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Emerging *Candida* species isolated from renal transplant recipients: Species distribution and susceptibility profiles

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ABSTRACT

Candidiasis is a major challenge among renal transplant recipients (RTRs) worldwide and is associated with high morbidity and mortality rates. Fluconazole is the most commonly used agent for *Candida* infections. However, frequent relapse and treatment failure are still reported among patients affected with this infection. In the present study, *Candida* species obtained from RTRs were characterized based on conventional and molecular assays. Furthermore, the antifungal susceptibility profiles of these species were determined. This study was conducted on a total of 126 RTRs within 2012–2016. The patients were categorized according to the referenced diagnostic criteria. The identification of *Candida* species was accomplished based on conventional examination, assimilation profile test, and polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method. The minimum inhibitory concentrations (MICs) of amphotericin B, fluconazole, itraconazole, voriconazole, posaconazole, and caspofungin were determined based on the guidelines of Clinical and Laboratory Standards Institute. The patients with *Candida* infection were diagnosed with urinary tract candidiasis (n = 17), peritonitis (n = 8), intra-abdominal candidiasis (n = 6), candidemia (n = 4), hepatosplenic candidiasis (n = 3), and *Candida* pneumonia (n = 3). A total of 41 *Candida* isolates, including *C. albicans* (n = 18), *C. famata* (n = 8), *C. kefyr* (n = 4), *C. tropicalis* (n = 4), *C. parapsilosis* (n = 3), *C. glabrata* (n = 2), and *C. lusitaniae* (n = 2), were isolated from 32.5% (41/126) renal transplant recipients. Fluconazole-resistance was observed in seven isolates, entailing *C. albicans* (n = 6) and *C. tropicalis* (n = 1). Fluconazole MIC for *C. lusitaniae* isolates was above the epidemiologic cut-off value (4–16 μg/ml). Furthermore, MIC range values of fluconazole against *C. famata* and *C. kefyr* were obtained as 4–32 μg/ml and 4–8 μg/ml, respectively. Posaconazole exhibited potent activity against *Candida* isolates, followed by caspofungin. The identification of *Candida* species, together with susceptibility testing, provides important data about the geographic trends of the fluconazole-resistance profiles of *Candida* species. It is necessary to maintain a consistent method for the implementation of early diagnosis and adoption of treatment regimen.

1. Introduction

Invasive fungal infections (IFIs) are associated with significant morbidity and mortality, especially among renal transplant recipients (RTRs) [1–3]. The RTRs are highly susceptible to the IFIs mainly caused by *Candida* species due to the prolonged use and high dose steroids,

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long-term immunosuppressive treatment, difficulty in primary diagnosis, and ineffective therapy [3,4]. Although *C. albicans* is the main agent of nosocomial fungal infections, non-*Candida albicans Candida* (NCAC) species have emerged in the recent years with diverse virulence and susceptibility profiles [5–8]. Owing to the increased use of azoles and echinocandins for prophylactic and therapeutic purposes, resistance to these agents in *Candida* species has recently become a serious clinical challenge [9,10]. Although there is no evidence regarding the efficacy of antifungal drugs in the prophylaxis and treatment of *Candida* infections in RTRs, they may significantly improve clinical outcomes and reduce healthcare costs [11]. In addition, prophylaxis and therapy may be important factors in explaining the observed changes in the distribution of etiologic agents and susceptibility pattern of each *Candida* species in different countries [12]. Azoles are therapeutic agents for *Candida* infections, among which fluconazole is the most commonly used agent with low toxicity, high solubility, and wide tissue distribution [10,13–16]. However, the use of fluconazole as prophylactic and treatment regimen in RTRs has been associated with frequent cases of relapse and treatment failure, which can be a potential risk factor leading to the gradual development of resistant species [17]. *Candida* infections can be a major challenge given the lack of effective therapeutic options and limited management experience. Consequently, potent antifungal agents and alternative antifungal strategies, including combination therapy, can be considered as effective approaches to improve the management of *Candida* infections [18,19]. Selected combinations of clinically licensed drugs (e.g., statins and antifungal agents) might be potential alternatives for the therapeutic management of these infections. However, all these approaches have remained at the laboratory experimental phase up to now [20,21]. The isolation, identification, and susceptibility testing of *Candida* species in RTRs have become an increasingly crucial issue for the management of fungal infections given the increased drug resistance scenario occurring during the last decades worldwide [10]. Therefore, the current study was conducted to characterize *Candida* species obtained from RTRs based on conventional and molecular assays to determine the epidemiology of *Candida* species. In addition, the present study sought to investigate the in vitro susceptibility profiles of six marketed antifungal drugs, as follows amphotericin B, fluconazole, itraconazole, voriconazole, posaconazole, and caspofungin against obtained *Candida* isolates.

