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Comparison of Three Methods of Determining MICs for Filamentous Fungi Using Different End Point Criteria and Incubation Periods

C. LLOP,[1] I. PUJOL,[2] C. AGUILAR,[1] J. SALA,[3] D. RIBA,[4] AND J. GUARRO[1]*

Unitat de Microbiologia, Facultat de Medicina, Universitat Rovira i Virgili,[1] Laboratori de Microbiologia, Hospital Universitari de Sant Joan de Reus,[2] and Unitat de Bioestadística,[3] Facultat de Medicina, Universitat Rovira i Virgili 43201 Reus, Tarragona, and Laboratori d'Estadística Aplicada i de Modelització, Universitat Autònoma de Barcelona, Bellaterra,[4] Spain

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Three different methods were used to determine the in vitro activities of amphotericin B, ketoconazole, itraconazole, and flucytosine against 30 isolates of different genera of filamentous fungi. MICs were determined visually, with or without agitation, and spectrophotometrically by using a broth microdilution method. For amphotericin B, there was one end point reading criterion (the minimum concentration of antifungal that inhibited 100% of growth), but for azoles and flucytosine there were two (the minimum concentrations that inhibited 50 and 75% of fungal growth, respectively) after 48 and 72 h of incubation. All tests were performed in triplicate. An intraclass correlation coefficient (ICC) was used to evaluate the reproducibility of each of the methods and the correlation among them. The reproducibility of the three methods was very high (ICC of 0.808 to 0.992), particularly in the case of azoles and flucytosine. In general, the degree of reproducibility was highest for azoles and amphotericin B after 72 h of incubation and for flucytosine after 48 h of incubation. The degree of correlation among the three methods was very high (ICC of >0.98) with all of the antifungals under all the conditions tested. The end point reading criteria and the time of incubation affected neither the reproducibility of the methods nor their correlation, and their effect on MICs was statistically significant.

The prevalence and severity of fungal infections in humans and the development of new antifungal agents have increased the interest in antifungal susceptibility testing of pathogenic and opportunistic fungi. Despite many efforts, there are still some methodological problems. One of the most important problems occurs in the susceptibility testing of imidazole derivatives and flucytosine because they partially inhibit fungal growth (1). Work on the development of standardized procedures for testing filamentous fungi has led to the recent publication by the National Committee on Clinical Laboratory Standards (NCCLS) of a proposed reference document (11). This standard considers the visual comparison of fungal growth in wells with growth in a control well as the conventional procedure for reading the MICs. However, it has been suggested that the spectrophotometric end point reading of MICs has the advantages of objectivity, rapidity, and even the elimination of "inoculum dependence" (6, 7). It has also been suggested that agitating the microtiter plates before reading the MICs in the broth microdilution method can help to improve the quality of the readings, at least when testing yeasts (2, 13). Espinel-Ingroff et al. (5) recently carried out an intra- and interlaboratory study and suggested that MICs be evaluated colorimetrically, with Alamar blue as an indicator, after incubation times of 48 to 72 h and that the inoculum density be approximately 10^4 CFU/ml for a reference method for testing filamentous fungi. Here we compare spectrophotometric and visual methods with or without agitation for reading MICs by using a wide range of species of filamentous fungi.

* 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. E-mail: umb@astor.urv.es.

MATERIALS AND METHODS

Test organisms. Thirty strains (most of which cause human infections) of filamentous fungi (8) were used in the study. They were: *Aspergillus fumigatus* (*n* = 5), *Aspergillus niger* (*n* = 2), *Cladophialophora bantiana* (*n* = 1), *Cladophialophora carrionii* (*n* = 3), *Cladosporium cladosporioides* (*n* = 1), *Cladosporium elatum* (*n* = 1), *Cladosporium macrocarpum* (*n* = 1), *Cladosporium sphaerospermum* (*n* = 2), *Exophiala dermatitidis* (*n* = 1), *Fusarium solani* (*n* = 3), *Paecilomyces brevicaulis* (*n* = 3), *Paecilomyces lilacinus* (*n* = 2) *Paecilomyces variotii* (*n* = 4), and *Scopulariopsis chartarum* (*n* = 1). *Paecilomyces variotii* ATCC 36257 was used as quality control.

Medium. RPMI 1640 medium powder with L-glutamine and without sodium bicarbonate (GIBCO BRL, Life Technologies), buffered to pH 7.0 with MOPS (morpholinepropanesulfonic acid; Sigma Chemical Co.) was used as culture medium.

