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Comparison of In Vitro Antifungal Susceptibilities of Conidia and Hyphae of Filamentous Fungi

J. GUARRO,[1]* C. LLOP,[1] C. AGUILAR,[1] AND I. PUJOL[2]

Unitat de Microbiologia, Facultat de Medicina, Universitat Rovira i Virgili,[1] and Laboratori de Microbiologia, Hospital Universitari de Sant Joan de Reus,[2] 43201 Reus, Tarragona, Spain

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The MICs and minimum fungicidal concentrations (MFCs) of amphotericin B, fluconazole, ketoconazole, flucytosine, miconazole, and itraconazole for 12 isolates of filamentous opportunistic fungi (*Scopulariopsis* sp., *Paecilomyces* sp., *Cladosporium* spp., and *Cladophialophora* sp.) were determined by a broth microdilution method with hyphal and conidial inocula. With hyphal inocula MICs and MFCs were practically always substantially higher. Only 25% of the 60 MIC comparisons showed discrepancies of twofold or less, while the remaining comparisons showed much larger differences.

Filamentous fungi are emerging as significant pathogens, particularly in immunocompromised patients (9); hitherto, more than 300 species have been reported to be potentially pathogenic for humans (2). Hence, antifungal susceptibility testing with these opportunistic pathogens is important in the clinical laboratory. It is important to develop methods for routine susceptibility testing of filamentous fungi so that the most appropriate antifungal agent can be chosen and used at the correct dose. Several published studies have defined the best conditions for obtaining a reference method (3–5, 10, 11, 13).

However, all these techniques use inocula consisting exclusively of conidia, which are usually not the morphological form of the infective fungus manifested in vivo. Even though several genera, such as *Acremonium*, *Fusarium*, and *Paecilomyces*, can occasionally produce conidia in vivo (14), most infections caused by filamentous fungi are characterized by the presence of hyphal elements in tissue. Conidial suspensions are easier to prepare and tests with conidial suspensions are more easily standardized, but the results can be very different from those obtained with hyphae (7). Therefore, it also seems appropriate to develop reliable methods for antifungal susceptibility testing of filamentous fungi with hyphal inocula and to compare their results with those obtained with conidial inocula.

Organisms. The panel of test organisms developed for the study were well-identified isolates belonging to the following genera: *Fusarium* (22 isolates), *Paecilomyces* (5 isolates), *Scopulariopsis* (10 isolates), *Cladosporium* (10 isolates) and *Cladophialophora* (3 isolates). Most of them were of clinical origin.

Inoculum preparation. Prior to the study the fungi were grown in slants of potato carrot agar (PCA). The inocula were prepared by flooding the surface of the agar slant with sterile distilled water and scraping the sporulated aerial mycelium with a loop. The suspension obtained was then filtered once through sterile gauze folded three times to remove the hyphae. The numbers of conidia in the suspensions were counted with a hemacytometer and were adjusted to 50,000 conidia/ml. Erlenmeyer flasks (150 ml) containing 10 ml of LCTA (Czapek broth [Difco] supplemented with 2% Tween 80 and 0.07% agar) (1) were inoculated with 250 μl of the conidial suspension and were incubated at 30°C without shaking. At between 16 and 72 h of incubation the cultures were examined about every 4 h (the dematiaceous fungi required the longest incubation periods) for the presence of hyphae. Only those suspensions with more than 90% hyphae were included in the study. The suspensions were filtered once through sterile gauze to remove hyphal mats and long filaments. The numbers of hyphal fragments in the cultures were counted with a hemacytometer and adjusted to 50,000 hyphal fragments/ml.

