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Article/Artigo

Species distribution and *in vitro* fluconazole susceptibility of clinical *Candida* isolates in a Brazilian tertiary-care hospital over a 3-year period

Distribuição de espécies e suscetibilidade *in vitro* ao fluconazol de isolados clínicos de *Candida* em um hospital terciário brasileiro num período de três anos

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ABSTRACT

Introduction: In this study, we aimed at identifying *Candida* isolates obtained from blood, urine, tracheal secretion, and nail/skin lesions from cases attended at the *Hospital Universitário de Londrina* over a 3-year period and at evaluating fluconazole susceptibilities of the isolates. **Methods**: *Candida* isolates were identified by polymerase chain reaction (PCR) using species-specific forward primers. The *in vitro* fluconazole susceptibility test was performed according to EUCAST-AFST reference procedure. **Results**: Isolates were obtained from urine (53.4%), blood cultures (19.2%), tracheal secretion (17.8%), and nail/skin lesions (9.6%). When urine samples were considered, prevalence was similar in women (45.5%) and in men (54.5%) and was high in the age group ≥61 years than that in younger ones. For blood samples, prevalence was high in neonates (35%) and advanced ages (22.5%). For nail and skin samples, prevalence was higher in women (71.4%) than in men (28.6%). *Candida albicans* was the most frequently isolated in the hospital, but *Candida* species other than *C. albicans* accounted for 64% of isolates, including predominantly *Candida tropicalis* (33.2%) and *Candida parapsilosis* (19.2%). The trend for non-*albicans Candida* as the predominant species was noted from all clinical specimens, except from urine samples. All *Candida* isolates were considered susceptible *in vitro* to fluconazole with the exception of isolates belonging to the intrinsically less-susceptible species *C. glabrata*. **Conclusions**: Non-*albicans Candida* species were more frequently isolated in the hospital. Fluconazole resistance was a rare finding in our study.

Keywords: Candidiasis. *Candida* spp. Anatomic sites.

RESUMO

Introdução: Neste estudo objetivamos a identificação de isolados de *Candida* obtidos de sangue, urina, secreção traqueal e de lesões de unha/pele, de casos atendidos no Hospital Universitário de Londrina num período de três anos. Avaliamos também a suscetibilidade dos isolados ao fluconazol. **Métodos**: Os isolados de *Candida* foram identificados pela reação em cadeia da polimerase (RCP) usando oligonucleotídeos iniciadores espécie-específicos. O teste de suscetibilidade *in vitro* ao fluconazol foi realizado segundo o procedimento de referência EUCAST-AFST. **Resultados**: Isolados foram obtidos de urina (53,4%), sangue (19,2%), secreção traqueal (17,8%) e lesões de unha/pele (9,6%). Considerando as amostras de urina, a prevalência foi similar em mulheres (45,5%) e em homens (54,5%) e foi alta no grupo de idade ≥ 61 anos do que em grupos mais jovens. Para amostras de sangue a prevalência foi alta em neonatos (35%) e idades avançadas (22,5%). Para amostras de unha e pele a prevalência foi maior em mulheres (71,4%) do que em homens (28,6%). *Candida albicans* foi a mais frequentemente isolada no hospital, mas outras espécies de *Candida* corresponderam a 64% dos isolados, incluindo predominantemente *Candida tropicalis* (33,2%) e *Candida parapsilosis* (19,2%). A tendência de *Candida* não-*albicans* como espécie predominante foi observada para todas as amostras clínicas, exceto para amostras de urina. Todos isolados de *Candida* foram considerados suscetíveis, *in vitro*, ao fluconazol com exceção dos isolados pertencentes às espécies intrinsecamente menos suscetíveis *C. glabrata*. **Conclusões**: Espécies de *Candida* não-*albicans* foram mais frequentemente isoladas no hospital. Resistência ao fluconazol foi rara no nosso estudo.

Palavras-chaves: Candidiasis. *Candida* spp. Sítios anatômicos.

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INTRODUCTION

Although ubiquitous in nature, *Candida* species can cause various infections primarily in hospitalized patients. With the evolution of intensive care medicine, it has become increasingly evident that critically ill patients represent a patient population susceptible to candidal infections. The increased susceptibility of these patients is largely due to the use of invasive devices, impaired immune mechanisms due to severe underlying illness, and widespread use of antibiotics[1, 2].