Antifungal drugs. Four antifungals were used: amphotericin B (Fungizone; E. R. Squibb & Sons, Barcelona, Spain), flucytosine (Hoffman-La Roche, Basel, Switzerland), and ketoconazole and itraconazole (Janssen Pharmaceutica, Beerse, Belgium). Ketoconazole, itraconazole, and flucytosine were provided as standard powders by the manufacturers. Stock solutions of amphotericin B and flucytosine were prepared at 1,000 and 5,000 μg/ml, respectively, with sterile distilled water. Ketoconazole was dissolved in 0.2 N HCl to a concentration of 50,000 μg/ml, and further dilutions with sterile distilled water were prepared to obtain a stock solution of 5,000 μg/ml. Stock solutions were stored for a maximum of 2 months at −20°C until ready to be used. A stock solution at 5,000 μg/ml of itraconazole was prepared in dimethyl sulfoxide at 75% and was immediately diluted with sterile distilled water and distributed into the microplates as described below.

Preparation of fungal inoculum. The fungal strains were cultured in oat meal agar slants at 25°C. The inocula were prepared by removing the sporulated fungi from the agar slant with a loop and suspending them in 10 ml of sterile water. The fungal suspensions were filtered once through a sterile gauze to remove hyphae. The resulting suspensions of conidia were vigorously vortexed and adjusted by adding sterile distilled water to a concentration of 10^5 CFU/ml by using a hemacytometer cell counting chamber, which was corroborated by a serial dilution plate count. These fungal suspensions were diluted 1:5 with RPMI to obtain 2× final suspensions. These conidial suspensions had a final concentration of 10^4 UFC/ml when mixed with antifungal solution.

Broth microdilution method. Broth microdilution testing was performed as described previously (14). Briefly, drug stock solutions were diluted with sterile distilled water to obtain 10× final desired concentrations. These 10× drug dilutions were diluted 1:5 with RPMI so that the concentrations were double. Aliquots of 100 μl of the 2× drug dilution were inoculated into the wells with a

TABLE 1. MICs of four antifungals against 30 isolates of filamentous fungi determined by three methods of end point reading

Genus	No. of strains	Antifungal agent[a]	Mean MIC[b] (μg/ml [range])			ICC (95% CI)
			V	VAA	S	
Cladosporium	5	Ampho B	0.66 (0.125–2)	0.66 (0.125–2)	0.57 (0.125–1)	0.989 (0.952–0.999)
	5	Keto	1.32 (1.0–2.0)	1.32 (1.0–2.0)	1.32 (1.0–2.0)	1
	5	Itra	1.00 (0.25–32.0)	1.00 (0.25–32.0)	0.76 (0.25–32.0)	0.995 (0.973–0.999)
	5	5-FC	3.48 (0.5–256.0)	3.48 (0.5–256.0)	4.59 (0.5–256.0)	0.990 (0.952–0.999)
Exophiala	1	Ampho B	0.06	0.06	0.06	ND
	1	Keto	0.25	0.25	0.25	ND
	1	Itra	0.125	0.25	0.25	ND
	1	5-FC	0.25	0.25	0.25	ND
Cladophialophora	4	Ampho B	0.29 (0.06–1.0)	0.29 (0.06–1.0)	0.50 (0.125–1.0)	ND
	4	Keto	0.07 (0.03–0.25)	0.07 (0.03–0.25)	0.09 (0.03–0.25)	ND
	4	Itra	0.06 (0.03–0.25)	0.06 (0.03–0.25)	0.04 (0.03–0.06)	ND
	4	5-FC	0.84 (0.25–4.0)	0.84 (0.25–4.0)	0.59 (0.25–2.0)	ND
Scopulariopsis	4	Ampho B	9.51 (4.0–16.0)	9.51 (4.0–16.0)	16.00 (8.0–32.0)	ND
	4	Keto	3.36 (2.0–4.0)	4.00 (4.0–4.0)	2.00 (1.0–4.0)	ND
	4	Itra	32.00 (32.0–32.0)	32.00 (32.0–32.0)	32.00 (32.0–32.0)	ND
	4	5-FC	256.00 (256.0–256.0)	256.00 (256.0–256.0)	256.00 (256.0–256.0)	ND
Paecilomyces	6	Ampho B	0.31 (0.03–8.0)	0.35 (0.03–8.0)	0.39 (0.03–16.0)	0.996 (0.982–0.999)
	6	Keto	0.50 (0.06–2.0)	0.50 (0.06–2.0)	0.17 (0.06–1.0)	0.893 (0.362–0.985)
	6	Itra	0.31 (0.03–32.0)	0.31 (0.03–32.0)	0.19 (0.03–32.0)	0.988 (0.951–0.998)
	6	5-FC	3.17 (0.25–256.0)	3.17 (0.25–256.0)	3.17 (0.25–256.0)	1
Aspergillus	7	Ampho B	0.37 (0.25–0.5)	0.37 (0.25–0.5)	0.37 (0.25–0.5)	1
	7	Keto	2.69 (2.0–8.0)	2.69 (2.0–8.0)	2.69 (2.0–4.0)	0.926 (0.714–0.986)
	7	Itra	0.07 (0.03–0.25)	0.07 (0.03–0.25)	0.07 (0.03–0.25)	0.985 (0.948–0.997)
	7	5-FC	23.78 (1.0–256.0)	21.53 (1.0–256.0)	9.75 (1.0–64.0)	0.975 (0.768–0.996)
Fusarium	3	Ampho B	1.0	1.0	1.0	ND
	3	Keto	32.0	32.0	32.0	ND
	3	Itra	32.0	32.0	32.0	ND
	3	5-FC	256.0	256.0	256.0	ND