Broth microdilution method. The broth microdilution method, described previously (10), was used to determine the MICs. The drug concentrations ranged from 16 to 0.03 μg/ml for amphotericin B, miconazole, itraconazole, and ketoconazole; 64 to 0.125 μg/ml for fluconazole; and 128 to 0.25 μg/ml for flucytosine and were obtained by using 10 twofold serial dilutions. In order to obtain the minimum fungicidal concentration (MFC), volumes of 10 μl were taken from every well showing inhibition and were spread onto PCA plates. Fungal colonies were counted after incubating the plates at 30°C for 48 h or until growth of the subcultures from the growth control well were apparent. The MFC of an antifungal agent was defined as the lowest drug concentration from which one colony or less was visible on the agar plate. All MICs and MFCs obtained with conidial inocula were compared with those obtained with hyphal inocula. Both on-scale and off-scale results were included in the analysis. The high off-scale MICs and MFCs (>128, >64, and >16 μg/ml) were converted to the next highest concentration (either 256, 128, or 32 μg/ml, respectively), and the low off-scale MICs (≤0.25, ≤0.125, and ≤0.03 μg/ml) were left unchanged. When the two values of a pair were off scale, this pair of values was not included in the study.

Of the 50 fungal isolates tested, we obtained useful hyphal inocula for only 12 of them (Table 1). Under the different conditions tested (inocula, temperatures, and incubation times), the remaining strains always gave mixtures of hyphae and conidia. The percentages of the latter were variable but were always higher than 20%. By this procedure 60 pairs of MICs and 29 pairs of MFCs were compared. The values obtained with hyphal inocula were higher than those obtained with conidial inocula except in two cases. The MICs and MFCs obtained with the two types of inocula were different in practically all cases.

Of the 60 MIC comparisons performed, only 15 (25%) showed differences of twofold or less. The rest of the pairs

* Corresponding author. Mailing address: Unitat de Microbiologia, Facultat de Medicina, Universitat Rovira i Virgili, Carrer Sant Llorenç, 21, 43201 Reus, Spain. Phone: 977-759359. Fax: 977-759322.

TABLE 1. Comparison of MICs and MFCs obtained with conidial and hyphal inocula of filamentous fungi

Strain	End-point	Concn (μg/ml)[a]											
		AMB		MON		ITRA		KETO		FLUCO		5-FC	
		C	H	C	H	C	H	C	H	C	H	C	H
Cladophialophora bantiana CBS 328.65	MIC	0.06	1	0.25	4	<0.03	0.0125	0.06	1	4	16	0.25	1
	MFC	>16	>16	>16	>16	8	8	>16	>16	>64	>64	>128	>128
Cladosporium cladosporioides CBS 171.54	MIC	1	4	1	8	0.5	2	0.5	2	32	>64	4	64
	MFC	1	8	>16	>16	8	>16	4	>16	>64		128	>128
Cladosporium cladosporioides FMR 5031	MIC	0.125	0.5	0.25	4	0.06	1	<0.03	0.5	16	32	0.125	0.125
	MFC	0.125	1	1	>16	0.06	4	<0.03	0.5	16	64	0.125	0.125
Cladosporium cladosporioides IFO 4459	MIC	0.25	8	1	>16	0.5	>16	1	>16	32	>64	0.25	64
	MFC	0.5	8	1	>16	>16	>16	>16		32	>64	0.25	128
Cladosporium elatum IFO 6372	MIC	0.5	4	2	8	0.5	4	1	2	32	>64	64	>128
	MFC	0.5	8	8	16	4	4	16	2	64			
Cladosporium sphaerospermum CBS 193.54	MIC	1	2	4	>16	>16	>16	4	>16	>64	>64	4	4
	MFC	1	2	>16						16		4	4
Cladosporium sphaerospermum FMR 5030	MIC	0.5	8	2	8	0.5	1	2	4	>64	>64	8	>128
	MFC			>16	>16	1	8	2	16				
Paecilomyces carneus FMR 5185	MIC	2	4	4	8	2	>16	2	8	64	>64	>128	>128
	MFC	>16	>16	>16	>16	>16		>16	>16	>64			
Paecilomyces farinosus FMR 641	MIC	>16	16	>16	>16	>16	>16	>16	>16	>64	>64	>128	>128
	MFC		>16										
Paecilomyces variotii FMR 3735	MIC	0.125	0.25	2	>16	<0.03	4	0.5	8	64	>64	<0.25	<0.25
	MFC	16	>16	>16		>16	>16	>16	>16	>64		64	>128
Paecilomyces variotii FMR 4647	MIC	0.25	2	0.25	>16	0.06	8	0.125	>16	4	64	2	>128
	MFC	2	>16	0.5		0.06	8	0.25		4	>64	>128	
Scopulariopsis chartarum FMR 3997	MIC	2	0.5	2	>16	1	>16	2	>16	64	>64	>128	>128
	MFC	2	4	2		1		2		>64			

