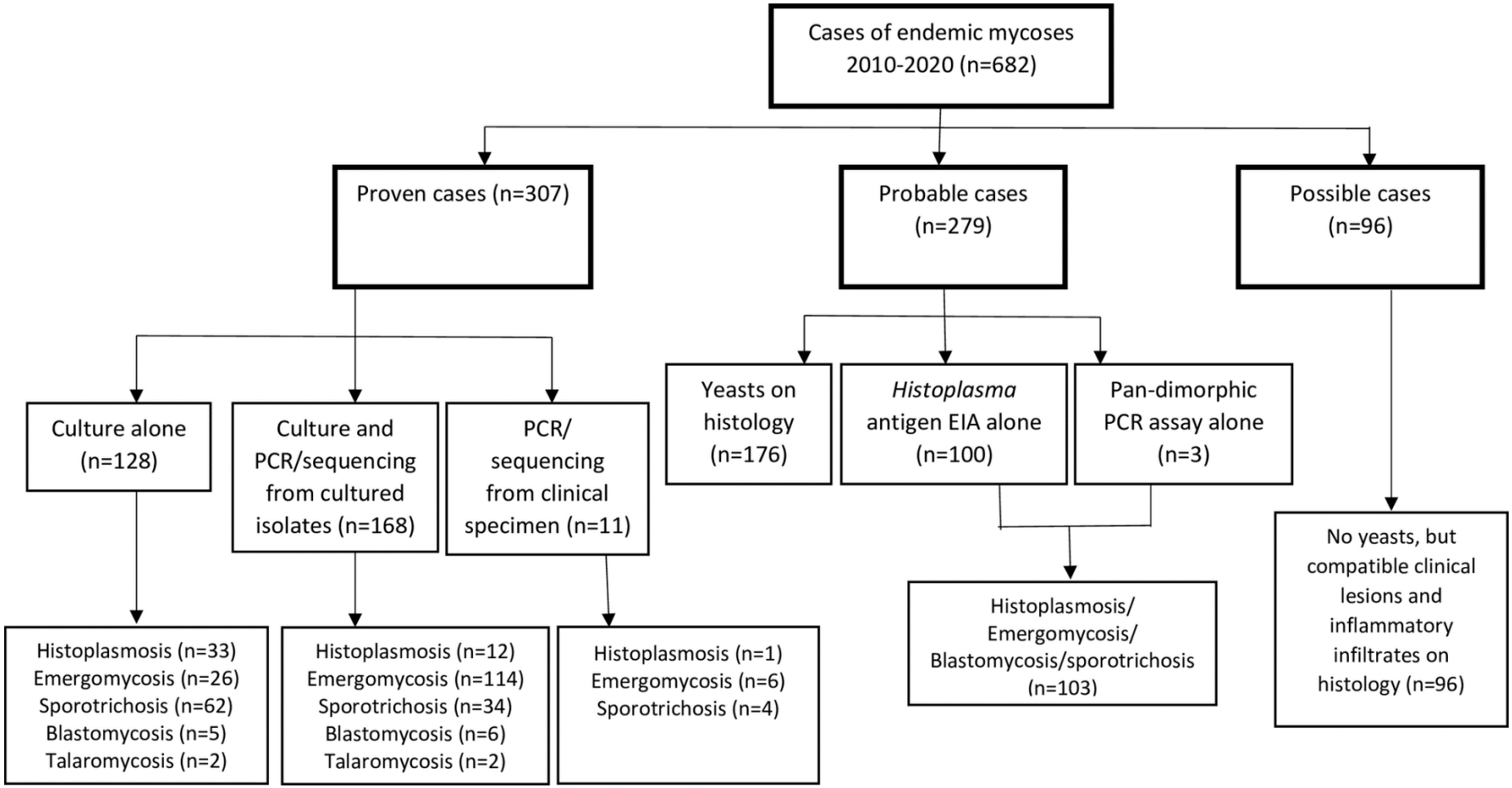

Figure 2: Flow chart showing levels of certainty for cases of endemic mycoses reported in South Africa from 2010-2020, n=682.

Table 1: Demographic and clinical characteristics of patients with proven, probable or possible laboratory-diagnosed endemic mycoses in South Africa (2010-2020), n=682.

Characteristics	All N=682	Unspecified endemic mycoses n=207	Sporotrichosis n=170	Emergomycosis n=154	Histoplasmosis n=133	Blastomycosis n=14	Talaromycosis n=4
Age, n	643	202	161	137	126	13	4
Age in years; median [IQR]	37 (31 – 45)	36 (30-44)	45 (34-57)	36 (30-41)	36 (31-41)	34 (29-51)	36 (26-40)
Missing data, n	39	5	9	17	7	1	-
Sex, n	670	204	170	147	131	14	4
Female, n (%)	234 (35)	74 (36)	46 (27)	48 (33)	60 (46)	4 (29)	2 (50)
Male, n (%)	436 (64)	130 (64)	124 (73)	99 (67)	71 (54)	10 (71)	2 (50)
Missing, n	12	3	-	7	2	-	-
Health sector	682	207	170	154	133	14	4
Public, n (%)	586 (86)	193 (93)	107 (63)	143 (93)	128 (96)	12 (86)	3 (75)
Private, n (%)	96 (14)	14 (7)	63 (37)	11 (7)	5 (4)	2 (14)	1 (25)
HIV status	377 (55)	125	48	122	72	7	3

Seropositive, n (%)	368 (98)	122 (98)	47 (98)	122 (100)	72 (100)	2 (29)	3 (100)
Seronegative, n (%)	9 (2)	3 (2)	1 (2)	-	-	5 (71)	-
Missing, n	305	82	122	32	-	7	1
Median CD4 cell [a]	181	23	38	100	17	2	1
Median CD4 count at diagnosis in cells/mm^3 (IQR)	24 (9-66)	23 (5-67)	59 (18-98)	14 (7-40)	50 (8-98)	78 (67-89)	5 (5-5)
Missing, n	501	184	132	54	116	12	3
ART[a]	85	-	19	56	8	2	
Naive, n (%)	14 (16)	-	3 (16)	9 (16)	1 (13)	1 (50)	-
Experienced, n (%)	71 (84)	-	16 (84)	47 (84)	7 (88)	1 (50)	-
Missing, n	597	207	151	98	125	12	4
Specimen type	569	188	154	140	72	11	4
Skin	276 (49)	75 (39)	86 (56)	54 (39)	52 (72)	9 (82)	-
Tissue	103 (18)	17 (9)	41 (27)	28 (20)	15 (21)		-
Urine	100 (18)	89 (47)	3 (2)	8 (6)	-	-	-

Blood	55 (10)	-	9 (6)	42 (30)	1 (0.6)	-	3 (75)
Bone marrow	12 (2)	5 (3)	1 (0.7)	4 (3)	2 (3)	-	1 (25)
Bone fragments	2 (0.4)	1 (0.5)	1 (0.7)	-	-	-	-
Pus abscess	9 (2)	-	7 (5)	-	1 (1.4)	-	1 (25)
Nails[b]	5 (0.9)	-	1 (0.7)	3 (2)	-	1 (9)	-
Sputum	3 (0.5)	1 (0.5)	1 (0.7)	1(0.7)	-	-	-
Wound swabs	3 (0.5)	-	3 (2)	-	-	-	-
Cerebrospinal fluid	1 (0.2)	-	1 (0.6)	-	-	-	-
Hair[c]	1 (0.2)	-	-	-	1 (0.6)	-	-
Missing	113	19	16	14	61	3	-
Outcome, n	57	-	4	47	1	4	1
Died, n (%)	30 (61)	-	4 (100)	24(51)	1 (100)	3 (75)	1 (100)
Survived, n (%)	19 (39)	-	-	23 (49)	-	1 (25)	-
Missing, n	633 (93)	207	166	107	132	10	3

a: ART status and CD4 counts only for HIV-seropositive patients on presentation; b and c: diagnostic laboratories reported positive culture of a thermally-dimorphic fungus from nail or hair specimens; the fungal identification was not confirmed at a reference laboratory and thus these may not truly be cases.

3.3.2. Sporotrichosis

Sporotrichosis was the most commonly diagnosed endemic mycosis occurring in 25% (170/682) of all cases. Of these, 73% (124/170) of the individuals were male, with a male to female ratio of 3:1 and an overall median age of 45 years (IQR, 34-57). Gauteng and KwaZulu-Natal provinces had the highest number of sporotrichosis cases with 38% (65/170) and 28% (48/170), respectively (S1 Fig). Fifty-nine per cent (101/170) of these cases were proven as *S. schenckii* by culture plus phenotypic and panfungal PCR/sequencing methods. Thirty-nine (23%) cases were categorised as probable and 29 (17%) as possible cases. The most common specimen types submitted for diagnosis of sporotrichosis were skin tissue (77/170, 45%) and unspecified tissue (41/170, 24%). HIV infection status was available for 28% (48/170) of the patients and of these, 98% (47/48) were HIV-seropositive. Forty-five of the HIV-seropositive cases were proven cases of sporotrichosis, 24 of these at NICD. Of the 47, 38 (81%) had a recorded CD4 count with a median of 59 cells/µl (IQR, 18-98 cells/µl). Forty per cent (19/47) of the HIV-seropositive cases had known ART status and of these, 84% (16/19) were ART-experienced. Seventy-eight per cent (25/35) of the patients with available data had multiple skin lesions, which were mostly located on the face and upper and lower limbs. Serum albumin levels were available for ten patients (6%) and all were below the specified reference ranges. Forty-four and forty-two patients had serum alkaline phosphatase and γ-glutamyl transferase results respectively and all had elevated levels for both.

3.3.2. Emergomycosis

Twenty-three per cent (154/682) of all patients in this case series had emergomycosis. Ninety-four per cent were proven cases (144/154) and 6% (10/154) were probable cases. Eighty-two per cent (119/154) of proven cases had species-level identification established using culture and phenotypic methods plus a panfungal PCR/sequencing assay. Of these, 98% (117/119) were infected with *Emergomyces africanus* and 2% (2/119) had *Emergomyces pasteurianus*. The male to female ratio was 2:1 (99:48). Diagnoses were mainly made at public hospitals (93%, 143/154). Patients had a median age of 36 years (IQR, 30-41) and were mostly diagnosed in the Western Cape Province (75/154, 49%), followed by Gauteng Province (45/154, 29%) (S1 Fig). Blood cultures (42, 29%), skin tissue (40, 29%) and unspecified tissue (28, 20%) were the main

specimen types for diagnosis of emergomycosis. Of the 154 patients, 79% (122/154) had a known HIV infection status and all were HIV-seropositive. Forty-six per cent (56/122) of the HIV-seropositive cases had their ART status recorded. Of these 56 cases, 47 (84%) were ART-experienced and nine patients (16%) were ART-naive. Eighty-two per cent (100/122) of the cases had a recorded CD4 count at the time of diagnosis and the median CD4 count was 14 cells/µl (IQR, 7-40 cells/µl). Eighty-eight per cent (108/122) had multiple skin lesions, 6% (6/108) had recorded pulmonary disease and 27% (29/108) had both cutaneous and pulmonary disease. Five per cent (5/108) of the patients had fungaemia. One patient with culture-confirmed *E. africanus* fungaemia had a history of a chronic headache, and brainstem lesions with no ring enhancement were visualised on a computer tomography scan with contrast. The remaining 22% (24/108) had no or limited available clinical information. Thirteen of these 24 patients had non-specific symptoms which included fever, weakness, weight loss, cough, nausea, diarrhoea and night sweats. Serum alkaline phosphatase results were available for 47/154 (30%) patients and the median value was high at 163 units per litre (IQR, 121-287). High levels of serum γ-glutamyl transferase were also reported in 48/154 (31%) with a median of 145 units per litre (IQR, 102-281). Clinical outcomes were available for 39 patients and of these, 54% (21/39) died.

3.3.3. Histoplasmosis

There were 133 (20%) cases of histoplasmosis during the study period. Patients had a median age of 36 years (IQR, 31-41) and a majority were male (71/133, 53%). Ninety-six per cent of cases were diagnosed at public hospitals. Although histoplasmosis was diagnosed in all nine provinces, most cases were reported from KwaZulu-Natal (39/133, 29%) and Western Cape provinces (32/133, 24%) (S1 Fig). Of the 133 cases, 46 (35%) were proven, 67 (50%) were probable and 20 (15%) were possible. Proven cases included a majority (72%; 33/46) identified by culture plus phenotypic identification methods alone, 26% (12/46) identified by culture plus phenotypic and panfungal PCR/sequencing methods and a single case confirmed by panfungal PCR/sequencing alone (directly from tissue). These proven cases were all caused by *Histoplasma capsulatum*. Among probable cases, all were diagnosed by histology and yeasts were observed with a morphology most consistent with *Histoplasma* species. Of the 133 patients with histoplasmosis, 64% (72/133) had a known HIV status and all 72 were HIV-seropositive. Only 17 cases had a known CD4 count at the time of diagnosis with a median CD4 count of 50

cells/µl (IQR, 8-98 cells/µl). Antiretroviral treatment status was available for 11% (8/72) and 88% (7/8) of patients were ART experienced. Of the 133 cases, clinical symptoms at presentation were recorded for only 6% (8/142). All eight patients had widespread skin lesions, while two were also reported to have TB. Twenty-two patients had full blood count results available, and all patients had evidence of pancytopenia. Overall, the blood chemistry and liver function test results were available for 17% (22/133) patients. Eight per cent (11/133) of the patients had results for serum levels of conjugated bilirubin and all were elevated; 14% (18/133) patients with results for serum alkaline phosphatase also had elevated levels (Table 2).

Table 2: Laboratory tests performed 2 weeks before or after the fungal diagnostic test for patients with proven, probable or possible laboratory-diagnosed endemic mycoses in South Africa, 2010-2020.

Characteristics	All	Emergomycosis	Sporotrichosis	Histoplasmosis	Blastomycosis	Talaromycosis
Blood chemistry & liver function tests						
Creatinine — µmol/litre (n)	130	57	46	19	4	3
Reference range	49-90					
Median	76	89	72	65	60	75
Interquartile range	53-111	61-128	52-105	45-87	53-1023	70-156
Albumin — µmol/litre (n)	36	16	10	5	4	1
Reference range	35-52					
Median	**26**	**24**	**31**	**26**	**26**	**14**
Interquartile range	19-31	18-28	26-39	18-29	21-35	14-14
Total bilirubin — µmol/litre(n)	114	50	42	17	3	2
Reference range	0-21					
Median	7	8	6	11	7	5
Interquartile range	5-13	5-16	4-10	7-14	3-31	4-5

Conjugated bilirubin — µmol/litre (n)	99	45	38	11	3	2
Reference range	0-5					
Median	4	5	3	**11**	3	2
Interquartile range	2-12	3-12	2-5	2-31	2-27	1-2
Alkaline phosphatase — U/litre (n)	114	47	44	18	3	2
Reference range	40-120					
Median	**152**	**163**	**157**	**143**	**152**	84
Interquartile range	107-243	121-287	87-240	94-231	70-158	79-89
γ-Glutamyl transferase — U/litre (n)	111	48	42	16	3	2
Reference range	0-35					
Median	**117**	**145**	**74**	**169**	**55**	**59**
Interquartile range	55-235	102-281	31-201	66-222	15-381	35-83
Alanine transaminase — U/litre (n)	132	58	47	22	3	2
Reference range	7-35					
Median	32.5	**40**	19	32.5	25	**115.5**
Interquartile range	19-56.5	30-78	13-35	23-61	18-33	33-198
Aspartate transaminase — U/litre(n)	102	45	40	12	3	2

Reference range	13-35					
Median	81.5	**153**	**35**	**77**	**39**	**245.5**
Interquartile range	35-173	91-272	23-61	37-115.5	33-44	157-334
C-reactive protein (n)	40	17	14	4	2	2
Reference range	<10					
Median	98	143	65	66	77	136.5
Interquartile range	42-175	67-210	32-116	52.5-172	3-151	136-137
Full blood count						
Haemoglobin ×10−9/litre (n)	160	69	61	22	4	3
Reference range	12.0-15.0					
Median	9.3	**8.1**	**10.8**	**8.3**	**9.2**	**11**
Interquartile range	7.25-11.2	6.6-9.9	9.1-12.4	6.8-10.2	8.75-11.25	7.4-11.3
Red cell distribution width % (n)	160	69	61	22	4	3
Reference range	11.6-14.0					
Median	16.2	**16.4**	**15.4**	**17.4**	**19.15**	**16.9**
Interquartile range	14.6-18.65	14.9-18.9	14.2-17.5	16.3-19.2	15.85-21.9	13.3-26.5

White-cell count — ×10−9/litre (n)	161	70	61	22	4	3
Reference range	4.00-10.00					
Median	5.32	4.96	6.07	**3.94**	5.39	**15.43**
Interquartile range	3.64-8.34	3.53-7.79	4.31-9.38	2.65-5.63	4.2-9.73	5.1-45.24
Red-cell count — ×10−9/litre (n)	160	69	61	22	4	3
Reference range	3.80-4.80					
Median	3.36	3	3.78	3.095	3.055	3.74
Interquartile range	2.63-3.915	2.28-3.54	3.1-4.18	2.63-3.77	2.62-3.805	2.58-14.2
Neutrophil count — ×10−9/litre (n)	94	40	39	12	2	1
Reference range	2.00-7.00					
Median	3.09	2.805	3.88	2.715	2.67	5.63
Interquartile range	2.18-5.8	1.525-4.305	2.4-10.21	1.49-5.055	2.24-3.1	5.63-5.63
Lymphocyte count — ×10−9/litre (n)	94	40	39	12	2	1
Reference range	1.00-3.00					
Median	0.73	**0.47**	1.1	**0.515**	1.14	**5.8**
Interquartile range	0.35-1.63	0.285-0.905	0.55-1.73	0.235-1.235	0.59-1.69	5.8-5.8

Monocyte count — ×10−9/litre (n)	94	40	39	12	2	1
Reference range	0.20-1.00					
Median	0.305	0.15	0.42	0.365	0.285	**1.9**
Interquartile range	0.14-0.6	0.09-0.405	0.2-0.74	0.22-0.56	0.2-0.37	1.9-1.9
Eosinophil count — ×10−9/litre (n)	92	39	39	12	2	
Reference range	0.00-0.40					
Median	0.075	0.09	0.08	0.035	0.085	
Interquartile range	0.02-0.195	0.02-0.2	0.03-0.26	0.01-0.27	0.03-0.14	
Basophil count — ×10−9/litre (n)	88	37	37	12	2	
Reference range	0.00-0.10					
Median	0.02	0.02	0.02	0.015	0.025	
Interquartile range	0.01-0.04	0.01-0.06	0.01-0.04	0.01-0.045	0.01-0.04	
Platelet count — ×10−9/litre (n)	159	68	61	22	4	3
Reference range	171-388					
Median	271	194.5	298	194.5	-	190
Interquartile range	148-386	122-351	211-402	61-419	-	173-484

3.3.4. Blastomycosis

Two per cent (14/683) of the cases were diagnosed with blastomycosis. Five cases were diagnosed by culture plus phenotypic identification methods and another five were diagnosed by culture and phenotypic identification plus panfungal PCR/sequencing. A single case was identified by PCR/sequencing alone. Three cases were classified as probable cases because yeasts consistent with *Blastomyces* species were observed during histology examination of tissue samples. Ten of the 14 cases were male with a median age of 34 years (IQR, 31-50) (Table 1). The cases of blastomycosis were diagnosed in six of the nine provinces. Most cases were diagnosed in the Eastern Cape (6/14, 43%) and Western Cape provinces (4/14, 29%). One case each was diagnosed in Free State, KwaZulu-Natal, Gauteng and North West provinces (S1 Fig). Species identification was available for five cases, of which four were *B. percursus* based on panfungal PCR/sequencing, and one was *B. emzantsi* on panfungal PCR/sequencing. Skin tissue was the most common specimen type submitted for diagnosis of five cases. Four of the five patients had multiple skin abscesses and pulmonary involvement, while one patient had a brain lesion seen on a computed tomography scan of the head. HIV infection status was known for seven patients and five were HIV-seronegative. All patients with proven *B. percursus* and *B. emzantsi* infections were HIV-seronegative. No further clinical details were available for the other cases. Low serum albumin levels were reported in four patients and three patients had high serum levels of alkaline phosphatase, γ-glutamyl transferase and aspartate transaminase.

3.3.5. Imported mycoses

Only four cases were diagnosed with *Talaromyces marneffei* infection. Three cases were reported in the Gauteng Province and one in KwaZulu-Natal Province. Two cases were diagnosed using culture plus phenotypic identification only, while the other two were diagnosed using culture and both phenotypic identification and PCR/sequencing. Two patients were male and the other two were female with a median age of 36 years (IQR, 29-39). Diagnosis was made from blood culture for three cases and pus from an abscess from one case. Of the four cases, three had a known HIV status and all were HIV-seropositive. Only one had a known CD4 count of 5 cells/µl. Two patients had an available travel history and confirmed recent travel to China. One was a South African woman who travelled to mainland China. She presented with

hepatosplenomegaly and skin lesions on the face. The second patient was also a South African woman who worked in Hong Kong and her infection was diagnosed from pus from an abscess. High peripheral white cell counts were reported in three patients and elevated serum levels of alkaline phosphatase, γ-glutamyl transferase and aspartate transaminase were reported in two patients.

3.3.6. Unspecified endemic mycoses

Most cases were not assigned to a causative fungal genus. Two hundred and seven cases were diagnosed as an unspecified endemic mycosis, possibly histoplasmosis or emergomycosis or blastomycosis because of the low or unknown specificity of the methods used. Seventy-seven per cent (160/207) were classified as probable cases and were diagnosed using the *Histoplasma* EIA (89/160, 56%), histopathology (68/160, 43%) and pan-dimorphic PCR assay (3/160, 2%) assay at NICD in 2020. None of these cases were culture-confirmed. Twenty-three per cent (47/207) were classified as possible cases. Among all patients with an unspecified endemic mycosis, 55% (114/207) had histology reports available and 79% (90/114) were reported as "deep fungal infections". An HIV status for these patients was available for 60% (125/207) and of these, 98% (122/125) were HIV-seropositive.

3.3.7. Laboratory survey on diagnosis of endemic mycoses at public hospitals

Of the 269 NHLS laboratories, only 21 (8%) microbiology and histopathology laboratories responded to the survey (S1 Text). From the 21 responses received, 15 indicated that they processed specimens from patients with a suspected endemic mycosis at least 1 to 5 times per annum and 3 reported diagnosing between 5 to 10 patients per year. All laboratories indicated regularly culturing *Sporothrix* and *Emergomyces*. Fifty per cent of the respondents indicated that they referred cases to other laboratories (such as the NICD) for confirmation. Tissue was the most common specimen type (75%) reported to be processed for diagnostic purposes. Specimens were reported to be processed in a class II biosafety cabinet by 81% of the respondents. Diagnostic methods used to identify endemic mycoses included culture (13, 62%) and histopathology (2, 10%).

3.4. Discussion

Our analysis of pathology laboratory data resulted in the identification of 682 cases of an endemic mycosis, most of which were proven. Approximately a third of cases were categorized as an unspecified endemic mycosis as the causative pathogen could not be identified to genus level due to the limitations of the available diagnostic methods. Sporotrichosis was the most common endemic mycosis followed by emergomycosis, histoplasmosis, blastomycosis and rarely, talaromycosis, an imported mycosis. A majority was diagnosed using histopathology and the main specimen type submitted for diagnosis was skin tissue.

Our understanding of the pathogens causing endemic mycoses is that they are found in specific environmental niches and thus exposure and subsequent infection is generally associated with outdoor activities [32]. Histoplasmosis, blastomycosis and presumably emergomycosis follow inhalation of tiny airborne microconidia that convert to the yeast form in the lungs with dissemination to the skin and other organs or systems mainly in immunocompromised individuals [33]. Sporotrichosis is usually caused by traumatic inoculation of vegetative material containing the fungus into the skin [34]. Detailed data from skin test prevalence surveys, which serve as a proxy for environmental exposure, are rarely reported in South Africa. A report by Lurie *et al* in 1955 indicated 12% positive histoplasmin skin reactions among laboratory staff working at the South African Institute for Medical Research [35]. Oladele *et al* reported 12 histoplasmin skin test surveys conducted in Africa until 2017 with a positivity ranging from 0% to 35% [36,37]. Mapping of laboratory-diagnosed cases does not generally indicate the source of infection, given the long incubation period between exposure and clinically apparent disease and the fact that patients may access healthcare facilities distant from their places of work, residence or outdoor activities. In our study, we only report case numbers per province with no residential address or patient history to help identify potential environmental exposures. The provinces with most reported cases were Gauteng, Western Cape and KwaZulu-Natal; this could be related to their relatively larger populations but also to better health resources for case-finding and diagnosis. The distribution of cases was not uniform across the country with the coastal Western

Cape Province reporting relatively more cases of emergomycosis; more cases of unspecified endemic mycoses and sporotrichosis in the high-altitude Gauteng Province; and relatively more cases of histoplasmosis in sub-tropical coastal province of KwaZulu-Natal. Based on very sparse underlying data, the burden of endemic mycoses in South Africa was roughly estimated at 100, 60, 40 and 10 cases per annum respectively for emergomycosis, histoplasmosis, sporotrichosis and blastomycosis [11]. As expected, using laboratory-diagnosed cases, we report even fewer average cases per annum (15, 13, 17 and 1 for emergomycosis, histoplasmosis, sporotrichosis and blastomycosis, respectively). This could be attributed to cases being missed or misdiagnosed as TB, for example, because of the overlapping clinical features [38]. *T. marneffei* infections were only reported from patients who travelled outside of South Africa to known endemic regions.

