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Effect of Increasing Inoculum Sizes of Pathogenic Filamentous Fungi on MICs of Antifungal Agents by Broth Microdilution Method

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Inoculum size is a critical variable in development of methods for antifungal susceptibility testing for filamentous fungi. In order to investigate the influence of different inoculum sizes on MICs of amphotericin B, 5-fluorocytosine, itraconazole, and miconazole, 32 clinical isolates (8 *Aspergillus fumigatus*, 8 *Aspergillus flavus*, 5 *Rhizopus arrhizus*, 8 *Pseudallescheria boydii*, and 3 *Fusarium solani* isolates) were studied by the broth microdilution method. Four inoculum sizes were studied: 1×10^2 to 5×10^2, 1×10^3 to 5×10^3, 1×10^4 to 5×10^4, and 1×10^5 to 5×10^5 CFU/ml. The National Committee for Clinical Laboratory Standards reference method for antifungal susceptibility testing in yeasts was modified and applied to filamentous fungi. The inoculum was spectrophotometrically adjusted, and all tests were performed in buffered medium (RPMI 1640) at pH 7.0 with incubation at 35°C for 72 h. MICs were read at 24, 48, and 72 h. Amphotericin B showed a minimum effect of inoculum size on MICs for all species with the exception of *P. boydii* ($P < 0.05$). A significant effect of inoculum size on MICs was observed with 5-fluorocytosine, for which there was an increase of more than 10-fold in MICs against all *Aspergillus* spp. between inoculum concentrations of 10^2 and 10^4 CFU/ml ($P < 0.001$). For itraconazole, the results showed a more species-dependent increase of MICs, most strikingly for *R. arrhizus* and *P. boydii*. Miconazole, which was tested only with *P. boydii*, did not demonstrate a significant effect of inoculum size on MICs. In summary, the effect of inoculum size on MICs for filamentous fungi was dependent upon the organism and antifungal compound tested. Thus, among antifungal compounds, itraconazole and 5-fluorocytosine demonstrated significant inoculum effects, while amphotericin B and miconazole showed comparatively minimum inoculum effects against pathogenic filamentous fungi. Moreover, among filamentous fungi, *P. boydii* and *R. arrhizus* exhibited the greatest inoculum effect.

The importance of infections due to filamentous fungi, particularly *Aspergillus* spp., *Fusarium* spp., *Rhizopus* spp., and *Pseudallescheria boydii* (1, 13, 15, 24), and the development of new antifungal agents, such as itraconazole (ITZ), have increased the demand for in vitro determination of MICs. This demand is especially urgent for immunocompromised patients with filamentous fungal infections, in whom treatment is difficult and often unsuccessful (10, 12). There have been major advances in development of reference methods by the National Committee for Clinical Laboratory Standards (NCCLS) for antifungal susceptibility determination for yeasts (14). While there is no reference method for filamentous fungi, studies addressing standardized methodology are being actively pursued (6, 8). The choice of assay media and incubation temperature and time as well as different approaches in scoring are important factors for determination of MICs against filamentous fungi. Studies with bacteria and yeasts clearly demonstrate that the concentration of the inoculum is also an important factor in determination of MICs (2). Little is known, however, about the effect of inoculum size on MICs for pathogenic filamentous fungi. We therefore studied the effect of a wide range of inoculum concentrations (10^2 through 10^5 CFU/ml) of a panel of medically important filamentous fungi on the MICs of amphotericin B (AmB), 5-fluorocytosine (5-FC), ITZ, and miconazole (MIZ).

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MATERIALS AND METHODS

Organisms. The following 32 clinical isolates were used in this study: *Aspergillus fumigatus* (8 isolates), *Aspergillus flavus* (8 isolates), *Rhizopus arrhizus* (5 isolates), *Fusarium solani* (3 isolates), and *P. boydii* (8 isolates). Two isolates of *Paecilomyces variotii* (ATCC 22319 and 93-PV-1) were used as quality controls. All fungi were subcultured onto potato dextrose agar (PDA) at 35°C and identified on the basis of colonial and microscopic morphologic characteristics (12, 22). All isolates were stored at −70°C on PDA (Wheaton, Millville, N.J.). For each experiment, strains were subcultured onto PDA slants (Remel, Lenexa, Kans.) for 48 h at 35°C and maintained 4 more days at room temperature (7 days for *P. boydii*). The numbers and origins of these isolates are outlined in Table 1.

