

## Identification and antifungal susceptibility of *Candida* species isolated from the urine of patients in a university hospital in Brazil

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### ABSTRACT

The aim of this study was to identify *Candida* spp. isolated from candiduria episodes at a tertiary hospital in the Midwest region of Brazil, and to determine their susceptibility profiles to antifungal compounds. From May 2011 to April 2012, *Candida* spp. isolated from 106 adult patients with candiduria admitted to the University Hospital of the Federal University of Mato Grosso do Sul were evaluated. Both, species identification and susceptibility testing with fluconazole-FLC, voriconazole-VRC, and amphotericin B-AmB were carried out using the Vitek 2. To discriminate species of the *C. parapsilosis* complex, a RAPD-PCR technique using the RPO2 primer was performed. From the total of 106 isolates, 42 (39.6%) *C. albicans* and 64 (60.4%) *Candida non-albicans* (CNA) - 33 *C. tropicalis*, 18 *C. glabrata*, 5 *C. krusei*, 4 *C. parapsilosis sensu stricto*, 2 *C. kefyr*, 1 *C. lusitanae*, and 1 *C. guilliermondii* were identified. All isolates were susceptible to AmB and VRC, whereas all *C. glabrata* isolates presented either resistance (5.6%) or dose-dependent susceptibility (94.4%) to FLC. The study of *Candida* spp. and their resistance profiles may help in tailoring more efficient therapeutic strategies for candiduria.

**KEYWORDS:** Urine infections. Antifungal agents. Nosocomial candiduria. *Candida* species. Antifungal susceptibility

### INTRODUCTION

The isolation of *Candida* spp. from urine cultures may indicate colonization or urinary tract infection (candiduria), but it may also be a sign of severe systemic candidiasis or candidemia<sup>1-3</sup>. *Candida* spp. can reach the urinary tract via the ascending route, from the urethra to the bladder, or by hematogenous spread, as *Candida* spp. is filtered by the kidneys and excreted in the urine.

The ability to form biofilm and the production of hydrolytic enzymes, which disrupt cell membranes, facilitate the spread of yeasts in the host, causing infection<sup>4-6</sup>. Lesions in the renal pelvis, tubules, and ureters, and formation of a “fungus ball”, which blocks and causes injury to the urinary system, are the main complications associated with candiduria, while development of candidemia is unusual<sup>1,4,7</sup>.

The source of candidiasis in humans is mostly endogenous, as *Candida* spp. are commensals in the digestive tract of a vast range of healthy people. Some conditions allow these commensal yeasts to become opportunistic, resulting in candidiasis in

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different sites of the body. Previous studies have shown that advanced age, female sex, urinary tract abnormalities, drainage catheters used in hospitalized patients, diabetes, malignancies, as well as the use of broad-spectrum antibiotics, corticosteroids and immunosuppressive agents are high-risk conditions for developing urinary tract infections<sup>3,8,9</sup>. Candiduria is frequently found in critically ill patients, in those who present with metabolic and immunosuppressive diseases, as well as in patients undergoing surgical procedures or facing long hospital stays<sup>4,10</sup>.

A study conducted in Spain revealed that 22% of critically ill patients hospitalized for more than seven days in intensive care units (ICUs) developed candiduria, and 10-15% urinary tract infections in the ICU were caused by the genus *Candida*<sup>11</sup>. In the United States, from years 1995-2001, it was estimated that there was an average of 25,000 cases of candiduria per year. Around one third of hospitalized patients with positive urine cultures for *Candida* spp were from the ICU, where the use of urinary catheters is frequent<sup>12,13</sup>. Moreover, it was found that a patient in a given ICU who is exposed to four different antibiotics has about a 35% risk of developing candidemia, and if *Candida* is isolated from another site, such as in urine, the risk increases to 80%<sup>13</sup>.

In severe systemic episodes of *Candida* infections, management of clinical conditions and enhanced patient survival depend on rapid interventions. Thus, correct identification of the pathogen and administration of specific antifungal therapies are crucial for patient recovery<sup>1,14,15</sup>.

