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Activities of Antifungal Agents against Yeasts and Filamentous Fungi: Assessment according to the Methodology of the European Committee on Antimicrobial Susceptibility Testing▽

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We compared the activities of antifungal agents against a wide range of yeasts and filamentous fungi. The methodology of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) for yeasts and spore-forming molds was applied; and a total of 349 clinical isolates of *Candida* spp., other yeast species, *Aspergillus* spp., and nondermatophyte non-*Aspergillus* spp. were investigated. The average geometric mean (GM) of the MICs of the various drugs for *Candida* spp. were as follows: amphotericin B (AMB), 0.55 μg/ml; liposomal amphotericin B (l-AMB); 0.35 μg/ml; itraconazole (ITC), 0.56 μg/ml; voriconazole (VRC), 0.45 μg/ml; posaconazole (POS), 0.44 μg/ml; and caspofungin (CPF), 0.45 μg/ml. The data indicated that the majority of *Candida* spp. were susceptible to the traditional and new antifungal drugs. For *Aspergillus* spp., the average GM MICs of AMB, l-AMB, ITC, VRC, POS, and CPF were 1.49 μg/ml, 1.44 μg/ml, 0.65 μg/ml, 0.34 μg/ml, 0.25 μg/ml, and 0.32 μg/ml, respectively. For the various zygomycetes, the average GM MICs of AMB, l-AMB, ITC, and POS were 1.36 μg/ml, 1.42 μg/ml, 4.37 μg/ml, and 1.65 μg/ml, respectively. Other yeastlike fungi and molds displayed various patterns of susceptibility. In general, the minimal fungicidal concentrations were 1 to 3 dilutions higher than the corresponding MICs. POS, AMB, and l-AMB showed activities against a broader range of fungi than ITC, VRC, and CPF did. Emerging pathogens such as *Saccharomyces cerevisiae* and *Fusarium solani* were not killed by any drug. In summary, the EUCAST data showed that the in vitro susceptibilities of yeasts and filamentous fungi are variable, that susceptibility occurs among and within various genera and species, and that susceptibility depends on the antifungal drug tested. AMB, l-AMB, and POS were active against the majority of pathogens, including species that cause rare and difficult-to-treat infections.

The frequency of invasive, opportunistic mycoses has increased significantly over the past two decades (2, 4, 10, 33). Among the myriad opportunistic fungal pathogens, *Candida albicans* and *Aspergillus fumigatus* cause the most well known infections (39, 42). Yet, the growing list of other opportunistic agents is of increasing importance. New and emerging fungal pathogens include species of *Candida* and *Aspergillus* other than *C. albicans* and *A. fumigatus*, opportunistic yeastlike fungi (e.g., *Trichosporon* species), the zygomycetes, hyaline molds (e.g., *Fusarium* and *Scedosporium* species), and a wide variety of dematiaceous fungi (1, 5, 18, 22, 24, 44). The diverse array of opportunistic fungi and their variable susceptibilities to both new and established antifungal agents have made the need for prompt identification and in vitro susceptibility testing more pressing.

The aim of this study was to investigate the in vitro activities of the antifungal agents available in Europe at the time of the study against a wide range of fungi, including yeasts and molds plus less common pathogens. Susceptibility testing of the yeasts and the spore-forming molds was performed according to the methods of the Antifungal Susceptibility Testing Subcommittee of the European Committee on Antimicrobial Susceptibility Testing (EUCAST) (40, 41).

