Resistance or Decreased Susceptibility to Fluconazole in *Candida* Species Isolated from Solid Organ Transplant Recipients: An Emerging Challenge

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ABSTRACT

Background: Invasive candidiasis are the most prevalent fungal infections in solid organ transplant (SOT) recipients. In this regards, emerging pathogens and antifungal resistance are concerning issues in transplantation medicine.

Objective: Regarding universal prophylaxis in SOT recipients, particularly with fluconazole, and the emergence of azole-resistant species, the present study was conducted to species identification and antifungal susceptibility profiles of yeasts isolated from SOT recipients.

Methods: All adults undergone solid organ transplantations between 21 March -22 September 2022 in Abu-Ali Sina transplant center, Shiraz, Iran, were included with 6 months follow-up. Species identification of isolated yeasts from different clinical specimens was performed by ITS1-5.8S-ITS2 gene sequencing. Antifungal susceptibility testing was determined according to the microbroth dilution method documented by CLSI.

Results: During the study period, 28 of 383 (9.8%) adult SOT recipients developed at least one positive culture of yeasts isolated from different clinical specimens. Candiduria was the most prevalent type of involvement by *Candida* species. The incidence rate of invasive candidiasis was 2.6%. Of 54 isolated yeasts, *C. albicans* was the most frequent species (22/54, 40.7%), followed by *C. glabrata* (11/54, 20.3%), and *C. parapsilosis* (9/54, 16.7%). Resistance or decreased susceptibility to fluconazole was found in 55% of isolates, and also 13% of isolates were known cross-resistant to different azole antifungal drugs.

Conclusion: Our results showed a high incidence of azole- resistant *Candida* strains causing candidiasis in SOT recipients. Indeed, the findings support the need to perform antifungal susceptibility testing of yeast isolates in immunocompromised patients to guide proper treatment.

KEYWORDS: *Candida*; Candidiasis; Antifungal resistance; Organ transplantation

INTRODUCTION

Invasive fungal infections (IFIs) are the primary cause of morbidity and mortality among solid organ transplant (SOT) recipients [1], which affect approximately 5 to 20% of SOT recipients [2]. Therefore, prevention and treatment of IFIs in SOT recipients is one of the important clinical aspects to have a successful organ transplant [3].

Invasive candidiasis (IC) is the most common type of IFI in SOT recipients, except for lung transplant recipients where invasive aspergillosis is more common [4]. IC in SOT recipients often present as candidemia, urinary tract infection, and peritonitis [5]. The median time to develop of IC is estimated three

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months post-SOT [6]. Risk factors for IC in SOT recipients are associated with the type of organ transplant. So, liver transplant recipients have a higher risk of developing IC [7, 8]. *Candida* (*C.*) *albicans*, *C. glabrata*, *C. tropicalis*, and *C. parapsilosis* are the most common *Candida* species causing IC in SOT recipients [7]. Nonetheless, emerging *Candida* species are known as causative pathogens in recent years.

Nowadays, due to the excessive use of azoles and echinocandins for prophylactic and therapeutic strategies, the resistance of *Candida* species to these antifungal agents has been observed. So, antifungal resistance is considered a serious challenge in the management of IC in SOT recipients [9, 10]. Fluconazole with low toxicity, high solubility, and wide tissue distribution is used as a prophylactic and therapeutic regimen in SOT recipients. However, in some cases has been associated with frequent relapse and treatment failure, especially in infections caused by *C. glabrata* and *C. krusei* [1, 11]. Echinocandins are mostly used in patients who have previously received azoles, and are involved in azole-resistant *Candida* species [12]. On the other hand, resistance to echinocandins has been reported in *C. glabrata* strains [3].

Regarding extensive use of antifungal prophylaxis in SOT recipients, evaluation of the species distribution and the antifungal susceptibility patterns of *Candida* species is an essential issue in transplant centers. Consequently, the findings could offer guidance to assist in choosing the appropriate treatment for SOT recipients who developed IC [11]. Hence, the present study aimed to identify *Candida* species isolated from adults receiving SOTs and assess their antifungal susceptibility profile.

MATERIALS AND METHODS

Study population and Definition

All adult SOT recipients (liver, kidney, heart, simultaneous pancreas-kidney (SPK), small intestine, multivisceral transplants, lung) were included with six months follow-up after transplantation. This study was conducted from 21 March 2022 to 22 September 2022 at Abu-Ali Sina transplant center, Shiraz, Iran. Located in the south of Iran and affiliated with Shiraz University of Medical Sciences, this center is recognized as one of the world's largest medical centers for organ transplantation, performing more than 600 SOTs per year. This center features 700 hospital beds, with over 10,000 kidney and liver transplants performed to date (https://abualisina.org).

