Molecular Characterisation of *Candida auris* Clinical Isolates at a Large Tertiary Academic Hospital

by

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A Dissertation submitted to the Faculty of Health Science, University of the Witwatersrand, in fulfilment of the requirements for the degree

Master of Science in Medicine

In

Clinical Microbiology and Infectious Diseases

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Co-supervisor: Dr Serisha Naicker

02 August 2022.

DECLARATION

The experimental work described in this dissertation was conducted under the supervision of Prof Nelesh Govender and Dr Serisha Naicker in the Centre for Healthcare-Associated Infections, Antimicrobial Resistance and Mycoses (CHARM), National Institute for Communicable Diseases (NICD), a Division of the National Health Laboratory Service (NHLS).

I, Dikeledi Mahlaku Mmakgabo Kekana, declare that this Dissertation is my own, unaided work. It is being submitted for the Degree of Master of Science in Medicine in Clinical Microbiology and Infectious Diseases at the University of the Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination at any other University.

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(Signature of candidate)

_____02_____day of___August____20_____22_____in_Sandringham__

DEDICATION

IN LOVING MEMORY OF MY MOTHER

RAISIBE LYDIA KEKANA

1966 - 2010

PRESENTATIONS

Dikeledi Kekana, Serisha D. Naicker, Liliwe Shuping, Sithembiso Velaphi, Firdose Nakwa, Jeannette Wadula, Nelesh P. Govender. Molecular Characterisation of *Candida auris* Clinical Isolates at a Large Tertiary Academic Hospital. Oral presentation: *National Institute for Communicable Diseases Research Forum*, Johannesburg, South Africa, 24 November 2021.

Dikeledi Kekana, Serisha D. Naicker, Liliwe Shuping, Sithembiso Velaphi, Firdose Nakwa, Jeannette Wadula, Nelesh P. Govender. Molecular Characterisation of *Candida auris* Clinical Isolates at a Large Tertiary Academic Hospital. Poster presentation: *31st Fungal Genetics Conference*, Asilomar Conference Grounds in Pacific Grove, United States of America, 15-20 March 2022.

ABSTRACT

Background: Candida auris is a multidrug-resistant, healthcare-associated fungal pathogen comprising of five known geographical clades. It was first reported in South Africa in 2014 and then detected in a previously misidentified case in 2009 through a retrospective study. C. auris has been reported in over 40 countries since its first detection and has caused nosocomial outbreaks in healthcare facilities including those in neonates. We describe the molecular epidemiology of C. auris, using whole-genome sequencing (WGS) over four years at a large tertiary academic hospital in South Africa with a focus on the hospital's neonatal unit which had a large outbreak during this period.

Methods: Cases of culture-confirmed C. auris infection or colonisation were identified through laboratory surveillance across the entire hospital from March 2016 through July 2020 and viable isolates were submitted to a reference laboratory. Phenotypic characterisation included the assessment of pseudohyphae production and aggregate formation using microscopy techniques. Antifungal susceptibility testing was performed using commercial broth microdilution and gradient diffusion methods. Molecular analysis was based on the WGS data of isolates; the quality of read data was assessed and bioinformatics analysis was performed. Phylogenetic analysis was used to confirm the clade assignments while phylodynamic analysis was used to determine the most common recent ancestor and transmission routes of the outbreak strains.

Results: Of 287 cases, 207 (72%) had viable isolates and 200 non-contaminated isolates were available for further phenotypic experimentation. Cases across the hospital had a median age of 1.4 years (interquartile range: 22 days – 21 years), with a large proportion diagnosed in the neonatal unit (91/287, 31.7%). Most isolates demonstrated moderate aggregation capabilities (124/200, 62%) while 33% (65/200) had extensive aggregation and only 6% showed no

aggregation. Only three isolates produced pseudohyphae. All strains grew at 37°C, 40°C and 42°C. Most isolates belonged to clade III (63%, 118/188) or clade IV (37%, 70/188). All 181 fluconazole-resistant isolates (minimum inhibitory concentration ≥32 μg/mL) had an ERG11 gene mutation. Additionally, 5 fluconazole-susceptible isolates also carried a mutation in the ERG11 gene. All clade III isolates had the VF125AL ERG11 mutation (118/118, 100%) while isolates in clade IV (68/70, 97%) had K177R/N335S/E343D mutations. We use Bayesian molecular clock phylogeny and dated the emergent time for the most recent common ancestor (TMRCA) for clade III in this hospital to early 2014 and in the neonatal unit to 2018. Phylodynamic analysis showed multiple introductions of C. auris into the neonatal unit.

Conclusion: Clades III and IV co-circulated in the hospital, with clade III causing all but one case in the neonatal unit. Most isolates contained previously-described clade-specific mutations related to azole resistance. The estimated emergence of the TMRCA for the hospital and neonatal unit clade III isolates was roughly consistent with the first cases reported. While the large neonatal unit outbreak may have originated from cross-unit transmission by infected/colonised patients, colonised healthcare workers or contaminated equipment, we could not exclude the possibility of transmission events from other healthcare facilities or from the community.

ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to my supervisors Prof Nelesh Govender and Dr Serisha Naicker for their continuous support, motivation, and guidance through this project. I would also like to extend a huge thanks to Prof Anne von Gottberg, Dr Nicole Wolter, Dr Mignon du Plessis and Prof Lee Harrison (Pitts) for this incredible opportunity of being a postgraduate fellow at the NICD. To the CDC mentors and Pittsburgh University mentors, Thank you for your assistance and guidance as well.

I am also grateful to the laboratory teams at both CHARM and CRDM for their guidance, assistance and support.

To my fellow SAPPHGene students, thank you so much, we struggle together, we win together. And to my unit 129 roommates (Rito and Buhle) you all are the ones.

Go Koko, Bo Malome, Bo mogatsamalome, Ba tswala, Sesi wa ka le Ngwana wa gešo. Ke le rata ka lerato la Bakorinthe 13. Ke a Leboga!

TABLE OF CONTENTS

DECLARATION **2**

DEDICATION **3**

PRESENTATIONS **4**

ABSTRACT **5**

ACKNOWLEDGEMENTS **7**

TABLE OF CONTENTS **8**

LIST OF FIGURES **12**

Chapter 1 ..... **12**

Chapter 3 ..... **12**

LIST OF TABLES **15**

LIST OF ABBREVIATIONS AND ACRONYMS **16**

CHAPTER 1: LITERATURE REVIEW **18**

1.1 Introduction **18**

1.2 Epidemiology of *Candida auris* **18**

1.2.1 Global epidemiology **18**

1.2.2 Prevalence in South Africa **20**

1.2.3 Neonatal outbreaks 21

1.3 Pathogen characteristics 22

1.3.1 Phenotypic characteristics 22

1.3.2 Natural habitat 26

1.3.3 Identification 27

1.4 Molecular epidemiology 28

1.4.1 Molecular typing of *Candida auris* 28

1.4.2 Whole genome sequencing 29

1.4.2 Molecular Geographic distribution 30

1.4.3 Emergence of *Candida auris* 32

1.5 Infection 34

1.5.1 Virulence and Pathogenicity 34

1.5.2 Clinical manifestations 36

1.6 Antifungal resistance and treatment 38

1.7 Infection prevention and control 42

1.8. Overall aim 43

1.9. Objectives 43

CHAPTER 2: METHODS AND MATERIALS 45

2.1. *Candida auris* surveillance and isolate collection 45

2.2 Description of the outbreak 46

2.4 Descriptive analysis of the cases of *C. auris* 47

2.5 Culturing of the isolates 47

2.6 Psuedohyphae production 47

2.7 Aggregation 48

2.8 Thermotolerance 48

2.9 Antifungal susceptibility testing 48

2.10 DNA extraction, quantification and identification 49

2.11 Library preparation and Whole Genome Sequencing 49

2.12 Single-Nucleotide Polymorphism (SNP) calling and phylogenetic analysis 50

2.15 Resistance mutation identification 52

CHAPTER 3: RESULTS 53

3.1 Descriptive epidemiology 53

3.1.1 Patient demographic information 53

3.1.2 Epidemic curve 57

3.2 Phenotypic characterization 60

3.2.1 Antifungal susceptibility 60

3.2.2 Aggregate formation, pseudohyphae production and thermotolerance 62

3.3 Molecular characterization 65

3.3.1 Phylogenetic analysis 65

3.3.2 Phylodynamic analysis 71

3.3.2 Outbreak reconstruction for clade III 75

CHAPTER 4: DISCUSSION 77

CHAPTER 5: CONCLUSION 85

Bibliography 87

Appendices 101

Appendix A: CANDIDA CRF for NICD/GERMS laboratory-based survey 2019 101

Appendix B: Ethical Clearance Certificate 103

Appendix C: Similarity Index 104

LIST OF FIGURES

Chapter 1

Figure 1.1: Global epidemiology of *Candida auris* (blue represents countries with reported cases) as of 05 February 2021 (US Centers for Disease Control and Prevention)…… **20**

Figure 1.2: *Candida auris* colonies on CHROMagar and Sabouraud dextrose agar**..…22**

Figure 1.3: Schematic depiction of *in vitro* and *in vivo* phenotypic switching in *Candida auris***.....24**

Figure 1.4: *Candida auris* single yeast cells and *Candida auris* aggregated yeast cells**..25**

Figure 1.5: Worldwide clade distribution of *Candida auris* (as of 28 February 2019)**….31**

Figure 1.6: Proposed events that might have led to the emergence of *Candida auris***…..34**

Figure 1.7: Schematic diagram of the mechanisms of antifungal drug action and resistance in *Candida auris***…….41**

Chapter 3

Figure 3.1: Sample distribution by sex and age group from a hospital in Gauteng, South Africa experiencing a *Candida auris* outbreak, 2016-2020 (n = 287)**……...56**

Figure 3.2.1: Epidemic curve by ward location of laboratory-confirmed cases of *Candida auris* infection or colonisation at an academic tertiary hospital in Gauteng Province from March 2016 to July 2020, n=287**……58**

Figure 3.2.2: Epidemic curve of laboratory-confirmed cases of *Candida auris* invasive infection or colonisation at an academic tertiary hospital in Gauteng, South Africa from March 2016 to July 2020, n= 287**…….59**

Figure 3.3: Aggregation formation. A, number of isolates with aggregate forming capabilities. B. Aggregation formation category (B1 - Extensive, B2 - Moderate and B3 - none). C. *Candida auris* growth under Dalmau culture. C1: An isolate that does not produce pseudohyphae, C2: An isolate that produces pseudohyphae**……64**

Figure 3.4: Phylogenetic tree depicting clade distribution and fluconazole resistance mutations of 188 invasive or colonising South African *Candida auris* strains isolated from patients admitted to a large metropolitan hospital in Gauteng, 2016-2020. The unrooted maximum parsimony tree was created using MEGA software using 287 338 single nucleotide polymorphisms (SNPs) based on 1000 bootstrap replicates, FCZ = Fluconazole**……..68**

Figure 3.5: Clade distribution of 188 South African *Candida auris* isolates from patients admitted to a large metropolitan hospital in Gauteng, 2016-2020 classified by the patients' ward location**……..69**

Figure 3.6: Specimen types by ward classified by clade of 188 South African *Candida auris* isolates from patients admitted to a large metropolitan hospital in Gauteng, 2016-2020**…….70**

Figure 3.7: Distribution of collection dates for 188 South African *Candida auris* isolates by ward location and clades**…….72**

Figure 3.8: Root-to-tip regression analysis of 67 South African *Candida auris* outbreak isolates from the neonatal ward of a large metropolitan hospital in Gauteng.

Genetic distance is plotted against sampling time and every data point represents a tip on the phylogeny. The R squared for the regression and the slope, reflecting the evolutionary rate (in substitutions per site per day) is also shown……..**73**

Figure 3.9: Maximum clade credibility tree of 67 South African Clade III *Candida auris* isolates from this study estimated using BEAST (Strict clock and coalescent model). Red tips represent cases from the neonatal ward. Blue bars represent 95% highest probability density. Black dashed line = Clade III TMRCA, Red dashed line = Outbreak strain TMRCA……..**74**

Figure 3.10: Reconstructed transmission tree of 118 South African Clade III *Candida auris* isolates from this study. Each case is represented by a node on the tree and arrows indicate plausible ancestries/transmissions. Mutations between the nodes are indicated by the colour arrows (red = no/few mutations; light grey= many mutations) and the numbers in blue represent the weight of mutations when compared to the ancestries. Time is represented by y axis (up: older; down: younger)……**76**

LIST OF TABLES

Table 3.1: Characteristics of patients (n = 287) with *Candida auris* infection or colonisation admitted to a large metropolitan hospital in Gauteng Province, South Africa, 2016-2020**……54**

Table 3.2: Minimum inhibitory concentration (MIC) distribution of *Candida auris* isolates (n = 207) from a tertiary hospital in Gauteng Province, South Africa from March 2016 to July 2020. MICs were determined using a commercial broth microdilution and E-test method for amphotericin B**……..61**

Table 3.3: Clade proportions and frequency of antifungal drug resistance within the study population among 188 South African *Candida auris* isolates (n (%))**…….67**

LIST OF ABBREVIATIONS AND ACRONYMS

µg/mL: Micrograms per millilitre

µl: Microlitre

AFLP: Amplified fragment length polymorphism

ATP: ATP-binding cassette

CDC: US Centers for Diseases Control and Prevention

CHARM: Centre for Healthcare Associated Infections, Antimicrobial Resistance and Mycoses

CLSI: The Clinical and Laboratory Standards Institute

CRDM: Centre for Respiratory Diseases and Meningitis

CRF: Case report form

CVC: Central venous catheter

DMP: Diagnostic Media Products

GTR: General time reversible

ICU: Intensive care unit

IPC: Infection prevention and control

ITS: Internal transcribed spacer

MALDI-TOF: Matrix-assisted laser desorption ionization–time of flight mass spectrometry

MFS: Major facilitator superfamily

MIC: minimum inhibitory concentration

MLST: Multilocus sequence typing

MRSA: Methicillin-resistant *Staphylococcus aureus*

NET: Neutrophil extracellular trap

NHLS: National Health Laboratory Service

NICD: National Institute for Communicable Diseases

NICU: Neonatal intensive care unit

OBP: Oxysterol- binding protein gene

PAP: Essential poly (A) polymerase gene

PCR: Polymerase chain reaction

PFGE: Pulsed-field gel electrophoresis

SNP: Single nucleotide polymorphism

TICU: Transitional intensive care unit

TPN: Total parenteral nutrition

USA: United States of America

WGS: Whole genome sequencing

YPD: Yeast extract peptone dextrose

CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

Candida auris is a multi-drug resistant yeast that causes invasive nosocomial infections and high mortality rates in healthcare facilities worldwide (Sardi *et al.*, 2013; Spivak and Hanson, 2018). It was first isolated in the year 2009 in Japan (Satoh *et al.*, 2009). Soon after that, retrospective studies on archived isolates found no cases before 2009, with the exception of one Korean case in 1996 (Lee *et al.*, 2011). Based on the above, *C. auris* was likely rare before 2009 (Lockhart *et al.*, 2017; Spivak and Hanson, 2018). *C. auris* can cause outbreaks in healthcare facilities due to its ability to adhere to environmental surfaces and colonise the skin of both patients and healthcare professionals. These factors, along with sub-optimal infection prevention and control (IPC) practices, promote the transmission of the pathogen (Das *et al.*, 2018). The rapid emergence and spread of *C. auris*, high crude mortality rates (30% to 60%) and antifungal resistance makes this pathogen a public health concern (Lockhart *et al.*, 2017).

1.2 Epidemiology of *Candida auris*

1.2.1 Global epidemiology

C. auris is a recently emerged fungus that causes nosocomial infection and outbreaks in patients admitted to healthcare facilities worldwide. It has been reported globally in over 40 countries across six continents including, the United States of America (USA), Japan, India, Venezuela, Kuwait, United Kingdom, Colombia, South Korea, Pakistan and South Africa (Figure 1.1) (Du *et al.*, 2020). The first case of *C. auris* was reported from Japan and was isolated from an ear of a 70-year-old woman in 2009. Satoh et al. (2009) were the first team to identify and describe this pathogen as a *Candida* species due to its phenotypic and genotypic characteristics (Satoh *et al.*, 2009). The very first case of *C. auris*, however, dates back to 1996 from South Korea, it was isolated from a blood culture of a one-year-old patient: it was misidentified as *Candida*

haemulonii, a close relative to *C. auris* and was detected using molecular identification through a retrospective study (Lee *et al.*, 2011).

The global epidemiology of *C. auris* has changed drastically in the last decade. Reports of sporadic cases in a few countries have now shifted to outbreaks involving a higher number of patients in healthcare facilities worldwide. Outbreaks caused by *C. auris* have been reported in over ten countries including South Africa, with an average crude mortality of 40% (Ahmad and Alfouzan, 2021). Fungal pathogens, particularly *Candida* species, are reported as the 4th leading cause of nosocomial bloodstream infections (BSI) and crude mortality in healthcare facilities following coagulase-negative staphylococci, *Staphylococcus aureus* and enterococci in the USA (Weiner *et al.*, 2016). Invasive *C. auris* infections have surpassed those of known nosocomial pathogens of the *Candida* genus including *Candida glabrata*, *Candida tropicalis,* and even *Candida albicans,* the dominant *Candida* species causing human infection (Ahmad and Alfouzan, 2021).

Figure 1.1: Global epidemiology of Candida auris (blue represents countries with reported cases) as of 05 February 2021 (US Centers for Disease Control and Prevention)

1.2.2 Prevalence in South Africa

The first cases of *C. auris* infection in South Africa were reported in 2014 which prompted a retrospective study that identified a case of *C. auris* dating back to 2009. *Candida parapsilosis* was the dominant cause of invasive bloodstream infection in 2009-2010 when *C. auris* was first detected (Govender *et al.*, 2016). By 2016-2017, *C. auris* was the third most common cause of candidaemia in South Africa (Van Schalkwyk *et al.*, 2019). Additionally, in recent years, *C. auris* has caused large outbreaks and has subsequently become endemic in many hospitals. From 2012-2016 (Govender *et al.*, 2018), most cases of *C. auris* in South Africa

were diagnosed in private hospitals in the Gauteng Province. This is hypothesised to be due to the availability and unrestricted use of antifungals and poor IPC practices which facilitates the transmission of the pathogen (Van Schalkwyk *et al.*, 2019). The prevalence of *C. auris* infection may, however, be underestimated due to the lack of resources and capacity to perform molecular identification of the pathogen in some laboratories or the misidentification of the pathogen through non-molecular/phenotypic testing in resource-limited areas (Govender *et al.*, 2019).

1.2.3 Neonatal outbreaks

C. auris outbreaks involving neonates have been documented in various hospitals in South Africa (Govender *et al.*, 2019; Naicker *et al.*, 2021). Little is known about the molecular and phenotypic characteristics of the causative *C. auris* strains. Globally, neonatal outbreaks caused by *C. auris* were observed in India, Colombia, and Venezuela while early outbreaks caused by *C. auris* were documented among adults in South Africa (Van Schalkwyk *et al.*, 2019). *C. parapsilosis* was the well-documented cause of nosocomial invasive infections in neonates due to its ability to survive in the environment and persist on hospital surfaces, characteristics that are also true for *C. auris* (Welsh *et al.*, 2017).

Candidaemia is one of the leading causes of death in the neonatal unit mainly due to the immature immune system of neonates. Very low birth weight infants have lower neutrophil and monocyte cell counts as well as lower antimicrobial peptides circulating in blood, including proteins of the complement system that are essential for opsonization and phagocytosis (Michalski, Kan and Lavoie, 2017). Candidaemia in neonates has been shown to result in impairments in neurodevelopment including blindness and deafness when compared to unaffected neonates (Hsieh, Smith and Benjamin, 2011).