MATERIALS AND METHODS

Microorganisms. The majority of the yeasts, yeastlike fungi, and molds were recovered by Innsbruck Medical University over a period of 10 years (1996 to 2006). The isolates were obtained from various specimens such as blood, respiratory tract, and biopsy specimens and specimens from other deep sites. In total, we evaluated the MICs and the minimum fungicidal concentration (MFCs) of 349 clinically relevant fungi, such as *Candida albicans* ($n = 59$), *Candida tropicalis* ($n = 10$), *Candida glabrata* ($n = 18$), *Candida krusei* ($n = 19$), *Candida parapsilosis* ($n = 18$), *Candida lusitaniae* ($n = 9$), *Cryptococcus neoformans* ($n = 13$), other yeasts ($n = 18$), *A. fumigatus* ($n = 29$), *Aspergillus flavus* ($n = 21$), *Aspergillus terreus* ($n = 34$), *Aspergillus niger* ($n = 13$), *Rhizomucor* spp. ($n = 17$), *Absidia* spp. ($n = 21$), *Rhizopus* spp. ($n = 21$), *Cunninghamella* spp. ($n = 4$), *Mucor* spp. ($n = 14$), and others ($n = 21$).

Drugs. The antifungal agents used in the study were amphotericin B (AMB; Sigma-Aldrich Vienna, Austria), liposomal AMB (l-AMB; Gilead Sciences GmbH, Germany), itraconazole (ITC; Janssen Research Foundation, Belgium), voriconazole (VRC; Pfizer, Ltd., Sandwich, United Kingdom), posaconazole (POS; Schering-Plough, Kenilworth, NJ), and caspofungin (CPF; Merck & Co., Inc., Rahway, NJ). The in vitro activity of l-AMB was investigated in this study, as only rare in vitro data are available for this compound.

Susceptibility testing. For *Candida* spp., the MICs were determined by using the reference procedure of the Antifungal Susceptibility Testing Subcommittee of EUCAST for the testing of fermentative yeasts (41). Briefly, testing was performed in flat-bottom microdilution plates with RPMI 1640 medium supplemented with 2% glucose and an inoculum size of 0.5×10^5 to 2.5×10^5 CFU/ml.

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TABLE 1. MFC ranges for the various antifungal agents against yeasts

Species	No. of isolates	MFC range (μg/ml)					
		AMB	l-AMB	CPF	ITC	VRC	POS
Candida species							
Candida albicans	59	2–4	0.5–2	0.15–1	0.06–8	0.06–8	0.12–8
Candida glabrata	18	0.5–8	0.5–4	0.15–4	4–>8	4–>8	4–>8
Candida parapsilosis	18	4–8	1–4	0.25–8	0.06–4	0.06–4	0.06–4
Candida krusei	19	0.5–8	1–8	2–8	0.5–8	0.5–8	0.5–2
Candida lusitaniae	9	4–8	1–4	4–8	0.25–2	0.06–8	0.06–1
Candida tropicalis	10	0.5–4	0.3–2	0.5–8	0.25–>8	0.12–8	0.12–>8
Candida guilliermondii	4	2–4	1–4	2–>8	2–8	0.25–0.5	0.25–0.5
Others							
Saccharomyces cerevisiae	3	4–>8	2–4	4–8	4–>8	4–>8	4–>8
Cryptococcus neoformans var. *neoformans*	10	1–2	0.25–1	>8	0.5–2	0.06–0.5	0.125–0.5
Cryptococcus neoformans var. *gattii*	3	1–2	0.5–1	>8	0.5–1	0.125–0.5	0.125–1
Trichosporon inkin	3	1–2	0.125–0.5	>8	0.25–0.5	0.03–0.5	0.5–1
Trichosporon asahii	4	4–8	0.15–2	>8	4–>8	>8	>8
Geotrichum candidum	4	4–>8	>8	>8	>8	4–>8	>8

MIC end points were determined spectrophotometrically at 24 h. For AMB, the MIC end points were defined as the lowest drug concentration that resulted in a reduction in growth of 90% or more compared with that of a drug-free growth control well. For the azoles and CPF, the MIC end point was defined as a 50% reduction in the optical density. The MFCs, which were considered the lowest drug concentrations that resulted in 99% killing, were determined as described previously (6).

For the dematiaceous fungi, the MICs were determined by using the reference procedure of the Antifungal Susceptibility Testing Subcommittee of EUCAST for spore-forming molds (40). Briefly, testing was performed in flat-bottom microdilution plates with RPMI 1640 medium supplemented with 2% glucose and an inoculum size of 2×10^5 to 5×10^5 CFU/ml. MIC end points were visually determined at 24 and 48 h. For the polyenes and the azoles, the MIC end points were defined as the lowest drug concentration that resulted in a 100% reduction in growth compared with that of a drug-free growth control well. For CPF, the minimum effective concentration (MEC) was evaluated (11, 15). Tests were performed in duplicate and were repeated twice.