The etiology of candiduria varies according to the geographic region, study period, and type of hospital. *Candida albicans* is the most prevalent candiduria agent; however, *Candida non-albicans* (CNA) species have been reported worldwide<sup>15,16</sup>.

The emergence of CNA species with reduced susceptibility or intrinsic resistance to antifungal compounds, mainly the azoles, is a major problem due to the increasing use of fluconazole (FLC) in candidiasis therapy<sup>7,17</sup>. In addition, studies conducted in ICU settings have reported that patients with candiduria have increased mortality rates when compared with similar patients without candiduria<sup>11</sup>. Therefore, early and accurate species identification, as well as investigations of antifungal susceptibility profiles of isolates, is indispensable for determining appropriate therapies for treating episodes of recurrent candiduria and/or persistent candidemia related to candiduria.

Therefore, the monitoring of epidemiological data in hospitals across different regions is important for establishing efficient control measures. The aim of this

study was to identify the various species of *Candida* isolated from candiduria episodes in a tertiary hospital in the Midwest region of Brazil, as well as to determine the *in vitro* susceptibility profiles of these species to various antifungal compounds.

## PATIENTS AND METHODS

### Patients

This study was conducted at the University Hospital, Federal University of Mato Grosso do Sul, Campo Grande, MS, from May 2011 to April 2012. It was approved by the institutional Ethics Committee in Research with Human Beings (process N° 86663, date of approval 08/30/2012). Adult female and male patients who were admitted to any hospital ward and presented with positive urine cultures for *Candida* spp. at least 1,000 CFU/mL were included in the study.

### Isolation and phenotypic identification

Urine sample sediments were initially seeded in Chromogenic *Candida* Selective Agar (CHROMagar, DIFCO; Becton Dickinson France S.A., Le Pont de Claix, France) for identification of *Candida* species. After inoculation, plates were incubated for 24-48 h at 30 °C and colonies were identified based on the chromogenic reaction produced, according to the standard instruction from the manufacturers.

The confirmation of *Candida* species identification and the antifungal susceptibility was performed using the automated Vitek 2 system (bioMérieux, Marcy-l'Etoile, France). From each sample, a yeast suspension was prepared in sterile saline (0.9%), using the densitometer (Densichektm Vitek 2). Yeast suspensions were added and incubated at 35.5 °C for 18 to 24 h. The equipment classified the final reading and biochemical differentiation reactions as excellent.

Differentiation of the *C. parapsilosis* complex species was performed by random amplified polymorphic DNA-polymerase chain reaction (RAPD-PCR) with the RPO2 primer (5'-GCGATCCCCA-3'), according to Rycovska *et al.*<sup>18</sup>.

*C. parapsilosis* isolates were re-suspended in saline with turbidity equivalent to 0.5 in the McFarland scale, centrifuged at 5000 x g and the pellets were subjected to DNA extraction. The final volume of reactions was 50 µL containing 20 ng of genomic DNA of each sample, 1.5 U of GoTaq DNA polymerase (Promega Co., USA), 10 µL of 5x concentrated buffer (1.5 mM MgCl<sub>2</sub>, 200 µM dNTP

and 1  $\mu\text{M}$  of primer RPO2). Amplifications were performed in a Veriti Thermocycler (Life Technologies, USA). After a first cycle of denaturation for 2 min at 94 °C, 35 cycles of denaturation at 94 °C for 1 min, annealing at 36 °C for 1 min, and extension at 72 °C for 75 s were performed, with a final extension step of 10 min at 72 °C.

Reference strains ATCC (American Type Culture Collection) were used as positive controls [*C. parapsilosis* (ATCC 22019), *C. orthopsilosis* (ATCC 96141) and *C. metapsilosis* (ATCC 96143)]. Amplified DNA products were separated by electrophoresis in a 1.5% agarose gel (Invitrogen, USA) with Tris-EDTA pH 8.0 buffer, stained with GelRed (Biotium, USA).

### Antifungal susceptibility testing

Determination of the minimum inhibitory concentration (MIC) was performed using the automated Vitek 2 (bioMérieux) system with the following antifungal drugs: amphotericin B (AmB), FLC, and voriconazole (VRC).

