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Analysis of the Influence of Tween Concentration, Inoculum Size, Assay Medium, and Reading Time on Susceptibility Testing of *Aspergillus* spp.

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The influence of several test variables on susceptibility testing of *Aspergillus* spp. was assessed. A collection of 28 clinical isolates was tested against amphotericin B, itraconazole, voriconazole, and terbinafine. Inoculum size (10^4 CFU/ml versus 10^5 CFU/ml) and glucose supplementation (0.2% versus 2%) did not have significant effects on antifungal susceptibility testing results and higher inoculum size and glucose concentration did not falsely elevate MICs. In addition, antifungal susceptibility testing procedure with an inoculum size of 10^5 CFU/ml distinctly differentiated amphotericin B or itraconazole-resistant *Aspergillus* strains in vivo from the susceptible ones. Time of incubation significantly affected the final values of MICs, showing major increases (two to six twofold dilutions, $P < 0.01$ by analysis of variance) between MIC readings at 24 and 48 h, but no differences were observed between antifungal susceptibility testing results obtained at 48 h and at 72 h. Significantly higher MICs were uniformly associated with higher concentrations of Tween ($P < 0.01$), used as a dispersing agent in the preparation of inoculum suspensions. The geometric mean MICs showed increases of between 1.5- and 10-fold when the Tween concentration varied from 0.1% (the geometric means for amphotericin B, itraconazole, voriconazole, and terbinafine were 1.29, 0.69, 1.06, and 0.64 μg/ml, respectively) to 5% (the geometric means for amphotericin B, itraconazole, voriconazole, and terbinafine were 1.97, 5.79, 1.60, and 4.66 μg/ml, respectively). The inhibitory effect of Tween was clearly increased with inoculum sizes of 10^5 CFU/ml and was particularly dramatic for itraconazole, terbinafine, and *Aspergillus terreus*. The inoculum effect was not observed when the Tween concentration was below 0.5% ($P > 0.01$).

The recent increased incidence of fungal infections and the growing number of new antifungal agents have multiplied the demand and interest for in vitro antifungal susceptibility testing (12, 20). For determination of MICs of antifungal agents two approved reference methods have been published by the National Committee for Clinical Laboratory Standards, the M27-A2 reference method for testing the susceptibility of yeast species (16), and the M38-A reference method for filamentous fungi (17). The M38-A methodology adopts some of the steps in yeast testing compiling the results of extensive collaborative studies on MIC test variables. However, this reference procedure exhibits some limitations such as long incubation periods for obtaining inoculum (7 to 10 days), problems for obtaining inoculum when molds present germinate to hyphal forms and do not form conidia, poor growth of some species with the assay medium recommended for susceptibility testing, and finally, the lack of correlation with clinical outcome (6, 7). Some studies have been conducted trying to overcome the limitations. Alternative assay media and modifications of inoculum size, incubation time, reading procedure, and endpoint determination have been assessed (3).

Taking into account the individuality of the behavior of fungi, minor variation of test variables can result in significant changes in MICs. Many sources of variation in antifungal MIC data have been published, such as the nature of the growth medium (14, 25), size of the inoculum (9), time of incubation (22), end-point criterion, pH (1, 13), and even the solvent used to prepare antifungal stock solutions (8) and the inoculum preparation procedure (21, 24). In addition, polyoxyethylene sorbitan monolaurate, Tween 20, a nonionic surfactant, has been widely employed as a dispersing agent in the preparation of conidial suspensions of hydrophobic fungi, particularly *Aspergillus* spp. It makes the dispersion of spores on water easier, yielding a more reliable procedure for inoculum preparation. However, surfactants can interact with both organisms and drugs affecting the activity in vitro of antimicrobial agents (2, 26). The M38-A document recommends the use of a drop of Tween 20 per ml (approximately 0.01 to 0.02 ml, 0.5 to 1%) (17) for inoculum preparation, but no standardized amounts of these agents have been employed in most of the reports published until now.