In Brazil, studies regarding the epidemiology of *Candida* infections in tertiary-care hospitals are still limited to few regions, with the majority being related to bloodstream infection[1, 3-13].

Although *Candida albicans* remains the main individual etiologic agent of candidemia, an increase in the number of cases caused by non-*albicans* species has been reported, including in Brazilian medical centers[1, 3-4, 7, 9-10]. The most common non-*albicans Candida* species causing bloodstream infections are *Candida parapsilosis* and *Candida tropicalis*[14].

Fluconazole is a systemic antifungal drug effective against most of the *Candida* species, although different degrees of susceptibility among species have been described; for example, *Candida krusei* has intrinsic resistance, and *Candida glabrata* shows less susceptibility than other *Candida* species[15]. Furthermore, the emergence of fluconazole resistance has been reported in *C. albicans*, *C. tropicalis,* and *C. parapsilosis*[16-20], including that observed in a Brazilian tertiary-care hospital[13, 21-22] that justifies the correct species identification and evaluation of their susceptibilities.

In this study, we analyzed for the first time the species distribution and fluconazole susceptibility of *Candida* isolates in the Hospital Universitário de Londrina, Parana State, Brazil.

METHODS

Candida isolates and cultures

We evaluated *Candida* sp. isolates obtained from patients admitted at the Hospital Universitário de Londrina (HU), a general tertiary-care hospital with 333 beds, including 43 intensive care unit (ICU) beds, in the southern Brazil from January 2005 to December 2007. The following data were recorded: sex, age, and patient location at the time of *Candida* detection.

Surveillance fungal cultures were obtained from several specimens during the trial. Cultures included in this study were those of the urine, blood, tracheal secretion, and superficial mycoses (nail/skin). For the urine specimens, *Candida* isolates were selected from significant candiduria (≥10^4 CFU/mL). The tracheal secretion isolates were colonization. The specimens were cultured using standard mycological procedures. Samples were inoculated on Sabouraud Dextrose Agar supplemented with chloramphenicol (100μg/ml). The plates were incubated at 28°C for 48 h. All the collected isolates were stored in yeast peptone dextrose (YPD) medium with 25% glycerol at -70°C and were deposited at the *Candida* culture collection of the Fungal Genetics Laboratory, Universidade Estadual de Londrina-Brazil.

Species identification

CHROMagar® *Candida* plates (CHROMagar Candida; CHROMagar®, France) were used for presumptive identification of *Candida* species. Definitive identification of all clinical isolates assayed in this work belonging to the genus *Candida* was performed by using a PCR-based method. PCR identification was carried out using species-specific forward primers (ITS1 and ITS2) corresponding to intergenic spacer regions and ITS4 as universal reverse primer located at the 26S rDNA as follows: CA (*Candida albicans*, 5´-TCA ACT TGT CAC ACC AGA TTA TT-3´), CT (*Candida tropicalis*, 5´-AAG AAT TTA ACG TGG AAA CTT A-3´), CGL (*Candida glabrata*, 5´-CAC GAC TCG ACA CTT TCT AAT T-3´), CP (*Candida parapsilosis*, 5´-GGC GGA GTA TAA ACT AAT GGA TAG-3´), and CK (*Candida krusei*, 5´-GAT TTA GTA CTA CAC TGC GTG A-3´)[23]. ITS4 (5´-TCC TCC GCT TAT TGA TAT GC-3´) was used as described previously[24]. The identity of *C. parapsilosis* isolates was confirmed by the employment of primers for *URA3* gene (orotidine-5´-phosphate decarboxylase) as described previously[25].

PCR was carried out using genomic DNA obtained as described by Furlaneto-Maia et al.[26]. For the optimum PCR conditions, a reaction volume of 20μL contained 0.8μL (2.5μM) of each deoxynucleoside triphosphate, 0.6μL (50mM) magnesium chloride, 0.5μL (20pmol/μL) of each primer, 10x Taq buffer and 1U of Taq polymerase (Invitrogen), and 2 l (5ng/μL) DNA template. Negative controls were performed with sterile deionised water in place of the template DNA. Reaction mixtures were subjected to an initial denaturing cycle of 5min at 96°C, followed by 34 cycles of 94°C for 1 min (denaturation), 60°C for 1 min (annealing), and 72°C for 1 min (elongation). The sizes of amplified DNA fragments were identified by comparison with molecular size marker DNA (100 bp DNA ladder). The identification of *C. dubliniensis* was based on phenotypic characteristics[27].