[a] AMB, amphotericin B; MON, miconazole; ITRA, itraconazole; KETO, ketoconazole; FLUCO, fluconazole; 5-FC, flucytosine; C, conidial inoculum; H, hyphal inoculum.

compared showed higher differences, and in some cases these were even of the order of 128- or 256-fold. In the case of MFCs, the discrepancies were slightly lower; i.e., in 9 of the 29 comparisons performed (31%), the differences were twofold or less (Table 2).

The main difficulty with the technique used in this study was the preparation of pure hyphal inocula, in which fully viable hyphae were uniformly dispersed without the presence of any mycelial mats. The method used to grow hyphae is based on that of Bezjak (1) and was not a great success, since of the 50 isolates tested we obtained suitable comparable suspensions of hyphal and conidial inocula for only 12 of them. Therefore, it seems evident that the procedure used in this type of study must still be perfected. Granade and Artis (6) standardized a method in which they prepared fragmented mycelium using a ground-glass tissue grinder, but it appears that this technique severely affects the viability of the fragmented hyphal elements.

The large discrepancies between the MICs and also between the MFCs obtained with hyphal and conidial inocula are in contrast to the results obtained in other studies (1, 12). Bezjak (1) found very similar MIC results when four strains of *Aspergillus* were tested with amphotericin B, with the differences never being more than 1 dilution. When there were differences, the MICs of the hyphal inocula were always the largest. Regli et al. (12) found that the susceptibility of one strain of *Aspergil-*

TABLE 2. Discrepancies between MICs and MFCs obtained with conidial and hyphal inocula

Difference (fold)	No. of isolates[a]											
	AMB		MON		ITRA		KETO		FLUCO		5-FC	
	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC	MIC	MFC
Same						2					2	2
2	4	3	1	1	1		2		3		2	1
4	3		2		2	1	2		4	2		1
8	2	2	2		1	1	1	3				
16	2	3	4		2		4	1	1		1	
32	1		1	2	1		1			1	1	
64					1	1						
128			1		2	1					1	
256							1				1	
512												1

[a] Data indicate the numbers of isolates for which the MICs or MFCs for the hyphal inoculum were either equal to (same) or 2- to 512-fold higher than the MICs or MFCs for the conidial inoculum. AMB, amphotericin B; MON, miconazole; ITRA, itraconazole; KETO, ketoconazole; FLUCO, fluconazole; 5-FC, flucytosine.

lus fumigatus to antifungal agents was similar when they used conidia and hyphal inocula. However, Bezjak (1) used a broth microdilution method and Regli et al. (12) used an agar diffusion method. Our results agree in part with those of Koenig and Kremer (7), who tested 13 isolates belonging to four species of *Aspergillus* with three antifungal drugs. They used an agar diffusion method and obtained considerable MIC discrepancies when the two types of inocula were used. Martin et al. (8) compared inocula of conidia and of germinating conidia of several filamentous fungi, and the results varied according to the antifungal agent tested. In general, the MICs of amphotericin B and itraconazole for the two fungal forms were similar, and the MFCs were higher for the germinated forms. The discrepancies found between results obtained with the two types of inocula seem, to some extent, to be logical, because the composition of the cell wall of many fungal species is not the same under all circumstances due to the different structural requirements of tubular and more or less spherical cells.

In conclusion, this study demonstrates the importance of the type of inoculum used to test the antifungal susceptibilities of filamentous fungi. However, the correlation of the results obtained with the two inoculum types in vitro with the clinical outcome will be a key factor in deciding what type of inoculum is the most adequate.

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