Identification and determination of virulence factors of invasive *Candida parapsilosis* sensu lato in paediatric patients at Universitas Academic Hospital

by

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Submitted in fulfilment of the requirements in respect of the Degree Doctor of Philosophy (School of Pathology) Medical Microbiology in the Faculty of Health Sciences at the University of the Free State, South Africa

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DEDICATIONS

This work is dedicated to my family.

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GLOSSARY AND ABBREVIATIONS

API: Analytical Profile Index

AST: Antifungal susceptibility testing

ATCC: American Type Culture Collection

BD: Becton Dickinson

BDG: 1,3-β-D-glucan

BMD: Broth Microdilution

CO_2: Carbon dioxide

CLSI: Clinical and Laboratory Standards Institute

CSF: Cerebrospinal fluid

DNA: Deoxyribonucleic acid

DNase: Deoxyribonuclease

ELISA: Enzyme Linked immunosorbent Assay

ELBW: Extremely low birth weight

FDA: Food and Drug Administration

FS: Free State

GM: Galactomannan

GERMS-SA: Group for Enteric, Respiratory and Meningeal disease Surveillance in South Africa

HCME: Hematogenous *Candida* Meningoencephalitis

ICU: Intensive Care Unit

IPC: Infection Prevention and Control

IV: Intravenous

ITS: Internal Transcribed Space

Late neonatal sepsis: Sepsis that occurs after one week of life

LBW: Low birth weight

MALDI-TOF MS: Matrix Assisted Laser Desorption/ionization Time of Flight Mass Spectrometry

MIC: Minimum Inhibitory Concentration

MLST: Multilocus sequence Typing

MLP: Microsatellite Length Polymorphisms

NAC: Non-*albicans Candida*

NCBI: National Center for Biotechnology Information

NHLS: National Health Laboratory Services

NICU: Neonatal intensive care unit

NaCl: Sodium chloride

NMR: Nuclear Magnetic resonance

OASIS: Online application for survival analysis

OPA: Orthophthalaldehyde

PCR: Polymerase chain reaction

PCR-REA: Polymerase chain reaction based restriction endonuclease analysis

PCR-RFLP: Polymerase Chain Reaction-Restriction Fragment Length Polymorphism

PGD_2: Prostaglandin D_2

PGE_2: Prostaglandin E_2

QACs: quaternary ammonium compounds

RAPD: Random Amplification of Polymorphic

RNA: Ribonucleic Acid

SA: South Africa

SDA: Sabauraud Dextrose Agar

STR: Short Tandem Repeats

T2MR: T2 Magnetic Resonance

UFS: University of the Free State

US: United States

USA: United States of America

VLBW: Very low birth weight

WHO: World Health Organisation

YM: Yeast Malt

YME: Yeast Malt Extract

YST: Yeast

LIST OF CONFERENCES AND PUBLICATIONS

Manuscript will be submitted for publication in relevant journals. The results will be presented at the 12th Annual Provincial Health Research Day hosted by the Free State Department of Health in collaboration with the University of the Free State and Central University of Technology on 22 November 2024.

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This chapter comprises the literature review. Motivation for the study is provided in this chapter. The chapter further introduces the reader to what is already known in the literature about *Candida parapsilosis sensu lato*. An overview of *Candida parapsilosis sensu lato* which forms part of the group comprising two other cryptic species is provided. The transmission and risk factors, virulence factors, pathogenesis, clinical manifestation, laboratory diagnosis, and management of the diseases caused by *Candida parapsilosis sensu lato* are also discussed. The chapter further describes the problem statement and outlines the study aims and objectives.

Chapter 2:

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Chapter 4:

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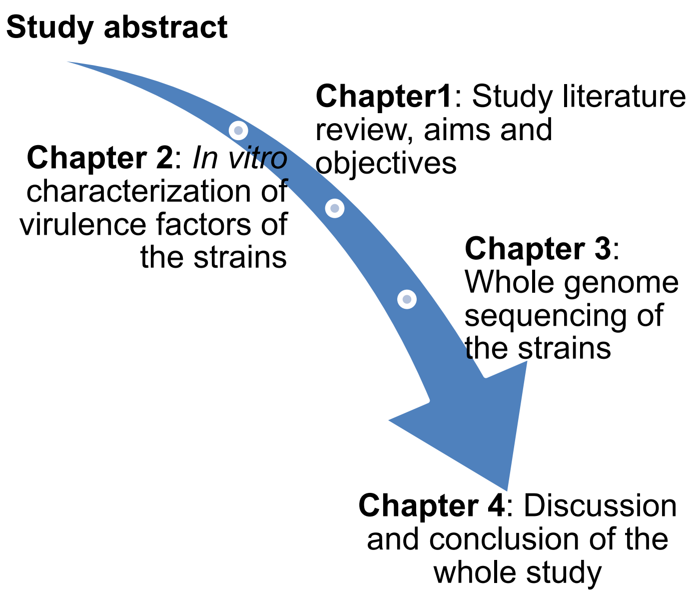
Flow diagram of the chapters:



DISSERTATION ABSTRACT

Introduction:

Invasive fungal infections contribute to a rise in morbidity and mortality, extended stay in hospital and higher health care cost. Due to the increased risk factors among the neonates, this population continues to bear the brunt as the morbidity and mortality due to *Candida parapsilosis* remains high. This is further complicated by the rising predominance of azole-resistant strains.

Aim:
This study aimed to identify the specific strains of *C. parapsilosis sensu lato* cultured from neonatal and paediatric patients at Universitas Academic Hospital and to determine their virulence potential. The clinical outcome data of the patients were correlated with the strains' data.

Methodology:
The study was conducted at the Department of Microbial, Biochemical and Food Biotechnology, University of the Free State, Bloemfontein. This was a retrospective cross-sectional laboratory-based study. *Candida parapsilosis* as identified on Vitek®2 (bioMérieux Inc, Marcy l'Etoile, France), from invasive clinical samples sent for routine laboratory diagnosis to the Medical Microbiology laboratory from Universitas Hospital's neonatal and paediatric wards, were used.

A sample size of approximately 30 was estimated based on the numbers from the previous year's however, only 21 strains were obtained, with one of the patients having two isolates. Therefore, 20 patients' strains were obtained. The *Candida parapsilosis* strains used for the study were those already stored in the laboratory at -80°C from the year 2018 to 2020. Data obtained from Vitek®2 (bioMérieux Inc, Marcy l'Etoile, France) as well as patients' demographic data and clinical information from patient clinical files were recorded on an Excel sheet for analysis. No patient names were recorded. Only study numbers were used.

D1/D2 sequencing was performed for species differentiation. Clinical records were reviewed, time to positivity, species identification, antifungal susceptibility testing results and patients' demographics were also retrieved from TrackCare. The QualiClean (QualiPharm, New Germany) used in the hospital setting at 1000 ppm for surface disinfection was tested against all the 20 strains to determine their susceptibility to the disinfectant. A crystal violet assay was conducted to determine biomasses of respective biofilms. For hydrolytic enzymes secretion, tributyrin agar was used for lipase activity, yeast carbon base bovine serum albumin agar for protease activity and the sabauraud dextrose agar supplemented with 8% egg yolk for phospholipase activity. Prostaglandin E_2 production was evaluated by using the enzyme-linked immunosorbent assay (ELISA; Cayman Chemicals, Ann Arbour, USA) according to the manufacturer's instructions. The biological method described by Brenner (Brenner, 1974) was followed for *in vivo* relative virulence in *Caenorhabditis elegans*. In addition, whole genome sequencing was conducted to study the ploidy of the study strains, genetic relatedness as well as the common antifungal resistance genes.

Results:

The strains were all *Candida parapsilosis sensu stricto.* Ninety percent of blood cultures had a time to positivity of 48 hours with 80% of the strains showing resistance to fluconazole and the intermediate resistance to voriconazole. For 70% of the strains, resistance to fluconazole and intermediate resistance to voriconazole was observed. In addition, the QualiClean disinfectant did not effectively inhibit any of the strains. Fifty-five percent of the patients were from neonatal intensive care unit followed by 35% from neonatal high care and 10% from paediatric wards. Eighty percent of the patients were males. Forty-one percent of the patients were categorized as very low birth weight and 55% delivered via caesarian section. The study population had multiple risk factors including the presence of invasive devices, total parenteral nutrition, gastrointestinal pathology, and administration of broad-spectrum antibiotics. Deaths were recorded for 47% of the patients. The strains produced biofilms, proteases, phospholipases, and prostaglandin E_2 in varying quantities. The

three *C. parapsilosis* strains tested for their virulence in *C. elegans*, killed the nematodes rapidly when compared to the *C. albicans* ATCC SC5314 strain.

All strains were haploid and were found to group into four related Clades, with majority of the strains in Clade 4. Clade 4 also housed 94% of the fluconazole-resistant strains. The *FKS1*, *MRR1* and *ERG11* genes were highly conserved between strains with none of the mutations previously associated with resistance patterns.

Conclusion**:**
Candida parapsilosis sensu stricto is circulating with majority of strains resistant to fluconazole, suggesting that fluconazole should be avoided as the empiric therapy among this population. All strains were virulent and resisted the action of the disinfectant. The contact time will need to be increased or a different disinfectant used. Multiple infection prevention and control interventions including hand hygiene are also to be intensified. Further studies are required to identify the resistance mutations or novel mutations prevalent at the study site, including those outside the common regions.

Keywords: Candidemia; *Candida parapsilosis*; Virulence factors; Hydrolytic enzymes; Prostaglandin E_2, Biofilm formation, Chlorine based disinfectant; *Caenorhabditis elegans*; Whole genome sequencing; Ploidy.

CHAPTER 1: LITERATURE REVIEW

1.1 MOTIVATION

Candidaemia is the presence of *Candida* species in the blood whereas invasive candidiasis refers to the serious infections following the invasion of *Candida* species in sterile sites such as blood and cerebrospinal fluid (CSF) (Pappas et al., 2018). Among the non-albicans species, *Candida parapsilosis* is the most common cause of invasive candidiasis especially among the vulnerable paediatric patients (Daneshnia et al., 2023a). In patients with candidaemia, *C. parapsilosis* is the second or third most commonly isolated species after *C. albicans* worldwide depending on the setting (Tóth et al., 2019b, Govender et al., 2016).

The prevalence and distribution of *C. parapsilosis* differs among the geographical location and age group. In the paediatric population, the neonates continue to bear the brunt as the morbidity and mortality remains high. Since 2016 to 2017, most cases of candidaemia in South Africa (SA), were due to *C. parapsilosis* (44%), followed by *C. albicans* (23%) and *C. auris* (14%) (van Schalkwyk et al., 2019, Qi et al., 2018a). Some of the studies in SA continue to show the predominance of *C. parapsilosis* as the leading cause of candidaemia (59.1%, 45.2% and 60.49%) among the neonates followed by *C. albicans* (30.9%, 29.0% and 28.40%) (Malunga et al., 2020, Pillay et al., 2021, Ramdin et al., 2023). Charsizadeh and colleagues found a *C. parapsilosis* mortality rate of 33% in a neonatal and paediatric intensive care unit (ICU) in Iran (Charsizadeh et al., 2018). In 2017, Free State (FS) was the second leading province after Northwest (NW) with *C. parapsilosis* infections (GERMS-SA, 2017). This increasing prevalence continues to be noted in SA from the neonatal wards (Shuping et al., 2021). According to a review by Daneshnia and colleagues, publications from SA between 2000 to 2022 indicate that about 30-35% of invasive candidiasis infections were caused by *C. parapsilosis* and more than 40-60% of the *C. parapsilosis* isolates were resistant to fluconazole (Daneshnia et al., 2023a).

Candida parapsilosis is a term comprising of three species, namely *C. parapsilosis sensu stricto*, *C. metapsilosis* and *C. orthopsilosis*. The current routine identification methods in the laboratories cannot differentiate the three species. Although *C. parapsilosis* is part of the human skin and gastrointestinal microbiome, it is not restricted to humans, as it is also found in animals and the environment (Trofa et al., 2008). Both the contaminated hands of the healthcare workers as well as the environment are possible sources of transmission for this pathogen to patients.

Resistance to azoles and the recent resistance to echinocandins by *C. parapsilosis* may have detrimental effects on patient care, increasing morbidity and mortality, particularly in the era of shortage of antifungals (Daneshnia et al., 2023a). In most low income countries, fluconazole is most commonly used as an empiric antifungal agent (Ferreras-Antolin et al., 2021), particularly in patients with no prior antifungal therapy or those who have renal impairment. Dependence on fluconazole is due to lack of more favourable antifungals such as liposomal amphotericin B that has less side effects compared to the readily available conventional amphotericin B deoxycholate (Maertens et al., 2022, Silver and Rostas, 2018). *Candida parapsilosis* may show high minimum inhibitory concentrations (MIC) to echinocandins (Moudgal et al., 2005) but successes have been recorded in some neonates through case studies (Ozkaya-Parlakay et al., 2014, Yalaz et al., 2006). Reports of outbreaks in neonatal units due to azole-resistant *C. parapsilosis* are increasing worldwide including Brazil, China and France (Fekkar et al., 2023, Qi et al., 2018b, Escribano and Guinea, 2022, Castro et al., 2023a, Thomaz et al., 2018), contributing to the inclusion of this pathogen in the World Health Organisation (WHO) fungal priority pathogen list (WHO, 2022). A South African surveillance study also found an increase in triazole-resistant *C. parapsilosis* from the laboratory based sentinel sites where only 37% of strains were susceptible to fluconazole and voriconazole (Govender et al., 2016), further highlighting concerns about the rise of fluconazole-resistant *Candida parapsilosis.*

The majority of *C. parapsilosis sensu lato* isolates cultured at Universitas Medical Microbiology laboratory are from neonatal wards. These are patients with risk factors such as prematurity, very low or low birth weight, exposure to broad-spectrum antibiotics, invasive devices and total parenteral nutrition. Speciation of *C. parapsilosis sensu lato* species at Universitas Medical Microbiology laboratory has not been performed before. It is therefore not known which of the three *C. parapsilosis sensu lato* species are circulating in the neonatal and paediatric wards. Differentiation of the circulating species will help to better understand the different virulence factors as well as antifungal susceptibility pattern or behaviour of the circulating strain(s). This might further assist in explaining why *C. parapsilosis* is outranking *C. albicans* in this population.

Correlation of the obtained data with clinical history, especially knowledge of the patient outcome, may assist in policy changes in terms of first line or empiric antifungals to be used at the study site. The results will also assist in strengthening the infection prevention control measures in both paediatrics and neonatal wards in order to reduce the possible horizontal transmission. Eventually, the information obtained from this study will help to decrease the mortality and morbidity, especially among the vulnerable patients infected with *C. parapsilosis* at Universitas Academic Hospital.

1.2 INTRODUCTION

Invasive fungal infections contribute to the increased morbidity and mortality, prolonged hospital stay and an increased health care cost. Although *C. albicans* has long been responsible for majority of candidaemia cases, non-*albicans Candida* (NAC) species are now leading worldwide (Hassan et al., 2019). The prevalence of *Candida parapsilosis* varies by setting and age (Lausch et al., 2019). Following its discovery in 1928, *C. parapsilosis* ranges from the first to the third most isolated species following *C. albicans* in the hospital setting worldwide (Tóth et al., 2019a). According to the SENTRY Antimicrobial Surveillance Program conducted between 2008-2009, *C. parapsilosis* was the third most common *Candida* species from blood cultures in intensive care unit (ICU) with higher resistance to fluconazole (6.8%) among the ICU patients than in non-ICU patients (4.3%) (Pfaller et al., 2011).

The adult ICU outbreaks due to *C. parapsilosis* have also been noted (Thomaz et al., 2022) but neonatal ICU remains the most affected with high mortality and morbidity due to drug resistance and multiple risk factors that are associated with prematurity. Lausch and coworkers found the 30-days mortality among those below 15 years to be 10.2% with higher percentage observed among the neonates (17%) in Denmark (Lausch et al., 2019). In a study from SA, where the neonates accounted for 49% of all paediatric cases, a higher mortality due to *C. parapsilosis* was also reported among the neonates with more than 50% of the isolates showing resistance to fluconazole (Shuping et al., 2021).

Risk factors associated with *C. parapsilosis sensu lato* infection include low birth weight, prolonged use of antibiotics, indwelling central or umbilical venous catheters, total parenteral nutrition, abdominal surgeries, neutropenia and mechanical ventilation (Liu et al., 2015). Most of the paediatric population have some of these risk factors during hospital admission, thus increasing their chance to acquire *C. parapsilosis* infections. The risk factors overlap with those seen in adults (Zuo et al., 2021), but the morbidity is worse in neonates due to their immature immune system as previously mentioned.

Earlier researchers discovered that *C. parapsilosis* species differ in terms of their virulence as well as the susceptibilities. This led to more research focusing on these differences and subsequently, three species of *Candida parapsilosis sensu lato* namely, *C. parapsilosis sensu stricto*, *Candida orthopsilosis* and *Candida metapsilosis* were identified (Tavanti et al., 2005). The species have different levels of virulence factors production. Amongst other factors that contribute to the virulence, the species form biofilm on medical devices, secrete hydrolytic enzymes and produce eicosanoids (Ataides et al., 2020).

Although phenotypic methods such as growth at 15% NaCl or pH 7.0 (Cordeiro et al., 2018) have been tested to differentiate the cryptic species, there is currently no available laboratory methods for routine identification in clinical settings that can differentiate the three related species (Gil-Alonso et al., 2016). The species are thus collectively identified as *C. parapsilosis*. As a result, most studies report on *C. parapsilosis* and do not differentiate the three species. However, since the diagnostic laboratories are slowly moving towards molecular diagnostics, future laboratories might be able to use methods such as polymerase chain reaction (PCR)-based restriction endonuclease analysis (PCR-REA) which are highly reproducible and reported to be 100% concordant to the internal transcribed spacer 1 (ITS1)/ITS2 sequencing (Neji et al., 2017).

Candida parapsilosis sensu stricto accounts for majority of clinical infections, while *C. metapsilosis* and *C. orthopsilosis* are cultured in minority of the clinical specimens (Romeo et al., 2012, Thomaz et al., 2018). Few South African publications differentiate between these species, and this could be driven by the cost factor. A surveillance study by Magobo and colleagues from both public and private hospitals detected *C. parapsilosis sensu stricto* from neonatal ICU (NICU) with no detection of other two cryptic species (Magobo et al., 2017). Whether the two other cryptic species have not been circulating in SA NICU settings is still to be confirmed.

Candida parapsilosis sensu lato is ubiquitous (Pammi et al., 2013). The organism is often cultured from hands and the gastrointestinal tract (Bonassoli et al., 2005, Pammi et al., 2013, Qi et al., 2018b) Initially, the organism was isolated from stools of a patient with diarrhoea (Ashford, 1928). Both the contaminated environment, and hands of the health care workers as well of the caregivers (de Paula Menezes et al., 2020) play a crucial role in nosocomial acquisition (Sabino et al., 2015) and this has been the finding in some outbreaks (Qi et al., 2018a, Asadzadeh et al., 2019) though not in some (Miranda et al., 2012). Bonnasoli and colleagues found a high rate (59.3%) of yeast colonisation on hands of both healthcare workers as well as community members with no prior exposure to the healthcare setting (Bonassoli et al., 2005). It is this presence on human hands that may be responsible for the transmission in paediatric wards.

As *C. parapsilosis* is transmitted by contact, intensified contact precautionary measures should be in place to limit its spread in the hospital wards or units. A series of infection prevention and control measures (IPC) measures, such as the routine screening of patients admitted in ICU (Fekkar et al., 2023), education, cohorting, environmental screening and cleaning (Qi et al., 2018a) are implemented to stop the spread of the organism in the wards.

Low to middle income countries may not have enough isolations rooms to action the positive screening results. Hand hygiene therefore remains the primary strategy in reducing health care associated infections (Haque et al., 2020). Alcohol hand rubs are advised as part of hand hygiene to prevent the spread of *C. parapsilosis* (Fekkar et al., 2023). In a study done in Brazil, candidemia continued to be a problem even after the use of alcohol-based hand sanitizers was encouraged and environmental cleaning measures were taken, (Thomaz et al., 2022) suggesting that they may not be effective against the resistant strains of *C. parapsilosis*.

Disinfectants are chemicals that inactivate microorganisms (McDonnell and Russell, 1999). Disinfection plays a crucial part in interrupting the transmission route of infections (Artasensi et al., 2021). Daily cleaning as well as terminal room cleaning after the patients vacate the room is crucial to prevent transmission and colonisation. Chlorine releasing compounds, glutaraldehyde, quaternary ammonium compounds (QACs), hydrogen peroxide and alcohol based products are among the disinfectants commonly used in hospitals, for environmental hygiene as well as for disinfection of medical devices (De Carolis et al., 2014). Chlorine releasing compounds, which are widely used, cheap and have a broad antimicrobial spectrum, include sodium hypochlorite or biocide (Artasensi et al., 2021). *Candida parapsilosis* is known to form biofilms on medical devices. The appropriate disinfectant should be able to inhibit biofilm production on medical devices. Even though sodium hypochlorite is still used in many settings, several studies have found QACs, acetic acid or hydrogen peroxide to be more effective than sodium hypochlorite against *C. parapsilosis* biofilms (Castro et al., 2023a, Pires et al., 2013).

Although *C. parapsilosis sensu lato* is not inherently resistant to azoles, polyenes or echinocandins, which are the drugs commonly used for invasive candidiasis, there is a documented increase in acquired resistance to these drugs worldwide (Thomaz et al., 2018) including in SA (Govender et al., 2016). This applies to both paediatric and adult populations, with the majority of bloodstream *C. parapsilosis* isolates resistant to fluconazole (Pammi et al., 2013). Echinocandins are recommended by national guidelines as first-line drugs for treatment of candidaemia (Pappas et al., 2016), but fluconazole and amphotericin B are often used in poor resource settings. In addition, studies have also shown differences in antifungal susceptibility of the *C. parapsilosis sensu lato* species (de Toro et al., 2011) (Demirci‐Duarte et al., 2021), highlighting the need to differentiate between the three species.

1.3 *Candida parapsilosis sensu lato*

1.3.1 Overview

The genus *Candida* contains species that are part of the human microbiome, and may cause invasive candidiasis. The ability to cause infections may have developed over time. Figure 1 shows various species that make up the genus *Candida,* dispersed among several evolutionary clades that also contain nonpathogenic species (Alves et al., 2020). Over the years, the landscape has changed from *C. albicans* to non-*albicans Candida* (NAC) being the leading cause of invasive candidiasis (Tóth et al., 2019a). The five most prevalent species known to cause invasive infections are *C. albicans, C. glabrata, C. parapsilosis, C. krusei* and *C. tropicalis* (Pappas et al., 2018). In addition, a new species, *C. auris,* has emerged as a pathogen in all parts of the world, with most SA cases reported in the Gauteng province (Govender et al., 2018).

