Molecular epidemiology of azole-resistant *Candida* species in South Africa

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Declaration

I, Rindidzani Edith Magobo, declare that this thesis is my own work. It is being submitted for the degree of Doctor of Philosophy in the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at this or any other University.

_____ 24 August 2020

Rindidzani E. Magobo Date

Dedication

I would like to dedicate this thesis to my parents, Malindi Johannes Magobo and Luvhengo Constance Magobo for their sacrifices and prayers, always stepping-in and standing in the gap to look after my children when I needed you the most, for all the opportunities they have given, for always reassuring me that I can achieve whatever I want if put my trust in the Lord. For this and many other sacrifices, I will forever be grateful.

Publications arising from this PhD

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2. Rindidzani E. Magobo, Shawn R. Lockhart, Nelesh P. Govender. Fluconazole-resistant *Candida parapsilosis* strains with Y132F substitution in *ERG*11 gene causing invasive infections in neonatal unit, South Africa. Mycoses. 2020. (In press) (**Chapter 4**)

3. Rindidzani E. Magobo, Craig Corcoran, Sharona Seetharam, Nelesh P. Govender. *Candida auris*-associated Candidemia, South Africa. Emerging Infectious Diseases. 2014; 20 (7): 1250-1252. (**Chapter 5**)

4. Nelesh P. Govender, Rindidzani E. Magobo, Ruth Mpembe, Mabatho Mhlanga, Phelly Matlapeng, Craig Corcoran, Chetna Govind, Warren Lowman, Martinus Senekal, Juno Thomas. *Candida auris* in South Africa, 2012-2016. Emerging Infectious Diseases. 2018; 24 (11): 2036-2040. (**Chapter 6**)

5. Rindidzani E. Magobo, Mabatho Mhlanga, Craig Corcoran, Nelesh P. Govender. Multi-locus sequence typing and antifungal susceptibility of *Candida auris* strains, South Africa. South African Journal of Infectious Diseases. 2020. (In press) (**Chapter 7**)

Presentations arising from this degree

1. Rindidzani E. Magobo, Craig Corcoran, Seetharam Sharona, Naicker Serisha, Nelesh P. Govender. Nelesh. *Candida auris*: An emerging, azole-resistant pathogen causing candidaemia in South Africa. 16th International Congress on Infectious Diseases. 2-5 April 2014, Cape Town, South Africa.

2. Rindidzani E. Magobo, Serisha D. Naicker, Jeanette Wadula, Maphoshane Nchabeleng, Yacoob Coovadia, Anwar Hoosen, Shawn R. Lockhart, Nelesh P. Govender for the TRAC-South Africa group. Detection of undiagnosed clusters of neonatal *Candida parapsilosis* fungaemia through sentinel surveillance, South Africa, 2009-2010. The 6th FIDSSA Congress. 5-8 November 2015. Drakensberg, South Africa.

3. Rindidzani E. Magobo, Nelesh P. Govender, Craig Corcoran. Molecular typing of multidrug-resistant *Candida auris* in South Africa. Faculty of Health Sciences Research day, University of Witwatersrand. 1 September 2016. Johannesburg, South Africa.

4. Rindidzani E. Magobo, Nelesh P. Govender, Craig Corcoran. Molecular typing of multidrug-resistant *Candida auris* in South Africa. The 6th Africa Society for Laboratory Medicine. 3-8 December 2016. Cape Town, South Africa.

5. Rindidzani E. Magobo, Mabatho Mhlanga, Craig Corcoran, Chetna Govind, Warren Lowman, Martinus Senekal, Juno Thomas, Nelesh P. Govender. The epidemiology of *Candida auris* in South Africa, 2012-2016. 7th FIDSSA Congress. 9-11 November 2017. Cape Town, South Africa.

Abstract

Introduction

With an increase in the prevalence of non-*albicans Candida* species associated with candidaemia and emergence of multidrug-resistant *Candida* species, we conducted a number of studies to address the epidemiology, genetic relatedness, and possible mechanisms of antifungal resistance in two *Candida* species (*Candida parapsilosis* and *Candida auris*) causing candidaemia in South Africa.

Methods

Study 1: *C. parapsilosis*

From February 2009 through to August 2010, cases of candidaemia were reported through national laboratory-based surveillance from public-sector hospital neonatal units in South Africa. Bloodstream *Candida* isolates were submitted to the National Institute for Communicable Diseases (NICD) in Johannesburg with corresponding laboratory reports containing patient demographic data such as age, sex, hospital and hospital unit. An incident case of candidaemia was defined as a patient of any age admitted to a sentinel hospital with first isolation of any *Candida* species from blood culture. A neonate was defined as a patient aged ≤28 days admitted to neonatal intensive care unit. Species identification was confirmed using ITS and D1/D2 sequencing, followed by genotyping using polymorphic microsatellite assay. Antifungal susceptibility results were analyzed using CLSI guidelines. To screen for mutations that confer resistance to azole antifungals, we sequenced the *ERG11* gene and transcription factor gene

MRR1 of *C. parapsilosis* isolates. The deduced nucleotide sequences were then compared to *C. parapsilosis* 22019 ATCC wild-type *ERG11* and CDC317 wild type *MRR1* strain.

Study 2: *C. auris*

We investigated the genetic relatedness of clinical *C. auris* isolates from patients admitted to either public- and private-sector hospitals in SA, which were submitted to a reference laboratory from 2012 through to 2015. Patient demographics and clinical details were recorded. We performed antifungal susceptibility testing, sequencing of the hotspot 1 and 2 regions of the *FKS1* and *FKS2* genes for all isolates with an echinocandin MIC of ≥1 μg/mL and cluster analysis using multilocus sequence typing. To address the epidemiology of *C. auris* in South Africa, we reviewed data from public- and private-sector diagnostic laboratories that reported confirmed and probable cases of invasive disease and colonization for October 2012-November 2016. We defined a case as a first isolation of *C. auris* from any specimen from a person of any age admitted to any healthcare facility in South Africa. We defined probable cases as cases where the diagnostic laboratory had used a nonconfirmatory biochemical identification method and *C. haemulonii* was cultured.

Results

Study 1: *C. parapsilosis*

Of 1671 cases with a viable *Candida* isolate, 393 (24%) occurred among neonates. Isolates from 143 neonatal cases were confirmed as *C. parapsilosis* sensu stricto. A large proportion of *C.*

parapsilosis isolates were resistant to fluconazole (77/143; 54%) and voriconazole (20/143; 14%). Of 79 closely-related genotypes, 18 were represented by ≥2 isolates; 61 genotypes had a single isolate each. Seven clusters, comprised of 82 isolates, were identified at 5 hospitals in 3 provinces. Isolates belonging to certain clusters were significantly more likely to be fluconazole resistant: all cluster 7 isolates and the majority of cluster 4 (78%), 5 (89%) and 6 (67%) isolates ($p<0.001$). A total of 73 isolates with antifungal susceptibility results from a single neonatal unit were analysed for mutations. Of these, 57 (78%) were resistant, 11 (15%) susceptible-dose-dependent and 5 (7%) susceptible. The most commonly identified amino acid substitution within the *ERG11* gene was Y132F in 68% (39/57) of fluconazole-resistant isolates and none in susceptible isolates. Three amino acid substitutions (R405K, G583R, and A619V) and 1 nucleotide deletion at position 1331 were identified within *MRR1* gene in 19 (26%) isolates. Microsatellite genotyping of 73 cases from a single neonatal unit grouped isolates into four clusters (50 isolates). Cluster 1 accounted for 23% (17/73) of all cases, cluster 2 for 22% (16/73), cluster 3 for 14% (10/73) and cluster 4 for 10% (7/73). We found an association between cluster type and fluconazole resistance (p-value=0.004). Isolates harboring the Y132F substitution were more likely to belong to a cluster than non-Y132F isolates.

Study 2: *C. auris*

Eighty five isolates were confirmed as *C. auris*. The median patient age was 59 years (IQR: 48-68 years), with males accounting for 68% of cases. Specimen types included: urine (29%), blood (27%), central venous catheter tips (25%), irrigation fluid (7%), tissue (5%), respiratory tract specimens (4%) and other (3%). Ninety seven per cent of isolates were resistant to fluconazole; 7% to both fluconazole and voriconazole; 8% to both fluconazole and echinocandins (considered multidrug-resistant) and all were susceptible to amphotericin B. Of 15 randomly-selected

fluconazole-resistant isolates, 14 had an isavuconazole MIC ≤1 μg/mL. No *FKS* mutations were detected. MLST analysis grouped isolates into two clusters: cluster 1 and cluster 2 comprising of 83 and 2 isolates respectively. To address the epidemiology of *C. auris* in South Africa, we analyzed 1,692 cases; 93% were from private-sector healthcare facilities, and 92% of cases from known locations were from Gauteng Province. Of cases with available data, 29% were invasive infections. The number of cases increased from 18 (October 2012-November 2013) to 861 (October 2015-November 2016).

Conclusion

We found that *C. parapsilosis*-associated candidaemia in public-sector neonatal units was caused by closely related genotypes, and there was molecular evidence of undetected outbreaks as well as intra-hospital transmission. Fluconazole resistance in *C. parapsilosis* strains from a single South African neonatal unit was predominantly driven by Y123F amino acid substitutions in the *ERG11* gene. Azole-resistant *C. auris* strains circulating in South African hospitals were related by MLST but the possibility of nosocomial transmission should be explored using a more discriminatory technique such as whole genome sequencing. Our results show a large increase in *C. auris* cases during the study period, centered on private hospitals in Gauteng Province.

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Table of contents

List of figures

List of tables

List of abbreviations and symbols

ABC	ATP binding cassettes
AFLP	Amplified fragment length polymorphism
BSI	Bloodstream infection
CDC	Center for disease prevention and control
CLSI	Clinical and Laboratory Standards Institute
CSF	Cerebrospinal fluid
CVC	Central venous catheters
DNA	Deoxyribonucleic acid
HIV	Human immunodeficiency virus
ICU	Intensive care unit
IPC	Infection prevention and control
ITS	Internal transcribed spacer
MDR	Multidrug resistant
MFS	Major facilitator superfamily
MIC	Minimum inhibitory concentration
MIC50	Minimum inhibitory concentration required to inhibit 50% of organisms
MLST	Multilocus sequence typing
NAC	Non-*albicans Candida*
NHLS	National Health Laboratory Service
NICD	National Institute for Communicable Diseases

NICU	Neonatal intensive care unit
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PFGE	Pulse field gel electrophoresis
PMM	Polymorphic microsatellite markers
QC	Quality control
RAPD	Random amplification of polymorphic DNA
RFLP	Restriction fragment length polymorphism
SDA	Sabourad Dextroxe Agar
SO	Surveillance officers
TPN	Total parenteral nutrition
UTIs	Urinary Tract Infections
WGS	Whole genome sequencing
µl	Microliter
µg/mL	Microgram per milliliter

Chapter 1: Introduction and literature review

1. Changing incidence of bloodstream infections due to *Candida* species

Bloodstream infections (BSIs) due to *Candida* species are a significant public health concern globally. Candidaemia is associated with high morbidity and mortality, as well as prolonged hospital stay and increased cost (1). The crude in-hospital mortality due to candidaemia was reported to be 25% in the USA, 38% in Europe and 43% in South Africa (2-4).

The incidence of BSI by *Candida* species differs by geographical regions. Over the past two decades, the incidence of candidaemia caused by non-*albicans Candida* species has increased significantly. However, *C. albicans* is still the most predominant species in most parts of the world including USA, North America, Latin America, Europe, and Asia-Pacific region (2, 3, 5). During the 2009-2010 surveillance period, *C. albicans* was the common species causing BSI in public-sector hospitals in South Africa. *Candida parapsilosis* was frequently isolated in the private-sector hospitals' patients (6). Data from the South African candidaemia surveillance from 2016 to 2017 showed that *C. parapsilosis* was the most commonly isolated *Candida* species among people with candidaemia (4). *C. parapsilosis*, which is usually susceptible to azole antifungal drugs, was found to be resistant mostly to azole antifungal agents (6). More recently, there has been a marked increase (14%) in isolation of pathogen *C. auris* in both public and private sector hospitals in South Africa (4). Van Schalkwyk found that *C. auris* accounted for 14% of candidaemia cases during the 2016-2017 surveillance period in South Africa (4). Frequency multidrug resistance in South African *C. auris* isolates has been found to be relatively low (0.0015%; 3/2000) (Personal communication).

1.1. Laboratory identification of *Candida* species

Laboratory identification of species causing invasive *Candida* infection is a very complex process. Several conventional, kit, or commercial and molecular-based methods are used for species-level identification of *Candida*. Conventional methods include; microscopy, culture and carbohydrates fermentation and assimilation which identifies morphological and physiological characteristics of different *Candida* species (7). For these tests to be carried out, *Candida* isolates must first be isolated on a primary medium, Sabouraud dextrose agar (SDA) or potato dextrose agar (PDA). *Candida* species produce white, creamy, smooth, and pasty colonies after 24 hours' incubation (8). CHROMagar *Candida* media (Mast Diagnostics, Merseyside, UK) can be used for selective and differential identification of *Candida* species. This media contains a chromogenic substrate that interacts with species-specific enzymes leading to the production of distinct colored colonies. Table 1 summarizes the species-specific colony colors obtained on CHROMagar *Candida* media. Other non-*albicans Candida* (NAC) species such as *C. famata, C. guilliermondii*, *C. kefyr*, *C. lusitaniae* and *C, norvegenesis* produce colonies with different shades of pink, lavender and ivory (off-white) which makes it difficult to distinguish them (7) (Table 1).

Table 1: CHROMagar *Candida* profiles for the most common *Candida* species (7)

Candida *species*	***CHROMagar* Candida**
C. albicans	Leafy-green
C. glabrata	Dark violet
C. tropicalis	Dark blue-gray with purple halo
C. parapsilosis	Off-white
C. krusei	Pink and dry
C. guilliermondii	Indistinguishable
C. dubliniensis	Dark green
C. rugosa	Blue with pale or white borders
Lodderomyces elongisporus	Turquoise

Other convenient and easy methods used to identify *Candida* species are strip or plate-based for carbohydrate fermentation and assimilation or enzyme detection. These tests are commercially available. The carbohydrate assimilation tests include but not limited to API 20C AUX, API 32C (bioMérieux, Marcy l'Etoile, France), and Auxacolor (Sanofi Diagnostics Pasteur, France). The principle behind these tests is the production of color or turbidity. On each plate or strip, each well contains a different substrate, which after incubation with specific *Candida* species, produces a particular assimilation profile. This profile can then be translated using a manual or computer-assisted numerical approach (9-12). API ID 32C assay tests for carbohydrate assimilation, organic acids, cycloheximide production, and esculin. It correctly identified 88.5-98% of yeast (13, 14). In addition to carbohydrates assimilation, Auxacolor also tests for cycloheximide and phenoloxidase produced by specific *Candida* species (14-16).

Automated platforms for identification of yeast include, but not limited to Vitek 2 YST (BioMérieux, Marcy l'Etoile, France) and Microscan walkaway (Beckman Coulter, Brea, CA, USA). Vitek 2 YST utilizes colorimetric substrates, which can only be read or analyzed automatically. With the emergence of rare *Candida* species such as *C. auris* which is difficult to identify with common commercially available biochemical platforms, there has been a move to utilize proteomic analysis using matrix-assisted laser desorption/ionization time of flight mass spectrophotometry (MALDI-TOF MS) for identification of pathogens that are difficult to identify with these platforms (17). Cellabos-Garzón et al. (2019) (18) compared the performance of MALDI-TOF MS versus Microscan. They found the percentage agreement for correct identification of common *Candida* species with these two platforms to be 93.5%. Overall, MALDI-TOF MS provides 100% accurate identification *Candida* species with the provision that the species profile is added in the database (19). Using routine diagnostic platforms, *C. auris* is incorrectly identified as *C. haemulonii* by Vitek 2 YST, *Rhodotorula glutinis* by API 20C Aux or API ID 32C, as *Saccharomyces cerevisiae* by Auxacolor or as *Candida famata* by Microscan walkaway (20).

The molecular-based assays provide more rapid and accurate identification of *Candida* species. The most commonly used method is the polymerase chain reaction (PCR) amplification of the D1/D2 domain of the 28S ribosomal DNA large subunit and the more variable internal transcribed spacer (ITS) regions ITS1 and ITS2 (17, 21, 22). Restriction fragment length polymorphism (RFLP) of 5.8S rRNA is a reliable alternative to sequencing of 28S rDNA (23, 24). Sequencing of the ITS has proven to be useful in accurate identification of clinically significant *Candida* species, including emerging species such as *C. auris*, which is difficult to

identify with biochemical methods currently used for diagnostic purposes. Cryptic species within common *Candida* species (*C. albicans* complex, *C. glabrata* complex, and *C. parapsilosis* complex) cannot be phenotypically distinguished using routine diagnostic assays. For instance, *C. parapsilosis* complex species (*C. parapsilosis* sensu stricto, *C. orthopsilosis*, and *C. metapsilosis*), show similar growth characteristics, cellular morphology, carbohydrate assimilation, and fermentation profiles (25). With *C. parapsilosis* sensu stricto being implicated in causing outbreaks in the hospital setting, it is vital to differentiate the species within the "*psilosis* complex accurately".

1.2. Azole resistance in *Candida* species

Before the introduction of antiretroviral therapy, the prevalence of fluconazole-resistant *C. albicans* isolated from mucosal lesions was high among HIV-infected patients. This was thought to be due to widespread use of itraconazole and fluconazole as primary treatment of patients with oropharyngeal or oesophageal candidiasis (26). Fluconazole resistance in common *Candida* species remains low in other regions including Latin America, North America, Europe, and Asia-western Pacific with reported prevalence ranging from 0.6% to 4.6% for *C. parapsilosis*, C. *albicans* (0.2% - 0.4%), *C. glabrata* (2.6% - 10.6%) and *C. tropicalis* (1.1% - 9.2%) (2,4). Findings from multicenter population-based surveillance in the USA demonstrated fluconazole resistance in *C. parapsilosis* to be 7.7% (2).

Global surveillance studies indicated that non-susceptibility to fluconazole is not common among cases of *C. parapsilosis* BSI (4). Similar findings were reported in the context of long-term fluconazole prophylaxis (27). However, the antifungal susceptibility data obtained during national laboratory-based surveillance between 2009 and 2010 in South Africa showed that 62% (336/538) of *C. parapsilosis* isolates were fluconazole non-susceptible, which differs from what is seen in other countries (2, 4, 28-30). In settings where *C. parapsilosis* is resistant to the azoles, which are commonly prescribed antifungal agents, inappropriate empiric therapy may potentially lead to even higher mortality. The molecular mechanisms of azole resistance in other *Candida* species have been established (31, 32, 33). Data on the molecular mechanisms of azole resistance for *C. parapsilosis* is emerging. However, most of these studies were conducted in areas where the prevalence of azole

resistance in *C. parapsilosis* BSI is very low compared to our setting, where fluconazole resistance is very high (34-38).

C. auris is resistant to multiple classes of antifungal drugs. Azole resistance is 44.3% and 12.7% for fluconazole and voriconazole respectively (39). Point mutations in the *ERG11* gene (Y126F, Y132F, and K143R), overexpression of the multidrug transporters, and *ERG11* gene, changes in the sterol biosynthesis pathway, mediate azole resistance in *C. auris*. The point mutations in *the ERG11* gene were shown to be clade-specific with Y126F found in South African clade, Y132F (Venezuela), and Y132F or K143R in India/Pakistan (39-41).

1.2.1. Mechanism of action of azole antifungal agents

Azoles are the largest group of antifungal agents in clinical use. They target the fungal cell membrane. Ergosterol is the major component of the fungal cell membrane, which provides stability by binding to the phospholipids and regulates the cell membrane fluidity, permeability, and activities of the enzymes that are attached to the membrane (42, 43). Lanosterol serves as a precursor for ergosterol formation. Although a number of enzymes are involved in the formation of ergosterol, C14α-demethylase is responsible for demethylation of lanosterol. The C14α-demethylase is an essential enzyme that serves as a target for azole antifungal agents. The major mechanism of action of azoles is by binding to cytochrome P450-mediated 14α-demethylase enzyme, thus inhibiting the demethylation of lanosterol and blocking the synthesis of ergosterol. The inhibition of 14α-demethylase leads to the formation of fungal cell membrane with modified structure and accumulation of sterol precursors [14-α-methyl fecosterol and 14α-methyl-ergosta-

8,24(28)-dien-3β,6α-diol] and depletion of ergosterol in the cell membrane (44). The lack of ergosterol in the cell membrane makes it very unstable, the cell membrane begins to breakdown leaking the cell content, and eventually, the fungal organism dies (Figure 1) (45-47).