Antifungal drugs. Four antimycotic drugs were used in this study: AmB (Bristol-Myers Squibb, Princeton, N.J.), ITZ (Janssen Research Foundation, Beerse, Belgium), MIZ (Janssen Research Foundation), and 5-FC (F. Hoffmann-La Roche & Co., Basel, Switzerland). MIZ was tested only against *P. boydii*. AmB, ITZ, and MIZ were provided by the manufacturers as powders. AmB was diluted in sterile distilled water, and stock solutions (5 mg/ml) were kept frozen at −70°C for further use. ITZ and MIZ were diluted in polyethylene glycol (molecular weight, 400; Sigma Chemical Co., St. Louis, Mo.) for stock solutions containing 5 and 10 mg/ml, respectively. 5-FC was provided as a working solution of 10 mg/ml. Additive drug dilutions to concentrations from 160 to 0.3 μg/ml for AmB, ITZ, and MIZ were made. For 5-FC, the concentrations were 640 to 1.25 μg/ml. All drugs except ITZ were diluted in RPMI 1640 (BioWhittaker, Inc., Walkersville, Md.) with L-glutamine and buffered with MOPS (morpholinepropanesulfonic acid; 0.165 M; final pH 7.0) without sodium bicarbonate. Because of the known poor solubility of ITZ, this drug was diluted 10-fold in polyethylene glycol 400. All drugs were then further diluted 1:5 in RPMI 1640. A volume of 100 μl of each concentration was dispensed in microtiter wells (96-U-bottom-well microtiter plate; Costar, Cambridge, Mass.), which resulted in final drug concentrations from 16 to 0.032 μg/ml for AmB, ITZ, and MIZ and concentrations from 64 to 0.125 μg/ml for 5-FC. All plates were prepared in advance, wrapped with aluminum foil to prevent dehydration, and maintained at −70°C.

Inoculum preparation. A spectrophotometric method, as described in detail previously (7), was used for the inoculum preparation. Briefly, fresh mature isolates on PDA slants were covered with 7 ml of saline and gently scraped with a sterile transfer pipette. Heavy particles were allowed to settle, and the supernatant was transferred to sterile tubes. The turbidities were measured with a spectrophotometer (Spectronic 20; Milton Roy, Rochester, N.Y.) at 530 nm. The

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TABLE 1. Filamentous fungi used to study effect of inoculum on MICs of antifungal agents

Organism	Isolate no.	Origin
A. fumigatus	AF-4A	BAL[a] fluid
	AF-7A	Sputum
	AF-27A	Sputum
	AF-28A	Lung biopsy
	AF-29A	Postmortem lung
	AF-30A	Skin biopsy
	AF-31A	Sinus drainage
	AF-33A	BAL fluid
A. flavus	AFI-8B	Sinus biopsy
	AFI-9B	Sputum
	AFI-10B	BAL fluid
	AFI-6	Sinus aspirate
	AFI-7	Nasal turbinate
	AFI-9	Nares culture
	AFI-50	Postmortem lung
	AFI-3066	BAL fluid
F. solani	FS-26	Foot ulcer
	FS-27	Cutaneous lesion
	FS-28	Sinus biopsy
P. boydii	PB-36	BAL fluid
	PB-194	Pharyngeal mass
	PB-197	BAL fluid
	PB-725	Bone biopsy
	PB-753	Sputum
	PB-895	Blood culture
	PB-1216	Leg biopsy
	PB-1434	Knee fluid
R. arrhizus	RA-16	BAL fluid
	RA-17	Sinus biopsy
	RA-18	Lung biopsy
	RA-19	Postmortem lung
	RA-20	Sinus drainage

