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ORIGINAL ARTICLE / ARTICLE ORIGINAL

In vitro antifungal susceptibility of clinical species belonging to *Aspergillus* genus and *Rhizopus oryzae*



Sensibilité in vitro des antifongiques d'isolats cliniques du genre Aspergillus et de Rhizopus oryzae

R. Kachuei^a, S. Khodavaisy^{b,*}, S. Rezaie^c, S. Sharifynia^c

^a Molecular Biology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran

^b Applied Microbiology Research Center, Baqiyatallah University of Medical Sciences, Tehran, Iran

^c Division of Molecular Biology, Department of Medical Mycology and Parasitology, School of Public Health, Tehran University of Medical Sciences, Tehran, Iran

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KEYWORDS

Antifungal susceptibility;
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Aspergillus spp.;
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Summary

Objective. — Among filamentous fungal pathogens, *Aspergillus* spp. and zygomycetes account for highest rates of morbidity and mortality among immunocompromised patients. Recently developed antifungal drugs offer the potential to improve management and therapeutic outcomes of fungal infections. The aim of this study was to analyse the in vitro activities of voriconazole, itraconazole, amphotericin B and caspofungin against clinical isolates of *Aspergillus* spp. and *Rhizopus oryzae*.

Material and methods. — The in vitro antifungal susceptibility of 54 isolates belonging to different clinical isolates of *Aspergillus* spp. and *R. oryzae* was tested for four antifungal agents using a microdilution reference method (CLSI, M38-A2). All isolates were identified by typical colony and microscopic characteristics, and also characterized by molecular methods.

Results. — Caspofungin (MEC range: 0.008–0.25 and MEC50: 0.0023 µg/mL) was the most active drug in vitro against *Aspergillus* spp., followed by voriconazole (MIC range: 0.031–8 and MIC50: 0.5 µg/mL), itraconazole (MIC range: 0.031–16 and MIC50: 0.25 µg/mL), and amphotericin B (MIC range: 0.125–4 and MIC50: 0.5 µg/mL), in order of decreasing activity. The caspofungin, voriconazole, and itraconazole demonstrated poor in vitro activity against *R. oryzae* isolates evaluated, followed by amphotericin B.

* Corresponding author.

E-mail addresses: kachueir@gmail.com (R. Kachuei), sadegh_7392008@yahoo.com (S. Khodavaisy), srezaie@Sina.tums.ac.ir (S. Rezaie), sharifynias@gmail.com (S. Sharifynia).

MOTS CLÉS

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Conclusion. — This study demonstrates that caspofungin had good antifungal activity and azole agents had better activity than amphotericin B against *Aspergillus* species. Although, azole drugs are considered ineffective against *R. oryzae*. This result is just from a small scale in vitro susceptibility study and we did not take other factors into consideration.

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Résumé

Objectif. — Parmi les agents pathogènes fongiques filamenteux, *Aspergillus* spp. et zygomycètes représentent les plus hauts taux de morbidité et de mortalité chez les patients immunodéprimés. Les antifongiques récemment développés offrent la possibilité d'améliorer la gestion et les résultats thérapeutiques des infections fongiques. Le but de cette étude était d'analyser les activités in vitro du voriconazole, de l'itraconazole, l'amphotéricine B et de la caspofungine contre des isolats cliniques d'*Aspergillus* spp. et *Rhizopus oryzae*.

Matériel et méthodes. — La sensibilité antifongique in vitro de 54 isolats appartenant à différents isolats cliniques d'*Aspergillus* spp. et *R. oryzae* a été testée par quatre agents antifongiques en utilisant une méthode de référence de microdilution (CLSI, M38-A2). Tous les isolats ont été identifiés par l'aspect des colonies typique et les caractéristiques microscopiques, et également caractérisés par des méthodes moléculaires.

Résultats. — La caspofungine (MEC gamme : de 0,008 à 0,25 et MEC50 : 0,0023 µg/mL) était la molécule la plus active in vitro contre *Aspergillus* spp, suivie par le voriconazole (gamme MIC : 0,031 à 8 et CIM50 : 0,5 µg/mL). L'itraconazole (gamme MIC : 0,031 à 16 et CIM50 : 0,25 µg/mL), et l'amphotéricine B (plage MIC : 0,125 à 4 et CIM50 : 0,5 µg/mL), par ordre décroissant de l'activité. La caspofungine, le voriconazole et l'itraconazole ont démontré une faible activité in vitro contre des isolats de *R. oryzae*, suivie par l'amphotéricine B.

Conclusion. — Cette étude démontre que la caspofungine a eu une bonne activité antifongique et que les agents azolés ont une meilleure activité que l'amphotéricine B contre des espèces d'*Aspergillus*. Toutefois, les médicaments azolés sont considérés comme inefficaces contre *R. oryzae*. Ce résultat est issu d'une étude limitée de la sensibilité in vitro et nous n'avons pas pris d'autres facteurs en considération.