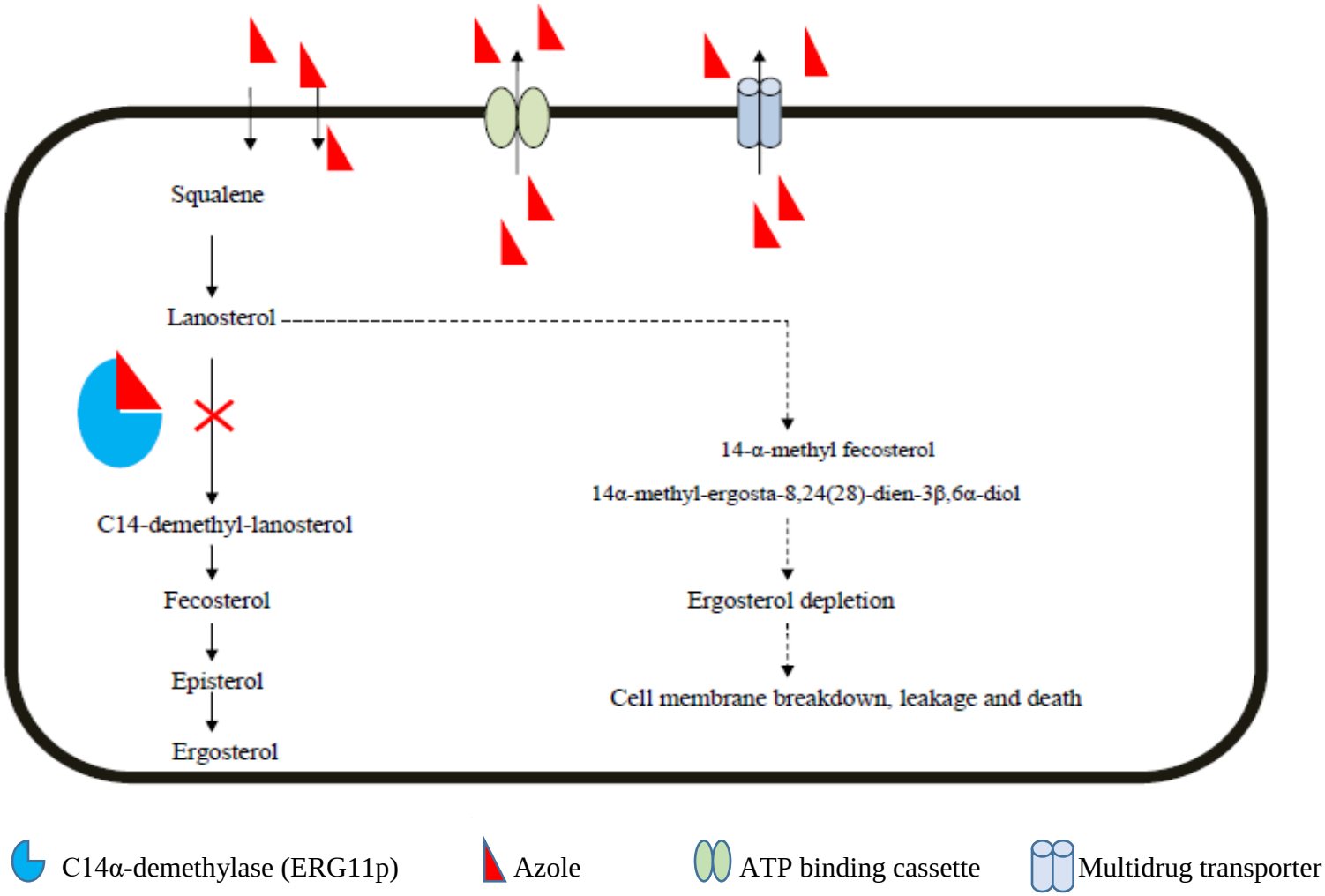

Figure 1: Ergosterol biosynthesis pathway depicting the azole antifungal mechanism of action in a susceptible *Candida*.

1.2.1. Mechanism of resistance to azole antifungal agents

Mechanisms leading to azole resistance are better characterized in *Candida albicans* than in other *Candida* species (48,49). Resistance may occur in one of the following ways, (**A**) Overexpression of the gene encoding multidrug transporters [ATP binding cassettes (ABC) and major facilitator superfamily (MFS)]. Overexpression of ABC (*CDR1* and *CDR2)* due to activating mutations in the transcription factor genes *TAC1* leads to efflux of all azole antifungal drugs (50). Overexpression MFS transporters (*MDR1*) due to activating mutations in the transcription factor genes *MRR1* leads to efflux of fluconazole and voriconazole (37,51-53); (**B**) point mutations in the 14α-demethylase, alters the protein structure and thus decrease the azole binding affinity; (**C**) overexpression of the drug target (*ERG11*) gene which encodes for the sterol demethylase, this is usually due to mutations in the transcription regulator gene *UpCs* (54,55); and (**D**) mutations in *ERG3* gene resulting in the inactivation of enzyme sterol desaturase which allows the cell to bypass the production of toxic methylated sterols in the presence of azoles (Figure 2) (48). Some of these mechanisms can be extrapolated to azole-resistant *C. parapsilosis* and *C. auris* isolates, and this will be discussed in detail below.

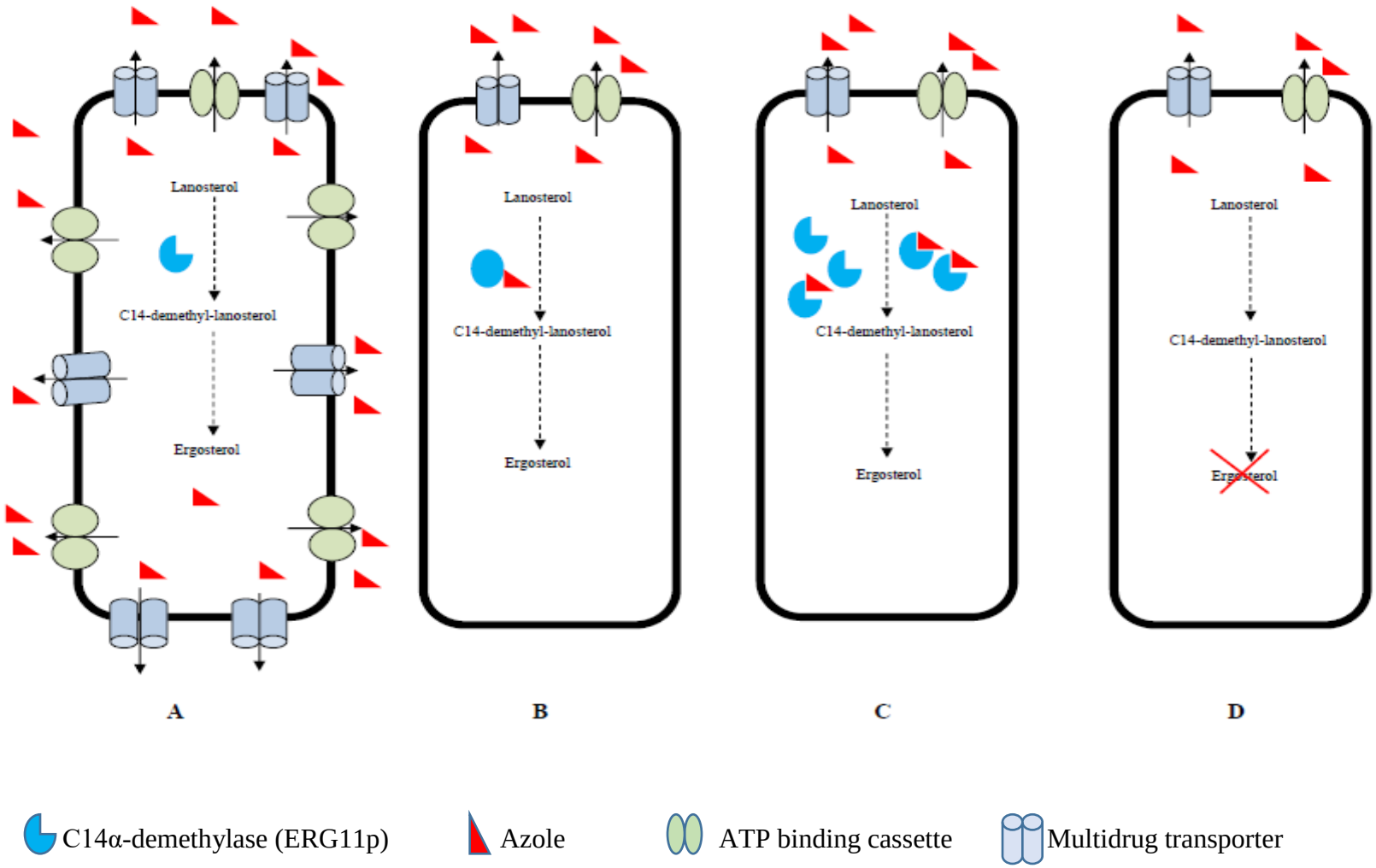

Figure 2: Summarized molecular mechanisms of azole resistance in Candida species. **A**. Overexpression of multidrug transporter genes **B**. Mutations in the drug target (*ERG11*p) **C**. Overexpression of the drug target (*ERG11*) **D**. Mutations in *the ERG3* gene.

1.3. Azole resistant non-*albicans Candida* species

1.3.1. *Candida parapsilosis*

1.3.1.1. *C. parapsilosis* as an emerging fungal pathogen

Recent data from the SENTRY program showed that in the last decade, *C. parapsilosis* was the third most common *Candida* species in North America, Europe, Latin America, and Asia-Pacific region after *C. albicans* and *C. glabrata* (4). Although *C. parapsilosis* ranked third, its frequency increased from 12.3% to 17.8% in 1997-2001 and 2009-2011 periods, respectively. In countries such as South Africa, India, and Latin America, *C. parapsilosis* and *C. tropicalis* ranks above *C. glabrata* (6,28,56). In South Africa, for instance, during the TRAC-SA surveillance period (2009-2010), Govender and colleagues (2016) reported that *C. parapsilosis* was leading non-*albicans Candida* species causing candidaemia in neonates versus adult population (6). During the 2016 to 2017 surveillance period, van Schalkwyk et al. (2019) showed that *C. parapsilosis* was the predominant *Candida* species followed by *C. albicans* and *C. auris* in the third place (5). The patients, mostly at risk, are those with cancer, diabetes, have undergone surgery and neonates. Risk factors associated with invasive *C. parapsilosis* infection are similar to those associated with other *Candida* species. These include admission to the intensive care unit (ICU), presence of central venous catheters (CVC), mechanical ventilation, total parenteral nutrition (TPN), administration of broad-spectrum antibiotics, prior antifungal therapy, premature birth, low birth weight and recent abdominal surgery (57-61). In neonates, invasive *C. parapsilosis* infection is associated with admission to neonatal intensive care unit (NICU), immature skin structure, presence of CVC, TPN, mechanical ventilation, and premature delivery, low birth

weight, and long-term use of antibiotics (57,58,60). The affinity of *C. parapsilosis* for foreign material has been shown by its isolation from peritoneal dialysis catheter and prosthetic heart valves, which could be linked to the ability to adhere and formation of biofilm on this material (62,63). The mortality associated with *C. parapsilosis* candidaemia in neonates was reported to be 10.02% between 1999 to 2012, according to a meta-analysis study (60).

1.3.1.2. Cryptic species within *C. parapsilosis* complex

A number of studies have described the species distribution within the *C. parapsilosis* species complex, with *C. parapsilosis* sensu stricto still accounting for a majority of cases of candidaemia. The prevalence of these cryptic species within the *C. parapsilosis* complex differs by geographical region, ranging from 0.6% to 6.8% for *C. metapsilosis* and 2.0% to 10.9% for *C. orthopsilosis* (64-67). The most comprehensive study on the prevalence of *C. parapsilosis* associated-candidaemia was conducted in the context of Spanish population-based surveillance of candidaemia, where *C. orthopsilosis* and *C. metapsilosis* accounted for 5.7% and 6.9% of the psilosis complex infections respectively (65). The high prevalence of these cryptic species may be because some patients had recurrent episodes, and therefore multiple isolates were analyzed. *C. orthopsilosis* and *C. metapsilosis* were shown to be susceptible to azoles and amphotericin B with lower minimum inhibitory concentrations versus *C. parapsilosis*, and also had an increased fluconazole MIC as compared to *C. parapsilosis*. However, MICs were still in the susceptible ranges for all species (64,65,68).

1.3.1.3. Hospital-associated outbreaks of *C. parapsilosis* fungemia in neonatal intensive care

Candida parapsilosis has relatively low virulence. However, it can adhere to and form biofilms on indwelling medical devices, produce aspartyl-proteinases and grows in TPN solutions (69-73). The hands of health care workers (HCW) have been implicated in the transmission of *C. parapsilosis* to neonates in hospital settings leading to outbreaks in NICUs and general ICU (72-79). In many of these outbreaks, isolates from the hands of HCWs have been found to be genetically similar to those isolated from patients (80). Genotyping can be used to unmask "smoldering clusters" of candidaemia over the years. An Icelandic group of researchers genotyped (PCR-fingerprinted) bloodstream isolates of *Candida* over 16 years and found that up to one-third of cases of candidaemia were cluster-associated (81). Most clusters involved 2 cases; NICUs were disproportionately affected, and the proportion of clustered isolates was highest for *C. parapsilosis*.

1.3.1.4. Molecular typing of *C. parapsilosis*

Molecular epidemiology, applied as an infection prevention and control tool, can show the persistence and transmission of similar strains in hospital settings. Several methods for differentiating *C. parapsilosis* at a molecular level have been used. This includes random amplification of polymorphic DNA (RAPD), multilocus sequence typing (MLST), electrophoretic karyotyping, restriction fragment length polymorphism (RFLP), and internal transcribed spacer region (ITS) sequencing (71,82). These methods have disadvantages, such as lack the ability to accurately determine the degree of genetic relatedness among strains, there is no standardized set of

primers, and therefore profiles are relatively unstable, *C. parapsilosis* is diploid or aneuploid and possesses little inter-strain sequence variation; therefore, its typing requires a technique that has a high degree of discrimination (83-85).

Microsatellite markers are polymorphic DNA loci consisting of a repeated nucleotide sequence that differs in a population; therefore, it creates multiple alleles for a microsatellite locus. Microsatellite analysis has an advantage over commonly used typing methods because of their behavior as co-dominant loci, evolves fast in a genome, and may be able to differentiate among isolates with low rates of sequence variation. Microsatellite analysis has been reported to have a high degree of discrimination and is reproducible (72). Because of its ability to detect micro-evolutionary changes of isolates from different body sites, it has been used as a typing tool for outbreak detection and in epidemiologic studies (76,77, 79,86-88). With the use of polymorphic microsatellite markers, Reiss and colleagues were able to demonstrate the persistence and transmission of a single cluster of C. *parapsilosis* in the neonatal unit and other general wards over three years (Reiss et al., 2012). Another study from Italy also observed that a single strain persisted and caused outbreaks in the neonatal unit over six years (76). Using microsatellite genotyping, Thomaz and colleagues reported that azole non-susceptible *C. parapsilosis* clone persisted and caused outbreaks in an adult ICU over a 2-year period in Brazil (89). However, with an increase in the use of whole-genome sequencing (WGS), the use of microsatellites as a genetic marker is decreasing. Several studies have used WGS to assess the genetic relatedness among *Candida* species causing outbreaks in hospital settings. However, most of the work has been on *C. auris* (40,90-93). In India, the use of WGS revealed that *C. auris* isolates causing outbreak were genetically related, suggestive of possible clonal transmission (40). The Centers for Disease Control and Prevention (CDC)

demonstrated that *C. auris* strains from three continents emerged almost simultaneously and that they belong to four major clades (39). Recently, Chow et al. (2019) identified a fifth major clade of *C. auris*, which was separated from other clades by >200 000 SNPs (93). WGS offers a high degree of discrimination and is a reliable tool to differentiate isolates with a low level of genetic differences and able to detect outbreaks. Currently, there is no standardized set of microsatellite loci for the typing of *C. parapsilosis* strains. Different studies analyzed four to six microsatellite loci for strain typing (76,77,86,87). Therefore, WGS can serve as a standardized genotyping method for epidemiologic purposes.

1.3.1.5. Molecular mechanisms of azole resistance in *C. parapsilosis*

A number of studies have recently explored and identified several possible mechanisms of azole resistance in *C. parapsilosis* strains. Point mutations within *ERG11*, particularly Y132F, was detected exclusively in fluconazole-resistant isolates. This mutation accounted for the majority (31-57%) of fluconazole resistance in *C. parapsilosis* (34,35,37,79). Grossman et al. (2015) identified Y132F as a major mutation responsible for resistance in fluconazole-resistant *C. parapsilosis* (34). In other studies, Y132F was mostly identified in combination with R398I (37). However, the direct impact of R398I polymorphism is not yet clear as it was also isolated from susceptible isolates. The gain of function mutations was also reported to influence azole resistance in *C. parapsilosis*. These mutations include G1747A and A2619C identified in transcription regulator gene *MRR1*, which were also shown to upregulate the expression levels of MFS transporter gene *MDR1* (34, 37,38). Branco et al. (2017) demonstrated that missense mutation in *ERG3* leads to complete loss of *ERG3* activity and subsequent resistance to posaconazole, suggesting that the mechanism responsible for

posaconazole resistance may be different to that induced by fluconazole and voriconazole (38). Using whole-genome sequencing, Rybak et al. (2017) demonstrated that *C. parapsilosis* strains, which were resistant to azoles had amino acid substitution G111R in *the ERG3* gene, which confirms the finding by Branco et al. (2017) that mutations in *ERG3* lead to resistance to azoles (38,94).

1.3.2. *Candida auris*

1.3.2.1. Epidemiology of *Candida auris*

Candida auris is an emerging multidrug-resistant fungal pathogen associated with clinical disease and was first isolated in 2009 from a Japanese patient with an external ear canal discharge (95). After this, in 2009, Kim and colleagues (2009) reported 23 cases of *C. haemulonii* later identified as *C. auris* from five hospitals in Korea from patients with candidaemia and chronic otitis media (96). In 2011, *C. auris* was isolated from three patients with candidaemia from South Korea. Two of these patients died, and one developed breakthrough infection despite fluconazole treatment (97). Twelve cases of fluconazole-resistant *C. auris* were reported from India in 2013 (98). The Indian cases were found to belong to a clade different from the Korean cases based on whole-genome sequencing single nucleotide polymorphism (39; 99). In 2014, cases were reported from India and Kuwait (100,101). Since its first isolation, cases of *C. auris* with varying clinical manifestations have been reported from Asia, South America, Europe, North America, and Australasia (96,97 100-103).

The global prevalence and geographic extent of *C. auris* disease is likely under-estimated, especially in low- and middle-income countries because conventional laboratory methods misidentify the fungus, and relatively few resource-limited countries have the capacity for identification by mass spectrometric or molecular methods (97,98,101). Figure 3 highlights the global distribution of *C. auris* with respect to the number of cases isolated to date. However, it does not differentiate whether the cases were reported from an outbreak situation or not.

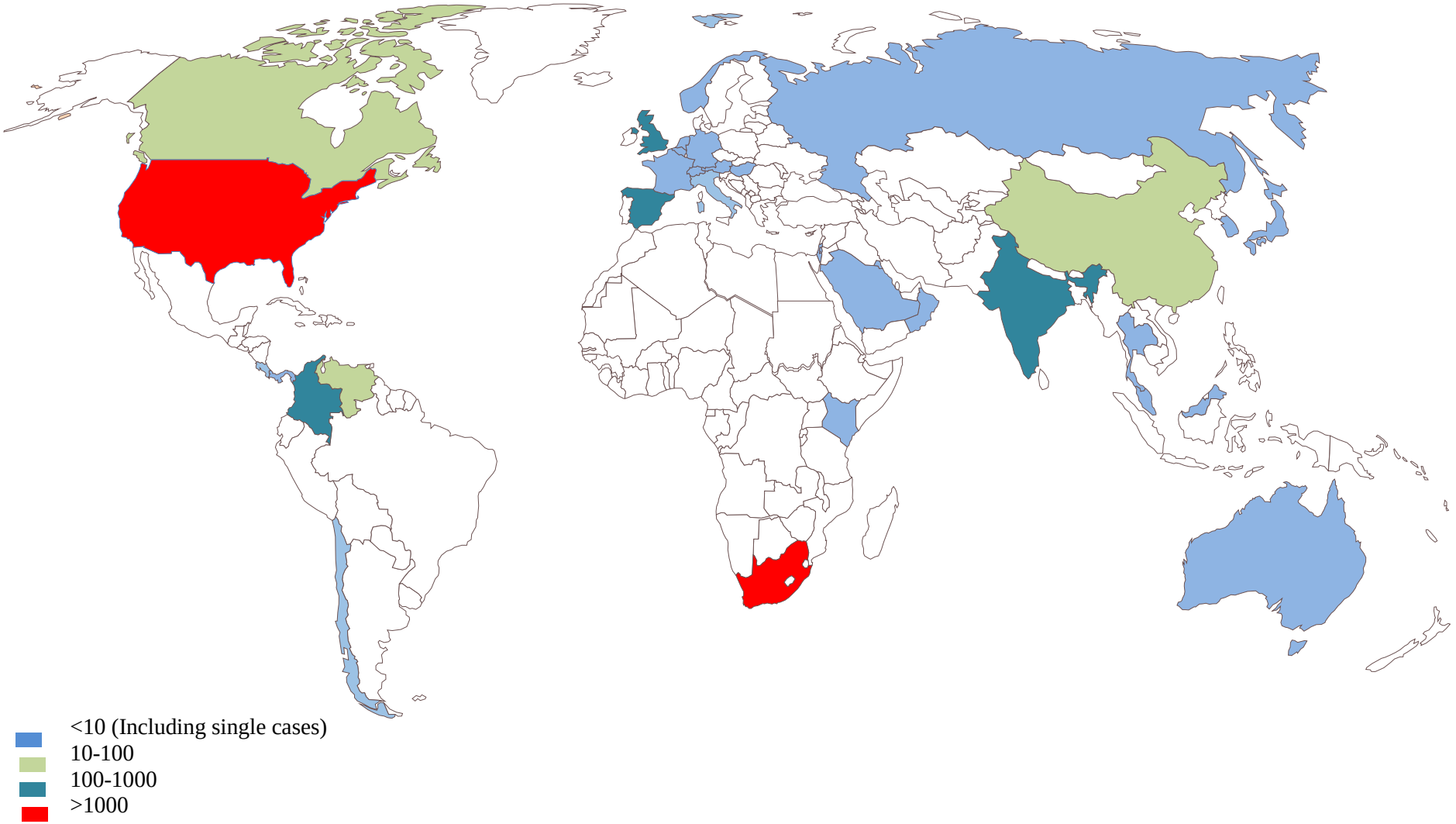

Figure 3: Global distribution of *C. auris* as of November 2019

1.3.2.2. Clinical characteristics, risk factors and outcome of *C. auris* infection

Clinical presentation of patients with *C. auris* infection is not different from those with other systemic *Candida* infections. *C. auris* has been isolated from different specimen types from both normally sterile and non-sterile body sites. Clinical manifestations include BSI, surgical wound infections, urinary tract infections (UTIs), respiratory tract infections, myocarditis, and meningitis (104,105). The risk factors associated with *C. auris* infection are prolonged ICU stay, abdominal and vascular surgery, prior exposure to antifungal drugs and broad-spectrum antibiotics, presence of CVC, urinary catheter, TPN, neutropenia, and chemotherapy (105,106,107,108). The estimated crude mortality associated with *C. auris* candidaemia in hospitalized patients ranges from 30% to 60% (39,97,105,109,110).

