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Germinated and Nongerminated Conidial Suspensions for Testing of Susceptibilities of *Aspergillus* spp. to Amphotericin B, Itraconazole, Posaconazole, Ravuconazole, and Voriconazole

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The effect of germinated and nongerminated conidia of *Aspergillus* spp. on the fungistatic (National Committee for Clinical Laboratory Standards document M38-P) and fungicidal activities (MICs and minimal fungicidal concentrations [MFCs] respectively) of amphotericin B, itraconazole, posaconazole (SCH56592), ravuconazole (BMS-207147), and voriconazole was evaluated. MFCs were the lowest drug dilutions that showed fewer than three colonies (99.9% killing). Overall, the MICs (0.12 to 4 μg/ml) and MFCs (0.5 to >8 μg/ml) of all of the agents tested with both inocula were the same or within 2 dilutions for the 72 isolates. Therefore, MICs and MFCs can be obtained with convenient and standardized nongerminated conidia.

The role of the laboratory in the selection and monitoring of antifungal therapy has gained greater attention with the increased incidence of systemic fungal infections and the growing number of new antifungal agents. The National Committee for Clinical Laboratory Standards (NCCLS) has proposed standard conditions for molds (document M38-P) (7, 8, 19, 21). Although the pathogenic form of most opportunistic molds is the hyphae, document M38-P (19) describes the more convenient and standardized preparation of nongerminated conidial inoculum suspensions. Prior studies have compared MICs obtained by employing either germinated conidia or hyphal suspensions to those obtained with nongerminated conidia for dematiaceous fungi (13), *Aspergillus* spp., and other opportunistic moniliaceous molds (2, 5, 13, 16–18, 22, 25). However, findings on the effect of hyphae on MIC determination (2, 5, 13, 17, 22, 25) have been more contradictory than those on the effect of germinated conidia (16, 18).

Although *Aspergillus fumigatus* is responsible for the majority (85 to 90%) of the different clinical manifestations of *Aspergillus* infections (4), other *Aspergillus* spp. also have been associated with severe infection in immunocompromised hosts (4, 21, 24, 25). The purpose of this study was to evaluate the effect of germinated and nongerminated conidia on MICs and minimal fungicidal concentrations (MFCs) of amphotericin B, itraconazole, posaconazole (SCH56292), ravuconazole (BSM-207147), and voriconazole for six *Aspergillus* spp. following NCCLS document M38-P for MICs (19).

Seventy-two isolates of *Aspergillus* spp., each from a different patient, were evaluated (Tables 1 and 2). *A. flavus* ATCC 204304 and *Candida parapsilosis* ATCC 22019 were included as controls; the MIC ranges for both controls were within established values (1, 8, 19). Stock inoculum suspensions were prepared as described in document M38-P (19) and adjusted spectrophotometrically to optical densities that ranged from 0.09 to 0.11 (78 to 82% transmittance) (6). For the nongerminated conidial inocula, the stock suspensions were diluted 1:50 in the NCCLS standard RPMI 1640 medium with morpholinepropanesulfonic acid (MOPS) buffer and without bicarbonate (RPMI). For the germination of conidia, the stock suspensions were incubated in RPMI at 35°C in a shaker incubator for 7 to 9 h at 180 rpm for five of the six species evaluated; germination of *A. terreus* conidia required 14 to 20 h of incubation. Conidia were considered fully germinated when the length of the germ tube was at least twice the length of the swollen conidia. After germination, the stock suspensions were also diluted 1:50 in RPMI. The final inoculum sizes for both conidial sources ranged from 1.0×10^4 to 3.6×10^4 CFU/ml.

MICs of amphotericin B (Bristol-Myers Squibb Pharmaceutical Research Institute, Wallingford, Conn.), itraconazole (Janssen Pharmaceutica, Titusville, N.J.), posaconazole (SCH56592; Schering-Plough Research Institute, Kenilworth, N.J.), ravuconazole (BMS-207147; Bristol-Myers Squibb), and voriconazole (Pfizer Pharmaceuticals, New York, N.Y.) were determined by the M38-P broth microdilution method (19). Drug dilutions were prepared at 100 times the final concentrations, followed by further dilutions (1:50) in RPMI to yield 2 times the final strength required (8 to 0.0078 μg/ml) for the test. Each microdilution well containing 100 μl of the diluted (two times) drug concentration was inoculated with 100 μl of the diluted (two times) inoculum suspensions (the final volume in each well was 200 μl). Both control strains were tested each time a set of isolates was evaluated. Microdilution trays were incubated at 35°C and visually examined at 48 h for MIC determination (19); MICs corresponded to either prominent (≥50%, azoles) or complete (amphotericin B) growth inhibition. The in vitro fungicidal activities each agent were determined as previously described (9); the MFC was the lowest concentration that showed fewer than three colonies. MIC AND MFC ranges and MICs and MFCs for 90% of the isolates tested (MIC_{90}s and MFC_{90}s, respectively) were obtained for each species-drug combination tested; MIC_{50}s and MFC_{50}s were obtained for *A. niger*.