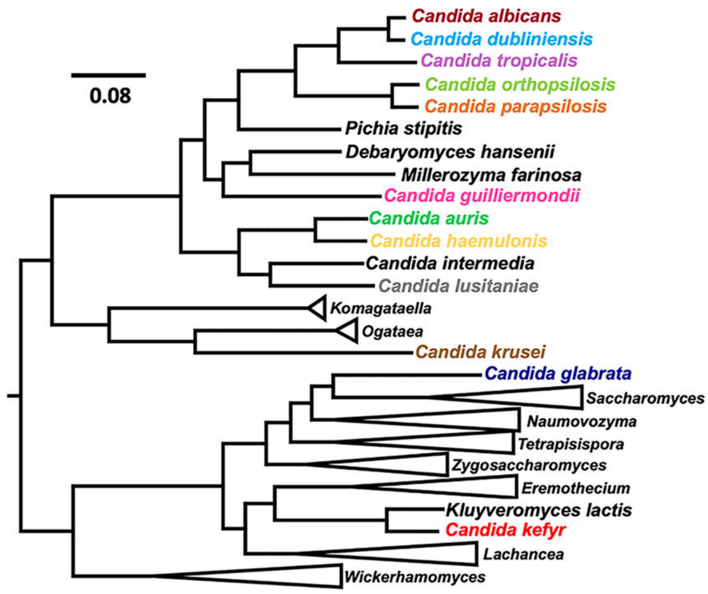


Figure 1: Evolutionary relationship between *Candida* species and other yeasts (Alves et al., 2020).

Of these species, *C. parapsilosis* (previously named *Monilia parapsilosis)* (Ashford, 1928)*,* has established itself as an important pathogen among neonates, the population with an immature immune system. The distribution of these species differs among countries. In SA, a laboratory based surveillance revealed 36% (143/393) *C. parapsilosis* cases among the neonates between the years 2009 to 2010 (Magobo et al., 2017).

Earlier studies reported *C. parapsilosis* strains to be more genetically heterogenous than other *Candida* species (Garcia-Effron et al., 2011, Barbedo et al., 2017). These studies divided *C. parapsilosis* strains into groups I, II and III based on differences of randomly amplified polymorphic DNA (RAPD), DNA sequencing of different genes and morphotyping (Lin et al., 1995). Tavanti and colleagues performed multilocus sequence typing (MLST) studies and suggested that the differences amongst the three groups warranted that they be replaced by species names (i.e. *C. parapsilosis sensu stricto*, *C. metapsilosis* and *C. orthopsilosis*) rather than groups, as illustrated in figure 1 (Tavanti et al., 2005). *Candida parapsilosis sensu stricto* is cultured from majority of clinical infections as compared to *C. metapsilosis* and *C. orthopsilosis* (Chakraborty et al., 2018). The above-mentioned finding differs across countries and settings and in other studies, *C. metapsilosis* is not found among clinical isolates at all (Ziccardi et al., 2015).

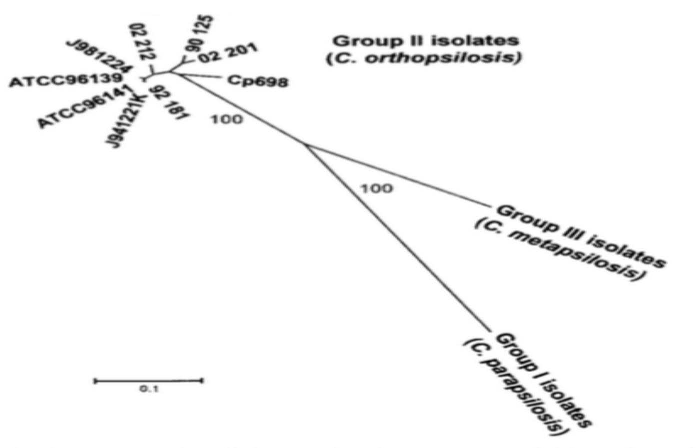


Figure 2: Unrooted radial tree distinguishing the three *Candida parapsilosis sensu lato* species (Tavanti et al., 2005).

1.3.2 Transmission and risk factors

Candida parapsilosis sensu lato is ubiquitous in the environment (Pammi et al., 2013). The organism is often cultured from hands and the gastrointestinal tract of humans (Bonassoli et al., 2005, Pammi et al., 2013, Qi et al., 2018a). Ashford isolated the organism from stools of a patient with diarrhoea years back (Ashford, 1928). Nosocomial acquisition is largely influenced by contact with the contaminated environment (Sabino et al., 2015) and the hands of healthcare providers and caregivers (de Paula Menezes et al., 2020); as has been the case in certain outbreaks (Qi et al., 2018b, Asadzadeh et al., 2019) though not in others (Miranda et al., 2012). Bonnasoli and colleagues found a high rate (59.3%) of yeast colonisation on hands of both healthcare workers as well as community members with no prior exposure to the healthcare setting (Bonassoli et al., 2005). It is this presence on human hands that may lead to the transmission in paediatric and neonatal wards. Besides nosocomial acquisition, of concern is the detection of this opportunistic pathogen in fruits and vegetables (Glushakova et al., 2023).

Studies specifically looking for risk factors of *C. parapsilosis* candidaemia have found the risk factors to be similar to other *Candida* species (King et al., 2017b). Risk factors associated with *Candida* infections include prematurity, low birth weight, prolonged use of antibiotics, indwelling central or umbilical venous catheters (Benjamin et al., 2010, Garzillo et al., 2017) including peritoneal dialysis catheters (Roy et al., 2021), parenteral nutrition and mechanical ventilation (Qi et al., 2018b, Juyal et al., 2014, Miranda et al., 2012). Liu and colleagues found maternal *Candida* vaginitis, repeated gastrointestinal surgery and prior use of antibiotics, especially glycopeptide antibiotics, to be associated with candidaemia in neonates (Liu et al., 2015).

Patients with hematological malignancy and transplants recipients are also at high risk of fungal infection (Ducassou et al., 2015). The risk is increased during the induction of chemotherapy in children with acute myeloid leukemia (King et al., 2017a). Colonisation before the solid organ transplant usually increases the risk (Danziger-Isakov et al., 2008). Gastrointestinal pathology that may be congenital, such as atresia or stenosis, and diseases, such as necrotising enterocolitis and intestinal obstruction, are common in neonates making them more at risk, because of the increased likelihood to undergo surgery and receive parenteral nutrition (Liu et al., 2015).

1.3.3 Virulence factors

Candida albicans virulence factors are well studied, compared to *C. parapsilosis sensu lato*. Biofilm formation and secretion of hydrolytic enzymes have been identified in both species (Pammi et al., 2013), however, different species of *C. parapsilosis senso lato* produce hydrolytic enzymes and biofilms at varying rates (Ataides et al., 2020, Németh et al., 2013). Furthermore, similar to *C. albicans*, *C. parapsilosis* produce eicosanoids which enhances colonisation (Mendoza et al., 2021).

Candida parapsilosis sensu stricto is generally regarded as the most virulent species. In the *Caenorhabditis elegans* infection model, *C. parapsilosis sensu stricto* was the highly virulent species, followed by *C. orthopsilosis* then *C. metapsilosis* (Brilhante et al., 2018). Brilhante and colleagues observed phospholipase and protease production in 55.1 % of *C. parapsilosis* sensu stricto, 42.1 % of *C. orthopsilosis* and 33.3 % of *C. metapsilosis* isolates (Brilhante et al., 2018). In contrast to the study by Brilhante and colleagues, the study by Souza and colleagues found all three species to exhibit similar virulence (Souza et al., 2018a). There was no statistically significant difference in the infection groups caused by the *C. parapsilosis* species complex (Souza et al., 2018a).

Biofilm formation

A biofilm is a complex layer of cells enclosed in extracellular matrix, that adheres to surfaces, providing the organism with protection from the host's defences, as well as antifungal activity (Borges et al., 2018). Biofilm formation follows adherence to medical devices. This may also be preceded by colonization of health care workers' hands, especially where there is a breach in infection prevention and control (IPC) measures. Biofilms promote persistent colonization leading to infections as well as increased resistance to antimicrobials and is the most remarkable factor in the virulence of *C. parapsilosis* (Branco et al., 2021).

Candida parapsilosis biofilms are less complex compared to *C. albicans* (Kuhn et al., 2002). The production of biofilms by *C. parapsilosis* species differs amongst the species, with majority of *C. parapsilosis sensu stricto* being biofilm producers. Cakir and colleagues found 75.6% of *C. parapsilosis sensu stricto* strains to be biofilm producers (Cakir et al., 2021) while da Silva and colleagues found 73% of *C. parapsilosis sensu stricto* strains to produce biofilms (da Silva et al., 2015). In the study by de Paula Menezes and colleagues, one isolates of the 11 *C. parapsilosis sensu stricto* did not produce biofilms while majority were able to form biofilms (de Paula Menezes et al., 2020). Of note, majority of the isolates in these studies are *C. parapsilosis sensu stricto*. This serves as a potential area for further research regarding other cryptic species. Da Silva and coworkers failed to establish a link between biofilm production and the specimen site nor the secretion of hydrolytic enzymes in the *C. parapsilosis* complex species (da Silva et al., 2015). Interestingly, strong biofilm forming strains of *C. parapsilosis* complex have a higher mortality rate (71%) than weak biofilm producing strains (28%) (Tumbarello et al., 2007).

Hydrolytic enzyme secretion

The secreted hydrolytic enzymes namely, phospholipases, aspartyl proteinases and lipases, facilitate adherence and tissue penetration and hence invasion (Paula-Mattiello et al., 2017, Pandey et al., 2018). Results regarding the variable hydrolytic enzyme activity in the three *C. parapsilosis* cryptic species is evident in literature.

Phospholipases refer to a diverse enzymes that are able to hydrolyze ester linkage in glycerophospholipids (Ghannoum, 2000). *Candida parapsilosis* secrete phospholipases, but their role in pathogenicity is not as clear as in *C. albicans* although they are thought to disrupt host cell membrane (Tóth et al., 2019a). As mentioned earlier, the secretion of phospholipases differ among the three cryptic species with some studies showing no production by *C.*

metapsilosis and C. orthopsilosis (Cakir et al., 2021, Ataides et al., 2020, da Silva et al., 2015).

Compared to *C. albicans, C. parapsilosis* produces less aspartyl proteinases (Trofa et al., 2008). Of the three aspartyl proteinases encoding genes (*SAPP1-3*), *SAPP1 and SAPP2* are mainly associated with evasion of the immune system (Tóth et al., 2019a). Similar to the phospholipase production, production of proteases differ among the three cryptic species (Cakir et al., 2021).

Lipases are enzymes that are widely distributed and can naturally catalyze the hydrolysis of triacylglycerols such as in fats and oils into free fatty acids and glycerol (Ruba et al., 2019). They contribute to the invasion of *C. parapsilosis,* especially products of the lipase encoding genes 1 and 2 (*LIP1 and LIP2*) (Renata et al., 2017). The absence of secreted lipases result in decreased pathogenicity and the inability to survive in macrophages (Tóth et al., 2014) indicating their role in the pathogenesis. This finding was also demonstrated by Gacser and co-workers through lipase locus deletion (Gácser et al., 2007).

In a study from Brazil*,* the majority (76%;22/29) *of C. parapsilosis sensu stricto* and *C. metapsilosis* isolates from fomites in hospitals produced proteinases, while only three were able to produce phospholipases (Paula-Mattiello et al., 2017). Contrary to the above, Ataides and colleagues found protease activity in all *C. parapsilosis sensu stricto* and *C. orthopsilosis* strains, with 50% of *C. metapsilosis* strains not showing any activity (Ataides et al., 2020). The above could have been as a result of the number of *C. orthopsilosis* strains (four) used in the study compared to *C. parapsilosis sensu stricto* (78) and *C. orthopsilosis* strains (five). The strains causing invasive diseases had higher proteinase activity in general from this study (Ataides et al., 2020). Similarly, phospholipase and esterase activity also differ among the three species (Cakir et al., 2021). Whether different habitats have an influence on the production of the enzymes is not clear, but species biological variations is thought to play a role as seen with *C. dubliniensis* and *C. albicans* (Ge et al., 2011).

Eicosanoid production

Eicosanoids, such as prostaglandins and leukotrienes, are immunomodulatory lipid metabolites derived from arachidonic acid, released from cell membranes by phospholipase (Tanmoy et al., 2018). Eicosanoids are produced by both mammalian cells and pathogenic fungi and are important for modulating cytokine signaling and anti-inflammatory activities (Chakraborty et al., 2019). Pathways leading to the synthesis of eicosanoids in mammals are different from pathogenic fungi. *Candida parapsilosis* is able to produce eicosanoids in the presence of arachidonic acid via the OLE2 independent pathway, with prostaglandin E_2 (PGE_2) and prostaglandin D_2 (PGD_2) being the most common (Chakraborty et al., 2019). Three homologous genes (*CPAR2_603600, CPAR2_807710, CPAR2_800020)* to a multicopper oxidase encoding gene *FET3*, an acyl-CoA oxidase (*POX1-3*) and an acyl-CoA thiolase (*POT1*) are important in the biosynthesis of eicosanoids by *C. parapsilosis* (Chakraborty et al., 2018, Mendoza et al., 2021). Deletion of these genes leads to a decrease in virulence when compared to the wild type (Chakraborty et al., 2018, Chakraborty et al., 2019).

1.3.4 Pathogenesis

The use of systemic antibiotics, the breach in the defense system and the immunosuppression promotes invasive candidiasis (Pappas et al., 2018). The paediatric population, especially neonates, are prone to invasive candidaemia due to their compromised skin integrity, their susceptibility to gastrointestinal infection and the need for invasive medical devices that breach the first line defenses (Trofa et al., 2008). While the broad-spectrum antibiotics deplete the normal microbiota that would normally prevent the *Candida* species overgrowth, the breach in the defense mechanisms promotes translocation to the bloodstream and the immunosuppression drives dissemination of the yeast to other organs (Pappas et al., 2018).

Contaminated hands and environment are the main sources in exogenous acquisition of *C. parapsilosis* (Delfino et al., 2014, da Silva et al., 2021) which is aggravated by the breach in IPC measures. *Candida parapsilosis* disease may occur with or without prior colonization and the contaminated external sources are responsible for the latter (Trofa et al., 2008). With regards to prior colonization, the skin (Trofa et al., 2008) and the gastrointestinal tract are the most colonized sites (Mesquida et al., 2023).

Adhesion is usually the first step involved in pathogenesis of the disease followed by invasion, evasion and localization or dissemination (dos Santos and Ishida, 2023) (Figure 3). *Candida parapsilosis* virulence factors are involved in the pathogenesis of invasive candidiasis. The agglutinin like sequence proteins (CPAR2_404800 and CPAR2_404780) assist with adhesion (Satala et al., 2023). Hydrolytic enzymes, including lipases, facilitate invasion (Renata et al., 2017), whereas proteinases contribute to evasion of the immune system (Tóth et al., 2019a). In some infections, the disease is localized while with others hematogenous dissemination to distant sites does occur such as the eye, brain, etc.

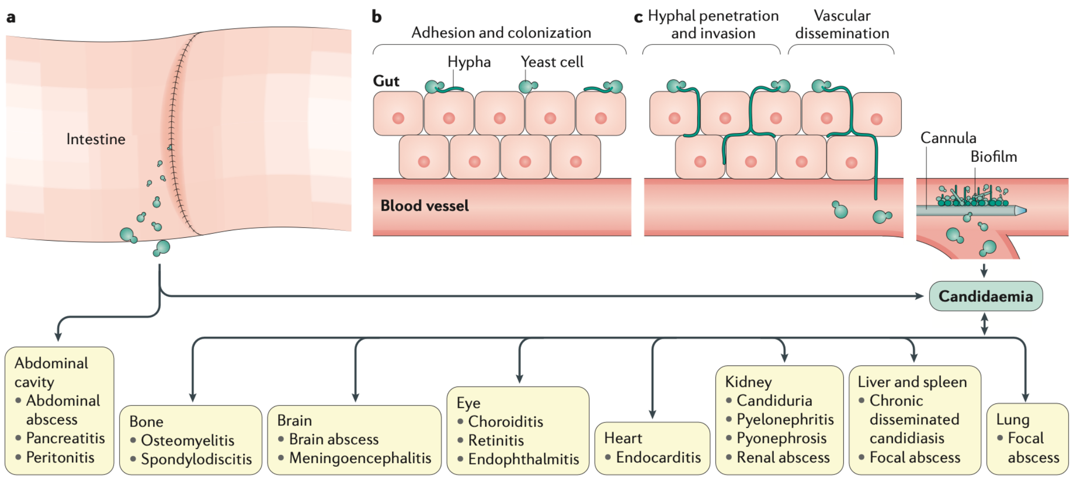


Figure 3: Pathogenesis of invasive candidiasis (Pappas et al., 2018).

The ability of *C. parapsilosis* to colonise skin, proliferate in glucose containing solutions as well as adhering to plastics promotes line related infections (Liu et al., 2015, Clancy and Nguyen, 2013). These infections present with higher blood burdens and thus detected earlier than *C. glabrata* (Pfeiffer et al., 2011). The lower *C. glabrata* burden is attributed to the liver filtration of *Candida* cells translocated across the gastrointestinal mucosa (Nguyen et al., 1996). In addition to line related infections, neonates usually receive lipid rich parenteral nutrition which promotes adhesion of *C. parapsilosis* to host cells and lysis of the competing microflora (Trofa et al., 2011) thus exposing the neonates to the invasiveness of this lipase secreting pathogen.

1.3.5 Clinical manifestations

The clinical manifestations of *C. parapsilosis* remains a challenge because signs and symptoms are non-specific and clinical manifestations resemble those of other *Candida* species. Symptoms such as severe sepsis, septic shock, disseminated candidemia (Yao-Sheng et al., 2023) to other sites leading to meningitis, peritonitis, ocular infections, otomycosis, onychomycosis, vulvovaginitis and urinary tract infections have been reported (Souza et al., 2018a). Shunt related infections (Bagheri et al., 2010) can occur as well as ocular manifestations (Li et al., 2016), endocarditis (Jones et al., 2002), and the involvement of the musculoskeletal system (Han et al., 2022, Gamaletsou et al., 2016).

In addition, a rare knee joint infection by *C. parapsilosis* was reported in an adult patient with no predisposing factors (Wang et al., 2019). Rare sites of dissemination in paediatric patients include hepatosplenic, cardiac and skeletal (King et al., 2017a).

A case-controlled study by Liu and colleagues (2015) found non-specific symptoms such as fever, abdominal pain, vomiting and oedema to be amongst the main clinical features in neonates with candidaemia (Liu et al., 2015). The study also found neonates with candidaemia to have high rates of shock, gastrointestinal and pulmonary haemorrhage as well as multiple organ failure. In addition, procalcitonin levels of >2 ng/mL, hemoglobin levels of <10 g/l, C-reactive protein levels of >50 mg/l, thrombocytopenia, liver dysfunction, hypoproteinemia were found to occur in the candidaemia group (Liu et al., 2015).

1.3.6 Laboratory diagnosis

Diagnosis of candidiasis can be divided into culture and nonculture methods. Culture methods include phenotypic identification tests that require cultured isolates. These include an automated VITEK®2 YST system (bioMérieux Inc, Marcy l'Etoile, France), matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) (bioMérieux Inc, Marcy l'Etoile, France) or (Bruker Daltonics, Germany), the analytical profile index (API) 20C (bioMérieux Inc., Hazelwood, Mo) or API 32C (bioMérieux Inc, Marcy l'Etoile, France).

Non culture methods include the serology based biomarkers such as 1,3-β-D-glucan (BDG) which is present in all *Candida* species, molecular assays such as multiplex polymerase chain reaction (PCR) methods as well as a novel T2 magnetic resonance *Candida* species panel which involves both the PCR and the magnetic resonance (Beyda et al., 2018, Clancy and Nguyen, 2018b).

Correct identification of the *C. parapsilosis sensu lato* species followed by susceptibility testing is important because the species are showing an increasing resistance to azole antifungals. Mortality and morbidity as a results of invasive *C. parapsilosis* will be decreased by early identification and early initiation of the appropriate therapy.

Culture methods

The recommended gold standard diagnostic test for candidaemia is blood cultures (Clancy and Nguyen, 2013). Automated microbial detection systems, such as BD Bactec (Becton Dickinson, USA) (Cherkaoui and Schrenzel, 2022) and BacT/Alert (bioMérieux Inc, Marcy l'Etoile, France) systems, are used in diagnostic laboratories (Horvath et al., 2004). The automated microbial detection systems use internal CO_2 sensors to detect the metabolic activity of microbes (Marais et al., 2004). Increased fluorescence is evidence for microbial growth with BD Bactec, whereas the BacT/Alert uses colour change, which is visualised at the bottom of the bottles (Reisner and Woods, 1999).

As indicated by Chibabhai, blood is inoculated in the anaerobic and aerobic bottles (BacT/Alert bottles, bioMerieux) for adults or a paediatric blood culture bottle (BacT/Alert bottles, bioMerieux) and incubated in the automated microbial detection system (Chibabhai, 2022). The optimal growth is usually achieved from the aerobic bottle since the *Candida* species are aerobic organisms (Horvath et al., 2004). When the two systems namely; BD Bactec 9240 and BacT/Alert were compared for aerobic and anaerobic media, the BacT/Alert system performed better than the Bactec in the detection of *Candida* species by detecting growth of 90% (135 of 150) of *Candida* pathogens, while Bactec detected 66% (100 of 150) (Horvath et al., 2004). Studies, comparing *Candida* species detection from paediatric bottles using the two above mentioned automated systems are lacking.

Isolation of *Candida* species from blood cultures is specific, although the sensitivity is low at 50% (Clancy and Nguyen, 2013). This poor sensitivity is even worse in children, as very low volumes are collected for laboratory investigations. Blood cultures will not detect *Candida* species in patients with deep-seated infections without candidaemia (Clancy and Nguyen, 2018a). In such patients, the representative specimen such as tissue or fluid aspirate from the infected site is required.

The selective sabouraud dextrose media is used if the fungal culture is requested or yeast, as evidenced by the budding oval yeast cells, seen on direct microscopy of the specimen (Kukhar et al., 2020). Switching of *C. parapsilosis* between different colony morphologies have been reported (Gómez-Molero et al., 2020). The colonies of *C. parapsilosis* on the selective sabouraud dextrose media appear white, creamy shiny smooth or may be wrinkled (Trofa et al., 2008). Another selective and differential medium, CHROMagar Candida Medium, which relies on the colour change to identify specific *Candida* species (Wang et al., 2019), whereby brown colonies indicate *C. parapsilosis* (Liu et al., 2015) may be used. Other media such as the cornmeal may be used to visualize the pseudohyphae of *C. parapsilosis* (Cordeiro et al., 2018).

Identification using the cultured colonies performed by the Vitek®2 yeast (YST) relies on the biochemical profile of the organisms on the database and the API system relies on assimilation of carbohydrates (Taverna et al., 2013). The Vitek®2 system has a yeast card for identification as well as a card for susceptibility testing (Graf et al., 2000). The possibility of identifying *C. parapsilosis* as *C. famata* is one of the downfalls of Vitek®2 (Kim et al., 2015, Sanguinetti et al., 2007). Identification of the organisms by this system takes about 15 hours (Graf et al., 2000) whereas susceptibility results mean is 16 hours (Cejudo et al., 2010). The Vitek®2 YST antifungal susceptibility testing (AST) card is used to test for the azoles: fluconazole and voriconazole, the polyene: amphotericin B, the echinocandins: caspofungin and micafungin and lastly the pyrimidine analogue: flucytosine where the spectrophotometry is used to determine the growth (Borghi et al., 2010).

