Original article

Genotypic variability and antifungal susceptibility of *Candida tropicalis* isolated from patients with candiduria

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Aims: The study aimed at characterizing genotypically *C. tropicalis* strains from patients with candiduria in a university hospital, and assessed the antifungal susceptibility profile.

Methods: The study was conducted with hospitalized patients who developed urinary tract infection from *C. tropicalis* from June 2010 to June 2011 at the Grande Dourados University Hospital of the Federal University, Dourados, MS, Brazil. Susceptibility to the antifungal agents amphotericin B and fluconazole was determined by broth microdilution. The genotypic variability of isolates of *C. tropicalis* was analyzed by microsatellite markers and RAPD-PCR.

Results: Only one isolate was resistant to amphotericin B (MIC → 16 μg/ml); the others were susceptible to fluconazole and amphotericin B. The genotypic variability by RAPD-PCR resulted in distinct profiles for RAPD markers. A total of 10 alleles were observed for the microsatellite loci URA3 and CT14, which were grouped differently, and four associations were observed for locus URA3 and eight for locus CT14.

Conclusions: *C. tropicalis* isolates from urine were susceptible to the antifungal agents tested. The genotyping techniques make possible proving the similarity and genetic diversity among isolates of *C. tropicalis* involved in nosocomial infections. This knowledge is important for the control and prevention of nosocomial infections caused by this yeast species.

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**Variabilidad genotípica y sensibilidad antifúngica de aislamientos de *Candida tropicalis* procedentes de pacientes con candiduria**

Antecedentes: *Candida tropicalis* es un patógeno humano emergente en las infecciones nosocomiales y es considerado la segunda o tercera especie de *Candida* más aislada en cultivos de orina.

Objetivos: El objetivo del estudio fue caracterizar genotipicamente aislamientos de *C. tropicalis* procedentes de pacientes con candiduria de un hospital universitario, y evaluar su perfil de sensibilidad a los antifúngicos.

Métodos: La investigación fue realizada con pacientes hospitalizados que desarrollaron una infección urinaria por *C. tropicalis* desde junio de 2010 hasta junio de 2011 en el Hospital Universitario de la Universidad Federal de Grande Dourados, Dourados, MS, Brasil. La sensibilidad a los agentes antifúngicos amfotericina B y fluconazol fue determinada mediante el método de microdilución en caldo. La variabilidad genotípica de los aislamientos de *C. tropicalis* se analizó mediante marcadores microsatélites y RAPD-PCR.

**Palabras clave:**
- Candiduria
- *Candida tropicalis*
- RAPD-PCR
- Microsatélite
- Sensibilidad antifúngica

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Nosocomial infections caused by *Candida* yeasts have increased significantly worldwide in recent years and have been the growing cause of morbidity and mortality in hospitalized patients, especially those severely ill and immunocompromised.\(^5\),\(^1\),\(^2\),\(^7\),\(^3\),\(^2\),\(^3\),\(^3\),\(^3\)

Nosocomial urinary tract infections caused by *Candida* spp. frequently occur in hospitalized patients, and account for 10–15% of all urinary tract infections.\(^5\) Episodes of candiduria in hospitalized patients increase morbidity and offer the risk of mortality.\(^1\),\(^2\),\(^4\) Furthermore, candiduria can be considered a risk factor for candidemia in adult patients.\(^1\),\(^6\)

*Candida tropicalis* is an emerging major human pathogen in nosocomial infections\(^5\),\(^2\),\(^1\),\(^2\),\(^2\),\(^3\),\(^1\) and it is considered the second or third *Candida* species most frequently isolated in urine cultures.\(^1\),\(^5\),\(^1\),\(^8\),\(^2\),\(^4\) This species has some important virulence characteristics such as increased resistance to azole antifungal drugs, significant adherence to the epithelium, more so than to the silicone, biofilm formation and expression of total hemolytic activity.\(^2\),\(^3\),\(^7\),\(^2\)