1.3.2.3. Laboratory identification of *C. auris*

Phenotypically, *C. auris* isolates appear as white to cream-colored colonies on SDA and pink on CHROMagar. On corn meal rice Tween 80 agar, they form ovoid to elongated budding yeast cells without pseudohyphae; grow at 37°C and 42°C (95,101, 111). On the other hand, *C. haemulonii* and *C. duobushaemulonii* strains (closely related) produce pseudohyphae, do not grow at 42°C and they do not assimilate the similar sugars. Several studies have shown that *C. auris* can be misidentified as *C. haemulonii* by Vitek-2 or BD Phoenix, as *Rhodotorula glunitis* API 20C, as *C. parapsilosis*, *C. lusi*taniae, *C. famata* or *C. guillermondii* by Microscan because their current databases lack

representative organisms (Table 2) (20,95,97, 98; 101). MALDI-TOF MS is a robust and reliable non-molecular based method for rapid identification *C. auris* from culture (111).

Table 2: Misidentification of *C. auris* by standard methods (20)

Standard identification method	***C. auris* misidentified as**
Vitek-2 YST (BioMérieux, Marcy l'Etoile, France)	*Candida haemulonnii*
API 20C (bioMérieux, Marcy l'Etoile, France)	*Rhodotorula glutinis, Candida Sake*
API ID 32C (bioMérieux, Marcy l'Etoile, France)	*Candida intermedia, Candida Sake, Saccharomyces klyveri*
BD Phoenix	*Candida haemulonnii, Candida catenulata*
Auxacolor (BioRad, Hercules, California, United States)	*Saccharomyces cerevisiae*
Microscan Walkway (Beckman Coulter, Brea, CA, USA)	*Candida parapsilosis*, *Candida lusi*taniae, *Candida famata* or *Candida guillermondii*
RapidID Yeast plus	*Candida parapsilosis*

1.3.2.4. Molecular typing of *C. auris*

Several assays have been used for genotyping of *C. auris* strains. Sequencing of ITS and 26S D1/D2 regions initially placed *C. auris* within the *Metschnikowiaceae* family, which is closely related to the Clavispora clade. Chowdhary et al. (2013) utilized a combination of sequencing of ITS and D1/D2 regions, M13 PCR fingerprinting, and AFLP to show that a clonal strain of *C. auris* was responsible for cases of candidaemia at Dehli hospital in India (98). Genetic relatedness among *C. auris* isolates has been previously reported. These studies demonstrated that the strains were clonal using pulsed-field gel electrophoresis (PFGE), MLST, AFLP and MALDI-TOF (100,112,113). A study of isolates collected from four different countries showed that isolates clustered by geographic regions when MLST was used (113). However, South African strains were scattered among different clusters suggesting that different genotypes may be circulating in the country. These were some of the initial studies to suggest geographical clustering. However, the molecular typing techniques employed displayed low discriminatory power.

Whole-genome sequencing (WGS) has proven to be a robust typing tool for *C. auris* with a high degree of discrimination. In India, WGS of multidrug-resistant *C. auris* isolates showed that these isolates were genetically related, suggested that they might have spread from a single source (40). In an international study which analyzed strains from four continents to assess the clonality of *C. auris*, WGS was able to cluster these strains by geographical region and also suggested that they emerged simultaneously (39). Subsequent studies show that *C. auris* isolates from the USA, Colombia, and the UK clustered with one of the four clades described by Lockhart and colleagues (2017) suggesting that they may have been introduced from other regions (39,90-92). Isolates from

the USA were related to all four major clades, suggesting that they were travel-related (90). Colombian *C. auris* isolates were genetically related to Venezuela isolates with only 140 SNP difference (92). In a recent study, a fifth major clade was described from an isolate from Iran. WGS showed that this isolate was genetically distinct with >200 000 SNP difference from other clades (93). This was not a case of introduction of strains into the country because this patient had never traveled outside of Iran.

1.3.2.5. Treatment of *C. auris* infections

Because most of the reported *C. auris* isolates are resistant to azoles, echinocandins are recommended as first-line treatment until susceptibility testing is done (114-116). In a setting where echinocandins are not available, amphotericin B is a treatment of choice and is recommended for treatment of eye, urinary tract, and central nervous system infections (117). Children below the age of 2 months are treated with amphotericin B deoxycholate (1 mg/kg daily). It is more effective and easily tolerated in neonates. For any patient above the age of 2 months, an echinocandin is recommended as a treatment of choice (118). However, an exception is given to patients with central nervous system, eyes, and urinary tract infections. These patients must be treated with amphotericin B deoxycholate. Liposomal amphotericin B may be considered in cases with persistent fungemia for ≥5 days or if patients are not responding to echinocandins (114,119). Treatment with fluconazole is not recommended because majority of *C. auris* reported are resistant to fluconazole. In the South African setting, fluconazole is not recommended for the treatment of *C. auris* infection because of the high prevalence of azole resistance among *C. auris* isolates circulating in South African hospitals (116).

1.3.2.6. Infection control and prevention

The rapid spread of *C. auris*, associated high mortality, high-level antifungal resistance has prompted the implementation of infection prevention and control (IPC) measures with suggestions from different parts of the world. The United Kingdom, USA, Europe, and South Africa have issued recommended IPC guidelines with regards to patient screening and isolation, contact precautions, hand hygiene, and general cleaning of the area that have come into contact with infected or colonized patients (110,115,116,120,121,122). The recommended infection prevention measures for *C. auris* in hospital settings are summarised in Table 2.

Table 3: Recommended *C. auris* infection prevention and control measures (110,122)

Institution	Transmission-based precautions	Hand hygiene	Environmental disinfection	Patient screening	Transfer of patients between facilities	Decolonization procedure
FIDSSA (SA)	Isolation of patients in single rooms within suites or in cohorts	Washing of hands with soap and water followed by alcohol hand rub; Use alcohol hand rub between care activities	Daily cleaning with neutral detergent followed by wiping with freshly-made sodium hypochlorite solution (1000 parts per million); Terminal cleaning and disinfection of beds after patient has left bed space; Terminal cleaning with hydrogen peroxide vapor; Dedicated equipment to be used if single-use equipment is unavailable; Thorough cleaning of multiuse devices; Dispose all waste and linen appropriately after patient has vacated the facility; Clean all contact areas.	Screening of patients or health care workers may be carried out in an outbreak setting among epidemiologically-linked patients.	Referral facility to inform receiving facility of the transferred patient's *C. auris* status (infected/colonized); Advice receiving facility on precautionary before and during transfer.	
PHE (UK)	Isolation of colonized or infected patients in single rooms with ensuite.	Washing of hands with soap and water followed by alcohol hand rub.	Use of chlorine-releasing agent at 1000 ppm is recommended; Terminal cleaning with hydrogen peroxide vapor after patient leaving; Cleaning of multiuse devices; Appropriate waste and line disposal; No washing or disposal of infected soiled material in clinical hand wash sinks.	Isolate all screen positive patients; 3 negative screens needed 24 hours apart to de-isolate; consider weekly screening of certain clinical areas due to possible recurrent colonization	Isolation of patients being transferred from other hospitals until screening results are available.	Decontamination of skin with chlorhexidine in critically ill; Catheter, venous, urinary bundle care; venous cannulas to be removed if sign of infection shows; Apply high standard aseptic technique for wound care; Use of chlorhexidine for mouth gargles; Use of topical nystatin and

					terbinafine for the topical management of the site of infection.
CDC (USA)	Isolation of infected patients into single rooms; If single rooms are insufficient, they should be reserved for high risk patient; Place patients with *C. auris* with other *C. auris* infected patients; Number of staff caring for patients with *C. auris* infection should be minimal.	Use of alcohol-based hand sanitizers; Washing of hands with soap and water; reduce hand contamination by wearing of gloves; should avoid touch area outside patient care environment with gloves; Hand hygiene before putting and after taking off gloves.	Use environmental protection agency-registered hospital-grade disinfectant.	Reassess patients for colonization every three months; Testing of swabs, groin, and sites which were previously *C. auris* positive; If the result are negative, retest once a week later.	
ECDC (Europe)	Isolation or cohorting of colonized or infected patients; Allocation of dedicated nursing staff;		Terminal cleaning after patient discharge with disinfectant with certified antifungal activity; Cross-sectional patient and environmental sampling in an outbreak setting.	Carry-out active surveillance in cross infection setting; sampling sites include nose/throat, axilla, groin, rectum, and venous catheter insertion site. Clinical samples include urine, feces, and wound draining fluid and respiratory specimens.	

1.4. Aim

To describe the molecular epidemiology of azole-resistant *Candida* species causing invasive infection in South Africa in order to:

1. Provide information on molecular epidemiology, including pathways of transmission, and thus assist in improving hospital IPC policies
2. Provide antifungal susceptibility data to assist in guiding appropriate empiric management, which in turn may reduce mortality.

1.5. Objectives

1. To characterize *C. parapsilosis* isolates from neonatal cases of candidaemia, diagnosed at Chris Hani Baragwanath hospital from 2009 to 2010. (Chapter 3)
2. To determine the epidemiologic and laboratory factors which are associated with strains belonging to a cluster (vs. non-cluster strains) among neonatal cases of candidaemia diagnosed at Chis Hani Baragwanath hospital from 2009 to 2010.(Chapter 3)
3. To determine the potential molecular mechanisms of azole resistance among *C. parapsilosis* BSI isolates diagnosed from 2009 to 2010. (Chapter 4)
4. To describe the identification (phenotypic and molecular) and antifungal susceptibility profiles of first cases *C. auris* candidaemia in South Africa, 2012-2014. (Chapter 5)
5. To describe the epidemiology of *C. auris* in South Africa from 2012 to 2016. (Chapter 6)
6. To characterize the *C. auris* strains using MLST. (Chapter 7)

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Chapter 2: Materials and methods

2.1. Study design and sample selection

This was a cross-sectional study to describe the molecular epidemiology and azole resistance of *Candida* species (*Candida parapsilosis* and *Candida auris*) isolates that were submitted as part of laboratory-based surveillance for candidaemia in South Africa between 2009 to 2017.

2.2. Sources of data and isolates

Isolates and data for secondary analysis were obtained from three surveillance programs conducted by the National Institute for Communicable Diseases (NICD), namely TRAC-SA (2009-2010), GERMS-SA (2012-2017) and passive electronic *C. auris* surveillance (2012-2016). These programs are described in detail below. The case definitions for candidaemia were the same for both programs, though the periods and participating hospitals differed.

2.3. Description of surveillance programs from which line list information and isolates were obtained

2.5.1. TRAC-SA (2009-2010)

In 2008, a laboratory-based sentinel surveillance project was designed by NICD in collaboration with public and private sector laboratory sites across South Africa. The primary objective was to describe the species distribution of *Candida* species causing BSI at a national level and to compare the species distribution between the public and private sectors. Another objective was to describe susceptibility to nine antifungal agents (fluconazole,

voriconazole, itraconazole, posaconazole, flucytosine, amphotericin B, caspofungin, micafungin and anidulafungin,) in 2009-2010, and to compare patterns between the public and private health sectors. A case of candidaemia was defined as a person with laboratory-confirmed candidaemia (based on a positive blood culture) diagnosed at any South African hospital. A case of recurrent candidaemia met the above criteria but had a positive blood culture >30 days after the first positive culture. The laboratory methods used to characterize *Candida* species included biochemical identification (using carbohydrate assimilation assays) and broth microdilution for antifungal susceptibility testing. During the 18-month surveillance period, 2164 cases of candidaemia were detected; 1382 (64%) cases were reported by sentinel laboratories, and 778 cases were detected by audit. Sixty-six percent of cases were detected by public sector laboratories. *C. albicans* was the most common species causing fungemia in the public sector. In contrast, *C. parapsilosis* was the most common species in the private sector.

2.3.2. GERMS-SA (2012-2017)

In 2012, the NICD's GERMS-SA platform was used to continue surveillance for candidaemia at selected sentinel hospitals. The sentinel sites changed over time. From 2012 to 2013, 1074 cases of candidaemia were detected from nine sentinel hospitals in Gauteng and Western Cape provinces. During the surveillance period 2014-2015, 864 cases were detected from 22 enhanced sentinel sites in eight provinces. From 2016 to 2017, 3817 cases were detected from enhanced sites (both public and private sector hospitals) from all nine provinces. Microbiology laboratories submitted clinical isolates to NICD for species confirmation and further characterization. Surveillance officers (SOs) collected additional clinical and epidemiological information on laboratory-confirmed cases. A case of candidaemia was

defined as a person with laboratory-confirmed candidaemia (based on a positive blood culture) diagnosed at a sentinel South African hospital. A case of recurrent candidaemia met the above criteria but had a positive blood culture >30 days after the first positive culture.

2.3.3. Passive electronic *C. auris* surveillance (2012-2016)

We conducted retrospective electronic surveillance for cases detected over four years since the first isolation of *C. auris* in South Africa. Line list specimen-level information was obtained from the four largest private-sector diagnostic pathology laboratories. The variables included age or date of birth, sex, date of specimen collection, location (province and admitting hospital), specimen type, and species-level identification. For *C. auris*, a case was defined as a patient of any age admitted to any South African hospital with first isolation of *C. auris* from any specimen (either infection or colonization) from January 2012 through to December 2016. A case of colonization was defined if isolate was cultured from normally unsterile sites (Urine, respiratory tract, CVC, and skin or mucosal specimen). A case of invasive disease was defined if isolate was cultured from a normally sterile site (blood, cerebrospinal fluid (CSF), serious fluid or tissue). We excluded all cases with high fluconazole MICs identified phenotypically as species other than *C. haemulonii*, *Rhodotorula glutinis*, *Saccharomyces cerevisiae*, *Candida sake*, *Candida lusitaniae*, or *Candida famata*.

2.4. Selection of isolates

2.4.1. *C. parapsilosis* isolates and cases

Inclusion criteria: All isolates identified as *C. parapsilosis* between 2009 and 2010 by ChromAgar *Candida* (Mast Diagnostics, Merseyside, UK) and Vitek-2 YST (bioMérieux Inc, Marcy l'Etoile, France) at the NICD were included in this study for the purposes of *C. parapsilosis* epidemiology, molecular characterization and mechanism of azole resistance. Isolates used in this study were identified before the introduction of MALDI TOF MS as part of the yeast identification platform at the NICD. However, the utilization of other methods (Virtek YST 2) for identification did not affect the isolate selection criteria.

Exclusion criteria: All stored isolates that were mixed (two or more *Candida* species) on ChromAgar *Candida* were not be included because *Candida* species have different virulence factors and may have various clinical manifestations. Cryptic species within *C. parapsilosis* species complex (*C. orthopsilosis* and *C. metapsilosis*) were not included in the cluster analysis sub-study.

2.4.2. *C. auris* isolates and cases

Inclusion criteria: All yeast isolates with high fluconazole minimum inhibitory concentration (MICs) and viable isolates initially identified as *C. haemulonii* by API 20C, *Rhodotorula glutinis* by API ID 32C (bioMérieux), as *Saccharomyces cerevisiae* by Auxacolor (BioRad, Hercules, California, United States) or as *Candida famata* by Microscan walkaway (Beckman

Coulter, Brea, California, United States) isolated from all anatomical sites from January 2012 through to December 2015.

2.5. Role of the candidate in surveillance data and isolate collection

The student was directly involved in the processing of isolates from sentinel hospitals, data entry, cleaning, and preparation of monthly reports for GERMS-SA surveillance during the 2012-2013 surveillance period. In the current study, the candidate extracted data from the TRAC-SA and GERMS-SA databases, cleaned and analyzed the data, undertook laboratory experiments, and produced results. For the passive electronic *C. auris* surveillance, the student cleaned the data and performed initial basic data analysis.

2.6. Species-level identification and antifungal susceptibility testing at NICD

2.6.1. *Candida parapsilosis* isolates and cases

Candida isolates were submitted to the NICD by the laboratories on Dorset transport medium (Diagnostic Media Products (DMP), Johannesburg, South Africa). Initial phenotypic identification was done using ChromAgar *Candida* medium (Mast Diagnostics, Merseyside, UK), Vitek 2 YST, API 20C Aux, and API ID 32C (bioMérieux, Marcy l'Etoile, France). Antifungal susceptibility testing (minimum inhibitory concentrations (MIC)) for fluconazole, voriconazole, itraconazole, posaconazole, caspofungin, anidulafungin, micafungin and flucytosine was performed using pre-prepared dried microbroth dilution panels containing Alamar Blue (Thermo Fisher Scientific, Cleveland, Ohio, USA); MICs were categorized using Clinical and Laboratory Standards Institute (CLSI) M27-S4 interpretive breakpoints.

Additionally, a 24 h epidemiologic cut-off values (ECVs) for *C. parapsilosis* and the Sensititre YeastOne test method were applied to MICs of all tested agents (1-3). MICs for amphotericin B were obtained using Etest (bioMérieux, Marcy l'Etoile, France) on RPMI 1640 plates, which contained 2% glucose (DMP, South Africa). *C. parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 strains were included as part of quality control (QC) runs on all days of testing, and MICs were found to be within QC range.

2.6.2. C. *auris* clinical isolates and cases

Suspected or confirmed cases of *C. auris* were submitted to NICD (national mycology reference laboratory) for species-level confirmation and antifungal susceptibility testing between 2012 and 2015. These isolates were mostly yeasts with high fluconazole MICs detected by Vitek 2 YST (version 7.0) or earlier versions (bioMérieux, Marcy l'Etoile, France). The yeasts were initially identified as *C. haemulonii*, as *Rhodotorula glutinis* by API 20C Aux or API ID 32C (bioMérieux), as *Saccharomyces cerevisiae* by Auxacolor (BioRad, Hercules, California, United States) or as *Candida famata* by Microscan walkaway (Beckman Coulter, Brea, California, United States) (4). Isolates were submitted on Sabouraud agar plates or Dorset transport medium (Diagnostic Media Products (DMP), Johannesburg, South Africa) to NICD. At NICD, phenotypic identification was performed using ChromAgar *Candida* medium (Mast Diagnostics, Merseyside, UK). All isolates submitted in 2015 were subjected to Matrix-Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) (Bruker, Bremen, Germany) mass spectrometric analysis using Biotyper v3.1 software (Bruker Ltd, Coventry, UK). We then confirmed the identification of *C. auris* isolates by PCR amplification and sequencing of the ITS domain of the ribosomal RNA gene and the

D1-D2 region of the 28S subunit using universal primers (5). AFST for fluconazole, voriconazole, itraconazole, posaconazole, caspofungin, anidulafungin, micafungin, flucytosine and amphotericin B was performed using microbroth dilution panels containing Alamar Blue (Thermo Fisher Scientific, Cleveland, Ohio, USA). Currently, there are no published or defined clinical breakpoints for *C. auris.* For epidemiological purposes, resistance was defined as follows: fluconazole ≥32 µg/mL, voriconazole ≥2 µg/mL (similar to CLSI breakpoints for *Candida krusei*), flucytosine ≥128 µg/mL and amphotericin B ≥2 µg/mL (adapted from Lockhart et al., 2017) (6). Echinocandin resistance was defined using the following tentative MIC breakpoints (adapted from CDC) (7): anidulafungin and micafungin ≥4 µg/mL. Caspofungin is an unreliable indicator of echinocandin resistance, and caspofungin MICs were not interpreted (8). Isavuconazole susceptibility testing was performed on a random sample of 15 fluconazole-resistant isolates using the Etest method. *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 strains were processed together with the samples as part of quality control (QC), and MICs were within the expected ranges.

2.7. Ethics

Approval for laboratory-based surveillance was obtained from the Human Research Ethics Committee (Medical), University of the Witwatersrand, Johannesburg. Ethics approval for this Ph.D. study was granted by the Human Research Ethics Committee (Medical), University of the Witwatersrand, Johannesburg (Clearance certificate no: M150256).

2.8. Statistical analysis

Continuous data were compared using the Mann-Whitney test for nonparametric data, and Pearson's chi-square test or Fischer's exact test was used for categorical variables. The analysis was done with Stata version 14.0 (StataCorp, College Station, TX, USA). *P* values of <0.05 were considered significant.

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Chapter 3: Detection of neonatal unit clusters of *Candida parapsilosis* fungaemia by microsatellite genotyping: Results from laboratory-based sentinel surveillance, South Africa, 2009-2010. Mycoses 2017; 60:320-327.

Introduction

Candida bloodstream infections (BSI) are a common cause of late-onset neonatal sepsis in intensive care units (ICU) (1). Incidence of candidaemia is several-fold higher in neonatal versus adult ICUs: 4.4 cases per 100 000 population in an Australian surveillance study and 1.48 cases per 1 000 patient-days in a US-based surveillance study versus 1.82 per 100 000 population in adults (1, 2). In the US, the overall incidence increased during the 1990s partly owing to improved survival of extremely low-birth weight neonates, with a concomitant decline in cases caused by *Candida albicans* and an increase in cases caused by *Candida parapsilosis* (1). Reports of neonatal candidaemia from several developed countries from 1991 through to 2004 show that approximately one-third of laboratory-confirmed cases were caused by *C. parapsilosis* (range, 16% to 65%) (3). *C. parapsilosis*-associated fungaemia is associated with a relatively lower mortality among neonates (16%) compared with *C. albicans*-associated fungaemia (44%) which may be related to the relatively higher virulence of *C. albicans* (4-6). However, *C. parapsilosis* adheres to and forms biofilms on indwelling plastic devices, produces secretory aspartyl-proteinases and proliferates in nutrient-rich solutions, including total parenteral nutrition (3, 8-11). This pathogen can also be easily transmitted from exogenous sources and the hands of healthcare workers to neonates (12). Outbreaks of candidaemia have thus been regularly reported from neonatal intensive care units (NICUs) (13-17). Azole-resistant *C. parapsilosis* has been recently described as an emerging agent of candidaemia in South Africa (18). We conducted sentinel laboratory-

based surveillance for candidaemia from 2009 through 2010 in South Africa and using microsatellite genotyping, aimed to provide molecular evidence of clusters of cases of *C. parapsilosis* fungaemia among neonates admitted to public-sector hospital NICUs.