When compared to the broth microdilution which is the gold standard for antifungal susceptibility testing (CLSI, 2008), the categorical agreement was between 80% up to above 95% (Cejudo et al., 2010, Borghi et al., 2010). The Sensititer YeastOne is another method for antifungal susceptibility testing and has been compared to the recommended CLSI BMD method. The Sensititer YeastOne was found to be less laborious with comparable results to the recommended BMD method except for a few drugs against certain *Candida* species (Altinbaş et al., 2020).

The MALDI-TOF MS is another rapid identification method that identifies bacteria and fungi. Species identification is accomplished by mass spectrum analysis of the crude cell extract within less than 10 minutes for the yeast (Zhao et al., 2018, De Carolis et al., 2014). The principle of the test is based on the use of laser to ionise the fungal proteins, creating a spectrum that is subsequently compared to the database (Natacha et al., 2022). The National Health Laboratory Services (NHLS) has recently introduced the MALDI-TOF MS (bioMérieux Inc, Marcy l'Etoile, France) in some high work volume laboratories. The test accurately identifies *C. parapsilosis* without differentiation of the three cryptic species. De Carolis and colleagues investigated the possibility of using the MALDI-TOF MS (Bruker Daltonics, Germany) as a potential method to identify the three cryptic species and concluded that through modification such as addition of mass spectrums for species in the database, the identification can be achieved (De Carolis et al., 2014). A review article by Chen and colleagues showed that most studies that looked at the accuracy of MALDI TOF MS found the accuracy to be above 90% (Chen et al., 2021).

The latest addition to the rapid identification methods is a BioFire® FilmArray® (bioMérieux Inc, Marcy l'Etoile, France) syndromic automated nested multiplex real-time PCR assay that is US FDA approved with the ability to identify *C. parapsilosis* from blood culture that flagged positive (Kang et al., 2020). The assay has a turnaround time of one hour, thus promoting early antifungal therapy initiation (Kang et al., 2020, Jones et al., 2022). Rhoads and colleagues recorded a sensitivity of 100% and specificity of 99.9% for the BioFire® bloodculture panel identification of *C. parapsilosis* (Rhoads Daniel et al., 2023).

The above-mentioned tests cannot differentiate the *C. parapsilosis sensu lato* species due to similar colony morphology and biochemical profiles. Cordeiro and co-workers (2018) studied the phenotypic driven strategies and found that *C. orthopsilosis* grows poorly at pH 7 and in 15% NaCl, compared to the other two species (Cordeiro et al., 2018). This method is however not sufficient to conclude on the identification of the three species.

Nonculture methods

Biomarkers exist as adjuncts to the diagnosis of invasive fungal infections. These include galactomannan (GM) for *Aspergillus* species and the 1,3-β-D-glucan (BDG) which is present in all *Candida* species (Huppler et al., 2017). There are few studies on BDG utility in paediatrics and the optimal cut-off for the BDG assay in paediatric patients is not as well defined as in adults (Ferreras-Antolin et al., 2022). The use of this biomarker in children is complicated by the detection of the higher BDG levels in some healthy children (Smith et al., 2007). However, the small amount of blood required for the test makes it more favorable for use in the neonatal population (Ferreras-Antolin et al., 2022).

In paediatric patients with hematogenous *Candida* meningoencephalitis (HCME), cerebrospinal fluid (CSF) BDG levels are useful. The levels correlate well with the infection and decrease with appropriate therapy (Walsh et al., 2019). The utility of these biomarkers were extensively studied in adults and extrapolation of the data to children should be considered with care (Ziccardi et al., 2015). The test also has low sensitivity in oncology patients who received preemptive echinocandins (Cohen et al., 2020) due to the drug inhibiting the BDG synthase (Guitard et al., 2016). While factors such as antibiotics use and the presence of bacteremia cause false positive results, the true positive results are not specific to candidiasis since BDG is also present in the cell wall of other fungi (Clancy and Nguyen, 2013). Furthermore, *C. parapsilosis* produces low levels of BDG (57 pg/mL) and this might reduce the sensitivity of the BDG assay (Mikulska et al., 2024).

The T2 magnetic resonance (T2MR) *Candida* species panel (T2 Biosystems, Lexington, MA) is one of the novel tests cleared by the United States Food and Drug Administration (U.S. FDA) for direct rapid diagnosis of invasive candidiasis (Hamula et al., 2016). This non culture polymerase chain reaction (PCR) cartridge based and nuclear magnetic resonance (NMR) system is used to identify *C. albicans* and the four NAC species, namely *C. parapsilosis*, *C. glabrata*, *C. tropicalis* and *C. krusei* (Clancy and Nguyen, 2019). The sensitivity of the test for *Candida* species ranges between 91% to above 95% (Krifors et al., 2022, Turner et al., 2017). About 3-5 mL of the whole blood is pipetted directly into the instrument and the results are available within five hours (Walsh et al., 2019).

Although it has been extensively studied on adults, few studies are conducted on paediatric patients with low volume specimens. Children between five and seven years were included in the study conducted by Hamula and colleagues (Hamula et al., 2016). The panel overcomes the challenges of small blood samples from paediatric patients, as it can efficiently diagnose candidaemia when small blood volumes are used. In addition, the test was shown to overcome other challenges such as low sensitivity , due to the patient having started antifungal treatment before specimen collection, as well as the prolonged turnaround time due to the incubation of the blood culture bottles (Lucignano et al., 2022). This test cannot differentiate between the three cryptic species (Mylonakis et al., 2015).

Typing methods

Different molecular methods exist to differentiate the three species, identify resistance genes, and study the genetic relatedness during the outbreaks. Microsatellite length polymorphism (MLP) is commonly used worldwide (Wang et al., 2016) and is highly reproducible, discriminant (99.9%) and easily automated (Cordeiro et al., 2018). The method is based on amplification of di- to pentanucleotide tandem repeats located at several loci in the genome (Stefaniuk et al., 2016). Four to six different species specific markers (CP1, CP4, CP6, B2, 3A, 3B, 3C, 6A, 6B, 6C) have been used in literature (Wang et al., 2016, Barbedo et al., 2017, Hilmioğlu-Polat et al., 2018).

Tavanti and coworkers used the multilocus sequence typing (MLST) to name the three species (Tavanti et al., 2005). The *Ban*I restriction pattern of the secondary alcohol dehydrogenase (SADH) gene allows the discrimination by generation of two fragments for *C. parapsilosis sensu stricto*, four fragments for *C. metapsilosis*, and no restriction site for *C. orthopsilosis* (Tavanti et al., 2005).

Other methods such as the randomly amplified polymorphic DNA (RAPD), the restriction fragment length polymorphism (RFLP) have been used in various studies (de Toro et al., 2011, Romeo et al., 2012, Pakshir et al., 2018). Several studies have used RAPD for *C. parapsilosis* speciation (Badali et al., 2017, Sakita et al., 2017) and for strain typing (Tay et al., 2009). The VII-1 type has been described as the most recovered from patients (van Asbeck et al., 2008). This method is less labour intensive than RFLP, less costly and can be performed on unknown DNA sequences (Alanio et al., 2017). A single short primer (Tay et al., 2009) or multiple primers (Alanio et al., 2017) are used for amplification.

Restriction fragment length polymorphism is based on deoxyribonucleic acid (DNA) digestion by restriction enzymes, southern blotting and radiolabelled probe hybridization (Wang et al., 2016). Since the discriminatory power of RFLP depends on the digestion profiles and number of used restriction enzymes, this method may not identify all yeasts (Karimi et al., 2015, Alanio et al., 2017). The method is also labour intensive and not reproducible between different laboratories (Alanio et al., 2017). In a study conducted by Thomaz and co-workers (2018) RFLP was used to identify *C. parapsilosis* species, followed by MLP to genotype the strains in an outbreak. Similarly, Barbedo and colleagues (2016) were able to quickly and accurately identify *C. parapsilosis* using RFLP.

Magobo and coworkers identified the *C. parapsilosis* using sequence analysis of the internal transcribed spacer (ITS) domain of the multi-copy ribosomal RNA gene and were able to show the dominant genotypes in certain hospitals using this method. Using Microsatellite/STR genotyping method the study showed molecular evidence of undetectable outbreaks and also evidence that certain clusters were more prone to fluconazole resistance (Magobo et al., 2017). Internal transcribed space sequencing was proposed to be the primary fungal barcode marker (Schoch et al., 2012). However, the D1/D2 sequencing seemed to be more successful than ITS region sequencing in a study by Taverna and colleagues where 12 isolates yielded an illegible ITS chromatograph (Taverna et al., 2013).

Whole genome sequencing is important in outbreak settings as it can detect the clusters (Guinea et al., 2021) as well as resistance genes (Daneshnia et al., 2023b). This methodology is however limited by costs for routine use in the diagnostic laboratories.

1.3.7 Management of infection

The appropriate use of antifungal agents, source control and the strict adherence to infection prevention and control measures are important in the management of fungal infections (Peixoto et al., 2023, Han et al., 2022). There are main classes of antifungals include the azoles, polyenes and echinocandins.

Azole drugs include the most commonly used fluconazole and voriconazole. They act by inhibiting ergosterol synthesis and have been used extensively in paediatric patients for empiric therapy and for fluconazole-susceptible isolates

(Lee et al., 2023). Mechanisms of resistance include drug target over expression, drug target alteration and efflux pumps (Berkow and Lockhart, 2017). Majority of resistance is however driven by alterations in the *ERG11* gene, mainly the Y132F amino acid substitution (Escribano and Guinea, 2022). Other triazoles, such as itraconazole, have not been well studied for invasive candidiasis (Pappas et al., 2016). Resistance to fluconazole among the *C. parapsilosis* is reported to be on the rise in different countries such as India (Singh, 2017), Brazil (Thomaz et al., 2022), United States of America (Moudgal et al., 2005) including SA (Govender et al., 2016, Magobo et al., 2017).

The polyene group of antifungals include amphotericin B deoxycholate and the liposomal formulations. The use of amphotericin B as an empiric drug is important, especially with the increasing fluconazole MICs observed amongst the *C. parapsilosis* isolates. Amphotericin B, act by binding to ergosterol, creating pores in the cell wall, eventually leading to leakage of ions and cell death. Mechanisms of resistance is rare and may be as a result of mutations in the ergosterol biosynthesis genes (Lee et al., 2023).

Echinocandins group includes caspofungin, micafungin and anidulafungin. They act by inhibiting 1,3-B- D-glucan synthesis in the fungal cell wall and their resistance is mainly by mutations in the drug target gene, *FKS1* (Lee et al., 2023). The use of echinocandins in the paediatric population is currently extensively researched. Both caspofungin and micafungin are FDA approved for use in children (Pappas et al., 2016). Furthermore, they have less side effects than amphotericin B (Mora-Duarte et al., 2002). Micafungin is well tolerated by neonates with invasive candidiasis and studies are forthcoming regarding the use of higher dosage to achieve good outcomes (Thomaz et al., 2018). Elevated caspofungin MICs have been reported against *C. parapsilosis* and although the drug is effective against biofilms *in vivo*, the effectiveness *in vitro* is questionable (Devrim et al., 2016). There is little information about anidulafungin, as more studies are needed regarding the safety of its use in neonates with invasive candidiasis.

Like the situation with antibacterial agents, there are few novel antifungal agents in the pipeline. A first member of the “gepix” antifungal groups with a novel mechanism of action called manogepix/fosmanogepix is currently in clinical development for treatment of invasive fungal infections (Covel et al., 2019, Hodges et al., 2023). The administration of this novel antifungal is both orally and intravenously with a broad-spectrum of activity against yeasts, moulds and multidrug resistant fungi (Hodges et al., 2023).

Studies have shown differences in antifungal susceptibility of the three related *C. parapsilosis sensu lato* species (van Asbeck et al., 2008, Alanio et al., 2017, Trobajo-Sanmartín et al., 2018). *Candida parapsilosis sensu lato* is not inherently resistant to azoles, polyenes or echinocandins, the drugs commonly used for invasive candidiasis. However, there is documented increase in minimum inhibitory concentration (MIC) of the antifungal drugs worldwide in both paediatric and adult populations (Thomaz et al., 2018).

Treatment of invasive candidiasis has changed dramatically in the past. Fluconazole and amphotericin B have been used for the treatment of candidiasis for many years. The Infectious Diseases Society of America (IDSA) guidelines recommend echinocandins as the first-line empiric therapy for fungal infections (Pappas et al., 2016). For neonatal candidiasis, amphotericin B and fluconazole are acceptable drugs for invasive candidiasis in the absence of echinocandins. Moreover, the risk of nephrotoxicity associated with amphotericin B does not seem to be high in neonates (Castro et al., 2023a).

Candida parapsilosis is transmitted by contact and therefore, intensified contact precautionary measures should be in place to limit its spread in the hospital wards or units. Prevention measures include hand hygiene, daily cleaning or disinfection of the surrounding area and medical equipment, terminal cleaning and cohorting of patients. Routine screening of patients admitted in ICU (Fekkar et al., 2023), education, cohorting, environmental screening and cleaning (Qi et al., 2018b) have been implemented to stop the spread of the organism in the wards. In low to middle income countries, hand hygiene may be the only option in reducing health care associated infections due to few isolation rooms (Haque et al., 2020). Alcohol hand rubs are advised as part of hand hygiene to prevent the spread of *C. parapsilosis* (Fekkar et al., 2023) but have been used in other outbreaks with no success against fluconazole-resistant *C. parapsilosis*

(Thomaz et al., 2022) suggesting that they may not be effective against the resistant strains of *C. parapsilosis*.

Studies on disinfectants resistance or antifungal action against *Candida* species have focused more on *C. albicans*. Studies directed specifically at *C. parapsilosis* are only starting to come up. Chlorine based disinfectant (biocide) is used at the study setting for disinfection of the surfaces. Abdolrasouli and colleagues demonstrated that chlorine-releasing compound called Chlor–clean, effectively killed *Candida* species at concentrations used in clinical practice (1000ppm) but failed to kill *C. parapsilosis* American type culture collection (ATCC) 22019 strain (Abdolrasouli et al., 2017). Another study indicated that the standard current biocide (sodium hypochlorite, 500 ppm or 0.5 g/liter) might not be effective to inhibit *Candida* biofilm production as compared to other disinfectants, such as acetic acid and hydrogen peroxide (Pires et al., 2013). A study by Castro and colleagues showed that the most effective sanitizers against *C. parapsilosis* biofilms are based on quaternary ammonium compounds (QACs) and orthophthalaldehyde (OPA) (Castro et al., 2023a). A greater focus on *C. parapsilosis* will be important especially in this era of disinfectants resistant and azole-resistant *C. parapsilosis*.

1.4 PROBLEM STATEMENT

Several studies indicate that the prevalence of *C. parapsilosis* cryptic species to differ according to the setting and age. Furthermore, some studies report that the extent of production of the virulence factors of the three cryptic species differ with *C. parapsilosis sensu stricto* being the most common and virulent, although there are contradictions in literature. Most studies are however in agreement that *C. parapsilosis* invasive disease is increasing among the paediatric population, especially the neonates with an increase in the triazole resistance.

Majority of *C. parapsilosis sensu lato* isolates cultured at Universitas Medical Microbiology laboratory are from neonatal wards. These are patients with risk factors such as prematurity, very low or low birth weight, exposure to broad spectrum antibiotics, invasive devices and total parenteral nutrition. Speciation of *C. parapsilosis sensu lato* species at Universitas Medical Microbiology laboratory has not been performed before. It is therefore not known which of the three *C. parapsilosis sensu lato* species are circulating in the neonatal wards. Differentiation of the circulating species will help to better understand the different virulence factors as well as antifungal susceptibility pattern or behaviour of the circulating strain(s). This might further assist in explaining why *C. parapsilosis* is outranking *C. albicans* in this population.

1.5 RESEARCH AIM

The aim is to identify the species and specific strains of *C. parapsilosis sensu lato* cultured from neonatal and paediatric patients at Universitas Academic Hospital and to determine their virulence potential. The data obtained and the clinical outcome of patients will be correlated.

1.6 RESEARCH OBJECTIVES

a) Collection of all stored *Candida parapsilosis sensu lato* blood culture isolates sent for routine laboratory testing and identified as *C. parapsilosis* using Vitek®2 (bioMérieux Inc, Marcy l'Etoile, France).
b) Species identification/differentiation using D1/D2 region sequencing.
c) Extraction and analyses of Vitek®2 (bioMérieux Inc, Marcy l'Etoile, France) antifungal susceptibility data, patients' demographics from National Health Laboratory Service (NHLS) TrakCare system and clinical information from the hospital clinical notes to obtain:
    - Gestational age
    - Birth weight
    - Congenital lethal conditions
    - Medical devices
    - Medication
    - Outcome (Mortality before discharge/discharged)
d) Determination of isolates' susceptibility to chlorine based disinfectant.
e) Determination of *in vitro* production of virulence factors:
    - Biofilm formation
    - Hydrolytic enzymes (phospholipase, lipase, proteases)
    - Prostaglandin E_2 production

f) Determination of relative virulence in the *Caenorhabditis elegans* infection model.
g) *Candida parapsilosis* strains whole genome sequencing to determine the relatedness and possible genetic determinants of drug resistance.

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CHAPTER 2: *IN VITRO* CHARACTERIZATION OF VIRULENCE FACTORS

2.1 ABSTRACT

Introduction:

Candida parapsilosis is one of the non-*Candida albicans* species affecting neonates because of the many risk factors neonates have such as being pre-term, having low birth weight, being on parenteral nutrition, having gastrointestinal pathology, the use of systemic antibiotics and invasive medical devices. The organism produces varying degrees of virulence factors.

Aim:

This study aims to identify the species and specific strains of *C. parapsilosis sensu lato* cultured from neonatal and paediatric patients at Universitas Academic Hospital and to determine their virulence potential. Correlation between the strains' data and the clinical outcome of patients will be examined.

Methodology:

Methodology included species identification using D1/D2 sequencing. The clinical records were reviewed, information of the time to positivity, species identification, antifungal susceptibility testing results and patients' demographics were also retrieved from TrackCare. The chlorine-based disinfectant, namely QualiClean (QualiPharm, New Germany) used in the hospital setting at 1000 ppm for surface disinfection was tested against all the 20 strains to determine their susceptibility to the disinfectant. Crystal violet assay was performed to determine the biomass of the respective biofilms. For hydrolytic enzymes secretion, tributyrin agar was used for lipase activity, yeast carbon base bovine serum albumin agar for protease activity and the sabauraud dextrose agar (SDA) supplemented with 8% egg yolk for phospholipase activity. The prostaglandin E_2 production was determined by using the enzyme-linked immunosorbent assay (ELISA; Cayman Chemicals, Ann Arbour, USA) according to the manufacturer's instructions. The biological

method described by Brenner (Brenner, 1974) was followed for *in vivo* relative virulence in *Caenorhabditis elegans*.

Results:

The strains were identified as *Candida parapsilosis sensu stricto.* Ninety percent of blood cultures had a time to positivity of 48 hours with 80% of the strains showing resistance to fluconazole as well as intermediate resistance to voriconazole. For 70% of the strains, resistance to fluconazole and intermediate resistance to voriconazole was observed. In addition, the QualiClean disinfectant did not effectively inhibit any of the strains. Fifty-five percent of the patients were from neonatal intensive care unit followed by 35% from neonatal high care and 10% from paediatrics wards. Eighty percent of the patients were males. Forty-one percent of the patients were categorized as very low birth weight and 55% delivered via caesarian section. The study population had multiple risk factors including the presence of invasive devices, total parenteral nutrition, gastrointestinal pathology, and the use of systemic antibiotics. The deaths were recorded for 47% of the patients. The strains produced biofilms, proteases, phospholipases, and prostaglandin E_2 in varying quantities. The three *C. parapsilosis* strains tested for their virulence in *C. elegans*, killed the nematodes rapidly when compared to the *C. albicans* ATCC SC5314 strain.

Conclusion:

The results imply that the *C. parapsilosis sensu stricto* is the circulating species with majority of isolates resistant to fluconazole, suggesting that fluconazole should not be used as the empiric therapy among this population. All strains were virulent and resisted the action of the disinfectant. The contact time will need to be increased or alternatively, a different disinfectant should be used. Hand hygiene is also to be intensified.

2.2 INTRODUCTION

Invasive fungal infections remain a leading cause of morbidity and mortality among preterm (< 40 weeks gestation) and low birth weight (<1500g) neonates (Zhou et al., 2023). These neonates have under-developed epithelial barriers, have been exposed to broad-spectrum antibiotics, have been on parenteral nutrition and frequently undergo invasive procedures (Weimer et al., 2022, Wang et al., 2023).

Blood cultures are still commonly used for the diagnosis of invasive fungal infections, despite their low sensitivity of about 50% (Fang et al., 2023). The time to positivity may be influenced by the fungal load, particularly since a small amount of blood is obtained from the neonates, subsequently leading to delayed positive blood cultures and the start of appropriate therapy (Wilson, 2020). In addition to yeasts being slow growers, prior antifungal use may further affect the recovery of fungal species, hence it is advisable to obtain cultures before initiating therapy (Rhodes et al., 2017). The VITEK®2 (bioMérieux Inc, Marcy l'Etoile, France) system or the matrix-assisted laser desorption/ionization time of flight (MALDITOF) (bioMérieux Inc, Marcy l'Etoile, France or Bruker Daltonics, Germany) analysers are the two systems currently used in the National Health Laboratory Services (NHLS) for the identification of pathogens, while only the VITEK®2 is used for susceptibility testing.

Candida parapsilosis sensu lato is a complex comprising *of C. parapsilosis sensu stricto, C. orthopsilosis and C. metapsilosis.* The former two species are more prevalent in critical care units (Peixoto et al., 2023). The currently available routine identification methods mentioned above, can only identify the organism as *C. parapsilosis* and does not differentiate between the three species. Although molecular methods are not routinely used for clinical diagnosis, they may be used for surveillance and research purposes to differentiate the three species. The deoxyribonucleic acid (DNA) sequencing of the D1/D2 region of the large subunit (LSU) of the *28S rDNA* is one of the methods that can differentiate the three species with high (> 98%) similarity percentages (Barbedo et al., 2017).

Candida parapsilosis has no known intrinsic resistance to the available antifungals such as the azoles, the polyenes and the echinocandins. However, *C. parapsilosis* has recently started to show increased minimum inhibitory concentrations (MIC) to azoles. From the South African laboratory-based sentinel study by Magobo and colleagues done between the year 2009 and 2010, 54% of the strains were resistant to fluconazole while 14% of the strains were resistance to voriconazole (Magobo et al., 2017). In another South African surveillance study by Govender and colleagues (2016), only 199 of the 531 (37%) *C. parapsilosis* strains were sensitive to fluconazole and voriconazole with cross resistance observed (Govender et al., 2016). This increase in the MIC of azoles is not only a finding in South Africa but also in other countries such as Spain (Escribano and Guinea, 2022), France (Fekkar et al., 2023) and Brazil (Thomaz et al., 2018) to mention a few.

