

# Genotyping of *Candida albicans* and Comparison of its Antifungal Resistance Pattern in the South Indian Region

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## Abstract

Recent studies have documented an increase in the incidence of antifungal resistance in newly emerging species closely related to *C. albicans*, and the coexistence of genotypic variants. Hence, an application of PCR-based molecular typing is crucial in identifying these fungi. Our study used molecular methods to characterize the latest genotypic subgroups of *C. albicans* and analysed if there was a relationship between the genotypes and the antifungal resistance pattern. The study was conducted in JSS Hospital, Mysuru, Karnataka between July 2018 and December 2020. A total of 1427 *Candida* species were isolated from clinical samples. *Candida albicans* were isolated and confirmed using Germ tube test, ID VITEK 2 and PCR (ITS primer). DNA extraction was done using the Hi-Media Yeast DNA Extraction Kit. The amplified products were analysed using Agarose gel electrophoresis (2%). Among 1427 *Candida* species, 282 were *Candida albicans*. The following resistance was exhibited to major antifungals - Caspofungin (3.5%), Amphotericin B (1.4%), flucytosine (2.8%) Fluconazole (6%) Micafungin (2.8%) Voriconazole (3.1%) and all were sensitive to miconazole. ABC genotyping showed Genotype A (450 bp) predominant (87.58%) followed by genotype B (840bp) (9.92 %) and genotype C (450bp and 840 bp) (0.2%). Genotype D and E were not observed. Our study showed the growing antifungal resistance in clinical isolates. Genotype A was predominant in South Karnataka region followed by Genotype B and C. There was no correlation between genotyping and antifungal resistance. However, a study with greater number of samples from diverse geographical locations may give more insight.

**Keywords:** Antifungal Susceptibility, Clinical Isolates, *Candida albicans*, Genotype A, Genotype B, Genotype C, Genotyping, Karnataka, South Indian Region

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**Citation:** Kumar KPP, Tejashree A. Genotyping of *Candida albicans* and Comparison of its Antifungal Resistance Pattern in the South Indian Region. J Pure Appl Microbiol. 2022 ;16(3):2123-2130. doi: 10.22207/JPAM.16.3.69

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## INTRODUCTION

*Candida albicans*, a ubiquitous commensal organism and the most prevalent and pathogenic *Candida* species, is responsible for majority of oral and systemic candidiasis,<sup>1-3</sup> community-onset and nosocomial candidiasis.<sup>4-6</sup> *Candida albicans* are responsible for the high morbidity and mortality rates affecting the immunocompromised and immunocompetent patients.<sup>3,5,7</sup> This is due to an increase in antimicrobial resistance and the restricted number of antifungal drugs.<sup>8</sup>

*C. albicans* can either grow as a yeast or in filamentous forms and is capable of forming true hyphae.<sup>9,10</sup> *C. dubliniensis* is a recently recognized species that is phenotypically and closely related to *C. albicans* and is susceptible to the same range of antifungal agents.<sup>11</sup> Recently documented studies have shown an increase in the incidence of newly emerging species closely related to *C. albicans* like *C. dubliniensis*, as well as the coexistence of genotypic variants and antifungal resistance during infections.<sup>11</sup>

Molecular typing of an infectious agent helps in epidemiological studies and in designing suitable infection control strategies.<sup>12</sup> PCR-based molecular typing methods are crucial in identifying these fungi<sup>13,14</sup> as they have a high discriminative power and are reproducible.<sup>15</sup> Molecular typing methods such as genome sequencing, multi-locus sequence typing, pulsed field gel electrophoresis, restriction fragment length polymorphism, and randomly amplified polymorphic DNA analysis have been developed for the characterization and deeper comprehension of *C. albicans*.<sup>16-18</sup> Several studies have identified the ABC genotypes of *C. albicans* from clinical specimens.<sup>19,20</sup> This method was developed based on the presence or absence of a transposable group I intron in the gene which encodes the 25S ribosomal RNA.<sup>21</sup> The use of molecular typing in the identification of *C. albicans* have provided vital information in supplementing the growing knowledge of yeast epidemiology. Ribosomal DNA is commonly used for PCR-based molecular methods for the species level identification of *Candida*. Ribosomal sequences have been widely used for genetic typing and identification of several fungal pathogens.<sup>22,23</sup>

In India, since no studies have been performed on genotyping of *C. albicans*, we

characterized the clinical isolates of *C. albicans* based on ABC genotyping and analysed its relation to the antifungal resistance. Identifying the different genotypes of *Candida albicans*, helps to track its relatedness with the emerging genetic variants. It also helps us identify antifungal susceptibility and virulence between different genotypes.