[a] BAL, bronchoalveolar lavage.

inoculum was adjusted with saline to achieve a starting inoculum concentration of 1×10^6 to 5×10^6 CFU/ml. The inoculum was further diluted 1:5 and then diluted 1:10 in RPMI 1640 three times, to obtain the desired concentrations after 1:1 dilution in the microtiter wells (100 μl) of 1×10^5 to 5×10^5, 1×10^4 to 5×10^4, 1×10^3 to 5×10^3, and 1×10^2 to 5×10^2 CFU/ml. The viability and inoculum sizes of all strains and the concentrations were checked by using quantitative colony counts. Following 1:1,000, 1:100, and 1:10 dilutions in saline for the three highest inoculum concentrations, 100 μl was plated in duplicate on Sabouraud agar. For the 10^2-CFU/ml suspension, 100 μl was directly used for the check plates. The plates were incubated for 48 h at 35°C, with the exception of *R. arrhizus* plates, which were incubated for 24 h.

Susceptibility testing. The MICs of all drugs against all isolates were determined in duplicate, and endpoint readings were performed by using the scoring criteria previously described in a collaborative study of susceptibility testing of yeasts (18). Each growth control was assigned 4+ (100%), and the turbidity of each well was classified as follows in comparison with the control: 3+, growth reduction of 25% or less (slight reduction in turbidity); 2+, reduction of as much as 50% (prominent reduction); and 1+, growth reduction of 75% (slightly hazy) or more. No visible growth was recorded as 0 (optically clear). Readings were performed after 24, 48, and 72 h of incubation at 35°C by using a microtiter reading mirror. Twelve microtiter trays were necessary for a panel of eight strains in four concentrations with three antifungal agents. The lowest drug concentration was dispensed in row 1. The wells of row 11 were used as a medium control, and row 12 served as a growth control for each strain. For AmB, the MIC was read as the lowest drug concentration which prevented visible growth. For 5-FC, ITZ, and MIZ, the MIC was considered the drug concentration which reduced the growth by 75% (1+) or more in comparison with the growth control. Different criteria for AmB versus azoles for endpoint determination are necessary, as AmB generally gives clear endpoints while azoles tend to produce less clearly defined endpoints.

Data analysis. The mean MIC ± standard error of the mean (SEM) was calculated for each species, inoculum concentration, and time point from two separate experiments. Off-scale results were included in the analysis, such that values of >64 or >16 μg/ml were converted to the next higher drug concentration (128 or 32 μg/ml, respectively). MICs of ≤0.032 or ≤0.125 μg/ml were assigned to the next lower concentration (0.016 or 0.062 μg/ml, respectively). The significance of differences between mean values for inocula was determined by using Student's *t* test. *P* values of <0.05 were considered statistically significant.

RESULTS

Inoculum quantification and growth. Turbidities for *A. fumigatus* and *P. variotii* were adjusted to 80% transmission (range, 80 to 81%), and that for *A. flavus* was adjusted to 78%. The transmission rate for *R. arrhizus*, *P. boydii*, and *F. solani* was 68% (range, 68 to 69%). The spectrophotometric inoculum preparation and quantification were reproducible throughout all experiments and for all organisms tested (data not shown). All 34 isolates tested, with the exception of *P. boydii*, grew in the microtiter plates to optically detectable turbidities within 24 h. Visible growth of *P. boydii* occurred at all inoculum concentrations after 48 h of incubation.

MICs. An inoculum effect was observed for all organisms. The magnitude of increasing MIC with increasing inoculum was dependent on the reading time and the antifungal compound.

AmB. The results for AmB are summarized in Table 2 and Fig. 1. There was a shallow linear two- to threefold increase of MICs across the range of inocula (10^2 to 10^5) at the same time points for all species tested other than *P. boydii*. The greatest difference (ninefold) was observed with *P. boydii*, ranging from 0.71 μg/ml for the 10^2-CFU/ml inoculum to 7.06 μg/ml for 10^5 CFU/ml, both of which were seen at the 48-h reading ($P \leq 0.001$).