Chlorine-based detergents are often used in healthcare facilities to clean the environment, so as to reduce the burden of organisms. The effectiveness of these chlorine-based detergents have been questioned, leading to the quaternary ammonium compounds (QACs) and orthophthalaldehyde (OPA) being suggested as the better options (Castro et al., 2023b).

Candida parapsilosis biofilms, comprising of pseudohyphae and yeast cells (Malinovská et al., 2023) allow the organism to successfully evade the action of antifungals (Fanning and Mitchell, 2012). In addition to being a successful biofilm producer, the organism secretes hydrolytic enzymes (Pandey et al., 2018) and produces immunomodulatory prostaglandin E_2 (Chakraborty et al., 2019). All these may assist the organism in the processes of pathogenicity such as the attachment, invasion, evasion and dissemination of the infection.

Several host models have been used and discussed in literature for studying fungal virulence (Junqueira, 2012, Casadevall et al., 2019, Fuchs and Mylonakis, 2006). *Candida parapsilosis* has shown *in vivo* virulence in *Caenorhabditis elegans*, a microscopic nematode that lives freely in soil (Feistel et al., 2019). The use of this nematode to measure host survival and rate of reproduction is relevant because of the homology of its innate immune system to that of humans (Irazoqui et al., 2010). In addition, the translucent nature of

C. elegans allows for the real-time tracking of infection and illness progression (Irazoqui et al., 2010).

In this study, the patients' clinical notes were analysed for demographics including risk factors and patient outcomes. Blood culture specimens' time to positivity for the previously stored *C. parapsilosis strains* was analysed as well as the strains' susceptibility to antifungal agents and chlorine-based disinfectant. The production of *in vitro* virulence factors of the strains were determined and the relative virulence of three selected strains in the *C. elegans* infection model was determined.

2.3 MATERIALS AND METHODS

2.3.1 Study setting

The study was conducted at the department of Microbial, Biochemical and Food Biotechnology, University of the Free State, Bloemfontein.

2.3.2 Study design

This was a retrospective cross sectional laboratory based study.

2.3.3 Study samples

Candida parapsilosis was identified on Vitek®2 (bioMérieux Inc, Marcy l'Etoile, France), from invasive clinical samples sent for routine laboratory diagnosis to the Medical Microbiology laboratory from Universitas Academic hospital's neonatal and paediatric wards, were studied.

2.3.4 Sample size and storage

A sample size of approximately 30 was estimated based on the numbers from the previous years however, only 21 strains were obtained. Of these 21 strains, two belonged to one patient. Therefore, 20 patients' strains were obtained. The *Candida parapsilosis* strains used for the study were those already stored in the laboratory at -80°C from the year 2018 to 2020.

The patients were admitted in the paediatric wards at the Universitas Academic Hospital, Bloemfontein, South Africa. They included neonates as well as other paediatrics patients. These were patients suspected to have candidemia and thus had blood cultures submitted to the laboratory for Microbiological investigations.

2.3.5 Data collection

Data obtained from Vitek®2 (bioMérieux Inc, Marcy l'Etoile, France) as well as patients' demographic data and clinical information from patient clinical files were recorded on an Excel sheet for analysis. No patient names were recorded. Only study numbers were used.

2.3.6 Ethical clearance

Ethical approval to perform the study was granted by the University of the Free State's Health Sciences Research Ethics Committee (Ethics Number: UFS-HSD2020/1856/2601). Permission to use the isolates was granted by the NHLS Universitas Laboratory Business unit, Head of pathology as well as the head of Medical Microbiology department and department of Biotechnology, Microbiology & Biochemistry. The Free State (FS) Department of Health (DoH) granted permission for access to clinical data. The Biosafety ethics committee granted approval to the study and there was already an authorization to keep, use or handle cultures or preparations of microorganisms (Ref J1/2/4/2 01/19).

2.3.7 Collection and identification of strains

Collection of strains

All the blood cultures submitted to the Department of Medical Microbiology, National Health Laboratory services, Universitas Academic Hospital, Bloemfontein, South Africa are routinely incubated in the BacT/Alert (bioMérieux Inc, Marcy l'Etoile, France) automated system. The system uses internal CO_2 sensors to detect the metabolic activity of yeasts and flag the positive blood cultures for further processing by the laboratory personnel to obtain the identification and susceptibility testing results using the cultured colonies.

All *Candida parapsilosis* strains as identified by Vitek®2 (bioMérieux Inc, Marcy l'Etoile, France) from blood culture sent for routine laboratory testing and stored at -80ºC were used for the study. These were in total 20 blood cultures from the neonatal and paediatrics wards.

Identification of strains

Genomic DNA extraction of all the presumptive *C. parapsilosis* strains was done using Quick DNA kit (Zymo Research). The extracted DNA was then referred to the DNA Sequencing Unit at the University of Stellenbosch for sequencing of D1/D2 region of the LSU *28S rDNA* gene. The D1/D2 sequences obtained from University of Stellenbosch sequencing facility were compared by nucleotide-BLAST (Blastn) with sequences available from National Center for Biotechnology Information (NCBI) GenBank.

2.3.8 Retrospective extraction of time to positivity of blood cultures, minimum inhibitory concentration (MIC) results for antifungals and demographics of patients

For the stored *C. parapsilosis* strains, data was extracted from the National Health Laboratory Service (NHLS) TrakCare system to obtain the time to positivity, patients' demographics, Vitek®2 identification, antifungal susceptibility testing results, as well as the blood cultures' time to positivity and recorded on the Excel sheet (Appendix A). The clinical and laboratory standards institute (CLSI) M60 guideline (2017) 1st edition was used to interpret the VITEK®2 susceptibility, minimum inhibitory concentration (MIC), results for antifungals (CLSI, 2017) .

Clinical notes were requested from the Universitas Academic hospital to extract the gestational age, birth weight, any congenital lethal conditions, risk factors such as use of medical devices, the prescribed antifungal therapy as well as the outcome of the patients (Mortality before discharge/discharged). These were all recorded on the Excel sheet.

2.3.9 Determination of susceptibility to chlorine-based disinfectant used in the hospital

The chlorine-based disinfectant, QualiClean (QualiPharm, New Germany) used in the hospital setting at 1000 ppm for surface disinfection, was tested against all the obtained strains of *C. parapsilosis* and *C. albicans* SC5314 by following the method of Abdolrasouli and colleagues with minor modifications (Abdolrasouli et al., 2017). In brief, the strains were sub-cultured onto sabauraud dextrose agar (SDA) (Thermofischer Scientific) and incubated at 35 ± 2°C in ambient air for 48 hours to ensure adequate growth. Four sachets of the 6 g QualiClean disinfectant powder were suspended in 1.125 L of sterile distilled water to prepare a stock concentration of 8000 ppm. The mixture was mixed well to completely dissolve the powder. Chlorine is the active ingredient in the disinfectant.

The QualiClean stock concentration was further diluted to a working concentration of 4000 ppm. A two-fold broth microdilution assay was used to dilute the disinfectant at a concentration ranging from 4000 ppm to 31.5 ppm in a flat-bottom 96-well microtitre plate (Lasec, South Africa). All the outer wells of the microtiter plates were filled with 200 µl of distilled water to prevent drying. Three to four colonies of the yeast isolates were diluted in 5 mL of 0.85% sterile saline to create a suspension and then adjusted to 0.5 McFarland standard corresponding to 1-5 $x10^6$ CFU/mL using a Densichek (bioMerieux, France). Twenty microlitres of the cell suspension of each yeast isolate was added to the wells containing different concentration of the disinfectant, mixed and incubated for 3 minutes, 30 minutes, 3 hours and 30 hours at 25°C on a continuous shaker. Wells 10 and 11 served as the positive control and negative control respectively. The *C. albicans* SC5314 was used at the positive control and the sterile distilled water as the negative control. At each time point, 5 µl aliquots were taken, plated onto the SDA , each plate wrapped with a parafilm and incubated for seven days at 35 ± 2°C. Viability of yeast cells was determined by the presence or absence of visible colonies. Each isolate was tested in duplicate. Minimum inhibitory concentration (MIC) of the disinfectant at each time point was defined as the lowest concentration at which no growth was observed (i.e. 100% inhibition).

2.3.10 *In vitro* production of virulence factors

Biofilm formation

The method used for this assay was modified from the methods described by Djordjevic and colleagues (Djordjevic et al., 2002) as well as Jin and colleagues (Jin et al., 2003). All the strains *of C. parapsilosis* as well as the *C. albicans* SC5314 were grown on the Yeast Malt (YM) agar (3 g/L malt extract, 3 g/L yeast extract, 5 g/L peptone, 10 g/L glucose and 16 g/L agar) for 24 hours at 30°C. Strains were subsequently inoculated into 10 mL yeast nitrogen base (YNB) broth (10 g/L glucose, 6.7 g/L YNB) and incubated at 30°C for 24 hours. Cells were harvested at 4000 *g* for five minutes and the supernatant removed. This was followed by washing the cells thrice with 10 mL phosphate buffered saline (PBS) (Sigma-Aldrich, USA).

Harvested cells were resuspended in 10 mL PBS, counted using a Neubauer hemocytometer (Marienfield, Germany) and standardised to 1 x 10^6 cells/mL in 0.2 mL filter sterilized (0.22 µM nitrocellulose filter, Merck Millipore, Ireland) RPMI-1640 medium (Sigma-Aldrich, USA). The 96-well plates were inoculated with the determined amount of cell suspension (per individual isolate) and filled to a final volume of 200 µL with RPMI-1640. Plates were wrapped with parafilm and incubated at 37°C for 48 hours. After incubation of the plates, the crystal violet assay was performed to determine the biomass of the respective biofilms. After 48 hours of incubation, the supernatants were removed from the 96-well plate. The biofilms were carefully washed twice with 200 µL sterile PBS and the wells air dried for 45 minutes. Cells were stained with 110 µL crystal violet for 45 minutes. This was followed by washing the cells three times with distilled water. De-staining the wells with 200µL of 95% ethanol for 45 minutes was subsequently performed. An aliquot (100µL) from each well was transferred to a new labelled 96-well plate and the absorbance measured at 595 nm. Strains were further categorised into low ($OD_{595nm} \leq 0.3$), moderate (OD_{595nm} = 0.31-

0.59) and high (OD_{595nm} >0.60) biofilm producers depending on their absorbance. All assays were performed in triplicate.

Hydrolytic enzyme secretion

A plate method as previously described by Price and colleagues was used for hydrolytic enzyme secretion (Price et al., 1982). A volume of 3 µL from the standardised cell suspension (1 x 10^6 cells/mL) was added in the centre of the following three media: tributyrin agar for lipase activity (Buzzini and Martini, 2002), yeast carbon base bovine serum albumin (YCB-BSA) agar for proteases activity (Ramos et al., 2015) and SDA supplemented with 8% egg yolk for phospholipase activity (Price et al., 1982, Williamson et al., 1986). Plates were incubated for five days at 37°C and checked regularly for the clearance/precipitation zones. The Pz value (i.e., diameter of the colony divided by diameter of the colony-plus-zone) was determined, with a Pz value of 1 indicating no enzyme activity and that of <1 suggesting some degree of activity (Riceto et al., 2015). The results were further divided into three categories: high activity (Pz = ≤0.40), moderate activity (Pz = 0.41 and 0.60) and the low activity (Pz = 0.61 and 0.99). All assays were performed in triplicate.

Prostaglandin E_2 production

Cells were grown, standardised and biofilms prepared as described above, in 90 mm polystyrene Petri-dishes. In addition to the cells, a final concentration of 500µM of arachidonic acid (Sigma-Aldrich, USA) (Stock of 1 g in 25 mL of absolute ethanol) was added to each Petri dish containing medium plus cells (Ells *et al*., 2011; Fourie *et al*., 2017). Petri dishes were covered with parafilm and incubated for 48 hours at 37°C to allow biofilm formation. Following incubation, biofilms were scraped off and filtered through pre-weighed filters (0.22 µM nitrocellulose). The supernatant was collected in 50 mL conical tubes (Corning Incorporation, USA) and kept on ice. Collected supernatants were acidified with 1M formic acid to a pH of less than 4. Solid-phase extraction (SPE) C18 cartridges (Waters, South Africa) were washed with 5 mL methanol (Merck, Germany) then 5 mL of deionised water. Subsequently, 10 mL of collected supernatants were applied and allowed to flow through the cartridges by gravity. This was followed by 5mL of deionised water to remove the nonbound impurities. Prostaglandin E_2 was extracted from the columns with 5 mL ethyl acetate, containing 1% methanol, and collected in vials covered with foil to protect from light. The eluent was dried under a stream of nitrogen gas, and dried samples were stored at - 80°C. The pre weighed filters containing the biomass were dried at 37°C for 48 hours and weighed to determine the biofilm biomass.

To determine the concentration of PGE_2 in the supernatant, the thawed supernatant was suspended in eicosanoid affinity (EIA) buffer and assayed for using enzyme-linked immunosorbent assay (ELISA; Cayman Chemicals, Ann Arbour, USA) according to the manufacturer's instructions. Two dilutions, done in triplicate, were assayed for individual strains. Values obtained from the control, consisting of only arachidonic acid without cells, were subtracted from experimental samples to remove values of non-enzymatic oxidation of arachidonic acid. Data was analyzed according to manufacturer's instruction.

2.3.11 Virulence in *Caenorhabditis elegans* infection model

Propagation of *Caenorhabditis elegans*

The method described by Brenner was followed in this study (Brenner, 1974). *Caenorhabditis elegans* AU37 [glp-4(bn2) I; sek-1(km4 X], obtained from the *Caenorhabditis* Genetic Centre (University of Minnesota), was propagated on Nematode Growth Media (NGM) (3 g/l NaCl, 2.5 g/l peptone, 5 mg/mL cholesterol,17 g/L agar) with *Escherichia coli (E. coli)* OP50 stock as a food source. About 10 to 15 reproductively active, synchronised *C. elegans* adult nematodes were transferred to a fresh NGM agar plate and incubated at 15 ^{0}C to allow reproduction of the nematodes. Adult nematodes were then removed from the plate. The L4 larvae were moved to a fresh NGM agar plate with *E. coli* OP50 and incubated at 15°C to allow nematodes to propagate. *Caenorhabditis elegans* nematodes were transferred to new NGM agar plates seeded with a lawn of *E. coli* OP50 every 6 days. All *C. elegans* stocks were kept at 15°C until further use. All manipulations were done at room temperature (20 ± 1°C)

Three *C. parapsilosis* strains (strains 19, 22 and 25) were selected based on the varying *in vitro* production of virulence factors. Nematodes grown on NGM were washed with M9 buffer (6 g/L Na2HPO4, 3 g/l KH2PO4, 5 g/l NaCl, 0.25 g/l MgSO4.7H2O) and placed on 24 hours old *C. parapsilosis* complex lawns [on Brain heart infusion (BHI) plates] for 4 hours at 25°C. After incubation, nematodes were washed from the plates with M9 buffer to remove all non-ingested *C. parapsilosis* cells, the nematodes were washed three times with M9 buffer. Approximately 60 nematodes were added to each well of a 6 well plate (Corning Incorporated, USA) containing 2 mL 80% M9 buffer and 20% BHI broth, with 90 µg/mL kanamycin and incubated at 25°C. Nematodes were monitored daily for survival and were regarded as dead when non-motile after mechanical stimulation with a sterile pipette tip. The number of dead and alive nematodes was recorded on an excel sheet. *Candida albicans* SC5314 was used as a control and the procedure was done in triplicate.

2.3.12 Statistical analysis

Data represents mean values and standard deviations of three independent biological replicates (unless stated otherwise), with three technical replicates. Chi-square testing was used to obtain the p values and p values of 0.05 or less were considered statistically significant. For the nematode survival assay an online application for survival analysis (OASIS) 2 (https://sbi.postech.ac.kr/oasis2/) was using for statistical analysis of the data.

2.4 RESULTS

2.4.1 Collection of strains and species identification

Collection of strains

A total of 26 strains were obtained from 2018 to 2020 from positive blood cultures that grew *Candida parapsilosis* as identified by Vitek®2. These were from both neonatal and paediatric wards sent for routine investigations at the Microbiology laboratory. Of these, 21 strains could be recovered, two of which were from the same patient (strain 3 and 11) and therefore, only one strain (strain 3) was included in the study (Table 1).

Table 1: Study strains distribution according to years and wards.

Years	Study strains	Wards
2018		
	1	NICU
	9	NHCU
	16	NHCU
2019		
	3	NICU
	4	NICU
	5	NICU
	6	NHCU
	7	NICU
	10	NHCU
	12	NICU
	13	NICU
	15	NHCU
	18	NICU
2020		
	19	Paeds 10A
	20	NHCU
	22	NHCU
	23	Paeds 10B
	24	NICU
	25	NICU
	26	NHCU

NICU= Neonatal Intensive Care Unit, NHCU= Neonatal High Care Unit, Paeds= Paediatrics.

Species identification/differentiation using D1/D2 region sequencing

All strains were *C. parapsilosis sensu stricto (C. parapsilosis)* by the sequencing D1/D2 of the LSU of the 28s rDNA gene. The percentage similarity ranged between 96% to more than 98%.

2.4.2 Data from National Health Laboratory Service TrakCare and hospital clinical notes

Distribution of patients from which *C. parapsilosis* was isolated

Most of the strains were from patients in the neonatal intensive care unit (NICU) [55% (11/20)], followed by the neonatal high care unit (NHCU) [35% (7/20)] and paediatric wards [10% (2/20)] .

Time to positivity of blood cultures

Twenty blood culture results from which the study strains were obtained were analyzed for the study. The time to positivity (TTP) for the study was defined as the period since the blood culture bottle was loaded into the instrument until the instrument flagged positive, indicating the growth of organisms in the bottle.

The median interquartile range (IQR) time to positivity (TTP) for the blood culture bottles was 35 hours (IQR, 28.25 - 42.25) hours. Most (90%, 18/20) of the blood cultures were positive within 48 hours while the remaining 10% (2/20) were positive after 48 hours (Figure 1). Of the 18, 22% (4/18) were positive within 24 hours and 78% (14/18) were positive after 24 hours but before 48 hours.

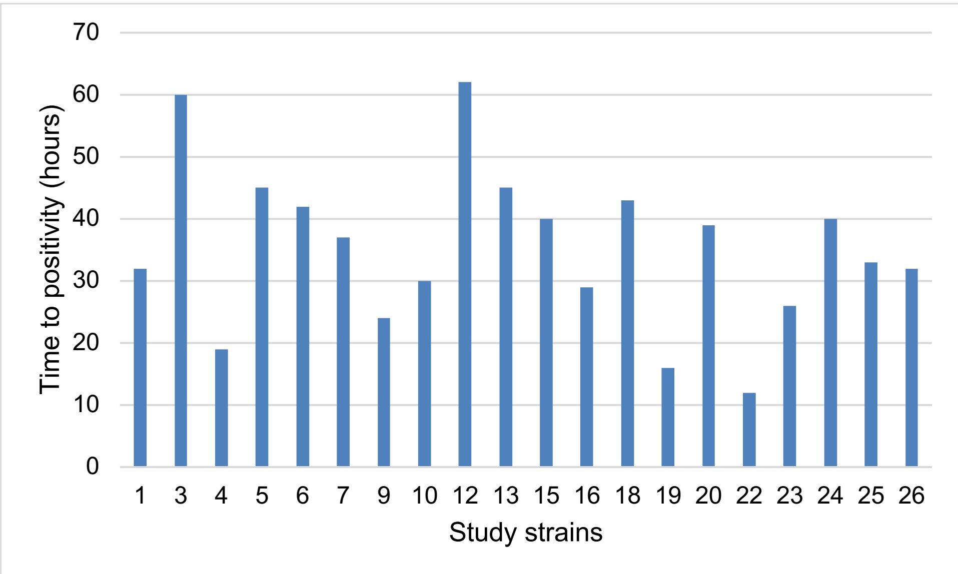

Figure 1: Time to positivity of the study strains showing that majority of the strains were positive within 48 hours

Antifungal susceptibility data obtained from Vitek®2

Resistance was observed for the azoles, namely fluconazole and voriconazole as shown in Table 2 and Figure 2. Eighty percent (16/20) of the strains were resistant to fluconazole (including the SDD=4) with only 20% (4/20) susceptible to fluconazole (MIC=≤2). With regards to voriconazole, 25% (5/20) of the strains were susceptible (MIC≤0.12), 70% (14/20) were intermediate (MIC=0.25-0.5) and 5% (1/20) resistant (MIC≥1). All voriconazole intermediate and resistant strains (70%,14/20) were also resistant to fluconazole. Some strains had increasing MICs (MIC=1-2) for caspofungin and micafungin with one strain (Strain 18) resistant to caspofungin at the MIC of ≥8. Of note for amphotericin B, the CLSI provides the epidemiological cut-off (ECOFF) values rather than the clinical breakpoints. The value, which is one for *C. parapsilosis*, is the cut-off for the sensitive wild-type population. The value corresponding to the ECOFF indicates the absence of mutations among strains.

Table 2: Strains obtained from different wards with their antifungal susceptibility testing results from Vitek®2.

Study strains	Antifungal susceptibilities				
	Fluc S=≤2 SDD=4 R=8	**Vori** S=≤0.12 I=0.25-0.5 R=≥1	**Caspo** S=≤2 I=4 R=≥8	**Mica** S=≤2 I=4 R=≥8	**Ampho B** No clinical breakpoints
1	R/SDD(4)	I(0.25)	S(1)	S(0.5)	MIC=1
3	S(≤0.5)	S(≤0.12)	S(0.25)	S(0.5)	MIC≤0.25
4	R(16)	I(0.5)	S(0.5)	S(0.5)	MIC≤0.25
5	R(8)	S(≤0.12)	S(0.5)	S(0.5)	MIC≤0.25
7	S(2)	S(≤0.12)	S(0.25)	S(0.5)	MIC=0.5
12	R(8)	I(0.25)	S(1)	S(0.5)	MIC=0.5
13	R(8)	I(0.25)	S(0.5)	S(0.5)	MIC≤0.25
18	R(8)	I(0.5)	R(≥8)	S(0.5)	MIC≤0.25
24	R(8)	I(0.25)	S(0.5)	S(0.5)	MIC≤0.25
25	R(8)	I(0.25)	S(0.25)	S(0.5)	MIC≤0.25

6	R(8)	I(0.5)	S(1)	S(2)	MIC=0.5
9	R(≥64)	R(1)	S(1)	S(0.5)	MIC≤0.25
10	R(8)	I(0.25)	S(0.5)	S(0.5)	MIC≤0.25
15	R(32)	I(0.25)	S(2)	S(1)	MIC=0.5
16	R(16)	I(0.5)	S(1)	S(0.5)	MIC≤0.25
20	S(≤0.5)	S(≤0.12)	S(0.5)	S(0.5)	MIC≤0.25
22	S(1)	S(≤0.12)	S(0.5)	S(0.5)	MIC≤0.25
26	R(8)	I(0.25)	S(2)	S(2)	MIC=0.5
19	R(8)	I(0.5)	S(0.25)	S(0.5)	MIC=0.5
23	R(8)	I(0.25)	S(0.25)	S(0.5)	MIC≤0.25

Fluc: Fluconazole, Vori: Voriconazole, Caspo: Caspofungin, Mica: Micafungin, Ampho B: Amphotericin B, R: Resistant, S: Susceptible, SDD: Susceptible dose dependent, I: Intermediate. The MICs are written in brackets.