The aim of this study was to investigate the effect of several test variables on susceptibility testing of *Aspergillus* spp. The study included strains from patients who had failed to respond to itraconazole or amphotericin B therapy and isolates exhibiting high MICs whose in vitro data correlated with the results of animal models of infection (4, 5, 15, 19, 27).

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MATERIALS AND METHODS

Fungi. A collection of 28 clinical isolates was tested. They were selected to represent ranges of susceptibilities in vitro as broad as possible. This collection included (i) seven isolates of *Aspergillus fumigatus* kindly provided by David W.

TABLE 1. Range of MIC values obtained for the 28 isolates

Strain	Species	MIC (μg/ml)											
		Amphotericin B			Itraconazole			Voriconazole			Terbinafine		
		Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}	Range	MIC_{50}	MIC_{90}
CNM CM-1242	*A. fumigatus*	0.06–2.0	0.5	1.0	0.015–8.0	0.5	4.0	0.12–2.0	0.5	1.0	0.25–>16.0	4.0	>16.0
CNM CM-1243	*A. fumigatus*	0.12–2.0	0.5	1.0	0.015–>8.0	1.0	8.0	0.12–2.0	0.5	2.0	0.50–>16.0	8.0	>16.0
CNM CM-1244	*A. fumigatus*	0.12–2.0	0.5	1.0	0.015–>8.0	0.5	5.2	0.12–4.0	0.5	2.0	0.50–>16.0	8.0	>16.0
CNM CM-1245	*A. fumigatus*	0.06–1.0	0.5	1.0	0.015–>8.0	0.75	4.0	0.25–2.0	0.5	2.0	0.50–>16.0	8.0	>16.0
CNM CM-1246	*A. fumigatus*	0.06–2.0	0.5	1.0	0.06–>8.0	>8.0	>8.0	0.12–0.50	0.25	0.5	0.50–>16.0	4	>16.0
CNM CM-1247	*A. fumigatus*	0.25–4.0	1.0	2.0	0.50–>8.0	>8.0	>8.0	0.25–4.0	1.0	2.0	0.50–>16.0	>16.0	>16.0
CNM CM-1252	*A. fumigatus*	0.25–4.0	1.0	2.0	0.25–>8.0	>8.0	>8.0	0.12–4.0	1.0	2.0	0.50–>16.0	>16.0	>16.0
CNM CM-1572	*A. terreus*	0.12–8.0	2.0	8.0	0.03–8.0	0.5	4.0	0.50–8.0	2.0	4.0	0.03–8.0	0.5	4.0
CNM CM-1573	*A. terreus*	0.12–8.0	2.0	8.0	0.06–8.0	0.5	4.0	0.50–8.0	2.0	4.0	0.03–8.0	0.5	4.0