2. Materials and methods

2.1. Patients

This study was conducted on 126 RTRs at the Nephrology and Kidney Transplant Research Center, Urmia University of Medical Sciences, Urmia, Iran, during 2012–2016. Data collected for the patients included age, gender, risk factors, clinical presentation, and mycological sample sites. The patients were diagnosed on the basis of clinical examination, and *Candida* infection was confirmed by direct examination, culture method, and histopathology [22–24]. The patients were grouped into candidemia, urinary tract candidiasis, *Candida* pneumonia, hepatosplenic candidiasis, intra-abdominal candidiasis, and peritonitis. A definitive diagnosis of invasive candidiasis was dependent on the recovery of *Candida* from bloodstream, peritoneal fluid or abscess material, biopsy, and urine [22–24]. The evidence of *Candida* pneumonia was based on visual inspection and histologic confirmation with a positive culture [24]. However, the patients with superficial fungal infections were excluded. Immunosuppressive regimen induction therapy consisted of the administration of cyclosporine (6 mg/kg) or tacrolimus (0.1–0.15 mg/kg) and mycophenolate mofetil (2 g/day) or mycophenolate sodium (720 mg bid) before the surgery, in addition to intravenous (IV) methylprednisolone pulse therapy (10–15 mg/kg) performed daily 1 h before the operation and after the operation for 3 days (triple therapy). The highly sensitive recipients and/or those suspected with delayed graft function were given prophylactic cytomegalovirus (CMV) and IV thymoglobulin (1 mg/kg daily up to 7 days), accompanied with IV gancyclovir (5 mg/kg bid), followed by valgancyclovir (900 mg/day), and *Pneumocystis jirovecii* prophylaxis (trimethoprim-sulfamethoxazole). This study was approved by the Research and Ethics Committee (nr. 2094) of Urmia University of Medical Sciences, Urmia, Iran. Written informed consent was obtained from the patient's next of kin for the publication of this report. The informed consent included the acceptance of policies for the management of personal information, procedures for data collection, and data management.

2.2. Fungal isolates/phenotypic characterization

The collected samples consisted of 41 isolates/patients from a variety of specimens, including urine (n = 17), biopsy (n = 9), peritoneal fluid (n = 8), blood (n = 4), and transbronchial lung biopsy (n = 3). The samples were sent to the mycology laboratory and examined by wet-mounted observation, followed by inoculation on Sabouraud dextrose agar (SDA, Difco) at 25 °C and 35 °C for 48 h. The grown isolates were preliminarily identified by standard mycological procedures, including colony color on CHROMagar *Candida* medium (CHROMagar Company, Paris, France), microscopic morphology on Corn Meal Agar (CMA, Difco, laboratories, Detroit, Mich., USA) with 1% Tween 80, and germ tube tests in serum at 37 °C for 2–3 h in dark. Additionally, the assimilation profile of all yeast isolates was performed by commercially available API strips (ID32C; bioMérieux, Marcy I'Etoile, France). All identified isolates were suspended in tryptic soy broth medium (TSB, Scharlau, Spain) containing 2% peptone, 2% glucose, and 20% glycerol at −80 °C for extra analysis and deposited at the reference culture collection of the Cellular and Molecular Research Center (CMRC), Urmia, Iran.