1.3 Pathogen characteristics

1.3.1 Phenotypic characteristics

C. auris is part of the Phylum Ascomycota and falls under the Saccharomycetes class (Bravo Ruiz and Lorenz, 2021). It is an oval-shaped budding yeast that is related to *Candida krusei*, *C. haemulonii* and *Candida lusitaniae* phylogenetically (Satoh *et al.*, 2009). *C. auris* is part of the CTG clade, yeasts that can translate the CTG codon as serine instead of leucine. The role of the amino acid switch is not fully understood (Santos *et al.*, 2011). It forms off-white/light pink colonies on commercial chromogenic agar (CHROMagar) and cream white colonies on Sabouraud dextrose agar (Figure 1.2) (Iguchi *et al.*, 2019). Contrary to characteristics of the well-studied *C. albicans*, *C. auris* cannot germinate, form filamentous hyphae or produce chlamydospores (Larkin *et al.*, 2017). Pseudohyphal structures are produced in high-stress conditions (de Jong and Hagen, 2019).

Figure 1.2: Candida auris colonies on commercial CHROMagar (left) and Sabouraud dextrose agar (Right). (Source: https://www.cdc.gov/drugresistance/solutions-initiative/stories/cdc-response-to-global-threat.html)

Phenotypic switching is a series of transitional proteomic events that allow pathogens to switch their morphology to rapidly adapt to their environment, such as when moving from the external environment into a host (Huang, 2012), Pathogens with this ability are often known to cause severe infection due to the ability to efficiently invade host tissue. Pathogens including *Candida albicans* can transform from single yeast cells in the external environment to a hyphal phenotype within a host organism (Figure 1.3) (Yue *et al.*, 2018).

The transition between the yeast phase and the filamentous phase can be triggered by several factors including host temperature, pH, carbon dioxide levels as well as the presence of serum (Biswas, Dijck and Datta, 2007). Filamentous cells are advantageous in invading host tissue and initiating entry while yeast cells can propagate within a host and travel to other organ systems at ease (Soll, 2009). There is evidence that *C. auris* can switch to a hyphal form in certain conditions (Wang *et al.*, 2018; Ruiz *et al.*, 2020). In a study conducted by Wang *et al* (2018), *C. auris* isolates produced pseudohyphae when grown in a yeast extract peptone dextrose (YPD) medium supplemented with 10% NaCl at temperatures of 37°C and 42°C (Shapiro *et al.*, 2009; Wang *et al.*, 2018). Other studies demonstrated that inhibiting the production of the heat-shock protein, Hsp60, stimulated the production of pseudohyphae (Kim *et al.*, 2019). When yeast cells of *C. auris* were cultured and inoculated into mice, filamentous colonies were isolated from the livers and kidneys of mice after a 24-hour systemic infection suggesting that filamentation occurs by passage through a mammalian host (Figure 1.3) (Yue *et al.*, 2018).

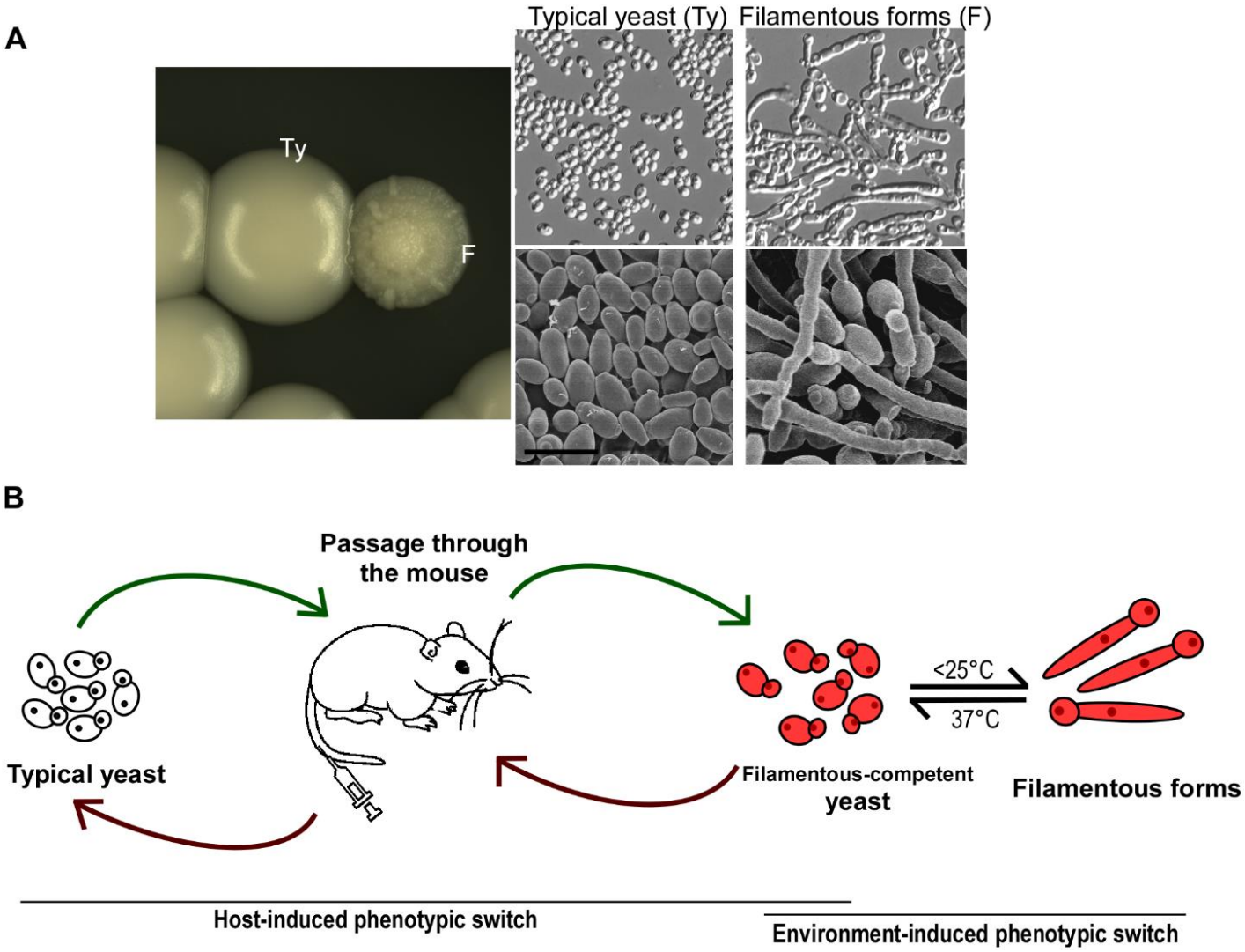


Figure 1.3: Schematic depiction of in vitro and in vivo phenotypic switching in Candida auris (Du *et al.*, 2020)

C. auris predominantly exists in the single-celled form but has been shown to form aggregates when parent and progeny cells do not fully detach during reproduction (Figure 1.4) (Borman, Szekely and Johnson, 2016). The ability of some *C. auris* isolates to form aggregates is not fully understood, and little is known about how aggregation influences the pathogen's interaction with the host, immune system response, biofilm formation as well as the tolerance to antifungal drugs (Brown *et al.*, 2020). Isolates display clade-specific phenotypic behaviour and morphological differences (Szekely, Borman and Johnson, 2019). Most strains of clade III (African) and some from clade II (East Asia) cluster together to form aggregates when cultured

which might allow them to persist longer in the environment than non-aggregating isolates belonging to other clades (Forsberg *et al.*, 2019; Szekely, Borman and Johnson, 2019).

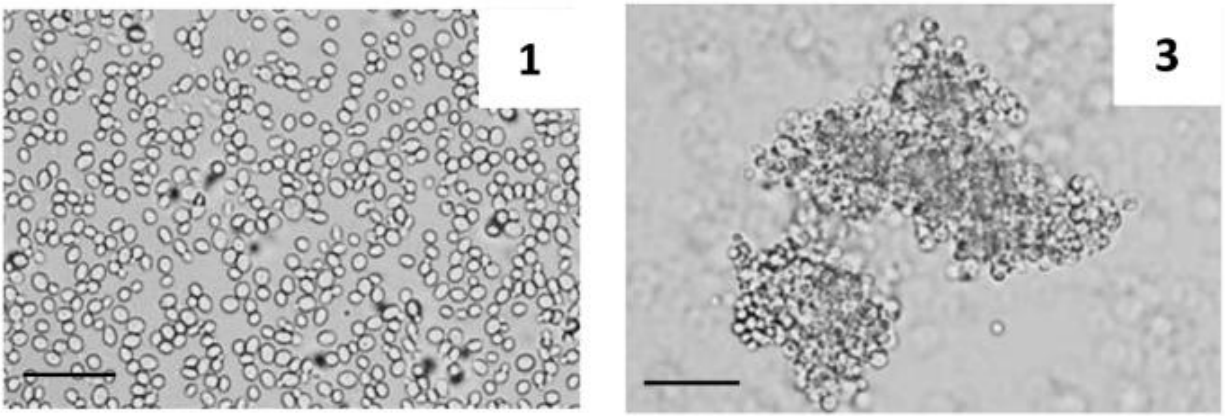


Figure 1.4: Candida auris single yeast cells (Left) and Candida auris aggregated yeast cells (Right) (Szekely, Borman and Johnson, 2019)

C. auris can form aggregates in certain environmental conditions including in the presence of triazole and echinocandin antifungals (Borman, Szekely and Johnson, 2016). Studies involving biological models investigating *C. auris* infection are rare, however, *Galleria mellonella* larvae and murine models have been used to assess the virulence and pathogenesis (Szekely, Borman and Johnson, 2019). When aggregating and non-aggregating strains of *C. auris* were used to infect a wound that was induced on a three-dimensional human skin model, aggregating strains were more cytotoxic, inducing a greater inflammatory response. Two proteins involved in tissue invasion in *C. auris* infection, ALS5 and SAP5, were upregulated in aggregate-forming strains compared to cells with the non-aggregating phenotype (Brown *et al.*, 2020). Aggregating strains have also been shown to form the greatest biomass of biofilm. Using RNA-sequencing to assess the transcriptional response, upregulated genes included those responsible for membrane and cell wall proteins, including *TSA1, ECM33, MP65, ALS* and *PHR1* (Brown *et al.*, 2020).

Most fungal species are unable to withstand physiological and fever temperatures and thus are unable to cause infection in humans. *C. auris,* however, grows at 38°C or 42°C and has a high tolerance for salt. Its ability to grow in these conditions can be used to distinguish it from other *Candida* species (except *C. albicans* that grows in similar conditions) (Cortegiani *et al.*, 2018; Rossato and Colombo, 2018). The ability of *C. auris* to thrive in unfavourable harsh environments sets it apart from the other *Candida* species as well as other human pathogens (Du *et al.*, 2020). To assess the effects of incubation temperatures on *C. auris* cultures, the yeast cells in the filamentous state were incubated at different temperatures after being isolated from mice. Isolates cultured at 25°C maintained their filamentous morphology while cultures incubated at other temperatures reverted to their yeast form (Yue *et al.*, 2018). However, the optimal temperature of hyphal formation in this study was less than 25°C which is lower than the physiological temperature (37°C) which hindered the filamentation of *C. auris* cells. This is contrary to the filamentation of *C. albicans* cells that optimally occurs at body temperature (Biswas, Dijck and Datta, 2007). This observation shows that *C. auris* might be able to switch to a filamentous state only in the external environment and not in a human host (Yue *et al.*, 2018).

1.3.2 Natural habitat

C. auris is part of the *Clavispora* clade within the Metschnikowiaceae family. Most species from this family have been isolated from non-human environments (Daniel, Lachance and Kurtzman, 2014). *Candida* species including *C. glabrata, C. parapsilosis* and *C. tropicalis* have been isolated from the environment including a rubber tree and a cassava (Ferreira *et al.*, 2010; Jackson *et al.*, 2019). *C. albicans* was isolated from an oak tree in the United Kingdom (Bensasson *et al.*, 2019). The environmental strains were found to be different from those interacting with human hosts (Carvalho *et al.*, 2014). *C. auris* was recently isolated in a marine ecosystem including a salt marsh and a sandy beach of the Andaman Islands in the Indian ocean

(Arora *et al.*, 2021); the discovery of *C. auris* in these wetlands supports the hypothesis that the microbe was a plant saprophyte before its introduction to humans (Casadevall, Kontoyiannis and Robert, 2019). One susceptible isolate was recovered from the salt marsh of the South Andaman Island suggesting that *C. auris* was formerly a susceptible strain that acquired resistance after its interaction with the human host (Arora *et al.*, 2021). Interestingly, most of the environmental isolates had high minimum inhibitory concentration (MIC) values for fluconazole and amphotericin B (Arora *et al.*, 2021). The presence of resistant strains in the environment could have been due to the use of fungicides in agriculture promoting the evolution of the strain or the re-introduction of resistant strains into the marsh and beach as human interaction with the environment increases.

1.3.3 Identification

Phenotypic identification methods include the use of chromogenic agar such as CHROMagar to differentiate from other *Candida* species, though its discriminatory ability remains unsatisfactory due to similarities in colony characteristics with other *Candida* species (Jeffery-Smith *et al.*, 2018). Conventional phenotypic and biochemical techniques of identification such as VITEK 2 (bioMérieux, Inc, Hazelwood, MO, USA), MicroScan (Siemens Healthcare, Sacramento, CA, USA) and API 20C (bioMerieux, Inc, Hazelwood, MO, USA) misidentify *C. auris* as closely-related *Candida* species such as *C. krusei*, *C. haemulonii*, *C. lusitaniae* and *Saccharomyces* spp. (Ademe and Girma, 2020). Accurate methods of identification include matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF), VITEK 2 with software version 8.01 or higher and molecular identification methods including sequencing the internal transcribed spacer (ITS) and large subunit (LSU) D1/D2 rDNA genes (Kordalewska and Perlin, 2019). Conventional non-selective culture remains the main method of detection in most laboratories especially those in resource-limited areas; it is both cost-effective and accessible but can miss *C. auris* especially from non-sterile specimens. Researchers have

developed a low-cost novel diagnostic broth that can select exclusively for *C. auris*. The broth contains 10% salt, gentamicin, mannitol and chloramphenicol in Sabouraud broth and is inhibitory to the growth of other microbes when cultured at 42°C (Welsh *et al.*, 2017).

The MALDI-TOF MS platform can identify *C. auris* with high accuracy with the Bruker Biotyper updated database and identification occurs in a few minutes (Kathuria *et al.*, 2015). The MALDI-TOF system identifies organisms through the time of flight mass spectrometer: samples are mixed with a matrix that ionizes and vaporises along with the sample when the mixture comes in contact with a laser. The charged ions of different sizes are propagated through a vacuum to a detector above the sample stage, the time of flight and velocity of the ions i.e. mass-charge ratio is measured and compared with the mass spectrum (peptide mass fingerprint) of organisms stored in the database (Singhal *et al.*, 2015). The accuracy of MALDI-TOF is comparable to genotypic identification methods such as the sequencing of the ITS gene, amplified fragment length polymorphism (AFLP) and multilocus sequence typing (MLST) (Girard *et al.*, 2016; Prakash *et al.*, 2016).

1.4 Molecular epidemiology

1.4.1 Molecular typing of *Candida auris*

The amplification of the ITS and D1/D2 sequences through the polymerase chain reaction (PCR) and the subsequent sequencing of the amplicon is the current gold standard for the identification and typing of *C. auris* (Cortegiani *et al.*, 2018). Common typing methods such as pulsed-field gel electrophoresis (PFGE), MLST, AFLP and microsatellite typing have been previously used to classify *C. auris* isolates. Through these methods, classification of *C. auris* into distinct clades was achieved; however, due to the insufficient discriminatory ability, the close relatedness of the isolates could not be determined (Jeffery-Smith *et al.*, 2018; Magobo *et al.*, 2020). Whole genome sequencing (WGS) provides a convenient method of identifying

an organism and also yielding information regarding its relationship with other isolates, evolutionary trends and emerging variants (Muñoz *et al.*, 2018).

1.4.2 Whole genome sequencing

C. auris has a haploid genome size of 12.5 Mb – 12.7 Mb, all compacted into five to seven chromosomes with a GC content of approximately 45%. The total gene pool of the *C. auris* genome is estimated to contain 5500 genes (Chatterjee *et al.*, 2015; Muñoz *et al.*, 2018). The *C. auris* genome is 85.9 % similar to its closest relative, *C. lusitaniae*. Several orthologous genes to *C. albicans* were found in the *C. auris* genome, including efflux transport genes such as the ATP-binding cassette (ABC) and the major facilitator superfamily (MFS) transporter families. These membrane transporters contribute to the resistance of antifungals in *C. albicans*; *C. auris* isolates have been shown to deploy the same mechanisms. Other *C. albicans* orthologous genes include proteins involved in the virulence of the pathogen (Sharma *et al.*, 2016)

The molecular epidemiology based on WGS provides an understanding of the dynamics and mechanism of transmissibility and possible source/s of infection in healthcare facilities (Chow *et al.*, 2018). WGS has been used in fungal outbreak investigations to determine the origin and source of outbreaks as well as epidemiological links between patients involved in infection outbreaks (Cuomo and Alanio, 2020). An *Exserohilum rostratum* infection outbreak was resolved using WGS; the fungal pathogen, which usually causes infections in plants, had only been reported in a few patients until this outbreak. The researchers found that the isolates from 19 cases were almost identical with fewer than two single nucleotide polymorphisms (SNPs) between any of the isolates, indicating a single source (Litvintseva *et al.*, 2014). In a study conducted by Sharma *et al*., (2016) on five *C. auris* isolates from five different hospitals in Delhi, India, WGS was used to show relatedness among the strains indicating a common source of infection in all hospitals or their recent differentiation (Sharma *et al.*, 2016). WGS has also

been used in healthcare-associated outbreak investigations for pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) in an outbreak involving 13 infants admitted to the neonatal intensive care unit (NICU) and three staff members at University Hospitals in Cambridge in the United Kingdom. SNP analysis of genomic data revealed clusters of MRSA infection within the outbreak while also identifying isolates not involved in the outbreak (Köser *et al.*, 2013). *Klebsiella pneumoniae,* another healthcare-associated pathogen, was investigated in a NICU outbreak using WGS. Transmission routes of the pathogen were established as well as tracing the source of infection and identifying "super-spreaders" within the outbreak (Wang et al., 2020).

