Susceptibility of *Candida* species isolated from immunocompromised patients to antifungal agents

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حساسية أنواع الـمُبْيَضَّات المستفرَدة من المرضى المنقوصي المناعة للعوامل المضادة للفطريات

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الخلاصـة: نفذت هذه الدراسة في المدة من تشرين الأول/ أكتوبر 2003 إلى آذار/ مارس 2007، وهدفت إلى استقصاء نماذج حساسية ذراري المبيضَّات المستفرَدة من 410 مرضى مَنْقُوصي المناعة بالعوامل المضادة للفطريات، وذلك في شيراز، جمهورية إيران الإسلامية. وقد تمَّ فحص المرضى بحثاً عن داء المبيضَّات الجهازي، ثم زُرعت العينات السريرية المأخوذة من مرضى الدراسة الذين ظهرت عليهم علامات سريرية للعدوى، للتعرف على درجة الاستعمار بالفطريات ولدراسة هذه المستعمرات. وقد درس الباحثون نماذج تمثُّل الكربوهيدرات في جميع المستفرَدات البالغ عددها 354 مستفرَدة. وعيَّن الباحثون حساسية المستفردات للعوامل المضادة للفطريات باستخدام الطريقة المرجعية للتخفيف المكروبي للمرق. ووجد الباحثون أن المبيضَّة البيضاء هي النوع الأكثر استفراداً. كما وجدوا أن الفاريكونازول كان ذا مفعول قويّ تجاه جميع المستفردات. كما وجدوا كذلك مقاومة كبيرة للإيتـراكونازول في جميع أنواع المبيضات. ويوصي الباحثون بقوَّة، بإجراء استقصاءات منتظمة للمقاومة لمضادات الفطريات في المراكز الطبية، لأن أمثال هذه الاستقصاءات تكفل معالجةً أكثر كفاءةً ونجاعةً لداء المبيضات الغزوي لدى المرضى المنقوصي المناعة.

ABSTRACT This study was carried out from October 2003 to March 2007 to investigate susceptibility patterns to antifungals of *Candida* strains isolated from 410 immunocompromised patients in Shiraz, Islamic Republic of Iran. Patients were checked for systemic candidiasis. Fungal colonization was determined and clinical samples collected from those patients with clinical signs of infections were examined. The carbohydrate assimilation patterns of all 354 isolates were studied. Susceptibility of the isolates to antifungal agents was determined using the reference broth microdilution method. *Candida albicans* was the species most often isolated. Voriconazole was highly active against all the isolates. Major resistance to itraconazole was observed in all *Candida* spp. Regular investigations into antifungal resistance in medical centres is highly recommended as this will result in more efficient management of invasive candidiasis in immunocompromised patients.

Sensibilité aux antifongiques des espèces de Candida isolées chez des patients immunodéprimés

RÉSUMÉ La présente étude, réalisée entre octobre 2003 et mars 2007, a recherché les évolutions de la sensibilité aux antifongiques des souches de Candida isolées chez 410 patients immunodéprimés dans la ville de Chiraz (République islamique d'Iran). Tous les patients ont été examinés à la recherche d'une candidose systémique. Une colonisation fongique a été constatée et les échantillons cliniques prélevés chez les patients présentant des signes d'infection ont été analysés. Le profil d'assimilation des hydrates de carbone de chacun des 354 isolats a été étudié. La sensibilité des isolats aux antifongiques a été déterminée aux moyens de la méthode de référence de microdilution en milieu liquide. Candida albicans s'est révélé être l'espèce la plus souvent isolée. Le voriconazole était hautement actif contre tous les isolats. Une résistance importante de toutes les espèces de Candida à l'itraconazole a été observée. Des analyses régulières de la résistance aux antifongiques dans les centres médicaux sont fortement recommandées, car les résultats permettront une prise en charge plus efficace de la candidose systémique chez les patients immunodéprimés.

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Introduction

There has been an increase in serious fungal infections in recent years, and there is a need for new antifungal agents. Unlike the development of antibacterial agents, relatively few drug targets in fungi have been used in the development of currently available antifungal agents [1]. Resistance among *Candida* spp. to antifungal drugs is an increasing problem in immunocompromised patients [2].