2.3. Molecular investigation

Genomic DNA was extracted from 2-day-old cultures as previously described [25]. Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) method was used for species identification [25,26], briefly, the PCR amplification of ITS-rDNA region was achieved using the universal primers ITS1 (5′-TCCGTAGGTGAACCTG CGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). The amplification was performed with a cycle of 5 min at 94 °C for primary denaturation, followed by 35 cycles at 94 °C (1 min), 56 °C (1 min), and 72 °C (1 min) with a final extension step at 72 °C for 7 min. Subsequently, the PCR products were digested by restriction enzyme *Msp*I at 37 °C for 4 h. The size of DNA fragments was directly determined with the comparison of molecular size marker and distinct banding patterns [17,18].

2.4. Antifungal susceptibility testing

Based on the guidelines of Clinical and Laboratory Standards Institute (CLSI) [27,28], the antifungal agents were diluted in RPMI-1640 medium (Sigma Chemical Co.) buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) (Sigma) with L-glutamine without bicarbonate to yield two times their concentrations and dispensed into 96-well microdilution trays with the final concentrations of 0.063–64 μg/ml for fluconazole (Pfizer, Groton, CT, USA), 0.008–8 μg/ml for caspofungin (Merck, Whitehouse Station, NJ, USA), 0.016–16 μg/ml for amphotericin B (Sigma, St. Louis, MO, USA), itraconazole (Janssen Research Foundation, Beerse, Belgium), voriconazole (Pfizer Central Research, Sandwich, United Kingdom), and posaconazole (Schering-Plough, Kenilworth, NJ). The plates were stored at −70 °C until they were used. Briefly, homogeneous suspensions were measured spectrophotometrically at the wavelengths of 530 nm to a percent transmission within the range of 75–77. Therefore, the final densities of the stock inoculum suspensions of the isolates ranged within 1×10^3–3×10^3 CFU/ml, as determined by quantitative colony

counts on Sabouraud glucose agar (SGA, Difco). After incubation at 35 °C for 24 h, minimum inhibitory concentration (MIC) values were visually determined. The MIC endpoints were determined with the aid of a reading mirror and defined as the lowest concentration of drug that prevents any recognizable growth (i.e., exerts 100% inhibition for amphotericin B) or significant (≥50%) growth diminution levels (all other agents), compared with the growth of a drug-free control. The MIC_{90} and MIC_{50} values were defined as the lowest concentration of the antifungals at which 90% and 50% of the isolates were inhibited, respectively. The MIC_{50}, $MIC_{90,}$ and geometric mean (GM) MIC were not determined when less than ten isolates were available. The fluconazole-resistant isolates were determined according to the new CLSI clinical breakpoint values for species-specific and non-species-specific *Candida* species [28]. *Candida parapsilosis* (ATCC 22019) and *C. krusei* (ATCC 6258) strains were used as the quality control isolates for each testing run.

2.5. Statistical analysis

The data were recorded using Microsoft Excel 2007 (Microsoft Corp, Redmond, WA, USA) and analyzed using SPSS software (version 16; SPSS Inc., Chicago, IL, USA). *P* value less than 0.05 was considered statistically significant.

