Journal of Molecular Science

Journal of Molecular Science
Volume 35 Issue 4, Year of Publication 2025, Page 1514-1519
DoI-10.004687/1000-9035.2025.196

Journal of Molecular Science

www.jmolecularsci.com ISSN:1000-9035

Antifungal susceptibility profile of *Candida glabrata* and *Candida parapsilosis* in a tertiary care centre - A pilot study

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Article Information
Received: 17-10-2025
Revised: 11-11-2025
Accepted: 26-11-2025
Published: 24-12-2025

Keywords
Candida, *Candida glabrata*, *Candida parapsilosis*, antifungal susceptibility testing, echinocandins, MIC.

ABSTRACT

Background:
Fungal infections caused by non-*albicans* have increased globally changing the epidemiology. Literature studies has suggested the rise in resistance of antifungal drugs since amphotericin B being the only used drug since 1950s.This study aims to focus on the antifungal susceptibility testing of the *Candida glabrata* and *Candida parapsilosis* and its resistance pattern to help with the treatment of these conditions.

Methodology
A total of 74 *Candida* isolates from various samples collected over a period of six months were considered for the study. All the isolates were subjected to conventional phenotypic and genotypic methods for confirmation. Antifungal susceptibility testing was performed using broth microdilution method according to the CLSI guidelines. 8 antifungal drugs; Amphotericin B, fluconazole, itraconazole, voriconazole, posaconazole, caspofungin, micafungin and anidulafungin were tested against the isolates.

Results:
Amphotericin B showed high resistance to *C. parapsilosis* and *C. glabrata* 64.9% and 45.9% respectively. Hence, not being the first drug of choice. In our study, 25 out of 37 isolates of *C. glabrata* were resistant to fluconazole (as per the breakpoints available in the CLSI), which is almost 50. Instead itraconazole, voriconazole or posaconazole can be used which showed 75.7%, 94.6% and 100% sensitivity respectively. Unlike C. *glabrata* ,the sensitivity of *C. parapsilosis* was better with fluconazole (75.7%) and voriconazole (94.6%) but had no major difference with amphotericin B (64.9%).

Conclusion:
We would like to conclude that echinocandins are to be started for *C. glabrata* and *C. parapsilosis* infections in patients with prolonged morbidity. In uncomplicated cases voriconazole can still be used. Judicious use of prophylactic agents and monitoring will prevent development of resistance among the *Candida* infections.

INTRODUCTION:

In recent years, fungal infections caused by *Candida* species have emerged as a significant public health concern, contributing to increased mortality and escalating healthcare costs [1]. While *Candida albicans* remains the most common cause of candidiasis globally, a notable shift has been observed with an increasing prevalence of non-*albicans Candida* (NAC) species such as *Candida glabrata* and *Candida parapsilosis* [1]. This epidemiological trend toward NAC species poses a

Journal of Molecular Science
Volume 35 Issue 4, Year of Publication 2025, Page 1514-1519
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challenge, particularly due to rising antifungal resistance, which complicates the selection of effective treatment regimens [2].

Antifungal susceptibility testing (AFST) plays a critical role in guiding clinical decisions, especially in infections caused by drug-resistant or uncommon *Candida* species. AFST involves the in vitro determination of the minimum inhibitory concentration (MIC) or minimum effective concentration (MEC)—the lowest concentration of an antifungal agent required to inhibit the visible growth of the organism [2].

Historically, amphotericin B was the only available antifungal agent following its introduction in the 1950s, and susceptibility testing was not routinely performed. However, with the advent of newer antifungal agents and the recognition of both intrinsic and acquired resistance, susceptibility testing has become essential for optimizing antifungal therapy [2].

Several variables, including incubation temperature, inoculum size, incubation time, and the medium used, can influence MIC results in vitro. Standardization of these parameters is crucial to ensure reproducible and clinically relevant results [3]. The Clinical and Laboratory Standards Institute (CLSI) M27-A2 guideline is commonly used for conducting antifungal susceptibility testing[4].

