Evaluation of the Gradient Concentration Strip Method for Antifungal Susceptibility Testing of Isavuconazole and Comparators for Mucorales Species

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SUSCEPTIBILITY

Evaluation of the Gradient Concentration Strip Method for Antifungal Susceptibility Testing of Isavuconazole and Comparators for *Mucorales* Species

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ABSTRACT MIC values for amphotericin B and three azoles determined by the EUCAST reference technique and by gradient concentration strips were compared for 30 *Mucorales* isolates belonging to clinically important species. Essential agreement (EA) within ±2 dilution steps at 24 hours between the techniques was 83.3% for isavuconazole. EAs for itraconazole, amphotericin B, and posaconazole were 86.7%, 73.3%, and 56.7%, respectively. A good agreement was obtained between visual and spectrophotometric readings for EUCAST.

KEYWORDS EUCAST, Etest, *Mucorales*, antifungal agents, antifungal susceptibility testing, filamentous fungi

Mucormycosis is associated with a high mortality rate, mostly affecting immunocompromised patients (1). *Mucorales* species show high *in vitro* MICs for several antifungals and are resistant to voriconazole (2–4), the first-line therapy for invasive aspergillosis. Recommended treatment for mucormycosis is high-dose liposomal amphotericin B combined with early surgery, if possible (5). Isavuconazole, a new broad-spectrum azole, is effective in patients with mucormycosis (6), generally well tolerated (7), and indicated for the treatment of mucormycosis when amphotericin B is not suitable (8). The recommended but sophisticated and time-consuming technique for antifungal susceptibility testing of molds is the broth microdilution method by CLSI or EUCAST (9, 10). Gradient concentration strips provide a fast and easy-to-handle tool for susceptibility testing of bacteria (11) and fungi (12). Whether antifungal MICs for *Mucorales* species obtained by the reference technique and the gradient concentration strips are comparable has been evaluated in a few studies with variable results (13–22). Data for isavuconazole are lacking.

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The aim of this study was to compare the MICs obtained by EUCAST and gradient concentration strip methodologies for isavuconazole, amphotericin B, posaconazole, and itraconazole for *Mucorales*.

Thirty *Mucorales* isolates, including 9 *Rhizopus arrhizus* (comprising 1 *R. arrhizus* var. *delemar*), 3 *Rhizopus microsporus*, 5 *Lichtheimia corymbifera*, 5 *Lichtheimia ramosa*, 4 *Mucor circinelloides*, and 4 *Rhizomucor pusillus*, were tested. Isolates were identified by sequencing of the internal transcribed spacer regions. Sequences were previously

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deposited in GenBank (23–26). Quality control isolates included *Aspergillus fumigatus* ATCC 204305, *Candida krusei* ATCC 6258, and *Candida parapsilosis* ATCC 22019.

MICs were determined for isavuconazole, itraconazole, posaconazole, and amphotericin B by gradient concentration strip and EUCAST techniques (9). Isavuconazole (Basilea Pharmaceutical, Ltd., Basel, Switzerland), itraconazole (Sigma-Aldrich, Saint-Quentin Fallavier, France), posaconazole (MSD, Kenilworth, NJ), and amphotericin B (Sigma-Aldrich) were obtained as pure powders, and stock solutions were prepared in dimethyl sulfoxide.

MIC determination was performed in flat-bottomed 96-well microtiter plates using RPMI 1640 (with L-glutamine, with pH indicator, but without bicarbonate) (Sigma-Aldrich) (9). Final concentrations ranged from 0.015 to 8 μg/ml. Isolates were cultured for 5 to 7 days, and spore suspensions were prepared in water. Spore suspensions were counted with a hemocytometer and adjusted to 2 to 5×10^6 CFU/ml. Trays were inoculated with 100 μl of the 1/10-diluted spore suspension. Plates were incubated at 37°C (30°C for *M. circinelloides*) for 24 h, and MICs were determined visually and spectrophotometrically (complete inhibition).