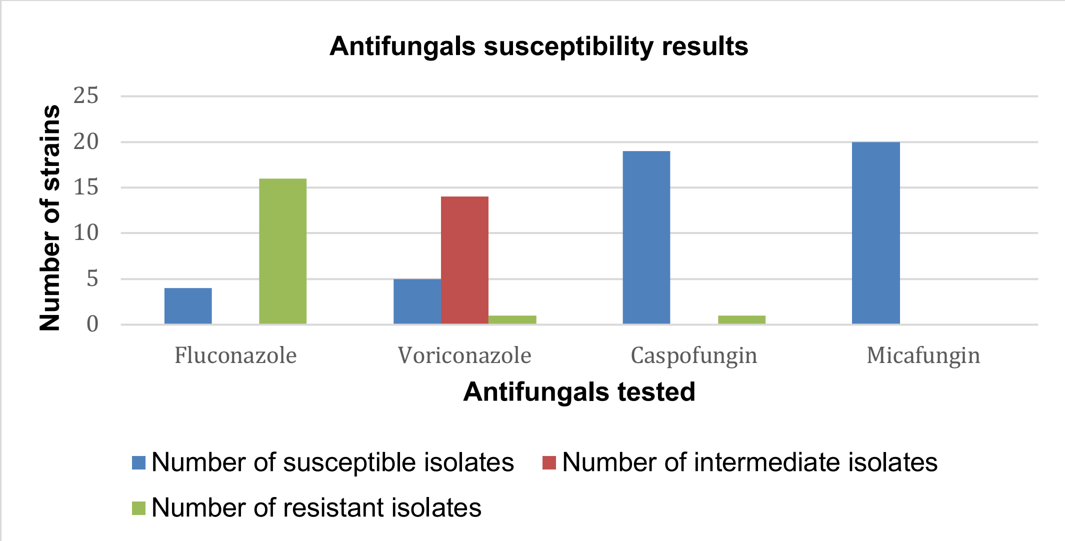

Figure 2: Antifungal susceptibility results of the tested 20 study strains for antifungals with CLSI breakpoints. This figure also shows strains resistant to fluconazole and intermediate to voriconazole including the one strain resistant to both azoles.

Data obtained from hospital clinical notes

For most patients [85% (17/20)] clinical notes were retrieved; however, the majority did not have the complete clinical information. Some missing information included the type of delivery [i.e., normal vaginal delivery (NVD) or caesarean section (C/S)]; gestational age; birth weight and the information of whether neonatal steroids were administered. The recorded patient data are summarized in Table 3.

- **Gender, mode of delivery, birth gestational age and birth weight**

Eighty percent (16/20) of the patients were males and 20% (4/20) were females according to the information recorded on the National Health Laboratory Service request form and the clinical notes.

Fifty-five percent (11/20) of the patients were delivered via C/S, 25% (5/20) via NVD and one patient had no information in the clinical notes. Only six percent (1/17) of the patients were recorded as full term, 82% (14/17) as preterm and there was no documentation for the remaining 12% (2/17), one of which was from paediatric ward and the other from the neonatology ward.

Table 3: Demographic data, clinical features, and outcomes of patients with *C. parapsilosis* candidemia

Patient characteristics	Values
Demographic data	
Birth weight (g),median (IQR). N=16	1320 (1078.0–1588.0)
Gestational age (weeks), median (IQR). N=17	31 (29.5–32.0)
Gender (male/female). N=20	16 (80%) /4 (20%)
NVD / CS. N= 17	5 (36.8%) /11 (57.89%)
Day of life at onset of candidemia (days), median (IQR)	20 (17-37)
Underlying comorbid diseases	17
Intraventricular hemorrhage (≥Stage II)	2/17 (12%)
Congenital anomalies (Trisomy 21)	1/17 (6%)
Respiratory distress syndrome	10/17 (59%)
Cardiovascular diseases (ASD, PDA, VSD, VPS, AHL)	6/17 (35%)
Gastrointestinal disease	12/17 (71%)
Renal disorders	2/17 (12%)
Presence of more than one co-morbidity	15/17 (88%)
Predisposing risk factors	17
Pre-term	14/17 (82%)
Receipt of systemic antibiotics	17/17(100%)
Presence of invasive lines	17/17 (100%)
Receipt of parenteral nutrition	13/17 (76%)
GI surgery	7/17 (41%)
Antenatal steroids administration at birth	7/17 (41%)
Mortality	8/17 (47%)
Early mortality (≤7 days)	0/17(0%)
Late mortality (>7 days)	8/17 (47%)

Of the patients with recorded birth weights, 18% (3/17) had extremely low birth weight (ELBW=<1000g), 41% (7/17) had very low birth weight (VLBW=<1500g), 29% (5/17) had low birth weight (LBW=<2500g) and 6% (1/17) were recorded as normal birth weight of 2600g. The remaining paediatric patient had no record of the birth weight in the clinical notes. Antenatal steroids were recorded for 35% (7/20) of the patients. These were the three patients who were categorized as ELBW and the four patients who were categorized as VLBW. The median (interquartile range, IQR) birth gestational age and weight of the neonates with candidemia were 31 weeks (IQR, 29.5 -32.0) and 1320 (IQR, 1078 - 1588) grams, respectively. The median onset of candidemia in the study was 26 days old (IQR, 17–37), and all were diagnosed after the first week of life (late neonatal sepsis).

- **Comorbid diseases, use of invasive devices and treatment administered**

All 17 patients whose clinical notes were retrieved had underlying comorbidities, and majority (88%,15/17) had more than one recorded comorbid disease. There were more [71% (12/17)] gastrointestinal manifestations followed by respiratory manifestations [59% (10/17)]. Other risk factors included abdominal surgery [41% (7/17)], endotracheal intubation [35% (6/17)], peripherally inserted central lines [88% (15/17)] and arterial lines [41% (7/17)]. Other invasive devices were central lines, a Hickman line for one full term patient who had acute myeloid leukaemia, and the nasogastric tubes. In addition, total parenteral nutrition (TPN) was recorded for 76% (13/20) of the patients.

The second paediatrics patient had nephrotic syndrome, was on TPN, had received multiple antibiotics including meropenem for five days, piperacillin-tazobactam for seven days, linezolid for 14 days, vancomycin for 14 days and ceftriaxone for 12 days.

All patients were on antifungal drugs. Amphotericin B was recorded for all patients while fluconazole was recorded for six patients. Micafungin, which is one of the echinocandins, was only recorded for one patient. This is no surprise as at the time of the study, echinocandins were only being introduced at the study setting. All these antifungals were preceded by systemic antibiotics such as ampicillin, piperacillin-tazobactam, gentamycin, amikacin, vancomycin, linezolid, meropenem and colistin.

2.4.3 Susceptibility to chlorine-based disinfectant

The *C. parapsilosis* strains as well as the *C. albicans* SC5314 demonstrated visible colonies on the SDA plate in the presence of the Qualiclean disinfectant after incubation at 3 minutes, 30 minutes and 3 hours across the 4000 ppm to 31.25 ppm concentration range (Table 4). The MIC at these time intervals was therefore more than 4000 ppm for all the strains. At 30 hours, only two strains (strain 7 and 9) had visible colonies on the SDA plates and turbidity on the microtiter plates. At this time interval, the MIC for isolate 7 and 9 was 125 ppm.

The MIC for the rest of the isolates were below 31.25 ppm meaning no growth was observed at the lowest concentration used in the study (31.25 ppm) at 30 hours. Thus, Qualiclean disinfectant was not effective against the yeast strains even after 3 hours contact time. The contact time of the disinfectant is 5 hours as indicated on Figure 3 below.

Table 4: Minimum inhibitory concentration of Qualiclean disinfectant against clinical strains of *Candida parapsilosis.* Effect of Qualiclean disinfectant against strains of *C. parapsilosis* using different concentrations between 4000 ppm to 31.25 over different time periods.

Study strains	**MIC (ppm)**			
	Contact time			
	3 min	30 min	3 hrs.	30 hrs.
C. albicans ATCC strain SC5314	> 4000	> 4000	> 4000	≤ 31.25
Candida parapsilosis 1	> 4000	> 4000	> 4000	≤ 31.25
C. parapsilosis 3	> 4000	> 4000	> 4000	≤ 31.25
C. parapsilosis 4	> 4000	> 4000	> 4000	≤ 31.25
C. parapsilosis 5	> 4000	> 4000	> 4000	≤ 31.25
C. parapsilosis 6	> 4000	> 4000	> 4000	≤ 31.25
C. parapsilosis 7	> 4000	> 4000	> 4000	125
C. parapsilosis 9	> 4000	> 4000	> 4000	125
C. parapsilosis 10	> 4000	> 4000	> 4000	≤ 32.25
C. parapsilosis 12	> 4000	> 4000	> 4000	≤ 31.25
C. parapsilosis 13	> 4000	> 4000	> 4000	≤ 31.25
C. parapsilosis 15	> 4000	> 4000	> 4000	≤ 31.25
C. parapsilosis 16	> 4000	> 4000	> 4000	≤ 31.25
C. parapsilosis 18	> 4000	> 4000	> 4000	≤ 31.25
C. parapsilosis 19	> 4000	> 4000	> 4000	≤ 31.25
C. parapsilosis 20	> 4000	> 4000	> 4000	≤ 31.25
C. parapsilosis 22	> 4000	> 4000	> 4000	≤ 31.25
C. parapsilosis 23	> 4000	> 4000	> 4000	≤ 31.25
C. parapsilosis 24	> 4000	> 4000	> 4000	≤ 31.25
C. parapsilosis 25	> 4000	> 4000	> 4000	≤ 31.25
C. parapsilosis 26	> 4000	> 4000	> 4000	≤ 31.25

MIC, minimum inhibitory concentration, >: more than, ≤ less or equal to.

Figure 3: The Qualiclean 6 g disinfectant sachet showing the recommended minimum contact time of 5 minutes.

2.4.4 *In vitro* production of virulence factors

Biofilm formation

All the study strains formed biofilms as shown in Figure 4. The amount of biofilm development exhibited by the strains varied, with a 9-fold variation between the strains that produced the highest and lowest biofilm. The strains were divided into low (OD_{595nm} ≤ 0.3), medium (OD_{595nm} = 0.31-0.59) and high (OD_{595nm} ≥0.60) biofilm producers and it was seen that most [90% (18/20)] of the strains were low biofilm producers. Strain 1 was a medium producer while strain 3 was a high biofilm producer, forming more biofilm biomass than the reference *C. albicans* strain.

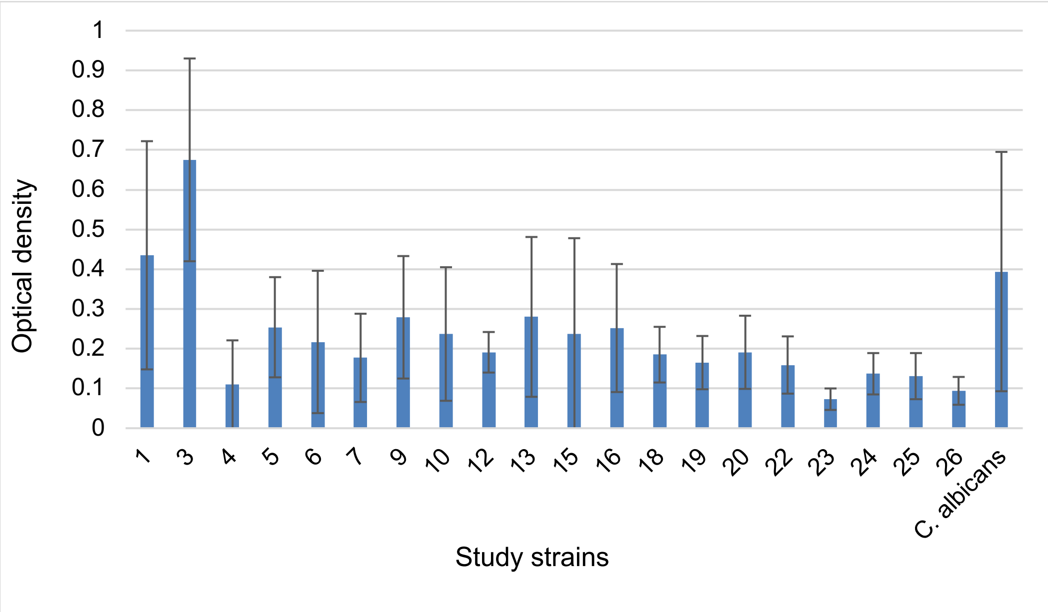

Figure 4: Biofilm biomass as determined by crystal violet biofilm assay. The data points are the averages of three independent repetitions and error bars represent standard deviations.

Hydrolytic enzyme secretion

All the studied strains showed protease and phospholipase activities, with Pz values below 0.5, but no lipase activity. The Pz values of proteases and phospholipases are shown in Figure 5. Protease activity was medium (Pz = 0.41-0.60) for 30% (6/20) of the isolates and high (Pz ≤ 0.40) for 70% (14/20) of the isolates. The average Pz value for proteases was 0.3675 (SD=0.058) with the highest activity seen for strain 25 (Pz = 0.22), followed by strains 24 and 22 (Pz = 0.31). The lowest activity was found in strains 18 and 26 (Pz = 0.43). *Candida albicans* SC5314 exhibited high protease activity (Pz = 0.24) as expected.

Phospholipase activity was classified as medium (Pz = 0.41-0.60) for 8 out of 20 strains (40%) and high (Pz ≤ 0.40) for 12 out of 20 strains (60%). The average Pz value for phospholipases was 0.4025 (SD=0.018). The phospholipase activity was highest in strain 5 (Pz = 0.36), followed by strains 7 and 12 (Pz = 0.38). The lowest activity was found in strain 26 (Pz = 0.44). *Candida albicans* SC5314 also showed a high phospholipase activity with Pz of 0.39. Of note, 45% (9/20) of the strains (i.e. strains 5, 6, 7, 9, 10, 15, 16, 19, 25) showed high activity for both enzymes. Of these nine strains, mortality was seen in 44% (4/9) of the patients, although this cannot necessarily be attributed directly to the *in vitro* enzyme activity.

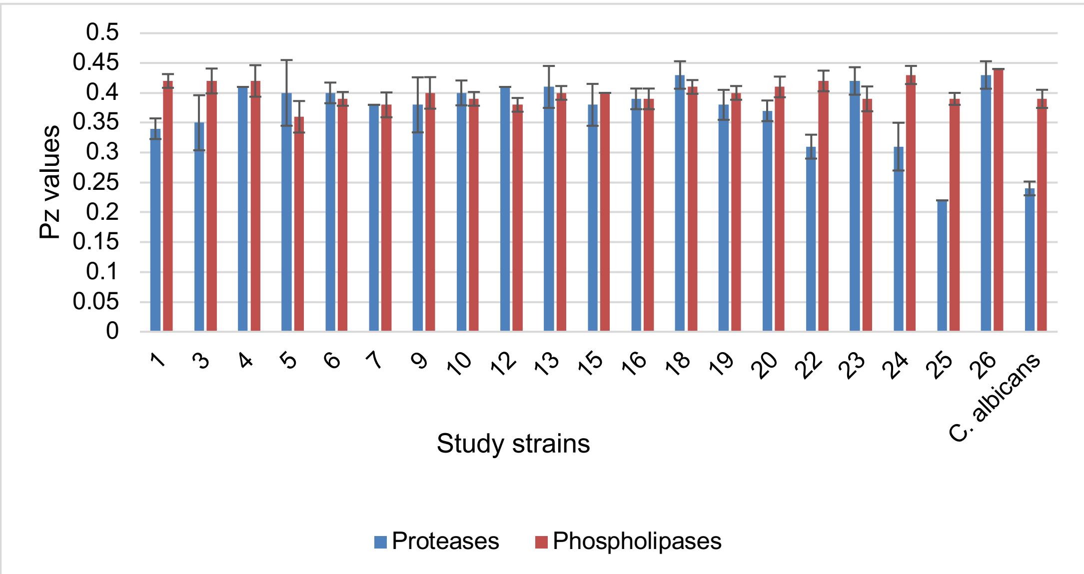

Figure 5: Pz values of strains for protease and phospholipase assays. The data points are the averages of three independent repetitions and error bars represent standard deviations.

Prostaglandin E_2 production

Prostaglandin E_2 (PGE_2) production from exogenous arachidonic acid was assessed from the study strains as well as the *C. albicans* SC5314 strain. Four strains generated PGE_2 levels exceeding 4000.00 pg/mg, while 11 strains produced PGE_2 above 2000.00 pg/mg but not exceeding 4000.00 pg/mg. The remaining five strains yielded PGE_2 levels below 2000.00 pg/mg. Most *C.*

parapsilosis strains produced high PGE_2 compared to the *C. albicans* SC5314 strain ($p<0.001$) (Figure 6). The two study strains with the highest production of PGE_2 were strains 6 and 25. The average of PGE_2 production by the *C. parapsilosis* strains was 2939.23 pg/mg (SD=1340.43). The strains that produced more than 2000.00 pg/mg PGE_2 (15/20) included 81% (13/16) of the fluconazole-resistant strains and only 13% (2/15) of the fluconazole-susceptible strains suggesting a possible relationship between biofilm production and the resistance pattern. The PGE_2 produced ranged from 838.53 pg/mg to 5871.14 pg/mg, with a 7-fold variation between the strains that produced the highest and lowest amounts of biofilm.

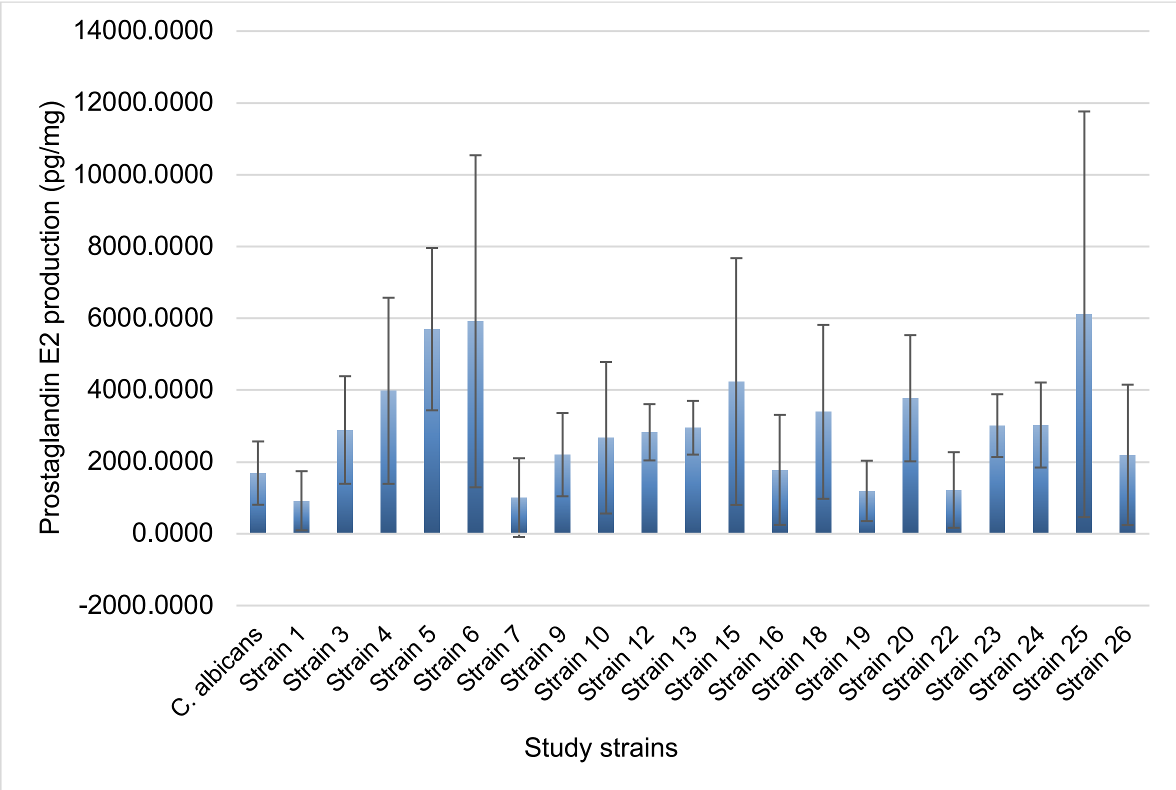

Figure 6: Prostaglandin E_2 production by *C. parapsilosis* strains and *C. albicans* SC5314. Concentration of PGE_2 was measured on supernatants after 24 hr-incubation at 37°C and normalised against biomass. The data points are the averages of two independent repetitions at two different concentrations and error bars represent standard deviations.

Strain characteristics and outcomes

In total, nine patients' outcome was recorded as being discharged while eight were recorded as having demised. Mortality was 47% (8/17) for the patients whose clinical notes were retrieved, and this occurred after seven days of hospital admission. Among the 80% (16/20) of the patients that had fluconazole-resistant isolates, 44% (7/16) demised, 37% (6/16) were discharged and the remaining 19% (3/16) had no outcome information in the clinical notes. From the 90% (18/20) of the patients whose blood cultures had TTP within 48 hours, 44% (8/18) demised, 39% (7/18) were discharged from the hospital and the clinical notes of 17% (3/18) could not be retrieved. Strains' characteristics and outcomes are summarised in Table 5 below.

Four of the patients who demised were from NICU, three patients from NHCU and one patient from paediatric 10B ward. Of the four patients from NICU, two were recorded as VLBW and the other two as LBW. All four patients underwent abdominal surgery. The TTP of the four strains was more than 24 hours but less than 48 hours. Three strains of the four patients were resistant to fluconazole while the other two were intermediate to voriconazole. The fourth strain was susceptible to both azoles. This patient with the susceptible strain had low birth weight, vertebral anomalies, acyanotic heart lesion, hypoplastic lung among other diseases; had undergone surgery for duodenal atresia type 3 in addition to other risk factors such as total parenteral nutrition, invasive devices and the use of broad-spectrum antibiotics. The three strains were low biofilm producers while the fourth strain was a medium biofilm producer. In addition, three of the strains were high phospholipase producers while the fourth was a medium producer. Three of the strains were high protease producers while the fourth was a medium producer. Concerning the PGE_2 production, half were low producers (<2000.00 pg/mg), while the other half medium producers (between 2000.00 and 4000.00 pg/mg).

Of the three NHCU patients who demised, the patients were recorded as ELBW, VLBW and LBW. Abdominal surgery was recorded for only one patient. The TTP of the ELBW, VLBW and LBW were 24 hours, 30 hours and 40 hours, respectively. The strains of the ELBW patient were resistant to both azoles while the other two strains were resistant to fluconazole and intermediate to voriconazole. All three strains were high phospholipase, protease producers and low biofilm producers. One strain was a low, the other medium and the third a high PGE_2 producer.

The paediatric 10B ward patient had no record of the birthweight nor surgical intervention. This patient had acute myeloid leukemia with neutropenic sepsis and acute respiratory disease syndrome. The blood culture's TTP was 26 hours, and the strain was resistant to fluconazole while intermediate to voriconazole. The strain was a low biofilm, medium protease, medium PGE_2 and high phospholipase producer.

