

Yeast Colonization and Drug Susceptibility Pattern in the Pediatric Patients With Neutropenia

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Background: Pediatric patients with neutropenia are vulnerable to invasive *Candida* infections. *Candida* is the primary cause of fungal infections, particularly in immunosuppressed patients. *Candida albicans* has been the most common etiologic agent of these infections, affecting 48% of patients

Objectives: The aim of this study was to identify *Candida* spp. isolated from children with neutropenia and determine the antifungal susceptibility pattern of the isolated yeasts.

Patients and Methods: In this study 188 children with neutropenia were recruited, fungal surveillance cultures were carried out on nose, oropharynx, stool, and urine samples. Identification of *Candida* strains was performed using germ tube and chlamyospore production tests on an API 20 C AUX system. Susceptibility testing on seven antifungal agents was performed using the agar-based E-test method.

Results: A total of 229 yeasts were isolated. Among those, *C. albicans* was the most common species followed by *C. krusei*, *C. parapsilosis*, *C. glabrata*, *C. tropicalis*, *C. famata*, *C. dubliniensis*, *C. kefyr*, and other *Candida* species. *C. glabrata* was the most resistant isolated yeasts, which was 70% resistant to fluconazole and 50% to itraconazole, 7.5% to amphotericin B and 14% to ketoconazole. All the tested species were mostly sensitive to caspofungin.

Conclusions: Knowledge about the susceptibility patterns of colonized *Candida* spp. can be helpful for clinicians to manage pediatric patients with neutropenia. In this study, caspofungin was the most effective antifungal agent against the colonized *Candida* spp. followed by conventional amphotericin B.

Keywords: Neutropenia; Candidemia; Antifungal; Susceptibility; Caspofungin; Amphotericin

1. Background

Opportunistic invasive *Candida* infection has considerably increased over the recent decades, with repercussions on morbidity and mortality and increases in related healthcare costs. *Candida* is the primary cause of fungal infections, particularly in immunosuppressed patients. *Candida* infection rates in hospital wards have increased from 3.2 cases per 1000 admissions during 1997–1999 to 5.5 per 1000 admissions during 2000–2002 and 6.9 per 1000 admissions during 2003–2005 (1). *Candida albicans* has been the most common etiologic agent of these infections, affecting 48% of patients (2), but recent studies have reported a relative shift toward non-*albicans* *Candida* spp. and a reduction in *C. albicans* infections. For example, *C. parapsilosis* was found to be the predominant species causing infections in pediatric ICU, suggesting nosocomial transmission (3).

In hospitalized patients, prior *Candida* colonization is considered as a major predisposing factor for the development of systemic candidiasis (4-6). The prophylac-

tic antifungal agents have been used to prevent fungal colonization and invasive infections (7), and the efficacy of isolation measures in colonized patients has been assessed as an adjunct to routine infection control to avert *Candida* transmission to patients (8). Nonetheless, the risk is compounded with increasing resistance of *Candida* spp. to available antifungal agents (4), as previously stated in several studies (9, 10). New antifungal agents have produced, but the effectiveness of various azoles, especially for the treatment of systemic candidiasis in pediatric patients has not been fully evaluated. In neutropenic patients with infection, invasive sampling to diagnose the etiologic agent is quite impossible because of the decreased platelet and white blood cell counts; hence, empirical therapy is often used in this population. Therefore, information on the distribution and susceptibility patterns of predominant *Candida* spp. colonization could be helpful for clinicians to manage these patients.

2. Objectives

The aim of this study was to identify *Candida* spp. isolated from pediatric patients with neutropenia and to determine the antifungal susceptibility pattern of the isolated yeasts.

3. Patients and Methods

This prospective study was conducted between March 2011 and March 2012 to investigate the fungal colonization and drug susceptibility patterns of isolated fungi from patients treated in Amir Hospital, a large tertiary-care referral pediatric hematology/oncology center in Shiraz university of medical sciences, Southern Iran. This study comprised of 188 pediatric patients (87 girls and 101 boys) with a mean white cell count of 3886 cell/mL (range: 170 - 20300 cell/mL). Most of the patients had acute leukemia including acute lymphoblastic leukemia (ALL) and acute myelogenous leukemia (AML), and underwent chemotherapy and/or hospitalizations. Many of patients had a history of antifungal prophylaxis administration.