For the gradient concentration strip method, RPMI agar plates were inoculated with the same spore suspension adjusted to 10^6 CFU/ml, and strips of isavuconazole (Liofilchem, Roseto degli Abruzzi, Italy) and the other drugs (Etest, bioMérieux, Marcy l'Etoile, France) were placed on the agar. MICs were determined after 24 h of incubation at 37°C (30°C for *M. circinelloides*). MICs were read where the inhibition ellipse intersected the MIC scale of the strip. Overgrowth by filaments bending into the ellipse was ignored.

Experiments were performed twice. Results of the two methods were analyzed by providing essential agreement (EA) values within ± 2 dilution steps. Agreement within ± 1 dilution step was also calculated. Categorical agreement was not calculated because there are currently no clinical breakpoints for *Mucorales* species.

Results of the *in vitro* susceptibility of *Mucorales* isolates determined by the EUCAST (visual reading) and gradient concentration strip methods are presented in Table 1. With the EUCAST method, isavuconazole MICs ranged from 0.25 to 16 μg/ml, with a geometric mean of 1.82 μg/ml. *In vitro* activity of isavuconazole depended on the species. The lowest and highest isavuconazole MICs were seen for *Lichtheimia* and *Mucor* isolates, respectively. With the gradient concentration strip method, isavuconazole MICs ranged from 0.25 to 16 μg/ml, with a geometric mean of 1.15 μg/ml. The EA between both techniques for isavuconazole was 83.3% (Table 2). When discrepant results were observed, gradient concentration strips showed lower MICs than the EUCAST method. Examples of results with the gradient concentration strips and the EUCAST inhibition curves are presented in Fig. 1.

For the comparators, MICs determined by the EUCAST method ranged from 0.125 to 2 μg/ml (geometric mean, 0.26 μg/ml) for amphotericin B, 0.0625 to 16 μg/ml (geometric mean, 1.66 μg/ml) for posaconazole, and 0.0625 to 16 μg/ml (geometric mean, 1.7 μg/ml) for itraconazole. MICs determined by gradient concentration strips ranged from 0.125 to 16 μg/ml (geometric mean, 0.81 μg/ml) for amphotericin B, 0.25 to 16 μg/ml (geometric mean, 0.45 μg/ml) for posaconazole, and 0.03125 to 16 μg/ml (geometric mean, 6.96 μg/ml) for itraconazole. EAs (± 2 dilution steps) for the comparators were 73.3%, 56.7%, and 86.7% for amphotericin B, posaconazole, and itraconazole, respectively (Table 2). Agreement within ± 1 dilution step was lower for isavuconazole, amphotericin B, posaconazole, and itraconazole at 66.7%, 53.3%, 40%, and 53.3%, respectively.

When MICs were determined spectrophotometrically with the EUCAST method, the EAs between the EUCAST and gradient concentration strip methods were 80%, 76.7%, 63.3%, and 73.3% for isavuconazole, amphotericin B, posaconazole, and itraconazole, respectively (data not shown).

The EAs between spectrophotometric and visual reading for the EUCAST technique were 93.3%, 100%, 86.2%, and 88.9% for isavuconazole, amphotericin B, posaconazole, and itraconazole, respectively (Table 3).

TABLE 1 Comparison of MICs determined visually by EUCAST broth susceptibility testing and by gradient concentration strip methodology for *Mucorales* isolates

Species	MIC (μg/ml) (range [geometric mean]) with[a]:							
	EUCAST method for:				Gradient concentration strip for:			
	ISA	AMB	PSZ	ITZ	ISA	AMB	PSZ	ITZ
Rhizopus spp. ($n = 12$)	1–8 (1.6)	0.25–2 (0.56)	0.125–2 (0.45)	4–16 (7)	0.25–4 (1.12)	0.5–16 (2.7)	0.25–16 (3.2)	0.5–16 (6.7)
Lichtheimia spp. ($n = 10$)	0.25–2 (1)	0.25–1 (0.15)	0.06–0.5 (0.134)	0.06–1 (0.33)	0.25–2 (0.6)	0.25–1 (0.66)	0.25–1 (0.57)	0.5–2 (0.9)
Mucor circinelloides ($n = 4$)	8–16 (11)	0.125–0.25 (0.17)	1–16 (4)	16	16	0.125	16	16
Rhizomucor pusillus ($n = 4$)	1–4 (1.6)	0.125–0.25 (0.15)	0.25–2 (0.5)	0.06–0.5 (0.15)	0.25–0.5 (0.42)	0.125–0.25 (0.25)	0.25–0.5 (0.35)	0.03–0.125 (0.07)
Total ($n = 30$)	0.25–16 (1.82)	0.125–2 (0.26)	0.06–16 (1.66)	0.06–16 (1.7)	0.25–16 (1.15)	0.125–16 (0.81)	0.25–16 (0.45)	0.03–16 (6.96)

[a]ISA, isavuconazole; AMB, amphotericin B; PSZ, posaconazole; ITZ, itraconazole.