Table 5: Summary of study strains hydrolytic enzymes, biofilm production, PGE_2 production, azoles susceptibility results and outcome of patients.

Strains	Proteases	Phospholipases	Biofilm	PGE_2	Fluc	Vori	Patient outcome
1	+++	++	++	+	R	I	D
3	+++	++	+++	++	S	S	DC
4	++	++	+	++	R	I	NC
5	+++	+++	+	+++	R	S	NC
6	+++	+++	+	+++	R	I	DC
7	+++	+++	+	+	S	S	D
9	+++	+++	+	++	R	R	D
10	+++	+++	+	++	R	I	DC
12	++	+++	+	++	R	I	DC
13	++	+++	+	++	R	I	NC
15	+++	+++	+	+++	R	I	D
16	+++	+++	+	+	R	I	D
18	++	++	+	++	R	I	D
19	+++	+++	+	+	R	I	DC
20	+++	++	+	++	S	S	DC
22	+++	++	+	+	S	S	DC
23	++	+++	+	++	R	I	D
24	+++	++	+	++	R	S	D
25	+++	+++	+	+++	R	I	DC
26	++	++	+	++	R	I	DC

PGE_2 = Prostaglandin E_2, Fluc = fluconazole, Vori = voriconazole, R = resistant, S = susceptible, D = Patient demised, DC = Patient discharged, NC = No clinical notes. + = Low production, ++ = Medium production, +++ = High production.

2.4.5 Virulence in the *Caenorhabditis elegans* infection model

Three *C. parapsilosis* strains (i.e., strains 19, 22 and 25) were selected based on the varying presence of virulence factors (Table 6) and their relative virulence in the *C. elegans* infection model was determined. The *C. albicans* SC5314 was included as a reference strain.

Table 6: Summary of the virulence characteristics of the three strains (19,22,25) selected for determining relative virulence in the *Caenorhabditis elegans* infection model.

Strains	Proteases	Phospholipases	Biofilm	PGE_2	Fluc	Vori	Patient outcome
19	+++	+++	+	+	R	I	DC
22	+++	++	+	+	S	S	DC
25	+++	+++	+	+++	R	I	DC

Fluc =fluconazole, Vori =voriconazole, R= resistant, S=susceptible, DC=Patient discharged, PGE_2=Prostaglandin E_2 production, +=Low production, ++= Medium production, +++= High production.

Figure 7A shows the killing of *C. elegans* by the strains while 7B shows the survival probability of *C. elegans*. Survival probability of all the infected nematodes were significantly reduced compared to control nematodes fed with *E. coli* OP50 (Figure 7B). The *C. albicans* ATCC SC5314 strain started killing the nematodes slowly over the first three days. More than 50% of the *C. elegans* were killed by *C. albicans* SC5314 strain on day 5, with 100% mortality on day 7 (Figure 7A). For the three *C. parapsilosis* strains 19, 22, and 25, the rapid killing was evident after day 2, except for strain 22, followed by slower rate of killing and 100% death of nematodes on day 5 while the 100% killing by strain 22 was only on day 6.

The two strains that demonstrated rapid killing after day 2, namely strain 19 and strain 25, were both high protease and high phospholipase producers. In addition, the strains were resistant to fluconazole and intermediate to voriconazole. In contrast, strain 19 was a high protease, medium phospholipase producer and susceptible to both azoles. The differences did not affect the outcome of the patients as all three patients were discharged from hospital. There was a statistically significant reduction of the nematodes by strain 19 on day 5 (p=0.01), by strain 22 on day 2 (p=0.0006), day 3 (p=0.0006) and day 4 (*p*=0.0137) and by strain 25 on days 4 (*p*=0151), 5 (*p*=0.0001) and 6 (*p*=0.0302) when compared to the *C. albicans* ATCC SC5314 strain.

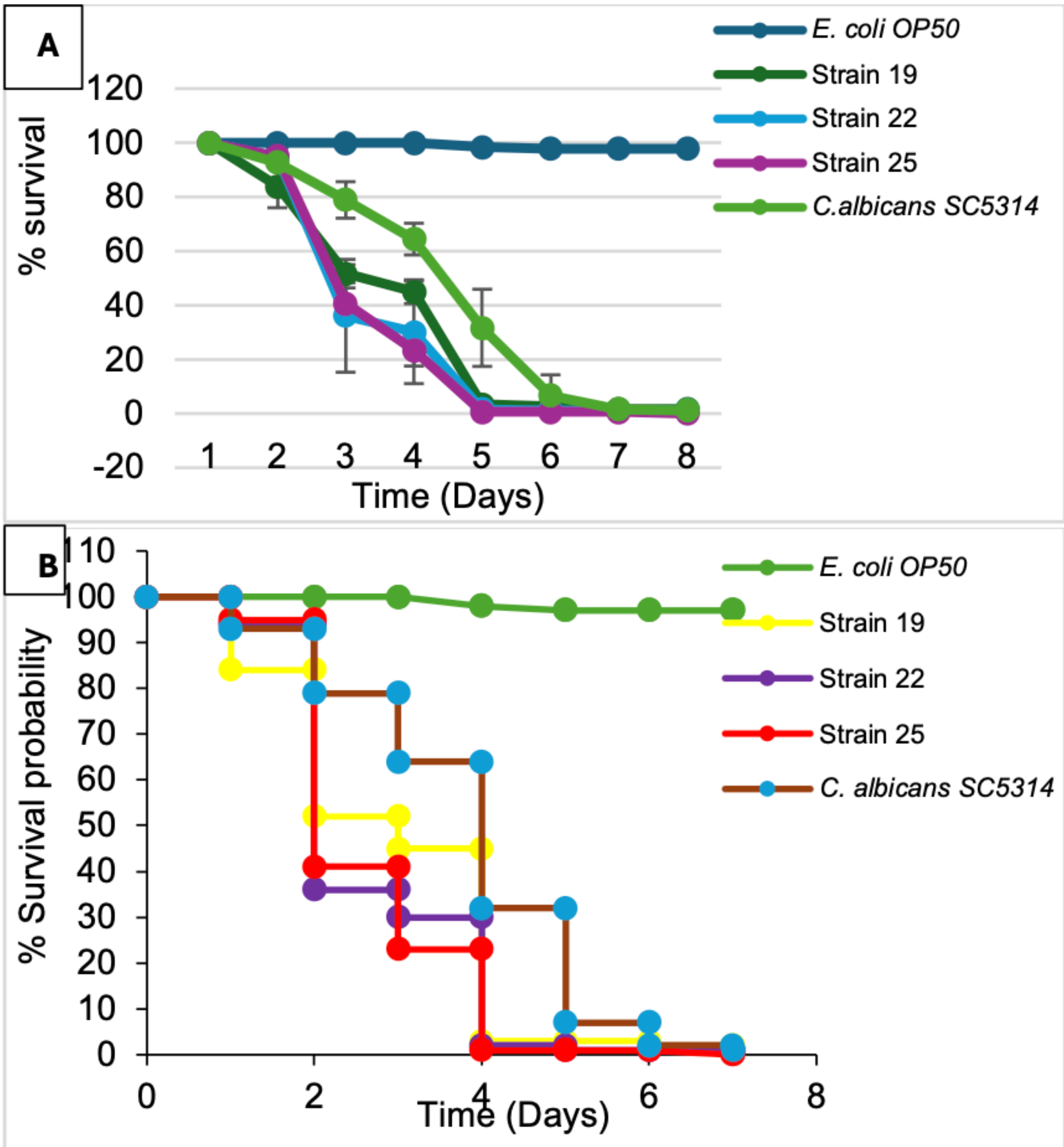

Figure 7: *Candida parapsilosis* and *Candida albicans* SC5314 killing compared to *Escherichia coli* OP50 killing of *Caenorhabditis elegans*. This experiment was done in triplicate. **A:** *Candida parapsilosis* killing and *Escherichia coli* OP50 killing of *Caenorhabditis elegans.* **B:** Kaplan-Meir graphs indicating the survival probability of *Caenorhabditis elegans*.

2.5 DISCUSSION

All the strains in this study were identified as *C. parapsilosis sensu stricto*. *Candida parapsilosis sensu stricto* is the species mostly recovered in clinical specimens such as blood cultures and catheter tips. The results of this study are similar to those from a South African surveillance study by Magobo and colleagues (2017) where they also identified all the strains from the neonatal unit clusters as *C. parapsilosis sensu stricto.* In addition, from Brazil, about 81.1% of the isolates were *C. parapsilosis sensu stricto* in Ziccardi and colleagues' study while Rodrigues and colleagues found 95.5% of the strains to be *C. parapsilosis sensu stricto* (Ziccardi et al., 2015, Rodrigues et al., 2022). Interestingly, the prevalence of *C. parapsilosis sensu stricto* in Italy from blood cultures was 48.1% in a survey by Franconi and colleagues (2023) surpassing even *C. albicans* (34.9%).

The distribution of strains in this study was expected since the neonates have more risk factors than the older children. The two paediatric patients in this study also had risk factors such as malignancy, use of systemic antibiotics and total parenteral nutrition thus increasing their risk factors for fungal infections. In a South African laboratory based surveillance by Shuping and colleagues, neonatal isolates contributed to most of the isolates obtained at 49% with paediatrics contributing only 20% to the total number and adolescents even the least number (4%) (Shuping et al., 2021). In addition, a European study by Warris and colleagues had neonatal strains contributing about 30% of the total strains with less from other wards including the paediatrics ICU (Warris et al., 2020).

The median TTP for fungal detection in blood cultures is about two days (Clancy and Nguyen, 2013) and may be more dependent of the fungal load. This however can also be influenced by the type of *Candida* species, and or higher growth rate which may lead to a shorter TTP (Lamy, 2019). Majority of the blood culture bottles were positive for growth within 48 hours in our study. In contrast to our findings, Kim and colleagues (2013) from Korea found the TTP of *C. parapsilosis* to be longer (50 hours) compared to *C. tropicalis* (27 hours) and

C. albicans (32 hours). In addition, other studies from India (Kaur et al., 2023), Australia (Keighley et al., 2023) and Taiwan (Chen et al., 2015) also found the TTP for *C. parapsilosis* to be longer.

As in other parts of the world, fluconazole-resistant *C. parapsilosis* is on the rise in South Africa. In this study, more than half of the strains exhibited intermediate susceptibility to voriconazole. Cross resistance between voriconazole and fluconazole due to mutations (G1747A, A2619C, and A3191C) in *MRR1* (encoding for a transcription factor of MDR1, which is a drug efflux pump) has been mentioned in literature (Branco et al., 2015, Branco et al., 2022). It is possible that the strains that were resistant to fluconazole and intermediate to voriconazole possess this mutation.

In contrast to the study by Garzillo and colleagues (Garzillo et al., 2017), only one strain was resistant to caspofungin. At the time of this study, amphotericin B was the most used antifungal for candidemia in the public sector followed by fluconazole, and as a result, the echinocandins were not routinely used especially in this population. Some strains had increasing MICs to echinocandins and there was already some resistance seen at the time in the private sector (Naicker et al., 2016). Monitoring of the MICs for echinocandins is warranted.

While antifungal prophylaxis refers to administration of antifungals in patients without signs and symptoms of fungal disease, pre-emptive therapy refers to administration of antifungals in patients with laboratory assays indicating invasive fungal disease whereas empiric therapy refers to administration of antifungals in patients with risk factors of fungal disease (Cornely et al., 2019). In our study, all patients had risk factors of fungal infections. Literature has suggested both the prophylactic use of fluconazole as well as the patient to patient transmission to be the possible drivers of fluconazole-resistant strains (Escribano and Guinea, 2022). Zhang and colleagues (2015) also suggested poor source control with subsequent prolonged suboptimal antifungal use as one of the factors driving fluconazole resistance (Zhang et al., 2015).

Only six patients received fluconazole therapy in this study. Among those with the resistant strains to fluconazole, the drug was empirically used in three patients while it was used for de-escalation after treatment with amphotericin B in one patient. Of note, 75% (12/16) of the patients were fluconazole naïve according to the clinical notes but still had fluconazole-resistant *C. parapsilosis*.

Resistance could therefore probably be attributed to the empiric use of fluconazole in the three patients mentioned above with risk factors for fungal disease. The other driver of fluconazole resistance could be patient to patient transmission or poor source control, since early source control is associated with better outcomes in patients with invasive candidiasis (Vergidis et al., 2016). Fluconazole-resistant strains through genetic alterations, are also known to have a better chance of adapting to host conditions explaining why they are more transmissible than the susceptible strains (Daneshnia et al., 2023b).

Gender and mode of delivery are not mentioned in literature as risk factors for candidemia in neonates. The predominance of males in this study could have been an incidental finding and also influenced by the small study size. Similar to our analysis, majority of studies have a higher proportion of male participants while having a smaller sample size (Qi et al., 2018b, Manzoni et al., 2006). Garzillo and colleagues from Italy had similar results as our study where majority of the neonates were males (13/17) in a small sample size study (Garzillo et al., 2017). Similarly, another study from Italy by Caggiano and colleagues had male to female ratio of 1.6:1 (Caggiano et al., 2017) while in a study by Menezes and colleagues from Brazil, males contributed 76.6% to the study population (Menezes et al., 2024).

The fact that more than half of the deliveries were via C/S could have been influenced by both maternal and neonatal health reasons. In addition, the study took place at a tertiary hospital where almost all mothers are referred because of high-risk pregnancy thus resulting in high rates of caesarian sections at tertiary hospitals. A retrospective observational study by Tunc and colleagues in Turkey found *C. albicans* rather than non-*albicans* species to be more associated with NVD (Tunc et al., 2023). The reasons for the mode of delivery were not looked at in this study. A study mentioned above by Menezes had half of the neonates delivered by C/S and the other half by NVD (Menezes et al., 2024).

Pre-term has been recorded as a strong risk factor for candidemia in neonates due to their immature immune system. As expected, in our study, 82% of the neonates were delivered pre-term. Miranda and colleagues from Brazil also found that the majority (80%) of their patients were pre-term neonates (Miranda et al., 2012). The findings are similar to another Brazilian study by Menezes and colleagues where they had at least 86.6% of neonates delivered before 36 weeks (Menezes et al., 2024). These neonates are often born with low birth weight, leading to the use of invasive devices, administration of systemic antibiotics and resulting in prolonged stay in order to increase the survival rate. It is therefore no surprise that in our study majority of the neonates had VLBW. Our findings however differ from those by Menezes and colleagues study from Brazil where 56.6% of the neonates had ELBW and 26.6% had VLBW (Menezes et al., 2024).

Antenatal steroids are known to improve outcomes in pre-term infants, but risk factors ought to be weighed against the benefits since the administration may lead to immune related diseases (Räikkönen et al., 2023). Four of the VLBW and three of the ELBW patients were reported to have received antenatal steroids. Only one of these patients demised. According to a cohort study by Yao and colleagues, children who had an antenatal course of steroids were more likely to experience infectious diseases during their first year of life (Yao et al., 2023). However, benefits of antenatal steroids are evident from a Cochrane review by Roberts and colleagues where steroids were shown to improve survival, reduce respiratory distress syndrome, necrotizing fasciitis and intraventricular hemorrhage (Roberts et al., 2017). Steroids in that review were not associated with any significant maternal or short-term fetal adverse effects. The neonates in our study were not monitored for infections following the administration of steroids.

Candida species are known to cause late onset sepsis (Gonia et al., 2017). The gastrointestinal tract is colonized with *Candida* species and as such, an increased colonization is directly proportional to an increased risk of invasive candidiasis (Mesquida et al., 2023). In this study, majority of the patients had more than one comorbid disease, but gastrointestinal manifestations were by far the most common with more than half of those patients having surgical interventions. Irrespective of the age, the risk of candidemia increases in patients who have undergone abdominal surgery. Kilic and colleagues performed a study among adults who underwent abdominal surgery and found 51% of the *Candida* isolates to be *C. parapsilosis* (Kilic et al., 2020). Similarly, the risk increased among the neonates from the Baltimore surveillance study by Shetty and colleagues (Shetty et al., 2005).

Even though *C. parapsilosis* is reported to not be as invasive as *C. albicans* to immature intestinal epithelial cells (Gonia et al., 2017), it can nonetheless result in invasive candidiasis due to the disruption of the protective barriers. Lines recorded in our patients' files included the PICC lines, A-lines, nasogastric tubes, CVP lines, umbilical venous catheters and Hickman lines. The presence of invasive lines in the study patients increased their risk factors to candidemia as *C parapsilosis sensu stricto* adheres strongly to medical devices (Lattif et al., 2010) and is able to form biofilms. These lines serve as a source of persistent seeding for *C. parapsilosis*. In addition, a neonate with colonised central venous line is known to have a ten-fold increased risk of developing fungal infection (Manzoni et al., 2006). Central venous catheter care bundles with continuous monitoring should be prioritized in order to reduce to risk of fungal infections.

As a result of their premature gastrointestinal tract, pre-term infants do require parenteral nutrition to reduce risks of developing diseases such as necrotising enterocolitis (Fusch et al., 2009). *Candida parapsilosis* infection has been recognised as one of the complications of using parenteral nutrition (Weems et al., 1987, Cano et al., 2005). In our study, TPN was recorded for thirteen patients adding to their risk factors for invasive candidiasis. Hyperglycemia is often observed in non-diabetic patients on TPN (Rosmarin et al., 1996). Since *C. parapsilosis* is known to thrive well in areas with high glucose concentration (Kuhn et al., 2002), Herek and colleagues could prove in their study that glucose highly promotes the growth as well as biofilm formation of *C. parapsilosis* (Herek et al., 2019).

Antibiotic exposure is known to be associated with candidemia although the exact mechanisms of how they predispose to candidiasis are not well understood (Kim et al., 2023). Interfering with lymphocytes function has been suggested by Drummond and colleagues in a study involving animal models (Drummond et al., 2022). All patients in our study received systemic antibiotics prior to developing invasive candidiasis. In a study by Zuo and colleagues from China, although done in adults, antibiotic use was strongly associated with invasive *C. parapsilosis* among patients in ICU (Zuo et al., 2021). The use of antibiotics was also more prevalent (91.1%) among the patients studied by Araujo and colleagues (Araujo et al., 2024).

The mortality in this study was 47% with majority [7/16;44%] of deaths associated with fluconazole-resistant strains. Similar results were found by Fekkar et al. in Paris where fluconazole-resistant strains resulted in higher mortality (40%) than the susceptible strains (Fekkar et al., 2023). In another study from Spain, mortality was 34% among the fluconazole-resistant strains (Alcoceba et al., 2022).

Time to positivity may assist in predicting the outcome of patients with invasive candidemia. The shorter TTP is associated with high mortality (Kim et al., 2013). A higher mortality (44%) was observed for the blood cultures that had TTP within 48 hours (p value =0.50). The study by Zeeshan and colleagues also found a mortality rate of 40% (Zeeshan et al., 2024). This is similar to findings by Keighley and colleagues (2023), who found that reduced TTP was linked to higher death rates (Keighley et al., 2023).

It is important to note that the longer TTP may lead to delayed start of appropriate therapy and delayed source control, thus resulting in dissemination of the infection and adverse patient outcomes, especially in neonates. As demonstrated by four patients who were discharged with strains producing both high phospholipases and high proteases, and by the other four patients who died with strains producing both high phospholipases and proteases, there was a 50% chance of death when the patients were infected with such strains.

Cleaning in the healthcare facilities depends on variables, such as the workforce, area cleaned, substance, technique as well as the equipment used (Peters et al., 2018). A lack of any of the above-mentioned leads to ineffective cleaning. Disinfectants refer to chemical agents that are capable of removing infectious microbes other than bacterial spores (Rutala and Weber, 2008). The disinfectant is therefore a variable among others that is required for effective cleaning. In addition, following the manufactures' instruction for the substance used will assist in effective cleaning. Contact times, which refer to the time the disinfectant should stay on the surface to effectively interact with the microbe, are critical for the effectiveness of the disinfectants and recommended contact times are dependent on adequate cleaning (i.e. removal of soil first), prior to disinfection since failure to do so will result in disinfection failure (Rutala and Weber, 2008).

Chlorine containing disinfectants, such as Qualiclean, are among the well-known and used disinfectants in health care settings (Assadian et al., 2021). Although the recommended contact time by the manufacturer of Qualiclean was a minimum of five minutes (Figure 8), this disinfectant was not effective against the strains even after 3 hours contact time.

Contact times are measured based on continuous wet contact and these times are significantly reduced in medical institutions under time constraints since disinfectants are applied to surfaces and allowed to air dry without being reapplied (Rutala and Weber, 2008). Frequent disinfection might be necessary with all the other variables mentioned above being in place.

Our disinfectant results are similar to the results reported by Abdolrasouli and colleagues (2017), who found that the *C. parapsilosis* in their study had consistently higher MICs. Similar results were also found when sodium hypochlorite solution was tested against pre-formed (24 hour) *C. parapsilosis* biofilms (Pires et al., 2013). It was found that the biofilm viability was not significantly affected by the disinfectant. Thus, the current practice in the hospital is deemed ineffective to clean and disinfect surfaces where *C. parapsilosis* may occur and a review of the practice as well a different disinfectant may be required.

One of the most significant virulence factors of *C. parapsilosis senso latu* is the formation of biofilms (Modiri et al., 2019). Attachment to foreign materials like invasive lines, protect the pathogen from the effects of the antifungal agents, resulting in infections that are persistent and difficult to treat. Crystal violet assay used in this study is one of the common methods to measure the biofilm mass. Regardless of the viability of cells, this method stains all the cells in the biofilm making the assay reliable for determining the total biofilm mass (Melo et al., 2011).

All patients in the study had invasive devices. The ability to attach to the devices increases the organism's virulence and persistence of the infection since the device acts as the source unless the source is addressed (Tumbarello et al., 2007). In addition, increased mortality is associated with biofilm producing *C. parapsilosis* (Rajendran et al., 2016). All the strains in our study formed biofilms at varying levels. The results are similar to previous findings where majority (72,5%) of *C. parapsilosis* strains were either in the low or medium biofilm producer category (Guembe et al., 2017, Marzucco et al., 2024).

According to literature*, C. parapsilosis* biofilm production correlate with the metabolic activity unlike the other *Candida* species (Marzucco et al., 2024). Though not tested in our study, and if true across all *C. parapsilosis* strains, then our strains had low metabolic activity. This means even the less compact biofilms of *C. parapsilosis* can reduce the effectiveness of fluconazole. In our study, these biofilms had no effect on other antifungals tested including amphotericin B, which was not the case in Melo and colleagues' study (Melo et al., 2011).

The secreted hydrolytic enzymes facilitate adherence and tissue penetration and hence invasion (Paula-Mattiello et al., 2017, Pandey et al., 2018). In this study, all 20 strains showed protease and phospholipase activity. However, in a study by Brilhante and colleagues (2018) with 49 *C. parapsilosis* strains, only

55.1% of the strains had protease and phospholipase activity (Brilhante et al., 2018). Similarly, in a study by Pandey and colleagues (2018) with nine *C. parapsilosis* strains, only three strains were positive for the former and five positive for the latter (Pandey et al., 2018).

