In vitro susceptibility and molecular characterization of *Candida* spp. from candidemic patients

Patricia Fernanda Herkert[^1], Renata Rodrigues Gomes[^1], Marisol Domínguez Muro[^2], Rosangela Lameira Pinheiro[^2], Gheniffer Fornari[^3], Vanânia Aparecida Vicente[^4],[^5], Flávio Queiroz-Telles[^6],[^7],[^8]  

[^1]: Postgraduate Program in Microbiology, Parasitology and Pathology, Biological Sciences, Department of Basic Pathology, LabMicro – Laboratory of Microbiology and Molecular Biology, Federal University of Paraná, Curitiba, Paraná, Brazil  
[^2]: Support and Diagnosis Unit, Mycology Laboratory, Hospital of Clinics, Federal University of Paraná, Brazil  
[^3]: Hospital de Clínicas, Federal University of Paraná, Brazil  
[^4]: Fellowship from Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil

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**A B S T R A C T**  
Background: *Candida* species are the main cause of hospital acquired fungal bloodstream infections. The main risk factors for candidemia include parenteral nutrition, long-term intensive care, neutropenia, diabetes, abdominal surgery and the use of central venous catheters. The antifungal drugs used to treat candidemia are mainly the echinocandins, however some isolates may be resistant to these drugs.  
Aims: This work aims to evaluate the in vitro susceptibility patterns of various *Candida* species isolated from blood samples and provide their identification by molecular characterization.  
Methods: Antifungal susceptibility testing was performed using the broth microdilution method. The sequencing of the ITS and D1/D2 regions of rDNA was used for molecular characterization.  
Results: Seventy-four of the 80 isolates were susceptible to anidulafungin, 5 were intermediate, and 1 was resistant. For micafungin 67 were susceptible, 8 were intermediate and 5 were resistant. All isolates were susceptible to amphotericin B. Lastly, 65 isolates were susceptible to fluconazole, 8 were dose-dependent and 4 were resistant. The molecular identification corroborated the phenotypic data in 91.3% of the isolates.  
Conclusions: Antifungal susceptibility data has an important role in the treatment of candidemia episodes. It was also concluded that the molecular analysis of isolates provides an accurate identification and identifies genetic variability within *Candida* species isolated from patients with candidemia.

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**Sensibilidad in vitro y caracterización molecular de aislamientos de *Candida* procedentes de pacientes con candidemia**

**R E S U M E N**  
Antecedentes: Los hongos del género *Candida* son la causa principal de infección micótica del torrente sanguíneo adquirida en el hospital. Entre los factores de riesgo asociados a la candidemia destacan la nutrición parenteral, la estancia prolongada en una unidad de cuidados intensivos, la neutropenia, la diabetes, la cirugía abdominal y la utilización de catéter venoso central. Los agentes antifúngicos más utilizados para tratarla son las equinocandinas, pero determinados aislamientos son resistentes a dichos componentes, por lo que algunos pacientes no responden al tratamiento.  
Objetivos: Este trabajo tiene como objetivo evaluar la sensibilidad in vitro de varios aislamientos de *Candida* procedentes de muestras de sangre y realizar su caracterización molecular.  
Métodos: Se hicieron pruebas de sensibilidad a los antifúngicos mediante el método de microdilución en caldo. Para la caracterización molecular se utilizó la secuenciación de las regiones ITS y D1/D2 del DNAr.

[^1]: Corresponding author.  
**E-mail address:** queiroz.telles@uol.com.br (F. Queiroz-Telles)

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Among the medically important fungi, Candida species are of great importance due to the high frequency of colonization and infection in human hosts.8 Under normal conditions, most do not cause damage to their hosts, and only cause tissue invasions and systemic infections when host defense mechanisms are weakened.26 Candida infections account for about 80% of the total fungal infections of the bloodstream, urinary tract and surgical site infections.8 The bloodstream infections caused by Candida have a high prevalence, morbidity and mortality,15 and have a profound economic impact due to the long hospitalization periods, intensive care and treatment.23

The incidence of candidemia in tertiary care hospitals in Brazil is 1.38 cases per 1,000 hospital admissions24 with a 54% mortality rate.9 The underlying conditions are cancer, neutropenia, surgery (mainly abdominal), mechanical ventilation, dialysis, parenteral nutrition and central venous catheter.8 In Brazil, Candida albicans is the leading agent, followed by Candida parapsilosis, Candida tropicalis, Candida guilliermondii, Candida glabrata and Candida krusei. Species as Candida intermedia, Candida haemulonii, Candida lusitaniae, Candida famata and Candida norvegensis are less frequent.31