The minimum inhibitory concentration (MIC) breakpoints used for fluconazole and voriconazole were those suggested by Laboratory Standards Institute's M27-S4 document<sup>19</sup>, for *C. albicans*, *C. tropicalis* and *C. parapsilosis*  $\leq 2 \mu\text{g/mL}$  were susceptible, 4  $\mu\text{g/mL}$  were susceptible dose-dependent (SDD) and  $\geq 8 \mu\text{g/mL}$  were resistant. For *C. glabrata* isolates,  $\leq 32 \mu\text{g/mL}$  were SDD and  $\geq 64 \mu\text{g/mL}$  were resistant. Due to a lack of consensus about the definition of MIC breakpoints for amphotericin B, we considered values suggested by previous publications<sup>20</sup>, MICs  $\leq 1 \mu\text{g/mL}$  as susceptible and MICs  $\geq 2 \mu\text{g/mL}$  as resistant.

From the growth on Sabouraud dextrose agar for 24 h, a yeast suspension with turbidity equivalent to 0.5 in the MacFarland was prepared to test the performance of the broth microdilution test.

The MIC values of FLC (Pfizer) were confirmed by the microdilution broth technique<sup>21</sup> using the RPMI-1640 culture medium (Vitrocell). The stock solution of FLC (5120  $\mu\text{g/mL}$ ) was thawed at room temperature and diluted in a twofold dilution ratio.

Flat-bottom microdilution plates containing 100  $\mu\text{L}$  of the twofold serial dilutions of FLC were inoculated with 100  $\mu\text{L}$  of *Candida* suspensions. The final inoculum concentration was 0.5 to  $2.5 \times 10^3$  CFU/mL and the final ranges of the tested FLC dilutions were 0.25 to 128  $\mu\text{g/mL}$ . Microdilution plates were incubated at 37 °C for 24 h. Each sample was tested in triplicate.

The quality control strains *C. krusei* (ATCC 6258) and *C. parapsilosis* (ATCC 22019) were provided by the Central Laboratory of Mato Grosso do Sul (LACEN-MS).

### Statistical analysis

Statistical analysis was performed using the Minitab software version 14 (Minitab Inc., State College, PA, USA). To determine the association between variables, the chi-squared ( $\chi^2$ ) or the Fisher's exact test were used; a  $p$ -value  $< 0.05$  was considered significant.

## RESULTS

### Patients' epidemiological characteristics

During the study period, 106 adult patients were diagnosed with candiduria; 58 (54.7%) patients were female and 48 (45.3%) were male. The patients' ages ranged from 19 to 93 years, with a median of 68 years. Twelve (11.3%) patients were aged between 18 and 30 years, 25 (23.6%) between 31 and 60 years, and 69 (65.1%) were older than 60 years. Regarding the hospital setting, 33 patients (31.1%) were admitted to the ICU, 33 (31.1%) to emergency medical care, 20 (19.0%) to internal medical clinical wards, eight (7.5%) to the coronary unit, five (4.7%) to the surgical units, four (3.8%) to the maternity ward, two (1.9%) to the infectious diseases ward, and one patient (0.9%) to the renal unit.