1.4.2 Molecular Geographic distribution

WGS has revealed five clades that are genetically distinct on different continents. Clade I is from South Asia, clade II is from East Asia, clade III is African, clade IV is from South America (Figure 1.5) and a newly-discovered clade V from Iran, all of which exhibit minimum genetic diversity within each other but are separated by plenty of SNPs (Muñoz *et al.*, 2018; Escandón *et al.*, 2019). Strains within a clade differ from each other by an average of two to 600 SNPs while inter-clade differences range from 40000 to 20000 SNPs (Muñoz *et al.*, 2018; Chow *et al.*, 2020)

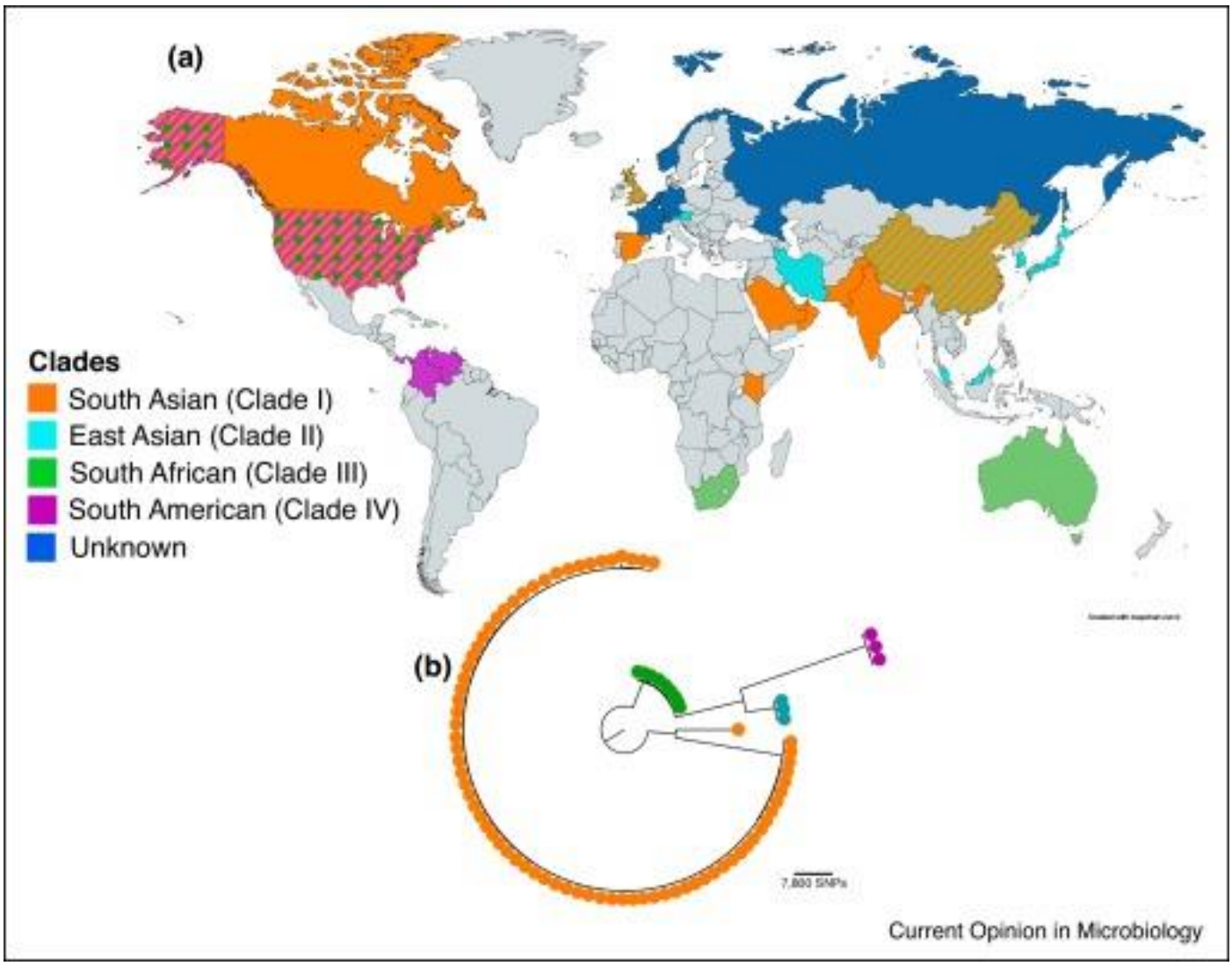


Figure 1.5: Worldwide clade distribution of Candida auris (28 February 2019) (Rhodes and Fisher, 2019)

In a phylogeographic study conducted by Chow *et al*., (2020), clade I was the most widespread, spanning ten countries in Europe, Africa, North America, Asia and the Middle East. Clade II was found in four countries in North America and Asia. Clade III was found in six countries in North America, Europe, Africa and Australasia. Clade IV was detected in six countries on the Asian, South and North American continents. Countries found to have diverse strains of *C. auris* were the United States (I, II, III, IV), Canada (Clade I, II, III) and Kenya (I and III). (Chow *et al.*, 2020). Other countries included Germany (Corsi-Vasquez and Ostrosky-

Zeichner, 2019) and the United Kingdom (Borman, Szekely and Johnson, 2016) which also have strains from different clades. This is probably due to the introduction of *C. auris* strains through international travel followed by local transmission of imported strains (Chow *et al.*, 2020). The clades possess different molecular and phenotypic characteristics as well as antimicrobial resistance profiles (Kordalewska and Perlin, 2019). The sequence analysis shows a simultaneous emergence of *C. auris* on four different continents that was independent (Lockhart *et al.*, 2017). The factors for this emergence are not fully understood; however, it is speculated to be due to the usage and selection pressures of the antifungal treatments in humans, animals and the environment (Lockhart *et al.*, 2017).

The *C. auris* genome has two mating types (i.e. genetic loci determining the sex of the fungus). Mating types contribute to creating genetic diversity within the species (Rodero *et al.*, 2002; Ross and Lorenz, 2020). Each clade possesses strains of the same mating type due to clonal expansion. Clade I and IV carry the MTL**a** mating type while MTLα mating type is found in clade II and clade III (Muñoz *et al.*, 2018). However, no evidence of mating and hybridization between strains from different clades (with different mating types) have been reported even in countries/health facilities where multiple clades exist. The presence of multiple clades in a single healthcare facility may increase the risk of patients being infected by strains of different mating types increasing the likelihood of mating; this could result in strains of *C. auris* with higher genetic diversity which might be advantageous with increased virulence, transmissibility and antifungal resistance (Chow *et al.*, 2020).

1.4.3 Emergence of *Candida auris*

C. auris is speculated to have existed for a long time; this is due to the high genetic diversity that exists within the species (Daniel, Lachance and Kurtzman, 2014). Genetic analysis of sequences obtained from different countries has shown that a common ancestor of the pathogen probably emerged 360 years ago, around the year 1626, through utilising divergence times and

the evolutionary rate of *C. auris* (Yadav *et al.*, 2021). Clade IV was shown to have been the most recent to emerge while clade II was the oldest (Chow *et al.*, 2020). The East Asian clade (II) which mostly causes non-invasive infection possesses the highest intra-clade diversity compared to any other clade, suggesting an earlier emergence (Rhodes *et al.*, 2018).

The factors for the emergence of *C. auris* are not fully understood; however possible reasons for the emergence have been proposed. Increased access to health care might have contributed to the emergence of *C. auris.* In recent years, more individuals are treated with antimicrobials and undergo invasive procedures (Ventola, 2015). The use of azoles for clinical use began in the 1980s, coinciding with the predicted time of emergence for the azole-resistant isolates that occurred approximately 38 years ago (Chow *et al.*, 2020). An increase in the number of immunocompromised individuals in short- and long-term healthcare facilities has also been observed; all of these factors create the perfect environment for an opportunistic multi-drug resistant pathogen such as *C. auris* to thrive (Jackson *et al.*, 2019). Human activity might have also influenced its emergence and spread. The increased interaction of human beings with nature could have increased contact between humans and *C. auris* from the environment (Jackson *et al.*, 2019). The increased use of fungicides in farming/agriculture, particularly triazole fungicides might have also had an effect. Fungicides probably decreased the population of triazole-susceptible fungal species in the environment and selected for azole-resistant species allowing for their expansion (Berger *et al.*, 2017).

Global warming has also been credited as the major influencer in the emergence of *C. auris*. Increased global mean temperatures, which are approaching physiological temperatures, are selective for thermotolerant pathogens such as *C. auris* (Garcia-Solache and Casadevall, 2010). The thermotolerance allows the pathogen to survive the external environment more so than related species. *C. auris* can survive during the host immune and inflammatory responses including fevers due to this ability (Figure 1.6) (Jackson *et al.*, 2019).

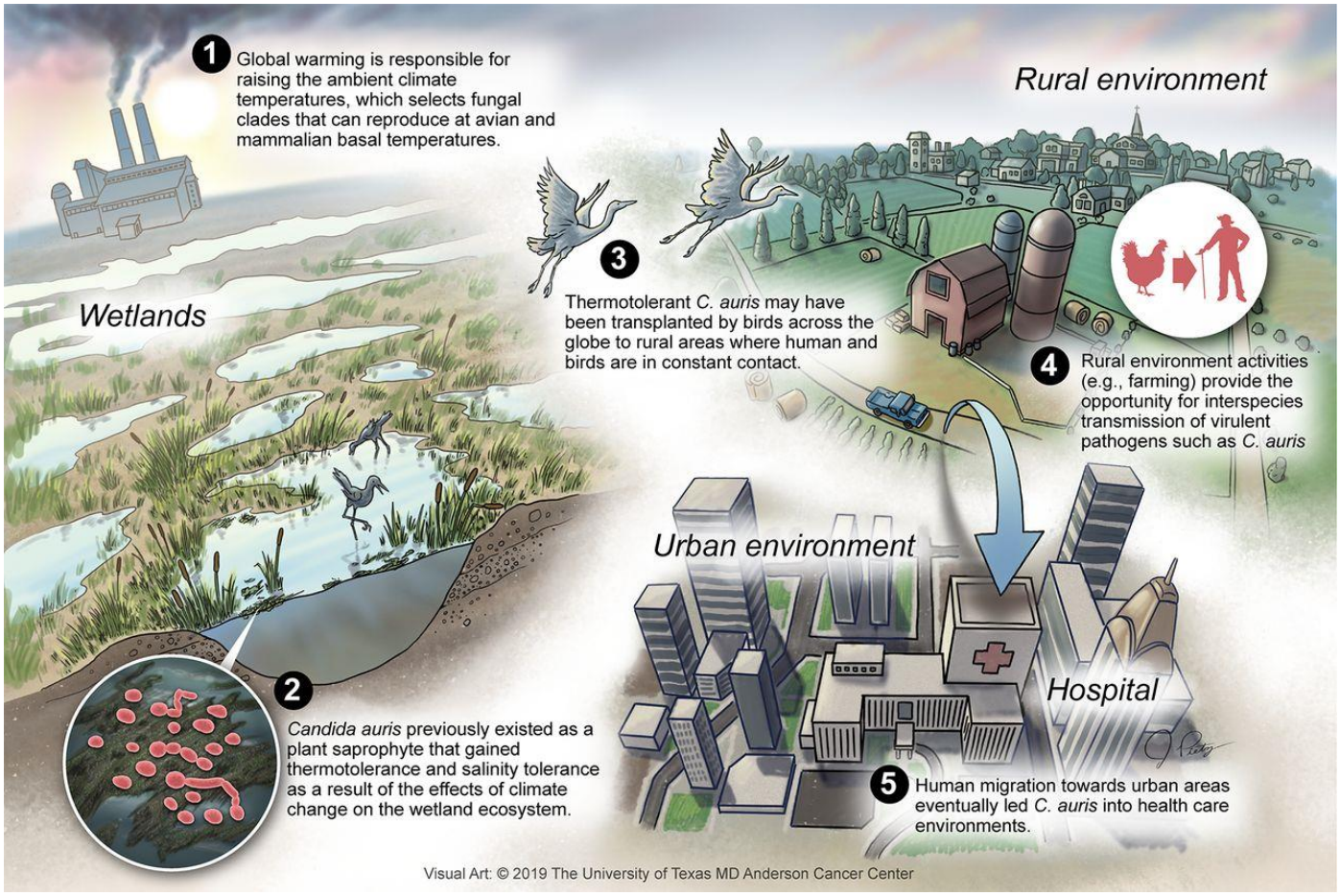


Figure 1.6: Proposed events that might have led to the emergence of Candida auris (Casadevall, Kontoyiannis and Robert, 2019)

1.5 Infection

1.5.1 Virulence and Pathogenicity

C. albicans is considered the most pathogenic *Candida* species; however, research has shown that *C. auris* isolates have comparable pathogenicity to *C. albicans*. The pathogenicity of *C. auris* is due to biofilm formation, oligopeptide transporters, proteinases, phospholipases and haemolysins depending on the strain (Forsberg *et al.*, 2019). Hydrolytic enzymes play a role in destruction of host tissues, invasion, adherence and tissue penetration (Silva *et al.*, 2012). These proteins and systems enable the pathogen to efficiently enter and invade the host; however, not all isolates of *C. auris* produce proteinases or phospholipases equally Therefore the virulence and pathogenicity of *C. auris* varies from strain to strain (Larkin *et al.*, 2017).

C. auris is less virulent than *C. albicans* but more virulent than its relatives, *C. glabrata, C. haemulonii* and *C. lusitanae*. One of the reasons for the differences in virulence between *C. auris* and *C. albicans* is the ability to form filamentous structures/hyphae that allow *C. albicans* to effectively invade host tissue during infection (Yue *et al.*, 2018). While the main virulence factor of *C. albicans* is its ability to form hyphal structures, these structures may instead assist *C. auris* in colonising the skin and mucous layers of patients and environmental surfaces rather than facilitating systemic infections (Yue *et al.*, 2018). *C. albicans* is a diploid species and has a greater genetic diversity while *C. auris, C. glabrata* and *C. lusitanae* are haploid organisms which might account for their lesser virulence (Hickman *et al.*, 2013).

Biofilms are communities of microorganisms that are surrounded by the matrix they produce; the polymeric matrix substance forms a biological diffusion barrier that protects the microbe against the penetration of antifungals and assists with adhesion and persistence on surfaces (Chen *et al.*, 2016). Genes responsible for biofilm formation in *C. albicans* such as apartyl proteases genes, the nonessential oxysterol-binding protein gene (OBP) and the essential poly (A) polymerase gene (PAP) have also been identified in the *C. auris* genome (Sharma *et al.*, 2016). *C. auris* produces significantly less biofilm when compared to the more prevalent *C. albicans* and *C. parapsilosis.* Therefore it adheres less to synthetic materials such as catheters but still significantly contributes to causing candidiasis associated with catheters (Larkin *et al.*, 2017).

The immune system response to *Candida* fungal infection includes macrophage-mediated phagocytosis by monocytes and neutrophils and the subsequent presentation of fungal antigens (S. Singh *et al.*, 2019)**.** Neutrophils engage and trap *Candida* species through the formation of neutrophil extracellular traps (NET) which are chromatin fibres containing antimicrobial proteins. They are released due to a microbial stimulus/inflammatory signal to engage and kill microbes (Yost *et al.*, 2009). However, neutrophils are unable to engage and release NET

during *C. auris* infection when compared to infection with *C. albicans*. This type of immune cell evasion mechanism is also seen with *C. lusitaniae*, a closely-related *Candida* species which suggests that this might be due to modification of surface molecules such as mannans that are necessary for neutrophil recognition (Johnson *et al.*, 2018). Aggregate and biofilm formation might also be a mechanism for evasion of the immune system, specifically neutrophil engulfment (Nett, 2019).

1.5.2 Clinical manifestations

Candida species are known to cause disease and death in the critically ill and immunocompromised. While the first reported *C. auris* case was from an ear infection, *C. auris* has been notorious for causing invasive bloodstream or deep tissue infections that are persistent. *C. auris* is difficult to clear from the bloodstream even in cases where the isolate is not resistant to antifungals (Ben-Ami *et al.*, 2017). It is often isolated from blood, cerebrospinal fluid, deep tissue samples and central venous catheter tips (Iguchi *et al.*, 2019). However, the pathogen also causes non-invasive infections involving surgical wounds and the lower urinary tract (Chowdhary, Voss and Meis, 2016). Very low birth weight neonates do not present with specific symptoms during *Candida* bloodstream infection which poses a challenge in diagnosis and subsequent treatment (Fu *et al.*, 2018).

Most patients at risk of candidaemia due to *C. auris* almost always have underlying chronic comorbidities (Osei Sekyere, 2018). Risk factors for infection in all age groups include underlying chronic illness, surgery, premature birth, exposure to broad-spectrum antimicrobials, prophylactic antifungal treatment, venous and urinary catheters, placement of drains post-operation, chemotherapy, blood transfusion, dialysis, low levels of white blood cells as well as prolonged intensive care unit (ICU) stays (more especially in the private sector in South African hospitals), organ transplant and other procedures that require immunosuppressive therapy (Azar *et al.*, 2017; Govender *et al.*, 2019; Van Schalkwyk *et al.*,

2019). Immunosuppressive agents such as steroids that are administered during some medical procedures including organ transplants and treatments for conditions such as cancer place patients at risk of infection. Immunocompromised patients are at greater risk for *C. auris* invasive infection and mortality due to their inability to fight infection and prevent its spread within the body (Azar *et al.*, 2017). Prolonged use of broad-spectrum antibiotics or antifungals as prophylaxis is one of the main risk factors for neonates; antimicrobials have been shown to increase colonization of pathogenic fungi such as *Candida* by decreasing the population of competing bacterial and fungal flora that are natural inhibiting competitors (Fu *et al.*, 2018). Other risk factors for neonatal patients also include a low birth weight, central line placement and total parenteral nutrition (TPN) (van Schalkwyk *et al.*, 2018).

The placement of indwelling medical devices is one way that *C. auris* can be introduced into the bloodstream of patients. *C. auris* is unable to penetrate an intact epidermis; infection and inflammation occur once the skin layer has been broken. This explains why catheters and indwelling medical devices are most important risk factors for invasive infection. The removal of these devices may help to resolve infection and should thus be considered in treatment (Ademe and Girma, 2020; Ahmad and Alfouzan, 2021).

The current COVID-19 pandemic also places hospitalised patients at risk for fungal infection including coronavirus-associated *C. auris* infection (CACa). The novel SARS-COV-2 can cause respiratory illness which lead to prolonged ICU stays in patients of older age, with underlying diseases or with a suppressed immune system, all risk factors for infection with *C. auris* (Adhikari *et al.*, 2020). Along with these factors, a *C. auris* BSI, the most prevalent form of CACa, can exacerbate the illness caused by COVID-19 leading to death. A study conducted in India reported infections caused by *C. auris* in COVID-19 patients in an Indian hospital ICU; 2.5% of the COVID-19 patients were diagnosed with candidaemia and 67% of the infections were caused by *C. auris*. The patients had risk factors that are common for both COVID-19

and *C. auris* infection including old age, underlying chronic illness, indwelling medical devices and a prolonged ICU stay. Hypertension was found to be the most prevalent comorbidy followed by diabetes mellitas and heart diseases. The case-fatality ratio among these patients was 60% (Chowdhary *et al.*, 2020).

1.6 Antifungal resistance and treatment

There are three main classes of systemic antifungals available for clinical treatment. These include azoles, polyenes and echinocandins (Frias-De-Leon *et al.*, 2020). The azole class includes antifungals such as fluconazole, posaconazole, and itraconazole. These agents inhibit the production of ergesterol¸ that forms part of the chemistry of the cell membrane, by binding to lanosterol 14 α-demethylase enzyme (Pasko, Piscitelli and Van Slooten, 1990). Drugs in the polyene class include amphotericin B; its mechanism of action includes an interaction with ergosterol which results in the formation of pores that causes monovalent ions to leak out of the cell (Stone *et al.*, 2016). The echinocandin class consists of micafungin, caspofungin and anidulafungin. They kill fungi by inhibiting the β-1,3-d-glucan synthase enzyme which is crucial for the production of glucan, a structural component fundamental to the integrity of the cell wall (Sucher, Chahine and Balcer, 2009). A novel antifungal called ibrexafungerp (SCY-078) also targets glucan synthase (Arendrup and Jørgensen, 2020). Other classes of antifungals include allylamines which disturbs membrane stability and flucytosine which inhibits DNA synthesis (Ghannoum and Rice, 1999; Frias-De-Leon *et al.*, 2020) (Figure 1.7).

The emergence and spread of the multi-resistant pathogen is underlined by a lack of antifungal stewardship as well as IPC programmes in most hospitals (Forsberg *et al.*, 2019). The resistance of *C. auris* to common disinfectants renders the pathogen a challenge to eradicate from the environment and its antimicrobial resistance renders it problematic to cure (Arendrup et al., n2017). Most strains from the African clade are resistant to fluconazole and have differing

resistance and susceptibility to other classes antifungals. Echinocandin-resistant strains are not as prevalent as fluconazole and amphotericin B resistant strains (Lockhart *et al.*, 2017). There are currently no definitive antifungal breakpoints for *C. auris*. Tentative breakpoints established using closely related *Candida* species are used (https://www.cdc.gov/fungal/candida-auris/c-auris-antifungal.html). Clade II has the fewest number of resistant isolates and clade I was found to have the highest number of resistant strains to fluconazole and amphotericin B. Clades differ in the mutations responsible for resistance against antifungals (Chow *et al.*, 2020). Echinocandins are used as the first line of treatment because only a small proportion of isolates are resistant to this antifungal class. Other antifungals can be used once the susceptibility profile of the isolate from the patient is determined (Lepak *et al.*, 2017). Liposomal amphotericin B is effective in the treatment of *C. auris*; the antifungal is even effective against biofilm formation (combined therapy with echinocandins). Amphotericin B deoxycholate is significantly more toxic than antifungals of other classes thus limiting its use to resource-limited settings (Azar *et al.*, 2017).