Understanding local antifungal susceptibility patterns is vital for effective patient management and infection control. In this context, we conducted a pilot study to evaluate the antifungal susceptibility profiles of *Candida glabrata* and *Candida parapsilosis*, two clinically relevant but less commonly studied NAC species, in a tertiary care center.

MATERIAL AND METHODS:

Ethical considerations: This study was conducted in the Microbiology Department, Mycology Lab for a period of six months from December 2023 to May 2024. Ethics approval was obtained (CSP/23/MAY/128/399) from Sri Ramachandra Institute of Higher Education and Research (DU), Chennai, India for collection of demographic details of the patients with fungal infection.

Inclusion criteria: Isolates from all patients with invasive candidiasis caused by *Candida glabrata* and *Candida parapsilosis* were included in the study.

Exclusion criteria: Isolates from patients affected with all other *Candida* infections other than *Candida glabrata* and *Candida parapsilosis* were excluded from the study.

Statistical analysis:
All the statistical analyses were performed using IBM SPSS Statistics software (version 28.0). Descriptive statistics were used to summarize the antifungal susceptibility profiles of *Candida glabrata* and *Candida parapsilosis*. Continuous variables, such as minimum inhibitory concentration (MIC) values, were expressed as mean MIC.

Comparative analyses between the two *Candida* species were carried out using Chi-square test to compare susceptible (S) or resistant (R) isolates. In addition, a repeated measures analysis of variance (ANOVA) was performed to evaluate the differences in MIC values across various antifungal drugs used in this study within each *Candida* species. A p-value of <0.05 was considered statistically significant for all tests.

Methodology:
Phenotypic identification was done for all the clinical isolates on chromogenic media (Hi-Chrome). Genotypic identification was done by Polymerase Chain Reaction – Restriction Fragment Length Polymorphism (PCR-RFLP) for all the isolates.

Antifungal susceptibility testing was performed by broth microdilution following the CLSI guidelines and tested against 8 antifungal drugs; Amphotericin B, fluconazole, itraconazole, voriconazole, posaconazole, caspofungin, micafungin and anidulafungin.

Phenotypic identification:
Chromogenic identification:
Chromogenic agar such as CHROMagar® *Candida* (Himedia) is a rapid method used for preliminary identification of *Candida* species. On CHROM agar, *C. parapsilosis* showed cream colour colonies *and C. glabrata* showed purple colour colonies. All the isolates were inoculated on the media and incubated at 37ºC for 24 – 48 hours to observe colour morphology.

Genotypic identification:
All the isolates which underwent phenotypic identification were confirmed by genotypic identification.

American Type Cell Culture Collection (ATCC) *Candida albicans* 90028 were used as the reference strains.

Genotypic identification:

Journal of Molecular Science
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Extraction and Amplification
DNA extraction of all the isolates was performed by in-house phenol chloroform method. The extracted DNA was checked using Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, USA) for the purity. PCR conditions were followed as previously described by **Vijayakumar et al** [5].

The primers used were Inter Transcribed Spacer (ITS) 1 and 4 regions and amplification was done using pre-programmed thermal cycler (Qiagen, Germany). DNA bands were visualized under UV trans-illuminator with (100 bp) DNA ladder as the marker (Figure 2). Bands were formed at an amplicon size of 871bp for *Candida glabrata* and 520bp for *Candida parapsilosis.*

RFLP was performed using confirmed PCR products of *C.glabrata* and *C.parapsilosis* and restriction enzyme *Msp*1 and visualized under UV trans-illuminator. Bands corresponding to C*andida parapsilosis* produced an amplicon size of 520bp and *C. glabrata* at 557, 314.

Antifungal susceptibility testing:
AFST was performed according to CLSI guidelines M27 A2 guidelines. All the isolates were sub-cultured on Sabouraud's Dextrose Agar (SDA) plates. *Candida albicans* ATCC 90028 was used as the quality control.

Drug concentration was prepared using antifungal drugs with the highest concentration as mentioned in Table 1. All the drugs were dissolved in Dimethyl sulfoxide (DMSO).