The differences in the epidemiology and therapeutic approach for the various Candida species justify identification of the species responsible for the disease. This information is essential not only for appropriate patient management, but also for the control of nosocomial infections. Additionally, this information provides hospital-specific data, as antifungal species and susceptibility patterns often vary between institutions.8 Therefore, this study was aimed to evaluate both the in vitro susceptibility patterns and the molecular characterization of those Candida species isolated from patients with candidemia.

Materials and methods

Isolates

This study assessed the in vitro susceptibility and molecular characterization of 80 Candida species obtained from blood samples that had been deposited into the mycology collection of the Laboratory of Mycology, UFPR Hospital, between January 2005 and June 2012 (Table 1).

Molecular characterization

For the DNA extraction, physical maceration performance with silica:celite (2:1) in CTAB (cetyl trimethylammonium bromide) and CIA (acidic solution of chloroform isooamyl alcohol)13 was used. The sequencing was performed on an ABI3500 sequencer. For ITS sequencing the primers ITS1 (5′-TCCGTAGTGAACTTCGCGG-3′) and ITS4 (5′-TCTTCCGCTTATTGATATGC-3′) were used.31 For the amplification of D1/D2 region primers NL-1 (5′-GATATCAATTAGGAGAATTG-3′) and NL-4 (5′-GTCGGTGTTCACGAGG-3′) were used,22 with the same conditions used as for the ITS sequencing. For the C. albicans ABC genotyping, primers CA-intL (5′-ATAAGGGAATCGGCAAAATAGATCCG-3′) and CA-int-R (5′-CCCTGGCTGTGTTCCGCTAGTATGAT-3′) were used.18

Alignment and phylogenetic construction

Sequences were edited with the BioEdit program,14 and compared with reference sequences for detection of similarity with the BLAST program.1 The alignment was performed with the MAFFT,16 and visual inspection by the MEGA 5.1 version.29 The clinical isolates included in the study and 36 reference sequences were used for the phylogenetic analysis (Table 1). An isolate of Neurospora crassa was included as an outgroup.3,10,30 The MEGA version 5.1 program29 was used to estimate the best-fitting evolutionary models for each data and the Maximum Likelihood analysis. Bootstrap support was estimated by 1000 replicates.

Antifungal susceptibility testing

The susceptibility tests against amphotericin B (Sigma–Aldrich Quimica, Madrid, Spain), fluconazole (Sigma–Aldrich Quimica, Madrid, Spain), micafungin (Mycamine®; Astellas Pharma Inc., Toyama, Japan) and anidulafungin (Ecalta–Pfizer, Kent, United Kingdom) were performed with the broth microdilution technique in accordance with the guidelines in CLSI document M27–A3.43 A reference strain C. albicans ATCC 10231 was included with each set of experiments for quality control. The MIC values for the echinocandins were verified by LEMI reference laboratory (UNIFESP – São Paulo – Brazil).

Results

The 80 isolates were identified as C. albicans (27 isolates), C. parapsilosis complex (24 isolates), C. glabrata (8 isolates), C. tropicalis (7 isolates), C. guilliermondii (5 isolates), C. krusei (3 isolates), C. pelliculosa (3 isolates), C. lusitaniae (2 isolates), and C. dubliniensis (1 isolate). Molecular identification was consistent with the phenotypic data in 91.3% isolates. Isolate LMICRO112 identified only as a Candida sp., was later confirmed as C. tropicalis with the molecular characterization. LEMICRO133 thought to be C. famata was identified as C. lusitaniae through molecular markers; and LEMICRO134, also thought to be C. famata, was reidentified as C. tropicalis. A single C. albicans isolate (LMICRO158) was molecularly characterized as C. dubliniensis, and C. guilliermondii LEMICRO180 was identified as C. parapsilosis through the molecular analysis. Two isolates in the C. parapsilosis complex, LEMICRO168 and LEMICRO175, were subsequently reidentified as C. metapsilosis and C. orthopsilosis, respectively.

According to the Maximum Likelihood analysis based on ITS sequencing regions, the isolates were clustered into nine different
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* Type.