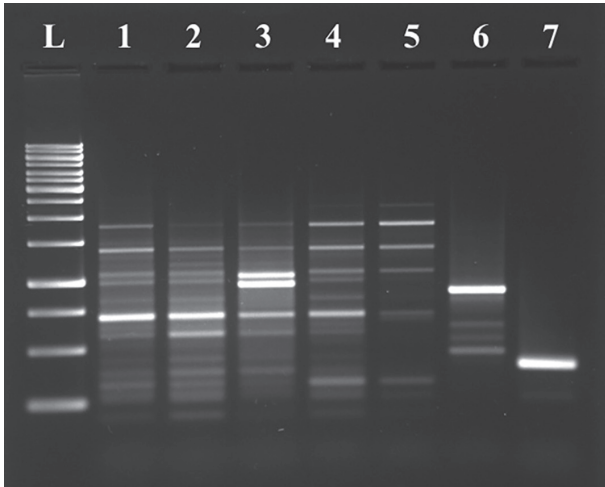
### *Candida* species

The CNA species predominated, occurring in 64 (60.4%) patients. There was no identification of more than one species in the same patient. CNA was more likely to be observed in patients older than 60 years when compared to those younger than 60 years (68.1% versus 48.9%, respectively;  $P=0.026$ ). *C. albicans*, *C. tropicalis*, and *C. glabrata* species were the most frequently isolated, accounting for 87.7% of all infections. The frequency of *C. albicans* (39.6%) and *C. tropicalis* (31.1%) cases did not differ from one another, and they were more frequently found than *C. glabrata* (17.0%) ( $[C. albicans = C. tropicalis] > C. glabrata$ ;  $P < 0.05$ ).

The four samples phenotypically identified as *C. parapsilosis* (sensu lato) were molecularly characterized as *C. parapsilosis* (sensu stricto) after performing the RAPD assay (Figure 1).

### Antifungal susceptibility

All isolates showed MIC = 1  $\mu\text{g/mL}$  for AmB and an MIC = 0.012  $\mu\text{g/mL}$  for VRC; therefore, they were considered susceptible. Regarding FLC, *C. krusei* and all *C. glabrata* isolates showed MIC values that were



**Figure 1** - Agarose gel showing the RAPD products obtained from *Candida parapsilosis* complex isolates and ATCC strains. Lane L - 100 bp molecular weight marker (GE Healthcare); lanes 1-4 - *Candida parapsilosis* complex isolates, all identified as *Candida parapsilosis sensu stricto*; lane 5 - *C. parapsilosis sensu stricto* (ATCC 22019); lane 6 - *C. orthopsilosis* (ATCC 96141); lane 7 - *C. metapsilosis* (ATCC 96143). RAPD= random amplified polymorphic DNA; ATCC=American Type Culture Collection

compatible with the SDD or R categories (Table 1). *C. kefyr*, *C. guilliermondii*, and *C. lusitaniae* were 100% susceptible (MIC = 2 µg/mL).

We did not observe associations among any of the studied variables with FLC resistance or SDD to FLC (Table 2). However, the mean number of *C. glabrata* isolated from ICU patients was higher than the numbers obtained from patients admitted to all the other hospital wards (22.7% versus 7.5%, respectively;  $P=0.043$ ).

## DISCUSSION

The correct identification of yeasts isolated from the

urine cultures of hospitalized patients, as well as the yeasts' antifungal susceptibility profiles, are relevant for determining appropriate treatments and urinary tract infection management<sup>1,3</sup>. This study revealed a greater proportion of CNA isolates from urine samples, which points to the emergence of strains resistant to FLC in our hospital.

There was a higher frequency of candiduria in women. Previous studies have shown that up to 30% of healthy women may experience persistent vulvovaginal colonization by *Candida* spp. This colonization, facilitated by the female anatomy, can ascend to the bladder and kidneys, causing urinary tract infections<sup>22-24</sup>.

Similar to other reports, most patients in our study were over 60 years of age. In general, older adults tend to exhibit natural modifications of the immune system, resulting in longer hospital stays in critical care units and the need of urinary catheters<sup>1,8,24-26</sup>.

Most patients in our study that tested positive for candiduria were hospitalized in ICU or emergency units, where most of them are generally submitted to invasive procedures and monitored with bladder catheters. These conditions may lead to the rupture of anatomical barriers, resulting in changes of the native host microbiota<sup>2,4,10,20</sup>.

It is generally accepted that urine cultures with at least 100,000 CFU/mL, with or without pyuria, can serve as an alert to a possible urinary tract infection, which is initially presumed as a bacterial infection<sup>5,8,27</sup>. There is no consensus among the authors regarding the cutoff point for interpreting urine cultures containing fungi; however, a minimum count of 1,000 CFU/mL in symptomatic patients has been considered a positive infection<sup>1,11</sup>. The presence of candiduria in critically ill patients has been regarded as an indicator of invasive candidiasis<sup>27</sup>.