Specimens were obtained from nose, oropharynx, stool, and urine of patients for fungal surveillance cultures. Samples were plated on Sabouraud dextrose agar (Merck, Darmstadt, Germany) and incubated at 24°C for 10 days. The purity of the isolate was evaluated by culturing the isolates on potato dextrose agar (OXOID LTD, Basingstoke, Hampshire, England) twice for 48 hours at 35°C. The identification of *Candida* strains was performed using germ tube, chlamyospore production tests, and by analyzing the carbohydrate assimilation reactions on the API 20 C AUX system (bioMérieux, Marcy l'Etoile, France). Two *Candida* spp., including *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258 were used as controls.

Susceptibility testing against amphotericin B, fluconazole, ketoconazole, voriconazole, itraconazole, caspofungin, and posaconazole was performed using an agar-based E-test method (bioMérieux, Solna, Sweden). Roswell Park Memorial Institute medium (RPMI) 1640, supplemented with 1.5% agar and 2% glucose at pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS), was used to then inoculated by dipping a sterile swab into an inoculum suspension adjusted to 0.5 McFarland standard turbidity (10^6 cells/mL) and streaking the agar in three directions. The minimum inhibitory concentration (MIC) of each drug was determined after 24 and 48 hours of incubation at 35°C. The tested concentrations of each drug ranged from 0.002 to 32 µg/mL, with the exception of fluconazole, with concentrations of 0.016 to 256 µg/mL. For caspofungin, itraconazole, voriconazole, posaconazole, fluconazole, and ketoconazole, the MIC was based on significant inhibition of 80% of growth. For amphotericin B, the MIC was determined as the point of complete inhibition (100%). The resistance breakpoints for the antifungals were as follows: fluconazole ≥ 64 , ketoconazole ≥ 4.0 , itraconazole ≥ 1.0 , voriconazole ≥ 8.0 , amphotericin B > 1.0 and caspofungin > 2.0 µg/mL (11-16).

The resistant breakpoint for posaconazole is not established as per CLSI document M27-A3 (12). The MIC₅₀ and MIC₉₀ (ie, the MIC at which 50% and 90% of isolates are inhibited) were also calculated. Statistical analysis was performed using SPSS software (version 16) and were analyzed using descriptive statistics and cross tabulation.

3.1. Ethical Considerations

The Ethics Committee of the Clinical Microbiology Research Center, Shiraz University of Medical Sciences, reviewed and approved the study. Written informed consents were obtained from the parents of all pediatric patients who participated in the study.

4. Results

In this study, 188 patients with neutropenia including acute lymphoblastic leukemia (n = 122), acute myeloid leukemia (n = 18), Burkitt lymphoma (n = 18), Hodgkin lymphoma (n = 13), megaloblastic anemia (n = 9), and aplastic anemia (n = 8) were entered. The mean age, white blood cell count and the number of chemotherapy treatment sessions of the patients were 6.6 years, 3886 cell/mL, and two times, respectively. These patients with clinical complains such as fever, bone pain, cough, pallor, headache, ecchymosis, epistaxis, vomiting, hematuria, and chest pain were admitted to the hospital. One hundred and two (54%) patients in at least one stage of previous admission received fluconazole or itraconazole for prophylaxis and nystatin as the mouth washing solution. But amphotericin B and voriconazole were received by 15 patients who were previously suspicious to have fungal infections. As all the patients in this study had the history of admission and neutropenia, the significant relationship between these factors and colonization in the patients cannot be determined.

During the study period, 88 of 188 children with neutropenia had colonized *Candida* spp. that is an overall colonization rate of 46.8%. Among the various hematologic disorders affecting the patients, the highest percentage of *Candida* colonization was found among those with acute lymphoblastic leukemia. Colonization of the oral cavity was significantly more frequent in comparison to the other investigated body parts. Characteristics of the patients are shown in Table 1.