Materials and methods

From February 2009 through to August 2010, cases of candidaemia were reported through national laboratory-based surveillance by 11 public-sector hospital laboratories and at least 85 private-sector hospitals referring specimens to five large private pathology laboratory networks in South Africa. Reporting laboratories submitted bloodstream *Candida* isolates to the National Institute for Communicable Diseases (NICD) in Johannesburg with corresponding laboratory reports containing patient demographic data such as age, sex, hospital and hospital unit. An incident case of candidaemia was defined as a patient of any age admitted to a sentinel hospital with first isolation of any *Candida* species from blood culture. For the purposes of this molecular genotyping sub-study, a neonate was defined as a patient aged ≤28 days and patients who were admitted to an NICU beyond this age were excluded. Incidence of neonatal candidaemia was calculated for a single large hospital using the annualized number of neonatal cases as a numerator and the annual number of admissions to the neonatal unit as a denominator. The annualized number of cases was also used to calculate the proportion of positive neonatal blood cultures that was attributed to *Candida* species at the same hospital.

Laboratories submitted *Candida* isolates to NICD on Dorset transport medium (Diagnostic Media Products (DMP), Johannesburg, South Africa). Phenotypic identification was initially performed using ChromAgar Candida medium (Mast

Diagnostics, Merseyside, UK), Vitek 2 YST, API 20C Aux and API ID 32C (bioMérieux, Marcy l'Etoile, France) (18). Minimum inhibitory concentrations (MIC) for fluconazole, voriconazole, itraconazole, posaconazole, caspofungin, anidulafungin, micafungin and flucytosine were determined using pre-prepared dried microbroth dilution panels containing Alamar Blue (Thermo Fisher Scientific, Cleveland, Ohio, USA); MICs were categorised using Clinical and Laboratory Standards Institute (CLSI) M27-S4 interpretive breakpoints. In addition, 24 h epidemiologic cut-off values (ECVs) for *C. parapsilosis* and the Sensititre YeastOne test method were applied to MICs of all tested agents (35-37). Amphotericin B MICs were determined by Etest (bioMérieux) on RPMI 1640 plates containing 2% glucose (DMP). *C. parapsilosis* ATCC 22019 and *Candida krusei* ATCC 6258 strains were included in quality control (QC) runs on all days of testing and MICs were consistently found to be within QC range.

Molecular identification of *C. parapsilosis* species complex was performed by PCR amplification and sequence analysis of the internal transcribed spacer (ITS) domain of the multi-copy ribosomal RNA gene using universal primers (19). Briefly, DNA was extracted from single colonies using Zymo ZR fungal/Bacterial DNA MiniPrep (Zymo Research Corporation, Irvine, California, USA). PCR amplification was performed using ITS1 and ITS4 primers (19). PCR amplicons were purified using Exonuclease I/ Shrimp alkaline phosphatase enzymes (Thermo Fisher Scientific). DNA sequencing was performed with the same primers used for PCR using BigDye terminator version 3.1 in an ABI 3500 genetic analyser (Applied Biosystems, Foster City, California, USA). Identification of cryptic species within the *C. parapsilosis* species complex was performed by pairwise sequence comparison using the NCBI BLAST algorithm (www.ncbinlm.nih.gov/blast). In addition, multiple nucleotide sequence alignment was

performed using ClustalW (46) to identify sequence differences between the three cryptic species within the species-complex: *C. parapsilosis* sensu stricto, *Candida orthopsilosis* and *Candida metapsilosis*. *C. parapsilosis* isolates from neonatal cases were then genotyped by polymorphic microsatellite marker analysis. The extracted DNA was amplified, targeting five microsatellite loci, using a method previously described (16). Following PCR amplification, fragment sizes were determined using capillary electrophoresis on an ABI 3500 genetic analyzer. Allele sizes were scored with respect to GenScan™ 500 ROX Size Standard using Peak scanner version 2.0. (Applied Biosystems, Foster City, California, USA). BioNumerics version 6.5 software was used to perform clustering using the Unweighted Pair Group Method with Arithmetic Mean (UPGMA) (Applied Maths, Sint-Martens-Latem, Belgium). A cluster was arbitrarily defined as a group of isolates with one allele difference between them plus a single neighboring isolate. Cluster analysis was restricted to isolates from neonates admitted to five public-sector hospitals.

Results

A total of 2172 cases of candidaemia were detected through surveillance, of which 1671 had viable isolates identified to species level at the reference laboratory. Of these, 393 cases (24%) occurred among neonates. The incidence of neonatal candidaemia at a large academic hospital in Soweto (Hospital 1: 23 440 neonatal unit admissions) was 49 cases per 10 000 admissions in 2009. During the same year, *Candida* species accounted for 8% (115/1376) of positive blood cultures in this hospital's NICU.

Thirty six per cent (143/393) of neonatal cases were caused by isolates initially identified by phenotypic methods as *C. parapsilosis*. Of these, the vast majority of cases (134/143; 94%) were from public-sector hospitals and 9 were from private hospitals. All isolates from these 143 cases were subsequently confirmed as *C. parapsilosis* sensu stricto by ITS sequencing; no *C. metapsilosis* or *C. orthopsilosis* isolates were detected. A large proportion of these isolates were resistant to fluconazole (77/143; 54%) and voriconazole (20/143; 14%) (Table 1).

Seventy nine closely-related genotypes were identified using microsatellite marker analysis. Eighteen genotypes were represented by 2 or more isolates (n=82) and 61 isolates had unique genotypes (Table 2). Seven clusters, comprised of 82 isolates, were identified at five hospitals in 3 South African provinces (Table 3). Thirty five isolates from neonatal cases diagnosed at these five hospitals belonged to unique genotypes and were not cluster-related. Cluster 1 isolates were unique to Hospital 2 accounting for 44% (8/18) of cases at this hospital. Cluster 2 isolates were detected at all 5 hospitals and were responsible for 39% (7/18) cases of neonatal candidaemia at Hospital 2 and 27% (4/15) of cases at Hospital 3. Cluster 3 was detected at 2 of 5 hospitals and accounted for 20% (3/15) of cases at Hospital 3 and only 1 case at Hospital 1. Clusters 4, 5, 6, and 7 together accounted for 68% (50/73) of cases at Hospital 1 and 63% (5/8) at Hospital 4 (Figure 1).

The number of cases of candidaemia was plotted for Hospital 1 by month and cluster type over the study period (Figure 2). Cluster 4 and cluster 5 isolates were responsible for 16 and 13 cases diagnosed between March and November 2009 respectively. A cluster 6 isolate was first detected from blood culture in February 2009 and re-emerged in January

2010. This cluster was responsible for majority of cases identified during the 2010 surveillance period (69%; 9/13). Cluster 7 isolates, which were unique to Hospital 1, caused a probable outbreak of 7 cases between October and December 2009. At Hospital 2, where 83% (15/18) of cases were caused by cluster 1 and cluster 2 isolates, an outbreak caused by cluster 1 probably occurred between April and August 2009, during which time 88% (7/8) of cases were diagnosed.

The overall proportion of fluconazole-resistant isolates at the 5 hospitals was 56% (65/117); the proportion varied significantly by hospital ($p<0.001$): 78% (57/73), 0% (0/18), 13% (2/15), 50% (4/8) and 67% (2/3) at Hospitals 1 through 5 respectively (Table 1). Isolates belonging to a cluster were no more likely to be resistant to fluconazole than non-cluster isolates (49/82, 60% vs. 16/35, 46%; $p=0.16$). However, isolates belonging to certain clusters were significantly more likely to be fluconazole resistant: all cluster 7 isolates and the majority of cluster 4 (78%), 5 (89%) and 6 (67%) isolates ($p<0.001$). There was no association between patient age (in weeks), sex or surveillance quarter and fluconazole resistance on univariate analysis [data not shown].

Table 1: Antifungal susceptibility profile of *Candida parapsilosis* isolates from cases of neonatal candidaemia (n=143**)**

Antifungal agent	**Range (mg/L)**	**MIC_{50} (mg/L)**	**MIC_{90} (mg/L)**	**Number of isolates (%):**				
				Susceptible	**Intermediate or Susceptible-dose-dependent**	**Resistant**	**Wild type[a]**	**Non-wild type**
Amphotericin B	0.12-1	0.5	1	-	-	-	140 (100.0)	0 (0.0)
Fluconazole	0.25-256	8	32	43 (30.0)	23 (16.0)	77 (54.0)	66 (46.0)	77 (53.0)
Voriconazole	0.008-8	0.12	1	85 (59.0)	38 (27.0)	20 (14.0)	82 (57.0)	61 (43.0)
Itraconazole	0.015-0.5	0.12	0.25	73 (51.0)	70 (49.0)	0 (0.0)	143 (100.0)	0 (0.0)
Posaconazole	0.008-0.5	0.12	0.25	-	-	-	66 (46.0)	77 (53.0)
Flucytosine	0.06-0.25	0.12	0.25	143 (100.0)	0 (0.0)	0 (0.0)	143 (100.0)	0 (0.0)
Caspofungin	0.25-2	1	2	143 (100.0)	0 (0.0)	0 (0.0)	143 (100.0)	0 (0.0)
Anidulafungin	0.25-2	1	2	142 (99.3)	1 (0.7)	0 (0.0)	143 (100.0)	0 (0.0)
Micafungin	0.5-2	2	2	142 (99.3)	1 (0.7)	0 (0.0)	143 (100.0)	0 (0.0)

[a]A wild-type isolate was defined as an isolate with a minimum inhibitory concentration ≤ published epidemiologic cut-off value for the specified agent using the Sensititre YeastOne method.

Table 2: *Candida parapsilosis* genotypes identified from neonatal cases of candidaemia (n=143)

Genotype	**No. (%) of cases**	**Microsatellite fragment length (base pairs) for each allele by locus:**					
		B	**G**	**PB**	**PC**	**PD**	
1	4 (2.8)	126/126	126/126	240/255	171/177	183/184 & 186/187	
2	2 (1.4)	126/126	126/126	239/254	171/177	183/184 & 186/187	
3	2 (1.4)	126/126	126/126	240/255	171/177	186/187	
4	14 (9.8)	128/128	126/126	213/243	171/177	183/184	
5	7 (4.9)	128/128	126/126	243/255	171/177	183/184	
6	7 (4.9)	128/128	126/127	213/243	170/176	183/184	
7	10 (7.0)	128/128	126/127	213/243	171/177	183/184	
8	2 (1.4)	128/128	126/127	240/255	171/177	183/184	
9	3 (2.1)	128/128	126/127	243/263	170/176	183/184	
10	2 (1.4)	128/128	126/127	243/255	170/176	183/184	
11	3 (2.1)	128/128	126/127	243/255	171/177	183/184	
12	2 (1.4)	130/130	126/126	183&186	165/165	144/145	
13	4 (2.8)	130/130	126/127	186/186	164/164	144/144	
14	2 (1.4)	130/130	126/126	186/186	165/165	144/144	
15	3 (2.1)	130/130	126/126	186/186	165/165	144/145	
16	6 (4.2)	130/130	126/127	186/186	164/165	144/145	
17	3 (2.1)	130/130	126/126	185/186	165/165	144/145	
18	6 (4.2)	130/130	126/127	186/186	165/165	144/145	
Unique genotypes	61 (42.7)	-	-	-	-	-	

Table 3: Number of *Candida parapsilosis* isolates and proportion with fluconazole resistance by cluster type for cases of neonatal candidaemia at five sentinel hospitals, n=117

Hospital	**City, Province**	**n**	**Number of *Candida parapsilosis* isolates (% fluconazole-resistant):**							
			Cluster 1	Cluster 2	Cluster 3	Cluster 4	Cluster 5	Cluster 6	Cluster 7	Non-cluster
1	Soweto, Gauteng	73	-	1 (0)	1 (0)	17 (76)	16 (88)	10 (70)	7 (100)	21 (76)
2	Ga-Rankuwa, North West	18	8 (0)	7 (0)	-	-	-	-	-	3 (0)
3	Durban, KwaZulu-Natal	15	-	4 (0)	3 (67)	-	-	-	-	8 (0)
4	Pretoria, Gauteng	8	-	1 (0)	-	1 (100)	3 (100)	1 (0)	-	2 (0)
5	Johannesburg, Gauteng	3	-	1 (100)	-	-	-	1 (100)	-	1 (0)
All	-	117	8 (0)	14 (7)	4 (50)	18 (78)	19 (89)	12 (67)	7 (100)	35 (46)

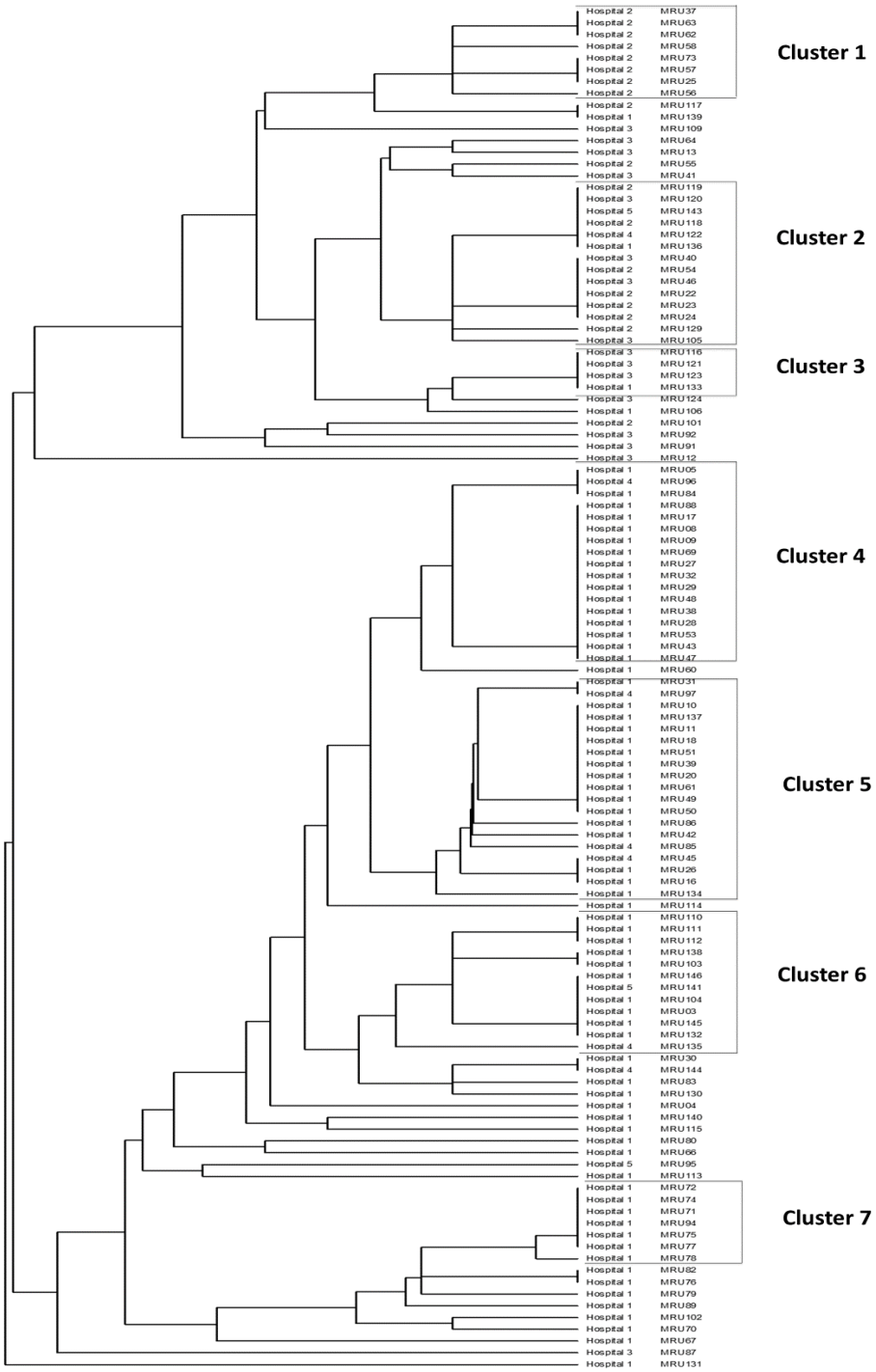

Figure 1: Cluster analysis of *Candida parapsilosis* isolates from five hospitals (n=117)

This phylogenetic tree shows clustering of *C. parapsilosis* isolates from 5 public-sector hospital NICUs based on microsatellite genotyping data, analysed by the Unweighted Pair Group Method with Arithmetic Mean (UPGMA). Seven clusters comprising of 82 isolates were identified; cluster 1 was unique to Hospital 2 and cluster 7 unique to Hospital 1 (Table 3).

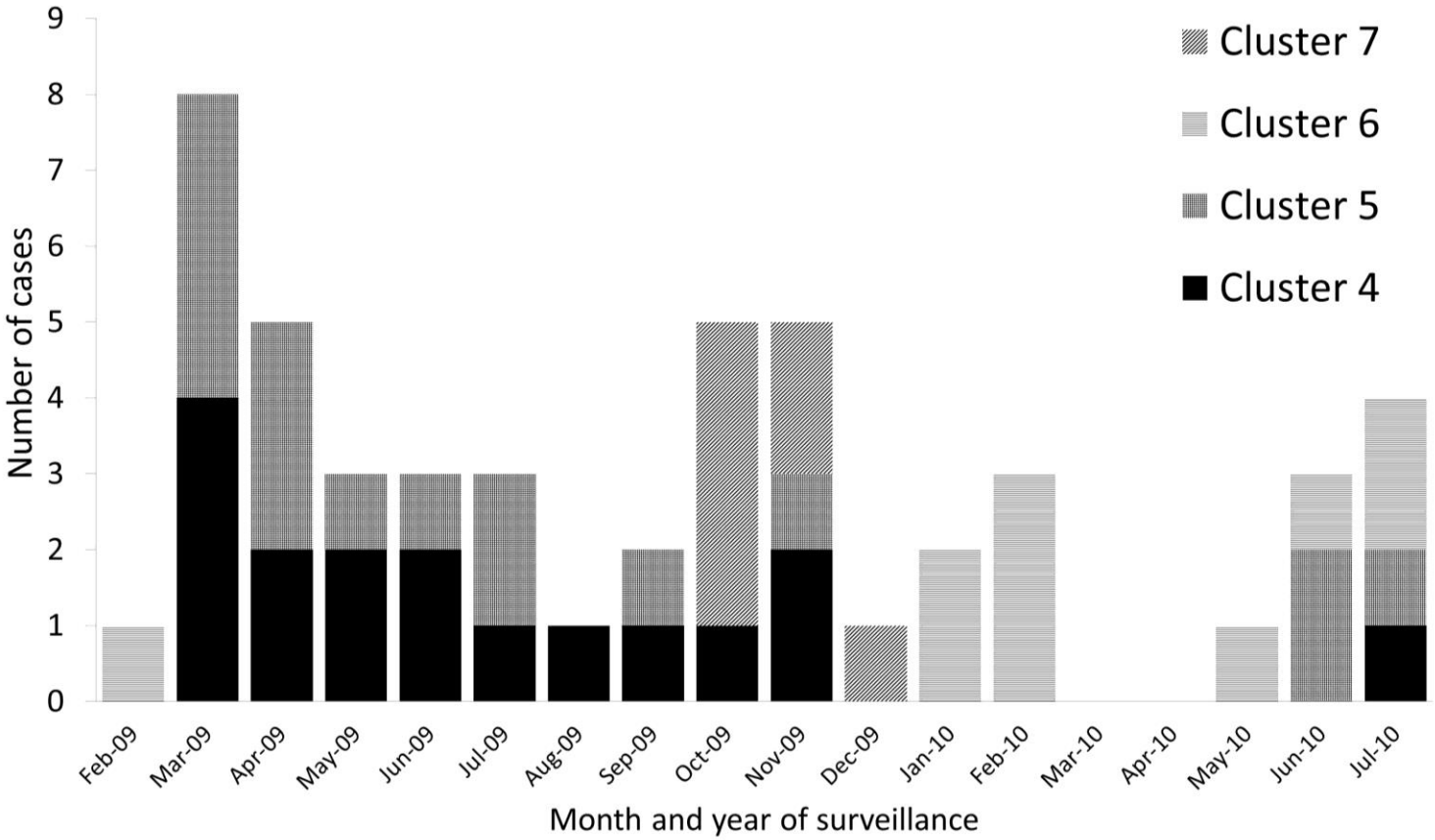

Figure 2: Cases of neonatal candidaemia caused by *Candida parapsilosis* at Hospital 1 (n=73) by surveillance month and cluster type

This bar graph shows the number of cases by cluster type detected at Hospital 1 during the surveillance period. Clusters 4 and 5 strains were predominantly detected in 2009. Cluster 7 strains were detected between October and December 2009. Cluster 6 strains were mostly detected in 2010.

Discussion

In this surveillance study in a resource-limited developing country, we found that neonates, most of whom were admitted to public-sector hospitals, accounted for almost a quarter of all cases of candidaemia. More than a fifth of neonatal cases were caused by *C. parapsilosis* sensu stricto; no *C. metapsilosis* and *C. orthopsilosis* isolates were detected. More than half of the *C. parapsilosis* isolates were resistant to fluconazole. Furthermore, we found evidence of clusters of closely-related genotypes at five public-sector hospitals using microsatellite genotyping. A significant association between cluster type and fluconazole resistance was observed.