Not much information is reported in literature about the secreted lipases however, they are thought to play a role in nutrition acquisition by the organism and therefore play a role in the pathogenesis (Tóth et al., 2014, Štefánek et al., 2023). In some studies, not all strains of *C. parapsilosis* produced all the hydrolytic enzymes and majority of those studied are the proteases and the phospholipases (Abi-chacra et al., 2013). In a study by Bramono and colleagues, lipase activity was found in all clinical *Candida* species studied including *C. parapsilosis* (Bramono et al., 2006).

The production of hydrolytic enzymes by *C. parapsilosis* strains in our study exhibited heterogeneity, with strains demonstrating low, medium, and high levels of enzyme production, alongside an inability to produce lipases. The nonproduction of lipases could possibly be explained by our small study size or differences in experimental conditions (Abi-chacra et al., 2013, Ziccardi et al., 2015).

Prostaglandins, which are examples of eicosanoids, are metabolites of C20 fatty acids, such as arachidonic acid. They serve a variety of biological purposes including modulating inflammation (Grózer et al., 2015, Funk, 2001). The production of prostaglandin by yeasts was first described in *C. albicans* and subsequently in other yeast, like *Cryptococcus neoformans* (Noverr et al., 2002). Similarly, *C. parapsilosis* is capable of producing prostaglandins (Prostaglandin E_2 and Prostaglandin D_2) from exogenous arachidonic acid (Chakraborty et al., 2019). Prostaglandin E_2 seems to promote the immune system evasion of *C. parapsilosis* by protecting the organism from phagocytic killing (Mendoza et al., 2021).

The production of prostaglandins in *C. parapsilosis* is not mediated by the *OLE2* as in *C. albicans* but by homologues of a multi copper oxidase encoding gene

(*FET3*), an acyl-CoA thiolase encoding gene (*POT1*) and an acyl-CoA oxidase encoding gene (*POX1-3*) (Chakraborty et al., 2018, Grózer et al., 2015).

While the exact role of the three genes mentioned above namely; *FET3, POT1* and *POX1-3* still need further research, their deletion or absence also lead to decreased virulence of *C. parapsilosis* (Chakraborty et al., 2019). Using macrophages that were derived from human peripheral blood monocytes, Chakraborty and colleagues further found that deletion of *FET3* reduces fungal burden (Chakraborty et al., 2018).

An invaluable tool for studying *Candida* species virulence is the infection model, *Caenorhabditis elegans*. The majority of clinically significant dimorphic fungi have been studied using *C. elegans*, which has shown to be a useful tool for identifying several virulence factors and immune-regulators as well as screening potent antifungal drugs (Ahamefule et al., 2021). Although *C. elegans* lacks adaptive immunity, and the understanding of its innate immunity still needs further understanding (Tran and Luallen, 2024), this soil nematode offers a great potential because of its rapid (3-day) life cycle, small size (1.5-mm-long adult), being a self-fertilizing hermaphrodite and the ease of laboratory cultivation (Brenner, 1974).

Similar to the results by Souza and colleagues (Souza et al., 2018b), *C. parapsilosis sensu stricto* resulted in higher mortality higher than that of *E. coli* OP50. Sousa and colleagues further reported on the presence of formation of filaments, the element associated with *C. elegans* killing (Souza et al., 2018b). Of note in this study, aggregation of hyphal filaments as described by Huang (Huang et al., 2014) were visualized on infected nematodes. The elements have been reported on *C. albicans* infection as well. A study by Pukkila-Worley indicated that some *Candida* species such as *C. lusitaniae* and some *C. albicans* mutant strains may not form hyphae and thus show decreased virulence (Pukkila-Worley et al., 2009). All the strains in our study were *C. parapsilosis sensu stricto* and could therefore not compare the *in vitro* virulence with that of *C. orthopsilosis* and *C. metapsilosis* strains. However, as expected from the study by Brilhante and colleagues, the strains were virulent (Brilhante et al., 2018).

CONCLUSION

In conclusion, the study demonstrated that virulent strains of *C. parapsilosis sensu stricto* continue to be the cause of high morbidity and mortality among the vulnerable population. Continuous surveillance of the circulating strains in healthcare settings should be emphasized and strengthened. More studies are needed to clarify the role of lipase production and the PGE2 production in the pathogenesis of *C. parapsilosis*. Furthermore, studies to identify other preventative measures in addition to surgical intervention for the neonates, especially those presenting with gastrointestinal infections common among the study population will add to the efforts to combat the spread of *C. parapsilosis.*

Resistance to azoles, increasing echinocandin MICs and the ineffectiveness of chlorine based disinfectants remains a concern. Amphotericin B and echinocandins where applicable, should be used as first line antifungal agents as recommended by the guidelines. Source control including management of invasive devices should be emphasized because of the nature of this organism's ability to form biofilms. A review of the disinfection practice, longer contact time of the QualiClean or a change of the disinfection may be warranted to decrease the burden of the organisms in the wards. Furthermore, molecular studies such as sequencing would be beneficial in determining the relatedness and resistance determinants of the strains.

Limitations of the study include the small number of strains. In addition to retrieval of only 17 of the hospital clinical notes, the duration of therapy could not be determined due to the paucity of information in the clinical notes. Furthermore, the study was done at one hospital, so no results could be extrapolated to other hospitals. Also, adult wards were not sampled to compare the strains with paediatric wards strains in order to draw a conclusion about what is circulating in the study site among both paediatrics and adult patients. There was no sampling of the environmental or healthcare workers hands to compare the strains circulating in the environment to the clinical strains.

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CHAPTER 3: WHOLE GENOME SEQUENCING OF STRAINS TO DETERMINE THE RELATEDNESS AND POSSIBLE GENETIC DETERMINANTS OF DRUG RESISTANCE

3.1 ABSTRACT

Introduction:

Fluconazole-resistant *Candida parapsilosis* has been dubbed a new threat among the paediatric population. The fluconazole-resistant *C. parapsilosis* strains are circulating in healthcare facilities globally. This poses a threat to empiric treatment of *Candida* species infections in resource limited countries with no availability of echinocandins or in cases where amphotericin B cannot be used. The whole genome sequencing was conducted to study the ploidy of the study strains, genetic relatedness as well as the common antifungal resistance genes.

Methodology:

Eighteen stored *C. parapsilosis* strains from Universitas hospital neonatal wards and two *C. parapsilosis* strains from paediatric wards with known antifungal susceptibility testing results were studied. These strains were from blood culture specimens sent for routine Microbiology laboratory investigations from the year 2018 to 2020. The DNA of all strains was extracted, followed by whole genome sequencing of the strains. After the genomes were assembled, the BBTools k-mer count was used to predict ploidy of the strains and the phylogenetic tree was created and visualized in FigTree. Blast databases were created for each assembly to identify the resistance genes (*FKS1*, *MRR1* and *ERG11*).

Results:

Interestingly, all strains were haploid and were found to group into four related clades, with majority of the strains in Clade 4. The *FKS1*, *MRR1* and *ERG11* genes were highly conserved between strains with none of the mutations previously associated with resistance patterns.

Conclusion:

The high level of relatedness of the different strains indicates that the population of *C. parapsilosis* circulating within the hospital is mostly clonal and constant over the three years. Since no mutations were found in the studied resistance genes, it is clear that resistance to antifungal drugs observed for these strains were as a result of other mechanisms. Continuous surveillance and intensified infection prevention measures are required

3.2 INTRODUCTION

Candida parapsilosis sensu lato is a complex of three cryptic species namely the *C. parapsilosis sensu stricto*, *C. orthopsilosis* and *C. metapsilosis* as discovered by molecular analysis because of their genotypic differences (Tavanti et al., 2005). The cryptic species belong to the clade that translate CTG as serine instead of leucine (Fitzpatrick et al., 2006, Mancera et al., 2019, Bergin et al., 2024).

Candida parapsilosis is an opportunistic pathogen that causes outbreaks in hospitals, especially among the critically ill neonates. The neonates have immature immune systems and have medical devices that promote biofilm formation. Furthermore, some have health conditions that predispose them to fungal infections such as gastrointestinal diseases and thus have to undergo surgery. The World Health Organisation (WHO) placed *Candida parapsilosis* under the high risk fungal priority list (WHO, 2022).

Clades of *C. parapsilosis* are analysed in outbreaks using molecular methods to support or dispute the relatedness of strains. Magobo and colleagues used polymorphic microsatellite marker analysis to identify the closely related genotypes in public hospitals (Magobo et al., 2017). In addition, whole genome sequencing (WGS), a highly discriminatory tool, can be used to analyse phylogeny as well as genetic mechanisms underlying resistance. Eight chromosomal pairs and about 13.1 megabyte (Mb) genome make up the diploid *C. parapsilosis* (Branco et al., 2023). The level of polymorphism is exceptionally low, with only one single nucleotide polymorphism (SNP) per 15,553 bases (Butler et al., 2009), raising the possibility of colonization by a single genotype in hospitals (West et al., 2021)

Although antifungal resistance was rare in *C. parapsilosis* until recently (Daneshnia et al., 2023a), multidrug resistant (MDR) strains, especially those resistant to azoles and echinocandins, have become a global healthcare problem, affecting both poorly resourced and well-resourced countries (Govender et al., 2016, Moudgal et al., 2005, Pristov and Ghannoum, 2019).

Different mechanisms of resistance to azoles exist for *Candida* species. These include the upregulation of *CDR1/CDR3* and *MDR1* (or the transcription factor, *MRR1*) encoding for efflux pumps, mutations or upregulation of the genes in the sterol synthesis pathway (*ERG11* and *ERG3*) as well the development of bypass pathways due to mutations (Pristov and Ghannoum, 2019, Rodrigues et al., 2022).

The most common gene responsible in clinical settings is the replacement of amino acid Y132F in the azole target gene, *ERG11*, encoding lanosterol14-alpha-demethylase (Brassington et al., 2024). Mutations in *ERG3* were also reported by Rybak and colleagues as the reason for azole resistance (Rybak et al., 2017) while Branco and colleagues identified mutations in the *MRR1* transcription factor, responsible for resistance to both fluconazole and voriconazole (Branco et al., 2022).

The Infectious Diseases Society of America (IDSA) guidelines recommend echinocandins as first line antifungals for treatment of yeast (Pappas et al., 2016). Among *Candida* species, *C. parapsilosis* have been reported to have higher minimum inhibitory concentrations (MICs) to echinocandins (Papp et al., 2018). Globally, echinocandin resistant *C. parapsilosis* has been noted in countries including China, (Ning et al., 2023b) and Iran (Davari et al., 2020). Naturally occurring variations in the *FKS1* gene are responsible for the decreased susceptibility to echinocandins.

Amphotericin B is another drug commonly used for invasive fungal infections. Resistance against this antifungal is very rare at about 1.3% (Branco et al., 2023). In addition to other mechanisms such as stress response, resistance may be as a result of upregulation in *ERG* genes (*ERG5, ERG6, ERG25),* leading to the modification in sterol synthesis, or mutations in *ERG* genes (*ERG2, ERG3, ERG6*), leading to the reduction of ergosterol (Sanglard et al., 2003, Ellis, 2002).

In order to gain a better understanding of the strain diversity as well as possible mechanisms of azole resistance in the studied strains, the genomes of the strains were investigated in this chapter.

3.3 MATERIALS AND METHODS

3.3.1 Study setting, design and population

The study setting, design and population are the same as described in Chapter 2.

3.3.2 Sample size and storage

As indicated in Chapter 2, 20 *C. parapsilosis* strains were used in this study. Three strains were from 2018, ten strains from 2019 and the remaining seven strains from 2020. The strains used for the study were stored at -80^{O}C.

3.3.3 Ethical clearance

Ethical approval to perform the study was granted by the University of the Free State's Health Sciences Research Ethics Committee (Ethics Number: UFS-HSD2020/1856/2601). Permission to use the strains was granted by the NHLS Universitas Laboratory Business unit, Head of pathology as well as the head of Microbiology department and department of Biotechnology, Microbiology & Biochemistry. The Free State (FS) Department of Health (DoH) granted permission for access to clinical data. The Biosafety ethics committee granted approval to the study and there was already an authorization to keep, use or handle cultures or preparations of microorganisms (Ref J1/2/4/2 01/19). No patients' names were recorded. Only study numbers were used.

3.3.4 Deoxyribonucleic acid extraction

The total genomic deoxyribonucleic acid (DNA) of the 20 strains was extracted using the Quick-DNA™ Fecal/Soil microbe Miniprep Kit (Zymo research Corporation) according to the manufacturer's instructions. The beta-mercaptoethanol was added to the genomic lysis buffer to a final dilution of 0.5% (500 µL per 100 mL). About 50 to 100 mg of fungal cells that had been resuspended in up to 200 µL of phosphate buffer solution was added to the ZR BashingBead Lysis Tube. The tubes were secured in a bead beater (Scientific Industries Inc) fitted with a 2 mL tube holder assembly and processed using optimized beat beater conditions (5 minutes). The ZR BashingBead Lysis Tube was centrifuged at 10 000 x g for 1 minute.

The supernatant (400 μL) was transferred to a Zymo-Spin III-F Filter in a collection tube and centrifuged at 8000 x g for 1 minute. To the filtrate, 1200 μL of the Genomic Lysis Buffer was added in the collection tube and mixed well. About 800 μL of the mixture was added to a Zymo-Spin IICR Column in a collection tube and centrifuged at 10 000 x g for 1 minute. The flow from the collection tube was discarded and the 800 μL of the mixture was transferred again to a Zymo-Spin IICR Column in a collection tube and centrifuged at 10 000 x g for 1 minute. DNA PRE-Wash Buffer was added to the Zymo-Spin IICR Column in a new collection tube and centrifuged at 10 000 x g for 1 minute. Then 500 μL g-DNA Wash Buffer was added to the Zymo-Spin IICR Column and centrifuged at 10 000 x g for 1 minute. The Zymo-Spin IICR Column was transferred to a clean 2 mL microcentrifuge tube and 100 μL of the DNA Elution Buffer was added directly to the column matrix. The solution was centrifuged at 10 000 x g for 30 seconds to elute the DNA.

The Zymo-Spin HRC Filter was placed in a clean collection tube, 600 μL prep solution added and thereafter centrifuged at 8 000 x g for 3 minutes. The eluted DNA was transferred to a prepared Zymo-Spin HRC Filter in a clean 2 mL microcentrifuge tube and centrifuged at 16 000 x g for 3 minutes. The filtered DNA concentration was then quantified using the nanodrop spectrophotometer (Labtron Equipment Ltd, UK).

3.3.5 Whole genome sequencing

The extracted DNA for all 20 strains was submitted for whole genome sequencing at the National Institute of Communicable Diseases (NICD) sequencing unit. Initial measurement of DNA concentration was done using a Qubit 4 fluorometer (Thermo Fisher Scientific) with the HS kit (Thermo Fisher Scientific). Input samples were then diluted to the required concentration and library preparation performed on a Hamilton NGS Star Plus (Hamilton Company). The Illumina DNA Prep kit (https://emea.illumina.com/products/by-type/sequencing-kits/library-prep-kits/illumina-dna-prep.html) was used to prepare normalised, sequencing-ready libraries as per the manufacturer's guidelines. Strains were sequenced on an Illumina NextSeq 2000 [Illumina (Pty) Ltd)] on a P2 flow cell. Each strain was sequenced to ≥ 200X coverage.

3.3.6 Whole genome sequencing data analyses

Quality assessment and genome assembly

Sequence quality was inspected using FastQC (v0.11.9) (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and combined results were obtained using multiQC (v1.6) (https://multiqc.info/). Trim Galore (https://github.com/FelixKrueger/TrimGalore) was used for quality and adapter sequence trimming with quality filtering set for q20 and a minimum read length of 50bp. In order to confirm species identification and to identify the presence of potential contaminants thus reducing the false positives (Breitwieser et al., 2018), KrakenUniq (v1.04) was applied to sequence reads for all samples using default settings against the KrakenUniq MicrobialDB database and all strains were identified as *C. parapsilosis*.

Genome assembly was performed using Spades (v3.14.1) (Prjibelski et al., 2020) in careful mode. Assembly statistics were generated using Quast (v5.0.2) (Gurevich et al., 2013). In addition, assembly quality was inspected using BUSCO (v5.2.2) (Manni et al., 2021) where *C. albicans* was used as a model for augustus (v3.4.0) (Stanke et al., 2008) and the fungalodb10 database.

Comparative genomics and phylogenetics

In order to assess the ploidy of *C. parapsilosis* strains, trimmed sequence reads were mapped to the masked reference sequence for *C. parapsilosis* (GCF_000182765.1_ASM18276v2_genomic.fna) using bwa-mem (v07.17) (Li, 2013). Samtools (v1.11) (Li et al., 2009) was used to convert the sam alignment file into a bam file and, thereafter, to sort the bam file by coordinates. The sorted alignment file was inspected for ploidy using ploidyNGS (Augusto Corrêa dos Santos et al., 2017) . This produced a text file that predicted the ploidy while also producing a histogram of heteromorphic variants. The results provided by the histogram were confirmed using BBTools kmer count (https://github.com/BioInfoTools/BBMap).

All *C. parapsilosis* genomes available on Genbank/RefSeq were downloaded from the National Centre for Biotechnology Information (NCBI – 7 August 2023). Furthermore, reference genomes for *C. albicans* (GCA_000182965.3_ASM18296v3) and *C. orthopsilosis* (GCA_000315875.1_ASM31587v1) were included in the first phylogeny.

The Benchmarking Universal Single-Copy Orthologs (BUSCO) was ran on all the RefSeq/Genbank genomes as per genomes generated in the current study. Thereafter, BUSCO_phylogenomics was used to generate a protein alignment of all core genes (https://github.com/jamiemcg/BUSCO_phylogenomics). A total of 512 single-copy and complete genes present in all strains were used for a MUSCLE (v5.1) (Edgar, 2004) alignment that spanned 317 848 amino-acids in length. The supermatrix alignment was inspected with modeltest-NG (v0.2.0) (Darriba et al., 2020) to identify the best model for constructing a maximum-likelihood tree. The Akaike information criterion (AIC), corrected AIC (AICc) and Bayesian information criteria (BIC) were in agreement that the model JTT-DCMUT+G4+F was the best model for the supermatrix maximum-likelihood tree. This model was used to reconstruct a phylogenetic tree using raxml-ng (v1.0.1) (Stamatakis, 2014). Bootstrap support was added to the best RAxML tree with 200 iterations performed. The tree was visualized in FigTree (https://github.com/rambaut/figtree/releases).

A second phylogeny was constructed using the same procedure except that *C. albicans* and *C. orthopsilosis* were removed from analysis thereby comparing only *C. parapsilosis* strains. Based on results from modeltest-NG for the second phylogeny the PMB+I+G4+FC samples model was used.

The SKA kmer-based algorithm (Harris, 2018), which is designed for haploid organisms, was applied to the genome assemblies. For SKA the reference *C. parapsilosis* was used for reference-based SNP-calling and as an outgroup. The SKA produced both a phylogenetic tree, a variant calling file per sample (VCF) as well as a table of all-vs-all SNP comparisons. Distances between strains were defined using various metrics such as the Jaccard Index, Mash-like distance, total number of SNPs and the SNP distance. Distance results can be used to define clades of related specimens through all the listed distance metrics. Furthermore, by comparing SNP counts between samples against a phylogenetic tree a defined number of SNPs between samples can be established and used as a cut-off for defining epidemiological clades.

In order to facilitate subsequent gene searches and for further comparative genomics applications all new assemblies were annotated. Annotations were not performed *de novo* but instead reference annotations were transferred from the reference genome annotations to each new assembly using liftoff (v1.6.3) (Shumate and Salzberg, 2021) with polishing. Gffread (v0.12.8) (Pertea and Pertea, 2020) was then used to extract all transcripts and proteins. The genomes were deposited in the National Center for Biotechnology Information database.

Identification and comparison of selected resistance-associated genes

Blast databases were created for each assembly and the FKS1 reference protein sequence (XP_036663785.1) was aligned to each assembly using NCBI BLAST tblastn (v2.10.1) (Altschul et al., 1990). Alignments for each assembly were inspected to ensure the correct sequence was identified. Thereafter, a bed file was created for each assembly that identified the location of BLAST alignments. The bed file was used to extract the nucleotide sequence using bedtools (v2.29.2) (Quinlan and Hall, 2010) from each respective assembly and where the alignment was identified on the reverse strand the sequence was reverse-complemented and thereafter, all sequences were translated in 3 forward frames and the correct frame selected for each assembly. A sequence alignment that included the reference sequence was created using MAFFT (v7.487) (Katoh and Standley, 2013) with the default auto setting and the alignment was inspected for mutations relative to the reference amino-acid sequence. The same process was followed for the genes, *MRR1* and *ERG11*.

3.4 RESULTS

3.4.1 Deoxyribonucleic acid extraction

Sufficient-quality DNA was obtained for all 20 study strains.

3.4.2 Ploidy of *Candida parapsilosis* strains

As per the histograms produced by ploidyNGS, all samples were identified as haploid using BBtools k-mer count.

3.4.3 Phylogeny of *Candida parapsilosis* strains

The genetic relatedness of strains was assessed by the phylogenetic tree as shown in the Figure 1. Four clades, separate from the reference strain (GCA000182765.2 ASM18276V2) were identified. The boostrap values of all the four clades were significant. All study strains had >2200 SNPs when compared to the reference, while the SNPs differences between all test strains ranged between 209 and 2858. The largest clade (Clade 4) contained 16 strains, isolated during all three years of the study. In comparison to the other three clades, this clade housed majority (15/16; 94%) of the fluconazole-resistant strains. In addition, Clade 4 had 12 voriconazole intermediate strains as well and one voriconazole-resistant strain. Clades two and three consisted of only fluconazole-susceptible strains while one of the Clade 1 strains was fluconazole-resistant. Both paediatric patients' strains were in Clade 4, showing close relatedness to some of the neonatal wards' strains in Clade 4 and suggesting the presence of a strain that has established itself in both the neonatal and paediatrics wards. By comparison the number of SNPs between

Clade 4 strains to strain 20 (Clade 3) ranged from 804-871 and >2452 between Clade 1 strains indicating that they were not closely related.

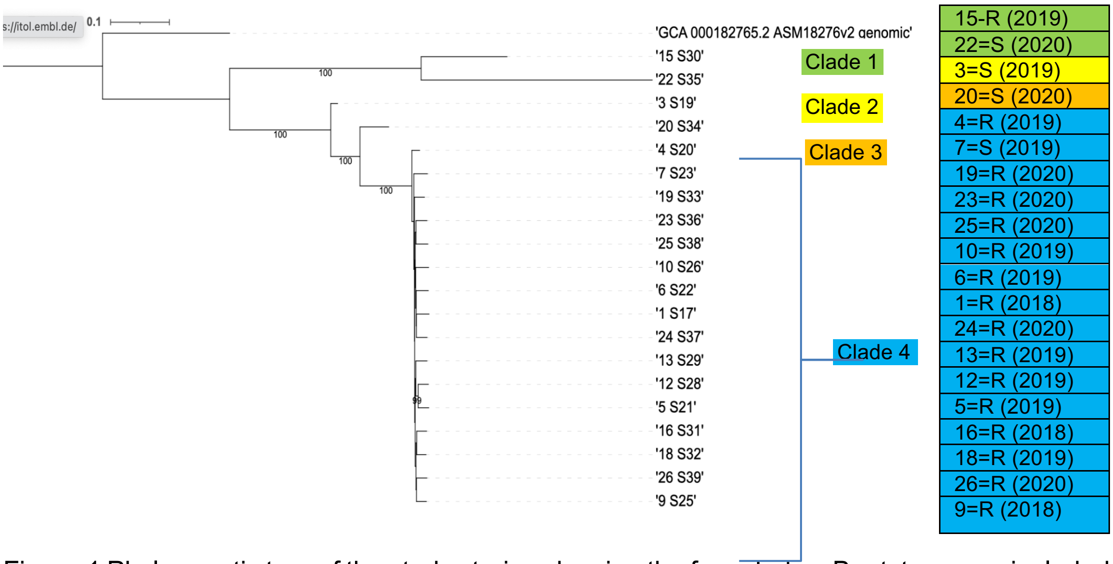

Figure:1 Phylogenetic tree of the study strains showing the four clades. Bootstraps are included on the tree. A reference strain is included among the strains. All the fluconazole-resistant strains are indicated with an R while fluconazole-susceptible strains are indicated with S. The year of strain isolation is indicated in brackets.