## MATERIALS AND METHODS

### Sample Collection

Samples were collected from patients attending tertiary healthcare (JSS Hospital) hospital in Mysuru, Karnataka between July 2018 to December 2020. Ethical clearance was obtained from the JSS Institutional Ethical Committee on 06/08/2018 (JSSMC/IEC/18/06/2018-19). Clinical samples like urine, blood, vaginal swab, pus, surgery wounds, and tracheal secretions were collected from the patients based on their clinical symptoms. A total of 73,878 samples were screened for *Candida* spp. and later confirmed as *Candida albicans* by phenotypic and genotypic methods. The samples were collected under sterile conditions and transported to the laboratory for analysis.

### Isolation and Identification of Isolates

The samples were cultured on Sabarauds Dextrose Agar at 37°C for 48 hrs. The identification of the suspected isolates were done by Chlamyospore production on cornmeal agar, germ tube test and differential culturing on *Candida* Hichrome agar.

### Differential Culturing

The suspected isolates were sub-cultured onto CHROMagar *Candida*<sup>®</sup> (CHROM -agar Himedia Bangalore) and incubated at 37 °C for 72 hours to obtain pure cultures and were screened for green colonies.

### DNA Extraction

The isolates were inoculated in 2ml of Yeast Extract Peptone Dextrose (YPD) and incubated at 30°C for 16 hrs in a gyratory shaker. DNA extraction was performed on the confirmed isolates using HiMedia Yeast DNA Extraction Kit following the instructions provided by the

manufacturers. Following DNA extraction, the concentration and purity of the DNA was checked using a Nanodrop (Thermo Scientific; Wilmington, DE, USA).

### Molecular Confirmation

Molecular confirmation of the samples confirmed as *Candida albicans* were done by internal transcribed spacer (ITS) PCR. The ITS region and the primers used for the molecular confirmation of *Candida albicans* were ITS1 (5'- TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATAT-3'). The cycling conditions followed for PCR are described below. Initial denaturation: 95°C - 5min  
Denaturation: 95°C - 1min  
No. of cycles: 30  
Primer annealing: 60°C – 1 min  
Extension: 72°C – 1min  
Final extension: 72°C – 5 min

Agarose gel electrophoresis was performed and the gel was observed under a Gel documentation system to view the different bands separated based on the size and molecular weight. ABC Genotyping: ABC Genotyping of the isolates were performed as described by McCullough et al.<sup>12</sup> The following primers were used for amplification CA-INT-L (5'-ATAAGGGAAGTCGGCAAATAGATCCGTAA-3'), CA-INT-R(5'-CCTTGGCTGTGGTTTCGCTAGATAGTATGAT-3').

Reaction components were assembled as described below. Thaw the reagents on ice. Take a 0.2ml PCR tube and add the reagents in the

following order: water (upto 25 µL), buffer (2.5 µL), dNTP's (0.2mM), MgCl<sub>2</sub> (5 µL), forward primers (1.0 µL), reverse primers (1.0 µL), Taq Polymerase and template DNA (1.0 µL). Gently mix the tubes by tapping it. Briefly centrifuge the tubes. Prepare a positive control and negative control. PCR program was set as

Initial denaturation: 95°C – 3 min

Denaturation: 95°C - 1min

No. of cycles: 30

Primer annealing: 60°C – 1 min

Extension: 72°C – 1min

Final extension: 72°C – 5 min

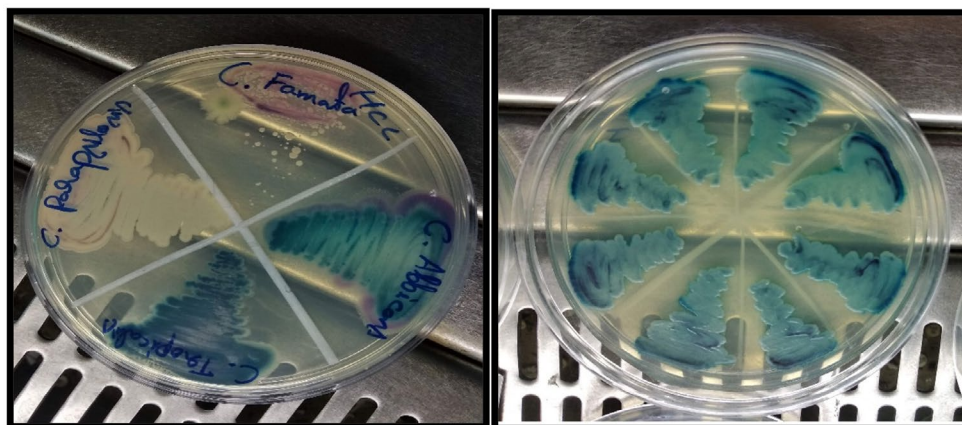
The amplified products were subjected to agarose gel electrophoresis (2%) and viewed under a Gel Doc System (BioRad Inc).