Almost two thirds of the patients in our study were male, confirming previous reports that endemic mycoses are more common in males [39-41]. The number of laboratory-diagnosed cases increased from 2010 to a peak in 2015. This increase was mainly noted for emergomycosis and may have been related to the description of a novel fungus, *E. africanus* in South Africa in 2013 and related studies that improved case finding and diagnostic testing. This supports the link between better clinician awareness, improved diagnosis and case reporting. In 2020, a decrease in the number of detected cases could have been associated with COVID-19 pandemic travel restrictions that discouraged movement of people and thus many patients could not seek medical attention.

Oladele *et al* reported an association between histoplasmosis and HIV in Africa [24]. More than half of the patients in our study were HIV-seropositive. Emergomycosis and histoplasmosis were mainly diagnosed in people with advanced HIV disease, as previously reported [23,42-46]. This highlights the need for simple screening methods to facilitate an earlier diagnosis in advanced HIV, analogous to the approach used for HIV-associated cryptococcosis. Furthermore, half of the culture-confirmed sporotrichosis cases in our study were also HIV-seropositive. HIV-infection worsens sporotrichosis, resulting in more severe disseminated cases, higher need for hospitalisation and risk of death [58]. However, some of these cases of sporotrichosis were only

proven by culture plus phenotypic methods; inexperienced laboratory workers might misidentify *Sporothrix* cultures as *Emergomyces* or vice versa due to the morphological similarities between these fungi. Other than the association of histoplasmosis with HIV, no other risk factors were discussed in our study as it was beyond our scope. We recommend that more studies be done in order to understand all the risk factors associated with endemic mycoses in South Africa or the whole continent.

The distribution of endemic mycoses globally has been described recently leading to redefined geographical boundaries of these diseases [20]. Reports on endemic mycoses were previously mainly from the Americas, Asia and some parts of Africa [9,18,24,45]. In Africa, few countries have surveillance systems for endemic mycoses [46-48]. A few outbreaks have been reported on sporotrichosis and histoplasmosis among mine workers and laboratory workers respectively [22,35]. Outbreaks of acute pulmonary histoplasmosis in South Africa have occurred mostly in individuals who had recently explored caves [49,50]. Thermally-dimorphic fungal infections are associated with significant mortality and morbidity in individuals with compromised immune systems, with up to 50% mortality being reported in patients with AIDS [11,40,43]. Of the 50 patients with available outcome data in our study, 62% died. Most of these outcome data were for 39 patients with HIV-associated emergomycosis, of whom 55% died. Similarly high mortality (50% case fatality among 52 patients) was reported in a previous case series from South Africa including many of the same cases [43]. Schwartz *et al* reported a case series of 25 patients with endemic mycoses in the Western Cape Province, of whom 24% died; this lower overall mortality was attributed to early diagnosis [40]. This mortality associated with endemic mycoses in South Africa is much higher than the reported rate in the United States of 5% and 7% in children and adults respectively [50] and this may be related to HIV as a major underlying condition, late diagnosis and lack of appropriate treatment.

The diagnosis of endemic mycoses can be challenging and requires a multifaceted approach [52-54]. In South Africa, endemic mycoses are diagnosed at most pathology laboratories by traditional methods such as culture with morphological identification or histopathology, the gold standard methods [12]. Difficult-to-identify fungi are submitted to the NICD for confirmation by

culture and molecular methods. A direct pan-dimorphic RT-qPCR or/and *Histoplasma* antigen EIA are performed on request at NICD. The histology laboratories employ stains such as periodic acid–Schiff (PAS), calcofluor white, Giemsa and Grocott's methenamine silver for identification of yeasts in tissue specimens. In this study, most cases were diagnosed using histology alone and resulted in most cases being classified as an unspecified endemic mycosis. Histology alone generally cannot clearly differentiate among thermally-dimorphic fungi due to morphological similarities between *Histoplasma*, *Emergomyces*, *Blastomyces* and *Sporothrix* [55-57]. The proven cases in this study were diagnosed using culture with phenotypic identification alone or with molecular methods in some cases. Identification of fungi requires expertise and biosafety level 3 facilities for culturing of pathogens such *Blastomyces, Emergomyces* and *Histoplasma* are not always available. Both public and private laboratories recorded proven cases after culture with identification of fungi often only to a genus level; considering the similarities in morphology among dimorphic fungi, some of the confirmed cases in our study might not be correctly identified as recorded. Diagnostic laboratories reported thermally-dimorphic fungi cultured from nail and hair specimens for a few cases which is highly unlikely. This further highlights the problem of misidentification and lack of expertise in correctly identifying dimorphic fungi. There is need for constant training in laboratories to ensure correct identification. Our survey indicated that most public-sector laboratories relied on culture methods and/or histology for diagnosis of endemic mycoses and most depended on referring cases to the NICD reference laboratory for confirmation. However, from 2012 to 2021, only 167 (24%) cases were referred to the NICD. Forty per cent of isolates were incorrectly identified or listed as a thermally-dimorphic fungus with no genus/species identification. This further emphasises the need for training. The NICD has one of very few mycology reference laboratories on the African continent, highlighting a general lack of surveillance and reference diagnostic capacity that may contribute to many cases being missed or misdiagnosed.

In our survey, rapid molecular and serological methods were not readily available at diagnostic laboratories. However, such assays are imperfect. Our pan-dimorphic PCR assay cannot differentiate among cases of infection caused by *Histoplasma, Blastomyces* and *Emergomyces* [29]. The close phylogenetic relationship of these different thermally-dimorphic fungi means that

pathogen-specific assays are difficult to develop. Delays in diagnosis have consequences that may be fatal, can lead to unnecessary antimicrobial use which can result in resistance or can result in longer hospital admissions. Up to 16% patients in our study had multiple skin lesions. Although non-specific, this should trigger suspicion of an endemic mycosis in the differential diagnosis and prompt ordering of appropriate laboratory tests. The mycoses endemic to southern Africa are all treated in the same way with initial amphotericin B in severe cases, followed by itraconazole; moderate or mild cases are treated with itraconazole only [16,30,46]. One of the limitations of our study was that we did not have access to treatments records for the patients that would have given more clarity on how the diseases were treated.

A major strength of this study was its national coverage and use of diagnostic pathology laboratory data from both public and private sectors and a national reference laboratory. Some of the limitations included the search strategy used in the laboratory information systems. For example, histology results were searched using SNOMED clinical terms or the names of the thermally dimorphic fungi or diseases and some cases could have been missed. Our mapping of laboratory-diagnosed cases by province cannot be used to infer areas of higher or lower endemnicity. We also collected very limited demographic and clinical data in this retrospective laboratory surveillance. Characteristics such as ethnic group, underlying medical condition and immunosuppressing medicines were not consistently available. Travel history and data on occupational and recreational pursuits were also generally not available. Outcomes were also available for only 7% of cases. The results from biochemical and liver function tests for patients closer to the time of diagnosis was also missing for most patients and this was a limitation in terms of conclusions that could be reached using this data. We did not store FFPE tissue or cultured isolates from proven cases identified only at diagnostic laboratories for further confirmatory testing at the NICD; if we had, this would have confirmed the diagnosis and furthermore identified the gaps in diagnostic capabilities.

Conclusion

This is the first attempt at determining the national epidemiology of endemic mycoses in South Africa. Although relatively few laboratory-diagnosed cases were identified, there is a large, immunosuppressed population at risk of severe disease and ours is probably a minimum estimate. There is a clear need for careful environmental mapping of the pathogen reservoirs and active human and veterinary surveillance of cases in South Africa in order to understand the true burden of these diseases. Rapid and accurate serology and molecular diagnostic tests, which are appropriate for resource-limited settings, will facilitate such surveillance. Mandatory reporting of endemic mycoses would also improve surveillance efforts but would also increase the reporting burden on diagnostic laboratories. More funding needs to be invested into neglected endemic mycoses to address the knowledge gaps in this field. Programs are also needed to improve laboratory diagnosis of endemic mycoses in Africa. Awareness of these diseases should also be increased in the general population and among healthcare workers.

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59. Supporting information
60. S1 Fig. Distribution of endemic mycoses in South Africa by province from 2010 to 2020
61. S2 Appendix. Endemic Mycoses in South Africa survey

CHAPTER 4: COMPARATIVE GENOMICS, POPULATION STRUCTURE AND PHENOTYPIC DESCRIPTION OF AFRICAN *HISTOPLASMA SPP.*

4.1. Introduction

Histoplasmosis, a disease caused by the fungus *Histoplasma* is one of the most prevalent fungal pulmonary diseases. In immunocompetent individuals, most cases are asymptomatic, self-limiting and usually resolve without treatment (1). Histoplasmosis is of particular importance in immunocompromised patients where it can progress to disseminated disease, frequently fatal (2–6). The treatment for the disease is by amphotericin B in severe cases followed by maintenance itraconazole or fluconazole (7–9).

Histoplasma was originally classified into three varieties defined by clinical manifestations, host-association, morphology when observed under the microscope in tissue, and geographical distribution: 1) *H. capsulatum* var. *duboisii* comprises African human pathogens, causes African histoplasmosis, characterised by granulomatous lesions mainly in cutaneous, subcutaneous and osseous tissue and by the larger size of the yeasts cells compared to the other two subspecies (10–14); 2) *H. capsulatum* var. *capsulatum* includes New World human pathogens and is found world-wide, especially in Latin America and in the mid-west of the United States; 3) *H. capsulatum* var. *farciminosum* is restricted to Old World equine pathogens, causes epizootic lymphangitis typified by ulcerated lesions of the skin, and pyogranulomatous subcutaneous nodules and has been reported in Northeast Africa, India, Pakistan, South Asia and, Europe (15–17).

The use of phylogenetics based on multi-locus sequence typing (MLST) was used to classify *Histoplasma* into at least eight clades, with additional studies expanding the number of *Histoplasma* clades that may be recognised as phylogenetic species (18,19). Most of the proposed eight phylogenetic species were geographically restricted. Analysis of whole-genome sequencing data combined with population genomic approaches confirmed at least four *Histoplasma* species that are genetically isolated and considered to be phylogenetic species therefore, a taxonomic reorganisation of the genus was proposed: (i) *Histoplasma capsulatum sensu stricto* Darling 1906, (ii) *Histoplasma mississippiense* sp. nov., (iii)

Histoplasma ohiense sp. nov., and (iv) *Histoplasma suramericanum* sp. nov.(20), with phenotypic characterisation confirming this reorganisation (18). Further studies revealed the existence of two additional phylogenetic species, one endemic to Southern Brazil (21) and another one endemic to the Indian subcontinent (22).

One blind spot of most efforts to characterise the genetic diversity of *Histoplasma* has been the paucity of African samples included in whole genome studies with only two isolates from the African continent that have been previously sequenced by Sepulveda and other in 2017 (18,20). Histoplasmosis causes a significant problem in Africa especially in HIV/AIDS patients with 470 cases reported between 1952 and 2017 and the disease often manifests as an extrapulmonary disease in skin, lymph nodes, and bones (12–14,23–25). The disease is endemic and widespread across the continent which might suggest the possibility of extensive genetic variation across Africa in the form of different species, or high diversity within species. Hypotheses indicate the existence of high genetic diversity in Africa. Classical taxonomic classifications suggested the presence of two different lineages of *Histoplasma* in Africa, *H. capsulatum var. duboisi*i (Hcd) and *H. capsulatum* var. *capsulatum* (Hcc). The neglected nature of histoplasmosis in Africa, due to a lack of accurate and timely diagnostic methods (26–29) has historically compounded this issue.

In this study, we address this gap using a population genomics approach to characterise isolates from South Africa and DNA from other African countries We found that South African strains form a monophyletic group that is differentiated enough to be considered a phylogenetic species. Next, we used data from African strains to understand their genetic diversity and population genetics compared to the previously described American and Indian strains. We compiled cases of histoplasmosis potentially caused by *H. capsulatum* var *duboisii* and we confirm that the disease is more frequently associated with extrapulmonary manifestations than other lineages of *Histoplasma*.

4.2. Materials and Methods

4.2.1. South African isolates collection

The samples included in this study came from two sources. First, we recovered isolates from the collection of *H. capsulatum* isolates archived between 1979 to 1998 at the National Institute for Communicable Diseases (NICD). A total of 36 isolates were stored in water at room temperature over these years but only 3 isolates were viable at the time of study. The second source of isolates was a passive laboratory-based surveillance for thermally dimorphic fungi associated with human and animal disease conducted from 2008 to date at all South African diagnostic pathology laboratories in the public and private sectors. A case of histoplasmosis was defined as a person or animal with an isolate, cultured from any specimen, which was confirmed as *H. capsulatum* by phenotypic or molecular methods. During this period, 13 human and two horse isolates were collected and described phenotypically and sequenced for further genomic analysis.

4.2.2 Morphological description and electron microscopy

We subcultured all samples directly onto actidione and Sabouraud dextrose agar (SabDex agar) (Diagnostic Media Products [DMP], Sandringham, South Africa) and incubated at 25°C for up to 6 weeks for the mould phase. To confirm dimorphism all isolates were transferred to brain heart infusion (BHI) agar (DMP, South Africa) and incubated at 35°C for up to 4 weeks for conversion to the yeast phase. All cultured isolates were handled in a biosafety level 3 (BSL3) laboratory. Yeast and mould phase isolates were examined using both light microscopy and electron microscopy. To study the mould phase, we sampled the cultures with cello tape and visualised them with lactophenol cotton blue stain. For the yeast phase, we used Gram staining for visualisation under the microscope.

4.2.3. Antifungal susceptibility

We studied the antifungal susceptibility of the yeast phase of the *Histoplasma* South African isolates using the broth microdilution (BMD) method (30). Only 16 human isolates were included in the antifungal susceptibility testing. Animal isolates were excluded because there

were only two isolates, and we intended on collecting more animal isolates and conducting a thorough analysis on them. We tested five antifungals: amphotericin B (Clinical Sciences Diagnostics, JHB, South Africa), fluconazole (Clinical Sciences Diagnostics, JHB, South Africa), itraconazole (Sigma, St Louis, MO, USA), 5-flucytosine (Sigma, St Louis, MO, USA), and micafungin (Sigma, St Louis, MO, USA). Briefly, we followed the BMD method described in the document M27-A3 of the Clinical and Laboratory Standards Institute (CLSI) for yeast phase with deviations as described previously by Kathuria and colleagues in 2014 (31,32). We grew the yeast form of *Histoplasma* for 7 days on BHI agar at 35ºC then on day 8 we used inocula from these cultures to prepare a 5 McFarland standard which was diluted in RPMI 1640 1 X 10^5 to 2.5 X 10^5 cfu/ ml. Endpoints for the BMD MICs were read at 50% inhibition for fluconazole, itraconazole, flucytosine, micafungin and 100% for amphotericin B and final drug concentrations ranged from 0.125 to 64 µg/ml for 5-flucytosine, micafungin and fluconazole, 0.03 to 16 µg/ml for amphotericin B and 0.015 to 8 for itraconazole. For quality control *Candida parapsilosis* American Type Culture Collection [ATCC] 22019, *Candida krusei* ATCC 6258 were included in each test run according to CLSI guidelines. All *Histoplasma* plates were incubated at 35°C and read after 7 and 10 days.

4.2.4. Genome sequencing

4.2.4.1 DNA extraction. First, we extracted DNA from the 18 isolates recovered as described immediately below. We grew isolates in their yeast form on BHI agar for four weeks. We collected a loopful of culture and extracted genomic DNA using the Zymo ZR fungal/bacterial DNA miniprep kit (Zymo Research Corp., USA) following the manufacturer's instructions.

4.2.4.2. Species identification with PCR. Before submitting isolates for whole genome sequencing, we used Sanger sequencing to verify the isolates were indeed *Histoplasma*. We amplified the *ITS* locus using primers and PCR as previously described by White et al 1990 (33). We then Sanger-sequenced the resulting amplicons (forward and reverse) using the same primers of the *ITS* region to confirm the isolates were *Histoplasma*. The samples were sequenced using a 3130 sequencer (Applied Biosystems, Life Technologies Corporation, USA. We visually inspected the chromatrograms using CHROMAS software (34). Finally, we used

the tool BLAST in GenBank (www.ncbi.nlm.nih.gov) to find the most similar sequences to each individual sequence.

4.2.4.3. Library construction. Eighteen isolates were processed for genomic analysis in South Africa at the NICD sequencing core facility Sandringham, Johannesburg. After DNA extraction, paired-end genomic libraries were built from high-molecular-weight DNA following the protocol described by Illumina's MiSeq System Denature and Dilute Libraries Guide (35). Approximately 10 µg of DNA was sonicated with a NextSeq DNA library kit to a mean fragment size of 150 base pairs (bp). We evaluated the concentration of each library with a Kapa library quantification kit (Kapa Biosystems, Wilmington, MA) on a 7900HT Instrument (Life Technologies).

4.2.4.4. Sequencing. We sequenced the libraries to a read length of 100 bp using v3 or v4 chemistries on an Illumina MIseq instrument. We obtained paired-end reads for all isolates and targeted an approximate coverage of 100X. The accession numbers for each isolate are on the NCBI website under PRJNA1109714. The quality of each read was determined using the NextSeq Control software 2.0.5 in combination with FASTQC and MultiQC Software (36). The target average coverage for each read was 100X. We used FASTQC 0.11.9 developed by Andrews et al. 2010 to check initial read quality and Trimmomatic 0.39 (37) to remove adapters and remove regions of low quality at the reads ends. We followed an iterative approach and after a first round of trimming, we re-checked individual read quality using FASTQC.

4.2.5. Publicly available datasets

We used previously published Illumina genome sequences and sequences from our collaborators at the Institut Pasteur, France and from the University of Brasília, Brazil. A total of 129 sequences were included in this analysis. These include 36 African isolates and 93 non-African isolates. The sources of African isolates are listed in Table 1 below. One hundred and sixteen sequences were from the genus *Histoplasma*. The isolates belong to five different phylogenetic species: 10 from *H. mississippiense,* 12 from *H. ohiense,* 21 from *H. suramericanum,* 21 from *H. capsulatum sensu stricto,* 16 from a lineage endemic to India (Accession number PRJNA1063273) and 36 African isolates (Accession number

PRJNA1109714 (20,55). Among the African sequences were 2 sequences from horse isolates. For rooting of the phylogenetic trees of *Histoplasma*, we used 13 genomes from the Family Ajellomycetaceae as outgroups: 8 from the genus *Emergomyces crescens* and *Emergomyces pasteurianus* and 5 genomes from the genus *Paracoccidioides brasiliensis* (38).

Table 1: Isolates from African human and animal patients with histoplasmosis used in whole genome sequencing data analysis showing countries of origin, n=36.

Name	**Clade and number**	**Country**	**Classification**
SECH_106-Hc_duboisii-A	Africa_1	unknown	*H. duboisii*
SECH_107-mis_Hc_duboisii-B	Africa_2	unknown	*H. duboisii*
SA1704	Africa_3	South Africa	*H. capsulatum*
SA1371	Africa_4	South Africa	*H. capsulatum*
SA1436	Africa_5	South Africa	*H. capsulatum*
SA1556	Africa_6	South Africa	*H. capsulatum*
SA297	Africa_7	South Africa	*H. capsulatum*
SA302	Africa_8	South Africa	*H. capsulatum*
SA355	Africa_9	South Africa	*H. capsulatum*
SA46	Africa_10	South Africa	*H. capsulatum*
SA47	Africa_11	South Africa	*H. capsulatum*
SA602	Africa_12	South Africa	*H. capsulatum*
SA811	Africa_13	South Africa	*H. capsulatum*
SA1556	Africa_14	South Africa	*H. capsulatum*
SA19	Africa_15	South Africa	*H. capsulatum*
SA562	Africa_16	South Africa	*H. capsulatum*
SA19VMG	*H_farciminosum*_1	South Africa	*H. farciminosum*
SA20VMK	*H_farciminosum*_2	South Africa	*H. farciminosum*
HISSP-CM7436-xx-CL-EGY	*H_farciminosum*_3	Egypt	*H. farciminosum*
HISSP-B05181-xx-CL-CIV	Africa_17	Côte d'Ivoire	*H. duboisii*
HISSP-CM7001-xx-CL-GNQ	Africa_18	Equatorial Guinea	*H. duboisii*
HISSP-CM7256-xx-CL-SEN	Africa_19	Senegal	*H. duboisii*

HISSP-CM7434-xx-CL-ZAF	Africa_20	South Africa	*H. duboisii*
HISSP-CM7704-xx-CL-GHA	Africa_21	Ghana	*H. duboisii*
HISSP-CM7767-xx-CL-NGA	Africa_22	Nigeria	*H. duboisii*
HISSP-CM8013-xx-CL-GHA	Africa_23	Ghana	*H. duboisii*
ES2_82_S60	Africa_24	Ivory Coast	*H. duboisii*
ES2_83_S61	Africa_25	Unknown country	*H. duboisii*
ES2_84_S63	Africa_26	Republic of Cameroon	*H. duboisii*
ES2_85_S64	Africa_27	Republic of Cameroon	*H. duboisii*
ES2_86_S66	Africa_28	Republic of Cameroon	*H. duboisii*
ES2_87_S68	Africa_29	Democratic Republic of Congo	*H. duboisii*
ES2_88_S70	Africa_30	Republic of Cameroon	*H. duboisii*
ES2_89_S72	Africa_31	Democratic Republic of Congo	*H. duboisii*
ES2_90_S74	Africa_32	Ivory Coast	*H. duboisii*
ES2_91_S76	Africa_33	Gabonese Republic	*H. duboisii*

4.2.6. Mapping, cleaning, filtering, and variant calling

Whole genome sequence data analysis was done following the method described by Jofre et al, 2022 (22). Briefly, FASTQ files were mapped to reference WU24 using BWA version 0.7.15.(39). The resulting BAM files were cleaned and sorted, and duplicate reads were marked using Samtools version 1.11. Single nucleotide polymorphisms (SNPs) were called using the GATK version 4.1.7.0 HaplotypeCaller function, setting the -ploidy option as 1. The resulting g.vcf files were merged using GATK GenomicsDBImport function and subsequently joint genotyped with the GATK GenotypeGVCFs function setting the -ploidy option as 1 as well. We used bcftools (40,41) to only include biallelic SNPS. Finally, we applied the following filters to the resulting multi-sample VCF files using GATK

VariantFiltration function: Quality depth (QD) < 2.0, FisherStrand (FS) > 60.0, Mapping Quality (MQ) < 30.0, Mapping Quality Rank Sum (MQRankSum) < -12.5, and Read Position Rank Sum (ReadPosRankSum) < -8.0. We generated one multi-sample VCFs for all sequences.

4.2.7. Phylogenetic reconstructions

We used phylogenetic analysis to determine whether the genetic clusters found in African *Histoplasma* satisfy the requirements to be considered a phylogenetic species. The phylogenetic species concept previously described was followed to identify species, defining species as genetic clusters that are reciprocally monophyletic (42). Population genetics framework was employed to quantitatively assess if the patterns of genetic variation were consistent with the existence of different species within *Histoplasma*. Briefly, we used the Python script vcf2phylip (43) to convert each multi-sample VCF into a concatenated genome wide alignment. We then built maximum likelihood (ML) trees from the genome-wide concatenated alignment using IQ-TREE 2 (44). We used GTR- gamma as the substitution model in all trees. To assess branch support, we used 1,000 replicates of ultrafast bootstrap (45).

4.2.8. Gene genealogy concordance

To measure the extent of genealogical concordance throughout the genomes in each population we split the supercontigs into 100-kb genetic windows. We weighed the topology of each window. Topology weighing is a method that measures the heterogeneity in a genome by using a set of gene genealogies. This method quantifies the contribution of each gene genealogy to the phylogeny of the whole genome tree. We identified each conserved orthologous gene in the database Fungi Odb10 BUSCO with 4000 Ajellomycetaceae genes and extracted the sequences of these genes from the multi-sample VCF. The gene sequences were converted to phylip format using the python script vcfphylip and we used IQTREE 2 to generate a ML tree for each BUSCO gene. Then we used the concatenated file of BUSCO gene trees in *twisst* to determine the proportion of gene genealogies that fit a given taxonomic population. True

species should show differentiation across the whole genome and not just in a few loci, (This shows whether different genes along the genome show the same evolutionary history).

4.2.9. Genetic differentiation and gene flow analysis

We formally tested whether all isolates from Africa form a single phylogenetic species, exploring two additional facets of genetic divergence. First, to study the extent of genetic differentiation between African isolates, we used principal component analysis (PCA) to measure allele frequencies across potential lineages of *Histoplasma*. This method summarises large high-dimensional datasets with possibly correlated variables into a reduced set of independent variables enabling easy visualisation. The principal components of all the samples are then projected on a two-dimensional space, and the axes show the different variables. Clustering is based on correlations between closely related individuals revealing population relations and within population stratification. We plotted the combinations of PC1/PC2 and PC3/PC4. We used cleaned and sorted BAM files with the program *angsd* (46) to generate a *beagle* file with estimated genotype likelihoods. We filtered sites with more than 20% missing data, mapping quality < 30, and a base quality < 20. Then we used the program PCAngsd (47) to compute the covariance matrix among sites. Then we used an R package *adegenet* (48) to visualise the principal components. We also calculated nucleotide diversity (π) within each population. We used vcftools to generate a VCF with variant and invariant sites (gvcf, --max-maf 0). Using this gvcf file as an input, we used *Pixy* (49) to calculate the π and Dxy between the monophyletic lineages identified in the phylogenetic tree (see above), but also to calculate the extent of genetic divergence and diversity along the genome in 1,00bp windows. To compare the values of π in each of these lineages with the magnitude of pairwise D_{xy}, we used an approximative Two-Sample Fisher-Pitman Permutation Test (R function *oneway_test*, library *coin*) with 1,000 subsampling iterations (50,51).