3. Results

Table 1 summarizes the clinical data of the patients. According to the results, 56.1% (n = 23) of the study population were male. The participants had the age range of 1–65 years at the time of diagnosis and included 3 children (1–12 years old), 5 adolescence (12–18 years old), 31 adult (18–60 years old) and 2 elderly (> 60 years old). The risk factors were the prolonged administration of corticosteroid and immunosuppressive therapy, kidney transplantation, renal failure, consumption of broad-spectrum antibiotics, Intensive Care Unit admission, abdominal surgery, hemodialysis, and indwelling bladder catheters. The patients with *Candida* infection were diagnosed with urinary tract candidiasis (n = 17), peritonitis (n = 8), intra-abdominal candidiasis (n = 6), candidemia (n = 4), hepatosplenic candidiasis (n = 3), and *Candida* pneumonia (n = 3). Based on the conventional and molecular techniques, 41 isolates of *Candida* species, including *C. albicans* (43.9%) and non-*Candida albicans* species (56.1%), were obtained from 126 RTRs. Among the various species identified, *C. albicans* (n = 18, 43.9%) was the leading agent, followed by *C. famata* (n = 8, 19.5%), *C. kefyr* (n = 4, 9.7%), *C. tropicalis* (n = 4, 9.7%), *C. parapsilosis* (n = 3, 7.3%), *C. glabrata* (n = 2, 4.8%), and *C. lusitaniae* (n = 2, 4.8%). Table 2 summarizes the MIC range, MIC_{50}, MIC_{90}, and GM MIC of six antifungal drugs against seven different species. Fluconazole-resistant isolates (n = 7, 17%) were determined according to the new CLSI species-specific and non-species-specific *Candida* species clinical breakpoints [27,28], including *C. albicans* (n = 6) and *C. tropicalis* (n = 1). *Candida lusitaniae* isolates showed a fluconazole MIC above the epidemiologic cut-off value (ECV; 4–16 μg/ml) according to the ECVs in antifungal susceptibility testing and interpretation for uncommon yeasts [29,30]. In addition, the MIC range values of fluconazole against *C. famata* and *C. kefyr* were 4–32 μg/ml and 4–8 μg/ml, respectively. In terms of the GM MIC values, posaconazole (0.02 μg/ml) exhibited potent activity against *C. albicans* isolates, followed by caspofungin (0.04 μg/ml). The widest range and highest MIC_{90} values for *C. albicans* against fluconazole were 0.25–64 μg/ml and 8 μg/ml, respectively. In addition, *C. famata* isolates appeared to be highly resistant to fluconazole (n = 3, MIC_{90} = 16 μg/ml), and fluconazole MIC was 3-log_2-dilution steps less active than amphotericin B MIC, which in turn was 5-log_2-dilution and 7-log_2-dilution steps less active than caspofungin and posaconazole, respectively. On the other hand, MIC ranges of fluconazole against *C. kefyr* (4–8 μg/ml), *C. tropicalis* (1–8 μg/ml), *C. parapsilosis* (1–2 μg/ml), *C. glabrata* (1–4 μg/ml), and *C. lusitaniae* (4-16 μg/ml) were not

Table 1
Clinical data of 41 kidney transplants recipients with candidiasis at the Nephrology and Kidney Transplant Research Center, Urmia University of Medical Sciences, Urmia, Iran, during 2012–2016.