CNM CM-1574	*A. terreus*	0.03–8.0	2.0	4.0	0.03–8.0	0.5	4.0	0.50–8.0	2.0	4.0	0.03–8.0	0.5	4.0
CNM CM-1575	*A. terreus*	0.12–16.0	2.0	4.0	0.015–8.0	0.5	4.0	0.25–8.0	2.0	4.0	0.03–8.0	0.5	4.0
CNM CM-1576	*A. terreus*	0.12–8.0	2.0	4.0	0.015–8.0	0.5	4.0	0.25–8.0	2.0	4.0	0.03–8.0	0.5	4.0
CNM CM-1578	*A. terreus*	0.12–>16.0	2.0	8.0	0.015–8.0	0.5	4.0	0.25–8.0	1.0	4.0	0.03–8.0	0.5	4.0
CNM CM-1579	*A. terreus*	0.12–16.0	2.0	8.0	0.03–8.0	0.5	4.0	0.25–8.0	1.0	4.0	0.03–8.0	0.75	4.0
CNM CM-459	*A. flavus*	1.0–>16.0	8	>16.0	0.12–8.0	0.75	4.0	0.25–2.0	1.0	2.0	0.12–4.0	0.37	2.0
CNM CM-890	*A. flavus*	0.50–8.0	1.0	2.0	0.06–8.0	0.5	4.0	0.12–4.0	1.0	2.0	0.03–4.0	0.25	1.0
CNM CM-900	*A. flavus*	0.25–8.0	2.0	2.0	0.015–8.0	0.5	4.0	0.25–4.0	1.0	2.0	0.03–2.0	0.25	1.1
CNM CM-1248	*A. flavus*	1.0–8.0	2.0	4.0	0.03–8.0	0.5	4.0	0.25–4.0	1.0	2.0	0.03–2.0	0.25	1.0
CNM CM-1264	*A. flavus*	0.25–8.0	1.0	2.0	0.03–8.0	0.5	4.0	0.12–4.0	1.0	2.0	0.03–2.0	0.12	1.0
CNM CM-1295	*A. flavus*	0.50–8.0	2.0	4.0	0.06–4.0	0.5	4.0	0.25–4.0	1.0	2.0	0.03–4.0	0.25	1.0
CNM CM-1357	*A. flavus*	0.25–2.0	1.0	2.0	0.03–4.0	0.5	4.0	0.25–4.0	1.0	2.0	0.03–1.0	0.06	0.5
CNM CM-152	*A. niger*	0.12–4.0	0.5	1.0	0.06–>8.0	1.0	4.0	0.12–1.0	0.5	0.5	0.03–2.0	0.25	1.0
CNM CM-519	*A. niger*	0.06–2.0	0.5	1.0	0.12–>8.0	>8.0	>8.0	0.12–2.0	1.0	2.0	0.06–4.0	1.0	2.6
CNM CM-794	*A. niger*	0.12–8.0	1.0	2.0	0.015–2.0	0.25	1.0	0.12–0.25	0.12	0.25	0.03–2.0	0.25	1.0
CNM CM-879	*A. niger*	0.03–16.0	2.0	4.0	0.015–>8.0	>8.0	>8.0	0.12–4.0	2.0	2.0	0.03–4.0	0.5	2.0
CNM CM-1524	*A. niger*	0.12–4.0	1.0	1.0	0.06–>8.0	1.0	8.0	0.12–1.0	0.5	0.5	0.03–2.0	0.25	1.0
CNM CM-1562	*A. niger*	0.06–2.0	0.5	1.0	0.06–>8.0	1.0	8.0	0.12–1.0	0.5	1.0	0.03–4.0	0.5	2.0
CNM CM-1607	*A. niger*	0.12–4.0	1.0	2.0	0.06–>8.0	1.0	>8.0	0.12–2.0	0.5	1.0	0.06–4.0	0.5	2.0