The incidence of candidaemia among immunocompromised patients including critically-ill patients, those on immunosuppressive regimens, transplant recipients and low birth weight infants has increased in recent years (39). The mortality attributed to candidaemia in paediatric patients is high (10%-14%) compared to 0.5% of those without candidaemia (44). In South Africa, previous studies on the incidence of candidaemia have been restricted to single hospitals and specific age groups (35, 42, 45). The incidence of neonatal candidaemia at a hospital in Johannesburg reportedly increased from 0.6% in 2007 to 1.8% in 2011 (35). In our study, *Candida* was a very common cause of neonatal sepsis with an incidence of 49 cases per 10 000 admissions at a one hospital, higher than previously reported from other developing countries (47, 48).

In recent years, *Candida* species other than *C. albicans* have increased as causative agents of candidaemia (40, 41). In a global surveillance study, *C. parapsilosis* was the second most common pathogen responsible for candidaemia after *C. albicans* (39). Several studies from South Africa have demonstrated a shift in the profile of species causing candidaemia among hospitalised patients. While *C. albicans* remains the dominant species causing BSI among adults, in more recent years (2005-2007), *C. parapsilosis* has emerged as the second most common species (45). However, *C. parapsilosis* was the most common species causing neonatal BSI, accounting for 54% of the cases (35). *C. parapsilosis* was the second most common species causing candidaemia after *C. albicans* in our study and was responsible for 1 in 5 cases of neonatal candidaemia. No cryptic species from the *C. parapsilosis* species complex were detected.

C. parapsilosis is an important cause of nosocomial infections because of its ability to colonize intravenous devices and the hands of health workers (14, 20, 21). These attributes allow *C. parapsilosis* to spread within a healthcare setting. Detection and elimination of this spread is paramount to infection prevention and control. Polymorphic microsatellite marker analysis (PMM) is a fast, reproducible, and archivable method for typing *C. parapsilosis* (10, 22-24). *C. parapsilosis* genotyping using PMM has a high discriminatory power (16). Using PMM, we were able to show that clusters of closely-related genotypes caused outbreaks in public-sector hospitals. At Hospital 1, we demonstrated that cluster 4 and 5 isolates were responsible for cases between March and November 2009 and likely constituted an outbreak since the closely-related genotypes were epidemiologically linked in place and time. Cluster 6 isolates were detected throughout the 2010 surveillance period with intermittent peaks and was the dominant cluster

causing disease at Hospital 1. Cluster 7 was unique to Hospital 1 with most cases detected over 3 months in late 2009. Using this genotyping method, we were thus able to retrospectively confirm outbreaks, some of which may not have been identified and managed in real-time (17, 25). Similar genotyping method was recently used in Austria, Portugal and Spain, and was found to be more discriminatory uncovering probable outbreaks and inter- and/or intra-hospital transmission of *C. parapsilosis* (49, 50,51)

Azole resistance in common *Candida* species remains low with reported prevalence ranging from 1.0% to 2.1% for *C. albicans*, 0.4% to 4.2% in *C. parapsilosis* and 1.4% to 6.6% in *C. tropicalis* (26). *C. parapsilosis* is usually susceptible to most antifungal agents including amphotericin B and the azoles (26-30). Furthermore, global surveillance studies indicate that resistance to fluconazole is uncommon among cases of *C. parapsilosis* BSI (31). Similar findings were reported in the context of long-term fluconazole prophylaxis (32). We found the prevalence of fluconazole resistance to be unusually high in this series of cases of neonatal candidaemia, although it varied by hospital. We also found an association between specific clusters and fluconazole resistance. This observation is of concern because in the South African public sector, fluconazole is often the antifungal agent of choice for treatment of candidaemia. Based on reported risk factors from *C. parapsilosis* outbreaks, cross-transmission of fluconazole-resistant *C. parapsilosis* strains among neonates was most likely related to contaminated medical devices and surfaces and due to suboptimal infection prevention and control practices, including poor adherence to hand antisepsis protocols (3, 21, 33, 34). In addition, we speculate that healthcare workers in NICUs, some of whom reside on the hospital premises, may become colonised by

fluconazole-resistant *C. parapsilosis* strains and potentially may be more likely to transmit this pathogen to patients.

Conclusion

We have shown that cases of *C. parapsilosis*-associated candidaemia in public-sector hospital NICUs were caused by closely-related genotypes and there was molecular evidence of undetected outbreaks as well as intra-hospital transmission. The high prevalence of fluconazole-resistant strains limits prophylaxis and treatment options at public-sector hospital NICUs.

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Chapter 4: Fluconazole-resistant *Candida parapsilosis* strains with Y132F substitution in *ERG*11 gene causing invasive infections in neonatal unit, South Africa. Mycoses 2020; 63: 471-477.

Introduction

Candidemia is associated with high morbidity and mortality in neonatal units (1, 2). *Candida parapsilosis* is the second most common non-albicans *Candida* species in South African public-sector hospitals and a common cause of candidemia in neonates, accounting for 53% of cases (3). The prevalence of azole resistance in South African *C. parapsilosis* strains is exceptionally high (58%-63%) versus other parts of the world (1%-7.7%) (3-7). Mechanisms leading to azole resistance are better characterised in *Candida albicans* than in other *Candida* species. Resistance may occur in one of the following ways, (i) point mutations in *ERG11* gene, resulting in reduced azole binding affinity; (ii) overexpression of the drug target (*ERG11*) gene which encodes for 14α demethylase; (iii) overexpression of the genes encoding multidrug transporters [ATP binding cassettes (ABC) and major facilitator superfamily (MFS)], (iv) overexpression of *CDR1* and *CDR* caused by activating mutations in the transcription factor genes *TAC1* leading to efflux of all azole antifungal agents (8) (v) overexpression of an MFS transporter (*MDR1*) caused by activating mutations in the transcription factor genes *MRR1* leading to efflux of fluconazole and voriconazole (9-11). and (vi) mutations in *ERG3* gene resulting in the inactivation of sterol desaturase. A number of studies from the United States of America, United Kingdom, Brazil, Portugal, South Korea and India have recently explored the possible mechanisms of azole resistance in *C. parapsilosis* strains (12-17). Souza et al

(2015) demonstrated that not only does upregulation of *MDR1* and amino acid substitutions in *ERG11* lead to resistance to fluconazole but upregulation of *ERG11* and *CDR1* are involved as well (14). The high prevalence of fluconazole resistance in South African *C. parapsilosis* bloodstream isolates provides a unique opportunity to investigate the mechanisms of fluconazole resistance in our setting. We describe the possible molecular mechanisms of fluconazole resistance in *C. parapsilosis* isolates causing invasive neonatal infections at Chris Hani Baragwanath Academic Hospital, Soweto, South Africa.

Materials and methods

We conducted laboratory-based surveillance at Chris Hani Baragwanath Academic Hospital through the TRAC-SA program from 2009 through to 2010. Reporting laboratories submitted bloodstream *Candida* isolates to the National Institute for Communicable Diseases (NICD) in Johannesburg with corresponding laboratory reports containing patient demographic data. A case of candidemia was defined as a person of any age admitted to any hospital with first isolation of any *Candida* species from blood culture. For the purposes of this molecular sub-study, a neonate was defined as a patient aged ≤28 days admitted to a neonatal unit at Chris Hani Baragwanath Academic Hospital. Any patient beyond this age was excluded. Chris Hani Baragwanath Academic Hospital is the third largest hospital in the world with approximately 3200 beds. The neonatal unit at this hospital forms part of the department of pediatrics, which has 408 beds. In 2000, 5645 children were admitted and the death rate was 9%. Half the admitted children were neonates. Incidence of neonatal candidemia was calculated using the annualized number

of neonatal cases as a numerator and the annual number of admissions to the neonatal unit as a denominator. The annualized number of cases was also used to calculate the proportion of positive neonatal blood cultures that were attributed to *Candida* species at the same hospital.

Candida isolates were submitted to National Institute for Communicable Diseases (NICD) from the laboratory at Chris Hani Baragwanath Academic Hospital. Phenotypic identification was initially performed using ChromAgar *Candida* medium (Mast Diagnostics, Merseyside, UK), Vitek 2 YST, API 20C Aux and API ID 32C (bioMérieux, Marcy l'Etoile, France). Minimum inhibitory concentrations (MIC) against nine antifungal agents were determined and categorized using Clinical and Laboratory Standards Institute (CLSI) M60 interpretive breakpoints. *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 strains were included in quality control (QC) runs on all days of testing and MICs were consistently found to be within QC range. Sequencing of the internal transcribed spacer (ITS) domain of the multi-copy ribosomal RNA gene was performed to confirm species identification using universal primers (18). Isolates that were confirmed as *C. parapsilosis sensu stricto* were then genotyped by polymorphic microsatellite marker analysis (19). To screen for mutations that confer resistance to azole antifungals, we sequenced the *ERG11* gene and transcription factor gene *MRR1* of *C. parapsilosis* isolates using a previously-described method (13). The deduced nucleotide sequences were then compared to *C. parapsilosis* 22019 ATCC wild-type *ERG11* and CDC317 wild type *MRR1* strain. Pearson's chi-square test or Student's t test was used to compare categorical or continuous variables. Analysis was performed with Stata version 14.0 (StataCorp, College Station, TX, USA). Approval for laboratory-based

surveillance was obtained from the Human Research Ethics Committee (Medical), University of the Witwatersrand, Johannesburg.

Results

During the surveillance period, 204 cases of candidemia occurred among infants aged <1 year admitted to Chris Hani Baragwanath Academic Hospital. Of these, 69% (140/204) of cases occurred among neonates. *C. parapsilosis* accounted for 59% (82/140) of cases of neonatal candidemia at this hospital, followed by *C. albicans* and *Candida glabrata* accounting for 31% (44/140) and 6% (8/140) respectively. Of the 82 neonatal *C. parapsilosis* cases, 73 cases had viable isolates; the remaining 9 were no longer viable for further molecular characterization. The incidence of neonatal candidemia at Chris Hani Baragwanath Hospital (23 440 neonatal unit admissions) was 49 cases per 10 000 admissions in 2009. During the same year, *Candida* species accounted for 8% (115/1376) of positive blood cultures in this hospital's neonatal unit.

Using ITS gene sequencing, all 73 isolates were confirmed as *C. parapsilosis sensu stricto*. Of these, 78% (57/73) were resistant to fluconazole, 15% (11/73) were susceptible-dose-dependent (SDD) and 7% (5/73) were susceptible (Table 1). Two *ERG11* amino acid substitutions, Y132F and R398I, were identified in 82% (47/57) of fluconazole-resistant isolates. The most commonly identified substitution was Y132F in 68% (39/57) of fluconazole-resistant isolates and none in susceptible isolates. Ninety-two per cent (36/39) of Y132F mutations were in combination with the R398I substitution. Three amino acid substitutions, R405K, G583R, and A619V, and 1 nucleotide deletion at position 1331 which caused a frameshift mutation were identified within *MRR1* gene in 19 of 73 (26%) isolates. The missense mutation, G583R, was exclusively identified in three (4%) resistant isolates. Another substitution, A619V was identified in 4 (5%)

resistant and 2 (3%) SDD isolates. One nucleotide deletion at position 1331 was identified in one SDD and one resistant isolate.

Four clusters comprising of 50 isolates were identified in this hospital by microsatellite genotyping. Cluster 1 accounted for 23% (17/73) of all cases, cluster 2 for 22% (16/73), cluster 3 for 14% (10/73) and cluster 4 for 10% (7/73). Twenty-three isolates (32%) did not belong to a cluster (Figure 1). Compared to non-cluster isolates, a larger proportion of isolates belonging to clusters were associated with fluconazole resistance (15/23 [65%] versus 41/50 [82%], p-value=0.004). All isolates belonging to cluster 4, a majority of cluster 1 (13/17; 76%), cluster 2 (14/16; 87%) and cluster 3 (7/10; 70%) isolates were resistant. Isolates with Y132F substitution had a 39% higher odds of belonging to a cluster than non-Y132F isolates (odds ratio 1.39; 95%CI, 0.48-4.00; p-value=0.6) (Table 2).

Table 1: Antifungal susceptibility profile of *C. parapsilosis* isolates from cases of neonatal candidaemia, TRAC-SA [2009-2010 (n=73)]

Antifungal agents	**Number of isolates with MIC (mg/L):**																	
	<0.00	**0.015**	**0.03**	**0.06**	**0.12**	**0.25**	**0.5**	**1**	**2**	**4**	**8**	**16**	**32**	**>64**	**Susceptible**	**Susceptible dependent**	**do**	**Resistant**	**Wild type**	**Non-wild type**
Amphotericin B					1		35	37							-	-		-	36 (49)	37 (51)
Fluconazole					1		1		3	11	10	26	19	2	5 (7)	11 (15)		57 (78)	5 (7)	68(93)
Voriconazole	2	2	1	13	5	16	10	21	3						26 (36)	31 (42)		16 (22)	18 (25)	55(75)
Itraconazole				4	23	50	4	1							26 (36)	47 (64)		0 (0)	71 (97)	2(3)
Posaconazole		3	8	14	21	27									-	-		-	11 (15)	62(85)
Caspofungin					1	1	1	34	39						73 (100)	0 (0)		0 (0)	34 (47)	39(53)
Micafungin					1	2	2	16	52						73 (100)	0 (0)		0 (0)	73 (100)	0(0)
Anidulafungin		1		1		1	2	56	11	1					72 (99)	1 (1)		0 (0)	72 (99)	1(1)
Flucytosine				40	30	3									73 (100)	0 (0)		0 (0)	73 (100)	0(0)

¶A wild-type isolate was defined as an isolate with a minimum inhibitory concentration ≤ published epidemiologic cut-off value for the specified agent using the Sensititre YeastOne method.

Table 2: Fluconazole-resistant *C. parapsilosis* isolates with *ERG11* Y132F substitution by cluster type and year (n=39)

Isolate ID	MIC (g/mL)		Year isolated	Microsatellite cluster
	Fluconazole	Voriconazole		
MRU05	16	0.25	2009	1
MRU09	16	1	2010	3
MRU10	32	0.25	2010	NC
MRU14	16	0.5	2010	3
MRU15	16	0.25	2010	NC
MRU16	32	1	2010	NC
MRU17	32	0.5	2010	NC
MRU18	16	0.25	2010	NC
MRU20	16	0.25	2010	3
MRU22	32	1	2010	2
MRU25	16	0.12	2010	3
MRU28	16	1	2010	3
MRU30	16	0.12	2009	2
MRU34	16	0.25	2009	2
MRU36	32	1	2010	1
MRU37	16	0.25	2009	1
MRU39	16	0.5	2009	2
MRU41	32	0.5	2009	1
MRU42	32	0.5	2009	2
MRU43	64	0.5	2009	2
MRU47	32	1	2009	2
MRU48	32	1	2010	2
MRU50	16	0.5	2009	1
MRU51	16	0.5	2009	1
MRU52	32	1	2009	2
MRU55	32	0.5	2009	1
MRU56	16	2	2009	NC

MRU58	32	1	2009	4
MRU59	16	1	2009	4
MRU60	32	1	2009	4
MRU61	16	1	2009	NC
MRU63	32	1	2009	4
MRU64	32	1	2009	NC
MRU66	16	1	2009	NC
MRU67	16	1	2009	NC
MRU68	16	0.5	2009	1
MRU70	32	0.015	2009	1
MRU72	32	1	2009	4
MRU73	64	2	2009	NC

NC- Non-cluster isolates

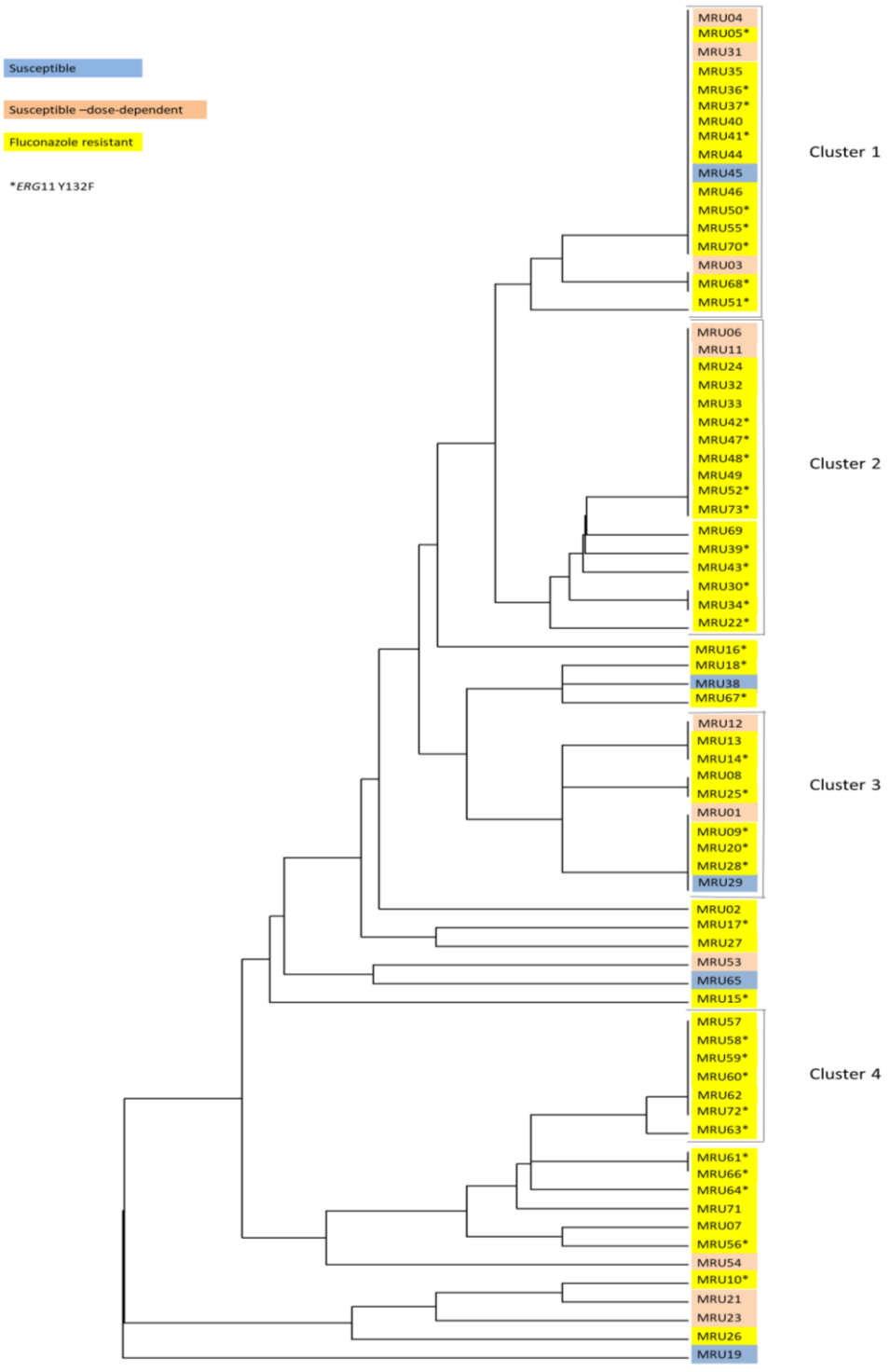

Figure 1: Dendrogram generated by UPGAM based on microsatellite analysis results showing clustering of fluconazole-resistant *C. parapsilosis* strains from a single neonatal unit.

Discussion

C. parapsilosis has emerged as the most common cause of nosocomial fungal bloodstream infections in neonates in South Africa (3,7). During the 2009-2010 surveillance period, we demonstrated that *C. parapsilosis* was the major cause of candidemia at Chris Hani Baragwanath Academic Hospital neonatal unit. Almost 80% of isolates were resistant to fluconazole. The predominant amino acid substitution was Y132F in the *ERG11* gene, accounting for 68% of fluconazole-resistant isolates. We found an association between fluconazole resistance mutations and cluster types. Y132F isolates were more likely to belong to a cluster than non-Y132F isolates. Gain of function mutations in *MRR1* gene (G583R and A619R) accounted for a small proportion of fluconazole resistance.

The prevalence of fluconazole-resistant *C. parapsilosis* in other parts of the world including the Latin America, USA (7.7%) and Asia-western Pacific (6%) is much lower (1%-6%) (4-6, 20). To date, South Africa has reported the highest fluconazole resistance in *C. parapsilosis* (58%-63%) globally (3, 7). The current study reports fluconazole resistance of 78%, which is the highest to date to be reported in South Africa compared to previous studies. In recent years, studies have shown that point mutations within the *ERG11* gene are major drivers of fluconazole resistance in *Candida* species. Similarly, our data showed point mutations within *ERG11* gene constitutes a majority (47/57; 82%) of mutations in fluconazole-resistant isolates. Of particular interest, Y132F substitution was found to be a major contributor to fluconazole resistance in South African *C. parapsilosis* isolates, accounting for 68% (39/57) of the fluconazole-resistant isolates,

which is considerably higher than reported in other countries. Recent studies showed that Y132F substitution accounts for 31%-57% of fluconazole-resistant *C. parapsilosis* isolates worldwide. This amino acid substitution Y132F was found exclusively in fluconazole-resistant isolates and none in susceptible and SDD isolates (12-14, 21). Our study findings confirm that Y132F is the predominant mechanism driving fluconazole resistance in *C. parapsilosis*. Y132F has also been reported in fluconazole-resistant *C. albicans*, *C. tropicalis* and *C. auris* isolates, though South African *C. auris* strains have the F126T substitution (22-24). The presence of Y132F and other substitutions at this position in *C. albicans* and *C. tropicalis* has been associated with reduced susceptibility to azole antifungals (20, 21). Another substitution, R398I was identified in 92% (36/39) of Y132F isolates. This was a similar occurrence in other studies, suggesting that R398I alone cannot drive or facilitate fluconazole resistance in *C. parapsilosis* isolates but may function to enhance resistance or to stabilize the conformational change derived from the other substitution (12, 13, 15, 25). Further analysis needs to be undertaken to ascertain the role played by R398I point mutation in fluconazole resistance.