Currently several molecular typing methods have been used for molecular characterization and correlation of *Candida* species in hospital infections. Among them, the Randomly Amplified Polymorphic DNA (RAPD) and microsatellite markers stand out.\(^5\),\(^1\),\(^0\),\(^0\) Genotyping of clinical isolates by RAPD technique involves the amplification of DNA fragments by polymerase chain reaction using short primers of random sequence.\(^5\) However, microsatellite markers also amplify DNA fragments by polymerase chain reaction and are co-dominant markers which allow the heterogeneous loci to be differentiated from the homozygous ones.\(^1\) The application of these molecular tools in infections within a hospital environment is to characterize genetic variations and demonstrate the degree of similarity between the isolates. Genotyping of different *Candida* species is important due to its prognostic and therapeutic significance, thus generating information for clinical epidemiology, allowing the correct treatment and control of infections in hospitals.

Studies aimed at assessing the genetic diversity of species of *Candida* non-*Candida albicans* (CNCA) isolated from hospitalized patients, as well as the evaluation of antifungal susceptibility profile, are relevant for improving therapeutic approaches and control hospital fungal infections. Therefore, the present study aimed at evaluating the molecular genetic diversity of *C. tropicalis* and studying its susceptibility to antifungal agents in patients with candididuria hospitalized in a university hospital.

**Materials and methods**

**Samples**

Urine samples from patients with urinary infection hospitalized at the University Hospital of the Grande Dourados Federal University (Dourados, State of Mato Grosso do Sul, Brazil) from June 2010 to June 2011, whose results were positive for *C. tropicalis*, were processed in this study.

In order to characterize a urinary tract infection caused by *C. tropicalis*, the presence of more than 10\(^5\) colony forming units per milliliter (CFU/mL) in urine was considered.

**Isolation and identification of isolates**

The yeasts were screened by cultivation in CHROMagar *Candida*\(^\text{(Difco, BD, Franklin Lakes, NJ, USA)}\) through routine laboratory analysis and stored in Sabouraud Dextrose Broth (Difco, BD, Franklin Lakes, NJ, USA) with 20% glycerol in a freezer at –70°C. Isolates of *C. tropicalis* were identified phenotypically by way of macroscopic, microscopic and biochemical features described in the classical method, including colony morphology, micromorphological analyses, and carbohydrate assimilation and fermentation tests.\(^3\)

**Antifungal susceptibility testing**

The antifungal susceptibility was determined by broth microdilution method, performed according to the document M27-A3 of the Clinical and Laboratory Standards Institute.\(^1\)

The antifungal agents used were amphotericin B and fluconazole, and the susceptibility cutoffs were in accordance to the parameters established by Yang et al.\(^3\) with MIC values ≤1 μg/ml considered susceptible and ≥2 μg/ml resistant to amphotericin, and by the supplement document M27-A3-M27-S3\(^8\) to fluconazole.

**Genomic DNA extraction**

Yeasts were cultured in Sabouraud Dextrose Broth (Difco, BD, Franklin Lakes, NJ, USA) and maintained overnight at 25°C. Genomic DNA was extracted as described by Chong et al.\(^6\) The amount and purity of genomic DNA were determined by optical density in a spectrophotometer (Genesys 10, Thermo Fisher Scientific, Waltham, Massachusetts, USA).

**Molecular identification by species-specific PCR primer**

The identification of the species *C. tropicalis* was confirmed by the amplification of the internal transcribed regions 1 and 2 of the rRNA gene of *Candida* species.\(^2\) The PCR reaction, adapted from Alves et al.,\(^7\) was performed with genomic DNA (10–20 ng), forward and reverse primers (10 pmol each), PCR Master Mix (Axygen Scientific, Union City, CA, USA) (12.5 μl), and water for a final volume of 25 μl. The primers used for the amplification reaction of the internal transcribed spacer 1 region (ITS1)-5.8S-ITS2 of the rRNA gene were: forward (C. tropicalis, 5-AAGAATTTAAAGTGGAAACTTA-3) and reverse (5-TCTCCCGCTTATGATATGC-3) (GenBank Accession...
RAPD-PCR assay

The molecular characterization of *C. tropicalis* isolates from urine was performed by RAPD-PCR based on the methodology of Bautista-Muñoz et al.\(^1\) The reaction contained 10 ng of genomic DNA, 0.4 µM of the primer, 2 mM of MgCl\(_2\), 1.2 U of Taq polymerase and 0.8 mM dNTP. The primers used for the reaction were OPA-18 (5-AGCTCGACCT-3), OPE-18 (5-GGACTGCGACA-3) and P4 (5-AAGAGCCCGT-3). The reaction conditions for RAPD-PCR were initial denaturation at 94 °C for 5 min, 45 cycles of denaturation at 94 °C for 1 min, annealing at 63 °C for 1 min, extension at 72 °C for 1 min, and final extension at 72 °C for 5 min.