Finally, we tested the magnitude of gene exchange between the putative phylogenetic species and other *Histoplasma* lineages. Species that are at an advanced stage along the divergence process show no or low amounts of gene flow between species to avoid merging into a single lineage. We estimated the extent of admixture by calculating the metric f_d published by Martin et al, 2015 (52). Previous simulations have shown that f_d is a powerful metric for detecting gene

flow between populations. We plotted the statistical values in R for visualisation. Finally, we calculated the f_b heuristic as previously described by Malinksy *et al*. 2018 which summarises the f_4-admixture ratios calculated by *Dtrios* to identify and plot introgression events between specific nodes/tips in a phylogenetically informed manner (53). We plotted the results from this analysis with f-branch, one of the programs included in *Dsuite* and then we used the ML tree described above.

4.3. Results

4.3.1 Phenotypic traits of the African *Histoplasma* isolates

4.3.1.1. Culture and microscopic phenotypic description

South African isolates were collected in a laboratory-based surveillance with isolates from both private and public hospitals between 2008 and 2021. Fifteen isolates were included from this period. Three isolates were included from an archived collection at NICD from 1979 to 1998. These eighteen isolates were available for morphological description and antifungal susceptibility testing. The colony morphology of all isolates was similar. At 25ºC, colonies were white, fluffy moulds that turned cream to brown with age on Sabouraud dextrose agar (Figure 1A). Electron microscopic images of *H. capsulatum* are shown *in* Figures 1C and 1D. Figure 1E shows microscopic structures that revealed septate hyphae with round microconidia and spiked macroconidia. At 37ºC, the fungus transitioned to cream or brown,

wrinkled, moist and heaped yeast-like colonies on BHI agar (Figure 1B). As expected, these colonies were composed of oval budding yeast cells (Figure 1F).

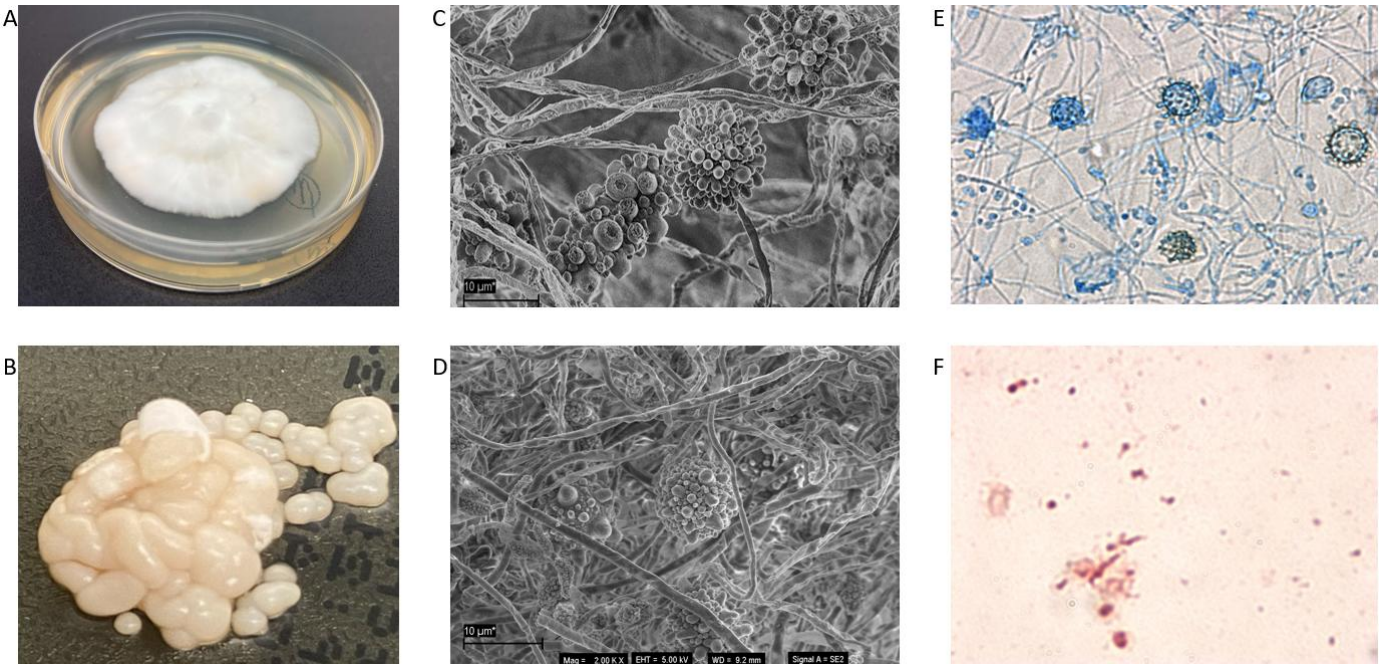


Figure 1: A: Morphology of a 3 week old *H. capsulatum* growing on a plate of Sabouraud dextrose agar at 25°C. B: Culture of a 2-week-old *H. capsulatum* growing on brain heart infusion (BHI) agar with 5% sheep blood at 35°C. C and D: Electron microscopy image of a 2-week-old *H. capsulatum* growing on Sabouraud dextrose agar at 35°C (Magnification = 2.00 K X, EHT = 5.00 Kv, WD = 9.2 mm, Signal A = SE2, Bar 10 µm). E: microscopic views of *H. capsulatum* after Gram staining at 1000X magnification. F: microscopic views of *H. capsulatum* after staining with lactophenol blue at 600X magnification.

4.3.2. Antifungal resistance

Only human isolates were studied for their antifungal susceptibility profiles. All isolates grew for minimum inhibitory concentrations (MIC) determination after 7 to 10 days and Table 2 summarises the MICs for five antifungal drugs for the yeast phase of the isolates. The yeast form of the isolates was inhibited by amphotericin b, itraconazole and fluconazole. Currently, there are no breakpoints for *H. capsulatum* therefore results are presented in terms of MIC50 and MIC90 as shown in table 2 below.

Table 2: In vitro antifungal susceptibility profiles of 16 *H. capsulatum* isolates.

	Susceptibility data (µg/ml) for:				
	Amphotericin B	Itraconazole	Fluconazole	Flucytosine	Micafungin
Isolate	MIC	MIC	MIC	MIC	MIC
SA4	<0.03	<0.015	0.25	>64	1
SA15	<0.03	<0.015	0.125	>64	1
SA19	<0.03	<0.015	0.5	>64	1
SA46	<0.03	0.0625	0.5	>64	1
SA47	<0.03	0.0625	0.5	>64	1
SA297	<0.03	0.0625	1	>64	1
SA302	<0.03	<0.015	0.5	>64	2
SA355	<0.03	0.0625	0.5	>64	1
SA562	<0.03	<0.015	0.25	>64	1
SA602	<0.03	<0.015	0.5	>64	1
SA811	<0.03	0.0625	0.5	>64	1
SA1356	<0.03	0.0625	0.5	>64	1
SA1371	<0.03	0.0625	0.5	>64	1
SA1436	0.0625	0.03	0.5	>64	8
SA1556	<0.03	0.03	0.25	>64	8
SA1704	<0.03	0.03	0.25	>64	2
MIC_{50}	<0.03	0.03	0.5	>64	1
MIC_{90}	<0.03	0.0625	0.5	>64	2

4.3.2. Quantitative analysis of demographic case data from South African diagnostic pathology laboratories in the public and private sectors.

Three of the archived isolates had no clinical data and a summary of clinical characteristics for the remaining 13 human isolates and 2 horse isolates are shown in Table 3. Seven out of the 13 human patients were females (54%). The median age of human patients was 36 years. Isolates were cultured from skin or skin punch biopsy n=8, unspecified tissue n=4, blood n=1.

Table 3: Clinical characteristics of South African human patients (n=13) and horses (n=2) with confirmed histoplasmosis (1979-2021).

Source	Year	Specimen ID	Gender	Age	HIV status	CD4 count	Specimen type
Human (n=13)	2010	SA46	Female	17	Unknown	Unknown	Skin punch biopsy
	2010	SA47	Male	30	Unknown	Unknown	Skin punch biopsy
	2013	SA297	Female	27	+	171	Skin punch biopsy
	2013	SA302	Female	28	+	67	Tissue
	2014	SA355	Male	46	+	194	Skin
	2014	SA562	Male	41	+	41	Skin punch biopsy
	2015	SA602	Male	40	+	Unknown	Tissue
	2015	SA811	Male	39	+	13	Tissue
	2018	SA1356	Male	42	+	Unknown	Skin
	2019	SA1371	Female	37	+	47	Skin
	2019	SA1436	Female	43	+	Unknown	Blood
	2020	SA1556	Female	39	+	Unknown	Tissue
	2021	SA1704	Female	38	+	8	Skin punch biopsy
Horse (n=2)	2019	SA19VMG	-	-	-	-	Swab/Pus aspirate
	2020	SA20VMK	-	-	-	-	Swab

4.3.2. Genomic analysis of African *Histoplasma* isolates

4.3.2.1 Africa harbors a well-differentiated lineage of *Histoplasma*

First, we explored the genetic diversity of *Histoplasma* in the African continent using phylogenomic approaches. All reads from this study were deposited in SRA under accession number PRJNA1109714. We generated a maximum likelihood phylogenetic tree to study the phylogenetic relationships between African *Histoplasma* and isolates from the rest of the world using whole genome sequences. The resulting phylogenetic tree, when we rooted with

17 genomes from the family Ajellomycetaceae as outgroups, shows that all the isolates from African formed a monophyletic group that represented both the Africa species described in Sepulveda et al. 2017, and the lineage previously known as *H. var. duboisii* (sometimes referred to simply as *H. duboisii*). This group is closely related to *H. capsulatum sensu stricto* as seen on the phylogenetic tree and, consistent with previous studies, belongs to the global clade of *Histoplasma* (Figure 2A). There was no differentiation between the variety duboisii and variety *capsulatum* from African patients. The two horse strains from South Africa did not cluster together with the clinical African *Histoplasma* strains. Instead, they grouped together with the *H. suramericanum* group. An ASTRAL tree which compiles the signal from gene genealogies generated from genomic windows also shows the African clade has a high concordance factor (77%) which suggests that a large portion of the genome shows the group as a monophyletic group. This level of concordance is expected in cases in which there is advanced divergence, and thus little admixture or incomplete lineage sorting. This clade is highly supported both by bootstrap and concordance factors based on BUSCO genes (Figure 2B).

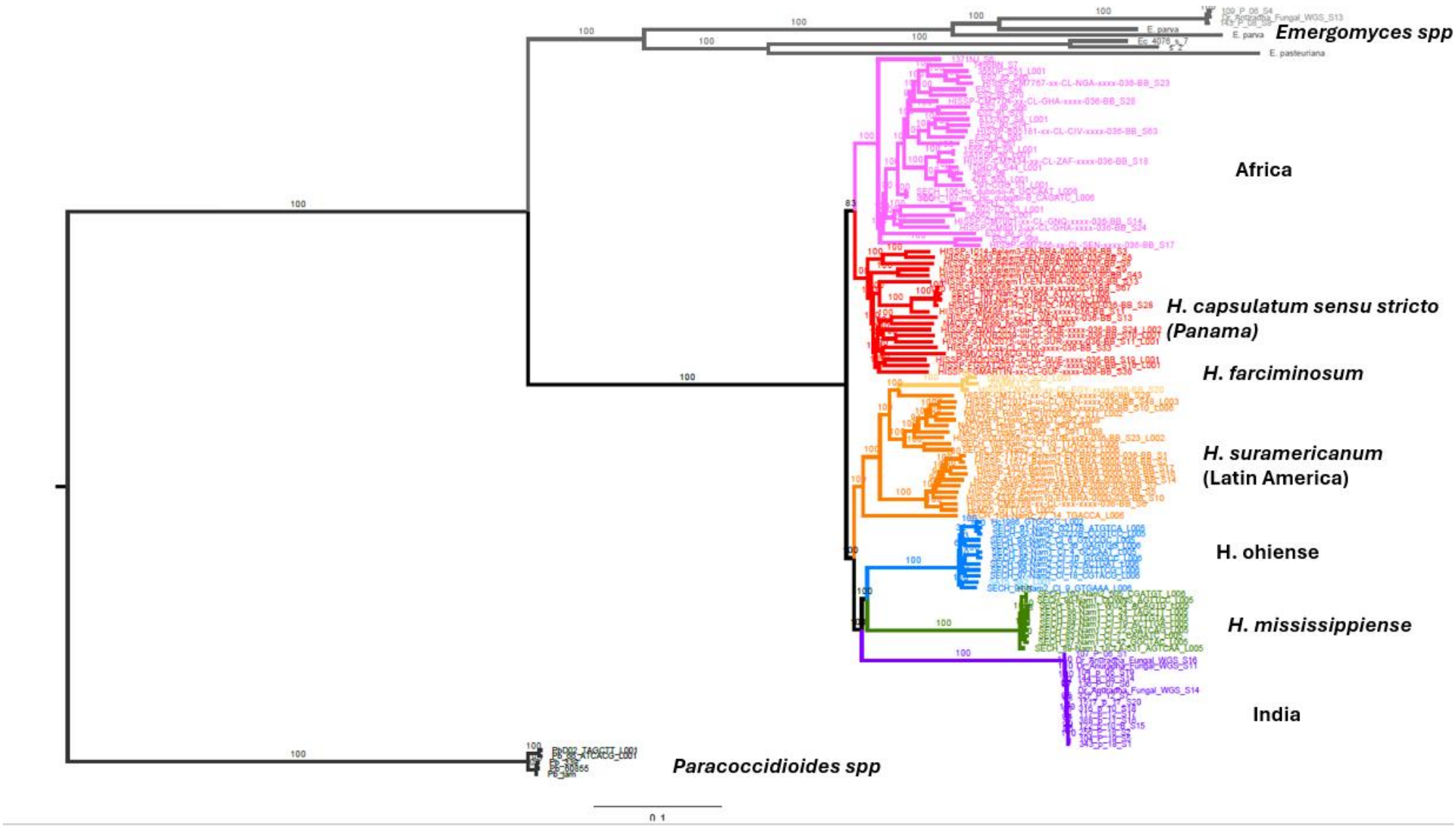

Emergomyces spp
E. parva
E. parva
E. pasteuriana
Africa
H. capsulatum sensu stricto
(Panama)
H. farciminosum
H. suramericanum
(Latin America)
H. ohiense
H. mississippiense
India
Paracoccidioides spp
0.1

Figure 2A: Neighbour joining phylogenetic tree constructed using IQ TREE software using single nucleotide polymorphisms from whole genome sequencing data n=129. *Histoplasma* strains from the African continent shown in pink were analysed compared to other global *Histoplasma* strains and closely related sequences were included for tree rooting. South African horse strains are coloured in yellow and circled in black clustered within the *H. suramericanum* group. Visualisation and annotation were done using iTol software.

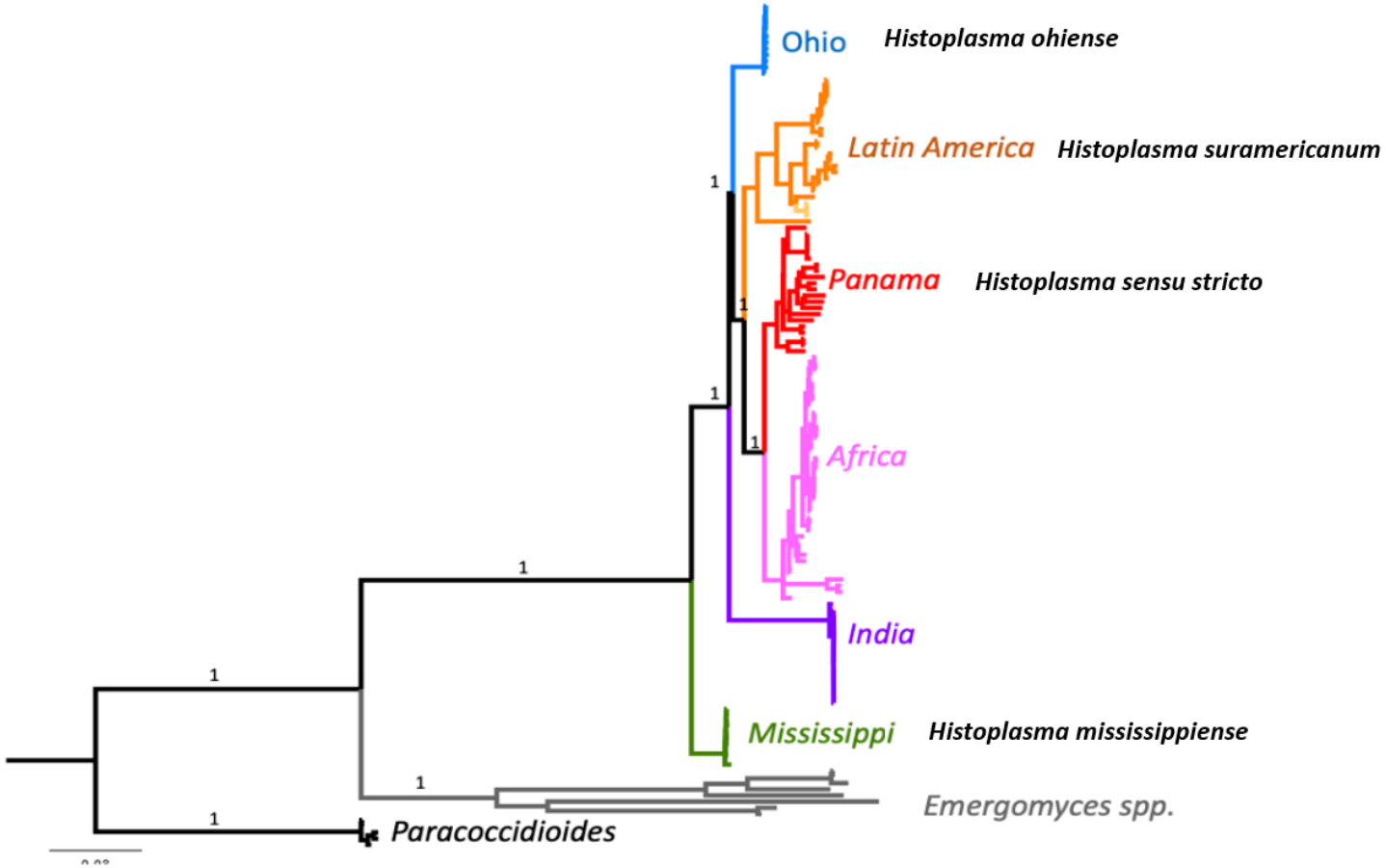
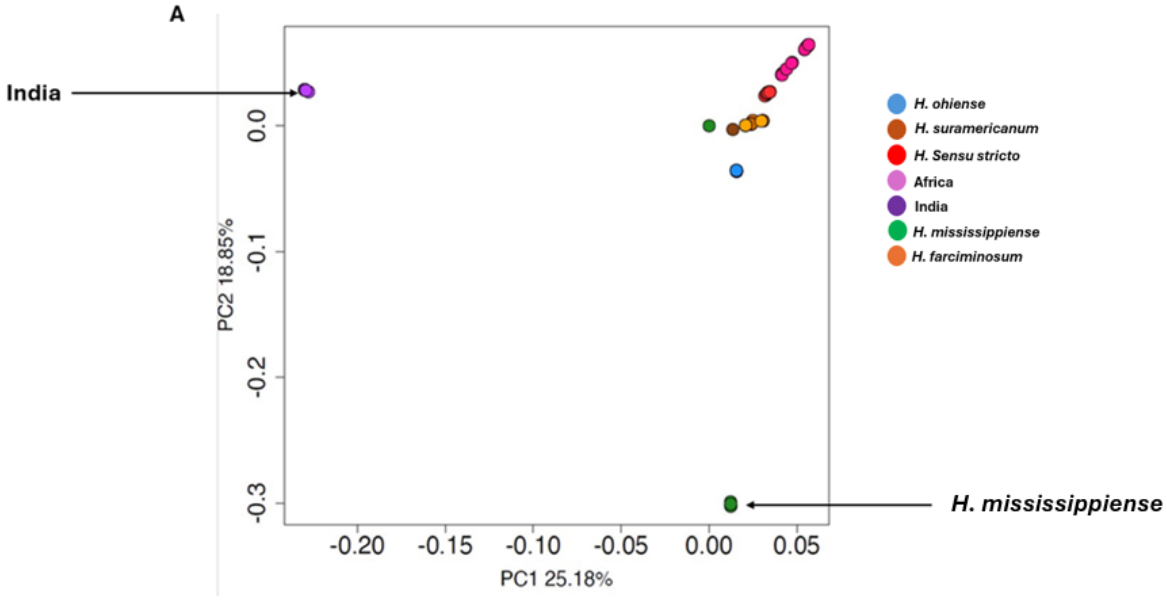


Figure 2B: ASTRAL trees with quadripartition support showing genomic concordance analysis measured using genetic windows.

4.3.2.2 Principal component analysis

Population-genetics based approaches also revealed that the African samples are genetically distinct. First, a PCA confirmed the existence of discrete genetic clusters in *Histoplasma*. *Histoplasma mississippiense* appears as the most differentiated clade in PC1, and the Indian lineage is the most differentiated clade in PC2 (Figure 3A). All the isolates from the African lineage cluster together in PC1 and PC2 and are differentiated from the other lineages of *Histoplasma* (Figure 3). The second figure 3B shows the group *H. ohiense* as the most differentiated from all other groups in PC3. The PCA analysis clearly differentiate the genetic populations as previously described without any genetic overlap.



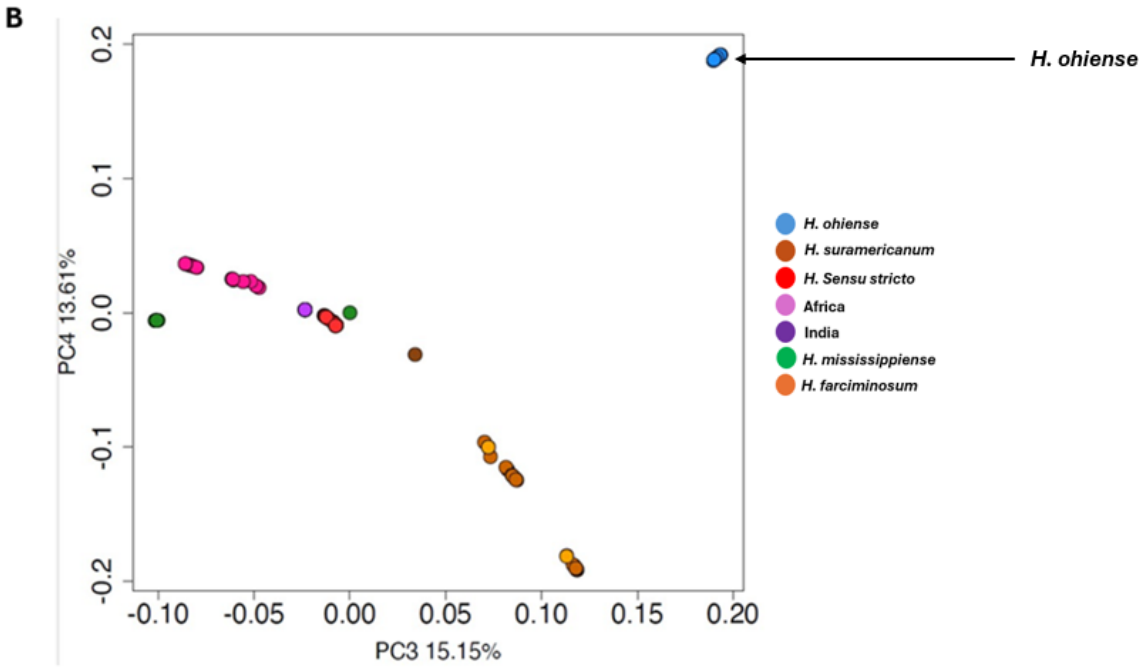


Figure 3: Principal component analysis (PCA) showing genetic variation assigned to different populations of *Histoplasma* isolates. PC1 and PC2 (A)in their own panel and PC3 and PC4 (B)in the second panel indicate the allelic frequencies in the different populations. Corresponding colours for populations are purple for India, Pink for Africa, green for Mississippiense, blue for Ohiense, brown for Suramericanum and yellow for Farciminosum sequences.

4.3.2.3. Genetic differentiation

Second, the study quantified the extent of genetic divergence (Dxy) between all pairs of *Histoplasma* lineages. The African lineage shows a high degree of differentiation from all other lineages (Figure 4A). We also calculated the extent of genetic diversity (π (pi)) within each lineage. π (pi) is a measure of nucleotide diversity, representing the average number of nucleotide differences per site between two randomly selected DNA sequences in a population. It is commonly used to quantify genetic variation within a species or population. Unlike all previous studies that sampled a limited number of isolates and inferred low polymorphism (N=2, pi=0.0213(20)), we find the existence of extensive genetic variation within the group (pi=0.05, CI=95%, Figure 4B) which is comparable with the other species of

Histoplasma. This result, in turn, suggests that the previous assessments were limited by their sample size.