Since nongerminated conidium suspensions are easier to

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TABLE 1. MICs for germinated and nongerminated conidia of *Aspergillus* spp. obtained by the NCCLS broth microdilution method[a]

Species (no. of strains tested) and antifungal agent	MIC (MIC_{90}) [μg/ml]	
	Germinated	Nongerminated
A. flavus (12)		
Amphotericin B	0.2–2 (2)	0.5–2 (2)
Itraconazole	0.03–0.2 (0.2)	0.03–0.5 (0.2)
Voriconazole	0.12–0.5 (0.5)	0.12–0.5 (0.5)
Posaconazole	0.12–1.0 (0.5)	0.03–0.5 (0.5)
Ravuconazole	0.12–0.5 (0.5)	0.12–1.0 (1.0)
A. fumigatus (30)		
Amphotericin B	0.2–2 (2)	0.5–2 (2)
Itraconazole	0.01–>8 (0.5)	0.03–>8 (0.2)
Voriconazole	0.12–1.0 (0.5)	0.06–1.0 (0.5)
Posaconazole	0.06–1.0 (0.12)	0.03–1.0 (0.2)
Ravuconazole	0.06–8 (0.5)	0.12–8 (1.0)
A. nidulans (10)		
Amphotericin B	0.2–4 (1.0)	0.2–4 (1.0)
Itraconazole	0.01–0.5 (0.2)	0.01–0.5 (0.5)
Voriconazole	0.01–2 (0.2)	0.01–0.5 (0.2)
Posaconazole	0.03–0.5 (0.12)	0.06–0.2 (0.12)
Ravuconazole	0.03–2 (0.2)	0.06–2 (0.5)
A. niger (7)		
Amphotericin B	0.2–0.5 (0.5)	0.5–1.0 (1.0)
Itraconazole	0.06–1.0 (0.5)	0.12–1.0 (0.2)
Voriconazole	0.12–0.5 (0.5)	0.12–1.0 (1.0)
Posaconazole	0.2–1.0 (0.5)	0.2–0.5 (0.5)
Ravuconazole	0.5–4 (4)	0.5–4 (4)
A. sydowii (1)		
Amphotericin B	0.12 (ND[b])	0.12 (ND)
Itraconazole	0.2 (ND)	0.5 (ND)
Voriconazole	0.12 (ND)	0.12 (ND)
Posaconazole	0.5 (ND)	0.2 (ND)
Ravuconazole	0.12 (ND)	0.2 (ND)
A. terreus (12)		
Amphotericin B	1.0–2 (2)	0.5–4 (4)
Itraconazole	0.01–0.5 (0.2)	0.03–0.5 (0.2)
Voriconazole	0.12–1.0 (1.0)	0.06–1.0 (1.0)
Posaconazole	0.03–0.5 (0.5)	0.01–0.5 (0.5)
Ravuconazole	0.2–1.0 (1.0)	0.12–1.0 (2)

[a] NCCLS M38-P method for antifungal susceptibility (MICs) testing of opportunistic mold pathogens.
[b] ND, not determined.

prepare, they have been traditionally employed for the antifungal susceptibility testing of molds. Because the measurement of conidial susceptibility could represent inhibition of conidial germination instead of hyphal growth by the antifungal agent, hyphae should be the fungal cells tested to evaluate the antifungal susceptibilities of *Aspergillus* spp. and other opportunistic molds. An alternative procedure is the use of germinated conidia. This study compared the in vitro fungistatic and fungicidal activities of five agents against nongerminated and germinated conidia of *Aspergillus* isolates. Conidial germination required 7 to 9 h of incubation for five of the six species; germination of *A. terreus* conidia required 14 to 20 h. Similar results (8 to 10 h) have been reported for *A. fumigatus* and *A. flavus* (16, 18). Overall, the MICs of the established and investigational agents obtained with both conidial suspensions were the same or within a 2-dilution range (Table 1). In prior studies, germinated conidida had no effect, or no significant effect, on the MICs of itraconazole, amphotericin B (for 3 to 10 *A. fumigatus* and *A. flavus* strains) (16, 18), voriconazole, and posaconazole (for *A. fumigatus*) (18). Therefore, the data obtained in this study are in agreement with those in previous reports for *A. fumigatus* and *A. flavus*. This report also suggests that germinated conidia had no substantial effect on the MICs for the other four *Aspergillus* spp. tested or on the MICs of the other new triazole, ravuconazole (Table 1).

The MFCs of the three new triazoles, amphotericin B, and itraconazole obtained with both types of conidia are listed in Table 2. Overall, both types of inocula also yielded similar fungicidal results. A prior study found no significant difference in the killing ability (killing curve experiments) of four antifungal agents against germinated and nongerminated conidia

TABLE 2. MFCs for germinated and nongerminated conidia of *Aspergillus* spp.