Sequence analysis of the transcription factor gene *MRR1* revealed three amino acid substitutions G478R, G583R and A619V which were exclusively identified in both SDD and resistant isolates. Although our study did not explore the effects of these substitutions on expression of *MDR1*, previous studies have shown that these substitutions have some form of gain-of-function activity in fluconazole-resistant *C. parapsilosis* isolates. Initial studies indicate these substitutions change fluconazole susceptibility in *C. parapsilosis* isolates as they were identified exclusively in SDD and resistant isolates (13, 26). Branco et al (2015) successfully demonstrated that G583R confers hyperactivity to *MRR1* transcription factor leading to upregulation of *MDR1* drug transporter (16). In the present

study, A619V was found in 2 SDD and 4 resistant isolates. This was similar to an observation in a multicenter US study, where this substitution was identified in a resistant isolate with 16-fold increased levels of *MDR1* and in an SDD isolate with 10-fold-increased levels of *MDR1*. The authors suggested that A619V has a moderate gain-of-function activity (13). Although we did not assess the expression levels of *MDR1* in the current study, based on the expression results of the same SNPs from other studies, our findings confirm that *MDR1* is an important role player in fluconazole resistance in *C. parapsilosis*.

Microsatellite analysis showed that Y132F isolates were more likely to belong to a cluster than non-Y132F isolates, suggesting that *C. parapsilosis* strains with or without a Y132F substitution isolated in this particular hospital's neonatal unit were equally responsible for candidemia outbreaks over an 18-month period. The fact that it appeared in multiple clusters also suggests that the mutation was acquired independently in multiple isolates rather than exclusively through clonal transmission. The presence of Y132F substitution in small clonal clusters in hospitals or communities from the US and South Korea studies may suggest that *C. parapsilosis* isolates harboring this substitution may have a competitive advantage over non-Y132F fluconazole-resistant isolates in a hospital setting (13, 15). The same Y132F substitution has been detected in clonal *C. auris* strains from Pakistan, India and Venezuela (24). This is concerning because both *C. parapsilosis* and *C. auris* have been implicated in nosocomial outbreaks of candidaemia in hospital settings, and they are resistant to fluconazole which is a treatment of choice for invasive candidiasis particularly in resource-limited countries where echinocandins are not available for public sector-hospitals. As such, it is important to conduct ongoing surveillance for fluconazole-resistant non-albicans *Candida* species because of their

ability to persist in the hospital environment, medical devices, hands of health care workers, and eventually causing outbreaks.

A number of limitations were identified in this study: First, we only analysed isolates and data from a single center to establish the association between clusters and mutations. Therefore, the data may not be representative of other medical centres in the country or region. Second, we had very limited demographic, clinical and treatment data for these cases. Lastly, we did not undertake quantitative real-time PCR to determine the expression levels of *ERG11* and *MDR1* in isolates with SNPs in the *ERG11* gene and *MRR1* transcription factor gene.

Conclusions

We demonstrated that fluconazole resistance in *C. parapsilosis* strains from a single South African neonatal unit was significantly associated with clusters and predominantly driven by Y123F amino acid substitutions in the *ERG11* gene. Although a large number of isolates had the Y132F substitution, it was not found exclusively in a single cluster.

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Chapter 5: *Candida auris*-associated candidemia, South Africa. Emerging Infectious Diseases. 2014; 20 (7): 1250-1252

To the Editor: We noted the report by Chowdhary et al. (1) and report *Candida auris* as a causative agent of candidemia in South Africa, with an estimated prevalence of 0.3% (N.P. Govender et al., unpub. data). First isolated in 2009, *C. auris* is an emerging species associated with clinical disease (2-6**)**. We analyzed 4 isolates submitted to the National Institute for Communicable Diseases (Johannesburg, South Africa) from 4 patients with candidemia who had been admitted to different public- and private-sector hospitals from October 2012 through October 2013.

Identification of the isolates was undertaken by using ChromAgar *Candida* medium (Mast Diagnostics, Merseyside, UK), Vitek-2 YST (bioMérieux, Marcy l'Etoile, France), API 20C AUX (bioMérieux), and sequencing of internal transcribed spacer (ITS) and D1/D2 domains of the ribosomal RNA gene (7), followed by microbroth dilution susceptibility testing (8). All isolates were misidentified as *C. haemulonii* and *Rhodotorula glutinis* by Vitek-2 YST and API 20C AUX assays, respectively (Table).

Similar to the findings of Chowdhary et al., all isolates assimilated N-acetyl-glucosamine (1). With the use of the CBS-KNAW database, pairwise sequence alignment of ITS region showed 99% sequence homology to Kuwait isolates, and alignment of D1/D2 domain showed 98%

homology to the Kuwait/India isolates (9). In a neighbor-joining phylogenetic tree based on ITS sequences, South Africa isolates formed a cluster with India and Kuwait isolates (Technical Appendix Figure).

Fluconazole MICs were high for all isolates (Table). Isolates 209 and 224 showed reduced voriconazole susceptibility with MICs of 1 μg/mL and 2 μg/mL, respectively, which is above the epidemiologic cutoff value for 11 *Candida* species (10). Isolates were susceptible to amphotericin B and echinocandins at low MICs Clinical data were available for 1 patient (Technical Appendix Table). Two *C. haemulonii* isolates were identified during laboratory-based sentinel surveillance for candidemia in South Africa; the ITS region of one isolate was sequenced and the isolate identified as *C. auris* (N.P. Govender, pers. comm.). In this study, *C. auris* was misidentified by routinely used tests and was accurately identified by sequencing, in keeping with previous findings *(*1**,** 3**,** 4**,** 6).

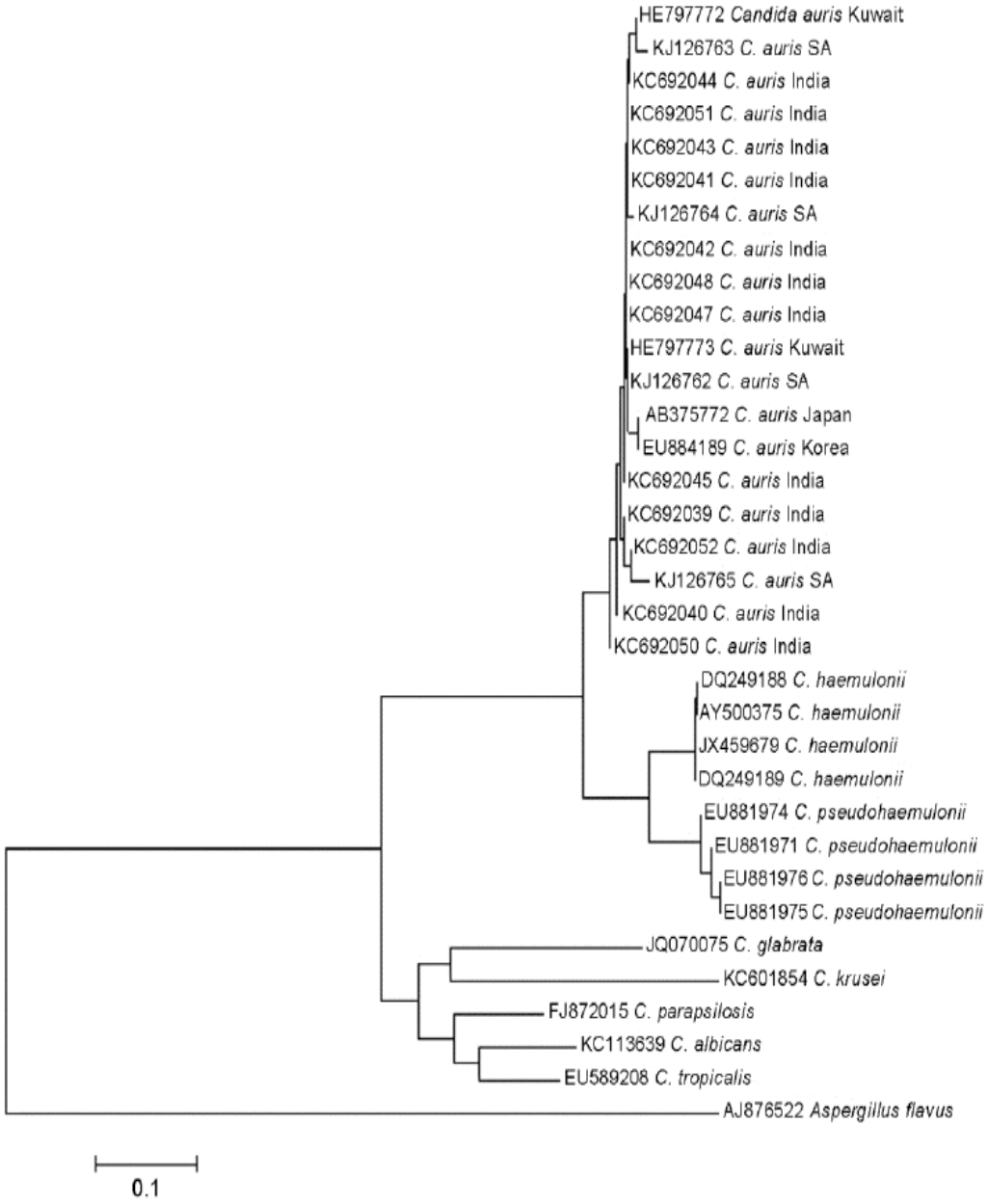
Table 1: Identification and antifungal susceptibility results of four *Candida auris* isolates from four patients with candidaemia (n=4)

Isolate ID	Age (years)	Sex	Hospital unit	Vitek-2 YST	API 20C AUX	DNA sequence analysis[a]	Minimum inhibitory concentration (µg/ml):								
							AMB	FLU	VRC	POS	ITC	5-FC	CAS	MFG	AFG
208	85	Male	High-care	*C. haemulonii*	*R. glutinis*	*C. auris*	1	>256	0.5	0.03	0.12	0.12	0.25	0.06	0.25
209	60	Male	Medical intensive care	*C. haemulonii*	*R. glutinis*	*C. auris*	0.5	>256	1	0.06	0.12	0.12	0.12	0.06	0.12
224	73	Male	Burns	*C. haemulonii*	*R. glutinis*	*C. auris*	1	>256	2	0.06	0.25	0.12	0.25	0.12	0.25
293	27	Male	Trauma intensive care	*C. haemulonii*	*R. glutinis*	*C. auris*	1	64	0.25	0.015	0.06	0.06	0.03	0.06	0.06

Abbreviations: AMB: amphotericin B; FLU: fluconazole; VRC: voriconazole; POS: posaconazole; ITC: itraconazole; 5- FC: flucytosine; CAS: caspofungin; MFG: micafungin; AFG: anidulafungin. [a]Sequence data for the four isolates have been deposited in the National Center for Biotechnology Information GenBank. The allocated accession numbers are KJ1236762 to KJ126765 and KJ126758 to KJ126761 for the ITS and KJ126762 to KJ126765 for the D1/D2 regions.

Technical Appendix Table: Clinical characteristics of a South African patient with candidemia due to *Candida auris*

Isolate ID	**Age**	**Sex**	**Risk factor/s**	**Antifungal treatment**	**Outcome**
224	73-year-old	Male	Referred to a public-sector specialist burns unit from a private-sector hospital 40% third degree burns with inhalational injury; required debridement, skin grafts and tracheostomy *In-situ*: central venous catheter/s, arterial line, urinary catheter Mechanically ventilated Multiple episodes of sepsis requiring broad-spectrum antibiotics including beta-lactams, colistin, linezolid and vancomycin Renal failure; required hemodialysis	Amphotericin B deoxycholate (only received 1 dose)	Died 35 days after first admission to hospital



Technical Appendix Figure 1: Phylogenetic relatedness of internal transcribed spacer (ITS) region of the ribosomal RNA gene of *C. auris* with closely-related *Candida* species.

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Chapter 6: *Candida auris* in South Africa, 2012-2016. Emerging Infectious Diseases. 2018; 24 (11): 2036-2040.

Introduction

The earliest reported case of infection with the yeast *Candida auris* in South Africa occurred in 2009; however, the pathogen was initially misidentified as *Candida haemulonii* (a closely related yeast), and *C. auris* was only confirmed retrospectively in 2014, when 4 other cases of *C. auris* candidemia were described in South Africa (1). Since descriptions in Southeast Asia in 2009, cases of *C. auris* have been reported from many countries on 6 continents (Asia, Africa, South America, Europe, North America, and most recently Oceania) (2).

C. auris has been associated with large healthcare-associated outbreaks because of its ability to be transmitted person-to-person by direct contact, form biofilms, persist in the hospital environment on surfaces and on shared equipment, and resist chemical disinfection by certain products (3-5)**.** Over the past 9 years, cases of *C. auris* have been detected at many hospitals in South Africa, causing large outbreaks at some facilities, and this pathogen now accounts for ~1 of every 10 cases of candidemia (6)**.** South Africa has a unique *C. auris* clade separated from Asian, Southeast Asian, and South American clades by tens of thousands of single-nucleotide polymorphisms, consistent with the hypothesis that *C. auris* emerged independently in Africa and simultaneously on several other continents (7). However, the prevalence and geographic extent of *C. auris* disease is likely underestimated, especially in low- and middle-income countries in

Africa, because conventional laboratory methods misidentify the fungus and relatively few resource-limited countries have the capacity for identification by mass spectrometric or molecular methods (8)**.** South Africa has an established national surveillance infrastructure for infectious diseases, including those caused by antimicrobial drug-resistant pathogens that is based on a large network of well-equipped diagnostic pathology laboratories. In light of an emerging epidemic of *C. auris* infections among hospitalized patients in parts of South Africa, we sought to describe the national epidemiology of laboratory-confirmed cases during 2012-2016.

Materials and Methods

We conducted national laboratory-based surveillance for *C. auris* retrospectively over a period of >4 years, from the earliest known reports of cases in South Africa in October 2012 through November 2016 (***1***)**.** We defined a case as a first isolation of *C. auris* from any specimen from a patient of any age admitted to any South Africa healthcare facility. We also included probable cases in which the diagnostic laboratory had used a nonconfirmatory biochemical identification method such as Vitek-2 YST (bioMérieux, Marcy l'Etoile, France) and *C. haemulonii* was cultured. The National Health Laboratory Service (NHLS) provides diagnostic pathology services to the public sector, serving ~83% of the population of South Africa, and has ~60 mostly hospital-based laboratories offering tests for fungal identification. NHLS laboratories performed species-level identification for *Candida* NHLS laboratories using several platforms during the surveillance period, namely Vitek 2 YST, API 20C Aux, or API ID 32C (bioMérieux); Auxacolor (Bio-Rad, Hercules, CA, USA); and Microscan (Beckman Coulter, Brea, CA, USA). Some of these diagnostic platforms are known to misidentify *C. auris* as other yeasts (9); however, we did

not include any other species in our probable case definition. Private pathology laboratory practices, which serve the remainder of the population with health insurance, have a centralized model of fungal identification; that is, central laboratories perform diagnostic tests for patient specimens referred from a large number of healthcare facilities across a region or province. These laboratories used the same yeast identification platforms; however, 1 private pathology practice introduced the Vitek MS system (bioMérieux) in 2013. In general, NHLS laboratories identified *Candida* to species level only for isolates from normally sterile sites, whereas private laboratories identified all *Candida* isolates to species level, regardless of the specimen source.

We obtained line list specimen-level data from 4 large private diagnostic pathology practices, which together serve almost the entire private health sector, for the surveillance period. We requested that these line lists included any specimens from which either *C. haemulonii* or *C. auris* was cultured. We deduplicated these laboratory data to patient level by applying the surveillance case definition and using a unique laboratory identifier. We then merged this dataset with a similar line list of cases of *C. auris* fungemia that were submitted to the National Institute for Communicable Diseases (NICD) from NHLS laboratories as part of candidemia surveillance. All these bloodstream isolates were confirmed as *C. auris* at NICD using the Bruker Biotyper system (Bruker, Bremen, Germany) or PCR amplification and sequencing of the internal transcribed spacer (ITS) domain of the ribosomal RNA gene using universal primers (10). Variables included in the final dataset were age or date of birth, sex, date of specimen collection, location (province and admitting hospital), specimen type, and species-level identification.

We defined a case as colonization if the isolate was cultured from central venous catheter tips (with no corresponding blood culture specimen), urine, respiratory tract specimens and skin or mucosal swabs. We defined a case as invasive disease if the source of the isolate was blood, cerebrospinal fluid, or serous fluid or tissue. Treating clinicians or hospital-based infection prevention and control (IPC) practitioners submitted the specimens that yielded these isolates; therefore, we may have detected cases of colonization because of active screening at some hospitals with outbreaks. There was no uniform practice for screening for colonization during the surveillance period, and this study preceded the interim guidance issued by NICD (11). To our knowledge, the number of healthcare facilities served by the laboratory network did not change over the surveillance period. We obtained approval for laboratory-based surveillance from the Human Research Ethics Committee (Medical), University of the Witwatersrand, Johannesburg.

Results

For October 2012-November 2016, we identified a total of 1,692 confirmed or probable *C. auris* cases at both public and private hospitals, although most patients (1,578/1,692, 93%) were admitted to private facilities. Of the private-sector cases, at least 647 (38%) had isolates that were identified as *C. haemulonii* and not confirmed as *C. auris***.** All 114 bloodstream isolates from public-sector cases were confirmed as *C. auris* at NICD's mycology reference laboratory. Of 1,579 case-patients with a recorded specimen type, 451 (29%) had invasive disease (Table), with isolates cultured from normally sterile sites: 344 (76%) from blood, 56 (12%) from fluid, 49 (11%) from tissue, and 2 (<1%) from cerebrospinal fluid. The remaining 1,128/1,579 (71%) isolates were cultured from sites of probable colonization: 622 (55%) from urine, 288 (26%) from

central venous catheter tips, 173 (15%) from respiratory tract, and 45 (4%) from skin, mucosal, or wound swabs.

Male patients accounted for 62% of cases. The median age of patients was 60 years (IQR 46–72 years); 38 patients (2%) were <18 years of age, and 9 (0.5%) were infants <1 year of age. Patients with invasive disease were younger than colonized patients: median age for invasive disease was 55 years (IQR 41–68 years) versus median 63 years (IQR 49–74 years) for colonized patients ($p < 0.001$). All cases of colonization were detected at private-sector hospitals; at least 39% (444/1,128) of these isolates had been identified as *C. haemulonii***.** In contrast, 25% (112/451) of invasive isolates had been identified as *C. haemulonii*.

Of cases with a known location, 92% (1,440/1,571) were reported from Gauteng Province, the most densely populated province and the economic and travel hub of South Africa. A median of 4 cases (interquartile range [IQR] 1-11) was reported from each of >94 hospitals. Of all cases with a known location, 80% (1,087/1,353) were reported from 20 private-sector hospitals, 17 of which were located in Gauteng Province (Figure 1). More than 80 cases were detected at each of 4 private hospitals in Pretoria (Gauteng Province). Large outbreaks (>75 cases) also occurred in a private hospital in Johannesburg (Gauteng Province) and another in Rustenburg (North West Province). The number of cases increased dramatically from 18 in the baseline period (October 2012-November 2013) to 861 in a later corresponding period (October 2015-November 2016) (Figure 2).