The European Organization for Research and Treatment of Cancer/Invasive Fungal Infections Cooperative Group (EORTC-MSG) criteria were used to define proven *Candida* infections [13]. So, the isolation of *Candida* species from sterile samples (blood, fluid) was defined as proven invasive candidiasis. Candiduria was defined as the isolation of > 104 CFU/mL *Candida* species from urine samples [14]. Heavy growth of *Candida* spp. in the bronchoalveolar washing or endotracheal aspiration samples was considered as respiratory tract colonization.

Fungal Culture

The collected specimens (urine, body fluids, bronchoalveolar washing, or endotracheal aspiration) were cultured on Sabouraud Dextrose Agar (SDA) (Merck, Denmark), and CHROMagarTM Candida (bioMérieux, Paris, France) with incubation for 24–72 hours at 35°C. Candidemia was diagnosed using the Becton Dickinson's BACTEC system followed by culture on SDA to isolate agents. All isolates were preserved in tubes containing 10% glycerol at -20°C for further investigation.

DNA Extraction

DNA extraction of isolates was performed using the method previously described [15]. Briefly, two or three colonies were transferred to tubes containing lysis buffer (100 mM Tris-HCl, pH 7.5, 10 mM EDTA, 0.5% w/v SDS, 100 mM NaCl), and placed in boiling water for 15 min. Then, sodium acetate (2.5 M) was added and kept at -20°C for 60 min. After centrifugation, the supernatant was transferred to a new tube. Followed by adding the same volume of cold isopropanol, the tubes kept at -20°C for 30 min. Then, 200–300 µl of cold

70% ethanol was added to the sediment which separated by centrifugation. After centrifugation at 10000 rpm for 5 min, the supernatant was removed, and the tubes were dried for 30 min at room temperature. Finally, 50 µl of sterile distilled water was added to the precipitated DNA.

Molecular Identification of Isolates

Identification of isolate was performed by the DNA-sequencing method following amplification of the ITS1–5.8S–ITS2 region using universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5′-TCCTCCGCTTATTGATATGC-3') [16]. The amplification was performed with an initial denaturation cycle for 5 min at 95°C, followed by 30 cycles of denaturation at 95°C for 30 s, annealing at 57°C for 45 s, and extension at 72°C for 45 s, with a final extension cycle at 72°C for 5 min.

The amplicons were sequenced by sanger sequencing method (Eurofins, Germany). After the sequencing of PCR products, the quality of the DNA sequences was checked using Chromas software version 2.6.6. In order to identify species, the ITS region sequence for each isolate was subjected to nucleotide BLAST analysis (https://blast.ncbi.nlm.nih.gov/Blast.cgi) and compared with similar reference sequences in the National Centre for Biotechnology Information Database (NCBI). The sequence data of ITS1–5.8S–ITS2 region have been deposited in GenBank.

Antifungal Susceptibility Testing

Antifungal susceptibility testing (AFST) was performed using the broth microdilution method according to the M27-A3 protocol documented by the Clinical and Laboratory Standards Institute (CLSI), considering the supplemental information of M27-S4 document [17]. Briefly, RPMI 1640 (Sigma, St. Louis, Missouri, USA) buffered to pH 7.0 using 0.165 N-morpholinopropanesulfonic acid (MOPS) (Sigma, USA) was used to perform susceptibility tests according to documented protocol. The minimum inhibitory concentration (MIC) values of all *Candida* isolates against antifungal drugs, including fluconazole (Sigma, USA), itraconazole (Sigma, USA), voriconazole (Pfizer, New York, USA), caspofungin (Sigma, USA), and amphotericin B (Sigma, Germany) were determined. The concentrations range of the tested antifungal agents was 0.03-16 µg/ml for itraconazole, voriconazole, and amphotericin B, 0.12 - 64 µg/ml for fluconazole, and also 0.015-8 µg/ml for caspofungin. The lowest concentrations of antifungals resulted in 50% decrease in fungal growth after 24h incubation compared to control wells (wells without antifungal agent) were considered the MIC values for azoles and caspofungin. Moreover, the lowest concentration that resulted in any visible growth of isolates (100% inhibition) was considered as the MIC for amphotericin B. *Candida krusei* (ATCC 6258) and *C. parapsilosis* (ATCC 22019) were included for quality control. MIC results were interpreted using clinical breakpoints described in the CLSI documents [18, 19]. The wild-type phenotypes of *C. dubliniensis*, *C. kefyr*, and *C. guilliermondii* were determined as described by Pfaller *et al.* [20].