Pathogenic fungi represent a serious threat to the lives and health of immunocompromised patients. Until recently, treatment of fungal infections was started with drugs, including azoles and polyenes, which induce severe side-effects in the hosts. Only recently has antifungal drug susceptibility testing become an important issue. There are 2 reasons for testing susceptibility to antifungals: therapeutic failure for amphotericin B has been reported for several *Candida* spp. and fluconazole-resistant fungi have also been reported. There is the potential for selection of non-albicans *Candida* spp. of yeasts, which may have implications for treatment since many of the non-*C. albicans* yeasts, such as *C. glabrata* and *C. krusei*, are inherently less susceptible than *C. albicans* to fluconazole [3].

The aim of the present study was to examine geographic trends in the activities of antifungal agents against *Candida* spp. isolated from immunocompromised patients admitted in a large teaching hospital, and by doing so to promote their effective management. The hospital is the only centre for solid organ transplant and chemotherapy in the south of the Islamic Republic of Iran.

Methods

From October 2003 to March 2007, 410 immunocompromised patients (from transplant and oncology units) were followed up for invasive fungal infections in Nemazi Hospital, a large teaching hospital in Shiraz University of Medical Sciences, Shiraz, southern Islamic Republic of Iran. We checked for fungal colonization of patients, and in those who had clinical signs of infection, clinical samples (blood, urine, oesophageal, oropharyngeal, vagina, biopsy and broncho-alveolar lavage) were examined for fungal infection using routine methods. The *Candida* species isolated were stored at –70 °C in 10% glycerol for varying periods of time.

All *Candida* spp. isolated (354 isolates) were cultured on potato dextrose agar (OXOID Ltd, United Kingdom) twice, for 24 h and 48 h, at 35 °C to ensure viability and purity. The carbohydrate assimilation patterns of all the isolates were studied using the API 32C system, according to the manufacturer's procedure (BioMérieux, France). *C. dubliniensis* was distinguished from *C. albicans* by molecular assay [4].

Susceptibility patterns of the isolates to fluconazole, amphotericin B, ketoconazole, nystatin (SIGMA-Aldrichemie GmbH-Steinheim, Germany), itraconazole (Jenssen Pharmaceutical, Belgium), and voriconazole (Pfizer, United Kingdom) were determined by a broth microdilution assay according to the Clinical and Laboratory Standard Institute (CLSI) [5]. Stock solutions of drugs were prepared in dimethyl sulfoxide or water.

Two CLSI quality control strains, *C. parapsilosis* ATCC 22019 and *C. krusei* ATCC 6258, were tested each time a set of clinical isolates was evaluated [6]. The yeast suspension and antifungal dilution were prepared according to CLSI M27-A2 guidelines [5]. Final concentrations of amphotericin B ranged from 8 to 0.016 μg/mL, fluconazole from 128 to 0.250 μg/mL, nystatin from 37 to 0.07 μg/mL and itraconazole, ketoconazole and voriconazole from 16 to 0.032 μg/mL. In each series, 1 positive control with no drugs and 1 negative control with no fungal suspensions were included. The plates were sealed and incubated at 35 °C. After 24 h and 48 h visual minimum inhibitory concentration (MIC) end-points were determined with the aid of a mirror. Visual end-points were determined as described in the CLSI guidelines [5]; these recommended end-points for azoles are the lowest drug concentration with a prominent decrease in turbidity (inhibitory concentration that gives 50% growth reduction), while for amphotericin B and nystatin, the MIC was the drug concentration showing complete inhibition of growth.

The amount of growth in each tube was compared to that of the growth in a positive control. Antifungal activity was expressed as the MIC of each isolate to the drug. The resistance cut-off points used were according to CLSI guidelines [5] or based on previous investigations [7,8]. MIC50 (the MIC at which 50% of the isolates are inhibited) and MIC90 (the MIC at which 90% of the isolates are inhibited) were also calculated. Data were entered into *SPSS*, version 11.5, and were subsequently analysed using descriptive statistics and cross tabulation.

The ethics committee of the Clinical Microbiology Research Centre at Shiraz University of Medical Sciences reviewed and approved the study regarding the patients, who gave written informed consent before participating in the study.