*: data not provided.
clades: Albicans, Dublieniensis, Tropicalis, Parapsilosis Complex, Glabrata, Pelliculosa, Guilliermondii, Lusitaniae and Krusei, supported by bootstrap values (Fig. 1). Sequencing analysis of the variable D1/D2 region was performed to confirm the identity of the isolates (LMICRO112, 133, 134, 158, 168, 175, and 180) that exhibited a discordance between molecular and phenotypic identification. The D1/D2 sequencing analysis confirmed the results from ITS sequencing, demonstrating that the isolates belonged...
Clade: Glabrata

Clade: Pelliculosa

Clade: Famata

Clade: Guilliermondii

Clade: Lusitaniae

Clade: Krusei

Fig. 1. (Continued)

Fig. 2. Phylogenetic tree of Maximum likelihood based on the alignment of D1/D2 region of rDNA built with 1000 bootstrap using the evolutionary model Tamura-Nei with gamma distribution, using mega version 5.1 program. *Neurospora crassa* (FGSC 8771) was used as an outgroup. T: type strain. Red: isolates with discordant molecular and phenotypic identification.
to the clades Tropicalis, Lusitaniae, Dubliiniensis, and Parapsilosis complex (Fig. 2). A low genetic variability rate among the C. albicans isolates was observed, and considerable interspecific differences were noted for the Parapsilosis complex, dividing it in three species: C. parapsilosis, C. metapsilosis and C. orthopsilosis.

The ABC genotyping of the C. albicans isolates distinguished between genotypes A and B, with a higher prevalence of genotype A (62%). It was noticed that both were susceptible to the tested antifungals, with the exception of isolate LMICRO145 of genotype B that was resistant to fluconazole. The mortality rate was similar between both genotypes, with seven deaths in both groups.

Antifungal susceptibility data showed several resistant isolates of C. glabrata, with 5 of them being resistant to micafungin and intermediate to anidulafungin, and 7 susceptible-dose dependent to fluconazole. The C. albicans species were the most susceptible to the antifungals tested, with only one isolate resistant to fluconazole. Among the C. parapsilosis complex, 5 isolates of C. parapsilosis had intermediate susceptibility to micafungin while the remaining isolates studied were susceptible to all the antifungals tested (Table 2).

Those isolates resistant to at least one antifungal (LMICRO112, 113, 115, 116, 117, 135 and 145) were further studied analyzing the relationship between the clinical data and the MIC values (Table 3). With regard to clinical response to treatment and the in vitro susceptibility tests results, it was observed that the LMI- CRO116 isolate (C. glabrata) was obtained from a patient whose blood cultures remained positive during micafungin (MCF) therapy, but was successfully treated after the addition of amphotericin B. The susceptibility tests revealed resistance of the isolate to MCF and anidulafungin (AND), but susceptibility to amphotericin B.

**Discussion**

The frequency of invasive mycosis by opportunistic fungal pathogens has increased significantly along the last years. Besides, more than 17 different Candida species have been identified as etiological agents of bloodstream infections.27

C. albicans is still considered the most common cause of candidemia in tertiary Brazilian hospitals, with a rate of about 40% of the episodes.11,13,21,25 The results obtained in this study corroborate this evidence. However, a rising incidence of infections caused by the non-C. albicans Candida species, especially C. tropicalis, C. parapsilosis, C. glabrata and C. krusei, has been noticed. These species were found in the present study as well. It is also difficult to phenotypically identify cryptic species within species complexes without the addition of molecular sequencing.8,20,34
### Table 3
Correlation between the clinical data of the patients and the in vitro susceptibility profile of the tested isolates.

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<th>Treatment</th>
<th>Response</th>
<th>Susceptibility profile</th>
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<td>Untreated(^a)</td>
<td>–</td>
<td>AND (I) MCF (R) AMB (S)</td>
</tr>
<tr>
<td>LMICRO116 C. glabrata</td>
<td>Burkitt lymphoma, dialysis</td>
<td>AMB</td>
<td>Favorable</td>
<td>AND (R) MCF (R) AMB (S)</td>
</tr>
<tr>
<td>LMICRO117 C. glabrata</td>
<td>Gastric ulcer, diverticular disease, abdominal surgery</td>
<td>Untreated(^b)</td>
<td>–</td>
<td>AND (I) MCF (R) AMB (S)</td>
</tr>
<tr>
<td>LMICRO135 C. guilliermondii</td>
<td>ALL, bone marrow transplantation</td>
<td>FLU, voriconazole</td>
<td>Undetermined</td>
<td>AND (S) MCF (I) AMB (S)</td>
</tr>
<tr>
<td>LMICRO145 C. albicans</td>
<td>Anorectal malformation, abdominal surgery</td>
<td>FLU, AMB</td>
<td>Favorable</td>
<td>AND (S) MCF (S)</td>
</tr>
</tbody>
</table>