C. parapsilosis ATCC 22019, *C. krusei* ATCC 6258, *A. fumigatus* ATCC 204306, and *A. flavus* ATCC 204304 were included as control isolates. The MFCs were evaluated by the method of Espinel-Ingroff (14, 16). Tests were run in duplicate and were repeated twice.

For *C. neoformans* and other species of nonfermentative yeasts, susceptibility testing was done according to the recommendations proposed by EUCAST, with the following minor modification: the microdilution plates were sealed and agitated at 350 rpm and 30°C for 48 h (8).

RESULTS AND DISCUSSION

The data obtained by the methodology of EUCAST show that the in vitro susceptibilities of yeasts and filamentous fungi are variable and that susceptibility occurs among and within various genera and species, and they further support the need for the genus-level identification of the pathogen for the appropriate management of infections. AMB, l-AMB, and POS were the drugs with the broadest in vitro activities, yet they also had high degrees of variability in their activities from strain to strain. The MICs were comparable to those determined by the CLSI for the various fungi tested (13, 30, 32, 34). The MFCs evaluated in this study were 1 to 3 dilutions higher than the corresponding MICs (Tables 1 and 2), and several pathogens were not killed by any drug.

Among the yeasts, species-specific variations and occasional resistance were encountered with the drugs tested (Table 3). *C. albicans* was the most susceptible and had MICs slightly different from those of the other yeasts. As shown by others (9, 29, 30, 31, 36), *C. krusei* and *C. glabrata* were the species with the highest MICs against ITC, VRC, and POS. The panel of yeasts tested included a number ($n = 18$) of fluconazole-resistant *C. glabrata* and *C. krusei* isolates (MICs > 4 μg/ml, according to a EUCAST technical note [17]). For this subset of isolates, ITC, POS, and VRC showed cross-resistance (MICs > 2 μg/ml) in 65% of the *C. glabrata* isolates and 35% of the *C. krusei* isolates. Such cross-resistance to the new drug POS was found in nearly 20% of strains, which is a proportion compatible with that published previously (29). Elevated MICs of AMB and l-AMB are rare, yet they were the highest in *C. krusei. C. lusitaniae* with primary resistance to polyenes (tentative MIC breakpoint > 1 μg/ml) was found infrequently; 98% of our strains were susceptible. A remarkable and somewhat contradictory finding was the fact that of the polyenes tested, l-AMB was more active against several yeast strains than AMB. The reasons for these differences are not fully clear and need to be clarified in more detail. As known from the CLSI method (27, 29, 30), we also observed relatively high CPF MICs for *C. parapsilosis.*

Among the yeastlike fungi, *Geotrichum candidum* was, in general, susceptible to the various drugs tested; higher MICs were observed for CPF. A similar lack of activity was obtained for *Trichosporon* spp. The MICs were consistently high, indicating that this agent might have practically no activity. Isolates of *Trichosporon asahii* are often resistant to AMB (MICs > 2 μg/ml) in vitro (35), which was also observed in our isolates, whereas l-AMB appeared to be active. This matter needs further clarification. POS completely lacked activity against *Trichosporon* spp.

C. neoformans isolates were susceptible to the azoles and the polyenes, and CPF lacked activity against *C. neoformans*. Other studies indicate that *C. neoformans* is also resistant to CPF (8).

