JOURNAL OF CLINICAL MICROBIOLOGY, Apr. 1993, p. 1009–1012
0095-1137/93/041009-04$02.00/0

Vol. 31, No. 4

Rapid and Highly Reproducible Method for Antifungal Susceptibility Testing of *Aspergillus* Species

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Received 29 September 1992/Accepted 16 January 1993

A new method for determining the MICs for *Aspergillus* species with slant-shaking incubation is described. The method was rapid (24 h), produced sharp visual end points, and showed high reproducibility. Additionally, MICs measured by this method correlated well with, as well as were two- to sixfold lower than, those by a reference method.

Aspergillus species are common opportunistic pathogens, especially in immunocompromised patients. Various methods for susceptibility testing of these fungi have been reported previously, including the agar (2) and broth (10) dilution MIC methods and simultaneous measurement of the MICs and minimal fungicidal concentrations with semisolid agar (13) or broth (12). Use of the relative inhibition factor (8) was reported as a novel approach which might give better prediction of in vivo antifungal activity than the MIC. However, a standard testing method has not yet been established. We designed a rapid and highly reproducible MIC method and compared our results with those of a reference method already reported.

Fungal strains and antifungal agents. The *Aspergillus* species tested were 29 clinically isolated strains, including 18 strains of *Aspergillus fumigatus*, 2 of *A. flavus*, 2 of *A. terreus*, 6 of *A. niger*, and 1 of *A. nidulans*. One strain of each species was randomly selected to examine the reproducibility of the test. Amphotericin B (AMB; Bristol-Myers Squibb), miconazole (MCZ; Janssen), itraconazole (Janssen), and fluconazole (FCZ; Pfizer) were dissolved and diluted twofold in series in dimethyl sulfoxide to provide a 100× concentrated stock solution, ranging from 10,000 to 1.25 μg/ml. For the itraconazole preparation, a small magnetic stirrer was employed to dissolve the powder.

Susceptibility testing method. The medium used for susceptibility testing was a modified YM broth, which consisted of 0.3% malt extract (Difco), 0.3% yeast extract (Difco), 0.3% polypeptone (Wako Pure Chemical Industries Ltd., Osaka, Japan), and 2% glucose, dissolved in 1/15 M phosphate-buffered saline (pH 7.0). The test strains were precultured at 30°C for 5 days on Sabouraud dextrose agar (BBL). Inocula were prepared by washing spores from the surface of the agar with sterile 0.75% saline containing 0.1% Tween 80. The spore suspension was filtered through sterile gauze and adjusted with sterile saline to a concentration of 1.0×10^7 cells per ml with a hemocytometer. Tubes (18 by 180 mm) containing 5 ml of YM broth were prepared, and 50 μl of serially diluted stock solution of each drug was added. The same volume of dimethyl sulfoxide was added to the control tube. Then, these test broths were inoculated with 100 μl of the spore suspension. After being mixed and covered with Parafilm, the test tubes were tilted at a 70° angle and incubated at 30°C for 24 h on a reciprocal shaker (model R-1; Taitec Corporation, Tokyo, Japan), with a shaking frequency of 100 times per min. The MIC was determined visually to be the lowest concentration at which no significant mycelial growth was observed (Fig. 1). A small amount of filamentous growth, a small amount of diminutive mycelial balls, or only a few irregular mycelial balls were judged as no growth. Interface growth, which was observed along the air-broth interface, was also interpreted as no growth. Finally, the pH values of the media after 24 h of incubation were recorded. For the next procedure to measure the mycelial protein amount, the specimens were centrifuged at 1,500 × *g* for 10 min and washed with distilled water three times to remove the medium components. To each was added 5 ml of 3% perchloric acid ($HClO_4$; Wako) solution, and each solution was mixed vigorously. Each mixture was then heated at 90°C for 10 min and cooled to room temperature. After the addition of 0.5 ml of 5% Celite no. 545 suspension (Wako), each suspension was centrifuged at 1,500 × *g* for 10 min at 4°C. The supernatant was discarded, and 5 ml of an ethanol-ether (3:1)-mixed solution was added to the sediment. After centrifugation at 1,500 × *g* for 10 min at 4°C, 1 ml of 1 N NaOH was added to the sediment and mixed. This specimen was then boiled for 30 min, and 5 ml of distilled water was added and mixed. The supernatant obtained after centrifugation under the conditions described above was used as the sample for protein measurement. The protein amount was measured by a dye-binding assay (1): namely, 0.2 ml of protein reagent (Protein Assay Kit 1; Bio-Rad) was added to 0.8 ml of the sample in a protein-free tube and mixed. The A_{595} was measured. After the blank absorbance was subtracted, the protein amount in the sample was determined from the standard curve values. The 90% inhibitory concentration (IC_{90}) was determined as the lowest concentration of each drug resulting in more than 90% inhibition of the mycelial protein content compared with that of the control. The experiment described above was repeated five times in the reproducibility evaluation.