RPMI-1640 medium with L-glutamine without sodium bicarbonate containing 0.2% glucose, MOPS in micro titer plate.

Inoculum suspension was prepared by taking 2-3 colonies of the isolate and dissolved in sterile distilled water. The turbidity was adjusted to a final concentration using 0.5 McFarland's standard (corresponding to $0.5x10^3$ - $2.5x10^3$ cells/ml).

and was dispensed into microdilution wells with various antifungal concentrations.
All the plates were incubated at 37°C for 24 hours and visually analyzed. The incubation was extended to 48 hours in case of insufficient growth.

Table 1 - Antifungal drugs displaying the range and mean MIC(Minimum inhibitory concentration)

Antifungal drug	Range (µg/ml)
Amphotericin B	0.125 – 32
Fluconazole	0.15 – 32
Itraconazole	0.06 – 16
Voriconazole	0.06 – 16
Posaconazole	0.06 – 16
Caspofungin	0.06 – 16
Micafungin	0.06 – 16
Anidulafungin	0.06 – 16

RESULTS:

A total of 74 isolates were collected from blood, urine, tissue and pus. The highest number were from urine specimens, followed by blood (Table 2).

Table 2 : Source and Total number of isolates

Sample source	Total (n)
Urine	39
Blood	28
Tissue	5
Pus	1
Others	1
Total	74

All the isolates underwent both phenotypic and genotypic identification ((Figure 1a, 1b, 2).

Out of the 74 isolates, 37 were identified as *Candida parapsilosis* and 37 were identified as *Candida glabrata.*

Figure 1a: Colour morphology of *Candida parapsilosis* on CHROMagar®

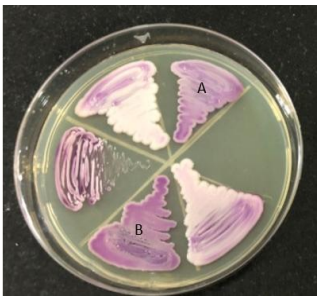

Figure 1b: Colour morphology of *Candida glabrata* (A,B) on CHROMagar®

Figure 2 – Banding pattern of different *Candida* species (Lane 1 – DNA marker, Lane 2 – *Candida auris*, Lane 3,6,14,17 *Candida parapsilosis*, Lane 4,6,8,9,11,12,16 – *Candida glabrata*, Lane 5,13 – *Candida guilliermondii*, Lane 7,10,15,18 – *Candida albicans*)

Journal of Molecular Science

Journal of Molecular Science
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Demographics:
The age of patients ranged from 1 to 82 years, where Male: Female ratio was 38:36. Predominant age group were between 41-60 years (38%) followed by 61-80 years (34%) (Figure 3)

Out of the 74 patients, only 43 of them had co-morbidities of which the predominant underlying condition was diabetes (DM) (35%), which is a risk factor for infection followed by hypertension (HTN) and coronary artery disease (CAD) and chronic kidney disease (CKD) (Figure 4)..

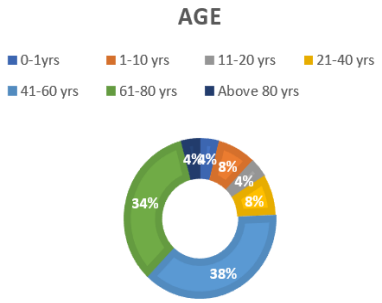

Figure 3: Pie chart representing the distribution of age among the sample.

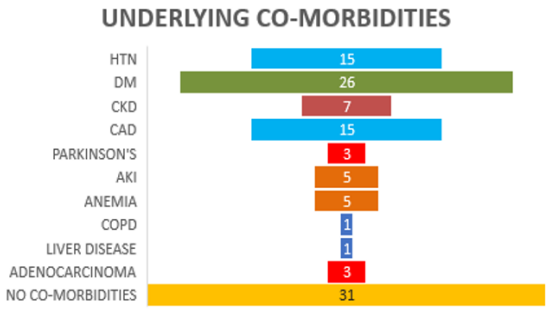

Figure 4: Graph representing the distribution of underlying co-morbid conditions among the sample patients

Antifungal susceptibility testing:
The CLSI M60 guidelines were used to check the breakpoints for various antifungal drugs used. The break points of *C.glabrata* to voriconazole and posaconazole are not available so epidemiological cut offs were taken. Similarly break points for itraconazole, voriconazole and posaconazole are not available for *C. parapsilosis* so epidemiological cut off were taken.