Next, we compared the magnitude of interlineage divergence with the magnitude of genetic variance within lineages. Instances of advanced speciation show larger Dxy than pi. Indeed, the African lineage shows a much larger Dxy than pi (Figures 4B and 4D). These metrics of genetic differentiation and diversity indicate that the African lineage is not only a monophyletic clade but also that it shows advanced differentiation from other lineages of *Histoplasma*. This group deserves a species designation based on the evidence from the analysis.

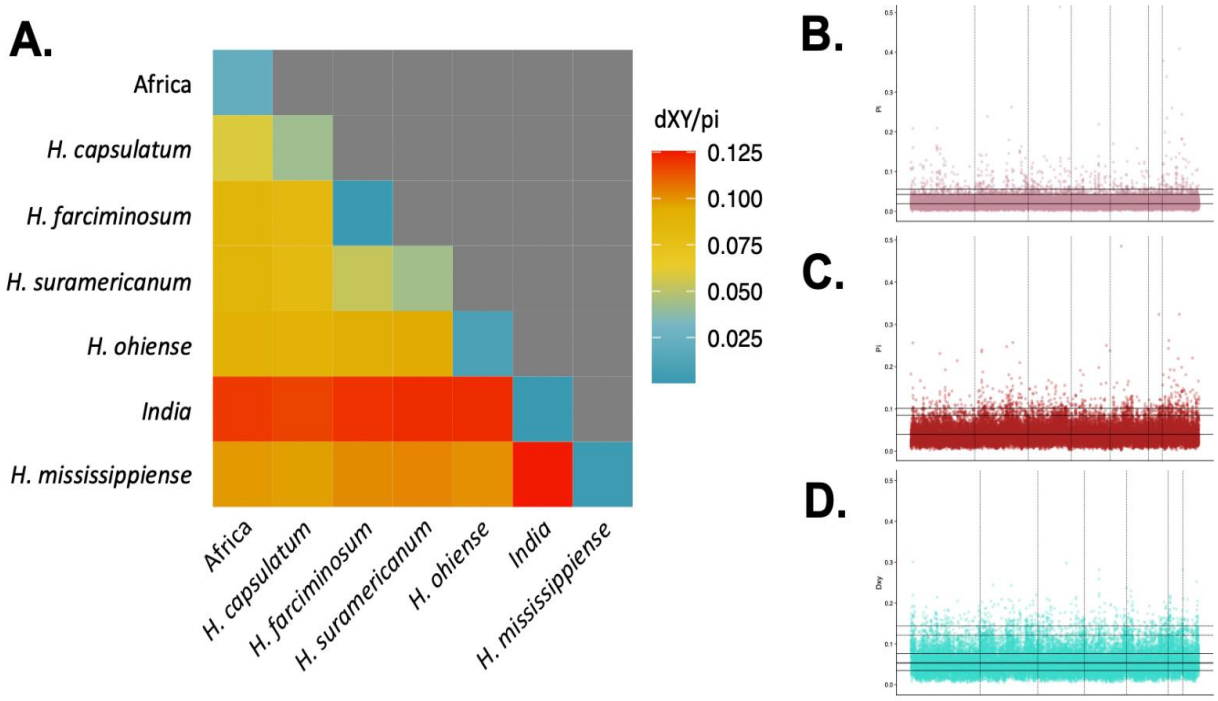


Figure 4: Genetic variation and differentiation between *Histoplasma* species. A. Nucleotide diversity analysis of seven *Histoplasma species*. B. π in African *Histoplasma* along the genome. C. π in *Histoplasma capsulatum sensu stricto*, the sister species of the African lineage along the

genome. D. Dxy between Africa and *H. capsulatum sensu stricto* shows extensive genome wide differentiation between the two clades.

4.3.2.4. Gene exchange between *Histoplasma* isolates

Finally, we studied whether the African lineage exchanged alleles with other closely related species of *Histoplasma*. f-branch, a derivative of the D-statistics, revealed evidence for gene exchange at low levels. Distinct species show little to no gene flow between populations. Low levels of gene exchange were observed between all populations (>2%) (Figure 5). African strains showed the highest allele sharing with *H. capsulatum mississippiense* strains. Slightly higher allele sharing was also observed between the *H. farciminosum* group and *H. capsulatum mississippiense*. The observed rates of gene exchange are too low, and this shows evidence of true species in the African population of clinical isolates, which can be separated from all the other strains supporting that African isolates form a distinct population. Note that F-branch is only effective at detecting evidence of gene exchange but does not precisely time the events of

introgression and thus is unable to precisely pinpoint the species involved in the admixture events.

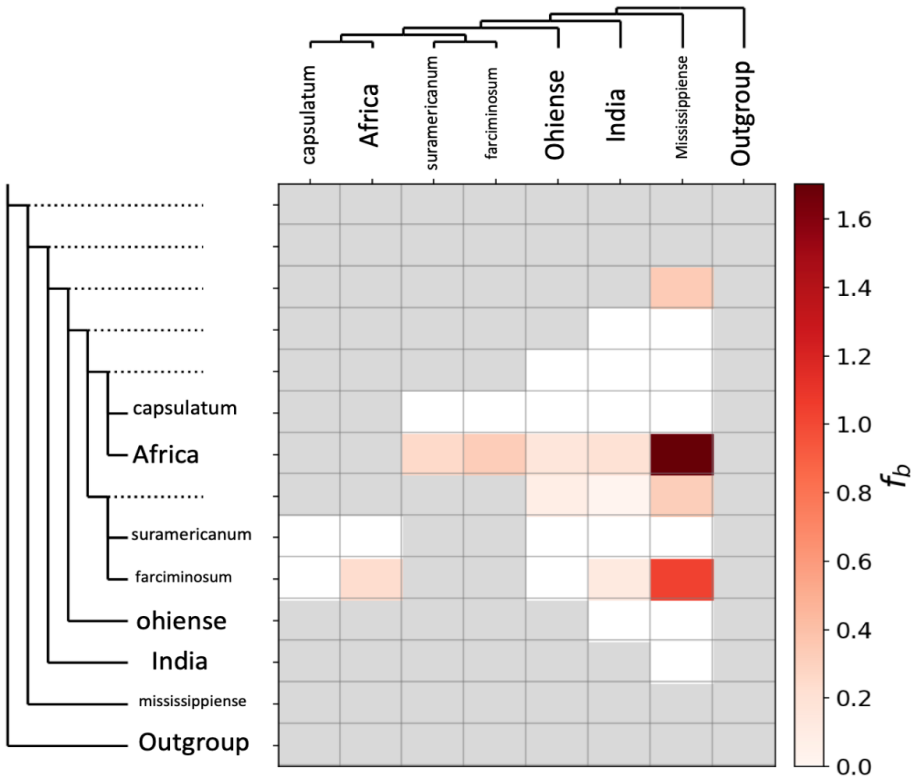


Figure 5: D-branch statistics. Low levels of gene exchange between Africa and other lineages of *Histoplasma* between 0 and 2%.

4.4. Discussion

The results from the analysis of the phenotypic characteristics of *Histoplasma* isolates from South African patients confirmed previously reported white, fluffy mould that turned cream to brown with age on SabDex agar and wrinkled, moist and heaped yeast-like colonies on BHI agar and gram staining revealed round, oval budding yeast cells (24). Low MIC values were recorded for amphotericin B, itraconazole and fluconazole. Micafungin had intermediate MIC values and Flucytosine showed high MIC values. The molecular characterisation of African isolates resulted in all clinical isolates from Africa forming one group distinct from all other previously described groups. The new monophyletic group formed by African isolates has enough evidence to be a novel species with a proposed name *Histoplasma africanus sp. nov*. The African group included

H. capsulatum and *H. duboisii* isolates and there was no genetic differentiation between them. The isolates from horses clustered together with the *H. suramericanum* group.

Antifungal susceptibility testing was performed on South African clinical isolates and low MIC values were recorded for amphotericin B and itraconazole as previously indicated in other studies. Intermediate MIC values were reported for fluconazole and micafungin and high MIC values were observed against flucytosine. We employed the yeast phase of the fungus to test for antifungal susceptibility of drugs that are mostly used for the treatment of histoplasmosis. The yeast phase of *Histoplasma* was utilised for antifungal susceptibility studies because it poses a lesser threat to laboratory infections, and this is the phase that causes human infection. Goughenour *et al*, 2015 optimised a standardised quantitative microplate-based growth assay for the determination of antifungal susceptibility of *Histoplasma capsulatum* yeasts (54). A study of 23 *Histoplasma* isolates by Kathuria et al, 2014 revealed good essential agreement with the MICs of both the mycelial and yeast forms, which were 87 to 100% for azoles, 100% for amphotericin B, and 96% for caspofungin (55). In a 2000 study by Kohler et al, antifungal susceptibility testing of 20 *Histoplasma* isolates compared caspofungin to amphotericin B. The mean MIC for caspofungin was 16.6 µg/ml (range: 8–32 µg/ml), while for amphotericin B, it was 0.56 µg/ml (range: 0.5–1.0 µg/ml) (56). We tested the isolates against a lower range of amphotericin B concentrations (0.03 to 0.5 µg/ml) and found a lower median MIC of <0.03 µg/ml. The median micafungin MIC of the isolates was also lower: 1 µg/. We observed high MIC values for flucytosine in *Histoplasma* isolates in this study, which is similar to what has been previously reported by Kathuria et al, 2014 (55). Low fluconazole MICs were reported in this study but treatment of HIV/AIDS patients with histoplasmosis with fluconazole failed in 10 out of 17 patients in a study by Wheat et al 2001, showing a 4 fold increase in MIC values from baseline (57).

Genomics has revolutionised our understanding of biological diversity, including for fungal pathogens. We utilised pathogen genomics to understand the speciation of African *Histoplasma* isolates in comparison with other global isolates. *Hcc* is the more common of the two species and is found worldwide, whereas *Hcd* is restricted to Africa (11,14,58). *Hcd* causes a form of

histoplasmosis known as African histoplasmosis, which is characterised by extensive tissue destruction and the formation of large granulomas (12). These differences in clinical manifestations observed in patients with African histoplasmosis might be associated with evolution of various virulence strategies in *Hcd* strains. The genus *Histoplasma* needed clarity especially for African isolates.

Previous molecular studies of geographically diverse *Histoplasma* isolates utilising mostly four protein-coding regions namely *ARF, H-ANTI, OLE* and *TUB1* led to unresolved relationships in the genus. Proposed phylogenetic species also needed increased taxon sampling and use of different phylogenetic and population genetics methods to resolve inconsistencies in the *Histoplasma* genus. The analysis by Sepulveda *et al,* in 2017 using whole genome sequencing data led to the proposal of four new *Histoplasma* species and the 2 African isolates formed their own distinct group (20). This study provided a blueprint for phylogenomic species identification using tools that are highly discriminatory within a hypothesis-testing framework. Typically, phylogenetic species recognition studies generally compare differences in nucleotide polymorphisms at specified regions which are usually less than 10 and monophyletic groups are supported by the concordance of these differences across genealogies inferred from different regions across the genome. The study by Sepulveda and others provided further analysis that can be done to support speciation and these included breaking down genomes into smaller partitions then accessing the relations in these partitions to see if they maintain the genetic relations shown by whole genome phylogeny. The genomic partitions studies we performed in this analysis reflected concordance in the genomes and the proposed species boundaries with the India, Mississippi, Latin America and Ohio populations (22). Genealogical concordance has been widely used as a principal criterion for delineating fungal species as described by Steenkamp and others in 2017 (60). The results from this study show that all human African isolates formed a distinctive population that is highly differentiated from all other populations.

Despite which part of the African continent the strains were collected from, all African sequences clustered together. There was no differentiation between the variety *duboisii* and variety *capsulatum* from African patients despite differences in diseases manifestations reported

in patients infected with these different isolates. Structure analysis showed close relations between the African population to the *Histoplasma* species sensu stricto although they did not form one population. The formation of a distinct *Histoplasma* population was also reported in Indian strains using the same population structure analysis methodology employed in this study published by Jofre et al, 2022 (22). Finding a single genetically distinct population on the African continent answers the question of whether two genetically different species (*Hcc and Hcd*) exist in Africa causing different disease manifestations. Overall, *Hcc* and *Hcd* isolates form one population with similar genetic characteristics.

The two horse strains from South Africa did not cluster together with the clinical African *Histoplasma* strains. Instead, they grouped together with the *H. suramericanum* group. Similar genetic relations were reported on 6 isolates from Ethiopia classified using 4 housekeeping genes and they clustered together with the Latin American (Lam A) *suramericanum* group (59). Whole genome analysis of more isolates is recommended to confirm these relations. The co-existence of all three varieties on the African continent warrants further genomic analysis of all three varieties to further understand these observations.

To further understand if different populations observed in the phylogenetic analysis of the sequences, we employed more methods to check if the populations were true species by measuring the amount of gene flow between the populations. Clearly distinct populations are supported by the lack of gene flow between true species (20,22). The homogenising effect of gene flow among species can lead to population evolving towards forming one species in future. Only a small fraction of gene flow between divergent species is expected and it should not be enough to break concordance among chromosomal genealogies. This pattern was observed in Indian strain studies by Jofre et al and it remains true in African strains as well. Low allele sharing was observed among all populations that is as expected in true species (60). Ohio and Latin America strains have the highest amount of allele sharing between populations in this study. Overall, allele sharing between populations was low (< 2%) and is comparable to the less than 5% introgression reported by Sepulveda et al in 2017 (20). Statistical analysis of the genetic differentiation of the *Histoplasma* populations showed that Mississippi, Ohio and India strains

are highly differentiated, and Africa and Panama strains are most similar. The Indian group has the highest genomic differentiation from the rest of the populations. The Mississippi, and Ohio groups share endemic geographical regions, yet are highly differentiated. Our study lacked the study of virulence factors from the whole genome sequencing data and such studies have not been documented specifically in the African isolates. It is important to study virulence factors as they often highlight selection pressure in important regions during natural selection though this was out of the scope of our study (24).

We have demonstrated that the phenotypic characteristics, phylogeny and genetic differentiation observed within the African population and in comparison, to other global isolates support the proposal of a novel species for African isolates, therefore we propose a new species *Histoplasma africanus sp nov* that has evolved separately from the previously described species.

In conclusion, the use of whole genome sequencing data has allowed a better understanding of the *Histoplasma* genus as more characterisations can be done using standardised methods that fulfil the classification of species in global isolates. Genomic analysis and speciation are important to understand the different disease variations observed in histoplasmosis. This study showed that enough genomic differentiation exists between the Panama group and Africa group to declare them separate species leading to the proposal of a novel species *Histoplasma africanus sp nov*. The evidence from this study refuses claims that *Hcd* and *Hcc* are genetically different, and the African group deserves a species designation. The evidence shown indicates independently evolving lineages that may warrant the development of species-specific diagnostic methods and treatment methods. Since *Histoplasma* has been understudied mostly on the African continent, efforts should be increased to characterise more pathogens from the continent. Sequencing whole genomes is becoming more affordable. Therefore, there is need for continued worldwide assessment of the genomic differentiation of this fungal pathogen.

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CHAPTER 5: LABORATORY VALIDATION OF *HISTOPLASMA* ANTIGEN AND MOLECULAR ASSAYS AND EVALUATION IN STORED SAMPLES FROM ADULTS WITH ADVANCED HIV DISEASE

5.1. Introduction

Histoplasma species were set down as high priority in the World Health Organization's (WHO) 2022 Fungal Priority Pathogen List (1). The WHO recognises histoplasmosis as a major global health problem owing to the diagnostic challenges and high mortality associated with this condition, particularly among people living with HIV; this requires renewed research and public health focus (2). Diagnosis of HIV-associated disseminated histoplasmosis is difficult because the clinical syndrome lacks specificity (with similarities to tuberculosis, for example) and multiple co-morbid conditions in people with advanced HIV disease may mask histoplasmosis. There are also limitations inherent to the currently available reference diagnostics, i.e. culture and histopathology, including a slow turn-around time and a requirement to obtain tissue samples for testing. The histoplasmosis diagnostic landscape has greatly improved since the introduction of rapid antigen assays (3–7). Relatively easy-to-obtain samples such as urine or blood can be used. The inclusion of *Histoplasma* antigen assays in the WHO essential diagnostics list in 2019 supports the drive that, by 2025, all Latin American countries should at least have antigen (or molecular assays) available (8). However, antigen assays are not currently available in most African countries because of inadequate demand for testing, inadequate laboratory infrastructure and lack of skilled personnel to perform complex assays and a lack of access to cheap, simple and accurate assays (9). Misdiagnosis and under-diagnosis are common in cases of histoplasmosis and there is a need to address this gap (10) (11). UNITAID funding (2006-2023) catalysed increased affordability for high-quality diagnostic tests such as the cryptococcal antigen (CrAg), lateral flow assay (LFA) for patients with advanced HIV disease in low-income countries; this needs to be expanded to histoplasmosis (12).

The burden of histoplasmosis in Africa is mostly unknown, owing partly to a lack of diagnostic assays. Most global reports on the disease are from the Americas. Only 470 cases of

histoplasmosis were reported in the literature from Africa over six decades (1952 – 2017), with people living with HIV accounting for 38% of the cases (11). In 2022, Ocansey *et al*. reported a steady increase in histoplasmosis cases on the African continent probably attributable to increased diagnosis (13). *Emergomyces africanus*, which is closely related to *Histoplasma capsulatum*, causes an indistinguishable clinical syndrome of disseminated disease in people living with HIV in southern Africa (14).

In South Africa, the National Institute for Communicable Diseases (NICD) offers antigen testing for patients with suspected histoplasmosis using a US Food and Drug Administration-approved enzyme immunoassay (EIA) (Clarus, IMMY, Norman, OK, USA); this assay has been reported to cross-react with *E. africanus* antigen (15). However, an LFA would provide more rapid results in a simpler format, requiring no specialised equipment and a long shelf life which is suitable for near-point-of-care testing in resource-limited settings. Molecular assays for histoplasmosis diagnosis have also been developed, though in-house assays lack standardisation and may cross-react with other dimorphic fungi (16–21). We aimed to validate and then evaluate a commercial LFA (versus an EIA) to detect *Histoplasma* antigen in urine samples. We also aimed to validate and then use a pandimorphic reverse-transcriptase quantitative polymerase chain reaction (RT-qPCR) assay, designed to target the mitochondrial small subunit (*mtSSU*) ribosomal gene of both *H. capsulatum* and *E. africanus,* to investigate the prevalence of histoplasmosis or emergomycosis among people living with HIV.

5.2. Materials and Methods

5.2.1. *Histoplasma* antigen enzyme immunoassay

Method: Urine samples submitted to the NICD from South African hospitals from patients with culture-confirmed endemic mycoses infections were used in this validation. The urine samples n = (201) were tested in duplicate using the Clarus EIA following the manufacturer's instructions. Detailed methods for the standard curve procedure have been previously described by Maphanga *et al* 2020 (15). Briefly, urine samples, which had been stored at -70°C, were thawed to room temperature before adding 100 µl to a plate with microtiter plate wells coated with capture anti-

Histoplasma monoclonal IgG antibodies. Plates were incubated at 37°C for 60 minutes. Horseradish peroxidase (HRP)-conjugated anti-*Histoplasma* monoclonal IgG secondary antibodies were added to enable detection, with incubation for another 45 minutes at 25°C. Plate washing was performed, 3,3,5,5-tetramethylbenzidine was added, followed by incubation for 30 minutes at 25°C. Finally, a stop solution was added before measuring the optical density of the reaction using a BioTek microplate reader (Analytical and Diagnostic Products, Randburg, South Africa) at a wavelength of 450 nm for the samples and 620 nm for the reference. A quantitative value was calculated from a standard curve equation, with a cut-off value of >0.2 ng/ml considered positive as recommended by the manufacturer.

Validation: The validation of this assay was previously described by Maphanga et al, 2020 (15).

5.2.2. *Histoplasma* lateral flow assay

Method: Urine samples submitted to the NICD from South African hospitals from patients with culture-confirmed endemic mycoses were infections used in this validation. The urine samples are kept frozen at -70 °C and they were vortexed after being thawed to room temperature. Then, 100 µl of urine was added to the sample reservoir of the *Histoplasma* antigen LFA device (Optimum Imaging Diagnostics [OIDx], Scarborough, ME, USA) and allowed to flow at room temperature for 15 minutes. Results were interpreted visually. A control line alone indicated a negative result, but a test and control line together indicated a positive result. No lines or only a test line was considered an invalid result. Results were read by two different individuals at separate times and both were blinded to each other's results.

Validation: The analytical performance of the LFA was assessed using 23 urine samples from patients with culture-confirmed fungal disease. The urine samples were from patients known to be infected with *E. africanus* (n=11), *H. capsulatum* (n=6), *Sporothrix schenckii* (n=4), *Acremonium* spp (n=1) and *Cryptococcus neoformans* (n=1). LFA results were read by two individuals. Results were also compared to EIA results on the same cohort of patients.

5.2.3. Pandimorphic reverse transcriptase quantitative polymerase chain reaction assay

Method: Nucleic acid extraction was performed following a method previously described (20). Whole nucleic acids were extracted from 1300 µl of whole blood, urine, serum or plasma, by adding the clinical specimen and 10 µl of an RNA virus internal control (DNA Virus Culture, 131 DICD-CY-L100, Diagenode, Seraing, Belgium) to a 2 ml tube containing glass fibre beads and vortexing for 2 minutes. The source of the samples was described in the cohorts included below. After bead beating, the sample was transferred to a new tube and centrifuged for 5 min at 10,000 x g. Whole nucleic acids were extracted from samples using High Pure viral nucleic acid kit (Roche Molecular Systems, Branchburg, United States) following the manufacturer's instructions. Nucleic acids were eluted in 35 µL of an elution buffer. All extracts were used for further analysis on the same day to avoid degradation of nucleic acids. The qPCR assay was performed with a probe and primers targeting the *mtSSU* gene of *H. capsulatum* and *E. africanus* using the LightCycler 480 Instrument II (Roche Molecular Systems, Branchburg, United States) and following the manufacturer's instructions (22). Amplification was performed in a 25 µl volume containing: 5 µl TaqMan Fast Virus 1-step master mix 1X (Thermo Fisher Scientific, Waltham, Massachusetts, USA), 0.1 µM Taqman probe 79P Pan (FAM-BBQ) for the *mtSSU*, 0.3 µM of 128F pan forward and 0.3 µM of 55R *Emergomyces* & 55R *Histoplasma mtSSU* reverse primers, 2.5 µl of mix primer Diagenode, 8 µl of the nucleic acid template and 8.625 of nuclease-free water. We used a Cq value of ≤45 as the cut-off for a positive assay.

Validation: We applied the assay to 268 DNA samples extracted from clinical isolates using the Zymo ZR fungal/bacterial DNA miniprep kit (Zymo Research Corp., Irvine, USA) following the manufacturer's instructions. Sanger sequencing was performed to confirm the identity of the samples using NCBI Blast tool. To determine the analytical sensitivity, we used 75 DNA samples extracted from clinical isolates of *E. africanus* (n=60), *E. pasteurianus* (n=2) and *H. capsulatum* (n=12) and *H. farciminosum* (n=1). The analytical specificity was determined using 193 fungal pathogens listed in Table 3. This list included 18 closely-related thermally-dimorphic fungi [(*Blastomyces dermatitidis* (n=1), *B. emzantsi* (n=7), *B. percursus* (n=10)], 5 non-related thermally-dimorphic fungal pathogens [(*S. schenckii* (n=4), *Talaromyces marneffei* (n=1)] and 126 different moulds and 44 yeasts. We also evaluated the performance of the pandimorphic RT-qPCR assay in detecting *Histoplasma/ Emergomyces* nucleic acids from a total of 47 clinical specimens including: 17 prospectively-collected fresh clinical specimens [tissue in saline (n=10),

bone marrow (n=2), blood (n=2), urine (n=1), serum (n=1), formalin-fixed paraffin embedded tissue (n=1)] and 30 frozen/thawed urine samples obtained from South African patients with suspected invasive fungal disease. The clinical cases were classified into three groups: proven (n=27), defined as confirmed histoplasmosis or emergomycosis by culture; probable (n=11), defined as a compatible histopathological appearance; and possible (n=9), defined as compatible clinical findings. The limit of detection was determined using 1:10 serial dilutions of 0.1 ng/µl *H. capsulatum* and *E. africanus* DNA. To simulate the detection of the pathogen from clinical samples, we spiked urine and whole blood from healthy volunteers with *E. africanus* and *H. capsulatum* yeast-phase isolates. Serial 1:10 dilutions from 1.5 X 10^3 were performed and the mixture was used to spike the samples to determine the limit of detection. Experiments were performed in triplicate.