### Antifungal Susceptibility Testing by VITEK 2 (Biomerix Inc)

3ml sterile saline was transferred to a test tube. Using a straight wire, a homogenous culture of *C. albicans* was transferred to the saline tube. The suspension was adjusted to 2 Mc Farland standards. The prepared suspension was placed in the VITEK 2 YST cassette for identification and

**Table 1.** Different genotypes based on the band sizes

No.	Genotypes	Basepair (bp)	Ref.
1.	Genotype A	450	McCullough, 1999 et al.
2.	Genotype B	840	
3.	Genotype C	450& 840	
4.	Genotype D	1080	
5.	Genotype E	1400	Tamura et al.



**Figure 1.** *Candida albicans* producing green colour colonies in HiChrome agar.

VITEK 2 YST08 for antifungal susceptibility. The results were then analysed.

### Controls

ATCC 90028 and SC 5314 were used as external control strains for identification and typing of our isolates.

### Storage

The isolates were then stored on YPD glycerol at -70°C.

## RESULTS

Among the 1427 *Candida* spp isolated from patients during the study period, 282 isolates were *Candida albicans*. The colonies of *Candida albicans* appeared as light green colonies on Hi CHROM-agar (Figure 1). Germ tubes were formed on incubation with human serum at 37°C (2.5-3

hrs). All the phenotypically identified *Candida albicans* were confirmed by PCR using the ITS primer PCR product of approximately 354bp, which was obtained as shown in Figure 2.

ATCC 90028 and ATCC 90029 were used as the reference strains for *Candida albicans* genotype A and genotype B and revealed band sizes of 450bp and 840bp respectively. CY 1123 produced two PCR products (450 and 840 bp) and was used as a reference strain for genotype C. Agarose gel electrophoresis revealed the different genotypes based on their band sizes (Table 1).

ABC genotyping of the 282 isolated strains of *Candida albicans* revealed genotypes A, B and C. However, we did not identify any Genotype D or E in our study. Genotype A, B and C accounted for 247 (87.58%), 28 (9.92%), 7(2.48%) respectively. Genotype A produced a band of 450bp while Genotype B produced a band size of 850bp (Figure 3). However, Genotype C revealed two

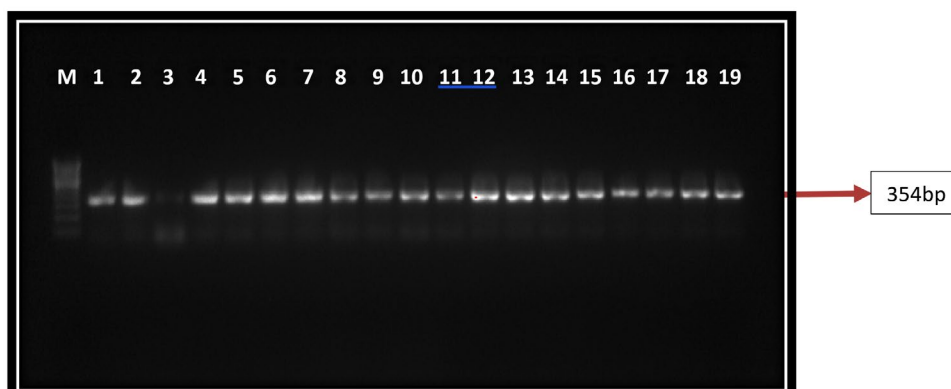


Figure 2. *Candida albicans* ITS Primer PCR with amplified product at 354bp.

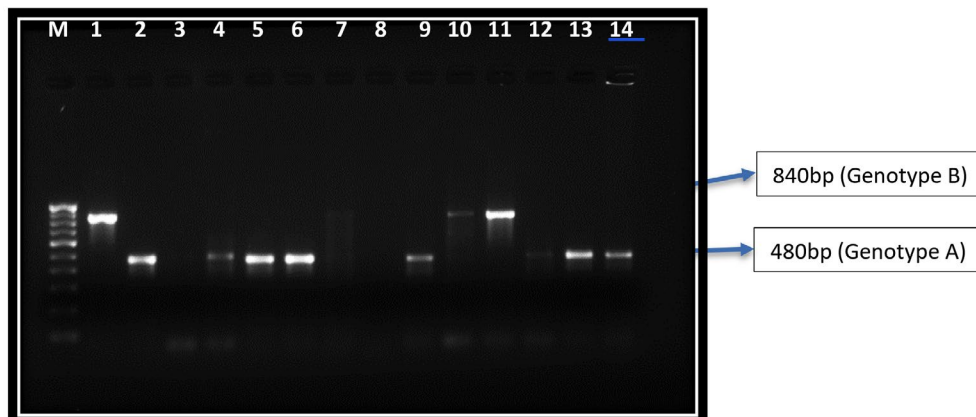


Figure 3. Agarose gel showing the Genotype A(480bp) and B(840bp).