Denning, CNM-CM-1242 (Mold Collection of the Spanish Center for Microbiology), CNM-CM-1243, CNM-CM-1244, CNM-CM-1245, CNM-CM-1246, CNM-CM-1247, and CNM-CM-1252; (ii) seven isolates of *Aspergillus terreus* (CNM-CM-1572 to CM-1579); (iii) seven isolates of *Aspergillus flavus* (CNM-CM-459, CNM-CM-890, CNM-CM-900, CNM-CM-1248, CNM-CM-1264, CNM-CM-1295, and CNM-CM-1357); and (iv) seven isolates of *Aspergillus niger* (CNM CM-152, CNM-CM-519, CNM-CM-794, CNM CM-879, CNM-CM-1524, CNM-CM-1562, and CNM-CM-1607). *A. fumigatus* ATCC 9197 and *Paecilomyces variotii* ATCC 22319 were included as control isolates in each set of experiments. All the strains were stored on slants or in water suspension at ambient temperature until used.

Antifungal drugs. The antifungal agents utilized were amphotericin B (Sigma-Aldrich Química, Madrid, Spain), itraconazole (Janssen Pharmaceutica, Madrid, Spain), voriconazole (Pfizer Ltd, Sandwich, United Kingdom), and terbinafine (Novartis, Basel, Switzerland). All of them were obtained as reagent-grade powders from their respective manufacturers. Stock solutions were prepared in 100% dimethyl sulfoxide (Sigma-Aldrich Química) at concentrations 100 times the highest concentration to be tested. All drugs were then diluted in the test medium and dispensed into 96-well flat-bottom microdilution trays and frozen at −20°C or −70°C (amphotericin B) until needed. The plates contained twofold serial dilutions of the antifungal drugs with a volume of assay medium of 100 μl. Two drug-free medium wells for sterility and growth controls were used. The range of concentrations tested was as follow: amphotericin B, from 16 to 0.03 μg/ml; itraconazole, from 8 to 0.015 μg/ml; voriconazole, from 64 to 0.12 μg/ml; and terbinafine, from 16 to 0.03 μg/ml.

Antifungal susceptibility testing. Antifungal susceptibility testing was performed simultaneously with different Tween concentration, assay medium, inoculum size, and incubation time. Tween 20 (Sigma-Aldrich Química) was added to facilitate the preparation of suspensions of spores of *Aspergillus* spp. Three sterile water-Tween solutions were used to assess their influence on MICs (5%, 0.5%, and 0.1%, corresponding to final volumes of 0.05, 0.005, and 0.001 ml, respectively, per ml of inoculum suspension). The surface of the colonies was covered with a sterile water-Tween solution and scraped with a sterile loop. Conidia were shaken vigorously for 10 s to break up clumps, transferred to a sterile tube, and then adjusted by microscopic enumeration with a cell-counting hemacytometer (Improved Neubauer Chamber; Merck, S. A., Madrid, Spain) to provide a final inoculum of 1×10^6 to 5×10^6 CFU/ml (24).

In addition, antifungal susceptibility testing was performed simultaneously with two assay media, (i) standard RPMI 1640, with glutamine and without bicarbonate, buffered to pH 7.0 with 0.165 M morpholinepropanesulfonic acid (MOPS) (Sigma-Aldrich Química), and (ii) RPMI 1640 supplemented with 18 g of glucose (Sigma-Aldrich Química) per liter to reach a final concentration of 2% (RPMI-2% glucose). Both media were prepared as double-strength solutions. Two different inoculum sizes were also tested, (i) 1×10^4 to 5×10^4 CFU/ml and (ii) 1×10^5 to 5×10^5 CFU/ml.

On the day of the test, each well was inoculated with 100 μl of the corresponding conidial inoculum suspension. All plates were incubated at 35°C. The MIC endpoint was defined as the lowest drug concentration that showed absence of growth (100% inhibition). Readings were made after 24, 48, and 72 h of incubation with the aid of a reading mirror. All the experiments were repeated three times on different days.

Data analysis. Statistical analysis was done with Statistical Package for the Social Sciences (version 12.0.) (SPSS S.L, Madrid, Spain). Both on-scale and off-scale results were included in the analysis. The off-scale MIC values were converted to the next concentration up or down. The effect of test variables on the MIC was analyzed with the analysis of variance test. In order to approximate a normal distribution, the MICs were transformed to $\log_2$ values. For some cases, there were clear outliers to the normal distribution after log transformation. Subsequently, results were also analyzed by the Mann-Whitney *U* test, a nonparametric test that permits the inclusion of data treated as outliers for the analysis of variance. $P < 0.01$ was considered significant.

RESULTS

Most of the isolates tested grew in the microtiter plates to detectable endpoints within 48 h. Table 1 summarizes the range of MICs obtained per isolate regardless of the combination of variables included in the study. Notably, a wide range of MICs was found for all the antifungal agents and species, advancing a great influence of variables on MICs.