5.2.4. Evaluation in Clinical Cohorts

Cohort 1: Urine samples were collected from 201 South African participants enrolled on a cohort study nested within a cryptococcal antigen (CrAg) screening programme between 2012 and 2016 (23). Patients were routinely tested for cryptococcal antigenaemia if their CD4 cell count was ≤100 cells/µl. We included 194 stored urine samples from this cohort; 67 participants had CrAg-positive blood results and 127 had CrAg-negative results, and no stored urine was available for the other 7 patients in the cohort. Baseline demographic and laboratory test data and six-month outcomes were available for this cohort. The urine samples collected at baseline were stored at -70°C, then batch-thawed and tested using the IMMY EIA. The same urine samples were later retrieved from -70°C storage in 2021, thawed and tested using the OIDx *Histoplasma* antigen LFA.

Cohort 2: 533 remnant blood samples from patients with a CD4 cell count of ≤100 cells /µl were tested with a pandimorphic RT-qPCR assay. These samples were randomly selected from 2700 blood samples from the national reflex CrAg screening programme submitted to the NICD for CrAg re-testing between April and July 2021. All these patients had CrAg-positive results. Only age and sex data were available for this patient group; we had no clinical or outcome data. Wake

et al, 2019 described 1 case of disseminated histoplasmosis among 67 CrAg-positive individuals from group 1 during 6 months of follow-up (23). Therefore, a prevalence for histoplasmosis of approximately 1.5% (0.5% to 2.5%) was estimated in cohort 2; with the precision of 0.005, we calculated a required sample size of 533.

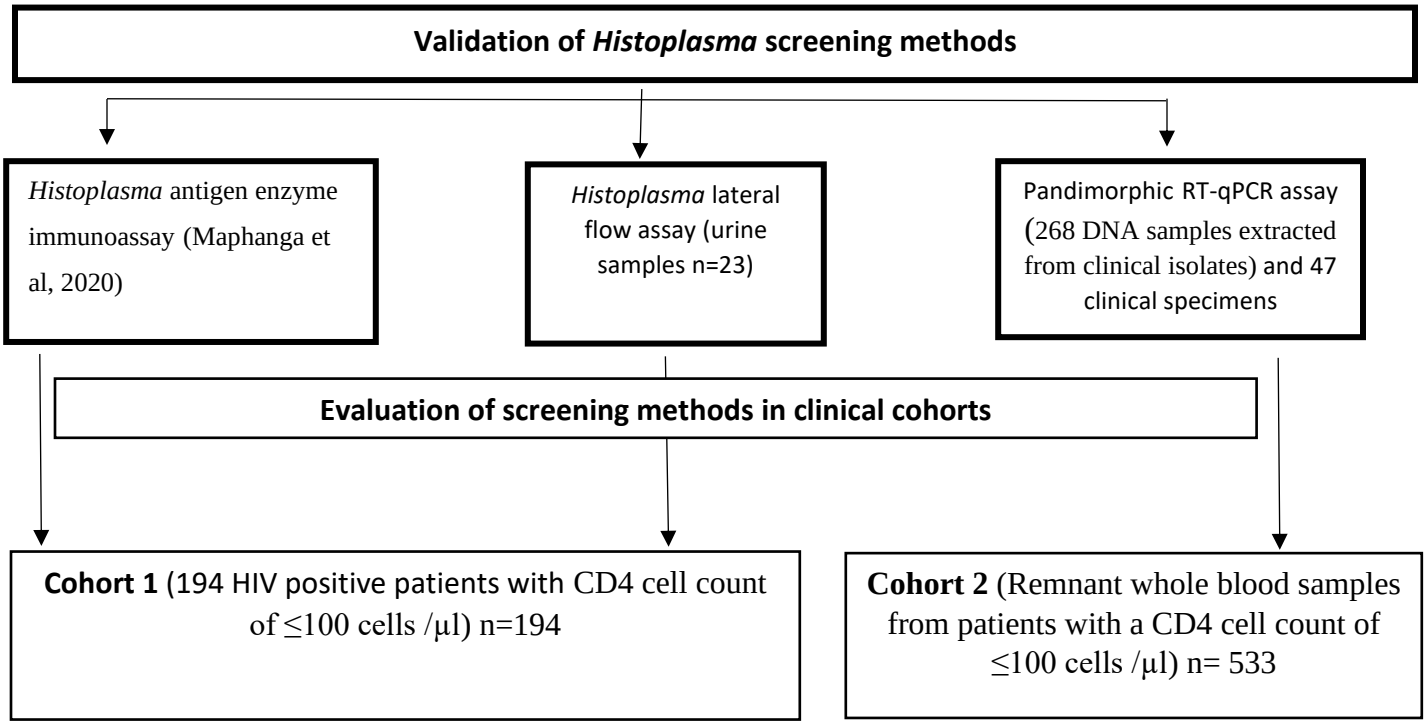

Figure 1. Flow chart showing summary of the methods followed in this study.

Ethics

The University of the Witwatersrand Human Research Ethics Committee (Medical) provided clearance for this study (M210109 and M201174).

5.3. Results

5.3.1. *Histoplasma* LFA validation: In the small laboratory validation of the LFA compared to the EIA and culture results, five of the 23 urine specimens tested LFA-positive (Figure 2). Four of the five LFA-positive urine samples were from patients with culture-confirmed histoplasmosis

while one had previously tested positive for cryptococcosis. The remaining 18 LFA-negative samples were from patients with confirmed emergomycosis (n=11), sporotrichosis (n=4), histoplasmosis (n=2) and *Acremonium* infection (n=1) (Figure 2). When the LFA was compared to a culture-confirmed diagnosis, the sensitivity and specificity for histoplasmosis were 67% (4/6, 95% CI 22-96%) and 94% (16/17, 95% CI 71-100%) (Table 1). When the LFA was compared to an EIA diagnosis, the sensitivity and specificity for histoplasmosis was 40% (4/10, 95% CI 12-74%) and 85% (11/13, 95% CI 55-98%) respectively.

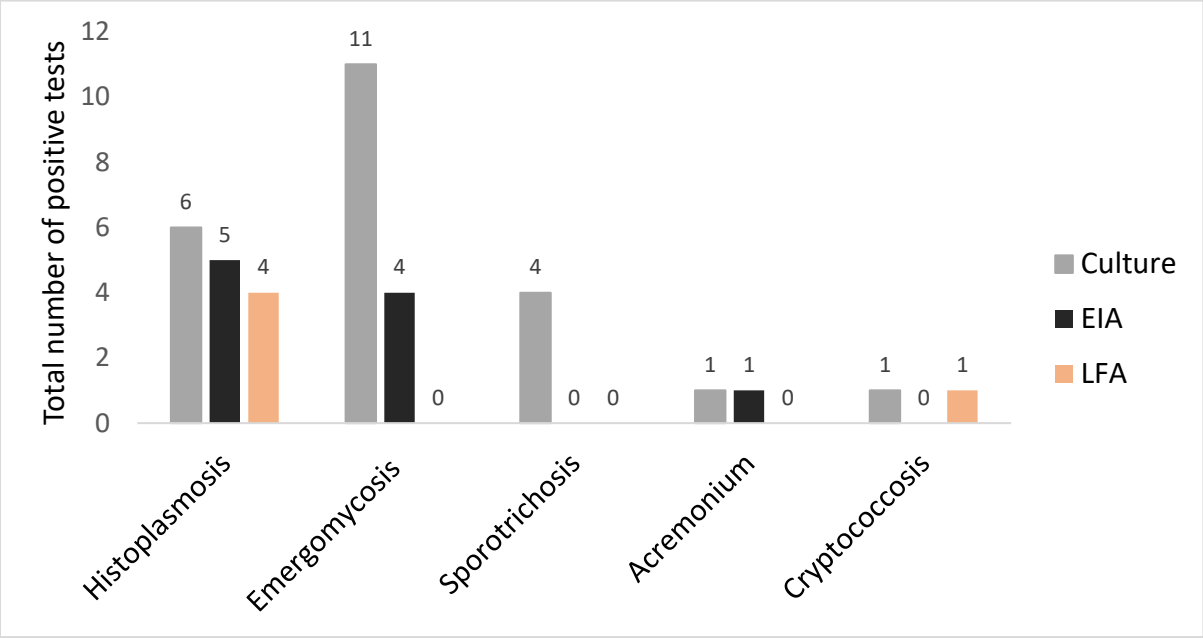

Figure 2: Results of testing urine samples from patients with suspected histoplasmosis for *Histoplasma* antigen using the LFA and EIA compared to culture results (n=23)**.**

Table 1: Summary of *Histoplasma* lateral flow assay (LFA) (OIDx) results compared to a culture-confirmed diagnosis or enzyme immunoassay (EIA) in a laboratory validation of urine samples (n=23).

	Culture-confirmed diagnosis of:	**Total**	***Histoplasma* EIA result:**	**Total**

		Histoplasmosis	Other fungal diseases*		Positive	Negative	
***Histoplasma* LFA**	Positive	4	1**	**5**	4	2	**6**
	Negative	2	16	**18**	6	11	**18**
Total		**6**	**17**	**23**	**10**	**13**	**23**

* Other fungal diseases included emergomycosis n=11, mycetoma n=1, sporotrichosis n=4, cryptococcosis n=1

**The 1 positive sample was a urine sample from a patient with culture-confirmed cryptococcosis.

5.3.2. Validation of the pandimorphic real-time quantitative PCR assay: The assay had an analytical sensitivity of 100% and an analytical specificity of 92% for *H. capsulatum /H. farciminosum* or *E. africanus/E. pasteurianus* when applied to DNA extracted from cultured fungal isolates (Table 2). Cross-reactivity was only noted with *B. emzantsi*, *B. dermatitidis* and *B. percursus* DNA (Supplementary Table 1). All *Cryptococcus* DNA samples were negative with the assay. When a genomic DNA concentration of 0.1 ng/µl was used, Cq values ranged from 23-25 for *B. emzantsi*, 28-30 for *B. dermatitidis*, and 26-28 for *B. percursus*.

Table 2: Sensitivity and specificity of pandimorphic reverse transcriptase–quantitative PCR assay using DNA samples from cultured fungal isolates (n=268).

	No. of DNA samples extracted from:		
Pandimorphic RT-qPCR assay	*Emergomyces* species/ *Histoplasma* species*	Other fungal pathogens	Total
Positive	75	18	**93**
Negative	0	175	**175**
Total	**75**	**193**	**268**
Sensitivity % (95% Cl)	100 (95-100)		
Specificity % (95% Cl)	91 (86-94)		

species:** ***Emergomyces africanus, Emergomyces pasteurianus, Histoplasma capsulatum, Histoplasma farciminosum

For the validation of the pandimorphic RT-qPCR assay in detecting *Histoplasma/ Emergomyces* in 47 clinical specimens, the pandimorphic RT-qPCR assay was positive in 19/47 (40.4%) samples (Table 3). Of the 27 proven cases of culture-proven histoplasmosis or emergomycosis, the pandimorphic RT-qPCR assay was positive for 27% (5/18) of urine specimens from those with emergomycosis and 22% (2/9) with histoplasmosis (Table 6). Of the 11 probable cases of histoplasmosis or emergomycosis diagnosed by histopathology, all had positive pandimorphic RT-qPCR results: urine (n=4), fresh tissue (n=3), whole blood (n=1), serum (n=1), formalin-fixed paraffin-embedded tissue (n=1) and bone marrow aspirate (n=1). A single patient with probable histoplasmosis or emergomycosis had whole blood, serum and urine samples, all of which were positive with the pandimorphic RT-qPCR assay. Nine patients had possible histoplasmosis or emergomycosis with clinically reported widespread skin lesions. Only 1 out of the 9 possible cases had a positive pandimorphic RT-qPCR with Cq value of 39.04 (Table 6).

Table 3: Clinical cases of suspected invasive fungal disease and results of the pandimorphic RT-qPCR assay (n=47).

Case category *	**Number of cases**	**Test method**	**Final diagnosis**	**Specimen type**	**Pandimorphic RT-qPCR assay positive, n/N (%)**	**Cq value**
Proven	27	Culture	Histoplasmosis (n=9)	Urine (stored)	2/9 (22)	38.39 – 39.23
			Emergomycosis (n=18)	Urine (stored)	5/18 (27)	38.2 – 41.19
Probable	11	Histopathology	Histoplasmosis/ emergomycosis (n=3)	Urine (stored)	3/3 (100)	38.57 – 41.27
			Not specified (n=6)	Bone marrow	1/1 (100)	30.47
				FFPE** tissue	1/1(100)	37.24
				Fresh tissue	3/3 (100)	33.09, 33.09, 35.25
				Whole blood	1/1 (100)	43.84
				Serum	1/1 (100)	38.57
				Urine	1/1 (100)	38.47
Possible	9	Compatible clinical presentation	Not specified (n=9)	Bone marrow	0/1 (0)	Negative
				Fresh tissue (fungal culture negative for 4 specimens and 3 samples of insufficient quantity for culture)	1/7 (14)	39.04

				Blood	0/1 (0)	Negative

*Proven cases were defined as confirmed histoplasmosis or emergomycosis by culture; *Probable cases were defined as cases with compatible histopathological appearance; *Possible cases were defined as cases with compatible clinical findings. **FFPE=formalin-fixed paraffin-embedded tissue

5.3.3. Limit of detection

The lowest detection limit for the spiked clinical specimens was 1.5 X 10^2 CFU/ml = 150 CFU/ml with a Cq value of 41 for urine and 1.5 X 10^2 CFU/ml=150 CFU/ml with a Cq value of 40.29 for whole blood (Table 4). The lowest detectable limit of the assay was 10^{-9} ng/µl for both *E. africanus* and *H. capsulatum* DNA (Table 5).

Table 4: RT-qPCR assay results for urine, plasma and blood specimens spiked with *E. africanus* DNA (Cq values).

DNA concentration of *E. africanus* (CFU/ml)	*E. africanus/ H. capsulatum* primers for *E. africanus* DNA (Cq value)		
	Urine	Plasma	Whole blood
1.5 X 10^5	27.00	**25.63**	27.39
1.5 X 10^4	38.00	No amplification	33.89
1.5 X 10^3	41.00	No amplification	39.63
1.5 X 10^2	**41.00**	No amplification	**40.29**
1.5 X 10^1	No amplification	No amplification	No amplification
1.5 X 10^0	No amplification	No amplification	No amplification

Table 5: RT-qPCT results for *E. africanus* and *H. capsulatum* DNA (Cq values) for samples spiked with *H. capsulatum* and *E. africanus* DNA

DNA concentration of *E. africanus* (ng/µl)	*E. africanus/H. capsulatum* primers for *E. africanus* DNA (Ct value)	DNA concentration of *H. capsulatum* (ng/µl)	*E. africanus/H. capsulatum* primers for *H. capsulatum* DNA (Ct value)
10^{-1} (0.1)	21.79	10^{-1} (0.1)	21.70
10^{-2} (0.01)	25.93	10^{-2} (0.01)	26.15
10^{-3} (0.001)	29.74	10^{-3} (0.001)	30.52
10^{-4} (0.0001)	33.93	10^{-4} (0.0001)	34.51
10^{-5} (0.00001)	37.44	10^{-5} (0.00001)	38.28
10^{-6} (0.000001)	38.13	10^{-6} (0.000001)	40.24

10^{-7} (0.0000001)	43.01	10^{-7} (0.0000001)	45.00
10^{-8} (0.00000001)	**45.00**	**10^{-8} (0.00000001)**	**45.00**
10^{-9} (0.000000001)*	No cycle threshold	10^{-9} (0.000000001)	No amplification
10^{-10} (0.0000000001)	No amplification	10^{-10} (0.0000000001)	No amplification
10^{-11} (0.00000000001)	No amplification	10^{-11} (0.00000000001)	No amplification

*lowest detectable limit was 10^{-9} ng/µl

5.3.4. *Histoplasma* EIA results for cohort 1: The cohort 1 included urine samples from 194 patients living with HIV. The median CD4 count at diagnosis was 34 cells/µl for 194 patients (Table 6). The most commonly recorded baseline symptoms were weight loss (143, 74%), cough (82, 42%), fever (68, 35%), night sweats (44, 23%), diarrhoea (25, 13%) and widespread skin lesions (13, 7%). Twenty-eight (14%) had died by 6 months. Of the 194 patients, 6/194 (3%) tested EIA-positive, five of whom were female. Three of the six also had a positive CrAg LFA result. Five of the six patients were managed for a suspicion of tuberculosis as histoplasmosis can mimic TB infection. Three patients had a cough and fever, and two reported night sweats and weight loss. Five were not on any antifungals at the time of testing and one was taking fluconazole. Two of the six patients died at 6 months and the cause of death was not established.

Table 6. Demographic and clinical characteristics of patients from cohort 1, n=194.

Characteristics	**All patients (n=194)**	**%**	**Positive *Histoplasma* EIA patients (n=6)**
Age			
Age in years; median [IQR]	39 (32-47)		37 (27-48)
Sex	**194**	**100**	**6**
Female	98	51	5
Male	96	49	1
Median CD4 count at diagnosis in cells/µl (IQR)	34(12-61)		64 (18)

AIDS-related conditions at baseline	**194**	**100**	**6**
Cryptococcal antigenaemia	67	35	3
Oesophageal candidiasis	2	1	0
Cervical cancer	1	1	0
Cryptosporidiosis, chronic intestinal	2	1	0
Herpes simplex infection	2	1	0
Kaposi's sarcoma	2	1	0
Disseminated or extra-pulmonary *Mycobacterium avium* or *Mycobacterium kansasii* infection	2	1	0
Mycobacterium tuberculosis infection	73	38	5
Pneumocystis pneumonia	11	6	1
Pneumonia, recurrent	4	2	0
Medications at baseline			
Antifungals*	61	31	1
Co-trimoxazole	70	36	3
TB prophylaxis (isoniazid)	1	1	0
Anti-tuberculosis treatment	29	15	2
Other antimicrobial agents	10	5	2
Corticosteroids (oral or IV)	14	7	0
Patient symptoms at baseline	**194**	**100**	**6**
Diarrhoea	25	13	0
Current cough	82	42	3
Fever (n=193)	68	35	3
Weight loss (n=193)	143	74	2
Night sweats	44	23	2
Vitals; median [IQR]	**194**	**100**	**6**
Temperature (n = 72)	36.3 (36.0 - 36.8)	range (26.3 - 38.6)	range 36(36.0 - 36.5)
Pulse (n = 189)	92 (80 - 101)	range (49 - 145)	range 82 (68 – 103)

Respiratory rate per min (n = 184)	20 (18 - 20)	range (10 - 24)	range 20 (16 – 20)
Systolic blood pressure (n = 189)	117 (106 - 130)	range (11 - 191)	range 115(99 – 124)
Diastolic blood pressure (n = 186)	75 (66 - 84)	range (50 - 101)	range 74 (61 – 97)
Chest examination findings	**62**	**32**	**6**
Abnormal	31	16	0
Normal	31	16	6
Skin examination findings	**194**	**100**	**6**
Normal	148	76	4
Cryptococcal-like lesions	1	1	0
Tinea-like lesions	5	3	0
Other localized lesions	29	14	2
Widespread lesions	13	7	0
Blood results at baseline	**194**	**100**	**6**
White cell count ×**10−9/litre (n)**	4.03 (2.99 - 6.2)	range (0 - 21.6)	range 5.11 (1.08 – 9)
Platelets ×**10−9/litre (n)**	249 (172 - 333)	range (0 - 917)	range 285 (190 – 412)
Creatinine **(µmol/litre)**	76 (62 - 97)	range (0.69 - 1331)	range 117 (50 – 240)
Albumin **(µmol/litre)**	26 (20 - 31)	range (11 - 43)	range 20 (20 – 30)
ALT **(µmol/litre)**	33 (20 - 57)	range (4 - 493)	range 29 (19 – 41)
Outcome at 6 months	**194**	**100**	
Died	28	14	2
Survived	166	86	4

*Antifungals included fluconazole, amphotericin B, flucytosine

5.3.5. *Histoplasma* LFA results for cohort 1: Applied to urine samples from cohort 1, the LFA had a 6% (12/194) positivity rate (Table 7). Only one of these 12 patients also had a positive urine *Histoplasma* EIA result; six had CrAg-positive blood results. Six of the twelve patients with positive LFA results had laboratory-confirmed TB-coinfection. Shortness of breath was reported by two patients, seven had a cough and eight reported weight loss. Of the 12 patients with positive LFA results, three died within 6 months of follow-up. When the LFA was compared to the EIA for the diagnosis of histoplasmosis, the sensitivity and specificity was 17% (1/6, 95% CI 42-64%) and 94% (177/188, 95% CI 90-97%), respectively.

Table 7: Comparison of the *Histoplasma* Clarus EIA and OIDx LFA assay results on urine samples from a cohort nested in a cryptococcal antigen screening programme (n=194).

		***Histoplasma* Clarus EIA**		**Total**
		Positive	**Negative**	
***Histoplasma* OIDx LFA**	Positive	1	11	12
	Negative	5	177	182
Total		**6**	**188**	**194**

5.3.6. Pandimorphic RT-qPCR results for cohort 2**:** Of 533 patients whose remnant CD4 whole blood samples were selected for RT-qPCR testing, 300 (56%) were male and their median age was 39 years (IQR, 12 – 83). Their median CD4 count was 37 cells/µl (IQR, 1-99). The RT-qPCR assay tested positive in 2% (10/533) of these patients and they all had confirmed cryptococcosis tested by CrAg LFA. Detailed clinical and microbiology results were not available and no follow up data on the patients was available either.

5.4. Discussion

Histoplasmosis diagnosis is difficult, time-consuming, and requires a certain degree of expertise. Methods that provide reliable results with short turn-around times constantly need to be developed and implemented, especially in low-resource settings (4,10,24). We

evaluated the LFA and the RT-qPCR diagnostic methods as alternatives to the culture gold standard for diagnosis of HIV-associated histoplasmosis. In-house validation of an LFA compared to an EIA and culture showed five positive tests out of 23 urine specimens. *Histoplasma capsulatum* culture was positive in 6 patients while the EIA tested positive on 10 urine specimens. The LFA is simple and can give results in 15 minutes but results from this study showed a lower positivity compared to both the EIA and culture. They could be because culture is performed using tissue or another specimen directly from an infection site, which increases the yield of this method for diagnosis compared to the LFA, which utilises urine samples to test for the presence of *H. capsulatum* galactomannan antigen. The *H. capsulatum* galactomannan antigen concentration is sometimes diluted to low concentrations in the urine, which could explain why in this study the LFA missed the 2 samples from culture-positive patients. The lower yield was also observed against the EIA during the evaluation which could be due to the less cross-reactivity reported with the LFA. The cross-reactivity reported with the EIA assay between *Histoplasma* and other closely related thermally dimorphic fungi such as *Emergomyces*, is not of major clinical concern because the treatment for diseases closely related to histoplasmosis is similar. The LFA performance was previously reported by the manufacturer when it was used to screen 266 clinical urine samples from people living with HIV with suspected histoplasmosis and showed 96% sensitivity and 89% specificity compared to culture results (25). The availability of tests such as LFA that give results in as little as 15 minutes can prompt further tests to confirm histoplasmosis diagnosis and guide clinicians with treatment decisions. We observed a false positive LFA result in a urine sample previously confirmed to be culture-positive for cryptococcosis similar to a discrepancy report by Caceres et al, 2021. MiraVista Diagnostics (Indianapolis, IN, USA) also developed an LFA for histoplasmosis and when it was compared to an EIA from the same company the two methods had the same sensitivity and specificity of 94% and 100% respectively (3). A panel of 100 urine samples, with 26 from people living with HIV, was also tested using the MiraVista LFA and EIA with sensitivity and specificity of 96% for the LFA and 96% sensitivity and 77% specificity for the EIA (26).

Validation of the pandimorphic RT-qPCR assay in cultured isolates yielded 100% sensitivity and 91% specificity proving that with training and availability of proper resources and laboratory infrastructure, this assay can be used in settings where facilities for culturing *Histoplasma* are lacking. There is still a need for culture tests but pandimorphic RT-qPCR

assay provides an alternative with a shorter turn-around time where results can be available within 24 hrs compared to culture that requires over 2 weeks to get results. In the current validation, the pandimorphic RT-qPCR assay was positive for only 22% of stored urine samples from cases with proven invasive fungal disease. Urine samples proved to not be the best candidates for the PCR as urine samples might not contain enough nucleic acids from *H. capsulatum* to allow detection. Alanio *et al* reported a higher sensitivity of 97.7% in at least one specimen tested and a specificity of 99% although in this study the authors used *Histoplasma* specific PCR. The study by Alanio included only 1 urine sample and reported that urine might not be the best sample for the *Histoplasma* RT-qPCR assay. The samples tested in the evaluation by Alanio, and colleagues included BAL, biopsy, skin, bone marrow aspirate, blood, digestive tract biopsy, adrenal gland biopsy, osteoarticular and CSF (27). In the same study, the *Histoplasma* RT-qPCR assay was positive in 76% of blood samples tested and indicated advantageous diagnosis from blood samples (27). In this study, the limit of detection of the assay for nucleic acids extracted from cultured isolates was 10^{-9} ng/μL, and for spiked clinical specimens (whole blood and urine) was $1.5X10^{2}$ CFU/ml. In our current study, the pandimorphic RT-qPCR assay showed cross-reactivity with *Blastomyces spp* similar to a previously report described by Wilmes et al. 2023 on RT-qPCR cross-reactivity of *Histoplasma* with *Blastomyces spp* (21). Although the assay was reported to have great sensitivity in the identification of cultured fungus, clinical presentation and histopathology results should also be considered when reporting the final diagnosis.

