In Vitro Antifungal Susceptibility of Clinically Relevant Species Belonging to *Aspergillus* Section *Flavi*

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The *in vitro* antifungal susceptibility of 77 isolates belonging to different clinically relevant species of *Aspergillus* section *Flavi*, including those of different phylogenetic clades of *A. flavus*, was tested for nine antifungal agents using a microdilution reference method (CLSI, M38-A2). Terbinafine and the echinocandins demonstrated lower MICs/MECs for all species evaluated, followed by posaconazole. Amphotericin B showed MICs ≥ 2 μg/ml for 38 (49.4%) of the 77 isolates tested.

Invasive aspergillosis is the most common cause of mortality resulting from infection by filamentous fungi in leukemic patients and hematopoietic stem cell transplant (HSCT) recipients worldwide (1). After *Aspergillus fumigatus*, *A. flavus* is the leading cause of invasive and noninvasive aspergillosis, particularly in infections of the respiratory tract, skin, mucosae, and eyes (2, 3). In contrast to *A. fumigatus*, *A. flavus* rarely causes invasive pneumonia and systemic infections in immunocompetent hosts (4). Voriconazole (VRC) is currently the drug of choice for the treatment of aspergillosis, although lipid formulations of amphotericin B (AMB), posaconazole (PSC), and caspofungin (CFG) have also been recommended to treat invasive aspergillosis refractory to or intolerant to other therapies (5). Human infections by *A. flavus* usually respond to treatment with AMB, VRC, and itraconazole (ITC), although failures of these drugs in cases of aspergillosis have already been reported (6–8). Invasive human aspergillosis caused by species of the section *Flavi* may involve several taxa, including *A. flavus*, *A. oryzae*, *A. tamarii*, *A. parasiticus*, *Petromyces alliaceus*, *A. nomius*, *A. qizutongi*, *A. beijingensis*, and *A. novoparasiticus* (3, 9–12). The high number of species of such a section that are potentially pathogenic for humans and their morphological similarity make it difficult to ascertain the clinical and epidemiological peculiarities of the infections they cause. In addition, cryptic species have been described within the taxon *A. flavus*, the most frequent infecting species (13, 14). Using molecular tools, we recently identified three phylogenetic species of *A. flavus* causing infections in humans (14). Considering that the antifungal susceptibility profiles of the members of the section *Flavi* are not completely known, in the present study, we evaluated the *in vitro* activity of nine antifungal drugs against 77 isolates representative of clinically relevant species, including *A. flavus* (belonging to our clades I, II, and III) (14), *A. oryzae* (belonging to our *A. oryzae* group) (14), *A. parasiticus*, *A. tamarii*, and *A. novoparasiticus* (Table 1).

The isolates were cultured on potato dextrose agar (PDA; Difco Laboratories, Detroit, MI) and incubated at 25°C for 7 days to prepare the fungal inocula. *Paecilomyces variotii* ATCC 36257, *Candida parapsilosis* ATCC 22019, and *Candida krusei* ATCC 6258 were included as control organisms. The isolates were previously identified by sequencing the acetamidase (*amdS*) and *O*-methyltransferase (*omtA*) genes and the internal transcribed spacer (ITS) region of ribosomal DNA (rDNA) (14, 15). Antifungal susceptibility testing was performed according to the Clinical

and Laboratory Standards Institute (CLSI) M38-A2 protocol (16). Briefly, 100-μl culture preparations in RPMI 1640 with 2% glucose were inoculated into the flat-bottom wells of 96-well microtiter plates containing 100 μl of the drug dilutions. The final inoculum concentration ranged from 0.4×10^4 to 5×10^4 CFU/ml. The drugs tested were provided by the manufacturers as pure powders and included the following: terbinafine (TRB), ITC, VRC, PSC, AMB, anidulafungin (AFG), micafungin (MFG), CFG, and 5-fluorocytosine (5FC). The MIC endpoints for the triazoles and AMB were defined as the lowest concentration that resulted in complete growth inhibition, while that of 5FC was defined as the lowest concentration that caused 50% growth inhibition. For the echinocandins, we applied the minimum effective concentration (MEC) endpoints, which were defined 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 results are shown in Table 1. In general, TRB and the echinocandins showed the lowest MICs/MECs for all species tested. Terbinafine showed a total geometric mean (GM) MIC of 0.03 μg/ml, while AFG and MFG showed a total GM MEC of 0.03 μg/ml. For CFG, the total GM MEC was 0.07 μg/ml. Posaconazole exhibited the lowest GM MIC among the azoles tested (0.35 μg/ml). Voriconazole and ITC showed a total GM MIC of 0.60 μg/ml. The three clades of *A. flavus* exhibited similar *in vitro* susceptibilities to all drugs tested. Despite some numerical differences that were observed among the MICs obtained with different triazoles, the GM MICs were quite similar and mostly within double dilutions, demonstrating the expected variability for the method. Regarding the other drugs evaluated, AMB exhibited MICs ≥ 2 for 38 (49.4%) of the 77 isolates tested, and 5FC showed poor *in vitro* activity against all species tested (GM MIC = 51.56 μg/ml). ITC,