The amplified products were subjected to electrophoresis in 1.5% agarose gel (110 V, 40 min), stained with ethidium bromide (0.5 mg ml\(^{-1}\)) and visualized under ultraviolet light. The size of the amplified product (149 bp), specific to *C. tropicalis*, was determined by using the molecular weight marker 50 bp.

**Microsatellite assay**

The reaction was adapted from the method described by Desse-Allibert et al.\(^1\). Two microsatellite markers were used, one upstream of the *URA3* genes (*URA3*, GenBank Accession No. EU288195.1) and one non-annotated sequence CT14. Each reaction contained 50 ng of genomic DNA, 0.1 µM of each primer pair, 5 mM of MgCl\(_2\), 1.25 U of Taq polymerase and 0.8 mM of dNTP. The forward and reverse primers used for the reactions were: URAF (5-ATTTGAGATCTCCCTTAAACTCTACATACT-3)/CTU2R (5-GTTGGAACATCAATTGCACACATAAAT-3) and CT14a (5-GTAATACTGTGATACCGGA-3)/CT14b (5-TAGCCCATTTCTAGTGGTC-3). The conditions for amplification were: 27 cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 7 s and final extension at 72 °C for 5 min.

The amplification products were subjected to electrophoresis in 8% polyacrylamide gel in 1 X TBE buffer (140 V, 5 h and 30 min), stained with ethidium bromide (0.5 mg ml\(^{-1}\)), and visualized under ultraviolet light. The determination of the sizes of the fragments was performed using the molecular weight marker 100 bp.

**Results**

The phenotypic analysis for the identification of the *Candida* species from the 12 patients was able to identify 15 isolates of *C. tropicalis* obtained from cases of candiduria. The method of species-specific PCR of the regions ITS1 and ITS2 of the rRNA gene confirmed the species *C. tropicalis* in all the 15 isolates.

**Antifungal susceptibility test**

The results for the susceptibility test are shown in Table 1. The range of the Minimal Inhibitory Concentration (MIC) value was 0.125–16 µg/ml for amphotericin B and 0.25–4 µg/ml for fluconazole. Only one isolate was resistant to amphotericin B and the others were susceptible to fluconazole and amphotericin B (Table 2).

**RAPD-PCR profiles**

The isolates showed six (A–F), two (A and B) and four (A–D) different molecular profiles for the markers OPA-18, OPE-18 and P4 respectively (Table 2 and Fig. 1).

The amplification using the primer OPA-18 allowed the formation of four clusters named I, II, III and IV, which grouped 13.3%, 26.7%, 20% and 26.7% of the samples respectively. Clusters I and II were more related to each other with a similarity coefficient of approximately 90%. Cluster IV correlated to the others in approximately 70%. Samples 14 and 15 had a correlation to the other with a similarity coefficient of 65% and 87.6%, respectively. The values found were: SAB 0.73 ± 0.11, mean 73.86 and standard deviation of 11.08 (Fig. 1a).

The results of amplified products by the primer OPE-18 led to the formation of two clusters (I and II), which grouped 33.3% and 66.6% of the samples, respectively. The similarity coefficient between the two clusters was 93.3%. The values found were: SAB 0.96 ± 0.33, mean 96.82 and standard deviation of 3.33 (Fig. 1b).