Among the aspergilli, *A. fumigatus* was the most sensitive species; comparable results were obtained for the polyenes and the azoles (Table 4). *A. terreus* displayed high MICs (geometric mean [GM] MICs of AMB and l-AMB, 2.82 and 2.84 μg/ml, respectively), which reflects the potential for primary polyene resistance (19, 25, 38). Overall, we failed to identify ITC-resistant *A. fumigatus* or *A. niger* strains, as has frequently been

TABLE 2. MFC ranges for the various antifungal agents against molds

Species	No. of isolates	MFC range (μg/ml)					
		AMB	l-AMB	CPF	ITC	VRC	POS
Aspergillus species							
Aspergillus fumigatus	29	0.5–4	0.5–8	>8	2–4	0.5–8	0.5–8
Aspergillus terreus	34	4–>8	1–>8	>8	0.5–4	1–4	0.5–2
Aspergillus flavus	21	4–>8	4–>8	>8	0.5–2	1–4	0.5–1
Aspergillus niger	13	4–>8	1–>8	>8	4–>8	2–4	1–2
Zygomycetes							
Rhizomucor species	17	1–4	0.5–2	>8	2–>8	>8	2–>8
Absidia corymbifera	4	1–2	0.5–2	>8	2–>8	>8	2–4
Absidia species	17	1–8	0.5–>8	>8	2–>8	>8	0.5–4
Rhizopus microsporus var. *oligosporus*	3	4–8	1–2	>8	>8	>8	>8
Rhizopus oryzae	6	4–8	>8	>8	>8	>8	2–8
Rhizopus species	12	2–8	0.5–>8	>8	4–>8	>8	2–>8
Mucor hiemalis	3	1–2	0.5–2	>8	>8	>8	2–4
Mucor species	11	1–2	0.125–2	>8	4–>8	>8	2–8
Cunninghamella species	4	8–>8	0.5–>8	>8	4–8	>8	2–4
Others							
Scedosporium prolificans	2	>8	>8	>8	>8	>8	>8
Scedosporium apiospermum	3	>8	>8	>8	4–>8	>8	1–2
Penicillium marneffei	2	4–>8	0.125–0.5	>8	0.25–1	>8	0.25–0.5
Penicillium species	2	4–>8	0.5–2	>8	2–>8	>8	0.5–2
Fusarium solani	2	>8	>8	>8	>8	>8	>8
Fusarium oxysporum	2	1–2	0.15–0.5	>8	0.25–4	>8	0.25–1
Sporothrix schenckii	2	8–>8	>8	>8	0.5–4	>8	0.5–1
Curvularia lunata	2	1–2	0.5–1	>8	0.5–1	>8	0.5–1
Bipolaris australiensis	2	1–2	0.125–0.5	>8	0.5–2	>8	0.125–0.5
Rhinocladiella aquaspersa	2	1–2	0.5–1	>8	0.5–1	>8	0.125–0.5

observed by Verweij et al. (43) and Mosquera and Denning (26), respectively. The MICs of the echinocandins against *Aspergillus* spp. were complicated by the fact that the MICs often exceed the concentrations safely achievable in plasma (20, 33). Therefore, evaluation of the MEC, defined as the lowest drug concentration that caused short, stubby, and highly branched hyphae, is recommended (3, 12, 20, 23, 28). The MECs were comparable among the various aspergilli tested.

TABLE 3. In vitro susceptibilities of yeast species to the various antifungal agents determined by the EUCAST methodology