TABLE 1 Activities of nine antifungal drugs against 77 isolates belonging to species of *Aspergillus* section *Flavi*[a]

Species (no. of isolates)	Antifungal agent[b]	MIC[c] or MEC[d] (µg/ml)																
		Range	GM	50%/90%	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128
A. parasiticus (3)	AMB	1–2	1.25								2	1						
	ITC	0.25–0.5	0.32						1	2								
	VRC	0.5	0.5							3								
	PSC	0.5–1	0.79							1	2							
	TRB	0.03	0.03			3												
	5FC	>64	>64														3	
	CFG	0.06	0.06				3											
	AFG	0.03	0.03			3												
	MFG	0.015	0.015		3													
A. novoparasiticus (4)	AMB	2–4	2.38									3	1					
	ITC	0.5–1	0.59							3	1							
	VRC	0.5–1	0.59							3	1							
	PSC	0.5	0.5							4								
	TRB	0.03	0.03			4												
	5FC	>64	>64														4	
	CFG	0.06–0.125	0.07				3	1										
	AFG	0.03	0.03			4												
	MFG	0.015–0.06	0.04		1	1	2											
A. flavus clade I (26)	AMB	1–2	1.31	1/2							16	10						
	ITC	0.25–1	0.44	0.5/1					7	17	2							
	VRC	0.25–0.5	0.47	0.5/0.5					2	24								
	PSC	0.5	0.50	0.5/0.5						26								
	TRB	0.03	0.03	0.030.03		26												
	5FC	8 to >64	54.54	64/>64										2			24	
	CFG	0.06–0.125	0.08	0.06/0.125			14	12										
	AFG	0.03–0.06	0.03	0.03/0.06		24	2											
	MFG	0.015–0.03	0.03	0.03/0.03	6	20												
A. flavus clade II (12)	AMB	1–2	1.19	1/2							9	3						
	ITC	0.5–1	0.71	0.5/1						6	6							
	VRC	0.5–2	0.71	0.5/1						7	4	1						
	PSC	0.25–0.5	0.45	0.5/0.5					2	10								
	TRB	0.03	0.03	0.03/0.03		12												
	5FC	64 to >64	64	64/>64													12	
	CFG	0.03–0.125	0.06	0.06/0.06		1	10	1										
	AFG	0.03	0.03	0.03/0.03		12												
	MFG	0.015–0.125	0.03	0.03/0.06	5	4	1	2										
A. flavus clade III (20)	AMB	1–4	1.62	2/2							7	12	1					
	ITC	0.25–2	0.96	1/2					2	2	11	5						
	VRC	0.25–2	0.84	1/2					2	4	11	3						
	PSC	0.25–1	0.45	0.5/1					5	13	2							
	TRB	0.03	0.03	0.03/0.03		20												
	5FC	64 to >64	64	64/>64													20	
	CFG	0.03–0.125	0.05	0.06/0.125		5	13	2										
	AFG	0.03–0.06	0.03	0.03/0.06		18	2											
	MFG	0.015–0.125	0.05	0.03/0.06	2	9	7	2										
A. oryzae group (10)	AMB	1–2	1.62	2/2							3	7						
	ITC	0.25–1	0.44	0.5/1					3	6	1							
	VRC	0.25–0.5	0.47	0.5/0.5					1	9								
	PSC	0.5	0.50	0.5/0.5						10								
	TRB	0.03	0.03	0.03/0.03		10												
	5FC	8 to >64	51.98	64/>64										1			9	
	CFG	0.06–0.125	0.08	0.06/0.125			5	5										
	AFG	0.03–0.06	0.03	0.03/0.03		9	1											
	MFG	0.015–0.03	0.03	0.03/0.03	2	8												