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Introduction

The incidence of fungal infections in immunocompromised populations has increased greatly over the past decades [5,11]. Among filamentous fungal pathogens, *Aspergillus* spp. and zygomycetes account for highest rates of morbidity and mortality among immunocompromised patients especially cancer, organ transplant, burn and surgical patients [10]. Recently developed antifungal drugs offer the potential to improve management and therapeutic outcomes of fungal infections [16]. Despite the availability of potent antifungal agents, systemic fungal infections continue to cause significant morbidity and mortality [1]. Itraconazole, voriconazole and posaconazole have been approved, particularly for the treatment of infections caused by *Aspergillus* and other filamentous fungi [17,22]. Due to the life threatening nature of these infections and reports of drug resistance, susceptibility testing of fungal pathogens has become very important [8]. The *in vitro* anti-fungal susceptibility testing of different pathogenic fungi is helpful in defining the activity spectrum of an antifungal and it can be a valuable guide for the clinician for treating infecting agents [24]. However, data about the *in vitro* antifungals against zygomycetes or other moulds are scarce in Iran. Nonetheless, there is little information regarding its susceptibility patterns against

currently available antifungal agents. The aim of this study was to analyse the *in vitro* activities of amphotericin B, itraconazole, voriconazole, and caspofungin against clinical isolates of *Aspergillus* spp. and *Rhizopus oryzae*.

Materials and methods

Fungal strains and culture conditions

During September 2010 until October 2014, 54 clinical isolates belonging to the *Aspergillus* genus and *R. oryzae* were collected from patients hospitalized at the teaching hospital in North of Tehran (the isolates obtained from samples are routinely sent to the hospital laboratory for diagnosis and present study was carried out on samples in coordination with the hospital, and according to the hospital it was not required to plan the hospital ethics committee). The isolates were sub-cultured onto potato dextrose agar and incubated at 48 h at 37 °C before testing. All isolates were identified by typical colony and microscopic characteristics, also characterized and determined by molecular methods. Two reference strains, *Candida parapsilosis* (ATCC 22019) and *Aspergillus flavus* (ATCC 204304) were included in all tests as references for quality controls and were tested by both methods in each series of assays.

Molecular characterization

The genomic DNA from the isolates was prepared as described previously [13] and used as template for PCR amplification. The species-specific identification of *Aspergillus* isolates and *R. oryzae* were studied by partial sequencing of β -tubulin gene fragments [2] and ITS1-5.8S-ITS2 region [13], respectively. The variable region of β -tubulin gene was amplified by using Bt2a (5'-GGTAACCAAATCGGTGCTGCTTTC-3') and Bt2b (5'-ACCCTCAGTGTAGTGACCTTGGC-3') primers. Also used universal primers for ITS regions (ITS1 primer: 5'-TCCGTAGGTGAACCTGCGG-3', ITS4 primer: 5'-TCCTCCGCTTATGATTATGC-3') for the amplification of *R. oryzae* and the amplicons were sequenced as described previously [13]. The quality of the sequences was examined by Phred/Phrap/Consed package. For the identification of the isolates, the nucleotide sequences obtained were compared with those already deposited in the data bank of the National Center for Biotechnology and Information (NCBI), using BLAST search tool (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>). The identification of the species was determined based on the best score. Multiple sequence alignments were performed with ClustalX version 2.0.

Antifungal susceptibility testing

In this study, minimum inhibitory concentration (MICs) were determined according to recommendations stated in the Clinical and Laboratory Standards Institute (CLSI) M38-A2 document [21]. Amphotericin B (Bristol-Myers-Squib, Woerden, The Netherlands), itraconazole (Janssen Research Foundation, Beerse, Belgium), voriconazole (Pfizer New York, NY, USA), and caspofungin (Bristol-Myers-Squib, Woerden, The Netherlands) were obtained as reagent-grade powders from the respective manufacturers for preparation of the CLSI microdilution trays. The antifungal agents were diluted in the standard RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) buffered to pH 7.0 with 0.165 M-morpholinepropanesulfonic acid (MOPS) (Sigma-Aldrich) with L-glutamine without bicarbonate to yield two times their concentrations and dispensed into 96-well microdilution trays at a final concentration of 0.016–16 $\mu\text{g/mL}$ for amphotericin B, itraconazole, and voriconazole; 0.08–8 $\mu\text{g/mL}$ for caspofungin. Plates were stored at -70°C until they were used. Briefly, all isolates were grown on potato dextrose agar plates at 35°C for up to 7 days to induce sporulation (good sporulation may be obtained after 48 hours of incubation), with the inoculum suspensions being prepared under appropriate biosafety methods by slightly scraping the surface of mature colonies with a loop and the resulting material suspended in sterile saline solution with Tween 20. If large aggregates existed, they were allowed to settle for several minutes, and then the homogeneous conidial suspensions were transferred to sterile tubes. The supernatants were adjusted spectrophotometrically at 530 nm wavelengths to an optical density (OD) that ranged from 0.09–0.13 for *Aspergillus* spp. and from 0.15–0.17 for zygomycet species. The final size of the stock inoculum suspensions of the isolates tested ranged from 0.4×10^4 – 3.1×10^4 colony forming units (CFU)/mL as determined by quantitative colony counts on Sabouraud glucose agar (SGA, Difco). The inoculum suspensions, including mostly non-germinated