ITZ. The MICs of ITZ are shown in Table 3 and Fig. 2. For all 16 isolates of *Aspergillus* spp., there was a moderate effect of inoculum size on MICs of 2- to 14-fold within the same incubation period. For example, there was a five- to sixfold increase in the MIC of ITZ for *A. fumigatus* and a 14-fold increase for *A. flavus* at 24 h ($P \leq 0.001$). For the isolates of *R. arrhizus*, the MICs increased more precipitously (10- to 40-fold) at the higher inoculum concentrations of $>10^4$ CFU/ml (Fig. 2) ($P \leq 0.001$). All *F. solani* strains showed resistance to ITZ (>16 μg/ml). Marked increases of 30- to >100-fold in MICs also were observed for *P. boydii* between 10^2 and 10^4 and between 10^2 and 10^5 CFU/ml ($P \leq 0.001$).

5-FC. The effects of inoculum size on MICs of 5-FC are given in Table 4. Values obtained for *A. fumigatus* and *A. flavus* showed a difference of more than 10-fold between the lowest and highest inoculum concentrations at the 24-h reading ($P \leq 0.001$). All MICs after 48 h of incubation were ≥64 μg/ml. 5-FC was not active against *R. arrhizus* and *F. solani* strains.

MIZ. MIZ, which was tested only with *P. boydii*, had a significant increase of MIC between 10^2 and 10^5 CFU/ml. The MIC at 48 h increased from 0.09 to 1.94 μg/ml ($P < 0.001$), and that at 72 h increased from 0.22 to 11.88 μg/ml ($P < 0.003$).

DISCUSSION

The NCCLS Subcommittee on Antifungal Susceptibility Testing has conducted numerous studies leading to proposed standards for antifungal susceptibility testing in yeasts (14). Within those studies, methodological questions, including assay media, inoculum quantification, incubation temperature and duration, and determination of endpoint criteria for different antifungal compounds, were evaluated (9, 11, 16, 17). The same questions for filamentous fungi are currently being addressed (6, 8). For the purposes of standardization, the inoculum concentration is a critical variable. The effect of increasing inoculum size causing an increase in the MIC of an antimicrobial compound is defined as the inoculum effect (2).

The present study demonstrates that individual antifungal compounds have different propensities for inoculum effect.

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FIG. 1. Effect of increasing inocula of filamentous fungi on MICs of AmB. Values are means ± SEM.

TABLE 2. Mean MICs of AmB at different inoculum concentrations and reading times

Organism (no. of isolates)	MIC (μg/ml) at the indicated inoculum concn and time[a]											
	1×10^2–5×10^2 CFU/ml			1×10^3–5×10^3 CFU/ml			1×10^4–5×10^4 CFU/ml			1×10^5–5×10^5 CFU/ml		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
A. fumigatus (8)	0.53 (0.03)	0.94 (0.04)	1.00 (0.00)	0.81 (0.06)§	0.94 (0.04)	1.22 (0.12)	0.88 (0.06)§	0.97 (0.03)	1.25 (0.11)†	0.94 (0.04)§	1.13 (0.09)	1.63 (0.13)§
A. flavus (8)	0.84 (0.06)	1.00 (0.08)	1.22 (0.12)	1.00 (0.00)†	1.19 (0.10)	1.56 (0.13)	1.13 (0.09)†	1.81 (0.19)§	2.19 (0.19)§	1.69 (0.12)§	3.50 (0.39)§	4.50 (0.56)§
R. arrhizus (5)	0.26 (0.03)	0.45 (0.03)	0.50 (0.06)	0.33 (0.04)	0.55 (0.08)	0.78 (0.09)†	0.43 (0.04)¶	0.53 (0.06)	0.85 (0.08)¶	0.80 (0.08)§	1.05 (0.12)§	1.85 (0.30)§
F. solani (3)	0.92 (0.08)	1.17 (0.17)	1.33 (0.21)	1.33 (0.21)	1.50 (0.22)	1.67 (0.21)	1.50 (0.22)†	2.00 (0.00)§	2.33 (0.33)†	2.00 (0.00)§	2.33 (0.33)†	2.67 (0.42)†
P. variotii (2)	NG	0.50 (0.18)	0.69 (0.19)	0.50 (0.00)	0.75 (0.14)	1.00 (0.00)	0.50 (0.00)	0.88 (0.13)	1.00 (0.00)	0.88 (0.13)	1.25 (0.25)	1.25 (0.25)
P. boydii (8)	NG	0.71 (0.09)	1.42 (0.18)	NG	1.18 (0.16)†	2.06 (0.27)	NG	2.16 (0.44)¶	4.19 (1.01)†	NG	7.06 (1.44)§	15.63 (3.38)§