The increased prevalence of CNA as a cause of

**Table 1** - *Candida* species and antifungal susceptibility in urine isolates (n=106)

<i>Candida</i> species (n; %)	Amphotericin B (%)			Fluconazole (%)			Voriconazole (%)		
	R	SDD	S*	R	SDD	S**	R	SDD	S***
<i>Candida albicans</i> (42; 39.6)	0	0	100	0	0	100	0	0	100
<i>Candida tropicalis</i> (33; 31.1)	0	0	100	0	0	100	0	0	100
<i>Candida glabrata</i> (18; 17.0)	0	0	100	5.6 <sup>(a)</sup>	94.4 <sup>(b)</sup>	0	0	0	100
<i>Candida krusei</i> (5; 4.7)	0	0	100	100	0	0	0	0	100
<i>Candida parapsilosis</i> (4; 3.8)	0	0	100	0	0	100	0	0	100
<i>Candida kefyr</i> (2; 1.9)	0	0	100	0	0	100	0	0	100
<i>Candida guilliermondii</i> (1; 0.9)	0	0	100	0	0	100	0	0	100
<i>Candida lusitaniae</i> (1; 0.9)	0	0	100	0	0	100	0	0	100

R = resistant; SDD = susceptible dose dependent; S = susceptible; MIC = minimal inhibitory concentration. \*(MIC ≤ 1 µg/mL); \*\* (MIC ≤ 2 µg/mL), \*\*\* (MIC ≤ 0.12 µg/mL); <sup>(a)</sup>(MIC > 64 µg/mL); <sup>(b)</sup>(MIC ≤ 32 µg/mL).



**Table 2** - Demographic variables and susceptibility to fluconazole (n=106)

Variables		Resistant or SDD (n=23)	Susceptible (n=83)	p-value
		n (%)	n (%)	
Inpatient unit	ER/ICU	18 (78.3)	48 (57.8)	0.074
	Other	5 (21.7)	35 (42.2)	
Age ≥60 years	No	6 (26.1)	31 (37.3)	0.316
	Yes	17 (73.9)	52 (62.7)	
Sex	Female	14 (60.9)	44 (53.0)	0.503
	Male	9 (39.1)	39 (47.0)	
CFU/mL	>100.000	22 (95.7)	70 (84.3)	0.294*
	≤100.000	1 (4.3)	13 (15.7)	
Leukocytes >5/field	No	2 (9.1)	13 (15.7)	0.732
	Yes	20 (90.9)	70 (84.3)	

\*Fisher's exact test. N= number of patients; SDD= susceptible dose-dependent; ER= emergency room; ICU= intensive care unit; CFU= colony-forming units.

candidiasis in hospital settings has been observed around the world at any site of infection, including the urinary tract<sup>3,28,29</sup>. The importance of this fact is that CNA species are likely to be resistant to antifungal agents, which reinforces the need to identify various *Candida* species, as well as to assess their susceptibility profile to antifungal agents in hospitalized patients<sup>13,17,30</sup>.

*C. albicans*, *C. tropicalis*, and *C. glabrata* were the main isolated species in our investigation. The comparison of the *Candida* species prevalence found in the present investigation with other eight Brazilian studies<sup>16,31-37</sup> on candiduria revealed a predominance of *C. albicans* in seven of them<sup>16,31-36</sup> and, in the second position, *C. tropicalis* in three studies<sup>16,33,35</sup>, the same behavior we observed in our study. In one study *C. tropicalis* was the most frequent<sup>37</sup>. The prevalence of *C. parapsilosis*, which was 4.7% in our study, in the other studies ranged from zero to 17.4%<sup>16,31-37</sup>. The evaluation of studies carried out in different regions of the country revealed a great similarity in the prevalence of *Candida* species among studies performed in the Midwest region<sup>16,31,32</sup> and those from the Southeast among themselves<sup>34,35,37</sup>. *C. albicans* is the most frequently found species in the digestive tracts of healthy people, and it has greater pathogenic mechanisms when compared to other *Candida* spp.<sup>5,38</sup>. Emerging *C. tropicalis* causes urinary tract infections primarily in patients presenting chronic degenerative diseases and/or trauma<sup>17,30,39</sup>.