Results

Of the 354 *Candida* species isolated, the most abundant were *C. albicans* (48.6%) followed by *C. krusei* (17.5%), *C. glabrata* (11.3%), *C. kefyr* (11.3%), *C. parapsilosis* (5.1%), *C. tropicalis* (1.7%) and *C. dubliniensis* (1.7%) and other *Candida* species (2.8%).

There was no significant difference between reading plates at 24 h and 48 h of incubation (24 h and 48 h MICs limits range for isolates were up to 94%

Table 1 Resistance rates of *Candida* spp. to antifungal agents

Species	Total No.	AMB		FLU		ITRA		KETO		VORI		NYS	
		No	%	No.	%	No.	%	No.	%	No.	%	No.	%
C. albicans	172	12	7.0	16	9.3	26	15.1	10	5.8	4	2.3	2	1.2
C. krusei	62	6	10.0	59	95.2	53	85.5	1	1.6	2	3.2	1	1.6
C. glabrata	40	6	15.0	38	95.0	34	85.0	0	–	0	–	0	–
C. kefyr	40	4	10.0	2	5.0	11	27.5	0	–	0	–	0	–
C. parapsilosis	18	4	22.3	5	27.7	10	55.5	0	–	0	–	2	11.1
Other[a]	10	0	–	4	40.0	2	20.0	0	–	0	–	0	–
C. tropicalis	6	2	33.3	2	33.3	0	–	0	–	0	–	0	–
C. dubliniensis	6	0	–	0	–	0	–	0	–	0	–	0	–
Total	354	34	9.6	126	35.5	136	38.4	11	3.1	6	1.7	5	1.4

[a] *2 cases each of C. famata, C. lusitanae, C. zeylanides and 4 cases of C. apicolata.*
Resistance was defined as: amphotericin B (AMB) > 1 µg/mL; fluconazole (FLU) ≥ 64 µg/mL; itraconazole (ITRA) ≥ 1 µg/mL; ketoconazole (KETO) ≥ 4 µg/mL; voriconazole (VORI) ≥ 8 µg/mL; nystatin (NYS) ≥ 16 µg/mL [5,7,8].

the same); we therefore report findings on plates incubated for 48 h. Table 1 summarizes the resistance to antifungal agents: overall, nystatin was the most effective against all *Candida* species (lowest resistance) but use of this agent is limited to mucosal infections.

Voriconazole was also highly active against all the isolates (Table 2). All *Candida* species except 2.3% of *C. albicans* and 3.2% of *C. krusei* were susceptible to voriconazole. *C. parapsilosis* was the most sensitive species to voriconazole (MIC90: 0.032 µg/mL and MIC50: 0.032 µg/mL) and *C. krusei* the least sensitive (MIC90: 2 µg/mL and MIC50: 0.250 µg/mL). Susceptibilities for all isolates are given in Table 2.

Among the 354 isolates studied, a total of 9 strains (3 *C. albicans*, 2 *C. glabrata*, 1 *C. kefyr*, and 3 *C. krusei*) were resistant to both itraconazole and amphotericin B. Six strains (4 *C. albicans*, 1 *C. glabrata*, 1 *C. krusei*) were resistant to fluconazole and itraconazole, 4 strains (3 *C. albicans*, 1 *C. krusei*) to itraconazole and ketoconazole, and 6 strains (2 *C. albicans*, 4 *C. krusei*) to itraconazole and voriconazole. One *C. albicans* isolate from the abdominal fluid of a male who had had a liver transplant was resistant to amphotericin B, fluconazole, ketoconazole, and itraconazole. He died 2 months after transplantation. In 26 strains highly resistant to fluconazole [MIC ≥ 64 µg/mL], voriconazole MICs were 1–4 µg/mL. In 15 strains highly resistant to itraconazole [MIC ≥ 1 µg/mL], voriconazole MICs were also consistently higher, ranging from 1 µg/mL to 16 µg/mL.

Discussion

Nearly half the cases of haematogeneous candidiasis are now reported to be attributed to *Candida* spp. other than *C. albicans* [9]. In Switzerland the distribution of *Candida* spp. remained stable between 1991 and 2000, with *C. albicans, C. glabrata* and *C. krusei* accounting for 66%, 14% and 2% of all cases of candidaemia respectively [10]. These data indicate that non-albicans *Candida* spp. and their susceptibility patterns must be considered when treating immunocompromised patients.