In South Africa, the true prevalence of histoplasmosis is unknown. From 2010 to 2020, 133 cases were reported and only 46 (35%) were proven cases (28). Relative to the high HIV and TB burden in the country, the case number was extremely low in this study and this under-estimation could be due to diagnostic limitations (28). The lack of information on the prevalence of histoplasmosis in South Africa is linked to the difficulties associated with the development of diagnostic assays that are specific to *H. capsulatum*. *H. capsulatum* is closely related to *Emergomyces* and *Blastomyces* making it difficult to differentiate these pathogens. In addition, the lack of awareness of such diseases makes epidemiological studies difficult to undertake. To understand the prevalence of histoplasmosis among people with HIV, we used the LFA to screen a cohort of 194 patients and 12 patients (6%) had positive results. The EIA was used to test the same patients but only 6 patients (3%) had positive results. These results showed poor concordance between the EIA and the LFA. A similar

study comparing the EIA and the LFA was conducted in Ghana on patients living with HIV regardless of symptoms at any stage of their HIV infection, CD4 counts and ART status. The results showed histoplasmosis prevalence of 4.7% and 5.6% respectively, similar to our study, and the 2 tests were also not compared to any gold standard (29). Patients in the Ghana study had a median CD4 count of 1049 cells/µL with an interquartile range of 258 – 1480 cells/µL and 52% of them were on ART at the time of recruitment, with 2.7% CrAg prevalence rate compared to patients in our current study who had an average CD4 count of 35 and 35% were CrAg positive. Of the 107 urine samples tested in the study in Ghana, the LFA tested positive for 6 samples while EIA tested positive for 5 of the same urine samples with a concordance of 99% for the EIA and LFA. The assays were performed side by side within 48 hours of specimen collection with no freezing (29), which contrasts with our analysis, where the assays were performed on the same urine samples but years apart following one or two freeze-thaw cycles, which might explain poor concordance observed between the same two assays in this study. Our samples were stored at -70°C for 3 years before testing using the LFA. We expected reduced sensitivity with the LFA since the assay was performed after prolonged freezing of urine, but we observed more positive results with this assay compared to EIA which was performed immediately after urine collection. Concordance between LFA and EIA has been reported from a different manufacturer MiraVista with 84% agreement between the two assays performed side by side (26).

Oladele and others reported a 7% prevalence rate for histoplasmosis in patients with advanced HIV disease in Nigeria using the IMMY Clarus EIA compared to the 3% reported in this study (30). Recruitment of patients in the study by Oladele was based on patients having 2 or more clinical features of histoplasmosis while our study included patients with positive CrAg results and CD4 counts of <100 cells/µL without any pre-selection related to histoplasmosis symptoms or signs, which may explain the lower prevalence. Among patients with HIV with a CD4 count of <100 cells/µL and all CrAg positive, comparable to when using IMMY Clarus EIA (3%) for screening for histoplasmosis in a similar cohort. The IMMY Clarus EIA was also validated in 2 cohorts including people living with HIV (446 from Guatemala and 123 from Colombia) and the sensitivity/ specificity/ PPV was 98%, 97% and 79% respectively (31). In a cohort of 391 patients from Mexico, the performance of the IMMY Alpha EIA was compared against the IMMY Clarus EIA resulting in reported sensitivity/ specificity/ PPV of 91%/ 91%/ 79% vs 67%/ 96%/ 86% (32). These studies

provide information on alternative screening methods for histoplasmosis, which should be used together with other tests like culture to provide quicker results. However, clinicians should be advised that both EIA and LFA assays cross-react with blastomycosis, although this should not generally affect patient treatment and blastomycosis is not common in persons with advanced HIV disease. A limitation of our study was that we did not have clinical data for the patients in cohort 2 of the whole blood samples, which would have been beneficial to this analysis.

The prevalence of histoplasmosis was also studied using the evaluated RT-qPCR assay screening for *H. capsulatum* nucleic acids in blood samples from a cohort of 533 patients, of which 10 samples were positive. The high sensitivity of the RT-qPCR assay during development allowed the detection of disseminated histoplasmosis in 92.3% of blood samples from immunocompromised individuals (20). A multiplex real-time PCR was tested on 24 clinical strains and 43 clinical samples from AIDS patients with proven fungal infection with a specificity and sensitivity of 100% in clinical strains and 90.7% in clinical samples (33). The detection of histoplasmosis in patients with HIV using a real-time PCR assay was also tested on 35 clinical serum samples and reported a sensitivity of 70% and specificity of 100% (34). Further studies need to be performed using clinical samples to provide data supporting whether the pandimorphic RT-qPCR assay can be used as an alternative assay for screening blood for histoplasmosis and other endemic mycoses in people living with HIV in South Africa to study the prevalence of these diseases. Previous evaluations of both conventional and real-time PCR targeting the *ITS*, *Hc100* and *mtSSU* genes for *Histoplasma* show that they provide alternatives that are easy to use in low-resource settings with high sensitivity and specificity compared to culture although there was evidence of cross-reactivity with closely related thermally-dimorphic fungi (24,27,35).

In conclusion, simple tests such as the OIDx LFA kit enable *Histoplasma* antigen testing directly from urine samples with the least amount of infrastructure and laboratory equipment needed. Molecular tests such as RT-qPCR assays can be implemented in diagnostic laboratories with training providing more sensitive results within a short time. By enabling early treatment initiation, tests that do not require cold chain storage and allow quicker diagnosing of individuals with histoplasmosis compared to culturing the fungus have a

potential public health impact. However, in light of our results, further work is required to evaluate the accuracy of the LFA in different contexts and to understand the poor concordance that we observed with the EIA, before its implementation as a diagnostic or screening assay in South African diagnostic laboratories.

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Supplementary Table 1: Clinical isolates used for the validation of the specificity of the pandimorphic RT-qPCR assay (n=193).

Clinical isolates ID	Clinical isolates	RT-qPCR result
Thermally-dimorphic fungi (n=23)		
831	*Sporothrix schenckii*	Negative
976	*Sporothrix schenckii*	Negative
979	*Sporothrix schenckii*	Negative
1126	*Sporothrix schenckii*	Negative
8	*Talaromyces marneffei*	Negative
BECN2	*Blastomyces emzantsi*	Positive
BECN3	*Blastomyces emzantsi*	Positive
BPCN7	*Blastomyces emzantsi*	Positive
BECNE6892	*Blastomyces emzantsi*	Positive
BECNE1992	*Blastomyces emzantsi*	Positive
BECN5	*Blastomyces emzantsi*	Positive
BESAIMR	*Blastomyces emzantsi*	Positive
BPCN13	*Blastomyces percursus*	Positive
BPCN17	*Blastomyces percursus*	Positive
BPX888	*Blastomyces percursus*	Positive
BSCNC	*Blastomyces percursus*	Positive
BPM124	*Blastomyces percursus*	Positive
BPNCPF4091	*Blastomyces percursus*	Positive
BPI777	*Blastomyces percursus*	Positive
BP222	*Blastomyces percursus*	Positive

BPOS	*Blastomyces percursus*	Positive
BPSD	*Blastomyces percursus*	Positive
ATCC10225	*Blastomyces dermatitidis*	Positive

Yeast (n=44)

VNI	*Cryptococcus neoformans*	Negative
VNIII	*Cryptococcus neoformans*	Negative
VNIV	*Cryptococcus neoformans*	Negative
VGII	*Cryptococcus gattii*	Negative
VGIII	*Cryptococcus gattii*	Negative
VGV	*Cryptococcus gattii*	Negative
1528	*Cryptococcus neoformans. var. neoformans*	Negative
1161	*Cryptococcus arrabidensis*	Negative
1189	*Naganishia albida*	Negative
1203	*Cryptococcus magnus*	Negative
1484	*Naganishia albida*	Negative
855	*Candida digboiensis*	Negative
864	*Magnusiomyces capitatus*	Negative
900	*Pichia fabianii*	Negative
902	*Pichia mississippiensis*	Negative
923	*Candida metapsilosis*	Negative
967	*Candida lipolytica*	Negative
978	*Candida pararugosa*	Negative
1017	*Wickerhamomyces edaphicus*	Negative
1026	*Wickerhamomyces anomalus*	Negative
1038	*Pichia myanmarensis*	Negative
1056	*Yarrowia lipolytica*	Negative
1059	*Wickerhamomyces anomalus (C. pelliculosa)*	Negative
1064	*Trichosporon montevideense*	Negative
1084	*Rhodotorula mucilafinosa*	Negative
1095	*Candida orthopsilosis*	Negative

1152	*Metschnikowia pulcherrima*	Negative
1156	*Candida duobushaemulonii*	Negative
1181	*Nakaseomyces bracarenis*	Negative
1293	*Clavispora lusitaniae*	Negative
1297	*Candida Sojae*	Negative
1298	*Wickerhamomyces anomalus*	Negative
1405	*Geotrichum candidum*	Negative
1410	*Pichia kudriavzevii*	Negative
1459	*Candida auris*	Negative
1465	*Candida tropicalis*	Negative
1529	*Candida tropicalis*	Negative
1495	*Meyerozyma guilliermondii*	Negative
1506	*Candida parapsilosis*	Negative
1508	*Candida dubliniensis*	Negative
1519	*Candida albicans*	Negative
1449	*Rhodotorula mucilaginosa*	Negative
1383	*Trichosporon inkin*	Negative
1443	*Nakaseomyces glabratus*	Negative
Moulds (n=126)		
764	*Pythomyces (Montagnulaceae)*	Negative
765A	*Monascus spp*	Negative
765B	*Aspergillus sclerotiorum*	Negative
767	*Lodderomyces elongisporus*	Negative
773	*Lichtheimia ramose*	Negative
775	*Mucor velutinosus*	Negative
776	*Acremonium spp*	Negative
778	*Scedosporium spp*	Negative
779	*Tilletiopsis spp*	Negative
780	*Rhizopus oryzae*	Negative
790	*Thielavia spp*	Negative
793	*Fusarium delphinoides*	Negative
800	*Curvularia spp*	Negative
808	*Sporisorium spp (Sphacelotheca spp)*	Negative
813	*Torulaspora delbrueckii*	Negative
815	*Sporothrix lignivora*	Negative
816	*Aspergillus deflectus*	Negative

818	*Chaetomium globosum*	Negative
819	*Pyrenoma spp*	Negative
822	*Fusarium solani*	Negative
832	*Cladosporium spp*	Negative
833	*Neocosmospora vasinfecta*	Negative
835	*Ophiostoma stenoceras*	Negative
837	*Trichoderms spp*	Negative
842	*Saksenaea oblongispora*	Negative
850	*Scedosporium prolificans*	Negative
874	*Cochliobolus lunatus*	Negative
879	*Leptosphaeria tompkinsii*	Negative
882	*Trichophyton rubrum*	Negative
885	*Cunninghamella bertholletiae*	Negative
887	*Clavispora lusitaniae*	Negative
928 (2)	*Curvularia (cochliobolus) hawaiiensis*	Negative
957	*Rhytidhysteron rufulum*	Negative
983	*Aspergillus nidulans*	Negative
985	*Bipolaris spicifera*	Negative
992	*Phialophora spp*	Negative
997	*Trichoderma citrinoviride*	Negative
1009	*Cadophora malorum*	Negative
1013	*Rhinocladiella basitona*	Negative
1073	*Rhizopus spp*	Negative
1080	*Penicillium roquefortii*	Negative
1100	*Rhizopus spp*	Negative
1101	*Chaetomium spp*	Negative
1110	*Curvularia spicifera*	Negative
1112	*Geosmithia lavendula*	Negative
1118	*Exserohilum rostratum*	Negative
1120	*Curvularia verruculoasa*	Negative
1127	*Rhizopus microspores*	Negative
1135	*Syncephalastrum spp*	Negative
1163	*Microsporum canis*	Negative
1165 (3)	*Syncephalastrum racemosum*	Negative
1168	*Roussoella percutanea*	Negative
1172	*Microsporum gypseum*	Negative

1177	*Fonsecaea spp*	Negative
1180	*Kodamaea ohmeri*	Negative
1190	*Fusarium chlamydosporum*	Negative
1195	*Scedosporium apiospermum*	Negative
1197	*Scedosporium apiospermum*	Negative
1200	*Scedosporium apiospermum*	Negative
1208	*Epicoccum nigrum*	Negative
1209	*Penicillium citrinum*	Negative
1214	*Acremonium percicinum*	Negative
1216 (2)	*Fonsecaea predosoi*	Negative
1227	*Curvularia spicifera*	Negative
1236	*Clasosporium cladosporiodides*	Negative
1255	*Scopulariopsis flava*	Negative
1260	*Trichoderma longibrachiatum*	Negative
1265	*Aspergillus Niger*	Negative
1269	*Epidermophyton floccosum*	Negative
1269 (2)	*Epidermophyton floccosum*	Negative
1270	*Trichophyton verrucosum*	Negative
1275	*Trichoderma orientale*	Negative
1278	*Sporothrix lignivora*	Negative
1280	*Fusarium proliferum*	Negative
1292	*Aspergillus nidulans*	Negative
1294	*Microsporum canis*	Negative
1305	*Phanerochaete spp*	Negative
1313	*Dichomitus squalens*	Negative
1316 R2	*Gliocladium viride*	Negative
1317	*Microsporum audouinii*	Negative
1336	*Sarocladium kiliense*	Negative
1338	*Exophiala phaeomuriformis*	Negative
1357	*Fusarium thapsinum*	Negative
1365	*Fusarium solani sp complex*	Negative
1366	*Cochliobolus cynodontis*	Negative
1384	*Cladosporium pulvericola*	Negative
1384B	*Cladosporium pulvericola*	Negative
1386	*Fusarium incarnatum equiseti*	Negative
1387	*Aspergillus flavus*	Negative

1389	*Mucor circinelloides*	Negative
1390	*Alternaria alternata species group*	Negative
1411	*Penicillium chrysogenum species complex*	Negative
1412	*Lichthemia corymbifera*	Negative
1415	*Nannizzia (microsporum) gypsea*	Negative
1416	*Scopuariopsis brevicaulis*	Negative
1423	*Nannizzia persicolor*	Negative
1424	*Exophiala dermatitis*	Negative
1425	*Aspergillus terrus*	Negative
1432	*Fusarium dimerum sp complex*	Negative
1433	*Aureobasidium pallulans*	Negative
1439	*Malassezia furfur*	Negative
1440	*Sporidiobolus salmonicolor*	Negative
1444	*Syncephalastrum racemosum*	Negative
1445	*Aspergillus versicolor*	Negative
1452	*Trichophyton mentagrophytes var. interdigitale*	Negative
1453	*Neoscytalidium hyalinum*	Negative
1454	*Chaetomium globosum*	Negative
1455	*Aspergillus flavus*	Negative
1457	*Aspergillus niger*	Negative
1466	*Fusarium venenatum*	Negative
1474	*Lasiodiplodia theobromae*	Negative
1486	*Rhizopus oryzae*	Negative
1487	*Purpureocillium lilacinum*	Negative
1500	*Aspergillus fumigatus*	Negative
1502	*Curvularia inequalis*	Negative
1507	*Aspergillus clavatus*	Negative
1509	*Aspergillus niger*	Negative
1510	*Neoscytalidium dimidiatum*	Negative
1516	*Aspergillus tamarrii*	Negative
1521	*Fusarium oxysporum sp complex*	Negative
1522	*Lomentospora prolificans*	Negative
1523	*Mucor arnelloinedis*	Negative
1524	*Curvularia lunata*	Negative
1525	*Aspergillus clavatus*	Negative
1526	*Trichophyton tonsurans*	Negative

1527	*Scedosporium boydii*	Negative

CHAPTER 6: MOLECULAR DETECTION OF *HISTOPLASMA CAPSULATUM* IN ENVIRONMENTAL SAMPLES COLLECTED FROM SOUTH AFRICAN CAVES, 2020-2021 (EXPLORATORY OBJECTIVE)

6.1. Introduction

Histoplasma capsulatum is a fungus that causes histoplasmosis in people living with HIV. The fungus is found in soil, especially in areas with bird or bat droppings. It is useful to be able to test soil for the presence of this fungus to identify hotspots for high-risk people to avoid. The environmental reservoir of *H. capsulatum* has not been properly established but it is known that the mycelial phase of this pathogen is harboured in the soil around river basins (1–4). The fungus thrives in areas with high nitrogen content and naturally occurs in caves, old buildings, and bird farms. Human infection is linked to occupational exposure and recreational activities as a result of fungal spores being released into the air when the soil is disturbed (5). Once the spores are inhaled from the air, the body temperature stimulates the change of the fungus from mycelium to yeast form (6). Disease manifestation depends on host factors such as the immune system and fungal factors like the amount of spores inhaled (7,8).

The first isolation of *H. capsulatum* from soil by inoculation of mice was reported by Emmons in 1949(9). Thereafter, bat cave-associated histoplasmosis has been reported in several studies (10–12). This includes outbreaks which have been reported in different regions of the world, including in South Africa (13–19). These outbreaks showed an association between *H. capsulatum* and birds/bat guano (20–22). They also helped delineate populations at higher risk from *Histoplasma* infections which include speleologists, eco-tourists, archaeologists, construction workers, bird farmers, and organic fertilizer handlers (22,23). South African caves in the Western Cape and Gauteng provinces have been documented as probable sources of exposure for speleologists, casual visitors, or guano miners with several outbreaks of acute pulmonary histoplasmosis being reported since 1957 among new members of speleological societies (1,19). These outbreaks were never confirmed in the laboratory. Generally, it is challenging to culture *Histoplasma* from the environment due to the high number of contaminants found in the soil (2). However, different studies have used molecular assays to test for the presence of *H. capsulatum* nucleic acids in soil and

demonstrated the usefulness of such tools in countries such as Brazil, Mexico, and the United States of America (2,24–27). The Hc100 nested PCR has shown a sensitivity of 54% and specificity of 100% in detecting *Histoplasma* in clinical samples compared to culture and serological tests (23,28). The *ITS* PCR has previously been used for screening of *Emergomyces* in soil samples in South Africa and 30% of the 60 samples were positive (29). Molecular assays used for *Histoplasma* screening in clinical samples can also be optimised for use in testing environmental samples. We utilised the RT-qPCR assay developed specifically for *Histoplasma* testing and the assay to screen for *Histoplasma/ Emergomyces* in soil samples collected from cave in South Africa. Such assays are useful in identification of high-risk areas such as bat-inhabited caves. They can also direct the development of strategies for preventing infection among people at increased risk of severe histoplasmosis. We, therefore, evaluated a conventional panfungal *ITS* PCR assay, conventional Hc100 nested PCR assay and a pandimorphic RT-qPCR assay to detect *H. capsulatum* directly from cave soil samples

6.2. Materials and methods

6.2.1. Environmental sample collection

Fifty-six environmental samples were collected from seven South African caves from December 2020 to September 2021. Three caves in the Western Cape province, three in Gauteng province and one in the Eastern Cape province (Exact locations could not be disclosed for safety reasons). Ten samples were collected per cave in three of the caves in the Gauteng and two of the caves in the Western Cape. Five samples and one sample were collected in the Eastern Cape and Western Cape, respectively. Sampling was by convenience as speleologists were exploring these caves during this same period. The environmental samples were labelled by the collectors as owl droppings (n=1), soil contaminated with bat guano (n=42) and soil (n=13).

6.2.2. Handling and processing of environmental samples

The samples were sent to the National Institute for Communicable Diseases (NICD) following International Carriage of Dangerous Goods by Road (ADR) safety regulations and the UN3373 – Biological substance Category B packaging requirements (30). Upon arrival, the samples

were stored at 4°C-8°C until processed. Handling and processing of the samples was done in a biosafety level 3 facility with personnel wearing protective equipment e.g. N95mask and gloves, as the environmental samples may pose a potential risk of infection.

6.2.3. In-house validation of molecular PCR assays

A total of 16 soil samples collected from different points around the National Institute for Communicable Diseases (NICD) were used during validation of the three molecular PCR assays (internal transcribed spacer (*ITS)* panfungal PCR, Hc100 nested PCR and pandimorphic reverse transcriptase quantitative polymerase chain reaction (RT-qPCR). The soil samples were assumed to harbour environmental fungi other than *H. capsulatum.* These soil samples were used to determine the specificity of the assays. One soil sample was randomly selected from the 16 to determine the limit of detection (LOD). A portion of the same soil sample was autoclaved. The autoclaved and non-autoclaved soil samples were spiked with $1.5X10^3$ CFU of *H. capsulatum* mould and thereafter, serial dilutions (1:10, 1:100, 1:1000) were made.

We also validated the assays using DNA stored in the -20°C freezer at NICD. Species identity from the DNA extracts was previously confirmed using Sanger sequencing of the internal transcribed spacer region (*ITS*)/ calmodulin/ beta-tubulin genes. DNA of *Histoplasma capsulatum* var. *capsulatum* (n=16), and *H. capsulatum* var. *farciminosum* (n=1) were used to determine the sensitivity of the assay. The LOD was determined using 1:10 serial dilutions of 0.1 ng/µl *H. capsulatum*. The specificity was determined using 66 fungal pathogens which included yeasts (n=18), moulds (n=25), closely related thermally-dimorphic fungi [(*Emergomyces africanus* (n=10), *Emergomyces pasteurianus* (n=1), *Blastomyces dermatitidis* (n=1), *B. emzantsi* (n=5), *B. percursus* (n=2)] and five non-related thermally-dimorphic fungal pathogens [(*S. schenckii* (n=4)].

6.2.4. DNA extraction from environmental samples

Genomic DNA was extracted from the environmental samples using a DNeasy PowerSoil Pro Kit (Qiagen, Hilden, Germany) as per the manufacturer's instructions. The DNA was stored at -20°C until further analysis. Briefly, 250 mg of soil was added to 800 µL of solution CD1 and vortexed gently to mix all reagents. The tubes were then secured to the vortex for harsh cell lysis for 10 minutes at high speed. Centrifugation of the mixture was done for 1 minute at 15000xg. The supernatant was then transferred to a clean micro centrifuge tube and 200 µL of solution CD2 was added and vortexed for 5 seconds followed by centrifugation for 1 minute at

15000 xg. Avoiding the pellet, 700 µL of the supernatant was removed and added to a clean microcentrifuge tube. The supernatant was then mixed with 600 µL of solution CD3 and vortexed for 5 seconds. The whole mixture was then loaded to an MB Spin column in two parts and centrifuged at 15 000 xg for 1 minute. The column was then placed into a clean collection tube and washed with 500 µL of EA and 500 µL of solution C5 with centrifugation after adding each solution at 15000 xg for 1 minute. 50 µL of solution C6 was then used to wash out the nucleic acids. The polymerase chain reaction (PCR) was performed immediately after nucleic acid extraction.

6.2.5. Conventional PCR assays

We used *ITS* 1 and 4 primers to amplify the DNA from the environmental samples as previously described by White et al., 1990. The Hc100 PCR was performed using two sets of primers according to Gómez et al. 2018 with minor modifications(23). The Hc100 PCR method included the use of two sets of primers designed by Bialek in 2001, the outer primer set Hc I (5_-GCG TTC CGA GCC TTC CAC CTC AAC-3_) and Hc II (5_-ATG TCC CAT CGG GCG CCG TGT AGT-3_) encoded 391-nucleotide sequence of a gene coding for a 100-kDa-like protein unique to *H. capsulatum* (accession number AJ005963). The inner primers Hc III (5_-GAG ATC TAG TCG CGG CCA GGT TCA-3_) and Hc IV (5_-AGG AGA GAA CTG TAT CGG TGG CTT G-3_) are complementary to positions 2291 to 2314 and 2500 to 2476, respectively. We used these primers to amplify the DNA from the environmental samples and clinical samples. Briefly, a total volume of 50 µl consisting of 2.5 µl buffer (10X), 0.75 µl $MgCl_2$ (25mM), 1 µl deoxynucleoside triphosphate (10 mM), 0.2µl Hotstart DNA polymerase (5 U/ul) (Thermo Fisher Scientific, South Africa), 38.55 µl nucleic free water, 1 µl each forward and reverse primer (10 uM) (Inqaba Biotechnical Industries (Pty) Ltd, South Africa) and 5 µl of DNA. The amplified product was diluted (1:10) and the 2nd PCR consisted of 0.83 µl buffer (10X), 0.25 µl $MgCl_2$ (25mM), 0.3 µl deoxynucleoside triphosphate (10 mM), 0.06 µl Hotstart DNA polymerase (5 U/ul) (Thermo Fisher Scientific, South Africa), 21.26 µl nucleic free water, 0.3 µl each forward and reverse primer (10 uM) (Inqaba Biotechnical Industries (Pty) Ltd, South Africa) and 1.7 µl of diluted DNA in a final volume of 25 µl. The thermal cycler conditions for both PCRs were set as described by Gómez et al. 2018 (23).

The *ITS* and nested Hc100 products were sequenced by Sanger sequencing on a 3130 sequencer (Applied Biosystems, Life Technologies Corporation, USA). Sequences were viewed and manually edited using Chromas lite. Species identification was done on the National Center for

Biotechnology Information (NCBI), nucleotide Basic Local Alignment Search Tool (BLAST) database (https://blast.ncbi.nlm.nih.gov/Blast.cgi).

6.2.6. Pandimorphic PCR assay

A pandimorphic RT-qPCR assay targeting the mitochondrial small subunit (mtSSU) of *Emergomyces africanus/ Histoplasma capsulatum* was used for the detection of *H. capsulatum* directly from environmental samples. PCR was performed according to Mapengo et al., 2022(31). A cycle threshold (Ct) value of ≤40 was considered positive, while any Ct above 40 was considered negative.