The mechanism of antifungal resistance in *C. auris* is not fully understood; however it has been linked to protein transporters, proteases, mannosyl transferases and antifungal target genes (Frias-De-Leon *et al.*, 2020). Alterations of the lanosterol 14 α-demethylase, encoded by *ERG11* gene mutations, result from amino acid substitutions such as Y132F, K143R, and F126L. The Y132F substitution is the most frequent, being identified in isolates from 11 countries and mostly in clades I and IV. The K143R substitution is mostly found in clade I and in some isolates of clade IV. The F126L substitution is exclusive to the African clade III (Spivak and Hanson, 2018). Resistance to azole antifungals varies between isolates. This varying resistance might be due to the presence of susceptible and non-susceptible *C. auris* strains in one circulating population. Another reason might be different antifungal resistance mechanisms against azoles that can exist in one population; this might include differences in

expression of the target proteins and/or varying expression of efflux pumps on the cell wall of the pathogen (Arendrup *et al.*, 2017; Osei Sekyere, 2018).

On the echinocandin target, 1,3-beta-glucan synthase enzyme (FKS1), a substitution called S639F is found in isolates of clade I and III that are resistant to micafungin (Chow *et al.*, 2020; Frias-De-Leon *et al.*, 2020). Resistance to antifungals has also been associated with the increased expression of ABC and MFS efflux pumps (Spivak and Hanson, 2018). Research has shown that resistant isolates possess mutated genes that are acquired instead of inherited. This is shown by the presence of closely related susceptible and resistant isolates as well as different resistant mutations in closely related isolates (Chow *et al.*, 2020).

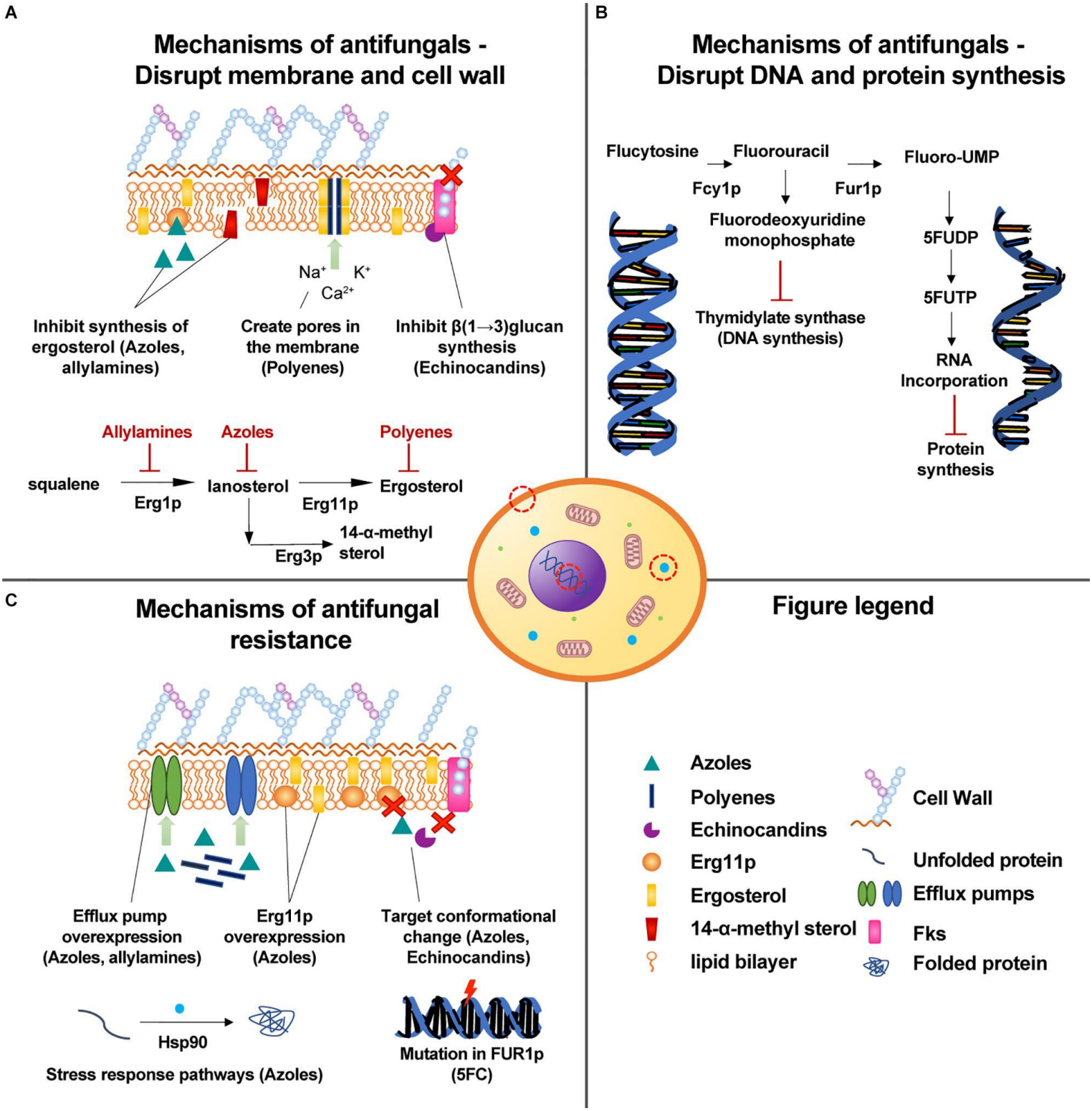


Figure 1.7: Schematic diagram of the mechanisms of antifungal drug action and resistance in *Candida auris* (Chybowska, Childers and Farrer, 2020).

The production of biofilms serves as a mechanism of antifungal resistance in some isolates of *C. auris* and other *Candida* species. Liposomal amphotericin B is the only antifungal shown to be successful in inhibiting the metabolic activity of the biomass by 90% (Sherry *et al.*, 2017). The antimicrobial resistance has also been linked to the formation of aggregates of the African clade III (Muñoz *et al.*, 2018; Szekely, Borman and Johnson, 2019). Echinocandins have been shown to penetrate biofilms formed by *Candida* species, which is why the aggregate forming isolates of the African clade are not more resistant to echinocandins than non-aggregate forming isolates of other clades (Szekely, Borman and Johnson, 2019). It is speculated that aggregate formation might assist isolates of the South African clade in limiting antifungal exposure by avoiding the penetration of antifungals to the centre of the cluster (Szekely, Borman and Johnson, 2019).

1.7 Infection prevention and control

The spread of *C. auris* in healthcare facilities is attributed to its ability to produce a biofilm. *C. auris* can survive for a long time on surfaces and is resistant to disinfectants such as quaternary ammonium (Spivak and Hanson, 2018). *C. auris* can be viable and metabolically active on surfaces but remain non-culturable for four weeks while culturable cells can be detected until two weeks. However, there is uncertainty as to whether the cells are still infectious in this non-culturable state (Welsh *et al.*, 2017). *C. auris* can persist on patient equipment including bed rails, bedding, floors, sinks and in the air around the infected or colonised patient. *C. auris* isolates grow better on moist surfaces compared to dry surfaces, even longer than *C. albicans* (Welsh *et al.*, 2017).

Patients colonized with the organism drive the transmission of the infection by contaminating their close environment, promoting the continuous spread of the pathogen, by shedding *C. auris* cells at a rate of approximately 10^6 cells/hour (Welsh *et al.*, 2017). It can, therefore, be transmitted to patients from the environment previously occupied by an infected patient

(Govender *et al.*, 2019). More effort needs to be applied concerning the control of colonisation; effective antiseptics to use on skin include iodine-based skin cleansers and chlorhexidine (Schelenz *et al.*, 2016; Spivak and Hanson, 2018) .

Prevention and control requires prompt identification of the pathogen upon isolation as well as the implementation of measures to prevent further spread of the pathogen (Chow *et al.*, 2018). Successful prevention protocols include recognition and isolation of confirmed cases and de-contamination of medical equipment serving as reservoirs (Cortegiani, et al., 2018). Prevention efforts should also include hand hygiene (Escandón *et al.*, 2019). Therefore, factors promoting transmission in hospital settings include colonisation of the skin of patients, delayed recognition of cases, overcrowding of units, suboptimal IPC practices in healthcare centres (Cortegiani *et al.*, 2018) and the increase in the number of invasive surgeries and procedures performed (Chowdhary, Voss and Meis, 2016) .

1.8. Overall aim

The aim of this project is to describe the molecular epidemiology of *C. auris* at a large tertiary academic hospital by using whole genome sequencing (WGS), with a focus on the hospital's neonatal department that has had an ongoing outbreak.

1.9. Objectives

- To describe characteristics of the *C. auris* outbreak at Chris Hani Baragwanath Academic Hospital (CHBAH) from 2016 through to 2020, with a focus on the neonatal department, using basic epidemiological methods.
- To describe demographic characteristics of neonates/infants with culture-confirmed *C. auris* infection and colonization admitted to the neonatal department at CHBAH.
- To describe *C. auris* isolates submitted through GERMS-SA surveillance from this hospital by phenotype (such as colony characteristics and aggregation) using

microscopic techniques and to determine the antifungal susceptibility using a commercial broth microdilution test and gradient diffusion method.

- To compare genome-wide variation and relatedness of clinical isolates of *C. auris* isolated from infants admitted to the neonatal department (namely, the transitional intensive care unit [TICU], NICU and ward 66) and patients admitted to other hospital wards (paediatric burns unit, adult burns unit, general ICU, etc.) using whole genome sequencing and SNP analysis.
- To determine chain(s) of transmission between neonates admitted to the neonatal department and patients admitted to other wards within the same hospital by using epidemiological information (e.g. dates of admission, ward location, date of positive culture) and genomic data to link cases.
- To determine the molecular mechanisms of antifungal resistance by identifying genes and mutations associated with antifungal resistance for a sub-set of antifungal-resistant isolates.

CHAPTER 2: METHODS AND MATERIALS

2.1. *Candida auris* surveillance and isolate collection

GERMS-SA is a surveillance project for infections of importance to the public health of South Africa, and is conducted by the National Institute for Communicable Diseases (NICD). Enhanced laboratory-based surveillance for *Candida* bloodstream infections was initiated in 2012 and ended in 2017. The case definition for the 2012-2017 candidaemia surveillance was any patient with a culture-confirmed *Candida* species from blood only. From 2012-2013, only enhanced surveillance sites from the Gauteng and Western Cape provinces were included. In 2014-2015, enhanced surveillance sites in provinces other than Gauteng and Western Cape were included. In 2016-2017, 25 enhanced surveillance sites in both private and public sectors across the country were included. Thus enhanced laboratory surveillance for candidaemia was conducted at CHBAH located in Gauteng Province in 2012, 2013, 2016 and 2017.

From August 2018 onwards, *C. auris*-specific surveillance was initiated due to the high incidence of *C. auris* infections during 2016-2017. The case definition for the *C. auris* surveillance was any patient admitted to any healthcare facility in South Africa where *C. auris* was cultured from any specimen type. For this surveillance programme, all private and public sector laboratories submitted isolates from all specimen types obtained from all patients with *C. auris* infection or colonisation. Chris Hani Baragwanath Academic Hospital participated in this surveillance between 2018 and 2020.

Isolates, from cases meeting the above specified definitions, were received on Dorset transport medium or agar plates (Diagnostic Media Products, NHLS, Johannesburg, South Africa) by the Mycology Reference Laboratory at the NICD. The isolates were cultured on

chromogenic agar (CHROMagar Candida, France) followed by identification using MALDI-TOF (Bruker Daltonics; Bremen, Germany) and antifungal susceptibility testing was performed and thereafter the isolates were stored in a -70°C freezer.

2.2 Description of the outbreak

During 2019, an outbreak of *C. auris* infections occurred in the neonatal department at CHBAH. The hospital is the third largest in the world and the largest in Africa. It occupies 173 acres and has approximately 3200 beds. The neonatal department has 185 beds with multiple sections including the 18-bed NICU, a 42-bed TICU, a six-bed surgical unit and other high care and low care sections. The first case of the outbreak occurred on the 12th of June 2019. In this study, *C. auris* isolates from all neonates admitted to the neonatal ward during the outbreak period (12 June 2019 – 30 May 2020) were included, as well as patients that were admitted to other wards in the hospital from March 2016 – July 2020. Based on the GERMS-SA candidaemia surveillance data (2012-2017), the first case of *C. auris* was identified in 2012 at CHBAH. According to a preliminary analysis of the GERMS-SA database, a total of 287 cases (207 with viable isolates) was reported from CHBAH between March 2016 and July 2020. These cases occurred in the adult and paediatric burns units, the neonatal department (NICU, TICU, wards 40, 66 & 67), surgical wards (wards 1, 8, 21 & H4), intensive care unit (ICU), outpatient clinics, internal medicine wards (ward G3), and neonatal theatre. Specimen types included blood, skin swabs, central venous catheter (CVC) tips and wound/burn swabs.

2.3 Case definitions for this study

C. auris cases included in this study were defined as any patient admitted to or seen at CHBAH from whom *C. auris* was cultured from any specimen type at the local National

Health Laboratory Service (NHLS) microbiology laboratory. Cases with multiple isolates were removed so that each patient was represented by one unique isolate.

2.4 Descriptive analysis of the cases of *C. auris*

Demographic and clinical data such as age, sex, ward location and site of *Candida* infection were sourced from the GERMS-SA database for 2016-2017. Demographic information for cases diagnosed in 2018-2020 was collected from the NHLS Trakcare database. GERMS-SA surveillance officers collected data for each case during the enhanced surveillance periods by completing a case report form (CRF) (Attached in Appendix A). Cases were described by age, sex and ward location. Patients were classified according to the following age groups: neonates, infants, children, adolescents, adults and those for whom age was unknown. All *C. auris* cases were plotted on an epidemic curve, categorised by invasive disease or colonisation.

2.5 Culturing of the isolates

Isolates were retrieved from the -70˚C freezer and cultured on Sabouraud agar (Diagnostic Media Products [DMP], NHLS, Johannesburg, South Africa) to confirm viability and thereafter cultured onto chromogenic agar (DMP, NHLS, Johannesburg, South Africa) (chromogenic powder sourced from MAST ID CHROMagar Candida; Mast Diagnostics, Amiens, France) to confirm purity. All *C. auris* isolates were identified by MALDI-TOF (Bruker Daltonics, Bremen, Germany) using the formic acid/acetonitrile extraction method (Lee *et al.*, 2017) before further tests were performed.

2.6 Psuedohyphae production

Pseudohyphae production of individual isolates were evaluated for this study through the Dalmau culture method (Szekely, Borman and Johnson, 2019). Individual isolates were streaked onto cornmeal agar plates (DMP, NHLS) and further incubation of the streak under

a sterile coverslip at 30°C for 48 h. Slides were examined under the microscope (400X) for any filamentation. *Candida albicans* (American Type Culture Collection (ATCC) 90028) was used as a positive control for pseudohyphae formation and *Candida glabrata* (ATCC 2950) was a negative control.

2.7 Aggregation

Aggregate formation was evaluated microscopically for this study by emulsifying a single *C. auris* colony in approximately 20 µl of sterile water on a microscope slide and microscopically examining the resultant emulsion at 400X magnification across the entire slide (Szekely, Borman and Johnson, 2019). Aggregation was scored as "none" (individual budding yeast cells), "moderate" (some yeast cells being present in small aggregates), or "extensive" (most cells being present as aggregates) (Szekely, Borman and Johnson, 2019). A known aggregating strain *C. auris* and a known non-aggregating *C. auris* strain were used as controls for this experiment: aggregating strains Col 215 and Col 222 from California and Santa Marta respectively and non-aggregating Col 54 from Popayan (all obtained from Hackensack Meridian Health Center for Discovery and Innovation, New Jersey, USA).

2.8 Thermotolerance

Thermotolerance was investigated for this study by sub-culturing isolates onto Sabouraud agar plates (DMP, NHLS) and assessing rate of growth after 24-48 h incubation at 35°C, 37°C, 40°C and 42°C (Ben-Ami *et al.*, 2017).

2.9 Antifungal susceptibility testing

Antifungal susceptibility testing (AST) had been performed by the Mycology Reference Laboratory at CHARM, NICD as part of the surveillance programme at the centre and the results were used in this study. AST's were performed using commercial gradient diffusion and/or broth microdilution methods (BMD). Isolates sub-cultured onto Sabouraud agar had

been tested using the Sensititre YeastOne Y09 method (Thermofisher, Waltham, Massachusetts, US); the following antifungals were tested and tentative breakpoints (µg/mL) for resistance listed in brackets were applied: fluconazole (≥32 µg/mL), caspofungin (≥2 µg/mL), anidulafungin (≥4 µg/mL), micafungin (≥4 µg/mL), amphotericin B (≥2 µg/mL). A gradient diffusion strip (E-test) (bioMerieux. France) was only performed for amphotericin B (≥2 µg/mL) (tentative breakpoints as proposed by the CDC) (Spivak and Hanson, 2018). All experiments included the quality control strains *Candida parapsilosis* (ATCC 22019) and *Candida krusei* (ATCC 6258) (CLSI guidelines M27-A3 and M60). Controls were selected because of the known and definite antifungal breakpoints of these strains. Multidrug resistant isolates were those that have reduced susceptibility to more than one antifungal while pan-resistance referred to reduced susceptibility to antifungals belonging to all three classes (azoles, echinocandins and polyenes).

2.10 DNA extraction, quantification and identification

C. auris isolates confirmed by MALDI-TOF were processed for extraction and quantification of genomic DNA for this study. DNA was extracted using the Zymo Research Quick-DNA Fungal/Bacterial miniprep kit (Zymo Research Corp, USA) using the manufacturer's instructions with exceptions to increase the concentration of the extract DNA. The DNA was extracted directly from fungal yeast cell cultures instead of resuspension of cells in water and 40 µL of elution buffer was used instead of 100 µL.

2.11 Library preparation and Whole Genome Sequencing

Sequencing for the 12.6 Mb sized genome was performed on of 188 samples. Genomic DNA was sent to the Sequencing Core Facility (NICD) for WGS. Genomic libraries were prepared using Nextera DNA Library Preparation (Illumina, San Diego, California, USA) and

sequenced on an Illumina NextSeq platform (Illumina, San Diego, California, USA) to produce paired end reads.

2.12 Single-Nucleotide Polymorphism (SNP) calling and phylogenetic analysis

The quality of read data was assessed using FastQC (v0.11.9, https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) while read filtering and trimming was performed using PRINSEQ (v0.20.4 , http://prinseq.sourceforge.net) (Schmieder and Edwards, 2011). The read data were aligned to a reference genome (B11221, accession number: PRJNA535510– a South African *C. auris* isolate that had been sequenced using PacBio by Lockhart et al., in 2016) using Burrows-Wheeler data transformation algorithm v0.7.5a (BWA) (Li and Durbin, 2009). Variant calling (SNP analysis) was performed using SAMtools v1.3 (Li *et al.*, 2009) through the Northern Arizona SNP Pipeline (NASP) (http://tgennorth.github.io/NASP/install.html) which is a well-published analysis pipeline for *C. auris* genome analysis. (Sahl *et al.*, 2016). Filtering parameters involved removing positions within the genome that had <10x coverage, <90% variant allele calls and those within duplicated regions of the reference (Escandón *et al.*, 2019). A maximum parsimony phylogeny tree was estimated using MEGA software (Version 10.0.5) and bootstrap analysis based on 1000 replicates using the bestSNP alignment produced by the NASP pipeline (Kumar *et al.*, 2008). External sequences representing each clade (I-V) were included in the analysis to confirm the clade assignment of the isolates. Also included in the analysis, was one sequence that was the very first isolate to be reported and sequenced from the hospital in 2009.