conidia, were diluted 1:50 in RPMI 1640 medium. All microdilution plates were incubated at 35°C after 21 to 26 hours for *Rhizopus* spp. and 46–50 hours for *Aspergillus* spp. to determine MICs. The MIC endpoints for amphotericin B, itraconazole, and voriconazole were determined with the aid of a reading mirror as the lowest concentration of drug that inhibited recognizable growth (100% inhibition). Also for *Rhizopus* spp. and *Aspergillus* spp., minimum effective concentration (MEC) endpoints for caspofungin were defined microscopically as the minimal antifungal concentration that caused visible morphological alterations of the hyphae. Tests were performed in duplicate, and when the results did not concur, the test was repeated and the mode of the MICs and MECs was considered. The MIC/MECs data obtained for clinical filamentous fungi were checked for significance at $\alpha = 5\%$ using Chi-squared (χ^2) test.

Results

In total, 54 filamentous fungi recovered and identification from clinical samples comprising 23 paranasal sinuses biopsy, 16 cutaneous infections, 14 respiratory secretion (include sputum, endotracheal secretion and bronchoalveolar lavage), and 1 ear swabs. Clinical isolates comprising 38 *A. flavus*, 8 *R. oryzae*, 5 *Aspergillus niger*, 1 *Aspergillus fumigatus*, 1 *Aspergillus terreus*, 1 *Aspergillus nidulans* were used for *in vitro* antifungal susceptibility test. The majority of *Aspergillus* isolates were obtained from respiratory tract specimens, followed by nasal biopsy, nail and ear samples. The most frequently species recovered from respiratory tract specimens was *A. flavus* (70.4%) and *R. oryzae* (14.8%) was significantly associated with paranasal sinuses biopsy ($P < 0.01$). The results of antifungal susceptibility of clinical isolates are presented in Table 1. In general, all tested antifungal agents showed good *in vitro* activity against *Aspergillus* spp. The MIC range and MIC/MEC₅₀ values for *Aspergillus* spp. were as follow: amphotericin B: 0.125–4 and 0.5 $\mu\text{g/mL}$; voriconazole: 0.031–8 and 0.5 $\mu\text{g/mL}$; itraconazole: 0.031–16 and 0.25 $\mu\text{g/mL}$; and caspofungin: 0.008–0.25 and 0.0023 $\mu\text{g/mL}$, respectively. Of the 46 *Aspergillus* spp. tested: 100% ($n = 46$), 91.3% ($n = 42$), 86.9% ($n = 40$), and 82.6% ($n = 38$), isolates were inhibited by caspofungin, voriconazole, itraconazole, and amphotericin B at MIC of $\leq 1 \mu\text{g/mL}$, respectively. *A. niger* appeared to be the specie most resistance to voriconazole (MIC₅₀: 2 $\mu\text{g/mL}$) and itraconazole (MIC₅₀: 4 $\mu\text{g/mL}$). *R. oryzae* isolates were significantly less susceptible to caspofungin, voriconazole, itraconazole, and amphotericin B than *Aspergillus* spp. ($P < 0.01$). The MIC/MEC₅₀ values and MIC range of *R. oryzae* isolates for antifungal agents were as follows: amphotericin B: 3 $\mu\text{g/mL}$ (range: 2–4 $\mu\text{g/mL}$); voriconazole: 16 $\mu\text{g/mL}$ (range: 2–16 $\mu\text{g/mL}$); itraconazole: 16 $\mu\text{g/mL}$ (range: 2–16 $\mu\text{g/mL}$); and caspofungin: 16 $\mu\text{g/mL}$ (range: 16–>16 $\mu\text{g/mL}$), respectively.

Discussion

Due to an increase in the numbers of patients at risk for fungal infections, such as transplant recipients and cancer patients who received chemotherapy, the incidence of systemic mycoses has increased [11,10]. Nowadays, drug

Table 1 *In vitro* activity of each antifungal drug against clinical isolates analyzed by source of isolation.
 Activité in vitro de chaque médicament antifongique contre les isolats cliniques.