Candida spp. were isolated from more than one body site of 26 (29.5%) patients, and in some cases, more than one species was isolated from each site. Overall, 229 yeasts were isolated. *C. albicans* was the most common species detected in 117 samples (51.2%), followed by *C. krusei* isolated from 18 patients (7.9%), *C. glabrata* from 14 (6.3%), *C. tropicalis* from 11 (4.7%), *C. famata* from 11 (4.7%), *C. parapsilosis* from 8 (3.5%), *C. dubliniensis* from 6 (2.4%), *C. kefyr* from 4 (1.8%), *Cryptococcus* including *C. terreus* and *C. laurentii* from 16 (7%) patients and the other *Candida* species, including *C. guilliermondii*, *C. rugosa*, *C. lusitanae*, *C. lambica*, as well as *Rhodotorula* spp. was isolated from 24

(10.5%) patients.

C. albicans, the most frequently isolated species, was sensitive to amphotericin B, fluconazole, voriconazole, itraconazole, ketoconazole, and caspofungin with the following sensitivities 97%, 88%, 94%, 72%, 92.4%, and 97%, respectively. *C. glabrata* was the most resistant isolated yeasts, with resistance rates of 70% to fluconazole, 50% to

itraconazole, 7.5% to amphotericin B and 14% to ketoconazole. All the fungal species exhibited highest sensitivity to caspofungin. The detected 16 *Cryptococcus* isolates were divided into two subspecies; *C. terreus* and *C. laurentii*. The results of antifungal susceptibility tests by E-test in *Candida* isolates are shown in Table 2. The lowest MIC₉₀ was observed against caspofungin.

Table 1. *Candida* Isolated from Different Specimens

Site of Body	Etiologic Agent (Isolates No.)
Oral	<i>C. albicans</i> (53), <i>C. dubliniensis</i> (6), <i>C. tropicalis</i> (3), <i>C. krusei</i> (3), <i>C. parapsilosis</i> (3), Other (20)
Stool	<i>C. albicans</i> (34), <i>C. famata</i> (5), <i>C. tropicalis</i> (5), <i>C. krusei</i> (6), <i>Cryptococcus spp.</i> (6), Other (21)
Nose	<i>C. albicans</i> (14), <i>C. dubliniensis</i> (1), <i>C. krusei</i> (2), <i>C. famata</i> (3), <i>C. glabrata</i> (4)
Urine	<i>C. albicans</i> (16), <i>C. famata</i> (4), <i>C. glabrata</i> (5), <i>C. krusei</i> (6), <i>Cryptococcus spp.</i> (3), Other (6)