2.13 Phylodynamic analyses

A root-to-tip regression plot was created using TempEst v1.5.3 (Rambaut *et al.*, 2016) to quantify the mutation rate and to assess the temporal signal for using the SNP alignment from

the NASP pipeline. Only the clade responsible for the outbreak in the neonatal ward was included in this analysis. A population genomic analysis approach was used to date the emergence of the common ancestor of the outbreak isolates using BEAST v1.8.4 (Drummond and Rambaut, 2007). The Bayesian phylogenies were created by applying a general time reversible (GTR) nucleotide substitution model under a strict molecular clock. A general time reversible (GTR) model was chosen due to its non-specific, general model of phylogenetic inference. The model uses different rates of substitution and as well as frequencies of nucleotides occurrence which is optimum for datasets with a non-linear transmission pattern (Choudhuri, 2014). A maximum clade credibility tree was generated with TreeAnnotator v1.8.4 and visualized using FigTree v1.4.4. The number of SNPs informed the time of introduction into the transmission route by using the calculated evolutionary rate. The isolates with a higher of number of SNPs (compared to the reference) and older collection dates were placed earlier in the transmission timeline and isolates with a lower number of SNPs were considered more recent in the transmission timeline/route. (Chow *et al.*, 2020)

2.14 Transmission routes reconstruction

To reconstruct potential transmission routes in the hospital, an algorithm called SeqTrack was used (Jombart *et al.*, 2011). SeqTrack is within the adegenet package in R, it uses a parsimony phylogeny approach to determine ancestries based on an alignment of SNPs and sampling dates of the isolates to create a schematic diagram of proposed transmission routes between the patients. The data from the isolates was assessed to investigate whether the isolates formed distinct clusters due to epidemiological variables such as year of isolation or ward location with regard to their genetic relationships.

2.15 Resistance mutation identification

Isolates that exhibited high MIC values to any antifungal agent using tentative MIC breakpoints mentioned in section 2.9 were assessed further for antifungal target gene mutations. The CLC Genomics Workbench (version 10, Qiagen, The Netherlands) was used to assess and identify mutations in the resistant *C. auris* isolates. The following genes were extracted from each resistant *C. auris* genome and assessed for any point mutations (i.e. substitutions, duplications etc.): *ERG11*, which is transcribed into an azole target and *FKS1*, an echinocandin target gene (Frias-De-Leon *et al.*, 2020). A strain of known susceptibility was used as a reference in the analysis to determine whether mutations were present (*C. auris* B8441 (Genbank accession PEKT00000000.2, 30559369). Susceptible isolates were also assessed for mutations in the same genes to confirm that these mutations were indeed responsible for reduced susceptibility in the isolates.

CHAPTER 3: RESULTS

3.1 Descriptive epidemiology

3.1.1 Patient demographic information

During the study period (March 2016 – July 2020), a total of 287 laboratory-confirmed cases, 207 (72.1%) of which had viable isolates, were reported from the hospital from different wards. A large proportion of the cases were from the neonatal unit (91/287, 31.7%), the paediatric 66/287 (23%) and adult burns units 57/287 (19.9%). The medical (adult; 11/287, 3.8% and paediatric; 9/287, 3.1%) and surgical (adult; 12/287, 4.2% and paediatric; 7/287, 2.4%) departments recorded the lowest number of cases (Table 3.1).

The median age (IQR) was 1.4 years (22 days – 21 years) with a range of 0 days to 85 years. The adult age group had the highest number of cases (74/287, 25.8%) followed by the neonates (62/287, 21.6%). The age group with the lowest number of cases was the adolescents (11/287, 3.8%). The study population was 54% (155/287) male and 42% (121/287) female while the sex of 4% (11/287) of the patients was unknown (Table 3.1, Figure 3.1). Most isolates were from blood (161/287, 56.1%) or skin swabs (33/287, 11.5%) (Table 3.1).

Table 3.1: Characteristics of patients (n = 287) with Candida auris infection or colonisation admitted to a large metropolitan hospital in Gauteng Province, South Africa, 2016-2020.

Characteristics	**Number of isolates (%)**
Sex	
Male	155 (54)
Female	121 (42.2)
Unknown	11 (3.8)
Age group	
≤ 28 days (Neonates)	62 (21.6)
29 days – 12 months (Infants)	56 (19.5)
1 year – 12 years (Children)	54 (18.8)
13 years – 17 years (Adolescents)	11 (3.8)
18 years – 85 years (Adults)	74 (25.8)
Unknown	30 (10.5)
Specimen type	
Blood	161 (56.1)
Skin swab	33 (11.5)
Arterial catheter tip	28 (9.8)
Central venous catheter tip	24 (8.4)
Urine	11 (3.8)
Tracheal aspirate	8 (2.8)
Tissue, not specified	6 (2.1)
Fluid aspirate, not specified	3 (1)
Skin scraping	1 (0.3)

Burn/wound swab	1 (0.3)
Unknown	11 (3.5)
Ward Location	
Neonatal unit	91 (31.7)
Paediatric burns unit	66 (23)
Adult burns unit	57 (19.9)
General intensive care unit (ICU)	30 (10.5)
Adult surgical	12 (4.2)
Adult medical	11 (3.8)
Paediatric medical	9 (3.1)
Paediatric surgical	7 (2.4)
Unknown	4 (1.4)

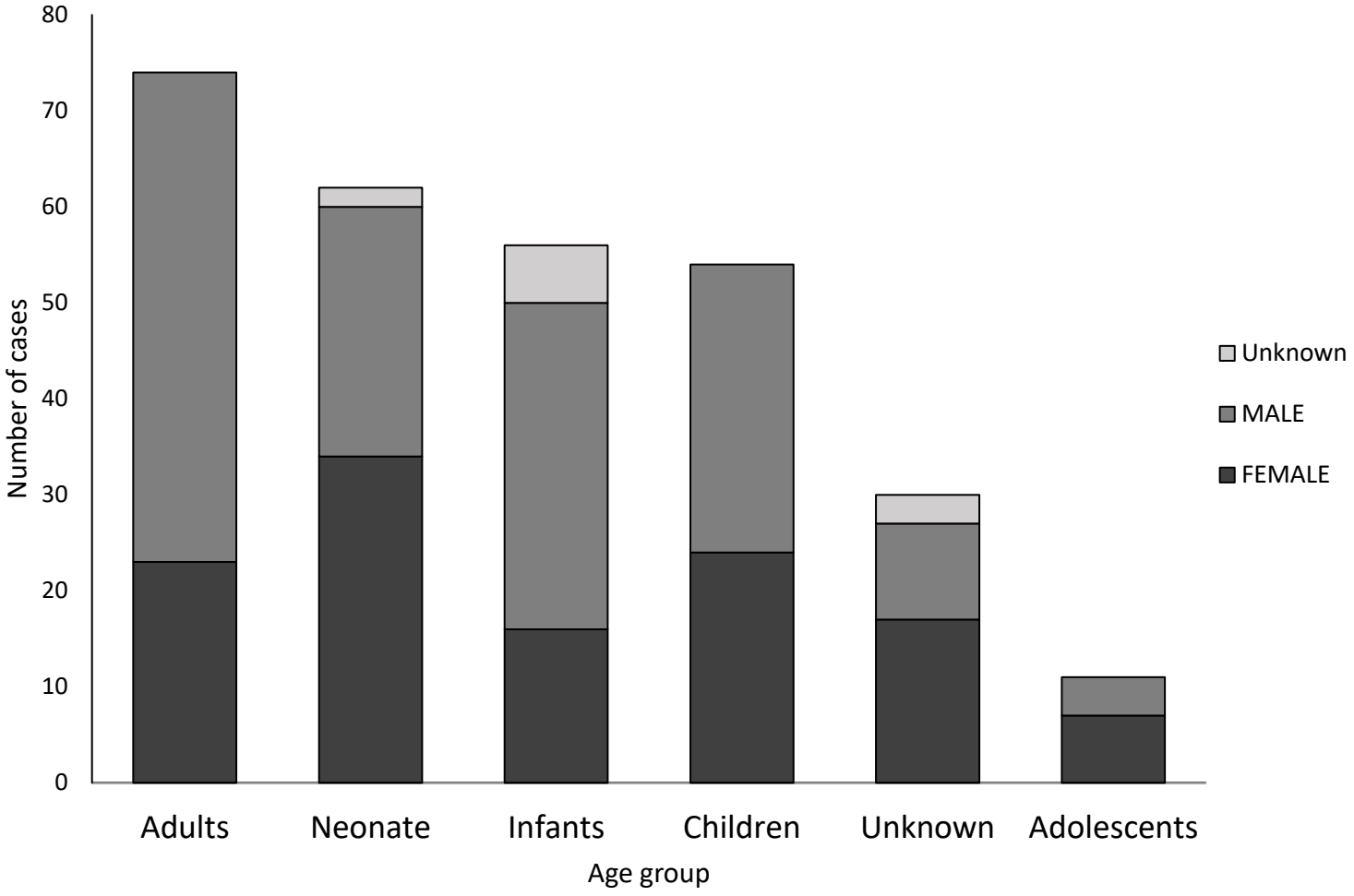


Figure 3.1: Sample distribution by sex and age group, from a hospital in Gauteng Province, South Africa experiencing a Candida auris outbreak, 2016-2020 (n = 287).

3.1.2 Epidemic curve

The epidemic curve shows the distribution of 287 culture-confirmed cases of *C. auris* invasive disease or colonisation admitted to CHBAH (Figures 3.2.1 and 3.2.2). Initial cases of *C. auris* occurred during 2016 in the paediatric burns unit, with a gradual introduction of cases into other wards, including the adult burns unit, the general ICU and the neonatal unit. By the end of 2017, at least one case had been identified in all wards. However, the number of cases remained relatively constant during 2016-2017, with an average of fewer than one case reported during each epidemiological week. The number of cases increased at the beginning of and throughout 2018, with this being due to an increase in cases in the paediatric burns unit. By the end of 2018, the number of cases admitted to the paediatric burns unit had declined with an even more substantial decline in 2019 (Figure 3.2.1).

In 2019, cases admitted to the paediatric burns unit accounted for 4.2% (12/287) of all cases while there was a notable increase in the number of cases occurring in the adult burns unit (21/287, 7.3%) and neonatal unit (28/287, 9.8%). While the number of cases admitted to the adult burns unit began to decline from July 2019, there was then a sustained increase of cases in the neonatal unit throughout 2019 and into 2020. Over the entire period, cases admitted to the neonatal unit accounted for a large proportion of the cases (91/287, 31.7 %). The introduction of *C. auris* in the neonatal unit was in August of 2017 with one case of invasive infection diagnosed. Subsequent to this introduction, only two cases were diagnosed during September 2017 and March 2018, respectively. The current outbreak in the neonatal unit began in July 2019 with at least two cases reported from the neonatal unit per week. The peak of the neonatal outbreak occurred in February 2020 with a total of nine cases in a single week (7th epidemiological week of 2020) (Figure 3.2.1).

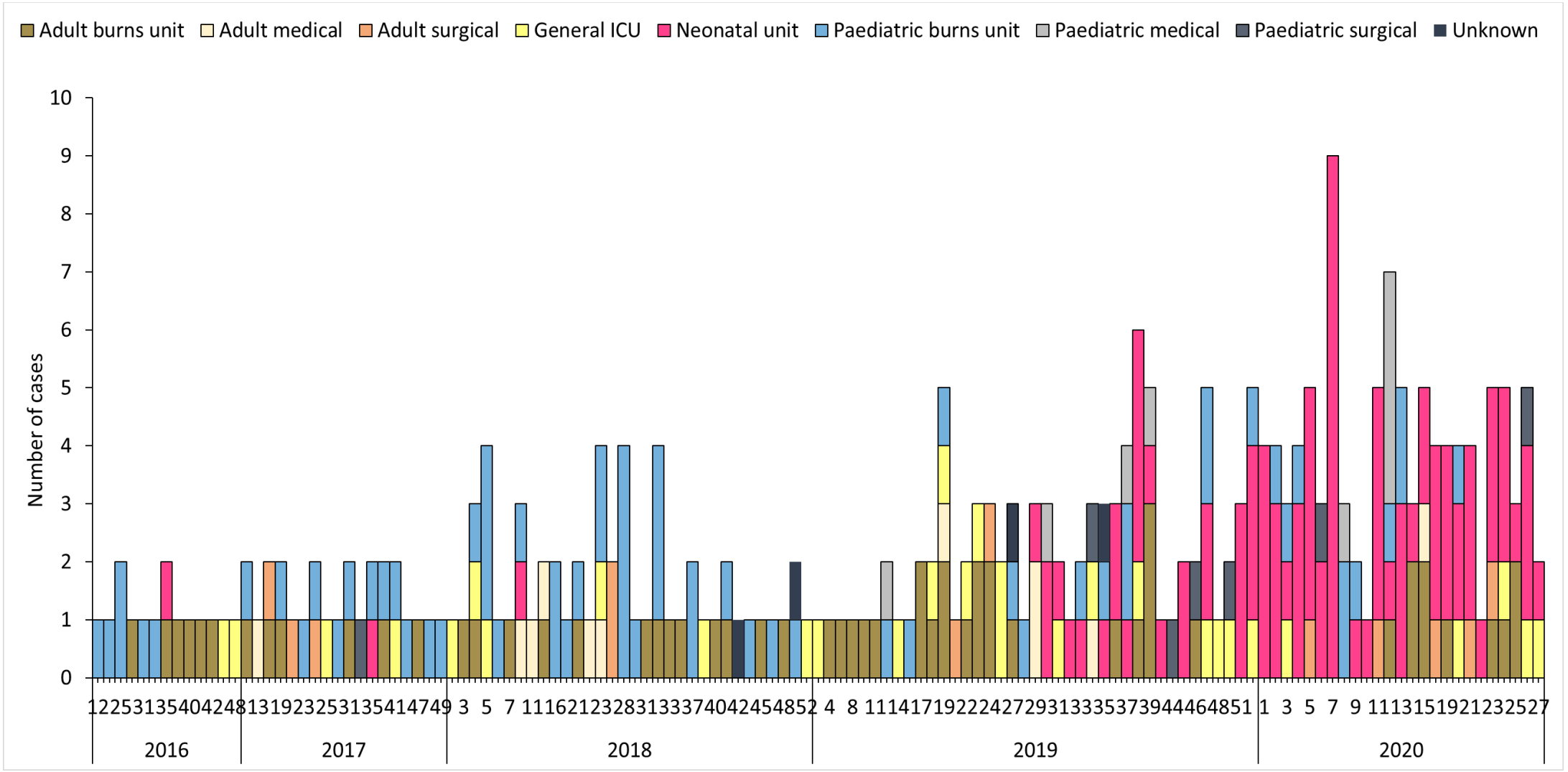


Figure 3.2.1: Epidemic curve by ward location of laboratory-confirmed cases of Candida auris infection or colonisation at an academic tertiary hospital in Gauteng, South Africa from March 2016 to July 2020, n= 287.

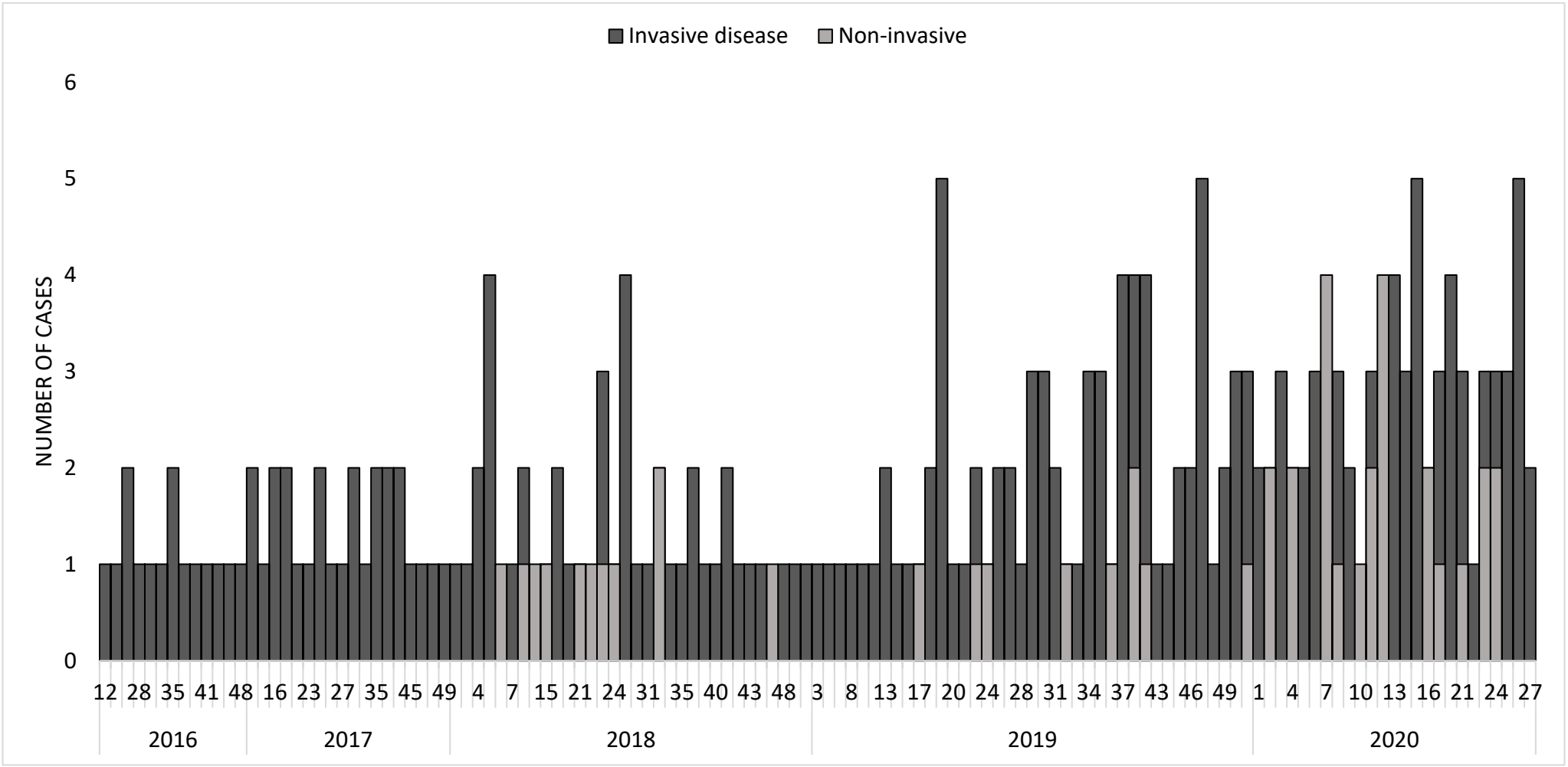


Figure 3.2.2: Epidemic curve of laboratory-confirmed cases of Candida auris infection or colonisation at an academic tertiary hospital in Gauteng, South Africa from March 2016 to July 2020, n= 287.

3.2 Phenotypic characterization

3.2.1 Antifungal susceptibility

The results of the antifungal susceptibility testing for ten antifungal agents are shown in Table 3.2. The MIC values were categorised according to the tentative antifungal breakpoints proposed by the CDC (https://www.cdc.gov/fungal/candida-auris/c-auris-antifungal.html) The majority (199/207; 96.1 %) of the isolates were found to be resistant to fluconazole (MIC ≥32 µg/mL) and only 6.2% (13/207) of the isolates were resistant to amphotericin (≥2 µg/mL).

Multidrug resistant isolates were also present. There were two isolates resistant to both caspofungin and fluconazole and three isolates resistant to both fluconazole and amphotericin B. No isolates in this study were pan-resistant.

Table 3.2: Minimum inhibitory concentration (MIC) distribution of Candida auris isolates (n = 207) from patients admitted to a tertiary hospital in Gauteng, South Africa from March 2016 to July 2020. MICs were determined using broth microdilution and E-test method for amphotericin B.