The antifungal susceptibility profiles of *Candida glabrata* and *Candida parapsilosis* isolates demonstrated the highest sensitivity to all the tested echinocandins and posaconazole, with a susceptibility rate of **100%** (**p < 0.01**), which was statistically significant followed by voriconazole, which showed a high sensitivity rate of **94.6%** (**p < 0.01**) also being statistically significant. In contrast, lower susceptibility rates were observed with itraconazole (**59.5%**), followed by fluconazole (**54.1%**), and amphotericin B (**44.6%**), none of which reached statistical significance (**p > 0.01**) (Figure 5,6).

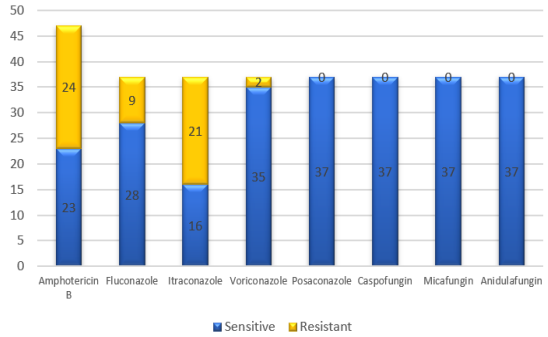

Figure 5: Bar diagram representing Candida *glabrata* sensitivity and resistance to the antifungal drugs. (x axis: antifungal drugs used, y axis: number of isolates)

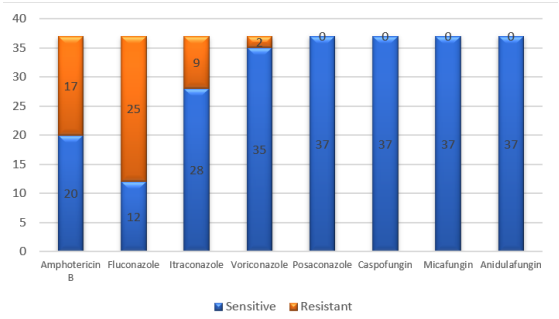

Figure 6: Bar diagram representing Candida *parapsilosis* sensitivity and resistance to the antifungal drugs. (x axis: antifungal drugs used, y axis: number of isolates)

Chi-square analysis revealed a statistically significant difference in susceptibility patterns between the two species. *C. glabrata* showed better susceptibility to itraconazole, while *C. parapsilosis* showed better susceptibility towards fluconazole (**75.7%**).

Repeated measures ANOVA demonstrated a significant variation in MIC values across different antifungal agents within each species (**p < 0.001**), indicating distinct susceptibility profiles depending on the antifungal tested.

Outcome:
Out of the 74 patients, 52 (70%) patients recovered from the infection and 14 (19%) left against medical advice and 8 (22%) succumbed to the infection.

DISCUSSION:
The age group in which the maximum isolates were in the age group of 51 to 70 years This is the age group where maximum number of diabetics are seen in India which is a significant factor for morbidity along with other risk factors in a

hospitalized patient. Diabetes is one of the risk factors which can lead to *Candida* infection [7]. The most common isolate we come across in India is *Candida tropicalis* followed by other non-*albicans Candida* like *Candida glabrata* and *Candida parapsilosis* [1].

Antifungal susceptibility has become a very important part of fungal diagnostics especially with the emergence of resistance to the commonly used antifungal agents. In our study, amphotericin B showed high resistance to *C. parapsilosis* (64.9%) and *C.glabrata* (45.9%) respectively. Hence, not being the first drug of choice.