Ethical Considerations

This study was approved by the Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran (IR.SUMS.REC.1400.433).

RESULTS

In the six-month study period during 21 March to 22 September 2022, 28 of 383 (9.8%) of solid organ transplant recipients in the adult population developed at least one positive culture of yeast species isolated from different clinical specimens. According to the EORTC/MSG definition, proven invasive candidiasis was diagnosed in 10 recipients (6 patients with positive culture of sterile body fluids,4 patients with positive culture of blood). Therefore, 2.6% (10/383) of invasive candidiasis were diagnosed in the studied duration. Liver transplant recipients had shown the most frequent episodes of *Candida* positive culture (20/28, 71.4%). Candiduria was the most frequent type of involvement found in 18 patients that 8 of them showed candiduria along with other types of involvement. Candidemia as the

Table 1: Distribution of *Candida* species isolated from SOT recipients according to type of specimen.

Candida species	Type of specimen				
	Urine	BW/ETA*	Fluid	Blood	Wound
Candida albicans [21]	13	3	4	2	-
Candida glabrata [11]	7	2	2	-	-
Candida parapsilosis [9]	6	-	-	3	-
Candida krusei (*Pichia kudriavzevii*) [4]	1	2	1	-	-
Candida kefyr (*Kluyveromyces marxianus*) [3]	3	-	-	-	-
Candida dubliniensis [3]	1	-	-	-	2
Candida guilliermondii (*Meyerozyma guilliermondii*) [2]	2	-	-	-	-

*BW: Bronchial Washing, ETA: Endotracheal Aspirate

most serious systemic *candida* infection was diagnosed in 4 patients, which 3 of them have previously shown candiduria.

In total, 52 clinical specimens had shown positive cultures, and 54 yeast species were recovered by culture method. According to DNA-sequencing results, 7 species of yeasts identified. The most prevalent species isolated from specimens was *C. albicans* (22/54, 40.7%), followed by *C. glabrata* (11/54, 20.3%), and *C. parapsilosis* (9/54, 16.7%). Rare species of *Candida* including *C. kefyr* (*Kluyveromyces marxianus*) and *C. guilliermondii* (*Meyerozyma guilliermondii*) were isolated from urine samples (Table 1). Accession numbers of gene sequences are available in Supplementary Table 1.

Antifungal Susceptibility Patterns

According to CLSI breakpoints, all *C. parapsilosis* and *C. glabrata* isolates were either SDD or resistant to fluconazole. Irrespective of *C. krusei* with inherent resistant to fluconazole, 30 of 54 isolates (55.5%) exhibited resistance or decreased susceptibility to fluconazole, and overall resistance rate to fluconazole was approximately 33%. Moreover, resistance to itraconazole was observed among *C. albicans* (n:4) and *C. glabrata* (n:4) isolates. Notably, *C. krusei* did not exhibit resistance to voriconazole and itraconazole. Additionally, the rates of resistance to itraconazole and voriconazole were found to be 14.8% and 5.5%, respectively. Caspofungin showed potent activity against all isolates, and all species were classified as susceptible for caspofungin. All *Candida* isolates were non-wild type for amphotericin B and caspofungin.

Cross-resistant species against fluconazole and itraconazole were found in two *C. albicans* and two *C. glabrata* isolates. One *C. parapsilosis* was cross-resistant to fluconazole and voriconazole. Moreover, two of *C. albicans* isolates were cross-resistant to fluconazole, voriconazole, and itraconazole. Overall, 7 of 54 isolates (13%) were known cross-resistant to different azole antifungal drugs.

C. kefyr (*Kluyveromyces marxianus*) showed low MIC values for all tested antifungal drugs. *C. guillermondii* (*M. guilliermondii*) exhibited MIC= 2 µg/mL for fluconazole, whereas the MICs for other azole antifungals were in the range of 0.06-0.25 µg/mL. Table 2 shows the results of the *in vitro* activity of 5 antifungal agents tested against 54 isolates of *Candida* species.