Antifungal	Breakpoint (µg/mL)	Number of isolates with the following MIC (µg/mL):																	MIC50	MIC90	Range	% resistant
		0.008	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128	256	unk				
Anidulafungin	≥4		1		28	129	42	7											0,12	0,25	0.015-0.5	0
Micafungin	≥4			2	96	94	12	1	2										0,12	0,12	0.03-1	0
Caspofungin	≥2			3	69	99	31	2	1			2							0.12	0.25	0.03-8	0.97
Flucytosine	≥128	3			67	125	10	2											0,12	0,12	0.008-0.5	0
Posaconazole	N/A	1	1	15	73	80	31	4	2										0,12	0,25	0.008-1	N/A
Voriconazole	N/A				2	8	21	58	103	13	2								1	1	0.06-4	N/A
Itraconazole	N/A			2	10	84	84	25	2										0,25	0,5	0.03-1	N/A
Fluconazole	≥32								1				7	13	34	73	79		128	256	1-256	96.1
Amphotericin B (E-test)	≥2		1	1	2	9	22	76	79	11	2							4	0,5	1	0.002-3	6.2

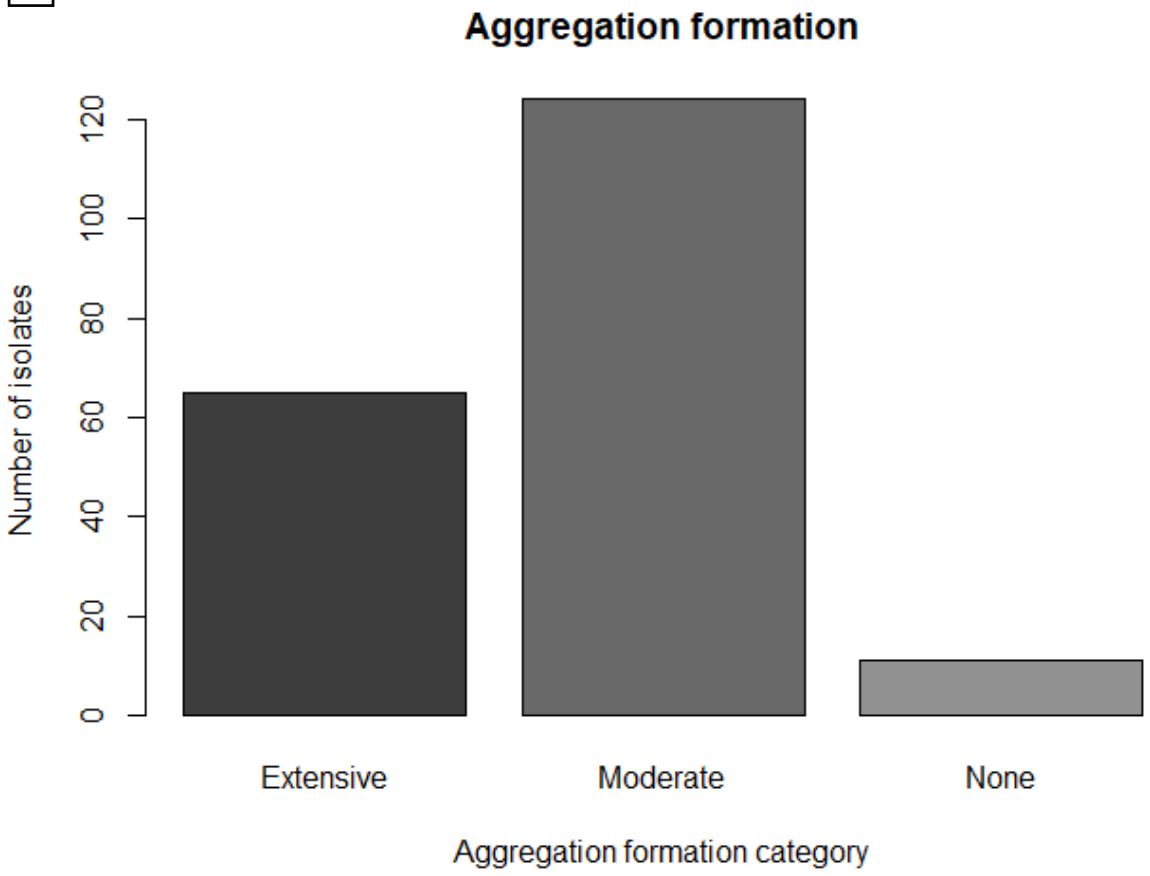
3.2.2 Aggregate formation, pseudohyphae production and thermotolerance

Of 207 viable isolates in this study, five were missing from storage while two were contaminated; therefore, only 200 were available for further experimentation for the purposes of this study.

Most isolates involved in this study had aggregate-forming capabilities (Figure 3, A). The majority of the isolates showed moderate aggregation, 124/200 (62%) (Figure 3, B2) while 32.5% (65/200) had extensive aggregation of the cells (Figure 3, B1) and only 5.5 % (add n/N) had no aggregating capability (Figure 3, B3).

Only three isolates in the study produced pseudohyphal filaments (Figure 3, C). All isolates grew at 37°C, 40°C and 42°C.

A


Aggregation formation
Number of isolates
0
20
40
60
80
100
120
Extensive
Moderate
None
Aggregation formation category

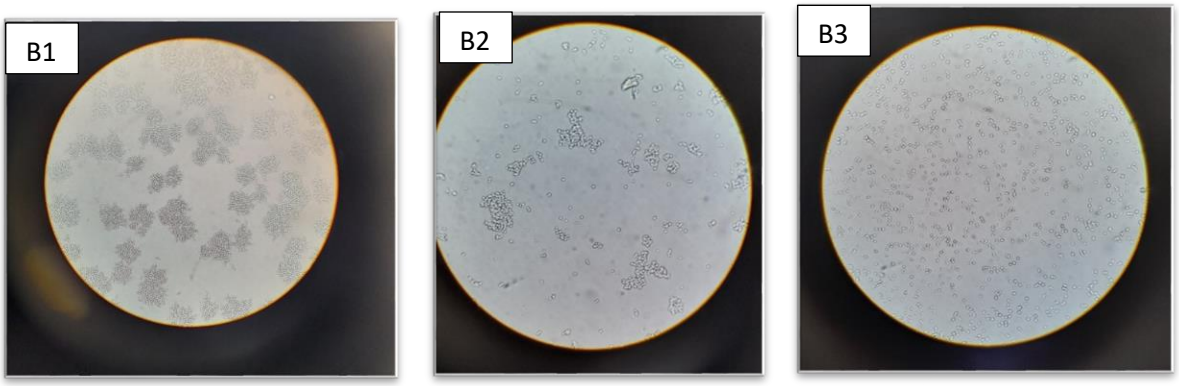

B1
B2
B3

Figure 3.3: Aggregation formation. ***A****, number of isolates with aggregate forming capabilities.* ***B****. Aggregation formation category (B1 - Extensive, B2 - Moderate and B3 - none).* ***C.*** *Candida auris growth under Dalmau culture.* ***C1****: An isolate that does not produce pseudohyphae,* ***C2****: An isolate that produces pseudohyphae*

3.3 Molecular characterization

3.3.1 Phylogenetic analysis

From March 2016 – July 2020, 207 viable *C. auris* isolates were collected from CHBAH. Of these, only 200 were available for WGS. Twelve sequences were of poor quality and were excluded from the analysis; hence the WGS data of 188 isolates was available for bioinformatics analysis. Overall, 63.1% (118/188) clustered with the clade III reference and therefore belonged to the African clade III and only 36.9% (70/188) sequences clustered with the clade IV reference (South American clade) (Table 3.3). Isolates in this study did not cluster in any other clade (Figure 3.4).

As mentioned, the majority of the isolates (181/188, 96.3%) were resistant to fluconazole and had an MIC value greater than 32 mg/L and the majority (186/188, 98,9%) had mutations in the *ERG11* gene that were known to contribute to resistance (five susceptible sequences also housed resistant mutations on the *ERG11* gene). Isolates for clade III had the VF125AL substitution that is specific to the clade. Isolates from clade IV had the following substitutions: K177R/N335S/E343D, which were documented in this clade previously. There were cases of susceptible isolates that possessed these substitutions especially in clade IV (one clade III isolate and four clade IV isolates). Some isolates with the above mentioned clade IV substitutions had an additional E102K substitution (27/70, 38 %). This substitution has not been documented previously or proven to contribute to decreased susceptibility in *C. auris* strains.

The neonatal ward, the main area of the outbreak from 2019 onwards, was dominated by clade III isolates while clade IV strains dominated the adult burns unit and paediatric burns unit and there was also some mixing of the clades in the burns units (Figure 3.5). Isolates from the neonatal ward constituted 36.8% (68/188) of the isolates in our phylogenetic tree. Of those, over half (53.6 %, 39/68) of the isolates were isolated from blood (38 belonged to

clade III and only one to clade IV) and skin swabs were the second most common specimen type in the neonatal ward (22%, 22/68). Bloodstream infections were also prominent in the adult burns unit (25/35, 75%) and paediatric burns unit (20/39, 51%) (Figure 3.6).

Table 3.3: Clade proportions and frequency of antifungal drug resistance within the study population among 188 South African Candida auris isolates (n (%)).

Clade	Number of isolates	Fluconazole resistant	Point mutation in *ERG11*	Point mutation in *FKS1*	Amphotericin B resistant
III	118 (62.5)	117 (99.1)	118 (100)	None	2 (2.8)
IV	70 (37.4)	64 (91.4)	68 (97.8)	None	1 (1.42)

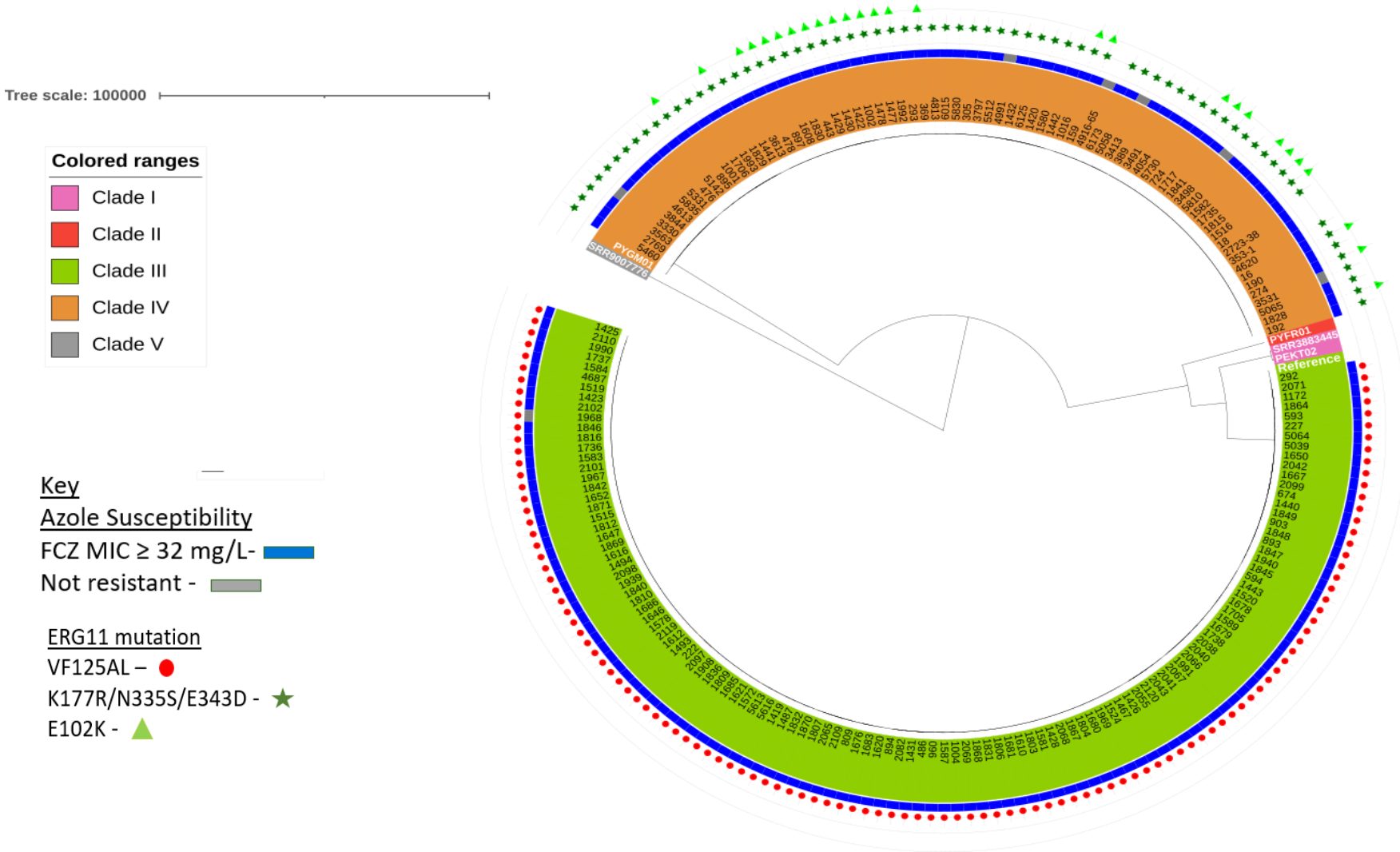


Figure 3.4: Phylogenetic tree depicting clade distribution and fluconazole resistance mutations of 188 invasive or colonising South African Candida auris strains isolated from patients admitted to a large metropolitan hospital in Gauteng, 2016-2020. The unrooted maximum parsimony tree was created using MEGA software using 287 338 single nucleotide polymorphisms (SNPs) based on 1000 bootstrap replicates. The tree scale represents SNP differences. FCZ = Fluconazole.

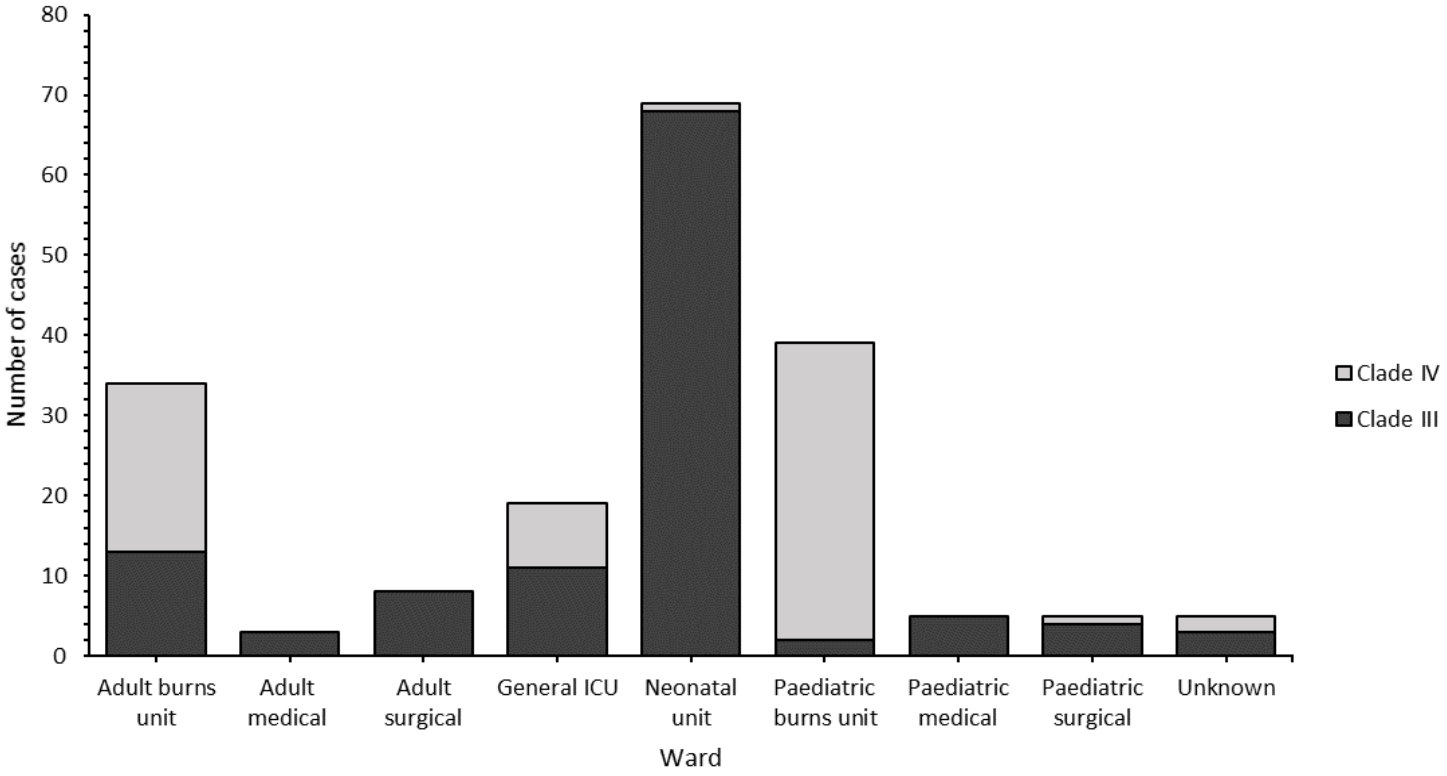


Figure 3.5: Clade distribution of 188 South African Candida auris isolates from patients admitted to a large metropolitan hospital in Gauteng, 2016-2020 classified by the patients' ward location.

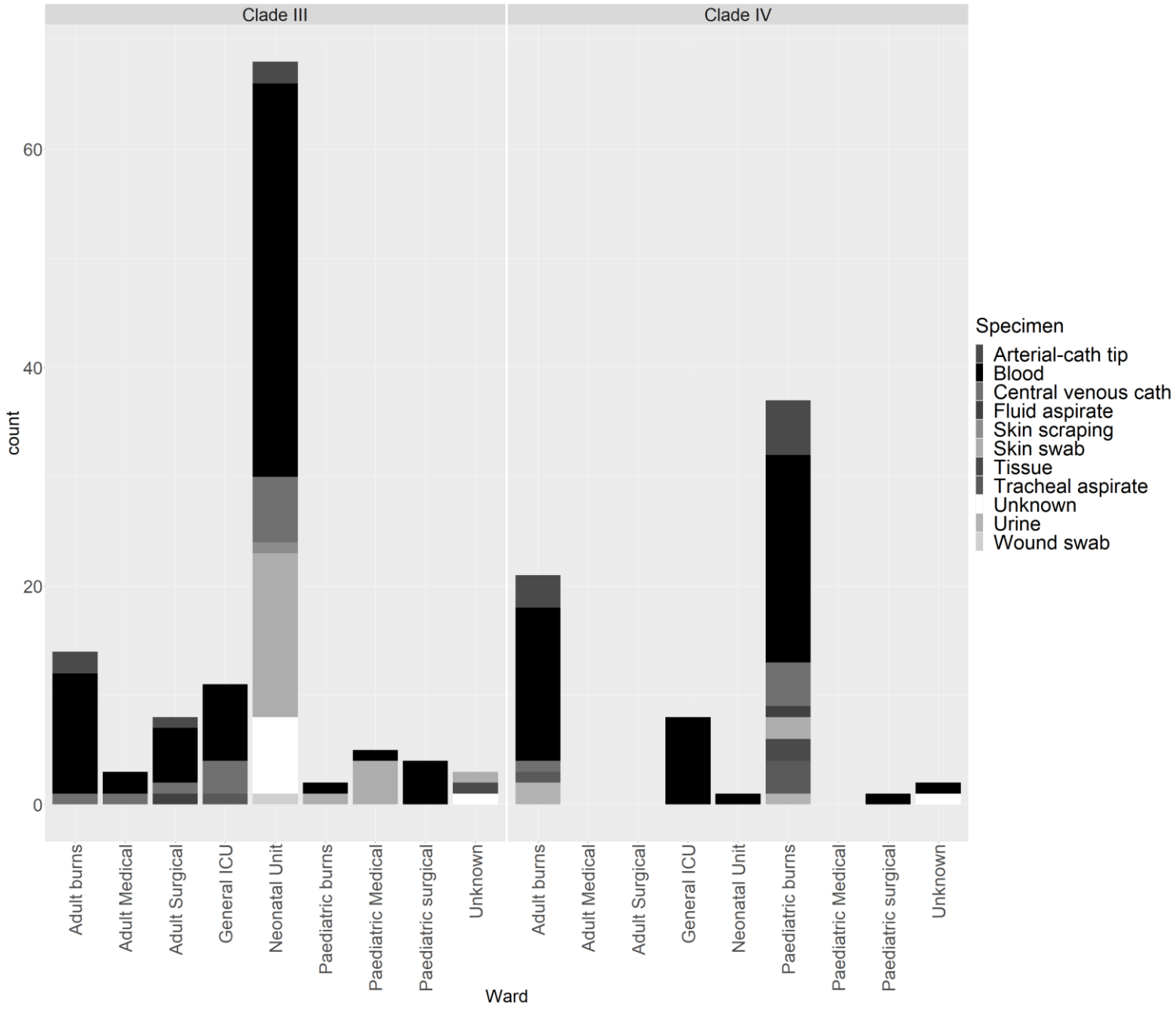


Figure 3.6: Specimen types by ward classified by clade of 188 South African Candida auris isolates from patients admitted to a large metropolitan hospital in Gauteng, 2016-2020

3.3.2 Phylodynamic analysis

The first *C. auris* isolated in South Africa, was isolated from CHBAH in 2009 from the adult burns unit and belonged to Clade IV (Naicker *et al.*, 2021) while the first case belonging to Clade III in the hospital was isolated on the 30th March 2017. *C. auris* was first introduced to the neonatal ward in August of 2017 (Figure 3.7).