**ALL:** acute lymphoblastic leukemia; **AND:** amiodafungin; **MCF:** micafungin; **AMB:** amphotericin B; **FLU:** fluconazole; **S:** susceptible; **SDD:** susceptible-dose dependent; **I:** intermediate; **R:** resistant.

\(^a\) Died before the beginning of the treatment.
\(^b\) Transient candidemia; undetermined: died.

methods. This was observed with the isolates of the Parapsilosis complex and those of *C. dubliniensis* and *C. lusitaniae* (Fig. 2), confirming the necessity to complement the presumptive identification based on morphology and biochemistry with molecular data. The identification of the Parapsilosis complex is necessary as these isolates vary in their antifungal susceptibility profiles.\(^{17,31}\) According to this study *C. parapsilosis* isolates showed intermediate susceptibility to micafungin, corroborating previous findings that suggest a decreased susceptibility in *C. parapsilosis*.\(^{3,12,17}\) The results of the present study show that the MIC values for amphotericin B among the three species of the Parapsilosis complex were similar. However, Lockhart et al.\(^{17}\) observed higher MIC values for *C. parapsilosis*. This supports the relevance of the in vitro susceptibility tests for appropriate patient management.

The ABC genotyping of the *C. albicans* isolates was also included in this study in order to provide additional discriminatory data regarding this species. According to McCullough et al.,\(^{38}\) genotype A is more frequent among the isolates of this species, and it has been correlated with lower susceptibility to fluconazole. In our study, genotype A was predominant (62%) among the analyzed isolates, and most of the isolates were susceptible to all of the antifungals tested regardless of the genotype, with the exception of one genotype B isolate which showed resistance to fluconazole. It was also observed that the mortality rates of the patients were similar, regardless of genotype involved. It remains unclear if genotype A is more virulent.\(^{39}\) Fluconazole has a good therapeutic activity against *C. albicans*, and has been used to prevent the systemic candidiasis for many years; in these cases, the susceptibility to fluconazole can reach 95%, being effective against most infections by this species.\(^{37}\) However, the repetitive and long-term use of fluconazole for chronic infections and its prophylactic use has favored the appearance of resistant isolates.

Infections due to *C. glabrata* have risen in the last few years and this appears to be related to the high usage of fluconazole in hospitals and the occurrence of resistant isolates to this antifungal.\(^{24}\) As a result, the echinocandins have been recently added as a first-line indication for the candidemia,\(^{8}\) but it has been noticed that some *C. glabrata* isolates are resistant to this agent as well.\(^{15}\) In our study, 62% of the *C. glabrata* isolates (5/8) exhibited resistance to micafungin and 87% (7/8) showed resistance to fluconazole. One of these highly resistant isolates (*C. glabrata* LMICRO116) was used by Bizerra et al.,\(^2\) to investigate the mutations associated with resistance. The authors confirmed the presence of a S663F mutation in the FKS2 gene, resulting in the production of a 1.3-β-glucan synthase enzyme with reduced susceptibility to the echinocandins, and a strong potential for clinical failure. Furthermore, species such as *C. albicans*, *C. parapsilosis*, *C. guilliermondii*, and *C. tropicalis* showed high MIC values in this study, a finding that is not consistent with a previous study conducted at the same hospital.\(^{11}\) This increased resistance to antifungals appears related to the rise of prophylactic and empirical therapies using fluconazole and echinocandins in this hospital.

Most of the *Candida* isolates showed susceptibility to the antifungal agents tested. However, *C. glabrata* presented the largest number of isolates resistant to the echinocandins and fluconazole. Accordingly, antifungal susceptibility testing has an important role in the treatment of candidemia, and the molecular analysis of isolates provides accurate identification of cryptic species in the *C. parapsilosis* complex and demonstrates the genetic variability between *Candida* species.

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### References

228