Table 2. Antifungal Susceptibility of *Candida* spp^a

Antifungal Agent	Species	Isolates No.	MIC ₅₀ (µg/mL)	MIC ₉₀ (µg/mL)	Range	Resistance No. (%)
Amphotericin	<i>C. albicans</i>	117	0.5	0.75	0.047 - 1.5	4 (3%)
	<i>C. krusei</i>	18	0.25	1	0.38 - 6	5 (1%)
	<i>C. glabrata</i>	14	0.25	0.75	0.25 - 6	1 (7.5%)
	<i>Cryptococcus spp.</i>	16	0.38	1	0.016 - 2	2 (11%)
Fluconazole	<i>C. albicans</i>	117	1.5	256	0.094 - 256	14 (12%)
	<i>C. krusei</i>	18	16	128	16 - 256	8 (40%)
	<i>C. glabrata</i>	14	16	128	16 - 256	10 (70%)
	<i>Cryptococcus spp.</i>	16	2	16	2 - 256	6 (33%)
Voriconazole	<i>C. albicans</i>	117	0.125	8	0.003 - 32	7 (6%)
	<i>C. krusei</i>	18	0.5	2	0.125 - 32	2 (10%)
	<i>C. glabrata</i>	14	1.5	2	0.75 - 32	3 (21%)
	<i>Cryptococcus spp.</i>	16	0.125	3	0.016 - 3	0
Itraconazole	<i>C. albicans</i>	117	0.032	32	0.016 - 32	36 (28%)
	<i>C. krusei</i>	18	0.75	4	0.19 - 32	6 (30%)
	<i>C. glabrata</i>	14	0.75	1	4 - 32	7 (50%)
	<i>Cryptococcus spp.</i>	16	0.75	32	0.023 - 32	8 (50%)
Ketoconazole	<i>C. albicans</i>	117	0.125	32	0.016 - 32	9 (7.6%)
	<i>C. krusei</i>	18	3	4	1 - 32	2 (11%)
	<i>C. glabrata</i>	14	1.5	4	2 - 32	2 (14%)
	<i>Cryptococcus spp.</i>	16	1.5	12	0.032 - 32	6 (33%)
Posaconazole	<i>C. albicans</i>	117	0.047	8	0.008 - 32	^b
	<i>C. krusei</i>	18	0.75	2	0.008 - 32	
	<i>C. glabrata</i>	14	32	8	1 - 32	
	<i>Cryptococcus spp.</i>	16	0.032	1	0.032 - 32	
Caspofungin	<i>C. albicans</i>	117	0.094	0.125	0.032 - 8	4 (3%)
	<i>C. krusei</i>	18	0.125	0.75	0.032 - 4	0 (0%)
	<i>C. glabrata</i>	14	0.094	0.19	0.064 - 0.19	0
	<i>Cryptococcus spp.</i>	16	0.094	1.5	0.032 - 4	1 (5%)

^a Species present in small numbers are not mentioned in the table.

^b As the breakpoint for posaconazole has not been established as per CLSI document M27-A312, the percent of resistant cases was not reported and only the MIC was reported.

5. Discussion

Patients with neutropenia and prior candidiasis are at high risk for developing systemic candidiasis (17). In this study, we investigated the major epidemiological characteristics of *Candida* colonization in children with neutropenia and their history of hospital admissions and chemotherapy. There is obvious that *Candida* colonization is a risk factor for developing invasive *Candida* infections (18) and candidemia in hospitalized patients. In one study, the reported rate of nosocomial candidemia increased more than 2-fold over the 9-year-study period (1), and in another, nosocomial candidemia was diagnosed in 6.9% of colonized neonates, compared with 0.76% of non-colonized neonates (19). The central nervous system, eyes, and other organs can also be infected by spreading of the infecting agent through the blood. In a previous study, the overall reported mortality rate associated with candidemia was 10.7% (20).

In the present study, 46.8% of pediatric patients were infected with *Candida* spp., 12.1% of neonates (19), 12.4% of infants (20), and 55.2% of adults with hematological malignancies were reported to have *Candida* colonization (6). The most commonly colonized sites in the present study were the oral cavity and rectum, which was in accordance to the findings of the other studies (6, 20). Among the *Candida* species isolated in the present report, 51.2% were identified as *C. albicans*. In other studies, *C. albicans* have been detected in 50% (21), 64.2% (20), 48.6% (22), 42% (19), and 55% (10) of cases. In blood cultures, *C. albicans* accounted for 37.2% (1) and 39.2% (23) of isolated *Candida* spp. In this study the isolation rate of non-*albicans Candida* was 48.8%, whereas 35.8% (20) and 78.2% (24) have been previously reported in the literature. The mortality associated with *C. albicans* (37.5%) is reported to be significantly higher than with non-*albicans* species (17.7%) (1); therefore, it is important to identify the etiologic species of this infections. In our study, *Cryptococcus* spp. accounted for 7% of the isolates. Colonization with this yeast has been less extensively described. The increased use of antifungals in immunocompromised patients, mainly for prophylaxis, is considered as the strongest causative factor changing the distribution of these agents, which have subsequently affected the mortality and the choice of empirical treatments (25).