In this sense, the concentration of Tween showed a dramatic effect on the MICs of antifungal compounds. The analysis of variance test applied to the data after log transformation pro-

TABLE 2. In vitro susceptibilities of Aspergillus spp. obtained with RPMI-2% glucose medium, an inoculum size of 10^5 UFC/ml, 48 of incubation, and different Tween 20 concentrations

Species (no. of isolates)	Tween concn (%)	MIC (μg/ml)							
		Amphotericin B		Intraconazole		Voriconazole		Terbinafine	
		Range	GM[a]	Range	GM	Range	GM	Range	GM
A. fumigatus (7)	5	0.50–4.0	1.18	4.0–>8.0	8.83	0.25–2.0	1.18	16.0–>16.0	27.13
	0.5	0.25–2.0	0.69	0.50–>8.0	3.28	0.25–4.0	1.22	4.0–>16.0	8.27
	0.1	0.25–2.0	0.57	0.25–>8.0	1.87	0.12–2.0	0.79	2.0–>16.0	5.94
A. terreus (7)	5	2.0–8.0	4.56	2.0–8.0	4.88	2.0–8.0	4.56	4.0	4
	0.5	2.0–8.0	4.27	0.06–1.0	0.48	1.0–4.0	1.94	0.50–2.0	0.88
	0.1	2.0–8.0	3.74	0.25–0.50	0.26	1.0–4.0	1.81	0.25–0.50	0.41
A. flavus (7)	5	1.0–>16.0	2.97	2.0–8.0	4.00	1.0–2.0	1.75	1.0–2.0	1.28
	0.5	0.50–8.0	1.94	0.50–1.0	0.65	1.0–2.0	1.35	0.12–0.50	0.33
	0.1	1.0–8.0	1.70	0.12–0.50	0.35	1.0–4.0	1.26	0.03–0.50	0.14
A. niger (7)	5	0.50–2.0	0.94	1.0–>8.0	6.56	0.12–2.0	0.69	1.0–4.0	2.21
	0.5	0.25–2.0	0.67	0.12–>8.0	1.75	0.12–2.0	0.67	0.25–2.0	0.61
	0.1	0.25–2.0	0.77	0.12–>8.0	1.34	0.12–2.0	0.69	0.25–1.0	0.41
Total (28)	5	0.50–>16.0	1.97	1.0–>8.0	5.79	0.12–8.0	1.60	1.0–>16.0	4.66
	0.5	0.25–8.0	1.40	0.06–>8.0	1.58	0.12–4.0	1.21	0.12–>16.0	1.15
	0.1	0.25–8	1.29	0.12–>8	0.69	0.12–4	1.06	0.03–>16	0.64

[a] GM, geometric mean.

vided significant differences among MICs obtained for each Tween concentration regardless of the inoculum size, the assay medium, and the time of reading.

The highest MICs were uniformly associated with the highest concentration of Tween (5%) when all the isolates were analyzed together (Table 2). This effect was particularly notable for itraconazole and terbinafine, for which the MICs obtained with the highest Tween concentration were significantly higher ($P < 0.01$). In addition, significant differences were found between the MICs of itraconazole and terbinafine achieved with concentrations of Tween of 0.5% and those achieved with the lowest concentration of Tween (0.1%, $P < 0.01$). The results for amphotericin B and voriconazole, however, were moderately influenced by the Tween concentration. The MICs for these agents were significantly higher ($P < 0.01$) only when the concentration of Tween used was maximal (5%).