[a] Values are means (±SEM) from two experiments for each species. Statistical significance in comparison to MICs of lowest inoculum concentration is indicated as follows: †, $P \leq 0.05$; ¶, $P \leq 0.01$; §, $P \leq 0.001$. NG, no growth.

Increasing inoculum size had a minimum effect on the MICs of AmB for all species except *P. boydii*. A significant inoculum effect was observed with 5-FC, for which there was an increase of more than 10-fold in the MICs against all *Aspergillus* spp. ITZ demonstrated a species-dependent increase of MICs, most strikingly for *R. arrhizus* and *P. boydii*. By comparison, there was no significant effect of the size of *P. boydii* inoculum on the MICs of MIZ.

This compound-dependent inoculum effect may be related to the different mechanisms of action of antifungal drugs. There also was a clear propensity of different species for inoculum effects, particularly as observed for *P. boydii*. Inoculum effects also have been found for bacteria and yeasts. At higher inoculum concentrations, bacteria which tend to be susceptible show a pattern of increasing resistance (2, 23). In previous studies with yeasts (21), increasing the size of test inocula also resulted in higher MICs of antimicrobial agents. Our results indicate that this principle applies also to filamentous fungi.

In analogy to susceptibility testing in bacteria, increasing the number of microbial targets may exceed the capability of a given drug amount to inhibit the growth of the organism in an in vitro test system. Inoculum concentration may particularly affect antifungal drugs whose antimicrobial activity is based on an enzymatic mechanism. For example, the activity of ITZ, which inhibits ergosterol biosynthesis by blocking the cyto-

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TABLE 3. Mean MICs of ITZ at different inoculum concentrations and reading times

Organism (no. of isolates)	MIC (μg/ml) at the indicated inoculum concn and time[a]											
	1×10^2–5×10^2 CFU/ml			1×10^3–5×10^3 CFU/ml			1×10^4–5×10^4 CFU/ml			1×10^5–5×10^5 CFU/ml		
	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h	24 h	48 h	72 h
A. fumigatus (8)	0.05 (0.02)	0.18 (0.02)	0.38 (0.05)	0.09 (0.02)	0.22 (0.01)	0.42 (0.05)	0.19 (0.02)§	0.37 (0.04)§	0.47 (0.02)	0.33 (0.03)§	0.45 (0.03)§	0.81 (0.06)§
A. flavus (8)	0.04 (0.01)	0.19 (0.02)	0.28 (0.04)	0.17 (0.02)§	0.31 (0.04)†	0.38 (0.04)	0.31 (0.04)§	0.51 (0.06)§	0.59 (0.06)§	0.55 (0.06)§	0.88 (0.06)§	0.91 (0.05)§
R. arrhizus (5)	0.41 (0.11)	1.45 (0.24)	2.55 (0.73)	1.04 (0.19)†	3.90 (1.51)	5.70 (1.44)	2.90 (0.87)†	>16.00§	>16.00§	>16.00§	>16.00§	>16.00§
F. solani (3)	≥16.00	≥16.00	≥16.00	≥16.00	≥16.00	≥16.00	≥16.00	≥16.00	≥16.00	≥16.00	≥16.00	≥16.00
P. variotii (2)	≤0.032	≤0.032	0.05 (0.03)	≤0.032	≤0.032	≤0.032	≤0.032	0.09 (0.06)	0.11 (0.05)	0.04 (0.01)	0.09 (0.06)	0.14 (0.06)
P. boydii (8)	NG	≤0.032	0.12 (0.04)	NG	0.09 (0.04)	0.59 (0.13)¶	NG	0.96 (0.20)§	3.59 (0.89)¶	NG	13.94 (3.65)§	>16.00§