The reference method. According to the method described by Schmitt et al. (12), antifungal agents were initially dissolved in dimethyl sulfoxide and then diluted in distilled

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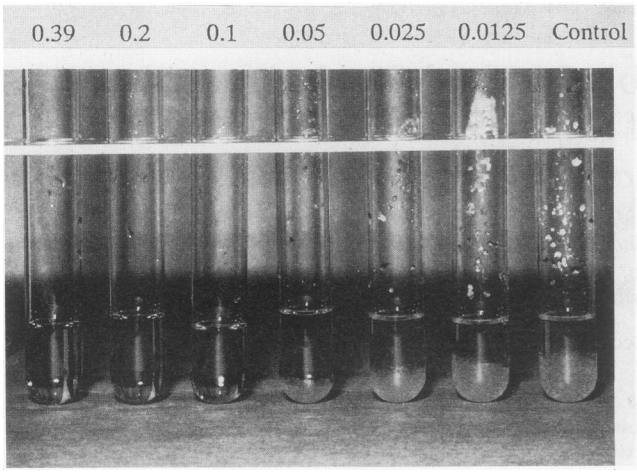

FIG. 1. AMB concentrations (in micrograms per milliliter) against *A. fumigatus* MF-27. A sharp end point was obtained after 24 h of incubation with shaking at 30°C in YM broth containing serially diluted AMB. The MIC was determined visually to be as 0.1 μg/ml.

water to produce twofold serial dilutions ranging from 50 to 0.025 μg/ml, and 0.1-ml portions of each concentration were added to the wells of microdilution plates. Spores suspended in distilled water were diluted in 2× yeast-nitrogen-glucose broth (Difco; 5% glucose) to produce the inoculum of 10^5 spores per ml. Portions of 0.1 ml of each spore suspension were added to microdilution tray wells that contained no drug (control), AMB, MCZ, or itraconazole. After 48 h of incubation at 35°C, the lowest concentration of each test drug that prevented visible growth was considered the MIC. *A. fumigatus* MF-27 was used as the reference strain for quality control.

The antifungal activities against the clinical isolates evaluated both by our method and by the reference method are compared in Table 1. Of the four drugs tested, itraconazole exhibited the most potent activity. By our method, MICs of 0.1 μg/ml or lower were evidenced for all species tested, with an especially low MIC of 0.0125 μg/ml for both *A. flavus* and *A. terreus*. By the reference method, the MICs were two- to sixfold higher than those found by our method. The Pearson correlation coefficients of both methods for AMB, MCZ, and itraconazole were 0.71, 0.59, and 0.64, respectively (Fig. 2). For FCZ, generally high MICs in excess of 100 μg/ml were evidenced for most strains.

Satisfactorily high reproducibility and agreement between the results with our method and the IC_{90}s were also demonstrated (Table 2). The Pearson correlation coefficients for AMB, MCZ, and itraconazole were 0.69, 0.84, and 0.83, respectively ($P < 0.05$).

The pH values of the YM broth after 24 h of incubation were between 6.5 and 6.8.