bands, one of 450bp band size and the other 840bp. (Figure 4)

Antifungal susceptibility testing was performed on 282 isolates of *C. albicans* against the commonly used antifungals: fluconazole, voriconazole Miconazole, flucytosine, caspofungin micafungin and Amphoterecin B. The results of the susceptibility pattern against the commonly used antifungals as well as the susceptibility exhibited by the different genotypes are summarised in Table 2. Miconazole was found to be the most effective antifungal being sensitive to all the antibiotics followed by Amphotericin B with the least resistance percentage of 1.4%. The samples showed the following resistance pattern to major antifungals like flucytosine and micafungin (2.8%) Voriconazole (3.1%) Caspofungin (3.5%) and fluconazole (6%).

Among all the identified A genotypes(n=247), 4%(n=10) were resistant to Fluconazole, while 2.8 % (n=7) % were resistant to caspofungin and Voriconazole. Only 2 % (n=5)

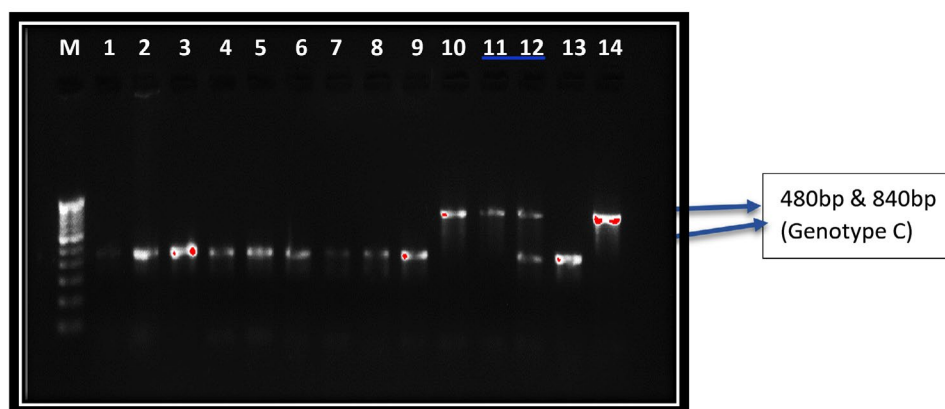
showed resistance to flucytosine and Micafungin. All were susceptible to Miconazole. Higher resistance was observed in Genotype B (n=28) compared to Genotype A, showing the most resistance 7.1 % (n=2) to Caspofungin, micafungin, fluconazole and Amphotericin B followed by 3.5 % (n=1) resistance to flucytosine and voriconazole. All were sensitive to Miconazole. Genotype C (n=7) were susceptible to all the antifungals.

## DISCUSSION

Molecular typing has nowadays been widely used and have proved a very useful tool for epidemiological analysis and have a deeper insight on microbial pathogens, trace the origin of a particular infection, and helps to establish the relationship between different commensal and infectious organisms. It also helps to track the emergence of antibiotic resistance strains and the genetic relatedness between different organisms of the same species.<sup>24</sup>

**Table 2.** AST pattern against the commonly used antifungal agents differentiated based on the genotypes

Antifungal agent	Overall, <i>Candida albicans</i> Antifungal resistance (%)	Resistance of Genotype A (n=247)	Resistance of Genotype B (n=28)	Resistance of Genotype C (n=7)
Caspofungin	3.5	2.7	7.1	0
Amphotericin B	1.4	7	7.1	0
Flucytosine	2.8	1.9	3.5	0
Fluconazole	6	3.9	7.1	0
Micafungin	2.8	1.9	7.1	0
Voriconazole	3.1	2.7	3.5	0
Miconazole	0	0	0	0



**Figure 4.** Agarose gel showing the Genotype C (480bp and 840bp).



Certain studies have used PCR techniques using primers that span highly variable sequences of ITS1 and ITS2, and conserved regions (18S, 5.8S and 28S rRNA genes) for differentiation of medically important *Candida* species.<sup>25-28</sup> Our study has also used these ITS primer sequences for the molecular confirmation of *Candida albicans*.<sup>27,28</sup>