TABLE 1 (Continued)

Species (no. of isolates)	Antifungal agent[b]	MIC[c] or MEC[d] (µg/ml)																
		Range	GM	50%/90%	0.015	0.03	0.06	0.12	0.25	0.5	1	2	4	8	16	32	64	128
A. tamarii (2)	AMB	1	1									2						
	ITC	1–2	1.41								1	1						
	VRC	2–8	4									1		1				
	PSC	0.5–2	1							1		1						
	TRB	0.03	0.03			2												
	5FC	>64	64														2	
	CFG	0.06	0.06				2											
	AFG	0.03	0.03			2												
	MFG	0.015	0.015		2													
Total (77)	AMB	1–4	1.43	1/2							39	36	2					
	ITC	0.25–2	0.60	0.5/1					12	36	24	5						
	VRC	0.25–4	0.60	0.5/1					7	49	17	4						
	PSC	0.25–2	0.35	0.25/0.5					44	29	3	1						
	TRB	0.03	0.03	0.03/0.03		77												
	5FC	8–64	51.56	64/64										5		32	40	
	CFG	0.03–0.125	0.07	0.06/0.125		6	49	22										
	AFG	0.03–0.06	0.03	0.03/0.03		71	6											
	MFG	0.015–0.03	0.03	0.03/0.06	22	42	10	3										

[a] Data for clades I, II, and III and the *A. oryzae* group are from Gonçalves et al. (14). GM, geometric mean.

[b] AMB, amphotericin B; ITC, itraconazole; VRC, voriconazole; PSC, posaconazole; TR, terbinafine; 5FC, 5-fluorocytosine; CFG, caspofungin; AFG, anidulafungin; MFG, micafungin.

[c] MIC$_{50}$, concentration at which 50% of the isolates were inhibited; MIC$_{90}$, concentration at which 90% of the isolates were inhibited.

[d] MEC$_{50}$, concentration at which 50% of the isolates showed morphological changes in the growing hyphae; MEC$_{90}$, concentration at which 90% of the isolates showed morphological changes in the growing hyphae.

VRV, and PSC showed MICs between 1 and 4 µg/ml for the two isolates of *A. tamarii*.

The treatment of invasive aspergillosis is a challenge due to the diagnostic difficulty, the severity of the clinical conditions of the patients, and the limited number of antifungal drugs available (8). Little is known about the prevalence of *A. flavus* and cryptic species in clinical samples or their susceptibility to antifungal drugs. One of the most interesting findings of this study was the demonstration that echinocandins generally exhibited higher *in vitro* activity than triazoles and AMB against all species. In addition, no differences in susceptibility were observed within the three *A. flavus* clades. Otherwise, *A. tamarii* appears to be less susceptible to azoles than other species of the section. *Aspergillus tamarii* has been primarily described to cause sinusitis, keratitis, and onychomycosis (11, 17, 18). In the case of azoles, the MICs of ITC and VRC for all isolates were higher than that of PSC, which is in agreement with other studies (19–21). Amphotericin B appears to have limited activity against most species of the section *Flavi*. In general, the AMB MIC values were at least 2-fold higher than those obtained with isolates of the section *Fumigati* (22, 23). Lass-Flörl et al. (24) correlated the *in vitro* antifungal susceptibility of 12 *A. flavus* strains isolated from bone marrow transplant recipients with their clinical outcome. Only the four patients infected with susceptible isolates (AMB MIC < 2 µg/ml; *n* = 4) survived, while those with resistant isolates (AMB MIC ≥ 2 µg/ml; *n* = 8) died. Despite showing excellent *in vitro* activity against some strains of *Aspergillus* spp., the therapeutic efficacy of TRB in the management of nondermatophytic mold infections is still unclear at present (25).

In summary, there is a great diversity of species belonging to the section *Flavi* that cause infections in humans. We emphasize the importance of using molecular methods to accurately identify *Aspergillus* at the species level because different species may vary in terms of susceptibility to antifungal agents.

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