We employed YM broth as a testing medium because it had the advantages of supporting good cell growth and of being easy to prepare and economical. Other investigators have recommended avoiding media containing high concentrations of glucose because acid production by the fungal organisms may ultimately inhibit antifungal activity (8). However, the modified YM broth (containing 2% glucose) showed a permissible final pH decrease. However, as is widely known, this medium is inappropriate for testing of flucytosine activity because it contains antagonists such as purines, pyrimidines, and other nucleosides. As for FCZ, since in vivo studies for this agent were not conducted, the interpretation of these high MICs was difficult. However, since a discrepancy between in vitro and in vivo activities

TABLE 1. Antifungal activities of four drugs against clinical isolates of *Aspergillus* species

Fungal species	No. tested	Antifungal agent	MIC range (μg/ml)		Geometric mean (μg/ml)	
			Our method	Reference method	Our method	Reference method
A. fumigatus	18	AMB	0.025–0.2	0.39–1.56	0.15	0.75
	18	MCZ	0.39–3.13	0.78–25	1.15	7.07
	18	Itraconazole	0.0125–0.1	0.1–1.56	0.03	0.37
	6	FCZ	50–400	ND[a]	158	ND
A. flavus	2	AMB	0.39–1.56	0.78–1.56	0.78	1.17
	2	MCZ	0.39	3.13	0.39	3.13
	2	Itraconazole	0.0125	0.1	0.0125	0.1
	2	FCZ	100	ND	100	ND
A. terreus	2	AMB	0.2–0.39	0.78–1.56	0.3	1.17
	2	MCZ	0.78	1.56–3.13	0.78	2.34
	2	Itraconazole	0.0125	0.1	0.0125	0.1
	2	FCZ	100–200	ND	150	ND
A. niger	6	AMB	0.025–0.1	0.39–0.78	0.05	0.71
	6	MCZ	0.39–3.13	3.13–25	1.30	8.85
	6	Itraconazole	0.0125–0.025	0.2–0.78	0.017	0.32
	3	FCZ	100–400	ND	200	ND
A. nidulans	1	AMB	0.78	1.56	0.78	1.56
	1	MCZ	0.39	3.13	0.39	3.13
	1	Itraconazole	0.025	0.2	0.025	0.2
	1	FCZ	25	ND	25	ND

[a] ND, not done.