We found that voriconazole was more active than fluconazole and itraconazole against all the *Candida* isolates tested. These results lend support to findings reported previously [11]. Fluconazole has a broad therapeutic range with low toxicity. Prolonged or repeated exposure to low-dose fluconazole was associated with increased frequency of fluconazole resistance in *C. albicans* isolates [12]. In our study, 64.5% of total *Candida* species were sensitive to fluconazole, and dose-dependent susceptibility was seen in 9 isolates.

Long-term itraconazole prophylaxis is associated with reduction in susceptibility. Resistance to fluconazole and itraconazole were mostly noted in *C. krusei* and *C. glabrata* isolates; in one study 72% of *C. glabrata* isolates from patients with advanced cancer were resistant to both fluconazole and itraconazole [13]. In our study, major resistance to itraconazole was observed in 85.5% of isolates of *C. krusei* and 85.0% of *C. glabrata*, and 102 strains showed dose–dependent susceptibility to itraconazole MICs of 0.25 and 0.5 µg/mL. Swinn et al. reported 55% and 65% of the non-albicans yeast isolates were susceptible to itraconazole and fluconazole respectively [14].

We found species were either resist to 2 antifungal agents or had high MIC for some antifungal agents. Consistent with some previous reports, isolates with elevated MICs for 1 azole were generally less susceptible to all the azoles. Cross-tabulation of voriconazole, fluconazole, and itraconazole MICs indicated that voriconazole MICs rose with fluconazole and itraconazole MICs [14,15].

In recent years, *C. glabrata* has become a significant cause of fungal infections [16]. This yeast has low intrinsic susceptibility to azole derivatives and it is able to acquire resistance during

Table 2 Antifungal susceptibilities of clinical yeast isolates (*Candida* species) (determined by the National Committee for Clinical Laboratory Standards microdilution reference broth method [5])

Organism[a]/antifungal agent	Minimum inhibitory concentration (mg/mL)		
	Range	50%	90%
C. albicans (172)			
Amphotericin B	0.032–8.0	0.064	1.000
Fluconazole	0.250–32.0	0.250	4.000
Itraconazole	0.032–16.0	0.250	0.500
Voriconazole	0.032–16.0	0.032	0.250
Ketoconazole	0.032–8.0	0.032	0.032
Nystatin	0.14–18.5	2.300	4.600
C. krusei (62)			
Amphotericin B	0.032–8.0	0.250	1.000
Fluconazole	1.0–64.0	64.000	64.000
Itraconazole	0.250–16.0	0.500	2.000
Voriconazole	0.032–32.0	0.250	2.000
Ketoconazole	0.032–8.0	0.250	4.000
Nystatin	0.290–18.5	2.300	4.600
C. glabrata (40)			
Amphotericin B	0.032–8.0	0.250	1.000
Fluconazole	0.125–64.0	32.000	64.000
Itraconazole	0.125–8.0	0.500	1.000
Voriconazole	0.032–2.0	0.125	0.250
Ketoconazole	0.032–4.0	0.125	0.500
Nystatin	0.580–9.25	2.300	4.600
C. kefyr (40)			
Amphotericin B	0.032–8.0	0.500	1.000
Fluconazole	0.125–16.0	0.250	8.000
Itraconazole	0.250–8.0	0.500	0.500
Voriconazole	0.032–1.0	0.032	0.064
Ketoconazole	0.032–1.0	0.032	1.000
Nystatin	0.580–9.25	2.300	2.300
C. tropicalis (6)			
Amphotericin B	0.250–2.0	0.500	0.500
Fluconazole	0.250–0.500	0.500	0.500
Itraconazole	0.250–0.500	0.250	0.500
Voriconazole	0.032–0.250	0.064	0.250
Ketoconazole	0.032–0.250	0.064	0.250
Nystatin	1.15–2.3	2.300	2.300
C. parapsilosis (18)			
Amphotericin B	0.032–16.0	0.250	0.500
Fluconazole	0.125–1.00	0.500	1.000
Itraconazole	0.250–4.0	0.500	0.500
Voriconazole	0.032–0.250	0.032	0.032
Ketoconazole	0.032–0.064	0.032	0.064
Nystatin	1.15–18.5	2.500	9.250
C. dubliniensis (6)			
Amphotericin B	0.125–0.250	0.125	0.250
Fluconazole	0.0125–0.5	0.125	0.500
Itraconazole	0.250–0.500	0.250	0.250
Voriconazole	0.032–0.250	0.064	0.250
Ketoconazole	0.032–0.064	0.032	0.064
Nystatin	2.3–4.6	2.300	4.600