Candida clinical presentations (no.)	Samples (no.)	Sign/symptoms	*Candida* species (no.)	Fluconazole-resistant isolates (no.)
Candidemia (n = 4)	Blood (n = 4)	SIRS +: T > 38 °C, H R > 90 beats/min, R R > 20 breaths/min, WBC > 12,000 cells/mm3	*C. albicans* (n = 2), *C. famata* (n = 1), *C. glabrata* (n = 1)	*C. albicans* (n = 1)
Urinary tract candidiasis (n = 17)	Urine (n = 17)	Asymptomatic or Pyuria	*C. albicans* (n = 6), *C. famata* (n = 4), *C. kefyr* (n = 2), *C. glabrata* (n = 1), *C. lusitaniae* (n = 2), *C. parapsilosis* (n = 2)	*C. albicans* (n = 3)
Candida pneumonia (n = 3)	Transbronchial lung biopsy (n = 3)	Fever, Cough, Dyspnea, Increased pulmonary secretion, Chest CT scan: Finely nodular, Diffuse infiltrate	*C. albicans* (n = 2), *C. parapsilosis* (n = 1)	
Hepatosplenic candidiasis (n = 3)	Biopsy (n = 3)	Fever, RUQ discomfort, Nausea, Elevation of liver enzyme	*C. albicans* (n = 1), *C. tropicalis* (n = 1)	
Peritonitis (n = 8)	Peritoneal fluid (n = 8)	Fever, Nausea, Vomiting, Abdominal pain	*C. albicans* (n = 4), *C. famata* (n = 1), *C. kefyr* (n = 1), *C. tropicalis* (n = 2)	*C. albicans* (n = 1), *C. tropicalis* (n = 1)
Intra-abdominal candidiasis (n = 6)	Biopsy (n = 3)	Fever, Abdominal pain	*C. albicans* (n = 3), *C. famata* (n = 2), *C. tropicalis* (n = 1), *C. kefyr* (n = 1)	*C. albicans* (n = 1)

Abbreviation: SIRS; Systemic Inflammatory Response Syndrome, T; Temperature, H R; Heart Rate, R R; Respiratory Rate, RUQ; Right Upper Quadrant.

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

Species (no.)	Antifungal Drug	Antifungal Drug susceptibility testing (μg/ml)			
		MIC range	MIC_{50}	MIC_{90}	GM
C. albicans (n = 18)	Amphotericin B	0.5–2	2	2	1.31
	Fluconazole	0.25–64	4	8	3.71
	Itraconazole	0.125–2	0.5	1	0.36
	Voriconazole	0.032–0.5	0.125	0.5	0.16
	Caspofungin	0.032–0.25	0.032	0.125	0.04
	Posaconazole	0.008–0.125	0.016	0.125	0.02
C. famata (n = 8)	Amphotericin B	1–4	ND	ND	ND
	Fluconazole	4–32	ND	ND	ND
	Itraconazole	0.032–2	ND	ND	ND
	Voriconazole	0.016–1	ND	ND	ND
	Caspofungin	0.016–1	ND	ND	ND
	Posaconazole	0.008–0.25	ND	ND	ND
C. kefyr (n = 4)	Amphotericin B	1–4	ND	ND	ND
	Fluconazole	4–8	ND	ND	ND
	Itraconazole	0.064–1	ND	ND	ND
	Voriconazole	0.032–0.25	ND	ND	ND
	Caspofungin	0.032–0.125	ND	ND	ND
	Posaconazole	0.008–0.032	ND	ND	ND
C. tropicalis (n = 4)	Amphotericin B	0.25–2	ND	ND	ND
	Fluconazole	1–8	ND	ND	ND
	Itraconazole	0.032–0.125	ND	ND	ND
	Voriconazole	0.032–0.064	ND	ND	ND
	Caspofungin	0.032–0.064	ND	ND	ND
	Posaconazole	0.008–0.032	ND	ND	ND
C. parapsilosis (n = 3)	Amphotericin B	0.25–0.5	ND	ND	ND
	Fluconazole	1–2	ND	ND	ND
	Itraconazole	0.064–0.125	ND	ND	ND
	Voriconazole	0.032–0.064	ND	ND	ND
	Caspofungin	0.032–0.064	ND	ND	ND
	Posaconazole	0.008–0.032	ND	ND	ND
C. glabrata (n = 2)	Amphotericin B	0.25–0.5	ND	ND	ND
	Fluconazole	1–4	ND	ND	ND
	Itraconazole	0.25–0.5	ND	ND	ND
	Voriconazole	0.125–0.5	ND	ND	ND
	Caspofungin	0.064–0.125	ND	ND	ND
	Posaconazole	0.008–0.064	ND	ND	ND
C. lusitaniae (n = 2)	Amphotericin B	0.125–1	ND	ND	ND
	Fluconazole	4–16	ND	ND	ND
	Itraconazole	0.25–1	ND	ND	ND
	Voriconazole	0.125–0.5	ND	ND	ND
	Caspofungin	0.032–0.125	ND	ND	ND
	Posaconazole	0.008–0.125	ND	ND	ND

Abbreviation: MIC; Minimum Inhibitory Concentrations, GM; Geometric Mean, ND; Not Determined because less than 10 isolates.

significantly different ($P > 0.05$). Based on the findings, posaconazole and caspofungin were more active than other antifungal agents.