In vitro antifungal susceptibility testing

A total of 201 samples of *Candida* spp. were tested. The *in vitro* antifungal susceptibility test was performed according to the EUCAST-AFST reference procedure (EDef 7.1)[28], using fluconazole (Sigma-Aldrich) as antifungal drug. Fluconazole powder was dissolved in sterile distilled water, in a stock solution at 6400ug/mL, and stored at -20°C overnight.

The yeast isolates were grown on Sabouraud dextrose agar for 24 h at 37°C. Suspensions were prepared in sterile saline (0.9%), adjusted to 1-5×10^5 cells/mL.

Serial dilutions of fluconazole were performed in RPMI-1640 medium, supplemented with glucose 2% (w/v) and buffered with 0.165M morpholinepropanesulfonic acid (MOPS-Sigma-Aldrich). The final ranges of the drug dilutions tested were 0.125 to 64 mg/L.

Flat-bottom microdilution plates containing 100uL of the two-fold serial dilutions of fluconazole were inoculated with 100uL of the inocula. The final concentration of cells was 0.5 to 2.5×10^5cells/mL. The microdilution plates were incubated at 37°C for 24h. Each sample was tested in triplicate. Minimal inhibitory concentration (MIC) endpoints were determined spectrophotometrically at 590 nm. MIC_{90} was defined as the concentration capable of reducing the growth of 90% of the total population. *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 standard strains were used as quality controls of the tests[28]. Interpretative breakpoints proposed by EUCAST for fluconazole (susceptible ≤2 mg/L and resistant ≥8 mg/L) were used[28].

Ethical considerations

This study was approved by the Ethics Committee of Hospital Universitário da Universidade Estadual de Londrina-PR.

RESULTS

Isolates identification

In this study, a total of 208 *Candida* isolates were obtained from individual patients, including: *C. albicans* (n=75), *C. tropicalis* (n=69), *C. krusei* (n=4), and *Candida* sp. (n=59) in CHROMagar® *Candida*-yeast differential medium. In our experiments, *C. albicans* colonies appeared green, *C. krusei* appeared dry and light pink with a whitish border, and *C. tropicalis* appeared dark blue to metallic blue. Further identification carried out using PCR method revealed high accuracy in specificity. The PCR amplification of rDNAs from the three *Candida* species, *C. albicans*, *C. tropicalis*, and *C. krusei*, resulted in amplification of a single DNA fragment of the expected size (data not shown).

The employment of species-specific primer corresponding to the intergenic spacer region (ITS) sequence from *C. parapsilosis* (CP) e *C. glabrata* (CGL) allowed the identification of *C. parapsilosis* complex (n=40) and *C. glabrata* (n=17) isolates incompletely identified by CHROMagar® *Candida* characteristics. Two isolates remained not identified at the species level. The identity of *C. parapsilosis* (formerly *C. parapsilosis* group I) isolates was confirmed by the employment of primers corresponding to *URA3* gene as previously described[25]. None of the isolates were *C. orthopsilosis* (formerly *C. parapsilosis* group II) or *C. metapsilosis* (formerly *C. parapsilosis* group III).

In this study, we have employed the tobacco agar medium for the differentiation of *C. dubliniensis* from *C. albicans*[27]. On this medium at 28°C, only one isolate produced yellowish-brown colonies with hyphal fringes (*C. dubliniensis*), whereas the remaining isolates formed smooth, white- to cream-colored colonies (*C. albicans*).

Sex- and age-specific distribution of *Candida* isolates

As shown in **Table 1**, the majority (86%) of isolates from urine specimen were obtained from patients at ICU. Prevalence was similar in women (45.5%) and in men (54.5%). When urine samples were considered, prevalence was high in the age group ≥61 years than that in younger ones.

Considering bloodstream infection, 90% of the patients were from intensive care units. For blood samples, prevalence was high in neonates (35%) followed by advanced ages (22.5%).