6.2.7. Quality control

Candida albicans ATCC 90028 was used as a positive control for the *ITS* panfungal PCR assay. *H. capsulatum* clinical NICD-MRL_603 was used as a positive control for Hc100 PCR, while *H. capsulatum* clinical NICD-MRL_603 and NCPF 40411 *Emergomyces africanus* were included as positive controls for the pandimorphic PCR assays. We included nuclease-free water as a negative control. Internal controls, such as Lambda DNA and RNA virus, were utilized during Hc100 and pan-dimorphic PCRs, respectively. These controls helped to monitor potential PCR failure that could have arisen from the presence of inhibitors in the environmental samples.

6.2.8. Statistical analysis

The sensitivity, specificity, positivity and negativity rate of the molecular methods in detecting *H. capsulatum* in the environmental samples were calculated using Stata version 14.0 (StataCorp, USA).

6.3. Results

6.3.1. In-house validation of molecular PCR assays

6.3.1.1. NICD soil samples

All 16 soil samples of various types collected around the NICD campus in Sandringham, Johannesburg were negative for *H. capsulatum* with the pandimorphic RT-qPCR, Hc100 nested PCR and *ITS* PCR. Only environmental fungi such as *Epicoccum nigrum, Macrophoma lageniformis, Lophiostoma corticola, Stagonospora cirsii, Pleosporales, Leptosphaerulina,*

Ascobolus (n=2), *Epicoccum italicum, Diymella s*pecies, *Phaeomoniella chlamydospora* and *Scleroderma capeverdeanum* were identified with the *ITS* PCR assay following Sanger sequencing. No PCR inhibitors were observed as the Lambda DNA and RNA virus were positive during Hc100 nested PCR and pan-dimorphic PCR respectively. The limit of detection determined using 1:10 serial dilution of soil spiked with *H. capsulatum* mould was as follows: pandimorphic RT-qPCR (autoclaved: $1.5X10^{-2}$ cell/ml [0.003 cell/ml] and non-autoclaved soil: 1.5×10^{1} [3,0 cell/ml]); Hc100 nested PCR (autoclaved and non-autoclaved soil: 1.5×10^{1} [3,0 cell/ml]; *ITS* panfungal PCR (autoclaved soil: 1.5×10^{2} [300 cell/ml]).

6.3.1.2. DNA from cultured isolates

The *ITS* PCR, Hc100 nested PCR and pandimorphic RT-qPCR all had a sensitivity of 100%, while the specificity was 100%, 99% and 92%, respectively (Table 1). Cross-reaction was noted with *H. farciminosum* DNA and an identity of *H. capsulatum* was obtained following sequencing of the Hc100 nested amplicon. No cross-reaction was observed with Hc100 nested PCR with DNA from any yeast and moulds including DNA of closely-related fungi e.g. *E. africanus, E. pasteurianus, B. dermatitidis, B. percursus* and *B. emzantsi.* The limit of detection obtained using DNA extracted from *Histoplasma* cultured isolates was as follows: pan-dimorphic RT-qPCR (DNA: 10^{-9} ng/µl [0.000000001 ng/µl] and Hc100 nested PCR (DNA: 10^{-4} ng/µl [0.0001 ng/µl).

6.3.2. Cultured isolates:

Table 1: Sensitivity and specificity for *ITS,* Hc100 nested PCR and pandimorphic RT-qPCR assays using DNA from cultured isolates.

	Conventional panfungal *ITS* PCR assay	**Conventional Hc100 nested-PCR assay**	**Pandimorphic RT-qPCR assay**
	Target: internal transcribed spacer gene	Target: gene encoding 100 kDa protein	Target: mitochondrial small subunit gene
Sensitivity (%)	100	100	100
	100	99	92

Specificity (%)			
Total tested	**83**	**83**	**83**

6.3.3. Environmental samples from South African caves

H. capsulatum was detected in 5 of the 7 caves with the molecular assays. These included three caves (caves 1, 2 and 3) in Gauteng province, one cave in the Western Cape province (cave 4) and one cave in the Eastern Cape (cave 6) (Table 2). The locations for the collection of cave samples are shown in the map in Figure 1.

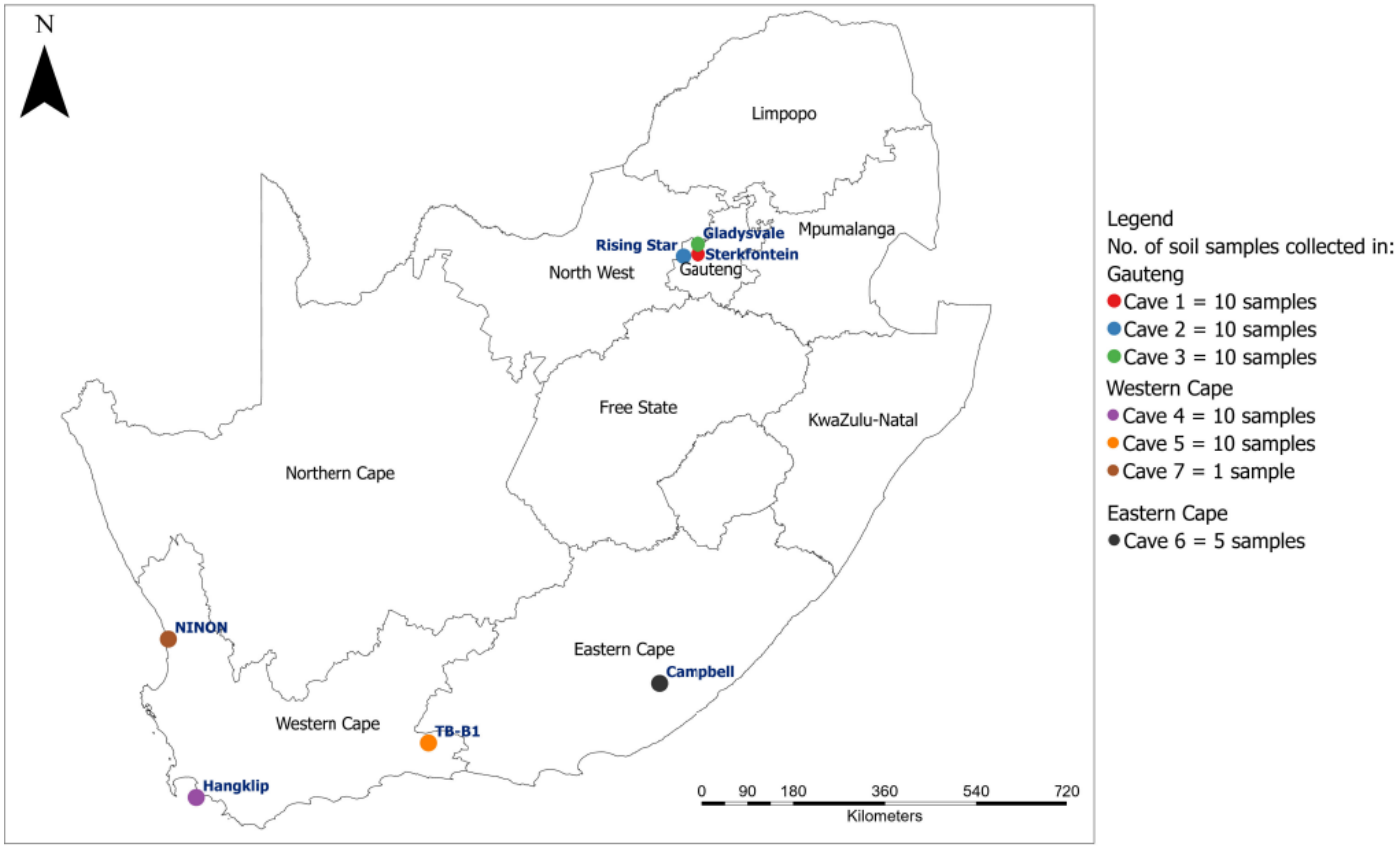

Figure 1: Map showing the location of the caves in the three South African provinces (Gauteng, Western Cape and Eastern Cape) where the environmental samples were collected.

Of 56 samples tested, 18 (32%) were positive from three caves in Gauteng [cave 1 (3/10); cave 2 (7/10); cave 3 (5/10)] and one cave in the Western Cape [cave 4 (2/10) and one in the Eastern Cape [cave 6 (1/5)] (Table 2). These samples were positive either by pandimorphic RT-qPCR or Hc100 nested PCR assays. The test positivity was 21% (12/56) with both pandimorphic RT-qPCR and Hc100 nested PCR assays. Seven percent (4/56) of samples were only pandimorphic RT-qPCR assay-positive and 4% (2/56) were only Hc100 nested

PCR-positive (Table 2). The 14 environmental samples that were positive with the Hc100 nested PCR assay had an identity of *H. capsulatum* with a similarity score of ≥90% based on pairwise alignment on BLAST. For the pandimorphic RT-qPCR assay, the 16 positive environmental samples that were positive had a median cycle threshold value of 35 with a range spanning from 26 to 40. None of the 56 samples tested positive for *H. capsulatum* with the *ITS* panfungal PCR assay. However, 55% (31/56) of the samples were negative for fungi while 45% (25/56) were positive for other fungi found in environment which included *Gamsia* species (n=6), *Chrysosporium* (n=3), *Cladosporium* species (n=2), *Ascogregarina* species (n=2), *Fusarium* species (n=1), *Penicillium* species (n=1), *Talaromyces* species (n=2), *Mucor* species (n=1), *Diploschiste* species (n=1), *Parengyodontium album* (n=1), *Cutaneotrichosporon* species (n=1), *Arthroderma uncinatum* (n=1), *Aspergillus versicolor* (n=1), *Alternaria* species (n=1) and uncultured *Trichosporonales* (n=1) (Table 3).

Table 2: Number of cave samples positive for *Histoplasma capsulatum* with three molecular assays.

Collection site	**Collection date**	**No. of environmental samples**	**Pan-dimorphic RT-qPCR assay**	**Hc100 Fungal PCR assay**	***ITS* panfungal PCR assay**	**Documented presence of *H. capsulatum***
Cave 1-UW-105 (GP)	19-08-2021	10	3	3	0	Yes
Cave 2 Raising star (GP)	17-09-2021	10	7	4	0	Yes
Cave 3 Gladysvale(GP)	22-09-2021	10	3	5	0	Yes
Cave 4 Hangklip (WC)	24-04-2021	10	2	1	0	Yes
Cave 5 TB-B1(WC)	05-12-2020	10	0	0	0	No
Cave 6 Campbell (EC)	28-03-2021	5	1	1	0	Yes
Cave 7 Ninon (WC)	16-04-2021	1	0	0	0	No

Total		56	16	14	0	

Table 3: Molecular results for the environmental samples collected from South African caves (2010 to 2021).

Cave	Collection Date	Sample #	ID	Material	PH Reading	Temperature	Pandimorphic RT-qPCR	Hc100 PCR	*ITS* PCR
U.W.105 (Gauteng)	**2021/08/19 14:15 - 15:30**	1	1	Owl droppings	38.70%	14.5°c	Negative	Negative	Negative
		2	2	Soil	43.10%	12.3°c	Positive	Positive	*Alternaria* species
		3	3	Gauno	53.50%	12°c	Negative	Negative	*Cladosporium/Penicillum* species
		4	4	Soil	47.40%	10.5°c	Positive	Positive	*Fusarium* species
		5	5	Soil	68.60%	12.2°c	Negative	Negative	Negative
		6	6	Soil	12.10%	19.6°c	Negative	Negative	Negative
		7	7	Soil	48.60%	12°c	Positive	Positive	Negative
		8	8	Soil	49.60%	11.7°c	Negative	Negative	*Aspergillus versicolor*
		9	9	Guano	42.80%	15.4°c	Negative	Negative	*Ascogregarina* species
		10	10	Guano	99.90%	17.7°c	Negative	Negative	*Chrysosporium pallidum*
Rising Star (Gauteng)	**2021/09/17 12:00 - 14:00**	1	11	Guano	57%	17.2°c	Negative	Negative	Negative
		2	12	Guano + fungus	83.40%	17.2°c	Positive	Positive	*Gamsia* species/ *Chrysosporium carmichaelii*
		3	13	Guano	92.40%	19.2°c	Positive	Negative	*Talaromyces* species
		4	14	Guano	90%	19.2°c	Positive	Positive	Negative
		5	15	Soil	71.50%	17.5°c	Positive	Positive	*Penicillium crustosum*
		6	16	Guano	79.30%	17.2°c	Negative	Negative	*Ascogregarina culicis*
		7	17	Guano + fungus	69.20%	15.3°c	Negative	Negative	*Gamsia* species
		8	18	Guano	92.90%	17.1°c	Positive	Negative	*Arthroderma uncinatum/ Trichophyton simii*
		9	19	Guano	95.20%	19.6°c	Positive	Negative	Negative
		10	20	Guano + fungus	52.70%	16.3°c	Positive	Positive	*Chrysosporium carmichaelii*

Gladysvale (Gauteng)	**22/09/2021 10:21 - 13:30**	1	21	Soil	57.10%	16.7°c	Negative	Negative	Negative
		2	22	Guano + fungus	72%	17°c	Negative	Negative	*Ascogregarina culicis*
		3	23	Guano + fungus	78.50%	17.5°c	Positive	Positive	Negative
		4	24	Soil	74.70%	15.8°c	Negative	Positive	Negative
		5	25	Soil	84.80%	16.8°c	Negative	Negative	Negative
		6	26	Guano	79.20%	16.9°c	Negative	Positive	*Gamsia* species
		7	27	Soil	72.20%	16.6°c	Negative	Negative	Uncultured Trichosporonales
		8	28	soil	66.30%	16.3°c	Positive	Positive	Negative
		9	29	Soil	73.90%	17.2°c	Negative	Negative	Negative
		10	30	Guano	73.90%	16.7°c	Positive	Positive	*Gamsia kooimaniorum*
Hangklip-10 (Western Cape)	**24-04-2021**	1	31	Guano + Soil	n/a	n/a	Positive	Positive	*Mucor flavus*
		2	32	Guano + Soil	n/a	n/a	Negative	Negative	Negative
		3	33	Guano + Soil	n/a	n/a	Negative	Negative	*Gamsia* species
		4	34	Guano + Soil	n/a	n/a	Negative	Negative	*Cutaneotrichosporon*
		5	35	Guano + Soil	n/a	n/a	Negative	Negative	Negative
		6	36	Guano + Soil	n/a	n/a	Negative	Negative	*Diploschiste scruposus*
		7	37	Guano + Soil	n/a	n/a	Negative	Negative	Negative
		8	38	Guano + Soil	n/a	n/a	Negative	Negative	Negative
		9	39	Guano + Soil	n/a	n/a	Positive	Negative	Negative
		10	40	Guano + Soil	n/a	n/a	Negative	Negative	Negative
TB-B1 (Western Cape)	**5-12-2020**	1	41	Guano	n/a	n/a	Negative	Negative	Negative
		2	42	Guano	n/a	n/a	Negative	Negative	Negative
		3	43	Guano	n/a	n/a	Negative	Negative	*Gamsia* species
		4	44	Guano	n/a	n/a	Negative	Negative	Negative

		5	45	Guano	n/a	n/a	Negative	Negative	Negative
		6	46	Guano	n/a	n/a	Negative	Negative	Negative
		7	47	Guano	n/a	n/a	Negative	Negative	Negative
		8	48	Guano	n/a	n/a	Negative	Negative	*Cladosporium cladosporioides*
		9	49	Guano	n/a	n/a	Negative	Negative	Negative
		10	50	Guano	n/a	n/a	Negative	Negative	Negative
Campbell Cave (Eastern Cape)	**28-03-2021**	1	51	Guano + Soil	n/a	n/a	Negative	Negative	Negative
		2	52	Guano + Soil	n/a	n/a	Negative	Negative	Negative
		3	53	Guano + Soil	n/a	n/a	Negative	Negative	*Talaromyces* species
		4	54	Guano + Soil	n/a	n/a	Positive	Positive	*Parengyodontium album*
		5	55	Guano + Soil	n/a	n/a	Negative	Negative	Negative
NINON (Western Cape)	**16-05-2021**	1	56	Guano + Soil	n/a	n/a	Negative	Negative	Negative

6.4. Discussion

Molecular methods are useful for rapid detection of *H. capsulatum* from clinical and environmental samples. These assays have a better yield than culture since it has been a challenge to culture the fungus directly from soil because it takes a long time to grow and is frequently overgrown by fast-growing fungi such as *Aspergillus* and other environmental fungi. We used three different molecular methods to screen for *H. capsulatum* nucleic acids in environmental soil samples. *H. capsulatum* was detected in five of the seven South African caves by two of these molecular assays.

The positivity rate was 29% with the pandimorphic RT-qPCR assay and 25% with the Hc100 nested PCR assay. Twelve samples were positive with both the pan-dimorphic PCR and Hc100 nested PCR, indicating a high (67%) degree of concordance in both tests and giving more confidence in the results. None of the environmental samples were positive with the *ITS* PCR assay. This is mainly because the *ITS* primers used in amplification of nucleic acids from direct soil samples targets all fungal pathogens in the sample. This makes it difficult to detect pathogens that are available is smaller quantities. The fungal pathogen detected with this assay will be the most abundant one No major difference was noted between the pandimorphic RT-qPCR and the Hc100 nested PCR assay in detecting *H. capsulatum* from the environmental cave samples but from the validation results the RT-qPCR had a lower LOD. However, generally, RT-qPCR assays are more sensitive than conventional PCR assays since they require a small amount of DNA for the detection of fungal pathogens from samples. In our study, the RT-qPCR assay LOD was 0.000000001 ng/µl and Hc100 nested PCR was 0.0001 ng/µl. The pandimorphic RT-qPCR assay could be more useful in detecting *H. capsulatum* from environmental soil samples because it has a shorter turnaround time when compared to the Hc100 nested PCR which requires the performance of two different PCR assays which is then followed by sequencing. We do not recommend the use of the *ITS* panfungal conventional PCR in testing environmental soil samples because it is not specific for *H. capsulatum* and the dominant fungi in the environment get amplified with the assay.

For the nested Hc100 PCR assay, an NCBI Blast search confirmed the presence of the *H. capsulatum* 100kDa region in the environmental soil samples. However, our in-house validation showed cross-reaction with *Histoplasma capsulatum var. farciminosum* DNA

reducing its specificity. A nested PCR targeting the *ITS* region of the rRNA operon was evaluated before and recommended for screening of *Histoplasma capsulatum var. farciminosum* in equines (32). No studies have been conducted to confirm the cross-reactions between the different species of *Histoplasma* with the nested Hc100 PCR. The pandimorphic RT-qPCR has been previously used for screening of clinical samples and cross-reactions have been reported with *Blastomyces* (27,33). All PCRs were negative in two of the seven caves tested. A negative PCR in these caves does not exclude *Histoplasma* contamination. There is still a possibility that these samples were contaminated by bacteria and other fungi limiting the detection of *H. capsulatum*. It is important that proper measures still be put in place to avoid cave visitors coming in contact with the contaminated soil.

Outbreaks can be detected and controlled with the use of molecular tests that have shorter turnaround times. The Hc100 nested PCR was previously used to trace the source of infection for an outbreak that occurred in a hotel in Acapulco, Guerrero, Mexico leading to control measures to prevent more outbreaks in this hotel (13). Our results showed that 25% of the environmental samples collected from South African caves were contaminated with *H. capsulatum* and this positivity rate is higher than the 10.5% recorded from testing 239 environmental soil samples including composted organic fertiliser, chicken manure and soil samples from cave floors, collected in Columbia tested using the Hc100 nested PCR (23). A previous study by Gomez *et al* in 2019 also documented the testing of 393 Colombian environmental samples using the nested Hc100 PCR with a positivity rate of 9.9% which supports the need for more sampling as our study only had 56 soil samples (34). A more sensitive assay the *Histoplasma* 100-kDa real-time PCR was optimised for testing environmental samples by Gomez and others in 2022. They tested 332 samples collected in Columbia and detected *Histoplasma* in 80% of raw organic materials and 62% of composted materials supporting the importance of optimising new molecular methods with short turnaround times to give results when testing environmental samples (28). In South Africa, soil samples need to be collected from all provinces and from non-cave sites contaminated by bird and bat guano to get a better understanding of the environmental niche for *H. capsulatum* countrywide. Our results are of major significance as they provide preliminary evidence confirming the presence of *H. capsulatum* in caves frequently visited by cave explorers.

A limitation of our study was sampling bias introduced by collecting samples based on the assumption that *H. capsulatum* might be present based on previously reported cases of histoplasmosis in these caves. The assumption was also based on the presence of bats in the caves. Other studies have suggested that cave explorers might have been infected during their visits to these caves (19). A histoplasmosis case was described in the second known outbreak in the Cape province and other cave explorers also reported a suspicion that when they coughed, it might have been due to histoplasmosis contracted after visiting the caves(19). Such outbreaks warrant more research into areas of possible infections. The results from our study provide confirmation of the presence of *H. capsulatum* nucleic acids in South African caves. This information will help alert speleologists to exercise caution and wear protective equipment when visiting the tested caves to prevent potential infections. The positive tests from our study should also be taken with caution as the amount of the fungus present was not quantified; therefore this is not equivalent to the infectivity and viability of the *H. capsulatum* present in these caves. Another limitation of the study was that during samples collection, certain important parameters such as pH were not recorded for some samples due to lack of equipment therefore this parameter was not reported on.

Caution must be taken with the interpretation of our results due to the lack of epidemiological data on actual infections that have occurred when people visited these caves. There is a need to improve the reporting of histoplasmosis infections in cave explorers as this helps with future studies. More studies on the environmental sampling and testing of *Histoplasma* in soil need to be conducted to add to the current available knowledge. We did not compare molecular methods to any culturing of fungus from soil samples which typically involves passaging of soil samples through animals such as mice. Recovery of *H. capsulatum* using a mouse model presents challenges due to the need for animal facilities, time and expertise.

We recommend future studies that passage positive soil samples through mice for culturing *H. capsulatum* to confirm and further classify this pathogen. Finally, the testing of *H. capsulatum* in the environment is particularly important at sites that are used for recreational or tourist purposes as they guide the relevant authorities in implementing the necessary prevention and control measures to reduce infections.

Conclusion

Environmental samples from five of seven caves were PCR-positive for *H. capsulatum* by two different methods. Negative PCR results do not exclude *Histoplasma* contamination in the other two caves. There is a need for more sampling from all provinces in South Africa and to collect non-cave samples for a better understanding of areas of possible *Histoplasma* infection. Appropriate measures such as wearing of mask or respirators should be taken when exploring these caves to avoid exposure and infection with the fungus, particularly when handling soil contaminated with guano.

6.5. References

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CHAPTER 7: DISCUSSION

7.1. Discussion

Although endemic mycoses (blastomycosis, emergomycosis, histoplasmosis, coccidiodomycosis, paracoccidioidomycosis, sporotrichosis and talaromycosis) cause significant mortality and morbidity, their true burden remains unknown, and these diseases are mostly neglected. Only 4 of these are endemic in southern Africa that is blastomycosis, emergomycosis, histoplasmosis and sporotrichosis. Proper disease management is required, and it can be mainly achieved through a better understanding of the risk factors, diagnostic methods, epidemiology, pathogen genetic diversity and treatment methods. Global experts in mycology outlined guidelines for the management of endemic mycoses through the initiative of the European Confederation of Medical Mycology in 2021(1). To further understand and address some of the gaps in this research field, this thesis discusses the epidemiology of endemic mycoses in South Africa with a major focus on histoplasmosis, the evaluation of current diagnostic methods for histoplasmosis, the analysis of genetic relations for global *Histoplasma* isolates and the environmental niche of *Histoplasma* in South Africa.