Species (no. of strains) and antifungal agent	MFC[a] (MFC_{90}) [μg/ml]	
	Germinated	Nongerminated
A. flavus (12)		
Amphotericin B	0.5–2 (2)	0.5–2 (2)
Itraconazole	0.2–8 (1.0)	0.06–8 (0.5)
Voriconazole	0.12–8 (2)	0.2–8 (2)
Posaconazole	0.06–2 (1.0)	0.12–1.0 (1.0)
Ravuconazole	0.2–4 (4)	0.12–2 (2)
A. fumigatus (30)		
Amphotericin B	0.2–8 (2)	0.5–8 (4)
Itraconazole	0.2–>8 (8)	0.12–>8 (8)
Voriconazole	0.06–8 (4)	0.06–>8 (2)
Posaconazole	0.12–>8 (2)	0.06–>8 (2)
Ravuconazole	0.5–>8 (>8)	0.2–>8 (>8)
A. nidulans (10)		
Amphotericin B	0.2–8 (1.0)	0.5–8 (1.0)
Itraconazole	0.12–>8 (0.5)	0.06–>8 (0.5)
Voriconazole	0.12–2 (1.0)	0.06–2 (1.0)
Posaconazole	0.03–2 (1.0)	0.06–2 (1.0)
Ravuconazole	0.06–2 (2)	0.06–2 (1.0)
A. niger (7)		
Amphotericin B	0.5–2 (1.0)	1.0–2 (1.0)
Itraconazole	0.5–8 (2)	0.2–4 (0.5)
Voriconazole	0.5–2 (2)	0.2–2 (1.0)
Posaconazole	0.2–1.0 (0.5)	0.2–0.5 (0.5)
Ravuconazole	2–>8 (8)	2–>8 (8)
A. sydowii (1)		
Amphotericin B	2 (ND[b])	2 (ND)
Itraconazole	>8 (ND)	>8 (ND)
Voriconazole	4 (ND)	2 (ND)
Posaconazole	2 (ND)	2 (ND)
Ravuconazole	0.5 (ND)	0.5 (ND)
A. terreus (12)		
Amphotericin B	1.0–4 (4)	1.0–>8 (>8)
Itraconazole	0.06–8 (2)	0.12–>8 (2)
Voriconazole	1.0–>8 (>8)	1.0–>8 (>8)
Posaconazole	0.06–4 (2)	0.12–4 (2)
Ravuconazole	4–>8 (8)	4–>8 (>8)

[a] Fewer than three colonies.
[b] ND, not determined.

of *A. fumigatus* (18). Although standard conditions are not available for determination of fungicidal activities against fungi, the fungicidal activities of voriconazole (3, 15, 23, 24), posaconazole (9, 20), and ravuconazole (10) against *Aspergillus* spp. have been evaluated. Although prior data have been obtained by nonstandardized MFC measurement procedures, the amphotericin B and voriconazole MFC_{90}s for *A. terreus* were higher (>8 μg/ml) than those for the other species tested (0.5 to 4 μg/ml) in this and other studies (20, 23). MFC ranges of the other agents similar to those listed in Table 2 have been published for *Aspergillus* spp. (3, 9, 10, 15, 20).

Reports of the testing of hyphal susceptibility to antifungal agents and unsuccessful attempts to standardize this procedure have been scanty (2, 11, 12, 14). A suitable hyphal suspension should contain pure, fully viable, and uniformly dispersed hyphae without mycelial mats (microcolonies). Otherwise, the density of the stock suspensions cannot be corrected or accurately diluted. Damage to viable hyphae also can occur during the appropriate grinding procedure (12), and 12 to 24 h of incubation is usually required. Unusually high amphotericin B MIC endpoints have been reported since the 1950s for *A. fumigatus* (11, 17) and later for *A. nidulans* (25) when hyphae were tested. Recently, MICs and MFCs for hyphae of other molds were substantially higher than those obtained with nongerminated conidia (13). However, hyphal and nongerminated conidial inoculum sizes were comparable for only 12 of the 50 inocula evaluated in that study. The prolonged incubation needed for hyphal growth probably increased the mycelial mass, thereby altering the size of the hyphal inoculum. In contrast, amphotericin B (2, 22) and itraconazole (5) MICs have been comparable when they were obtained by employing conidial and hyphal inocula of *A. fumigatus* and *A. flavus*. The lack of a standardized procedure by which to obtain suitable hyphal inoculum suspensions has precluded meaningful evaluations of in vitro results with a hyphal inoculum.

In conclusion, the data obtained in this and other studies indicate that MICs for isolates of *Aspergillus* spp. can be obtained by using a nongerminated conidial inoculum. Preparation of such suspensions is a faster, more convenient, and more economic procedure for use in the clinical laboratory than that for germinated conidial inocula. The MICs and MFCs obtained in this and other studies also suggest that interlaboratory evaluations are warranted to investigate the reliability and clinical usefulness of the determination of MFCs of both established and investigational agents for molds.

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