Species	No. of isolates	Range and GM MIC (μg/ml)											
		AMB		l-AMB		CPF		ITC		VRC		POS	
		MIC range	GM MIC	MIC range	GM MIC	MIC range	GM MIC	MIC range	GM MIC	MIC range	GM MIC	MIC range	GM MIC
Candida species													
Candida albicans	59	0.06–1	0.12	0.015–0.12	0.03	0.06–0.25	0.14	0.06–0.5	0.23	0.06–0.25	0.13	0.06–0.5	0.14
Candida glabrata	18	0.125–2	0.41	0.5–1	0.75	0.125–1	0.32	0.5–4	0.83	1–4	1.66	0.5–4	1.58
Candida parapsilosis	18	0.125–1	0.30	0.5–1	0.42	0.5–2	0.82	0.125–1	0.32	0.06–0.5	0.08	0.03–0.125	0.05
Candida krusei	19	0.25–2	0.51	0.5–2	0.51	0.06–1	0.58	0.5–4	1.10	0.5–2	0.53	0.5–2	0.68
Candida lusitaniae	9	0.5–2	1.16	0.06–0.125	0.27	0.25–1	0.32	0.03–0.125	0.07	0.06–0.5	0.07	0.06–0.5	0.06
Candida tropicalis	10	0.125–1	0.38	0.25–1	0.32	0.125–1	0.34	0.125–0.5	0.56	0.06–0.5	0.48	0.06–0.5	0.12
Candida guilliermondii	4	1	1	0.06–0.12	0.10	0.5–1	0.68	0.5–1	0.87	0.03–0.125	0.24	0.06–0.5	0.45
Others													
Saccharomyces cerevisiae	3	0.5–1	0.66	0.03–0.06	0.04	1–2	1.30	1–4	2.00	0.125–0.5	0.29	0.5–1	0.66
Cryptococcus neoformans var. *neoformans*	10	0.5–2	0.75	0.06–0.12	0.07	2–4	2.04	0.06–0.5	0.11	0.01–0.5	0.05	0.06–0.5	0.14
Cryptococcus neoformans var. *gattii*	3	0.5–1	0.66	0.03–0.06	0.06	4–8	5.33	0.06–0.5	0.22	0.01–0.06	0.05	0.06–0.125	0.06
Trichosporon inkin	3	0.5	0.5	0.03–0.06	0.06	4–8	5.33	0.03–0.5	0.21	0.01–0.03	0.05	0.25–0.5	0.28
Trichosporon asahii	4	1–2	1.5	0.01–0.03	0.02	4–8	4.00	0.03–0.5	0.17	0.03–0.06	0.04	4–8	6.00
Geotrichum candidum	4	1–2	1.5	0.06–0.25	0.14	2–4	3.00	0.5–2	1.45	0.125–0.5	0.25	0.25–0.5	0.37

TABLE 4. In vitro susceptibilities of the molds to various antifungal agents determined by the EUCAST methodology

Species	No. of isolates	Range and GM MIC or MEC (μg/ml)											
		AMB		l-AMB		CPF		ITC		VRC		POS	
		MIC range	GM MIC	MIC range	GM MIC	MEC range	GM MIC	MIC range	GM MIC	MIC range	GM MIC	MIC range	GM MIC
Aspergillus species													
Aspergillus fumigatus	29	0.5–2	0.69	0.5–2	0.31	0.25–1	0.45	0.5–1	0.46	0.25–1	0.36	0.5–1	0.37
Aspergillus terreus	34	2–4	2.82	2–4	2.84	0.125–1	0.19	0.5–1	0.58	0.25–1	0.29	0.125–0.5	0.19
Aspergillus flavus	21	1–4	1.51	1–4	1.38	0.25–1	0.28	0.5–2	0.59	0.5–2	0.41	0.125–0.5	0.21
Aspergillus niger	13	0.5–4	1.00	1–2	1.23	0.25–1	0.36	2–4	0.98	0.5–2	0.33	0.25–0.5	0.26
Zygomycetes													
Rhizomucor species	17	0.25–1	0.58	0.3–0.125	0.39	>8		>8		>8		1–4	2.23
Absidia corymbifera	4	1–1	1.00	0.125–1	0.43	>8		1–2	2.50	>8		0.5–1	0.29
Absidia species	17	0.5–1	0.80	0.5–2	0.72	>8		4–>8		>8		0.5–2	0.33
Rhizopus microsporus var. *oligosporus*	3	1–1	1.00	0.03–0.25	0.13	>8		8–>8		>8		4–8	5.33
Rhizopus oryzae	6	2–4	3.00	1–4	2.00	>8		4	4.00	>8		1–2	1.89
Rhizopus species	12	1–4	2.08	1–4	2.50	>8		>8		>8		2–4	0.78
Mucor hiemalis	3	0.5–1	0.86	0.03–0.5	0.21	>8		8–>8		>8		1–2	1.33
Mucor species	11	0.5–1	0.73	0.03–0.5	0.38	>8		4–8	4.36	>8		2–4	0.72
Cunninghamella species	4	0.5–4	2.25	0.5–4	1.47	>8		1–4	1.75	>8	>8	1–2	2.00
Others													
Scedosporium prolificans	2	>8		>8		>8		>8		>8		>8	
Scedosporium apiospermum	3	2–4	2.66	1–2	1.33	>8		2–4	2.66	2–>8		0.5–2	1.00
Penicillium marneffi	2	1–2	1.5	0.03–0.25	0.14	>8		0.01–0.5	0.25	>8		0.06	0.06
Penicillium species	2	0.5–1	0.75	0.5–1	0.75	>8		0.5–8	4.25	2–>8		0.5	0.05
Fusarium solani	2	4	1.00	4–8	6.00	>8		4–8	6.00	>8		1	1
Fusarium oxysporum	2	1–4	2.5	0.03–0.5	0.10	>8		1–8	4.50	4–>8		0.125	0.12
Sporothrix schenckii	2	1–4	2.5	1–2	1.50	>8		0.125	0.12	>8		0.25	0.25
Curvularia lunata	2	0.5–1	0.75	0.125–0.5	0.31	>8		0.03	0.03	>8		0.25	0.25
Bipolaris australiensis	2	1	1.00	0.01–0.06	0.03	>8		0.125	0.12	>8		0.125	0.12
Rhinocladiella aquaspersa	2	1	1.00	0.5–1	0.75	>8		0.125	0.12	>8		0.06	0.06