Resistance to antifungal agents is associated with high mortality rate in immunocompromised and at-risk hospitalized patients. These agents could be categorized into primary, acquired, and clinical resistant ones. Primary resistance to antifungal agents is known as intrinsic and occurs when the organism is naturally resistant to the antifungal agent, such as *C. krusei*, which is known to be universally resistant to fluconazole (26). Acquired resistance develops during treatment, and is often the result of genetic mutations (27). Clinical resistance, i.e., failure of anti-fungal therapy, depends on a variety of factors, such as the host immune system, pharmacokinetics of the antifungal agent, and the species involving in fungal

infection. Intrinsic resistance to amphotericin B is rare and acquired resistance during therapy is even less common (28, 29). *C. glabrata* and *C. krusei* tend to have higher MICs than *C. albicans*, and a small proportion of them have been found to be resistant to amphotericin B with MIC ≥ 2 $\mu\text{g/mL}$ (30). *C. glabrata* with amphotericin B MIC ≥ 2 $\mu\text{g/mL}$ was reported in less than 1% of USA and in 4.4% of European isolates (31). Furthermore, difficulty in the treatment of infection with *C. glabrata*, which is often resistant to many azole antifungal agents, especially fluconazole, is also reported (32). Recent studies have revealed that the MICs of triazoles, voriconazole, itraconazole and fluconazole, for *C. glabrata* were higher than those by most other *Candida* species (10, 21, 22).

Amphotericin B is recommended as the first-line therapy for invasive mycoses, and is commonly used in pediatric wards (24). The use of this drug is limited by the toxicity of the conventional formulation and the high amount of the lipid emulsions. In the present study, susceptibility testing revealed that all isolates of *C. albicans* were more sensitive to amphotericin B and caspofungin than to the other studied antifungal agents. However, resistance to amphotericin B was seen in 1% of *C. krusei* and 7.5% of *C. glabrata* isolates. In one study, amphotericin B resistance was found in nearly 20% of *C. parapsilosis* isolates (33).

Triazole alters the fungal cell membrane by inhibiting ergosterol synthesis through an interaction with 14-demethylase, which leads to alterations in cellular permeability and a loss of membrane fluidity and integrity (34). The currently available triazole antifungals include fluconazole, voriconazole, itraconazole, ketoconazole, and posaconazole. Resistance to azoles was found in all *Candida* spp., with species-specific trends. Gene mutations related to ATP dependent pumps CDR genes in *Candida* spp. appears to confer resistance to multiple azoles and have been associated with fluconazole treatment, and cross-resistance with other azoles may also be possible (34, 35), which was well documented by molecular methods (36). According to previous studies, resistance to older azoles is commonly reported by *C. krusei* and *C. glabrata* (21, 22).

In the present study, fluconazole resistance was observed in 12%, 40%, and 70% of *C. albicans*, *C. krusei*, and *C. glabrata* isolates and resistance to itraconazole was found in nearly 28%, 30% and 50% of *C. albicans*, *C. krusei*, and *C. glabrata* strains, respectively, these amounts are consistent with the findings reported in previous studies (2, 21). Cross-resistance between the new and former azoles is a concern, such as fluconazole-voriconazole and itraconazole-posaconazole (37, 38). In the present study, voriconazole and fluconazole resistance was observed in 10% of *C. krusei* and 21% of *C. glabrata* isolates. Posaconazole is the newest orally administered triazole antifungal with an extended spectrum of activities. Due to cross activity between this antifungal agent and other azoles, the MICs for many *Candida* spp. were higher than 2 $\mu\text{g/mL}$ in this study.

Our findings showed that the new antifungal agents are effective for the treatment of yeast infections. Echinocan-

dins such as caspofungin are active against many fungal species and have been approved by the U.S. Food and Drug Administration (FDA) to be used for the treatment of candidemia and invasive candidiasis. In our study, caspofungin was the most effective agent, with lower MIC₅₀ and MIC₉₀ values against all the *Candida* spp. studied. Several prophylactic antifungal agents are produced to reduce the *Candida* species colonization and associated morbidity and mortality in patients at risk of developing this infection. Knowledge about the susceptibility patterns of colonized *Candida* spp. can be helpful for the clinicians who chose the best therapeutic option to manage the high-risk patients. We found that caspofungin was the best antifungal agent against *Candida* colonization for pediatric patients with hematological disorders and neutropenia, followed by conventional amphotericin B.

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Author's Contributions

None declared.

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