[a] Ampho B, amphotericin B; Keto, ketoconazole; Itra, itraconazole; 5-FC, 5-flucytosine.
[b] Geometric mean of the three values obtained after 48 h with growth inhibitions of 75% for azoles and flucytosine and 100% for amphotericin B. 95% Cl, confidence interval at 95%; V, visual reading without previous agitation; VA, visual reading after agitation; S, spectrophotometric reading; ND, not determined.

multichannel pipette, with the first well of each row containing the lowest concentration. Well, 12 of each row served as the growth control. All the microplates were stored at −20°C until used, except the itraconazole plates, which were stored at 4°C for a maximum of 1 month. When the susceptibility tests were performed, 100 μl of the diluted inoculum suspensions was added to each well to bring the drug dilutions to the final test concentrations. For amphotericin B, ketoconazole, and itraconazole these concentrations were 0.03 to 16 μg/ml and for flucytosine they were 0.25 to 128 μg/ml. The microplates were incubated without agitation at 30°C. Readings were made at 48 and 72 h of incubation.

Susceptibility tests. The microplates were first read visually without agitation (V) with the aid of an inverted reading mirror. A second reading was performed after 5 min of agitation on a microdilution plate shaker set at 50 rpm (VA). Finally, the microplates were read with a spectrophotometer (Lambda 2; Perkin-Elmer) at 570 nm (S). The MIC of amphotericin B was defined as the lowest concentration that inhibited 100% of growth. For the two azoles and flucytosine two end point criteria were used, i.e., the lowest concentration which led to a 50 or a 75% inhibition of growth in comparison to controls. Every strain was tested in triplicate, and a new inoculum was prepared for each test.

Statistical analysis. Both on- and off-scale results were included in the analysis. The high off-scale results were converted to the hypothetical next highest concentration, and the low off-scale results were left unchanged. To perform this study, the MICs were transformed to logarithmic values. The means of the log data were back-transformed (antilog) to get the geometric mean.

The reproducibility of the results obtained from all the possible combinations of the different reading methods (V, VA, and S), end points (50 and 75% of inhibition of growth for azoles and flucytosine, and inhibition of 100% for amphotericin B), and incubation times (48 and 72 h) was evaluated by using the intraclass correlation coefficient (ICC) (10, 17) among the successive readings (three readings for every possible combination). A one-way random-effect model was assumed to calculate this ICC. Results were expressed over a maximum value of 1.

The degree of correlation among the three methods was evaluated assuming a two-way mixed effect model and using an absolute agreement definition of the ICC among the median value of the three MICs obtained for each strain. This analysis was performed globally, i.e., all of the strains were considered together and also for each genera. In the latter case, only the reading at 48 h, the 75% growth inhibition for azoles and flucytosine, and the 100% inhibition for amphotericin B were considered. The correlation among the methods was only analyzed when five or more strains of the genus were tested. Results were also expressed out of a maximum value of 1.

The effect of incubation time and end point definition on the MICs obtained by each reading method was determined by applying the Student's *t* test and Bonferroni correction to all possible pairs of conditions. When the effect of one variable was studied, the other one was fixed as a constant. The statistical analyses were performed with the statistical SPSS package (version 8.0) for the PC.