To better understand the introduction/emergence of *C. auris* in the neonatal ward, we used sampling dates as well the single nucleotide polymorphism (SNP) alignment to estimate divergence times. The rate of evolution, which is defined as the number of substitutions per polymorpic site per year, was estimated using TempEst v1.5.1. A root-to-tip regression plot (Figure 3.8) was created to determine whether there was a linear relationship/positive correlation between the time the isolates were sampled and the number of substitutions along the tree topology. Clade III sequences from the neonatal ward were used for this analysis. The relationship between genetic distances/nucleotide substitution along the tree and sampling dates was sufficient (Correlation coefficient = 0.5), the data set demonstrated a clock-like/timed evolution across the outbreak timescale and therefore had a sensible temporal signal for further bayesian molecular dating. The evolutionary rate, which is the gradient of the regression plot, equated to 1.3471e-5 substitutions per polymorphic site per year. The root-to-regression plot predicted that the emergence of the most recent ancestor was in 2018 (X-intecept) (Figure 3.8).

Molecular clock analysis of a phylogeny was perfomed using a Bayesian strict clock coalescent model and a maximum clade credibility tree was created. The estimated emergence time for the most common recent ancestor (TMRCA) for Clade III in this hospital dated back to early 2014 (95% highest posterior density, 2013 – mid 2015) while the TMRCA for the outbreak emerged near 2018 (95% highest posterior density, mid 2017 and mid 2018), corresponding to the

estimation from the root-to-regression analysis (Figure 3.9). The ancestor for clade III was most probably introduced into the hospital through an external source.

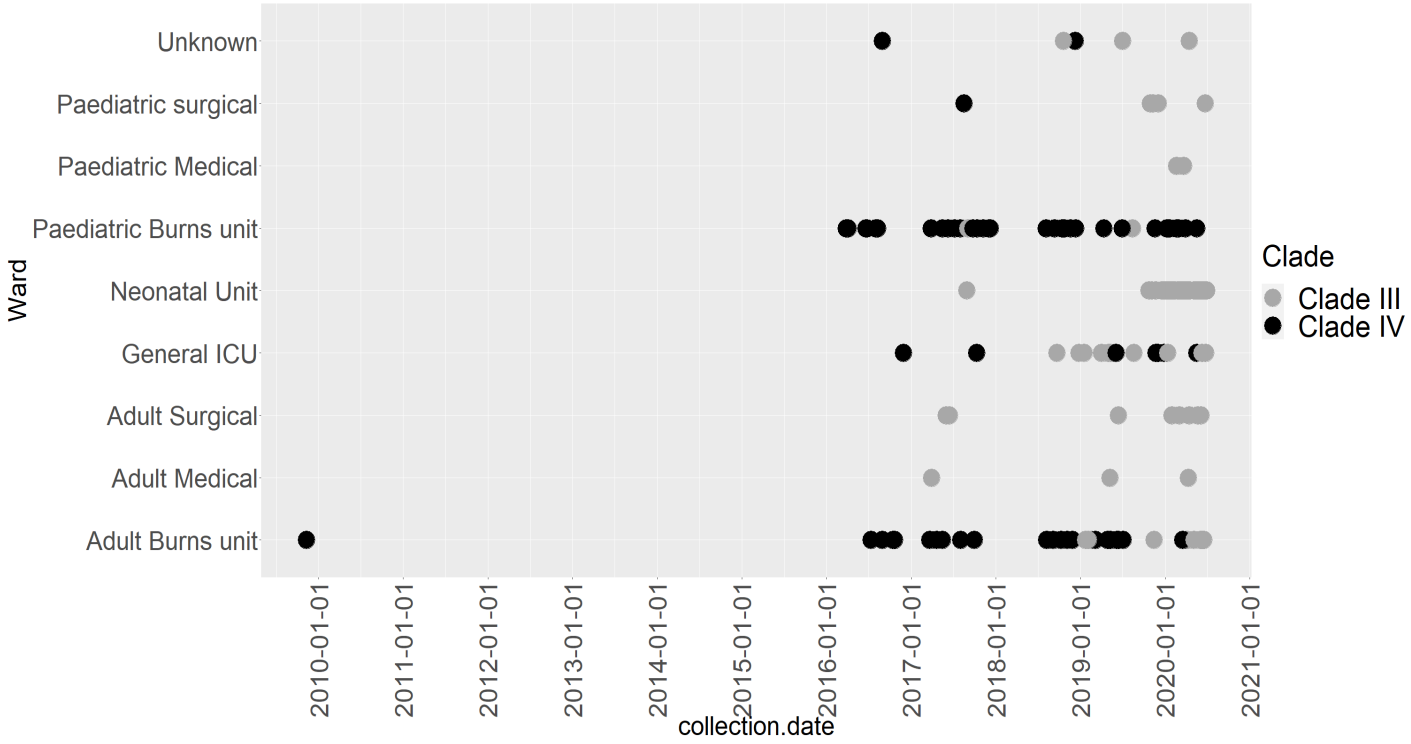


Figure 3.7: Distribution of collection dates for 188 South African Candida auris isolates by ward location and clades.

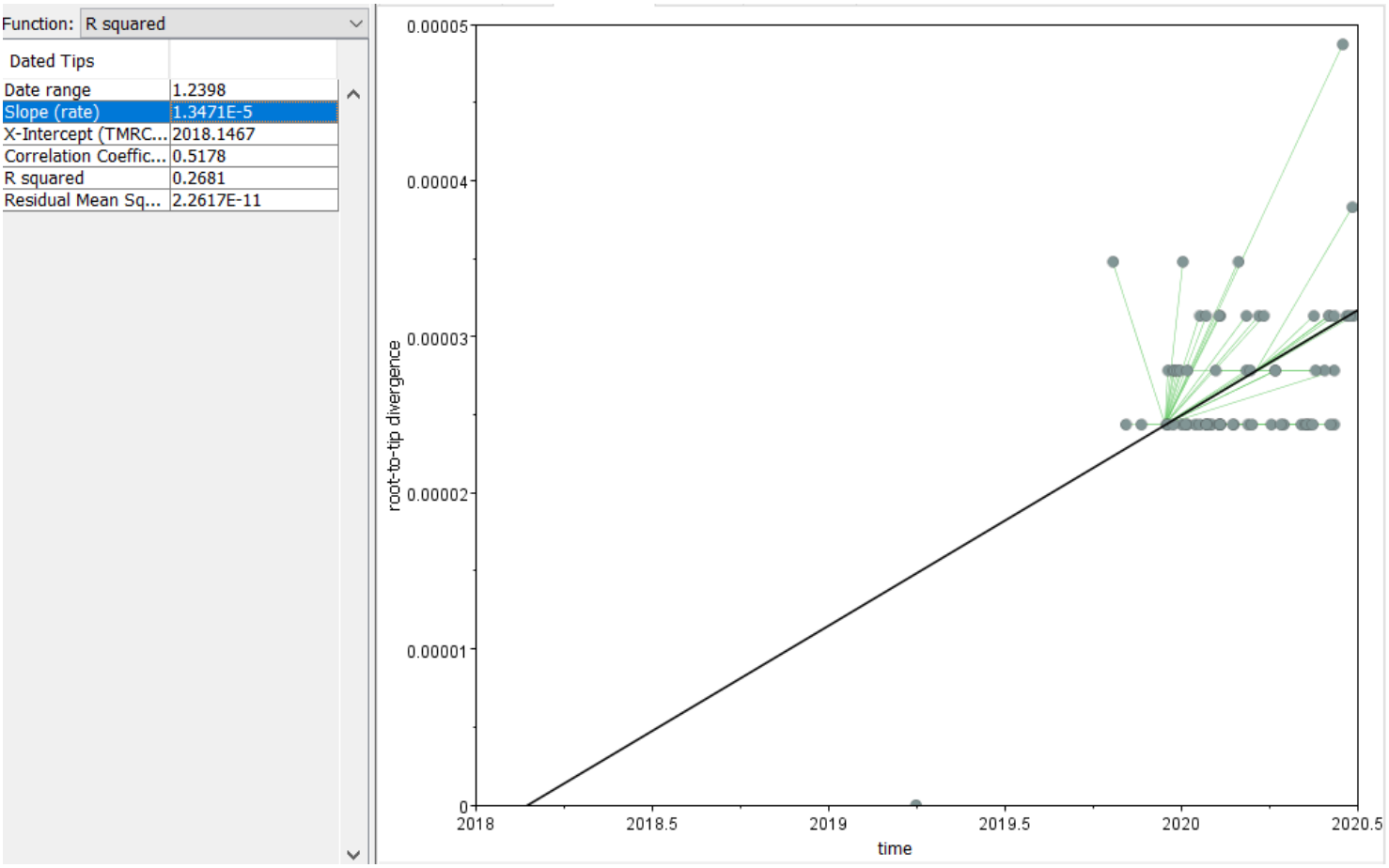


Figure 3.8: Root-to-tip regression analysis of 67 South African Candida auris outbreak isolates from the neonatal ward of a large metropolitan hospital in Gauteng. Genetic distance is plotted against sampling time and every data point represents a tip on the phylogeny. The R squared for the regression and the slope, reflecting the evolutionary rate (in substitutions per site per day) is also shown.

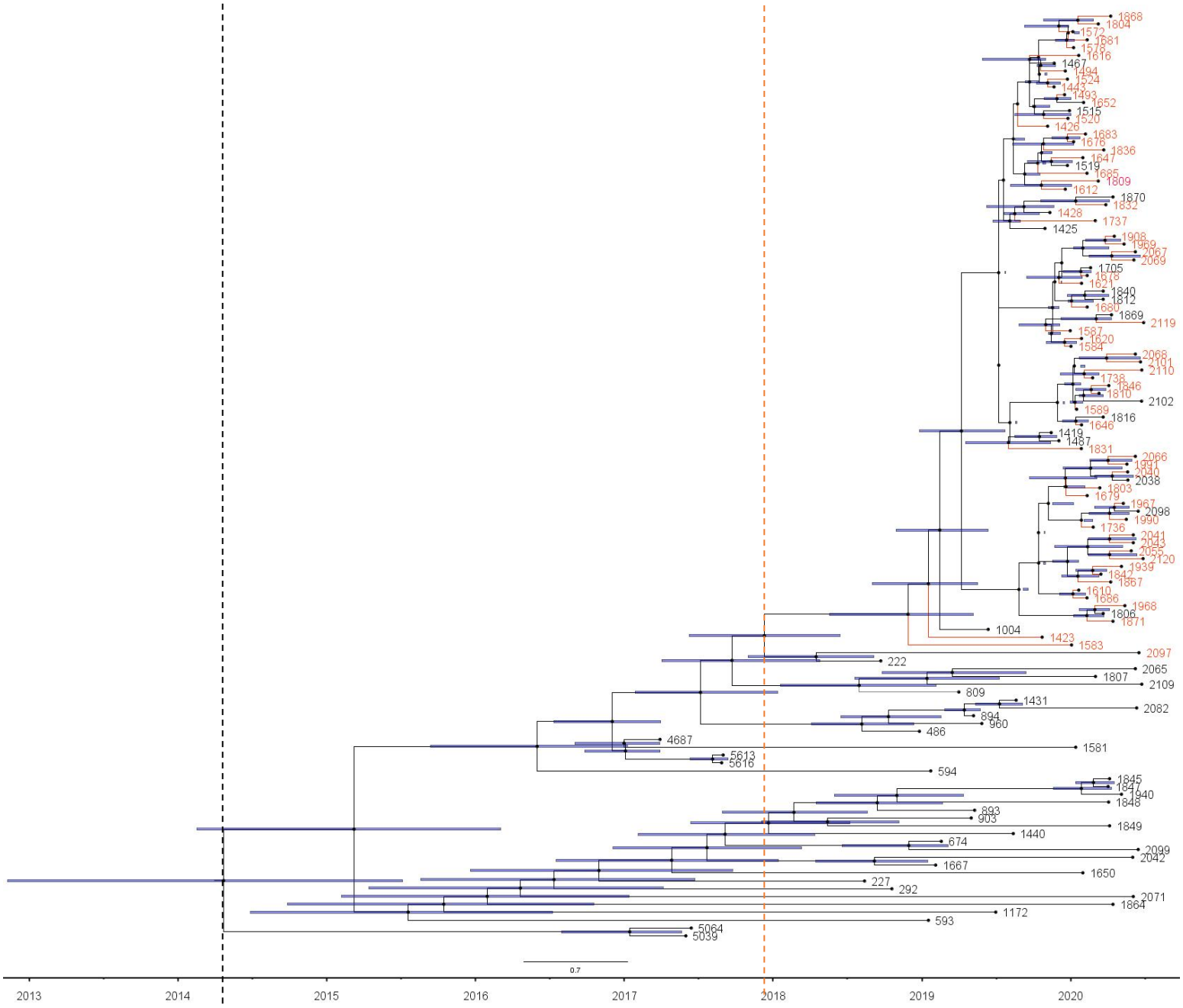


Figure 3.9: Maximum clade credibility tree of 67 South African Clade III Candida auris isolates from this study estimated using BEAST (Strict clock and coalescent model). Red tips represent cases from the neonatal ward. Blue bars represent 95% highest probability density. Tree scale represents branch length. Black dashed line = Clade III TMRCA, Red dashed line = Outbreak strain TMRCA.

3.3.2 Outbreak reconstruction for clade III

The potential infection chains or transmission route of *C. auris* clade in the hospital were determined using SeqTrack. Only isolates from clade III were included in this analysis, which is the clade responsible for the outbreak in the neonatal ward. This analysis allowed us to track clade III strains across the hospital wards and introduction into the neonatal ward.

There were multiple introductions of *C. auris* into the neonatal ward (Figure 3.10), which also supports the non-linear root-to-tip-regression plot of the clade III isolates in Figure 3.8. Case 1 (Isolate 4867 on the phylogenetic trees) was the index case of this tree and was isolated from a patient in an adult medical ward. Two transmission events occurred from Case 1 that gave rise to two clusters. The first cluster begins with Case 37 (1172) from an unknown ward. This cluster contained 18 cases with eight infections in the neonatal ward. The second cluster from Case 1 begins with Case 6 (227) from the adult burns unit. This cluster contained infections from the neonatal ward outbreak. Case 18 (594) seems to be the index case/ancestral case for most infections involved in this outbreak. From this ancestor, the weight is as low as zero in most branches, which indicates rapid transmission of the pathogen between the cases. This is contrary to the high number of mutations between the cases earlier in the transmission tree; these cases were sporadic and are further apart in their sampling dates. Overall, infections were introduced into the neonatal unit from the adult burns unit, adult medical department and an unknown ward.

The Bayesian analysis using Beast estimated that the TMRCA of the outbreak strain occurred in 2018. This estimation was supported by the date of isolation for Case 6 (227) and Case 10 (292), ancestors of the neonatal ward outbreak, which were both isolated in 2018.

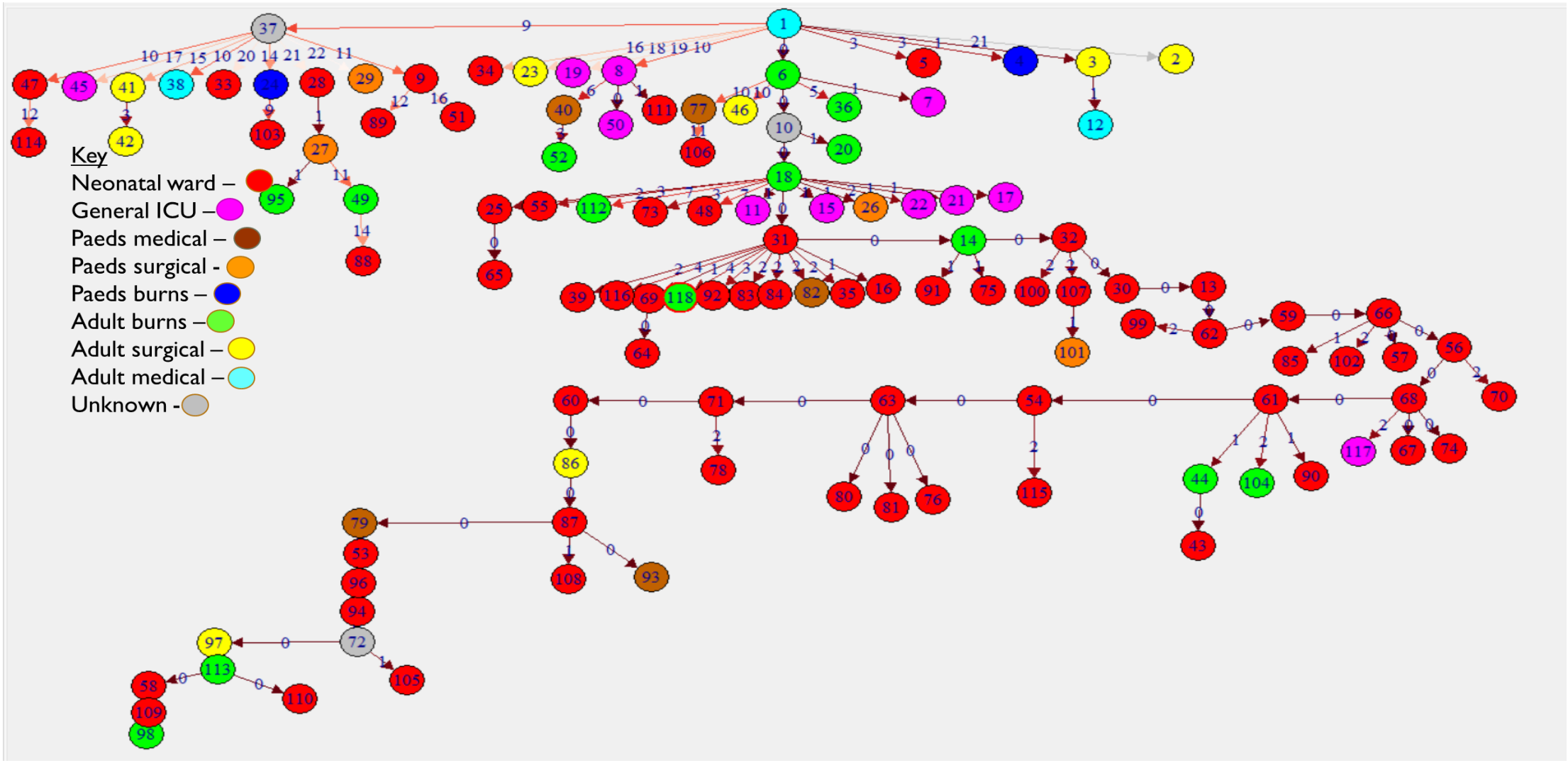


Figure 3.10: Reconstructed transmission tree of 118 South African Clade III Candida auris isolates from this study. Each case is represented by a node on the tree and arrows indicate plausible ancestries/transmissions. Mutations between the nodes are indicated by the colour arrows (red = no/few mutations; light grey= many mutations) and the numbers in blue represent the weight of mutations when compared to the ancestries. Time is represented by y axis (up: older; down: younger).