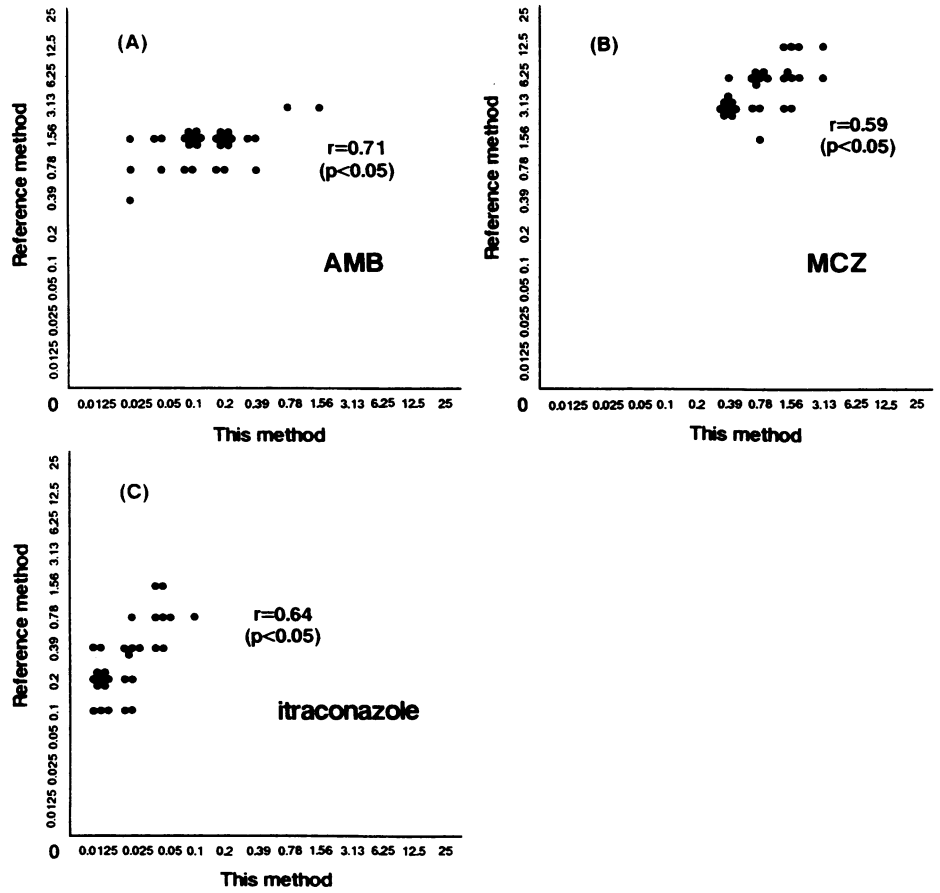

FIG. 2. Correlation of the median values among triplicate determinations by our method and by the reference method described by Schmitt et al. (13) for AMB, MCZ, and itraconazole.

was previously reported (9, 11), in vivo activity might not be reflected by this testing method.

Other important factors for this method were suggested to be the angle of tilting of the tubes used and the frequency of shaking. An angle of 70° and a frequency of 100 times per min allowed for the optimal formation of mycelial balls, and interface growth was minimal.

For susceptibility testing, sufficient contact between the drug and the organism is essential; by our testing method, the antifungal agent and conidia were thoroughly mixed in the liquid medium by shaking during the testing procedure. Recently, Denning et al. (3) reported a broth dilution method with shaking incubation. Our method differed from theirs mainly in the following: (i) shaking was more vigorous in our method and (ii) we did not take the interface growth as positive growth, because it was considered to be the result of insufficient mixing of the fungus and the drug.

With regard to the MIC ranges of AMB reported for *A. fumigatus*, the agar dilution method produced results of 0.14 to 0.6 μg/ml (2); the broth dilution method without shaking generated results of 0.2 to 0.8 μg/ml (12), 0.25 to 12.5 μg/ml (7), or 0.1 to 1.0 μg/ml (6); and the semisolid agar method produced results of 0.39 to 1.56 μg/ml (13). These values were generally higher than those generated by our method. A similar tendency in the MCZ (5) and the itraconazole (4, 5, 14) assays was also observed. Although a fair comparison was impossible, our results suggest that constant and sufficient contact between the conidia and the drug in the liquid medium incubated with shaking contributed to drawing the potency of the antifungal agent fully and to producing a relatively low MIC. Additionally, the high degree of agreement between the results by our method and the IC_{90}s by the reference method suggested that decisions based on visual MICs were satisfactorily objective in our setting.

The reproducibility of our MIC method was shown to be excellent in five repeated experiments with the three antifungal agents. The high degree of contact between the conidia and the antifungal agents might also have contributed.

TABLE 2. Reproducibility and agreement between the results by our method and IC_{90}s

Test method	No. (%) of results within one twofold dilution for five fungi in five separate assays			
	AMB (n = 25)	MCZ (n = 25)	Itraconazole (n = 25)	Total (n = 75)
Our method	24 (96)	25 (100)	25 (100)	74 (99)
IC_{90}	20 (80)	24 (96)	25 (100)	69 (92)
A[a]	23 (92)	22 (88)	25 (100)	70 (93)

[a] A, agreement within one twofold dilution between the results by our method and IC_{90}s for the same isolate on the same day.

We conclude that our new susceptibility testing method is a useful alternative for testing *Aspergillus* species.

We gratefully acknowledge Hideyo Yamaguchi for many helpful suggestions, Amanda Nishida for her assistance in the preparation of the manuscript, Janssen Pharmaceutical Ltd. for kindly supplying the MCZ and itraconazole, Bristol-Myers Squibb for providing the AMB, and Pfizer Pharmaceutical Ltd. for offering the FCZ.

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