The prevalence of *C. glabrata* in our study (17%) was higher than the one found in other earlier Brazilian reports, which ranged from 0 to 12.5 %<sup>16,31-35,37</sup>. Our data corroborate previous findings, which have shown that *C. glabrata* has emerged in tertiary hospitals in recent years, both in Brazil and in other countries<sup>8,40</sup>.

The *C. parapsilosis* complex has emerged as a primary agent of urinary tract infections in hospitalized patients due to its capacity to form biofilm<sup>36,41</sup>. Not all members of the *C. parapsilosis* complex are virulent; among them, *C. metapsilosis* appears to present the lowest virulence, but this evidence is still limited<sup>42</sup>. This reinforces the importance of differentiating *C. parapsilosis* in clinical studies, including isolates from other anatomic sites<sup>43</sup>. In our study, the four (3.8%) isolates that belong to the *C. parapsilosis* complex were identified as *C. parapsilosis (sensu stricto)*.

Several studies have shown the emergence of antifungal resistance, especially FLC resistance in *C. albicans*<sup>44</sup>; however, all of the *C. albicans* isolates were susceptible to AmB, FLC, and VRC. Moreover, the non-albicans species were also susceptible to the tested drugs, except *C. krusei*, which is intrinsically resistant to FLC. Furthermore, all isolates of *C. glabrata* were also resistant or SDD to this azole. Resistance to FLC or AmB has already been described in *C. glabrata*<sup>27</sup> and *C. tropicalis*<sup>30</sup>.

In Brazil, five studies from different regions evaluated the susceptibility to FLC of *Candida* species isolated from urine<sup>5,32-34,45</sup>. Using the broth microdilution method to evaluate *C. albicans* isolates, one study showed no resistance to FLC<sup>34</sup>, as did ours, and another showed 15.0% of resistance<sup>5</sup>. Using the disk diffusion, 7.5%<sup>5</sup> and 75%<sup>33</sup> of resistance to FLC were observed. The isolates of *C. tropicalis* were 100% sensitive to FLC, using the broth microdilution method, in two studies<sup>34,45</sup> and 12.5% of resistance in one<sup>5</sup>, confirming our findings. Using the disk diffusion method, high rates of FLC resistance were observed<sup>32,33</sup>. Regarding *C. parapsilosis*, there was no resistance to FLC<sup>34</sup>, as in the present study. We have also observed variations in the susceptibility profile of *Candida*

species to the most common antifungal agents of hospital use, especially to FLC, depending on the *Candida* species, methodology used, number of cases studied and year of publication.

The increasing prevalence rates of these species, which feature high MIC values for FLC, have already been reported in North America and Europe<sup>1,8,44,46</sup>. Some reports have demonstrated that this change in the susceptibility profile to FLC may be a consequence of the indiscriminate use of this antifungal agent in nosocomial settings<sup>8,44,47</sup>. In this study, the patients' previous use of FLC was not investigated. In fact, all *C. parapsilosis (sensu stricto)* isolates were susceptible to AmB, FLC, and VRC, corroborating the findings of other authors<sup>42,48</sup>.

In serious and systemic infections, the patient's survival depends on the early intervention and specific antifungal therapy. Delays to initiate an effective antifungal therapy among hospitalized patients is associated with high mortality<sup>1</sup>. Candiduria in seriously ill patients should be carefully evaluated, as this medical condition may be the only indicator of invasive candidiasis. Its detection increases the chances of successful treatment and enhances the patients' survival<sup>4,17</sup>.

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## AUTHORS CONTRIBUTIONS

GMEL: study design; collection, analysis, interpretation of data; paper writing. MON: data collection and analysis. MRC: data interpretation and paper writing. RAST: data collection and analysis. JON: data collection and analysis. CLT: molecular biology data analysis and interpretation. DYT: molecular biology data analysis, interpretation and paper writing. GMBDN: data analysis, interpretation and paper writing. RPM study design, data analysis, interpretation and paper writing. AMMP study design, data analysis, interpretation and paper writing.

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