CHAPTER 4: DISCUSSION

Candida auris causes severe illness in patients admitted to healthcare facilities but has been responsible for few reported neonatal outbreaks worldwide since its emergence. In this study, we describe the molecular and phenotypic characteristics of *C. auris* isolates from a hospital in Gauteng Province, South Africa which had a large outbreak in the neonatal ward from July 2019 to July 2020. The study involved WGS and epidemiological data to link cases from the outbreak and construct possible transmission routes of cases within the hospital. A large proportion of the cases were reported from the neonatal unit during the study period. One hundred and eighteen of 188 sequenced isolates from patients across the hospital were classified as clade III; this clade was also responsible for the neonatal unit outbreak. In addition, the majority of isolates were resistant to fluconazole and carried clade-specific mutations on the target gene for fluconazole.

By 2016, *C. auris* was reported in over 100 South African hospitals and caused outbreaks in some of these hospitals. The pathogen has replaced other *Candida* pathogens and was the third most common cause of candidaemia in South Africa behind *C. albicans* and *C. parapsilosis* (Van Schalkwyk *et al.*, 2019). Over the entire study period (March 2016 – July 2020), cases admitted to the neonatal unit accounted for a large proportion of the cases. *C. auris* outbreaks involving neonates have been reported previously in other hospitals in South Africa (Van Schalkwyk *et al.*, 2019). The true prevalence of *C. auris* is unknown in some parts of South Africa, especially in resource-limited rural settings. This is due to the need for accurate identification of the pathogen which ideally requires molecular identification through PCR or expensive machines such as MALDI-TOF mass spectrometry (Kathuria *et al.*, 2015).

Neonatal *C. auris* infections have been reported in other parts of the world as well. A tertiary care NICU in India reported 17 neonates who developed a *C. auris* infection; the risk factors for neonates in the cohort included low birthweight, treatment with broad-spectrum antibiotics and presence of central lines (Chandramati *et al.*, 2020). Alvarado-Socarras et al. (2021) reported a cluster of eight neonatal *C. auris* infections in Colombia with similar risk factors (Alvarado-Socarras *et al.*, 2021). Most cases in our study had candidaemia. Symptoms of *C. auris* bloodstream infection are non-specific in neonates which further complicates diagnosis (Chandramati *et al.*, 2020).

Most isolates in our study belonged to clade III, the African clade (63%, 118/188) or clade IV (37%, 70/188), the South American clade. Molecular epidemiology of *C. auris* in South Africa was determined in a previous WGS study from 2009-2018, which suggested that the majority (85%) of isolates circulating within the country belong to clade III, with 12% belonging to clade I and only 3% belonging to clade IV (Naicker *et al.*, 2021). However, the clade IV contributed to a proportionally larger case load in this hospital compared to the overall proportion of clade IV isolates in the national study (Naicker *et al.*, 2021). An earlier global molecular epidemiology study reported 51 isolates that only belonged to clade III in South Africa (Chow *et al.*, 2020) while, interestingly, the first reported isolate in South Africa in 2009 belonged to clade IV (Naicker *et al.*, 2021). These observations from previous studies might have been due to sampling bias, a small sample size or a rapid clonal expansion of clade IV in the hospital (CHBAH) that might have occurred between 2018 and 2020.

Clade III strains were isolated in countries including the United Kingdom, Australia and Spain while clade IV was found in Colombia, Israel, Panama and Venezuela (Chow *et al.*, 2020). Multiple *C. auris* clades in South Africa may be explained by the continuously increasing global travel. Furthermore, phylogeographic mixing of *C. auris* clades has been observed more often in recent years. Countries including Canada (containing clades I, II and III), Kenya (clades I and III) and the United States (clades I, II, III and IV) had isolates

corresponding to multiple *C. auris* clades. A genomic epidemiology study conducted in a hospital in Shanyang City, Shaanxi Province, China reported a total of 93 *C. auris* isolates that all belonged to the African clade III; according to their analysis, the ancestor of these isolates was hypothesised to have been imported into the hospital from the United Kingdom (Tian *et al.*, 2021) Outbreaks of clade III infection have also been reported in the USA and the UK (Borman and Johnson, 2020; Price *et al.*, 2020). In Kenya, clades III and IV circulated in the same healthcare facility (Chow *et al.*, 2020) similar to what we observed in our single-hospital study.

In agreement with previous studies, most isolates in our study were resistant to fluconazole, an antifungal of the azole class. Fluconazole-susceptible isolates of *C. auris* are very rare worldwide especially within the African clade III which limits the treatment options (Naicker *et al.*, 2021). Fluconazole-resistant *C. auris* as a cause of healthcare-associated infections is an added burden to public health since fluconazole is the least expensive and most accessible antifungal especially in rural/resource-limited settings (Govender *et al.*, 2019; Miot *et al.*, 2021). *C. auris* has varying susceptibility to the other antifungals in the azole class and therefore these azoles could be an alternative, if susceptibility for these drugs has been determined, although cross resistance can occur between antifungals of the same class (Panackal *et al.*, 2006). However, side effects including periostitis, myositis, hyponatremia and hypokalemia due to voriconazole and itraconazole have been reported (Benitez and Carver, 2019). Amphotericin B resistance has been reported among *C. auris* isolates but remains low while echinocandin resistance also has been reported, especially in clade I isolates (Bravo Ruiz and Lorenz, 2021). Echinocandin antifungals are therefore recommended as first-line treatment in adults in South Africa though amphotericin B deoxycholate is recommended for initial treatment in neonates (Govender *et al.*, 2019) because this antifungal can effectively penetrate the central nervous system and thus treat meningoencephalitis (Ascher *et al.*, 2012). A recent study involving six patients

colonised/infected with *C. auris* revealed clade III *C. auris* isolates that were all resistant to both fluconazole and amphotericin B in a healthcare facility in Los Angeles, USA (Price *et al.*, 2020). We did not observe isolates resistant to all classes of antifungals in this study; however, pan-resistant isolates (0.5%) have been reported in South Africa in a previous study (Maphanga *et al.*, 2021). *C. auris* isolates resistant to more than one antifungal class are a great public health concern.

C. auris has three major mechanisms of resistance to azole drugs: overexpression of efflux pumps, upregulation of the target gene and mutations on the target gene (Frias-De-Leon *et al.*, 2020). The majority of our fluconazole-resistant isolates had mutations in the *ERG11* gene that are known to contribute to resistance. We also observed clade-specific variations in mechanisms of azole resistance. Clade III isolates had the VF125AL substitution while clade IV isolates possessed the K177R, N335S, E343D substitutions. The clade III *ERG11* mutation (with a VF125AL substitution) seems to be universal across resistant isolates within the clade and has been seen in other clade III isolates from previous studies, even those isolated from other countries outside of Africa (Chow *et al.*, 2020; Price *et al.*, 2020). The mechanism of resistance against fluconazole on the *ERG11* gene for clade IV isolates show polymorphic clade differences. Clade IV isolates from different geographical areas seem to have different mutations on the *ERG11* gene that produce high MICs to azole drugs (Healey *et al.*, 2018). Sequences in our study had the same mutation as isolates originating from Colombia, which contained all three ERG11 substitutions (K177R, N335S, E343D), while the azole resistance in clade I Indian/Pakistan and some clade IV Colombian isolates was due to the Y132F or K143R substitution (Healey *et al.*, 2018). Interestingly, the K177R, N335S, E343D clade IV substitutions have not been shown to contribute to increased azole resistance even in the Colombian isolates. Instead, other Colombian isolates had the following substitutions: I466M, Y501H, that have been shown to contribute to resistance, which we did not see in our isolates (Healey *et al.*, 2018). Some sequences with the above mentioned clade

IV substitutions had an additional E102K substitution; this substitution has not been documented or proven to contribute to decreased susceptibility in *C. auris* strains. There were cases in our study where susceptible isolates possessed resistant mutations especially in clade IV. It appears that clade IV isolates might have a more complex mechanism of resistance.

Other molecular mechanisms of resistance to azole drugs that have been reported include the upregulation of the *CDR1* gene that is responsible for the expression of critical efflux pumps including the ABC and the MFS. The transcriptional upregulation of *CDR1* has been shown to increase the MIC values of *C. auris* isolates (Ostrowsky *et al.*, 2020). Other mechanisms include the involvement of HSP90 proteins, a heat shock protein, that regulates cellular responses due to stress induced by antifungals when overexpressed (Kim *et al.*, 2019). Duplication of chromosome 5, which houses the *ERG11* gene has also been shown to increase MIC values (Lockhart, 2019). The constant availability and use of fluconazole for prophylaxis and non-selective treatment in healthcare facilities of South Africa might have contributed to the evolution of a fluconazole-resistant population of *C. auris* isolates. More research would be beneficial to determine whether the above mentioned mechanisms are involved in resistance within the strains circulating in the country and the antifungals most affected by these mechanisms.

Some *C. auris* isolates exist as single-cells while others do not separate from the parent yeast cells and thus exist as clusters of aggregates (Rossato and Colombo, 2018). Most isolates involved in this study had aggregate-forming capabilities. Aggregation formation allows the pathogen to persist in the environment despite disinfection efforts. In a study conducted by Chatterjee et al., (2020); clade III isolates demonstrated a resistance to broad-spectrum ultraviolet C light (UV-C) exposure even after 30 minutes of exposure to the light as recommended by the manufacturer, due to their aggregating ability (Chatterjee *et al.*, 2020). The aggregates impede the penetration of light to the centre of the clump; this principle of impedance is also responsible for the resistance of such *C. auris* strains against other

disinfectants (Chatterjee *et al.*, 2020). Aggregating strains are also more tolerant to antifungal agents than their non-aggregating counterparts. The protective effect of aggregation is also assisted by biofilm formation (R. Singh *et al.*, 2019) that in turn assist with adherence to surfaces (Borman, Szekely and Johnson, 2016). Their ability to persist and adhere on environmental surfaces proves to be the driver of many transmissions within facilities (Rossato and Colombo, 2018).

Clade-specific aggregate forming abilities of *C. auris* isolates have been documented elsewhere. A study carried out on Southern Asian and South African lineage isolates showed that all isolates from the latter lineage formed aggregates while isolates from the South Asian lineage did not (Szekely, Borman and Johnson, 2019). Additionally, no isolates from the South African lineage from this previous study, were able to form any pseudohyphal structures (Szekely, Borman and Johnson, 2019) whereas in the current study, we reported only one isolate of clade III that produces psuedohyphae (we did not confirm that this isolate was pure in culture). It should be noted that the variability in experiment preparation techniques and methods might have affected the outcome. Phenotypic characteristics of *C. auris* isolates could have an impact on the virulence and pathogenicity, which further impacts the clinical manifestation and mortality rates of infection by the pathogen (Brown *et al.*, 2020). More research is imperative to determine the heterogeneity among the phenotype of *C. auris* isolates, including other phenotypic characteristics such as biofilm formation, to establish the type of strains circulating in a country or healthcare facility in order to successfully mitigate this impact.

Chow and colleagues used collection dates of clinical and environmental isolates and their corresponding genomic data to better understand the emergence of *C. auris* (Cuomo and Alanio, 2020)(2020). They estimated divergence times for the four major clades (I, II, III, IV) using molecular clock analysis. They estimated that the time to most recent common ancestor (TMRCA) for *C. auris* emerged 360 years ago. The clade IV ancestor was estimated to have

emerged relatively earlier, 39 years ago; while the ancestor for clade III emerged 188 years ago (Chow *et al.*, 2020). Both these clades diverged from their common ancestor decades ago but were seemingly introduced into the hospital in our study in the last 10 years. The mutation rate estimated in our study (1.3471e-5 substitutions per site per year) was similar to that of a Kenyan outbreak caused by clade III isolates, the calculated mutation of the outbreak strains was 1.8695e−5 substitutions per site per year (Adam *et al.*, 2019; Chow *et al.*, 2020); therefore the rate at which *C. auris* isolates mutate and evolve seems constant in outbreak situations.

Furthermore, phylodynamic analysis showed support for a polyphyletic population of isolates within the hospital, which suggested multiple external introductions of antifungal resistant *C. auris*. Although the mode of introduction into the neonatal unit is unknown and we cannot exclude sources from outside the hospital (community or other facilities), the outbreak may have originated from cross-unit transmission within the hospital by infected/colonised patients, colonised healthcare workers or contaminated equipment. Patient environments have served as resevoirs of infection; in one study done at a chest hospital in India, patient colonization was linked to several frequent contact surfaces including bed railings, sheets, pillows and bed side trollies while TMRCA analysis also linked a patient isolate to one found in the environment (Yadav *et al.*, 2021). Infection prevention and control practices in a Colombian centre experiencing *C. auris* infections in their NICU included chlorhexidine baths of colonised patients, contact precaution and constant cleaning and disinfection of frequently touched environmental surfaces to curb infections (Alvarado-Socarras *et al.*, 2021).

The main limitation of our study was that we only had a small subset of isolates that were circulating in the hospital; the analysis only includes cultured isolates from patients with invasive infections and only a few with colonisation. An extensive collection of isolates using systematic sampling in a prospective study, including those isolated from the environment

would be needed to determine the extensive transmission routes involved with a higher resolution and to avoid missing links. In addition, travel or hospital transfer histories of patients in our study was not known; this information would have allowed us to potentially determine how the pathogen was introduced into the hospital.

Chapter 5: Conclusion

Our study allowed us to characterise the strains circulating in a major metropolitan public hospital in South Africa. We saw that a large proportion of the isolates were among neonatal patients. Most of the patients in our study were diagnosed with invasive disease due to *C. auris* because of the surveillance methodology that we used.

The use of microscopic techniques allowed us to determine the phenotypic characteristics of the strains circulating within the hospital. Through these techniques, we saw that most of the isolates formed aggregates; this quality assists the pathogen with adhering to surfaces and persisting on surfaces regardless of disinfection efforts. This characterisation also included the assessment of antifungal susceptibility profiles of isolates, which revealed the resistance of the majority to fluconazole, which informs the empiric treatment of patients at the hospital.

The use of WGS allowed for the molecular characterisation of the pathogen with high resolution. Our data showed that two *C. auris* clades populated the hospital: clade III (the African clade) and clade IV (the South American clade) which co-circulate. Clade III was largely responsible for the outbreak in the neonatal unit of the hospital. Most isolates that were resistant to fluconazole carried previously published clade-specific mutations related to azole resistance in some cases.

The use of epidemiological and genomic data allowed us to link the cases and determine transmission chains. Phylodynamic analysis showed a polyphyletic population of isolates within the hospital, suggesting multiple external introductions of the strains into the hospital. The estimated emergence of TMRCA for the hospital and neonatal unit clade III isolates was roughly consistent with the first cases reported or documented on the epidemic curve. We also saw transmission events of clade III isolates into and out of the neonatal unit. This was speculated to be due to healthcare workers colonised with the pathogen or contaminated

equipment brought into and out of the ward. Although the manner of introduction into the neonatal unit is undetermined, temporal analysis of the outbreak strains placed TMRCA as early 2018, suggesting a recent introduction.

These results highlight the need for stringent IPC practices within the investigated hospital, including isolation of infected or colonised patients, strict hand hygiene protocols by staff attending to patients, screening and testing of close contacts and disinfection of frequently touched surfaces. Future research is necessary to determine the molecular epidemiology of *C. auris* in South African communities and acute- and long-term healthcare facilities, as previous studies carried out in the country, including the present one, suggest a greater diversity of *C. auris* isolates in circulation. Effective treatment for *C. auris* is also imperative; therefore, further research is necessary to fully characterise other mechanisms of resistance of isolates (including the involvement of efflux pumps, chromosome modifications and others) circulating in the country.

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Appendices

Appendix A:

CANDIDA CRF for NICD/GERMS laboratory-based survey 2019

Date completed: _____ **Name of person completing form**: _____

Candida Case Report Form for NICD/GERMS Lab-based Surveys

SA ID number_____ **Lab specimen ID number**_____ (attach lab report to this form)

Age _____ □days □months □years □unknown **Sex** □ M □ F □ Unknown

Institution/Hospital _____ **City** _____ **Country: ZA**

Date of admission (e.g. 1 Jan 2015) _____ **Reason for admission** _____ □ Unknown

Date of discharge (e.g. 1 Jan 2015) _____ **Patient status at discharge** □ Alive □ Dead □ Unknown **If patient died, date of death** (e.g. 1 Jan 2015) _____ □Unknown

Major underlying medical conditions (Check all that apply) or □ None □ Unknown

□ Diabetes mellitus	□ Liver disease (specify):_____
□ Solid tumour	□ HIV/AIDS
□ Haematologic malignancy	□ Chronic kidney disease
□ Bone marrow transplant	□ Haemodialysis
□ Solid organ transplant	□ Surgical procedure in the last 90 days (specify):_____
□ Steroid use (specify condition:_____)	□ Other (specify):_____

Date of collection of first _Candida_ culture (e.g. 1 Jan 2015) _____

Dates of collection of subsequent positive _Candida_ cultures (if available): _____ _____

Site of _Candida_ infection

□ Bloodstream	□ Urine
□ Respiratory tract (specify: sputum, BAL)_____	□ Central venous catheter tip
□ Other (specify):_____	

Did the patient have any other positive fungal cultures in the 90 days before the first _Candida_ culture date?

□ Yes □ No □ Unknown

If yes, list organism, site, and date specimen was obtained

Organism	Site of Infection	Date of specimen collection (dd/mm/yyyy)

Was the patient hospitalized in the 90 days before the current admission? □ Yes □ No □ Unknown

Did the patient have a central venous catheter at any time in the 7 days before *Candida* culture date? □ Yes □ No □ Unknown

Did the patient have a urinary catheter at any time in the 7 days before *Candida* culture date? □ Yes □ No □ Unknown

Did the patient receive systemic antibiotics in the 14 days before *Candida* culture date?

□ Yes □ No □ Unknown

Did the patient receive any antifungal treatment in the 90 days before *Candida*-positive culture?

□ Yes □ No □ Unknown

If yes, specify antifungal drug, dose, start date, stop date, indication

Antifungal drug	Dose	Start date	Stop date	Indication

Did the patient receive antifungal treatment after *Candida*-positive culture? □ Yes □ No □ Unknown if yes, specify drug(s), dose, and duration:

_____

Date of first negative culture after detection of *Candida* infection (e.g. 1 Jan 2015)

_____ □ Unknown

Version 2.0 2019-2023

Appendix B:

Ethical Clearance Certificate

UNIVERSITY OF THE WITWATERSRAND, JOHANNESBURG

R14/49 Ms Dikeledi Kekana

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M210298

NAME: (Principal Investigator)	Ms Dikeledi Kekana
DEPARTMENT:	Clinical Microbiology and Infectious Diseases School of Pathology National Institute of Communicable Disease National Health Laboratory Service
PROJECT TITLE:	Molecular Characterisation of Candida auris Clinical Isolates at a Large Tertiary Academic Hospital
DATE CONSIDERED:	Ad hoc
DECISION:	Approved unconditionally
CONDITIONS:	Approval issued to access data collected under M160667 and M1809107 (NICD Surveillance and GERMS-SA)
SUPERVISOR:	A/Prof N. Govender and Ms S. Naicker
APPROVED BY:	CB Penny Dr CB Penny, Chairperson, HREC (Medical)
DATE OF APPROVAL:	12/03/2021

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 **February** and will therefore be due in the month of **February** each year. Unreported changes to the application may invalidate the clearance given by the HREC (Medical).

DM Kekana
Principal Investigator Signature

01/04/2022
Date

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix C:
Similarity Index

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