AMB, l-AMB, and POS showed satisfactory in vitro activities against the zygomycetes (Table 4). The most active drug was l-AMB, but it lacked activity against some strains of *Rhizopus* spp. and *Cunninghamella* spp. By contrast, those strains were susceptible to POS. POS showed a lack of activity against some strains of *Rhizomucor* spp., *Rhizopus* spp., and *Mucor* spp.; yet it showed global activity against *Absidia* spp. The meaning of these in vitro findings is not fully clear, as we lack breakpoints and know from earlier studies that mycological resistance in vitro does not always mean clinical failure (7, 21). None of the other azoles or CPF showed activity against the zygomycetes.

Within the class of rare fungi, *Scedosporium prolificans* was multidrug resistant; similar results were obtained for *Fusarium solani*, which showed high MICs of ITC, VRC, and the polyenes; the drug of choice would be POS. The MICs of l-AMB for *Fusarium oxysporum* were lower than those of AMB. So far, the limited number of isolates analyzed prevents significant conclusions from being drawn.

Whether MICs are the best in vitro predictors of the in vivo or clinical response to antifungal therapy is uncertain. Several authors suggest that the MFCs have the potential to be more relevant to the clinical outcome, especially in the context of profoundly immunosuppressed hosts (37). The clinical value of either MFCs or MICs as predictors of resistance in fungal infections remains to be established. The CLSI Subcommittee for Antifungal Susceptibility Tests conducted two collaborative studies and identified conditions for the determination of MFC end points for mold isolates (14, 16). The polyenes are fungicidal for many pathogens, yet they lacked activity against several non-*A. fumigatus* strains, *Penicillium* spp., and zygomycetes (Tables 1 and 2). Among the azoles, POS was active against most of the fungi tested, whereas ITC, VRC, and CPF killed the fungi only at pathophysiological concentrations. It is obvious that many emerging pathogens, e.g., *S. cerevisiae* and *G. candidum*, are not killed by any agent. The role of MFCs needs to be elucidated, especially when the MIC reflects susceptibility while the MFC indicates resistance, as was the case for, e.g., *G. candidum* with all drugs tested, *Scedosporium apiospermum* and *Rhizopus oryzae* with the polyenes, *A. niger* with ITC, and the zygomycetes with POS.

In summary, the methodology of EUCAST provided data similar to those provided by the CLSI methodology, was able to detect reliable MICs, and generated consistent data. AMB, l-AMB, and POS were active against the majority of strains, including species that cause rare and difficult-to-treat infections.

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