Table 1: Characteristics of cases of *C. auris* invasive disease versus colonization, n=1579[a]

Characteristic	**Cases with available data (n=1579)**	**Invasive disease (n=451)**	**Colonization (n=1128)**	**p value**
Age in years (median, interquartile range)	n=1576	55 (41-68)	63 (49-74)	<0.001
Sex (n, %)	n=1540	n=442	n=1098	
Male	998 (65)	314 (71)	684 (62)	0.001
Female	542 (35)	128 (29)	414 (38)	
Health sector (n, %)	n=1549	n=439	n=1110	
Private	1435 (93)	325 (74)	1110 (100)	<0.001
Public	114 (7)	114 (26)	0 (0)	
Province (n, %)	n=1465	n=424	n=1041	0.22
Gauteng	1336 (91)	380 (90)	956 (92)	
Mpumalanga	72 (5)	25 (6)	47 (5)	
North West	20 (1)	7 (2)	13 (1)	
Other provinces	37 (3)	12 (2)	25 (2)	

[a]Specimen data were unavailable for 116 cases

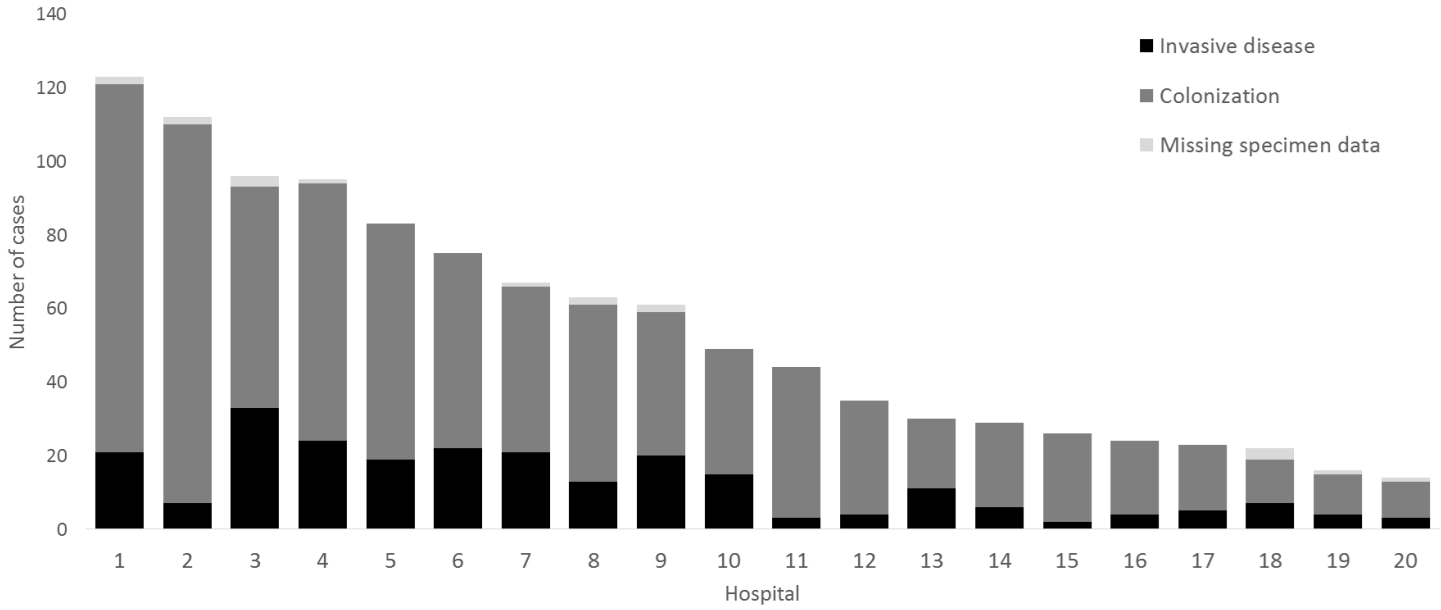

Figure 1: Distribution of cases of *C. auris* by invasive disease versus colonization from the top 20 private hospitals reporting cases, n=1087

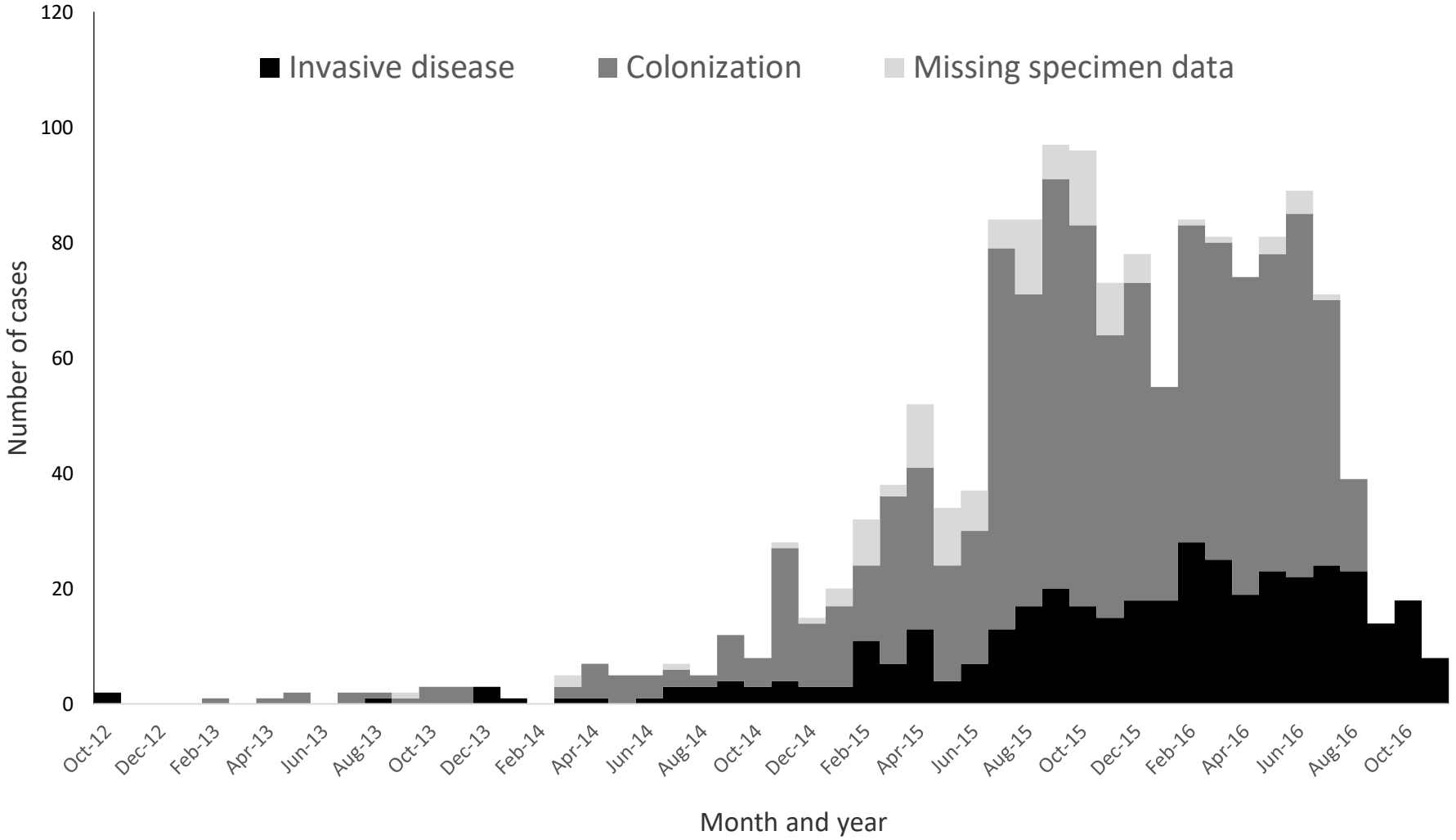

Figure 2: Distribution of cases of *C. auris* by month and year from October 2012 through September 2016, n=1306

Discussion

We have demonstrated a dramatic increase in the number of confirmed or probable cases of *C. auris* over 4 years in South Africa. Of concern, *C. auris* was detected at a large number of hospitals; most patients were admitted to private-sector hospitals in Gauteng Province. Most cases were probably caused by colonization, but *C. auris* is now also a common cause of invasive disease, accounting for 10% of all cases of candidemia in recent national surveillance (6). We collected minimal patient information through this surveillance but were able to document that most cases occurred among adults rather than children.

The factors that have led to the emergence and rapid spread of a unique clade of *C. auris* in hospitals in South Africa are not well established. Azole-resistant *C. parapsilosis* is already endemic in private-sector hospitals in Gauteng Province (12)**.** We have previously hypothesized that these strains of azole-resistant *C. parapsilosis* initially emerged as a consequence of indiscriminate use of fluconazole for prophylaxis and treatment and that suboptimal adherence to IPC practices caused the transmission of the pathogen within hospitals. This setting is the same one in which *C. auris* has become endemic and has caused large hospital outbreaks.

The trend we observed is consistent with increased detection of *C. auris* in other regions of the world. For instance, India has seen a large increase in the number of *C. auris* cases, from 12 in 2013 to >350 in 2017 (13)**.** By the end of May 2018, the United States had 311 cases and 29 probable cases of *C. auris* (14)**.**

Although *C. auris* has now been isolated from >94 hospitals across South Africa, most cases were detected at a small number of hospitals in a restricted geographic region. Focused attention on antifungal stewardship and multimodal IPC interventions at these facilities could limit further outbreaks and minimize transmission of this pathogen within South Africa and across South Africa's borders. Cross-border transmission from South Africa has already been documented (15). However, *C. auris* is notoriously difficult to eradicate from hospitals or units once it has become endemic, in part because of its ability to adhere to polymeric surfaces and form biofilms (16)**.** Thorough decontamination has been recommended to reduce the environmental bioburden (2**).**

Early detection of *Candida* species is recommended to facilitate appropriate treatment and implement IPC measures; however, standard biochemical platforms cannot reliably identify *C. auris* in microbiology laboratories. Many diagnostic pathology laboratories in South Africa used methods that could not reliably identify *C. auris* during the surveillance period, but these methods have been largely replaced in 2018 with more accurate methods of identification, including Vitek 2 YST-ID with version 8.01 software (bioMérieux), matrix-assisted laser desorption/ionization-time of flight mass spectrometry or internal transcribed spacer, and D1/D2 sequencing (9)**.**

Our study provides a national picture of the emergence of *C. auris* in public and private hospitals. However, the surveillance was limited in several respects. We may have underestimated the

number of cases in the public sector because NHLS laboratories did not routinely identify isolates from nonsterile sites to species level; even so, the geographic distribution of cases in the private sector is likely to be accurate. The 4 large private pathology practices that contributed data to the study have a combined national coverage of the private health sector. In addition, we have observed a similar geographic distribution of cases of *C. auris* candidemia detected through our national active population-based surveillance (6)**.** We believe that we have observed a real increase in cases over time; this finding is particularly true for private laboratories where *Candida* was routinely identified to species level even from nonsterile sites across the surveillance period. However, some laboratories may have changed their species-level identification practices in response to the emergence of *C. auris.* We included *C. haemulonii* in the case definition because many laboratories used the Vitek 2 YST system without a software update at the time of surveillance, but we did not include yeasts for which other diagnostic platforms can mistake *C. auris*. In addition, cases were diagnosed at some but not all facilities on the basis of clinicians submitting appropriate specimens for fungal culture or of IPC practitioners performing active screening for colonization. We extracted routine laboratory data, often with missing data elements, from several sources and applied a specific surveillance case definition. Deduplication of data to patient level may have been flawed because unique identifiers are not universally used in the healthcare system.

Conclusion

In conclusion, we report a large increase in cases of *C. auris* invasive disease and colonization since the first isolation of this yeast in South Africa in 2009. The increase is mostly attributable to cases in private hospitals in Gauteng Province.

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Chapter 7: Multi-locus sequence typing and antifungal susceptibility of *Candida auris* strains, South Africa. Southern African Journal of Infectious Diseases. 2020; 35 (1),a116.

Introduction

Since its first description in Japan in 2009, cases of *Candida auris* with varying clinical manifestations have been reported from Asia, Africa, South America, Europe, North America and Australasia (1-8). However, the global prevalence and geographic extent of *C. auris* disease is likely under-estimated especially in low- and middle-income countries because conventional laboratory methods misidentify the fungus and relatively few resource-limited countries have the capacity for identification by mass spectrometric or molecular methods (4-7).

Of concern, *C. auris* is almost universally resistant to fluconazole and some isolates have reduced susceptibility to voriconazole, amphotericin B and the echinocandins (4, 6, 9). As expected, persistent or breakthrough fungaemia has been reported among patients initially receiving fluconazole therapy, resulting in death in some cases (2, 4). *C. auris* has been associated with large healthcare-associated outbreaks owing to its ability to be transmitted person-to-person, persist for long periods in the hospital environment and resist chemical disinfection by certain products (10-14).

A whole genome sequencing study showed that *C. auris* strains from each of three continents were almost clonal, with simultaneous independent emergence of clonal groups on each

continent being hypothesized (10). Multilocus sequence typing (MLST) and amplified fragment length polymorphism (AFLP) typing has also demonstrated strain clustering by geographical region (11, 14). More recently, it has become clear that patients and strains have crossed borders, seeding outbreaks in new locations (15, 16). In light of an emerging epidemic of *C. auris* infections among hospitalised patients in parts of South Africa, we sought to describe the antifungal susceptibility profile and genetic relatedness of a convenience sample of *C. auris* strains referred to a South African reference laboratory.

Materials and methods

Laboratory surveillance

A case was defined as a patient of any age admitted to any South African hospital with first isolation of *C. auris* from any specimen (representing either infection or colonisation) from January 2012 through to December 2015. Referring laboratories submitted suspected or confirmed isolates of *C. auris* to the National Institute for Communicable Diseases (NICD) in Johannesburg. Only cases with viable isolates referred to NICD were included in this study. These cases are thus not necessarily representative of all diagnosed cases of *C. auris* over this time period. Corresponding laboratory reports, containing patient demographic information such as age, sex, hospital, specimen type and date of specimen collection, were also submitted.

Species-level identification and antifungal susceptibility testing

Laboratory identification systems regularly misidentify *C. auris* in a predictable way. Referring laboratories, therefore, submitted isolates to the national mycology reference laboratory at NICD when *C. auris* was suspected, for species-level confirmation and antifungal susceptibility testing. These were most often yeasts with high fluconazole minimum inhibitory concentrations (MICs) which had been identified by Vitek 2 YST using software version 7.0 or earlier versions (bioMérieux, Marcy l'Etoile, France) as *C. haemulonii*, as *Rhodotorula glutinis* by API 20C Aux or API ID 32C (bioMérieux), as *Saccharomyces cerevisiae* by Auxacolor (BioRad, Hercules, California, United States) or as *Candida famata* by Microscan walkaway (Beckman Coulter, Brea, California, United States) (17). Isolates were submitted on Sabouraud agar plates or in sealed bottles containing Dorset transport medium (Diagnostic Media Products (DMP), Johannesburg, South Africa) to NICD. We initially performed phenotypic identification using ChromAgar Candida medium (Mast Diagnostics, Merseyside, UK). We subjected all isolates to Matrix-Assisted Laser Desorption Ionization-Time of Flight (MALDI-TOF) (Bruker, Bremen, Germany) mass spectrometric analysis using Biotyper v3.1 software (Bruker Ltd, Coventry, UK). Identification of *C. auris* isolates was then confirmed by PCR amplification and sequencing of the internal transcribed spacer (ITS) domain of the ribosomal RNA gene and the D1-D2 region of the 28S subunit using universal primers (18). Antifungal susceptibility testing for fluconazole, voriconazole, itraconazole, posaconazole, caspofungin, anidulafungin, micafungin and flucytosine was performed using commercially-available microbroth dilution panels containing Alamar Blue (Thermo Fisher Scientific, Cleveland, Ohio, USA). Neither the European Committee on Antifungal Susceptibility Testing (EUCAST) nor the U.S.-based Clinical and Laboratory Standards Institute (CLSI) have defined clinical breakpoints for

C. auris. For epidemiological purposes, resistance was conservatively defined as follows (adapted from Lockhart et al (19)): fluconazole ≥32 μg/mL, voriconazole ≥2 μg/mL (similar to CLSI breakpoints for *Candida krusei*), flucytosine ≥128 μg/mL and amphotericin B ≥2 μg/mL. Echinocandin resistance was defined using the following tentative MIC breakpoints (adapted from CDC (20)): anidulafungin and micafungin ≥4 μg/mL. Caspofungin is an unreliable indicator of echinocandin resistance and caspofungin MICs were not interpreted (21). Isavuconazole susceptibility testing was performed on a random sample of 15 fluconazole-resistant isolates using the Etest method. *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 strains were included in quality control (QC) runs on all days of testing and MICs were found to be within the expected QC ranges.

FKS gene sequencing of *C. auris* isolates

We sequenced the hotspot 1 and 2 regions of the *FKS1* and *FKS2* genes for all isolates with an echinocandin MIC of ≥1 μg/mL (22-23). All sequences were aligned with the *FKS* sequences of a hypothetical protein obtained from the draft genome sequence of *C. auris* strain VPCI 479/P/13 (accession number CVRJ00000000) from India (24) and a wild-type *Candida albicans* strain ATCC 90028 (GQ456066).

Genotyping of *C. auris* isolates

To determine the genetic relatedness among the *C. auris* strains, we sequenced four loci (ITS, D1-D2, RPB1 and RPB2) using a previously-published method (14). GenBank accession numbers for sequence data of ITS, D1-D2, RPB1 and RPB2 loci are attached in the supplementary table. Briefly, we extracted DNA from single colonies using Zymo ZR fungal/Bacterial DNA MiniPrep (Zymo Research Corporation, Irvine, CA, USA). PCR amplification was performed using primers adopted from Prakath et al (14); the following control strains were included: MRL208 (KJ126758) and MRL209 (KJ126759). PCR amplicons were purified using Exonuclease I/ Shrimp alkaline phosphatase enzymes (Thermo Fisher Scientific). DNA sequencing was performed with the same primers used for PCR using BigDye terminator version 3.1 in an ABI 3500 genetic analyser (Applied Biosystems, Foster City, CA, USA). Identification of *C. auris* was performed by pairwise sequence comparison of the ITS region using the NCBI BLAST algorithm (www.ncbinlm.nih.gov/blast). We performed multiple sequence alignment of concatenated sequences using ClustalW (25). Using the final MLST dataset, we generated a phylogenetic tree by a neighbour-joining statistical method using 2000 bootstrap replications on MEGA 6 (26) (Figure 1). A cluster was defined as isolates from different patients which shared a common ancestor, with a bootstrap value between 90% and 100%. Ethical clearance was obtained from the Human Research Ethics Committee (Medical), University of the Witwatersrand, Johannesburg.

Results

From January 2012 through to December 2015, 86 viable isolates from 77 patients were referred to NICD for identification and antifungal susceptibility testing. One case of *C. haemulonii* infection (1 isolate) was excluded. Cases of *C. auris* infection/ colonisation were diagnosed at 32 hospitals, 27 of which were located in Gauteng province and most of these in the City of Johannesburg or Tshwane. The remaining cases were diagnosed at hospitals located in seven other provinces which included; North West, Limpopo, KwaZulu-Natal, Free State, Eastern Cape, Western Cape and Mpumalanga. Seventy two (85%) isolates were from patients admitted to private-sector hospitals; the remainder were from public-sector hospitals. The median age of cases was 59 years (inter-quartile range (IQR), 48-68 years). Males accounted for 68% (50/74) of the cases. Twenty nine per cent (22/75) of isolates were cultured from urine followed by 27% (20/75) from blood, 25% (19/75) from central venous catheter tips, 7% (5/75) from irrigation fluid, 5% (4/75) from tissue, 4% (3/75) from respiratory tract specimens and the remainder from miscellaneous sites. Twenty four (32%) of 76 patients had hospital ward information available. Of these, 12 (57%) were admitted to an intensive care unit, 6 (25%) to a trauma unit, 3 (13%) to a burns unit, 2 (8%) to general wards and 1 (4%) to a neurosurgery ward. No further clinical or outcome information was available.

Antifungal susceptibility testing

Antifungal susceptibility testing was performed on all 85 *C. auris* isolates (Table 1). Of these, 82 (97%) were resistant to fluconazole with an MIC_{50} and MIC_{90} of 128 µg/mL and 256 µg/mL

respectively. Six (7%) isolates were resistant to voriconazole and seven (8%) to echinocandins. All six voriconazole-resistant isolates were resistant to fluconazole though none of these were resistant to echinocandins. All seven echinocandin-resistant isolates (8%) were resistant to fluconazole and were thus considered multidrug-resistant, i.e. resistant to ≥2 classes of antifungal agents. No isolates were resistant to flucytosine or amphotericin B. Posaconazole and itraconazole had potent in vitro activity with an MIC_{50} and MIC_{90} of 0.25 µg/mL and 0.12 µg/mL respectively (Table 1). The MIC_{50} (range) for isavuconazole was 0.19 µg/mL (0.06-4 µg/mL) for 15 fluconazole-resistant strains.

FKS sequencing

We sequenced the hot spot regions of the *FKS1* and *FKS2* genes of 14 isolates with an echinocandin MIC ≥ 1 µg/mL. One isolate was resistant to both anidulafungin and micafungin and 6 isolates were resistant to micafungin. No resistance-associated mutations were detected within the hot spot regions of the *FKS1* and *FKS2* genes.

Genotyping of *C. auris* isolates

Eighty five isolates from 76 patients were confirmed as *C. auris* using ITS sequencing. MLST was performed on all 85 isolates. Phylogenetic analysis grouped the isolates into 2 clusters, cluster 1 comprising of 83 isolates and cluster 2 comprising of 2 isolates (Figure 1). The two isolates belonging to cluster 2 had single nucleotide polymorphisms within the RPB1 locus,

when compared to cluster 1 isolates and had been cultured from patients at two separate hospitals. Five patients had more than one isolate; isolates cultured from each of these individual patients had similar MLST profiles. A phylogenetic tree based on ITS sequences showed that South African isolates were closely related to Indian and Kuwaiti isolates (Figure 2).

Table 1: Antifungal susceptibility profile of *Candida auris* isolates (n=85)

Antifungal agent	**No. of isolates with MIC (µg/mL) of:**																**Susceptible (N/%)**	**Resistant[a] (N/%)**	**MIC_{50} (µg/mL)**	**MIC_{90} (µg/mL)**
	<0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256				
Amphotericin B						2	42	41									85 (100)	0 (0)	0.5	1
Fluconazole												3	**5**	**23**	**27**	**27**	3 (3)	82 (97)	128	256
Voriconazole	2	6	3	16	24	11	11	6	**2**	**3**	**1**						79 (93)	6 (7)	0.12	1
Posaconazole	38	22	13	6	2	2	1	1									-	-	0.015	0.06
Itraconazole		11	40	23	6	5											-	-	0.03	0.12
Isavuconazole[b]				1	5	5	2	1		1							-	-	0.19	0.38
Flucytosine		1	1	67	11									5			85 (100)	0 (0)	0.06	0.12
Caspofungin	8		26	26	6	6		3	3	6	1						-	-	0.06	2
Anidulafungin			43	18	4	7	2	8	2	**1**							84 (99)	1 (1)	0.03	1
Micafungin		11	31	21	8	2		1	4	**5**	**2**						78 (92)	7 (8)	0.06	2

[a]In the absence of breakpoints, resistance was arbitrarily defined as: fluconazole ≥32 µg/mL, voriconazole ≥2 µg/mL, anidulafungin/ micafungin ≥4 µg/mL, flucytosine ≥128 µg/mL and amphotericin B ≥2 µg/mL, Isolates tested for Isavuconazole [b]n=15

Figure 1: Phylogenetic tree based on multi-locus sequence typing (MLST) data of South African *C. auris* (n=85) isolates using the neighbour-joining analysis method with 2000 bootstrap replications. Sequences of South Korean reference strains (KCTC17809 and KCTC17810) were retrieved from the GenBank.

Figure 2: Phylogenetic tree of isolates of the *C. auris* (n=85) obtained by using neighbour-joining phylogenetic analyses and 2,000 bootstrap replications based on ITS sequences.