Analyzing the clusters constructed by means of amplified products of the primer P4, the formation of clusters I and II, which grouped 13.3% and 73.3% of the samples, respectively, was observed. The similarity coefficient between the two clusters was 80%. Samples 14 and 1 correlated to the others with a coefficient of similarity of 78% and 63.1%, respectively. The values found were: SAB 0.74 ± 0.08, mean 74.63 and standard deviation 8.66 (Fig. 1c).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Susceptibility test to amphotericin B and fluconazole for 15 isolates of <em>C. tropicalis</em> obtained from 12 patients with candiduria.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Variables</td>
<td>MIC values (µg/ml)</td>
</tr>
<tr>
<td></td>
<td>Amphotericin B</td>
</tr>
<tr>
<td>MIC range</td>
<td>0.125 – 16</td>
</tr>
<tr>
<td>Geometric mean MIC</td>
<td>0.91</td>
</tr>
<tr>
<td>MIC(_{12})</td>
<td>1</td>
</tr>
<tr>
<td>MIC(_{50})</td>
<td>1</td>
</tr>
</tbody>
</table>

\(\text{MIC}_{12}\): lowest concentration able to inhibit the growth of 50% of the isolates.

\(\text{MIC}_{50}\): lowest concentration able to inhibit the growth of 90% of the isolates.
### Table 2
Data from isolates of *C. tropicalis*, RAPD profiles and microsatellite genotypes.

<table>
<thead>
<tr>
<th>Patients</th>
<th>Isolated</th>
<th>Date of isolation</th>
<th>MIC</th>
<th>RAPD</th>
<th>Microsatellites</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>AMB</td>
<td>FLU</td>
<td>OPA-18</td>
</tr>
<tr>
<td>P1</td>
<td>1</td>
<td>21/06/2010</td>
<td>0.5</td>
<td>0.5</td>
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</tr>
<tr>
<td>P2</td>
<td>2</td>
<td>20/07/2010</td>
<td>1</td>
<td>1</td>
<td>D</td>
</tr>
<tr>
<td>P3</td>
<td>3</td>
<td>22/09/2010</td>
<td>&gt;16</td>
<td>0.5</td>
<td>B</td>
</tr>
<tr>
<td>P4</td>
<td>4</td>
<td>19/12/2010</td>
<td>1</td>
<td>1</td>
<td>B</td>
</tr>
<tr>
<td>P5</td>
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<td>24/01/2011</td>
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<td>1</td>
<td>B</td>
</tr>
<tr>
<td>P6</td>
<td>6</td>
<td>03/02/2011</td>
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<td>0.25</td>
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</tr>
<tr>
<td>P7</td>
<td>7</td>
<td>07/02/2011</td>
<td>0.5</td>
<td>4</td>
<td>A</td>
</tr>
<tr>
<td>P8</td>
<td>8</td>
<td>04/03/2011</td>
<td>0.5</td>
<td>2</td>
<td>C</td>
</tr>
<tr>
<td>P9</td>
<td>9</td>
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<td>0.5</td>
<td>D</td>
</tr>
<tr>
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<td>0.5</td>
<td>2</td>
<td>D</td>
</tr>
<tr>
<td>P11</td>
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<td>30/03/2011</td>
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<td>C</td>
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<tr>
<td>P12</td>
<td>12</td>
<td>05/04/2011</td>
<td>0.5</td>
<td>1</td>
<td>C</td>
</tr>
<tr>
<td>P13</td>
<td>13</td>
<td>25/04/2011</td>
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<td>1</td>
<td>D</td>
</tr>
<tr>
<td>P14</td>
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<td>0.5</td>
<td>F</td>
</tr>
<tr>
<td>P15</td>
<td>15</td>
<td>05/05/2011</td>
<td>1</td>
<td>1</td>
<td>E</td>
</tr>
</tbody>
</table>

**Fig. 1.** Dendrogram of cluster isolates from candiduria by UPGMA determined by RAPD-PCR using primers (A) OPA-18, (B) OPE-18, and (C) P4. The similarity among genotypes was determined with the Dice coefficient and calculated by the BioNumerics software version 4.6. The roman algorithms (I–IV) represent the cluster, and the letters (A–F) represent profiles.
Microsatellite analysis

Amplified products from microsatellites were observed for all *C. tropicalis* isolates. The differences in size of the amplified fragments between the two markers are related to the different number of repeats of microsatellites. Isolates that showed two fragments were heterozygous and those that showed one fragment were homozygous.