By species, comparisons between the MICs obtained with 5% and 0.1% Tween also resulted in significant differences for the majority of them ($P < 0.01$), with major increases between MICs for itraconazole and terbinafine. *A. terreus* appeared as the species most influenced by Tween concentration, since the MICs for most of the antifungals tested were significantly different ($P = 0.00$). Table 2 exemplifies the MIC results per species for each Tween concentration, fixing the medium (RPMI-2% glucose), the inoculum size (10^5 CFU/ml), and the time of reading (48 h) as constants. The geometric mean MICs showed increases of between 1.5- and 10-fold when the Tween concentration varied from 0.1% (the geometric means for amphotericin B, itraconazole, voriconazole, and terbinafine were 1.29, 0.69, 1.06, and 0.64 μg/ml, respectively) to 5% (the geometric means for amphotericin B, itraconazole, voriconazole, and terbinafine were 1.97, 5.79, 1.60, and 4.66 μg/ml, respectively). In summary, the concentration of Tween emerged as having a significant influence on antifungal susceptibility testing of *Aspergillus* spp. but the magnitude of the effect was species and antifungal dependent.

Regarding inoculum size, changes in MICs were moderate when the inoculum size varied from 10^4 to 10^5 CFU/ml. In fact, higher inoculum sizes increased the MICs for all the antifungal agents tested, but these increases were not statistically significant ($P > 0.01$) if other test variables were fixed as constants. Most of the MICs rose one to two twofold dilutions (data not shown). However, a higher inoculum effect was observed when MICs were analyzed, taking into account the combined effect of inoculum size and Tween concentration. In this sense, significantly higher MICs were observed for antifungal susceptibility tests including both the highest inoculum size (10^5 UFC/ml) and the highest Tween concentration (5%). The combined effect of Tween concentration and size of inoculum on MICs is exemplified on Table 3, which exhibits MICs of antifungal agents after 48 h of incubation. The geometric mean MIC increased from 2- to 14-fold when the inoculum was adjusted to 10^5 UFC/ml and prepared with the highest concentration of Tween (5%). Again, the highest increases were observed for itraconazole and terbinafine. By species, *A. terreus* was the most influenced by the size of the inoculum.

Analyzing MICs by assay medium, antifungal susceptibility testing results for all the isolates grown in RPMI were identical to those obtained with RPMI supplemented with glucose. In addition, glucose supplementation did not affect the MIC values whatever the final Tween concentration used for the inoculum preparation ($P > 0.01$).

Time of incubation significantly affected the final MICs (data not shown). A major increase (two to six dilutions) was observed between 24 and 48 h ($P < 0.01$), a difference that could be explained by the fact that many isolates needed 48 h for exhibiting detectable growth. However, differences between MIC readings at 48 and 72 h were minimal (no more than two dilutions) for most of the antifungals analyzed. A major dependency on the length of the period of incubation was noted for amphotericin B, whose MICs were significantly higher for 72 h ($P < 0.01$), but the MICs after 48 and 72 h of

TABLE 3. MIC results showing the combined effect of Tween concentration and inoculum size after 48 h of incubation

Species (no. of isolates)	Tween concn (%)	Inoculum (CFU/ml)	MIC (μg/ml)							
			Amphotericin B		Intraconazole		Voriconazole		Terbinafine	
			Range	GM[a]	Range	GM	Range	GM	Range	GM
A. fumigatus (7)	0.1	10^4	0.25–1	0.49	0.06–>8.0	1.36	0.12–1.0	0.52	1.0–>16.0	3.03
	5	10^5	0.5–4.0	1.18	4.0–>8.0	8.83	0.25–4.0	1.24	16.0–>16.0	27.13
A. terreus (7)	0.1	10^4	0.5–2.0	1.16	0.06–0.50	0.11	0.25–1.0	0.67	0.12–0.50	0.30
	5	10^5	2.0–8.0	4.27	2.0–8.0	4.72	2.0–8.0	3.87	4.0	4.0
A. flavus (7)	0.1	10^4	0.50–>16.0	1.20	0.06–0.50	0.17	0.25–2.0	0.69	0.03–0.25	0.09
	5	10^5	0.50–>16.0	2.69	2.0–8.0	4.06	1.0–2.0	1.66	0.50–4.0	1.22
A. niger (7)	0.1	10^4	0.06–2.0	0.42	0.03–>8.0	0.80	0.12–2.0	0.51	0.03–1.0	0.18
	5	10^5	0.50–2.0	0.82	1.0–>8.0	6.25	0.12–2.0	0.65	1.0–4.0	1.97
Total (28)	0.1	10^4	0.06–>16.0	0.73	0.03–>8.0	0.39	0.12–2.0	0.59	0.03–>16.0	0.36
	5	10^5	0.50–>16.0	1.83	1.0–>8.0	5.71	0.12–8.0	1.51	0.50–>16.0	4.50

[a] GM, geometric mean.

incubation were comparable ($P > 0.05$) for the rest of the antifungal agents evaluated.