Discussion

C. auris is an emerging antifungal-resistant pathogen causing outbreaks globally. In South Africa, *C. auris* has reportedly caused large outbreaks in several hospitals, predominantly in private-sector hospitals in Gauteng province (27). Almost all isolates in our case series were resistant to fluconazole and a smaller proportion were resistant to both fluconazole and voriconazole or both an azole and echinocandin. Despite being cultured from patients admitted to >30 hospitals, isolates were largely clonal by MLST. A more discriminatory technique, such as WGS, probably needs to be used to explore isolate relatedness in more detail.

Azole-resistant *C. parapsilosis* is already endemic in private-sector hospitals in Gauteng province (28). We have previously hypothesized that these strains of azole-resistant *C. parapsilosis* initially emerged as a consequence of indiscriminate use of fluconazole for prophylaxis/ treatment and transmission of this pathogen within hospitals then occurred owing to suboptimal adherence to infection prevention and control practices (28, 29). This is the same setting in which *C. auris*, another azole-resistant pathogen, emerged several years ago (6), has become endemic and has recently caused several large hospital outbreaks (27).

Several studies have reported multi-drug resistance in *C. auris* (2-7). Our study confirms that majority of isolates have elevated fluconazole MICs. In a recent whole genome sequencing study, fluconazole resistance among 10 South African strains was mediated by an F126T substitution in the *ERG11* gene (10). The emergence of yet another fluconazole-resistant

pathogen in our setting is worrisome, especially in public-sector South African hospitals where echinocandins are only available in tertiary centres. Eight per cent of the studied isolates were both fluconazole- and echinocandin-resistant. Multi-drug resistance is concerning because this limits treatment to amphotericin B or a combination of antifungal agents. There is no current evidence that strains with elevated echinocandin MICs are associated with clinical failure, with the exception of central nervous system and urinary tract infections (owing to poor penetration of echinocandins into these compartments). Interestingly, we were not able to document any mutations in the *FKS1 or FKS2* genes, even among strains with relatively high echinocandin MICs. Phenotypic echinocandin resistance in these isolates may be mediated by other mechanisms such as enhanced chitin expression and other point mutations outside the hot spot regions of *FKS1* and *FKS2* genes (30-32). Mutations outside the hot spot regions may have a compensatory effect on the gene leading to alteration in the protein structure that causes variation in the MIC (31, 32). Echinocandin resistance-associated point mutations have been recently identified in *FKS1 HS1* (S639F/P) of *C. auris* strains exhibiting MIC levels ≥4 µg/mL (*21, 33, 34*). We found some evidence of elevated isavuconazole MICs despite this agent not being available in South Africa. While isavuconazole is efficacious for treatment of invasive aspergillosis and mucormycosis, there is limited evidence of its efficacy in treating invasive candidiasis (35). Five of our tested strains had flucytosine MICs of 64 µg/mL; in contrast, 47% of isolates were flucytosine non-wild type in an Indian study (9). This may reflect differing patterns of flucytosine use. Flucytosine is neither registered nor available in South Africa (36). Amphotericin B will continue to be recommended as a first-line treatment option for *C. auris* infections in public-sector hospitals where echinocandins are not accessible (13, 37, 38).

Combined with the results of classic epidemiologic investigations, molecular data can provide evidence of horizontal transmission in healthcare settings and guide infection prevention and control efforts. Genetic relatedness among *C. auris* isolates has been previously reported. These studies demonstrated that the strains were clonal using pulsed-field gel electrophoresis (PFGE), MLST, AFLP and MALDI-TOF (11, 14). Another study of isolates from 4 different countries showed that isolates clustered by geographic regions, when MLST and MALDI-TOF were used. However, South African isolates scattered among different clusters suggesting that different genotypes may be circulating in the country (14). Whole genome sequencing of 10 strains of *C. auris* strains from South Africa (strains also included in the current study) demonstrated that these strains were highly clonal with a few single nucleotide polymorphisms (<70 SNPs) (*10*). Using MLST, we have demonstrated that *C. auris* strains are highly related despite being isolated from patients admitted to a large number of hospitals; however, this assay was unable to discriminate strains from different hospitals. This either suggests that clonal strains are circulating or that the method is insufficiently discriminatory. Therefore, a more robust and discriminatory method such as WGS may be needed to demonstrate intra-species variability and demonstrate nosocomial transmission. Microsatellite genotyping has been used extensively in detection of *C. parapsilosis* outbreaks in neonatal ICUs and was shown to be highly discriminatory in uncovering undetected outbreaks (39-42). However, it is difficult to create a microsatellite system for largely clonal pathogen and this is also a labour-intensive process. Thus, whole genome sequencing may offer a convenient, though relatively more costly, approach (10).

This passive surveillance system was limited in several respects. Cases were diagnosed based on clinicians submitting appropriate specimens for fungal culture. Diagnostic laboratories only referred a small proportion of suspected or confirmed *C. auris* isolates to NICD and referral of suspected antifungal-resistant strains may have biased the reported antifungal susceptibility profile (27). NICD's national active surveillance system for candidaemia (GERMS-SA) will provide a more representative picture of the scale of *C. auris* infection in South Africa (43). We envisage undertaking further molecular genotyping using whole genome sequencing as this is currently the widely-used technique to type *C. auris* isolates.

Conclusion

Azole-resistant *C. auris* strains circulating in Gauteng hospitals were related by MLST but the possibility of nosocomial transmission should be explored using a more discriminatory technique such as whole genome sequencing.

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Chapter 8: Discussion

This thesis discusses two azole-resistant *Candida* species causing healthcare-associated infections in both public and private-sector hospitals in South Africa. In the last decade, *C. parapsilosis* emerged as the second common non-*albicans Candida* species causing invasive infection in hospitalized patients and was the prominent species causing neonatal candidaemia in South Africa (1). With the emergence of *C. auris* we have further noticed a shift in the epidemiology of candidaemia with *C. albicans* being the most dominant species isolated from hospitalized patients followed by *C. parapsilosis, C. glabrata* and *C. auris* in public-sector hospitals and *C. parapsilosis* and *C. auris* in private-sector hospitals (2). We speculate that these strains of azole-resistant *C. parapsilosis* and *C. auris* initially emerged as a consequence of indiscriminate use of fluconazole for prophylaxis and treatment and that suboptimal adherence to IPC practices caused the transmission of the pathogen within hospitals. Patients most likely to be at risk of infection with both *C. parapsilosis* and *C. auris* are those with compromised immune system including, patients with underlying medical condition, those who have undergone surgery, admission to ICU, presence of central venous catheter, mechanical ventilation, TPN, administration of broad spectrum antibiotics and prior antifungal treatment (3-10). Findings from this study demonstrated that a fifth of the patient with *C. parapsilosis* candidaemia were neonates. Govender et al. (2016), also demonstrated that *C. parapsilosis* was the main etiology causing neonatal candidaemia in South Africa during the 2009/2010 surveillance period (1). However, when comparing the age groups of patients mostly likely to be infected by these pathogens, adults were found to be more likely to be infected to *C. auris* infection than neonates and children (1, 2).

Transmission dynamics for both *C. parapsilosis* and *C. auris* have been reported in some studies. They adhere to plastic medical devices such as intravenous catheters and hands of health care workers (11-13). This characteristic allows these pathogens to be easily transmitted and spread in hospital settings. Using polymorphic microsatellite genotyping, we demonstrated that clusters of *C. parapsilosis* strains are circulating and causing outbreaks at South African public-sector hospitals. This was consistent with finding from other studies where they found that strains of *C. parapsilosis* persisted in the environment over a long period and caused hospital-associated outbreaks in NICUs (14-16). Our speculation is that these strains were likely being transmitted to the neonates through contaminated medical device and/or surfaces and suboptimal infection prevention and control practices, including poor adherence to hand antisepsis protocols (17-20). We investigated the genetic relatedness of *C. auris* isolates, which were submitted to the reference laboratory for species level identification. MLST clustering indicated that *C. auris* strains circulating in South Africa were closely related. This method however, has a low discriminatory power. Therefore, a technique with high discriminatory power such as WGS may be superior approach, though relatively more costly (21).

The prevalence of azole resistance in *C. parapsilosis* is usually low in most part of the world (22-25). South Africa has since reported the highest prevalence of fluconazole resistance in *C. parapsilosis* to date ranging from 58-63% (26). Our study showed that 54% of *C. parapsilosis* causing neonatal candidaemia at 5 public-sector hospitals were resistant to fluconazole and 14% to voriconazole. Data from a single center neonatal unit showed that 78% of *C. parapsilosis*

isolates were resistant to fluconazole. We further demonstrated that azole resistance in *C. parapsilosis* was mostly driven by point mutations within the *ERG11* gene. Similarly, we demonstrated that *C. auris* was highly resistant to fluconazole and had reduced susceptibility to echinocandins. Fluconazole resistance in both pathogens was mostly driven by point mutations within *ERG11* gene with specific reference to Y132F and F126T in *C. parapsilosis* and *C. auris* respectively. Y132F has also been reported in fluconazole-resistant *C. albicans, C. tropicalis* and *C. auris* isolates, though South African *C. auris* strains have the F126T substitution (27-29). The presence of Y132F and other substitutions at this position in *C. albicans* and *C. tropicalis* has been associated with reduced susceptibility to azole antifungals (30, 31). This is concerning because these pathogens been implicated in nosocomial outbreaks of candidaemia in hospital settings, and they are resistant to fluconazole which is a treatment of choice for invasive candidiasis particularly in resource-limited countries where echinocandins are not available for public sector-hospitals. As such, it is important to conduct ongoing surveillance for fluconazole-resistant non-albicans *Candida* species because of their ability to persist in the hospital environment, medical devices, hands of health care workers, and eventually causing outbreaks.

Focused attention on antifungal stewardship and multimodal IPC interventions at these facilities could limit further outbreaks and minimize transmission of these pathogen within South Africa. A number of infection prevention and control strategies have been published by different public health organisations including FIDSSA, PHE, CDC and ECDC. IPC interventions such as environmental disinfection, hand hygiene, cohorting of infected and colonised patients should be implemented in South African hospitals to reduce the incidence of nosocomial fungal infections and potential outbreaks (32-36).

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Chapter 9: Conclusion, future research, limitations and recommendations:

This thesis addressed the molecular epidemiology, antifungal susceptibility profiles and possible mechanisms of azole-resistance of two non-albicans *Candida* species causing nosocomial infections in South African hospitals. We found that cases of *C. parapsilosis*-associated candidaemia in public-sector hospital neonatal units were caused by closely-related genotypes and that there was molecular evidence of undetected outbreaks as well as intra-hospital transmission. Almost 60% of these cases were resistant to fluconazole. This unusually high prevalence of fluconazole-resistant *C. parapsilosis* strains limits prophylaxis and treatment options in public-sector hospital neonatal units in Gauteng province. We further demonstrated that fluconazole resistance in *C. parapsilosis* strains from a single South African neonatal unit was significantly associated with clusters and predominantly driven by Y123F amino acid substitutions in the *ERG11* gene. Although a large number of isolates had Y132F substitution, we found no association with clusters. With the emergence of *C. auris*, a multidrug-resistant pathogen causing healthcare-associated outbreaks globally, we described the for the first time the emergence of multidrug-resistant *C. auris* in Africa. We further demonstrated an exponential increase in cases of *C. auris* invasive and colonisation infections in South Africa since its first isolation in 2009. This increase was mostly attributable to cases in private hospitals in Gauteng Province. We could not determine the risk factors and routes of transmission due to a lack of clinical and epidemiological information. MLST and WGS showed that *C. auris* strains circulating in South African hospitals were largely clonal and belong to a distinct African clade. Fluconazole resistance among South African strains was driven by F126T amino acid substitution in the *ERG11* gene, which was different from India, Pakistan, and Venezuela.

Limitations

A number of limitations were identified in the sub-studies:

1. Due to limited demographic, clinical and treatment data, we could not establish the association between *C. parapsilosis* cluster types and clinical outcomes of the patients. In addition to this, we could not determine the source of infection because no samples were collected from hands of health care workers or the hospital environment.

2. Data from a single center was analyzed to establish the association between *C. parapsilosis* clusters and mutations. Therefore, the data may not be representative of other medical centres in the country or region. We also did not undertake quantitative real-time PCR to determine the expression levels of *ERG11* and *MDR1* in isolates with SNPs in the *ERG11* gene and *MRR1* transcription factor gene.

3. Using MLST, we have demonstrated that *C. auris* strains are highly related despite being isolated from patients admitted to a large number of hospitals; however, this assay was unable to discriminate strains from different hospitals. This either suggests that clonal strains are circulating or that the method is insufficiently discriminatory. Therefore, a more robust and discriminatory method such as WGS may be needed to demonstrate intra-species variability and demonstrate nosocomial transmission.

4. Passive surveillance system for *C. auris* was limited in several respects. Cases were diagnosed based on clinicians submitting appropriate specimens for fungal culture. Diagnostic laboratories only referred a small proportion of suspected or confirmed *C.*

auris isolates to NICD and referral of suspected antifungal-resistant strains may have biased the reported antifungal susceptibility profile. NICD's national active surveillance system for candidaemia (GERMS-SA) will provide a more representative picture of the scale of *C. auris* infection in South Africa. We envisage undertaking further molecular genotyping using whole genome sequencing as this is currently the widely-used technique to type *C. auris* isolates.

Recommendations

In light of the observed clusters of *C. parapsilosis* causing outbreaks in neonatal units at five South African public-sector hospitals, which is an indication of poor adherence to IPC measures, it will be pertinent to conduct regular IPC audits in these wards. Multidrug-resistant strains *C. auris* have been recorded in five continents, including Africa. These strains become untreatable, and in populations where they are detected, they are reported to cause a being deadly infection. The Federation of Infectious Diseases Society of Southern Africa has published evidence-based guidelines on best-practice in prevention, diagnosis, and management of *C. auris* infections in both public- and private-sector hospitals. These guidelines should be implemented as part of routine IPC measures in both private- and public-sector hospitals in order to reduce the incidence of nosocomial infections and possible outbreaks.

Based on some of the findings from this thesis, the NICD started the *C. auris* surveillance programme (GERMS-SA) in August 2018. The main objective of this surveillance is to understand the molecular epidemiology of clinical and environmental *C. auris* strains isolated collected from both private-and public sector hospitals in South Africa, using WGS, to implement appropriate measures to limit the rapid spread of this pathogen in healthcare settings. This will be achieved through sequencing entire genomes of *C. auris* strains to determine if there is genetic diversity within the SA clade, compare relatedness of outbreak strains, to determine if genome level differences occur among sequentially-isolated strains from the same patient and identify the presence of antifungal resistance mutation.

Appendix A- Ethics approval

R14/49 Ms Magobo Rindidzani Edith

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M150256

NAME: (Principal Investigator)	Ms Magobo Rindidzani Edith
DEPARTMENT:	School of Pathology Anatomical Pathology Division National Institute for Communicable Diseases
PROJECT TITLE:	Molecular Epidemiology of Candida Parapsilosis in South Africa, 2009 to 2013
DATE CONSIDERED:	27/02/2015
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr Nelesh Govender

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

DATE OF APPROVAL: 02/03/2015

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

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix B- Ethics approval for *C. auris* study

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

R14/49 Dr Vanessa Quan

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M1809107

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

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

DECLARATION OF INVESTIGATORS

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

Principal Investigator Signature

12/11/2018
Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix C-Change of title approval

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

Private Bag 3 Wits, 2050
Fax: 027117172119
Tel: 02711 7172076

Reference: Mrs Sandra Benn
E-mail: sandra.benn@wits.ac.za

07 April 2016
Person No: 0307319H
TAA

Ms RE Magobo
House 631
Waterfall View
Midrand
1685
South Africa

Dear Ms Magobo

Doctor of Philosophy: Change of title of research

I am pleased to inform you that the following change in the title of your Thesis for the degree of Doctor of Philosophy has been approved:

From:	**Molecular epidemiology of Candida parapsilosis in South Africa, 2009 to 2013**
To:	**Molecular epidemiology of azole-resistant: Candida species in South Africa**

Yours sincerely

Mrs Sandra Benn
Faculty Registrar
Faculty of Health Sciences

Appendix D- Declaration: Student's contribution to articles and agreement of co-authors

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

I, Rindidzani Edith Magobo, student number [0307319H], declare that this Thesis is my own work and that I contributed adequately towards research findings published in the article(s) stated below which are included in my Thesis.

Signature of Student: Rindidzani Edith Magobo **Date**: 24 February 2020

Name of Primary Supervisor: Prof Nelesh P. Govender

Signature of Primary Supervisor ..... **Date** 24/2/20

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

Article 1: Title: Detection of neonatal unit clusters of *Candida parapsilosis* fungaemia by microsatellite genotyping: Results from laboratory-based sentinel surveillance, South Africa, 2009-2010

Journal name, year, volume and page numbers: Mycoses 2017; 60:320-327

Authors	Name	Signature	Date
2nd author	S.D. Naicker		25/2/2020
3rd author	J. Wadula		26/2/2020
4th author	M. Nchabeleng		
5th author	Y. Coovadia		27/2/2020
6th author	A. Hoosen		2/3/2020
7th author	S.R. Lockhart	Shawn R. Lockhart -S Digitally signed by Shawn R Lockhart -S Date: 2020.02.25 08:09:39 -03'00'	
8th author	N.P. Govender		24/2/20

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

Appendix D- Declaration: Student's contribution to articles and agreement of co-authors

Article 1: Title: Detection of neonatal unit clusters of *Candida parapsilosis* fungaemia by microsatellite genotyping: Results from laboratory-based sentinel surveillance, South Africa, 2009-2010

Journal name, year, volume and page numbers: Mycoses 2017; 60:320-327

Authors	Name	Signature	Date
2nd author	S.D. Naicker		25/2/2020
3rd author	J. Wadula		26/2/2020
4th author	M. Nchabeleng		03/03/2020
5th author	Y. Coovadia		
6th author	A. Hoosen		
7th author	S.R. Lockhart	Shawn R. Lockhart -S Digitally signed by Shawn R. Lockhart -S Date: 2020.02.25 08:08:44 -05'00'	
8th author	N.P. Govender		

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

Article 2: Title: Fluconazole-resistant *Candida parapsilosis* strains with Y132F substitution in *ERG*11 gene causing invasive infections in neonatal unit, South Africa

Journal name, year, volume and page numbers: Mycoses, 23 February 2020 (Accepted for publication)

Authors	Name	Signature	Date
2nd author	S.R. Lockhart	Shawn R. Lockhart -S Digitally signed by Shawn R. Lockhart -S Date: 2020.02.25 08:08:44 -05'00'	
3rd author	N.P. Govender		24/2/20

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

Appendix D- Declaration: Student's contribution to articles and agreement of co-authors

Article 3: Title: *Candida auris*-associated Candidemia, South Africa.

Journal name, year, volume and page numbers: Emerging Infectious Diseases 2014; 20 (7):1250-1251.

Authors	Name	Signature	Date
2nd author	C. Corcoran		25/2/20
3rd author	S. Seetharam		27/2/20
4th author	N.P. Govender		27/2/20

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

Article 4: Title: *Candida auris* in South Africa, 2012-2016

Journal name, year, volume and page numbers: Emerging Infectious Diseases 2018; 24 (11): 2036-2040

Authors	Name	Signature	Date
1st author	N P Govender		24/2/20
2nd author	R.E. Magobo		25/2/2020
3rd author	R. Mpembe		25/02/20
4th author	M. Mhlanga		25/2/20
5th author	P. Matlapeng		25/02/20
6th author	C. Corcoran		25/02/20
7th author	C. Govind		
8th author	W. Lowman		5/3/20
9th author	M. Senekal		2·3·20
10th author	J. Thomas		06/03/20

Comments by primary supervisor: The student performed a preliminary cleaning and a preliminary analysis of the dataset. She assisted with writing sections of the manuscript and with the other authors, reviewed the final drafts of the manuscript.

Appendix D- Declaration: Student's contribution to articles and agreement of co-authors

Article 5: Title: Multi-locus sequence typing of azole resistant *Candida auris* strains, South Africa

Journal name, year, volume and page numbers: South African Journal of Infectious Diseases, January 2020 (Accepted for publication)

Authors	Name	Signature	Date
1st author	M. Mhlanga		25/2/20
2nd author	C. Corcoran		25/2/20
3rd author	N.P. Govender		24/2/20

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

Appendix E- Turnitin report

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

FACULTY OF HEALTH SCIENCES

PLAGIARISM DECLARATION TO BE SIGNED BY ALL HIGHER DEGREE STUDENTS

SENATE PLAGIARISM POLICY: APPENDIX ONE

I Rindidzani Edith Magidi (Student number: 1309319H) am a student registered for the degree of Doctor of philosophy in the academic year 202.

I hereby declare the following:

- I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.
- I confirm that the work submitted for assessment for the above degree is my own unaided work except where I have explicitly indicated otherwise.
- I have followed the required conventions in referencing the thoughts and ideas of others.
- I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.
- I have included as an appendix a report from "Turnitin" (or other approved plagiarism detection) software indicating the level of plagiarism in my research document.

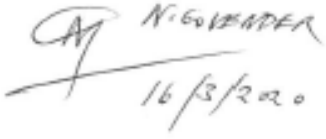
Signature: [signature] Date: 24/08/2020

Appendix E- Turnitin report

Ms

by Rindi Magobo

Submission date: 06-Mar-2020 03:37PM (UTC+0200)
Submission ID: 1270519169
File name: 0307319HMagobo06032020_Without_References.doc (1.16M)
Word count: 7771
Character count: 46354



Appendix E- Turnitin report

Ms

ORIGINALITY REPORT

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SIMILARITY INDEX	INTERNET SOURCES	PUBLICATIONS	STUDENT PAPERS

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★ Submitted to University of Utah

Student Paper

Exclude quotes	On	Exclude matches	< 1%
Exclude bibliography	On		

N. GOLDBERGER

16/3/2020