The Biosample accession numbers of the strains are listed in Table 1. They were uploaded to the NCBI sequence and are available under BioProject ID PRJNA1137595 using the link: https://urldefense.com/v3/__http://www.ncbi.nlm.nih.gov/bioproject/1137595__;!!LRJdiIM!DQVwrmVdquqLofS0XGGszgng25nyQAR35H-DygHEhF-TcQ5nrT98cwbE9G3jeB86ICLj4KnPlTf2VkRuapBDhyQ$

There are now 44 strains available on the NCBI (https://www.ncbi.nlm.nih.gov/datasets/genome/?taxon=5480) as shown on Table 3, but at the time of collection of study strains, there were 32 genomes available. The phylogenetic tree did not indicate exactly which of the previous genomes the study strains clustered with.

Table 1: The Biosample accession numbers of the study strains.

Accession	**Sample Name**	**SPUID**	**Organism**	**Tax ID**	**Strain**
SAMN42623213	C_para_1	C_para_1	*Candida parapsilosis*	5480	*Candida parapsilosis sensu stricto*
SAMN42623214	C_para_3	C_para_3	*Candida parapsilosis*	5480	*Candida parapsilosis sensu stricto*
SAMN42623215	C_para_4	C_para_4	*Candida parapsilosis*	5480	*Candida parapsilosis sensu stricto*
SAMN42623216	C_para_5	C_para_5	*Candida parapsilosis*	5480	*Candida parapsilosis sensu stricto*
SAMN42623217	C_para_6	C_para_6	*Candida parapsilosis*	5480	*Candida parapsilosis sensu stricto*
SAMN42623218	C_para_7	C_para_7	*Candida parapsilosis*	5480	*Candida parapsilosis sensu stricto*
SAMN42623219	C_para_9	C_para_9	*Candida parapsilosis*	5480	*Candida parapsilosis sensu stricto*
SAMN42623220	C_para_10	C_para_10	*Candida parapsilosis*	5480	*Candida parapsilosis sensu stricto*
SAMN42623221	C_para_12	C_para_12	*Candida parapsilosis*	5480	*Candida parapsilosis sensu stricto*
SAMN42623222	C_para_13	C_para_13	*Candida parapsilosis*	5480	*Candida parapsilosis sensu stricto*
SAMN42623223	C_para_15	C_para_15	*Candida parapsilosis*	5480	*Candida parapsilosis sensu stricto*
SAMN42623224	C_para_16	C_para_16	*Candida parapsilosis*	5480	*Candida parapsilosis sensu stricto*
SAMN42623225	C_para_18	C_para_18	*Candida parapsilosis*	5480	*Candida parapsilosis sensu stricto*
SAMN42623226	C_para_19	C_para_19	*Candida parapsilosis*	5480	*Candida parapsilosis sensu stricto*
SAMN42623227	C_para_20	C_para_20	*Candida parapsilosis*	5480	*Candida parapsilosis sensu stricto*
SAMN42623228	C_para_22	C_para_22	*Candida parapsilosis*	5480	*Candida parapsilosis sensu stricto*
SAMN42623229	C_para_23	C_para_23	*Candida parapsilosis*	5480	*Candida parapsilosis sensu stricto*
SAMN42623230	C_para_24	C_para_24	*Candida parapsilosis*	5480	*Candida parapsilosis sensu stricto*

SAMN42623231	C_para_25	C_para_25	*Candida parapsilosis*	5480	*Candida parapsilosis sensu stricto*
SAMN42623232	C_para_26	C_para_26	*Candida parapsilosis*	5480	*Candida parapsilosis sensu stricto*

Table 2: Genomes from other strains in South Africa.

Assembly	**GenBank**	**Scientific name**	**Release**	**WGS accession**
ASM18276v2	GCA_000182765.2	*C. parapsilosis*	Oct 2011	
ASM3628897v1	GCA_036288975.1	*C. parapsilosis*	Jan, 2024	
canParSR23v1	GCA_963989715.1	*C. parapsilosis*	Mar, 2024	
ASM1534487v1	GCA_015344875.1	*C. parapsilosis*	Nov, 2020	JADLIH01
ASM3056877v1	GCA_030568775.1	*C. parapsilosis*	Jul, 2023	JAJMGO01
ASM1404949v1	GCA_014049495.1	*C. parapsilosis*	Aug, 2020	JABWAC01
ASM1404944v1	GCA_014049445.1	*C. parapsilosis*	Aug, 2020	JABWAB01
ASM1404958v1	GCA_014049585.1	*C. parapsilosis*	Aug, 2020	JABVZZ01
canParB	GCA_903989565.1	*C. parapsilosis*	Oct, 2021	CAJEJE01
CUCEI_Cparap_1.0	GCA_037952885.1	*C. parapsilosis*	Apr, 2024	JBAJNE01
CPARA-Y9	GCA_947184175.1	*C. parapsilosis*	Nov, 2022	CAMXCU01
ASM1404945v1	GCA_014049455.1	*C. parapsilosis*	Aug, 2020	JABWAA01
ASM3271479v1	GCA_032714795.1	*C. parapsilosis*	Oct, 2023	JAUTWI01
90-137_Cse4-HA_2.0	GCA_011316035.2	*C. parapsilosis*	May, 2020	VUYR02
CBS6318.1	GCA_000982555.2	*C. parapsilosis*	Mar, 2014	CBZQ02
CBS1954.1	GCA_900004165.1	*C. parapsilosis*	Oct, 2014	CBZP02
GA1.1	GCA_000982675.1	*C. parapsilosis*	Oct, 2014	CBZX02
ASM2576798v1	GCA_025767985.1	*C. parapsilosis*	Oct, 2022	JAMFPA01
ASM2576788v1	GCA_025767885.1	*C. parapsilosis*	Oct, 2022	JAMFPD01
ASM2576799v1	GCA_025767995.1	*C. parapsilosis*	Oct, 2022	JAMFOZ01
ASM2576790v1	GCA_025767905.1	*C. parapsilosis*	Oct, 2022	JAMFPB01
ASM2576771v1	GCA_025767715.1	*C. parapsilosis*	Oct, 2022	JAMFPH01
ASM2576777v1	GCA_025767775.1	*C. parapsilosis*	Oct, 2022	JAMFPG01
ASM2576804v1	GCA_025768045.1	*C. parapsilosis*	Oct, 2022	JAMFOX01

ASM3704109v1	GCA_037041095.1	*C. parapsilosis*	Mar, 2024	JAXQHX01
ASM3704112v1	GCA_037041125.1	*C. parapsilosis*	Mar, 2024	JAXQIA01
FS367	GCA_008764215.1	*C. parapsilosis*	Oct, 2019	VTQU01
ASM2576791v1	GCA_025767915.1	*C. parapsilosis*	Oct, 2022	JAMFPE01
ASM1673578v1	GCA_016735785.1	*C. parapsilosis*	Jan, 2021	JADCQT01
ASM2576782v1	GCA_025767825.1	*C. parapsilosis*	Oct, 2022	JAMFPF01
ASM2576800v1	GCA_025768005.1	*C. parapsilosis*	Oct, 2022	JAMFOY01
ASM2576789v1	GCA_025767895.1	*C. parapsilosis*	Oct, 2022	JAMFPC01
ASM3704071v1	GCA_037040715.1	*C. parapsilosis*	Mar, 2024	JAXQHE01
ASM1673582v1	GCA_016735825.1	*C. parapsilosis*	Jan, 2021	JADCQS01
ASM3235709v1	GCA_032357095.1	*C. parapsilosis*	Oct, 2023	JASKDP01
ASM4143060v1	GCA_041430605.1	*C. parapsilosis*	Aug, 2024	JBFMME01
CPARA-4418	GCA_964199955.1	*C. parapsilosis*	Jul, 2024	CAXMZS01
ASM2553188v1	GCA_025531885.1	*C. parapsilosis*	Oct, 2022	JAMFRL01
ASM2929064v1	GCA_029290645.1	*C. parapsilosis*	Mar, 2023	DAGWRK01
ASM2002712v1	GCA_020027125.1	*C. parapsilosis*	Sep, 2021	JAHPZS01
SRR1779153_bin.70_CONCOCT_v1.1_MAG	GCA_937873065.1	*C. parapsilosis*	Jan, 2023	CALMVX01
ASM402644v1	GCA_004026445.1	*C. parapsilosis*	Jan, 2019	SCGV01
ASM402628v1	GCA_004026285.1	*C. parapsilosis*	Jan, 2019	SCGQ01
ASM2002707v1	GCA_020027075.1	*C. parapsilosis*	Sep, 2021	JAHPZQ01

Blank spaces indicate non availability of the WGS accession.

3.4.4 Resistance genes detected

The strains were evaluated for resistance genes against azoles, amphotericin B and echinocandins. The *ERG11* gene responsible for resistance to azoles and amphotericin B was highly conserved in all study strains. The most common mutation, which may be due to the silent mutations (T591C), or the missense mutations (A396T), leading to the replacement of amino acid Y132F, was not detected. Mutations in the *MRR1* transcription factor were also not detected as well as the mutations in the multidrug resistance 1 gene (*MDR1*).

The *ERG3* gene mutations which have been reported in the literature were not studied.

Similarly, the *FKS1* gene responsible for resistance in echinocandins was identified in the newly sequenced *C. parapsilosis* assemblies. All strains carried mutations at known locations that confer fungal echinocandin resistance in various fungal species i.e. *C. parapsilosis* P660A in the hotspot 1 of the *FKS1* gene. Amino-acid sequences did not vary between strains for the Fks1 protein and no differences were identified between the study strains and the reference strain. Mutations in the hotspot 2 or outside the hotspot such as the V595I, F1386S conferring resistance to the echinocandins, were not studied. There were few differences identified between study strains and the genes from the reference strain and none of the mutations correlated with resistance patterns.

3.5 DISCUSSION

Ploidy refers to the number of chromosomal sets in an organism (Todd Robert et al., 2017). An organism's ploidy level is crucial for understanding its evolution, populations, and genomes (Augusto Corrêa dos Santos et al., 2017). Errors in the replication can affect an organism's ploidy status, resulting in the loss or an increase in fitness (Todd Robert et al., 2017). Aneuploidy, the imbalance in the genome, is used as a response mechanism to stress by *Candida* species (Yang et al., 2021).

Fungal genomes are known to vary in terms of ploidy, and this may play a role in their pathogenicity. Although *C. parapsilosis* is typically diploid, the predicted ploidy values produced unexpected results for our strains with values e.g. ploidy >=6. This rate of heterozygosity is sufficiently low that k-mer based analysis called all *de novo* genomes in our study as haploid. A low level of heterozygosity has previously been reported for *C. parapsilosis* (Pryszcz et al., 2013). Similar to our study, Tavanti and colleagues' study supported the haploid status with low variation in the genome of *C. parapsilosis* (Tavanti et al., 2010).

Different ploidy states for *C. parapsilosis* strains have also been discussed in literature with some articles referring to *C. parapsilosis* as diploid, haploid or aneuploid (Fundyga et al., 2004). These variations are among individual strains of the same species and are also influenced by the environmental conditions (Todd Robert et al., 2017). Whelan and Kwon-Chung suggested that *C. parapsilosis* is either a diploid or aneuploid since it is disomic for at least one chromosome (Whelan and Kwon-Chung, 1988). The aneuploidy was also supported by Fundyga and colleagues who found that the natural isolates of *C. parapsilosis* may be aneuploid (Fundyga et al., 2004).

Outbreaks due to fluconazole-resistant strains have been detected in Bloemfontein, though without the detection of the most common mutation, Y132F (Bergin et al., 2024). Four different clades were circulating at the time of this study as shown in figure 1. Single nucleotide polymorphisms refer to a genomic variant at a single base position in the DNA and are used to determine the relatedness of strains. Most of the strains were housed in clade four which had SNPs ranging from 209 to 313 indicating a single source outbreak. This clade shows closely related strains from 2018 to 2020 supporting the persistence of the source in the unit. Clade 1 was responsible for cases in 2019 and 2020.

The closely related strains in clade 4 might have persisted throughout the three years with the introduction of some other strains in 2019 and 2020 that are not closely related to clade 4. Clade 4 was associated with resistant strains. This is similar to Magobo and colleagues' study where certain clades were also associated with resistant strains (Magobo et al., 2017). Similar to the study by Misas and colleagues, most resistant strains were grouped in one clade suggesting the existence of a single resistant strain population that most likely spread by transmission (Misas et al., 2024).

Currently, guidelines like IDSA recommend echinocandins as first line antifungals for *Candida* infections followed by amphotericin B (Pappas et al., 2016). While our study strains were susceptible to echinocandins, the resistance to echinocandins have been reported from other parts of the world such as China (Ning et al., 2023a). The echinocandins MICs tend to be higher in *C. parapsilosis* since the organisms has a naturally occurring polymorphisms in the *FKS1* gene (Martí-Carrizosa et al., 2015). Mutations in the gene *FKS1* have previously been associated with echinocandin resistance in *C. parapsilosis* (Khalifa et al., 2024). In our study, all strains carried mutations in the *FSK1*, despite the strains being susceptible to the echinocandins. In a South African study by Magobo and colleagues, there was no detection of any mutations in the *FKS* genes of the *C. auris* strains that were resistant to echinocandins suggesting involvement of other mechanisms (Rindidzani et al., 2020). Mutations outside the two hotspot regions of the *FKS1* gene have been identified in literature with some conferring resistance to echinocandins (V595I, F1386S) and some not (S745L, M1328I, A1422G) as mentioned in a study by Carrizosa and colleagues (Martí-Carrizosa et al., 2015). Our study strains were also susceptible to amphotericin B supporting the rare occurrence of resistance to this antifungal in *C. parapsilosis* isolates.

The reporting of fluconazole resistance among the *C. parapsilosis* strains continues worldwide (Thomaz et al., 2022, Govender et al., 2016, Zeeshan et al., 2024). The resistance has been largely associated with the Y132F mutation in the *ERG11* gene (Escribano and Guinea, 2022). Others mutations include K143R, M178T, N283Y and T591C mutations (Ceballos-Garzon et al., 2022). What is also clear in the literature is that not all fluconazole-resistant isolates harbour the Y132F mutation. In a study by Asadzadeh and colleagues, only five of the eleven fluconazole-resistant isolates harboured this mutation (Asadzadeh et al., 2017). The *ERG11* mutation was also reported by Corzo-Leon and colleagues in 12 of their 15 clinical isolates in Mexico (Corzo-Leon et al., 2020) and a study by Zeeshan and colleagues also detected the mutation in six out of seven fluconazole-resistant *C. parapsilosis* isolates (Zeeshan et al., 2024), while Ceballos-Garzon and colleagues only detected the mutation in 58% of the resistant isolates (Ceballos-Garzon et al., 2022). In our study, *ERG11, MDR* and *MRR1* genes were highly conserved between strains with no mutations correlating with resistance patterns.

The absence of mutations correlating with resistance to fluconazole and voriconazole in our study strains also suggest that other mechanisms of resistance were responsible for the resistance to fluconazole and voriconazole observed in our study strains. These data suggest that factors such as gene regulation rather than specific mutations may account for differences in drug-resistance. Furthermore, the results suggest that the common mutations mentioned in literature might not be prevalent at the study setting.

3.6 CONCLUSION

Whole genome sequencing remains a widely used tool to identify possible outbreaks. The findings support a need for continuous surveillance of resistance among *C. parapsilosis* strains. Infection prevention measures need to be intensified to break the chain of transmission from medical devices, the environment and possibly personnel. Further studies are required to identify the resistance mutations or novel mutations prevalent at the study site, including those outside the common regions.

The study limitations included the small sample size as well as the inability to study other resistance genes and the expression of resistance genes in the strains. In addition, strains from adult units were not included in the study. Environmental or healthcare workers' samples were not obtained to establish a correlation with the strains from the patients.

3.7 RECOMMENDATIONS AND FUTURE RESEARCH DIRECTIONS

The study highlighted the risk factors, virulence factors produced by the strains, and the clinical outcome of the patients. The resistance pattern and the ineffectiveness of the disinfectants are also highlighted. Strict infection prevention and control measures need to be reinforced, a change of disinfectant is recommended as well as a continuous monitoring of antifungal susceptibility patterns. Future studies are therefore required to identify the resistance mutations or novel mutations prevalent at the study site, including those outside the common regions.

3.8 REFERENCES

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CHAPTER 4: GENERAL DISCUSSION AND CONCLUSION

4.1 GENERAL DISCUSSION AND CONCLUSION

Morbidity and mortality due to *Candida parapsilosis* among neonates is on the rise globally (Hassan et al., 2019, Çetin Fatma et al., 2024). The prevalence of *C. parapsilosis* and the rise of azole-resistant strains complicate the situation further. In South Africa, several studies show the increasing prevalence of *C. parapsilosis* over *C. albicans* (Malunga et al., 2020, Pillay et al., 2021, Ramdin et al., 2023). In the latest study by Ramdin and colleagues, the incidence of candidemia ranged between 1.2 -5.1 per admissions over a period of seven years (2013-2019) (Ramdin et al., 2023). Understanding the circulating strains, their virulence, relatedness, resistance determinants, as well as the risk factors prevalent in the population under study, is essential to combating the strain's rising prevalence.

4.1.1 Risk factors among the studied population

All patients had varying risk factors mentioned in the literature. A total of 82% of the patients were born preterm, similar to Miranda and colleagues' study from Brazil (Miranda et al., 2012). In addition, 42% were very low birth weight, 29% low birth weight and 18% extremely low birth weight. The above-mentioned risk factors often lead to the use of invasive devices, and administration of systemic antibiotics, resulting in prolonged stay to increase the survival rate. Gastrointestinal diseases were by far the most common (71%) followed by respiratory disease (59%). Gastrointestinal surgery was performed on 41% of the patients, further increasing the risk which is estimated to be 51% according to the study performed by Kilc and colleagues from Turkey (Kilic et al., 2020).

Different invasive medical devices, such as lines and endotracheal tubes, were recorded for all patients with total parenteral nutrition (TPN) recorded for 76% thus possibly promoting the adherence and biofilm formation by the pathogen. Invasive devices are the source of infection with persistent seeding of the organism (Manzoni et al., 2006). Education, monitoring and documentation of device management to reduce continuous seeding should be strengthened.

Immunocompromised conditions such as malignancy have been recorded as risk factors and from this study one paediatric patient had acute myeloid leukemia. In addition to the above mentioned risk factors, multiple systemic antibiotics including meropenem, piperacillin-tazobactam, vancomycin, linezolid and colistin were administered to patients, which could also promote fungal infections (Kim et al., 2023).

Besides the presence of risk factors, and although not investigated in our study, possible environmental sources need to be taken care of and studies looking at these sources will benefit the study site. Sinks were implicated as the source of *C. parapsilosis* in a study by Baba and colleagues from Japan (Baba et al., 2024). These may often be overlooked when sources of infection are searched for in an outbreak setting. Proper disinfection of such areas including milk preparation areas as well as communal areas for healthcare workers are important in reducing the environmental colonization. These measures should be coupled with the most basic and often neglected hand hygiene and hand hygiene education. Periodic audits with feedback ought to be done in intensive care units to reduce the risk of nosocomial infections in the already compromised patients.

4.1.2 Strains virulence factors

Candida parapsilosis sensu stricto was responsible for candidemia at the study site with more than half of the strains being azole-resistant. Literature suggest that both the prophylactic use of fluconazole as well as the patient to patient transmission are the possible drivers of fluconazole-resistant strains (Escribano and Guinea, 2022). However, only three patients in this study received fluconazole empirically, indicating that patient-to-patient transmission may have been responsible in this instance rather than the empiric use. In addition, Zhang and colleagues suggested poor source control with subsequent prolonged suboptimal antifungal use as one of the factors driving fluconazole resistance (Zhang et al., 2015). Amphotericin B and echinocandins should be used as first line antifungal agents as recommended by the guidelines (Pappas et al., 2016).

Further studies are needed to evaluate the factors that promote the ease of transmission of the azole-resistant strains in neonatal intensive care units.

The heterogenous production of hydrolytic enzymes by the *C. parapsilosis* strains was evident in our study, since strains varied from low, medium and high production of hydrolytic enzymes and in addition failure to produce lipases. The nonproduction of lipases could be explained by our small study size or differences in experimental conditions (Abi-chacra et al., 2013, Ziccardi et al., 2015). With all study strains being biofilm producers, their ability to attach to the devices increase the virulence and persistence of the infection since devices act as the source of infection (Tumbarello et al., 2007). In addition, increased mortality is associated with biofilm producing *C. parapsilosis* (Rajendran et al., 2016).

Prostaglandin E_2 produced by the study strains could have promoted the immune system evasion of *C. parapsilosis* by protecting the organism from phagocytic killing, adding to the virulence of the strains (Mendoza et al., 2021). In line with previous findings (Souza et al., 2018a), strains of *C. parapsilosis sensu stricto* studied resulted in higher *C. elegans* mortality when modelling the *in vivo* host-fungus interaction. Source control such as management of invasive devices should be emphasised because of the nature of this organism's ability to form biofilms and also to combat the spread of this virulent strains among vulnerable populations.

Our disinfectant results are in line with the results reported by Abdolrasouli and colleagues (2017), who found that the *C. parapsilosis* in their study had consistently higher minimum inhibitory concentrations. Similar results were also seen when sodium hypochlorite solution was tested against pre-formed (24 hour) *C. parapsilosis* biofilms (Pires et al., 2013). The current practise in the hospital is deemed ineffective to clean and disinfect surfaces where *C. parapsilosis* may occur and a review of the practice as well a different disinfectant may be required.

4.1.3 Whole genome sequencing

Four clades were detected with 98% of the resistant strains in the fourth clade. The paediatric and neonatal strains were closely related in the fourth clade, suggesting a common source in wards that are not situated close to each other. The *ERG11, MRR1* and the *FKS1* genes were highly conserved among the strains studied. In addition, the strains had the most common substitution the hotspot 1 of the *FKS1* gene (Garcia-Effron et al., 2008), although they were not resistant to echinocandins. None of the other studied mutations correlated with the resistance pattern. Thus, further studies are required to explore the other resistance genes and the regions outside the hostpot areas.

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