[a] Values are means (± SEM) from two experiments for each species. Statistical significance in comparison to MICs of lowest inoculum concentration is indicated as follows: †, $P \leq 0.05$; ¶, $P \leq 0.01$; §, $P \leq 0.001$. NG, no growth.

FIG. 2. Effect of increasing inocula of filamentous fungi on MICs of ITZ. Values are means ± SEM.

chrome P-450-dependent C-14-demethylation of lanosterol, against *R. arrhizus*, *P. boydii*, and *Aspergillus* spp. decreased substantially (19). A decline in antifungal activity with increasing inoculum size also was seen with MIZ against *P. boydii*. The MICs of 5-FC showed a precipitous increase, perhaps related to increased amounts of thymidylate synthetase, the enzymatic target for 5-FC.

Mechanisms other than increasing the amount of enzymatic target, such as that which occurs with penicillin-binding proteins in bacteria, are associated with increasing MICs of β-lactam antibiotics (23). Similarly, as the population of fungi increases, a population of heteroresistant organisms may expand. AmB, by comparison, exhibited little inoculum effect against most fungal species, with the exception of *P. boydii*. The principle mechanism of action of AmB is a direct interaction with ergosterol in the fungal cell membrane rather than with an enzymatic target. This mechanism reduces the likelihood of alteration of the fungal target despite increasing numbers of fungal cells. Nevertheless, an inoculum effect was still observed with AmB against *P. boydii*, suggesting perhaps that other mechanisms attenuate the action of AmB. For example, production of catalase by fungi may diminish the lipoperoxidation produced by AmB, leading to higher MICs. The limited in vitro activity of 5-FC against *Aspergillus* spp. has also been shown by

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TABLE 4. Mean MICs of 5-FC at different inoculum concentrations

Organism (no. of isolates)	MIC (μg/ml) at the indicated inoculum concn[a] (CFU/ml)[a]			
	1×10^2–5×10^2 CFU/ml	1×10^3–5×10^3 CFU/ml	1×10^4–5×10^4 CFU/ml	1×10^5–5×10^5 CFU/ml
A. fumigatus (8)	3.38 (1.03)	14.88 (6.10)	44.50 (9.60)§	>64.00§
A. flavus (8)	0.46 (0.05)	1.73 (0.22)§	11.69 (2.33)§	>64.00§
R. arrhizus (5)	>64.00	>64.00	>64.00	>64.00
F. solani (3)	>64.00	>64.00	>64.00	>64.00
P. variotii (2)	0.06	0.06	0.22§	0.66§

[a] Values are means (± SEM) from two experiments for each species. All results were determined at 24 h. Statistical significance in comparison to MICs of lowest inoculum concentration is indicated as follows: †, $P \leq 0.05$; ¶, $P \leq 0.01$; §, $P \leq 0.001$.

Denning et al. (4), using a lower inoculum concentration of 10^3 CFU/ml and a different method. *Fusarium* spp. appear to be intrinsically resistant to ITZ and 5-FC. These findings are supported by the studies of Reuben et al. (20).