[a]*Candida species with less than 6 isolates are not included in the table.*

treatment. Resistance to itraconazole was mostly noted in *C. glabrata* and *C. krusei* isolates. In a study by Seifert et al., all *C. glabrata* were resistant to itraconazole [17]. All the isolates except 5.8% of *C. albicans*, and 1.6% of *C. krusei* were susceptible to ketoconazole. Because systemic ketoconazole has a greater propensity to inhibit mammalian cytochrome P450, it has fallen out of clinical use. We found no serious problem with regard to the susceptibility patterns of *C. tropicalis*, *C. dubliniensis*, and other *Candida* spp.

Our study covered severely immunocompromised patients, hospitalized mainly in the oncology and transplantation units. The prevalence of invasive fungal infection ranges from 5% to 50% in kidney and liver transplants [18]. *Candida* spp. and *Aspergillus* spp. are responsible for more than 80% of all fungal infections in solid organ transplant recipients and febrile neutropenic patients [18,19], in whom these infections are the major causes of morbidity and mortality [20].

It is common practice to administer triazoles as prophylaxis for fungal infections in our hospitals, and this may explain the high rates of resistance of the clinical *Candida* strains (all species) to itraconazole (38.4% resistant), fluconazole (35.5%) and amphotericin B (9.6%). For many years, amphotericin B deoxycholate has been a standard therapy for invasive fungal infections [21]. Unfortunately, a side-effect of this agent is nephrotoxicity, and it is often poorly tolerated.

As routinely practised, in empiric therapy antibiotics are given most often to patients before the specific microorganism causing an infection is identified. Empiric antibiotics are typically broad-spectrum, that is, they are effective against a wide variety of possible microorganisms. As shown in our study, itraconazole and fluconazole, with resistance rates of 38.4% and 35.5%, cannot be good choices for empirical therapy in our region, although they are easily available and cost-effective. The increased loads of intrinsically resistant strains to these 2 antifungal agents may account for the high resistance rates. It seems that voriconazole, although very costly in our region, could serve as a more effective agent against systemic candidiasis in immunocompromised patients.

Conclusions

As demonstrated, itraconazole and fluconazole resistance are growing concerns in our region; thus, performing regular investigations into antifungal resistance in medical centres for more efficient management of invasive candidiasis in immunocompromised patients is highly warranted. In doing so, determining the sensitivity profile of community strains is possible only through periodic surveillance studies. A more cost-effective and efficient therapy also needs to be considered.

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Combat drug resistance: No action today, no cure tomorrow

Antimicrobial resistance is not a new problem but one that is becoming more dangerous. Many countries are taking action, but urgent and consolidated efforts are needed to avoid regressing to the pre-antibiotic era.

On World Health Day 2011 on 7 April, the World Health Organization (WHO) issued a call for action to halt the spread of antimicrobial resistance by introducing a six-point policy package for all countries to combat antimicrobial resistance.

WHO is calling on everyone to think, act and take responsibility for combating drug resistance including: policy-makers and planners, the public and patients, practitioners and prescribers, pharmacists and dispensers, the pharmaceutical industry.

Although governments need to take the lead and develop national policies to combat drug resistance, health professionals, civil society and other groups can also make important contributions. For example, doctors and pharmacists can prescribe and dispense only the drugs that are required to treat a patient, rather than automatically giving either the newest or best-known medicines. Patients can stop demanding that doctors give them antibiotics when they may not be appropriate. Health professionals can help rapidly reduce the spread of infection in health care facilities.

Further information about WHO's six-point policy package to combat drug resistance can be found at: http://www.who.int/world-health-day/2011/en/index.html