The precise global prevalence data for endemic mycoses is not available due to underreporting and varying levels of awareness and challenges associated with the diagnosis of such infections. We conducted a comprehensive analysis of laboratory records of all endemic mycoses reported in South Africa for a period between 2010 and 2020 in one major private and all public pathology laboratories. Our analysis resulted in the identification of 682 cases of endemic mycoses. Approximately a third of cases were categorised as an unspecified endemic mycosis as the causative pathogen could not be identified to genus level due to the limitations of the available diagnostic methods. Sporotrichosis was the most common endemic mycosis followed by emergomycosis, histoplasmosis, blastomycosis and rarely, talaromycosis, an imported mycosis. Reports on endemic mycoses were previously mainly from the Americas, Asia and West Africa especially, from Nigeria and Ghana (2–5). This could be because, in Africa, most countries do not have disease surveillance systems for endemic mycoses since they are not notifiable diseases (6,7). The availability of a centralised reporting database for all endemic mycoses diagnosed and confirmed may assist in the early detection of outbreaks. Clinical manifestations of endemic mycoses depend highly on the host's immune status and patients with immunocompromised host defences such as those

living with HIV and transplant recipients who are on immunosuppressive therapy can progress to disseminated disease. Diseases such as histoplasmosis and sporotrichosis have the potential to cause outbreaks (8–10). Several outbreaks associated with endemic mycoses have been reported in South Africa (11–13). The diagnosis of endemic mycoses is more challenging especially in cases of disease reactivation when patients have departed from areas of endemicity and now reside in geographic areas where the pathogens are rarely encountered. Pathogens causing endemic mycoses can stay dormant in the immune system for as long as 50 years if healthy individuals get infected and will only get reactivated once the immune system of the individual becomes compromised. For HIV-associated infections such as histoplasmosis and emergomycosis, the high burden of AIDS has increased the cases of endemic mycoses on the African continent and this leads to complications with the treatment of such HIV-infected patients as they may have co-infections and do not respond well to medications (14–17). In the attempt to determine the national epidemiology of endemic mycoses in South Africa, we estimated that there was an underreporting of cases even though we just looked at laboratory records without any other reference to the true burden of disease because of the disproportionately high burden of HIV-AIDS in the country, lack of access to proper health care and unavailability of effective antifungal drugs. Immunocompromised individuals are at a higher risk of infections by opportunistic infections such as *Histoplasma spp* and *Emergomyces spp*. However, we observed few cases reported yearly. Of note is the rise in cases reported coinciding with the study on AIDS-related endemic mycoses that was performed at a large hospital in Western Cape and decreases in cases reported after the study was finished which could have been driven by better reporting of emergomycosis which was a major focus during the period of the study (14). A population-based national study was performed in the United States in 2002 looking at a database containing hospitalisation records for patients with endemic mycoses with results showing 22.5 cases per million requiring hospitalisation and fatality rates of 4.6 and 28.7 cases per million highlighting the importance of studying these diseases (18). The prevalence of endemic mycoses was compared to other diseases in the United States of America and in 2022 alone, the USA reported approximately 1.9 million new cancer cases and 609,360 cancer-related deaths—figures that are significantly higher than those for endemic mycoses (20) .Blastomycosis has been reported at 1-2 cases per 100 000 population with paracoccidiodomycosis being reported mainly in Brazil (80% of cases). In the Americas 2 cases of sporotrichosis per 1 million population (20). In South Africa, enhanced surveillance was conducted in the Western Cape province from 2008 to 2011 on infections caused by

systemic and dimorphic fungi and 24 cases caused by thermally dimorphic fungi were identified (21). We also surveyed how pathology laboratories were conducting the actual diagnosis of these cases through a questionnaire. The results from this survey indicated a lack of skills and facilities for proper diagnosis of endemic mycoses as indicated by responses to our survey where most laboratory workers were not keeping culture plates for long periods which is a requirement for the growth of some endemic fungi like *Histoplasma*. Public health efforts should be increased to decide which diseases among endemic mycoses to be included on mandatory reporting to reduce unnecessary morbidity and mortality based on the incidence rates. More funding needs to be invested into neglected endemic mycoses to address the knowledge gaps in this field. Awareness campaigns of these diseases should also be increased in the general population and among healthcare workers.

Timely diagnosis of diseases is important as it allows clinicians to initiate early and appropriate treatment for patients. There are also challenges that are associated with the diagnosis of histoplasmosis. The main challenges include the low suspicion index as well as the lack of tools for rapid and accurate diagnosis. Diagnosis of histoplasmosis is also challenging because symptoms in people living with HIV lack specificity and also present with symptoms similar to opportunistic infections caused by *Mycobacterium* species. Histoplasmosis diagnosis is associated with long turn-around times when using culture methods, lack of biosafety level 3 facilities, lack of skilled people for accurate identification of the pathogen, lack of funds to purchase antigen detection tests, lack of antigen and antibody tests that can easily be implemented in pathology laboratories, lack of standardisation of molecular tests, and the misidentification of *H. capsulatum* in both culture and histology due to its similarities with other closely related fungi. Identification using histopathology and culture requires personnel who are skilled. Other routine laboratory diagnostic methods such as the MALDI-TOF system can provide presumptive identification (22,23). Relatively easy-to-obtain samples such as urine, blood, serum and plasma can be used for antigen detection and molecular tests, improving the turn-around time for diagnosis (24–26). Currently, antigen detection tests are considered the quickest and most sensitive and are useful in the diagnosis of disseminated histoplasmosis (27). At the moment antigen-detecting assays and real-time PCR assays are not readily available in the majority of the diagnostic laboratories in South Africa and the rest of Africa because of high cost, for example, a single IMMY Clarus EIA kit with 96 tests costs R21 505 (US$1228) which is

unaffordable in most settings (6,28,29). In South Africa, the National Institute for Communicable Diseases, Mycology Reference Laboratory (NICD, MRL) serves as a reference centre for all diagnostic laboratories and currently the Clarus *Histoplasma* antigen assay (IMMY, Norman, United States) is South African National Accreditation System (SANAS) accredited and currently being used for diagnosing of disseminated histoplasmosis from suspected patients' urine samples. We validated the rapid LFA from Optimum Diagnostics and the *Histoplasma/Emergomyces* RT-qPCR for diagnosis of histoplasmosis and used these tests to screen for histoplasmosis in the South African population of people living with HIV. The results showed that 6% of the 194 patients included in the study had a positive test with the LFA assay and 3% has a positive result with the EIA assay. In our study we concluded that diagnostic assays with shorter turn-around times such as the LFA and EIA assays are useful and should be implemented in pathology laboratories. These assays do not need expensive infrastructure and give reliable results and can be used to inform decisions on preliminary treatment while waiting for culture results which is the current gold standard in histoplasmosis diagnosis. Simple assay such as LFA and EIA make use of easy to obtain specimens such as urine and blood and can be used in prevalence studies to stay informed on disease trends (30–33). Clear improvement in the management of histoplasmosis has been reported when using antigen detection tests and they have been included in the WHO list of essential diagnostic tests (34).

The usefulness of LFA and EIA were shown in various validations in patients from the United States of America and Colombia and comparisons have been previously done versus the EIA (33,35,36). Such quick and easy-to-use diagnostic tools are relevant for public health as they provide quicker results compared to culture and help reduce costs, although they may need to be used together with other confirmatory tests. Standardisation of RT-qPCR assays is required as the field now has evidence of development of many such assays targeting various genes such as the *ITS* multicopy region of the ribosomal gene, genes coding for the M antigen, mtSSU and 100 kDa protein which are validated and used specifically in different laboratories (24,25,28,37). The techniques for these assays include both conventional and nested PCR as well as real-time PCR. In Spain, a reference laboratory developed various real-time PCR assays that had a superior sensitivity of 89% compared to culture results (73%) and antigen detection (40%) (37). The validation of the pandimorphic RT-qPCR assays adds support that this assay can be used as a diagnostic tool. So far there is no standardisation on

PCR tests available for histoplasmosis diagnosis and no such test has been commercialised for in-vitro diagnosis but results from evaluations of available tests are promising. Caceres et al (2019), reported overall sensitivity of 95% and specificity of 99% for PCR tests in people living with HIV after a meta-analysis of molecular assays targeting different *Histoplasma* genes following a literature search (38). Adequate training will need to be provided to support the use of these tests. Cross-reactions have been reported but they do not require alteration of treatment plans as mycoses caused by most thermally dimorphic fungi are treated with the same anti-fungal drugs such as histoplasmosis and emergomycosis. Our results provide further evidence that antigen tests and RT-qPCR assays can be important in reducing the turn-around time for histoplasmosis diagnosis in South African patients.

There has been a lack of studies on the comparative genomics of *Histoplasma* isolates from the African continent to isolates from other parts of the world. Most studies on the genetic differences of *H. capsulatum* isolates have been focused on strains from the Americas. The most widely recognised clades are the North American and the Latin American clades, which are geographically separated and have distinct phenotypic and molecular characteristics. The African continent is home to two varieties that cause histoplasmosis in humans, that is *H. capsulatum* var *duboisii* (*Hcd*) *and H. capsulatum* var *capsulatum (Hcd)*. *Hcd* and *Hcd* are distinguished by their geographic distribution, clinical presentation, and phenotypic characteristics when observed inside tissues although genetically they all clustered into one clade (39). A study by Sepulveda was the first to characterise *Histoplasma* isolates using whole genome sequences, leading to the renaming of *Histoplasma* cryptic species (44). In our study, we compared the genetic similarities of African isolates with global isolates and the results showed that African isolates form a distinct population which was different from the rest of the global isolates. These African isolates were closely related to *Histoplasma sensu stricto* species. The evidence from this study showed that the African isolates form a new species *Histoplasma africanus sp nov*. The new cluster described shows lack of gene flow between species and enough evidence of genetic variation within and across populations of species. Most importantly, this thesis shows that whole genome sequencing can distinguish between isolates from different geographical regions. A similar study on genomic analysis of Indian isolates revealed a distinct monophyletic clade and this is similar to our observation with the African isolates (40). A study on *Histoplasma* isolates in Alberta, Canada using whole genome sequences included isolates from 3 local outbreaks and a comparison of these

isolates showed that they were genetically distinct from isolates from imported cases into Canada indicating local acquisition of histoplasmosis. *Histoplasma* had not been previously reported in this region and the study indicated an expansion of the geographical range for *Histoplasma* in this country (41).

There is published evidence that *Histoplasma* is found in soil especially contaminated with birds and bat droppings (42–44,44,45). No evaluation and screening for histoplasmosis in soil samples using molecular methods has been reported in South Africa although screening for *Emergomyces* nucleic acids has been performed on South African soil samples with 30% (18/60) positivity rate (46). Therefore, we evaluated molecular methods for screening for *Histoplasma* nucleic acids in environmental samples and testing samples from frequently visited caves in South Africa. We chose cave samples due to the high probability of finding *Histoplasma* nucleic acids in these samples because of the association of *Histoplasma* with bat droppings. Seventy-one percent (5/7) of the caves screened for *Histoplasma* in our study were found to be positive either by nested Hc100 or the pan-dimorphic RT-qPCR. Traditionally, screening for *H. capsulatum* in soil samples was conducted by passaging soil samples using mice and then culturing their lungs and livers for *Histoplasma* growth (42,46). This method has long turnaround times and requires established animal facilities to perform the experiments and a study for screening for Emergomyces nucleic acids by passaging soil samples through mica failed due to contamination (46). PCR tools can potentially be used to provide rapid sensitive and specific detection of *Histoplasma* in soil samples. While the tests used in this study have been evaluated in clinical samples to a larger extent, the positive results from our study showed the usefulness of these tools in environmental screenings. This study lacked a true reference making it difficult to rule out false positives and that was a limitation noted. To confirm the positives from this study we performed Sanger sequencing on the Hc100 PCR products from the nested PCR and confirmed the identity of *Histoplasma* using nucleotide BLAST and they all were similar to the *Histoplasma* 100 kDa gene. The Hc100 PCR and pan-dimorphic RT-qPCR may be useful in testing environmental samples for *Histoplasma* nucleic acids providing knowledge on areas contaminated with *Histoplasma* and measures can be put in place when visiting such areas, especially for people with compromised immune systems. Therefore, there is a need to test a wider range of soil Samples in South Africa to acquire more information on the coverage of this pathogen. The Hc100 nested PCR has been previously evaluated for use in testing soil samples versus the

100 kDa real-time PCR by Gomez and others in 2022 and the real-time PCR was able to identify 60% more positive results (45). Such high sensitivity is expected since real-time PCR does not rely on the visualisation of products on agarose gel allowing detection of low concentrations of *Histoplasma* nucleic acids. Conventional PCR amplifying the *ITS* gene was previously used to detect similar pathogens such as *Emergomyces* in soil samples in South Africa and positivity rates up to 30% were reported (46). The *ITS* PCR used in the *Emergomyces* study by Schwartz and others in 2018 was followed by amplification with *Emergomyces*-specific primers and this is different from our study in which we used only *ITS* primers to record the dominating fungus in the sample. To get more specific results we recommend performing *ITS* amplification followed by *Histoplasma* specific primers as well. The soil study results from this thesis reinforce the importance of studying sources of infection to prevent or reduce infections and inform on policies on proper protection guiding people visiting areas considered as high risk for infection.

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CHAPTER 8: CONCLUSION, LIMITATIONS AND RECOMMENDATIONS

8.1. Conclusion

This thesis addressed the epidemiology of endemic mycoses in South Africa. We also studied the comparative genomics of *Histoplasma* isolates from the African continent with other global isolates, various diagnostic methods for histoplasmosis were evaluated and used to screen for *Histoplasma* antigen in HIV-positive patients and environmental samples from South African caves. The true global burden of histoplasmosis remains unknown and this study of reported cases over a decade indicated underestimation and misdiagnosis of cases of endemic mycoses in South Africa especially for histoplasmosis and emergomycosis due to their association with HIV considering the high burden of HIV in the country. Due to challenges associated with the diagnosis of histoplasmosis and the lack of awareness of this disease, it remains hard to know the actual prevalence and incidence of the disease. This information is important especially in South Africa since a few outbreaks have been reported especially in cave explorers and the fact that the disease is associated with HIV and causes significant morbidity and mortality in immunocompromised individuals. Furthermore, the country has a high burden of HIV disease which is highly associated with histoplasmosis and emergomycosis. The African continent hosts two varieties of *H. capsulatum* causing disease in humans; therefore, we performed genomic analysis and speciation studies to help understand the different disease variations observed. Evidence from this study indicates that African isolates form a single clade regardless of the country of origin, with no genetic distinction observed between *Histoplasma capsulatum* var. *capsulatum* and var. *duboisii*, as all clustered within a single genetic populationThe evidence from the study of whole genome sequences of African isolates supports the description of a novel species *Histoplasma africanus sp nov* which has not been described before. Since African isolates form a distinct group, there is a need to perform further studies into the virulence factors of these isolates in comparison with other global isolates to gain an understanding if there is need for the development of clade-specific diagnostic methods and treatment methods. Current knowledge shows that African histoplasmosis has a few different symptoms to the histoplasmosis reported in other part of the world such as, mainly presenting as skin lesions and affecting the bones, then there is need to further investigate the virulence factors in this group of isolates. *Histoplasma* has generally been understudied on the African continent and efforts should be

increased to better understand the impact the pathogen has on the continent. Generally, sequencing whole genomes is becoming more affordable therefore, there is a need for continued worldwide assessment of the genomic differentiation of this fungal pathogen. This study led to the identification and description of a new species, *Histoplasma africanus* sp. nov., based on detailed sequence analysis and its distinction from other global isolates. This is important as it adds knowledge to the understanding of the classification of this genus and resolving inconsistencies previously brought by genotyping using only housekeeping genes.

Histoplasmosis remains a neglected infection in South Africa and the rest of the African continent and in many cases, it is misdiagnosed or underreported, due to the lack of skilled personnel and clinical awareness. The validation of easy-to-implement assays with short turn-around times for histoplasmosis diagnosis in this study and their usefulness in South African patients to allow better management of this disease and avoid a higher burden on public health resources by reducing the hospitalisation time for patients. Our screening for *Histoplasma* nucleic acids in HIV patients' blood provides an alternative screening method that can be used together with the gold standard testing method of culturing while guiding clinicians on how to treat patients while waiting for the long turnaround time results from culture. Several strategies need to be implemented in the South African health system to curb the devastating impact of this disease including implementing a decentralised model for antigen testing allowing easy access to treatment for patients. Studying the sources of infection for histoplasmosis provided evidence to support the need for taking extra caution when visiting areas with confirmed *H. capsulatum* presence. The testing for soil samples was important because proper measures will be put in place for cave explorers and tourists who visit caves harbouring *H. capsulatum*. Such results support the hypothesis that soil is a reservoir for pathogens such as *H. capsulatum*. Future studies need to be carried out on soil from areas suspected to be harbouring *H. capsulatum* such as old buildings infested with bats, chicken coops and more caves frequently visited by tourists and cave explorers throughout the whole country to further knowledge of the ecological niche of *H. capsulatum*. Overall, concerted efforts need to be made to address all these challenges discussed in this thesis presented by histoplasmosis and other endemic mycoses. It is also important to increase awareness of endemic mycoses in clinicians in areas of low endemnicity for better diagnosis and management of the diseases.

8.2. Limitations

Several limitations were observed in the sub-studies, and they are listed below:

1. In our study of the epidemiology of endemic mycoses in South Africa, we searched laboratory databases for cases reported between the period of 2010-2020. A limitation was the search strategy used in the laboratory information systems. Some cases could have been missed due to the terminology used during the search. For example, histology results were searched using the systemised nomenclature of medicine SNOMED clinical terms or the names of the thermally dimorphic fungi or diseases and some cases could have been missed.
2. The epidemiology study also lacked information on proper reporting of areas of infection versus areas where patients received diagnosis and treatment as patients sometimes travel to larger hospitals with better resources to seek treatment. We did not have any records of previous travel for patients and had no access to addresses for patients to see if there was a pattern in certain areas visited previously by patients and the infections diagnosed linking cases reported to areas where patients got infected. Mapping data is generally difficult to link with infections as endemic mycoses stay in the patient's immune system lying dormant for many years at times if they are immunocompetent, only to resurface once their immune system is compromised again. This makes it difficult to map sources of infection based on areas where hospitalisations are reported. Therefore, our mapping of laboratory-diagnosed cases by province cannot be used to infer areas of higher or lower endemicity.
3. We also collected very limited demographic and clinical data in this retrospective laboratory surveillance. Characteristics such as ethnic group, underlying medical condition and immunosuppressing medicines are important to report together with epidemiological reports for diseases but in our study, these data were not consistently available. Travel history and data on occupational and recreational pursuits were generally not available.
4. A study limitation observed was that we did not manage to culture and test all isolates included in the comparative genomics study for antifungal susceptibility in order to get more comprehensive data due to challenges with moving *Histoplasma* isolates across borders. We conducted antifungal susceptibility testing on 15 isolates that were cultured at NICD in South Africa and for the rest of the African isolates, we only had

access to DNA sent from the Institut Pasteur for sequencing and sequences available from collaborators in Brazil; antifungal susceptibility testing was not conducted on these isolates. This was largely due to challenges associated with moving hazardous biological substances across borders since *H. capsulatum* is considered a high-risk pathogen requiring high containment.

5. The evaluation of different diagnostic tests using urine samples from HIV-positive patients had a limitation that the testing was not done side by side due to the unavailability of the lateral flow assay when the enzyme immunoassay was used for testing. Therefore, the results reported were tested at different time points but it is still important to report such data as it informs future research.
6. In the study for the presence of *Histoplasma* nucleic acids in soil samples, we managed to collect samples only in Gauteng, Western Cape and Eastern Cape provinces due to the challenges with accessing caves throughout the country. This was noted as a limitation because for a better understanding of the infection sources for histoplasmosis, more samples need to be collected across the country and the data needs to be availed to all cave explorers in order for them to take proper precautionary measures when visiting caves with reported *Histoplasma* presence.
7. The collection of soil samples in our study was also biased as it was based on assumptions of areas where previous infections might have occurred. There was no proper documentation of the previous infections, and we used knowledge and reports from speleologists in the areas where the soil samples were collected.
8. The molecular methods used for the screening of *Histoplasma* in soil samples were also not compared to the gold standard method which is passaging soil samples through mice and then culturing their livers and lungs. This was because we did not have an animal model set up for such experiments and this was beyond the scope of our study. Direct culturing of soil samples is challenging because there are usually high numbers of contaminants in the soil and since *Histoplasma* is a slow-growing fungus, it gets overgrown by the other contaminating fungi.

8.3. Recommendations

We recommend that awareness campaigns for endemic mycoses for clinicians and training of laboratory personnel on the diagnosis of these diseases be carried out throughout South Africa for both private and public hospitals to increase the number of cases being reported.

During the years 2010 to 2020, a spike in cases reported was observed in 2014-2015 and this was largely attributed to a sub-study that was conducted in the Western Cape focussing on patients living with HIV who were infected by pathogens causing endemic mycoses. This result indicates that many cases are being missed generally due to challenges that are associated with the diagnosis of endemic mycoses. The analysis of *Histoplasma* whole genome sequencing data also highlighted the lack of isolates from African patients. The recommendation would be to have collaborations throughout Africa on how to collect or form an isolate bank and for sequencing to be done and data to be shared and publicly available for such neglected infections. Collaborations will help with the sharing of ideas between researchers who deal with infections from endemic mycoses as the knowledge on the molecular analysis of these isolates is largely lacking. During this study, we also observed that African researchers (bioinformaticians) with the knowledge to analyse *Histoplasma* whole genome sequencing data are scarce - we collaborated with researchers in the United States of America. Histoplasmosis has similar symptoms to TB but studies on the association of these two diseases are lacking in South African patients. We recommend more studies be carried out for further understanding of the association rates between these two diseases. The recommendation would be to fund such training and promote the sharing of knowledge throughout the whole African continent. We also recommend storage of isolates at both room temperature in water and at -80°C in glycerol for all *H. capsulatum* isolates from the whole continent to avoid contamination as they will be needed for future studies. The diagnosis of histoplasmosis is generally challenging, and we recommend the standardisation of methods with short turn-around times such as RT-PCR assays in order to decrease the rates of unnecessary morbidity and mortality. In South Africa, introducing point-of-care testing methods such as lateral flow assays and enzyme immunoassays in all diagnostic hospitals and clinics to decentralise the testing currently being mainly performed only at the reference laboratory will also be useful as our study showed that most diagnostic laboratories cannot carry out such tests. We also recommend the collection of fresh samples and immediate testing of the collected samples for better and more reliable results. We recommend the collection of more environmental samples from bats infected caves, old buildings and farms or individual households with chicken coops for the screening of *Histoplasma* nucleic acids around South Africa as such a study will help with providing information necessary for the implementation of measures for controlling infections when people visit endemic areas such as tourist caves. A similar study was conducted in South Africa screening for *Emergomyces* nucleic acids in 60 soil samples and 18 of them were found positive, therefore, we

recommend screening for *Histoplasma* in these same samples for convenience and access the positivity rate in the same soil samples. This will add on to the knowledge on the prevalence of *Histoplasma* in these caves as they were collected from Gauteng, Eastern Cape, KwaZulu Natal, and Northern Cape, thereby covering more South African provinces in this soil screening. Lastly, we recommend the evaluation of the recently published *Histoplasma* 100 kDa real-time PCR because of this higher sensitivity and this assay has a short turn-around time to give results and is less prone to cross contamination when compared to the nested PCR.

Appendix A- Ethics PhD study approval

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

R49 Ms RE Mapengo

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)
CLEARANCE CERTIFICATE NO. M210109

NAME: (Principal Investigator)	Ms RE Mapengo
DEPARTMENT:	School of Pathology Dept of Clinical Microbiology & Infectious Diseases Medical School University
PROJECT TITLE:	*Investigating diagnostic methods for histoplasmosis and the ecological niche and comprehensive population structure genomic analysis of African Histoplasma capsulatum*
DATE CONSIDERED:	2021/01/29
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Professor N Govender
APPROVED BY:	Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	2021/05/19

This Clearance Certificate is valid for 5 years from the date of approval. An extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Research Office secretariat on the 3rd floor, Phillip Tobias Building, Parktown, University of the Witwatersrand, Johannesburg.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated from the research protocol as approved, I/we undertake to submit details to the Committee. **I agree to submit a yearly progress report**. When a funder requires annual re-certification, the application date will be one year after the date when the study was initially reviewed. In this case, the study was initially reviewed in **January** and therefore reports and re-certification will be due in the month of **January** each year. Unreported changes to the study may invalidate the clearance given by the HREC (Medical).

Signature of Principal Investigator

21/05/2021
Date

Appendix B- Ethics approval for *Diagnostics* study (CICAS urine Samples)

R14/49 Dr Rae Wake et al

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M141177

NAME: (Principal Investigator)	Dr Rae Wake et al
DEPARTMENT:	National Institute of Communicable Disease Tambo Memorial Hospital, Helen Joseph Hospital, Free State Primary Care HIV Clinics and Chris Hani Baragwanath Academic Hospital
PROJECT TITLE:	Optimising the Clinical Impact of Cryptococcal Antigen Screening and Management of Asymptomatic Human Immunodeficiency Virus Infected Individuals
DATE CONSIDERED:	28/11/2014
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr Nelesh Governder, Prof Thomas Harrison and Dr Joseph Jarvis
APPROVED BY:	Professor P Cleaton-Jones, Chairperson, HREC (Medical)
DATE OF APPROVAL:	22/06/2015

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

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report**.

Principal Investigator Signature Date 22.6.15

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix C- Ethics approval for *Diagnostics* study (Blood Samples)

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

Office of the Deputy Vice-Chancellor (Research and Postgraduate Affairs

TO: Professor NP Govender
School of Pathology
National Institute for Communicable Diseases
Sandringham

E-mail: neleshg@nicd.ac.za

CC: Supervisor: Not applicable
<>
and <HREC-Medical Research Office@wits.ac.za>

FROM: Mr Iain Burns
Human Research Ethics Committee (Medical)
Tel: 011 717 1252

E-mail: Iain.Burns@wits.ac.za

DATE: 2021/03/09

REF: R14/49

PROTOCOL NO: **M201174** (This is your ethics application reference number. Please quote it in all enquiries, oral or written, relating to this study.)

PROJECT TITLE: *Quality assurance of a cryptococcal antigen-screening programme for people living with advanced HIV*

Please find attached the Clearance Certificate for the above project. I hope it goes well and that an article in a recognized publication comes out of it. This will reflect well on your professional standing and contribute to Government funding of the University.

MSWorks2000/Iain0007/Clearscan.wps

Appendix D- Turnitin report

Rutendo_Mapengo_PHD THESIS 14-10-2024_v4-1.docx

by Rutendo Mapengo

Submission date: 14-Oct-2024 04:34AM (UTC+0200)
Submission ID: 2484400541
File name: Rutendo_Mapengo_PHD_THESIS_14-10-2024_v4.docx (4.1M)
Word count: 27689
Character count: 156658

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