Isolates from tracheal secretion and superficial (nail and skin) lesions represented 17.8% and 9.6% of the isolates obtained in this 3-year survey, respectively. Concerning sex, for tracheal secretion samples, prevalence was higher in men (66%). On the other hand for nail and skin samples, it was higher in women (71.4%) than in men (28.6%). For both body-site samples, prevalence was significantly higher in the age group ≥61 years than that in younger ones **(Table 1)**.

Species distribution

In a total of 208 *Candida* cultures, *C. albicans* represented 36%, while non-*albicans Candida* accounted for the majority (64%) of these isolates obtained from distinct clinical samples. **Table 2** shows the species distribution of *Candida* isolates with regard to clinical samples. Urine was the most common source of the *Candida*, accounting for 53.4% of the total isolates, with the following colony counting: 10^4 CFU/mL (20.7%), 10^5-10^6 CFU/mL (37.7%), and 10^7 CFU/mL (20.7%). Most *C. albicans* strains were isolated from urine samples (45%). Distribution of identified non-*albicans* species from urine was: *C. tropicalis* — 36.1%; *C. glabrata* — 12.6%; *C. parapsilosis* — 3.6%; *C. krusei* — 1.8%; and *C. dubliniensis* — 0.9%, as shown in **Table 2**. In this study, we found a low rate of *C. dubliniensis* isolates among a total of 75 isolates previously identified as *C. albicans*. Of the isolates obtained from blood cultures, 75% were non-albicans *Candida* species, the most common being *C. tropicalis*, followed by *C. parapsilosis* **(Table 2)**. Prevalence of non-*albicans Candida* species was also observed from nail/skin infection specimens, the most common being *C. parapsilosis* (45%) and *C. tropicalis* (35%). Most *C. parapsilosis* strains were isolated from tracheal secretion samples (46%).

Susceptibility tests

Susceptibility tests for fluconazole were performed on 205 isolates of *Candida* species. **Table 3** shows the MICs at which 50% (MIC50) and 90% (MIC90) of the isolates tested were inhibited for fluconazole. The majority of *Candida* isolates were considered susceptible to fluconazole (MIC of ≤2 mg/L), with the exception of *C. glabrata* isolates that are intrinsically less susceptible to fluconazole **(Table 3)**. MIC readings for quality control strains (ATCC 90028 and ATCC 22019) were within the limits described in the (EDef 7.1)[28].

TABLE 1 - Characteristics of patients with candidiasis according to specimen type of yeast isolation.

	Specimen sample^a			
Characteristics	urine (n=111)	blood (n=40)	tracheal secretion (n=37)	nail/skin lesions (n=20)
Gender (M/F)^b	(54.5/45.5)	(40.0/25.0)	(66.0/34.0)	(28.6/71.4)
Age$^{b, c}$	≤1-18 (10.0) 19-35 (20.5) 36-60 (25.5) ≥61 (44.0)	Neonate (35.0) ≤1-18 (10.0) 19-35 (15.0) 36-60 (17.5) ≥61 (22.5)	≤1-18 (2.7) 27-60 (24.3) ≥61 (73.0)	38-61 (43.0) ≥61 (57.0)
Patient location^b	Adult ICU (86.0) Hospitalized (14.0)	Adult ICU (50.0) Neonate ICU (35.0) Pediatrics ICU (5.0) Others (10.0)	ND	ND

M: male, **F:** female, **ICU:** intensive care unit, **ND:** not determined, ^{a}number of isolates according to specimen sample, ^{b}values in parentheses are percentages, ^{c}ages are given in years.

TABLE 2 - Distribution of *Candida* species and the clinical samples.

	Candida species^a							
Specimentype	*albicans*	*tropicalis*	*parapsilosis*	*glabrata*	*krusei*	*dubliniensis*	*Candida* sp.	Total
Urine	50 (45.0)	40 (36.1)	4 (3.6)	14 (12.6)	2 (1.8)	1 (0.9)	-	111
Blood	10 (25.0)	14 (35.0)	10 (25.0)	2 (5.0)	2 (5.0)	-	2 (5.0)	40
Tracheal secretion	12 (32.4)	8 (21.6)	17 (46.0)	-	-	-	-	37
Nail/skin lesions	3 (15.0)	7 (35.0)	9 (45.0)	1 (5.0)	-	-	-	20
Total	**75 (36.0)**	**69 (33.2)**	**40 (19.2)**	**17 (8.2)**	**4 (1.9)**	**1 (0.5)**	**2 (1.0)**	**208**

^{a}Values are given in n (%).