TABLE 2 MIC dilution differences between EUCAST and gradient concentration strip methodologies for 30 *Mucorales* isolates

	Etest-EUCAST dilution difference							EA (%)	
Drug[a]	<−2	−2	−1	0	+1	+2	>2	±1 dilution step	±2 dilution steps
ISA	5	4	5	8	7	1	0	66.7	83.3
AMB	0	0	2	7	7	6	8	53.3	73.3
PSZ	1	0	1	7	4	5	12	40	56.7
ITZ	2	4	1	7	8	6	2	53.3	86.7

[a]ISA, isavuconazole; AMB, amphotericin B; PSZ, posaconazole; ITZ, itraconazole; EA, essential agreement.

First-line treatment for mucormycosis is liposomal amphotericin B (27); isavuconazole may be used when amphotericin B is inappropriate (8). Amphotericin B, isavuconazole, and posaconazole show good *in vitro* activity against *Mucorales* (2, 4, 26). Activity of itraconazole is limited to *Lichtheimia* spp., *Rhizomucor* spp., and some *Syncephalastrum racemosum* isolates (2, 4, 26). Here, the best *in vitro* activity, determined by the EUCAST technique, was seen for amphotericin B, with the lowest MICS for *Lichtheimia* spp. and *R. pusillus*. MICs for azole antifungals were generally higher and in accordance with previous studies (2, 4, 26).

Recommended techniques for antifungal susceptibility testing of molds are the reference techniques of CLSI and EUCAST. The gradient concentration strip method has shown good correlation with the reference techniques for yeasts and filamentous fungi (28). Nevertheless, only a few studies evaluated the correlation between gradient concentration strips and CLSI (14, 16–22) or EUCAST (13, 15) methodology for *Mucorales* species. One study compared isavuconazole results obtained by the CLSI and gradient concentration strip techniques (17), but correlation between the EUCAST and gradient concentration strip methods has never been evaluated. Here, the EA between the gradient concentration strip and EUCAST techniques for isavuconazole was 83.3%. When discrepant results were observed, except for *M. circinelloides*, gradient concentration strips gave lower MICs. The EAs between the two techniques were 73.3%, 56.7%, and 86.7% for amphotericin B, posaconazole, and itraconazole, respectively. Agreement