4. Discussion

Invasive fungal infections have been associated with significant morbidity and mortality in the organ transplant recipients. In this regard, candidiasis is the most common IFIs among the RTRs [1–3,31–33]. In line with the other studies, in the present research, *C. albicans* (43.9%) was the main species isolated from RTRs [3,8,34]. Nevertheless, NCAC have been concerned as the etiological agents of candidiasis [35–37]. In the current study, the most common NCAC species was *C. famata*, followed by *C. kefyr* and *C. tropicalis*. It seems that the alteration of *C. albicans* to non-*Candida albicans* species may be associated with the use of fluconazole for prophylactic regimen and the development of fluconazole-resistant isolates [38]. However, intrinsic resistance to fluconazole and echinocandins can be noted as another reason for the enhanced prevalence of NCAC isolates [39]. In addition, the use of medical devices and improvement of the diagnostic tools available for the identification of *Candida* species may be other factors [40,41]. Susceptibility testing may help to choose an appropriate therapy and improve the outcome of infections. Moreover, the proper identification of *Candida* species with susceptibility testing provides significant data about geographic trends in the resistance profiles of *Candida* species [42]. The data presented here suggested posaconazole and caspofungin as active drugs against *Candida* species that maybe available in the market for a short period of time. In contrast, 17% of the isolates were resistant to fluconazole. In the current study, fluconazole showed high MICs (MIC_{90} = 16 μg/ml) against *C. famata* isolates. In line with this finding, Pfaller et al. [43] and Beya et al. [44] reported high MICs for azoles and polyenes agents against *C. famata*, *C. lusitaniae* [45], and *C. kefyr* [46]. They also demonstrated that limited data are accessible on the therapy against those emerging opportunistic fungal infections, which has become a severe clinical challenge. Although fluconazole is the drug of choice for prophylaxis and treatment of patients suffering from candidiasis, the prolonged use of this agent has contributed to the development of drug resistance in *Candida* species [47]. The emergence of new species and antifungal resistance has raised the issue of using alternative therapeutic strategies [19]. Echinocandins are the recommended therapeutic options for patients with potent activity, excellent safety profile, and favorable pharmacokinetics [48,49]. On the other hand, micafungin is used for prophylaxis and treatment with a broad spectrum of activity in both neutropenic and non-neutropenic patients [13,50,51]. It seems that lower concentrations of drugs cause fewer side effects and improve the treatment outcomes. Remarkably, in vitro antifungal profiles for the non-*albicans*

Candida species are relatively scarce and based on low numbers of test strains in RTRs. One of the limitations of our study was the use of a single center retrospective design with a small sample size. However, we provided new data related to the local epidemiology in RTRs in order to carry out surveillance studies targeted toward the prevention and control of candidiasis, which would be of interest for antifungal stewardship. To add to the existing knowledge, it is required to conduct further studies regarding the epidemiology of *Candida* infections in transplant recipients to control such infections. Fungal infections are uncommon among kidney transplant recipients; however, these infections remain an important reason of morbidity and mortality in this group. The identification of *Candida* species, together with susceptibility testing, provides important data about the geographic trends of fluconazole-resistance profiles of *Candida* species. In addition, it is necessary to maintain a consistent method for the implementation of early diagnosis and determination of treatment regimen among the kidney transplant recipients.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.micpath.2018.09.026.

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