TABLE 3 - *In vitro* susceptibilities to fluconazole of *Candida* species isolates

Candida species	MIC values (mg/L)			resistant (%)[a]
(Number isolates)	range	50%	90%	(N of isolates)
albicans (75)	0.125-8.0	0.5	2.0	1.3 (1)
tropicalis (69)	0.125-32	1.0	2.0	7.2 (5)
parapsilosis (40)	0.125-16	1.0	2.0	2.5 (1)
glabrata (17)	0.25-32	8.0	16.0	64.7 (11)

[a]resistance breakpoints MIC of ^{3}8 mg/L, **MIC:** minimal inhibitory concentration.

DISCUSSION

Infection represents a frequent complication among patients admitted to tertiary hospitals. In particular, the incidence of candidiasis has been increasing during the past years. The largest multicentric study conducted in Latin America reveals a large burden of candidemia in Brazilian tertiary-care hospitals[1].

In this study, the isolate identification in species level by CHROMagar® *Candida* medium was in agreement to what was found in molecular identification. The PCR approach for species identification that was employed in this study is a reliable and sensitive method for the diagnosis of the most commonly encountered clinical-relevant *Candida* species. Furthermore, the employment of phenotypic method based on differential growth on tobacco agar[27] allowed the identification of one *C. dubliniensis* isolate (from urine sample). *Candida dubliniensis* is a newly emerging opportunistic pathogen that shares many phenotypic similarities with *C. albicans*. These similarities pose problems in the identification of isolates and have previously led to misidentification of these species[29].

The frequency of non-*albicans* species from distinct clinical specimens observed here was greater than *C. albicans*, which is consistent with the results of previous studies in Brazilian tertiary hospitals[21, 30]. *C. tropicalis* was the most frequent species isolated from candidemia (35%), which frequency was higher than that observed in the Brazilian multicenter study (16% to 29%)[1]. The second-most frequent species were *C. albicans* and *C. parapsilosis*. These data confirm the increasing importance of non-*albicans* species as agents of fungemia in Brazil. Besides, the low frequency of *C. glabrata* and *C. krusei* is in agreement with a previous report that consolidates the concept that candidemia due to these species is rare in Brazil[1].

An increase of candiduria among hospitalized patients has been reported[31-34]. Although the significance of *Candida* isolated from urine of patients is still unclear (reviewed in Kauffman[35]), the overall mortality associated with ICU candiduria can reach 50%[35]. In this study, identification of yeasts obtained from urine revealed *C. albicans* to be the most common species, followed by *C. tropicalis* and much less commonly by other species. Studies conducted in Brazilian medical centers have also shown increased rates of isolation of *C. tropicalis* from urine[30, 34, 37-38].

Candida parapsilosis was the species most often isolated from superficial mycoses (nail and skin), followed by *C. tropicalis*. This is in agreement with other studies that identified *C. parapsilosis* as the prevalent species in the processes of onychomycosis[39-40]. *C. parapsilosis* is a common inhabitant of normal skin, and this presumably serves as a reservoir of infection for the nails. Recently, we showed the capability of *C. parapsilosis* cells to adhere and grow as biofilm on human nail surfaces[41].

Candida parapsilosis was also prevalent in tracheal secretion specimens, followed by *C. albicans*. In a multicenter study of immunocompetent patients receiving mechanical ventilation performed in France, Azoulay found that *C. albicans* was the most common species and that the *Candida* colonization of the respiratory tract may predispose to bacterial ventilator-associated pneumonia[42].

In the present study, most of the isolates were susceptible to fluconazole. As expected, high resistance rate (64.7%) was observed in *C. glabrata*, which is an intrinsically less susceptible species. This resistance rate was similar to that observed by Bruder-Nascimento et al.[21] who also evaluated the resistance of *Candida* isolates obtained from distinct clinical specimens in a Brazilian tertiary hospital.

Although much has been reported regarding the epidemiology of *Candida* infections, the present work is the first report about the distribution of *Candida* species in invasive and non-invasive candidiasis in our hospital. These data suggest the need for continuous surveillance of candidiasis and antifungal susceptibility trends to adopt treatment strategies applicable to particular healthcare institutions.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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