DISCUSSION

Candida species are important causes of fungal infections in organ transplant recipients, leading to significant mortality rate and increased costs of patient care and duration of hospitalization. Candidiasis is associated with the complexity of transplantation surgical procedures, broad-spectrum antibiotics, long-term hospitalization, and the extensive immunosuppressed status of organ recipients.

Table 2: *In vitro* susceptibility patterns of *Candida* species to antifungal drugs.

Candida species	Antifungal	MIC Range	GM	MIC50	MIC90	Mode	S	SDD/I	R	NWT
C. albicans [21]	Fluconazole	0.25->64	3.2	2	64	1	12	2	8	6
	Itraconazole	0.03->16	0.10	0.06	2	0.03	17	1	4	4
	Voriconazole	0.03-4	0.11	0.06	0.5	0.06	14	6	2	9
	Caspofungin	0.015-0.12	0.04	0.06	0.06	0.06	22	0	0	0
	Amphotericin B	0.015-0.12	0.04	0.06	0.12	0.06	ND	ND	ND	0
C. glabrata [11]	Fluconazole	4->64	9.6	8	64	8	0	9	2	8
	Itraconazole	0.25->16	0.77	0.5	2	0.5	0	7	4	2
	Voriconazole	0.12-4	0.29	0.12	4	0.12	ND	ND	ND	3
	Caspofungin	0.03-0.12	0.06	0.06	0.06	0.06	11	0	0	0
	Amphotericin B	0.12-0.25	0.15	0.12	0.25	0.12	ND	ND	ND	0
C. parapsilosis [9]	Fluconazole	4-32	13.71	ND	ND	8	0	1	8	8
	Itraconazole	0.03-0.06	0.03	ND	ND	0.03	9	0	0	0
	Voriconazole	0.06-1	0.22	ND	ND	0.12	4	4	1	9
	Caspofungin	0.5-1	0.54	ND	ND	0.5	9	0	0	0
	Amphotericin B	0.06-0.5	0.16	ND	ND	0.25	ND	ND	ND	0
C. krusei (*Pichia kudriavzevii*) [4]	Fluconazole	8-32	13.45	ND	ND	8	0	0	4	0
	Itraconazole	0.06-0.25	0.12	ND	ND	0.12	4	0	0	0
	Voriconazole	0.12-0.25	0.17	ND	ND	-	4	0	0	0
	Caspofungin	0.12-0.25	0.14	ND	ND	0.12	4	0	0	0
	Amphotericin B	0.25	0.25	ND	ND	0.25	ND	ND	ND	0
C. kefyr (*Kluyveromyces marxianus*) [3]	Fluconazole	0.25	0.25	ND	ND	0.25	ND	ND	ND	0
	Itraconazole	0.03	0.03	ND	ND	0.03	ND	ND	ND	ND
	Voriconazole	0.03	0.03	ND	ND	0.03	ND	ND	ND	3
	Caspofungin	0.015	0.015	ND	ND	0.015	ND	ND	ND	0
	Amphotericin B	0.25	0.25	ND	ND	0.25	ND	ND	ND	ND
C. dubliniensis [3]	Fluconazole	0.12-0.5	0.31	ND	ND	0.5	ND	ND	ND	0
	Itraconazole	0.03	0.03	ND	ND	0.03	3	0	0	0
	Voriconazole	0.03	0.03	ND	ND	0.03	ND	ND	ND	0
	Caspofungin	0.03-0.06	0.04	ND	ND	0.06	ND	ND	ND	0
	Amphotericin B	0.03	0.03	ND	ND	0.03	ND	ND	ND	0
C. guilliermondii [2]	Fluconazole	2	2	ND	ND	2	ND	ND	ND	0
	Itraconazole	0.06-0.25	0.12	ND	ND	-	ND	ND	ND	0
	Voriconazole	0.06-0.12	0.08	ND	ND	-	ND	ND	ND	0
	Caspofungin	0.25-0.5	0.35	ND	ND	-	ND	ND	ND	0
	Amphotericin B	0.03-0.06	0.04	ND	ND	-	ND	ND	ND	0

GM: geometric mean, MIC: minimum inhibitory concentration, S: susceptible, SDD: susceptible-dose-dependent, I: intermediate, R: resistant, NWT: non-wild type (MIC>ECV), ND: not determined.

Several studies have reported the incidence rate of *Candida* infections in transplant recipients ranged between 1.9% - 3.8 % in USA [5, 6]. Another studies have declared the incidence rate of 1.1-8.2% for invasive candidiasis in liver transplant recipients in different European centers and Brazil [21, 22].Our data show that in our center the incidence of

invasive candidiasis was 2.6% in agreement with previous studies.