In our study, 25 (67.6%) out of the 37 isolates of *C. glabrata* were resistant to fluconazole (as per the breakpoints available in the CLSI), which is almost 50 %. Hence, not a drug to be used for treating infections. Instead itraconazole, voriconazole or posaconazole can be used which showed 75.7%, 94.6% and 100% sensitivity respectively. According to the study by **Panackal AA et al**.[8] secondary resistance to other available antifungal classes is common resulting in poor treatment outcomes. The incidence of *Candida* infection caused by fluconazole-resistant strains and derivatives is high in India, this may be due to prolonged exposure as prophylaxis or treatment [9]. Fluconazole is often recommended and administered because of its availability for oral administration with minimal toxicity, and lower cost [10]. These findings agreed with the view of **Spreghini et al.** [11] that newer azole, such as posaconazole and voriconazole, replaced fluconazole in prophylaxis routines and have raised similar concerns about resistance and drug-drug interactions as observed in azoles.

Infections caused by *Candida glabrata* are often complicated to treat due to their inherent resistance to most of the antifungals including the azoles. In this case, speciation of *Candida* is crucial to initiate appropriate antifungal treatment.

In our study all the isolates were susceptible to posaconazole and echinocandins. This is in concordance with a study by **Singh et al.** from Delhi [12]. Only 1% of patients were exposed to echinocandins, since the fact that usage of echinocandins in many hospitals in India is limited due to financial limitations, contributing to the absence of echinocandin resistance in the present study.

According to CLSI M27-A2, the interpretative breakpoints for fluconazole for in vitro testing of *Candida* species using broth microdilution method is ≤8 μg ml −1 for susceptible (S), 16–32 μg ml −1 for susceptible-dose dependent (S-DD), and ≥64 μg/mL −1 for resistant (R)[6].
The prevalence of echinocandin resistance among *Candida* species remains low ranging from 1-4% with a negligible intrinsic resistanc [12]. However, echinocandins resistance can become an emerging scourge in *Candida* species. According to SENTRY surveillance program conducted between 2006 and 2010 reported 11% echinocandin resistance among Multidrug resistant (MDR) *Candida glabrata* [13]. In our study, echinocandins resistance was not observed.

Unlike *C. glabrata* the sensitivity of *C. parapsilosis* was better with fluconazole (75.7%) and voriconazole (94.6%) but no major difference with amphotericin B (35.1%).

Invasive candidiasis in low-birth-weight neonates, transplant recipients, critical care patients, and those receiving parenteral nourishment is caused by *Candida parapsilosis*, a prevalent species within NACs [14]. The high prevalence of *Candida parapsilosis* is also endorsed due to the ability to persist and thrive in the hospital environments for long periods. Its exceptional capacity to adhere to abiotic surfaces, such as catheters, and to form biofilms constitutes a doorway to systemic colonization [15]. Antifungal resistance in this pathogen is known to be mostly caused by the widespread use of antifungals, both therapeutically and prophylactically.

CONCLUSION:

To conclude, antifungal susceptibility testing helps clinicians in choosing appropriate drug to prevent morbidity and mortality. Acquired and intrinsic resistance to azoles is of a clinical concern. Based on our study, echinocandins are preferred for *Candida glabrata* and *Candida parapsilosis* infections in patients with prolonged morbidity. In uncomplicated cases voriconazole can still be used. Judicious use of prophylactic agents and surveillance of antifungal susceptibilities with improved diagnostics are necessary to prevent development of resistance among the *Candida* species.

Study limitation:
This study was conducted from a single centre for a shorter period. There is a need of multicentric centric study with a larger sample size for a better understanding of the antifungal susceptible profile.

ACKNOWLEDGEMENT: Nil

FUNDING:
Ms. Swetha Raghavendra Prasad has received Chancellor's Summer research grant (RICERCA)

Journal of Molecular Science
Volume 35 Issue 4, Year of Publication 2025, Page 1514-1519
DoI-10.004687/1000-9035.2025.196

from Sri Ramachandra Institute of Higher Education and Research.
CONFLICT OF INTEREST: Nil

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