DISCUSSION

A great deal of progress has been achieved in antifungal susceptibility testing of filamentous fungi. A reproducible reference methodology has been developed (17). However, the reference method cannot be the best technique for testing all organisms and it is possible that new antifungal agents require modified methods or new assays show a better correlation between in vitro and in vivo susceptibility results and patient outcome. Modifications have been proposed, but minor variations can have significant influences on MICs and should be analyzed in depth before being put into clinical practice. Significant influences on MICs have been described depending on time of incubation and other test variables (23), and some reports have pointed out that even large inoculum size and glucose supplementation could falsely elevate the MICs (18).

However, Denning et al. demonstrated that inoculum sizes higher than those proposed, 1×10^4 to 5×10^4 CFU/ml, generate reproducible in vitro susceptibility data for *Aspergillus* spp. that can predict clinical outcome (4). They were able to identify test conditions in vitro that consistently differentiated resistant *Aspergillus* strains in vivo from the susceptible ones. The former had high itraconazole MICs and demonstrated no benefit over untreated controls in a murine model when treated with different itraconazole doses. Regarding glucose supplementation, previous reports suggest no effect on *Aspergillus* growth rate when RPMI is supplemented with 2% glucose, even after incubation for 100 h (14).

Our results agree with previous findings since no changes in MICs were found with RPMI or RPMI supplemented with glucose, and MICs were not falsely elevated when an inoculum size of 10^5 UFC/ml was used. In addition, the antifungal susceptibility testing procedure with an inoculum size of 10^5 UFC/ml distinctly identified resistant *Aspergillus* strains in vivo as the MICs of amphotericin B and itraconazole were >2 μg/ml and >8 μg/ml, respectively.

A significant effect of surfactant on the MIC results of *Aspergillus* was observed. We have consistently obtained higher MICs when major concentrations of Tween were used for the inoculum preparation. Some reports have described the effect of nonionic detergent on the activity in vitro of antimicrobial agents (10, 11). A great influence was found by Komatsuzawa et al. even with a concentration of the surfactant as low as 0.015%. Apparently, the effect of Tween is antifungal dependent, and we postulate that it could be related to the solubility of the antifungal in the medium used. It has been suggested that some surfactants interfere with antimicrobial activity by solubilizing molecules of the antimicrobial agent within surfactant micelles, preventing the microorganism-agent interaction. The surfactant could modify the antifungal solubility index on RPMI and favor agent precipitation, allowing MICs to be increased. This study demonstrates that the inhibitory effect of Tween was dramatic for itraconazole and terbinafine and particularly significant for *A. terreus* isolates.

The use of Tween for inoculum preparation for antifungal susceptibility testing of *Aspergillus* spp. has been universally accepted. The results of the present study confirm the importance of standardizing the concentration of surfactant to use on inoculum preparation. Changes in MICs as great as four dilutions could change the classification of a strain from susceptible to resistant. This effect is clearly increased with an inoculum size of 10^5 UFC/ml. However, significant inoculum effect was not observed when Tween concentration was below 0.5%. Glucose supplementation of assay medium did not have a significant influence on MICs of the four antifungal agents tested. We conclude that special attention must be taken to the addition of Tween 20 for inoculum preparation of *Aspergillus* spp., in order to allow reproducible antifungal susceptibility testing results to be obtained and to avoid falsely elevated MICs.

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