Inoculum preparation by spectrophotometer for antifungal susceptibility testing in yeasts has been widely studied (3, 9, 11, 16). Recently, the same issue was addressed for filamentous fungi (7). Depending on different optical properties of fungal elements, adjustment to species-specific transmission rates read at 530 nm revealed reproducible results in CFU for several species of filamentous fungi. Spectrophotometric inoculum preparation was evaluated also in a multicenter study with different species of pathogenic filamentous fungi (8). A mean of 1.4×10^6 CFU/ml was demonstrated in 95% of 180 preparations at transmission rates from 68 to 82%. Similar values were found in this study (data not shown).

The length of incubation also affected MICs in our study. For example, a sevenfold increase in the MICs of ITZ against isolates of *A. fumigatus* and *A. flavus* was observed between the 24- and 72-h reading points at the 10^2-CFU/ml concentration. More strikingly, MICs of 5-FC increased to >64 μg/ml for almost all species after 48 h of incubation. Perhaps extended incubation times allow resistant organisms to overgrow the initially susceptible subpopulation, leading to higher MICs. On the other hand, during prolonged incubation a degradation of antifungal compounds also may occur. Alternatively, a change in the capacity of antifungal azoles or 5-FC to bind to enzymatic targets, such as lanosterol C-14-demethylase or thymidylate synthase, may develop.

This study utilized a broth microdilution method. Espinel-Ingroff et al. (6), who tested AmB, ITZ, MIZ, fluconazole, and ketoconazole against *Aspergillus* spp., *P. boydii*, *R. arrhizus*, and *Sporothrix schenckii* with broth macro- and microdilution, found good intra- and interlaboratory agreement between the two tests (>90%) among the six participating centers. All other test conditions were adjusted to the NCCLS reference method for yeasts. Reuben et al. (20) used microdilution for susceptibility testing of 44 clinical isolates of *Fusarium* spp. Although a different method (medium, inoculum preparation, and scoring system) was used, the MICs obtained for AmB and ITZ were comparable to our results. While no inoculum effect was observed with AmB, there was a significant effect of inoculum concentration on the MIC of ITZ, resulting in MICs of ≥16 μg/ml at an inoculum size of 10^5 conidia (20).

The standardization of antifungal susceptibility testing not only will provide accurate and reproducible results among different laboratories but also will provide the basis for treatment of severe fungal infections in immunocompromised hosts. The interpretation of MICs of antifungal compounds for this purpose may vary, depending on the inoculum size. Fungi considered to be susceptible under low-inoculum conditions may be considered resistant under higher-inoculum conditions, even when the same breakpoint is used.

The effect of increasing inoculum on increasing MICs in vitro also may parallel treatment failures associated with delayed diagnosis. A delay in diagnosis of invasive fungal infection permits growth of higher local tissue concentrations of organisms. These higher concentrations of organisms in tissues in vivo may parallel the higher inoculum concentrations in vitro, resulting in organisms for which the MICs are higher and thus resulting in greater resistance to antifungal therapy. Depending on the localization of fungal infections and the impaired immune response of the host, the probability of an unsuccessful treatment increases as diagnosis is delayed and organisms proliferate. Denning and Stevens (5) reviewed 2,121 cases of treatment of invasive aspergillosis and found an overall response for AmB of only 55% (176 of 320 sites), despite therapy for >14 days. The impact of delay in diagnosis, inoculum effect, and host response may play important roles in outcome of this infection. Clearly, further studies correlating in vitro, in vivo, and clinical responses to antifungal therapy are needed in order to provide a foundation for interpretation of MICs against medically important filamentous fungi.

In summary, the effect of inoculum size of medically important filamentous fungi on MICs of antifungal compounds was dependent on the organism and compound tested. Among antifungal compounds, ITZ and 5-FC demonstrated significant inoculum effects, while AmB and MIZ had comparatively minimum inoculum effects against pathogenic filamentous fungi. Moreover, among filamentous fungi, *P. boydii* and *R. arrhizus* exhibited the greatest effect of inoculum size on MICs of antifungal compounds. These findings further contribute to a foundation to guide the development of future methods for standardizing antifungal susceptibility testing against filamentous fungi and for further elucidating in vitro-in vivo correlations.

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