A total of 10 alleles were observed for the 15 isolates, five for each of the URA3 locus and CT14. Four allelic combinations were observed for the locus URA3 and 8 for the locus CT14 (Table 2).

Discriminatory power based on the Simpson index for the URA3 marker was 0.372, and 0.86 for CT14, while combining the two markers the discriminatory power provided was 0.88.

Discussion

The occurrence of candiduria may be due to factors such as anatomical abnormalities in the urinary tract, comorbidities, urinary drainage devices, abdominal surgery, admission to the Intensive Care Unit (ICU), use of broad spectrum antibiotics, diabetes mellitus, increasing age and belonging to female gender.\textsuperscript{1,25} *C. tropicalis* has been featured among nosocomial infections as a global emerging pathogen among species of CNCA.\textsuperscript{15} It is a commensal microorganism in the human gastrointestinal tract with the potential to cause invasive infections due to virulence factors, which have greater potential to spread and cause mortality in ICU patients than *C. albicans* or other CNCA species.\textsuperscript{15,19,23}

Studies have demonstrated the need to perform antifungal susceptibility testing for *C. tropicalis*, because some isolates may present a resistance to antifungal agents.\textsuperscript{15,26,32} However, the results of our study indicate that the isolates of *C. tropicalis* from urine were susceptible to the antifungal agents tested, except for one isolate which was resistant to amphotericin B. This profile may be related to microbiota established in this hospital or to the clinical and pathological profile of patients.

The analysis of RAPD-PCR allowed us to evaluate the genotypic profile of each isolate, making it possible to differentiate them genotypically. This method has been used for the study of clinical isolates of *Candida* spp., with the purpose of identification, correlation and phylogenetic analysis.\textsuperscript{28,30} The primer OPA-18 was more discriminatory than OPE-18 and P4 for genotypic analysis by RAPD-PCR; however, all indicated similarity among isolates.

With regard to microsatellites, the marker CT14 showed greater generic genetic difference between the isolates, as eight allelic combinations were observed. The combination of the two markers revealed nine different genotypes among the isolates, with the predominance of genotypes 172:172 and 145:151 for URA3 and CT14, respectively. However, the discriminatory power (0.88) obtained from the combination of markers is not favorable to explain the typing results with confidence.\textsuperscript{17}

The occurrence of two isolates in one patient with different genotypic profiles by RAPD-PCR and microsatellite techniques was observed. Genotypic variation of these isolates may be associated to the reproduction of microorganisms during the in vivo or in vitro growth. A considerable alteration in the MIC of the isolates from patient 6 (isolates 6 and 7) to fluconazole points out the importance of antifungal susceptibility testing and warns about the tendency of decreased sensitivity of CNCA species.

Nosocomial infections caused by *Candida* may be due to the presence of yeasts in the patient's own microflora (endogenous) or the transmission to the patient from the microbiota in health professionals, on inanimate surfaces, catheters and probes (exogenous).\textsuperscript{37} The combination of the two techniques for genotyping revealed that isolates 4 and 5, from different patients and with different periods of hospitalization, had genotypic similarities, confirming the possibility of a spread, exogenously, of the infectious agent of a single strain from patient to patient. Thus, knowledge of the source of infection can help to prevent the spreading of resistant microorganisms and, therefore, help with the appropriate prophylactic treatment.

The results show the possibility of assessing the genetic similarity among isolates of *Candida* species involved in nosocomial infections, and comparing the genotypes among isolates from different sites, as already reported by other authors,\textsuperscript{3,10} which aids in the investigation of outbreaks. The RAPD-PCR and microsatellite techniques allowed us to visualize the genetic diversity and molecularly define the isolates, as it was observed by other authors who used these techniques of molecular genotyping to assess the genetic diversity of species of *Candida*.\textsuperscript{3,10–12} This information regarding genetic diversity is important for the control and prevention of nosocomial infections of endogenous or exogenous origin caused by yeasts, especially those isolates of *C. tropicalis*, which have emerged as an isolated species of CNCA in hospital infections.

Conflicts of interest

The authors have no conflicts of interest to declare.

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References