RESULTS

Correlation among the reading methods. Table 1 shows the geometric mean and the range of MIC of the four antifungals tested against the 30 strains of seven genera of filamentous fungi. It also shows the degree of agreement among the MICs obtained with the three methods assayed after 48 h of incubation with an end point of 75% growth inhibition (azoles and flucytosine) or 100% inhibition (amphotericin B). In general, the activity of the different antifungals was quite variable among the genera tested. The highest MICs were those against *Scopulariosis* and *Fusarium* spp. The agreement among the three reading methods was very high in all cases, and for ketoconazole in *Cladosporium*, flucytosine in *Paecilomyces* and amphotericin B in *Aspergillus* it was complete (ICC = 1). The results were worse for ketoconazole in *Paecilomyces* (ICC = 0.893). Table 2 shows the degree of correlation among the MICs obtained with the three reading methods when all of the fungal strains were considered globally under the different combinations of end point definition and time of reading. In all cases the ICC was very high with the lowest value being 0.982, indicating that agreement was good among the three methods tested. In the case of azoles and flucytosine, there were no

TABLE 2. Degree of correlation of MICs, measured by ICC, obtained by the three methods of reading (visually, visually with agitation, and spectrophotometrically) with three different breakpoints and two times of reading

% Growth inhibition	Time of incubation (h)	ICC (95% CI)[a] with:			
		Keto	Itra	5-FC	Ampho B
50	48	0.982 (0.966–0.991)	0.995 (0.990–0.997)	0.995 (0.991–0.998)	
	72	0.992 (0.985–0.996)	0.995 (0.987–0.997)	0.996 (0.992–0.998)	
75	48	0.987 (0.979–0.994)	0.999 (0.998–0.999)	0.996 (0.994–0.998)	
	72	0.997 (0.994–0.998)	0.998 (0.997–0.999)	0.996 (0.992–0.998)	
100	48				0.990 (0.982–0.995)
	72				0.998 (0.996–0.999)

[a] Antifungal agents are as defined for Table 1.

important differences between the ICC obtained with the two types of end point definition or those obtained for the two periods of incubation. For amphotericin B, the differences between the ICC values obtained for the two periods of incubation were also minimal.

Reproducibility. Table 3 shows the reproducibility of the three methods used for MIC determination. Reproducibility was very high for all the antifungals under each condition. For azoles and flucytosine, the degree of reproducibility was highest with flucytosine with the spectrophotometric method and an end point of 75% of growth inhibition at 72 h. The reproducibility was worse with ketoconazole also with the spectrophotometric method, with an end point of 75% of inhibition at 48 h. In general, reproducibility was slightly lower for amphotericin B when the spectrophotometric method was used. Very few differences were observed between the MICs that inhibited 50 or 75% of fungal growth in each of the three reading methods for both azoles and flucytosine. The reproducibility for flucytosine was best at 48 h, whereas for azoles and for amphotericin it was best at 72 h of incubation.

Influence of the end point definition and length of incubation on MICs. Both the end point definition and the length of incubation had a significant effect on MICs ($P < 0.05$) in each of the three reading methods. The MICs were highest when an end point of 75% of growth inhibition after 72 h of incubation was considered (data not shown).

DISCUSSION

The definition of the end point in MIC determinations is one of the most striking problems in standardizing antifungal susceptibility tests for azoles and flucytosine (1–3, 10). The NCCLS (11) has recently made some specific recommendations when these drugs are tested against filamentous fungi. For example, the end point reading criterion is less stringent than that used for testing amphotericin B, i.e., a slight turbidity is allowed instead a total absence of fungal growth. Alternative methods for improving the quality of MIC readings by using both different methodologies and different criteria have been published (1–4, 6, 7, 12, 13), and they include comparisons among visual readings with or without previous agitation and spectrophotometric readings. However, these works are usually devoted to yeasts.

We have compared the influence of three reading methods on the MICs of several antifungals under different testing conditions. In our study, we used only a few strains from a variety of genera (rather than many strains from only a few genera) in order to reach the maximum variability in the microorganisms tested. In all cases the degree of correlation was very high. This suggest that agitation in the visual reading and spectrophotometric method do not improve the results obtained with the conventional visual method of reading MICs. Previous studies with yeasts have shown that there are considerable differences

TABLE 3. Degree of reproducibility of three different methods of reading, measured by ICC, among three serial MICs of four antifungals against 30 strains of filamentous fungi with three end point criteria and two times of reading