According to our results, candiduria was the most prevalent type of involvement by *Candida* species in our study. The significance of candiduria in clinical setting is uncertain. While candiduria is usually considered as colonization, it should be particularly taken important in immunocompromised patients due to the possibility of this condition for the indication of systemic or invasive infections [23]. Though *C. albicans* was the most commonly recovered species from urine samples in accordance with previously published data [24, 25], the isolation of less common species is growing, potentially as a result of selection pressure from antifungals like fluconazole administered as prophylaxis treatment.

In line with the other studies, *C. albicans* (40.7 %) was the most frequent species isolated from SOT recipients in the present research [5, 6, 21]. Nevertheless, non-albicans *Candida* species have accounted for 60% of isolates as the etiological agents of candida colonization or infection. In the current study, the most common non-albicans *Candida* species was *C. glabrata* followed by *C. parapsilosis* similar to previous study by Andes et al. [6]. Moreover, rare *Candida* species including *C. kefyr* and *C. guillermondii* were isolated from SOT recipients in accordance with previous reports [11, 24, 26, 27]. Despite the isolation of these rare species as colonized yeasts in the urinary tract, cases of invasive infections have also been reported [28, 29].

The emergence of *Candida* strains with resistance or decreased susceptibility to antifungal drugs is of considerable issue. Despite the mounting concern, there are insufficient data on antifungal resistance patterns in SOT recipient. The frequent isolates with resistance or SDD patterns to fluconazole are one of the main findings of our study. Our results revealed a 33% fluconazole resistance rate, which is remarkably higher than previous studies on SOT recipient [11, 21, 30]. Besides, 22% of isolates showed decreased susceptibility to fluconazole. These findings could be explained by extensive prophylaxis regimens with fluconazole in our center that may represent with the emergence of resistant strains due to impose significant selective pressure [31]. Moreover, cross-resistant between azole antifungal drugs was observed in the current study. Since azole antifungal agents are first-line treatment for candidiasis, cross-resistant strains are getting of worrisome concern. According to earlier research, *C. guilliermondii* isolates have high MIC for azole antifungals and particularly fluconazole [26, 32]. Although no breakpoints was determined for categorization of *C. guillermondii* to susceptible or resistant, species isolated in this study seem to be generally susceptible to tested antifungal drugs that are in consistent with previous results [24].

Candida kefyr (*Kluyveromyces marxianus*) occasionally isolate from dairy products [33], and has also been isolated from a variety of clinical samples or from the hands of health care workers [34]. Recent reports suggest that *C. kefyr* is an emerging pathogen in immunocompromised patients. Similar to other investigation in Iran, *C. kefyr* had shown susceptibility to common antifungals [24].

Collectively, evidence from resistance and decreased susceptibility of *Candida* species to antifungal agents, azoles in particular, supports the need to perform antifungal susceptibility testing of isolates. For immunocompromised patients such as recipients of SOT, the choice of proper treatment must be guided by susceptibility testing as well as species identification.

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Supplementary Table 1: Accession numbers of sequences for *Candida* species isolated from SOT recipients deposited in NCBI GenBank.

Species	Accession numbers
Candida albicans [21]	OQ363164, OQ134720, OQ145112, OQ134709, OQ134713, OQ134731, OQ145119, OQ134732, OQ134708, OQ134711, OQ145118, OR991998, OQ145115, OQ134734, OQ145128, OQ363140, OQ363160, OQ363151, OQ134725, OQ363159, OQ134730, OQ134724
Candida glabrata [11]	OQ134723, OQ134717, OQ134718, OQ134726, OQ145117, OQ134715, OQ145126, OQ363139, OR991999, OQ363150, OR992000
Candida parapsilosis [9]	OQ363149, OQ024929, OQ024932, OQ145116, OQ145121, OQ145120, OQ363145, OQ145122, OQ024937
C. krusei (*Pichia kudriavzevii*) [4]	OQ134728, OQ134710, OQ134714, OR991997
C. kefyr (*Kluyveromyces marxianus*) [3]	OQ134722, OQ145114, OQ134716
Candida dubliniensis [3]	OQ363148, OQ145123, OQ363143
Candida guilliermondii (*Meyerozyma guilliermondii*) [2]	OR992001, OR992002

CONFLICT OF INTEREST:

None declared.

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