Species	Antifungal agents	Ranges	MIC/MEC ₅₀	MIC/MEC ₉₀	GM
<i>Aspergillus flavus</i> (38)	Amphotericin B	0.125–4	0.5	1	0.432
	Itraconazole	0.063–2	0.25	0.5	0.259
	Voriconazole	0.031–1	0.5	1	0.435
	Caspofungin	0.008–0.25	0.031	0.125	0.0315
<i>Aspergillus niger</i> (5)	Amphotericin B	0.125–0.5	0.25	ND	0.287
	Itraconazole	0.5–16	4	ND	4
	Voriconazole	0.5–8	2	ND	1.741
	Caspofungin	0.008–0.063	0.008	ND	0.0182
Other <i>Aspergillus</i> spp. (3)	Amphotericin B	0.5–1	0.5	ND	0.629
	Itraconazole	0.031–16	0.5	ND	0.628
	Voriconazole	0.062–8	1	ND	0.79
	Caspofungin	0.008–0.063	0.016	ND	0.020
<i>Rhizopus oryzae</i> (8)	Amphotericin B	2–4	3	ND	2.828
	Itraconazole	4– >16	16	ND	10.767
	Voriconazole	2–16	16	ND	8.724
	Caspofungin	16– >16	16	ND	16

GM: geometric mean; MIC₅₀: concentration at which 50% of the isolates were inhibited; MEC₅₀: concentration at which 50% of the isolates showed morphological changes in the growing hyphae; ND: not determined.

resistance of fungal pathogens has become very important, but susceptibility tests for effective antifungal agents are not routinely performed [18]. The use of *in vitro* antifungal susceptibility testing can be a valuable guide for the clinician to appropriately treat their patients [3]. As shown in this study, caspofungin was very good *in vitro* activity against *Aspergillus* spp., and inhibited the growth of 100% of isolates at MEC \leq 1 μ g/mL, followed by voriconazole (91.3%), itraconazole (86.9%), and amphotericin B (82.6%). However, 17.4%, 6.5%, and 4.3% isolates showed MICs of >2 μ g/mL for amphotericin B and >4 μ g/mL for itraconazole and voriconazole, respectively. Also in Pfaller et al.'s study the caspofungin (MEC range: 0.031–8 and GM: 0.5 μ g/mL) showed the lowest MECs for all *Aspergillus* species tested [20]. Parisa et al. in a study about determined antifungal susceptibility of 108 *Aspergillus* species isolated reported that voriconazole (MIC₉₀ \leq 0.250 μ g/mL) was highly active and inhibited the growth of 100% of against of *Aspergillus* spp. [18]. Pfaller et al. reported that voriconazole inhibited 98% of isolates at MIC \leq 1 μ g/mL, followed by amphotericin B (89%), and itraconazole (72%) [20]. The antifungal activity of voriconazole was superior to that of itraconazole, in agreement with a report by Mallie et al. [14]. Among the most prevalent *Aspergillus* species in our study, *A. flavus* exhibited the highest resistance to amphotericin B and *A. niger* showed the highest resistance to itraconazole and voriconazole. Also in Parisa et al.'s study, 36 of the 66 *A. flavus* were resistant to amphotericin B [18]. Goncalves et al. reported that amphotericin B appears to have limited activity against most species of the *Aspergillus* section *Flavi* [9]. In agreement with other studies, our study demonstrates that *Rhizopus* species are the most common causes of mucormycosis [19,23]. In our study, caspofungin, voriconazole, and itraconazole appear to have limited activity against *R. oryzae* isolates. High voriconazole and itraconazole MICs of ≥ 2 mg/L were found for *R. oryzae* isolates, and the overall MIC₅₀ was

16 mg/L. These results are consistent with previous studies [4,7,12,15]. Dannaoui et al.'s study showed that azole drugs are considered ineffective against zygomycetes and amphotericin B MICs ranging from 0.06–1 mg/L have been reported for *Rhizopus* spp. [4]. In agreement with our study, Diekema et al. reported that caspofungin is not generally considered to be active against *Rhizopus* spp. [6]. The zygomycetes have been demonstrated to be a heterogeneous group with regard to *in vitro* antifungal susceptibility [4,6], and more data are needed in order to determine which new or investigational agents are active against individual species.

Conclusions

In summary, *in vitro* antifungal susceptibility in this study showed caspofungin had excellent *in vitro* antifungal activity and azole agents had better activity than amphotericin B against of *Aspergillus* species. This result is just from a small scale *in vitro* susceptibility study and we did not take other factors into consideration. Clinical effectiveness in the treatment of infection remains to be determined for these promising drugs.

Disclosure of interest

The authors declare that they have no competing interest.

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