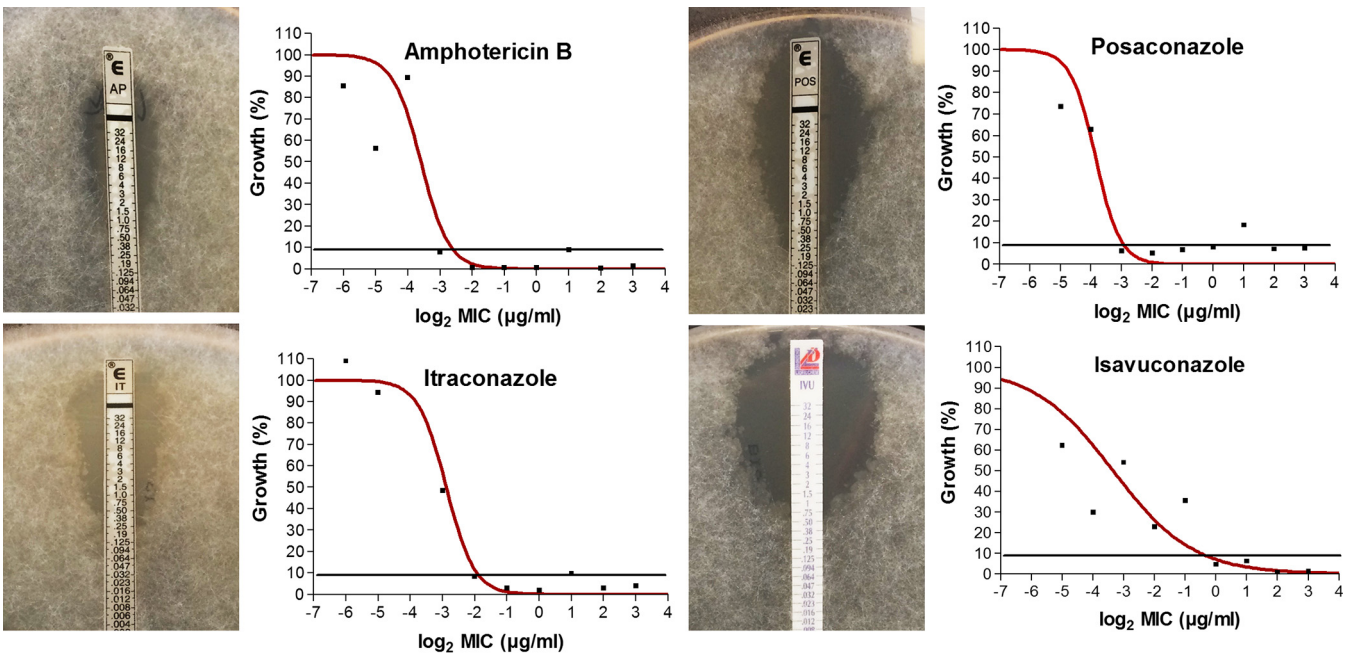

FIG 1 Examples of gradient concentration strip results and EUCAST inhibition curves obtained by spectrophotometric reading for an isolate of *L. corymbifera* (CBS 120581) for isavuconazole and comparators. Spectrophotometric curves were modeled by nonlinear regression based on a maximum-effect model with GraphPad Prism.

TABLE 3 Visual versus spectrophotometric reading of MICs determined by EUCAST methodology at 24 h

	Visual vs spectrophotometric MIC reading							EA (%)	
Drug[a]	<−2	−2	−1	0	+1	+2	>2	±1 dilution step	±2 dilution steps
ISA	0	1	1	13	11	2	2	83.3	93.3
AMB	0	1	0	19	9	1	0	93.3	100
PSZ	1	0	3	17	6	0	3	86.7	86.7
ITZ	0	0	0	18	6	2	4	80.0	86.7

[a]ISA, isavuconazole; AMB, amphotericin B; PSZ, posaconazole; ITZ, itraconazole; EA, essential agreement.

rates for the ±1 dilution step were lower and in accordance with previous studies (13, 15, 17, 21, 22). Various results have been obtained for EAs between gradient concentration strip and CLSI or EUCAST techniques for *Mucorales* species (13–22). EAs ranged from 70.5% to 96.5% for amphotericin B (13–16, 19, 21, 22), 50% to 83% for itraconazole (18, 20–22), and 77% to 100% for posaconazole (13–16, 18, 19, 22). For isavuconazole, comparison of gradient concentration strips and the CLSI method resulted in an EA of 84.4% (17), similar to our results. Why the correlation between the techniques is sometimes not optimal remains unknown. Others have reported MIC reading difficulties for *Mucorales* isolates, because hyphae frequently overgrow the strip (13, 29). Incubation time may also be important, because better correlation was obtained for posaconazole with shorter incubation time (16), possibly related to different optimal growth temperatures of *Mucorales* species (30).

One of the drawbacks of the EUCAST technique for testing molds is the visual determination of MICs. Previous reports demonstrated that spectrophotometric reading is a good alternative for *Aspergillus* (31–33) and *Mucorales* (4) species. Here, correlation between visual determination and spectrophotometric reading for *Mucorales* isolates was high. Incorporation of spectrophotometric reading would lead to more objective MIC determination and automation of the process.

In conclusion, the EA between gradient concentration strips and EUCAST methodology for determination of isavuconazole MICs against *Mucorales* isolates was high (83.3%), although it did not reach the threshold of ≥90%. Further work is warranted to test the technical parameters that may improve the correlation between the two techniques.

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