An old and simple molecular method for the differentiation of *C. albicans* was analysing the RFLPs of cellular DNA, dividing them based on the position of a dimorphic band 12 and then subdividing them into types. It was a reliable and reproducible method and hence used for large epidemiological studies in US and UK.<sup>19</sup>

McCullough et al.<sup>12</sup> reported that a PCR primer designed to span the 25S rRNA gene (rDNA) region classified *C. albicans* into four genotypes. Based on the length of the PCR product, the genotypes were inferred as genotype A (450bp), genotype B (840bp), genotype C (450 & 840bp), and genotype D (1,080bp). They also confirmed that *C. albicans* genotype D belonged to the same taxon as *C. dubliniensis*. This was the widely used method for typing of *C. albicans* isolates and also for differentiating *C. albicans* from *C. dubliniensis*.<sup>16,29,30</sup> In our study, most of the *Candida albicans* were Genotype A (87.58%) followed by Genotype B (9.92%). Only a small percentage belonged to Genotype C (2.48%). Our study correlates with the studies done in other countries by Bii, C. et al. (Kenya),<sup>31</sup>; Emmanuel, N.N., et al., (Nigeria),<sup>32</sup> Watanabe et al. Japan<sup>16</sup> Sawadago<sup>33</sup> where, genotype A of *C. albicans* was the most predominant followed by Genotype B and Genotype C the least prevalent. This method was unique and reproducible and helped in the identification of the different genotypes of *Candida albicans* namely Genotype A, B, C, D and E.

Tamai IA et al.<sup>34</sup> isolated *Candida albicans* from HIV-infected patients and identified that 66% of the samples were genotype A.<sup>34</sup> In two other studies in Turkey performed by Karahan<sup>35</sup> among 190 *Candida* strains, nearly 51% were genotype A. Another study in Brazil, Chaves et al.<sup>36</sup> also reported genotype A as the most common genotype. From all these studies it was concluded that Genotype A was the most common genotype in majority of the studies.

In contrast to all these studies, a study by Abdul Rahim<sup>37</sup> from Dublin, Ireland and Moron 38 Malaysia found Genotype A (n=65) the predominant one, but Genotype C (n=20) was more prominent than Genotype B (n=15). This may be attributed to the geographical location. It was observed that the percentage of Genotype B and C were almost similar to each other in studies done in Iraq by Dalvand et al.<sup>38</sup> and Samaka et al.<sup>39</sup>

Although resistance is rare in *Candida albicans*, long term use of antifungals during recurrent infections has led to emergence of resistance.<sup>40</sup> Our study noted a growing resistance in *Candida albicans* against the commonly used antifungal agents. The highest resistance was exhibited against fluconazole being 6%, which is worrisome.

Our study showed that Genotype B showed more resistance compared to Genotype A & C. Although most of the *Candida albicans* were Genotype A (n=247), Genotype B (28) showed the most resistance. Genotype B showed a resistance of 7.1% each to the commonly used antifungals Amphotericin B, Fluconazole, caspofungin and Micafungin. However, Mercure et al.<sup>41</sup> reported an association of *Candida albicans* genotype A with a high resistance to flucytosine. Studies by Dalvand,<sup>38</sup> Samaka<sup>39</sup> also analysed if there was an association between the antifungal resistance pattern and the *Candida* genotypes, however, found no association.

To conclude, our study showed a growing antifungal resistance in clinical isolates. ABC genotyping revealed that genotype A was predominant than B and C in the South Karnataka region. As genotyping techniques have high efficiency, in future, genotyping can be attempted with a greater sample size considering different geographical locations. This would help us analyse if there was any association between the genotype's antifungal resistance and the pathogenesis. Our study showed Genotype B to have a higher resistance compared to Genotype A, inspite of most of the *Candida albicans* being Genotype A. All genotype C isolates were susceptible to the major antifungals. Most of the studies done on genotyping of *Candida albicans* focused on the isolates obtained from infected lesions of oral cavity, vaginal mucosa and stool samples. A

genotyping method with high resolution would be useful for identifying the dissemination area, source and route of Candidiasis and for designing proper management strategies for Candidiasis.

#### ACKNOWLEDGMENTS

The authors would like to thank JSS Academy of Higher Education and research for providing all the facilities for conducting the study.

#### CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

#### AUTHORS' CONTRIBUTION

All authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

#### FUNDING

None.

#### DATA AVAILABILITY

The datasets generated during and/or analysed during the current study are available from the corresponding author on reasonable request.

#### ETHICS STATEMENT

This study was approved by JSS Ethical Clearance Committee (Reference number JSSMC/IEC/18/06/2018-19)

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