Reading type	Breakpoint (% growth inhibition)	Time of incubation (h)	ICC (95% CI)[a] with:			
			Keto	Itra	5-FC	Ampho B
Visual	50	48	0.951 (0.912–0.974)	0.976 (0.957–0.988)	0.974 (0.953–0.986)	
		72	0.970 (0.945–0.984)	0.984 (0.970–0.992)	0.965 (0.937–0.982)	
	75	48	0.960 (0.929–0.979)	0.975 (0.956–0.987)	0.986 (0.973–0.993)	
		72	0.960 (0.929–0.979)	0.974 (0.957–0.988)	0.975 (0.955–0.987)	
	100	48				0.825 (0.709–0.905)
		72				0.916 (0.853–0.956)
Visual after agitation	50	48	0.961 (0.931–0.980)	0.976 (0.957–0.988)	0.982 (0.968–0.991)	
		72	0.966 (0.939–0.983)	0.981 (0.966–0.990)	0.970 (0.947–0.985)	
	75	48	0.955 (0.920–0.977)	0.976 (0.957–0.988)	0.984 (0.972–0.992)	
		72	0.964 (0.936–0.981)	0.971 (0.948–0.985)	0.974 (0.953–0.987)	
	100	48				0.811 (0.688–0.897)
		72				0.915 (0.850–0.956)
Spectrophotometric	50	48	0.860 (0.763–0.925)	0.948 (0.908–0.973)	0.970 (0.945–0.984)	
		72	0.918 (0.857–0.957)	0.957 (0.924–0.978)	0.967 (0.941–0.983)	
	75	48	0.850 (0.747–0.919)	0.894 (0.818–0.944)	0.971 (0.948–0.985)	
		72	0.936 (0.888–0.967)	0.982 (0.967–0.991)	0.992 (0.986–0.996)	
	100	48				0.808 (0.683–0.895)
		72				0.896 (0.819–0.946)

[a] Antifungal agents are as defined in Table 1.

between the MICs obtained with visual and spectrophotometric methods, mainly for amphotericin B and flucytosine, but also for azoles under certain conditions (3, 4).

It has been suggested that agitation before the visual reading would be helpful in the susceptibility testing of yeasts (10), and several studies have shown that results improve after agitation (1–4, 13). Previous agitation also improved the correlation between visual and spectrophotometric methods (1) and between intra- and interlaboratory agreement and even gave more on-scale results in susceptibility tests against fluconazole (2). In general, the main cause of nonagreement among the methods was that visual reading gave higher MICs. However, another study tested several isolates of *Candida* species against five antifungals, and the results were similar to ours, i.e., the correlation among visual, visual-with-agitation, and spectrophotometric readings was very high (13).

We expected that the results would be improved by agitating the microplates when testing filamentous fungi because the hyphal clumps that may have formed would disgregate. However, it did not significantly affect the MICs. It would be interesting to corroborate this in studies with hyaline filamentous fungi. In our study, the reproducibility of the three methods tested was very high in all cases and under the different testing conditions. Previous studies which tested *Candida* spp. against several antifungals by using different methods and end point reading criteria showed that the spectrophotometric method had the best reproducibility. In these studies, the MICs of azoles and flucytosine had slightly better reproducibilities when the end point was the 50% growth inhibition and not the 75% inhibition (3, 12). In susceptibility testing of different filamentous fungi against amphotericin B and itraconazole, Espinel-Ingroff et al. (5) obtained a high reproducibility with the colorimetric method by using Alamar blue as an indicator as well as by using the conventional method.

The effect of incubation time on MICs was significant for all reading methods and both end points. This does not agree with previous results which showed that MICs obtained spectrophotometrically were independent of incubation time when testing yeasts (1). However, our results do agree with other studies with yeasts (3) and filamentous fungi when MICs were determined by a visual method without agitation (5, 15). In one of these studies we demonstrated that incubation time produced changes in MICs when the temperature increased (15).

Our study also showed that the breakpoint had an important effect on the MICs of azoles and flucytosine under all combinations of reading method and incubation time. Other studies have also compared the effect that different end points have on MICs (3, 4, 12), but the results have not been statistically analyzed and so they cannot be compared with ours.

The main conclusion of our study is that the reading method has no effect on MICs when filamentous fungi are tested. We also concluded that a minimum incubation time of 72 h for azoles and amphotericin B slightly improves the reproducibility of results in the fungi we tested. The reproducibility of flucytosine improves at 48 h, as was shown in another study (C. Llop, J. Sala, M. D. Riba, and J. Guarro, unpublished data). However, these results cannot be generalized to all filamentous fungi because of the wide variety of the morphology and biology of these microorganisms. Further studies with other species are needed to confirm our results.

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